Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

DOTTORATO DI RICERCA IN SCIENZE MEDICHE SPECIALISTICHE
Ciclo XXVIII

Settore Concorsuale di afferenza: 06/D2

Settore Scientifico disciplinare: MED 14

TITOLO TESI

Histological study on double line of intravenous Tacrolimus infusion in SLA defined pig model

Presentata da: **Federico Zacchini**

Coordinatore Dottorato   **Relatore**

Prof. Roberto Di Bartolomeo   **Prof. Gaetano La Manna**

Esame finale anno 2016

INDEX

ABSTRACT

1 – Abstract

The immunosuppressive therapy still remains the only therapeutic strategy to control excessive immune activation following renal transplantation, but remain the problems related to excessive immunosuppression and in particular the toxicity due to high doses of immunosuppressive drugs such as calcineurin inhibitors. The present study has the aim of documenting, in a porcine animal model, the histological damage from calcineurin inhibitors using incremental doses of Tacrolimus, achieved in a limited amount of time, until it reaches toxic blood concentrations. We perform the study under different condition, like oral administration, intravenous infusion and with or without kidney transplant. It is noted that the damage is early, predominantly vascular and that affects different organs in addition to the kidneys. We also observe that the functional damage underestimates the structural damage.

The search for non-invasive methods for the identification of biomarkers of nephrotoxicity and rejection, and to better characterize the inflammation status, led us to conduct analysis of the exosomal content, allowing us to observe the presence of serum cytokines which, although in low amounts, suggest a possible role of these in the inflammatory process mediated by exosomal vesicles.

Finally, following the genetic SLA typing for the determination of the donors and the recipients, in an accessory project, we got a controlled and stable colony of pigs with SLA defined in homozygosis through the coupling of specific pathogen free pigs (SPF) for DQB-1 and SLA-1 genes.

INTRODUCTION

2.1 - Calcineurin Inhibitors

Nowadays, renal transplantation is the best therapeutic option for end-stage kidney disease patients, but despite the evident amelioration of renal transplant results in the short and mid-term (5-years graft survival >90%) [Ojo et al., 2013], the outcomes in the long term are not yet fully satisfying. The main cause of graft loss is mainly due to the activation of host immune system against the allograft [Gastor et al., 2010; Sellarés et al., 2012]. The increased activity of immune cells causes an extensive parenchymal damage, leading to short- or long-term organ loss. Immunosuppressive therapy is still the only strategy to control immune activation, but the chronic use of these drugs can lead to severe complications due to an excessive immunosuppression, potentially giving rise to tumours, infections or high-dose drug toxicity.

Calcineurin inhibitors (CNI), represented in the clinical use of cyclosporine and Tacrolimus (TAC), are an important class of drugs used as immunosuppressants in the process of organ transplant or to treat certain kidney diseases or immune-mediated diseases. After their use, since the 80s, there has been a significant reduction in kidney transplant rejection episodes associated with improved survival of both patient and graft. Today, 94% of the kidney transplant recipients are discharged after transplantation with a CNI-based immunosuppressive regimen [Andreoni et al., 2007].

The immunosuppressive properties of cyclosporine and Tacrolimus result from inhibition of calcineurin, a calcium-dependent and calmodulin-dependent phosphatase. Intracellularly, these molecules bind to cyclophylin for cyclosporine and FKBP12 for Tacrolimus [Takahashi et al., 1989; Harding et al., 1989; Siekierka et al., 1989]. The competitive binding of cyclosporine-cyclophylin and tacrolimus-FKBP12 complexes to calcineurin, inhibits phosphatase activity of calcineurin. This inhibits the dephosphorylation and translocation of the nuclear factors of activated T-cells [Flanagan et al., 1991], which regulates IL-2 transcription and thus T-cell activation [O'Keefe et al., 1992]. Calcineurin is not T-cell specific, and inhibition of this pathway by cyclosporine and Tacrolimus gives rise to toxicity beyond immunosuppression [Liu et al., 2007].

However, their extensive use has shown side effects, both early and late, out of the kidney. In particular they can lead to acute CNI nephrotoxicity or chronic CNI nephrotoxicity. In literature it is not known the

relationship between acute and chronic renal damage in the absence of other possible factors, which influence renal function (hyperglycaemia, obesity, drugs, etc.).

2.1.1 - Acute CNI Nephrotoxicity

The acute nephrotoxicity is primarily due to hemodynamic and tubular toxicity mechanisms [Krejci et al., 2010]. The functional damage is secondary to vasoconstriction of the afferent arteriole [Murray eta al., 1985], mediated by the activation of the renal angiotensin-aldosterone system that induces an up-regulation of vasoconstrictor factors such as endothelin 1 and thromboxane, and simultaneously a down-regulation of vasodilator factors like prostacyclin, prostaglandin E2 and nitric oxide [Olyaei et al., 2001; Burdmann et al., 2003; Hortelano et al., 2000]. The marked vasoconstriction leads to hypoxic damage with the release of free radicals and superoxide anions [Diederich et al., 1994]. These superoxides decrease NO bioavailability that can lead to decreased vasodilation and unopposed vasoconstriction, which is a main mechanism of cyclosporine-induced hypertension and decreased GFR (Glomerular Filtration Rate) [Roullet et al., 1994]. The CNI also induce an increase both in vivo and in vitro of the levels of TGF-β (Transforming Growth Factor beta), cytokine able to promote, along with the endothelin 1, renal interstitial fibrosis through the extracellular matrix synthesis [Khanna et al., 2002]. In native kidneys, stimulating effects of cyclosporine on sympathetic nerve activity could play a role in the acute effects of cyclosporine by increased renal vascular resistance and secondarily decreased GFR [Zhang et al., 2000]. In transplanted kidney, which lack innervation, the sympathetic effects of cyclosporine were not observed [Hausberg et al., 2006]. The effects of cyclosporine on sympathetic nerve activity could be explained by the fact that calcineurin is ubiquitously expressed in neural tissue [Hemenway & Heitman, 1999; Naesens et al., 2009].

At tubular level, in response to the action of glomerular vasoconstriction, can observe the appearance of isometric vacuolization of the cytoplasm of tubular cells characterized by swelling of the endoplasmic reticulum and increased of lysosomes [Mihatsch et al., 1985; Morozumi et al., 2004]. These structural changes are the consequence of hypoxic/ischemic damage by the reduction of renal plasma flow [Du et al., 2009]. Moreover, the acute nephrotoxicity of CNI with renal dysfunction associated to the graft, is often present in absence of these morphologic lesions. In contrast, isometric tubular vacuolization associated with the use of cyclosporine and Tacrolimus can be found in the absence of renal dysfunction. The isometric

tubular vacuolization is not associated with progression to chronic CNI nephrotoxicity [Naesens et al., 2007a].

Furthermore, inclusion bodies are also sometimes noted in the tubular cytoplasm in association with the cyclosporine use [Kim & Suh, 1995]. Most of that are giant mitochondria, but it is currently not known what triggers their formation, but it is clear that cyclosporine has important effects on mitochondrial functioning [Simon et al., 2003].

Finally, thrombotic microangiopathy can occur after renal transplantation as result of many factors, including ischemia-reperfusion endothelial injury, renal infections, rejection, malignancies, and various drugs. The use of the CNI is clearly an important risk factor for post-transplant thrombotic microangiopathy [Ponticelli, 2007]. Furthermore it has been suggested that cyclosporine and Tacrolimus can increase platelet aggregation and activate prothrombotic factors.

2.1.2 - Chronic CNI Nephrotoxicity

The chronic damage associated with CNI therapy was first demonstrated by Myers el al., showing that cyclosporine not only induces reversible alterations in renal vascular resistance, but is associated with irreversible damage of the renal architecture [Myers et al., 1984].

Irreversible changes to every component of the renal parenchyma would be at the basis of chronic nephrotoxicity, whose relationship with the acute injury is not clear. The three kidney compartments can be irreversibly injured by CNI: vessels (arteriolar hyalinosis), tubulointerstitium (tubular atrophy and interstitial fibrosis), and glomeruli (thickening and fibrosis of Bowman's capsule and focal segmental or global glomerular sclerosis). These structural changes could be the outcome of the constant hemodynamic stimulus and tubular toxicity [Naesens et al., 2007a]. From the histopathological point of view they affect all of the kidney compartments. Characteristic morphological aspect is the presence of granular hyaline deposits in the tunica media of the afferent arterioles, defined arteriolar hyalinosis, which is always observed in chronic CNI nephrotoxicity [Nankivell et al., 2004]. Arteriolar hyalinosis is characterized by the replacement of necrotic smooth muscle cells by focal or circular lumpy protein (hyaline) deposits at the periphery of the wall of afferent arterioles. Arteriolar hyalinosis is commonly regarded as irreversible [Naesens et al., 2007a].

The finding of segmental glomerular sclerosis is very common and reflects the damage of glomerular hyperfiltration. In more advanced stages chronic nephrotoxicity the severe arteriolar hyalinosis contributes to glomerular sclerosis and the tubular degeneration. If not recognized and treated promptly, after these histopathological aspects corresponds clinically progressive decline in kidney function values and the onset of high blood pressure with possible progressive loss of the graft. As mention before, the use of CNI can lead to the release of free radicals and superoxide anions, as a result of renal vasoconstriction that can lead to renal hypoperfusion and hypoxia, and subsequently to the formation of reactive oxygen species or free radicals, which then causes cellular injury and promotes cellular death by apoptosis [Longoni et al., 2001].

Calcineurin inhibition may also directly activate apoptosis genes and increase apoptosis in tubular and interstitial cells, thus inducing tubular atrophy [Servais et al., 2008; Shihab et al., 1999]. Moreover, inflammation with macrophage infiltration has been associated with chronic CNI nephrotoxicity [Young et al., 1995].

The production of TGF-β, that is directly upregulated by cyclosporine, induces the development of focal interstitial fibrosis and tubular atrophy through the synthesis of extracellular matrix proteins, inhibition of their degradation and the activation of the migration of mesenchymal epithelial cells [Border & Noble, 1997; Wolf, 2006].
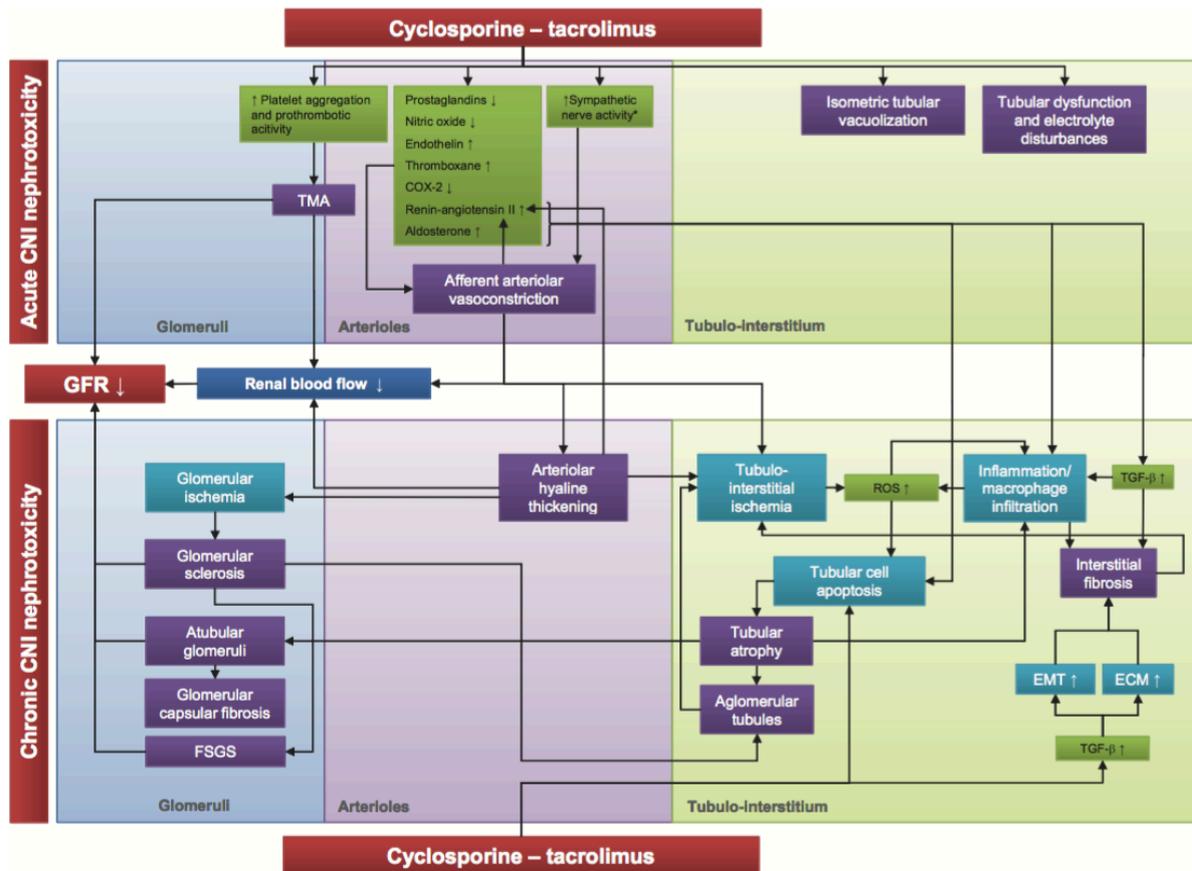
Figure 1: Schematic representation of the etiology of calcineurin inhibitor nephrotoxicity. CNI, calcineurin inhibitor; TMA, thrombotic microangiopathy; EMT, epithelial mesenchymal transition; ECM, extracellular matrix; GFR, glomerular filtration rate; FSFS, focal segmental glomerulosclerosis; ROS, reactive oxygen species. Figure from Naesens et al., 2007a.

2.1.3 - Electrolyte Disturbance

As we already mentioned, the use of CNI impact renal hemodynamics and structure, but lead to tubular functional alteration and ion homeostasis disturbance like hyperkalemia, hypomagnesemia and magnesium wasting, hyperchloremic metabolic acidosis (distal tubular acidosis), and hyperuricemia [Heering & Grabensee, 1991; Clive, 2000; Alexander et al., 2008]. The hyperkalemia induced by CNI is likely multifactorial and refers to inhibitory effects on Na^+-K^+-ATPase in collecting ducts [Lea et al., 1994] and to distal tubular acidosis. Furthermore, in 75% of patients treated with cyclosporine, there is evidence of decreased numbers of mineralcorticoid receptors that lead to hyperkalemia and metabolic acidosis as a result of aldosterone resistance [Heering et al., 2004]. The hypomagnesemia relating to reduce expression of paracellin-1 in thick ascending limb cell by cyclosporine [Chang et al., 2007], that resulting in decreases in

magnesium transport and contributes to magnesium wasting. Finally, it was shown that cyclosporine indirectly opens ATP-sensitive K^+ channels by inhibition of calcineurin, which could contribute to the CNI-associated hyperkalemia [Wilson et al., 2000].

2.1.4 - Histologic Diagnosis

There are a lot of difficulties to have good diagnosis that can distinguish between CNI-related nephrotoxicity and other injury phenomena. Different pathologies share the same clinical picture of gradual decrease in renal graft function and lead to nonspecific tubular atrophy, interstitial fibrosis and loss of renal allograft [Farnsworth et al., 1984]. Tubular vacuolization and progressive arteriolar hyalinosis, that can be considered more specific for CNI nephrotoxicity, are also seen secondary to other processes. To making a positive diagnosis of CNI nephrotoxicity is mandatory to combine several histologic features and exclusion of other processes that lead to similar histologic findings. The study of CNI nephrotoxicity in native kidneys may be less troublesome [Ojo et al., 2003], but it should be kept in mind that an essential difference between native kidneys and transplanted kidneys is the absence of innervation of transplanted kidneys; this innervation plays a prominent role in the regulation of renal vascular resistance. Therefore, the consideration done for the native kidney may not be true for the transplanted kidney and should be interpreted cautiously.

2.1.5 - Systemic Levels of CNI

The main issue after renal transplantation is to maintain a reasonable balance between efficacy, to avoid rejection, and toxicity of the immunosuppressive agents used. Al lot of studies correlate rejection and concentration-toxicity to CNI dosage [Laskow et al., 1996; Kershner & Fitzsimmons. 1996]. The dosage used in these studies was very broad (5 to 25 ng/ml), because Tacrolimus is drug with narrow therapeutic windows. Maintaining the concentrations of the CNI within preset target ranges may be complicated by the high inter- and intraindividual pharmacokinetic variability [Staatz & Tett, 2004], due to the high variability in absorption, distribution, metabolism and elimination of these compounds. Also the intestinal absorption of Tacrolimus is low, variable and influenced by concomitant ingestion of food, diabetes, uremia, ethnicity, gastrointestinal problems and diarrhea. After absorption, Tacrolimus and cyclosporine is distributed in erythrocytes, and is bound to plasma proteins like albumin, globulines and lipoproteins. Metabolism of CNI

occurs mainly in the liver and the gastrointestinal tract and then can be almost completely eliminated in the bile [Fahr, 2003]. This high variability in the pharmacokinetics and concentration-toxicity associations suggests that therapeutic drug monitoring of CNI is useful. Despite that routine monitoring of cyclosporine and Tacrolimus is common practice, the therapeutic application of such monitoring has not prevented kidney transplant recipients from developing acute rejection, or avoiding nephrotoxicity, which is virtually universal by 10 years after transplantation [Nankivell et al., 2003]. Today, is not clear whether the rate of development of lesions that can be associable to chronic CNI nephrotoxicity, correlate to the dose of cyclosporine or Tacrolimus therapy. Some studies suggest association of chronic CNI nephrotoxicity lesion with higher CNI exposure [Nankivell et al., 2004; Nakamura et al., 2007], other studies have found association of lower levels of CNI with higher increase in chronic tubulo-interstitial damage [di Paolo et al., 2004; Naesens et al., 2007b].

2.2 – Pig as Animal Model

The pig is very similar to humans in terms of anatomy, genetics and physiology and can vary from miniature to large breeds. Choosing of the right breed and age allows various surgical and non-surgical procedures used in human medicine including catheterization, heart surgery, valve manipulation, endoscopy and broncho-alveolar lavages. These procedures are particularly difficult or impossible to perform in many animal models including rodents [Meurens et al., 2011]. The anatomy of the pig is comparable to human; for example the size of kidneys is very similar to human kidneys [Ibrahim et al., 2006]. Moreover, pigs have also multirenculate and multipapillate kidneys with true calices. In terms of genetics, the size and the composition of the porcine genome are comparable to those of humans [Hart et al., 2007]. Pigs are also remarkably similar to humans in terms of physiology. Both species are omnivorous and their organs generally share common functional features [Swindle et al., 2012]. Highlighting these similarities, pig-to-primate organ transplantation models are being used successfully [Cooper et al., 2016].

2.2.1 − Pig as Animal Model for Transplant

Study and comprehension of organ transplant biology need the employment of animal models, and several works have been realized organs transplant on different kind of animals, such as mouse [Skoskiewicz et la., 1973; Ge & Gong, 2011], rats [Zhu et al., 2009], swine [Utsugi et al., 2001], dogs [Mathews et al., 2000] and non-human primates [Vial et al., 2000]. For studies that involve analysis of Major Histocompatibility Complex (MHC) compatibility for organ transplantation, it is preferable to use mid-size animal models that share similarity to human, like pigs, instead of murine or rat models, even though the latter are easier to handle. For example, pigs have high percentages of neutrophils in the peripheral blood like humans (50-70%) in contrast to rodents [Fairbairn et al., 2011]. The swine immune system more closely resembles humans for more than 80% of analysed parameters, whereas mice were more similar to humans in less than 10% [Dawson, 2011]. Furthermore, rodents are not suitable for kidney transplant experiments and immune studies because, unlike pigs, they do not constitutively express class II MHC antigens on vascular renal endothelium [Natali et al., 1981]. Swine are considered as a suitable model for transplant biology studies also because MHC organization is well established and controlled. In particular, they were extensively used to evaluate MHC matching for tolerance or immunological activation against the graft [Madariaga et al., 2015; Loss et al., 2007]. The advantage of regular pigs is that growth rate and attainment of a proper weight is faster. Moreover, the creation of a new herd with well-defined characteristics in SLA (Swine Leukocyte Antigen) loci could be useful for many other applications than transplantation, i.e. studies on resistance to infectious diseases that is correlated to SLA.

Also there are few studies that have investigated the mechanisms of nephrotoxicity by CNI in animal models [Utsugi et al., 2001; Cibulskyte et al., 2005]. Most of the studies that employed small rodents have not been able to document the same damage from CNI highlighted in man; on the contrary, the pig is suitable as an animal model affine to the man thanks to the size of the organs and the similar cardiovascular and renal physiology [Bollen et al., 1997; Terris, 1986].

There are some differences between man and pig, in particular in the pharmacokinetics of the molecules with immunosuppressive action: Frey et al. have documented the need to employ doses of steroid and CNI up to six times more in the pig, either orally or intravenously, compared to man, to achieve the same blood levels and accordingly the same pharmacological action [Frey et al., 1988]. Finally it is to be considered in the

animal the presence of endemic and frequent infections in swine that can contribute to kidney damage especially in immunosuppressed subjects.

2.2.2 – Swine Leukocyte Antigen

Like previously said, in recent years is increased the interest in pigs as an ideal model for large animals [Lunney, 2007], especially as animals adapted to xenotransplantation of tissues or organs [Dehoux & Gianello, 2007]. Following these considerations, knowledge of the genetic background of the major histocompatibility complex (MHC) that contains the swine leukocyte antigens (SLA) is very important. The SLA genes, that encoding for the MHC molecules, are highly polymorphic [Smith et al., 1995]. This important evolutionary feature adapts to their indispensable role in the immune response of the pig [Potts & Slev, 1995], as it allows the MHC to recognize an almost unlimited number of non-self antigens. The complex of SLA genes is one of the most gene-dense regions of the pig genome and mapped on chromosome 7, spanning the centromere, and are divided into three regions: class I, class II and class III (figure 2a-2b).

Figure 2a: Comparative genomic organization of the human and swine major histocompatibility complex class I region. The swine map is based on fully sequenced haplotype Hp-1.1 [Renard et al., 2006]. Notwithstanding the presence of numerous class I genes, only SLA-1, SLA-2 and SLA-3 are functional and constitutively expressed. Figure from Lunney et al., 2009.
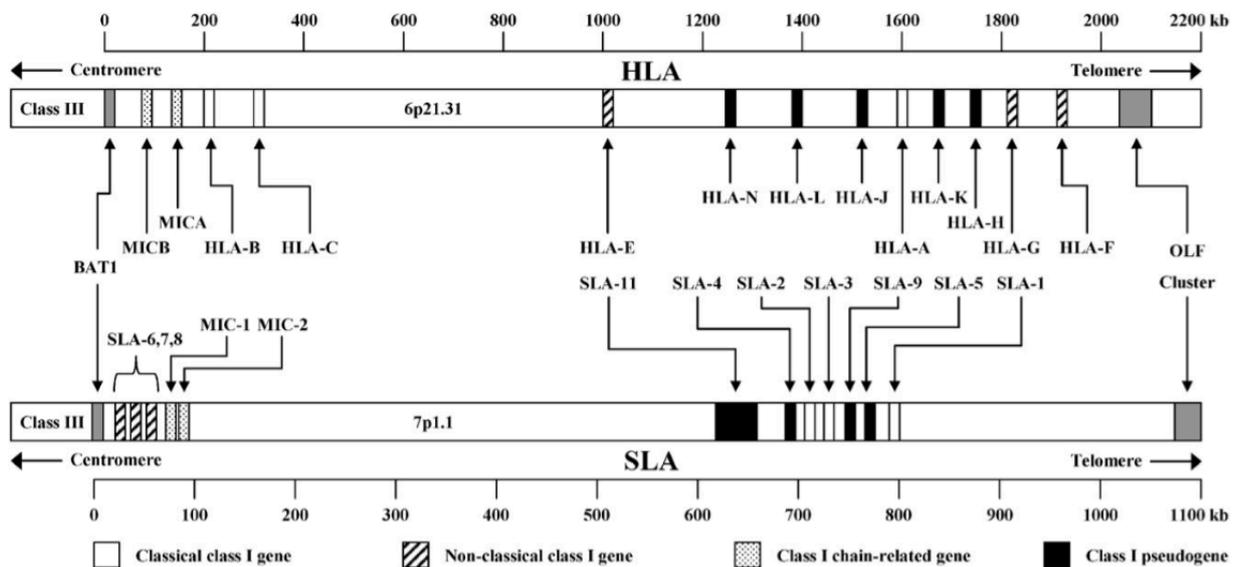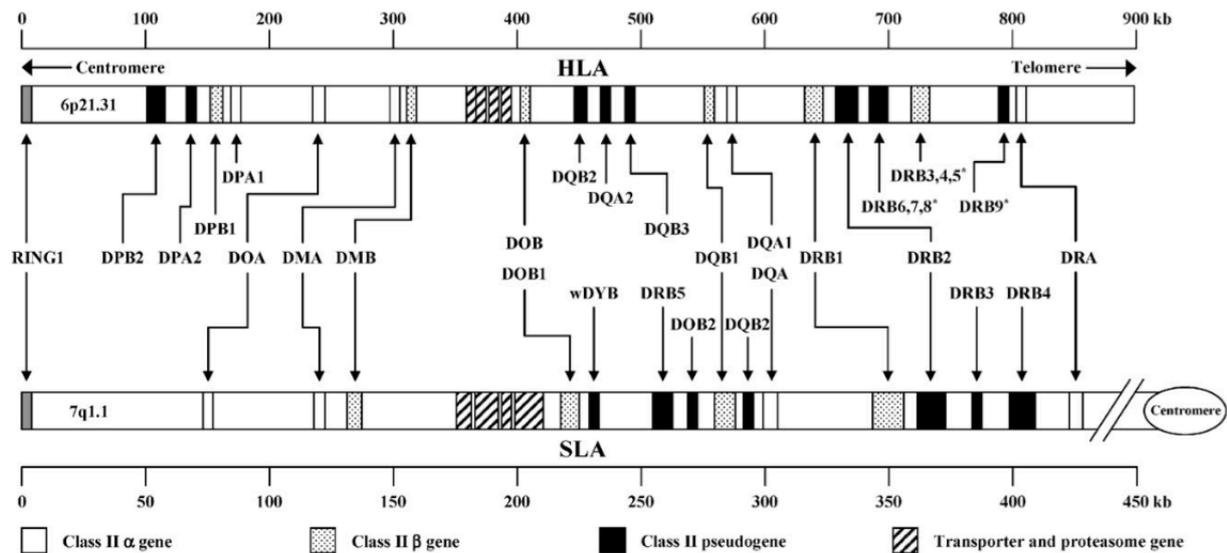
Figure 2b: Comparative genomic organization of the human and swine major histocompatibility complex class II region. The swine map is based on fully sequenced haplotype Hp-1.1 [Renard et al., 2006]. Figure from Lunney et al., 2009.

