STUDY PROTOCOL AND STATISTICAL ANALYSIS PLAN

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STUDY PROTOCOL.

HYPOTHESIS

Steroids form one of the pillars of immunosuppression in transplant patients. However, their use is associated with an increase in post-transplant comorbidity. Although steroid withdrawal post-transplant can improve the metabolic and cardiovascular profile in transplant patients, it is not really known whether this therapeutic strategy results in a greater risk for the de novo appearance of donor-specific antibodies (DSA), which are associated with a reduction in kidney graft survival. Also unknown is the causal relationship between the appearance of DSA and histological lesions in stable kidney transplant patients after steroid withdrawal in the third post-transplant month. Finally, another unknown is whether this immunosuppressive strategy is associated with changes in the balance between effector and suppressor lymphocyte subpopulations that selectively infiltrate the graft via peripheral homing receptors.

This randomized controlled study of stable kidney transplant patients aimed to determine the natural history after steroid withdrawal in the third month post-transplant of the de novo production of anti-HLA antibodies (DSA), by means of the sequential measurement of DSA during the first two years post-transplant. At the same time we wished to determine the repercussion of this therapeutic strategy on the kidney graft histology by performing protocol biopsies, as well as the risk factors for the appearance of de novo DSA in these patients. Finally, we aimed to monitor sequentially the balance of the lymphocyte (CXCR3highCD4+/CCR4highCD4+) and regulatory cell (Treg CCR4high) subpopulations in peripheral blood and samples of the graft tissue obtained by fine-needle aspiration puncture (FNAP) after the withdrawal of steroids versus continuation of conventional triple therapy.

The lack of prospective serological, histological and lymphocyte subset studies in kidney transplant patients in whom steroids are withdrawn makes this study very pertinent. The findings will doubtless give rise to an immunosuppressive strategy that optimizes the results of a transplant in terms of survival.

OBJECTIVES

Primary objective

To compare the incidence of de novo DSA in stable kidney transplant patients after withdrawal of steroids in the third month post-transplant as compared with patients who continue to receive conventional triple immunosuppression: steroids, tacrolimus (prolonged-release –Advagraf, or immediate-release –generic or Prograf, according to their availability in each Spanish Community) plus mycophenolate mofetil (MMF) or mycophenolic acid (MPA).

Secondary objectives

1. To determine the course of the histological findings in the two treatment groups.

2. To determine the course of the balance in the subpopulations of lymphocytes (CXCR3highCD4+/CCR4highCD4+) and regulatory cells (Treg CCR4high) using flow cytometry in samples from peripheral blood and FNAP after withdrawal of steroids in the third month post-transplant versus conventional triple immunosuppression.

3. To analyze and compare the incidence of immunological dysfunction in the two treatment arms, as well as graft and patient survival at one and two years of follow-up.

4. To examine the potential metabolic and cardiovascular benefit of steroid withdrawal in terms of renal function, hypertension, diabetes and hyperlipidemia.

5. To assess the adherence to treatment with immunosuppressors in the two treatment groups and its impact on the generation of DSA.

STUDY DESIGN AND DURATION

This was a randomized, open-label clinical trial with two treatment arms and a follow-up of two years in patients who received a first deceased-donor kidney transplant. The combinations of immunosuppressors in the study arms were those approved in the technical documentation and used routinely in clinical practice. This was therefore a phase IV clinical trial in the usual conditions of use. The patient recruitment period was 12 months. The minimum follow-up period was 24 months.

Inclusion criteria: a) Both male and female patients, 18 years of age or over with no immunological risk (PRA <25% and absence of DSA) who received a first deceased or living donor kidney transplant. b) Patients who for the first three months post-transplant were receiving tacrolimus plus MPA or MMF and steroids and who had stable plasma levels of tacrolimus. c) Absence of clinical or histological immune dysfunction before randomization. d) Absence of de novo DSA at the time of randomization. e) Patients who wished and were able to give their written informed consent to participate in the study. f) Female patients who agreed to use efficient contraceptive methods during the study.

