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UNDERLYING MECHANISMS OF
IDIOPATHIC NEPHROTIC SYNDROME IN CHILDREN:
EVIDENCE OF A CIRCULATING PERMEABILITY FACTOR

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CHAPTER 1. INTRODUCTION

1. NEPHROTIC SYNDROME IN CHILDREN

1.1 DEFINITIONS AND CLASSIFICATION

Nephrotic syndrome (NS) is the commonest form of glomerular disease in children, defined by the presence of [1,2] :

- Heavy proteinuria: ≥40 mg/sqm/hr, or urine protein/creatinine ratio >2 mg/mg

- Hypoalbuminaemia: ≤25 g/L

- Oedema

The central abnormality in all cases of NS is massive proteinuria, due to a structural and functional defect of the glomerular filtration barrier (GFB). Nephrotic syndrome can be primary or secondary in nature[1,2] . Primary NS occurs in the absence of systemic disease and constitutes the majority of childhood NS. It represents around 95% of cases in children. In most of them the disease can be considered idiopathic, while in few cases, usually refractory to therapy, an underlying mutation in genes encoding podocyte associated proteins, resulting in structural or functional disruption of the GFB can be identified. NS is usually a genetic disease when it presents within the first three months of life, and it is defined as congenital nephrotic syndrome (CNS)[3] . Secondary NS, in which a systemic disease

processes causes renal injury, occurs very rarely in children and is attributable to various aetiologies, including, but not limited to, infections (malaria, HIV, schistosomiasis, hepatitis B and C), malignancies (leukaemia and lymphoma), toxins, Henoch-Schönlein purpura, sickle cell disease and systemic lupus erythematosus[1].

Classically, idiopathic NS in children has been further described, based on its histopathological findings on renal biopsy, as minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), mesangio-proliferative glomerulonephritis (MePGN), membrano-proliferative glomerulonephritis (MPGN), membranous glomerulonephritis (MGN), and focal and global glomerulosclerosis (FGS). MCD, followed by FSGS, are the types seen most commonly [4]. However, in recent times, there is a growing consensus that MCD and FSGS are part of a spectrum of disease, where the earlier stage is represented by MCD and is more responsive to treatment, while, FSGS corresponds to an advanced stage of disease progression that is less likely to respond to treatment [5].

Although there are various ways of classifying idiopathic childhood NS, the most widely used classification is based on disease response to corticosteroid therapy. Steroid responsiveness provides the best guide to therapy and is the chief determinant of the clinical course and likely outcomes in children presenting with NS [1,3]. Depending on the response to steroid therapy, patients can be described as steroid sensitive, steroid resistant, steroid dependent or frequently relapsing[1] . Patients who enter

remission within 4 weeks of commencing steroids are referred to as having steroid sensitive nephrotic syndrome (SSNS), whereas those who fail to achieve complete remission despite 8 weeks of corticosteroid therapy are said to have steroid resistant nephrotic syndrome (SRNS), where complete remission is characterised by a marked reduction in proteinuria (uPCR <0.2 mg/mg or <1+ on urine dipstick) for 3 consecutive days. Some patients are initially classified as SSNS, but their subsequent course of illness varies and further differentiates them into steroid dependent and frequently relapsing. Patients who develop a relapse either while still receiving steroids, or within 2 weeks of discontinuation of treatment following a steroid taper, are said to be steroid dependent (SDNS), while frequently relapsing nephrotic syndrome (FRNS) is characterised by more than two relapses within the first 6 months or at least four relapses within a year (Table 1)[1] .

	Definition
Nephrotic syndrome	Oedema, uPcr ≥2 mg/mg or 3+ protein on urine dipstick, hypoalbuminaemia ≤25 g/l
Complete remission	uPcr <0.2 mg/mg or <1+ on urine dip-stick for 3 consecutive days
Initial responder (SSNS)	Attainment of complete remission within initial 4 weeks of corticosteroid therapy
Initial resistance (SRNS)	Failure to achieve complete remission after 8 weeks of corticosteroid therapy
Relapse	uPcr ≥2 mg/mg or ≥3+ protein on urine dipstick for 3 consecutive days
Frequent relapse	>2 relapses within 6 months of initial relapse, or ≥4 relapses in any 12-month period
Steroid dependence	two consecutive relapses during corticosteroid therapy or within 14 days of ceasing therapy

Table 1. Definitions of nephrotic syndrome in children[1]

1.2   EPIDEMIOLOGY

The annual incidence of nephrotic syndrome is reported as 2-7 cases per 100,000 children, with the cumulative prevalence being nearly 16 per 100,000 children[1,2]. 90% of the NS cases have an age of onset below 7 year of age, with the average age range of 3-4 years[2]. In childhood, the disease has a male preponderance, with the male to female ratio of nearly 2:1, however, this gender disparity fades by adolescence, whereupon, the incidence of NS in males and females becomes equal. Although the

statistic pertaining to the incidence of NS has remained relatively stable over the past 2 decades, there is evidence from some retrospective and cross-sectional studies demonstrating that geographical location and ethnic background cause considerable variations in the incidence [3,6]. European literature indicates a higher incidence of NS among South Asians than among their European counterparts[7] and historical data from USA studies shows that African-American children have a higher incidence of NS as compared to children of European descent [8,9].

1.3   CLINICAL PRESENTATION

The characteristic presenting symptom in NS is oedema, which is an abnormal accumulation (>3% of child's body weight) of fluid in the interstitial compartment. It is typically soft and pitting, and initially appears in a periorbital distribution, before spreading to dependent areas such as the lips, scrotum, lower back and lower extremity [1,10]. NS patients have an excess of total body sodium, and two hypotheses have been proposed for the development of oedema in NS: the underfill and the overfill hypotheses[11]. The underfill hypothesis proposes that excessive urinary protein loss leads to hypoalbuminaemia, causing fluid shift from intravascular compartment into interstitial space due to low oncotic pressure. The homeostatic response to hypovolaemia then activates the renin-angiotensin-aldosterone system (RAAS) and secondary sodium

retention occurs[12]. While this theory explains the commonly observed clinical picture of hypotension, oliguria, and tachycardia, not all clinical circumstances fit this hypothesis, and it fails to explain why albumin replacement alone is often insufficient to stimulate diuresis without the addition of a diuretic[13]. The alternative overfill hypothesis postulates that an intrinsic defect enhances sodium and water reabsorption at the distal tubules, causing intravascular volume expansion. It should be noted that both hypotheses are not mutually exclusive, and that there is probably some overlap between the two. The volume status of a child may depend on the stage of disease when a child is being evaluated. Establishing whether a child is overfilled or underfilled can be clinically important because the management of oedema may be different for both volume-expanded and volume-contracted patients. One suggested method to distinguish between the two patients involves measuring the fractional excretion of sodium (FENa) and the relative urinary potassium excretion [UK/(UK + UNa)], where patients with a low FENa (<1%) and high urinary potassium excretion (>60%) would be expected to have a low intravascular volume.

Hyperlipidaemia is a hallmark of NS. It occurs together with changes in the composition of serum lipoproteins. Various underlying mechanisms have been described, such as the increased synthesis of cholesterol, triglycerides and lipoproteins in the liver as a compensatory response to reduced serum oncotic pressure. The urinary excretion of an unknown regulatory protein could be involved in the down- and up-regulation of

different enzymes required for lipid metabolism and of lipoprotein receptors described in NS, including but not limited to lecithin-cholesteryl acyltransferase (LCAT) deficiency, downregulation of LDL and VLDL receptors, cholesterol ester transfer protein (CETP), and acetyl-Coenzyme A acetyltransferase (ACAT)-2[14].

1.4   INITIAL INVESTIGATIONS AND MANAGEMENT

Evaluation of NS at first presentation primarily consists of the following baseline investigations:

(i)   urinalysis and urine microscopy.

(ii)   serum electrolytes, albumin, renal function, complete blood count and cholesterol.

(iii)   quantified protein:creatinine ratio (uPCR) on a spot sample or on 24 h collection.

While the gold-standard quantitative measurement of proteinuria involves urine collection over 24 hours so that variations in protein excretion due to circadian rhythm, posture and physical activity are accounted for, it can often be difficult to perform this test in children,

hence, uPCR using a random urine sample is used as a surrogate, where uPCR > 2 mg protein/mg creatinine is indicative of nephrotic range proteinuria[15]. In addition, a general clinical assessment, including a thorough history (questioning about any family history of kidney disease) and physical examination should be conducted, and relevant parameters should be measured (table 2).

Clinical parameters	Oedema	Signs and symptoms of hypovolaemia	Signs and symptoms of infectious/ systemic disease
• heart rate • respiratory rate • blood pressure • O2 saturation • Body weight	• Periorbital • Pretibial • Genital • Ascites • Bowel wall oedema • Pleural effusion • Pulmonary oedema • Anasarca	• Abdominal pain • Tachycardia • Col hands/feet • Oliguria • Capillary refill >2s	• Fever • Skin rash • Purpura • Arthritis

Table 2. Physical examination parameters to look for in NS[16]

These evaluations are needed not only to distinguish primary NS from secondary NS, but are also required to exclude other conditions presenting with edema and/or hypoalbuminemia, for example protein-

losing enteropathies, heart failure, glomerulonephritis and the state of severe protein-malnutrition known as kwashiorkor. Additional serum studies to exclude secondary causes of nephrotic syndrome may be indicated and include C3 and C4 complement levels; antinuclear antibody (ANA) and possibly anti-double-stranded DNA; HIV antibody; hepatitis A, B, and C serologies; and consideration of other viral serologies such as HIV antibodies.

Once a presumptive diagnosis of idiopathic nephrotic syndrome is made on typical clinical findings, it warrants prompt management with corticosteroid therapy[17]. According to several guidelines[1], the standard corticosteroid therapy generally includes 60 mg/sqm/day (or 2 mg/kg per day) of prednisone for 4 to 6 weeks, followed by 40 mg/sqm every other day for a minimum of 6 weeks, with a further tapering of the dose until it is discontinued. The Italian Society for Pediatric Nephrology recommends a similar scheme but without any tapering of the dose[2] (Table 3).

Prednisone (PDN)	Dosage	Duration
Treatment of the 1st episode:		
60 mg/m² (maximum 60 mg)	In single or 2 divided doses	6 weeks
40 mg/m² (maximum 40 mg)	On alternate days	6 weeks
Treatment of the 1st relapse:		
60 mg/m² (maximum 60 mg)	In single or 2 divided doses	Until remission for 5 days
40 mg/m² (maximum 40 mg)	On alternate days	6 weeks

Table 3. Steroid protocol according to Italian guidelines[16]

Symptomatic management in those with signs and symptoms of hypovolaemia should also be sought. General management of oedema include salt restriction, moderate fluid restriction, and cautious use of diuretics in those with severe oedema and after significant intravascular depletion has been either excluded or corrected. Albumin infusion of 0.5-1g albumin/kg body weight, typically using a 20% human albumin solution can be done to correct intravascular depletion[2]. The most optimal therapeutic approach should be tailored to the clinical needs of the individual patient, in order to limit the adverse effects of diuretic use and optimise the benefits of albumin infusion.

1.5   COMPLICATIONS

Infections and thromboembolic events are two major complications of NS in children.

Intercurrent infections are one of the most serious complications of NS. In addition to treatment with immunosuppressive medications, other risk factors for infections include low serum IgG levels due to urinary loss of IgG, abnormal T lymphocyte function, and decreased levels of factors B and D of the alternative complement pathway. In the past, serious bacterial infections and sepsis were the chief cause of high NS mortality rates, reportedly up to 40% before 1940. Today, the most common and serious type of infection is primary bacterial peritonitis, with an estimated incidence of 2-5% in children with NS [18]. Cellulitis, sepsis, meningitis, and pneumonia are the other bacterial infections that can occur in children with NS, while the most commonly encountered viral infection is varicella. Prophylactic antibiotic use to prevent infections in children with nephrotic syndrome is a topic of debate, with certain studies recommending its use in high risk patients (<2 years old), those with SRNS and FRNS, and those

with a previous pneumococcal infection[19], while others arguing against its use due to development of resistance [2,20].

Thromboembolism is another serious complication of NS, with a risk of approximately 1.8% to 5% and reportedly higher in children with SRNS [21,22]. NS is considered a hypercoagulability state and carries an increased risk of both arterial and venous thrombosis, such as deep vein thrombosis (DVT), pulmonary embolism, cerebral sinus venous thrombosis, and renal vein thrombosis. Abnormalities of the coagulation cascade, such as increased clotting factor synthesis in the liver (factors I, II, V, VII, VIII, X, and XIII), and loss of coagulation inhibitors such as antithrombin III in the urine are some of the likely factors contributing to the risk of clotting in children with NS. Other risk associated with an excessive thromboembolic risk include increased platelet aggregability, hyperviscosity due to increased fibrinogen levels, hyperlipidemia, prolonged immobilization, and use of diuretics [23].

