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Detection of Antinephrin Antibodies in Childhood Idiopathic Nephrotic Syndrome

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INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most frequent glomerular disease in children entailing an intense treatment with steroids and other immunosuppressive drugs. Based on the response to first-line treatment with steroids, INS in children is classified as steroid-resistant (SRNS) or steroid-sensitive (SSNS).[1] Most INS cases have multifactorial pathogenesis involving immunological, genetic, and epigenetic factors.[S1,S2] Currently, a biomarker, associated with INS pathophysiology, that is able to predict the clinical course of the disease (response to treatment and occurrence of relapses) is lacking.

The role of autoantibodies as circulating permeability factors, able to directly damage the glomerular barrier, leading to proteinuria, has been recently explored.[2,3,S3] A subset of specific autoantibodies against 7 podocyte proteins was initially identified in 66% of children with INS.[4] Notably, each patient showed positivity for multiple autoantibodies correlating with proteinuria levels. The presence of circulating antinephrin (anti-NPHS1) autoantibodies was initially highlighted in 29% of patients with minimal change disease by Watts *et al.*[5] in 2022. A new multicenter study recently identified the presence of antibodies against nephrin in 44% of adult patients with minimal change disease, as well as in 52% of children with INS, thus defining the clinical entity of nephrin-associated podocytopathies.[6] High pretransplant anti-NPHS1 antibody levels were also found in pediatric patients with posttransplant recurrent focal segmental glomerulosclerosis with elevated IgG deposition in graft biopsies, colocalizing with nephrin at the slit-diaphragm.[7,8] Here, we evaluate the presence of anti-NPHS1 autoantibodies in a homogenous cohort of pediatric patients with INS. We also correlate the anti-NPHS1 autoreactivity with the clinical spectrum of INS and the response to steroids.

RESULTS

Patients Demographics

The clinical characteristics of the patient cohort are described in Supplementary Table S1. Out of the 69 recruited patients with podocytopathies, 60 were diagnosed with INS. Of them, 83% were classified as SSNS. Twenty samples were collected at their disease onset and free of therapy, whereas 24 were sampled from children at relapse with half of them during immunosuppressive treatment. Sixteen SSNS samples were collected at remission. The remaining 17% of the patients with INS did not respond to the initial steroid treatment and were classified as nongenetic SRNS (Supplementary Table S2). Nine patients presenting a