Exclusion criteria: a) Recipients of a multiorgan transplant. b) Retransplants. c) Presence of DSA prior to transplant or at the time of randomization. d) Cold ischemia time >30 hours. e) Patients with serum creatinine levels above 2 mg/dL or proteinuria above 1g/day at the time of randomization. f) Previous episode of severe rejection (II-B-III in the Banff/09 classification) before randomization. g) Presence of subclinical rejection in the protocol biopsy before randomization. h) Patients with BK-polyomavirus nephropathy at the time of randomization. i) Patients with recurrent or de novo glomerulonephritis. j) Patients receiving treatment with immunosuppressive drugs other than those of the clinical trial in question. k) Patients who were positive against the human immunodeficiency virus (HIV) or who had a severe systemic infection and who the investigator deems the need for continued therapy. l) Patients who had had any previous malignant disease during the previous five years, except basal cell or excised squamous cell carcinoma.

Selection procedure and coding: Once written informed consent was obtained at the screening visit, the baseline data were collected to determine the eligibility of the patients to participate in the study. Those patients who fulfilled the inclusion criteria were randomized in a proportion 1:1 to one of the two treatment arms.

Randomization was done locally at each center via the case report form (CRF) to assign the eligible patients to one of the two treatment arms. This randomization was done using a computer program after introducing the patient data into the electronic database (CRF) and determining the

status of the anti-HLA antibodies (DSA) and the histological findings at the third month post-transplant.

Description of the treatment groups: After verifying the inclusion/exclusion criteria, the patients were distributed randomly in the two study groups:

Group A: steroids, tacrolimus (prolonged-release –Advagraf, or immediate-release tacrolimus – generic or Prograf, according to their availability in each Community) plus MMF or MPA.

Group B: tacrolimus (prolonged-release –Advagraf, or immediate-release tacrolimus –generic or Prograf, according to their availability in each Community) plus MMF or MPA.

Treatment schedule:

Group A: Tacrolimus 0.15 mg/Kg/day to maintain levels of 8-12 ng/ml for the first two months. With effect from the third month tacrolimus 0.1 mg/kg/day to maintain levels of 5-8 ng/ml; **Mycophenolate mofetil (MMF)** 2 gr/day or mycophenolic acid (MPA) 1440 mg/day for the first two weeks post-transplant. With effect from this point MMF 1-1.5 gr/day or MPA 720-900 mg/day; **Steroids:** 0.5 gr of methyl prednisolone intraoperatively and 125 mg on day 1; prednisone 30 mg for the first two weeks post-transplant, then tapering 5 mg/week to reach the dose of 5 mg/day at the second month post-transplant. After this the same dose of prednisone was continued.

This treatment was maintained throughout the whole study period in Group A.

Group B: These patients received the same treatment schedule as those of Group A up to the time of randomization (third month post-transplant). After this they received: **Tacrolimus** 0.1 mg/kg/day to maintain levels of 5-8 ng/ml; **MMF** 1-1.5 g/day or **MPA** 720-900 mg/day; withdrawal of steroids between the third and fourth month post-transplant as per usual practice in the center.

Both treatment groups received pre-operatively tacrolimus 0.12 mg/Kg orally and MMF 1 g. or MPA 720 mg. Both treatment groups could receive induction therapy with anti-CD25 monoclonal antibodies (Basiliximab 20 mg iv on days 0 and 4) or polyclonal antibodies (Thymoglobulin 1 mg/kg/day for 4-7 days) in patients at risk of delayed graft function and delayed introduction of tacrolimus.

Other treatments: Cases of acute rejection were treated with 3 boluses of 500 mg/day of i.v. methylprednisolone. Cases of steroid-resistant rejection were treated with a standard cycle of 7-10 days of rabbit thymoglobulin. Cases of acute humoral rejection were treated in accordance with the protocol at each center: plasmapheresis, immunoglobulins, and rituximab, either alone or in combination.

