Urine: A New Tool for Molecular Diagnosis of Chronic Rejection in Kidney Transplantation?

Dissertação desenvolvida sob orientação científica:

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On the cover: Kidneys digital illustration.

(Withdrawn from: https://www.uab.edu/medicine/news/images/News/greg_kidney.jpg)

"You don´t have to be great to start, but you have to start to be great".

Zig Ziglar

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Abbreviations

°C – Celsius degree

18SRNA – Ribosomal subunit 18S

ACTB – β-actin

AHR – Acute Humoral Rejection

APC – Antigen-Presenting Cells

AUC – Area Under the Curve

B2M – β-2-microglobulin

BCR – B-cell Receptor

BMI – Body Mass Index

Breg – Regulatory B cell

C4d – Complement component 4d

CCL2 – Chemokine (C-C motif) ligand 2

CCL3 – Chemokine (C-C motif) ligand 3

CCL4 – Chemokine (C-C motif) ligand 4

CCL5 – Chemokine (C-C motif) ligand 5

CCR2 – C-C Chemokine receptor type 2

CCR6 – C-C Chemokine receptor type 6

CCR5 – C-C Chemokine receptor type 5

CD10 – Cluster of Differentiation 10

CD103 – Cluster of Differentiation 103

CD19 – Cluster of Differentiation 19

CD19 – Gene encoding the Cluster of Differentiation 19

CD25 – Cluster of Differentiation 25

CD27 – Cluster of Differentiation 27

CD28 – Cluster of Differentiation 28

CD3 – Cluster of Differentiation 3

CD30 – Cluster of Differentiation 30

CD3E – Cluster of Differentiation 3E

CD4 – Cluster of Differentiation 4

CD40 – Cluster of Differentiation 40

CD40L – Cluster of Differentiation 40 ligand

CD68 – Cluster of Differentiation 68

CD79B – Gene encoding the Cluster of Differentiation 79B

CD79B – Cluster of Differentiation 79B

CD8 – Cluster of Differentiation 8

CD80 – Cluster of Differentiation 80

CD86 – Cluster of Differentiation 86

CD95 – Cluster of Differentiation 95 ligand

CD95 – Cluster of Differentiation 95

cDNA – Complementary Deoxyribonucleic Acid

CGN – Chronic Glomerulonephritis

CMVab – Cytomegalovirus antibody

CP – Chronic Pyelonephritis

CsA – Cyclosporin A

CTLA4 – Cytotoxic T-lymphocyte-associated protein 4

CTLs – Cytotoxic T cells

CTOT-O4 – Clinical Trials in Organ Transplantation

CX3CR1 – Chemokine (C-X3-C motif) receptor 1

CXCL10 – C-X-C motif Chemokine 10

CXCL10 – Gene encoding the C-X-C motif Chemokine 10

CXCL9 – C-X-C motif Chemokine 9

CXCL9 – Gene encoding the C-X-C motif Chemokine 9

CXCR3 – Chemokine (C-X-C motif) receptor 3

CYC1 – Cytochrome C-1

Dc – Dendritic cell

DNA – Deoxyribonucleic Acid

DSA – Donor Specific Antibody

EDTA – Ethylenediaminetetraacetic Acid

ESRD – End-Stage Renal Disease

FASL – FAS ligand

FK506 – Tacrolimus

FOXP3 – Forkhead box p3

FOXP3 – Gene encoding the transcription factor FOXP3

GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase

GATA3 – Gene encoding the transcription factor GATA3

HLA – "Human Leukocyte Antigen"

IFNα – Alpha Interferon

IFNβ – Beta Interferon

IFNγ – Gama Interferon

Ig – Immunoglobulin

IgG – Immunoglobulin G

IL1 – Interleukin 1

IL10 – Interleukin 10

IL10 – Gene encoding Interleukin 10

IL12 – Interleukin 12

IL13 – Interleukin 13

IL15 – Interleukin 15

IL17 – Interleukin 17

IL17A – Interleukin 17 alpha

IL1b – Interleukin 1 beta

IL2 – Interleukin 2

IL21 – Interleukin 21

IL22 – Interleukin 22

IL23 – Interleukin 23

IL25 – Interleukin 25

IL26 – Interleukin 26

IL31 – Interleukin 31

IL33 – Interleukin 33

IL35 – Interleukin 35

IL4 – Interleukin 4

IL4 – Gene encoding Interleukin 4

IL5 – Interleukin 5

IL6 – Interleukin 6

Kg – Kilograms

mg – milligrams

MHC – Major Histocompatibility Complex

mRNA – Messenger Ribonucleic Acid

NGE – Normalized Gene Expression

NK – Natural Killer cell

nTh – naïve T helper cell

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Reaction

PE – Phycoerythrin

PKD – Polycystic Kidney Disease

PRA – Panel Reactive Antibody

qPCR – Quantitative Polymerase Chain Reaction

RCF – Relative Centrifugal Force

RNA – Ribonucleic Acid

RNFW – Rnase Free-Water

ROC – Receiver Operating Characteristic

RQ – Relativity Quantity

RT-PCR – Real-Time Polymerase Chain Reaction

SAPE – Streptavidin-Phycoerythrin Conjugate

SD – Standard Deviation

SF3A1 – Subunit 1 of Splicing Factor 3a

STAT4 – Signal transducer and Activator of Transcription 4

STAT6 – Signal transducer and Activator of Transcription 6

STRs – Short Tandem Repeat

Tc – T cell

TCR – T cell Receptor

TE – Tris-- Ethylenediaminetetraacetic Acid

Tfh – T follicular helper cell

TGF-β1 – Gene encoding the Tumor Growth Factor β1

TGF-β1 - Tumor Growth Factor β1

Th –T helper cell

TNF3 – Tumor Necrosis Factor 3

TNF3 – Gene encoding Tumor Necrosis Factor 3

TNFR2 –Tumor Necrosis Factor Receptor 2

TNFα – Tumor Necrosis Factor alpha

TNFβ – Tumor Necrosis Factor beta

Treg – Regulatory T cells

UBC – Ubiquitin C

XCL1 – Chemokine (C motif) ligand (XCL1)

Abstract

Renal transplantation has become the treatment of choice for patients with end-stage renal disease. Recognizable improvements in early graft survival and long-term graft function have made kidney transplantation a more cost-effective alternative to dialysis. Moreover, the discovery of new immunosuppressive agents has made a significant impact on short-term graft survival.

Despite these improvements a substantial portion of grafts develop progressive dysfunction and fail within a decade by a process known as chronic rejection. Ongoing monitoring of kidney transplants is crucial to avoid the development of this condition. The most common approaches to monitor renal allograft function are the measurement of the serum creatinine levels, whose variations are not specific for rejection and sometimes there is a need to perform renal biopsies, which is a risky process and only diagnoses rejection once it is installed.

The main objective of this study was to create an immunological and cellular profile associated with chronic dysfunction establishment that could serve as a diagnostic tool. For this purpose, a series of different analyses were carried out on 71 patients with stable/normal renal function after the transplant and the results compared to the same tests performed on 27 patients who have been diagnosed with chronic graft rejection.

The development of an antibody screening method in the urine of renal transplant patients was one of the most relevant points in the study with more than half the patients diagnosed with chronic rejection presenting anti-HLA Class I antibodies in urine.

The normalized gene expression values found in the urinary sediment of patients diagnosed with chronic rejection confirm the involvement of inflammation in the development of chronic rejection. Furthermore, the increased normalized gene expression levels for B cells markers (*CD19* and *CD79B*) in chronic rejection group suggest the existence of B cells clusters that can function as a tertiary lymphoid tissue which can harbour B cell maturation into memory B cells and antibody producing plasma cells.

According to the receiving operating characteristic curves, *CD19*, *CXCL10* and *TNF3* were the genes with the highest diagnostic values for chronic rejection.

This study demonstrates that analysis of the urine of renal transplant patients could give valuable information that may allow the monitoring of the transplant without resort to invasive methods. Nevertheless, only the combination of results obtained in the urine and blood samples can provide a complete and accurate assessment of the allograft condition.

Keywords: Renal transplant; Chronic rejection; Biomarkers.

Resumo

A transplantação renal tornou-se a terapia de eleição para doentes com insuficiência renal crónica terminal. As melhorias, em termos de sobrevivência do enxerto e da função renal a longo prazo, fizeram com que a transplantação renal tenha uma melhor relação custo/benefício do que a diálise. A acrescentar a isto, a introdução de novos agentes imunossupressores teve um impacto significativo na sobrevivência do enxerto a curto prazo.

Apesar destas melhorias, uma quantidade substancial dos enxertos desenvolvem uma disfunção progressiva com perda de função total no prazo de uma década, através de um processo denominado rejeição crónica. A monitorização funcional dos rins transplantados torna-se crucial para tentar evitar o desenvolvimento desta condição. Os métodos mais comuns para realizar a monitorização da função do enxerto são as medições dos níveis de creatinina no soro, sendo que estas variações não são específicas para a rejeição e ainda, por vezes, é necessário realizar biopsias renais, que é um processo arriscado e que só diagnostica esta rejeição depois de esta estar instalada.

O principal objetivo deste estudo foi desenvolver um perfil imunológico e celular, associado ao estabelecimento da disfunção crónica, que possa servir como ferramenta de diagnóstico. Tendo isto em vista, um conjunto de análises foram efetuadas em 71 transplantados renais com uma função renal estável e os resultados, comparados com os mesmos testes efetuados em transplantados renais que tinham sido diagnosticados com rejeição crónica.

O desenvolvimento de um novo método de seleção de anticorpos na urina de transplantados renais foi um dos pontos mais importantes neste estudo, sendo que mais de metade dos transplantados com rejeição crónica apresentou anticorpos anti-HLA Classe I na urina.

Os valores da expressão génica normalizada encontrados para as células do sedimento urinário dos transplantados renais, confirmaram o envolvimento da inflamação no desenvolvimento da rejeição crónica. Além disso, os valores mais elevados de expressão génica normalizada para os marcadores da célula B (*CD19* e *CD79B*), no grupo da rejeição crónica, sugerem a existência de aglomerados de células B que funcionam como um órgão linfoide terciário, capaz de albergar a maturação da célula B em células de memória e em células do plasma produtoras de anticorpos.

De acordo com a análise feita das curvas características de operação do recetor, os genes *CD19*, *CXCL10* e *TNF3* foram os que obtiveram maior valor de diagnóstico para a rejeição crónica.

Este estudo comprova que a análise da urina de transplantados renais, no futuro breve, pode fornecer informações valiosas, permitindo assim, a monitorização funcional destes doentes sem recurso a procedimentos invasivos. No entanto, só a combinação dos resultados obtidos na urina e no sangue pode oferecer uma avaliação completa e precisa da condição do enxerto.

Palavras-chave: Transplantação renal; Rejeição crónica; Biomarcadores.

1. Introduction

1.1 Transplantation

The term transplantation, in immunology, refers to the act of transferring healthy cells, tissues or organs from one person (donor) to another (receptor/recipient) in order to re-establish a lost function. The organs, tissues or cells transplanted are called grafts (1, 2).

Organ transplant has revolutionized the treatment options for various diseases. Patients with end-stage renal disease (ESRD) only had dialysis as possible treatment. Nowadays kidney transplant has become the treatment of choice for most patients with this condition, mainly due to the better quality of life and increased survival that it offers when compared to dialysis (3, 4).

The first fruitful experimental kidney transplants were performed at the Vienna Medical School in Austria with animals in 1902. Only in 1954, Joseph E. Murray and his colleagues at Peter Bent Brigham Hospital in Boston performed the first truly successful kidney transplant in humans from one twin to another identical twin. So we can consider transplantation as a recent phenomenon since the biggest developments have taken place within the past 60 years (5).

Presently, there are two main barriers that need to be overcome in organ transplantation: first the disproportion between the number of patients waiting for the transplant and the number of organs available for transplant, the second and most important barrier is the immune system, as it recognizes the graft as foreign and initiates a series of mechanisms that lead to graft rejection (6, 7).

Nowadays the methods available to monitor and evaluate graft function are the measurement of blood creatinine levels or invasive and painful procedures like biopsies. The main concerns with these methods are that variations on the creatinine levels are not specific of rejection and biopsies stand, as an expensive and difficult medical procedure that can lead to inaccurate diagnoses, since it only analyses a small piece of tissue that may not show any signs of rejection, when the rejection could be already installed on different regions of the graft. Furthermore, biopsies often detect pathological changes at advanced and irreversible stages of graft damage (8).

Therefore, there is a need to develop more sensitive and specific methodologies based on donor and recipient genotyping, transcriptional and proteomic profile to differentiate and detect early stages of graft injury (9).

The answer may well lie on the use of biomarkers in kidney transplants. Currently a biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses

to a therapeutic intervention" by the Biomarker Definitions Working Group (10). Indeed, the validation of pre and post-transplant biomarkers can lead to better outcomes in graft survival as they can help understand the distinct molecular and cellular mechanisms triggered by allotransplants. The ideal biomarker should reflect alterations of allograft function as they happen, allow better matching between donor and recipient and get an insight of a person's susceptibility to graft rejection (11).

1.1.1 Transplant Types

The origin of the graft determines the type of transplant. When the graft is transferred from one part of the body to another in the same individual it is called an autotransplant. When the graft is transferred between two genetically identical individuals from the same species is named isotransplant, this type of transplant is characteristic of transplants between twins. While a transplant concerning two individuals of the same species but genetically different is known as allotransplant. This is the most common type of transplant and the one studied in this work. At last, when a graft is transferred between individuals from different species is called xenotransplant (1, 2).

Each type of transplant elicits a different immune response. For autologous and isotransplants the immune response is practically non-existent. In contrast, the xeno and allotransplants trigger a strong immune response due to the presence of cells from the donor that are recognized as foreign by the recipient's immune system, that ultimately may lead to graft rejection. The different types of immune responses and the mechanisms involved in the process of graft rejection will be explained later.

1.2 Graft rejection

The process of rejection, in transplantation, is defined by the time post-transplant the rejection occurs and also, according to the histopathological characteristics presented by the graft on biopsies. There are 3 types of graft rejection: hyperacute rejection, acute rejection and chronic rejection.

The hyperacute rejection occurs in the first 24 hours after transplant and it is characterized by thrombotic occlusion of the graft vasculature. Acute rejection usually happens on the first 6 months after transplant and the main feature is a rapid graft dysfunction due to inflammation, however, in the past three decades, there has been a dramatic reduction in the incidence of acute rejection with introduction of potent immunosuppressive drugs (1, 2).

Finally, chronic rejection is a result of a progressive functional decline of the graft over a period of months to years after transplant. Currently, chronic rejection is the most prevalent cause of late renal allograft loss and, as there is no cure for this condition, it is considered a major concern in the field (1, 2). The mechanisms and molecules involved in graft rejection will be described throughout the thesis.

1.3 Major histocompatibility complex (MHC)

The major histocompatibility complex (MHC), located on the short arm of Chromosome 6 (6p21), is a large region of DNA containing over 200 coding *loci*. In humans, the MHC is known as the "Human Leukocyte Antigen" system (HLA) and was first described in the 1950's, when it was revealed that HLA antigens present on human leukocytes could react with antibodies produced during blood transfusions and pregnancies (12, 13) (Figure 1).

At the present time, the involvement of these HLA molecules in the recognition and presentation of self and foreign antigens is well described (14). Moreover the HLA system is inherited as a HLA haplotype in a Mendelian fashion system, as you receive a haplotype from each parent, genetic recombination can lead to new allelic combinations (15). In clinical transplantation, they play a crucial role in the alloimmune response leading to graft acceptance or graft rejection (16). The new allelic combinations dramatically reduce the probability of two unrelated individuals to have identical HLA genes making the HLA the major immunological barrier for organ transplant as the number of HLA disparities between donor and receptor increases the risk of graft failure (14).

The HLA system is divided into two regions encoding two different Classes of molecules, HLA Class I and HLA Class II. There is a third region between HLA Class I and Class II known as MHC III region that does not belong to the HLA system (17).

The HLA Class I glycopeptide antigens are virtually expressed on the surface of most nucleated cells where they bind and present peptides to circulating CD8+ T cells, also known as cytotoxic T cells (Tc). The Class I region contains the classical *HLA-A*, *HLA-B*, and *HLA-C* genes that encode the heavy chains (α) of Class I molecules (18, 19).

