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De novo anti-HLA antibody study in patients with kidney transplants in the Valencian Region

Bachelor's Thesis in Biotechnology

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This work has been carried out in the Transfusion Center in the Valencian Region, center which collects data from patients of the transplantation centers in Valencia (Hospital Universitario y Politécnico La Fe and Hospital Universitario Doctor Peset).

Title: De novo anti-HLA antibody study in patients with kidney transplants in the Valencian Region.

Summary

Kidney transplantation is one of the treatments of choice for certain renal diseases. When the transplant is produced between members of the same species (with genetic differences between them), the donor organ is called allograft and the molecules of this, which as a result of the transplant may cause an immune response in the recipient, are known as alloantigens. Antibody mediated rejection is one of the immune responses in the transplant related to allograft loss, it appears mainly as a result of the exposure to molecules of the histocompatibility system (HLA, in humans). HLA system is a very polymorphic and polygenic group of genes in the human species which codifies for surface molecules present in all nucleated cells in the organism (HLA class I), and in a more specialized manner, in antigen presenting cells (APC)(HLA class II). HLA molecules are the first detectors of foreign molecules (antigens) and therefore, generators of signals for a possible immune response. The high variability of HLA within the individual, provides the ability of discrimination between the possible external threats, nonetheless in the context of transplantation between donor and recipient, it can produce an immune response against the allograft.

This work is based on the hypothesis that the development of post-transplant (those we call ''de novo'') anti-HLA antibodies is crucial for the adequate monitoring of kidney transplant recipients, as they play an essential role in acute and chronic rejection and that particularly donor specific antibodies (DSA) are an important risk factor for antibody mediated rejection (ABMR). The major objective of this project is, thus, the study of de novo anti-HLA antibody formation comparing patient baseline characteristics of a kidney transplant cohort to try to establish possible patterns in the differences between patients in our cohort in development of the immune response after transplantation. Overall, we have analysed several factors and their relation to differences in patient alloimmunization in a determined population (kidney transplant patients in the Valencian Region), proving some interesting relationships that may help to predict the probability of generating post-transplant antibodies.

Key words: Anti-HLA antibody; kidney; transplant; rejection; donor specific antibodies

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Título: Estudio de anticuerpos anti-HLA formados de novo en pacientes trasplantados de riñón en la Comunidad Valenciana

Resumen

El trasplante de riñón es uno de los tratamientos de elección para determinadas enfermedades renales. Cuando el trasplante se produce entre individuos de la misma especie (con diferencias genéticas entre ellos), se denomina aloinjerto al órgano del donante y aloantígenos a las moléculas de éste, que, como resultado del trasplante, pueden generar una respuesta inmune en el receptor. El rechazo mediado por anticuerpos es una de las respuestas inmunes en el trasplante relacionada con la pérdida de aloinjerto, y aparece principalmente como resultado de la exposición a moléculas del sistema de histocompatibilidad (HLA, en humanos). El sistema HLA es un conjunto de genes muy polimórfico y poligénico en la especia humana que codifica para moléculas de la superficie celular de todas las células nucleadas del organismo (HLA clase I), y de una manera más especializada de células presentadoras de antígeno (APC en inglés) (HLA Clase II). Las moléculas HLA son el primer detector de moléculas extrañas (antígenos), y, por tanto, generador de señales para una posible respuesta inmune. La alta variabilidad del HLA en el individuo proporciona la capacidad de discriminación entre las posibles amenazas externas, aunque estas diferencias, en el contexto del trasplante entre donante y receptor, pueden desencadenar una respuesta inmune contra el aloinjerto.

Este trabajo está basado en la hipótesis de que el desarrollo de anticuerpos anti-HLA post-trasplante (esos que llamamos "de novo") es esencial para la correcta monitorización de los pacientes trasplantados de riñón, ya que estos juegan un papel muy importante en el rechazo agudo y crónico, y en que en especial los anticuerpos específicos de donante (DSA) son un factor de riesgo para el rechazo mediado por anticuerpos. El principal objetivo de este proyecto es, pues, el estudio de anticuerpos anti-HLA formados de novo comparando características de pacientes de una cohorte determinada para intentar establecer patrones de las diferencias entre pacientes de la cohorte en la respuesta inmune producida post-trasplante. En general, hemos analizado varios factores y su relación con las diferencias en aloinmunización entre pacientes de una población determinada (pacientes trasplantados de riñón en la Comunidad Valenciana), demostrando algunas relaciones interesantes que podrían ayudar a predecir la probabilidad de generar anticuerpos post-trasplante.

Palabras clave: anticuerpo anti-HLA; riñón; trasplante; rechazo; anticuerpo donante específico.

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Nomenclatures and abbreviations

ABMR: Antibody Mediated Rejection
BD: Brain Death
CKD: Chronic Kidney Disease
DCD: Donation after Cardiac Death
dnDSA: de novo Donor Specific Antibody
DSA: Donor Specific Antibody
ERSD: End Stage Renal Disease
HLA: Human Leukocyte Antigen
IQR: Interquartile Range
MFI: Mean Florescence Intensity
MHC: Major Histocompatibility Complex
ND: No data
OPTN: Organ Procurement Transplant Network
PCR-SSO: Sequence Specific Oligonucleotide PCR
PCR-SSP: Sequence Specific Priming PCR
PRA: Panel Reactive Activity
RR: Relative Risk

1. Introduction

Renal Transplantation

Kidney transplantation is the treatment of choice in patients with certain renal diseases that require substitution therapy [1]. It is the solution with the best cost/effectivity relation for chronic kidney disease, meaning a notable improvement in the patient's life quality and an important economic saving to the National Health System [2]. We understand with chronic kidney disease (CKD) the gradual loss of the organ's function, stratified into stages of which 5, kidney failure or end-stage renal disease (ESRD), is the most severe [1], [3]. When reached, a series of measures become necessary to compensate for the kidney's functionality deficit as the disease has an important effect in illness-caused mortality (morbi-mortality), especially cardiovascular [1]. According to the data provided by the *Organ Procurement Transplant Network* (OPTN), the reasons for kidney transplants are: glomerular diseases; diabetes; polycystic kidneys; hypertensive nephrosclerosis; renovascular and other vascular diseases; congenital, rare, familial and metabolic disorders; tubular and interstitial diseases; neoplasms and retransplant/graft failure [4]. When the transplant is produced between members of the same species (with genetic differences between them), the donor organ is called allograft and the molecules of this, which as a result of the transplant may cause an immune response in the recipient, are known as alloantigens. The availability of organs is a limiting factor for transplantation as a solution to CKD and patients are generally included in a dialysis program, that provides an extrarenal depuration system, until there is organ availability [1].

Different kind of organ donors exist, on one hand there are living donors and on the other cadaveric, from which we can distinguish between donation after irreversible loss of brain function known as encephalic or brain death (BD) and donation after cardiac death (DCD) or asystole [5], [6]. These last have been denominated *Non Heart Beating Donors*, *Donors after Cardiac Death* or, the most recent denomination, which is more accurate with respect to the diagnosis of death, *Donors after the Circulatory Determination of Death*. Living donation was initially reduced as the use of deceased donors increased; however, it has a series of advantages over cadaveric donation which have made it an attractive option again since the beginning of the century, the first being the results obtained [2], [5]. As stated by the OPTN, the survival of the allograft after the first year is of 89% for renal transplants from cadaveric donors versus 95.1% for those from living donors. These differences become even more significant after five years where durability is of 66.5% compared to 79.7% respectively. As regards to patient survival, living transplant also improves the outcomes with 5-year survivals of 82% for recipients of deceased donors versus 90.2% in the case of living renal transplants [5]. The second reason that explains the renewed interest in this kind of donation is it can, at least, palliate the shortage of organ donors [5], [6], especially in younger patients [2]. In 2019, in Spain: 7356 patients were in waiting lists of which only 3423 were transplanted (46.5%). When analyzing data in the Valencian Region, the gap was even larger; the estimated transplant waiting list per million persons was 166.8 pmp (834 persons) but only 59.8 pmp (299) transplants were performed, a 35.9% of the waiting list. [7].

The first completely successful renal transplant, which took place in 1954 by the research group headed by Joseph Murray, was a living transplant between identical twins [5], [8]. It solved the main challenge encountered during transplant attempts until then: HLA disparity between donors and recipients and the subsequent deterioration and posterior graft loss caused by an aggressive acute immune response [5].

HLA System

The Major Histocompatibility Complex (MHC), system known as Human Leukocyte Antigen (HLA) in humans, is a group of genes located on the short arm of chromosome 6, on band 6p21.3. This organized genetic system encodes for a great variety of molecules with key roles in biological pathways [9]. It exhibits great diversity between individuals and species that provide HLA the ability of discrimination between the possible external threats, nonetheless, in the context of transplantation between donor and recipients, it can produce an immune response against the allograft [10]. Being the largest cluster in the human genome, it is divided into three regions known as class I, class II and class III according to the function and structure of its gene products, all involved in immune response and suppression [9]. Within class I, we can distinguish 3 main genes: HLA-B, HLA-C and HLA-A that encode for proteins with the same name and other loci that encode for non-classical HLA molecules: HLA-E, HLA-F, HLA-G, HLA -H. Similarly, the class II region has various loci that codify for classic HLA class II proteins: HLA-DP, HLA-DQ, HLA-DR and non-classic HLA class II isotypes: HLA-DM, HLA-DO (they carry out different functions) [10]. Finally, more than 50 genes have been identified in class III region of which C3, C4 and C5 of the complement, heat-shock proteins and the TNF family (including TNF- α) are worth highlighting. For what concerns us, we will focus on HLA-A, -B, -C from class-I antigens and HLA-DP, -DQ- DR from class-II antigens *(figure 1).* The first are expressed in all nucleated cells and platelets (except for those of the central nervous system) while the second, are present in a more specialized manner, in antigen presenting cells (APC). Examples of these are B lymphocytes, macrophages, monocytes, dendritic cells, Langerhans cells, endothelial cells and thymic epithelial cells [9].