The functional class I genes are the constitutively expressed SLA-1, SLA-2 and SLA-3 genes, while others are pseudogenes [Lunney et al., 2009]. In fact, there is evidence that some SLA haplotypes possess a duplicate SLA-1 locus [Ho et al., 2009; Tanaka-Matsuda et al., 2009]. Classical SLA class I genes (the functional ones) consist of eight exons (figure 3): exon 1 encodes the leader sequence, exon 2–4 encode extracellular domains, exon 5 the transmembrane domain, and exon 6–8 the cytoplasmic domain [Satz et al., 1985]. The functional SLA class I genes code for 45 kDa transmembrane glycoprotein, consisting of three extracellular domains, $\alpha 1$, $\alpha 2$ and $\alpha 3$ [Blicher et al., 2005; Zhang et al., 2009]. This is the domain that plays the most important role in immune recognition. Precisely, the $\alpha 1$ and $\alpha 2$ domains form the peptide-binding groove, whereas the $\alpha 3$ domain is a binding site for the CD8 co-receptor. These proteins are constitutively expressed on the surface of all nucleated cells [Lunney et al., 2009].

The class II genes are discriminated in those for alpha and beta chains of antigens for the SLA-DR, -DQ, -DM, and -DO [Lunney et al., 2009]. There are several pseudogenes for the genes of the beta chain of SLA class II haplotypes, but have not been identified pseudogenes for DQB-1. The class II genes α chain and β chain have essentially the same molecular structure (figure 3) except for additional exons encoding the cytoplasmic domain [Gustafsson et al., 1990]. The expressed SLA class II antigens (DR an DQ) are found primarily on the surface of macrophages, B cells and dendritic cells [Chamorro et al., 2000; Summerfield et

15

al., 2003]. The SLA class II antigens are heterodimeric proteins, which consist of an α chain non-covalently bound to a β chain, and together form the peptide-binding groove.
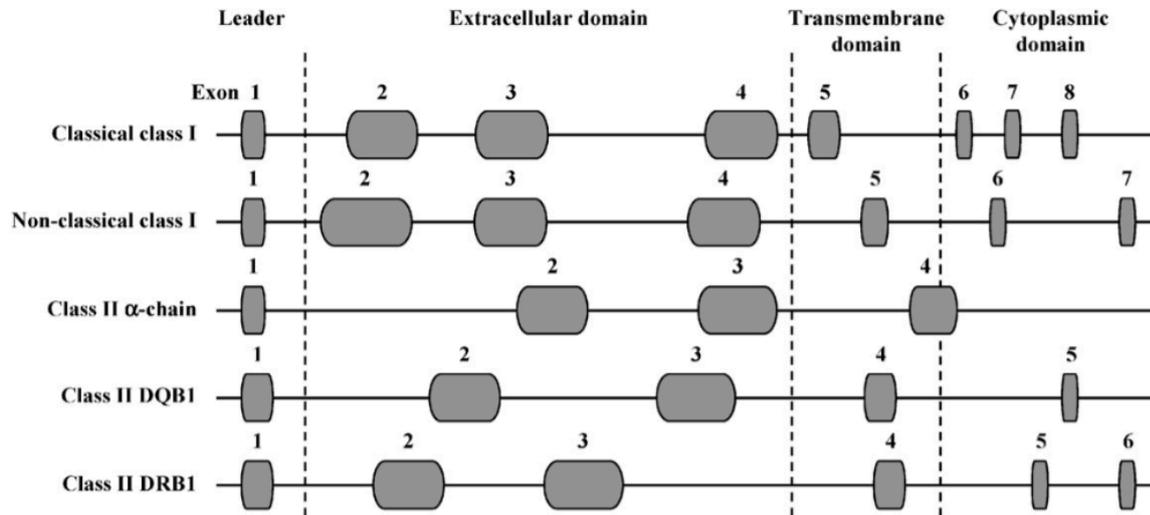
Figure 3: Schematic molecular organization of the SLA genes. Exons are represented by the grey ovals and introns by lines. Gene length is approximate based on fully sequenced haplotype Hp-1.1 [Renard et al., 2006]. Figure from Lunney et al., 2009.

2.2.3 – Xeno-recognition of SLA Molecules

The uses of pig xenografts for human transplantation have shown that there is a direct recognition of the SLA molecules from human cells. In particular SLA class II antigens are recognized directly by human T-cells [Yamada et al., 1995; Shishido et al., 1998], which may mediate the xenoimmune response. Natural killer cells [Sullivan et al., 1997] and CD8+ [Shishido et al., 1997] can recognize class I antigens and can lead to the killing of porcine cells and the destruction of the organ. In addition, the SLA protein can inhibit the cytotoxicity mediated by human natural killer cells [Kwiatkowski et al., 1999]. Studies of antibodies against SLA antigens have shown that can induce acute rejection, and the antibodies of the human leukocyte antigen (HLA) class I of sensitized patients cross-react with the antigens of the SLA class I [Naziruddin et al., 1998]. These xeno-recognitions suggest that there is high structural homology between the human and swine MHC proteins.

2.3 – Pig Blood Groups

There are 16 recognized porcine blood groups and the S gene that affects blood group AO expression [Andersen, 1962; Simon et al., 2003]. The blood group factors are defined by antisera derived from sensitized pigs, but five of the blood groups have at least one antigen that is not detectable with current antisera and thus may have a "blank" phenotype. Most of these blood group antigens are present only in erythrocytes, but there is evidence that some group antigens are distributed on other leukocytes or tissues [Hala, 1967]. The gene products have been identified for only four of the blood groups.

The pig is known to have AO blood group system, comparable with the ABO blood group system of humans, for which scientific community have focused his efforts, even for the interest in defining of blood group for xenograft studies. The major erythrocyte antigen is A substance which presence was confirmed in the 1950s and 1960s [Sprague, 1958; Rasmusen, 1964]. Also O groups have also been described [Andresen, 1962]. Pigs do not have a B blood group antigen. In pigs now we can recognize four phenotypes: A, A^w (weak A), O and blank. The O phenotype can react with anti-s antisera (equivalent to human anti-H substance), while lack of reactivity with either A or anti-s indicated the blank phenotype. Most A phenotyping is now done using monoclonal antibodies that are commercially available for human blood typing or using microtyping cards [Martínez-Alarcón et al., 2010], because of the similar structure of pig A substance to that of human A substance [Sako et al., 1990]. This method makes it possible to identify pigs with A phenotype but does not discriminate O or blank phenotype, even using available monoclonal anti-H antibodies.

Erythrocyte antigen A (EAA) alleles located on chromosome 1 are encoded by UDP-N-acetylgalactosamine transferase, which adds the terminal sugar to create pig A substance [Ellegren et al., 1994]. The blood groups O has a major deletion in EAA alleles, thus, none of the functional enzymes are produced [Yamamoto & Yamamoto, 2001]. Thanks to these knowledge and genetic information, Nguyen et al. developed a multiplex genotyping method for the porcine AO blood group gene [Nguyen et al., 2011]. This method is based on a multiplex PRC to screen gene exons to find the deletion carried by the O group allele (figure 4).
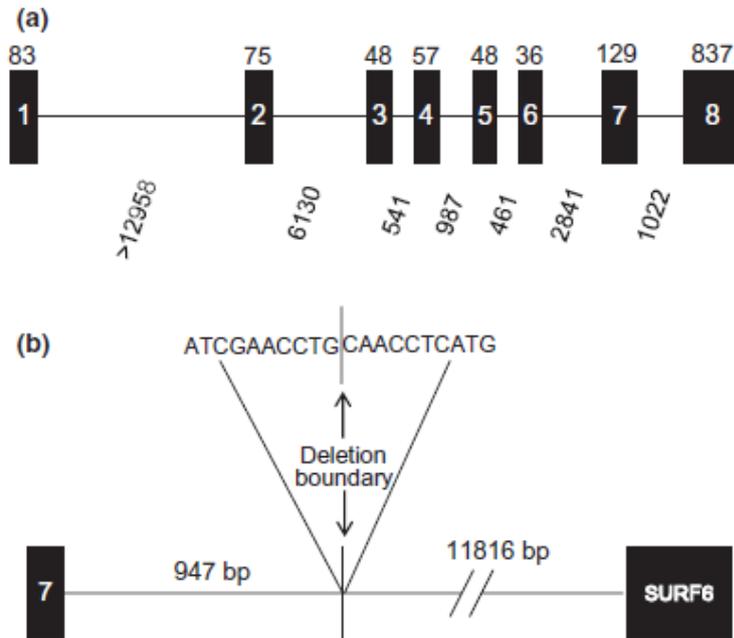
Figure 4: The exon/intron structure of the porcine AO blood group gene allele A (a) and characterization of the deletion junction involved in allele O (b). (a) The rectangles and horizontal lines indicate exons and introns, respectively. The numbers indicate the size (bp) of exons and introns. (b) Ten nucleotides each before and after the starting point of the deletion are shown. Figure from Nguyen et al., 2011.

2.4 – Exosomes

Many studies have focused their attention on some particular structures of cellular origin, called the exosomes, involved in the mechanisms of communication between cells. The exosomes are microparticles of endocytic origin that are released into the extracellular microenvironment by several cell types, like dendritic cells [Zitvogel et al 1998], macrophages [Théry et al., 2009], lymphocytes [Simons & Raposo, 2009], epithelial [Lin et al., 2005] and tumour cells [Wolfers et al., 2001]. They currently have been identified in many cell types in culture as well as in different biological fluids including blood [Caby et al., 2005], urine [Pisitkun et al., 2004], saliva [Ogawa et al., 2011], breast milk [Admyre et al., 2007] and cerebrospinal fluid [Vella et al., 2007]. This ubiquitous characteristic hypothesize that it is a physiological mechanism common to all cells. Due to their nature, they create a microenvironment inside them that represents only a portion of the cytosolic proteins and nucleic acids, a feature that makes them so interesting for studying.

2.4.1 − Exosomes Structure and Biogenesis

Exosome are approximately 30-100 nm in diameter and present a cup-shaped morphology at electron microscopy [Heijnen et al., 1999]. Their topology is the same as the cell from which originate, meaning that the outer side of the vesicle, from the point of view of structure and composition, corresponds to the outer side of the cell, while the internal corresponds to the cytosol. Exosomes contain many proteins some of which are common to all exosomes such as transport and fusion proteins (annexins, flotillin), the tetraspanins (CD9, CD24, CD63), heat shock protein (Hsp70, Hsp90), cytoskeletal proteins and proteins involved in the biogenesis of multivesicular bodies (Alix, TSG101) [Théry et al., 2001; Simons & Raposo, 2009]. Most of that are good markers for exosome detection, because expressed on multivesicular bodies but almost absent on plasmatic membrane. They also contain a number of proteins that are derived from the cytosol of the cell of origin. The lipid composition refers to lipid rafts [Subra et al., 2008], whereas the composition of nucleic acids is enrichment of microRNAs and mRNA [Valadi et al., 2007]. The DNA was found only in a few studies, as it notoriously is absent or present in small amounts in exosomes [Thakur et al., 2014].

The biogenesis of exosomes derived from endosomal maturation pathway [Lakkaraju et al., 2008]: starting from the mono-ubiquitinated membrane proteins, they are invaginated through vesicles called early endosomes. From here they mature in late endosomes that can come back to the plasma membrane, allowing the recycling of membranes, or join into other endosomes and undergo a further process of invagination, leading to the formation of multivesicular bodies. It's during this process that these vesicles may introduce in their lumen cytoplasmic components. Finally the multivesicular body may terminate in a lysosome, degrading its content, or fuse with the plasma membrane resulting in the release of the vesicles under the name of exosomes [Théry et al., 2002].
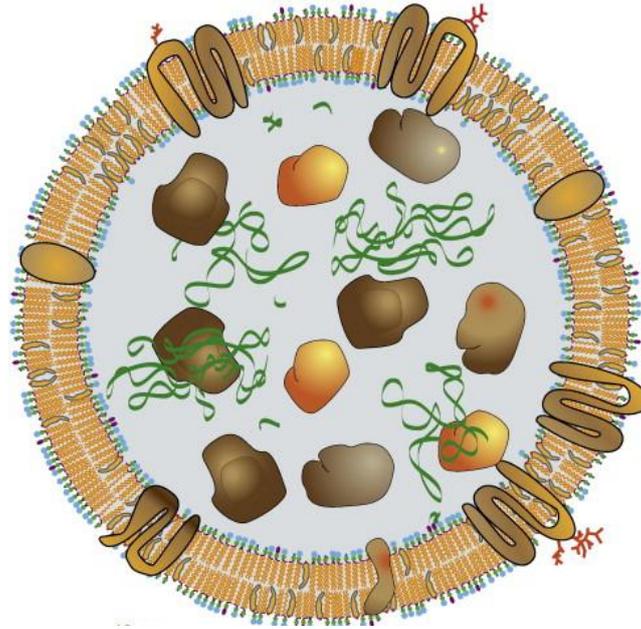
Figure 5: Simplification of the structure and composition of the exosome Inside there are proteins and nucleic acid. The membrane contain cholesterol and phospholipids and membrane proteins.

2.4.2 – Biological Function of Exosomes

The exosomes, following their release into the extracellular space, they can spread in the environment or in the microcirculation and act as active biological signals. Their biological functions ranging from regulating the immune response [Théry et al., 2009], to a role in apoptosis and angiogenesis [Janowska-Wieczorek et al., 2005], inflammation [van Niel et al., 2001; Prado et al., 2008], morphogenesis [Beckett et al., 2013] and coagulation [Heijnen et al., 1999], as well as in the spread of oncogenes by exosomes secreted by cancer cells [Parolini et al., 2009; Hood et al., 2011], or diffusing prions [Vella et al., 2007] and viruses from cell to cell [Pegtel et al., 2011]. All these features show that the predominant role of exosomes may be intercellular communication.

Thanks to these characteristics, exosomes appear to be a means of different communication compared to the more well-known methods, such as the autocrine, paracrine, endocrine, exocrine and synaptic, which are based purely on the interaction of single molecule-receptor. Because of his structure and composition, exosome allows the diffusion of a more complex message, in terms of quantity of information, due to its nature of biomolecules container, that allowing to obtain both specific and different targets, offering therefore more extensive response, both in the number of cells and type of response, such as growth, division, survival

and apoptosis. It has been demonstrated that exosomes play a role in the regulation of immune response; animal studies highlighted a role in tolerance induction through the decrease in the production of pro-inflammatory cytokines and in the proliferation of regulatory T-cells [Li et al., 2012]. Moreover, it seems that exosomes contain mRNA and miRNA involved in modulation of immune system [Montecalvo et al., 2012], as it was demonstrated that mRNA could be translated into proteins by target cells [Valadi et al., 2007] and miRNA can influence cells response [Mittelbrunn et al 2011; Montecalvo et al., 2012].

In recent years exosomes have aroused great interest in the scientific community, especially in regard to the search for biomarkers of pathological conditions; in particular in the field of clinical proteomics and miRNomics, where the search for biomarkers of disease in biological fluids, which is economical and effective for an early diagnosis of the disease, appears to be the desired goal. In this respect exosomes promise to be an excellent source thanks to the fact that they can give information about the cells from which they originate, thanks to their role as communication between cells, and the ability to analyse low abundance proteins that would otherwise be masked by the most numerous proteins present in bio-fluid from which they are derived. Furthermore, the obtaining of the bio-fluids often is performed by non-invasive or minimum invasive techniques to the patients, allowing greater comfort and less risk for them.

AIM OF THE STUDY

Main Project

The present study has set itself the aim of documenting, in an animal pig model, the histological damage by calcineurin inhibitors using incremental doses of Tacrolimus obtained in a limited interval of time, up to reach blood concentrations able to determine high toxicity. Furthermore the goal of the project is to evaluate the alterations in the immune system and the graft after kidney transplantation, through the analysis of classic soluble molecules present in the urine and plasma of SLA-defined pigs. Three treatment regimens will be set up: normal immunosuppression, high-dose and low-dose, in order to evaluate respectively the conditions of tolerance, drug-toxicity and transplant rejection.

Accessory Project

The pigs that underwent renal transplantation are also analysed in the exsosomal content to see some correlation to the inflammatory condition following the transplant and the Tacrolimus administration.

The second objective is the development of a controlled colony of pigs with a defined SLA genes (SLA-1, DQB-1), through the coupling of specific pathogen free pigs (SPF).

METHODS

4.1 - Study Design

4.1.1 − Treatment Schedules Design

The figure 6 summarizes the study design, which consist in three different treatment schedules (table 1) with Tacrolimus (TAC) performed on a total of 7 pigs (table 2), plus two pig kidney donors.

FEATURES OF PIGS

Sex	Female
Age	7 months
Mean body weight	79.6 ± 2.5 kg
Race	5 pigs purebred Large White (LW) and 2 hybrid Large White/Duroc (LW/D)

Table 1 −Features of pigs

TREATMENT SCHEDULES

Group A	Oral administration of TAC to a pig (A1) of LW breed, "not subject to renal transplantation"
Group B	Intravenous administration of TAC to two pigs (B1 and B2), both hybrids LW/D, "not following kidney transplantation"
Group C	Intravenous administration of TAC of 4 pigs (C1, C2, C3 and C4), LW breed, "kidneys transplant".

Table 2 − Treatment schedules

The animals defined as C1 and C2 have received grafts from genetically different pigs (LW/D) while the animals C3 and C4 from pigs genetically identical (LW), to have genetically different subjects, and then with greater biological complexity.
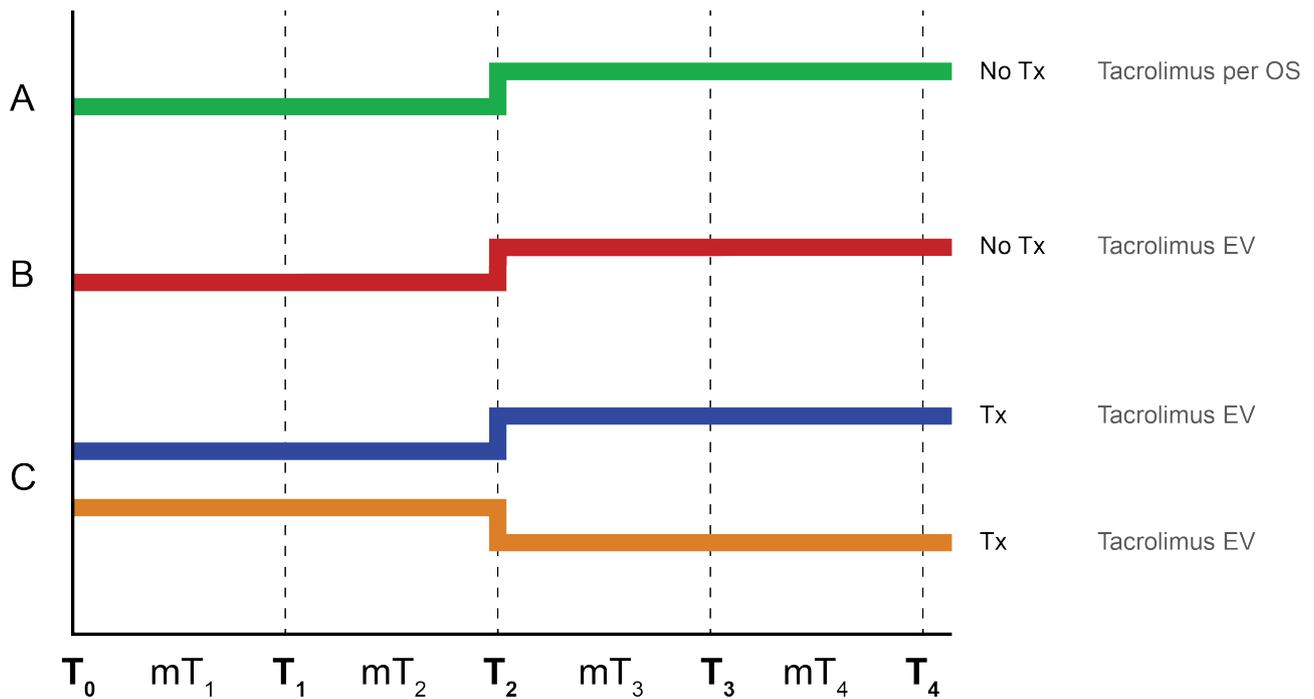
Figure 6 – Study design. Every group follow a therapeutic doses for the first two weeks. The last two weeks in the groups A, B and C-HD the dose of drug has been increased until reaching a toxic blood concentration of Tacrolimus. The group C-LD undergo a progressive reduction of the treatment up to the gradual suspension of the drug.

4.1.2 - Time Schedule Design

The study design, according to the scheme described above, includes the daily analysis of clinical chemistry parameters, the immunosuppressive state assessment using Cylex ImmunKnow procedure twice a week and histological examination of kidney tissues, at the beginning, at the 14th and 28th day (end of study) of the experimentation.

The study duration was 4 weeks and were considered the following times:

T0:   start of experimentation (drug administration);

T1:   7 days from the start of drug administration;

T2:   14 days from start of drug administration;

T3:   21 days from start of drug administration;

T4:   28 days from the start of drug administration / end of the study.

The weekly average values of the studied parameters have been calculated for each animal group:

MT1:   mean until T1: corresponds to the time interval between T0 and T1 (first week);

MT2:   mean until T2: interval between T1 and T2 (the second week);

MT3:   mean until T3 interval between T2 and T3 (third week);

MT4:   mean until T4: interval between T3 and T4 (fourth week).

In order to reach a growing blood Tacrolimus level we design a progressive administration of oral or intravenously immunosuppressive drug. For the groups A and B we wanted to reach toxic blood concentration of TAC over four weeks. For the group C we decided to use two different regimens: for each pair of pigs, the first two week was administered a immunosuppressive drug to reach the therapeutic range for immunosuppression. The following two weeks, in one pig the dose of drug has been increased until reaching a toxic blood concentration of TAC (Group C " High Dose" – HD). The other pigs undergo a progressive reduction of the treatment up to the gradual suspension of the drug (Group C " Low Dose" – LD).

The therapeutic range for adequate immunosuppression resulted between 30-40 ng/mL of Tacrolimus, as described by Utsugi and collaborators [Utsugi et al., 2001]. The toxicity was achieved by administration of the drug to higher levels.

Are considered **input** data:

1)   Tacrolimus administration (oral/ intravenously).

2)   Blood Tacrolimus level.

Are considered **output** data:

1) Blood and urine samples   Blood samples: haemoglobin (g/dL), white blood cells (10^3/mmc), blood creatinine (mg/dL); urine samples: urine proteins (mg/dL), urine sodium (mEq/L), urine magnesium (mEq/L) all compared with urine creatinine (mg/dL).

| 2) Lymphocytes | Evaluation of the CD4+ T lymphocytes activation for the determination of cell-mediated immunity with Cylex ImmuKnow test. |
| 3) Biopsies | Biopsies of native kidney and also on graft in group C. |

The blood samples and urine were performed daily and represented as average values in the MT1, MT2, MT3 and MT4.

The Cylex Test was performed 2 times per week. Biopsies of native kidney and graft were performed whenever possible at T0, T1 and T2, T4. The last biopsies coincided with the necropsy of the subjects and the sampling of tissues of interest. The collection of urine and blood for the extraction of the exosomal vesicles was performed 3 times per week.

4.2 - Surgical Technique

The pigs were catheterized to the outer right jugular vein for the intravenous infusion of the immunosuppressive drug and the left external jugular vein for blood sampling. This has prevented the contamination problems of the drug during the sampling. The urinary biochemical parameters were obtained through bladder catheter and ureter nephrostomy catheter in the transplanted pigs. The appropriate dose of intravenously Tacrolimus over the 24 hours, was administered using elastomeric pumps (Easypump 60 mL/h, B/Braun) (Prograf 5 mg/mL, infusion solution, Astellas) in the jugular vein. The test ended after 28 days with complete necropsy and sampling of kidney and other organ tissues with standardized methods.

4.3 - Pigs Therapy

Because of oral administration, for A1 pork it was not possible to reach toxic levels of blood Tacrolimus for the reduced compliance. This situation has arisen from the 3rd week.

In B and C groups pigs were permitted to reach toxic levels with intravenous administration of TAC. During the study the pigs were supported with fluid intermittent therapy at 1-2 mL/kg/h with sodium chloride 0.9% or ringer lactate solution, infused through the vascular access of the intravenous catheter. For the pigs of group C, the day before renal transplantation was administered antibiotic therapy based on ampicillin-

sulbactam intravenously. Anaesthetic therapy involved the use of ketamine, midazolam, eptadone, atropine, sodium pentothal. Assisted ventilation was performed with the animal paralyzed and isoflurane to 2-4% in air. The heat loss during the surgery has been countered by the use of heated mat and the utilization of the infusion liquid at body temperature. In the first week after transplantation was administered subcutaneously calciparine.

4.4 - Pathological and Clinical Investigations

For the blood count, serum and urinary clinical chemistry, were performed a serial of blood and urine samples. Urinary chemistry was performed after the urinary routine analysis, which includes the evaluation of the specific weight, chemical and physical investigation with dipstick and microscopic evaluation of the sediment.

