Changes in Autoreactive Memory B Cells as Biomarker of Response to Adrenocorticotropic Hormone in Patients With Membranous Nephropathy
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1. Hypothesis:

We hypothesize that adrenocorticotropic hormone (ACTH, Acthar) reduces proteinuria in patients with membranous nephropathy by inhibiting autoreactive memory B cells and by increasing regulatory T cells. Therefore, the number of circulating autoreactive B cells can be used as a biomarker of disease activity and response to Acthar therapy.

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2. Rationale for the study:

Membranous nephropathy (MN) is an autoantibody-mediated glomerular disease and is the major cause of nephrotic syndrome (NS) in adults worldwide. The disease is caused by the formation of immune deposits on the outer aspect of the glomerular basement membrane. These immune deposits contain podocyte antigens and circulating antibodies specific to those antigens, resulting in complement activation, podocyte injury, proteinuria, and renal failure. In 2009, podocyte phospholipase A2 receptor (PLA_2R) was reported as the antigenic target in over 70% of adult patients with MN [1]. The disease shows a benign or indolent course in the majority of cases, but about 30-40% of affected patients progress to end-stage kidney disease (ESKD) 5-10 years after diagnosis [2].

Treatment with steroids in association with immunosuppressants including alkylating agents and calcineurin inhibitors has been suggested for patients with persistent NS [3]. However, these medications are burdened by substantial toxicity with an excess risk of serious adverse events. Starting from the evidence that B cells play a crucial role in the pathogenesis of the disease, both as precursors of autoantibody-producing cells and as antigen-presenting cells, we performed a series of prospective studies showing that anti-CD20 B-cell depleting monoclonal antibody rituximab safely reduces proteinuria in MN patients [4-7]. About 30% of patients treated with rituximab or other immunosuppressive agents, however, do not respond to these therapies or relapse after initial response, possibly because these therapies (including rituximab) are only partially effective in inhibiting memory B cells, a B-cell subset important in autoantibody production.

Adrenocorticopropic hormone (ACTH) has been recently proposed as an alternative therapy for MN, after the incidental finding by Berg *et al*. that this hormone had antiproteinuric effects in 14 MN patients [8]. This observation was thereafter confirmed by other retrospective [9] and prospective [10] studies. ACTH is produced by the anterior pituitary gland and induces cortisol production by the adrenal glands. Its antiproteinuric effects, however, seem not to be mediated by the increased cortisol synthesis, since steroid therapy alone has been proven to be ineffective in inducing remission or delaying end-stage renal disease for MN [11]. ACTH is a universal agonist for the five melanocortin receptors (MC1R-MC5R). MC1R has been implicated in mediating ACTH protective effects on podocytes by reducing cell oxidative stress, apoptosis, injury and loss in the proteinuric kidney disease models [12]. The podocyte protective effects of ACTH are at the basis of its antiproteinuric effects in a wide range of glomerular diseases, including, besides MN, IgA nephropathy, focal segmental glomerulosclerosis (FSGS), lupus nephritis, and minimal change disease.

More importantly in the context of MN, published data by others and our preliminary data presented below show that ACTH has also direct anti-inflammatory and immunomodulatory functions on T and B lymphocytes, including reduction of pro-inflammatory cytokines, nitric oxide, adhesion molecules, and production of anti-inflammatory IL-10 [13]. In patients with West syndrome [14], ACTH administration resulted in helper T cell and total B cell inhibition. Evidence is also available that ACTH inhibits the recruitment of memory B cells in the cerebral fluid of patients with opsoclonus-myoclonus syndrome [15]. In a mouse model of autoimmune encephalomyelitis

[16], ACTH inhibited autoreactive Th17 cells and promoted a significant increase in regulatory T cells (Treg), a T cell subset with important immune protective roles in autoimmune diseases, including MN [17]. These immune modulating effects are consistent with the reported reduction of anti-PLA_2R autoantibodies described in MN patients treated with ACTH [10].

Building on this evidence, **we hypothesize that the antiproteinuric effects of ACTH in MN are mediated by the inhibition of autoreactive memory B cells and the promotion of Treg**. These unique effects, together with ACTH direct nephroprotective activity on podocytes, make ACTH a valuable option for MN patients, including those who do not respond or relapse after other therapies targeting different pathways involved in MN pathogenesis. The number of autoreactive memory B cells could also represent a biomarker of disease activity and response to ACTH therapy.

