UNIVERSITY OF IOANNINA

Faculty of Medicine — School of Health Sciences

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STUDY PROTOCOL

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Translator's note: *This document is an English translation and structural revision of the original Greek-language study protocol. Content has been reorganized to mirror the data fields required by the ClinicalTrials.gov Protocol Registration and Results System (PRS) for observational studies, to facilitate registration and assignment of an NCT number. Scientific content has been clarified and tightened, but no data, criteria, or endpoints have been altered in substance.*

1. Study Identification

1.1 Brief Title

Immunophenotyping profiling in patients with primary nephrotic syndrome

1.2 Official Title

Immunophenotyping profiling in patients with nephrotic syndrome: Emphasis on the diagnostic and prognostic value of immune cells in the different phenotypes of nephrotic syndrome.

2. Brief Summary

Primary nephrotic syndrome caused by membranous nephropathy (MN), focal segmental glomerulosclerosis (FSGS), or minimal change disease (MCD) is, in each case, associated with dysregulation of specific innate and adaptive immune cell populations. However, the relationship between circulating immune cell subsets and key clinical outcomes, specifically treatment response and disease relapse, remain incompletely defined, and the classical clinical and laboratory markers currently used to monitor these diseases are non-specific and have limited predictive value.

This prospective, single-center cohort study will enroll adult patients with biopsy-proven (or, for MN, anti-PLA2R-antibody-positive) primary nephrotic syndrome followed at the Nephrology Clinic of the University Hospital of Ioannina. Peripheral blood immune cell subsets, including monocyte subsets, NK cells, regulatory T cells, Th17 cells, senescent CD8+ and CD4+ T-cell subsets, naïve and memory T and B cells, and B-cell subsets, will be quantified by multicolor flow cytometry at baseline, before treatment initiation, and at months 3, 6, and 12 after treatment. Patients will be followed clinically for a total of 24 months.

The study aims to determine whether baseline levels and longitudinal treatment-related changes in these immune cell subsets are associated with treatment response and disease relapse and to correlate them with standard clinical and laboratory markers of renal function and disease activity.

3. Background and Rationale

3.1 Nephrotic syndrome overview

Nephrotic syndrome is among the most common clinical presentations of glomerular disease. It is characterized by increased permeability of the glomerular filtration barrier, resulting in marked proteinuria, hypoalbuminemia, edema, hypercholesterolemia, and lipiduria[1]. The most frequent causes of primary nephrotic syndrome are primary membranous nephropathy (MN), minimal change disease (MCD), and primary focal segmental glomerulosclerosis (FSGS). Other causes associated with systemic disease include diabetic nephropathy, amyloidosis, and lupus podocytopathy[2].

3.2 Immune mechanisms in membranous nephropathy

A substantial body of literature implicates the immune system in the pathogenesis of nephrotic syndrome[3]. In primary MN, B-cell involvement is well established: pathogenic anti-phospholipase A2 receptor (anti-PLA2R) autoantibodies drive subepithelial immune-complex formation and complement-mediated podocyte injury[4]. This discovery underpins B-cell-depleting therapy with rituximab, an anti-CD20 monoclonal antibody, now a guideline-endorsed treatment option for MN[5]. The contribution of T cells to MN pathogenesis is less well characterized, though regulatory T cells (Tregs) and CD4+ Th2 cells have been implicated[6].

3.3 Immune mechanisms in minimal change disease

In MCD, the underlying pathophysiological trigger remains unclear. T-cell dysfunction leading to increased glomerular permeability has historically been the leading proposed mechanism[7]. Tregs may also contribute: in experimental models, induction of regulatory T cells attenuates disease, and peripheral CD4+CD25+FoxP3+ Tregs increase with remission[8]. The role of B cells in MCD was long considered negligible; however, the efficacy of rituximab suggests that a glomerular permeability factor may be produced by B cells, or by T cells acting through B-cell-dependent or B-cell-regulated pathways. That rituximab is effective in glucocorticoid-responsive but not in glucocorticoid-resistant or glucocorticoid-dependent MCD may reflect distinct underlying pathogenic mechanisms across these phenotypes[9].

