"PRECISION MEDICINE IN LUPUS NEPHRITIS: A MULTICENTER PROOF-OF-CONCEPT STUDY FOR HISTOPATHOLOGICAL BIOMARKERS ANALYSIS IN RENAL BIOPSY"

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INTRODUCTION

Lupus nephritis (LN) may affect approximately half of patients with Systemic Lupus Erythematosus (SLE). LN is a major cause of morbidity and the most important predictor of mortality in patients with SLE (1,2). Some 5-20% of patients with LN may develop end-stage renal disease within 10 years of follow-up from the time of diagnosis (3). Other studies have described progression to end-stage renal disease in 10-30% of patients with LN (4,5).

The European League Against Rheumatism (EULAR), the European Renal Association (ERA) and the European Dialysis and Transplant Association (EDTA) have recently updated their recommendations for the management of LN. These recommend the use of intravenous (IV) methylprednisolone boluses followed by lower doses of oral glucocorticoids (GC) and place mycophenolate mofetil (MMF) and the European regimen of cyclophosphamide (CYC) as the immunosuppressive drugs of first choice, with the IV CYC regimen for certain more aggressive cases. They also consider the use of "multitarget therapy" based on the combination of tacrolimus (TAC) and MMF and GC in patients with proteinuria in the nephrotic range who have not responded to the first line of treatment. For refractory active renal disease, they recommend as an alternative the use of rituximab (RTX) 1000 mg IV repeated after 15 days (6).

Recently, belimumab has been shown to be significantly more effective than placebo in the treatment of patients with active LN (7). This finding will lead to positioning belimumab in the therapeutic algorithm for LN.

However, in clinical practice these immunosuppressive drugs are not always effective in the treatment of LN, and even one in 3 patients with an initial favorable response may experience renal recurrence (3).

The choice of the appropriate treatment for LN and its early initiation are key to improve the prognosis of these patients and to avoid progression to chronic renal failure.

The identification of biomarkers capable of predicting the response (or lack thereof) to one or another therapy at the time of LN diagnosis would allow to implement precision medicine, thus constituting a revolution in the treatment of patients with LN. Being able to make personalized therapeutic decisions, based on the biological characteristics of each patient's renal sample, allows more targeted treatments with greater specificity to be established.

The **objective** of this project is to analyze histopathological biomarkers in the renal biopsy to predict the renal response to the different drugs used in the treatment of LN. This would contribute to a more specific and cost-effective therapeutic strategy.

PATIENTS AND METHODS

Inclusion criteria:

Patients with a diagnosis of SLE according to the 1997 American College of Rheumatology (ACR) criteria (8).

Diagnosis of lupus nephritis type 3,4,5 or mixed forms of the above according to the International Society of Nephrology (ISN)/Renal Pathology Society (RPS) classification of LN (9).
Date of performance of the renal biopsy: year 2000 or later. The reason for this time frame is the theoretical homogenization in the therapeutic management of LN with the introduction of MMF into the therapeutic armamentarium of LN and the extension of the use of the European intravenous cyclophosphamide guideline.

Availability of the patient's first renal biopsy specimen preserved for re-evaluation.

Availability of essential clinical data to carry out the study.

Signature of the informed consent by the patient.

Exclusion criteria:

Refusal by the patient to sign the informed consent.

Sample size

For this proof-of-concept study, our objective is to analyze around **60 renal biopsy** samples (expandable according to the results obtained).

Methods:

The following **general variables** will be collected:

- demographic data: age, sex, ethnicity.

- clinical data on SLE:

    - diagnosis and chronological data: time of SLE diagnosis, time of LN diagnosis, time form SLE diagnosis to LN.

    - 1997 ACR SLE classification criteria,

    - activity: involvement of different organs and systems by SELENA-SLEDAI (10) at the time of LN diagnosis and at the time of the last evaluation of the patient (or death, if applicable).

    - damage: by item and by domain according to the Systemic Lupus International Collaborating Clinics Damage Index (SDI) (11) at the time of LN diagnosis and at the time of the last evaluation of the patient (or death, if applicable).

    - comorbidity: arterial hypertension, diabetes, dyslipemia, smoking habit, severe infections, neoplasms, etc. at the time of LN diagnosis and at the time of the last evaluation of the patient (or death, if applicable).

- laboratory data at the time of LN diagnosis and at the time of the last evaluation of the patient (or death, if applicable):

    - blood (general tests): acute phase reactants (erythrocyte sedimentation rate and C-reactive protein), full blood count, creatinin, glomerular filtration rate, blood urea nitrogen, liver function tests, lipid profile.

- blood (serological tests): complement (C3 and C4), levels of anti-dsDNA antibodies, antiphospholipid antibodies: anticardiolipin (Ig M and Ig G), anti-beta2GP-1 (Ig M and Ig G), lupus anticoagulant.

- urine: hematuria, piuria, proteinuria, casts.

- histopathological markers of renal biopsy:

  - histological class according to the 2003 ISN/RPS classification (9).

  - National Institutes of Health (NIH) activity index (12): score (maximum 24) and by item (endocapillary hypercellularity, neutrophils/karyiorrhexis, hyaline deposits/wire loops, fibrinoid necrosis, cellular o fibrocellular crescents, interstitial inflammation).

  - National Institutes of Health (NIH) chronicity index (12): score (maximum 12) and by item (global glomerulosclerosis, fibrous crescents, tubular atrophy, interstitial fibrosis).

  - markers related to B lymphocytes which may include but is not limited to: CD19, CD20 and CD138.

  - markers related to BLyS (B lymphocyte stimulator) and its functional consequences which may include but is not limited to expression of BLyS and its receptors: BAFF-R, BCMA and TACI.

