Cover Page

Official Title of the Study:

Biomarkers and outcome predictors of pediatric nephrotic syndrome: a genetic, transcriptomic and secretome multiomics study

Acronym:

PRECISE

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Fondazione IRCCS Ca' Granda
Ospedale Maggiore Policlinico
Sistema Socio Sanitario
Regione Lombardia
ERKNet The European Rare Kidney Disease Reference Network

Dipartimento Area Materno - Infatile
SC NEFROLOGIA E DIALISI PEDIATRICA – TRAPIANTI DI RENE–Direttore: Prof. Giovanni Montini
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Biomarkers and outcome predictors of pediatric nephrotic syndrome: a genetic, transcriptomic and secretome multiomics study

Acronimo: PRECISE

Promoter: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico,
Via Sforza 28, 20122 Milano, Italia

Coordinating Centre: Struttura SC Nefrologia e Dialisi Pediatrica – Trapianti di Rene
Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico,
Via della Commenda 9, 20122 Milano, Italia

Principal Investigator:
Prof. Giovanni Montini
Firma _____

Study design: biological non-pharmacological observational study**.**

Numero della versione: v.1.0

Data: 31/07/2024

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Index

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1. Schedule of assessments

A. Schedule of assessments: main population (incident INS)

	Enrolment (Onset)	6-weeks visit (End of metil-prednisolone pulses*)	6-months visit	12-months visit	First relapse
Informed consent	x				
Inclusion/exclusion criteria	x				
Demographic data	x				
Physiscal examination	x	x	x	x	x
Assessment of oedema	x	x		x	x
Medical history	x			x	
Vitals sign [1]	x	x	x	x	x
Clinical blood sample[2]	x	x	x	x	x
Clinical urine sample[3]	x	x	x	x	x
Blood for genetic analysis	x				
Blood for mononuclear cells analysis	x	x		x	x
Urine for molecular analysis	x	x	x	x	x
Serum for molecular analysis	x	x	x	x	x

[1] Height, weight, blood pressure

[2] BUN, sCr, albumin, Na, K, total cholesterol, IgG, triglycerides, (local laboratory) serum complement C3 (local laboratory, only at enrolment)

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[3] Urine protein/creatinine ratio, urinalysis (local laboratory)

* Only in patients not achieving remission at week 4

B. Schedule of assessments: control group

	Enrolment
Informed consent	x
Inclusion/exclusion criteria	x
Demographic data	x
Physiscal examination	x
Vitals sign [1]	x
Clinical blood sample[2]	x
Clinical urine sample[3]	x
Blood for PBMC extraction	x
Urine	x
Serum	x

[1] Height, weight, blood pressure

[2] BUN, sCr, albumin, Na, K, total cholesterol, triglycerides, IgG, IgM, IgA (at local site)

[3] urine protein/creatinine ratio, urinalysis (at local site)

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C. Schedule of assessments: validation cohort (prevalent INS)

	Enrolment (Relapse)	Remission
Informed consent	x	
Inclusion/exclusion criteria	x	
Demographic data	x	
Physiscal examination	x	x
Assessment of oedema	x	x
Medical history	x	
Vitals sign [1]	x	x
Clinical blood sample[2]	x	x
Clinical urine sample[3]	x	x
Blood for PBMC extraction	x	x
Urine	x	x
Serum	x	x

[1] Height, weight, blood pressure

[2] BUN, sCr, albumin, Na, K, total cholesterol, triglycerides, IgG, IgM, IgA (at local site)

[3] urine protein/creatinine ratio, urinalysis (at local site)

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2. Abbreviations

INS	Idiopathic nephrotic syndrome
PBMC	Peripheral blood mononuclear cells
SSNS	Steroid sensitive nephrotic syndrome
SRNS	Steroid resistant nephrotic syndrome
SDNS	Steroid-dependent nephrotic syndrome
FRNS	Frequent relapsing nephrotic syndrome
IRNS	Infrequent relapsing nephrotic syndrome
BUN	Blood Urea Nitrogen
Ig	Immunoglobulins
Th17	T helper 17
Treg	T regulatory
UMI	Unique Molecular Identifier counts
uPr	Proteinuria
C3	Serum complement 3
PBS	Phosphate Buffered Saline
2D-DIGE	two-dimensional difference gel electrophoresis
TEM	transmission Electron microscopy
LC-MS	Liquid chromatography-tandem mass spectrometry
RedCap	Research Electronic Data Capture
ANOVA	analysis of variance

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3. Responsability

The promoter of the study is the IRCCS Ca' Granda Ospedale Maggiore Policlinico Foundation. The coordinating center will be the SC Nephrology and Pediatric Dialysis – Kidney Transplants of the IRCCS Ca' Granda Ospedale Maggiore Policlinico Foundation. The role of Principal Investigator (PI) will be covered by Prof. Giovanni Montini, who will coordinate the clinical study and manage the consortium. Dr. William Morello will assume the role of Sub-investigator and will be directly responsible together with Prof. Giovanni Montini for patient enrollment.