2.   STEROID SENSITIVE NEPHROTIC SYNDROME (SSNS)

2.1   CLINICAL COURSE

Patients who reach disease remission with initial corticosteroid therapy are diagnosed as having SSNS, the most common form of childhood nephrotic syndrome (NS). The clinical course of SSNS in children varies

from a single episode to infrequent or frequent relapses. 80–90% of the SSNS patients that responded to initial treatment with steroids will go on to have relapses that require repeated courses of steroid therapy[24]. Approximately one third of these are definitively cured after a single relapse, 40-50% are dependent on steroids to remain in remission (SDNS), and 20-30% will experience four or more relapses within a year, meeting the criteria for frequently relapsing nephrotic syndrome (FRNS)[25]. The main predictors of relapses and their frequency are reported to be the age of onset of the disease, time to respond to steroids, length of treatment, infections, and rapid steroid tapering [26].

2.2 PHARMACOLOGICAL MANAGEMENT AND LONG-TERM OUTCOME

While the mainstay of management of the relapses of SSNS still remains corticosteroid therapy, according to KDIGO, the treatment regimen differs depending on whether the patient has frequent or infrequent relapses [27,28]. Infrequent relapsers, described as patients who have a relapse within 6 months of initial response, or one to three relapses in any 12-month period, are usually managed with a short course of steroids. According to the KDIGO clinical practice guidelines[28], the treatment of infrequent relapses is a single-daily dose of prednisone 60 mg/sqm/day (maximum of 60 mg/day) until the patient has been in complete remission for at least 3 days, and then a single dose of prednisone 40mg/sqm (maximum 40mg)

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on alternate days for at least 4 weeks until tapering. Frequent relapsers on the other hand are those who experience ≥2 relapses within 6 months of initial therapy or ≥4 relapses in a year. The treatment strategy employed for these patients is 60 mg/sqm of prednisone until the child is in remission for >3 consecutive days, followed by prednisone given on alternate days at the lowest dose needed to maintain remission without adverse effects. Patients who are unable to stop steroids and relapse very early after steroid discontinuation are the most challenging are defined as steroid-dependent NS (SDNS). Relapses in this patient are usually managed in the same way as frequent relapsers[1].

Prolonged corticosteroid use is known to have several side effects in children with NS, including growth impairment, development of cataracts and substantial weight gain[29]. As such, children with SDNS or FRNS are usually treated with several steroid-sparing agents when low-dose alternative-day steroid therapy fails or when severe adverse effects of steroids develop or simply to reduce the burden of side effects. Their mechanisms of action are mostly thought to involve suppression of the immune system; however, they are not devoid of significant side effects either. Cyclophosphamide is the first steroid-sparing agent used for SSNS often in combination with steroids to maintain remission. It is thought to act as an inhibitor of T-cell function and is associated with gonadal toxicity, as well as hair loss, leukopenia, and infections[30]. Calcineurin inhibitors (CNIs) such as cyclosporine (CsA) and tacrolimus, are also used and as steroid sparing agents in relapsing SSNS (and as first line therapy in SRNS).

They are able to mantain remission in 60-85% of those treated but are associated with relapses upon discontinuation [31,32]. Common side effects of CsA include gingival hypertrophy and hypertrichosis while tacrolimus is associated with diabetes mellitus[31]. Mycophenolate mofetil (MMF) has a similar therapeutic efficacy but is associated with fewer side effects than CNIs. It is an inhibitor of T-cell and B-cell proliferation. However, it also requires prolonged therapy due to probable relapse upon discontinuation [33]. Finally, Rituximab, a chimeric anti-CD20 monoclonal antibody is another option that allows for discontinuation or reduction of steroids and other steroid-sparing agents in NS[34]. However, it is associated with some rare but life-threatening side effects that limit its clinical use. The side effects include acute infusion reactions, fatal pulmonary fibrosis, Pneumocystis pneumonia, malignancy, and progressive multifocal leukoencephalopathy [35,36].

Overall, SSNS is mostly considered a benign condition with excellent long-term outcomes. It is rarely thought to be continued past puberty, with a recent study reporting that among 104 cases of SSNS in children, only 14% experienced relapse in adulthood at a follow up of 30 years [37]. However the chronic course, the side effects of steroids and steroid-sparing immunosuppressive agents can be associated with a major impact on the quality of life of affected children and caregivers [38].

3. STEROID RESISTANT NEPHROTIC SYNDROME (SRNS)

3.1 DEFINING STEROID RESISTANCE

Steroid residence is defined as a failure to achieve disease remission with initial corticosteroid therapy. Children with SRNS makes up only 10-20% of NS, however, it represent the most difficult clinical challenge, with over 50% children refractory to therapy progressing to end-stage renal disease (ESRD)[39] and 50% of them recurring after renal transplantation[40]. The appropriate initial therapy needed to define steroid resistance is unclear. The International Study of Kidney Disease in Children (ISKDC) defines resistance as no urinary remission within 4 weeks of prednisone therapy 60 mg/sqm/day, while the Italian guidelines state that no urinary remission following 4 weeks of prednisone 60 mg/sqm/day followed by three intravenous pulses of methylprednisolone at 500 mg/m2 and two further weeks of prednisolone at 60 mg/m2/day [2]. It is agreed upon however, that almost all patients with SRNS who respond will do so within 4 weeks and only a small percentage will respond later and are often called late responders.

3.2 ROLE OF RENAL BIOPSY IN SRNS

More than 80% of the children with idiopathic nephrotic syndrome will respond to steroid therapy by entering complete remission, therefore, the

use of renal biopsy is indicated mainly in individuals with SRNS. It is also performed in the setting of atypical features such as age at onset <1 or >12 years old, gross or persistent microscopic haematuria or presence of red cell casts, abnormal serologies, or significant persistent renal failure[2].

The most dominant lesions seen on the renal biopsies of children with SRNS are FSGS and MCD [41]. It was believed that MCD is more likely to respond to treatment as compared to FSGS, with historical studies providing evidence that >50% of children with SRNS associated with FSGS do not respond to initial steroid therapy would progress to ESRD. Nevertheless, there is a growing consensus among paediatric nephrologists that both these histological findings represent a continuum of disease severity. According to this theory, MCD represents the milder early stage of the disease that is characterised by a normal appearance or glomeruli at the light microscope, but podocyte foot process effacement seen across at least 50% of the glomerular surface at electron microscopy while FSGS is the later, more severe stage of the disease with focal scarring affecting some but not all glomeruli, and segmental scarring affecting part of an individual glomerulus [3,41].

Considering this debate over the 2 most commonly seen histologies of SRNS, along with a 4.1% risk of complications (such as significant haemorrhage) associated with renal biopsies [42], their real clinical significance remains unclear. Nonetheless, their role in identifying the rare cases of idiopathic membranous glomerulopathy (IMN) and

membranoproliferative glomerulonephritis (MPGN), conditions which differ in their aetiology and management from MCD/FSGS does appear to be of some clinical importance[2].

3.3   PHARMACOLOGICAL MANAGEMENT

After failure to respond to corticosteroid therapy, there are 2 main strategies employed for the management of SRNS in children:

1.   Immunosuppression: to induce disease remission;

2.   Symptomatic management: to reduce the severity of proteinuria.

In practice, the recommended management of SRNS begins with a combination of an immunosuppressant (CNIs mostly) and prednisone, followed by the addition of an antiproteinuric agent if there is no improvement[43].

The most commonly used immunosuppressants in the management of SRNS are CNIs, which include mainly cyclosporine (CsA) and tacrolimus. They have been shown to successfully induce complete or partial remission in 60-80% of individuals, where partial remission is defined as >50% reduction in proteinuria[43,44]. CsA suppresses the immune response by inhibiting T cell activation in 2 ways: it downregulates the transcription of interleukin-2 and has an inhibitory effect on antigen-presenting cells

(Langerhans and dendritic cells), which are the main agents of T cell stimulation. Moreover, cyclosporine has also been shown to act directly on the podocyte cytoskeleton to stabilise cell shape[45]. Tacrolimus, which also works by inhibiting T cells, serves as an alternative CNI in SRNS with emerging success rates over time. Several recent studies have reported the beneficial effects of tacrolimus in inducing remission in children with SRNS, including this study by Roberti et al.[46], where partial and complete remission with tacrolimus was achieved in 81% of children with SRNS and this study by Gulati et al. that documented excellent remission rates in 18 of 19 patients [47]. The optimal dose of both CsA and tacrolimus in not known but the recommended dose of cyclosporine in SRNS is 5 mg/kg/day (or 150 mg/sqm) given in two doses; however, the dose is often increased in non-responders. It is also recommended that children with SRNS receive a 6-month trial of CNIs [44]. It should be noted however, that the dose and duration of CNI required to maintain remission should be minimised in order to limit the occurrence of side effects such as nephrotoxicity, infection, hyperkalaemia, renal tubular acidosis, glucose intolerance, and hirsutism, that are associated with both the CNIs. Overall, CNIs remain the mainstay of the management of SRNS in children.

Ofatumumab, a humanised anti-CD20 monoclonal antibody approved for the treatment of chronic lymphocytic leukaemia (CLL) that is resistant to other therapeutic options, has recently emerged as a novel biology therapy in otherwise resistant cases of SRNS. Basu in 2014[48] described first the reduction and then the remission of proteinuria after the 6th dose of

ofatumumab in a patient originally being managed for multidrug resistant CLL with ofutumumab. Following this observation, ofutumumab was administered to four children with rituximab resistant SRNS and was able to achieve disease remission in all of them. Few pilot studies have confirmed the efficacy of ofatumumab in SRNS [49].

Other immunosuppressive agents like cyclophosphamide and rituximab that have proven to be useful in the management of SSNS have not yielded similar results for management of children with SRNS [81]. Nevertheless, they continue to be used in the management of SRNS in some places around the world. Some non-controlled trials have hinted at the benefits of MMF in SRNS management, and it has been considered a good choice due to its non-nephrotoxic profile, but there is a lack of sufficient controlled data, and therefore its use cannot be recommended yet [43].

Regarding symptomatic treatment, the use of nonimmunological therapy has shown to reduce the severity of protein loss. Antiproteinuric agents such as Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARBs) have effectively lowered proteinuria in various diseases involving proteinuria[50]. They act through the reduction of intraglomerular pressure by inhibiting angiotensin II–mediated efferent arteriolar vasoconstriction. The importance of antiproteinuric therapy in the management of SRNS is based on evidence that a reduction in proteinuria is associated with slower progression of CKD and ultimately

better long-term renal outcomes[51]. In addition to proteinuria, oedema also warrants symptomatic management, which, as discussed before, is possible with salt restriction, moderate fluid restriction, and a judicious use of diuretics in certain severe cases.

3.4   PROGNOSIS

Among children with INS, SRNS patients has a poorer long-term outcome. In the past, SRNS in children was associated with a poor outcome due to a poor response to steroid therapy, with >50% of children with SRNS reported to have progressed to ESRD[39]. With the emergence of newer therapeutic options, the situation has improved considerably for these patients, with several studies reporting a drop in the progression rate to ESRD and an increase in the remission rates of SRNS with the use of CNIs [41]. While the short-term outcome of children with SRNS looks positive, not much is known about the long-term prognosis of these patients. Few recent studies have been conducted that imply a good long- term outcome[52], however, further evaluation of the long-term prognosis of SRNS is required.