The blood count was performed using the automated system SIEMENS ADVIA 2120 (manufacturer: ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown NY, USA), set for the swine. The examination was completed by an evaluation of the blood smear on a slide under a light microscope.

The chemical analysis were performed using the automated analyser Beckman Coulter/Olympus AU400 with dedicated reagents of the same manufacturer (manufacturer: AU 400, Olympus/Beckman Coulter, Brea, CA, USA) and using validated methods for the swine.

4.5 - Cylex ImmuKnow Test

To measure the activation of T lymphocyte we performed the Cylex ImmuKnow test that allows determining the intracellular quantity of ATP of lymphocytes. The test requires a whole blood sample of the animal treated with TAC to recover the lymphocytes. The sample remains intact for 30 hours at room temperature after sampling. Whole blood, collected into tubes containing heparin, is diluted 1:4 with 100 µL of Rosewell Park Memorial Institute Medium (RPMI). Sample was incubated in quadruplicate for 15-18 hours with or without 25 µL of phytohemagglutinin-L (PHA) 2.5 µg/mL in an incubator at 37°C and 5% CO_2. Subsequently, the CD4+ T cells stimulated and unstimulated were selected in the wells using magnetic beads coated with monoclonal antibodies (Dynabeads, Dynal, Oslo Norway) and a powerful magnet (Cylex Magnet Tray 1050, Cylex Inc. Columbia, MD). The conjugation of the monoclonal mouse anti-pig CD4

antibody and magnetic beads was performed using Dynabeads Antibody Coupling kit (Life Technologies, Dynal). After washing of the CD4+ lymphocytes, it was added a reagent able to lyse the cells in order to determine the release of intracellular ATP. Then, a mixture of luciferin/luciferase was added to the cell lysate, and after 30 minutes the level of bioluminescence was measured with a luminometer. The amount of emitted light (maximum emission at 562 nm) is compared with a calibration curve generated with ATP calibrators (0, 1, 10, 100, or 1000 ng/mL). The ATP concentration (ng/mL) in each sample is calculated from the calibration curve using an Excel program provided by Cylex. The level of the animal's immune response is assessed according to the amount of ATP produced.

4.6 - Monitoring of Blood Tacrolimus Level

The quantitative analysis of the TAC on blood samples collected on EDTA tubes was performed with an high performance liquid chromatography tandem mass (LC/MS/MS) as previously described [Meinitzer et al., 2010].

A sample of 100 µL of blood was deproteinized with 200 µL of a solution containing zinc-sulfate eptahydrate and methanol in ratio 1:4. The sample was centrifuged at 12.800 rcf for 12 minutes and 200 µL of supernatant was transferred into plate. Calibrators and controls (at known concentration) were treated in the same way. 15 µL of each sample was injected into the analytical system. The two-dimensional chromatography includes a first step of online sample purification on polymer column Poros R1/20, and a second step of chromatographic separation on reversed phase column Luna Phenyl-Hexyl 50x2.1. The analytes were ionized using electrospray positive ESI source by capturing NH_4^+ and identified based on their mass to charge ratio.

4.7 - Urine Collection

Urine was collected from catheterized non-transplanted pigs and transplanted pigs directly in a sterile container added with protease inhibitor mixture (P8340, Sigma-Aldrich). Immediately after the collection urine was centrifuged at 3000 rcf for 30 minutes at 4°C. The supernatant was saved and stored at -80°C.

4.8 - Isolation of Urinary Exosomes by Ultracentrifugation

Urine was thawed and centrifuged at 17.000 rcf for 20 minutes at room temperature to remove urinary sediment including whole cells, large membrane fragments and other debris. The supernatant was saved and the 17.000 rcf pellets were suspended in an isolation solution (250 mM sucrose, 10 mM triethanolamine, pH 7.6) followed by incubation with DTT (final concentration of 200 mg/mL) at 37°C for 5–10 minutes. When the pellets were completely solubilized it were centrifuged again at 17.000 rcf for 20 minutes at room temperature. The two supernatants from the 17.000 rcf spins were pooled and ultra-centrifuged at 200.000 rcf for 1 hour at room temperature (Beckman XL-70 ultracentrifuge; Beckman Coulter, Rotor: 70.1 Ti; Beckman Coulter). The exosomal fraction was washed with isolation solution and ultra-centrifuged again at 200.000 rcf for 1 hour at room temperature. Pellets were solubilized in 1:2000 of buffer depending on usage. For microscopy study the pellets were solubilized in PBS, for proteomics study were solubilized in lysis buffer (RIPA buffer or REB II buffer) with protease inhibitors, and then stored at -80°C.

4.9 - Isolation of Serum Exosomes by Ultracentrifugation

The serum was obtained from whole blood with a centrifugation at 3000 rcf for 15 minutes at 4°C. The serum was used fresh or stored at -80°C. The serum, fresh or thawed, was centrifuged at 1500 rcf for 15 minutes at 4°C to remove cell debris. The supernatant was saved and centrifuged at 17.000 rcf for 20 minutes at 4°C. The 17.000 supernatant was filtered with 0.22 µm filter. The filtrated solution was ultra-centrifuged at 200.000 rcf for 1 hour at 4°C (Beckman XL-70 ultracentrifuge; Beckman Coulter, Rotor: 70.1 Ti; Beckman Coulter). Pellets were washed with PBS and ultra-centrifuged again at 200.000 rcf for 1 hour at room temperature. Pellets were solubilized in 1:2000 of buffer depending on usage. For microscopy study the pellets were solubilized in PBS. For proteomics study the exosomal fraction was first solubilized in PBS and then albumin was depleted with affinity removal column (Agilent Human 14 Multiple Affinity Removal System Spin Cartridges) to remove the proteins co-precipitated with the exosome purification process. The depleted exosomes were concentrated with Agilent Concentrators Spin 5K MWCO in a minimum volume of PBS and then stored at -80°C.

4.10 - Electron Microscopy for Exosomes Identification

Exosomal pellets were solubilized in PBS and then quantified with Bradford Assay (Biorad). 10 µg of exosomal protein were applied to formvar-nickel grids for 60 minutes. After washing with PBS the sample was fixed with glutaraldehyde 2.5% for 10 minutes. After washing membranes underwent negative staining with 0.5% uranyl acetate and incubated for 15 minutes. After that the sample was air-dried for 5 minutes. After drying, the grids were examined with an electron microscope operated at 60 kV.

4.11 - Atomic Force Microscopy for Exosomes Identification

The Atomic Force Microscopy (AFM) analysis were performed with the atomic force microscope NT-MDT (Smena Nova, Moscow, Russia). The analysis were conducted in dry, semi-contact mode in the following conditions: scanning speed 0.5 Hz, NSG11 golden silicon probes (NT-MDT) with tips of diameter of 10 nm, the cantilever with the resonance frequency of 115-325 Hz. The images (512 x 512 pixels) have been developed with the software Gwiddion 2.17.

4.12 - Gel Electrophoresis and Western Blot

Urinary exosome pellets were solubilized in lysis buffer (RIPA buffer: 25 mM Tris-HCL pH 7.6, 150 mM NaCl, 1% NP40, 0,1% SDS) and then added Laemmli sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl pH 6.8). 30 µg of protein samples were separated by one-dimensional SDS–PAGE electrophoresis and then eventually stained with Colloidal Blue Staining (Biorad). Other gels were transferred to nitrocellulose membranes (Amersham Biosciences) for western blotting, which were blocked with TBS-Tween 0,1% in 5% milk blot (1 hour) and probed with antigen-specific primary antibodies overnight at 4°C: monoclonal antibodies to Heat-Shock Protein (Hsp70) (1:3000), CD9 (1:1000), policlonal antibodies to Acquaporin 2 (AQ2) (1:1000) (Thermo Scientific), CD63 (1:1000) (Gene-Tex). Finally the membrane was incubated with the secondary antibodies conjugated with horseradish peroxidase. The antibody-antigen reactions were visualized by using ECL plus western blotting detection system (Amersham Biosciences). The images were acquired with ChemiDoc Molecular Imager with Image Lab Software (Biorad).

4.13 - Proteomic Characterization of the Exosomal Content

Exosomal pellets were solubilized in lysis buffer (RIPA buffer: 25 mM Tris-HCL pH 7.6, 150 mM NaCl, 1% NP40, 0.1% SDS,) for 30 minutes at 4°C. The proteins were precipitated in 4 volumes of cold acetone (-20°C) and placed overnight at -20°C. Subsequently, the solution was centrifuged at 18.000 rcf for 20 minutes at 4°C, the pellet is dried with speedvac and re-suspended in a minimum volume of urea 8 M. After that, 50 µg of proteins was chemically reduced with DTT (Sigma-Aldrich) in 10 mM Ammonium Bicarbonate 100 mM (AMBIC) for 30 minutes at 56°C. The proteins are alkylated with 55 µM iodoacetamide (Fluka) in 100 mM AMBIC and then digested with 1 µg of trypsin in 1 mM HCl overnight at 37°C. Finally, the reaction is stopped with Formic Acid 5% and the proteins were dried with speedvac and stored at -80°C until mass analysis. Samples, solubilized in 10 µL of H_2O:acetonitrile:formic acid - 95:3:2 were diluted 1:5 with 0.1% TFA and centrifuged to remove insoluble components. 3 µL of each solution were analysed by LC-MS/MS with a QTOF Agilent 6510 equipped with an Agilent 1200 nanoHPLC (60 min gradient) and CHIP-Cube. The eight peptides more intense for scanning have been isolated and sequenced; mass/mass spectra obtained were analysed by database search with the software Mascot Server (v.2.4, Matrix Science, UK).

4.14 - OFFGEL

For isoelectric-point based peptide separation, the 3100 OFFGEL Fractionator and the OFFGEL Kit pH 3–10 (both Agilent Technologies) with a 12-well setup was used according to the protocol of the supplier. Ten min prior to sample loading, 12-cm-long IPG gel strips with a linear pH gradient ranging from 3 to 10 were rehydrated in the assembled device with 25 µL of focusing buffer per well. One hundred µg of the exosomal protein extract was diluted in focusing buffer to a final volume of 1.8 mL, and 150 µL of sample was loaded in each well. The sample was focused with a maximum current of 50 µA and typical voltages ranging from 500 to 4000 V until 50 kVh was reached after 12 h. The 12 recovered fractions were subjected to buffer exchange to remove glycerol by ultrafiltration in a centrifuge with Amicon (Millipore) with a 3 kDa filter. The buffer was replaced with milli-Q H_2O; the purification has required 4 h of centrifugation. The samples were reduced with 5 µL of DTT in 10 mM AMBIC 100 mM, at 57°C for 30 sec; alkylated with 5 µL of iodoacetamide in 55 mM AMBIC 100 mM; digested with 0.5 µg of trypsin, at 37°C over night. The samples was dried and re-suspended 30 µL of H_2O + 0.1% formic acid and analysed by LC-MS/MS.

4.15 - Luminex Assay

The first use of flow cytometry for analysis of microsphere immunoassay was published in 1997 (Fulton et al., 1997). More recently high-level multi-analyte profiling (xMAP) have been successfully developed to complement single-analyte assays. xMAP Technology enables multiplexing of biological tests. Systems using xMAP Technology perform discrete assays on the surface of color coded beads known as microspheres, which are then read in a compact analyser. Using multiple lasers or LEDs and high-speed digital-signal processors, the analyser reads multiplex assay simultaneously results by reporting the reactions occurring on each individual microsphere.

For the evaluation of the exosomal content was performed immune-analysis for recognition of 13 antigens (GM-CSF, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL -8, IL-10, IL-12, IL-18, TNF). Luminex assay was performed with Porcine Cytokine-Chemokine Magnetic Bead Panel (Millipore) for serum, adapted for exosomal matrix and reading the results using the Magpix (Luminex) system for reading. For each sample were loaded 30 μg of protein extract and normalized to this value.

4.16 - Pathological Anatomy

Biopsies obtained on native and transplant kidneys were studied by light microscopy (LM), electron microscopy (EM), immunofluorescence (IF) and immunohistochemistry (IHC).

4.16.1 - Light Microscopy

Samples for light microscopy were fixed in 10% buffered formalin for 24 hours, cut to automatic tissue processor and embedded in paraffin. The inclusion was cut with the microtome for the preparation of serial sections of 3 micron thickness for each sample. The sections were then stained with hematoxylin-eosin (EE), Periodic acid-Schiff (PAS) and Masson's trichrome stain. The preparations were observed under an optical microscope (Nikon Labophot-2). The evaluation of the vascular damage was done using the vascular score described by Karpinski et al. (1999) modified as follows: the arteries were evaluated observing the narrowing of the lumen, evaluating the grade: 0 - absent; 1 - "Increase wall thickness but to a degree that is less than the diameter of the lumen"; 2 - "That wall thickness is equal or slightly greater than the diameter of the lumen"; 3 - "That wall thickness to xceeds the diameter of the lumen, with extreme luminal narrowing or occlusion".

4.16.2 - Electron Microscopy:

The fresh samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and then in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2 for 1 hour at 4°C. The samples, dehydrated in alcohol, were included in epoxy resin (Araldite). By ultramicrotome were obtained semi-thin sections of 1 and 0.09 µm thick. Thin sections, counterstained with uranyl-acetate and lead-citrate were observed at the transmission electron microscope Zeiss EM 109.

4.16.3 - Immunofluorescence

The samples for the immunofluorescence was immediately frozen in OCT Compound (SAK 4583, Sakura Finetek Europe B.V.) in a plastic tray disposable Cryo-Mold, and stored at -80°C. The inclusion was then cut with a cryostat, with sections of 5 micron thickness, and then collected on charged slides. The sections was then incubated with fluorescent antisera directed against IgG, IgM, IgA, the complement factors of C3c and C1q and fibrinogen (Dako Polyclonal Rabbit Anti-Human IgG-AM-C3c Complement C1q-Complement-Fibrinogen-Kappa Light Chains-Lambda Light Chains/FITC). The dilution expected for all antibodies is 1:30 and the antibodies were diluted in PBS. Incubation lasts 1 hour and the slides are mounted in glycerin. The sections was then observed under a fluorescence microscope Nikon Eclipse 80i and acquired with the NIS-Elements BR Software 3.2.

4.16.4 - Immunohistochemistry

For formalin-fixed immunohistochemistry, sections of 3 µm of paraffin-embedded tissue have been cut, mounted on polarized slides, deparaffinized with diaphanous and gradually hydrated with increasing concentrations of alcohol. The endogenous peroxidase activity was blocked with 30 minutes of incubation with 3% H_2O_2 in methanol solution. The sections were washed with Tris Buffer and antigens were retrieved with Pronase-E 0,05%, pH 7.5 for 7 min at 37°C. The non-specific binding was blocked with incubation with 20% normal goat serum (NGS) and 1% BSA in PBS for 30 minutes at room temperature (IgG and IgM) and with Protein Block solution without serum for 15 min at room temperature (IgA). The slides were incubated over night at 4°C with a rabbit polyclonal antibody anti-IgM, diluted 1:30000 in 20% NGS and 1% BSA in Tris and with an antibody anti-IgG rabbit polyclonal, diluted 1:40000 in 20% NGS and 1% BSA

in Tris, and polyclonal anti-IgA goat, diluted 1:4000 in 1% BSA in Tris. All antibodies were made from Novus Biologicals. The slides were stained for 2 minutes at room temperature with 3,3-diaminobenzide tetrahydrochloride (DAB Chromogen/Substrate Kit Tablet format Cat. K 001, Diagnostic BioSystem, Pleasanton, CA). The sections were counter-stained with a Papanicolaou solution. Negative controls were performed in parallel, substituting the primary antibodies with 10% NGS in PBS. For positive controls were used pork sections of formalin-fixed, paraffin-embedded.

4.17 − SLA Typing

For the SLA typing we perform specific molecular techniques using the same pigs for the CNI experiments, and also a different pigs for the SLA homozygous haplotypes determination.

4.17.1 - Animals and Breeding for Homozygous Determination

The specific pathogen free (SPF) pigs herd has been maintained at IZSLER research laboratories. The animal was stabled at SPF condition, under the guidelines of Federation of Laboratory Animal Science Associations (FELASA) [Guillen, 2012]. In this study, a total of 31 pigs were evaluated for their SLA-1 and DQB-1 alleles. 15 SPF pigs were typed in order to select the ones with shared SLA haplotypes to generate offspring with some piglets homozygous. The mating was done using oestrus synchronization techniques and natural service. As a result were obtained three different offspring, called farrow 1 (F1), farrow 2 (F2) and farrow 3 (F3), from one boar and two different sows for a total of 16 piglets.

4.17.2 - SLA Typing by DNA Sequencing

The SLA typing was performed by direct sequencing of the exons 2 and 3 of the polymorphic regions of genes SLA-1 and DQB-1, coding for class I antigens and class II antigens respectively. PCR and sequencing primers were designed in the intronic conserved regions flanking exon 2 and 3 of SLA-1 and oDQB-1 genes using Primer3 software (http:/frodo.wi.mit.edu).

4.17.3 - DNA Extraction

DNA was extracted from whole blood by Blood Purification Kit using Maxwell 16 System (Promega Corporation, Madison, WI USA). The extracted DNA was quantified using the spectrophotometer Nanodrop 3.0.0 (Thermo Scientific). Each sample was normalized to a concentration of 30-50 ng/µL and stored at -20°C.

4.17.4 - PCR and DNA Sequencing

For each sample the fragments of interest were amplified by PCR reactions set up in a final volume of 25 µL using the AmpliTaq Gold Polymerase kit (Thermo Fisher) and GC-RICH PCR System (Roche) for GC rich sequences. The PCR were performed in the thermocycler GeneAmp PCR System 9700 (Applied Biosystem) with the annealing temperatures present in table 3.

The amplified fragments were assessed by gel electrophoresis in agarose and compared with two markers of different molecular weight. Subsequently, the amplified DNA was purified using the QIA quick PCR Purification Kit (250) (QIAGEN Inc. Valencia, CA). The concentration of the purified DNA fragments was assessed by 2% agarose gel electrophoresis by determining the optimum amount of amplified sequence for subsequent PCR. The PCR for DNA sequencing were set up in a final volume of 10 µL using the BigDye Terminator v1.1 Cycle Sequencing kit (ABI) and conducted through the thermal cycler GeneAmp PCR system 9700 with the following thermal profile: 96°C for 30 s, 25 cycles at 95°C for 10 s and 60°C for 3 min.

To avoid allele dropout and to be more accurate in the analysis the sequences we design two pair of primers for the exon 2 and 3 of SLA-1 because it's locus is highly polymorphic. The primer used for PCR were listed below (table 3):

Target regions	Sequences (5'-3')	Annealing temperature (°C)
SLA-1 Exon 2	F1: GGGCCCTTCTCCGACCT	62
	R1: CCCAAACTGAAAACGAAACC	
	F2: TTCTCCGACCTACACCCCTT	62
	R2: ACGAAACCGGGcGACAGTCC	
SLA-1 Exon 3	F1: CCGGTTTCGTTTTCAGTTTG	60
	R1: TGTCTTGTGGAGAAGCTGGA	
	F2: GTTTCGTTTTCAGTTTGGGTTG	60
	R2: TCCTCTCCgTGTGGGAAGGC	
DQB-1 Exon 2	F1: CGGGTTTCAGGTGGATGG	64
	R1: CAAGTCTTTCGCCTGCTGTG	
DQB-1 Exon 3	F1: GTCCAGGGATCTTTCCACCT	62
	R1: CTGATGCCCTTGCTATGAGG	

Table 3 – Primers and target regions for the SLA typing.

4.17.5 - Sequences Analysis

The sequence of PCR were purified with Millipore kit plate and loaded onto 48 capillaries automated sequencer 3730 DNA Analyser (Applied Biosystems). The sequences were aligned and compared to reference alleles available on IPD-MHC SLA sequence database (https://www.ebi.ac.uk/ipd/mhc/sla/) [Robinson et al., 2005]. Alignments were performed using Sequencer software (Gene Codes corp., Ann Arbor, MI), amino acid sequences were deduced from the nucleotide sequence using Sequencer translation utility.

4.18 – Blood Group Typing

The blood groups of these animals were determined by traditional serological method involving typing microcards for human anti-A and anti-B antibodies. For the PCR analysis, genomic DNA was isolated from peripheral blood samples and extracted by Blood Purification Kit using Maxwell 16 System (Promega Corporation, Madison, WI USA). The extracted DNA was quantified using the spectrophotometer Nanodrop 3.0.0 (Thermo Scientific). PCR amplification was performed in a 25 µL reaction containing 50-

100 ng of DNA using AmpliTaq Gold Polymerase kit (Thermo Fisher). The multiplex PCR conditions and primers was used as described by Nguyen and collaborators [Nguyen et al., 2011]: PCR had an initial denaturation at 95°C for 3 min followed by 35 cycles of 1 min at 94°C, 1 min annealing at specific temperatures for each set of primers, and extension at 72°C. We use only the primer for recognize the exon 8 and the intron 7 deletion, used together, for discriminate the A group from O group. The primer used for PCR were listed below (table 4):

PRIMERS FOR AO TYPING

Target regions	Sequences (5'-3')	Annealing temperature (°C)
Exon 8	F: CGCCAGTCCTTCACCTACGAAC	66
	R: CGGTTCCGAATCTCTGCGTG	
Intron 7 deletion junction	F: AATGTCCTTATGCTGGCCTGG	66
	R: AACAACACACTCCTGAACAACAGA	

Table 4 – Target regions of the primer were the pig AO blood group gene.

RESULTS

Main Project

5.1 - Clinical Parameters Results

The trend of the Tacrolimus is maintained below 60 ng/mL in the first two weeks of treatment (figure 7, table 5). The assumption of the oral drug is characterized by a slower achievement of the target level of blood Tacrolimus, that is realized in mT2 onwards.

In all other cases the therapeutic target is achieved in mT1. These concentrations are changed from the third week highlighting a correlation with the dose administered. In particular, the B group and the C-HD group reach toxic levels ≥ 80.0 ng/mL. Otherwise, the A and the C-LD groups, after a phase of maintenance of therapeutic levels of TAC, shown not more therapeutic concentrations in mT4. This was clear in C-LD group, that was underwent a progressive reduction of the treatment up to the gradual suspension of the drug.
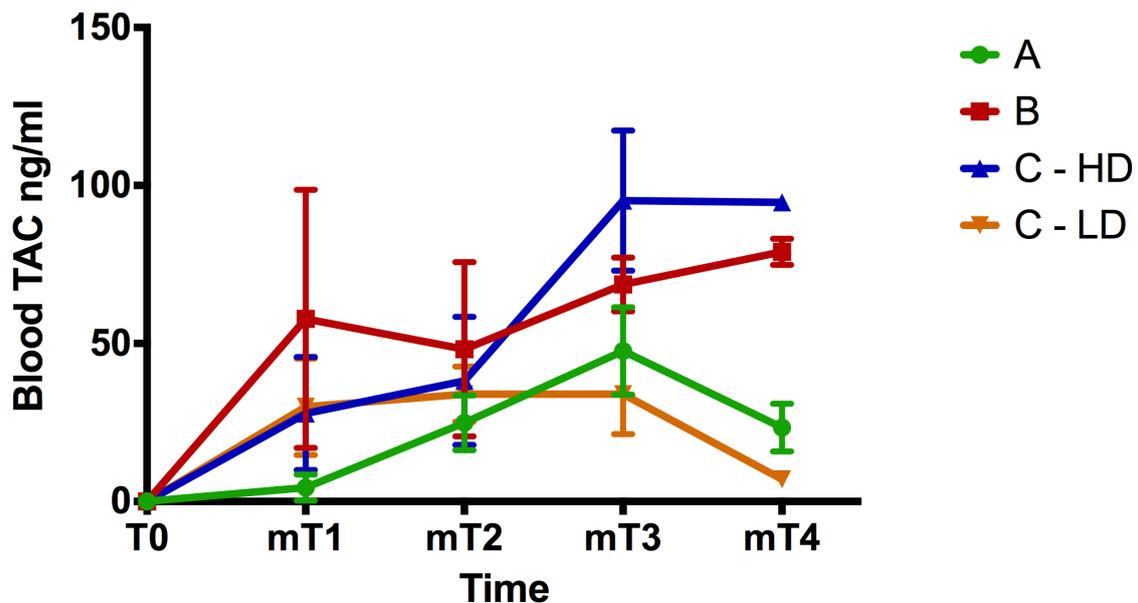
Figure 7 − Average of Tacrolimus administered in the time intervals in the different groups.

	Tacrolimus Administered (mg/kg/die)				
	T0	mT1	mT2	mT3	mT4
A	0,00	0,60	1,21	1,83	1,25
B	0,00	0,19	0,18	0,24	0,28
C – HD	0,00	0,14	0,18	0,28	0,38
C – LD	0,00	0,14	0,18	0,07	0,01

Table 5 – Average of Tacrolimus dose administered in the time intervals in the different groups.