Internal collaborations

Affiliation	*Name*	*Study Role*
IRCCS Ca' Granda Ospedale Maggiore Policlinico	Dr. William Morello	Sub-Investigator

External collaborations

Affiliation	*Department*	*Name*	*Study Role*
University of Milano	Department of Clinical Sciences and Community Health	Dr. Federica Collino	Biological sample processing, biochemical and coordination of single cell RNAseq experiments and single vesicles studies.
Heidelberg University Hospital	Division of Pediatric Nephrology, Center for Pediatrics and Adolescent Medicine	Prof. Franz Schaefer	Patients recruitment, biological sample processing, management of the consortium.

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Cologne University Hospital	Pediatric Department of Pediatrics, Division of Pediatric Nephrology and Center for Molecular Medicine	Dr. Max Liebau	Patients recruitment, biological sample processing, proteomics and phospho-proteomics and bioinformatic analysis.
Medical University Gdansk	Rare Diseases Centre and Department of Biology and Medical Genetics	Prof. Beata S. Lipska-Ziętkiewicz	Biological sample processing, exome sequencing analysis
University of Istanbul	Division of Pediatric Nephrology.	Prof. Alev Yilmaz	Patients recruitment, biological sample processing, epigenetics analysis
Vilnius University Faculty of Medicine	Clinic of Children's Diseases	Prof. Augustina Jankauskiene	Patients recruitment and bioinformatic analysis

External services

Name	*Type of service*
University of Torino, Prof. B. Bussolati	EV data analysis

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Eurofins, Italy	Novaseq 6000
MacroGen UE	Sample shipping and exome sequencing

4. Amendments

Not applicable.

5. Timelines

Stato dello studio	***Pianificazione***
Inizio della raccolta dati	30/10/2024
Fine della raccolta dati	30/10/2027
Report finale dello studio	30/03/2028

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6. Rationale and background

Idiopathic nephrotic syndrome (INS) is the most common glomerular disease in children, with an annual incidence of 2-7 cases per 100,000 children. INS is characterized by proteinuria, edema, hypoalbuminemia, hyperlipidemia, and thrombotic complications (1). The disease has an extremely variable clinical behavior, and the first-line treatment is based mainly on steroids and other immunosuppressive (IS) drugs. The response to steroids guides the actual classification. Approximately 80–90% of patients with nephrotic syndrome are initially steroid sensitive (SSNS), while 10–20% will not achieve remission despite second or third-line treatments and are defined as steroid resistant (SRNS) (2). The SSNS population exhibits an uncertain clinical course, characterized by various relapses and requiring immunosuppressive treatment, prolonged into adulthood in 1/3 of patients. These treatments have major side effects and come with an increased socio-economic burden as seen all over Europe.

There is no biomarker able to predict the INS clinical course (response to treatment and occurrence of relapses) and to tailor the treatment for most patients. INS, at least in the steroid-sensitive forms, shows an immune pathogenesis. Potential causative circulating factors have been described, however none has been unequivocally shown to be pathogenic. The recent identification of genetic risk loci associated with HLA Class II region among the most represented in SSNS patients (3) emphasizes the central role of adaptive immunity in the NS pathogenesis. From the cellular side, various experimental evidence implies that dysfunction in some populations of T lymphocytes may be related to the pathogenesis of the disease (4). Among these, a Th17/Treg imbalance has been described in patients with minimal change glomerulopathy (5). Furthermore, the remission after the use of Rituximab, a monoclonal antibody against the CD20 surface antigen, suggests that B cells may also be involved in the pathophysiology of NS (6). The role of autoantibodies as circulating permeability factors able to directly damage the glomerular barrier, leading to proteinuria in INS, has been recently explored. Antibodies against Annexin A2, actin and ATP-synthase, ubiquitin-C-terminal hydrolase L1, and CD40 antibodies were detected across both pediatric and adult INS patients in a variable percentage of INS patients highlighting the complexity of this pathology. Recently, the presence of anti-nephrin antibodies in post-transplant recurrent focal segmental glomerulosclerosis patients (7), as well as in the blood and kidney biopsies of patients with minimal change disease (8).

As shown, INS displays a multifactorial and complex pathogenesis, where immunological, genetic, and epigenetic factors are involved. There is therefore an urgent medical need to find early biomarkers to allow for a precise selection and duration of the treatment, avoiding unnecessary severe side effects, and to characterize sub-cohorts for clinical studies. Biofluid secretome can harbor promising disease biomarkers (9). These biomarkers will also favor the understanding of the pathogenesis, allowing for an alternative classification and stratification of the patients.