4   PATHOPHYSIOLOGY

4.1   DYSFUNCTION OF GLOMERULAR FILTRATION BARRIER

The central abnormality in all cases of NS is massive proteinuria, i.e., the excessive glomerular filtration of macromolecules, especially albumin, and their excretion in urine, due to a structural and functional defect of the glomerular filtration barrier (GFB). The filtration of blood by glomeruli is one of the key roles of kidney as it allows excretion of fluid and waste products while most of the blood proteins and all blood cells within the vasculature are retained. This process of filtration is made possible by the GFB, which is a highly specialized blood filtration interface that sieves depending on the size and the charge of molecules. It is composed of three distinct layers: fenestrated capillary endothelium, a shared extracellular matrix called the glomerular basement membrane (GBM), and podocytes with foot processes that line the epithelial side of the GBM. All the layers have specialised features that contribute to the filtering function of the GFB under normal physiological conditions. The fenestrated endothelium is covered by a highly negatively charged glycocalyx over its luminal surface that promotes a first selection of molecules passing through the barrier by electric charge[53]. The GBM is composed of a complex network of negatively charged aminoglycosides and fibrous proteins including laminin and collagen type IV and acts a charge- and size- selective filtration barrier [54]. Finally, podocytes are

specialized epithelial cells in the external layer of the GFB that possess numerous foot processes. Neighboring foot processes are connected to each other by networks of specialized cell-to-cell junctions known as slit diaphragms. The slit diaphragm aids the filtration function of GBM by allowing the passage of smaller molecules and retaining the larger ones. Furthermore, slit diaphragm is filled with two transmembrane proteins important for the correct functioning of the GFB- nephrin and podocin[54].
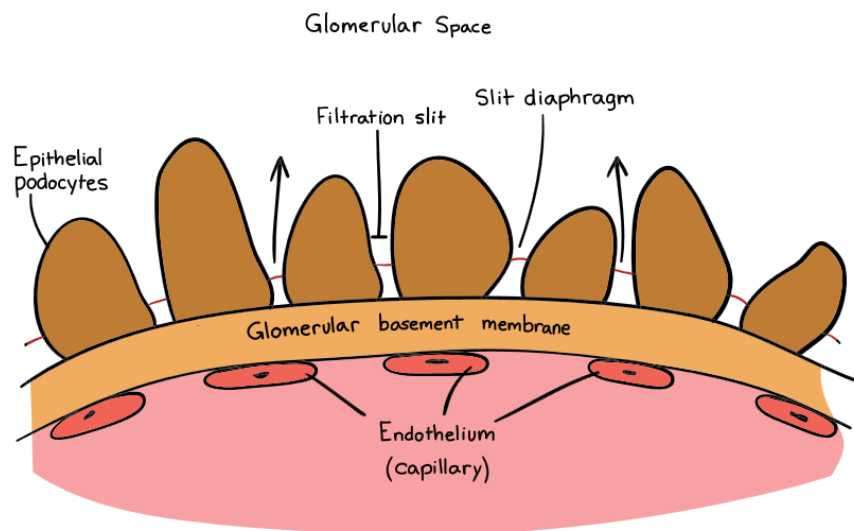

Figure 1 - schematic representation of glomerular filtration barrier

Abnormalities in any of the above components of GFB, especially GBM and podocyte foot processes, lead to the massive proteinuria seen in NS. In NS, there is a loss of the negative chare of GBM which leads to the impaired filtration by the glomeruli. Aside from this, podocyte foot process effacement (FPE) is an invariable hallmark of not just NS, but also other glomerular diseases involving proteinuria[1]. FPE is a pathological derangement believed to be an adaptive response of podocytes to injury and stress[55]. The reason FPE is associated with proteinuria is unclear however, it is speculated that the detachment of interdigitating foot processes in FPE may be responsible for the protein leakage.

4.2 GENETIC FORMS OF NEPHROTIC SYNDROME IN CHILDREN

In addition to podocyte foot process abnormality, recent experimental studies have shown that mutations in genes coding for the proteins that fill the slit diaphragm, such as nephrin and podocin, can alter the filtration barrier and probably are the cause of nephrotic syndrome.

The first genetic form was identified in 1998 in children with congenital nephrotic syndrome (Finnish type), an autosomal recessive disease characterized by the appearance of massive proteinuria shortly after birth, unresponsiveness to steroids and homozygous mutations in the NPHS1 gene, encoding for Nephrin[56].

Since then, mutations in an increasing number of genes encoding different proteins of glomerular slit diaphragm or signal transduction molecules have been associated to the pathogenesis of INS in children (NPHS1, NPHS2, LAMB2, WT1, etc)[57] .

The likelihood of finding a causative gene mutation is higher in patients unresponsive to corticosteroids, with around 30% of children with SRNS having a gene mutation, while no mutations have been yet identified in steroid sensitive patients[58,59]. Consequently, a genetic mutation can explain the disease in only a minority of cases, whereas the totality of steroid sensitive forms and 70% of SRNS recognize a different cause.

4.3 CIRCULATING PERMEABILITY FACTORS AND IMMUNE DYSREGULATION

The pathogenetic mechanisms of non-genetic INS (both steroid sensitive and resistant) remain poorly defined. Immunological processes involving the podocyte play a key role in the occurrence of protein permeability of the glomerular membrane. A circulating permeability factor responsible for glomerular barrier dysfunction has long been advocated, but not identified.

The presence of an immune-related circulating factor with a central role in the pathogenesis of INS in children is supported by the following observations:

1) the high response rate to immunosuppressive treatments, eg corticosteroids, calcineurin inhibitors, MMF and rituximab[1];

2) the evidence of disease recurrence after kidney transplantation [60];

3) the efficacy of immunosuppressive drugs and/or plasmapheresis in treating recurrence after transplant in a variable percentage of patients [61]

4) INS patients plasma can induce proteinuria in rats[62];

5) A report of transient proteinuria in a child born from a mother with INS [63]

Different approaches have been used to identify the so-called "permeability factor", without conclusive results.

In 1996, using an in vitro model based on the variation of volume in rat glomeruli according to different albumin concentrations and incubation with human sera, Savin et al. observed an increased albumin permeability when sera from INS patients who relapsed after transplant were tested. The effect of sera was reduced after plasmapheresis. Their hypothetical permeability factor was between 30-50 kDa and soluble in 70% ammonium sulfate[64] (12).

In 2008, the same group identified Cardiotrophin-Like Cytokine Factor-1 (CLCF-1), a member of the IL-6 family, by galactose affinity chromatography and mass spectrometry. In their in vitro model CLCF-1 was able to increment albumin permeability and a CLCF-1 antibody abolished this effect. In murine podocytes CLCF-1 alters the normal cellular structure and may act through the JACK/STAT pathway. In their preliminary and unpublished data, CLCF-1 concentration in INS patients can be up to 100 times higher than in controls, but available assays are not sensitive enough to measure of CLCF-1 in patient samples [65]. These results have not been replicated by different groups, nor validated in appropriate patient cohorts. The potential role of CLCF-1 as a permeability factor has still to be verified.

Soluble Urokinase Plasminogen Activator Receptor (suPAR), the soluble form of the glycoprotein uPAR, was recently proposed as a candidate permeability factor. uPAR interacts with integrins and could be involved in podocyte motility in proteinuric diseases, moreover suPAR has been described as a prognostic biomarker for cardiovascular and kidney diseases. Despite initial enthusiasm regarding the first clinical reports on suPAR as biomarker for INS, further studies did not support this hypothesis[66,67]. Particularly, suPAR is inversely related to glomerular filtration rate and its increment could be associated to the deterioration of renal function. Moreover conflicting data are now available on the potential role of suPAR on podocyte damage in animal models[68].

Delville et al. using a high-density protein array data followed by an enzyme-linked immunosorbent assay identified the presence of pretransplant antibodies anti-CD40, as predictor of disease recurrence in a cohort of INS patients undergoing kidney transplantation. CD40 is a member of the TNF receptor superfamily with a role in immunity and inflammation. They hypothesized anti-CD40 play a pathogenetic role in INS recurrence through interaction with suPAR[69]. Again, these initial observations need to be validated by other groups and in larger and well-characterized clinical setting, also in light of the new conflicting data on suPAR.

Therefore, while strong evidence suggests the presence of a circulating permeability factor, the molecular mechanisms of INS in children are still unknown. This is the gap this project aims to fill.

Finally, it is also suspected that dysfunction or dysregulation of T lymphocytes may also play a role in the pathogenesis of NS. Firstly, many known inhibitors of T lymphocyte function including, corticosteroids, alkylating agents, calcineurin inhibitors, and mycophenolate mofetil have successfully been used in treating most forms of NS, and this provides strong support to this theory. In addition, remission of NS has been induced following infections with measles and malaria, diseases known to depress cell-mediated immunity, which further suggests that an immunological abnormality might contribute to the disease

pathogenesis[70] and different studies support the presence of T-cells and B-cells dysfunction in children with INS[71].

5. SCOPE OF THE THESIS

This project aim is to clarify molecular mechanisms involved in INS in children, focusing on the role of genetic mutation in relapse, the identification of patients whose sera contain the circulating permeability factor able to induce dysfunction of GFB, and looking for innovative biomarkers for the disease.

Chapter 2. We performed a retrospective, multicentre, national cohort study to address the long-term prognosis of renal graft, the risk factors for recurrence and the predictors for response to therapy following recurrence in paediatric patients undergoing renal transplantation because of a SRNS. We were able to demonstrate that genetic forms of NS do not relapse after renal transplantation.

Chapter 3. At the bench side, in order to detect sera of patients with SRNS containing the so-called permeability factor, we identify patients with different forms of SRNS, collected their sera and tested the samples by means of a novel method which assess the permeability to bovine serum albumin (BSA) through a three-layer device (3LD).

Chapter 4. A preliminary proteomic analysis of sera from SRNS children relapsed after renal transplantation and tested through the GFB model

described in chapter 3 was performed in order to identify the proteins involved in the pathogenesis of disease and recurrence.

Chapter 5. INS children with different response to the initial steroid treatment were screened for cytokine plasma level at the diagnosis. We were able to demonstrate that macrophage migration inhibitory factor (MIF) was a good predictor of steroid response.

Chapter 6. Protein content of urinary extracellular vesicles in INS children with different response to the available treatment was screened in order to provide biomarker of response and prognosis and to shed light on molecular mechanism of the disease.

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CHAPTER 2

POST-TRANSPLANT RECURRENCE OF STEROID RESISTANT NEPHROTIC SYNDROME IN CHILDREN: THE ITALIAN EXPERIENCE

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ABSTRACT

Background: Steroid resistant nephrotic syndrome (SRNS) is a frequent cause of end stage renal disease in children and post-transplant disease recurrence is a major cause of graft loss.

Methods: We identified all children with SRNS who underwent renal transplantation in Italy, between 2005 and 2017. Data were retrospectively collected for the presence of a causative gene mutation, sex, histology, duration of pre-transplant dialysis, age at onset and transplant, HLA matching, recurrence, therapy for recurrence, and graft survival.

Results: 101 patients underwent a first and 22 a second renal transplant. After a median follow-up of 58.5 months, the disease recurred on the first renal transplant in 53.3% of patients with a non-genetic and none with a genetic SRNS. Age at transplant >9 years and the presence of at least one HLA-AB match were independent risk factors for recurrence. Duration of dialysis was longer in children with relapse, but did not reach statistical

significance. Overall, 24% of patients lost the first graft, with recurrence representing the commonest cause. Among 22 patients who underwent a second transplant, 5 suffered of SRNS recurrence. SRNS relapsed in 5/9 (55%) patients with disease recurrence in their first transplant and 2 of them lost the second graft.

Conclusions: Absence of a causative mutation represents the major risk factor for post-transplant recurrence in children with SRNS, while transplant can be curative in genetic SRNS. A prolonged time spent on dialysis before transplantation has no protective effect on the risk of relapse and should not be encouraged. Retransplantation represents a second chance after graft loss for recurrence.

INTRODUCTION

Steroid resistant nephrotic syndrome (SRNS) is the most common acquired cause of end stage renal failure (ESRD) requiring transplantation in children. Advances in genetic screening have allowed the identification of a monogenic cause of SRNS in one third of cases[1]. Genetic SRNS are associated with an underlying mutations in genes encoding podocyte associated proteins, resulting in structural or functional disruption of the glomerular filtration barrier [2]. The pathophysiology of SRNS without underlying mutations remains poorly explained and is thought to involve an unknown circulating permeability factor [3] which may also be implicated in the recurrence soon after transplantation [4].

Unfortunately, in up to 50% of patients, SRNS relapses after transplantation and disease recurrence is a major cause of graft loss [4–6]. Genetic SRNS have been reported to have a low rate of recurrences [7–9]. On the other hand, previous studies have suggested non-African race, rapid progression to ESRD (<3 years) and previous recurrence after transplantation to be associated with SRNS relapse [8, 10, 11], but no

established risk factors can actually predict the outcome. A longer time on dialysis before transplantation was believed to decrease the risk of relapse, but not confirmed by large reports [12, 13]

There is currently little consensus regarding the best management of post-transplant SRNS recurrence, which represents a devastating complication for families and physicians, and poses a significant threat to allograft survival. Plasma exchange (PE), steroids and rituximab are the most common strategies to treat the recurrence[14].

The objective of this study was to identify factors affecting the risk of recurrence and graft loss in children with SRNS, by stratifying the population according to their genetic status.

METHODS

We performed a retrospective, multicentre, observational cohort study to address the long-term prognosis of renal graft, the risk factors for recurrence and the predictors for response to therapy following

recurrence in paediatric patients undergoing renal transplantation because of a SRNS.

We identified patients who underwent renal transplantation at all five Italian paediatric transplant centres, between 2005 and 2017, with a primary diagnosis of SRNS and onset before 18 years. Patients were included if a clinical diagnosis of SRNS was made in an individual with otherwise unexplained nephrotic-range proteinuria refractory to standard steroid therapy and subsequently confirmed by renal biopsy showing a histological picture of focal segmental glomerulosclerosis (FSGS), minimal change disease (MCD), or diffuse mesangial sclerosis (DMS). Clinical records, pathology reports and genetic screening results were reviewed for the purposes of this study. Data were also collected about sex, age of disease onset, duration of pre-transplant dialysis, age at transplant, immunosuppression, allograft donor characteristics, disease recurrence, therapy for recurrence, and graft survival.