Drugs to treat the most usual infections, like Pneumocystis jirovecii or CMV, were used in accordance with the protocol at each center. Dyslipidemia was treated with statins or with ezetimibe in the event of the appearance of adverse events. Fibrates were avoided, except for gemfibrozil, as they are associated with worsening renal function. Hypertension was treated according to the protocol at each center to obtain a target BP of <130/80 mmHg. As beta blockers have been associated with new onset post-transplant diabetes, they were initially avoided unless they were indicated for another condition (ischemic heart disease, arrhythmia, or other) or in patients with refractory hypertension.

Criteria for withdrawal of subjects from the study: Any participant could withdraw from the study at any time, withdrawing their consent, with no need to justify their decision. The investigator could also withdraw patients from the study in the event of the appearance of any concomitant disease or adverse event that, in the judgement of the investigator, warranted withdrawing the patient from the study, or if there were violations of the protocol or other reasons. The investigator attempted to follow any patient with an adverse event at the time of withdrawal and provide the appropriate treatment in the event of any problem.

Patients who developed acute humoral or steroid-resistant rejection during the study were treated with the best maintenance immunosuppression in the opinion of the investigator and were withdrawn from the study. This could consist of adding steroids or an anti-mTOR (sirolimus or everolimus). All these patients were followed in the same way as those who remained in the protocol in order to have a more robust intention-to-treat analysis.

If the study medication was interrupted for any reason for more than 21 consecutive days or for more than two periods of 7 days or more the patient was considered to have abandoned the

treatment prematurely and was only included in the intention-to-treat analysis and excluded from the per protocol analysis.

Variables:

Efficacy variables:

The *primary variable* was the presence of donor-specific anti-HLA antibodies (DSA) at 24 months.

The *secondary variables* were:

a) Graft and patient survival.

b) Rate of acute clinical rejection, confirmed by biopsy, calculated glomerular filtration rate (determined by aMDRD) and proteinuria at 3, 6, 12 and 24 months.

c) BP figures and number of hypertensive patients, lipid levels and need for lipid-lowering drugs, and increase in weight and BMI.

d) Rate of post-transplant diabetes mellitus (PTDM) or glucose intolerance one year post-transplant according to the criteria of the ADA (PTDM: baseline glycemia ≥126, or ≥200 mg/dl after glucose overload, or need for treatment; glucose intolerance: glycemia after overload of 140-199 mg/dL).

e) Determination of the lymphocyte subpopulations (CXCR3highCD4+; CCR4highCD4+ and Treg CCR4high) by flow cytometry at 1, 3, 6, 12,18 and 24 months.

f) Appearance of acute or chronic pathological lesions of the graft using:

*Kidney graft biopsy. Biopsies were done before randomization (month 3 post-transplant) and in month 24 post-randomization (protocol biopsies). In the case of suspected acute rejection a biopsy of the graft was done before or within 48 hours of starting anti-rejection treatment. Likewise, a graft biopsy was done at any time if graft dysfunction was detected, as evidenced by proteinuria 0.5 g/day or an increase in the serum creatinine of 25% above its baseline level. The biopsies were evaluated according to the Banff´09 classification (*Sis B, et al Am J Transplant 2010;10: 464-71*). Biopsies done for clinical indication and those done prior to randomization were evaluated locally at each participating center. The local pathologist interpreted the biopsy for the presence of clinical or subclinical rejection, borderline lesions or progression of chronic lesions that

explained a possible graft failure to thus provide the most opportune treatment. The biopsies done and the pre-randomization and final visits were also sent to the pathologist at Carlos Haya Hospital, Malaga, who also interpreted and classified them using the same Banff'09 criteria. The biopsies were processed in each center and sent to the central pathologist for study. The slides comprised at least 2-3 slices of the biopsy. The staining was done locally (hematoxylin eosin, periodic acid Schiff, Masson trichrome and methenamine silver).