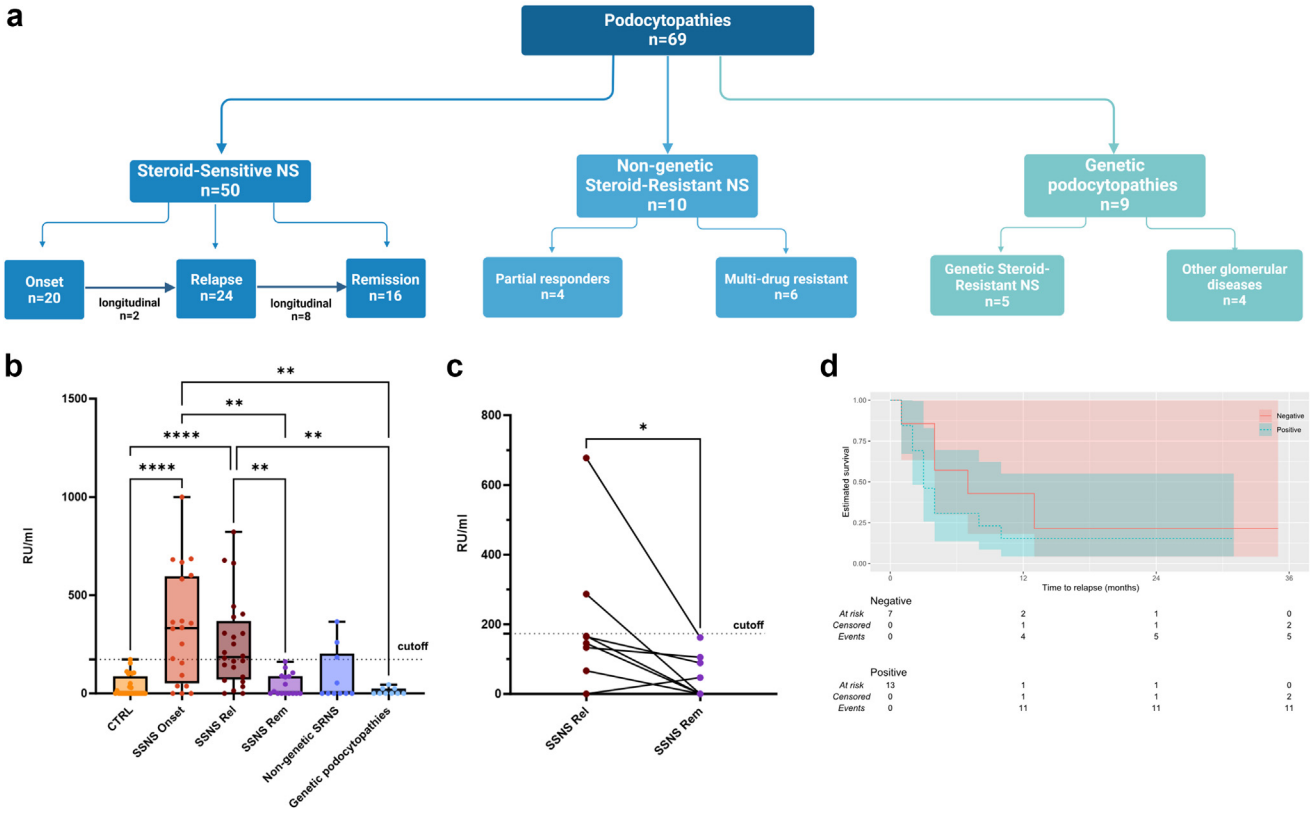

Figure 1. (a) Flowchart of podocytopathies' patient recruitment and detection of antinephrin antibodies. A total of 69 subjects with podocytopathy were included in the study. Of these, 50 were classified as steroid-sensitive nephrotic syndromes (SSNS), among whom 20 were tested at onset, 24 at relapse, and 16 during remission. Of these, 2 patients at onset were also tested during relapse and 8 relapsing patients were longitudinally evaluated at remission. Fifteen patients with nephrotic syndrome were diagnosed with steroid-resistant forms of the disease (SRNS). Among these, 10 had a nongenetic SRNS (4 partial responders and 6 multidrug resistant, MDR), whereas 5 had a genetic form of SRNS. Four genetic glomerular diseases with a different etiopathology were also selected. (b) Analysis of anti-NPHS1 antibody levels. Anti-NPHS1 levels were measured by using indirect enzyme-linked immunosorbent assay after subtraction of patient-specific serum backgrounds. OD_{450} values were converted into U/ml by interpolation with a standard curve. A threshold for the definition of anti-NPHS1 positivity was set at 173 RU/ml, based on the value obtained from the most autoreactive control. Anti-NPHS1 levels for every patient subgroup are reported in the box plot as the median (minimum–maximum) of the converted values. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. Nonparametric Kruskal-Wallis test with Dunn's *post hoc*. CTRL, healthy pediatric controls ($n = 28$); Genetic podocytopathies, patients with podocytopathies with a genetic origin ($n = 9$); Nongenetic SRNS, patients with nongenetic SRNS ($n = 10$); SSNS Onset, patients with SSNS at disease onset ($n = 20$); SSNS Rel, patients with SSNS during relapse ($n = 24$); SSNS Rem, patients with SSNS at remission ($n = 16$). (c) Histogram showing anti-NPHS1 concentration in 8 patients with SSNS analyzed longitudinally. Anti-NPHS1 antibody titers were measured at relapse (SSNS Rel) and at the following remission (SSNS Rem). The anti-NPHS1 antibody titer is presented as RU/ml. $^{*}P < 0.05$. Wilcoxon matched pairs signed rank test. (d) Survival analysis for relapsing events in patients with SSNS onset before treatment. Estimated survival refers to the relapse-free period. The red line indicates the probability of relapsing for patients with anti-NPHS1 negative SSNS onset ($n = 7$), whereas the blue line refers to patients with anti-NPHS1 positive SSNS onset ($n = 13$). The tables below refer to the absolute number of patients with SSNS who were negative (above) and positive (bottom) for anti-NPHS1, being either at risk, right-censored, or experiencing the event (relapse) in 12 months. Curves are reported with their relative 95% confidence intervals. $P = 0.298$.

genetic form of podocytopathies were enrolled (5 with genetic SRNS, 4 with other genetic glomerular diseases) (Supplementary Table S3) and represented the disease control group (Figure 1a). A cohort of 28 age-matched children with no kidney-related or immunological disease, were also recruited as healthy controls. Details on patient recruitment and data analysis are reported in Supplementary Methods section.