The PRECISE proposal aims to address unmet needs in INS children, searching for biomarkers/indicators/predictors of this rare kidney primary disease. In this context, we hypothesize

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that biomarkers obtained from liquid biopsies (blood and urine) will improve the stratification and prognosis of INS patients, allowing for accurate tailoring of treatments. As the pathogenesis of the disease is multifactorial, an integrated cellular and secretome multi-omics approach will allow for the identification of biomarkers across multiple levels, to be then validated in the routine clinical setting.

7. Objectives

The **PRIMARY OBJECTIVE of the PRECISE** study is the generation of a predictive model to differentiate children with SRNS or SDNS from patients with IRNS at the onset through the integration of -omics, genetic, and clinical data (biomarker signature).

The following **4 SECONDARY OBJECTIVES** will be pursued in the study:

1. Definition of the genetic/epigenetic modifications participating in the pathogenesis of INS.
2. Evaluation of adaptive immune system alterations in INS children.
3. Identification of specific protein patterns in serum and urine in different INS subtypes.
4. Description of the molecular characteristics in healthy children.

8. Methods

8.1 Study design

Open, multicenter, non-pharmacological observational study.

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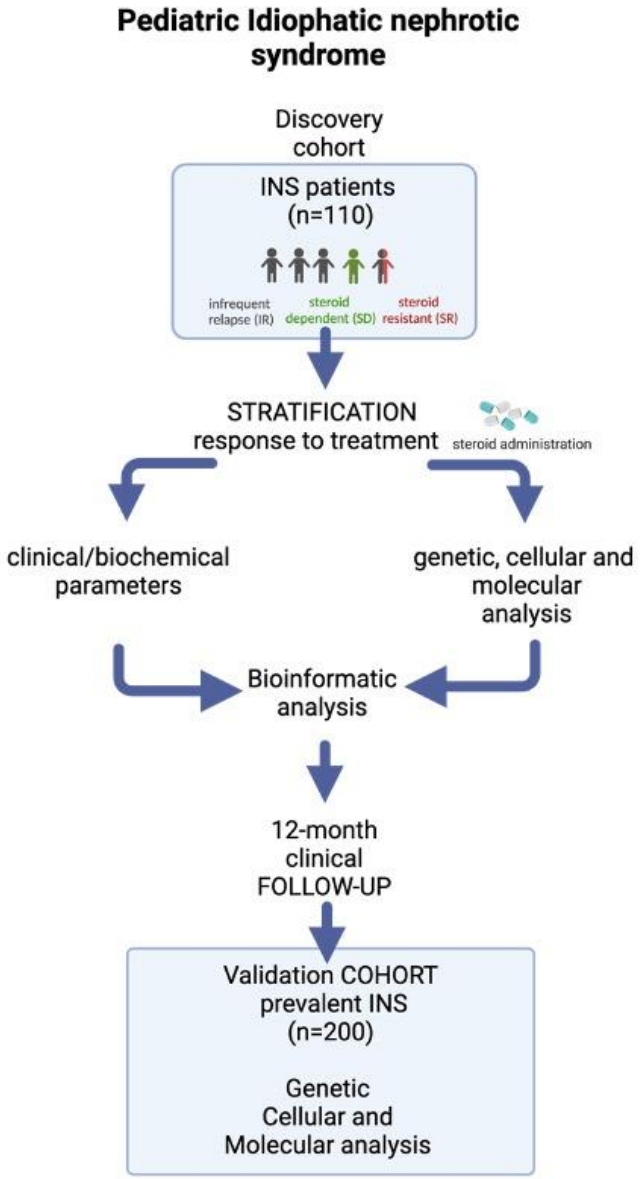

The PRECISE study is an open, multicenter, non-pharmacological biological study. The study will recruit a total of 310 INS patients and 40 controls. In details, the study will recruit 110 treatment-naive childhood INS patients as a discover cohort, who will be followed for 12 months. At enrolment urine and blood samples will be collected as for normal clinical practice for the planned analysis. At the 12-month follow-up, patients will be classified according to international guidelines as steroid-dependent (SDNS)/frequent relapsing (FRNS) and infrequent relapsing nephrotic

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email: nefrologiapediatrica@policlinico.mi.it

syndrome (IRNS) (10). Additional assessments and biological samples will be performed according to the study flowchart and as described in the study procedure chapter. A homogeneous control group (n=40) by age of children undergoing sampling for minor urological surgical correction procedures will be also enrolled. In the validation phase, biological samples from an independent group of prevalent INS (200 cases) cases will be collected.

8.2. Primary endpoint

To describe different molecular signatures (characteristics), predictive of the progression of INS, which would be able to discriminate among SRNS/SDNS and IRNS groups, based on genetic/epigenetic background, immune status, and vesicular proteome/transcriptomic profile.

8.3. Secondary endpoints

Related to secondary objective 1:

1- Genetic kidney- and immune-related risk factors measured as number of variants in kidney- and immune-related genes, associated to INS incidence, progression, and poor response to therapy.