For the measurement of C4d an unstained slide was sent to the reference center, at ambient temperature.

*The interpretation of the central pathologist was used mainly to analyze the progression of the chronic graft nephropathy. For each patient, the sum of the classification of the chronic lesion was obtained from the 5 histological categories: 1) cg: chronic glomerulopathy. 2) ci: chronic interstitial. 3) ct: tubular atrophy (chronic). 4) cv: arteriolar hyalinosis (chronic). 5) mm: mesangial matrix.

Confounding variables: Donor and recipient age, HLA compatibility and PRA, delayed graft function, BMI, CMV infection, concomitant treatment (statins, ACEIs/ARA, beta blockers, diuretics, and ASA), scale of treatment compliance and levels of tacrolimus or CsA.

Monitoring of donor-specific anti-HLA antibodies (DSA):

Monitoring of DSA was done with the Luminex assay and X-MAP technology. The presence of DSA was determined pre-transplant and in months 3, 6, 12, 18 and 24.

Before transplant the presence of anti-HLA antibodies was determined in all the patients by Luminex Mixed, which detects the presence of antibodies against HLA-class I and class II antigens. In positive cases the specificity of the antibodies was determined by Luminex SA (*single antigen*). Likewise, in patients with a high risk of sensitization (previous pregnancy or transfusion) the Luminex SA was also done, even if they had had a negative Luminex Mixed result. Before transplant a serum sample was taken from each patient and frozen in three aliquots of 0.5 ml in Eppendorf tubes.

If the patients had no anti-HLA antibodies pre-transplant, monitoring for these antibodies was done throughout the study using the Luminex Mixed assay. When this assay was positive for any of the measurements established in the study a Luminex SA was done to detect the specificities. If, on the other hand, a patient had a positive Luminex Mixed pre-transplant only the Luminex SA assay was done at all the successive study visits.

Any determination with a mean fluorescence intensity (MFI) >500 was considered positive. All determinations of DSA were centralized at the immunology laboratory of Carlos Haya hospital, Malaga.

All the samples obtained from the various participating centers for determination of anti-HLA DSA were stored at -80º or -40º C until being sent. During transit the samples were stored in dry ice.

Analysis of the lymphocyte subpopulations in peripheral blood by flow cytometry:

Determinations were done with the same periodicity as for the DSA. Blood samples anticoagulated with EDTA (10 ml) were obtained. A volume of 50 ml was incubated in Falcon polypropylene tubes 12 x 75 mm within 48 hours of extraction with the following monoclonal antibodies: CD4, CD127, CD25, CD183 and CD194 labeled with the fluorochromes FITC, PE, APC, APC-Cy7 and Per-CP. In the window for the CD4+ lymphocytes we studied the expression for the markers CD183 and CD194 and in the window for the CD4 +CD25high CD127 lymphocytes we studied the expression of CD183 and CD194 in the Treg cells. The acquirement and analysis was done in at least 5000 lymphocyte cells with a FACScanto II dual-laser cytometer with FACSdiva software. Each sample of the aspiration cytology underwent the same analysis in a sample of blood anticoagulated with EDTA (3ml) for the comparative analysis.

FNAP for morphological analysis and flow cytometry:

The FNAP was done according to the technique of Hayry (Kidney Int. 1989;36:130-141) with the same periodicity as that for the DSA and if there was any suspicion of immune dysfunction. Aspirate samples were obtained with a Ham solution (10 ml). With the 0.8 mm / 25G needle implanted in a Cameco-Gun device the cortical region of the graft was punctured with local anesthesia, and aspirating by vacuum about 20 µL of cell material in a Hepes buffer culture

medium with heparin and human serum albumin (Ham solution). At the same time, a blood sample was obtained to calculate the corrected index (CI), which was the result of the differential in the leukocyte count between the FNAP specimen and the peripheral blood sample. The numerical differences for each cell subtype were multiplied by a correction factor and the sum of the different corrected increases described the intensity of the inflammation. A CI greater than 3 was considered suggestive of acute rejection. The aspirate was processed by flow cytometry to analyze the lymphocyte subpopulations in the same way as these were analyzed in peripheral blood, described above.