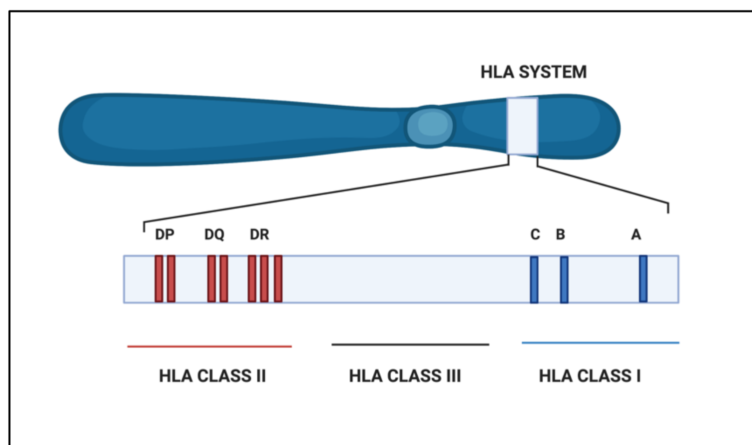

Figure 1. *Location and Organization of HLA system in chromosome 6*

MHC was first discovered in 1948 by Snell and Gorer as a genetic locus associated with acceptance or rejection of transplanted grafts in mice [9]. Years later, through skin transplant studies, Medawer and Billingham identified the existence of a genetic basis for allograft recognition and named the genes responsible for this process histocompatibility genes [10]. In 1954, Jean Dausset described the same system in humans (HLA) when identifying the presence of antibodies against leukocytic antigens in patients with previous blood transfusions that where later also discovered in women with previous pregnancies and people with previous transplants [9].

Class-I and class-II molecules play an essential role in T-cell mediated adaptive immunity. HLA molecules are the first detectors of foreign molecules (antigens) and therefore, generators

of signals for a possible immune response. We can summarize the main functions of HLA molecules in antigenic recognition, antigen presentation to immunocompetent cells and the regulation of the immune response. Class-I and class-II HLA proteins carry out each of these functions differently [10]. With respect to antigenic recognition and presentation, both type of proteins collect antigenic peptides from inside presenting cells (produced by intracellular protein degradation [9]) and transport them to the cell surface where they will later be recognizes by CD4+ or CD8+ T cells *(figure 2).* However, the nature of the peptides that bind the different HLA classes is different. In general, endogenous peptides from intracellular virus or bacteria and (more frequently) from deteriorated self-molecules are associated with HLA class I and are recognized by cytotoxic T lymphocytes (CD8+ T Cells). Meanwhile, exogenous antigenic peptides are associated with HLA class II and recognized by CD4+ T cells (often called helper T cells) [9], [10]. Once activated, CD8+ and CD4+ T cells have different effector functions; the cytotoxic function of the first allows them to kill virus-infected cells and cancer cells, while CD4+ T cells have a wider range of effector functions which involve the targeted delivery of cytokines to activate other cells in charge of the production of antibodies (like macrophages or B lymphocytes) and the humoral immune response [9].

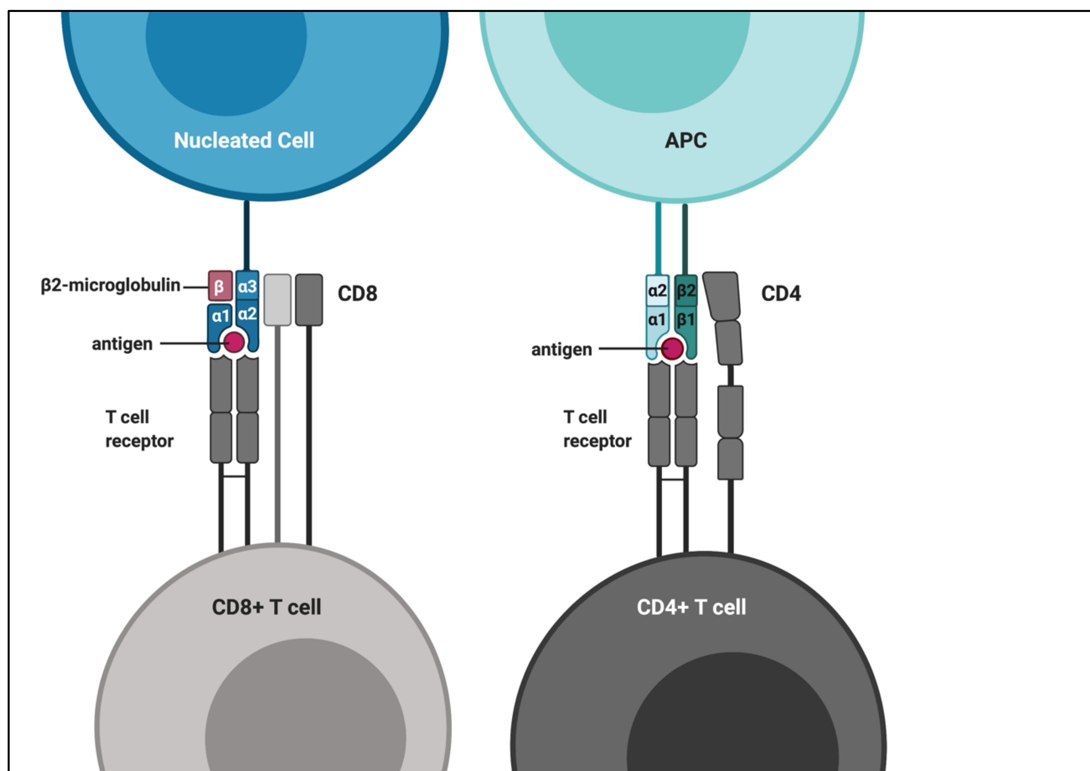

Figure 2. *Antigen presentation through HLA class I and class II molecules to the corresponding T cells.*

Class-I molecules are composed of two polypeptide chains: a heavy α chain (45 kD) and a light β2-microglobulin chain (12.5 kD) joined by non-covalent bonds. The light chain is not polymorphic and not encoded within the HLA system but is located on chromosome 15 [10]. The α chain is highly variable between individuals of the same species [10]. It has three extracellular domains ($\alpha 1, \alpha 2, \alpha 3$), a transmembrane region composed of hydrophobic amino acids that anchor the molecule to the cell membrane and a cytoplasmic tail. $\alpha 1$ and $\alpha 2$ helices compose a peptide-binding grove which can bind peptides of 8-10 amino acids in length. This is the region with highest variability and is resultantly responsible for the high polymorphism (different

forms) of HLA class I proteins [9]. Class-II molecules are formed by two non-covalently linked polypeptide chains: a heavy or α chain (33 kD) and a light or β chain (29 kD). This time, the genes encoding both chains are polymorphic and located in the HLA locus. Both chains have a similar organization constituted by extracellular domains $\alpha 1$ and $\alpha 2$; $\beta 1$ and $\beta 2$ [10]; and anchored to the cell membrane by a transmembrane region of hydrophobic amino acids with a cytoplasmic tail. In HLA class-II molecules, the peptide-binding groove is formed by the $\alpha 1$ and $\beta 1$ domains. Unlike class I, they can accommodate longer peptides of 12-24 amino acids (and even longer) *(figure 3).*

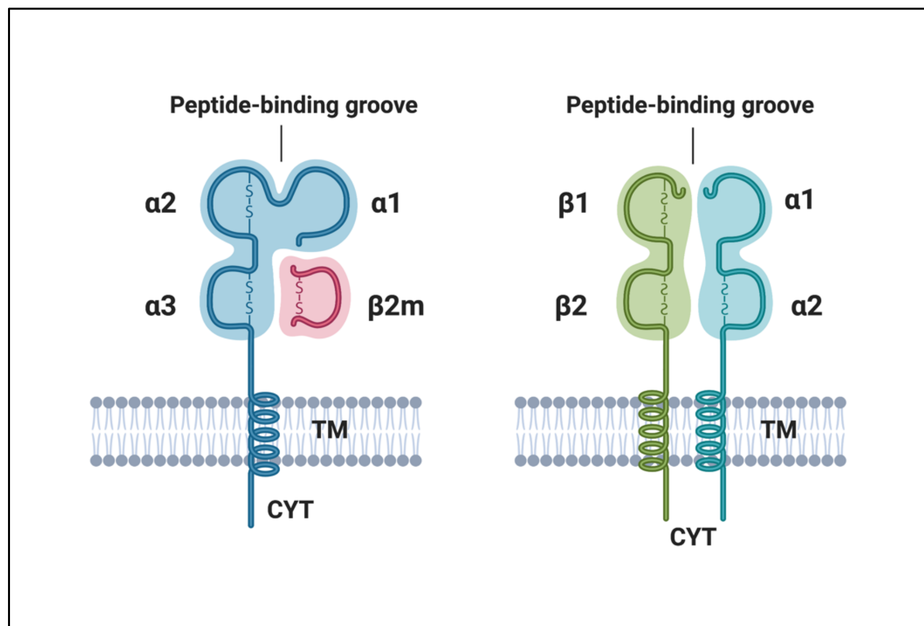

Figure 3. *Schematic diagram of the structure of HLA class I and class II molecules. TM: transmembrane region. CYT: cytoplasmic tail.*

There are three important characteristics of the HLA system that make it specific and extremely variable. As we have already mentioned it is polygenic (composed by many different genes). In transplantation, this increases difficulty to find an adequate compatibility between donor and recipient to avoid rejection [11].