The trend of renal function expressed as serum creatinine (figure 8) in the four groups of animals is within the normal values until the time mT2, and then worsen, that is more highlights in groups B and C-LD in the two weeks of a high dose. Specifically, the B group, which reached the highest Tacrolimus dosages, develops the most kidney damage with higher creatinine values than the other groups; C-HD group shows a similar pattern but with creatinine values smaller in mT4. It should be considered that such functional deviations in the latter group are made in the presence of native kidney kept in place.
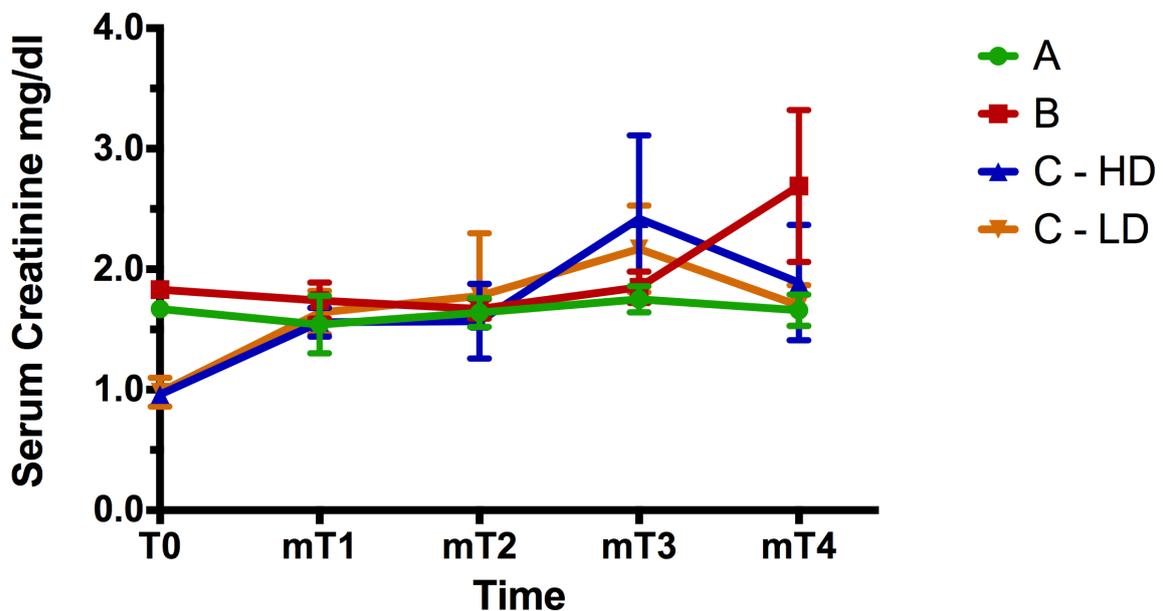
Figure 8 – Average amount of plasma creatinine observed in the time intervals in the different groups.

The trend of urine creatinine (figure 9), for a high dose groups, appears specular to that of the serum creatinine, showing in all treated animals a reduction of the values in relation to creatinine.
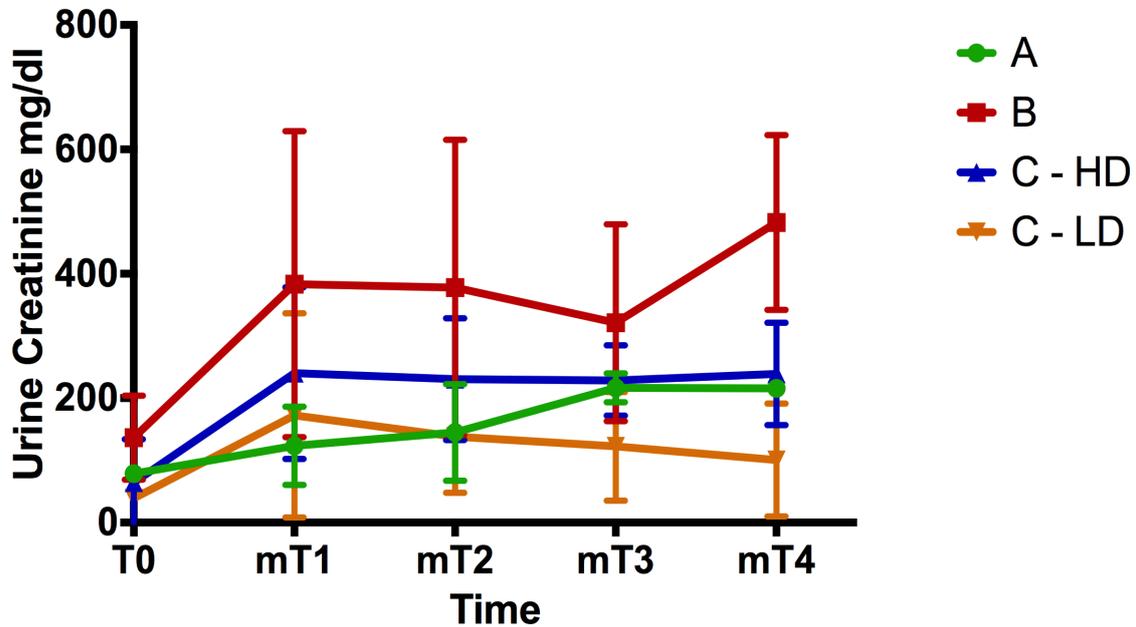
Figure 9 – Average amount of urinary creatinine observed in the time intervals in the different groups.

We represented the value of urine sodium and urine magnesium as ratio to urine creatinine to normalize the value to the excretory rate. The urine sodium (figure 10) appears more related to the stress of surgery than to the drug levels of the Tacrolimus. Moving away from T0 time, in mT2, there was greater urine sodium in C-LD group until it returns to normal level in mT4. Except for this outline value, the quantity of urine sodium showed no significant difference during the trial.

The same considerations can be made for the urine magnesium (figure 11), considered a marker of tubular damage, which may as a result for Tacrolimus toxicity damage. Despite the tendency to a decrease of the amount in the last weeks, there are no significant differences.

The level of serum sodium and serum magnesium showed no difference during the trial (data not show).
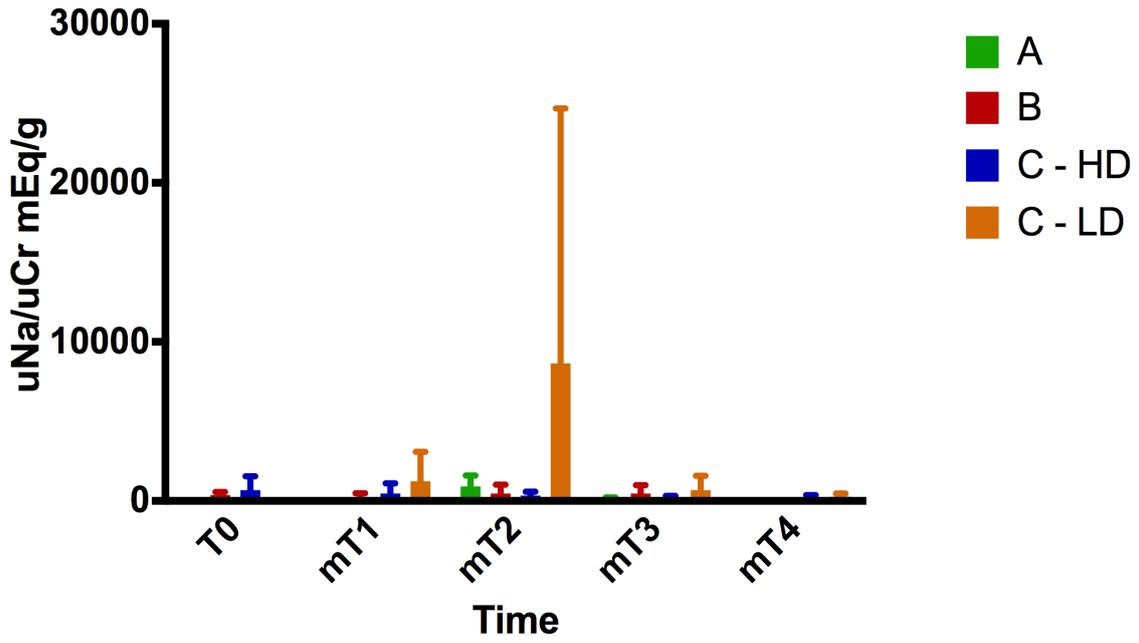
Figure 10 – Average of the ratio of urine sodium/urine creatinine seen in the time intervals in different groups.
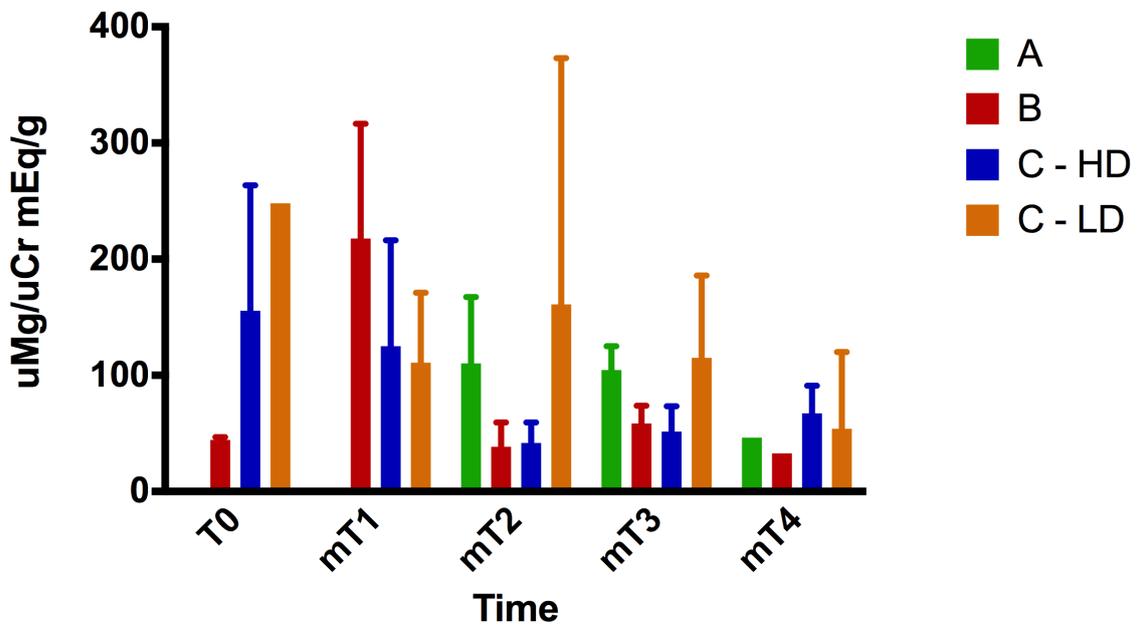
Figure 11 – Average of the ratio of urine magnesium/urine creatinine seen in the time intervals in different groups.

We wanted to observe specifically the trend of Group C, consisting of 4 transplanted pigs and specifically the different functional contribution of the native kidney and transplanted kidney. These pigs underwent a progressive administration of immunosuppressive drug injected intravenously to reach the therapeutic range of immunosuppression in the first two weeks. The following two weeks, in one pig the dose of drug has been increased until reaching a toxic blood concentration of Tacrolimus. The other pigs underwent a progressive reduction of the treatment up to the gradual suspension of the drug. The renal function, expressed with urine creatinine, is mainly supported by the native kidney (figure 12). This data is reflected in the measurement of diuresis in the early post-transplant days, that it is produced by the native kidney.

In the later stages, for the same given fluid therapy, this value differs between the two groups: in C-HD group remains a decreased excretion of urine; otherwise, in the C-LD group we saw a recovery in urine output (figure 13).
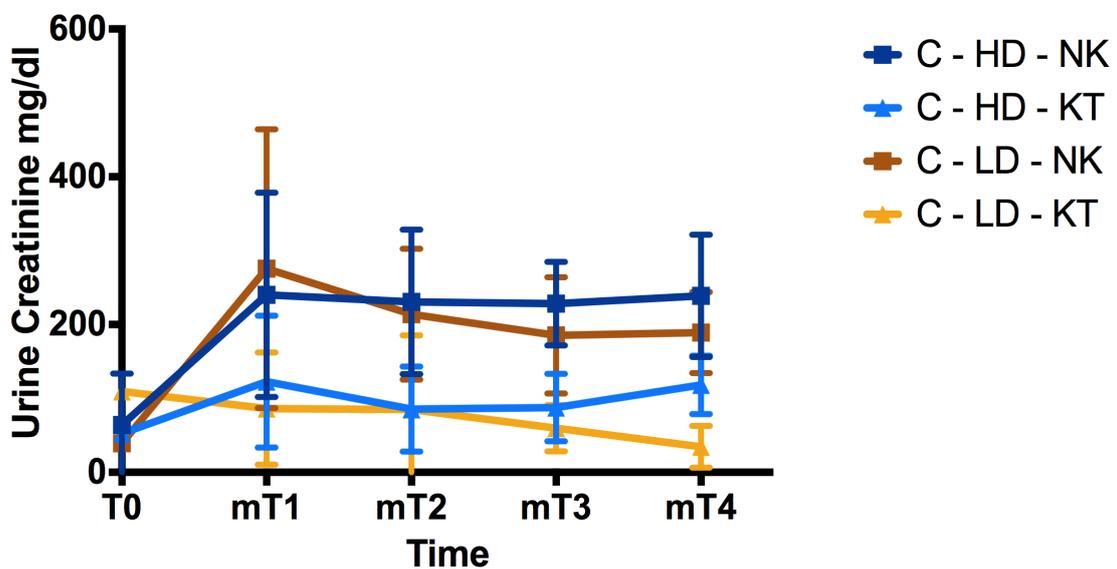
Figure 12 – Average amount of urine creatinine observed in the time intervals in native and transplanted kidneys of pigs undergoing kidney transplantation.
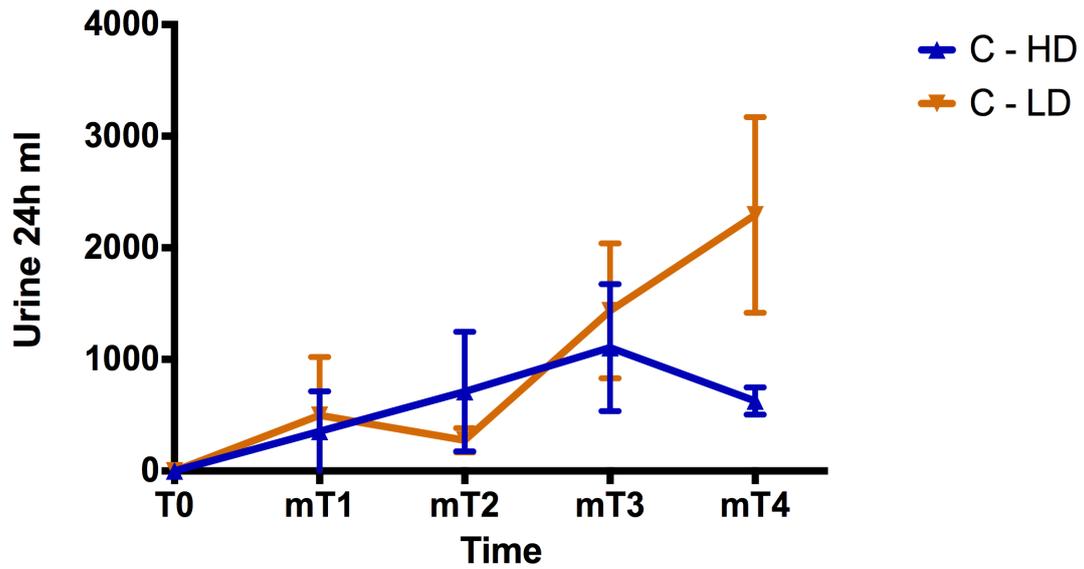
Figure 13 – Average amount of urine output of 24 hours observed in the time intervals in pigs undergoing kidney transplantation.

The trend of the ATP levels measured by the Cylex ImmuKnow test shows an inverse correlation between blood levels of Tacrolimus and activation of CD4+ lymphocytes. All the groups who have suffered an increase of the dose of the immunosuppressive drug have consistently ended the trial with lymphocyte activation values lower than the beginning of experimentation. The C-LD group, subjected to reduced Tacrolimus dosages, shows ATP levels similar to the beginning of the therapy (figure 14, table 6).
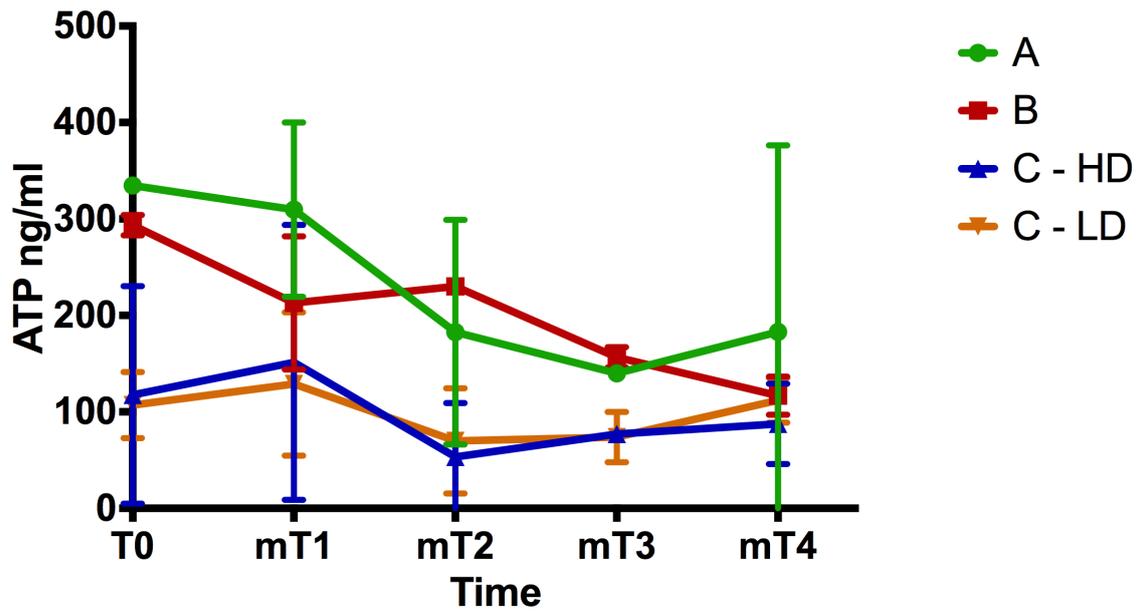
	CILEX (ATP ng/ml)				
	T0	mT1	mT2	mT3	mT4
A	335	310	183	140	183
B	294	213	230	157	117
C – HD	118	151	53	77	88
C – LD	107	129	70	74	112

Figure 14 - Table 6 – Cylex ImmuKnow test. Average amount of ATP produced by lymphocytes, observed in the time intervals in the different groups.

5.2 – Pathological Anatomy Results

5.2.1 - Light and Electron Microscopy

The images of the histological slides are shown at the end of this chapter.

Group A

At the time T2 in the renal biopsy is present multifocal atrophy of glomeruli and mild mesangial proliferative glomerulonephritis. In the epithelium of the tubules there is acute cellular swelling, mild interstitial fibrosis, lymphoplasmacytic interstitial nephritis and mild histiocytic interstitial nephritis. Also we see a multifocal proliferation of capillaries and arterioles.

At necropsy (T4), in the kidney is present a glomerular multifocal atrophy and mild membranoproliferative glomerulonephritis, acute cellular swelling of the epithelium of the tubules, multifocal and moderate histiocytic lymphoplasmacytic interstitial nephritis. There is also mild fibrosis and edema around the arteries.

Group B

The anatomical and pathological results of the two subjects are shown together as they are very similar. At T0 in the renal biopsy there is mild membranoproliferative glomerulonephritis, and glomerular atrophy. There is mild and acute diffuse epithelial cell swelling of the tubules, but the vessels were normal. There is marked marrow fibrosis in a subject.

At the time T2 in the renal biopsy there is multifocal atrophy of the glomeruli and mild membranoproliferative glomerulonephritis, mild diffuse lymphoplasmacytic interstitial nephritis and acute tubular epithelial cell swelling; arterial vessels have a score vessel 2 in a subject and another 3 in which there is also marked and multifocal proliferation of capillaries.

At the time T4 was performed the necropsy, the kidney present membranoproliferative glomerulonephritis, multifocal glomerular atrophy, acute tubular epithelial cell swelling, mild multifocal lymphoplasmacytic interstitial nephritis and interstitial fibrosis. There was an increase in the number of arteries and arterioles, score 3 of vascular damage. In other organ, vascular damage it was marked with a grade 3 in the spleen, bladder and heart, and a grade 2 in the lung arteries.

Group C

The histological findings of the four subjects (two treaties with high-dose and two at low doses) are shown together as they are very similar.

In the native kidney biopsy at T0 are some not fully mature glomeruli, mild lymphoplasmacytic interstitial nephritis and mild multifocal fibrosis with normal arterioles. In the transplanted kidney there is a slight tubular dilation with normal arterioles.

In the native kidney biopsy at T0 there is multifocal and mild glomerular atrophy, mild membranoproliferative glomerulonephritis, moderate ectasia of the proximal tubules and multifocal cellular degeneration in hyaline droplets. Mild interstitial fibrosis and mild fibrosis around the small arteries, arterioles were normal.

In the transplanted kidney biopsy is observed multifocal glomerular atrophy, mild membranoproliferative glomerulonephritis, lymphoplasmacytic interstitial nephritis, mild interstitial fibrosis, tubular ectasia with moderate presence of hyaline droplets. Severe perinephric fibrosis and mild perivascular fibrosis.

In the native kidney at T2 there is mild glomerulonephritis membranoproliferative, mild lymphoplasmacytic interstitial nephritis, mild and multifocal fibrosis and arteries is normal. In kidney transplant biopsy there is mild membranoproliferative glomerulonephritis, interstitial fibrosis, severe interstitial nephritis with multifocal lymphoplasmacytic fibrosis around the arterial vessels. The vessels were normal. In a pig treated with low doses there is a vascular damage of grade 1.

At T4 necropsy was performed, in the native kidney there is mild membranoproliferative glomerulonephritis, mild multifocal lymphoplasmacytic interstitial nephritis. The vascular damage is estimated grade 3 in the two subjects treated at high doses and in grade 1 and 2 in 2 patients treated with low doses. In the transplanted kidney there is always a slight membranoproliferative glomerulonephritis, moderate multifocal lymphoplasmacytic interstitial nephritis, vascular damage of grade 3 with perivascular edema (in 3 subjects), and grade 2 (in one of two subjects treated in low doses). In the other organs examined, vascular damage ranged from 2 in the lymph node, ureter, lung, and grade 3 in the myocardium and bladder.

5.2.2 - Periodic Acid-Schiff and Masson's Trichrome Staining

Periodic Acid-Schiff (PAS) and Masson trichrome special stains were performed on all the kidney samples. The PAS staining always shown a slight thickening of the glomerular basement membrane, and Masson's trichrome staining shown the presence of interstitial fibrosis, both perivascular and capsular.

5.2.3 - Immunohistochemistry

The immunohistochemistry was performed only for subjects of group C. At time T2, the native and transplanted kidneys show a multifocal and segmental glomerular positivity for anti-IgG and anti-IgA antibody. The positivity for anti-IgM antibody is segmental and diffuse in the glomeruli. At necropsy the positivity is much reduced or absent for IgA and IgG; for the anti-IgM antibody they are positive in the glomeruli and in the vessel, but only in transplanted kidneys.

5.3 - Clinical Aspects

In all the animals that were given incremental dose of the drug, corresponding to an increase in the plasma concentration of Tacrolimus, there has been the appearance of clinical symptoms initially nonspecific (fatigue, loss of appetite) then more evident, with increasing plasma concentration of drug. In particular, at levels of blood Tacrolimus 10.4 ng/mL appeared weakness, fatigue, loss of appetite; at plasma levels of 32.5 ng/mL tremors, hyperthermia and diarrhoea. In group A, starting from the third week, these symptoms led to the rejection of the meal containing the drug. All the animals have been supported with fluid therapy and in case of hyperthermia was administered antibiotic.

5.4 − Blood Group Typing

The blood typing was a fundamental step for the correct transplant study. Thanks to the discovery of a deletion of exon 8 in AO blood group gene in pigs that have the O allele, it was possible to set up a multiplex PCR for typing of blood AO group. We used this technique in addition to the usual blood group typing by microcards to have reliable results.

In figure 15 shows some screened pigs before transplantation in order to identify the blood group. For this screening a multiplex PCR was performed using both primer pairs to identify both the allele A and allele O.

The results was compared with the ones of classical serological typing using microcard, which is based on the human ABO system, but, because the allele B was absent in swine, the kit only determines the presence of the A or its lack; although sometimes it is positive for the B allele, pointing out the fact that it is not a completely reliable tool for typing. The result shows that in some pigs there are inconsistencies: in the pig-2 and pig-15 the kit showed a blank allele while typing with PCR showed an A allele. The rest of the PCR data are consistent with kit, but not show the presence of heterozygous.

	1	2	3	4	5	M	6	7	8	9	10	M	11	12	13	14	15	16	17
KIT	A	/	A	/	/		/	/	A	A	A		/	A	A	A	/	A	A
PCR	A	A	A	/	/		/	/	A	A	A		/	A	A	A	A	A	A

← Allele O

← Allele A
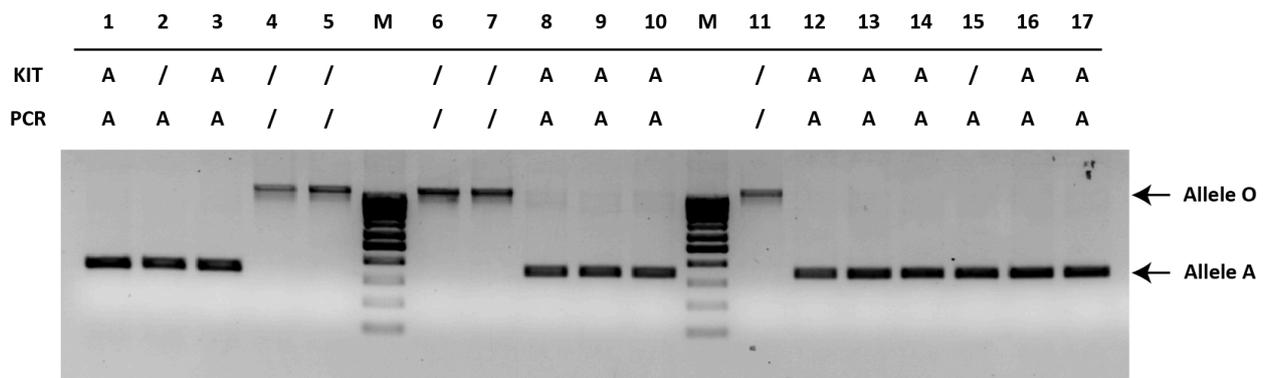
Figure 15 – The representative genotyping results of multiplex PCR for the porcine AO blood group gene. The serological typing results are indicated in line named "kit", the genotyping results are indicated in line named "PCR". The band representing allele A was obtained with exon 8 primers, resulting in ~340 bp band, for the allele O the band was obtained with intron 7 primers resulting in ~1070 bp band.