To address our hypothesis, we designed a mechanistic prospective, open-label study to evaluate the effects of Acthar on autoreactive memory B cells and regulatory T cells in MN patients. This study is expected 1) to provide crucial knowledge on the mechanism of action of Acthar in MN, 2) identify autoreactive memory B cells as a biomarker of response to Acthar, and 3) collect additional information on the safety/efficacy of this treatment.

3. Patient population:

This mechanistic, open-label study will include patients with histological diagnosis of primary membranous nephropathy (with renal biopsy performed no longer than 3 years prior) and persistent (>3months) proteinuria >3.5g/24h despite at least 3 months of ACE inhibitor or angiotensin receptor blocker therapy. The study will include both subjects naïve to previous treatments and subjects who received previous immunosuppressive treatment with steroids + alkylating agents (for at least 6 months), calcineurin inhibitors +/- steroids (6-12 months), or rituximab (6 months).

Inclusion criteria

- Age 18 to 70 years
- Free of immunosuppression for at least 3 months
- Capability of understanding the purpose of the study
- Written informed consent

Exclusion criteria

- eGFR < 30ml/min/1.73m^2
- Kidney Transplant
- Secondary MN (defined on the basis of clinical criteria)
- Type 1, uncontrolled Type 2 diabetes mellitus glycosylated hemoglobin (HbA1c) >8% (prior diagnosis of gestational diabetes mellitus is not an exclusion)
- History of previous use of Acthar for treatment of nephrotic syndrome
- Prior sensitivity to Acthar or other porcine protein products
- Contraindication to Acthar per Prescribing Information
- Planned treatment with live or live attenuated vaccines once enrolled in the study
- More than three previous treatment regiments
- Participation to other clinical trials over the previous 12 months
- History of cancer, except carcinoma in situ and treated basal and squamous cell carcinomas
- Pregnancy
- Lactation
- Current substance abuse
- Any clinically relevant condition that might affect study participation and/or study results

4. Study design

Research strategy

Clinical study design

This will be a prospective-cohort study in which all eligible patients will receive Acthar therapy. After inclusion, patients will undergo monthly evaluations of clinical parameters (body weight and BP), biochemistry, proteinuria and serum creatinine up to month 3, then at month 6, 9, and 12 after treatment start. At baseline and at 6 and 12 months we will also collect blood (30ml) to measure circulating anti-PLA_2R reactive memory B cells, Treg numbers, and anti-PLA_2R autoantibodies (**Fig**).

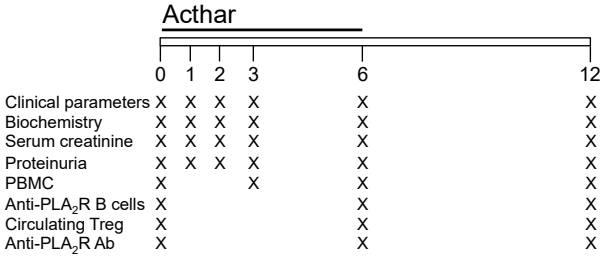

Fig . Study design.

Treatment. ACTH (Acthar) will be administered subcutaneously (SC) at the dose of 80 units for the first week and then 80 units SC twice weekly to complete 6 months of therapy, with a cumulative drug exposure of 3,920 units.

- *Data handling:* A password-protected and coded, electronic database will be created to include patient demographics, physical and laboratory results including BP, serum creatinine, immunosuppressive drug doses and trough levels. Acthar doses will be also recorded at the time of each blood withdrawal, to monitor response to Acthar therapy.

Ex vivo studies

- *T and B cells.* PBMC will be isolated by Ficoll gradient centrifugation. Flow cytometry assays will be performed on fresh cells. We will use standardized multicolor flow cytometry to phenotype $CD4^+$ and $CD8^+$ T cells and B cells, including regulatory ($CD127^{lo}FoxP3^+$) , naïve ($CD45RA^+CD27^+$), central memory ($CD45RO^+CD27^+$), effector memory ($CD45RO^+CD27^-$) and T cells, naïve B cells ($CD19^+CD27^-IgD^+$), non-class-switched memory B cells ($CD19^+CD27^+IgD^+$), class-switched memory B cells ($CD19^+CD27^+IgD^-IgM^-CD20^+CD38^{+/-}$), and $CD3^+CD4^+CXCR5^+$ and follicular helper T cells. Appropriate isotype matched monoclonal antibodies will be used as controls. Phenotyping will be performed using 3-color flow cytometry on a Canto analyzer (Becton Dickinson, Franklin Lakes, NJ).