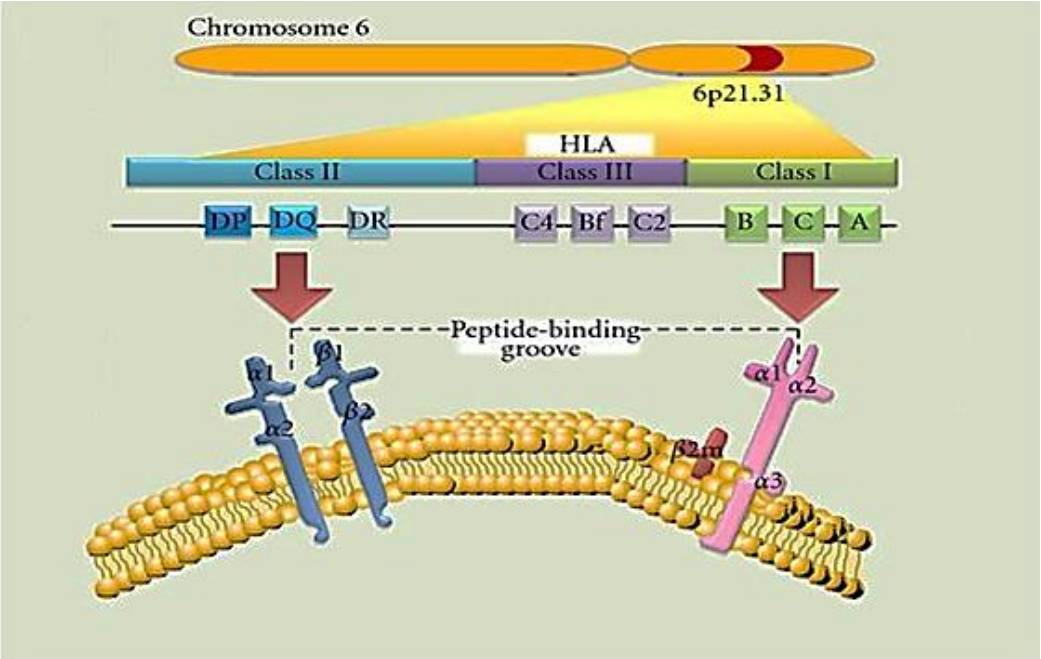

Figure 1- Localization of the MHC complex on chromosome 6. The HLA system represents the most polymorphic genes of the human genome. The HLA genes are divided in HLA Class I and HLA Class II according to their structure, expression and function of the molecules they express. The structure of both Classes is also represented here. (Adapted from: AYALA GARCIA, M.A. [et al.]. - The major histocompatibility complex in transplantation. Journal of transplantation.2012 (2012) 842141.)

The cell surface glycopeptide antigens of the *HLA-DP*, *HLA-DQ* and *HLA-DR loci* establish the Classic HLA Class II molecules and their function is to present peptides to T helper cells (Th). The expression of HLA Class II antigens is restricted to the antigen-presenting cells (APCs), including dendritic cells (Dcs), B-lymphocytes, macrophages and endothelial cells (20), however they can also be expressed in other cells after stimulus. The products of the Class II genes *DR*, *DP*, and *DQ*, are heterodimers of two non-covalently associated glycosylated polypeptide chains; α and β, as shown in Figure I (7).

The MHC Class III region of the genome encodes several molecules that take part in inflammation processes, including some complement components (21).

The HLA Class I molecules display an elevated degree of polymorphism mainly due to the variable amino acid sequence of the α1 and α2 domains, which determine the antigenic specificities of the HLA Class I molecules .The polymorphisms of Class II molecules occur in the first amino terminal β1 domain of *DRB1*, *DQB1*, and *DPB1* gene products. This polymorphism is essential to an efficient adaptive immune response since it allows a large diversity of peptide antigen recognition and presentation to T cells (22).

Conversely, the extremely high polymorphism contributes to the HLA disparities between donor and receptor emphasizing the importance of the HLA system in organ

transplant. There are a series of methodologies available to achieve donor receptor compatibility, including HLA Typing, Cross Matching and Antibody Screening (5). The best match to a certain donor is obtained by an algorithm defined by law in each country and is always changing mainly due to technological advances that help developing new ways to get closer to the perfect match but also due to ethics/moral concerns.

1.4 The Immune System

The immune system has the capacity to respond to external threats including microorganisms or peptides. This response can be divided into two types: innate response with a large spectrum of specificity or adaptive response with a restricted specificity. These two types of response are different but present a close collaboration between them to enhance the efficiency of the immune response (23).

The first non-specific line of defence is provided by the innate immune system which includes the epithelial barrier, phagocytic cells such as neutrophils and macrophages, dendritic cells that capture and present antigens, cells that release inflammatory mediators such as mast cells and natural killer cells (NK) (24). The molecules that participate in the innate immune response are cytokines and plasma proteins such as complement factors and acute phase proteins (25). The innate immune response stimulates the adaptive immune response creating an important cooperation that makes the immune system able to respond to almost any type of aggression (25).

Adaptive immune responses are initiated when antigen-receptors on lymphocytes recognize a foreign antigen. T-lymphocytes mediate cellular immunity and B-lymphocytes mediate humoral or antibody-mediated immunity. The adaptive immune system has an immunological memory, making the immune response faster and stronger to the same antigen (26).

1.4.1 T cell activation

Alloantigens refer to the antigens expressed exclusively by the donor. These alloantigens are considered the main cause for graft rejection and the HLA allogeneic molecules stand as the major target for immune response due to their extremely high polymorphism associated with their extraordinary capacity to generate polyclonal T-cell responses.

As described before one of the functions of the HLA molecules expressed on the surface of the APC is to bind and present antigens to the T cells (27). These antigens are recognized by the T cell receptors (TCR) expressed on the surface of T cells. This is the first

step to initiate an immune response (28). The TCR consists of one α-chain and one β-chain that together directly recognize peptide-HLA ligands and, it is associated with CD3 subunits (29). Depending on the antigen, the HLA Class that binds the allogeneic peptide will differ as well as the core receptor on the T cell. The core receptor CD8 will help recognize HLA Class I molecules bound to the allogeneic peptide and CD4 will assist in the recognition of the peptide bound to the HLA Class II molecule (30).

The recognition of the peptide-HLA molecule is the first signal to initiate an immune response but alone is not enough to completely activate T cells. Two more signals are necessary to promote T-cell proliferation: cytokine secretion and effector function after T cell activation (18).

The second signal can be costimulatory or coinhibitory and a variety of molecules can be receptors for this signal (31). The strongest receptor of costimulatory signals is CD28 that is expressed on T cells (CD4 and CD8) and has two known ligands: CD80 and CD86. These ligands are expressed on activated APCs. The most common coinhibitory signal receptor is cytotoxic T-lymphocyte-associated protein 4 (CTLA4) that also can bind to CD80 and CD86 as it presents a similar structure as CD28 (32). However, when CTLA4 binds to the APCs, sends an inhibitory signal that ends the immune response. Another molecule that has a crucial role in the immune response is CD40 ligand (CD40L) expressed on activated T cells. The CD40L binds to CD40, expressed on APCs, inducing the expression of CD80 and CD86, improving the efficacy of T cell activation (33).

A third signal is required to complete the activation of T cells. Recent studies have shown that interleukin 12 (IL12) and interferon (IFN) α/β are the most important sources for this signal in various responses. There have also been some reports suggesting that IL1can provide a third signal, especially for CD4 T cells (34).

All the mechanisms involved in T cell activation are schematized on Figure 2.

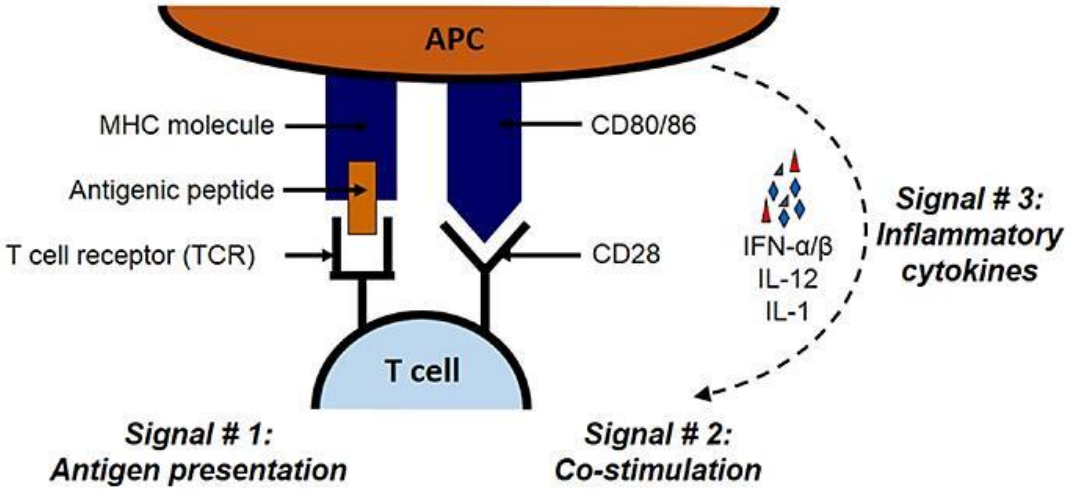

Figure 2- The three signals required for the stimulation of antigen-specific T cell. First, antigenic proteins go through antigen processing and then the peptides are presented through MHC Class I or II molecules for CD8 and CD4 T cells, respectively. The second signal is known as co-stimulation and is provided by molecules from B7 family member proteins CD86 and CD80 expressed on APCs. These B7 proteins interact with their receptors such as CD28 present on T cells. Inflammatory cytokines such as IFN-α/β, IL12, and IL1 constitute the third signal. (Source: Gujar SA, Lee PW. Oncolytic virus-mediated reversal of impaired tumor antigen presentation. Frontiers in oncology. 2014;4:77)

1.4.2 Direct and Indirect allorecognition pathway

Alloreactive T cells recognise alloantigens via two distinct, but not mutually exclusive pathways: direct and indirect (35). Direct recognition occurs when recipient T cells recognise intact donor MHC molecules complexed with peptide on donor APCs, without processing by recipient APCs. This happens, essentially because the majority of the grafts contain resident APCs like macrophages and dendritic cells (36). Allorecognition via the indirect pathway requires that recipient APCs migrate to the graft and process the donor-HLA antigen before presenting it to recipient T cells. Both pathways contribute to allograft rejection (36).

The direct pathway has been associated with acute rejection and the indirect pathway appears to have a bigger involvement in chronic rejection (37). A third pathway, which may serve as a link between the direct and indirect pathways, has been proposed. Recipient Dendritic cells can acquire intact HLA molecules from donor cells or tissues and at the same time have the capacity to activate recipient T cells leading to direct anti-donor alloimmune responses (Figure 3)(38).

The indirect pathway activates naïve CD4+ (T-helper cells) as the foreign antigen is presented by a HLA Class II molecule. The direct pathway can activate both naïve T-helper

cells and naïve CD8+ cells depending on the HLA Class that presents the allogeneic peptide (25).

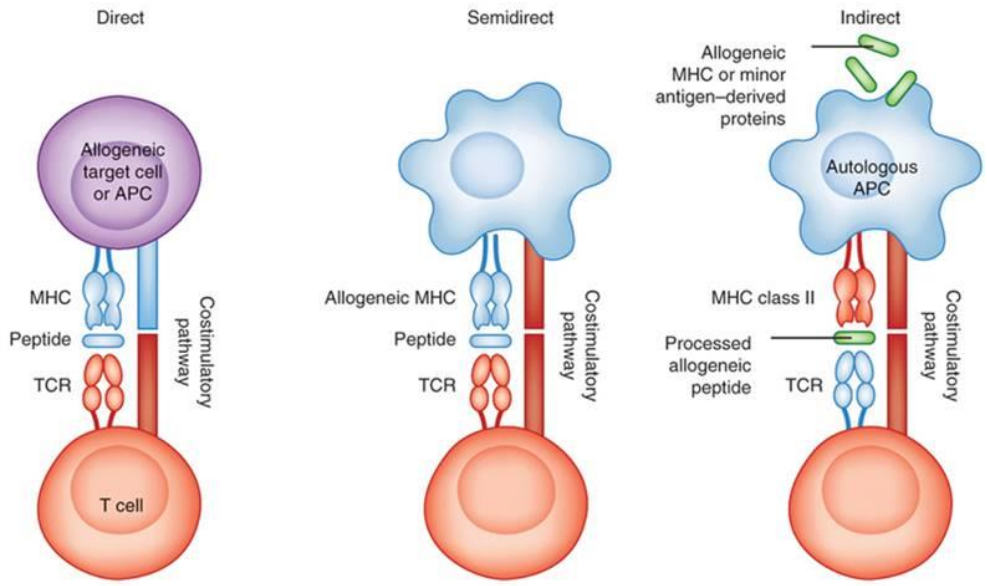

Figure 3- The three pathways of allorecognition by T cells. In direct allorecognition, as the name suggests, T cells are activated directly by APCs or any cell expressing allogeneic MHC. The occurrence of indirect recognition relies on foreign antigen processing by autologous APCs. Then, peptides derived from these allogeneic antigens are cross-presented by autologous MHC Class II on autologous APCs. In semidirect allorecognition, allogeneic MHC Class I or MHC Class II molecules are assimilated and MHC-peptide complexes presented by autologous APCs. (Adapted from: ZAKRZEWSKI, J.L.[et al.]. - Overcoming immunological barriers in regenerative medicine. Nature biotechnology.32 (2014) 786-794.)

Naïve CD8+ T cells differentiate into CD8+ cytotoxic T cells (CTLs) after foreign peptide–HLA Class I presentation, causing the release of cytokines, mostly IL12. The CTLs are effector cells of the immune system and their activation leads to the formation of granules that contain perforin and granzyme B (39). These molecules are released on the synaptic cleft between the effector cell and the target cell (39).

Recent studies proved that perforin is the key molecule, since it forms transient pores on the surface of target cells, providing a short window of time for direct entry of granzyme B into the cytosol (40, 41). Once inside the target cell, granzyme B activates cell-death pathways that operate through the activation of caspases (particularly caspase-3), but it also leads to cell death in the absence of activated caspases, by engaging aggregation of target cell death receptors, like Fas, by their cognate ligands, such as Fas ligand (FasL), on the killer-cell membrane, which results in classical caspase-dependent apoptosis (Figure 4) (35, 42).

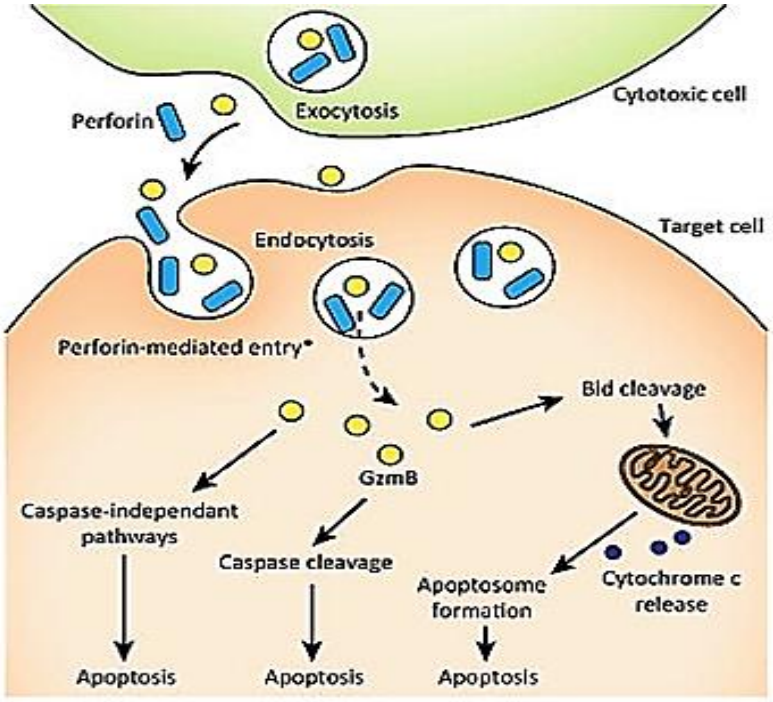

Figure 4- The granzyme B–Perforin cytotoxic pathway. Granzyme B and perforin are released from the granules of a cytotoxic cell to the synaptic cleft. Then perforin forms transient pores allowing granzyme B to enter the target cell. Once inside the target cell cytoplasm, granzyme B can initiate apoptosis through both caspase-dependant and caspase-independent pathways by cleaving a number of substrates. (Adapted from: HIEBERT, P.R., GRANVILLE, D.J. - granzyme B in injury, inflammation, and repair. Trends in molecular medicine.18 (2012) 732-741.)