To ensure that the result was not an artefact, the PCR was repeated for some of the pigs, using the two pairs of primers not together (Figure 16), but instead performing two single PCR. We choose some pigs that in the multiplex PCR show only allele A (pigs 8, 10 and 16), some that shows only allele O (pig 4), and finally the two that are inconsistent with the kit results (pigs 2 and 15). The result shows first of all that the multiplex PCR not occurred correctly, because some pigs that in the multiplex PCR showing only the A allele (pigs 8, 10, 16), in this single PCR show both alleles (heterozygosity). Other pig shows only the O allele (pig 4) confirming his homozygosity. Finally, the pigs that gave inconsistencies with the kit results (pigs 2 and 16) were confirmed as heterozygotes, thus showing a different result than that of microcard. No pigs have been found homozygous for the allele A.
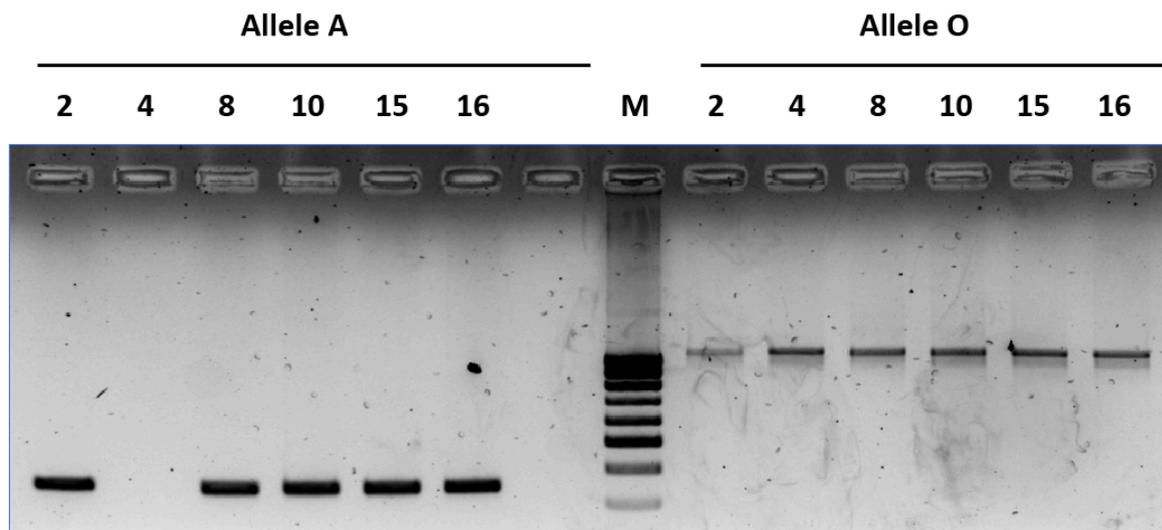
Figure 16 – Two single PCR for the porcine AO blood group gene. The left part of the gel is the PCR done with exon 8 primers, the right part of the gel is the PCR done with intron 7 primers.

Accessory Project

5.5 - Exosomes Results

5.5.1- Exosomes Purification

It was developed a method for the extraction/purification of exosomes from pig urine and serum samples. The most suitable protocol to isolate vesicles from the urine is the ultracentrifuge, as it results in a higher yield and purity, in acceptable time duration. The development of the method of urine exosomes purification was based on the depletion of uromodulin or Tamm-Horsfall protein (THP) of about 90 kDa, which is the most abundant soluble protein in the urine, which interferes with the proteomic methods for the identification of exosomal proteins [Fernández-Llama et al., 2010]. Its polymerization can trap the urinary exosomes, but with heat treatment and the reducing agents (DTT), exosome vesicles can be released in solution and thus to an increase in the concentration of proteins revealed.

The optimal treatment found for the release of exosomes was that which provides the incubation of the pellet obtained after centrifugation at 17.000 rcf, with DTT at 37°C for 10 minutes (figure 17).
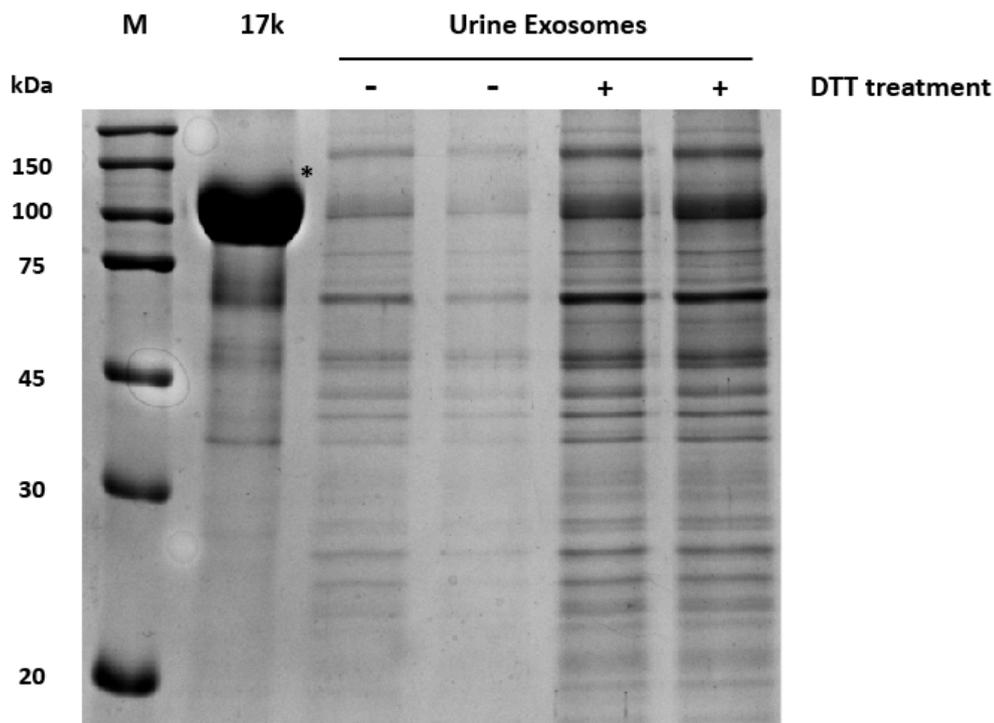
Figure 17 – SDS-PAGE with Comassie Blue Stain. In 17K lane are loaded the proteins from the 17.000 rfc pellet without the DTT treatment, almost all of the proteins is the THP, marked with the asterisk. The urine exosome protein contents was loaded twice both for the exosomes isolated with or without the treatment with DTT at 37°C.

To develop a method of purification from the serum exosomes we focused on the depletion of albumin, the most abundant soluble protein in the serum, using different kinds of columns for the depletion of albumin and of the most abundant proteins in serum-plasma. The kit that have given the best result for the pig serum were the Agilent Human 14 Multiple Affinity Removal System Spin Cartridges (figure 18).
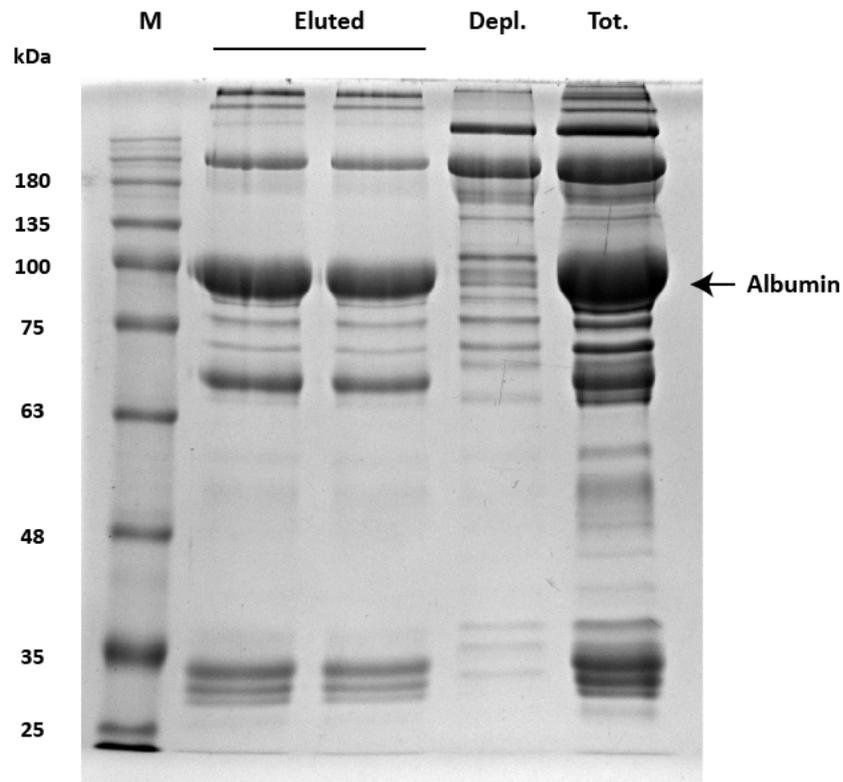
Figure 18 – SDS-PAGE with Comassie Blue Stain, serum exosomes purificated with an albumin depletion kit. The first two lanes refer to the proteins kept in the purification column and then eluted. Most of the albumin was found there. The "depl." lane refers to the same exosomes sample after the depletion with the kit. "Tot." lane refer to a total protein content of serum exosomes without the depletion of the most abundant proteins.

To date there is no single protein marker that identifies exosomes and distinguishes them from other larger vesicles, such as apoptotic bodies, the endoplasmic reticulum vesicles or other contaminants. This is why we need to use different methods to characterize exosomal vesicles.

5.5.2 - Exosomes Microscopy

Electron microscopy and AFM are precious tools to verify the purity of the sample, the size and morphology of the identified vesicles. The exosomes observed by electron microscopy show a typical spheroidal morphology, with diameter of about 30-100 nm (figure 19 A-B). Additional details on the morphology and size of exosomes were obtained by the AFM. Dimensional analysis by line analysis revealed the existence of two types of vesicles with different sizes: a smaller with height of 10 nm and width of 80 nm, a larger with 16 nm and width of 120 nm (Figure 19 C-D).
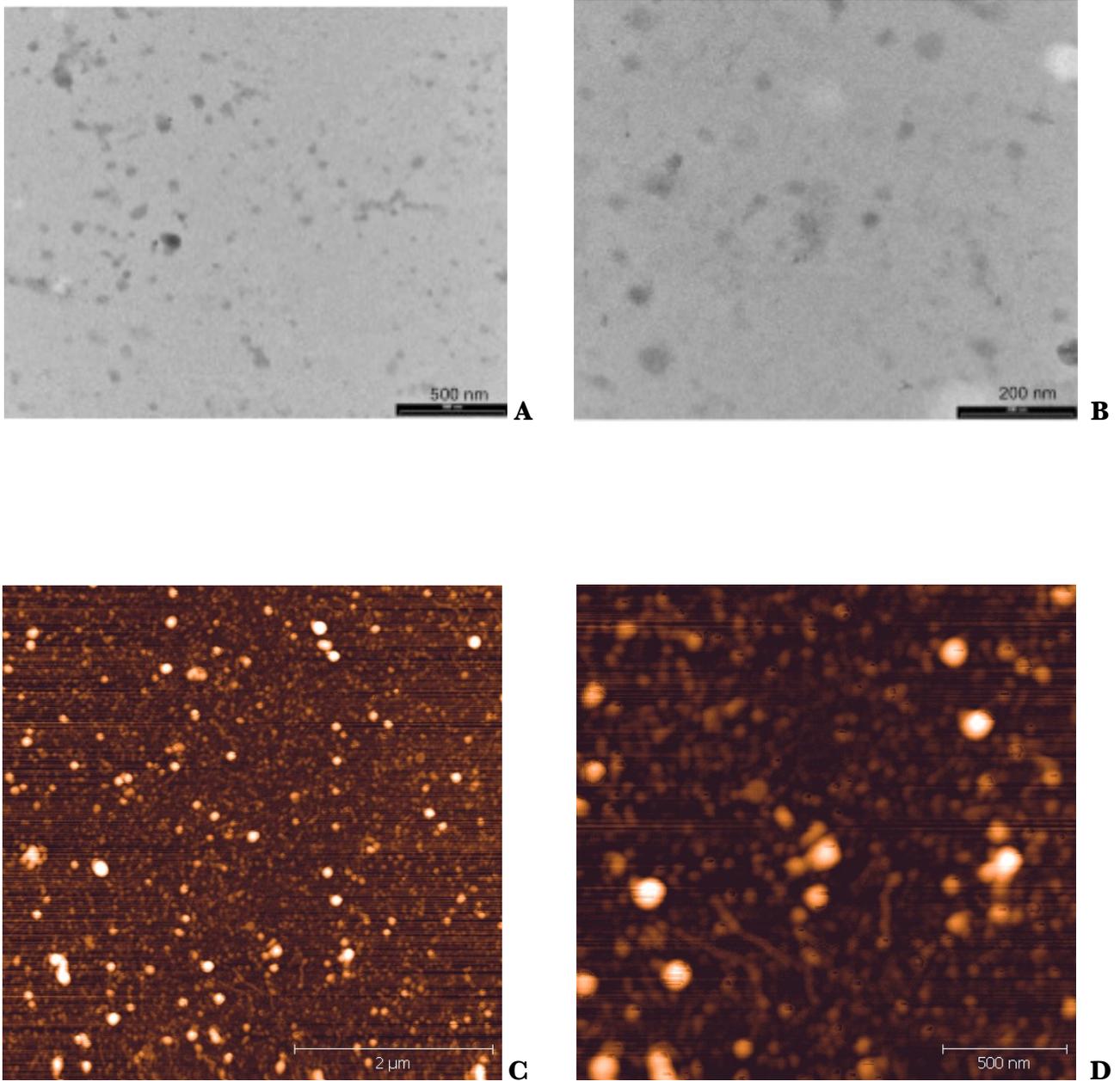
Figure 19 – **A-B**: Urinary exosomes observed by transmission electron microscopy. **C-D**: Urinary exosomes observed by atomic force microscopy (AFM).

5.5.3 - Proteomics

We analyse the exosomes content using the common exosomal protein markers by Western Blot: Hsp70, CD9, CD63 and AQ2 for urine exosomes and HSP70, CD9 and CD63 for serum exosomes (Figure 20).
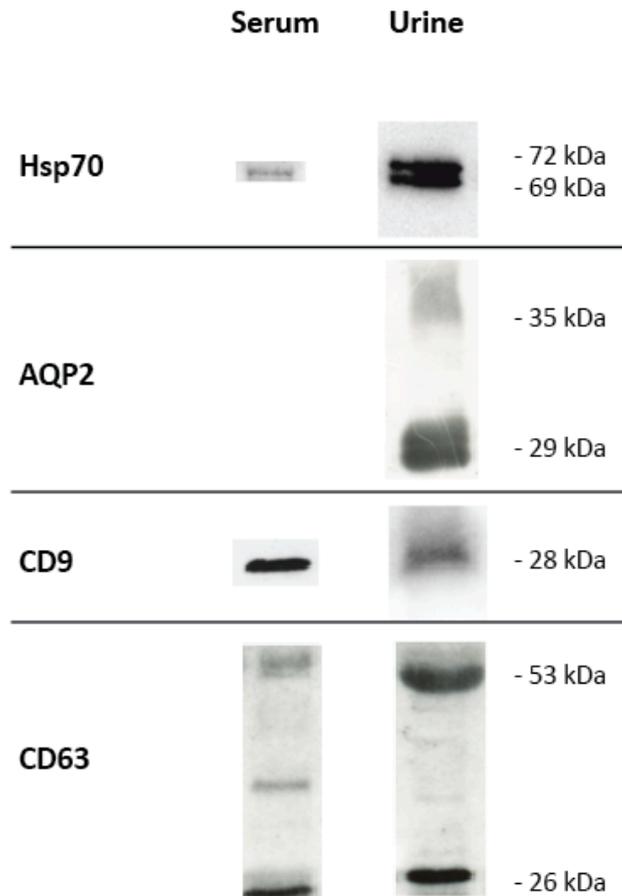
Figure 20: Western Blot analysis for some exosomal markers. Hsp70 (Heat-Shock protein), CD9 and CD63 are marker for all of the exosomes, while AQP2 (Aquaporin 2) is marker only for urine exosomes.

Following the isolation, the identification and characterization of exosome vesicles by microscopy and with the main surface markers, exosomes have been studied for their protein content.

To better characterize the different isolation technique, we compared two types of isolation of the vesicles: with ultracentrifuge and ExoQuick method, a commercial kit for the isolation of exosomes. The proteomic analysis of exosomal vesicles from the urine by LC-MS/MS method has resulted in the identification of 88 proteins by the ultracentrifuge method and 53 proteins by ExoQuick method. The second method has numerous advantages compared to the first, as it is very fast and does not require the use of ultracentrifuges, but has not proven to be the best method for the analysis of protein content, since it has a loss of exosomal proteins. Some of the proteins identified by ultracentrifuge method are listed in table 7.

Urinary proteins	Exosomal proteins	Immunoglobulin and serum proteins	Proteins involved in cell motility, endocytosis and exocytosis
ALBU	HSP72	LAC	ACTB
UROM		HVM17	ANXA1
AQP1		KV204	ANX11
FETUA		TRFE	LRP2
S12A3		ANGL2	IST1
S12A1		SOCS7	S10A6
EGF			SDCB1
XPP2			EZRI
BHMT1			K2C8

Table 7 – Proteins found in exosomes by LC-MS/MS. Most of them are typical exosomal proteins

The sample fractionation steps conducted prior to mass detection are critically important for the comprehensive analysis of complex protein mixtures. The shotgun technique used to a rapid identification of exosomal content can't identify a large number of proteins, and preferentially find the most abundant ones. The effective way to overcome the complexity of these proteomes is to combine several fractionation steps. OFFGEL fractionation, provides the ability to pre-fractionate peptides into discrete liquid fractions and demonstrated high efficiency and repeatability necessary for the analysis of such complex proteomes [Hörth et al., 2006]. The proteomic analysis of urine exosomes has resulted in the identification of 170 proteins. Some of the proteins identified by ultracentrifuge method are listed in table 8.

Urinary proteins	Exosomal Proteins	Immunoglobulin and serum proteins	Proteins involved in cell motility, endocytosis and exocytosis
UROM	HSP27	IGJ	PSAP
ALBU	HSPB1	PIGR	ANXA5
AQP1	TNS1	LAC2	VTA1
ASSY	CD9	IGHM	ANXA4
PODXL	CD59	MUCB5	MXRA8
DPEP1		CNTN4	ANX11
CUBN		IGHG3	ANXA1
		PIGR	ANXA2
			SDCB1
			EZRI
			CO6A1

Table 8 – Proteins found in exosomes by OFFGEL fractionation and LC-MS/MS. Most of them are typical exosomal proteins

5.5.4 - Luminex Analysis

We performed the analysis of cytokines and chemokines in the exosomes of the pigs subjected to different doses of Tacrolimus, with kidney transplantation, by the Luminex assay. The results of the analysis shown the presence of some analytes in serum exosomes. The trend of the cytokines after the drug administration, or after the surgery, shows no strong statistical correlation, probably because of the small number of subjects taken into consideration.

As can be seen in table 9, refers to the group of transplanted pigs C-HD, the GM-CSF and TNFα analytes are completely absent, while the analytes IL-8 and IL-18 are present in minimal amounts in only few times, but because of that it is not possible to take into account these values. The values of the other cytokines are always relatively low and not significant from a statistical point of view.

Table 10 shows the group of the transplanted pigs C-LD. In this case, the absent analytes are GM-CSF, IL-1α, IL-2, IL-4, IL-6, IL-18 and TNFα. The other cytokines do not have a significant value.

Despite the absence of most of the analytes and the statistical aspect, many cytokines and chemokines have been identified, demonstrating the presence in exosomal conten.

Luminex – Group C-HD
Analytes

	GM-CSF	IFNγ	IL-1α	IL-1β	IL-1ra	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IL-18	TNFα
T0	<0.02↓	<0.18↓	0.02	0.32	0.14	0.15	0.43	0.01	<0.01↓	0.35	0.60	<0.04↓	<0.01↓
mT1	<0.02↓	0.57	0.02	0.34	0.15	0.14	0.39	0.02	0.03	0.33	0.64	<0.04↓	<0.01↓
mT2	<0.02↓	0.57	0.03	0.42	0.16	0.16	0.46	0.06	<0.01↓	0.32	0.38	0.12	<0.01↓
mT3	<0.02↓	0.20	0.02	0.38	0.22	0.11	0.25	0.04	<0.01↓	0.27	0.31	0.06	<0.01↓
mT4	<0.02↓	<0.18↓	0.00	0.16	0.16	0.05	0.08	<0.01↓	<0.01↓	0.14	0.41	<0.04↓	<0.01↓

Table 9 – Means of the values of 13 cytokines and chemokines, expressed in ng/ul, of the group of pigs that has undergone to increase of the Tacrolimus administration.

Luminex – Group C-LD

Analytes

	GM-CSF	IFNγ	IL-1α	IL-1β	IL-1ra	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IL-18	TNFα
T0	<0.02↓	8.05	<0.00↓	<0.04↓	0.07	<0.01↓	<0.05↓	<0.01↓	0.36	0.02	0.60	<0.04↓	<0.01↓
mT1	<0.02↓	7.86	<0.00↓	<0.04↓	0.07	<0.01↓	<0.05↓	<0.01↓	0.36	0.02	0.44	<0.04↓	<0.01↓
mT2	<0.02↓	6.50	<0.00↓	0.07	0.24	<0.01↓	<0.05↓	<0.01↓	<0.01↓	0.04	0.40	<0.04↓	<0.01↓
mT3	<0.02↓	5.38	<0.00↓	0.07	0.23	<0.01↓	<0.05↓	<0.01↓	0.26	0.03	0.37	<0.04↓	<0.01↓
mT4	<0.02↓	4.90	<0.00↓	0.05	0.15	<0.01↓	<0.05↓	<0.01↓	<0.01↓	0.03	0.42	<0.04↓	<0.01↓

Table 10 – Means of the values of 13 cytokines and chemokines, expressed in ng/ul, of the group of pigs that has undergone to reduction of the Tacrolimus administration.

5.6 – SLA Typing

In this study, a total of 4 alleles at 2 SLA loci were identified in 31 pigs by direct sequencing. The low genetic diversity is due to the inbreeding conditions. The gene sequences and deducted protein sequences were compared with other published to IPD-MHC SLA sequence database, indicated that two alleles were novel. These novel alleles were found on SLA-1 gene and we temporarily called it AL1 and AL2. The other two alleles on DQB-1 gene refer to DQB1*04sp16 and DQB1*0801 alleles, found on IPD-MHC SLA database.

In the first 15 pigs (referring to the parental generation), the analysis of the pig sequences yielded two distinct patterns for the SLA-1 gene and two distinct patterns for DQB-1 gene, with two possible haplotypes. To see how haplotypes segregate in the offspring and get their correct distribution, we mating two sows with identical haplotypes and one boar with different pattern. We obtained two farrows (F1 and F2) from one sow and one other farrow (F3) from the second sow (figure 21). Thanks to that we had identified two new haplotypes called Hp1 and Hp2 (table 11). Hp1 has the following alleles: SLA1*AL1, DQB1*04sp16; Hp2 has the following alleles: SLA1*AL1, DQB1*0801 (shown the schematic distribution in figure 22).

A

Hp1/Hp2   Hp2/Hp2

Hp1/Hp2   Hp1/Hp2   Hp1/Hp2   Hp2/Hp2

B

Hp1/Hp2   Hp2/Hp2

Hp1/Hp2   Hp1/Hp2   Hp2/Hp2   Hp1/Hp2   Hp1/Hp2   Hp1/Hp2   Hp2/Hp2   Hp2/Hp2

C

Hp1/Hp2   Hp2/Hp2

Hp1/Hp2   Hp2/Hp2   Hp2/Hp2   Hp2/Hp2
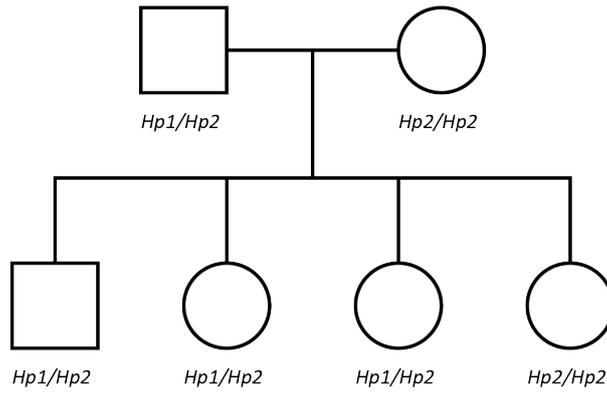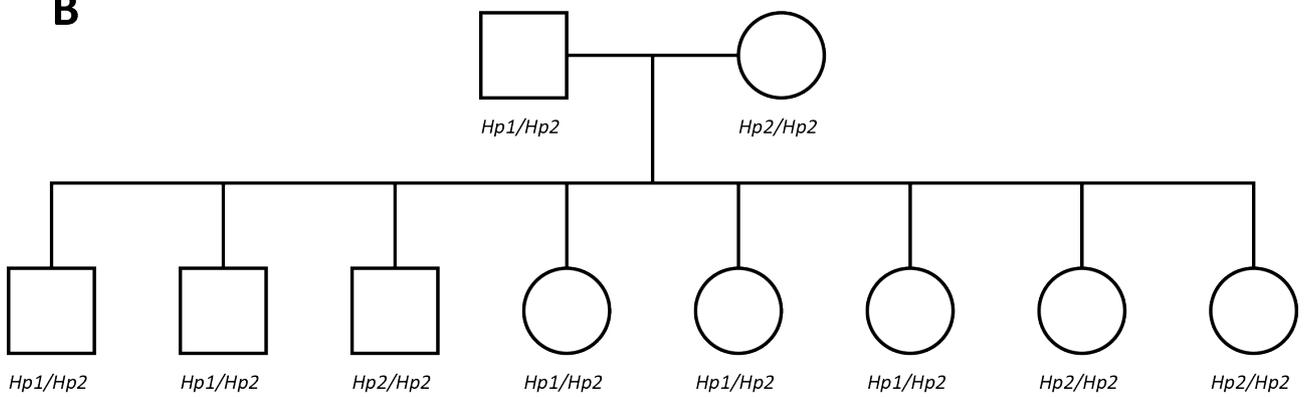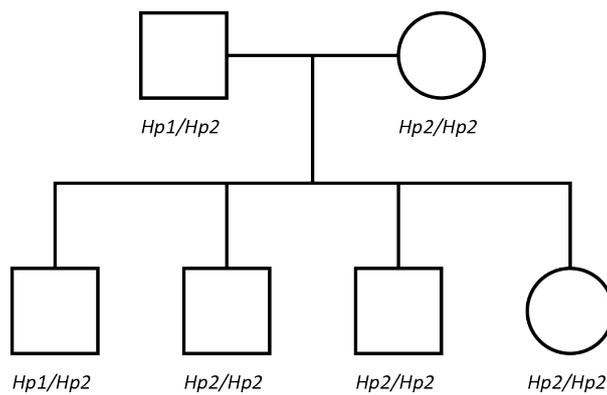
Figure 21: Family tree of three farrows. The squares represent males, while the rounds are the females. Below each shape are showed the identified genotypes. The boar used for the mating was the same for the three farrows, but we use two different sows genetically identical for SLA locus.