Antinephrin Antibody Serological Measurement

The anti-NPHS1 titers were significantly increased at onset (median: 332.3, interquartile range [IQR]: 51.5–596.5 RU/ml) and at relapse (185.1, IQR: 71.2–368.8 RU/ml) compared with the control group (0.0, IQR: 0.0–87.7 RU/ml) ($P < 0.0001$ vs. SSNS onset and SSNS relapse) (Figure 1b). The antibody levels were almost undetected in the genetic podocytopathies group, measured as 0.0 (IQR: 0.0–25.1 RU/ml; $P < 0.01$ vs. SSNS onset and SSNS relapse) as well as in the nongenetic SRNS group (0.0, IQR: 0.0–203.4 RU/ml; $P > 0.99$ vs. healthy controls). The levels of the anti-NPHS1 antibodies followed the disease activity, by decreasing significantly in patients with SSNS after remission (4.9, IQR: 0.0–88.4 RU/ml; $P < 0.01$ and

Table 1. Patient characteristics stratified by antinephrin antibodies positivity in the podocytopathies group

Characteristics	Anti-NPHS1 Negative $n = 42$	Anti-NPHS1 Positive $n = 25$	P value
Group, *n* (%)			$P = 0.25$[a]
Nongenetic SRNS	6 (14.3)	3 (12.0)	
SSNS	31 (73.8)	22 (88.0)	
Genetic podocytopathies	5 (11.9)	0 (0.0)	
Sex, *n* (%)			$P = 0.62$[a]
Male	23 (54.8)	10 (40.0)	
Female	19 (45.2)	15 (60.0)	
Race, *n* (%)			$P = 0.15$[a]
Caucasian	31 (73.8)	16 (64.0)	
Asian	5 (11.9)	1 (4.0)	
North-African	2 (4.8)	4 (16.0)	
African	4 (9.5)	2 (8.0)	
Other	0 (0.0)	2 (8.0)	
Age at sampling (yr), median (IQR)	10.5 (5.0–15.0)	11.0 (3.7–16.3)	$P = 0.92$[b]
Age at onset (yr), median (IQR)	3.5 (2.0–9.0)	4.0 (3.0–12.7)	$P = 0.34$[b]
Protein-to-creatinine ratio, median (IQR)	1.4 (0.2–6.9)	6.5 (2.4–9.6)	$P < 0.01$[b]
IgG immunoglobulins (mg/dl), median (IQR)	551.0 (228.0–771.2)	232.0 (149.0–610.7)	$P = 0.02$[b]
Drugs treatment at the time of collection, *n* (%)			$P = 0.18$[a]
Immunosuppressants	20 (47.6)	8 (32.0)	
Others	7 (16.7)	2 (8.0)	
NT	15 (35.7)	15 (60.0)	

Immunosuppressants, prednisone, mycophenolate, tacrolimus; IQR, interquartile range; *n*, the number of patients positive or negative for anti-nephrin antibodies whose complete clinical information where available; nongenetic SRNS, nongenetic steroid-resistant nephrotic syndrome; NT, not treated; Other, ramipril; SSNS, steroid-sensitive nephrotic syndrome.
[a]Pearson.
[b]Wilcoxon.

$P < 0.05$ vs. SSNS onset and SSNS relapse, respectively). Raw data of the enzyme-linked immunosorbent assay (ELISA) are shown in Supplementary Table S4.

Next, we established a cut-off value (173 RU/ml) for anti-NPHS1 positivity, generated on the most autoreactive subject identified in the control population (Supplementary Figure S1). Anti-NPHS1 antibodies exceeded the cut-off value in 65% of patients with SSNS at disease onset and 54% at relapse. Of the nongenetic SRNS patients, 30% tested positive for anti-NPHS1 presence. The distribution of anti-NPHS1 autoantibodies among the podocytopathies population is reported in Supplementary Figure S2. A longitudinal sample at remission was available for 8 patients. Among them, 25% tested positive for anti-NPHS1 antibody presence during relapse, with a 100% reduction rate when remission was achieved ($P < 0.05$ vs. SSNS Rel) (Figure 1c).

Anti-NPHS1 positive patients were characterized by increased proteinuria ($P < 0.01$) and decreased circulating IgG levels ($P < 0.05$) compared to antibody-negative subjects (Table 1). In patients with INS during active disease (proteinuria > 0.2 mg/mg), a positive correlation was observed between the levels of anti-NPHS1 antibodies and proteinuria (Rs $= 0.258$, $P < 0.05$). Furthermore, the anti-NPHS1 antibody positivity was associated with steroid sensitivity, measured by Pearson's' chi-square test (odds ratio $= 5.78$, 95% confidence interval: 1.42–23.4). No differences in the relapse rate comparing anti-NPHS1 positive and negative SSNS subjects at onset before treatment were observed, with 85% and 57% of individuals experiencing the event at the 12-month time point, respectively ($P = 0.298$) (Figure 1d).