The naïve T-helper cells (nTh) are one of the first immune cells to be activated post-transplant, playing a key role in rejection. When activated the nTh cells have the capacity to differentiate into one of several T helper cell lineages, including the classical T-helper 1 (Th1) and T-helper 2 (Th2), T-helper 17 (Th17) and regulatory T cells (Treg), each with a characteristic cytokine profile, transcription factor, and signalling pathway through which their differentiation is mediated (Figure 5) (43).

The presentation of the antigen to naïve CD4+ T cells with the presence of IL12 and NK cell-derived interferon gamma (IFNγ) induces Th1 cells activation (44). This activation requires T-bet (T-box expressed in T cells) and signal transducer and activator of transcription 4 (STAT4) (45). When activated, they predominantly produce IL2, IFNγ and tumour necrosis factor α (TNFα).Th1 cytokines collectively promote cell-mediated immune responses (46).

Th2 activation is stimulated by IL4 and IL33 and depends on GATA3 and STAT6 as transcription factors (47). Cytokines produced by Th2 activated cells include IL4, IL5, IL6, IL9, IL10 and IL13 (48). Some of these Th2 cytokines downregulate Th1 cytokines

productions, while others, in conjunction with interleukins released from tissue-derived and dendritic cells (IL25, IL31, IL33), facilitate humoral responses (49-51).

Th17 cells are involved in humoral immune responses, but may also contribute to the cell-mediated immune responses due to their ability to produce interleukin-17 (IL17). Th17 polarization in humans is dependent upon IL1b, IL6, IL21, and IL23 and the transcription factor, RORγt (52). Once activated, Th17 cells secrete IL17A, IL26 and IL22 that stand as potent pro-inflammatory mediators. Furthermore, the release of these cytokines causes chemokine secretion by resident cells stimulating the recruitment of neutrophils and macrophages to inflammation sites (53).

The activation of macrophages by Th1 and some Th17 cells will trigger cell-mediated immune responses. The macrophages are remarkable phagocytic cells with huge plasticity, so naturally, they are considered one of the most important immune effector cells (54).

Th2 and Th17 cells activate B cells that mature into plasma cells that are known by their ability to produce antibodies against the foreign antigens. These antibodies are called immunoglobulins (Ig) and they can be divided into five classes, each of which mediates a characteristic biological response following antigen binding (46).

Regulatory T cells are a heterogeneous group of T lymphocytes that control potentially harmful autoreactive T cells in the periphery. In transplantation, they seem to be involved in the establishment of tolerance (55). Their development is dependent on the expression of the transcription factor forkhead box p3 (FOXP3). IL2 and CD25 also seem to have an important part in the development of natural Treg cells. The induction of Treg cells is favoured by environments where transforming growth factor (TGF)-β1 is present. Thymus-derived Tregs suppress alloreactive Th1 and Th2 cells as well as alloreactive CD8+ Tcells and alloantibody-producing B cells by secreting immunosuppressive cytokines including TGF-β1, IL10 and IL35. This ability is crucial to maintain immune homeostasis (51).

It should be noted that, more than describe all the mechanims of the immune system in detail, this introduction was intended to show the key agents involved in immunological responses as well as their immunological diversity and above all, ilustrate the cooperativeness of the different types of immune responses responsible for graft rejection.

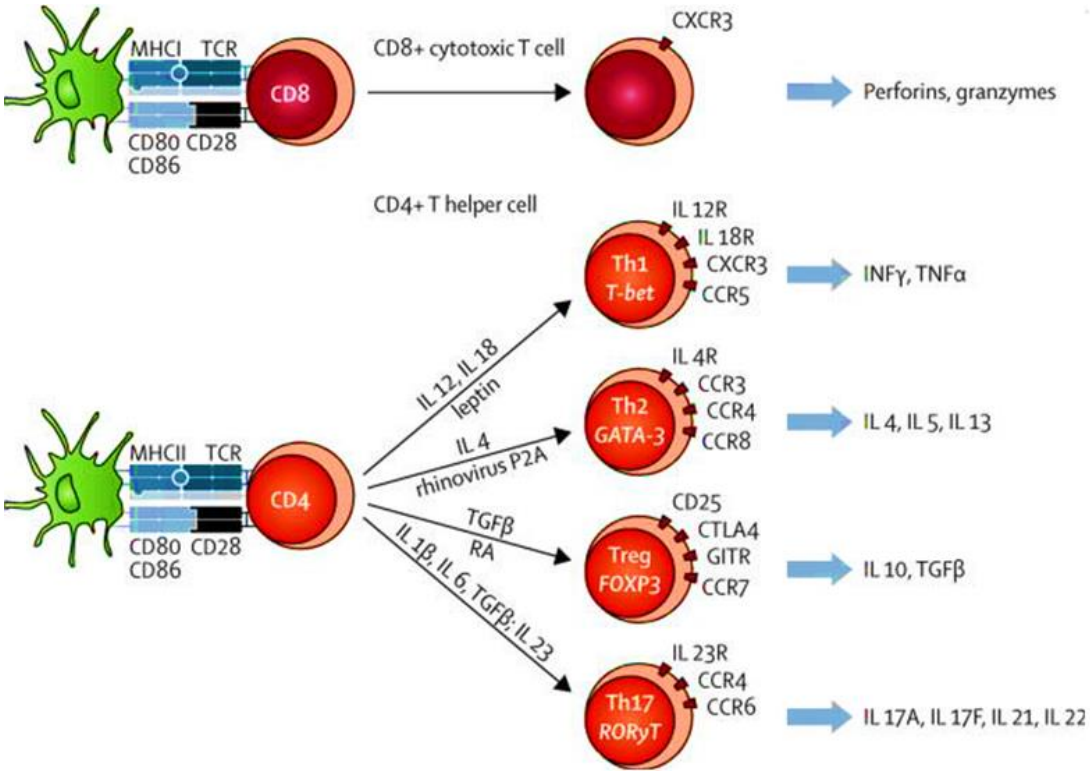

Figure 5 - CD4+ T helper cell differentiation and CD8+ T-cell cytotoxicity. HLA Class I molecules induce CD8+ T-cell cytotoxicity whereas HLA Class II APCs conduct the differentiation of naive CD4+ T cells towards T helper (Th) 1, Th2, Th17 cells or regulatory T cells depending on the cytokine balance in the local microenvironment. The different Th cell subsets and Treg cells have different characteristic cytokine profiles, transcription factors necessity and signalling pathways.
(Withdrawn from: BRUSSELLE, G.G. [et al.] - New insights into the immunology of chronic obstructive pulmonary disease. Lancet.378 (2011) 1015-1026.)

1.5 B cells and their role in transplanted organ rejection

As explained before, the main role of B-cells is to serve as precursors of antibodies. The activation of B-cells takes place when the antigen binds to the B-cell receptor (BCR) that generates a signalling process that culminates in presentation to CD4+ cells of the antigen and BCR by the HLA Class II molecules, as described above. In this interaction there is another subtype of T-cells that is essential to B cell maturing: T follicular helper cells (Tfh). These Tfh deliver the cytokines necessary, predominantly IL21 (56, 57). This creates the proper environment to B cells to differentiate into two cells types: memory B cells and antibody-producing plasma cells.

The antibodies produced by these plasma cells are specific for the donor cells, increasing the immune response against the graft cells and lowering the graft survival (58). This the primary role of B cells in transplant rejection. Still, a considerable number (30%–50%) of kidney and heart allograft recipients undergoing chronic rejection do not seem to

have detectable circulating HLA antibodies or complement deposits in the graft. Moreover some recent studies have shown that B cells may also contribute to transplant rejection, perhaps by facilitating cellular immune responses (59).

In addition to producing antibodies, B cells influence T cell responses by mechanisms such as antigen presentation, cytokine production and costimulation for productive immunity (59).

Memory B cells contribute to serological immunity by rapidly differentiating into plasma cells when exposed to the same antigen as when they were formed. CD27 is the known marker of memory B cells (60, 61).

In contrast, a specific subset of B cells, regulatory B cells (Breg), seem to have the ability to induce tolerance by either blocking the antigen recognition or by suppressing immunity by secreting IL10 (55, 62).

1.6 Hyperacute Rejection

Hyperacute rejection is humorally mediated and happens due to the existence of preformed donor-specific anti-HLA antibodies. These antibodies bind to blood vessels and activate the complement system, which initiates a cascade of events leading to coagulation of platelets and immediate circulation blockage that ultimately results in loss of graft function (63, 64). These preformed anti-HLA antibodies can be induced by prior blood transfusions, multiple pregnancies or even previous transplants (12, 13, 65).

1.7 Acute Rejection

1.7.1 Cell-mediated Immune response in acute rejection

A sudden increase in serum creatinine, fluid retention, and occasionally fever and graft inflammation are the symptoms that patients with acute cellular rejection often exhibit.

Since the current therapy is mostly directed at T cells, the incidence of acute rejection is approximately 5%–10% in the first year in unsensitized patients (66). Clinically, acute cellular rejection is characterized by the gathering of mononuclear cells in the interstitium associated with inflammation of the tubules and, occasionally, of the arteries (63).

The mononuclear cells that infiltrate the interstitial space around tubules are essentially CD4+ and CD8+ T cells (67). T cells contain cytotoxic granules (perforin and granzyme A and B) or the cytotoxic effector ligand, FasL that are able to trigger apoptosis by the mechanisms described above. This is naturally accompanied by an increase in the CTL

associated transcripts mRNA: granzyme B, perforin, and FasL, as well as T-bet (68-70). There are other cytokines and chemokines selectively expressed in acute rejection like IFNγ, TNFβ, TNFα and chemokine (C-C motif) ligand 5 (CCL5) (71).

Another important feature of acute rejection is the invasion of the tubular epithelium by infiltrating T cells and macrophages, clinically known as tubulitis (63). In severe cases, graft dysfunction and progressive tubular loss can occur due to rupture of the tubular basement membrane causing the release of proteins into the interstitium (72). The degree of apoptosis correlates with the number of cytotoxic cells and macrophages recruited to the tubular epithelium (72).

There are other molecules present in tubular epithelium like CD103 and Tregs (73). CD 103 seems to play a role in fixating T cells in the epithelial layer by binding to E-cadherin. Until now, the function of Tregs in the tubules is not clear yet. It is only natural that the levels of mRNA for CD103, perforin, granzyme B, and FOXP3 are increased in the urine of patients with acute rejection (74).

During acute rejection, a variety of chemokines are produced in the graft, including C-X-C motif chemokine 10 (CXCL10), CCL2, CCL3, CCL4, CCL5, and XCL1 that are postulated to participate in T cell recruitment to the graft and cytokines TNFα, TGF-β1, and IL6 (75-78).

TGF-β1 is the central molecule that regulates epithelial-mesenchymal transition and its expression by tubular cells, leads to abnormal phenotypes usually seen in grafts suffering from rejection (79).

Infiltrating cells express several chemokine receptors, including CCR2, CCR5, CXCR3, and CX3CR1. The pattern of expression suggests a predominance of Th1 over Th2 cells (75). CCR5 is probably important in the pathogenesis of rejection, as humans who are homozygous for inactive 32 forms have a greater graft survival than those with the active form (80).

Glomerulitis is, sporadically, a visible feature of acute cellular rejection. The cells in the glomeruli are fundamentally a mixture between CD3 T cells and a small number of CD68 macrophages (81). The mechanisms proposed for cell mediated injury to the graft are schematized on Figure 6.

1.7.2 Humoral Immunity in Acute rejection

Approximately 25% of acute rejection episodes are due, at least in part, to anti-HLA antibodies against the donor. Risk factors include presensitization and reduced

immunosuppression (82). However, despite the immunosuppression treatment chosen, there is a possibility to develop an acute humoral rejection (AHR), even with strong depleting T cell therapy (78).

The binding of anti-donor antibodies to the endothelium of the graft activates complement, which triggers the recruitment of cellular infiltrates, neutrophils and macrophages in the capillaries (83). Both cellular and antibody-mediated acute rejection episodes are more frequent in sensitized patients than in patients without circulating antibodies (84).

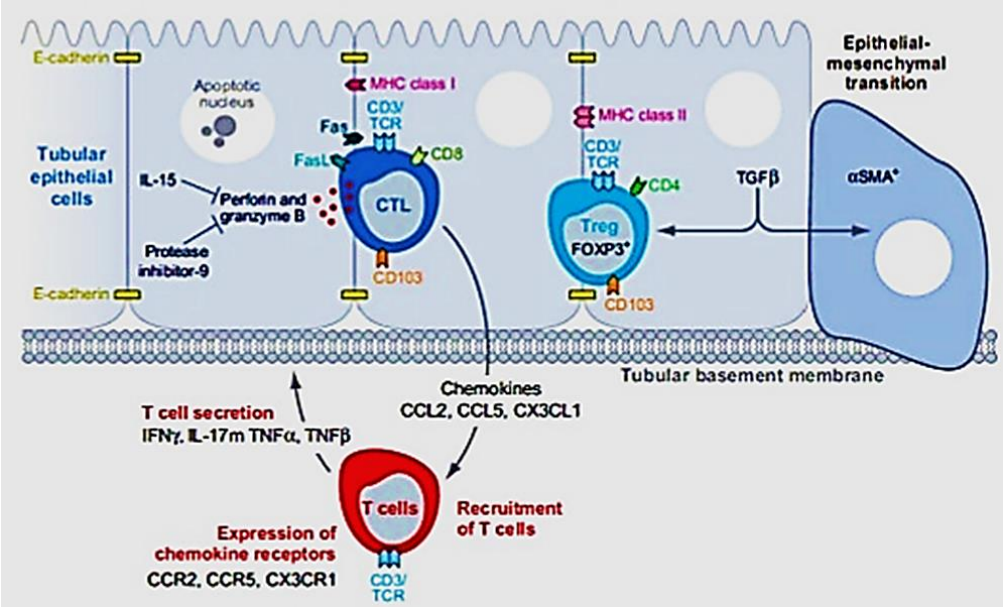

Figure 6 – Proposed mechanisms of cell-mediated graft injury. Cytokines released by inflammatory cells (IL17 and TNFα) induce the release of chemokines (CCL2, CCL5, CX3CL1) that recruit T cells to the graft. These recruited T cells, in particular cytotoxic T lymphocytes are thought to promote apoptosis either by granzime-perforin pathway or by the engagement of Fas by FasL on activated T cells. T cells are kept on the tubular epithelium by CD103 binding to E-cadherin. The production of TNFβ by the tubules stimulates FOXP3 and IL15 expression. IL15 has the ability to inhibit perforin production. Epithelial-mesenchymal transition is a phenotype seen in various grafts suffering from acute rejection and results from the exposure of tubular cells to TGFβ, which is a crucial protein in regulation of this transition. (Withdrawn from: CORNELL, L.D. [et al.]. - Kidney transplantation: mechanisms of rejection and acceptance. Annual review of pathology.3 (2008) 189-220)

1.8 Chronic Rejection

Chronic rejection is characterized by a fairly slow, but unpredictable rate of decay in renal function after the initial 3 months post-transplant (85).

Grafts suffering from chronic rejection exhibit many features seen in healing wounds, including fibroblast, endothelial cell, or epithelial cell proliferation and collagen deposition

within the graft parenchyma and blood vessels; all of these processes result in interstitial fibrosis, ischemia, and the loss of graft function (86, 87). Other histopathological characteristics of chronic rejection are fibrous intimal thickening of the arteries, glomerulosclerosis and tubular atrophy. These symptoms are often found in combination with an increase in creatinine levels and also with aggravation of hypertension (85, 88).

Through the years some risk factors of chronic rejection have been identified, where the most relevant is the existence of previous acute rejection episodes as the half-life of cadaveric allografts decrease in patients who had episodes of acute rejection (89).

The timing of the acute rejection episodes also influence the outcome of the graft, as the episodes of acute rejection that take place within the first 3 months after transplant have a lower impact on chronic rejection comparing with the ones occurring after 3 months transplant (90).