  - markers related to other cell lineages which may include but is not limited to: CD3 for T cells and CD68 for macrophages.

  - markers whose determination in urine has proved useful in the diagnosis and follow-up of LN which may include but is not limited to the proinflammatory cytokine Monocyte Chemoattractant Protein-1 (MCP-1) and the Neutrophil Gelatinase Associated Lipocalin (NGAL).

- SLE therapeutical data (including duration of the treatment):

  - treatments for SLE prior to the diagnosis of LN: antimalarials, glucocorticoids (maximum dose), immunosuppressants: azathioprine (AZA), mophetil mycophenolate (MMF), cyclophosphamide (CYC); biological therapies: belimumab, rituximab.

  - treatment of LN: glucocorticoids (maximum dose), immunosuppressants: AZA, MMF, CYC; biological therapies: belimumab, rituximab.

  - therapeutical data of comorbidities (including duration of the treatment): antihypertensive agents, oral antidiabetics, insulin, hypolipidemic drugs.

- Evolution/prognosis of SLE: accumulated damage by using SDI (11) (by item and by domain), comorbidities accrual, severe infections, organ failure, death.

Variables will be collected to establish different patterns of response to treatment and evolution of LN:

a) complete renal response, defined according to EULAR/ERA-EDTA recommendations (13): proteinuria <0.5 g/24 hours and (near) normal estimated GFR.

b) partial renal response, defined according to EULAR/ERA-EDTA recommendations (13): ≥50% proteinuria reduction to subnephrotic levels and (near) normal eGFR

c) no response: all the other cases.

d) proteinuria levels at 12 months of treatment,

e) renal relapse as defined as reproducible increase in uPCR to >1 g if the baseline value was <0.2 g, to >2 g if the baseline value was between 0.2 g and 1 g, or more than twice the value at baseline if the baseline value was >1 g AND/OR reproducible decrease in GFR of >20%, accompanied by proteinuria (>1 g), and/or RBC and/or WBC cellular casts (yes/no and number of flares), e) time to first flare, f) chronic renal failure,

f) end-stage renal disease requiring dialysis and/or renal transplantation.

Data collection will be based on the clinical history of the patients included in the study.

Confidentiality will be respected in accordance with RD 1720/2007 and the Data Protection Laws. Approval will be requested from the **Galician Clinical Research Ethics Committee (CEIC)** as well as from the CEIC of each center, if necessary.

EXPERIMENTAL STRATEGY AND RATIONALE

In this project we will use renal biopsies from patients with LN. These samples, preserved in 10% formaldehyde and embedded in paraffin blocks by the corresponding Anatomic Pathology Services, correspond to patients with LN who underwent renal biopsy in the centers participating in the project.

BLyS (B lymphocyte stimulator) plays a key role in the pathophysiology of LN (14). Therefore, in this study we will focus on markers related to BLyS and its functional consequences. On the one hand, we will analyze among others the expression levels of BLyS and its receptors (BAFF-R, BCMA and TACI), since the expression levels of BLyS and its receptors are elevated in serum and renal biopsies of patients with LN and are associated with disease progression and severity (15-17).

On the other hand, because BLyS induces B cell survival (14), we will analyze the expression levels of different B cell markers such as CD19 and CD20 among others. Finally, we will analyze the plasma cell marker CD138, since plasma cell infiltration is associated with increased severity of lupus nephritis (18). We will analyze the expression levels of markers of other cell lineages such as CD3 for T cells and CD68 for macrophages.

We will also analyze at least 2 biomarkers whose urinary levels in patients with LN have been associated with a worse prognosis of LN: the chemokine MCP-1 (monocyte chemoattractant protein-1) and the enzyme NGAL (neutrophil gelatinase-associated lipocalin) (19).

The expression of these markers will be initially determined by immunohistochemical staining of renal biopsies, which are preserved in formaldehyde. The samples will be stained and analyzed mainly in the Anatomic Pathology Service of the "Complejo Hospitalario Universitario de Vigo" and in the laboratories of the "Instituto de Investigación Sanitaria Galicia Sur" (IISGS), although the different participating centers will participate in this work to the extent possible and cost-effective for the project. Different methods of quantification will be used in function of the stains of the different markers.

STATISTICAL ANALYSIS

In the descriptive study, numerical variables will be expressed as mean ± standard deviation (SD) or median and interquartile range (IQR), depending on whether the distribution is normal or not, respectively. We will establish 2 groups of patients, according to the response to treatment and the clinical evolution of the patient with LN (i.e., complete remission yes/no). To establish differences between patients in these 2 groups, we will use the $\chi 2$ test for categorical variables or Fisher's exact test when the expected frequencies are small, the t-Student test for normal continuous variables, and the Mann-Whitney U test for variables with non-normal distribution.

Different methods to analyze and/or mitigate the missing data problem will be used.

The percentage of positive cells and staining intensity will be correlated with the different patterns of response to treatment and evolution of LN.

Univariate and multivariate linear logistic regression analyses will be performed to explore the relationships between the different variables studied (clinical, histopathological, therapeutic...) and the presence of the different renal outcomes (dependent variable). Values of $p < 0.05$ will be considered significant.

We will carry out other different statistical methods according to the results that we observe from our initial analysis.

Statistical analyses will be performed by the Statistical Specialist of the IRIDIS Group.

TIMELINES

Development of the definitive protocol: month 1.

Submission and Approval by Ethics Committee: months 1-3.

Renal samples identification and preparation: months 4-5.

Informed consent signatures: months 4-6.

Shipment of renal samples: months 4-6.

Anatomo-pathological and laboratory studies: months 7-12.

Review of clinical charts: months 7-12.

Monitoring of the database: months 13-15.

Statistical analysis: month 16.

Elaboration of the final report: month 17-18.

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