3.4 Immune mechanisms in focal segmental glomerulosclerosis

The pathophysiology of FSGS is similarly incompletely defined, with reported associations to T-lymphocyte dysfunction and dysregulation[10]. Immunophenotyping of peripheral blood in FSGS patients has demonstrated an imbalance between Th1 and Th2 populations, Th17 overexpression, and a significant increase in CD8+ T cells — including activated CD8+ subsets — alongside a reduction in HLA-DR+ activated Tregs in steroid-sensitive disease; these abnormalities normalize with treatment. Monocyte counts are also significantly reduced compared with healthy controls[11].

3.5 Limitations of current clinical biomarkers

The principal parameter used clinically to monitor treatment response remains 24-hour urinary protein excretion. Complete remission is defined as a reduction in proteinuria to below 0.3 g/24h, and partial remission as a reduction of ≥50% from baseline together with total proteinuria below 3.5 g/24h. Non-response is defined as persistent proteinuria above 3.5 g/24h despite guideline-directed therapy, and relapse as recurrence of proteinuria above 3.5 g/24h in a patient who had previously achieved complete remission. In MN specifically, serum anti-PLA2R antibody titers provide an additional marker of immunological disease activity: a decline in antibody levels can precede and predict clinical response, while re-emergence or a rise in titer in a patient previously in remission can predict relapse[12, 13]. Anti-PLA2R1 antibody titers and proteinuria capture humoral activity and glomerular injury, but only partially explain this heterogeneity, are informative in only 70–80% of patients and frequently lag behind cellular events [14].

3.6 Knowledge gap

Despite the established contribution of specific immune cell populations to primary nephrotic syndrome, the literature remains comparatively sparse regarding abnormalities in regulatory T-cell subsets, monocyte subsets, B-cell subsets (including CD5+ B cells), T-helper subsets, and NK cells, and their potential role in disease pathogenesis across MN, MCD, and FSGS. Furthermore, classical clinical and immunological biomarkers of disease activity and treatment response are non-specific, correlate poorly with the degree of underlying renal injury, and in most cases cannot predict renal outcome. Whether changes in specific circulating immune cell subsets are associated with clinical outcomes in primary nephrotic syndrome, and whether they add predictive value beyond classical markers, remains an open question that this study is designed to address.

4. Study Objectives and Novelty

4.1 Primary objective

To characterize innate and adaptive immune cell subsets in patients with primary nephrotic syndrome (MN, FSGS, MCD), and to determine their association with treatment response and disease relapse.

4.2 Secondary objective

To correlate the immune cell subsets under study with classical clinical and laboratory indices of renal function, and with markers of the other organ systems affected by nephrotic syndrome (lipid metabolism, coagulation, cardiovascular status).

4.3 Novelty

The prospective component of the study is designed to establish whether these immune cell subsets can function as diagnostic and prognostic biomarkers in primary nephrotic syndrome and whether their baseline levels or early treatment-induced kinetics can forecast subsequent treatment response or disease relapse, across three histologically and immunologically distinct causes of primary nephrotic syndrome studied within a single prospective cohort.

5. Study Design

Design parameter	Value
Study Type	Observational
Observational Study Model	Cohort
Time Perspective	Prospective, with a cross-sectional characterization at baseline
Setting	Single center
Institution / Site	Nephrology Clinic, University Hospital of Ioannina, University of Ioannina, Greece
Number of cohorts	3 (MN, FSGS, MCD)
Estimated enrollment	60 participants
Follow-up duration per participant	24 months from treatment initiation
Biospecimen retention	Yes - serum/plasma biobanked at -80C
Sampling method	Non-probability sample (consecutive patients meeting eligibility criteria at a single clinic)

6. Study Setting and Population

All patients who meet the eligibility criteria below and are followed at the Nephrology Clinic of the University Hospital of Ioannina will be considered for enrollment. There is no randomization or investigational product: patients receive standard-of-care treatment for their underlying disease per current international guidelines, and immune cell profiling is performed alongside routine clinical care.

7. Eligibility Criteria

7.1 Sex

All (male and female).

7.2 Age

Adults (18 years and older).

7.3 Inclusion Criteria

1. Patients with nephrotic syndrome and a biopsy-confirmed diagnosis of membranous nephropathy, primary focal segmental glomerulosclerosis, or minimal change disease.
2. Patients with membranous nephropathy and positive serum anti-PLA2R antibodies, in the absence of histological confirmation by renal biopsy.
3. Patients with a previously confirmed diagnosis of a primary nephrotic syndrome cause, who present with disease relapse requiring re-induction therapy.