2- A methylation profile of target genes measured as number of hypomethylated and hypermethylated immune-related gene regions, related to a faster progression to kidney failure and altered immune profile.

Related to secondary objective 2:

3- Difference in terms of % and absolute number of subpopulations of B cells and T cells among patients classified as SRNS/SDNS, IRNS, and healthy children.

4- Changes in immune cell subsets genes, expressed as the number of expressed genes per cell (log-transformed Unique Molecular Identifier counts, UMI), among patients classified as SRNS/SDNS, IRNS, and healthy children.

5- Signaling dynamics changes, measured as number of phosphosites for each target protein expressed in the subpopulation of primary B and T cells, implicated in the development of kidney damage in INS.

***Related to secondary objective 3**:*

6- Difference in the urine EV concentration expressed as total number/urine creatinine levels, and size as mean diameter, among patients classified as SRNS/SDNS, IRNS, and healthy children.

7- Difference in the urine and serum EV surface proteome phenotype, in terms of the ratio of the average of label-free quantification intensities for each detected EV protein, among patients classified as SRNS/SDNS, IRNS, and healthy children.

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8- Difference in the serum proteome, in terms of the ratio of average of label-free quantification intensities for each detected protein, among patients classified as SRNS/SDNS, IRNS, and healthy children.

***Related to secondary objective 4**:*

9- To describe different molecular signatures (characteristics) for healthy children group in the same way done for the primary endpoint.

9. Enrollment and inclusion/exclusion criteria

The recruiting centers that will be involved in the study are listed in Appendix 1.

9.1 STUDY POPULATION (incident patients)

Inclusion criteria

1. Clinical diagnosis of idiopathic nephrotic syndrome (nephrotic range proteinuria uPr/uCr ratio> 2mg/mg, hypoalbuminemia < 3.0 g/dl, oedema);
2. No previous treatment for INS
3. age between 1 and 18 years at onset
4. signed informed consensus

Exclusion criteria

1. Congenital or infantile nephrotic syndrome (age < 1 year old); secondary nephrotic syndrome; glomerulonephritis; autoimmune diseases; vasculitis.
2. Absence of signed informed consensus
3. previous treatments with prednisone or prednisolone for INS

9.2 CONTROL GROUP

Inclusion criteria

1. Clinical diagnosis of minor congenital abnormalities of kidney and urinary tract
2. Absence of proteinuria (uPr/uCr < 0.2 mg/mg)
3. age below 18 years
4. signed informed consensus

Exclusion criteria

1. Immune-mediated diseases not associated with proteinuria (glomerulonephrities)
2. age > 18 Years

9.3 VALIDATION COHORT

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Inclusion criteria

1. Previous clinical diagnosis of idiopathic nephrotic syndrome (nephrotic range proteinuria uPr/uCr ratio> 2mg/mg, hypoalbuminemia < 3.0 g/dl, oedema);
2. age between 1 and 18 years at onset
3. signed informed consensus

Exclusion criteria

1. Congenital or infantile nephrotic syndrome (age < 1 year old); secondary nephrotic syndrome; glomerulonephritis; autoimmune diseases; vasculitis.
2. Absence of signed informed consensus

10. Study procedure

10.1 Study visits

Population 1 (main population_incident INS):

At the enrollment visit, prior informed consent the following assessments will be performed: physical examination, assessment of oedema, measurement of vital signs, blood and urine samples collection (see A. Schedule of assessments for more details). Afterwards, patients will be assessed at 6 weeks, 6 months and 12 months according to A. Following the international guidelines, at the 12 months visit patients will be clinically classified as SRNS, SDNS, or IRNS according to their medical history. Schedule of assessments: main population (incident INS).

Patient will come back to the center in case of relapse (see definitions) to perform the 'first relapse visit' according to A. Schedule of assessments: main population (incident INS).

Population 2 (control group):

This population will perform after informed consent only the enrollment visit, according to B. Schedule of assessments: control group.

Population 3 (validation cohort):

Patient will be enrolled after informed consent at relapse (enrolment visit). During this visit, a physical examination, assessment of oedema, measurement of vital signs and collection of blood and urine samples will be performed according to C. Schedule of assessments: validation cohort. At the remission (see definitions), patients will come back to the center to perform the remission visit according to C. Schedule of assessments: validation cohort.

10.2 Informed Consent and inclusion/exclusion criteria

The Investigator or designee will ensure that each subject and/or subject's parent/legal guardian is given full and adequate oral and written information about the nature, purpose, possible risks, and

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benefits of the study. Subjects/parents/legal guardians will be notified that they are free to discontinue participation in the study at any time and will be given the opportunity to ask questions and allowed time to consider the information provided. After informed consent and assent (as appropriate) is obtained, subjects will undergo enrollment assessments. Once it is determined that they are eligible for the study, they will be enrolled in the study and baseline assessments will be completed. A copy of the signed ICFs will be provided to the subject and/or subject's parent/legal guardian.