Patients were divided in 3 groups: Group A (Genetic SRNS): patients with an identified causative genetic mutation and/or a first degree relative with SRNS and/or extra-renal disease manifestations pathognomonic of

SRNS, Group B (Idiopathic SRNS): patients with a negative or heterozygous recessive genetic test result and without a first degree family history or associated extra-renal manifestations pathognomonic of SRNS, Group C (unknown genetic status): patients with no genetic analysis performed and no family history or extra-renal manifestations typical of SRNS.

Definitions. Nephrotic-range proteinuria, urine protein:creatinine ratio (uPr/uCr) ≥2 mg/mg. Age at disease onset, age at first clinical presentation of nephrotic syndrome. Steroid resistance, persistence of nephrotic range proteinuria following 4 weeks of daily 60 mg/sqm prednisone therapy. Post-transplant disease recurrence, an otherwise unexplained persistent nephrotic range proteinuria after renal transplantation, when rejection was excluded. Graft loss, functional failure of the renal allograft, necessitating renal replacement therapy. Remission after recurrence, complete resolution of proteinuria (uPr/uCr <0.2 mg/mg). Partial remission after recurrence, persistent reduction of proteinuria (uPr/uCr <2 mg/mg) with preserved renal function.

Statistical analysis. Categorical variables were compared using the Chi-squared test for independence. The distribution of continuous variables in groups was compared using the Wilcoxon signed-rank test and the Kruskal-Wallis test. Linear regression models were used to compare continuous variables. For multivariate analysis, multiple logistic regression models were used. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using the open source software R. (R Core Team, 2014. R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study cohort. During the study period, a total of 728 (618 deceased and 110 living donors) renal grafts were performed at the 5 Italians pediatric transplant centres, of whom 123 in patients with ESRD secondary to SRNS. 101 patients received a first renal allograft and 22 a second renal transplant (12 failures of the original cohort and 10 failures of a first

51

transplant that occurred before the study period). The number of patients who received a first transplant at each center is as follows: Istituto G. Gaslini, Genova - 31, Bambino Gesù Children's Hospital, IRCCS, Rome - 23, Fondazione IRCCS Ca' Granda, Ospedale Maggiore, Milan - 20, Regina Margherita Children's Hospital, Turin - 15, University Hospital of Padua- 12. The study cohort is summarized in Figure 1.
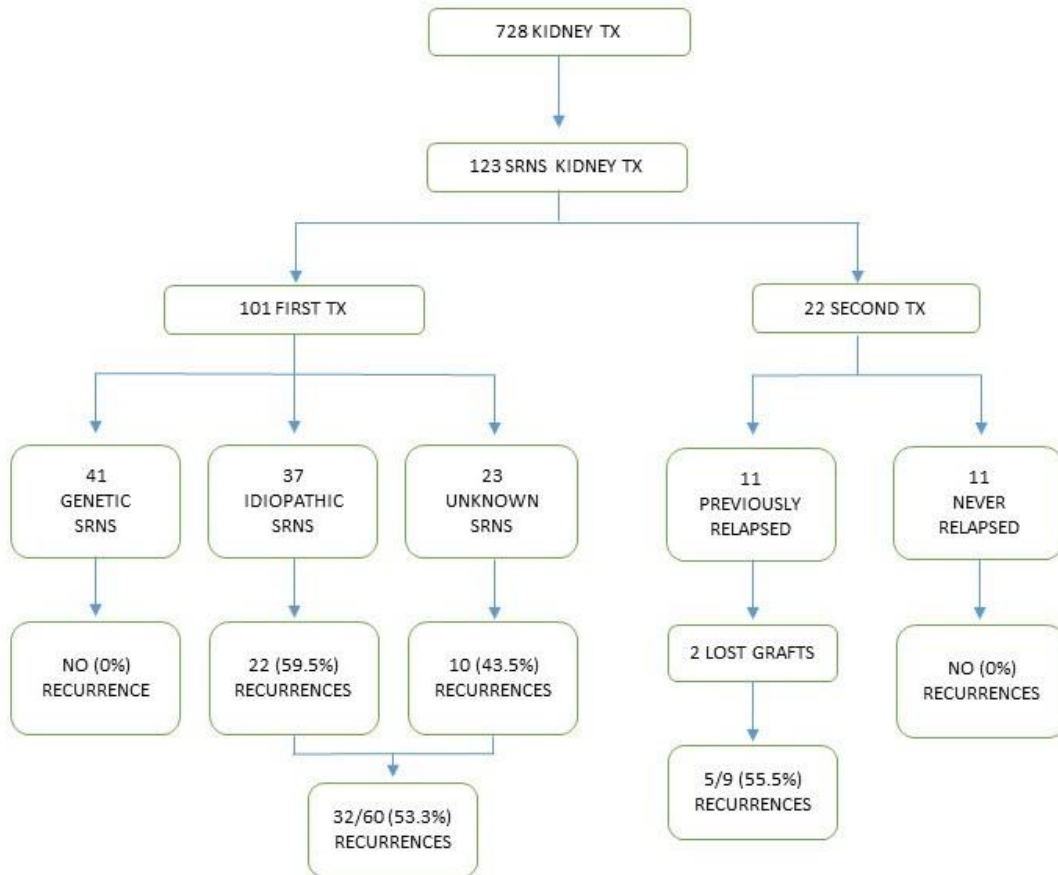
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41 GENETIC SRNS	37 IDIOPATHIC SRNS	23 UNKNOWN SRNS	11 PREVIOUSLY RELAPSED	11 NEVER RELAPSED
NO (0%) RECURRENCE	22 (59.5%) RECURRENCES	10 (43.5%) RECURRENCES	2 LOST GRAFTS	NO (0%) RECURRENCES
	32/60 (53.3%) RECURRENCES		5/9 (55.5%) RECURRENCES	

Figure 1 – Study cohort

First renal graft. 101 patients (52.5% males) underwent a first renal transplant. The median age at onset was 2.8 years of age (range 0 – 17.2); 24 individuals (25.2%) presented with congenital SRNS, defined as onset of disease within the first three months of life. Renal histology was consistent with FSGS in 85 cases, MCD in 14 and DMS in 2. Main demographic and clinical characteristics are summarized in Table 1. At transplant all patients received an induction therapy with basiliximab and immunosuppression with steroids, calcineurin inhibitors and mofetil mycophenolate. 2 patients were treated with plasmapheresis pre-transplantation.

Characteristics	First renal Tx	Second renal Tx
Total	101	22
Gender		
Male	53 (52.5%)	13 (59.1%)
Female	48 (47.5%)	9 (40.9%)
Genetic disease		
Yes	41 (40.6%)	5 (22.7%)
No	37 (36.6)	13 (56.6%)
Unknown	23 (22.8%)	4 (18.2%)
Age at onset (years) median (range)	2.8 (0 – 17.2)	4.45 (0-14.29)
Age at transplant (years) median (range)	11.8 (2.6 – 20.8)	16.71 (4.56-31.1)
Time to ESRD (years) median (range)	3.3 (1.7 – 14.3)	2.5 (0-7.5)
Time on dialysis before transplantation (years) median (range)	2 (0 – 9)	Not available
Donor type		
Living	6 (5.9%)	2 (9.1%)
Deceased	95 (94.1%)	20 (90.9%)
Follow-up (months) median (range)	58.5 (0.7 – 157.8)	40 (0-148)

Table 1: Main demographic and clinical characteristics of SRNS children transplanted between 2005 and 2017

Genetic testing results were available for 76 individuals (75.2%) (Table 2): 39 had an autosomal dominant mutation or were homozygous for a recessive mutation, 8 were heterozygous carriers and 29 had a negative genetic test. Genetic results were unavailable for 25 patients, among whom we were able to identify 2 additional patients with genetic SRNS: one had a sibling with established genetic SRNS and another showed extra-renal disease manifestations suggesting a genetic disease. The patient cohort was therefore comprised of: Group A (genetic SRNS): 41 individuals (40.6%), Group B (idiopathic SRNS): 37 individuals (36.6%), and Group C (unknown genetic status): 23 individuals (22.8%). Age at the onset was similar between Group B (idiopathic) and Group C (unknown), while it was younger for patients with a genetic disease (Group A) ($p < 0.0001$).

NPHS1, encoding nephrin, was the single most commonly mutated gene and accounted for one third (33.3%) of positive genetic results, followed by WT1, encoding Wilms tumour protein and NPHS2, encoding podocin.

Mutations in these genes were responsible of 28.2% and 20.5% of genetic SRNS, respectively. Taken together, mutations in NPHS1, NPHS2, and WT1 accounted for 82% of identified genetic cases. Pathogenetic mutations were also identified in the following genes: PLCE1, ACTN4, COL4A5, SMARCAL1, LMX1B, COQ2 (Table 2). In 8 cases renal disease was associated with a syndromic presentation, as follows: Denys Drash syndrome in 4 cases and one case each of Frasier syndrome, WAGR syndrome, Leopard Syndrome and Schimke immuno-osseous dysplasia.

Gene	Encoded protein	Mode of inheritance	Genetic tests n = 76
NPHS1	Nephrin	recessive	13
WT1	Wilms tumour protein	dominant	11
NPHS2	Podocin	recessive	8
ACTN4	α-Actinin	dominant	2
PLCE1	Phospholipase C	recessive	1
COL4A5	Type IV collagen α5 chain	x-linked recessive	1
SMARCAL1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like protein 1	recessive	1
LMX1B	LIM homeobox transcription factor 1 β	dominant	1
COQ2	Coenzyme Q2	recessive	1
Heterozigous carriers		recessive	8
No mutations			29

Table 2: prevalence of mutations among patients with available genetic results

<u>Post-transplant disease recurrence</u>. Median follow-up is 58.5 months

(range 0.7 – 157.8). SRNS recurred in 32 individuals (31.7%) after the first

renal transplant, at a median time of 2 days post-transplantation. When stratified by genetic status, the incidence of post-transplant disease recurrence was 59.5% in Group B (idiopathic SRNS) and 43.5% in Group C (unknown genetic status). No Group A (genetic SRNS) child experienced disease recurrence and this group was therefore excluded from further analysis (Table 3).

Characteristics	Total, n = 101	Recurrence, n = 32	No recurrence, n = 69
Genetic results *n, %*			
Negative	37	22 (59.5)	15 (40.5)
Unknown	23	10 (43.5)	13 (57.5)
Positive	41	0 (0.0)	41 (100.0)

Table 3: incidence of recurrence, stratified by genetic testing results

The difference in post-transplant disease recurrence between group B and C, however, was not significant (p = 0.23). Risk factors for recurrence were evaluated in the remaining 60 patients (group B and C). Overall, SRNS recurred in 32/60 (53.3%) non-genetic patients.

As all relapses except one (identified 10 years after transplantation) occurred within 8 months from transplant, the analysis was made at 8 months of follow-up and included all evaluable patients (54 patients). Age at transplant was categorized as $\geq$ 9 years, following a ROC analysis identifying it as the best cut-off for relapse prediction in our dataset (Figure 2).
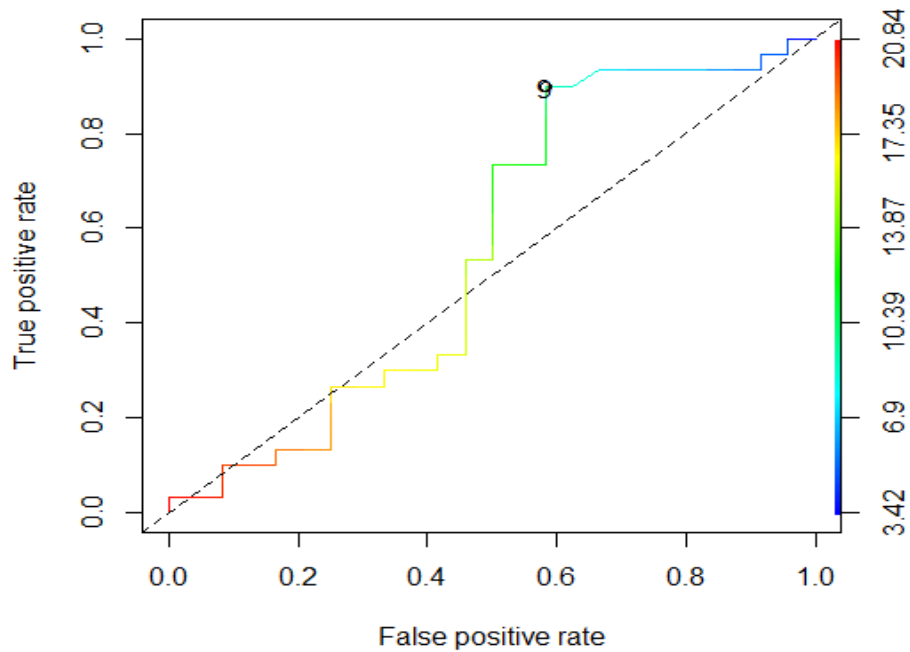
Figure 2 – ROC curve identifying the best cut-off for age at transplant with FPR = 0.58333333, TPR (sensitivity) = 0.86666667, Specificity = 0.41666667,p value = 0.01823

Bivariate analysis was performed by Wilcoxon test for independent samples. Multivariate analysis was performed by a logistic regression model.