7.4 Exclusion Criteria

4. Patients with nephrotic syndrome in whom membranous nephropathy, primary FSGS, or MCD has not been confirmed by renal biopsy (and who do not meet Inclusion Criterion 2).
5. Patients with secondary causes of nephrotic syndrome (malignancy, autoimmune disease, drug-induced, etc.).
6. Patients with active malignancy.
7. Patients with severe heart failure (NYHA class IV) or hepatic failure.
8. Patients with active infection or inflammation.
9. Patients receiving, or who received within the preceding 6 months, immunomodulatory agents for an unrelated condition.

7.5 Sampling Method

Non-probability sample - consecutive eligible patients presenting to the Nephrology Clinic.

8. Study Groups / Cohorts [ClinicalTrials.gov: Groups/Cohorts]

Cohort	Definition
Cohort 1 - Membranous Nephropathy (MN)	Biopsy-proven primary MN, or anti-PLA2R-antibody-positive MN without histological confirmation
Cohort 2 - Focal Segmental Glomerulosclerosis (FSGS)	Biopsy-proven primary FSGS
Cohort 3 - Minimal Change Disease (MCD)	Biopsy-proven MCD

Patients presenting with disease relapse who meet Inclusion Criterion 3 are assigned to the cohort corresponding to their underlying histological diagnosis.

9. Sample Size and Power Calculation

Treatment response varies substantially across the three entities. In primary MN, immunosuppressive therapy induces complete or partial remission in roughly 60% of high-risk patients by 24 months; in the MENTOR trial, rituximab achieved complete or partial remission in 60% of patients at 24 months (35% complete), while spontaneous remission occurs in up to a third of untreated patients[1,5]. In adult MCD, corticosteroids induce remission in approximately 75-90% of patients, although the majority subsequently relapse[9,15]. In primary FSGS, response is less favorable: complete or partial remission is achieved in roughly 40-60% of adults with prolonged corticosteroid therapy, with steroid resistance reported in a substantial proportion of patients[16].

Reported relapse rates in the literature were used to inform the power calculation. In MN, relapse occurs in approximately 25-30% of patients achieving complete remission and in up to 50% of those achieving only

partial remission[17]. In FSGS, relapse rates are approximately 25-35% after complete remission and up to 50% after partial remission[18]. In MCD, relapse occurs in 50-75% of glucocorticoid-responsive patients achieving complete remission, and approximately 25% of patients experience frequent relapse (>4 episodes/year) [14, 19]. Based on these incidence and prevalence estimates, a power analysis was performed using SPSS v.27. For 80% power, the minimum sample size required was 50 patients with primary nephrotic syndrome. Accounting for sample bias and anticipated attrition, the target enrollment was set at 60 patients.

Management and treatment of enrolled patients will follow current international guidelines for CKD and glomerular disease presenting as nephrotic syndrome - specifically for MN, MCD, and FSGS - per the KDIGO Clinical Practice Guideline for the Management of Glomerular Diseases (2021 edition, or its most current update at the time of treatment)[20].

10. Study Procedures

10.1 Baseline (Cross-Sectional) Assessment - Month 0

Performed prior to initiation of treatment for the underlying disease.

Demographic and clinical data: sex, age, smoking status, alcohol use, obesity, family history, and comorbidities, with particular attention to hypertension, diabetes mellitus, dyslipidemia, coronary artery disease, and coexisting autoimmune disease. A full clinical examination will be performed (systems review, blood pressure, pulse, weight, height, body mass index), and current medications will be recorded.

Routine laboratory assessment (at baseline and at regular intervals thereafter, per current standards of care for primary nephrotic syndrome): complete blood count, INR, comprehensive metabolic panel (glucose, urea, creatinine, potassium, sodium, calcium, phosphate, AST, ALT, CPK, GGT, alkaline phosphatase, total serum protein, HDL and LDL cholesterol, triglycerides, HbA1c, iron, TIBC, ferritin), inflammatory markers (CRP, ESR), urinalysis, and 24-hour urine collection (creatinine, urea, protein, albumin, sodium). Estimated glomerular filtration rate will be calculated using the CKD-EPI creatinine equation.