MC1R surface expression on T and B cells will be also assessed using AMNIS Imaging Flow Cytometer MKII (EMD Millipore, Darmstadt, Germany). PBMC from age- and gender-matched healthy subjects will be used as controls.

- *Anti-PLA_2R B cells and antibodies.* Anti-PLA_2R reactive B cells will be measured according to the protocol described in the preliminary data. Anti-PLA_2R circulating autoantibodies in the serum will be measured by ELISA using the EURIMMUNE kit (EA 1254-9601 G).

- *MC1R polymorphisms.* The human *MC1R* gene is highly polymorphic, with >35 genetic variants identified at present. We will perform MC1R genotyping on our cohort of patients to start testing the hypothesis that different genotypes are associated with different responses to Acthar therapy. We will also compare changes in immunological parameters (Treg, Teff, anti-PLA_2R B cells and antibodies) after Acthar therapy according to the MCR1 polymorphisms.

We have standardized both flow cytometry assessments [18] for use in clinical trials. We will perform all assays under good laboratory practice (GLP) compliance; previous work shows high reproducibility. We have published experience in performing serial measurements of Teff and Treg numbers and function in dialysis patients [19], which documents the feasibility of the proposed study.

5. **Primary endpoint**:
   - Primary endpoint of the study will be the change in proteinuria from baseline (before Acthar therapy) to 12 months after therapy

Secondary endpoints will include both clinical and mechanistic endpoints.

Secondary clinical endpoints:

- Complete (proteinuria reduction ≤500mg/24h) or partial (proteinuria reduction <3.5g/24h but >500mg/24h and >50% reduction than baseline, with stable renal function) remission at 12 months after inclusion
- Time to complete or partial proteinuria remission
- 24h proteinuria at 6 and 12 months after inclusion
- Serum albumin at 6 and 12 months after inclusion
- Estimated GFR at 6 and 12 months after inclusion
- Safety variables

Secondary mechanistic endpoints:

- Number of anti-PLA_2R memory B cells at baseline and 3, 6, and 12 months after inclusion
- Number of circulating $CD4^+CD25^+CD127^{int}FoxP3^+$ Tregs at baseline and at 3, 6, and 12 months after inclusion
- Levels of anti-PLA_2R antibodies at baseline and 3, 6, and 12 months after inclusion
- Changes in T cell subsets after ACTH treatment
- Changes in MC1R in T cells and B cells at baseline.

Analysis plan and statistics: The clinical analyses will primarily evaluate the change in proteinuria and its relationship with autoreactive Bmem after Acthar therapy and secondarily they will assess rate of complete or partial remission changes in serum albumin, and eGFR over the 12-month follow-up period. Mechanistic, *ex vivo* studies will measure changes in total Tregs after Acthar therapy and their relationship with changes in proteinuria and anti-PLA_2R Abs.

Analyses will be performed initially in an intention-to-treat fashion. Thereafter, to exclude patients who stopped Acthar therapy during the follow-up period, we will consider in secondary analyses only those subjects who will have received the entire course of Acthar treatment. A p value of <0.05 will be considered statistically significant. We have access to appropriate biostatistical support through the Icahn School of Medicine to assist with these analyses (http://icahn.mssm.edu/research/grant-application-resource-center/statistical-support).

Sample size estimation. This will be a pilot, explorative study and the sample size will not be calculated on the basis of the expected treatment effect. However, according to previous reports, we expect that approximately 70% of patients will reach complete or partial remission during the first year of follow-up and that 20 subjects will be enough to detect as statistically significant a change in proteinuria between baseline and 12 months [20]. The same number of patients will be enough to detect a significant difference in PLA_2R antibodies [10]. To account for possible drop-outs, we will include 25 patients. The data generated from this study will form the basis for the sample-size estimation of a larger prospective study testing the utility of anti-PLA_2R memory B cells as a biomarker for response to Acthar therapy in MN patients.

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