Detection of anti-HLA antibodies, both before and after transplant, is associated with chronic allograft rejection. The requirement of dialysis during the first week after transplantation, known as delayed graft function, is also consider a risk factor of chronic rejection (58). Finally, the recipient age and race may also have an effect on the development of chronic rejection (91, 92).

The majority of risk factors identified are related to recognition of foreign antigens on the graft by the recipient, which triggers cellular and humoral responses, as explained above. In chronic rejection, the humoral immunity appear to have the biggest role, but both types of immune responses are responsible for graft rejection (86).

Nowadays the complement component 4d (C4d) deposits have been taking attention as in situ marker for humoral rejection. C4d is one of the degradation products of complement component C4, that remains covalently linked to the tissue after activation. In renal allograft biopsies with chronic rejection C4d deposits have been found in the peritubular capillaries in 34% of late allograft biopsies (93).

Another aspect that confirms the impact of humoral responses in chronic rejection is the presence of antibodies in circulation. These antibodies can be against HLA or non-HLA antigens. Renal transplant recipients with anti-HLA antibodies are, in theory, 5–6 times more likely to develop chronic rejection and, eventually, lose their grafts (94). Still, there are a large number of acute and chronic rejection episodes that occur in the absence of circulating anti-HLA antibodies. This is consistent with the data of recent studies suggesting that the antibodies found in circulation might not reflect what really is being produced locally in the graft (95).

There is some data reporting non-specific antibodies binding to endothelial cells in renal transplant recipients, suggesting the involvement of antibodies against non-HLA antigens in chronic rejection (96).

In conclusion, chronic rejection is the term used to describe the long-term loss of function in transplanted organs as consequence of an accumulation of different injuries to the graft, befalling in both early and post-transplant period. Nevertheless, the mechanisms behind chronic rejection are far from clear and more comprehensive studies covering the pathogenesis are obligatory (97).

1.9 Tolerance in Transplantation

1.9.1 The concept of tolerance in Transplantation

There are two ways to define tolerance in human transplantation: clinical and immune tolerance. The term clinical tolerance refers to the survival of the graft in the absence of non-specific immunosuppression agents (98). On the other hand, immune tolerance is related to the absence of a visible immune response against the graft without non-specific immunosuppression agents (99). Evidently, the achievement of tolerance is the ultimate goal in transplantation and it is an area of intensive study with numerous tolerogenic protocols being attempted in humans, but the majority has failed to accomplish the desirable effect (100).

The alloimmune response can be separated into central and peripheral tolerance, according to the mechanisms that induce a tolerance state. These are related and not mutually exclusive (101).

Central tolerance consists on the removal of T and B autoreactive lymphocytes by a process known as clonal deletion. T and B cells mature in the thymus and in the bone marrow, respectively (102-104).

In some cases, T or B cells with self-reactivity escape from the thymus or bone marrow and numerous mechanisms take action to try to control or eliminate these cells. These mechanisms constitute the Peripheral tolerance, including deletion and apoptosis, anergy and regulation or suppression (105).

The deletion mechanism of self-reactive T lymphocytes, in both thymus and periphery, is achieved through apoptotic cell death. Fas (CD95) with its ligand (Fas-L or CD95L) mediate this process on T cells, and can occur in developing thymocytes as well as in mature T cells (106).

Anergy is a state of immune unresponsiveness of T or B cells to further antigenic stimulation as a consequence of absent costimulation (107, 108).

Lastly, the third mechanism of peripheral tolerance is called regulation or suppression of the immune response where the most important cells known, until now, are the regulatory T cells. These cells control the immune response to foreign antigen to make sure the host remains unharmed (109). The hallmark of Treg cells is the transcription factor FOXP3, who controls the molecular programs involved in mediating Treg function (110). Nonetheless, the mechanisms by which Treg cells exert their effects are not completely understood (111).

In recent years, an interaction between Tregs and regulatory B cells (Bregs) has been discovered and it has been suggested that these subtypes collaborate to promote tolerance in organ transplants. Lee et al. (112) established that adoptively transferred Bregs require the presence of Tregs to maintain tolerance, and that adoptive transfer of Bregs increases the number of Tregs. They had previously demonstrated the ability of Bregs to transfer tolerance to untreated, transplanted animals and also that Bregs are antigen specific. As a final point, they suggest TGF-β1 as the possible intermediate to Bregs promoting Treg activation, supporting graft survival (112.

1.9.2 Accommodation

Accommodation refers to acquired resistance of an organ or tissue to immune-mediated injury. Accommodation can also be defined as a condition in which an allograft has a normal function, despite the presence of antibodies in the recipient specific against the graft (113, 114).

The first time that accommodation was observed was in AB0-incompatible renal transplants, which were surprisingly functional and able to subsist after anti-blood group -A or –B antibodies were momentarily removed from circulation of graft recipients (115, 116).

There were proposed 3 mechanisms by which accommodation could be achieved: a change in antigen so that less antibody would bind, modification of antibodies so that they would be less cytotoxic or an adjustment in the graft so that it became resistant to humoral immunity (117). After some studies, the hypothesis with more supporting evidence was the one proposing that the graft could become unaffected by the antibodies against it. The major proof of this theory is C4d deposition found in biopsies without other signs or symptoms of rejection. Complement fixation, advocates that antibody binding is intact in accommodated

organs, but at the same time the absence of lysis suggests that some form of regulatory pathway is the basis for graft survival in accommodation (118).

Accommodation can be considered as a positive response to transplantation as it prevents acute types of humoral injury. Conversely, by preventing acute injury, accommodation allows chronic processes to develop, producing damage to the graft (119). There is the possibility that the same proteins and pathways responsible for preventing initial harm to the graft might be the ones causing the impairment later in time (114).

The prevalence of accommodation after renal transplantation maintain as the fundamental question awaiting answer. Developments in the management of patients with antibodies against AB0 and HLA antigens, will possibly bring progress in the understanding of how frequent is accommodation and what consequences it carries. Clinical studies may also provide hints concerning manipulations that permit or even induce accommodation (114).

1.9.3 Immunosuppression in transplantation

The objective of immunosuppression is to prevent or treat allograft rejection and at the same time minimize drug toxicity responsible for infection and malignancy (120).

Combinations of several agents are used concurrently and in different immunosuppressive regimens that can be classified as induction, maintenance, or anti-rejection regimens. Induction regimens provide intense early post-operative immune suppression, while maintenance regimens are used throughout the patient's life to prevent both acute and chronic rejection (120). Induction immunosuppressive agents consist of depleting and non-depleting protein drugs (polyclonal and monoclonal antibodies) and maintenance immunosuppressive therapies include small molecule drugs with two central categories: calcineurin inhibitors and antiproliferatives (120).

Calcineurin inhibitors suppress the immune system by preventing IL2 production in T cells. The most commonly used and better described calcineurin inhibitors are Cyclosporine and Tacrolimus. The main side effects of these agents are hypertension, nephrotoxicity, neurotoxicity, and lipid abnormalities giving rise to an increased risk of death from cardiovascular complications (121). The antiproliferatives more frequently applied are Sirolimus and Everolimus and they exert their function by inhibiting IL2 and IL15 driven proliferation of B and T cells and also vascular smooth muscle cells (122). Furthermore Sirolimus seem to have the ability to decrease antibody production by B cells. The most common side effects are hyperlipidaemia, cytopenias and dyslipidaemia (123).

The main challenge with immunosuppression strategies is to get the right balance between the immunosuppressive action that avoid rejection episodes and the drug toxicity that leads to undesirable side-effects as explained above. The ideal immunosuppressive strategy would ideally be the one that stimulates the development of tolerance to alloantigens and at the same time promotes successful withdrawn of immunosuppression (124).

1.10 Biomarkers in Renal Transplantation

Efficient application of biomarker identification can allow personalized therapy for renal transplant patients, since it might permit early detection and identification of renal graft status, guide clinicians in minimizing the risk of graft rejection events and offer vital information concerning withdrawal of immunosuppression (125, 126).

Needle biopsy still is the most common diagnostic tool to detect rejection of renal allografts. As previously mentioned, it is an invasive procedure that is always subjected to a certain percentage of error and even the interpretation of the results can vary between pathologists (127, 128).

Nucleic acid-based assays (principally micro-array assays and quantitative PCR (qPCR), proteomics and metabolomics-based approaches represent alternatives for the development of biomarkers of allograft status (124).

Through the years various studies regarding biomarkers of rejection (acute and chronic) have been executed. Sarwal et al. (129) revealed extensive differences in gene expression profiles of RNA isolated from 67 biopsy specimens. They suggested that the variances in gene profile were associated with differences in immunologic and cellular features and clinical course (130).

Furthermore, in a report from Desvaux et al. (69) 43 human renal-allograft biopsies were quantified for mRNA expression of granzyme B, Fas ligand (FasL), IFN-γ, IL4 and IL6 with a qPCR method. They concluded that upregulated granzyme B and FasL mRNA expression was correlated with all allograft rejection types ($p < 0.01$).

Another important work done on biopsies of kidney transplant patients was the one done by Oliver Thaunat et al. (95) where it is stated that the inflammatory infiltrate, present in chronic rejection, becomes organized into an ectopic lymphoid tissue, which harbors the maturation of B cell clusters responsible for humoral responses against the graft. Additionally they suggested that this maturation could be impeded by blocking certain genes (95).

The chance to measure gene-expression profiles in the peripheral blood and/or urine from kidney transplant patients allows the testing of numerous hypotheses by non-evasive methods and will have a direct impact on clinical practice. In a small study of 25 transplant recipients, granzyme B, Fas ligand (FasL) and perforin mRNA were significantly elevated in peripheral blood and graft tissue from patients with acute rejection, compared with their levels in allografts without rejection (131).

Conversely, the multicentre Clinical Trials in Organ Transplantation (CTOT) -04 study was designed to detect a urinary-cell mRNA signature from a panel of nine candidate genes identified in previous studies. granzyme B and perforin were both included in the nine candidate genes in the study, but the best-fit model did not include these genes. Moreover, this study showed that a signature derived from CD3E mRNA, CXCL10 mRNA and 18S ribosomal RNA could discriminate between biopsy samples showing acute rejection versus those that had no rejection (132). CXCL10 together with CXCL9 are IFNγ-dependent chemokines and they are critical regulators of leucocyte trafficking and activation which are dynamically expressed in multiple transplant scenarios (133).

Multiple studies have shown an association between these chemokines and allograft injury (134, 135). This association was a confirmed on a previous work done in our lab where CXCL10, CXCL9 and TGF-β1 were selected as decent biomarkers for chronic rejection by the analysis of the receiving operating characteristic (ROC) curves (data not published).

1.11 Objectives

Chronic rejection is currently the most prevalent cause for kidney allograft failure and the mechanisms involved in this condition are far from clear. So the need to determine a genetic, genomic and humoral profile associated with chronic rejection, in kidney transplant patients, is evident.

In order to assess this profile, the following specific objectives were delineated:

- Evaluate the impact HLA mismatches in graft function and in humoral responses;
- Perform the screening of anti-HLA antibodies in both urine and blood samples;
- Establish a genomic profile of immune response mediators, related to chronic rejection, in the urine of renal transplant patients;
- Assess the predicting value of the genetic, genomic and humoral changes in the establishment of chronic rejection.

2. Methods

2.1 Population in study

The urine and blood samples necessary for this work were collected from patients submitted to renal transplant for more than two years, under regular clinical consultation on the Renal Transplant Unity from the "*Centro Hospitalar Universitário de Coimbra*" E.P.E.

The patients in this study can be divided into two groups: one with patients with a stable renal function and other with patients who have been diagnosed with chronic dysfunction. The demographic features that allowed the samples distribution to each group are displayed on Table I. The most relevant clinical events of both groups are exhibited on Table II. A total of 99 samples were gathered thanks to the collaboration of Pathology Unit. It was also included a control group consisting of 9 healthy individuals.

Table I – Demographic features of both group of patients present in the study.

	Group NF N=72 (Normal Function)	**Group CR N=27 (Chronic Rejection)**
Sex (M/F)	42/30	20/7
Age (Years; Mean ± SD)	55,79 ± 11,37	49,04 ± 12,92
BMI (Mean ± SD)	26,42 ± 3,904	24,33 ± 4,768
Organ Source	Cadaveric	Cadaveric
PRA (%)	[0-20%]-95,84 [21-50%]-4,16	[0-20%]-96,3 [21-50%]-3,70
Immunosuppression CsA/ FK506 (mg/Kg)	CsA - 0,15 FK506 – 8	CsA - 0,15 FK506 - 8
Time post-transplant (Years; Mean ± SD)	9,43 ± 5,71	11,8 ± 5,60

Legend: M - male; F - Female; BMI - Body mass index; CsA - Cyclosporin A; FK506 - Tacrolimus; PRA - Panel Reactive Antibody; mg - milligrams; Kg - Kilograms; SD - Standard Deviation.

Table II – Most relevant clinical events of both groups.

	Group 1 N=73 (Normal Function)	Group 2 N=27 (Chronic rejection)
Original disease (%)	PKD-13.89 CGN-15.27 CP-6.94 Undetermined-63.90	PKD-11.11 CGN-7.40 CP-11.11 Undetermined-70.38
Creatinine Levels (mg/ dl; Mean ±SD)	1,17 ± 0,26	2,71 ± 1,16
Acute rejection episode within the first month after transplant (%)	5.56	11.1
Cold ischemia (hours; Mean)	19	18
Diabetes after transplant (%)	16.7	14.81
Infections- Positivity for CMVab (%)	95.83	92.60

Legend: PKD - Polycystic Kidney Disease; CGN - Chronic Glomerulonephritis; CP - Chronic Pyelonephritis; CMVab - Cytomegalovirus antibody; SD - Standard Deviation.

2.2 Processing peripheral blood samples

2.2.1 DNA extraction

The blood samples were collected to *vacuette*® blood collection tubes. Then 200 µL of each sample were withdrawn to proceed to the DNA extraction according to MagAttract® DNA Blood Midi M48 kit (Qiagen®) protocol.

This protocol is based on the DNA binding to magnetic particles coated with silica in the presence of a chaotropic agent. The process begins with the addition of ML buffer resulting in cell lysis, then *MagAttract B* (solution that contains the magnetic particles in suspension) is added and the DNA binds to the magnetic particles. The isolation of the DNA is achieved due to the presence of a magnet that allows the magnetic particles with DNA to be isolated.

There were some washing steps during this method and in the final step the DNA was eluted in 300 µL of Tris- Ethylenediaminetetraacetic Acid (TE). The tubes containing the DNA of each sample were stored at 4°C.

2.2.2 Blood serum isolation

The blood was collected to *vacuette*® blood collection tubes that were submitted to 1465 RCF centrifugation during 8 minutes (Kubota 5910) allowing serum separation from the other blood components. The serum was collected into 1,5 mL *Eppendorf* tubes and stored at 4°C.

2.3 Processing urine samples

There were collected about 15 mL of urine into *vacuette*® urine collection tubes. The tubes were centrifuged during 25 min at 1465 RCF. The supernatant was transferred into 2mL *Eppendorf* for antibody screening and stored them at -25°C. The pellet (urinary sediment) was resuspended in 1 ml of Phosphate Buffer Saline (PBS) 1x and 100 µL were transferred to a 1,5 mL *Eppendorf* tube for DNA extraction.

The remaining solution was submitted again to another spin but this time at 17226 RCF for 5 minutes. The supernatant was discarded and the urinary sediment suspended in 600 µL of RLT Buffer + β-mercaptoethanol (10%) in order to lyse the cells. Samples were stored at -80°C.

2.3.1 DNA extraction from cells of the urinary sediment

Performed with the DNA extraction kit by MagAttract ® DNA Blood Midi M48 (Qiagen ®). The elution volume of DNA was 75µL on Tris-EDTA (TE). The protocol is the same as described above for DNA extraction from peripheral blood section.

2.3.2 RNA extraction from cells of the urinary sediment

Frozen samples, previously stored at -80°C, were thawed at 4°C, homogenized and subjected to centrifugation at maximum speed for 3 minutes. The supernatant (350µL) was placed on the shaker QIACube (Qiagen ®).

RNA was extracted using the RNeasy Mini Kit. RNA (Qiagen ®) was eluted in 50 µL of RNase-free water (RNFW) and stored at -80°C.