We were mating one boar SLA Hp1/Hp2 with two sows SLA Hp2/Hp2. The genetic of the three farrows are represented in figure 21. We obtained 16 piglets, which 9 were SLA Hp1/Hp2 and 7 SLA Hp2/Hp2. Thanks to the presence of both male and female piglets we can maintain the homozygous condition in the future farrows of at least one haplotype.
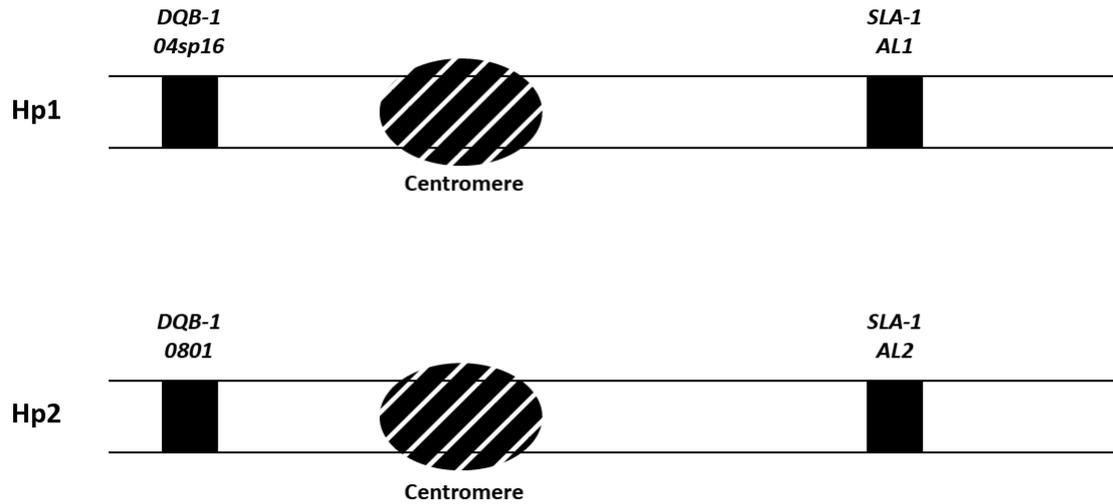
Figure 22 – Schematic representation of the two haplotypes identified. Both of the loci are on the chromosome 7, spanning the centromere.

Haplotype	SLA-1	DQB-1
Hp1	AL1	04sp16
Hp2	AL2	0801

Table 11 – Haplotype identified for the two genes.

Because the locus of SLA-1 gene is highly polymorphic [Smith et al., 1995] we set up the sequencing PCRs with two pair of primers for the exon 2 and 3 of SLA-1 gene a to avoid allele dropout. The result was identical. The identification of the sequences was done with the reading of the chromatograms, like the example of a heterozygous allele in figure 23.
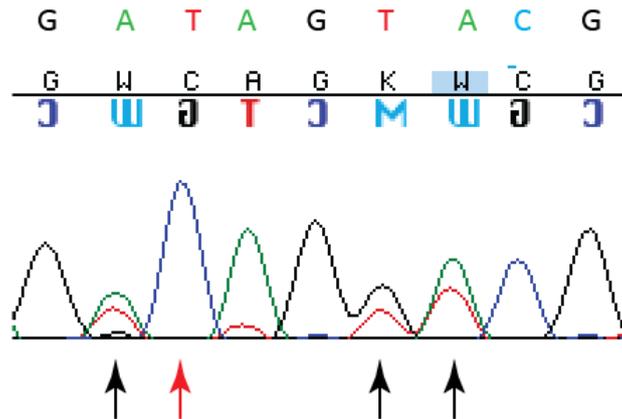
Figure 23 – Chromatogram of a portion of SLA-1 exon 3 gene of a heterozygous sequence. The upper sequence is the reference sequence to the fully sequenced haplotype Hp-1.1 registered on SLA sequence database. The second line is the 5'-3' sequence founded with the PCR, while the third line is the same sequence but complementary. A= Adenine, C= Cytosine, G= Guanine, T= Thymine, W= A and T, K= G and T. The black arrows highlight the heterozygous bases, while the red one is refer to a homozygous mutation.

Afterwards we deduced the amino acid sequence of the AL1 and AL2 alleles of SLA-1 gene.

The deduced protein sequence for the residues 1 to 223 of the two new alleles. In red are shown the amino acid residues different from the reference sequence.

SLA-1*AL1:

```
1          11         21         31         41         51         61         71
M GPGALFLLLS GTLALTGTQA GPHSLSYFYT AVSRPDRGDS RFFIVGYVDD TQFVRFDSDA PNAKMEPRAQ

        81         91
WIQQEGPEYW DRETQISKET


        101        111        121        131        141        151        161
AQTYRVGLKT LRGYYNQSEA GSHTLQSMFG CYLGPDGLLL RGYRQDAYDG ADYLALNEDL RSWTAADMAA

        171        181        191        201 203
QISKRKREAA DEAEQERSYL QGTCVEWLQK YLEMGKDTLQ RAE
```

59

SLA-1*AL2:

```
1          11         21         31         41         51         61         71
M GPGALFLLLS GTLALTGTQA GPHSLRYFDT AVSRPDRGNP RFIEVGYVDD TQFVWFDSDA PNPRMEPRAP


        81         91
WIEKEGQEYW DRETQISKDN




       101        111        121        131        141        151        161
AQTYRVKLNN LRGYYNQSEA GSHTLQSMYG CDVGPDGLFL RGYSQFAYDG ADYIALNEDL RSWTAADTAA

       171        181        191        201 203
QITKRKREAA DAAEQMRSYL EGACVEGLQK YLEMGKDTLQ RAE
```

The DQB-1 sequences of DQB1*04sp16 allele and DQB1*0801 was just deposited on SLA sequence database; we show here the deduced protein sequence for the residues 1 to 120. In red are shown the amino acid residues different from the reference sequence.

DQB1*04sp16

```
      10         20         30         40         50         60
RDSPQDFVYQ FKGECYFYNG TQRVWSVDRY IYNQEEFLRF DSDMGEYRAV TPLGRPDADY
      70         80         90        100        110        120
LNGQKEALEQ KRAELDTVCK HNYQIEEGTT LQRRVQPTVT ISPSKAEALN HHNLLVCAVT
```

DQB1*0801

```
      10         20         30         40         50         60
RDSPQDFVYQ FKFECYFFNG TQRVRLVTRY IYNQEEYARF DSDVGEYRAV TPLGRPAADY
      70         80         90        100        110        120
WNSQKDILEQ TRAELDTVCK HNYQIEEGTT LQRRVQPTVT ISPSKAEALN HHNLLVCAVT
```

This sequences highlight that the DQB-1 locus is less polymorphic than the SLA-1 locus.

Group A: Electron Microscopy of Renal biopsy. The ultrastructural analysis shows diffuse mesangial deposits.
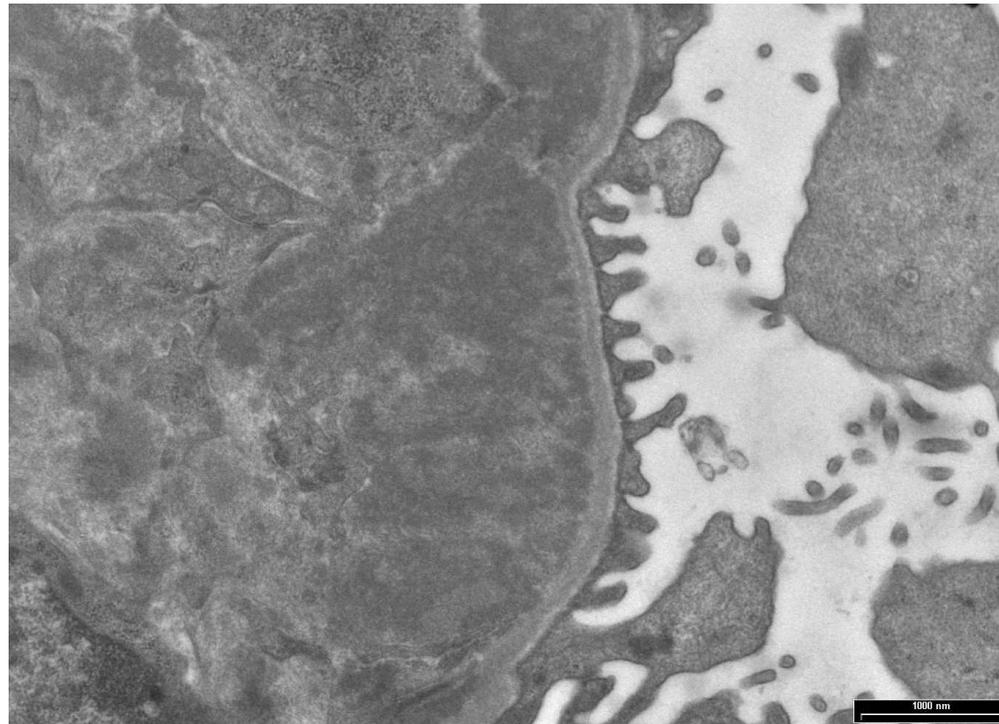
Light Microscopy: T0

Group C - HD

Group C - LD

Native Kidney

Fig. 1

Native Kidney

IgG

Fig. 3

Transplanted
Kidney

Fig. 2

Transplanted
Kidney

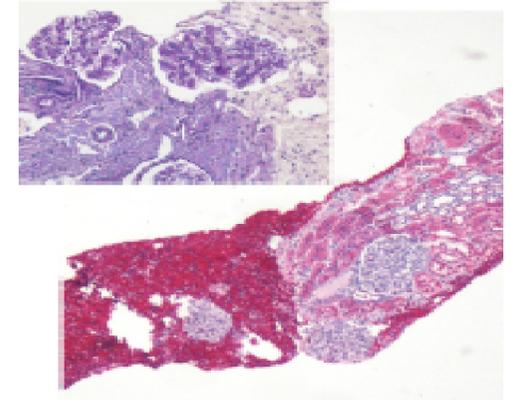Fig. 4
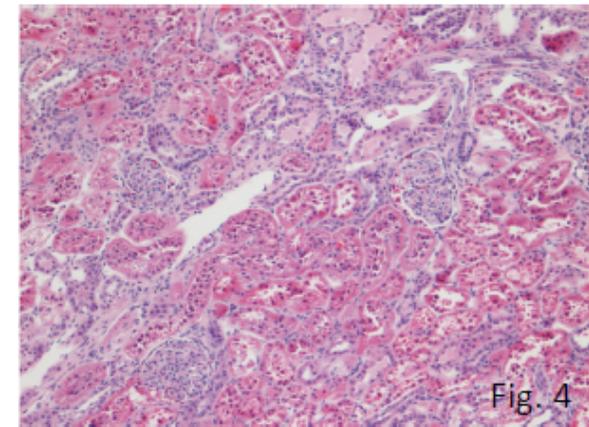
Light Microscopy: T2

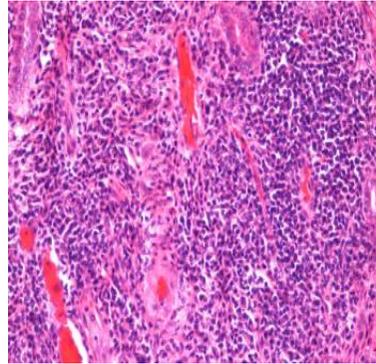| Group A | Group B | Group C - HD | Group C - LD |
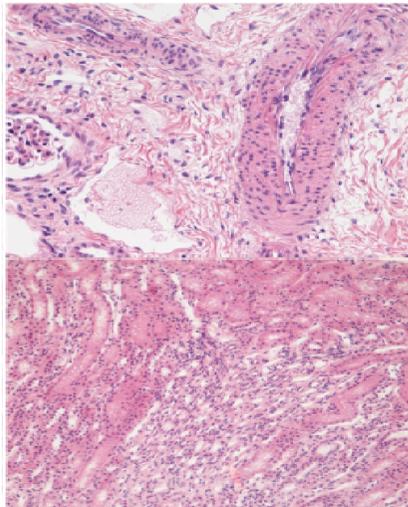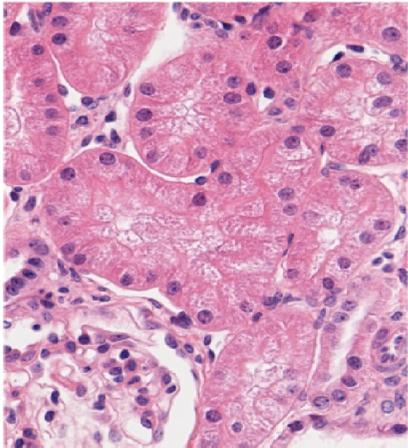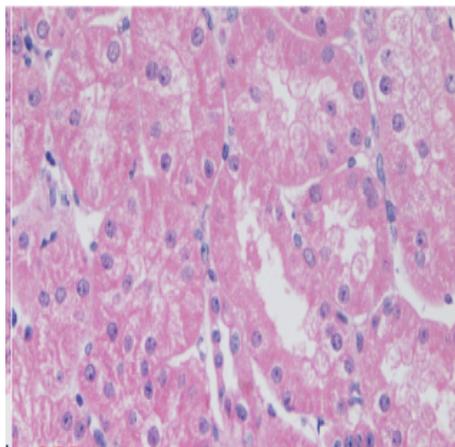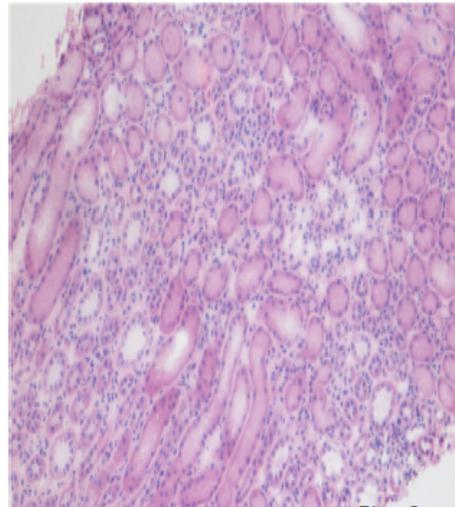
Native Kidney

Native Kidney

IgG

IgM

Ig A

Fig. 9
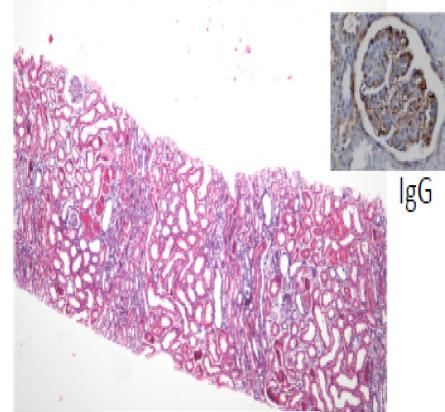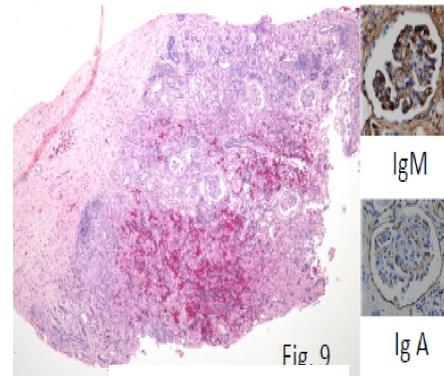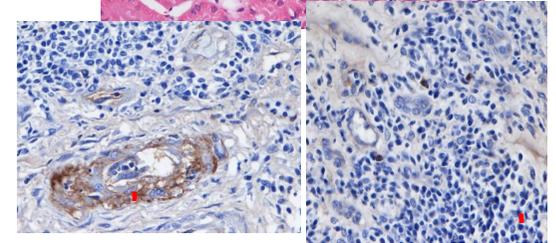
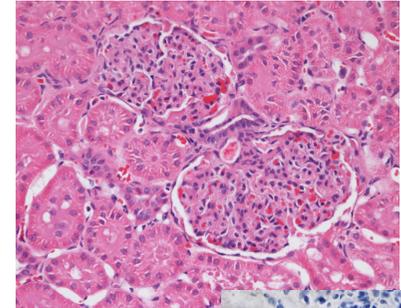Transplanted
Kidney
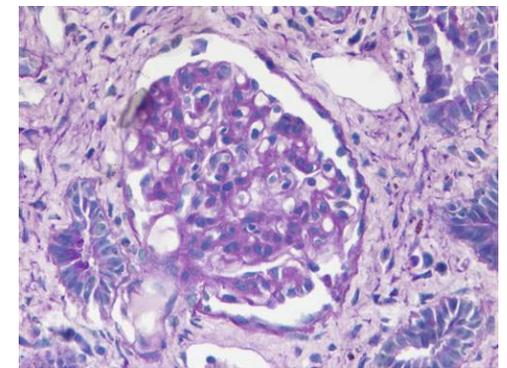
Transplanted
Kidney

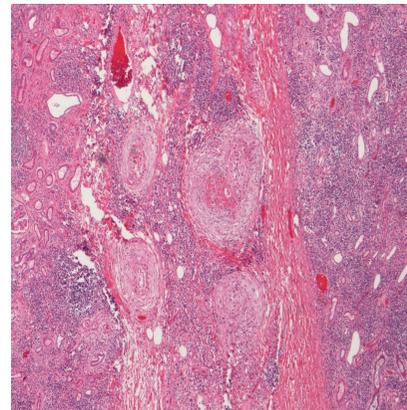Light Microscopy: T4

Group A	Group B	Group C - HD	Group C - LD
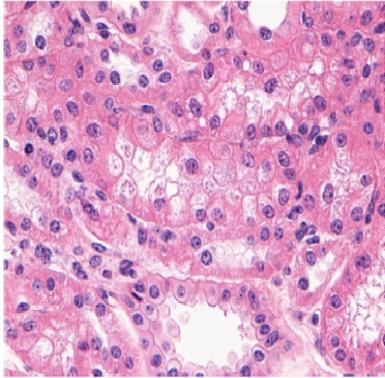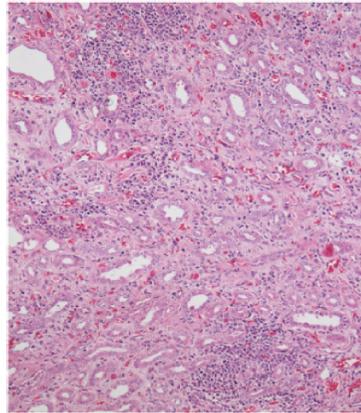
Native Kidney

Native Kidney

IgM

Ig A

Fig. 9

Transplanted
Kidney

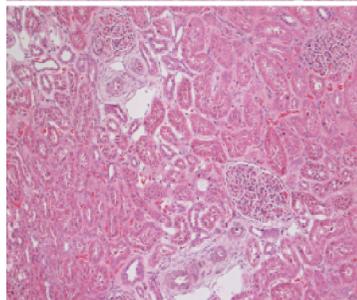Transplanted
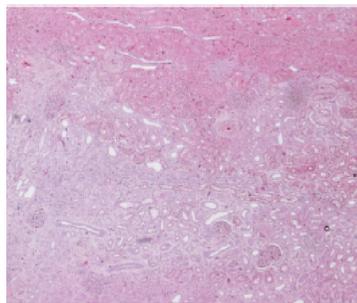Kidney

Electron Microscopy: T0
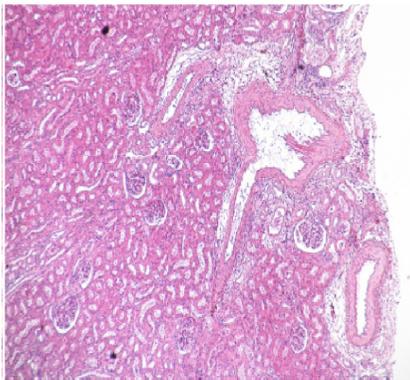
| Group B | Group C - HD | Group C - LD |

Native Kidney

Native Kidney

Transplanted
Kidney

Transplanted
Kidney

Electron Microscopy: T2

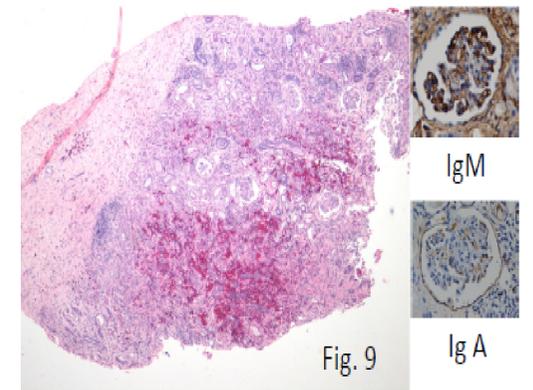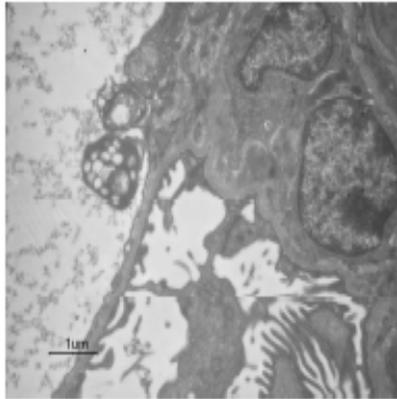| Group A	Group B	Group C - HD	Group C - LD
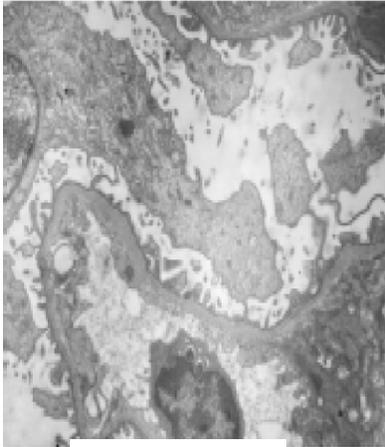
Native Kidney