At bivariate analysis, the following variable were significantly associated to relapse:

- Age $\geq$ 9 years (p = 0.01823)

- At least one HLA AB match (p = 0.01752)

- At least one HLA DR match (p = 0.01763)

Gender, donor age and donor type (living or deceased) did not affect the risk of recurrence. Time to ESRD and duration of dialysis before transplant were not significantly associated with relapse; anyway, they were both longer in children with relapse (median = 4.6 vs 2.7 years, p = 0.2673 and 2.4 vs 1.8 years, p = 0.06582 respectively).

We were not able to assess the role of different induction schedules, since all patients were homogeneously treated with basiliximab and immunosuppressive therapy. However, among the two patients treated with pre-transplantation PE, one experienced relapse the day after transplant.

Multivariate analysis included all the variables associated with recurrence at the bivariate analysis with a p-value <0.1 (Table 4).

Variables
Age $\geq$ 9 years
At least one HLA AB match
At least one HLA DR match
Duration of dialysis before transplant

Table 4. Variables included in the multivariate analysis

Age at transplant > 9 years and HLA-AB match were the only independent risk factors for recurrence after transplant (p = 0.01017 and p = 0.02465, respectively). However, the best prediction model for relapse, characterized by the lowest residual deviance and lowest AIC, included also a longer duration of dialysis before transplant (Null deviance: 68.029 on 49 degrees of freedom; Residual deviance: 49.584 on 46 degrees of freedom; AIC: 57.584; p = 0.06994). The model including the

aforementioned 3 variables has a likelihood ratio test p value of 0.000356,

and a pseudo R squared value of 0.271136 (McFadden method). The risk

factors for recurrence in group B and C are summarized in Table 5.

Variables	Total	Recurrence, n (%)	No recurrence, n (%)	Univariate analysis *P*-value	Multivariate analysis *P-value*
Gender					
Male	30 (55.6%)	18 (60%)	12 (40%)	0.4624	0.26990
Female	24 (44.4%)	12 (50%)	12 (50%)		
Age at transplant, *yr*					
>9	40 (74.0%)	26 (65%)	14 (35%)	**0.01823**	**0.01017**
< 9	14 (26.0%)	4 (28.6%)	10 (71.4%)		
HLA-AB matching				0 vs >0	0 vs >0
0	7 (13.5%)	1 (14.3%)	6 (65.7%)	**0.01752**	**0.02465**
1	18 (34.6%)	13 (72.2%)	5 (27.8%)		
2	17 (32.7%)	9 (52.9%)	8 (47.1%)		
3	10 (19.2%)	6 (60%)	4 (40%)		
HLA-DR matching					
0	16 (30.8%)	5 (31.2%)	11 (68.8%)	**0.01763**	0.46309
1	34 (65.4%)	24 (70.6%)	10 (29.4%)		
2	2 (3.8%)	0 (0%)	2 (100%)		
	Recurrence		No recurrence		
Duration of dialysis median, range	2.4,, 0.6 - 9		1.8, 0.1 - 5	0.06582	0.06994
Time to ESRD median, , range	4.6, 0 - 12		2.7, 0 - 13	0.2673	0.72323
Donor Age median, , range	14, 1 - 63		11, 1 -56	0.1609	0.84874

Table 5: Risk factors for post-transplant disease recurrence in Group B (idiopathic SRNS) and Group C (unknown genetic status)

Disease recurrence was treated in all patients with PE with a median of 20 sessions (range:4-79). 22 were treated with rituximab and 9 with high dose steroids. The use of other therapeutic agents was as follows: ofatumumab (3), mesenchymal stromal cells (2), intravenous immunoglobulins (2), abatacept (2), cyclophosphamide (2), cyclosporin (1) and thymoglobulin (1). Overall, a complete or partial remission was achieved in 15 and 4 patients respectively, 13 patients (40.6%) failed to achieve sustained disease remission, despite treatment and 11/13 subsequently lost the graft. Use of rituximab or high dose steroids did not influence the response rate (p= 0.3574). The remission was persistent with preserved renal function in 13/15 patients. One patient had second untreatable relapse 10 years after transplant and lost the kidney. One additional patient with partial remission, following experienced rejection and lost his graft.

Graft loss. 24 patients (23.8%) experienced loss of a first renal graft. The causes of graft loss were as follows: disease recurrence in 12 (50%), rejection in 6 (25%), primary non-functioning graft in 3 (12.5%),

thrombosis in 2 (8.3%) and chemotherapy toxicity for post-transplant thrombo-proliferative disease in one case (4.1%). In addition, death with a functional graft due to sepsis occurred in 2 patients.

Second renal transplant. During the study period, 22 SRNS patients received a second renal graft; 11 of them had had a recurrence in the first graft, while 11 lost their transplant for different reasons. Among patients with a previous recurrence, 2/11 patients lost their graft immediately after the transplantation for reasons different from relapse (death and surgery complications) and were not included in the following analysis. After a median follow-up of 40 months, 5 patients relapsed on the second transplant. All of them have had a recurrence in the first graft. Therefore, in our population, only 5 out 9 (55.5%) evaluable patients with a previous relapse experienced recurrence of proteinuria after the second kidney transplantation. Among 5 relapsed patients, only 2 subsequently lost the second graft. None of the 11 patients who lost the first transplant for different reasons suffered of relapse, with 4/11 having a genetic disease. Outside SRNS recurrence, 2 patients experienced a graft rejection and lost

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the second kidney graft and 1 patient died after a post-transplant lymphoproliferative disease.

DISCUSSION

SRNS is a leading cause of ESRD in children. Post-transplant recurrence is a common complication, associated with an increased risk of graft loss. Many efforts have been made to identify the risk factors for recurrence in order to improve prevention and treatment strategies [5, 6, 15].

Our study gives a clear picture of the Italian experience with kidney transplantation in children with SRNS, during a period of over 10 years, encompassing all the recent acquisition regarding the etiopathology and therapeutic options for SRNS.

The overall incidence of post-transplant disease recurrence (53% of non-genetic patients) is consistent with the available scientific literature, stretching back almost three decades [16, 17]. Recurrence is confirmed to be a very early event, with a median time from transplant of 2 days and 30/32 events occurring within the first 2 months after transplantation. By

stratifying the cohort according to genetic status, we have been able to confirm that genetic SRNS does not recur after transplant. While previous reports have identified the genetic status as an important risk factor for disease recurrence, most available retrospective studies are unable to account for the genetic status of the majority of their cohorts [17, 18]. To the best of our knowledge, no previous studies were able to assess the risk of recurrence in an equally characterized population, as regards genetic disease. In our study, genetic results were indeed available for 76/101 patients (75.2%), furthermore since the remaining individuals (Group C, unknown genetic status) closely resemble Group B (Idiopathic SRNS) in key clinical features, including similar age of onset and rate of recurrence (43.5%), it is likely that most of them also represent cases of idiopathic SRNS. Indeed, we believe that patients with early onset or congenital SRNS were more likely to be tested for a genetic disease, while genetic testing was less performed in older children and adolescents with a clinical picture of idiopathic SRNS.

Our observation is in line with previous studies that report no or very low relapse rate after transplantation in children with genetic SRNS [9, 17, 19].

Few old reports have suggested a risk of relapse for genetic SRNS, but they are almost all related to NPHS2 mutation, including heterozygous individuals [20–23]. The causative role of the variants included in these reports should be reconsidered, as exquisitely suggested in a recent review by Bierzynska [15].

The rate of recurrence in idiopathic SRNS (group B) was 59.5%. The result is slightly superior than previously reported. When genetic patients are excluded, Ding [17] and Pelletier [19] found a relapse rate of 46.3% and 47%, respectively. The lower recurrence rate described by these groups could be justified by the presence of unknown genetic SRNS patients. Indeed, when both group B and C are considered, the overall rate of recurrence was 53.3 % in our cohort. Therefore, our data underline the importance of a genetic evaluation for SRNS genes in order to plan transplantation, as it represents the principle risk factor for recurrence.

Aside from absence of a genetic aetiology, our study identified age at transplant greater than 9 years and HLA-AB match as independent risk factors for recurrence. The best prediction model for recurrence included also a longer duration of dialysis.

Nehus et al reported a higher rate of recurrence in younger children, among a cohort of 327 patients, though genetic results were not reported for any participants [24]. No significant difference in relapse rate according to the age at transplant were detected by Tejani et al [12] and in the more recent studies by Ding et al and Pelletier et al [17, 19]. Again, unavailability of genetic testing for the majority of their patients could justify the different findings.

HLA-AB match was independently related to recurrence in our cohort, in contrast HLA AB or DR match did not influence the risk of relapse in the study by Tejani et al [12] and did not affect transplant outcome in adolescent with SRNS in a retrospective study of the NAPRTCS registry [25].

Following the evidence that a circulating factor is responsible for recurrence, it has been suggested that a prolonged dialysis prior to renal transplantation would have a protective effect as far as the risk of relapse is concerned. The results of our study do not support this hypothesis. Indeed, in our study cohort, a longer duration of dialysis was associated with an increased risk of relapse. Even if this variable did not reach

significance, its inclusion identifies the best prediction model for recurrence (R squared 0.271136). Among the few studies which investigated the association between duration of dialysis and disease recurrence, no significant differences were found in a single centre experience of 43 patients by Senggutuvan [16]. In a larger cohort of 132 paediatric renal transplants, found no relationship between disease recurrence and duration of dialysis was found [12]. Hence, since no protective effect was proven by others and our data show a longer duration of dialysis in patients with recurrence, even if not statistically significant, it is not justified to prolong the duration of dialysis before transplantation in children with SRNS.

Whether donor type (living vs deceased) is significantly associated with disease recurrence remains controversial. Data from old registries [26, 27] found no increased recurrence rates according to the type of donors. Other studies have suggested living donor as an independent risk factor for recurrence [17, 28, 29]. Our study cohort included only 6 living donor recipients (5.9%), reflecting the reluctance of paediatric nephrologists to use living donors in SRNS patients, due to the risk of recurrence and graft

loss. On the other hand, since in our cohort no relapses occurred in genetic SRNS, another important clinical implication of our study is that living kidney donors can be safely used in genetic SRNS patients.

All patients from our cohort were treated with PE, following SRNS recurrence. Complete or partial remission was achieved in 19/32 (59.4%) children, with a functioning graft after a median follow-up of 39.5 months. Similar rates of response were previously reported. Kashgary et al in their meta-analysis identified a remission rate of 70.2% in children treated with PE [14]. A lower response rate was reported by Pelletier [19], but remission information was available only for 49/64 (77%) relapsed patients and the detailed immunosuppressive strategy is missing. According to our results, PE is confirmed as an effective treatment for recurrence. Even the small numbers, rituximab and high dose steroids did not influence the response rate in our cohort.

On the other hand, disease recurrence was the leading cause of graft loss in non-genetic SRNS and the rate of graft loss after relapse (34.3%) in our study is consistent with previous data [26].

Among the small number of retransplanted individuals included in our study, the overall incidence of relapse in a second renal graft after a first recurrence is not significantly different from the first transplant (55%). In 4/9 patients who experienced a relapse in their first transplant, proteinuria did not recur after the second transplantation. This contrasts with reported small cohorts in whom the incidence of recurrence approaches 100% once the first transplant was lost for recurrent SRNS [10, 12, 28, 30]. We are not able to identify the factors responsible of the different outcome, but according to our data retransplantation after relapse can be considered in children with SRNS.

CONCLUSIONS

Twelve years of the Italian experience with post-transplant SRNS recurrence allows us to reach different important conclusions. Firstly, the absence of underlying genetic mutations predicts a high risk of post-transplant recurrence, therefore genetic screening must be performed in all children with SRNS before transplantation in order to best plan their care in the post-transplant period. Age >9 years is an independent risk factor for recurrence, while a prolonged time spent on dialysis before

transplantation has no protective effect on the risk of relapse and should not be encouraged. Living donor did not influence the risk of relapse and can be safely used in genetic SRNS patients. PE based treatment strategies are effective in the majority of relapsed patients. Finally, in those who experience graft loss, even for recurrence, it is appropriate to consider retransplantation, as it maybe curative in the long term.