2.4 HLA typing by Luminex® (LABType® SSO Typing Tests)

The HLA typing began with the amplification of the coding regions of HLA-A, HLA-B, HLA-C and HLA-DR alleles using the polymerase chain reaction (PCR).

The primers used for the amplification are biotinilated. The single stranded product is then hybridised with a multiplex of up to 100 beads, all of which can be uniquely identified by their internal dyes and all of which are selectively coated with specific oligonucleotide sequences. These beads are made of polystyrene and each one is internally dyed with a unique combination of red and infrared dye.

The combination of different intensities of the two dyes, allows the identification of each bead by its unique signature when excited by a laser beam. A red laser is used to excite and therefore identify the specific bead and a green laser is used to excite and therefore identify any reporter dyes captured on the beads during the assay. In this case, the molecule chosen was Streptavidin – Phycoerytherin (SAPE) conjugate, with Streptavidin binding to the biotin used to label the primers and Phycoerytherin serving as the reporter dye.

2.4.1 HLA genes amplification

The amplification of the HLA genes was accomplished with One Lambda® amplification kits, more specifically, the D-mix (LABType® Primer Sets and D-mix) with the respective amplification primers and TaqPolimerase (5U/µL) from ABgene.

A 96 well plate was prepared with 9 µl of the Master Mix and 1 µl of DNA in each well. After sealing the plate, the amplification occurred in the C1000TM Thermal Cycler with the amplification program showed in Table III.

Table III - HLA genes PCR amplification program.

Step	Temperature(°C)	Time (min)	Cycles
1	96	03:00	1
2	96	00:20	5
	60	00:20	
	72	00:20	
3	96	00:10	30
	60	00:15	
	72	00:20	
4	72	10:00	1
5	4	∞	1

2.4.2 Denaturisation/Neutralization and Hybridization

At the end of the amplifications, the resulting products were denatured and neutralized with the denaturing buffer and neutralizing buffer, respectively. As described above, the next step was the hybridization with the beads that occurred at 60°C during 15 minutes. These beads are coated with specific oligonucleotide HLA sequences for each locus: *locus A* – RSSOH1A; *locus B* – RSSOH1B; *locus C* – RSSOH1C; *locus DR* – RSSOH2B1.

Subsequently, 2 washing steps were needed to proceed to the addition of Streptavidin – Phycoerytherin (SAPE) solution (Stock SAPE and SAPE Buffer), as explained above it allows us to detect the biotinilated PCR products. In order to allow biotin and Streptavidin bonding, an incubation at 60°C for 5 minutes was required.

Finally, after some more washing steps, 60 µl of washing buffer were added to each well and all products were transferred into an ELISA plate with conical bottom.

The plate was read on the LABScan™ 100 and the fluorescence variations were interpreted on the LabTools program from One Lambda®.

2.4.3 Anti-HLA Class I and Class II Antibody screening in blood serum

Beads coated with anti-HLA Class I and Class II purified antigens were used to detect anti-HLA antibodies in the serum. The information provided by these beads can be deduced in LABScan™100 (Luminex®).

The serum samples were defrosted and centrifuged at maximum speed (Eppendorf, Centrifuge 5415R). The following step was to incubate the samples (diluted 1:2) with the LABScreen® Mixed (2,5 µL) beads in the dark and with moderate stirring on an ELISA plate of 96 wells for 30 minutes. This step allows the antigens to bind to the respective antibodies present in the blood serum of each sample.

When the incubation was completed, there was a need to execute some washing steps to remove the beads that remain unbounded. This wash was done with washing buffer 1X.

The analysis is based on fluorescence so there is a need to add a fluorophore, in this case was Phycoerytherin (PE) conjugated with anti-IgG, that binds to the antibodies that were bound to the antigens present in the beads. Another incubation was required under the same conditions as the first one.

Again, after the incubation, it was necessary to complete some washing steps and following the last wash, 80 µL PBS 1x were added. The plate was placed on the

LABScan™100 equipment and the results analysed on the HLA fusion program from One Lambda®.

2.4.4 Anti-HLA Class I and Class II Antibody screening in urine

The screening of antibodies in the urine was completed by taking 1 mL of supernatant and adding 1mL of PBS 2x. The next step was the incubation overnight, in the same conditions as before, with the LABScreen® Mixed beads but this time with 10 µL. The next steps are exactly the same as in the antibody screening in the serum after the incubation.

This was a method developed in our lab and might need some small changes.

2.5 Relative quantification in cells from the urinary sediment

2.5.1 Reverse-transcription PCR

This type of PCR is used to obtain complementary DNA (cDNA) from extracted RNA, in this case, RNA from urinary sediment cells. This is possible because the polymerase present in this reaction is a reverse transcription polymerase.

The reverse transcription Kit used was the *iScript™ Reverse Transcription Supermix* (Bio-Rad). The process begins with the addition of the extracted RNA (8 µl) to the 5X *iScript™ Reverse Transcription Supermix* (4 µl) and RNFW (8 µl). The synthesis reactions were performed in the DNA Engine® Thermal Cycler (Bio-Rad) with the PCR program displayed on Table IV.

Table IV- PCR program used in the reverse transcription reaction using the *iScript™ Reverse Transcription Supermix*.

Steps	Temperature (°C)	Time (min)
1	25	10:00
2	42	60:00
3	85	05:00
4	4	∞

2.5.2 Normalization

Data normalization in real-time RT-PCR is a major step in gene quantification analysis since it compensates intra and inter-kinetic RT-PCR variations (sample-to-sample and run-to-run variations).

The normalization was achieved with the evaluation of gene expression levels of reference genes. It is assumed that the expression of these reference genes does not differ between reads.

In order to select the two reference genes, random samples were used in the reactions with each one of 7 genes that could be used as reference gene: *β- Actin* (*ACTB*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *Ubiquitin C* (*UBC*), *β -2- microglobulin* (*B2M*), *subunit 1 of splicing factor 3a* (*SF3A1*), *ribosomal subunit 18S* (*18S rRNA*) and *Cytochrome C -1* (*CYC1*) and proceeded to Real-Time PCR reactions.

The results obtained in these reactions were analysed on the GeNorm Program (PrimerDesign) that selected the two reference genes suitable for the samples being studied. Then, Real-Time PCR (RT-PCR) reactions of all samples with reference genes were performed and the results were analysed again on the GeNorm Program but this time to acquire the normalization factor for each sample. The normalization factor is crucial to calculate the Normalize gene expression values (NGE) when examining the genes of interest.

2.5.3 Real-Time PCR reactions

Real-time Polymerase Chain Reaction allows monitoring of the progress of the PCR as it occurs, therefore the data is collected throughout the amplification process, rather than at the end of the PCR. In this type of PCR, a fluorescent reporter is used and an increase in the reporter fluorescence signal is directly proportional to the number of amplicons generated. The more starting copies of the nucleic acid target, sooner a significant increase in fluorescence is observed.

The fluorescent marker selected in this work was SYBR® Green I (Bio-Rad), which evenly binds to double-stranded DNA molecules, emitting a fluorescent signal with a defined wavelength. The signal is detected during the extension step of the PCR reaction in real time. Signal intensity increases with increasing number of cycles due to the accumulation of the PCR product.

However, SYBR® Green I have some disadvantages as it may generate false positive signal as the SYBR Green I dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences.

The RT-PCR reactions were executed on the Light Cycler® 480 (Roche) equipment and the Master Mix for these reactions contained 2x QuantiTect SYBR Green PCR Master Mix (QIAGEN) (5 µl), RNFW (2 µl) and 10x QuantiTect Primer assay (QIAGEN) (1µl).

Then the cDNA of each samples was added to a well of a 96 Light Cycler® 480 Multiwell Plate (2 µl) making the final volume of each reaction 10 µl. All interest genes studied as well as the respective reference genes are listed below on Table V and the amplification program is presented on Table VI.

Table V - List of all the interest genes studied as well as the respective reference genes.

Reference Genes		Interest Genes			
GAPDH (QT00079247)	CYC1 (QT00209454)	CXCL10 (QT01003065)	CXCL9 (QT00013461)	TGF-β1 (QT00000728)	GATA3 (QT00095501)
		IL4 (QT00012565)	CD19 (QT00203826)	CD79B (QT00203651)	IL10 (QT00041685)
		TNF3 (QT01079561)	FOXP3 (QT00048286)	IL17 QT00009233	

Table VI - Real-Time PCR amplification program.

Steps	Temperature (°C)	Time (min)	Cycles
1 - Enzime Activation	95	15:00	1
2 – Amplification	95	00:15	50
	55	00:30	
	72	00:30	
3 - Melting curve	65 - 95	-	1

The results were analysed on the Light Cycler® 480 software (Roche) and the two main aspects of analysis were the melting curves and the crossing points obtained. The melting curves reflects the specificity of the amplification and the crossing points are used to determine the relative quantity (RQ) of each gene by applying the RQ= 2-ΔCp formula. The Normalized gene expression (NGE) values are given by the quotient between the RQ for the gene of interest and the normalization factor for each sample.

2.6 Statistical analysis

The presentation of results is done using the mean ± standard deviation for each group. The results were submitted to *U Mann-Whitney* test or *Fisher´s* exact test and considered statistically significant when $p<0.05$. The normalized gene expression values were also analysed by the receiving operator characteristics (ROC) curves for specificity and sensitivity. The software used was the IBM SPSS® Statistics 20.

3. Results

3.1 Demographic Data and Clinical Features of the population in study

The demographic data displayed on Table I reveals a similarity between both groups despite the sole criterion applied being the fact that the patients have been submitted to renal transplant for more than two years. As a result of this homogeneity, the influence of characteristics like, body-mass index or even the time-post transplant, in the results obtained is greatly reduced.

Table II exhibits the clinical features of both groups of patients. The distribution of patients into both groups was based on the diagnostic made by the clinicians and mainly by the serum creatinine levels that are clearly elevated on chronic rejection group. In terms of diabetes and ischemia both groups show similar incidence and until the samples collection none patients revealed any signs of active infections by cytomegalovirus. The increased percentage of patients with previous episodes of acute rejection on chronic rejection group confirms the importance of acute rejection in the development of this condition.

3.2 Donor/Receptor mismatches

The Donor/Receptor compatibilities are shown on table VII. The compatibilities refer to the HLA match typing of both donor and receptor for each group. The HLA Class I *loci* analysed were "*locus A*" and "*locus B*" with 4 maximum possible mismatches, two for each locus. The HLA Class II locus tested was "*locus DR*" with a maximum of two possible matches between donor and receptor.

Table VII - Donor/Receptor compatibilities.

Normal Function Group		Chronic Rejection Group	
HLA Class I (A/B) Mismatches (Mean)	**HLA Class II (DR) Mismatches (Mean)**	**HLA Class I(A/B) Mismatches (Mean)**	**HLA Class II(DR) Mismatches (Mean)**
2,62	0,69	2,64	0,62

Concerning HLA Class I *loci*, the two groups presented an average of 2,62 mismatches for normal function group (NF Group) and 2,69 for chronic rejection group (CR group) out of four possible matches, as explained before.

In contrast the average mismatches for HLA Class II *locus* were 0.69 for NF group and 0,62 for CR group indicating that the majority of the patients of both groups were compatible with their donor for HLA Class II *locus*.

It is important to refer that there were five patients in the NF Group that had 6 mismatches with their donor showing no compatibility with their donor. In CR group only one patient exhibited this condition.

3.3 Anti-HLA antibody screening in the serum

The antibody screening in the serum of renal transplant patients with normal function (NF Group) revealed a low percentage of anti-HLA Class I antibodies and a slightly higher percentage of anti-HLA Class II antibodies (Table VIII).

Regarding the renal transplant patients who have been diagnosed with chronic rejection (CR group) the percentage of anti-HLA Class I antibodies was even lower comparing to NF group. In contrast, the percentage of anti-HLA antibodies Class II is on a small scale higher in CR group (Table VIII).

Table VIII - Percentage of anti-HLA antibodies found in the serum of renal transplant patients.

	Anti-HLA antibodies Class I		Anti-HLA antibodies Class II	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
NF Group	12,7	87,3	16,9	83,1
CR Group	3,7	96,7	18,5	81,5

Legend: NF - Normal Function group; CR - Chronic Rejection group.

3.4 Anti-HLA Antibodies vs. HLA Mismatches

Table IX shows the association between the number of HLA Class I mismatches and the presence of anti-HLA Class I antibodies in the serum of renal transplant patients. It is clear that the higher number of mismatches is linked with the detection of circulating anti-HLA Class I antibodies. In the CR group, with only one patient with anti-HLA Class I antibodies, no relevant information can be withdrawn.

Table IX- Relationship established between the number of HLA Class I mismatches and the number of patients with presence of anti-HLA Class I antibodies.

N° of mismatches	Normal Function Group		Chronic Rejection Group	
	N° of patients with Class I mismatches	N° Patients with anti-HLA Class I antibodies	N° of patients with Class I mismatches	N° Patients with anti-HLA Class I antibodies
4	12	2	6	0
3	26	4	6	1
2	21	2	12	0
1	5	1	2	0
0	2	0	0	0

Table X- Relationship established between the number of HLA Class II mismatches and the number of patients with presence of anti-HLA Class II antibodies.

N° of mismatches	Normal Function Group		Chronic Rejection Group	
	N° of patients with Class II mismatches	N° Patients with anti-HLA Class II antibodies	N° of patients with Class II mismatches	N° Patients with anti-HLA Class II antibodies
2	7	1	4	0
1	29	9	9	2
0	24	2	13	3

The association between the number of HLA Class II mismatches and the number of patients with presence of HLA Class II antibodies is represented in Table X. The number of patients with no mismatches for Class II is clearly superior comparing to Class I mismatches.

3.5 Anti-HLA antibody screening in urine of renal transplant patients

The antibody screening in the urine of renal transplant patients revealed that more than half of the patients in the chronic rejection group exhibited anti-HLA Class I antibodies (Table XI). The percentage of these antibodies in normal function group stayed around 20%.

The percentage of anti-HLA Class II antibodies for the CR Group in urine is around 22% whereas in NF group is 24%.

Table XI- Anti-HLA antibodies percentage found in urine of renal transplant patients

	Anti-HLA Class I antibodies		Anti-HLA Class II antibodies	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
NF Group	24	76	15,5	84,5
CR Group	51,89	48,11	22,2	77,8

Legend: NF- Normal Function group; CR- Chronic Rejection group

3.6 Comparing anti-HLA antibodies screening in urine with anti-HLA antibodies screening in the serum

The different results obtained in antibody screening in the serum and in urine are displayed on Table XII. The variances of percentages for anti-HLA Class I antibodies are the most obvious, with special emphasis for the CR group where the percentage goes from 3,7% in the serum to 51,89% in urine. These differences are statistically significant.

Regarding anti-HLA Class II antibodies the differences are less evident with the biggest change happening in CR group where the percentage of anti-HLA Class II antibodies goes from 18,5% in the serum to 22,2% in urine. The results had no statistical significance (Table XII).

Table XII - Comparison of HLA antibody percentage in the serum and urine

Group	HLA Class I antibodies (%)			HLA Class II antibodies (%)		
	Serum	Urine	*p* value	Serum	Urine	*p* value
FN group	12,7	24	*p*=0,03	16,9	15,5	*p*>0,05
CR group	3,7	51,89		18,5	22,2	

Legend: The differences were considered statistically significant when p<0.05 for the Fisher's exact test. NF- Normal Function group; CR- Chronic Rejection group.

Table XIII demonstrate that the patients with anti-HLA antibodies in the serum are not necessary the same with anti-HLA antibodies in the urine. This becomes evident for the

NF group when the number of patients with anti-HLA antibodies in urine is 15 and in serum is only 9. The difference is even greater in CR group where 12 patients have anti-HLA antibodies in urine whereas in the serum this number is only 4.

Table XIII - Comparison of anti-HLA antibody detection in the serum and urine for each group of renal transplant patients.

	NF group		CR group	
Urine / Serum	Patients with anti-HLA antibodies	Patients without anti-HLA antibodies	Patients with anti-HLA antibodies	Patients without anti-HLA antibodies
Patients with anti-HLA antibodies	9	6	4	2
Patients without anti-HLA antibodies	15	41	12	9

Legend: NF - Normal Function group; CR - Chronic Rejection group.