Disease-specific serology: in patients with confirmed membranous nephropathy, serum anti-PLA2R antibody titers will be measured per the KDIGO 2021 Clinical Practice Guideline for Glomerular Diseases[20].

Annual assessments: viral serology and tumor markers (HBsAg, HBsAb, HBcAb, anti-HCV, AFP, CA 19-9, CA 15-3, CEA, CA-125, PSA); lipid panel (apo-A, apo-B, Lp(a)) and fasting insulin.

Cardiac evaluation: all patients will undergo cardiology assessment with transthoracic echocardiography.

Exploratory biobanked assays: additional inflammatory, coagulation, and lipid-pathway biomarkers beyond the routine panel above may be assessed at a later stage using biobanked serum/plasma samples stored at -80C.

10.2 Immunophenotyping Panel

The following peripheral blood immune cell subsets will be quantified by multicolor flow cytometry at baseline, prior to treatment initiation:

- **B-cell compartment:** total CD3−CD19+; CD19+CD27+IgD+ (non-switched memory); CD19+CD27+IgD− (class-switched memory); CD19+CD27−IgD+ (naïve mature); CD19+CD5+ (regulatory B cells, Bregs).
- **T-cell compartment:** CD3+; CD3+CD4+; CD3+CD8+; CD4/CD8 ratio; CD4+CD25+FoxP3+ (regulatory T cells, Tregs); CD4+CD45RA+ (naïve); CD4+CD45RO+ (memory); CD45RA+RO+ (transitional).
- **Monocyte compartment:** total monocytes; CD14++CD16− (classical); CD14++CD16+ (intermediate); CD14+CD16++ (non-classical); CD14+HLA-DR+ (activated).

- **CD16+CD56+ (NK cells)**

10.3 Prospective Follow-up

Prospective follow-up will include reassessment of all immune cell subsets listed in the above section at Months 3, 6, and 12 after treatment initiation. Thereafter, for patients in remission, clinical follow-up will continue for an additional 12 months (through Month 24) using standard clinical and laboratory monitoring per current guidelines.

11. Outcome Definitions

Outcome	Definition
Complete remission	Proteinuria < 0.3 g/24h
Partial remission	>= 50% reduction in proteinuria from baseline AND total proteinuria < 3.5 g/24h
Non-response	Persistent proteinuria > 3.5 g/24h despite guideline-directed therapy
Relapse	Recurrence of proteinuria > 3.5 g/24h in a patient who had previously achieved complete remission

12. Outcome Measures

12.1 Primary Outcome Measures

Measure	Description	Time Frame
Treatment response	Complete or partial remission vs. non-response, defined per Section 11; correlated with baseline and serial immune cell subset levels	Baseline to Month 12
Disease relapse	Recurrence of proteinuria > 3.5 g/24h after complete remission; correlated with immune cell subset levels at relapse and preceding time points	Baseline to Month 24

12.2 Secondary Outcome Measures

Measure	Description	Time Frame
Composite renal outcome	>=50% decline in eGFR (CKD-EPI) from baseline, and/or initiation of kidney replacement therapy	Baseline to Month 24
Cardiovascular events	Incident cardiovascular events during follow-up	Baseline to Month 24
Thromboembolic events	Incident venous or arterial thromboembolic events	Baseline to Month 24
Infections	Incident infections requiring medical evaluation or treatment	Baseline to Month 24
Hospitalizations	All-cause hospitalization	Baseline to Month 24

13. Statistical Analysis Plan

The following analyses will be performed at study completion:

10. Survival analysis (Cox proportional hazards regression), with complete/partial remission and/or non-response as endpoints, to evaluate their association with the immune cell subsets under study.

11. Multivariate analysis to identify differences between patients who relapse and those who remain in remission.
12. Multivariate analysis to identify differences between patients presenting with nephrotic-range versus non-nephrotic-range proteinuria.
13. Logistic regression modeling to predict disease relapse based on patient characteristics and the immune cell subsets under study.
14. Logistic regression modeling to predict non-response to treatment based on patient characteristics and the immune cell subsets under study.

14. Study Timeline

Total follow-up per participant: 24 months from treatment initiation for the underlying disease (baseline, Months 3, 6, 12, and clinical follow-up to Month 24, with relapse-triggered reassessment as needed).