In addition, it is highly polymorphic, term which describes the variability in amino acid sequences in proteins coded by allelic genes (genes with several forms). To date the IMGT/HLA Database has reported 26,887 HLA alleles (of which 19,586 are class-I and 7,301 are class-II). Furthermore, not all loci are equally polymorphic, for example in class-I, gene HLA-B is the most polymorphic with 7,255 alleles, followed by HLA-A (6,082 alleles) and HLA-C (5,842 alleles). In class-II, the most polymorphic genes are DRB (3,357 alleles), DQB1 (1,826 alleles) and DPB1 (1,556 alleles) [12]. In transplanted allografts, polymorphism of HLA-molecules determines the outcome (acceptance or rejection) [10]. These variations of HLA molecules affect their specificity by determining which amino acids are in the peptide binding groove [9]. In class-I, variability is nearly exclusive of the $\alpha 1$ and $\alpha 2$ domains of the heavy chain while in class-II, polymorphism occurs mainly in the $\beta 1$ domain [10]. The evolutionary reason for the presence of multiple HLA alleles in the population is to ensure recognition of a large number of particles and/or microorganisms, so it is impossible for a single pathogen to evade host defenses in all individuals of a species [9]. However, this multiplies exponentially the difficulty to find a compatible donor recipient pair.

Finally, the expression of these genes is codominant, so expression of both alleles of a single locus is simultaneous [11]. The set of HLA alleles present in each chromosome is known as haplotype [10]. All heterozygous individuals have two HLA haplotypes (one paternal and one maternal), each of which contains three class-I (-A,-B,-C) and three class-II (-DP, -DQ, -DR) loci [9].

Compatibility and anti-HLA antibodies

HLA incompatibility between non-identical donor and recipients is considered the main barrier to successful transplantation, mainly due to antibody mediated rejection (ABMR) triggered by the production of antibodies with specificity towards non-self HLA proteins (humoral sensitization) [13]. Due to the high levels of expression of HLA in donor cells, non-self HLA molecules facilitate allorecognition and are a strong stimulus of the humoral immune response [14]. These anti-HLA antibodies, depending on the time of appearance, can be classified in pre-transplant, when the exposure to HLA antigens has been given before the transplant, which occurs mainly for three reasons: a previous transplant, a pregnancy or a transfusion, and post-transplant ("de novo" antibodies), when their appearance has been a consequence of the actual transplant. After the development of the anti-HLA antibodies we consider a patient is alloimmunized. In addition, antibodies can be donor or non-donor specific (DSA and non-DSA respectively), if they are complementary or not to a region of a peptide in the donor (which is not present in the recipient). Specially DSA have been identified as central components of ABMR in kidney transplants and this has been recognized as a major cause for renal allograft loss [15], [16]. Donor specific anti-HLA antibodies can appear any time post-transplantation, usually due to insufficient immunosuppression or non-adherence to immunosuppressive therapy [13]. Anti-HLA class II DSA are the more frequently de novo produced antibodies in patients unsensitized before transplantation [16].

Several factors deserve consideration when matching the donor kidney with the recipient, as the donor organ can act as an alloantigen. When transplanting tissues or cells from a genetically different individual to the graft recipient, the alloantigen of the donor induces an immune response in the recipient against the graft [17]. Mismatched amino acids in HLA proteins can trigger DSA formation. However, not all epitope mismatches have the same immunogenicity and therefore not all mismatches will induce the same immune response.

Rejection

We understand by allograft rejection the pathologic changes, with or without disfunction of the allograft, caused by the inflammation induced by the recipient's immune system as it recognizes the non-self antigens in the allograft. Both innate and adaptive immune systems are involved in rejection. Although T lymphocytes are the main cells involved in allograft recognition, other costimulatory molecules like cytokines are also implicated in the rejection process.

Among the immunological responses that can activate during a transplant, a humoral immune response characterizes for the production of anti-HLA antibodies which may be donor specific (DSA). One of the processes of this response is the activation of an immunological memory whereby a specific, faster and more aggressive response will take place if a re-exposure to the antigen is produced (for example in a new transplant). This will increase the possibility of rejection and, in more severe cases, cause graft loss.

The renal transplant rejection can be classified as follows:

1. **Hyperacute rejection**
   Occurs minutes after transplantation and happens as a result of preformed antibodies or ABO incompatibility; it is rarely seen now a days.

2. **Acute rejection**
   Can happen any time after transplant, although it usually takes place within days to weeks from the intervention. This can be:

   -Antibody mediated Rejection (ABMR): Caused by circulating donor specific alloantibodies (DSA), mainly against HLA antigens, it produces antibody-mediated injuries to the kidney through the activation of the complement dependent pathway and other innate immunity mechanisms.

   -Acute T cell rejection: Lymphocytic infiltration in the tubules, interstitium and arteria intima. The recipient's lymphocytes are activated by APC recognizing non-self donor antigens.

3. **Chronic Rejection**
   Usually takes place more than three months post-transplantation. It can be chronic ABMR or chronic T cell rejection (uncommon).

4. **Acute rejection superimposed on chronic rejection**

Factors that correlate with an increased risk of rejection are prior sensitization, type of transplant (deceased vs living), advanced donor age, prolonged cold ischemia time, HLA mismatch, ABO incompatibility, recipients age (young compared old) and race (African American compared to White race), delayed graft function, therapy non-adherence, inadequate immunosuppression and previous episodes of rejection.
Until now, most studies have focused in the emergence of pretransplant antibodies, given that their analysis will help to avoid the more immediate rejection (hyperacute). However, the investigation of antibodies that form post-transplant is essential to prevent acute or chronic rejection and establish when necessary the strategies to prevent graft loss.

Analytical strategies

Given the well-established relationship between HLA compatibility and allograft evolution, determination of HLA antigens in both donor and recipient is compulsory for renal transplantation. Before, typing was usually determined through serological methods, but each time more, molecular biology methods are replacing these as they allow a better definition at allelic level, which enable a better control of humoral immune response [18].

Typing by serological methods consists in determining the alleles of the HLA system expressed in an individual. Recipient lymphocytes were classically crossed with serums containing known HLA specificities. Rabbit complement was added, and cell lysis was produced when binding had been efficient (Terasaki microlymphocytotoxicity).

Thanks to the polymerase chain reaction methodology (PCR), typing can now be done with a higher specificity and resolution (although usually an equivalent resolution to serological tests is used). Molecular techniques focus on the polymorphic exons 2 and 3 of HLA-I and exon 2 in HLA-II. Different molecular methods can be distinguishing depending on the basis of sequence recognition. In renal transplantation the most frequently used techniques are PCR-SSP (sequence specific priming) and PCR-SSO (sequence specific oligonucleotide). The first uses primers with sequences complementary to sequences specific to known HLA specificities, while the second amplifies the whole locus of HLA-I and HLA-II exons. Posteriorly, amplified DNA is hybridized with oligonucleotide probes in a solid support, although from the last years Luminex technology has been adapted to carry out a variation of this solid phase test. DNA probes are fixed to Luminex microspheres so that amplified DNA can hybridize with the microspheres and is marked with a fluorochrome. Each microsphere has a specific colour, which in combination with the DNA fluorochrome allows reading with flow cytometry. Results are interpreted by a typing program [19].

Once known the typing of donor and recipient, HLA compatibility between both can be determined. The higher this is the less risk of an immune response and therefore possible allograft damage. Compatibility is studied especially for loci A, B and DR, although C and DQ are getting introduced. At the moment DP is not considered. The main reason for not carrying out a more detailed study is the limited time between the organ extraction and the transplant. A cross match test is compulsory before a possible transplantation to study recipient specific humoral immunization against the potential donor. This is usually done by cytotoxicity incubating receptor serum with donor lymphocytes in the presence of rabbit complement. If cellular lysis is produced, transplantation will not be carried out as there is high risk of hyperacute rejection. If a higher sensibility is required the cross match can also be performed through flow cytometry [19].

Periodic study of antibody presence has to be done on patients in waiting list, especially after sensitizing events like pregnancies, transfusions or other transplants, to detect the existence pre-formed antibodies and allow a correct management of these patients. Detection and characterization of preformed antibodies increases transplant success possibilities, yet, the more antibodies detected the harder it is to find a compatible donor. The identification of post-transplant anti-HLA antibodies is also important to prevent acute or chronic rejection and establish, when necessary, the strategies to prevent graft loss. Antibody detection can be carried out by cytotoxicity or flow cytometry. The first, and more traditional is based in the microlymphocytotoxicity Terasaki test, where the panel reactive activity (PRA) against which a patient was sensitized was achieved crossing patient serum to a lymphocyte panel representative of the population. Now a days, calculated PRA (cPRA) is obtained with the Luminex detected specificity results, without the need for complement fixing. An advantage of this second method is it allows to objectively quantify anti-HLA antibody concentration by mean fluorescence intensity (MFI) [19]. When a patient presents an elevated rate of antibodies against external tissue or molecules it is considered hypersensitized. A special transplant program exists for patients in waiting list with a PRA higher or equal to 98% [20].