3.7 Gene expression in urinary sediment

3.7.1 *CXCL10*, *CXCL9* and *TGF-β1*

The normalized gene expression (NGE) values of genes encoding the chemokine's *CXCL10* and *CXCL9* in chronic rejection group (CR group) are increased in comparison with normal function group (NF group) and the differences are considered statically significant with a *p* value of p=0.03 and p=0.04 (Figure 7). The control group exhibits NGE values higher than both groups for *CXCL9* gene and lower in *CXCL10* gene.

Regarding the gene encoding *TGF-β1*, NGE levels are also found higher in CR group comparing to NF group, obtaining significant differences with a p=0.02. NGE levels of the control group for the gene encoding *TGF-β1* are lower than both NF and CR groups.

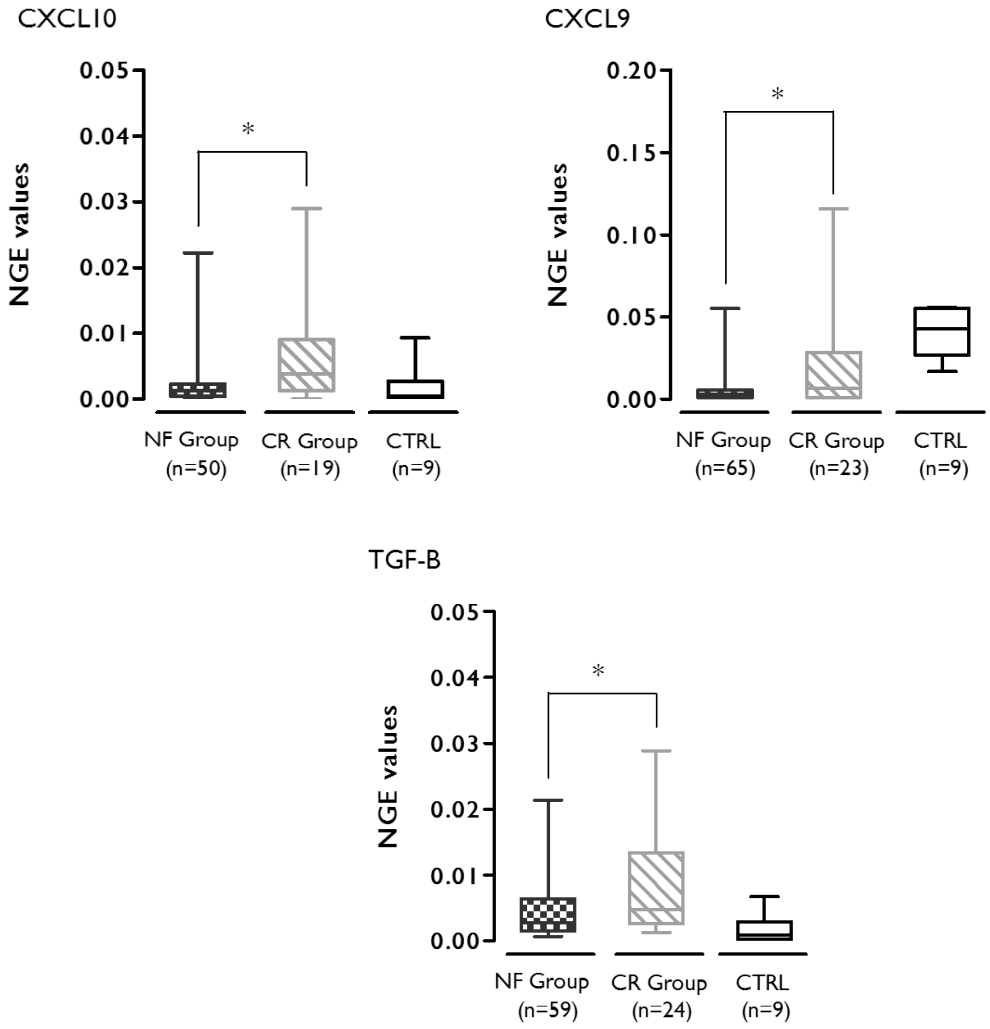

Figure 7- Box and whiskers plots of mRNA levels, in the urinary sediment, of *CXCL10*, *CXCL9* and *TGF-β1*. The horizontal line within each box represents the median, the bottom and top represent the 25th and 75th percentile values, and the thin vertical bars extend to the minimum and maximum values 0,03, 0,04 and 0,02 respectively .The NGE values of *CXCL10, CXCL9* and *TGF-β1* are superior for CR group, with a *p* value of * represents the results considered statistically significant for *U Mann-Whitney test* ($p<0,05$). NGE – Normalized Gene Expression; NF – Normal Function Group; CR – Chronic Rejection Group; CTRL – Control Group; *TGF-β1* – Gene encoding Tumour Growth Factor-β; *CXCL10* – Gene encoding C-X-C motif chemokine 10; *CXCL9* – Gene encoding C-X-C motif chemokine 9.

The analysis of receiver operating characteristic (ROC) curves, in terms of specificity and sensitivity, allows the assessment of the predicting value for chronic rejection in each gene. *CXCL10* gene displayed an area under the curve (AUC) of 74,73% and the best association of specificity and sensitivity was 87,0% and 62,5%, respectively.

The area under the ROC curve for *CXCL9* was 68,04% with the top correlation between specificity and sensitivity being 96,4% and 45,0%, respectively. Regarding TGF-β1,

the finest association for specificity and sensitivity was 53,0% and 72,0%, in that order. The AUC for *TGF-β1* was 63,09%.

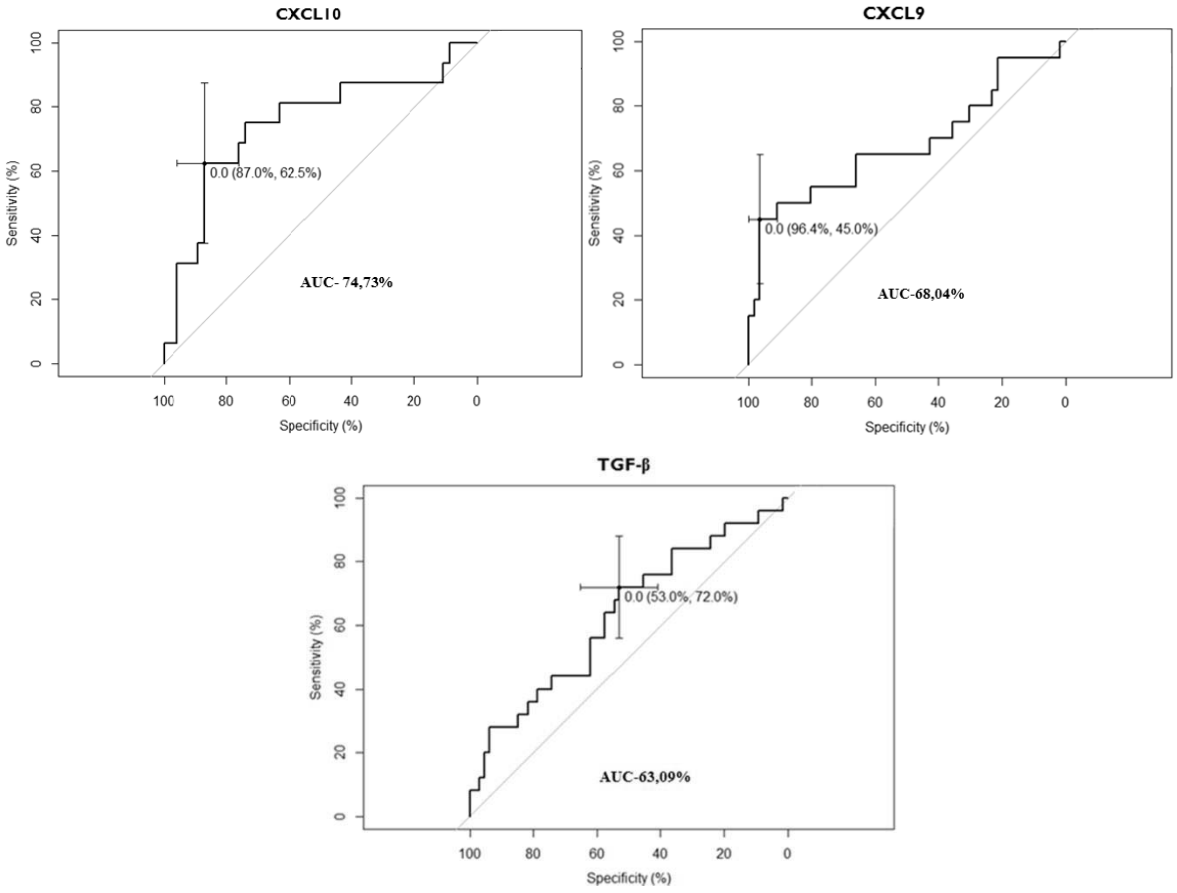

Figure 8- ROC curves (95% confidence interval) for sensitivity and specificity of *CXCL9*, *CXCL10* and *TGF-β1* to evaluate their diagnostic value for chronic rejection. CXCL10 gene showed the biggest predictive potential among the three genes with an AUC of 74,73% and a specificity of 87,0% and a sensitivity of 62,5. ROC, receiver operating characteristic; AUC- area under the curve.

3.7.2 *GATA3* and *IL4*

The NGE values of the T-cell-specific transcription factor *GATA3* are found superior for NF group rather than for CR group with a significant *p* value = 0.04. The Control Group unveils a higher expression than both CR and NF groups. In contrast, the gene encoding interleukin 4 showed a higher prevalence in CR group comparing to NF group, however, no statistical significance was found. No amplification was verified for *IL4* in control group (Figure 9).

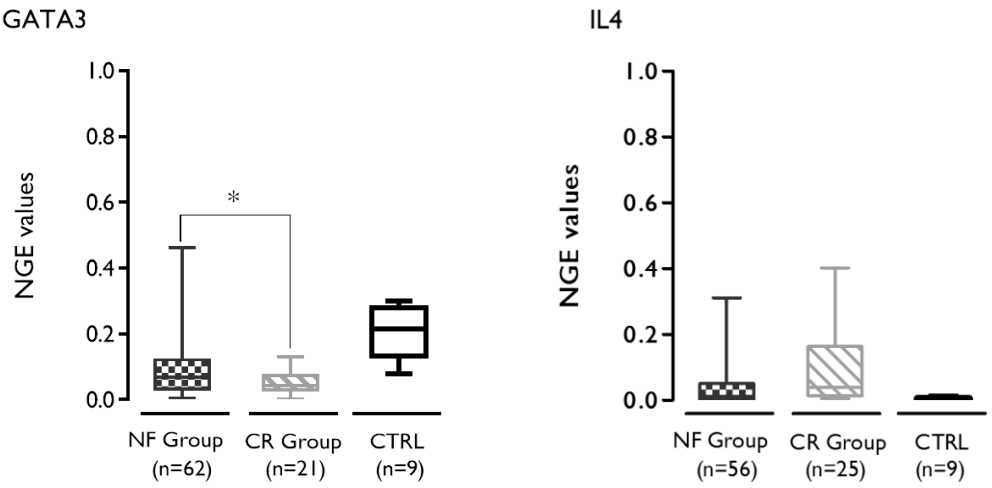

Figure 9- Box and whiskers plots of mRNA levels, in the urinary sediment, of *GATA3* and *IL4*. The horizontal line within each box represents the median, the bottom and top represent the 25th and 75th percentile values, and the thin vertical bars extend to the minimum and maximum values. *GATA3* NGE values are increased for NF group with statistical significance (p=0,04).On the other hand, NGE levels of *IL4* are higher for CR group but no statistical significance was achieved.
* represents the results considered statistically significant for *U Mann-Whitney test* (p<0,05).
NGE – Normalized Gene Expression; NF – Normal Function Group; CR – Chronic Rejection Group; CTRL – Control Group; *GATA3* – Gene encoding T-cell-specific transcription factor GATA3; *IL4* – Gene encoding interleukin 4.

Concerning the ROC curves for *GATA3* and *IL4*, the AUC found were 61,96% and 50,94%, respectively. In terms of specificity and sensitivity, the optimal correlation for *GATA3* was 44,3% in specificity and 61,8% in sensitivity. *IL4* ideal association was 30,2% for specificity and 92,3% for sensitivity.

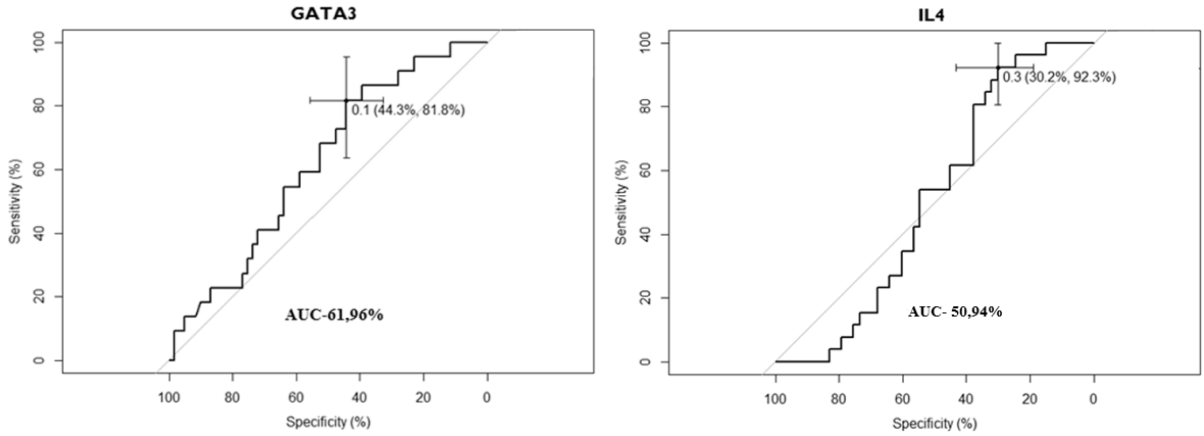

Figure 10- ROC curves (95% confidence interval) for sensitivity and specificity of *GATA3* and *IL4* to evaluate their diagnostic value for chronic rejection. Neither of the two genes exhibited a significant diagnostic value for chronic rejection. The AUC for *GATA3* was 61,96% and for *IL4* this value was 50,94%. ROC – receiver operating characteristic; AUC – area under the curve.

3.7.3 *CD79B* and *CD19*

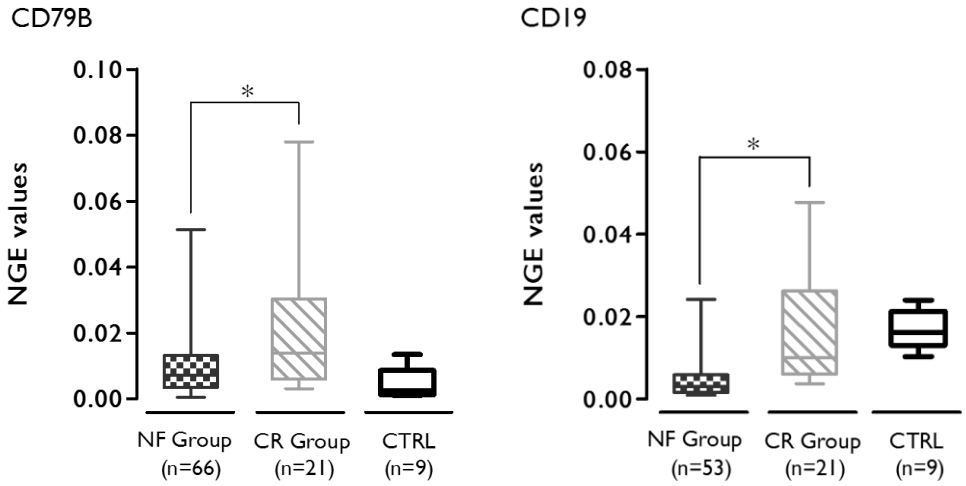

Figure 11- Box and whiskers plots of mRNA levels, in the urinary sediment, of *CD79B* and *CD19*. The horizontal line within each box represents the median, the bottom and top represent the 25th and 75th percentile values, and the thin vertical bars extend to the minimum and maximum values. *CD79B* and *CD19* show increased NGE values for CR group comparing to NF group. The differences found are statistically significant (p=0.03 and p<0.0001, respectively).
* represents the results considered statistically significant for U Mann-Whitney test (p<0,05).
NGE – Normalized Gene Expression; NF – Normal Function Group; CR – Chronic Rejection Group; CTRL – Control Group; *CD79B* – Gene encoding the Cluster of Differentiation 79B ; *CD19* – Gene encoding Cluster of Differentiation 19.