10.3 Demographic data

Demographic data (age, sex, and ethnicity, appearance age) and response to therapy will be collected for all participants during the enrollment visit.

10.4 Medical history

Medical history will be recorded at enrollment visit. Investigators should document the occurrence, signs, and symptoms of the participant's preexisting conditions, including all prior significant illnesses. A detailed medical history about disease indication will be requested and will include prior surgeries, renal biopsies, hospitalizations, dialysis, transplantation, laboratory data, and extrarenal manifestations of the disease. Available disease medical history for the 3 years preceding screening will be requested.

10.5 Physical examination

A physical examination, performed by the investigator or designee, will include, at a minimum, assessment of the following: general, head, ears, eyes, nose, throat, heart, chest, lungs, abdomen, skin, extremities, back/neck, musculoskeletal, and lymph nodes. Additional symptom-driven physical examinations may be performed at any time, as deemed necessary by the investigator. See the schedule of assessments for the details regarding at which visit physical examination will be performed.

10.6 Assessment of oedema

Edema will be assessed at enrollment, 6-week visit, 12-month visit and at the first relapse. Assess for peripheral edema by pressing the area firmly with the thumb or index finger for 5 seconds and then release.

10.7 Vital signs

Vital signs including height, weight and systolic and diastolic blood pressure measurements will be evaluated at the visits indicated in the schedule of assessments.

10.8 Routine laboratory tests (local laboratory)

Blood samples. Blood samples for clinical assessments will be collected at the visits specified in the

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SC NEFROLOGIA E DIALISI PEDIATRICA – TRAPIANTI DI RENE–Direttore: Prof. Giovanni Montini
Tel. 0255032883
email: nefrologiapediatrica@policlinico.mi.it

schedule of assessments. The following analytes will be measured: BUN, sCr, albumin, Na, K, total cholesterol, triglycerides, IgG, IgM, IgA and serum complement 3 (C3). These exams will be conducted at each recruiting site.

Urine samples. Urine for clinical assessments will be collected at the visits specified in the schedule of assessments. The following analytes will be measured: urine protein/creatinine ratio, and urinalysis. These exams will be conducted at each recruiting site.

10.9 Sample collection for molecular analysis

The following samples are needed:

1. Blood sampling: for INS patients and controls, two additional aliquots of blood (a maximum of 10-12 mL at a single time; body weight ＞12 kg) based on the patient's age and clinical condition during the sampling will be collected in the occasion of routine laboratory tests.
2. Urine sampling: spot urines (20-30 mL) from residual urine from routine laboratory tests.

Detailed sample processing:

- ***Whole blood.*** Blood collection for genetic and epigenetic will be performed for all NS patients recruited into the study. Whole blood will be collected and stored at 4°C for analysis. Shipment of samples will be done at 4°C.
- ***Mononuclear cells from peripheral blood.*** Whole blood will be isolated using the BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube. Peripheral blood mononuclear cells (PBMCs) will be isolated by Ficoll procedure followed by washing step with PBS, and freezed in Cell Storage Media at density of 10^6 cells/vial and stored in liquid nitrogen or -80°C. Shipment of samples will be done by dry ice.
- ***Urine.*** Urine samples will be centrifuged at 3,000 g for 15 minutes to remove large membrane fragments and other debris. A protease inhibitor cocktail will be added immediately (PI inhibitor). Urine aliquots will be stored at -80°C. Shipment of samples will be done by dry ice.
- ***Serum.*** The serum will be obtained by direct cascade activation of coagulation from whole blood. The blood will then be centrifuged at 3,000g for 20 minutes to remove corpuscular components along with clots and debris. Serum aliquots will be stored at -80°C. Shipment of samples will be done by dry ice.

Urine and serum will be centralised at Heidelberg University Hospital. The storage of peripheral blood mononuclear cells (PBMCs) will be centralized at the coordinating centre.

Any residues of biological material that will not be used for the study will be eliminated at the end

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of the study.

11. Detailed technical methodologies

Exome sequencing (collaborator involved: Medical University Gdansk). DNA samples extracted from blood leukocytes will be subject to sequencing using TruSeqExome Enrichment Kits according to the manufacturer's instructions (Illumina). Data analysis will be carried out as previously described (11).