C1q

The complement system plays an essential role in increasing pathogenicity of DSA. When DSA bind the graft endothelial, the classical complement pathway will activate several mechanisms that can result in endothelial injury and allograft damage. The capacity of DSA to fix the complement depend on their class (IgM and IgG are the most effective activators). Within IgG, subclasses IgG1 and IgG3 are the strongest initiators of the classical complement response [14]. Circulating donor specific anti-HLA antibodies that bind complement in kidney transplant recipients can be detected using antigen bead assays that detect C1q-binding [13]. If the de novo DSA (dnDSA) studied has complement fixing ability it will bind C1q, forming an antigen-antibody association, thus activating the C1 complex and resultantly initiating the classical pathway of the complement system.

2. Objectives

This work is based on the hypothesis that the development of post-transplant (those we call ''de novo'') anti-HLA antibodies is crucial for the adequate monitoring of kidney transplant recipients, as they play an essential role in acute and chronic rejection and that particularly DSA are an important risk factor for antibody mediated rejection.

The major objective of this project is, thus, the study of de novo anti-HLA antibody formation comparing patient baseline characteristics of a kidney transplant cohort to try to establish possible patterns in the differences between patients in our cohort in development of the immune response after transplantation. Particular objectives include:

1. Assessing characteristics that favour the development of post-transplantation anti-HLA antibodies.
2. Identifying risk profile in the patients of this cohort.
3. Identifying the characteristics of the more deleterious antibodies.

3. Materials and Method

3.1. Data obtention

We conducted a retrospective observational study of ''de novo'' anti-HLA antibody formation in 2320 patients who had a kidney transplantation in the Valencian Region over a 40 - year period, from 01/07/1979 to 22/01/2020. Information was extracted from the Transfusion Center in the Valencian Region, as an Excel sheet, using a self-created program. This was transferred to MySQL, a relational database management system (RDBMS) that allows to retrieve, modify and administrate a database using SQL. Initially, 2849 records were extracted from the center's program but patients that were still in waiting list and had not yet received a transplant were excluded.

The clinical data of interest was collected and introduced into MySQL to create a new database. The following information was recorded: age, sex, ABO group, Rh group, date of first dialysis session, pregnancies, number of transfusions, dates of up to three transplants per patient, graft state and the result of the LAB Screen Single Antigen assay (SNG). When available, C1q test results were also recorded. On reviewing the data, each category was ordered to identify missing data or wrong records.

3.2. Descriptive statistics

The descriptive statistics of our population of study were prepared for each independent variable (age, sex, blood group, time in dialysis, number of pregnancies, number of transfusions, number of previous transplants, graft state, SNG distribution, HLA class and loci towards which de novo formed antibodies were produced and C1q test results) using MYSQL, excel and GraphPad Prism 8 software. This allowed us to check the distribution of our population and reliability of the later statistical analysis.

Age

Our population of study was divided into different age groups: 0-9 years old, 10-19 years old, 20-29 years old, 30-39 years old, 40-49 years old, 50-59 years old, 60-69 years old, 70-79 years old and from 80 years old onwards. The number of total patients in each subgroup were counted and results were plotted for better visualization.

Gender

Frequency distribution of our population by gender was also measured and plotted. Because some data was not available, we split our records into three groups: female, male and no data (ND).

Blood group

With the ABO group and Rh data, the frequency distribution of the different blood groups (0+, 0-, A+, A-, B+, B-, AB+, AB-) was determined. Several records were missing for both the ABO group and the Rh group; we considered as ND patients with missing Rh data (as this was more common and all patients missing ABO data were also missing Rh data).

Transfusions

There was a high variability within the possible number of transfusions a patient could have had. As a result, we considered zero and one as cut-off points, hence having three groups:

patients that had not had previous transfusions, patients that only had one and patients with two or more previous transfusions. Frequencies were calculated and results were plotted.

Previous transplants

Another of the recorded data categories was the dates of up to three transplants per patients. With this, we determined the number of previous transplants each individual had: zero when talking of a first transplant, one when analyzing a second transplant and two when dealing with a third transplant. Frequencies for this groups were plotted.

Pregnancies

To measure pregnancy frequencies, only female patients were taken into account so unlike for the other variables, the total cohort here was 901. Like with previous transfusions, there was a wide range of possible outcomes so, again, cut-off points were used (0, 1 ,2 ,3 and over 3 pregnancies).

Time in dialysis

The time in dialysis was calculated (when relevant) with the date of the first transplant posterior to the dialysis date and the dialysis date. To decide the appropriate time unit, we looked at our data and opted to use years. We generated groups of time intervals, recorded and plotted frequencies in each. A group of ND was also used when there was no data available. The groups were the following: No dialysis, (0-1], (1-3], (3-5], > 5 and ND.

Graft state and SNG distribution

Frequencies of graft state (functional, F ; rejected R ; no data, ND) and SNG distribution (No antibodies generated post-transplantation (SNG -), preformed antibodies (SNG +) which we assumed did not generate antibodies post-transplantation and de novo formed antibodies (SNG+)) were measured and plotted.

With the number of patients with positive SNG, the total number of tests (SNG), the number of rejected transplants, the total number of transplants performed and the same information about patients with DSA within patients with de novo formed antibodies, the following was calculated and graphed:

$$\% \, SNG+ = \frac{SNG(+) \; patients}{SNG \; tests \; performed} * 100$$

$$\% \, Rejections = \frac{Rejected \; transplants}{total \; transplants \; performed} * 100$$

$$\% \, Rejections \; in \; SNG(+) patients = \frac{SNG(+) \; patients \; with \; rejected \; trasplants}{SNG(+) \; patients} * 100$$

$$\% \, Rejections \; in \; SNG(-) patients = \frac{SNG(-) patients \; with \; rejected \; trasplants}{SNG(-) \; patients} * 100$$

$$\% \, DSA+ = \frac{DSA(+) \; patients}{Patients \; with \; de \; novo \; generated \; antibodies} * 100$$

$$\% \, Rejections \; in \; DSA(+) \; patients = \frac{DSA(+) \; patients \; with \; rejected \; trasplants}{DSA(+) \; patients} * 100$$

$$\% \, Rejections \; in \; DSA(-) \; patients \; = \frac{DSA(-) \; patients \; with \; rejected \; trasplants}{DSA(-) \; patients} * 100$$

SNG (+) tests include both patients with preformed antibodies and patients with de novo formed antibodies. However, for the purpose of our study, when referring to DSA, we only include patients with de novo formed antibodies. Patients with preformed antibodies were not further studied to differentiate between having DSA or non-DSA (NDSA). The representations allowed to visualize the difference in graft rejection between patients with and without anti-HLA antibodies and the effect of DSA within the de novo formed antibodies.

Class and Loci

De novo formed antibodies can be generated only against class I, only against class II or against both class I and II donor HLA molecules. From the patients that developed post-transplantation antibodies, we differentiated and plotted this information to see if antibodies were predominantly produced against a specific class. The same was done with the loci against which they reacted; we were left with eight groups: A, B, Cw, DP, DQ, DR, DRw and no data.

C1q

C1q tests are rarely performed in our center. In fact, only 81 tests have been carried out; various to the same patients (to test for different class antibodies or at different times). If taking into account persons instead, the total is only 36. To visualize the results, we organized the recorded data so that tests were shown per patient. We then distinguished between positive and negative test results and plotted this data for better visualization.

3.3. Statistical Analysis

Using GraphPad Prism 8, the normality and homoscedasticity of our data was analyzed with four different normality tests (D'Agostino & Pearson omnibus normality test, Shapiro-Wilk normality test, KS normality test and Anderson-Darling normality test) and two homoscedasticity tests (Bartlett's test and brown-Forsythe test). Because our populations of study did not follow the Gaussian distribution, we proceeded to use non-parametric tests to carry out univariant analysis with the objective of comparing three different groups (patients that did not generate antibodies post-transplantation, patients that had pre-formed antibodies but still did not generate new ones and patients that generated de novo antibodies). These gave us an insight on the variability of each factor by identifying the similarity or differences between the groups. Records have been expressed as median (IQR) for quantitative variables and n (%) for categorical variables; Kruskal-Wallis test and Chi square test were used respectively. We considered statistically significant values those with a P value < 0.05. Association studies were latter performed.

1.3.1. Univariant analysis

Quantitative data

The Kruskal-Wallis test does not directly address hypothesis about the medians of the groups and was therefore used for all quantitative data (age; previous pregnancies, transfusions and transplants and time in dialysis) despite not being homoscedastic, as was the case of previous transfusions, transplants and time in dialysis. Instead, it determines statistically significant differences between groups using mean ranks. The null hypothesis supports samples are taken from identical populations and observations are independent within each sample and

among groups. If this was not the case, the null hypothesis was rejected. To determine which groups are different from the others, the Dunn test was conducted as post-hoc testing in quantitative variables.