Figure 11 shows the normalized gene expression levels for *CD79B* and *CD19* genes. Both genes reveal a greater prevalence in CR group instead of NF group. These difference

are considered statistically significant with a value of p=0.03 for *CD79B* and p<0.0001 for *CD19*.

The NGE levels of control group are lower than NF and Cr groups in *CD79B* gene, however, in *CD19* gene the normalized gene expression values are increased comparing to NF and CR group NGE values.

The ROC curve For *CD79B* gene showed an AUC of 59,97% and the prime correlation point for specificity and sensitivity was 72,9% and 50,0%, respectively. The optimum association between specificity and sensitivity, for *CD19* gene, was 72.5% specificity and 77,8% sensitivity. *CD19* also exhibited an AUC of 79,15 %.

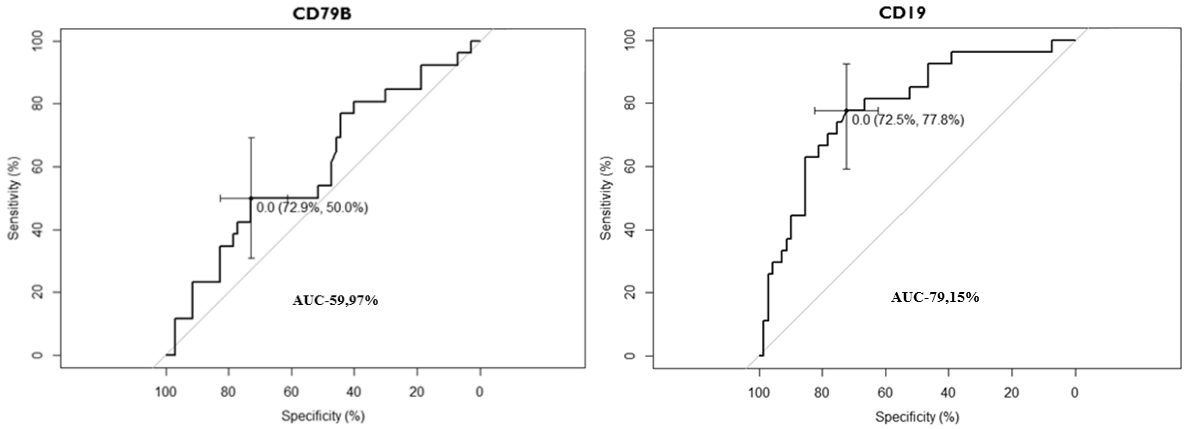

Figure 12- ROC curves (95% confidence interval) for sensitivity and specificity of *CD79B and CD19* to evaluate their diagnostic value for chronic rejection. *CD19* gene displayed the best predictive value among all genes evaluated with the specificity and sensitivity values being 72,5% and 77,8%, respectively. The AUC was 79,15%. ROC-receiver operating characteristic; AUC-area under the curve.

3.7.4 *IL10*, *TNF3* and *FOXP3*

The gene encoding *IL10* shows a greater propensity for CR Group and, despite the low number of samples that had amplification, the differences between the NF and CR groups are statistically significant with a value of p=0.02. None of the samples in control group had amplification for *IL10* gene.

TNF3 gene also had increased NGE values for the chronic rejection group with statistical significance, presenting a p value =0.0002. The control group exhibited NGE values superior than normal function and chronic rejections groups.

Finally, *FOXP3* gene revealed higher NGE levels for the normal function group when compared to CR group but no statistical significance was achieved. The NGE values of control group gene were higher than both groups (Figure 13).

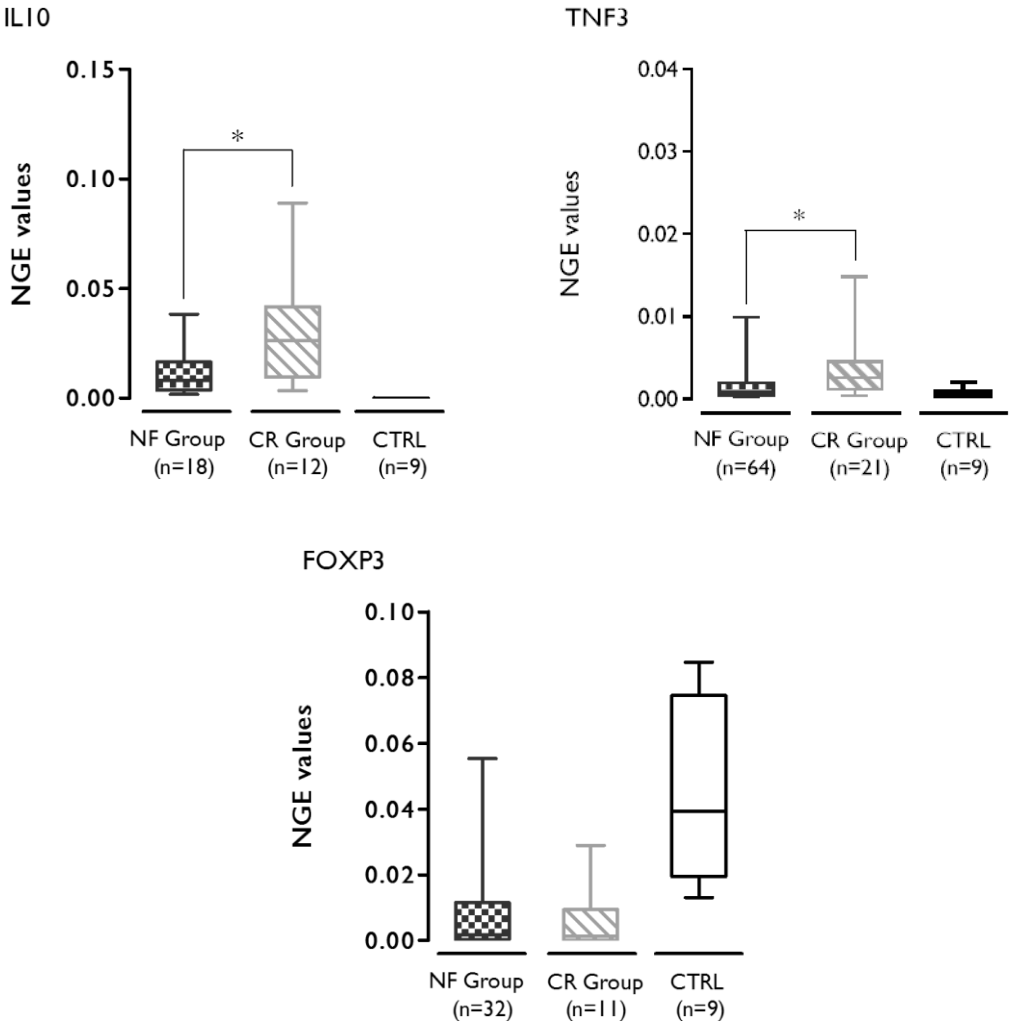

Figure 13- Box and whiskers plots of mRNA levels, in the urinary sediment, of *IL10*, *TNF3* and *FOXP3*. The horizontal line within each box represents the median, the bottom and top represent the 25th and 75th percentile values, and the thin vertical bars extend to the minimum and maximum values. The NGE values of both *IL10* and *TNF3* are superior for CR group comparing to NF group. The results are statistically significant with a p value of 0.02 and 0.002, respectively. Oppositely, The NGE values of *FOXP3* gene are increased in NF group, however, no statistical significance was observed.

* represents the results considered statistically significant for U Mann-Whitney test (p<0,05). NGE- Normalized Gene Expression; NF – Normal Function Group; CR – Chronic Rejection Group; CTRL – Control Group; *IL10* – Gene encoding Interleukin-10; *TNF3* – Gene encoding Tumour Necrosis Factor-3; *FOXP3* – Gene encoding the transcription factor FOXP3.

IL10 ROC curve revealed an area under the curve of 76,23%. The best correlation for specificity and sensitivity was 82,4% and 66,7%, respectively. These values for *TNF3* were 76,2% and 68,0%. The AUC for *TNF3* was 72,25%.

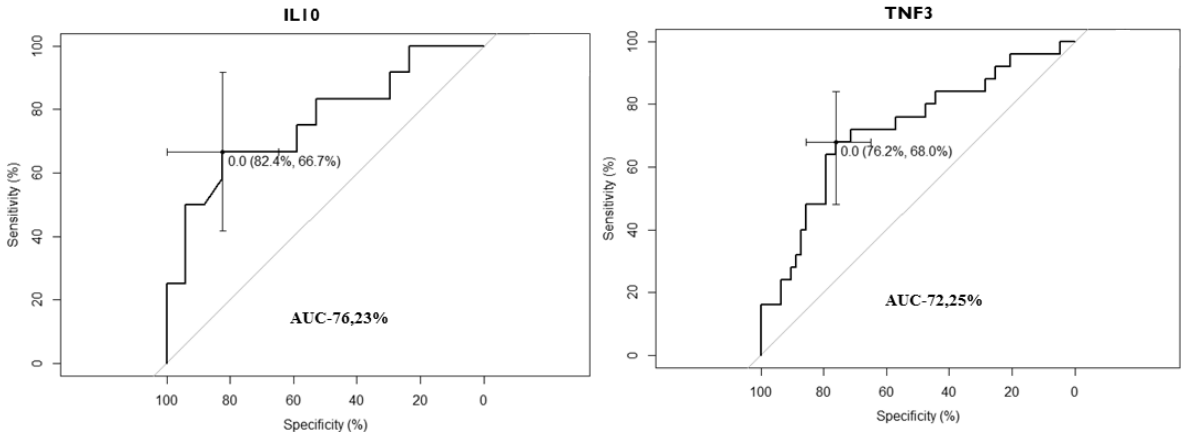

Figure 14- ROC curves (95% confidence interval) for sensitivity and specificity of *IL10* and *TNF3* to evaluate their diagnostic value for chronic rejection. Despite the low amplification, *IL10* gene exhibited decent predictive values for chronic rejection presenting an AUC of 76,23% with the specificity and sensitivity values being 82,4% and 66,7%, respectively. *TNF3* gene also showed a decent predictive value for chronic rejection with an AUC of 72,25% with a specificity of 76,2% and a sensitivity value of 68,0%. ROC – receiver operating characteristic; AUC – area under the curve.

The *IL17* gene was also tested, however, very few samples or even none amplified so it was not possible to proceed to any statistical analysis.

4. Discussion

In the last 20 years, the short-term results of kidney transplantation have improved considerably, mainly due to the use of modern immunosuppressive agents in doses sufficient to prevent acute rejection (136, 137). Despite these improvements, a significant percentage of allografts develop a progressive decline of renal function and fail within a decade making chronic rejection the main cause for late graft loss (138).

The mechanisms behind chronic rejection are not completely understood, therefore the extensive studies to better characterize this condition are extremely important and bring us closer to create strategies for improving kidney transplant in terms of survival, monitoring and prognostic (55).

Currently, renal transplant monitoring is done by regular clinical consultation. The samples evaluated in this study were from patients followed by the Renal Transplant Unity from the "*Centro Hospitalar Universitário de Coimbra*" E.P.E. As explained above, the only criterion for inclusion of the samples in the study was that the patients have undergone kidney transplant for more than two years. Posteriorly, the samples were divided into two groups: patients who have a normal graft function and patients with chronic rejection. This division was based on the creatinine levels and on the diagnostic of chronic rejection made by the clinicians. However it is important to note that sometimes the adverse effects of immunosuppressive drugs are mistaken with chronic rejection clinical manifestations leading to incorrect diagnostics.

Apart from the resemblance intra and inter-group, one important aspect to retain from Table I and II, is the higher percentage of previous acute rejection episodes in the CR group which is in agreement with current literature arguing that acute rejection facilitates the development of chronic rejection (64).

Despite the satisfactory homogeneity found in both groups, in future works more restrictive criteria should be applied when collecting the samples, in order to reduce the impact factor of demographic and clinical features in the results obtained. Moreover, future studies should include a higher number of patients with chronic rejection to balance the number of samples in each group.

Another limitation of this study lies on the control group only being composed by healthy individuals. A more suitable control group would be a set of individuals that were submitted to a kidney transplant and have a functional graft, but do not require any immunosuppressive agents to survive. These patients are registered in a worldwide database but it was impossible to get blood and urine samples from these patients.

As explained before, the HLA system is highly polymorphic making the search for a total compatible donor very challenging. It has been verified that HLA mismatches, specially the HLA-mismatches at the *HLA-A*, *B* and *DR loci*, influence renal transplant recipients outcomes (139, 140).

It is only natural that the amount of HLA mismatches was taking into account when choosing the suitable donor, explaining why the number of HLA mismatches found in both groups was not significant and the total of donor/receptor HLA mismatches between the two groups for either Class I and Class II *loci* was very similar (Table VII). This is crucial since the recognition of mismatched donor histocompatibility antigens is the key event that, eventually, triggers an immune response initiating allograft rejection (141).

Furthermore, until some years ago the first criterion for donor eligibility in terms of donor/receptor mismatches was the number of mismatches in HLA Class II *"DR" locus*, explaining the low number of mismatches for this *locus* found in both groups (Table VII).

The importance of humoral sensitization against foreign HLA antigens has been recognized since the beginning of transplant medicine (142). Over the past two decades, the advances in molecular approaches and in the technology available, resulted in implementation of sensitive assays for the screening and identification of anti-HLA antibodies (143).

Several studies demonstrated that the development of anti-HLA antibodies, pre or post-transplant, has a negative effect on graft survival (144-147). Most of these studies focus on donor-specific anti-HLA antibodies and on their ability to bind HLA antigens on allograft endothelium activating either the classic complement pathway or induce antibody-dependent cell-mediated cytotoxicity (148, 149).

Nevertheless, the contribution of non-donor specific antibodies cannot be ignored as in some patients the levels of these antibodies rise in rejection episodes suggesting a non-specific immune upregulation (150, 151).

In this study, the frequency of anti-HLA antibodies Class I detected in the serum of renal transplant patients was surprisingly higher in the normal function group when compared to the same frequency in chronic rejection group which was particularly low. The anti-HLA Class II antibodies frequency are considerably higher than anti-HLA Class I antibodies frequency in both groups. This goes in agreement with the literature suggesting that anti-HLA Class II antibodies detected in the serum have a more predictive value for injuries in microcirculation causing loss of graft function (152).

The association between a higher number of HLA mismatches and the amount of patients with anti-HLA Class I antibodies was validated for NF group (Table IX) showing that HLA mismatches are able to induce antibody production.

On the other hand for HLA Class II antibodies this association was not confirmed suggesting that HLA mismatches have different immunogenicity's. This has been described in some studies and also has been discussed the ability of some HLA mismatches to promote graft tolerance, under specific clinical conditions (153, 154). Additionally, Platt (155) theorized that the antibodies detected in the serum may be the ones with low affinity that are unable to bind to the graft, therefore not the ones causing graft rejection.

Considering the HLA mismatches different immunogenicity's, the low frequencies of HLA antibodies detected in the serum and also the theory that antibodies detected might not be the ones acting on the graft, there was a need to take a different approach and urine came as a natural alternative since it is produced in the kidneys and is easily collected by non-invasive methods.

The antibody screening methodology applied in the urine of renal transplant patients was developed from the standard procedures in our lab, normally used to perform antibody screening in the serum. Surprisingly decent results were obtained from the screening in urine, with a statistically significant increase of anti-HLA Class I antibodies frequency when compared to the frequency of the same antibodies detected in the serum of patients diagnosed with chronic rejection (Tables XII and XIII).

In 2005, Yi-Ping et al. (156) proposed a mechanism where anti-HLA Class I antibodies bind to HLA Class I molecules on the surface of graft endothelial cells stimulating cell proliferation and up-regulating cell survival genes promoting chronic rejection development.