Whole-Genome Bisulfite Sequencing Method (collaborator involved: University of Istanbul). For epigenetics analysis on CD45 lymphocytes, 5 µg of genomic DNA (OD260/280: 1.8–2.0) will be used as starting material. Bisulfite treatment of genomic DNA will be performed, and non-methylated cytosine nucleotides will be converted to uracil and read as thymine (T) when sequenced. Methylated cytosines protected from conversion are still read as cytosine. The EpiGnome libraries will be diluted and loaded onto the cBot DNA Cluster Generation System. After cluster generation, the flow cell will be transferred to the HiSeq 2500 System for sequencing using 75 bp paired-end reads. The HiSeq 2500 generates approximately 500 Gb of sequence data per flow cell or about 62 Gb per lane allowing a coverage of approximately 120 Gb of data per sample. Additional sequencing can be completed for higher coverage. Data analysis of whole genome bisulfite sequencing will be conducted. FastQC tool will be used to check the raw data quality. Alignment will be done with the Bismark tool to identify the methylated regions (methylation calling). Statistical analyses will be performed with a methyl Kit.

Cytofluorimetric analysis (collaborator involved: the University of Milano). Purified PBMCs will be discriminated by incubation with the dead cell Stain Buffer (BD) and surface staining will be then performed at 4°C for 20 min. Cells will be then fixed and permeabilized, stained at room temperature for 20 min with antibodies against intracellular antigens. For cell activation and intracellular cytokines staining, PBMCs will be stimulated with PMA/Ionomycin (1 µg/mL) for 2 hours. Brefeldin A (10 ng/mL) will be added as a protein transport inhibitor. Cells will be stained with Fixable Viability Stain 780 (BD) followed by cell-surface marker (BD Pharmingen) and analyzed with the BD FACS Symphony flow cytometer.

Single cell RNAseq experiments combined with CITE-seq (collaborators involved: IRCCS Ca' Granda Ospedale Maggiore Policlinico and the University of Milano). CD3+ and CD19+ cells from peripheral blood will be sorted from purified PBMCs. CD3+ cells will be labeled using barcoded versions of anti-CD4 and anti-CD8 (TotalSeqC – _Biolegend). After cell number and viability determination by automated cell count, the appropriate volume of CD3+ and CD19+ cells will be loaded onto a 10x Genomics chip to recover 10.000 cells/sample. RNA-Seq and single-cell repertoire analyses will be performed according to 10x Genomics 5' V(D)J protocol. Library sequencing will be performed using the Illumina NovaSeq 6.000 (Eurofins facilities, https://eurofinsgenomics.eu/en/custom-dna-sequencing/).

scRNA-Seq data analyses (collaborators involved: IRCCS Ca' Granda Ospedale Maggiore

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Policlinico and the University of Milano). Raw fastQ files will be aligned to the human reference genome GRCh38, subjected to quality control filtering, and data normalization high variable genes will be identified and used in a principal component analysis (PCA). Downstream analyses of scRNAseq data on different unsupervised algorithms (UMAP, tSNE) will allow the spatial visualization of the single-cell dataset and features. T- and B- -cell clusters will be defined using the Leiden algorithm, and differentially expressed genes (DEGs) between distinct cell clusters and experimental groups will be identified (P value ≤0.05, log2 fold change ≥1.5). The procedure will generate an enrichment in immune cell subsets with a specific molecular signature that will be analyzed bioinformatically. Gene Ontology (GO) analyses will also be performed. For scTCR-seq and scBCR-seq data analysis, CDR3 sequences and V(D)J gene rearrangement will be obtained using the Cell Ranger VDJ 10X pipeline. T/B cells showing consistent CDR3 amino acid sequence will be defined as clonotypes.

EV isolation and characterization (collaborator involved: the University of Milano). Purification of EVs will be obtained by size exclusion chromatography using 70 nm qEV columns (IZON) after a 100 kDa centrifugal filter (Sartorius). The concentration and size distribution of EVs will be measured using NanoSight LS300 (NanoSight Ltd). EVs integrity will be characterized by transmission Electron microscopy (TEM) by adherence to glow-discharged formvar-coated copper grids 300 mesh.

Single vesicles studies (collaborator involved: the University of Milano). EV evaluation at single levels will be performed following the pipeline described by (12). Super-resolution microscopy will be performed using the Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging, Oxford, UK) equipped with a 100X. Tetraspanins and selective markers will be tested using the Apex Antibody Labelling Kit (Invitrogen) according to the manufacturer's protocol. Samples will be also analyzed with ExoView Plasma Tetraspanin kit on an ExoView R100 instrument (NanoView Biosciences) following the manufacturer's instructions.

EV protein corona and lipid evaluation (collaborator involved: the University of Milano). EVs surface phenotype will be analyzed using the MACSplex kit (Miltenyi Biotec) on a BD FACSCanto II (Becton Dickinson) as (12). Proteomic studies will be then performed by two-dimensional difference gel electrophoresis (2D-DIGE) followed by liquid chromatography-tandem mass spectrometry (LC-MS) as (13). Surface proteins will be separated from the ones from the lumen. Lipidomic profile will be also evaluated by gas chromatography (GC)-MS.