DISCUSSION

This monocentric cross-sectional study evaluates the presence of anti-NPHS1 antibodies in a homogeneous population of children with INS, most of whom are of Caucasian ethnicity. Using an enzyme-linked immunosorbent assay, we found that 65% of patients with SSNS at disease onset and 54% at relapse have elevated levels of circulating autoantibodies against nephrin compared with healthy individuals and those diagnosed with genetic podocytopathies. The prevalence of anti-NPHS1 positive patients in our cohort is among the highest recently reported. Conversely, our data highlighted the absence of nephrin antibodies in a portion of proteinuric children with INS. These data show a partial divergence from a recently published study, that found an 80% prevalence of anti-NPHS1 antibodies in pediatric patients with INS with nephrotic range proteinuria (>2 mg/mg), reaching 90%, considering subjects without prior immunosuppressive treatment.[6] However, differences in the detection methods, such as the absence of an immunoprecipitation step, the use of a cutoff in our analysis, as well as the employment of different recombinant forms of NPHS1, may technically explain the differences in percentages of positivity reported in our study. These variations highlight the need for further studies analyzing the interactions responsible for nephrin recognition to standardize and optimize anti-NPHS1 autoantibody detection for clinical applications.

In our study, anti-NPHS1 titers in patients with SSNS significantly decreased at remission, confirming previous data generated from a heterogenous NS population with minimal change disease, mainly with adult-onset[5] and from a more homogeneous pediatric INS cohort.[6] The anti-NPHS1 titers directly correlated with proteinuria. Our data are

in contrast with a previous study, which reported no differences in proteinuria levels between anti-NPHS1 antibody positive and negative in an adult cohort.[7] The latest publication of Hengel *et al*.[6] also identified a direct correlation between anti-NPHS1 levels and albumin-to-creatinine concentration in urine. In our cohort, the levels of anti-NPHS1 IgG negatively correlated also with the global serum IgG levels. All these findings suggest a clear connection between anti-NPHS1 IgG production and retention in the serum and INS severity, classically defined by high proteinuria followed by hypogammaglobulinemia.

Our data corroborate previous studies showing the connection between anti-NPHS1 autoantibodies and INS. However, past studies were limited by their patient selection. Chebotareva *et al*.[9] analyzed anti-NPHS1 antibodies in a cohort of patients with adult-onset NS, whereas Watts *et al*.[5] used samples from the NEPTUNE study, including both adult- and children-onset, 97% of whom were already under immunosuppressive therapy preventing conclusions about anti-NPHS1 antibodies' role. In our study, elevated anti-NPHS1 antibody levels were detectable in over half of pediatric patients with SSNS during relapse in which 50% of the population received treatment. However, because of the small size cohort of patients and the heterogeneity in the treatment, conclusions about the role of immunosuppressive therapy on anti-NPHS1 antibody titer are still unclear. For this reason, a prospective study defining such correlation is mandatory for the future.

Here, we also described a 5.78-higher likelihood for subjects with SSNS to be anti-NPHS1 positive at relapse compared with children with SRNS. This study has some limitations, including the lack of a prospective evaluation of anti-NPHS1 levels at disease onset and during disease progression in SSNS. Moreover, the absence of patients with SRNS at onset does not allow assessment of the prognostic value of anti-NPHS1 antibodies in identifying children with SRNS at onset, before receiving steroid therapy.

Lastly, we detected variable background signals obtained from the different patient sera by enzyme-linked immunosorbent assay that could limit the experimental reproducibility of this assay. However, this highlights the importance of their removal to increase the assay's stringency as well as the specificity of the reported signals.

DISCLOSURE

All the other authors declared no competing interests.

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DATA AVAILABILITY STATEMENT

All the data to support the findings are included in the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Figure S1. Antinephrin antibody curve converting OD450 values (on the Y-axis) to relative units per ml (RU/ml, on the X-axis).

Figure S2. Flowchart showing the distribution of antinephrin autoantibodies among the podocytopathies population based on the cut-off value (173 RU/ml) established for anti-NPHS1 positivity.

Table S1. Clinical characteristics of the cohort of podocytopathies.

Table S2. Clinical and histological analysis of nongenetic steroid-resistant nephrotic syndrome.

Table S3. Histological and genetic details of patients with a genetic form of podocytopathies.

Table S4. Raw data of the ELISA showing the antibody signal and the negative serum background in each sample tested.

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