Furthermore, Ali et al. (154) confirmed the capacity of anti-HLA Class I antibodies to induce endothelial cells proliferation after transplantation by a mechanism that facilitates the development of an inflammatory response due to the release of inflammatory cytokines and chemokines, like CXCL10 and CXCL8 by endothelial cells and it also favors cellular rejection, as a result of expression of cell-surface adhesion molecules resulting in increased addition of monocytes to the kidney endothelium (157, 158).

The high frequency of anti-HLA Class I antibodies found in urine of CR group patients can result from the mechanism described above, with the anti-HLA Class I antibodies moving into the urine when they lose adherence to the endothelium allowing their detection in urine but not in the serum.

In Table XIII, it is possible to observe the discrepancies in both urine and serum analyses, with 15 patients in NF group and 12 in CR group with detectable antibodies in the urine but not in the serum.

The 15 patients in NF group should be followed and their urine and serum tested for anti-HLA antibodies in the next few years, to understand if they are in a process of developing chronic rejection. If so, this could be a breakthrough in terms of chronic rejection diagnostic as it would be a simple, non-invasive, low cost method that would allow a premature diagnostic of chronic rejection, contrasting with the current methods available (essentially biopsies) that frequently diagnose this condition when it is irreversible.

Another point of interest is the production of these anti-HLA antibodies found in the urine of renal transplant patients. This has brought back B cells to the center of transplant immunology as they are the major producers of alloantibodies.

Thaunat et al. (95) noticed that the inflammatory infiltrated of kidney grafts, explanted because of terminal chronic rejection, had turn into organized ectopic lymphoid tissues, harbouring the maturation of a local immune response responsible for tissue destruction.

The formation of this functional ectopic germinal center, a process known by lymphoid neogenesis, permits the maturation of B cells into memory B cells and plasma cells, the last ones being the cells responsible for alloantibodies production. Oppositely, if the formation of the ectopic tertiary lymphoid tissue was incomplete the maturation of B cells was also blocked.

The fact that this maturation can be inhibited, unveils new targets for novel therapeutic approaches. In the same study, it was also verified that the circulating antibodies were different from the ones produced locally in the graft, denoting the existence of two different immune responses targeting different antigens.

The immune system has the capacity to produce a local adaptive immune response in the presence of secondary lymphoid organs. This immune response leads to the release of inflammatory chemokines who recruit naïve B cells into the graft originating the tertiary lymphoid tissue also known as B-cell cluster. Then, as explained above, if the neogenesis is completed, the ectopic germinal center becomes functional and the accumulated B cells can mature into memory B cells and to plasma cells.

Moreover, the B cells from this tertiary tissue can also locally promote T-cell response, as they function as antigen presenting cells, creating all the conditions for tissue destruction and for development of even more chronic inflammatory processes (159).

Alternatively, other studies show that B cells can promote tolerance but the mechanisms behind it are not entirely understood (160, 161).

The results obtained by qRT-PCR seem to go accordingly to the mechanism proposed above. We evaluated the gene expression in the cells of the urinary sediment of the immunological mediators of inflammatory state and fibrosis of renal tissue: CXL10 and CXCL9 and also one of the most important cytokines of the immune system TGF-β1 to confirm the aggravated inflammation of the graft in CR group.

The NGE levels of all three genes came out with superior values in the chronic rejection group with significant differences towards NF group (Figure 7). These results go in agreement with previous work done in our laboratory and also with the literature available (162-164). In fact, CXCL10 has been sustained as a decent biomarker for rejection, mainly, due to their role in regulating chemotaxis during the inflammatory response resulting from allograft rejection after transplantation (165).

The value of *CXCL10* as a suitable biomarker for chronic rejection was verified by the ROC curve analysis of the NGE values where *CXCL10* proven to be the best among the three genes with a specificity of 87,0% and a sensitivity of 62,5% corresponding to an AUC of 74,73% (Figure 8) which is a very satisfying value for a marker.

Although the information available suggest that *CXCL9* can have more value as a marker for acute rejection, the ROC curves analysis of *CXCL9* revealed an extremely high value for specificity (96,4%) but a low sensitivity value (45,0%) with an AUC of 68,04% (Figure 8) making *CXCL9* a decent biomarker for chronic rejection. The NGE values of the control group for both chemokines should be higher than NF and CR group, but that condition is only verified for *CXCL9*.

The NGE levels of *TGF-β1* gene are also in accordance to what was expected due to their aptitude to promote interstitial fibrosis, one of the hallmarks of chronic rejection, by increasing extracellular matrix proteins and blocking their destruction (166, 167). Szeto et al. (168) has already suggested that *TGF-β1* gene expression in the urinary sediment may serve as an instrument to evaluate the degree of renal injury in chronic kidney diseases. This is confirmed by the ROC curve analysis obtained for *TGF-β1* where a AUC value of 63,09% can be considered acceptable, however, a value of 53,0% (Figure 8) in terms of specificity can be consider inadequate in terms of diagnostic value.

The expression of the *CXCL10* and *CXCL9* genes in both groups suggest an involvement of Th1 pathway which culminates in the release of these chemokines.

The transcription factor T-bet primer was not available in the Lab, so we had to pick the transcription factor GATA3, associated with a Th2 pathway. Normally if one pathway is activated the other is downregulated.

Taking this into account, the NGE values for *GATA3* gene were found increased for the normal function group with statistical significant differences (Figure 9), implying that the development of chronic rejection is more likely associated with a Th1 response rather than with Th2, despite CR group having some *GATA3* gene amplification.

This goes in agreement with the results obtained for the inflammatory chemokines and with the some studies stating that Th1-like cytokine and chemokine production, triggered by indirect allorecognition, can induce chronic rejection while production of Th2 cytokines is linked with sheltering from the development of chronic rejection (169).

The control group displayed higher NGE values than both groups (Figure 9) suggesting a prevalence of a Th2 pathway in the cells of urinary sediment of healthy individuals. It is important to refer that the *IL17* gene had no amplification in neither group, suggesting that for this samples the T helper subtype Th17 was not present in the urinary sediment.

The NGE values obtained for *IL4* gene were found increased for the CR Group (Figure 9), which was surprising as IL4 is one of the interleukins responsible for promoting a Th2 differentiation, it was expected that the expression of this gene would be higher for NF group considering the results obtained for *GATA3* gene (39). The ROC curves for *GATA3* and *IL4* genes exhibited very low values of specificity (44,3% and 30,2%, respectively) (Figure 10) exposing a low predictive value for chronic rejection development.

The NGE values obtained for *CD79B* and *CD19* genes may help explain the apparent contradiction of *IL4* gene expression values. *CD79B gene* encodes a subunit of the B cell receptor and *CD19* encodes a protein found on the surface of B cells, so by evaluating the expression of these two genes we are assessing the presence or absence of B cells in the urinary sediment of both group of patients (170, 171).

The normalized gene expression values of both genes were found higher for CR group, with statistically significance differences when compared to NF group (Figure 11), indicating a greater presence of B cells during chronic rejection.

These results can reinforce the results obtained by Thaunat et al. in explanted chronic rejected kidney grafts, claiming the existence of a tertiary lymphoid tissue formed by B-cell clusters where B cells can mature. Furthermore, B cells seem to have the ability to produce IL4 which might explain the high expression of *IL4* in CR group (172).

The *CD19* gene ROC curve unveiled *CD19* has the gene with highest prediction value for chronic rejection in this study, with a specificity value of 72,5% and 77,8% for sensitivity corresponding to an AUC of 79,15% (Figure 12). The ROC curve acquired for *CD79B* gene unveiled a low sensitivity value (50,0%) and a decent specificity value (72,9%) with an AUC of 59,9% (Figure 12) indicating a poor value of diagnostic for chronic rejection.

Finally, the last 3 genes whose expression was evaluated in the urinary sediment were *IL10*, *TNF3* and *FOXP3*. IL10 is an anti-inflammatory cytokine and normally is produced by regulatory T cells (173). FOXP3 is a transcription factor necessary for the development and function of regulatory T cells (110). FOXP3 and IL10 are often associated with allograft acceptance as regulatory T cells play a crucial part in the development of tolerance (55).

TNF3 encodes a part of the all TNFα that plays a crucial role in inflammation processes by inducing a variety of cellular responses, which include cell death, survival, differentiation, proliferation and migration(174).

Inflammatory processes are part of chronic rejection disease so TNF3 is an important gene to evaluate. The NGE values of TNF3 confirmed this propensity for allograft inflammation in chronic rejection as they were significantly increased in CR group (Figure 13).

The NGE values for *FOXP3* genes were, as expected, superior for NF group (Figure 13), as explained above there are numerous studies associating *FOXP3* expression with tolerance and extended graft survival, however no significant differences were achieved for this gene (74, 175).

Interestingly, the expression of the same gene in a previous study done in our lab was also found increased for NF group with statistical significance, but the analysis was performed in peripheral blood samples indicating that peripheral blood analysis would be the best fit for *FOXP3* gene expression analysis (data not published).

The NGE values for *IL10* gene were unexpectedly increased in the CR group with statistical significance (Figure 13). A possible explanation for this result is the low number of samples that had amplification for this gene which can lead to inaccurate results. Moreover, through the years some studies have reported the role of IL10 in B cell activation and proliferation, so the high expression of *IL10* gene can be linked to the greater presence of B cells in the urinary sediment of patients diagnosed with chronic rejection (176-178).

IL10 gene expression was also evaluated in the peripheral blood in a prior study and the NGE values were also found higher for CR group with statistical significant differences between NF and CR group (data not published).

The ROC curve analyses for *IL10* gene showed an AUC of 76,23% with specificity and sensitivity values of 82.4% and 66,7% (Figure 14). These are decent predictive values, however, due to the low number of samples that had amplification IL10 cannot be chosen, in this study, as a suitable biomarker, for chronic rejection.

At last, the *TNF3* gene exhibited an AUC of 72,25% with a specificity value of 76,2% and a sensitivity value of 66,0% (Figure 14) which are respectable values for diagnosis of chronic rejection. *CD19*, *TNF3* and *CXCL10* genes showed the greatest potential as a biomarker for chronic rejection.

In my opinion, the best way to integrate the qRT-PCR as a standard diagnostic procedure for chronic rejection in renal transplant patients, would be to perform the gene expression evaluation in both urine and blood samples, as it becomes clear from this study and previous works that changes in the expression of certain genes are detectable in the urine (*CD19, TNF3 and CXCL10*, for example) and others are only visible in the blood (*FOXP3*). By doing both analysis, the results obtained would be more reliable and could lead to early and accurate diagnosis of this condition that still is the major problem in transplantation.

5. Conclusion

This study intended to perform a complete evaluation of the urine of renal transplant patients, in order to find potential biomarkers of chronic rejection, that may allow transplant monitoring without recourse to invasive techniques but, above all, it was meant to contribute to increase the knowledge of the mechanisms behind chronic rejection as this disease still is a major concern in transplantation.

Regardless of the minimum criteria applied for sample selection, the two groups (patients with a stable/normal graft function and patients diagnosed with chronic rejection) exhibited a decent homogeneity in their clinical and demographic features with the main difference being the higher percentage of previous acute rejection episodes in the chronic rejection group reinforcing the significance of acute rejection in the development of chronic rejection.

In the present study, the relationship between the presence of anti-HLA antibodies and the gradual decline of allograft function was not totally verified. Indeed for anti-HLA Class I antibodies the percentage of patients was higher for NF group and even though the percentage of patients with detectable anti-HLA Class II antibodies was higher in CR group the differences were not significant.

The relationship established, by various studies, between the number of HLA mismatches and the detection anti-HLA antibodies in the serum could only been verified for anti-HLA Class I antibodies in NF group where the number of patients with anti-HLA antibodies was higher for 2 or more HLA mismatches.

One of the most relevant points in this study was the development of an antibody screening method in the urine of renal transplant patients. This method allowed the detection of anti-HLA antibodies in renal transplant patients who had not showed any evidence of having anti-HLA antibodies in circulation, particularly, in CR group where more than half the patients presented anti-HLA Class I antibodies in urine. This increased percentage reinforces the relevance of these antibodies in the development of chronic rejection by inducing inflammatory responses and by their capacity to stimulate proliferation of endothelial cells.

This method still needs technical validation but the results obtained showed that urine can really be crucial to assess all features involved in graft dysfunction. Furthermore, the follow up of the patients with a normal graft function that had anti-HLA antibodies could validate this method as diagnostic tool for chronic rejection in renal transplant, if these patients develop this condition. If so, the antibody screening method could be implemented as a standard procedure and together with the screening in the serum, the information

generated would be more precise and could lead to an early diagnose of chronic rejection or at least would help identify the patients more susceptible to develop this condition.

In terms of gene expression in the urinary sediment of renal transplant patients, the normalized gene expression values for the inflammatory chemokines, CXCL10 and CXCL9, were higher for CR group verifying the role of inflammation in chronic rejection development and showing that both can be potentially decent biomarkers for rejection, specially *CXCL10*.

The higher NGE values for *TGF-β1* gene for CR group tend to show the involvement of this molecule in loss of graft function as it is able to promote the establishment of interstitial fibrosis, a hallmark of chronic rejection.

The increased NGE values for *GATA3* gene found in NF group, suggest that Th2 pathway may be involved in the development of graft tolerance. On the other hand, the lower expression of *GATA3* gene in chronic rejection group indicates a preference of Th1 differentiation pathway in the development of the disease. In future works, it would be important to evaluate the expression of the transcription factor T-bet and IL12 in order to confirm that Th1 differentiation is more active in the urinary sediment of renal transplant patients diagnosed with chronic rejection.

Concerning the NGE values obtained for *CD19*, *CD79* and *IL4* genes, the increased expression in chronic rejection patients indicates a greater presence of B cells in the urinary sediment of these patients. This goes in agreement with the studies performed by Thaunat et al. that report the existence of B cells clusters, similarly to a lymphoid tissue, capable of harbouring B cells maturation that may have a crucial role in the development of chronic rejection.

The higher presence of B cells in the urinary sediment of chronic rejection patients can also support the increased frequency of anti-HLA antibodies found in the urine of these patients as B cells are the major precursors of alloantibodies. Moreover, Thaunat et al also stated that the maturation of B cells in these clusters could be impeded by blocking the expression of certain genes, so these genes may constitute potential drug targets for treatment of chronic rejection or at least, for causing a delay in the development of this condition, increasing the survival rates of renal transplant patients.

Regarding the NGE values of the transcription factor FOXP3, characteristic of regulatory T cells, the increased levels for the normal function group confirms the involvement of these cells in induction of graft tolerance as these values were found.

Finally, *TNF3* gene expression levels were higher for CR group proving once again that allograft inflammation is a characteristic of chronic rejection development. The *IL10* gene expression values were also increased for CR group, but for this gene very few samples amplified so the results might be misleading.

The analysis of the receiving operating characteristic (ROC) curves revealed *CD19*, *CXCL10* and *TNF3* as the genes with the highest significant predicting value for chronic rejection in the sample studied, so in future works this should be taking into account.

The ideal control group, for this type of study, should be composed of patients who were submitted to a renal transplant but do not require immunosuppressive agents to survive in order to identify the gene expression values that result from renal transplant alone. However it is almost impossible to get blood and urine samples from these patients so to overcome this barrier, In future works more restricted criteria should be applied in sample selection to get even more homogeneity in the groups.

Another important point is the identification by "Single Antigen" assays of the anti-HLA antibodies found in the urine and serum samples, whose results were not presented here due to the lack of sufficient reagents to proceed to the analysis of all the samples. It would be interesting to know if the detected anti-HLA antibodies were donor-specific or not. Also in the future, the study design should be reconsidered, for example, the urine and blood samples should be taken every 3 months for a period of 3 years to evaluate the differences of gene expression and antibody screening over time

In conclusion, the results obtained in the antibody screening and gene expression analysis of the urine of renal transplant patients prove that the urine can be an important biological material to examine and can give vital information to assess the clinical condition of renal transplant patients.

However, the analysis of peripheral blood samples cannot be excluded or discarded. In fact, only the conjugation of both analyses provides a complete evaluation of the allograft as most of the clinical and histopathological manifestations of chronic rejection happening locally are also reflected in the surrounding regions.

Future works, may include flow cytometry analysis of the urine of these patients which should add vital information like, the number of cells present in each sample and even more importantly, could help identify the type of cells in the urinary sediment which would have a significant impact in the interpretation of the other results.

It is important to underline that the contribution of this work is very small, given the complexity of the mechanisms wrapped in the development of chronic rejection.

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