Proteomics and phospho-proteomics (collaborator involved: Cologne University Hospital). For expression profiling and characterization of cellular phosphor-proteomes, a fully established standard pipeline at the Cellular Stress Responses in Aging-Associated Diseases and the Centre for Molecular Medicine Cologne will be used. Proteins are purified, alkylated, and digested. Peptides are purified after acidification by a Stage-Tip protocol using an SDB-RPS-Matrix (Styrene Divinyl Benzene reverse phase sulfonate-Matrix and measured in a Q Exactive Plus Tandem or a LTQ Orbitrap XL spectrometer. Raw data is processed using the MaxQuant software, is compared to reference datasets, and is analyzed and displayed using Perseus software. To obtain insights into the

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activation of specific signaling cascades by posttranslational modification (phosphorylation) we will also characterize the phospho-proteome. Phosphorylated peptides are enriched after standard isolation and purification with a special kit (High-Select Fe-NTA Phospho-peptide Enrichment Kit). For an unbiased serum proteomics approach, we can rely on the established fully automated clinical pipeline using liquid handling robots and state-of-the-art mass spectrometry platforms, including the latest generation Orbitrap Exploris 480. The approach enables the detection and quantification of on average 300 plasma/serum proteins per hour using a data-independent acquisition approach from a 5 µl sample volume.

12. Source Documents

Medical history and all clinical data collected during the study will be collected from each visit's medical records.
Local laboratory records will be used to collect laboratory analysis results.

13. Sample Size

As this is a descriptive and explorative study without inference in the primary endpoint, and given the incidence of childhood idiopathic nephrotic syndrome, which is considered a rare disease, the number of recruiting centers, we foresee enrolling in this study 110 treatment-naive INS children. A homogeneous age control group of healthy children undergoing minor urological surgical corrections will be enrolled as a control group (n=40 children in 3 years). These numbers are the maximum we can recruit, but at the same time, we think that this study could serve as an important basis for appropriate studies in the future.

14. Data Management

A consortium was created under the direction of the Lead Unit (IRCCS Ca Granda of Milano, Prof. G. Montini, Proposal Coordinator) with the Partners involved in the study. At the enrollment visit, a unique code will be assigned to each participant. The de-identification of the data will take place in such a way that people who access the database will not be able to trace the identity of the subjects in any way. Only local investigators will be able to trace the identity of the enrolled subjects.

The data necessary for the study will be recorded in a specific eCRF in a Data Management System validated according to national legislation, provided by the Scientific Management of the Foundation. The platform used will be RedCap (Research Electronic Data Capture).

The REDCap Consortium is made up of >1000 institutional partners around the world (research institutions, universities, ministries, etc.). The consortium supports a secure web application (REDCap) designed exclusively to support data capture for research studies. The REDCap application allows users to create and manage online databases quickly and securely and is currently in use for more than 110,000 projects with approximately 150,000 users covering numerous areas of research interest across the consortium.

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Through REDCap, for this study the following will be implemented: a) identification at user level, with specific restrictions based on role in the study b) validation and control of data integrity in real time c) de-identification of patients before data export d) centralized data storage with daily backup, a secure server within the Foundation's IT structure.

15. Statistical analysis

15.1 Primary endpoints: analysis

Statistical analysis using linear mixed effect models will ensure that data from the same individuals over time are properly considered. Network-based analysis using a linear mixed effect model LASSO implementation will allow us to identify key features associated with various clinical endpoints. To identify subsets of features, across all OMICs, associated with disease progression and treatment response, we will use a genetic algorithm-based feature selection approach. Unlike LASSO, this approach can identify non-linear relationships between features and can be linked to almost any predictive modeling approach. The feature subsets will further be evaluated in already available public domain data and then applied to a much larger cohort within the PRECISE consortium.

15.2 Secondary endpoints: analysis

Differences between two groups of patients for all the analysis conducted will be evaluated using Student's parametric T-test (in case of normality of variables distribution and homoscedasticity of variance) or Mann – Whitney's non-parametric test. The normality of the data will be evaluated by the Shapiro-Wilk test and the homoscedasticity assumption of the variance will be evaluated by the Hartley F-max test. In cases where more features are available than samples, regularized regression approaches, such as LASSO, or feature selection approaches will be used to identify potential combinations of features associated with a given outcome. Analyses of differences in continuous outcome measures between 3 or more levels of categorical features will take place through analysis of variance (ANOVA) using multiplicity-adjusted post-hoc tests by Dunnett or Tukey, as appropriate. Data analysis will be performed using GraphPad Prism software and P-values of 0.05 will be accepted.