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CHAPTER 3

ASSESSMENT OF INCREASED GLOMERULAR PERMEABILITY ASSOCIATED WITH RECURRENT FOCAL SEGMENTAL GLOMERULOSCLEROSIS USING AN IN-VITRO MODEL OF GLOMERULAR FILTRATION BARRIER

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ABSTRACT

The presence of circulating permeability factors (cPFs) has been hypothesized to be associated with recurrence of focal segmental glomerulosclerosis (rFSGS) in renal allografts. The available methods to detect cPFs are complex, not easily repeatable and inappropriate to represent the anatomical characteristics of the three-layer glomerular filtration barrier (GFB). Here we describe a novel method which measures the permeability to bovine serum albumin (BSA) through a three-layer device (3LD). The 3 layers comprise: (1) conditionally immortalized human podocytes (HCiPodo), (2) collagen type IV coated porous membrane and (3) human glomerular endothelial cells (HCiGEnC). Using this method, we found that sera from all rFSGS patients increased albumin permeability, while sera from non recurrent (nrFSGS) and genetic (gFSGS) forms of FSGS did not. The mechanisms underlying the increase of albumin permeability are probably due to endothelial cell dam- age as an initial event, which was demonstrated by the decrease of Platelet endothelial cell adhesion molecule (PECAM-1 or CD31), while the podocytes' expressions of

synaptopodin and podocin were normal. Furthermore, we also found that the plasmapheretic treatment (PPT) eliminated the effect of increasing BSA permeability in sera from rFSGS patients. These preliminary data suggest that our in vitro GFB model could not only be useful in predicting the recurrence of FSGS after renal transplantation (RTx), but also be a valuable in vitro model to study podocyte and endothelial cell biology.

INTRODUCTION

Primary focal segmental glomerulosclerosis (pFSGS) is a glomerular disease of unknown etiology, often characterized by heavy proteinuria, which in more than 50% of cases does not response to the available therapies and progresses toward end stage kidney failure [1, 2]. It has long been hypothesized that the presence of circulating permeability factors (cPFs) which damage the glomerular filtration barrier (GFB) could contribute to the pathogenesis of pFSGS [3].This hypothesis was based on the frequent recurrence of FSGS after renal transplantation (RTx, 30–50%), the reported proteinuria resolution when an FSGS kidney is transplanted in an unaffected recipient, the appearance of proteinuria in the new- born of a mother affected by FSGS, the regression/reduction of proteinuria after plasmapheresis (PP) in some affected patients [4–12].

The traditional method to determine the circulating permeability factors was based on the measurement of albumin reflection coefficient with isolated rat glomeruli [13], but this method is very complex and requires animal sacrifice. Pegoraro et al. proposed a simpler method using cultured

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rat glomerular epithelial cells grown on the Millicell filters, which allow sampling of apical and basolateral media. Through measuring the 125I-labeled human serum albumin across the epithelial cell monolayer, they tested the circulating permeability activity of sera from idiopathic nephrotic syndrome patients [14]. In a recent paper, Kachurina et al. described a novel method based on cultured mice podocytes challenged with sera from FSGS patients, using immunofluorescence microscopy followed by computerized image-processing analysis [15]. More recently, Srivastava et al. developed a reporter-based assay, using transfected podocyte cells for the study of activated genes after exposure to the plasma from recurrent FSGS (rFSGS) patients [16]. However the main limitation of all these models, except the first one [13], is that the assays are based on only one epithelial monolayer, which is far from the in vivo physiological structure of GFB which is a three-layer structure with glomerular endothelial cells, podocytes and the basement membrane in between. Here we described a method of co-culture of conditionally immortalized human podocytes and glomerular endothelial cells on the opposite of a porous membrane, which does not rely on special

instruments nor on the synthesis of devoted biomaterials, so it may be widely adopted by the scientific community and is more easily performed than the original method based on isolated rat glomeruli [13]. Moreover this device not only permits us to study separately the events occurring in podocytes and/or endothelial cells, but also to detected the albumin permeability activity of sera from patients through a simple colorimetric analysis of BSA across the three-layer device (3LD).

MATERIALS AND METHODS

Patient samples

Seven recurrent FSGS (rFSGS), 10 non recurrent FSGS (nrFSGS), and 5 genetic forms of FSGS (gFSGS) sera from adult and pediatric patients (Table 1), who signed an informed consent [M. 02. F (A)], were collected in Adult and Pediatric Nephrology, Dialysis and Renal Transplant Units (Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico-Milan). 8 healthy blood donor sera were collected in the Blood Transfusion Center of the same hospital. rFSGS was diagnosed when renal transplanted (RTx) patients with a histological confirmed diagnosis of FSGS in their native kidneys (5 patients) or with a history of steroid resistant nephrotic

syndrome (SRNS) as the cause of native kidney failure (2 patients), developed significant proteinuria (> 3 g/ day) and/or had histologic finding of FSGS at the renal graft biopsy within the first year after RTx. All the seven rFSGS had been submitted to PP treatment. In each rFSGS patient, pre- and post-PP sera were collected before and immediately after PP treatment.

In 6 of rFSGS cases, the blood sample was collected at the first or second PP therapy, performed immediately after the FSGS recurrence (within first 2 months), while in one patient (subject number 2), transplanted in 2011 and affected by an early FSGS recurrence, who required chronic PP therapy, the blood collection was performed after 6 years of RTx. PPs were performed using albumin in most patients (subjects 1, 2, 4, 5, 6), while plasma was utilized in patients 3 and 7.

Serum samples were processed under sterile laminar flow hood (Heraeus, Hanau, Germany) and divided into small volume aliquots to avoid multiple freeze/thaw cycles and stored at − 80 °C. Before use, sera were thawed on ice immediately before experiment and diluted to 2% with cell culture medium.

Table 1 Patient clinical information

No.	G	Ethnicity	Renal disease in native kidneys	Age at the last Tx, yr	No. of Tx	Diag. post TX	Time of recur. after last Tx, day/ year	Time of sampling day/ year	Mutation	P_{BSA} %
1	F	Caucasian	pFSGS	29	2	rFSGS in 2 allografts	42 d	43 d after Tx	ND	33.6
2	M	Caucasian	pFSGS	31	1	rFSGS	150d	6 y after Tx	Neg	25.5
3	M	Caucasian	pFSGS	32	2	rFSGS in 2 allografts	5 d	2 d after Tx	ND	40.2
4	M	Caucasian	pFSGS	44	2	rFSGS in 2 allografts	60d	62 d after Tx	ND	32.3
5	M	Caucasian	pFSGS	25	2	rFSGS in 2allografts	60d	61 d after Tx	ND	28.9
6	F	Asian	SRNS	10	2	rFSGS in 2 allografts	16d	16d after Tx	ND	22.03
7	F	Caucasian	SRNS	21	1	rFSGS	18 d	20 d after Tx	ND	35
8	M	African	sFSGS DM	57	1	nrFSGS	NA	1 yr after Tx	ND	Neg
9	F	Caucasian	sFSGS HT	45	1	nrFSGS	NA	6 yr after Tx	ND	Neg
10	M	Caucasian	sFSGS HT	50	1	nrFSGS	NA	2 yr after Tx	ND	Neg
11	M	African	sFSGS DM	54	1	nrFSGS	NA	1 yr after Tx	ND	Neg
12	M	Caucasian	uFSGS	10	1	nrFSGS	NA	7 yr after Tx	ND	Neg
13	M	Asian	sFSGS HT	59	1	nrFSGS	NA	68 d after Tx	ND	Neg
14	M	Caucasian	sFSGS HT	58	1	nrFSGS	NA	28 d after Tx	ND	Neg
15	M	Caucasian	sFSGS DM	67	2	nrFSGS	NA	65 d after Tx	ND	Neg
16	M	Caucasian	sFSGS HT	40	1	nrFSGS	NA	317 d after Tx	ND	Neg
17	M	Caucasian	sFSGS DM	54	1	nrFSGS	NA	74 d after Tx	ND	Neg
18	F	Caucasian	gFSGS	15	1	nrFSGS	NA	2 m before Tx	NPHS2 htz Compoud	Neg
19	M	Caucasian	gFSGS	14♦	0	NA	NA	14 yr▲	SMARCAL1—ex3. c.740G>C (p.R247P) htz/ex.4 c.831G>A (p.W277*) htz	Neg
20	M	Caucasian	gFSGS	1 m♦	0	NA	NA	2 yr▲	Homozygous deletion of Exons(12–22) in NPHS1	Neg
21	F	Caucasian	gFSGS	8♦	0	NA	NA	9 yr▲	COL4A4 htz (C.4889C>A) CRB2 htz (C.3078.3093del) ADCK4 (C.187C>T)	Neg
22	M	Caucasian	gFSGS	10♦	0	NA	NA	10 yr▲	NPHS2—c.855_856 delAA p.(Arg286Thrfs*17) Omozigote	Neg

G gender, *Diag* diagnosis, *Tx* renal transplant, *Recu* recurrence, P_{BSA} BSA permeability, *M* male, *F* female, *gFSGS* genetic focal segmental glomerular sclerosis, *nrFSGS* non recurrent FSGS, *pFSGS* primary FSGS, *rFSGS* recurrent FSGS, *sFSGS* secondary FSGS, *uFSGS* undefined FSGS, *SRNS* steroid-resistant nephrosic syndrome, *DM* diabetes mellitus, *HT* hypertension, *NA* non applicable, *Neg* negative, *ND* not determined, *m* month, *yr* year, *d* day; ♦ age at onset, yr, ▲ age of sampling

Cell cultures

Conditionally immortalized human glomerular endothelial cells (HCiGEnC) and conditionally immortalized human podocytes (HCiPodo) (both from University of Bristol, Bristol, UK) were cultured as previously described [17, 18]. Briefly, for propagation, cells were grown at 33 °C and 5% CO_2 in endothelial growth medium 2-microvascular (EGM2- MV, Lonza, Walkersville, MD USA) containing 5% fetal calf serum (FCS) and growth factors as supplied, excepting vascular endothelial growth factor (VEGF) or in RPMI-1640 medium containing 10% Fetal bovine serum (FBS), 5 µg/ml transferrin, 5 ng/ml sodium selenite, 0.12 U/ml insulin, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Sigma Aldrich, Milan, Italy). HCiGEnC and HCiPodo were utilized within 35 and 20 passage respectively.

In-vitro model of glomerular filtration barrier (GFB) and assessment of permeability

The in vitro model of GFB is assembled according to a modified previously described methodology [19]. Briefly, using the Millicell hanging cell

culture inserts with Poly- ethylene Terephthalate (PET) microporous (1 µm diameter) membrane (Millipore, Milan, Italy) coated on both sides with collagen type IV (Sigma), 75,000 endothelial cells are seeded on the lower side of the membrane and allowed to adhere to it for 4 h; then the endothelial cells are cultured in EGM2-MV medium containing 5 ng/ml of VEGF (Sigma) at 37 °C and, after 3 days, 65,000 podocytes are seeded on the upper side of the membrane and cultured by their own medium. Podocytes and endothelial cells are co-cultured in their respective medium for an additional 5 days before experiment. To assess albumin permeability, after carefully washing 3LD with PBS, RPMI-1640 only and RPMI-1640 plus 40 mg/ml BSA are added in the upper (podocyte) and lower (endothelial) compartment respectively. After 2 h incubation, the concentration of BSA is assessed in the upper compartment and taken as the basal level (BSAb). Thereafter, both compartments are emptied, washed again, and the lower compartment is filled with the experimental solution (i.e.: adriamycin at concentration of 0.8, 1.4 and 2 µM; or 2% serum to be tested, added to the medium), while the upper compartment with podocytes' medium. The 3LD is then incubated for 24 h with

adriamycin or 48 h with patient's serum. At the end of the incubation, the two compartments are emptied again, washed and BSA permeability assay is repeated as before (BSAexp). The changes in BSA permeability are expressed as: (BSAexp-BSAb)/ BSAb %. BSA has been measured by spectrometry using the DC protein assay kit (Bio-Rad, Milano, Italy). Each patient serum and each ADR concentration were tested in 3 differ- ent devices contemporarily and the results are expressed as mean ± SD. Healthy control experiments were obtained by incubating the device with sera from 8 healthy blood donors in triplicate (Fig. 1a).

Immunofluorescence

After removing the membrane from the insert using a sharp scalpel and placing it onto a microscope slide, cells to be studied (pocodytes or endothelial cells) were washed and fixed with 10% neutral buffered formalin for 10 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS. Sequentially, the cells are incubated overnight at 4 °C with the primary antibody (rabbit anti-CD31, 1:100, Abcam, Cambridge, UK; rabbit anti-synaptopodin, 1:50 and rabbit anti-podocin, 1:50, Sigma), followed by the addition of the appropriate fluorescent-labelled

secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, 1:200 Invitrogen, S. Giuliano Milanese, Italy) and 4',6-diamidin-2-fenilindolo (DAPI, 1:1000, Sigma). The specificity of the primary antibody was demonstrated by the lack of staining after substituting the primary antibody with proper control immunoglobulin (rabbit IgG isotype control, Zymed, California, USA. Images were acquired by AxioObserver microscope and recorded by AxioVision software 4.8 (all from Zeiss, Milan, Italy). The parameters for image acquisition were first established with cells exposed to healthy sera and then were applied to all samples treated with patients' sera. The percentage of the membrane surface covered by cells and mean fluorescence intensity (FI) from 10 images of each sample were analysed by Image J software. The results were expressed as percentage of FI of healthy controls.