Other determinations: The patients were monitored for renal function, levels of calcineurin inhibitors, blood count and other parameters, and imaging tests as per clinical practice in Spain. The concomitant treatment was also as usual. The fasting glucose figures were monitored daily during the first week, at least twice during the second week, and at least weekly until the end of the first month. During the second and third months they were monitored at least once every two weeks. With effect from the third month and until the end of the first year they were monitored at least once a month. The HbA1c was measured and a standard oral glucose tolerance test of 75 gr was done at 3, 12 and 24 months.

Pregnancy tests: These tests were done for all women who were potentially considered to be of child-bearing age in phases I (screening) and II (randomization) of the study. The pregnancy tests were done locally in serum using detection of the human chorionic gonadotropin hormone.

STATISTICAL ANALYSIS PLAN.

Descriptive statistics are presented together with the various analyses. Both an intention-to-treat analysis and an analysis according to the final treatment were done. The categorical variables were analyzed using the chi-square test or Fisher's exact test, according to needs. The quantitative data were analyzed using the Student t test or the Mann-Whitney U test, according to needs. Changes over time in the DSA and lymphocyte subsets in the two study groups were analyzed using a general linear model for repeated measures, adjusting for confounding variables. Correlations between the various subsets were analyzed using Pearson correlation. The graft and patient survival curves were calculated with the Kaplan-Meier method. The rates of survival of the groups were compared with the log-rank test. Finally, a linear regression analysis was done to determine the factors associated with the generation of de novo DSA, including steroid withdrawal. The data were analyzed using SPSS 20.0.

PRELIMINARY RESULTS

A total of 230 patients were initially recruited between March 2015 and November 2017 for the clinical trial. The patients were from 5 centers with a high transplant activity: Carlos Haya Hospital, 159 patients (69.1%); Canarias University Hospital, 33 patients (14.3%); Vall de Hebron Hospital, 17 patients (7.4%); Bellvitge Hospital, 14 patients (6.1%) and Dr. Peset Hospital, 7 patients (3%).

Of the 230 patients, 125 (54.3%) were not randomized for various reasons: screening failure, 74 (59.2%); patient wishes, 16 (12%); acute rejection, 9 (7.2%); graft loss, 9 (7.2%); died, 3 (2.4%); lost to follow-up, 1 (0.8%) and other causes, 13 (11.0%). The main causes of screening failure were: creatinine >2 mg/dl (41.9%) and subclinical rejection (20.3%) in the protocol biopsy at the third month.

Finally, 105 patients were randomized to one of the two treatment arms: Group A: tacrolimus+MMF/MPA+prednisone (n=52) and Group B: tacrolimus+prednisone (n=53). The mean age was 54.8 years for Group A and 52.1 years for Group B. Most patients were aged 18 to 65 years (82.69% Group A vs 83.02% Group B). Most patients were also male (Group A 75% vs Group B 71.7%) and Caucasian (Group A 90.38% vs Group B 96.23%).

Appearance of donor-specific anti-HLA antibodies (DSA) and acute rejection.

Steroid withdrawal at the third month post-transplant did not result in the appearance of DSA in patients with a low immunological risk, at least during the first two years of follow-up and using a MFI >500. No significant differences were found in the number of patients who experienced acute rejection after randomization between Group A (7.14%) and Group B (11.54%).

Histological study of the protocol biopsies

The results of the protocol biopsies showed that the patients who had subclinical inflammation of the graft at the third month post-transplant had more chronic lesions (p=0.008) and worse renal function than the patients who had no inflammatory lesions. Likewise, the patients who had subclinical tubulo-interstitial inflammation (borderline lesions) 24 months post-transplant had more chronic lesions than those who had no subclinical inflammation (p=0.023) and this was more relevant in those patients who continued to receive prednisone (p=0.020).