Categorical data

The Chi-squared test was used to determine independence/dependence of categorical measurements from the different groups. A contingency table was created for each categorical variable and the test was then performed. When pertinent, Fisher's exact test was performed on our groups of study by pairs to determine the group or groups responsible for the relationship.

1.3.2. Association studies

To study if a significant relationship existed between rejection outcome and antibody presence, class against which antibodies appeared and loci against which they appeared and C1q positive tests, Cramer's V coefficient (V) was calculated with excel. For this, contingency tables were prepared, and the Chi-square test was performed. Cramer's V was calculated with the following formula:

$$V = \sqrt{\frac{X^2}{n * (q-1)}}$$

Where V is Cramer's V coefficient, X^2 is Pearson's Chi-square value, n is the total number of values and q is the minimum of the number of columns and number of rows in the contingency table.

The relative risk (RR) of rejection happening in SNG (+) patients, DSA (+) patients and C1q (+) patients, and the relative risk of different class antibodies to bind C1q, was also measured to depict the probability of occurrence of each event (it shows direction and magnitude of the association, if any). It was calculated as follows:

$$RR_{SNG} = \frac{Relative\ rejections\ in\ SNG +}{Relative\ rejections\ in\ SNG -}$$

$$RR_{DSA} = \frac{Relative\ rejections\ in\ DSA +}{Relative\ rejections\ in\ DSA -}$$

$$RR_{CLASS} = \frac{Relative\ rejections\ in\ class\ I}{Relative\ rejections\ in\ class\ II}$$

$$RR_{C1q} = \frac{Relative\ rejections\ in\ C1q +}{Relative\ rejections\ in\ C1q -}$$

Where RR_{SNG} is the relative risk of rejection due to the presence of any de novo formed antibody, RR_{DSA} is the relative risk of rejection with the presence of de novo DSA, RR_{CLASS} is the relative risk of class I antibodies binding C1q with respect to class II, and RR_{C1q} is the relative risk of rejection in patients with antibodies that bind the C1q subunit of the complement system.

Development time and allograft state

Anti-HLA antibody development time was calculated with of the first SNG positive test post-transplantation and the transplant date. One was use as the boundary point so that we were left with two groups, patients that developed antibodies up to one-year post-transplantation and patients that took more than a year to generate them. To study the association between the factors a Fisher's exact test and Crammer's V coefficient were calculated.

C1q, allograft state and class

Apart from the Crammers V coefficient and the Relative Risk calculated to analyze the association between DSA C1q binding ability and allograft state, we also performed a Fisher's exact test. The association between DSA C1q binding ability and antibody class was studied with a Chi-square test.

2. Results

In this study, we report the results of our retrospective observational study on the antibodies produced against the HLA mismatches in patients with kidney allografts in the Valencian Region.

4.1. Baseline characteristics

From the 2320 patients studied, 491 (21.2%) had positive LAB Screen Single Antigen assays after kidney transplantation. Of these, 329 (14.2% of the total) did not have pre-formed antibodies and yet developed de novo antibodies after the intervention. 208 patients generated DSA, 45 generated non DSA and we had no data available to determine specificity towards donor antigens in 76 patients. The median time in generation of post-transplantation antibodies was 10 years (IQR: 3-18). From the DSA (+) group, a total of 27 patients developed C1q binding antibodies *(figure 4).*

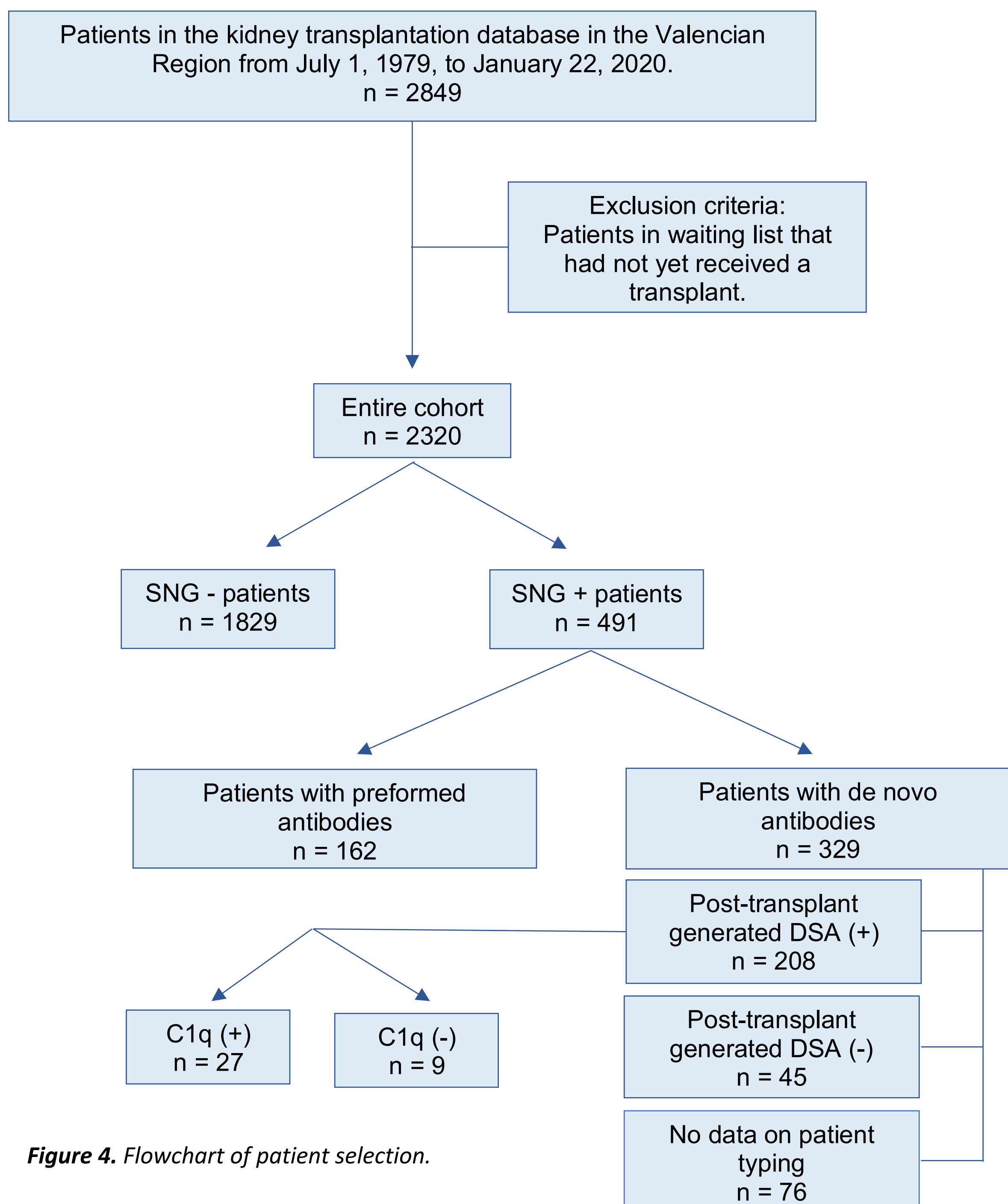

Figure 4. *Flowchart of patient selection.*

The distribution of our population by age, showed the predominant age group in transplantation patients was 60-69 years old with 25.99 % of our cohort, followed closely by age groups surrounding this: 50-59 (22.24 %) and 70-79 years old (21.47 %). Age groups of 30-39 and 40-49 years old, also had a substantial number of patients in them (7.89 % and 13.88 % respectively). However, the frequencies of groups in the tails of our distribution were lower as a result of the unusual occurrence of transplantation in young and elderly individuals (0.39% in the 0-9 years old group, 2.03 % in the 10-19 years old group, 3.10 % in the 20-29 years old group and 3.02 % in the ≥ 80 years old group). The majority of our population was male (60.86 % vs 38.84 %) with only a 0.3 % of unknown genders (ND). With respect to blood groups, the frequencies observed show notably larger numbers for A+ (36.51 %) and 0+ (33.45 %) groups, followed by B+ (6.29 %), A- (7.16 %) and 0- (6.29 %) groups, and very small frequencies for AB+ (3.41 %), B- (1.51 %) and AB- (0.86 %) groups; no data was available for 3.06 % of the records. This distribution matches that determined in our country by the Spanish Red Cross, with very similar percentages for the more popular groups, but some variation when looking at less popular groups (the Spanish Red Cross manifests the 0- group is the third more frequent with 9 % over B+ (8 %) and A- (7 %)), although the frequencies of the AB+, B- and AB- groups were fairly similar [21].

Previous transfusions or transplants were not common; 65.52 % of the patients studied had not had transfusions, 30.39 % had one, and only 4.09 % had more than one transfusion; 88.71 % did not have previous transplants, 10.22 % of the allografts were second transplants and 1.08 % were third transplants. The organization of our cohort by previous pregnancies did not result as expected, due to the large number of missing data (ND) (30.85 %). Nonetheless, the available data showed most women in our cohort had two pregnancies (23.53 %). Similar percentages were found for women with one (13.65 %), three (13.54 %) and four or more pregnancies (13.76 %) and the lowest record was for women with no pregnancies (4.66 %). However, as mentioned before, this distribution has a large uncertainty associated to the missing records. According to time in dialysis, most of our patients had been in dialysis before transplantation. 31.64 % and 31.34 % were distributed in the groups corresponding to have been in dialysis for up to a year and for one to three years respectively. 14.35 % had been in dialysis for three to five years and only 7.84 % were in dialysis for more than five years. 13.71 % were not in dialysis at all and we were only missing this information for 1.12 % of the patients.