Native Kidney

Transplanted
Kidney
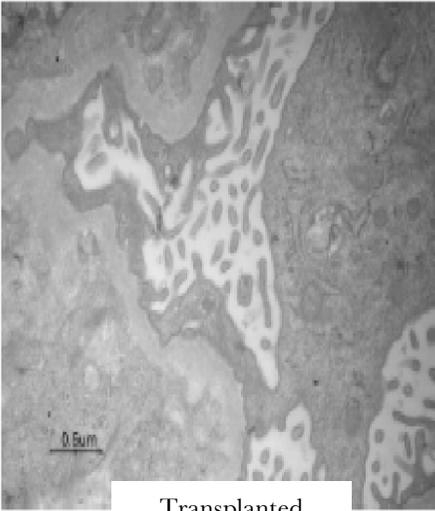
Transplanted
Kidney

Electron Microscopy: T4

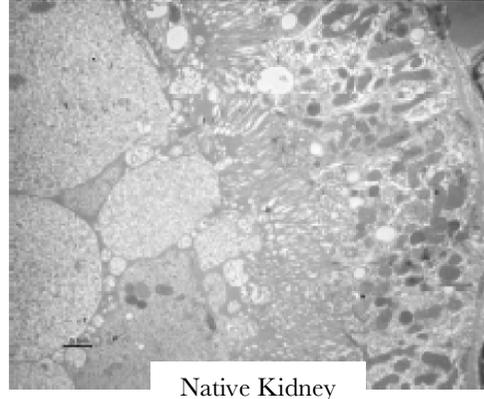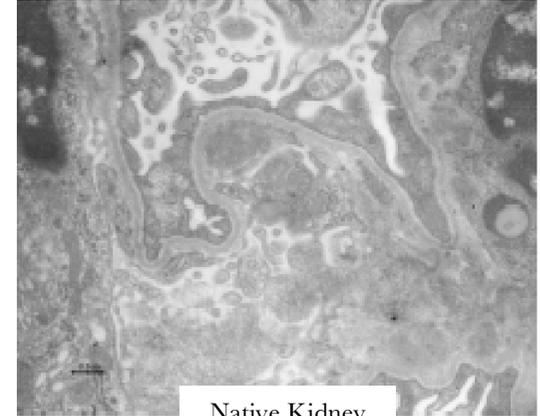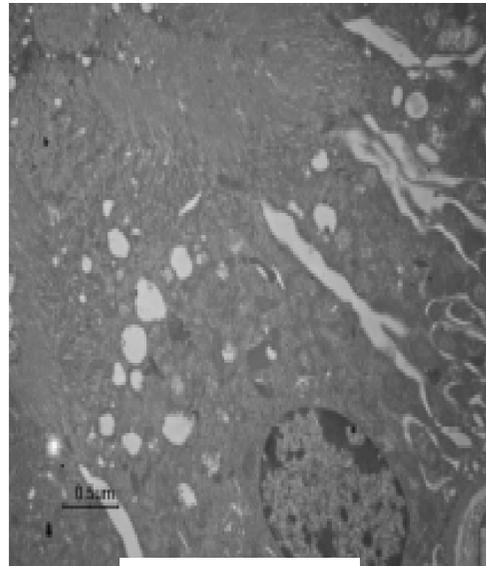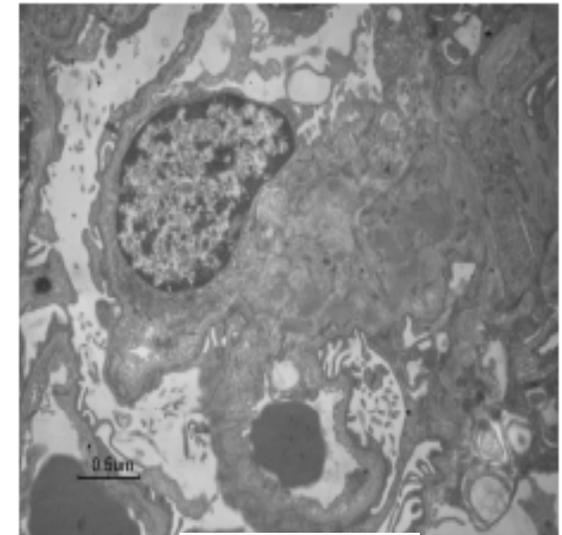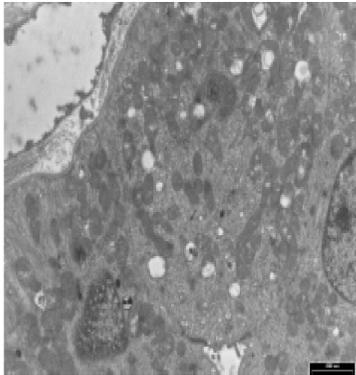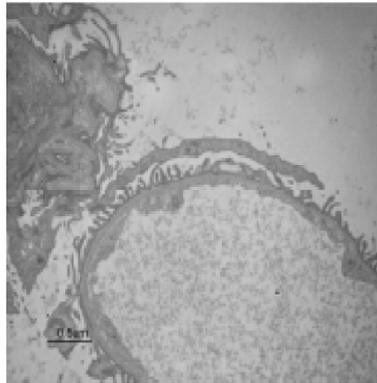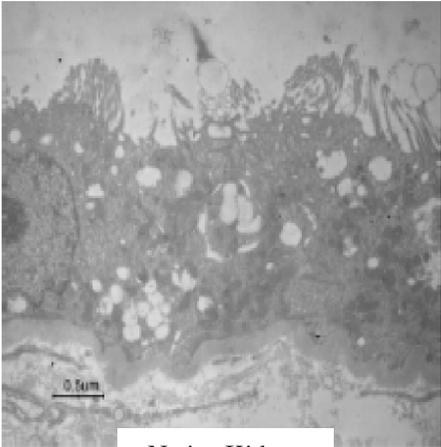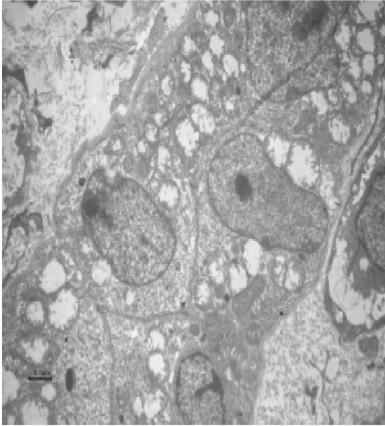| Group A | Group B | Group C - HD | Group C - LD |
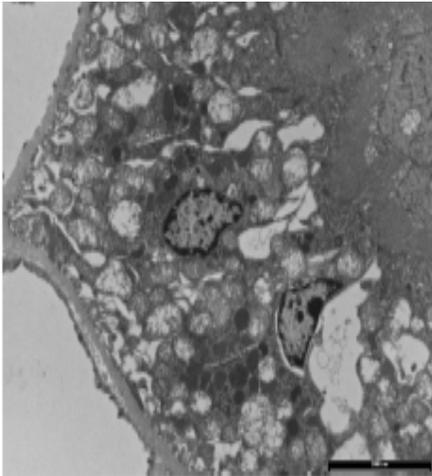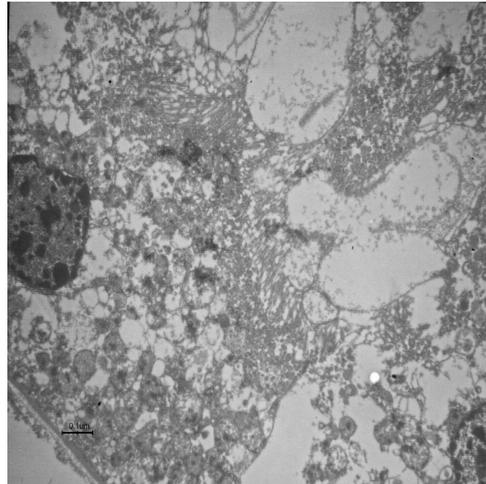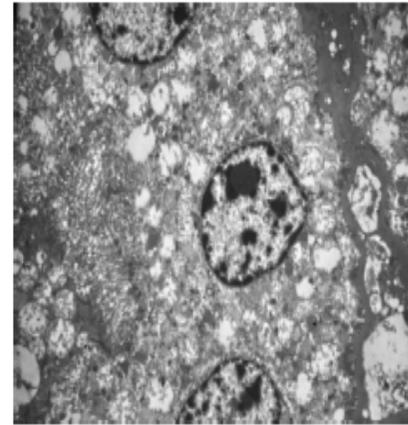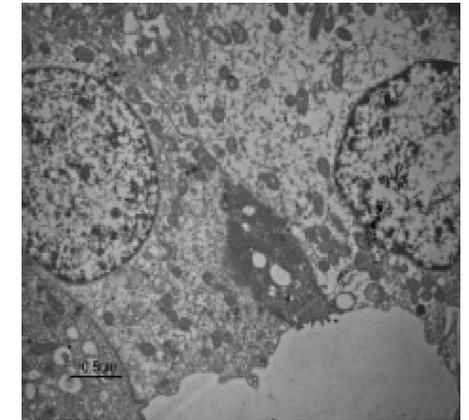
Native Kidney

Native Kidney
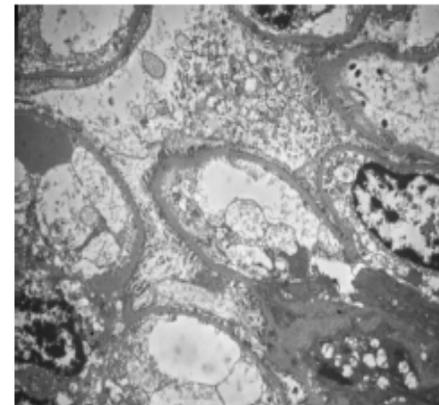
Transplanted
Kidney
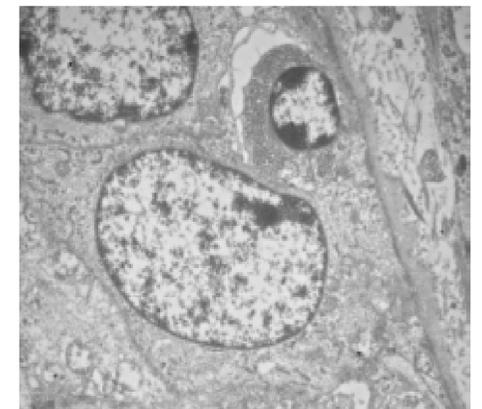
Transplanted
Kidney

DISCUSSION

Main Project

6.1 – General Discussion

Our study aimed to evaluate, in pig animal model, the kidney damage (functional and histological) induced by calcineurin inhibitors using incremental doses of Tacrolimus, to reach high blood levels toxicity in a limited time interval.

Our results show that:

1. Kidney damage from CNI is precocious;

2. The damage is not only for the kidneys, but also for other organs, like spleen, hearth and liver.

3. The clinical course is not representative of the histological damage. The dosage of blood creatinine is confirmed of little clinical utility as an early evaluation tool of renal function due to the latency with which changes after a specific renal insult (in our case the toxicity of Tacrolimus). The persistence of a preserved diuresis does not allow estimating the underlying renal insult; however, the reduction of toxic damage linked to the action of CNI is associated with an increase in urine output values.

4. Parameters such as the blood sodium and the blood magnesium, used in clinical practice for the estimation of renal tubular function, are difficult to interpret in animal models studied as observed in wide ranges of variability and difficult comparison between the different groups.

5. There is no difference, in terms of toxicity, between the methods of drug administration (oral versus intravenous) in the animals studied.

Regarding the blood pressure, a non-invasive monitoring method has not been possible due to the characteristics of the animal model. The data for any signs of high blood pressure, investigated by visual pathologic analysis, was negative. This leads us to conclude that the kidney damage from CNI is supported primarily by sustained vascular hypertension mechanisms.

The present study, even with the limitations due to the small size of the sample considered, allows some interesting observations:

1. The use of TAC to induce a rapid and stable immunosuppression in healthy pigs is recommended to dose 0.21 mg/kg/day responsible for a blood level equal to 30-40 ng/ml. The data is in line with those reported in the literature [Utsugi et al., 2001].

2. From a technical point of view, the intravenous administration of the drug, compared with the oral route, was found, even with the difficulties of managing an infusion catheter in animals, the convenient.

3. The oral route does not allow to evaluate the correct administration and therefore absorption of the drug; however, both routes of administration have ensured an adequate dose of drug to induce renal and systemic toxicity.

4. The use of Cylex test remains a valuable tool for monitoring the immune response to immunosuppressive therapy administered. There is a linear relationship between Tacrolimus administered doses and blood levels of the drug. The evaluation of the activity of the immune system is considered the best system to adjust the dosage of immunosuppressive drugs. Today there are numerous in vitro methods for this evaluation, but the United States Food and Drug Administration approved the Cylex ImmuKnow assay in 2002 [Zeevi, Lunz, 2013] for its use in the clinic as a tool to assess the cell-mediated immunoreactivity in immunocompromised patients and to optimize the drug regimen [Uemura et al., 2011]. For this reason the test was used flanked to the quantification of intravenous Tacrolimus during the whole experimentation. For the first time the Cylex ImmuKnow test was applied to the pig animal model and to do this it was necessary to modify it by using monoclonal antibodies of murine anti-swine CD4a to allow its use on an animal pig model. Although this test suffers from a high interindividual variability [Schulz-Juergensen el al., 2012], in the analysis we observed that the increasing of suppression of lymphocyte is generally consistent with the progress of therapy while is reversed when it is reduced (C-LD group). This confirms the achievement of a state of immunosuppression in the high dose of drug groups, and a recovery of immune activity in the low drug dosage group. This trend reflects the data of blood Tacrolimus, which however appears

that, by itself, is insufficient to determine the biological effects immunosuppressive action [Sampson et al,. 2008]. The use of Cylex ImmuKnow test is therefore found to be necessary and appropriate tool for monitoring even in animal pig model.

5. In all pigs, at T0 and in the absence of any therapy, were found renal glomerular and tubular lesions characterized by deposits both optical microscopy and electronic microscopy. With the immunofluorescence study the deposits appeared composed mainly of immunoglobulin, perhaps of repeated subclinical viral infections, typical of pig life (zoonoses).

6. All pigs were sacrificed at the end of the study. The necropsy study showed lesions compatible with CNI toxicity not only in the kidneys but also in other organs such as the heart, spleen and liver. As mentioned previously, this finding suggests that the toxicity of CNI is systemic and difficult to identify in the absence of specific tests.

6.2 - Pathological Anatomy

In all the kidney samples examined, from the first biopsy was performed we observed a kidney maturation of some glomeruli not yet complete, and in the other a slight membranoproliferative glomerulonephritis, highlighted not only at eosin and haematoxylin stain, but also with the PAS, ME, and the IHC. In pigs, this disease is an almost always-present condition as in all herds are endemic infections such as the PCV2 infection. The infection was also evidenced by the constant presence of lymphoplasmacytic interstitial nephritis in all samples. The most interesting lesions of this study were those in the vascular level. Subject of group A in which the administration of Tacrolimus was oral and had not undergone transplantation, we did not have lesions in luminal narrowing of the arteries, but there were multifocal proliferation of capillaries and arterioles, indicative of a probable previous drug-induced hypoxia. In the two pigs of group B not undergoing transplantation and intravenous administration of the drug, the vessels in the kidney showed a score of 2 to 3, and even proliferation of capillaries already at time T2. At necropsy, the vascular lesions were present with score from 2 to 3 also in the spleen, bladder, heart and lung, indicating an effect of the drug not only in kidneys but in the whole organism. In the 4 pigs underwent transplantation and in intravenous administration of the drug, the vascular lesions began to be present at time T2 and at a low grade, would then become evident with a grade 2 to 3, both in native and transplanted kidney, and in the other organs

such as lymph node, ureter, lung, bladder and myocardium, indicating in this case also a systemic effect of the drug. In transplanted kidneys were no visible damage from rejection.

6.3 – Blood Typing

The identification of blood group for the transplantation of a pig organ has always aroused discussion in scientific community for to the correct determination [Martínez-Alarcón et al., 2010; Smith et al., 2006], because of the incompatibilities of the classical serological human-derived typing methods. Also it's fundamental for transplantation study had a good blood typing to correct determine the donors and recipients. The use of the genetic technique optimized by Nguyen et al., allowed us to have both the tools (serologic and genetic) to guarantee greater reliability. The results showed us how this genetic typing is more correct because it reveals blood groups that are masked in the serological typing. Strangely, as already noted in the literature [Nguyen et al., 2011], have not been identified pigs homozygous for the allele A. Moreover, the frequency of animals with blood group A (genotyped as AO) was lower of those with blood group O. Thanks to this typing method, and after SLA typing, we select the sows for the transplant study.

Accessory Project

6.4 - Characterization of the Exosomal Content

Exosomes are aroused great interest in the scientific community because of their communication features. The analysis and characterization of their contents is continuously progressing, in order to find any correlation between the exosomal content and the specific physiological state of the organism from which they are obtained. For this purpose we develop a good protocol for isolation of the exosomal vesicles from the urine and serum using ultracentrifuge, resulting in high yield and purity. This protocol is an adaptation of the most protocols used for the exosomes purification [Chen et al., 2013; Fernández-Llama et al., 2011; Zhou et al., 2006], for our purposes and for the pig model. The key point for the optimal purification was the depletion of the most abundant proteins in urine (THP) and serum (albumin), achieved thanks to the heat treatment and reducing agents (DTT) for the THP, and the use of the albumin depletion column kit for the

albumin. For the correct characterization of exosome shape, the electron microscopy was the usual technique [Lässer et al., 2012]. To be even safer and scrupulous we perform another identification with a different type of microscopy, the atomic force microscopy (AFM), already used in the past for exosome characterization [Palanisamy et al., 2010]. The dimensional analysis shows the presence of spheroidal shape vesicles of about 30-100 nm, typical of the dimension of the exosomes. The absences of vesicles of more than 150 nm ensure us of a good exosome purification, without contaminants.

After having guarantee the exosomal nature of the purified vesicles by microscopy and western blotting we had performed some explorative proteomics analysis. The purpose of the western blotting is yet to demonstrate the correct exosomal insolation, thanks to the use of specific antibodies. The proteomic analyses with shotgun LC/MS/MS also was done to better characterize the different isolation technique, because we compared the ultracentrifuge and ExoQuick method. In both analyses we found typical exosomal proteins, like the ones involved in the cell motility, endocytosis and exocytosis. Also we found some immunoglobulin. Since the number of identified proteins was low, we tried to fractionate the sample with the OFFGEL technique. This allowed us to identify 170 proteins, even those with similar characteristics to those identified by the shotgun method. Although we had optimized the technique of purification, the presence of uromodulin and albumin is still present in both mass techniques, misleading the interpretation of the data.

To perform a preliminary proteomic test, quick but accurate, we set up a Luminex assay to evaluate the 13 most studied cytokines in the blood, with pro and anti-inflammatory characteristics, to evaluate a possible trend during the weeks of the transplant experiments. The kit has been optimized to use a purified exosomes, because it is the first time that this matrix was used in Luminex assay. We analyse the cytokines and chemokines in serum exosomes of transplanted pigs subjected to different doses of Tacrolimus (C-LD, C-HD groups), in order to see some variation of these molecules during the treatment with Tacrolimus, that can correlate with the structural and functional damage. The data obtained have little statistical significance due to the small number of animal employed, and because of the variability associated with the exosomes purification method, that although it was optimized, the mechanical and human intervention has an impact on the outcome of purification. However, the presence of cytokines and chemokines in the exosomal content may suggest a role in the transmission of these important inflammatory mediators through the exosomal transport mechanism.

Other tests could highlight the link between the presence and amount of cytokines and the state of inflammation due to the Tacrolimus toxicity or graft rejection, but because of the low number of pigs employed, it was not possible to use more sophisticated proteomic techniques for the detection of possible biomarkers. To obtain a trusted data, in fact, it would have been necessary to greatly increase the number of animals involved. Furthermore, investigative proteomics would require more volumes of blood and urine samples, which would be incompatible with the experiment logistics. In addition, exosomes are characterized by an intrinsic variability, yet to be verified by the scientific community, for which the use of a greater number of subjects would be necessary.

6.5 − SLA Typing

As for the blood typing, the SLA typing was a fundamental step for the correct transplant study. Known the genotype and phenotype of the SLA nature of the pigs let us to choose the desired donator and the desired recipient for the transplants. In fact, after the typing we select the sows that share similar SLA proteins structure to have easy therapeutic management of the graft and not have uncontrolled immune response due to the genetic incompatibility.

 Furthermore, in order to develop a controlled colony of SPF pigs with defined SLA, we perform an optimization of the direct DNA SLA typing technique. The end goal was to obtain a stable homozygous condition with some saws and boars with identical SLA genes. For the typing we focused only in exon 2 and 3 of SLA-1 gene and DQB-1 gene, because these exons encode for peptide-binding domains as well as interact directly with the immune cell receptors and are therefore considered to be functionally vital [Lunney et al., 2009]. In particular, we expected extreme polymorphisms concentrated in exons 2 and 3 for the SLA-1 gene [Smith et al., 1995] and exon 2 for DQB-1 gene [Smith et al., 2005]. The first typing run helped us to select the pigs with shared SLA haplotypes. The three farrows obtained after the mating of the two sows with one boar, shows that 16 piglets which 9 have haplotype SLA Hp1/Hp2 and 7 SLA Hp2/Hp2, near the Mendelian patters of inheritance, as we expected. Also we obtain 3 male and 4 female piglets homozygous for the Hp2 that can maintain the pure homozygous line.

Based on the IPD-MHC SLA database a total of 116 SLA class I alleles and 167 SLA class II alleles have been identified to date. We have identified two known DQB-1 alleles (DQB1*04sp16 and DBQ*0801) and

two unknown SLA-1 alleles called AL1 and AL2. This has led, then, to the discovery of two new haplotypes called Hp1 and Hp2. The deduced amino acid sequences show the high grade of polymorphism compared with the reference sequence of Hp-1.1 haplotype. Despite more than a decade that are collected sequencing data of SLA genes, they have been identified yet few haplotypes for a region so highly polymorphic. No wonder the discovery of two new alleles not yet registered in the database, and further research, using other genotyping techniques, such as PCR-SSP (Single Specific Primer) and PCR-RFLP (Restriction Fragment Length Polymorphism), will allow us to confirm the new sequences and then later we will sending them to the database.

CONCLUSIONS

75

Main Project

Our study highlight how the use of Tacrolimus is effective, once reached the therapeutic target and regardless of the route of administration (both oral or intravenous), to reach a valid immunosuppression. For what concerns the outcome of the administration of calcineurin inhibitor in pigs, the laboratory results, in particular pathological and clinical aspects, have shown that the kidney damage from calcineurin inhibitors is early (observable already at 15 days of administration of the drug). Incremental doses cause rapid toxic action, not only in the kidney but also in other organs such as the heart, liver and spleen. The toxic action of Tacrolimus appears predominantly tubular and vascular. We confirm the limited clinical use of blood creatinine as an early evaluation tool of renal function, because the change in its value occurs late in relation to the damage. These assessments have revealed significant features of the role of the calcineurin inhibitors in toxicity: it was multi-organ and it was precocious to functional damage, because the functional damage underestimates the structural damage. These considerations have been made possible thanks to the use of porcine animal model, for which it has been possible the serial sampling of blood and urine samples, as well as clear and depth biopsies and necropsies, for which it would not be possible in a human trial.

Our study confirms, for the first time in pigs, that the use of Cylex test for the evaluation of immunosuppression, after the necessary adaptations for the pig model, is a reliable tool also in the swine model. Furthermore, unlike the histological sampling, is a good non-invasive tool for early detection of the degree of immunosuppression of the animal studied.

The use of a genetic technique for the pig blood group typing has permitted more reliably screening for the choice of donor and recipient pigs.

Accessory Project

The analysis of exosomal vesicles allowed better characterization of them, demonstrating the robustness and reliability of the purification technique, although, inevitably, some contaminant is co-purified with them. The characterization has been confirmed both from the microscopic technique and with the protein content detected by mass spectrometry. The analysis of content of serum exosomes by Luminex technique, have documented the presence of certain cytokines and chemokines, although in low amounts. This suggests a possible role of these cytokines and chemokines in the inflammation process mediated by exosomal vesicles, whose role in the cell-to-cell communication is very important but still under study.

Finally, the sequencing of the SLA-1 and DQB-1 genes of genetically stable SPF pigs colony, has allowed the identification of two new alleles for the SLA-1 gene and two known alleles for DQB-1 gene, and then follow the allelic segregation in the offspring through specific mating of boars and sows. Thanks to that we obtain different pigs homozygous for one of the SLA haplotypes for both males and females pigs, thus obtaining a swine genic line that is defined, pure and stable.

BIBLIOGRAPHY

Admyre C, Johansson SM, Qazi KR, Filén JJ, Lahesmaa R, Norman M, Neve EP, Scheynius A, Gabrielsson S. Exosomes with immune modulatory features are present in human breast milk. J Immunol. 2007 Aug 1;179(3):1969-78.

Alexander RT, Hoenderop JG, Bindels RJ: Molecular determinants of magnesium homeostasis: Insights from human disease. J Am Soc Nephrol 19: 1451–1458, 2008.

Andresen E. Blood groups in pigs. Ann NY Acad Sci1962; 97: 205.

Andreoni KA, Brayman KL, Guidinger MK, Sommers CM, Sung RS: Kidney and pancreas transplantation in the United States, 1996 –2005. Am J Transplant 7: 1359 –1375, 2007.

Beckett K, Monier S, Palmer L, Alexandre C, Green H, Bonneil E, Raposo G, Thibault P, Le Borgne R, Vincent JP. Drosophila S2 cells secrete wingless on exosome-like vesicles but the wingless gradient forms independently of exosomes. Traffic. 2013 Jan;14(1):82-96.

Blicher T, Kastrup JS, Buus S, Gajhede M. High-resolution structure of HLA-A*1101 in complex with SARS nucleocapsid peptide. Acta Crystallogr D Biol Crystallogr. 2005 Aug;61(Pt 8):1031-40.

Bollen P, Ellegaard L. The Gottingen minipig in pharmacology and toxicology. Pharmacol Toxicol 1997; 80 (suppl 2):3.

Border WA, Noble NA: Transforming growth factor {beta} in tissue fibrosis. N Engl J Med 331: 1286 –1292, 1994.

Burdmann EA, Andoh TF, Yu L, Bennett WM: Cyclosporine nephrotoxicity. Semin Nephrol 23: 465– 476, 2003.

Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol. 2005 Jul;17(7):879-87. Epub 2005 May 20.

Chamorro S, Revilla C, Alvarez B, Lo´ pez-Fuertes L, Ezquerra A, Domı´nguez J. Phenotypic characterization of monocyte subpopulations in the pig. Immunobiology 2000;202:82–93.

Chang CT, Hung CC, Tian YC, Yang CW, Wu MS: Ciclosporin reduces paracellin-1 expression and magnesium transport in thick ascending limb cells. Nephrol Dial Transplant 22: 1033–1040, 2007.

Chen CY, Hogan MC, Ward CJ. Purification of exosome-like vesicles from urine. Methods Enzymol. 2013;524:225-41.

Cibulskyte D, Kaalund H, Pedersen M, Hørlyck A, Marcussen N, Hansen HE, Madsen M, Mortensen J. Chronic cyclosporine nephrotoxicity: a pig model. Transplant Proc. 2005 Oct;37(8):3298-301.

Clive DM: Renal transplant-associated hyperuricemia and gout. J Am Soc Nephrol 11: 974 –979, 2000.

Cooper DK, Ezzelarab MB, Hara H, Iwase H, Lee W, Wijkstrom M, Bottino R. The pathobiology of pig-to-primate xenotransplantation: a historical review. Xenotransplantation. 2016 Jan 27.

Dawson, H. A comparative assessment of the pig, mouse, and human genomes: structural and functional analysis of genes involved in immunity and inflammation. In The Minipig in Biomedical Research. 2011. (McAnulty, P.A., ed.), CRC Press, Taylor & Francis Group, pp. 321–341.

Dehoux JP, Gianello P. The importance of large animal models in transplantation. Front Biosci. 2007 Sep 1;12:4864-80.

Diederich D, Skopec J, Diederich A, Dai FX: Cyclosporine produces endothelial dysfunction by increased production of superoxide. Hypertension 23: 957–961, 1994.