The patients with and without subclinical tubulo-interstitial inflammation at the third month post-transplant experienced a significant increase in inflammation (t, i) ($p<0.001$ and $p=0.058$) and chronic parameters (ct, ci) ($p=0.017$ and $p=0.017$) at 24 months post-transplant.

Finally, at 24 months post-transplant the increase in acute and chronic lesions ($p=0.019$ and $p=0.009$) was more relevant in those patients who had borderline lesions at the third month and who ceased steroids.

Diabetes.

Although the increase in the incidence of post-transplant diabetes mellitus was not significant ($p=0.359$) between Group A (25.6%) and Group B (16.7%), the HbA1c values rose significantly ($p=0.013$) in Group A (6.3±1.2%) compared to Group B (5.7±0.6%) at the end of the follow-up.

Lipid profile.

No significant differences were noted between the groups in values of total cholesterol (164±28 vs 161±27 mg/dL; $p=0.677$), HDL cholesterol (51±14 vs 44.7±10 mg/dL; $p=0.095$), LDL cholesterol (86±21 vs 92±18 mg/dL; $p=0.337$) or triglycerides (140±54 vs 76±9.7 mg/dL; $p=0.469$) at the end of the follow-up.

Blood pressure.

No significant differences were noted between the groups in diastolic blood pressure values (74.9±11 vs 76±9.7 mmHg; $p=0.715$), although the systolic blood pressure values rose significantly in Group A versus Group B (133±16 vs 125±15 mmHg; $p=0.055$) at the end of the follow-up.

Renal function.

No significant differences were noted between the groups in creatinine (1.34±0.4 vs 1.4±0.4 mg/dL; $p=0.458$) or proteinuria (209±185 vs 148±82 mg/24h; $p=0.303$) at the end of the follow-up.

Lymphocyte subpopulations.

A significant increase was seen in lymphocyte subpopulations in the FNAP samples compared to the peripheral venous blood samples. Likewise, the patients with borderline lesions showed an

increase in the CXCR3highCD4+ and CD14+CD16+ subpopulations in venous blood and FNAP samples compared to the patients with no subclinical inflammation in the biopsy.

Additionally, a direct correlation was found between the CD14+CD16+ subset obtained by FNAP at 3 months and the presence of IFTA at 24 months. Likewise, the CCR4highCD4+ lymphocyte subset obtained at 3 months post-transplant correlated inversely with the interstitial (r=-0.431; P=0.007) and tubular (r=-0.436; P=0.007) inflammation at 24 months post-transplant.

Graft and patient survival.

After randomization both graft and patient survival were 100% in the two treatment arms.

Safety assessment.

All severe adverse events (SAE) after randomization were monitored and recorded. These SAE were classified according to their nature into three groups: infections of any type (Group A 9.6% vs Group B 22.6%), cardiovascular disease (5.7% vs 3.8%) and neoplasm (3.8% vs 1.9%). No significant differences were seen between the two treatment arms (p=0.110, p=0.677, and p=0.618).

There was a tendency in Group B to a higher number of severe infections (urinary sepsis, complicated urinary infections, opportunistic infections, CMV infection) requiring hospitalization. This was possibly because these patients received a higher dose of mycophenolate mofetil at 6 months (954±162 vs 1041±211 mg; P=0.08) and 12 months (937±162 vs. 1005±174 mg) after steroid withdrawal. All the infections resolved satisfactorily with the opportune treatment.

Five patients experienced cardiovascular events. Two patients in Group B had ischemic heart disease that was revascularized and three in Group A had ischemic heart disease, ventricular dysfunction and atrial fibrillation, respectively. Finally, three patients developed neoplasms, two skin cancer (basal cell 1 and squamous cell 1) in one patient in each group and one patient in Group A developed cancer in the native kidney.