Allograft outcome was ‘functional’ in 88.53 % of transplantation cases and ‘rejected’ in 10.56 %; no data was available for 0.91 % of the cohort. With respect to the SNG results, 78.84 % had negative test, 6.98 % had positive SNG tests as a result of preformed antibodies and 14.18 % generated de novo antibodies after transplantation (in concordance with McCaughan & Tinckam [14]). Only these last were considered as newly generated, and patients with preformed antibodies were assumed to have persistent antibodies instead of de novo formed. Overall, 21.16 % of the 2320 patients had positive SNG tests. When analyzing SNG (+) and SNG (-) patients separately, the rejection percentages were 26.90 % and 6.29 % respectively. From the SNG (+) tests of patients with de novo formed antibodies, 63.22 % were DSA and 13.68 % were confirmed ‘Non DSA’ (data was missing for 23.10 % of the patients). Rejection within patients with de novo DSA was 44.23 % and 13.64 % in patients with NDSA. This data is summarized in *figure 5* and shows rejection was notably higher within SNG (+) patients (as compared to the total cohort and specially to only SNG- patients), and inside the de novo SNG (+) group, it was exceptionally high within patients with DSA.

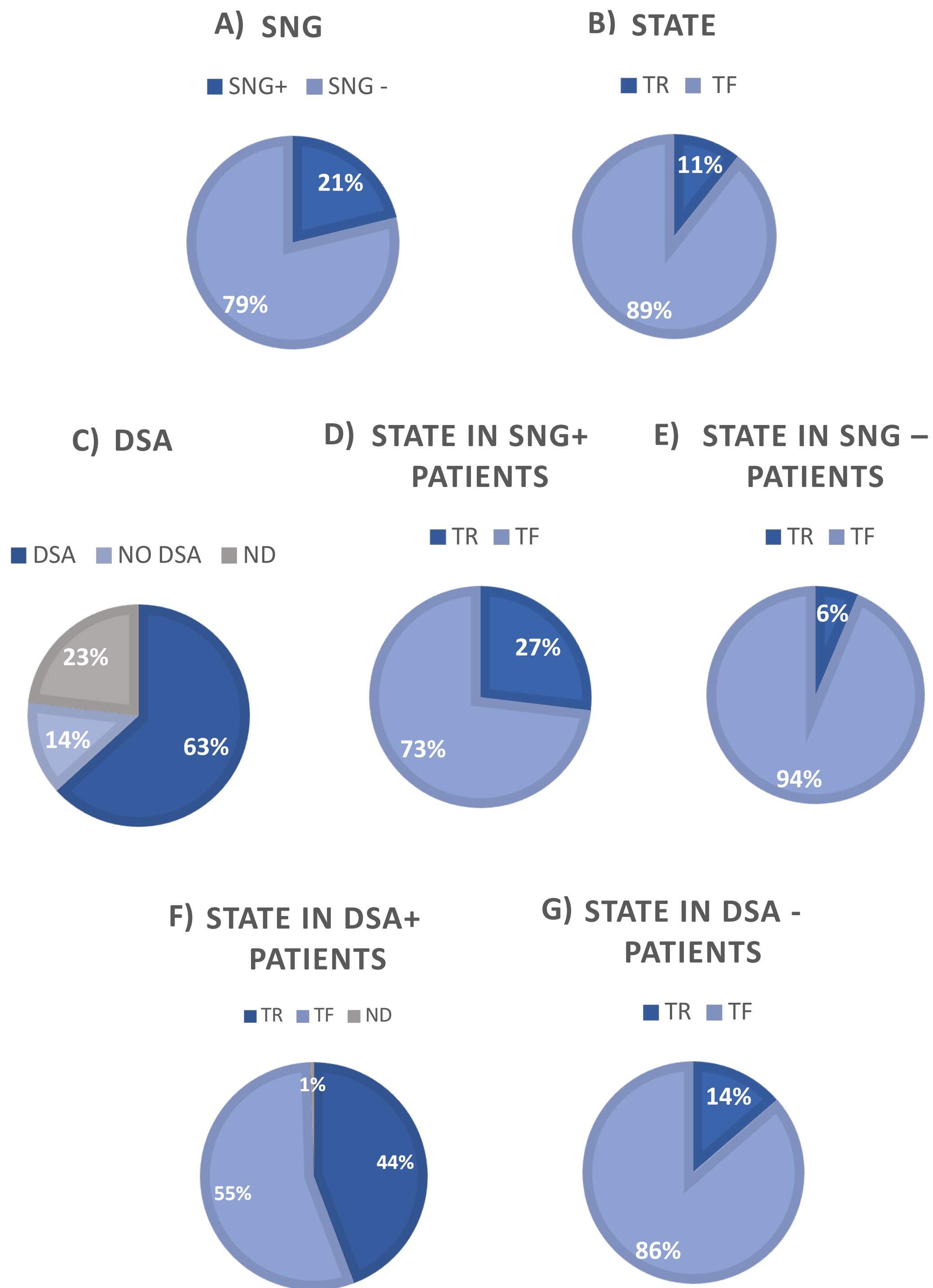

Figure 5. *Graphical representation of our population by de novo antibody generation and allograft state to visualize how rejection varies in different groups of patients. A) Distribution of SNG tests. B) Distribution of allograft state in our cohort. C) DSA distribution within patients with de novo formed antibodies. D) Allograft state in SNG + patients (including preformed and de novo generated antibodies). E) Allograft state in SNG – patients. F) Allograft state in DSA + patients. G) Allograft state in DSA – patients with de novo formed antibodies.*

We observed from the de novo antibody class distribution, most patients either generated antibodies only against class II (40.12 %) HLA antigens or against both class I and class II (39.82 %). A lower percentage presented antibodies only against class I HLA antigens (19.76 %) and we did not have data available for only 0.3 % of the patients. When looking at organization by loci, distribution seems more homogeneous. 20.69 % of our cohort generated antibodies against DQ, 17. 23 % against DR, 16.83% against A and the same against B, 12.28 % against DRw and 8.12 % against Cw. No data was available for 2.08 %.

From the patients to which the C1q tests was performed (36), 71.8 % gave positive results in at least antibodies against one HLA-class binding the C1q subunit of the complement system (versus 28.2 % negative tests).

The descriptive statistics of our cohort are summarized in *Figure 6*.

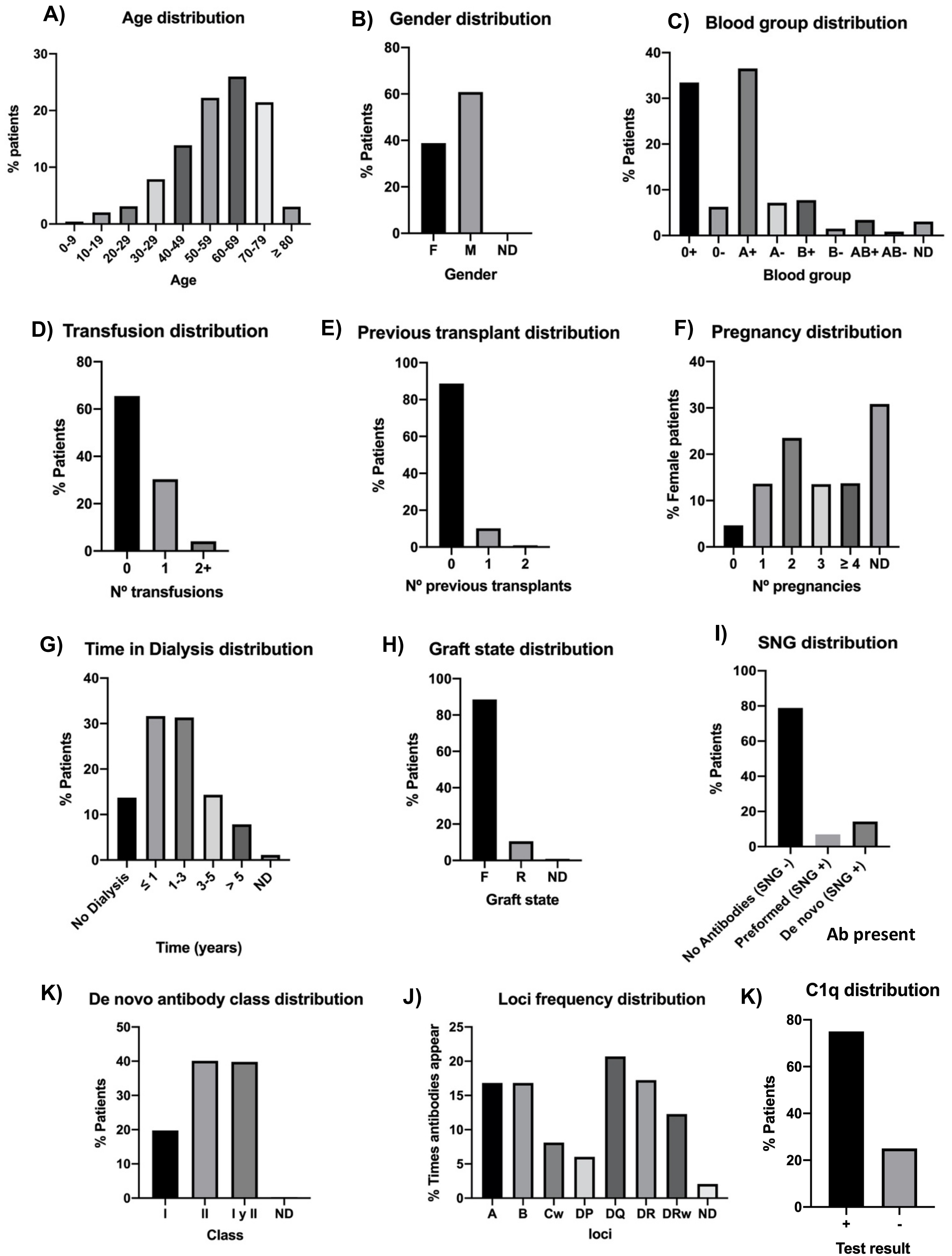

Figure 6. *Distribution of our population according to different independent variables. A) Patient distribution by age. B) Patient distribution by gender. C) Patient distribution by blood group. D)*