Statistical analysis

Data are presented as mean ± SD. At least three replicates were performed for each sample. Two-tail Student's t test was used for analysis of data when two groups of data were compared. ANOVA test was applied

when comparing more than 2 groups of data. P values <0.05 were considered significant.

RESULTS

Assembly of the in-vitro model of GFB

Based on our previously published method [19], we successfully substituted the immortalized mouse podocytes and mouse capillary endothelial cells with the condition- ally immortalized human podocytes and human glomerular endothelial cells. As shown by Fig. 1b, immunofluorescence staining of cells grown on the membrane with anti-CD31, a specific endothelial cell marker (left) and anti-podocin, a specific podocyte marker (right) demonstrated the homogeneous formation of the cell monolayer on the two different sides of the membrane (average area covered by endothelial cells and podocytes 91 ± 4.9% and 89.9 ± 1.9%, respectively) (Fig. 1b).

Validation of the albumin permeability assay

For validation of the albumin permeability assay, we used adriamycin to induce cell damage, which in turn must result in increasing the BSA

passage across the three-layer structure. In physiological conditions (medium alone), BSA permeability was 1.3 ± 4.1%. Incubation with adriamycin at concentration of 0.8, 1.4 and 2 µM for 24 h induced a statistically significant and dose dependent increase of BSA permeability as compared with cells incubated with medium alone (18.95 ± 2.05%, *P < 0.05; 31.5 ± 1.98%, **P < 0.01;

41.75 ± 5.59%, **P < 0.01 respectively) (Fig. 1c). Immunofluorescence Staining showed that treatment with ADR induced both endothelial cells and podocytes damages. As seen in supplementary Fig. 2, there was a remarkable decrease of CD31 expression on endothelial cells, while podocytes presented not only the decrease of podocin and synaptopodin immunostaining intensity but also the changes of their distribution (podocin: from cytoplasmic and mem- brane expression to weak cytoplasmic expression; synap- topodin: from cytoplasmic, along acting filaments and cell processes expression to cortical, near to the cell membrane expression.).

Effects of sera from patients with diverse forms of FSGS on the GFB

To check if the in vitro GFB model can be used to detect the circulating permeability factors, we added in the endothelial cell compartment the sera from 7 patients with early post- transplant rFSGS, 10 from nrFSGS, 5 from pediatric patients with gFSGS and 8 from healthy blood donors (HBD). As shown in Fig. 2a, all rFSGS samples increased significantly the BSA permeability (27% ± 5.27), while the nrFSGS, gFSGS and HBD samples did not (− 2.1% ± 6.2; 3.0% ± 3.5; 0.4% ± 5.2 respectively). The differences between the effects on BSA permeability of rFSGS vs nrFSGS, gFSGS and HBD sera were statistically significant (**P < 0.01).

To verify the reproducibility of BSA permeability assay, serum from 3 patients with rFSGS was used in three independent different experiments at 2 weeks distance of each other, each experiment was performed in triplicate at the same time. As shown in Fig. 2b, the variation among experiments was not statistically significant (Sample1: 40.3 ± 7.2%; 34.7 ± 6.5%; 32.2 ± 0.9%; ANOVA P = 0.39. Sample 2: 23.6 ± 6.3; 23.8 ± 1.1; 20.1 ± 3.3; ANOVA P = 0.69. Sample3: 20.6 ± 1.5; 17.9 ± 2.2; 20.8 ± 1.1; ANOVA P = 0.22).

Effects of pre- and post- plasmapheresis sera on the GFB

To verify if PP can change the GFB permeability to albumin, we compared the effects of sera collected pre- and post PP from the 7 rFSGS patients. As shown in Fig. 2c, incubation with pre-PP sera induced a consistent increase in BSA permeability, but this effect was no longer evident with sera collected immediately after PP treatment. (26.1 vs − 9.5; 23.6 vs − 9.4; 32.3 vs − 6.7; 20.6 vs − 6.0; 28.7 vs -0.63; 22 vs 1.5; 22.6 vs 1.7). The differences between pre- and post PP sera were statistically significant ($*P < 0.05$; $**P < 0.01$).

Immunofluorescence staining

Then we assessed if the changes in BSA permeability induced by rFSGS sera were associated with any change in the expression of some molecules specific for either podocytes or endothelial cells. Figure 3a showed that sera from the majority of patients with rFSGS (5 out of 7) induced an evident decrease of Platelet endothelial cell adhesion molecule (PECAM-1 or CD31) expression on endothelial cells, while sera from nrFSGS and gFSGS did not; the normalized mean fluorescence intensity (% of healthy sera) was: rFSGS, 47.7% ± 34.6; nrFSGS, 100% ± 9.7; gFSGS, 98.1% ± 12.5 respectively) (Fig. 3b). Staining with anti-

synaptopodin presented the expression along acting filaments and cell processes (arrows) (Fig. 4a–d, i) and anti-podocin showed a filamentous cytoplasmic, cell surface and cell processes distribution (arrows) (Fig. 4e–h, j). Incubation on endothelial cells with sera from different patients, which is presumably the initial event occurring in vivo, didn't change the intensity or the distribution of these molecules.

DISCUSSION

We successfully assembled a system of HCiPodo and HCiGEnC co-culture which mimics the in vivo GFB struc- ture (Fig. 1a, b), whose permeability to BSA was highly increased in a dose-dependent way by adding adriamycin (Fig. 1c), a chemotherapeutic nephrotoxic drug already utilized for inducing chronic proteinuric nephropathy in rodents [20, 21]. Although some clinical aspects might predict recurrence of FSGS after RTx [2], a reliable and repeatable laboratory methodology could be useful in the clinical practice. Testing the sera drawn from 7 patients with rapid rFSGS after RTx (from 5 to 150 days), we found a significant increase of BSA permeability in all these patients (Fig. 2a). The genetic or secondary

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forms of FSGS are not expected to recur at all (genetic) or need a long time before recurrence (metabolic) after RTx [4]. In fact, we were not able to find any evi- dent change in BSA permeability with sera collected from those patients, some of whom received a RTx, but did not recur, suggesting a high specificity of the test for the rFSGS (Fig. 2a).

The repeatability of our method was confirmed by the overlapping results obtained by three independent experiments performed in the same serum sample (Fig. 2b).

PP has been reported to have beneficial effects in some patients with rFSGS [5, 8, 9, 22]. In fact, sera collected before PP in 7 rFSGS patients induced a consistent increase in BSA permeability; these effects were no longer evident when 3LD was challenged with sera collected immediately after PP (Fig. 2C). These results reinforce the suggestion of the presence of cPFs in most patients with FSGS who recurred after RTx [4–12].

Although it has long been hypothesized that rFSGS is the consequence of podocyte damage due to various pathogenic CPFs [23–25], the initial events occurring when the glomerular endothelial cells come in contact with the hypothesized cPFs are still unknown. Figure 3 showed that 48 h

incubation on endothelial cells with the majority of rFSGS patients sera (5 of 7) induced a noticeable decrease of CD31, while at the same time point the podocytes still presented a normal expression of synaptopodin (Fig. 4a–d,

i) and podocin (Fig. 4e–h, j). CD31 is a cellular adhesion and signalling receptor that is highly expressed at endothelial cell–cell junctions and have implications in the maintenance of vascular barrier integrity [26]. More than 15 years ago, Ferrero and colleagues reported that PECAM-1-specific monoclonal antibody augments transit of 125I-labelled albumin across endothelial cell junctions, both in cultured cells and in mice [27]. Our results suggest that the hypothesized cPFs might trigger the pathological events first by damaging the endothelial cells, followed by an increase of

GFB permeability and consequently a podocyte damage. It is worth underlining that not all sera from our rFSGS patients (2 of 7) induced a decrease of CD31 expression, implying that the possible mechanism(s) may well be characterized by different pathogenic pathways. So, the

purpose for future research could be that of better understanding and defining these possible different pathways.