16. Quality control

Only after the approval from ethics committee and regulatory authorities, centers will enroll patients who meet inclusion and exclusion criteria and will propose them to participate to the study signing the informed consent. The coordinating center will have access to all registered data to verify any anomalies and report them to the participating center. All data will be handled, registered, and reported in accordance with the good clinical practice. As Promoter, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico can carry out a quality check on the study at its discretion. In this case, the investigator should allow the monitor to directly access all relevant documentation and devote some of his or her staff time to the reviewer to discuss the results of the monitoring and any other aspects of the study. In addition, the regulatory authorities can carry out inspections. In

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this case, the investigator must authorize the inspector direct access to all relevant documentation and devote part of his time and his staff to the inspector himself to discuss the results of the monitoring and any other aspects of the study.

17. Study limitation

This study is a pilot study and shows some risks associated with it. The following limitations can be highlighted:

1. Due to the nature of the pathology (rare disease), one of the risks could be not reaching the number of recruited patients (annual incidence of 2-7/100,000 children). Our contingency plan will be the integration of additional ERKnet and ESCAPE Network centers through an addendum to the protocol.
2. The other limitation could be the absence of sample stability. Our contingency plan will be to define specific isolation workflow protocols, centralize the biobanking system, and perform sample stability tests for the different applications.
3. Another problem could be a difficult blood draw based on the age of the new-onset children recruited in the study. We will exclude Infantile N from the study to overcome this problem.

From the statistical side, all the data will be extensively scrutinized to ensure that misreporting is minimized. Potential confounders will be identified and assessed routinely throughout the analysis. Where possible, confounding factors will be adjusted for in the analysis. Where < 10% of missing data are present in a variable we will aim to impute missing data from other available data sources using a random Forest regression or classification approach. Variables with >20% missing values within a group will be kept aside and used only for descriptive quantitative evaluations. Statistical analysis using linear mixed effect models will ensure that data from the same individuals over time are properly considered.

18. Other aspects

The study will be conducted by the Standards of Good Clinical Practice, the ethical principles deriving from the Declaration of Helsinki.

The study and the related documentation will be presented to the competent Ethics Committee of each center. The study will begin only after receiving the authorizations requested according to the institution's internal procedures.

The Ethics Committee must also approve any changes to the protocol and advertising used to recruit subjects for the study, according to local regulations.

18.1 Informative note to subjects and consensus module to personal data treatment.

The investigators have the responsibility to obtain a signed informed consent by parents or legal

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guardians before collecting data. Signed documents must be stored by the investigators and a copy must be given to them. Parents, legal guardians or patients (if = 18 years old) will have all the time necessary for the evaluation of the information received before providing their informed consent to the use of sensitive data of minors. The investigator will have to obtain spontaneous informed consent from them before using it in any way for the study. The written consent to the handling of sensitive data must be subscribed by the date and signature of parents, legal guardians or patients (if = 18 years old) and by the investigator or his representative. The investigator has to give to them a signed copy of informed consent; the original form will be retained with the other documents of the study protocol; the module for the acquisition of informed consent to the treatment of sensitive data will be attached to the clinic folder.

The Principal Investigator guarantees that the data of the patients enrolled during the study will be kept, archived, and processed in full compliance with the regulations on privacy under art. 13 of Regulation No. 2016/679/EU and the national privacy legislation in force, the Code of Ethics regarding data processing for statistical-scientific purposes, and the "Guidelines for the processing of personal data in the context of clinical trials of medical products" published in the Gazzetta Ufficiale n. 190 of 14 August 2008.

Patient data will be accessible only to internal and external personnel specifically appointed and bound by an obligation of secrecy in relation to any information learned during the study.

The Principal Investigator also guarantees that he has implemented the minimum security measures required by the aforementioned legislation for data processing using electronic and non-electronic tools, in order to avoid illicit data processing.

18.2 Insurance

Considering the observational nature of the study, extra insurance is not mandatory in addition to those already provided in normal good clinical practice.

19. Disclosure plan and communication of study results

The Principal Investigator of the study will undertake to draft a final report and make the results public at the end of the study. The data will be made public anonymously and presented as required in the aggregate model.

20. Publication and Intellectual property

Foundation as Promoter will guarantee the dissemination and publication of the study results, even in the event of negative results, without any constraints and guarantee the collaborating center visibility proportional to actual participation. Each scientific publication containing the results and data of the study must indicate the role and participation of the Centers and the Foundation, in a

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manner proportional to the actual contribution made in the study and the role played by each party. The data will be published in aggregate form or in any case anonymized, so as not to allow in any way the identification of the interested party to whom the data refers.

The Parties recognize that for the conduct of the collaboration within the scope of the study, data, information, know-how, inventions (patentable or not) owned by each party may be used and shared, which remains the exclusive owner even if it grants them to the other a right of access and use, non-exclusive and free, for the sole purpose of carrying out the activities covered by the study and limited to its duration. It is understood that this right of use does not include the right to sublicense to third parties. In compliance with current legislation, the data and results generated within the Study will be the property of the Sponsor, unless specific agreements are made between the Sponsor and the Centres.

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Appendix 1. List of recruiting centers.