15. Biospecimen Retention and Data Management

Serum and plasma samples will be biobanked at -80C at baseline and at each follow-up time point. In addition to the assays specified in Section 10, banked samples may be used for exploratory analysis of additional inflammatory, coagulation, and lipid-pathway biomarkers not included in the routine panel, at a later stage of the study.

16. References

16.1 References (cited in order of appearance)

1. Couser WG. Primary membranous nephropathy. Clin J Am Soc Nephrol. 2017;12(6):983-997.
2. Politano SA, Colbert GB, Hamiduzzaman N. Nephrotic syndrome. Prim Care. 2020;47(4):597-613.
3. Tipping PG, Kitching AR. Glomerulonephritis, Th1 and Th2: what's new? Clin Exp Immunol. 2005;142(2):207-215.
4. Beck LH Jr, Bonegio RGB, Lambeau G, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med. 2009;361(1):11-21.
5. Fervenza FC, Appel GB, Barbour SJ, et al. Rituximab or cyclosporine in the treatment of membranous nephropathy. N Engl J Med. 2019;381(1):36-46.
6. Kuroki A, Iyoda M, Shibata T, Sugisaki T. Th2 cytokines increase and stimulate B cells to produce IgG4 in idiopathic membranous nephropathy. Kidney Int. 2005;68(1):302-310.
7. Shalhoub RJ. Pathogenesis of lipoid nephrosis: a disorder of T-cell function. Lancet. 1974;2(7880):556-560.
8. Le Berre L, Bruneau S, Naulet J, et al. Induction of T regulatory cells attenuates idiopathic nephrotic syndrome. J Am Soc Nephrol. 2009;20(1):57-67.
9. Vivarelli M, Massella L, Ruggiero B, Emma F. Minimal change disease. Clin J Am Soc Nephrol. 2017;12(2):332-345.
10. Salfi G, Casiraghi F, Remuzzi G. Current understanding of the molecular mechanisms of circulating permeability factor in focal segmental glomerulosclerosis. Front Immunol. 2023;14:1247606.
11. Ye Q, Zhou C, Li S, et al. The immune cell landscape of peripheral blood mononuclear cells from PNS patients. Sci Rep. 2021;11(1):13083.

12. Liang Y, Wan J, Chen Y, Pan Y. Serum anti-phospholipase A2 receptor (PLA2R) antibody detected at diagnosis as a predictor for clinical remission in patients with primary membranous nephropathy: a meta-analysis. BMC Nephrol. 2019;20(1):360.

13. Ruggenenti P, Debiec H, Ruggiero B, et al. Anti-phospholipase A2 receptor antibody titer predicts post-rituximab outcome of membranous nephropathy. J Am Soc Nephrol. 2015;26(10):2545-2558.

14. Logt AV, Justino J, Vink CH, et al. Anti-PLA2R1 Antibodies as Prognostic Biomarker in Membranous Nephropathy. Kidney Int Rep. 2021;6(6):1677-1686. Published 2021 Apr 22. doi:10.1016/j.ekir.2021.04.002

15. Waldman M, Crew RJ, Valeri A, et al. Adult minimal-change disease: clinical characteristics, treatment, and outcomes. Clin J Am Soc Nephrol. 2007;2(3):445-453.

16. Rosenberg AZ, Kopp JB. Focal segmental glomerulosclerosis. Clin J Am Soc Nephrol. 2017;12(3):502-517.

17. Du Buf-Vereijken PWG, Wetzels JFM. Efficacy of a second course of immunosuppressive therapy in patients with membranous nephropathy and persistent or relapsing disease activity. Nephrol Dial Transplant. 2004;19(8):2036-2043.

18. Troyanov S, Wall CA, Miller JA, Scholey JW, Cattran DC. Focal and segmental glomerulosclerosis: definition and relevance of a partial remission. J Am Soc Nephrol. 2005;16(4):1061-1068.

19. Nakayama M, Katafuchi R, Yanase T, Ikeda K, Tanaka H, Fujimi S. Steroid responsiveness and frequency of relapse in adult-onset minimal change nephrotic syndrome. Am J Kidney Dis. 2002;39(3):503-512.

20. Rovin BH, Adler SG, Barratt J, et al. KDIGO 2021 Clinical Practice Guideline for the Management of Glomerular Diseases. Kidney Int. 2021;100(4S):S1-S276.