Patient distribution by number of previous transfusions. E) Patient distribution by number of previous transplants. F) Patient distribution by number of pregnancies, only female patients were taken into account. G) Patient distribution by time in dialysis. H) Patient distribution by state of the allograft. I) Positive SNG distribution. J) Distribution of post-transplantation formed antibodies by class of HLA against which they appear. K) Loci against which post-transplantation antibodies appear. L) Distribution of C1q test results.

4.2. Univariant Analysis

Univariant Analysis are summarized in Table 1.

4.2.1. Age

As shown in table 1, the median age of kidney transplantation patients was 60 years old (IQR: 48-70). This differed between groups being higher when only considering patients that did not generate post-transplant antibodies (62, IQR: 50-70) and notably lower in patients with de novo formed antibodies (51, IQR: 39-61). The group of patients with preformed antibodies had a median of 57 years old (IQR: 48-66). As explained above, these patients were assumed to have persistent antibodies and, therefore, not considered as de novo forming antibody patients for our study. This way, we could have included them in the no antibody generator group; however, the presence of preformed antibodies was likely to have an effect on the variables of study. Although our analysis did not focus in this group of patients, it was worth differentiating to avoid 'contaminating' our control group of no antibody generator patients. The statistical tests performed returned a p value < 0.0001 and determined all groups were significantly different among them. However, the greatest contribution to this variability was the significant difference between the control group and the 'de novo antibodies' group.

4.2.2. Gender

Within our total cohort 60.9 % of kidney transplant patients were male; in the control group, this percentage was 64.1 %; in the post-transplant generated antibodies group it was 59.9 % and in the preformed antibodies group only 25.9 %. After performing Fisher's exact test by pairs, we found this last was the only responsible for the high significant difference between the groups shown in table 1. We, therefore, could not establish a true significant relationship between values in our groups of interest (control and de novo generated antibodies groups).

4.2.3. Blood group

The blood group distribution was constant throughout the different groups with A+ and 0+ being the more frequent blood groups, followed B+, A-,0- and further by 0-, AB+ and AB-. The chi-squared test returned a p value of 0.9946 which being greater than 0.05 suggested our variables of study were independent and there was no significant relationship between blood group and the generation of post-transplant antibodies.

4.2.4. Pregnancies

The median of pregnancies was two (IQR: 1, 3) for our total cohort and all our study groups. The p value obtained with the chi-square test was as expected by this distribution, greater than 0.05 (0.5876). As with blood group, this demonstrates independency between our variables and no relationship between the number of pregnancies and the generation of post-transplant antibodies.

4.2.5. Transfusions

Although there is no apparent variation between the different group medians (table 1) the p value obtained with the Kruskal-Wallis test was <0.0001. Quantiles can be discontinuous as estimators when there are many repeated values within the data, which can lead to misleadingly thinking the test results are incorrect. However, the null hypothesis can also be rejected for lack of independence or groups not having the same shape distribution, which is what happened here. Looking at the descriptive statistics of the different groups we observed, although they all showed positive skewness, the difference in magnitude of this was important between the group that did not generate antibodies and the patients that generated post-transplantation antibodies and those having preformed antibodies.

4.2.6. Previous transplants

Similarly, medians do not change between groups when analyzing previous transplants. Again, this is probably due to the large amount of repeated values. Nonetheless, the p value obtained was < 0.0001. In addition, on looking at IQR, all groups maintain the value zero except for the group of post-transplant generated antibodies, which's upper limit is one. From this data we observe patients that generate antibodies have been exposed to previous transplants more frequently than other groups. If a patient is exposed to non-self HLA proteins, he will generate an immune response against these. An already activated immune system may unleash a faster response when the event of transplantation is repeated. The skewness values for this analysis also show important differences, especially between the group that did not generate antibodies and the patients that generated post-transplantation antibodies and between this last with the group having preformed antibodies.

4.2.7. Time in Dialysis

Results showed a median of two years in dialysis before transplantation except for patients with preformed antibodies (for who this value increases to three years). IQR of the non-antibody generator group was the same as that of the total cohort. The range (like the median) was also larger for patients with preformed antibodies; it makes sense to think it is harder to find a suitable donor for sensitized patients. The IQR of our group of interest (patients that generate post-transplant antibodies) was lower. Looking at skewness value, the significant relationship between the groups (p value < 0.0001) seems to be caused by the difference in values between the control group and the groups with antibodies.

4.2.8. State of graft

88.5 % of grafts were functional at the moment of study in the total cohort. In our control group this incremented to 92.8 %. Within patients with preformed antibodies, 91.4 % had functional allografts and within patients with de novo formed antibodies this percentage was as low as 63.2 %. The Chi-square test gave a p value < 0.0001 demonstrating relationship between our variables of study. Fisher's exact test by pairs clarified high significant difference between all groups except between control group and the preformed antibodies group, which had no effect over our conclusion as we considered this second as a 'no post-transplant antibodies generator' group.

Table 1. *Baseline characteristics of the entire cohort and comparison between with and without post-transplant generated antibodies (differentiating preformed antibodies).*

Baseline characteristics	Entire cohort N=2320	No Antibodies generated post-transplant	Preformed antibodies	Post-transplant generated antibodies	P value*
Age, years, median (IQR)	60 (48.0,69.9)	62 (50.0, 70.0)	57 (48.0, 66.3)	51 (39.0,61.0)	< 0.0001
Gender					< 0.0001***
male, n (%)	1412 (60.9)	1173 (64.1)	42 (25.9)	197 (59.9)	
Female, n (%)	901 (38.8)	650 (35.5)	120 (74.1)	131 (39.8)	
**No data, n (%)	7 (0.3)	6 (0.3)	0 (0.0)	1 (0.3)	
Blood group					0.9946
0+, n (%)	776 (33.5)	620 (33.9)	54 (33.3)	102 (31.0)	
0-, n (%)	146 (6.3)	116 (6.3)	10 (6.2)	20 (6.1)	
A+, n (%)	847 (36.5)	656 (35.9)	63 (38.9)	128 (38.9)	
A-, n (%)	166 (7.2)	131 (7.2)	12 (7.4)	23 (7.0)	
B+, n (%)	180 (7.8)	142 (7.8)	12 (7.4)	26 (7.9)	
B-, n (%)	35 (1.5)	29 (1.6)	2 (1.2)	4 (1.2)	
AB+, n (%)	79 (3.4)	59 (3.2)	6 (3.7)	14 (4.3)	
AB-, n (%)	20 (0.9)	19 (1.0)	0 (0.0)	1 (0.3)	
**ND, n (%)	71 (3.1)	57 (3.1)	3 (1.9)	11 (3.3)	
Pregnancies, median (IQR)	2 (1.0, 3.0)	2 (1.0, 3.0)	2 (1.0, 3.0)	2 (1.0, 3.0)	0.5876
Transfusions, median (IQR)	0 (0.0,1.0)	0 (0.0,1.0)	0 (0.0,1.0)	0 (0.0,1.0)	< 0.0001
Previous transplants, median (IQR)	0 (0.0,0.0)	0 (0.0,0.0)	0 (0.0,0.0)	0 (0.0,1.0)	< 0.0001
Length of dialysis before transplant, years, median (IQR)	2 (1.0,4.0)	2 (1.0,4.0)	3 (2.0, 5.0)	2 (1.0, 3.0)	< 0.0001
State of graft, n (%)					< 0.0001
Functional	2054 (88.5)	1698 (92.8)	148 (91.4)	208 (63.2)	
Rejected	245 (10.6)	114 (6.2)	12 (7.4)	119 (36.2)	
**ND	21 (0.9)	17 (0.9)	2 (1.2)	2 (0.6)	

*Compared between three groups: no antibodies generated post-transplant, preformed antibodies and post-transplant generated antibodies. P values for quantitative variables with median (IQR) are result of Kruskal-Wallis test, and categorical variables with n (%) are chi-square test.

** No data (ND) is shown when records were missing and is included in the percentage distribution of the population; It was, however, not used in the contingency tables generated to perform the chi-square test.

*** p value of statistical test < 0.05 but statistical significance proven not applicable to our study by post-hoc tests.