Di Paolo S, Teutonico A, Stallone G, Infante B, Schena A, Grandaliano G, Battaglia M, Ditonno P, Schena PF: Cyclosporin exposure correlates with 1 year graft function and histological damage in renal transplanted patients. Nephrol Dial Transplant 19: 2107–2112, 2004.

Du S, Hiramatsu N, Hayakawa K, Kasai A, Okamura M, Huang T, Yao J, Takeda M, Araki I, Sawada N, Paton AW, Paton JC, Kitamura M. Suppression of NF-kb by cyclosporine A and tacrolimus (FK506) via induction of the C/EBP family: implication for unfolded protein response. J Immunol 2009; 182:7201-7211.

Ellegren H, Chowdhary BP, Fredholm M, Høyheim B, Johansson M, Bräuner Nielsen PB, Thomsen PD, Andersson L. A physically anchored linkage map of pig chromosome 1 uncovers sex- and position-specific recombination rates. Genomics. 1994 Nov 15;24(2):342-50.

Fahr A: Cyclosporin clinical pharmacokinetics. Clin Pharmacokinet 24: 472– 495, 1993.

Fairbairn L, Kapetanovic R, Sester DP, Hume DA. The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. J Leukoc Biol. 2011 Jun;89(6):855-71.

Farnsworth A, Hall BM, Duggin GG, Horvath JS, Tiller DJ: Interstitial fibrosis in renal allografts in patients treated with cyclosporin. Lancet 2: 1470 –1471, 1984.

Fernández-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA. Tamm-Horsfall protein and urinary exosome isolation. Kidney Int. 2010 Apr;77(8):736-42.

Flanagan WM, Corthesy B, Bram RJ, Crabtree GR: Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352: 803– 807, 1991.

Frey BM, Sieber M, Mettler D, Gänger H, Frey FJ. Marked interspecies differences between humans and pigs in cyclosporine and prednisolone disposition. Drug Metab Dispos. 1988 Mar-Apr;16(2):285-9.

Fulton, R.J., McDade, R.L., Smith, P.L., Kienker, L.J., Kettman Jr., J.R., 1997. Advanced multiplexed analysis with the FlowMetrix system. Clin. Chem. 43 (9), 1749.

Gaston RS, Cecka JM, Kasiske BL, Fieberg AM, Leduc R, Cosio FC, Gourishankar S, Grande J, Halloran P, Hunsicker L, Mannon R, Rush D, Matas AJ. Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure. Transplantation. 2010 Jul 15;90(1):68-74.

Ge F, Gong W. Strategies for successfully establishing a kid-ney transplant in a mouse model. Exp Clin Transplant 2011.

Guillen J. FELASA guidelines and recommendations. J Am Assoc Lab Anim Sci. 2012 May;51(3):311-21.

Gustafsson K, LeGuern C, Hirsch F, Germana S, Pratt K, Sachs DH. Class II genes of miniature swine. IV. Characterization and expression of two allelic class II DQB cDNA clones. J Immunol 1990;145:1946–51.

Hala K. The occurrence of erythrocyte antigens on cells from various tissues of the pig. Folia Biol (Praha) 1967; 13:189.

Harding MW, Galat A, Uehling DE, Schreiber SL: A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. Nature 341: 758 –760, 1989.

Hart EA, Caccamo M, Harrow JL, Humphray SJ, Gilbert JG, Trevanion S, Hubbard T, Rogers J, Rothschild MF. Lessons learned from the initial sequencing of the pig genome: comparative analysis of an 8 Mb region of pig chromosome 17. Genome Biol. 2007;8(8):R168.

Hausberg M, Lang D, Levers A, Suwelack B, Kisters K, Tokmak F, Barenbrock M, Kosch M: Sympathetic nerve activity in renal transplant patients before and after withdrawal of cyclosporine. J Hypertens 24: 957–964, 2006.

Heering P, Grabensee B: Influence of ciclosporin A on renal tubular function after kidney transplantation. Nephron 59: 66 −70, 1991.

Heering PJ, Klein-Vehne N, Fehsel K: Decreased mineralocorticoid receptor expression in blood cells of kidney transplant recipients undergoing immunosuppressive treatment: Cost efficient determination by quantitative PCR. J Clin Pathol 57: 33−36, 2004.

Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. Blood. 1999 Dec 1;94(11):3791-9.

Hemenway CS, Heitman J: Calcineurin. Structure, function, and inhibition. Cell Biochem Biophys 30: 115−151, 1999.

Ho CS, Franzo-Romain MH, Lee YJ, Lee JH, Smith DM. Sequence-based characterization of swine leucocyte antigen alleles in commercially available porcine cell lines. Int J Immunogenet. 2009 Aug;36(4):231-4.

Hood JL, San RS, Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer Res. 2011 Jun 1;71(11):3792-801.

Hortelano S, Castilla M, Torres AM, Tejedor A, Bosca L: Potentiation by nitric oxide of cyclosporin A and FK506-induced apoptosis in renal proximal tubule cells. J Am Soc Nephrol 11: 2315−2323, 2000.

Hörth P, Miller CA, Preckel T, Wenz C. Efficient fractionation and improved protein identification by peptide OFFGEL electrophoresis. Mol Cell Proteomics. 2006 Oct;5(10):1968-74.

Ibrahim Z, Busch J, Awwad M, Wagner R, Wells K, Cooper DK. Selected physiologic compatibilities and incompatibilities between human and porcine organ systems. Xenotransplantation. 2006 Nov;13(6):488-99.

Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, Ratajczak MZ. Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. Int J Cancer. 2005 Feb 20;113(5):752-60.

Karpinski J, Lajoie G, Cattran D, Fenton S, Zaltzman J, Cardella C, Cole E. (1999) Outcome of kidney transplantation from high-risk donors is determined by both structure and function. Transplantation 1999;Vol 67(8), pp 1162-1167.

Kershner RP, Fitzsimmons WE: Relationship of FK506 whole blood concentrations and efficacy and toxicity after liver and kidney transplantation. Transplantation 62: 920 − 926, 1996.

Khanna A, Plummer M, Bromberek C, Bresnahan B, Hariharan S. Expression of TGF- β and fibrogenic genes in transplant recipients with tacrolimus and cyclosporine nephrotoxicity. Kidney Int 2002; Vol 62: 2257-63.

Kim JY, Suh KS: Light microscopic and electron microscopic features of cyclosporine nephrotoxicity in rats. J Korean Med Sci 10: 352−359, 1995.

Krejci K, Tichy T, Bachleda P, Zadrazil J. Calcineurin inhibitor-induced renal allograft nephrotoxicity. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub.2010; 154(4): 297-306.

Kwiatkowski P, Artrip JH, John R, Edwards NM, Wang SF, Michler RE, Itescu S. Induction of swine major histocompatibility complex class I molecules on porcine endothelium by tumor necrosis factor-alpha reduces lysis by human natural killer cells. Transplantation. 1999 Jan 27;67(2):211-8.

Lakkaraju A, Rodriguez-Boulan E. Itinerant exosomes: emerging roles in cell and tissue polarity. Trends Cell Biol. 2008 May;18(5):199-209.

Laskow DA, Vincenti F, Neylan JF, Mendez R, Matas AJ: An open-label, concentration-ranging trial of FK506 in primary kidney transplantation: A report of the United States Multicenter FK506 Kidney Transplant Group. Transplantation 62: 900 −905, 1996.

Lässer C, Eldh M, Lötvall J. Isolation and characterization of RNA-containing exosomes. J Vis Exp. 2012 Jan 9;(59):e3037.

Lea JP, Sands JM, McMahon SJ, Tumlin JA: Evidence that the inhibition of Na+/K(+)-ATPase activity by FK506 involves calcineurin. Kidney Int 46: 647– 652, 1994.

Li X, Li JJ, Yang JY, Wang DS, Zhao W, Song WJ, Li WM, Wang JF, Han W, Zhang ZC, Yu Y, Cao DY, Dou KF. Tolerance induction by exosomes from immature dendritic cells and rapamycin in a mouse cardiac allograft model. PLoS One. 2012;7(8):e44045.

Lin XP, Almqvist N, Telemo E. Human small intestinal epithelial cells constitutively express the key elements for antigen processing and the production of exosomes. Blood Cells Mol Dis. 2005; 35: 122–8.

Liu EH, Siegel RM, Harlan DM, O'Shea JJ: T cell-directed therapies: Lessons learned and future prospects. Nat Immunol 8: 25–30, 2007.

Longoni B, Boschi E, Demontis GC, Ratto GM, Mosca F: Apoptosis and adaptive responses to oxidative stress in human endothelial cells exposed to cyclosporin A correlate with BCL-2 expression levels. FASEB J 15: 731–740, 2001.

Loss M, Vangerow B, Schmidtko J, Kunz R, Jalali A, Arends H, Przemeck M, Rückholt H, Leuwer M, Kaup FJ, Rensing S, Cozzi E, White DJ, Klempnauer J, Winkler M. Acute vascular rejection is associated with systemic complement activation in a pig-to-primate kidney xenograft model. Xenotransplantation. 2000 Aug;7(3):186-96.

Lunney JK. Advances in swine biomedical model genomics. Int J Biol Sci. 2007 Feb 10;3(3):179-84.

Lunney JK, Ho CS, Wysocki M, Smith DM. Molecular genetics of the swine major histocompatibility complex, the SLA complex. Dev Comp Immunol. 2009 Mar;33(3):362-74.

Madariaga ML, Michel SG, La Muraglia GM 2nd, Sekijima M, Villani V, Leonard DA, Powell HJ, Kurtz JM, Farkash EA, Colvin RB, Allan JS, Cetrulo CL Jr, Huang CA, Sachs DH, Yamada K, Madsen JC. Kidney-induced cardiac allograft tolerance in miniature swine is dependent on MHC-matching of donor cardiac and renal parenchyma. Am J Transplant. 2015 Jun;15(6):1580-90.

Martínez-Alarcón L, Ramis G, Majado MJ, Quereda JJ, Herrero-Medrano JM, Ríos A, Ramírez P, Muñoz A. ABO and RH1 blood group phenotyping in pigs (Sus scrofa) using microtyping cards. Transplant Proc. 2010 Jul-Aug;42(6):2146-8.

Mathews KA, Holmberg DL, Miller CW. Kidney transplantation in dogs with naturally occurring end-stage renal disease. J Am Anim Hosp Assoc 2000;36(4): 294–301.

Meinitzer A, Gartner G, Pilz S, Stettin M. Ultra fast liquid chromatography-tandem mass spectrometry routine method for simultaneous determination of cyclosporin A, tacrolimus, sirolimus, and everolimus in whole blood using deuterated internal standards for cyclosporin A and everolimus. Ther Drug Monit. 2010 Feb;32(1):61-6.

Meurens F, Summerfield A, Nauwynck H, Saif L, Gerdts V. The pig: a model for human infectious diseases. Trends Microbiol. 2012 Jan;20(1):50-7.

Mihatsch MJ, Thiel G, Basler V, Ryffel B, Landmann J, von Overbeck J, Zollinger HU: Morphological patterns in cyclosporine-treated renal transplant recipients. Transplant Proc 17: 101–116, 1985.

Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, González MÁ, Bernad A, Sánchez-Madrid F. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat Commun. 2011;2:282.

Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood. 2012 Jan 19;119(3):756-66.

Morozumi K, Takeda A, Uchida K, Mihatsch MJ: Cyclosporine nephrotoxicity: how does it affect renal allograft function and transplant morphology? Transpl Proc 36: S251–S256, 2004.

Murray BM, Paller MS, Ferris TF: Effect of cyclosporine administration on renal hemodynamics in conscious rats. Kidney Int 28: 767–774, 1985.

Myers BD, Ross J, Newton L, Luetscher J, Perlroth M: Cyclosporine-associated chronic nephropathy. N Engl J Med 311: 699 –705, 1984.

Naesens M, Kambham N, Concepcion W, Salvatierra O, Jr., Sarwal M: The evolution of non-immune histological injury and its clinical relevance in adult-sized kidney grafts in pediatric recipients. Am J Transplant 7: 2504 –2514, 2007a.

Naesens M, Lerut E, Damme BV, Vanrenterghem Y, Kuypers DR: Tacrolimus exposure and evolution of renal allograft histology in the first year after transplantation. Am J Transplant 7: 2114 –2123, 2007b.

Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. Clin J Am Soc Nephrol. 2009 Feb;4(2):481-508.

Nakamura T, Nozu K, Iijima K, Yoshikawa N, Moriya Y, Yamamori M, Kako A, Matsuo M, Sakurai A, Okamura N, Ishikawa T, Okumura K, Sakaeda T: Association of cumulative cyclosporine dose with its irreversible nephrotoxicity in Japanese patients with pediatric-onset autoimmune diseases. Biol Pharm Bull 30:2371–2375, 2007.

Nankivell B, Richard J, Borrows R, Fung CL-S, O'Connell PJ, Allen R, Chapman JR: The natural history of chronic allograft nephropathy. N Engl J Med 349): 2326 –2333, 2003.

Nankivell BJ, Borrows RJ, Fung C L-S, O'Connell PJ, Chapman JR, Allen RD: Calcineurin-inhibitor nephrotoxicity: Longitudinal assessment by protocol histology. Transplantation 78: 557–565, 2004.

Natali PG, Quaranta V, Nicotra MR, Apollonj C, Pellegrino MA, Ferrone S. Tissue distribution of Ia-like antigens in different species: analysis with monoclonal antibodies. Transplant Proc. 1981 Mar;13(1 Pt 2):1026-9.

Naziruddin B, Durriya S, Phelan D, Duffy BF, Olack B, Smith D, Howard T, Mohanakumar T. HLA antibodies present in the sera of sensitized patients awaiting renal transplant are also reactive to swine leukocyte antigens. Transplantation. 1998 Oct 27;66(8):1074-80.

Nguyen DT, Choi H, Jo H, Kim JH, Dirisala VR, Lee KT, Kim TH, Park KK, Seo K, Park C. Molecular characterization of the human ABO blood group orthologus system in pigs. Anim Genet. 2011 Jun;42(3):325-8.

Ogawa Y, Miura Y, Harazono A, Kanai-Azuma M, Akimoto Y, Kawakami H, Yamaguchi T, Toda T, Endo T, Tsubuki M, Yanoshita R. Proteomic analysis of two types of exosomes in human whole saliva. Biol Pharm Bull. 2011;34(1):13-23.

Ojo AO, Held PJ, Port FK, Wolfe RA, Leichtman AB, Young EW, Arndorfer J, Christensen L, Merion RM: Chronic renal failure after transplantation of a nonrenal organ. N Engl J Med 349: 931–940, 2003.

Ojo AO, Morales JM, González-Molina M, Steffick DE, Luan FL, Merion RM, Ojo T, Moreso F, Arias M, Campistol JM, et al. Comparison of the long-term outcomes of kidney transplantation: USA versus Spain. Nephrol Dial Transplant. 2013;28:213–220.

O'Keefe SJ, Tamura J, Kincaid RL, Tocci MJ, O'Neill EA: FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature 357: 692– 694, 1992.

Olyaei AJ, de Mattos AM, Bennett WM: Nephrotoxicity of immunosuppressive drugs: New insight and preventive strategies. Curr Opin Crit Care 7: 384 –389, 2001.

Palanisamy V, Sharma S, Deshpande A, Zhou H, Gimzewski J, Wong DT. Nanostructural and transcriptomic analyses of human saliva derived exosomes. PLoS One. 2010 Jan 5;5(1):e8577.

Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, Coscia C, Iessi E, Logozzi M, Molinari A, Colone M, Tatti M, Sargiacomo M, Fais S. Microenvironmental pH is a key factor for exosome traffic in tumor cells. J Biol Chem. 2009 Dec 4;284(49):34211-22.

Pegtel DM, van de Garde MD, Middeldorp JM. Viral miRNAs exploiting the endosomal-exosomal pathway for intercellular cross-talk and immune evasion. Biochim Biophys Acta. 2011 Nov-Dec;1809(11-12):715-21.

Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci USA. 2004 Sep 7;101(36):13368-73. Epub 2004 Aug 23.

Ponticelli C: De novo thrombotic microangiopathy. An underrated complication of renal transplantation. Clin Nephrol 67: 335–340, 2007.

Potts WK, Slev PR. Pathogen-based models favoring MHC genetic diversity. Immunol Rev. 1995 Feb;143:181-97.

Prado N, Marazuela EG, Segura E, Fernández-García H, Villalba M, Théry C, Rodríguez R, Batanero E. Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction. J Immunol. 2008 Jul 15;181(2):1519-25.

Rasmusen BA. Gene interaction and the A–O blood-group system in pigs. Genetics 1964; 50: 191.

Renard C, Hart E, Sehra H, Beasley H, Coggill P, Howe K, et al. The genomic sequence and analysis of the swine major histocompatibility complex. Genomics 2006;88:96–110.

Robinson J, Waller MJ, Stoehr P, Marsh SG. IPD--the Immuno Polymorphism Database. Nucleic Acids Res. 2005 Jan 1;33.

Roullet JB, Xue H, McCarron DA, Holcomb S, Bennett WM: Vascular mechanisms of cyclosporin-induced hypertension in the rat. J Clin Invest 93: 2244 –2250, 1994.

Sako F, Gasa S, Makita A, et al: Human blood group glycosphingolipids of porcine erythrocytes. Arch Biochem Biophys 278:228, 1990.

Sampson VB, Dunn SP, Rymeski B, Malatack J, Rong NH, Flynn L, Krueger LJ. Failure of immunosuppressive drug levels to predict T-cell reactivity in pediatric transplant patients. J Pediatr Surg. 2008;43(6):1134-41.

Satz ML, Wang LC, Singer DS, Rudikoff S. Structure and expression of two porcine genomic clones encoding class I MHC antigens. J Immunol 1985;135:2167–75.

Schulz-Juergensen S, Burdelski MM, Oellerich M, Brandhorst G. Intracellular ATP production in CD4+ T cells as a predictor for infection and allograft rejection in trough-level guided pediatric liver transplant recipients under calcineurin-inhibitor therapy. Ther Drug Monit. 2012;34(1):4-10.

Sellarés J, de Freitas DG, Mengel M, Reeve J, Einecke G, Sis B, Hidalgo LG, Famulski K, Matas A, Halloran PF. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant. 2012 Feb;12(2):388-99.

Servais H, Ortiz A, Devuyst O, Denamur S, Tulkens PM, Mingeot-Leclercq MP: Renal cell apoptosis induced by nephrotoxic drugs: Cellular and molecular mechanisms and potential approaches to modulation. Apoptosis 13: 11–32, 2008.

Shihab FS, Andoh TF, Tanner AM, Yi H, Bennett WM: Expression of apoptosis regulatory genes in chronic cyclosporine nephrotoxicity favors apoptosis. Kidney Int 56: 2147–2159, 1999.

Shishido S, Naziruddin B, Howard T, Mohanakumar T. Recognition of porcine major histocompatibility complex class I antigens by human CD8+ cytolytic T cell clones. Transplantation. 1997 Jul 27;64(2):340-6.

Shishido S, Naziruddin B, Xu XC, Howard T, Mohanakumar T. Indirect recognition of porcine xenoantigens by human CD4+ T cell clones. Transplantation. 1998 Mar 15;65(5):706-12.

Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH: A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. Nature 341: 755–757, 1989.

Simon N, Morin C, Urien S, Tillement JP, Bruguerolle B: Tacrolimus and sirolimus decrease oxidative phosphorylation of isolated rat kidney mitochondria. Br J Pharmacol 138: 369 –376, 2003.

Simons M, Raposo G. Exosomes--vesicular carriers for intercellular communication. Curr Opin Cell Biol. 2009 Aug;21(4):575-81.

Skoskiewicz M, Chase C, Winn HJ, Russell PS. Kidney trans-plants between mice of graded immunogenetic diversity. Transplant Proc 1973; 5: 721.

Smith TP, Rohrer GA, Alexander LJ, Troyer DL, Kirby-Dobbels KR, Janzen MA, Cornwell DL, Louis CF, Schook LB, Beattie CW. Directed integration of the physical and genetic linkage maps of swine chromosome 7 reveals that the SLA spans the centromere. Genome Res. 1995 Oct;5(3):259-71.

Smith DM, Lunney JK, Martens GW, Ando A, Lee JH, Ho CS, Schook L, Renard C, Chardon P. Nomenclature for factors of the SLA class-I system, 2004. Tissue Antigens. 2005 Feb;65(2):136-49.

Smith DM, Newhouse M, Naziruddin B, Kresie L. Blood groups and transfusions in pigs. Xenotransplantation. 2006 May;13(3):186-94.

Sprague L. On the recognition and inheritance of the soluble blood group property "Oc" of cattle. Genetics 1958; 43: 913.

Staatz CE, Tett SE: Clinical pharmacokinetics and pharmacodynamics of tacrolimus in solid organ transplantation. Clin Pharmacokinet 43: 623– 653, 2004.

Swindle MM, Makin A, Herron AJ, Clubb FJ Jr, Frazier KS. Swine as models in biomedical research and toxicology testing. Vet Pathol. 2012 Mar;49(2):344-56.

Subra C, Laulagnier K, Perret B, Record M. Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. Biochimie. 2007 Feb;89(2):205-12. Epub 2006 Nov 16.

Sullivan JA, Oettinger HF, Sachs DH, Edge AS. Analysis of polymorphism in porcine MHC class I genes: alterations in signals recognized by human cytotoxic lymphocytes. J Immunol. 1997 Sep 1;159(5):2318-26.

Summerfield A, Guzylack-Piriou L, Schaub A, Carrasco CP, Taˆche V, Charley B, et al. Porcine peripheral blood dendritic cells and natural interferon-produ- cing cells. Immunology 2003;110:440–9.

Takahashi N, Hayano T, Suzuki M: Peptidyl-prolyl cistrans isomerase is the cyclosporin A-binding protein cyclophilin. Nature 337: 473– 475, 1989.

Tanaka-Matsuda M, Ando A, Rogel-Gaillard C, Chardon P, Uenishi H. Difference in number of loci of swine leukocyte antigen classical class I genes among haplotypes. Genomics. 2009 Mar;93(3):261-73.

Terris JM. Swine as a model in renal physiology and nephrology: an overview. Swine in biomedical research 1986.

Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, Zheng Y, Hoshino A, Brazier H, Xiang J, Williams C, Rodriguez-Barrueco R, Silva JM, Zhang W, Hearn S, Elemento O, Paknejad N, Manova-Todorova K, Welte K, Bromberg J, Peinado H, Lyden D. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell Res. 2014 Jun;24(6):766-9.

Théry C, Boussac M, Véron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol. 2001 Jun 15;166(12):7309-18.

Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002 Aug;2(8):569-79.

Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol. 2009 Aug;9(8):581-93.

Uemura T, Riley TR, Khan A, Hollenbeak C, Schreibman I, Ghahramani N, Reeves, B, Domen RE, Zander DS, Kadry Z. Immune functional assay for immunosuppressive management in post-transplant malignancy. Clin Transplant. 2011;25(1):E32-7.

Utsugi R, Barth RN, Lee RS, Kitamura H, LaMattina JC, Ambroz J, Sachs DH and Yamada K. Induction of transplantationtolerance with a short course of tacrolimus (FK506) Transplantation 2001; Vol 71, 1368-1379.

Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007 Jun;9(6):654-9.

Vella LJ, Sharples RA, Lawson VA, Masters CL, Cappai R, Hill AF. Packaging of prions into exosomes is associated with a novel pathway of PrP processing. J Pathol. 2007 Apr;211(5):582-90.

Van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bensussan N, Heyman M. Intestinal epithelial cells secrete exosome-like vesicles. Gastroenterology. 2001 Aug;121(2):337-49.

Vial CM, Ostlie DJ, Bhatti FN, et al. Life supporting function for over one month of a transgenic porcine heart in a baboon. J Heart Lung Transplant. 2000;19:224-229.

Wilson AJ, Jabr RI, Clapp LH: Calcium modulation of vascular smooth muscle ATP-sensitive K() channels: Role of protein phosphatase-2B. Circ Res 87: 1019 −1025, 2000.

Wolf G: Renal injury due to renin-angiotensin-aldosterone system activation of the transforming growth factor-beta pathway. Kidney Int 70: 1914 −1919, 2006.

Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, Flament C, Pouzieux S, Faure F, Tursz T, Angevin E, Amigorena S, Zitvogel L. Tumorderived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med. 2001; 7: 297−303.

Yamada K, Sachs DH, DerSimonian H. Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. J Immunol. 1995 Dec 1;155(11):5249-56.

Yamamoto F, Yamamoto M: Molecular genetic basis of porcine histo-blood group AO system. Blood 97:3308, 2001.

Young BA, Burdmann EA, Johnson RJ, Alpers CE, Giachelli CM, Eng E, Andoh T, Bennett WM, Couser WG: Cellular proliferation and macrophage influx precede interstitial fibrosis in cyclosporine nephrotoxicity. Kidney Int 48: 439 − 448, 1995.

Zeevi A, Lunz J. Cylex ImmuKnow Cell Function Assay. Methods Mol Biol.2013;1034:343-51.

Zhang W, Victor RG: Calcineurin inhibitors cause renal afferent activation in rats: A novel mechanism of cyclosporine-induced hypertension. Am J Hypertens 13: 999 − 1004, 2000.

Zhang N, Qi J, Feng S, Gao F, Liu J, Pan X, Chen R, Li Q, Chen Z, Li X, Xia C, Gao GF. Crystal structure of swine major histocompatibility complex class I SLA-1 0401 and identification of 2009 pandemic swine-origin influenza A H1N1 virus cytotoxic T lymphocyte epitope peptides. J Virol. 2011 Nov;85(22):11709-24.

Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, Gross P, Knepper MA, Star RA. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int. 2006 Apr;69(8):1471-6.

Zhu L, Gong D, Zou Y, et al. Cervical heterotopic kidney transplantation in rats using non-suturing and preserving-bag techniques. Nephrol Dial Transplant 2009; 24: 2708.

Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G, Amigorena S. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med. 1998; 4: 594–600.