4.3. Association studies

4.3.1. Antibody presence and rejection

The value for the calculated V_{SNG} (Cramer's V coefficient for SNG+ patients) was 0.3, which describes a moderate association between antibody presence and rejection. The V_{DSA} was 0.2 determining a lower association between rejection and DSA+ patients (compared to DSA – patients). The calculated relative risk was 4.28 for SNG+ patients (compared to SNG- patients) and 3.24 for DSA+ (as compared to DSA-). These values represent how much larger the probability is for the event (rejection) to occur with the positive factors (positive tests). Being greater than one, we can establish, for both, a positive association; the presence of the factor is thus associated to a higher occurrence of the event. Results of RR support those obtained with the Cramer's V coefficient.

Once established the association, the amount each factor contributed to the event was studied. Corresponding to only 21.16 % of the total cohort, SNG (+) patients described 53.47 % of the rejection cases. Further breaking down this group, we observed most of this rejection was due to patients with de novo DSA (comprising only a 3.97 % of the total cohort they responded for 37.55 % of the total rejection). Patients with de novo NDSA (0.26 % of the total cohort) contributed 2.45 % to the total rejection and patients with preformed antibodies (1.42 % of the total cohort), 13.47 %. The remaining 78.83 % of our population of study explained the other 46.53 %. Note although the percentages contributed to the total rejection were similar, the proportion of the group constituted by SNG (+) and SNG (-) patients were largely different *(table 2)*.

Table 2. *Table representing differences in rejection between groups of patients*

Group	% of total cohort	% contributed to total rejection
SNG (+) patients	21.2	53.5
Patients with de novo DSA	4.0	37.6
Patients with de novo NDSA	0.3	2.4
Patients with preformed Ab	1.4	13.5
SNG (-) patients	78.8	46.5

4.3.2. Class, Loci and Rejection

A low association between antibody class and rejection was demonstrated by the Cramer's V coefficient calculated ($V_{CLASS} = 0.3$) and The RR obtained. We found rejection was 1.2 times more common in class I antibodies than in class II. V_{LOCI} resulted in a very small value (0.09) with little if any association between loci and rejection. We therefore did not perform any subsequent analysis.

4.3.3. Development time and allograft state

The P value obtained with the fisher's exact test was <0.0001 demonstrating a statistically significant difference between time for development of anti-HLA antibodies and allograft state. Our results showed a lower rejection rate in patients that developed antibodies before a year. The Cramer's V coefficient returned low evidence for this same result ($V_{TIME} = 0.2$). As a result, the same test was repeated only taking into account DSA. This time the p value obtained was 0.7459 undoing the previous relationship.

4.3.4. C1q, allograft state and class

The Fisher's exact tests performed to study the association between C1q binding antibodies and rejection and the first and class incidence, did not result in statistically significant relationships. We had a major limitation with the lack of availability of C1q data, having very reduced number of records to obtain reliably conclusions from them. The Cramer's V coefficient obtained ($V_{C1q} = 0.04$) did not show association either. The RR had a value of one, which again, indicates no relationship. Because of the data limitation the Chi-square test performed to study the relationship between antibody class and C1q has little reliability and we did not consider this results.

3. Discussion

In this retrospective cohort study, the development of de novo formed anti-HLA donor and non-donor specific antibodies (DSA and NDSA respectively) was significantly associated with a worst transplantation outcome. This result supports the conclusion obtained by many previous studies that describes the degree of HLA mismatches is directly associated to antibody mediated rejection (AMBR) [15][13][16][22]. DSA represented the cause for the majority of the allograft rejection in positive LAB Screen Single Antigen assay (SNG+) patients. The median time contemplated for the appearance of the post-transplantation antibodies was 10 years. We also found an association between de novo DSA (dnDSA) class and rejection (weak evidence), with this outcome being 1.2 times more frequent in the presence of class I than class II antibodies, although the presence of these last was more common. Anti HLA-class II DSA have been contemplated as the predominant post-transplantation formed antibodies in unsensitized kidney transplant recipients [16]. Because the statistical significance of this result was quite weak we turned to the literature and found, despite class I de novo DSA being generated less frequently, they usually develop earlier in post-transplantation as compared to class II de novo DSA [15], [16], which could explain why rejection seemed more common in presence of class I antibodies. Several studies have shown different time intervals for rejection to happen after the appearance of anti-HLA antibodies: from 3 to 105 months [16], [23]. In addition, many publications relate class I dnDSA to acute (earlier) ABMR while associate class II dnDSA to a greater risk of chronic (long-term) ABMR [15][22][16]. Taking into account we only considered the first positive SNG test per patient, that class II antibodies usually develop later than class I and that we did not have access to the exact dates rejection was determined, it would be reasonable to assume this may have been caused by antibodies we did not take into account for our study (what would have subtracted importance to rejection caused by class II antibodies).

Comparing the data of the different groups established (control, preformed and de novo), we were able to determine statistically significant relationships between the development of post transplantation formed antibodies and various factors of study in the univariant analysis. Because our data did not follow the Gaussian distribution, the Chi-square test was performed for categorical data and the Kruskal Wallis test for quantitative variables. Instead of comparing the medians, this last compared the sum of the ranks and could therefore also be used for heteroscedastic data. We found anti-HLA antibodies are more commonly produced in younger recipients which is in concordance with Yamamoto et al. and Polanco et al. [15], [24]. Aging affects innate and adaptive immune responses resulting, among other things, in a decreased ability to generate specific antibodies [25], [26].

Transfusions or other transplants were also found to have a significant effect on de novo anti-HLA antibody production. In addition to being well known risk factors in the development of these when patients are exposed to the events pre-transplantation, Ferrandiz et al., observed early post-transplant blood transfusions were associated with an increased immunological risk which may be responsible for the production of DSA [27].

Finally, we observed time in dialysis had a significant effect over the production of antibodies. Various studies have been done on whether dialysis directly affects the functionality and survival of the allograft, and the topic still results controversial in the literature, with some publications describing time in dialysis has a negative effect on these and others defending no such relationship exists [1][28]. Authors like Losappio et all., illustrate the complex interplay between the innate and adaptive immune dysfunction in hemodialysis exploring the pathophysiology of this treatment. On the whole, patients in dialysis are susceptible to early and long-term complications like chronic inflammation, which together with the effects of ESRD can result in a

pattern of immunosenescence [29]. This explains why our group of patients with de novo antibodies seemed to be less time in dialysis (if analysing IQR). A compromised immune system will have more difficulties in generating an immune response and resultantly in antibody production.

No significant relationship was found with the other variables studied. Sex, blood group, and pregnancies seemed to be independent on the generation of de novo post-transplantation antibodies. Although we cannot be certain on the conclusion of this last. Together with previous transplants and transfusions, pregnancies have a well-known effect on the development of preformed antibodies. Whether, like the two first this may also influence post-transplantation antibody formation favouring the appearance of new antibodies could not be determined as a result of the large quantity of missing data. Similarly, loci against which dnDSA appeared did not result related to a worst outcome. Nevertheless, -DQ and -DR appeared to be the loci towards most patients resulted immunized. With respect to the C1q analysis to study the effect of complement binding on allograft rejection and see the predominance of any antibody class, we did not have access to enough data to obtain reliable conclusions. The non-significance of our statistical tests was most certainly due to the reduced observations we had for this study. Various publications have already demonstrated, when comparing complement-binding DSA with those unable to do so, complement-fixing DSA have a greater risk of AMBR (both acute and chronic), together with a more severe histological damage and a bigger risk of allograft loss [15]. Because data shortage was a major limitation, we could not perform following analysis in this field.

Although more investigation has been done in the last years in the field of post-transplant development of anti-HLA antibodies, and specially dnDSA, more work is still vital to be able to use this information to foresee and prevent acute and/or chronic rejection and resultantly graft loss. For example, despite having strong evidence of the adverse effects of complement binding DSA, most laboratories do not perform this test routinely. It will be necessary to increase the number of C1q tests performed to provide a better monitoring of kidney transplant recipients [13]. In addition, it will be important not only to achieve as much HLA matching as possible, but also to do this precisely at epitope level [30].

Overall, we have analysed several factors and their relation to differences in patient alloimmunization in a determined population (kidney transplant patients in the Valencian Region), proving some interesting relationships that may help to predict the probability of generating post-transplant antibodies. Nonetheless, more analysis should be carried out to study the development of de novo anti-HLA antibodies. Most studies focus in the emergence of pretransplant antibodies, given that their analysis will help to avoid the more immediate rejection (hyperacute). However, the further study of antibodies that form post-transplant (those we call ''de novo'') is crucial to prevent acute or chronic rejection and establish, when necessary, the strategies to prevent graft loss.

4. Conclusion

At present, the study of de novo formed anti-HLA antibodies to understand their impact on allograft function and help develop efficient therapies for their control, represents an expanding field in the area of renal transplantation. Understanding the more favorable conditions for the development of de novo anti-HLA antibodies, identifying risk patients and distinguishing factors that provide these antibodies with a greater damage capacity is crucial for the adequate monitoring of kidney transplant recipients.

The main objective of this study was to study de novo anti-HLA antibody formation comparing patient baseline characteristics of a kidney transplant cohort to try to establish possible patterns in the differences between patients in development of the immune response after transplantation. The conclusions drawn from this analysis are the following:

- Statistically significant relationships exist between age, previous transplants, transfusions and time in dialysis and the presence of de novo anti-HLA antibodies.
- Therefore, younger patients, recipients of previous transplants and transfusions and patients with long dialysis treatments have higher risk of developing post-transplantation antibodies.
- De novo anti-HLA DSA are associated to a worst transplantation outcome.

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