In conclusion, these preliminary data suggest that the assessment of BSA permeability by our in vitro 3LD could be a valuable method for predicting the recurrence of FSGS after RTx. Moreover this device could be a worthwhile instrument to study separately the biology of podocytes and endothelial cells and their cross-talking during physiological and pathological processes.
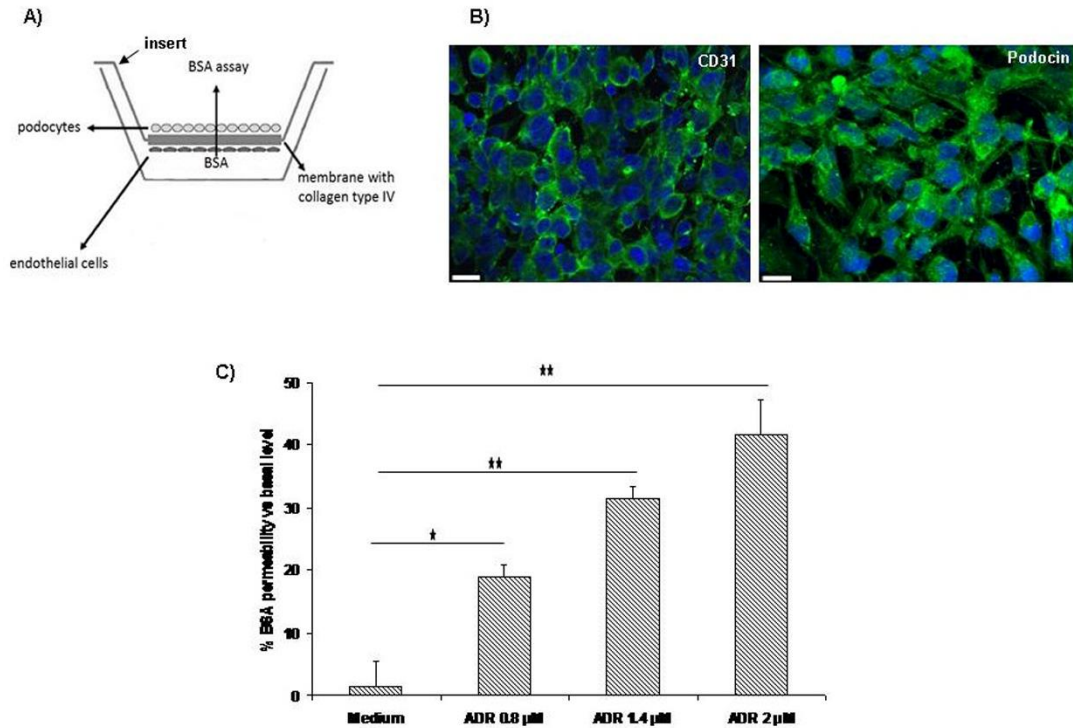
Fig. 1. Co-culture assembly and BSA permeability after adriamycin (ADR) treatment. A) . Schematic drawing of the preparation of the in vitro GFB and BSA permeability assay. HCiGEnC and HCiPodo were grown on the opposite of the PET membrane coated both side with Collagen type IV. The three-layer structure divided the system into a lower (endothelial) and upper (podocyte) compartment. For BSA permeability assay, the lower compartment was filled with 40 mg/ml of BSA in cell culture medium while the upper compartment with medium alone. **B)** Immunofluorescence staining with anti- CD31 (left, green), anti-podocin (right, green) and 4', 6-diamidin- 2-fenilindolo (DAPI, blue) on cells grown on the membrane. Scale bar: 20 μm. **C)** Incubation on endothelial cell compartment with ADR at different concentration for 24 h induced a dose-dependent increase of BSA permeability. *P < 0.05, **P < 0.01. Each bar represents the mean ± SD of triplicate experiments.
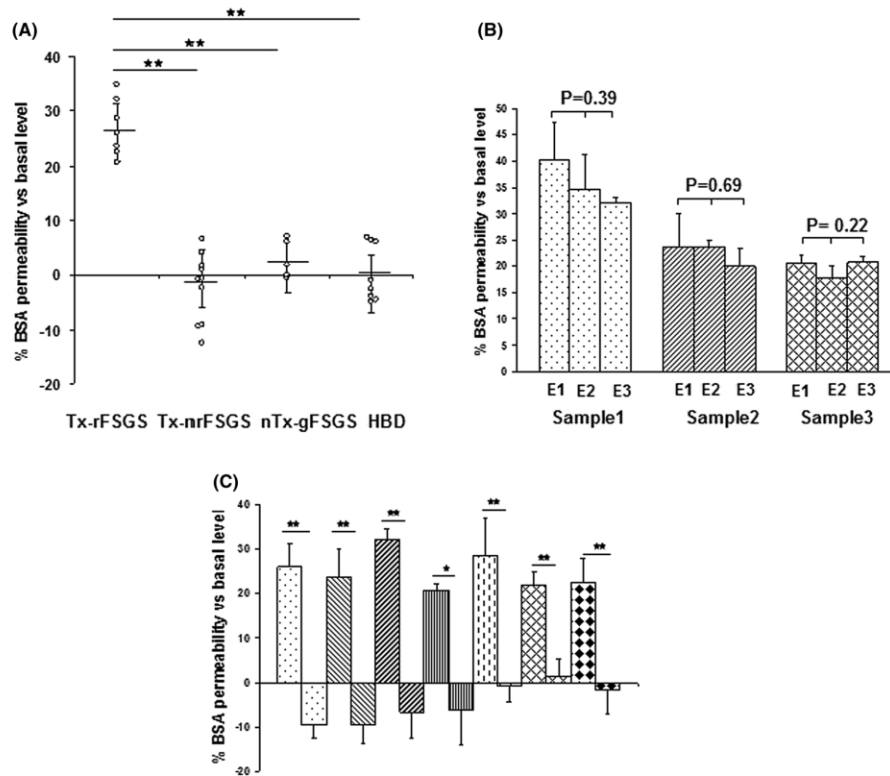
Fig. 2. Effect of sera incubation on BSA permeability. a Effect of sera from patients with diverse forms of FSGS on BSA permeability. Incubation on lower (endothelial) compartment with 2% of sera from recurrent (n = 7), non recurrent (n = 10), genetic (n = 5) FSGS and healthy blood donors (n = 8) for 48 h. BSA permeability was con- sistently increased in all the 7 rFSGS patients, without any relevant increase in the other cases. Tx-rFSGS, renal transplantation with recurrent FSGS; Tx-nrFSGS, renal transplantation without recurrent FSGS; nTx-gFSGS, non renal transplantation with genetic FSGS; HBD, healthy blood donor. **b** Reproducibility. Variation of BSA permeability among three independent experiments with serum from each of 3 rFSGS patients. The results, expressed as percentages of basal condition, demonstrate no statistically significant difference. E1, E2, E3: experiment 1, 2, 3. **c** Effect of pre- and post-plasmapheresis sera on BSA permeability. Incubation of sera collected immediately before and after PP showed the complete disappearance of the effect of pre-PP sera on albumin permeability by PP. All results were expressed as percentages of basal condition. Each bar represents the mean ± SD of triplicate experiments. *P < 0.05, **P < 0.01
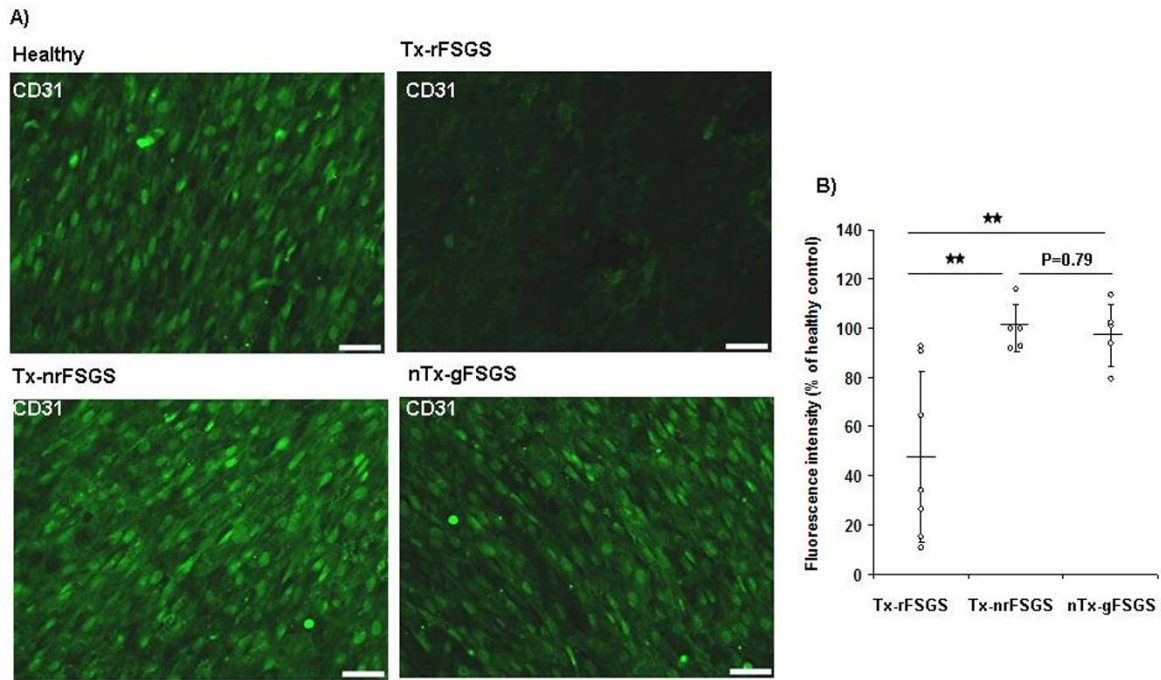
101

Fig. 3. Immunofluorescence staining on endothelial cells after sera incubation.
A) Representative images of CD31 expression. After 48 h incubation of endothelial cell compartment with 2% sera from recurrent (n = 7), non recurrent (n = 5), and genetic (n = 5) FSGS patients, CD31 expression was considerably decreased in 5 out of 7 rFSGS patients, while there were no evident change in all the others. **B)** Semi quantitative analysis of the mean fluorescence intensity (FI) showed that sera from majority of rFSGS patients decreased the mean FI, while sera from nrFSGS and gFSGS did not. The results were expressed as percentages of FI of healthy control. Scale bar: 50 μm.
$**P < 0.01$
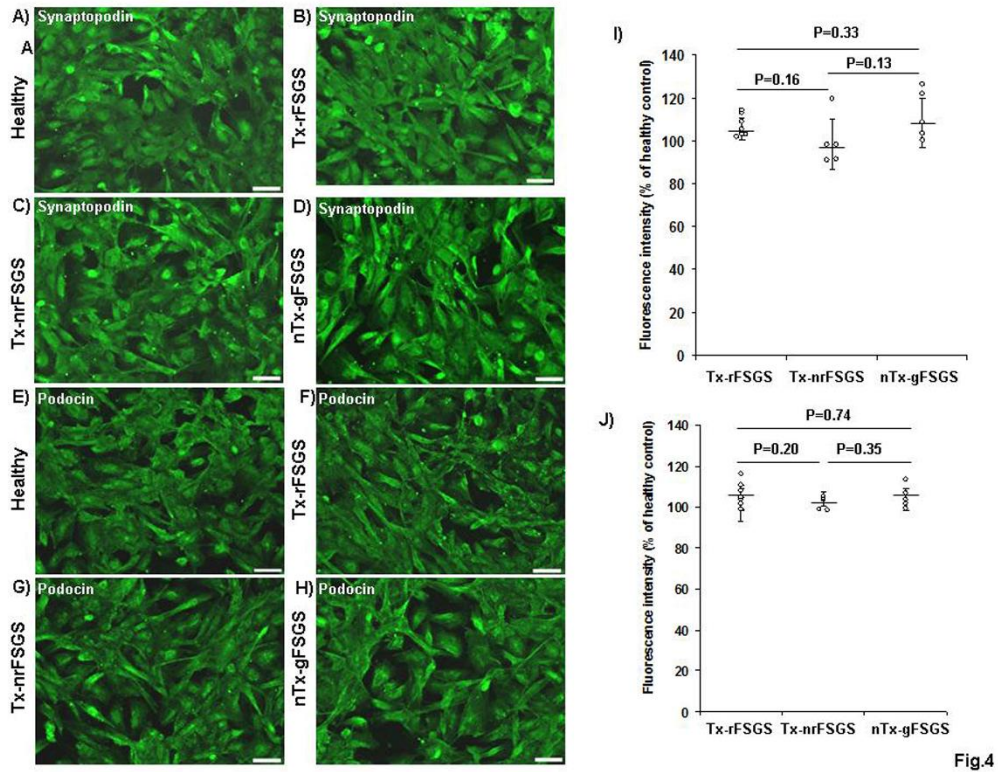
Fig. 4. Immunofluorescence staining on podocytes after sera incubation. Representative images of expression of synaptopodin (a–d) and podocin (e–h). After 48 h incubation of the endothelial cell com- partment with 2% sera from recurrent (n = 7), non recurrent (n = 5), genetic (n = 5) FSGS patients, the intensity as well as the distribution of synaptopodin and podocin were not changed. Semi quantitative analysis of FI of synaptopodin (i) and podocin (j) showed that there were no significant variations among the different groups. Scale bar: 50 μm

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CHAPTER 4

PRELIMINARY PROTEOMIC ANALYSIS OF SERA FROMSTEROID-RESISTANT NEPHROTIC SYNDROME PATIENTS RECURRING AFTER RENAL TRANSPLANTATION AND ABLE TO INDUCE ALBUMIN PERMEABILITY USING AN IN-VITRO MODEL OF GLOMERULAR FILTRATION BARRIER

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ABSTRACT

Steroid resistant Nephrotic Syndrome (SRNS) is a glomerular disease characterized by a poor long-term renal outcome. Genetic SRNS account for around 30% of cases and are associated with mutations in genes encoding for components of the glomerular filtration barrier (GFB). The pathogenesis of the disease is unknown in the other cases. A so-called permeability factor has been advocated but never identified and it is thought to mediate relapses after renal transplantation. We have previously described a novel in vitro model of GFB composed by three-layer device (3LD) and able to assess albumin permeability induced by sera from SRNS recurring after renal transplant.

Here we performed a preliminary proteomic analysis of sera from SRNS patients recurring after renal transplantation, tested positive on the GFB model and compared the results with sera obtained after plasmapheresis and from genetic SRSN children.

INTRODUCTION

Steroid Resistant Nephrotic Syndrome (SRNS) is a glomerular disease characterized by a poor long-term renal outcome in case of resistance to steroid treatment[1,2]. The pathogenesis of the disease has been characterized in only about 30% of cases, in which mutations in genes encoding for components of the glomerular filtration barrier (GFB) can be identified[3]. The underlying mechanisms of non-genetic SRNS remain poorly defined[4]. Although evidence suggests the presence of an immune-related circulating factor[5–8], responsible for glomerular barrier dysfunction, increased albumin loss in the urine, and relapse after renal transplantation any previous attempts to unravel these mechanisms and to identify the so-called "permeability factor" have been inconclusive[9]. Previous studies were indeed flawed by the absence of a homogeneous group of patients, with the inclusion of patients with genetic and non-genetic diseases, the absence of a reliable and reproducible in vitro model to test the biologic effects of patient sera and obstacles preventing the in depth characterization of patient sera.

We have previously described a novel in vitro model of GFB composed by three-layer device (3LD) and able to assess albumin permeability induced by sera from SRNS recurring after renal transplant (Chapter 3).

Here we performed a preliminary proteomic analysis of sera from SRNS patients recurring after renal transplantation, tested positive on the GFB model and compared the results with sera obtained after plasmapheresis and from genetic SRSN children.

MATERIALS AND METHODS

Patient samples

Patients were recruited at the Pediatric Nephrology Dialysis and Transplant unit of Fondazione IRCCS Ca'Granda OSpedale Maggiore Policlinico – Milano. Sera were collected from all patients with SRSN relapsed after renal transplantation and undergoing plasmapheresis and from patients with genetic SRNS. Steroid resistance was defined as persistence of nephrotic range proteinuria following 4 weeks of daily 60 mg/sqm prednisone therapy. Post-transplant disease recurrence, was

defined as an otherwise unexplained persistent nephrotic range proteinuria after renal transplantation, when rejection was excluded.

The in-vitro model of GFB was assembled as previously described [CHAPTER 3, submitted to Journal of Nephrology].

Samples were collected from all recruited patients and controls at any time during the clinical follow-up. Samples were centrifuged to obtain sera and frozen immediately at -80°C. Sera will be stored until the subsequent evaluation on the GFB in vitro model.

Protein Extraction, Quantification and analysis

Collected sera were suspended and supplemented with a mix of protease inhibitors and nucleases according to the manufacturer's instructions.

The supernatants were collected and the protein concentration in the samples was determined using Bradford assay (Bio-Rad protein assay, Bio-Rad, Hercules, CA, United States). Absorbance was measured using a spectrophotometer (Gene Quant 100, GE Healthcare) at 595 nm. The extracted proteins were stored at −80∘C until use. Desalination was performed by filtration (Amicon 3K device). Samples were depleted of

human serum albumin (HAS) and antibody components by agarose resins (Thermo Scientific Kit).

Proteins were quantified again (Bradford assay) after desalination and depletion, then digested by trypsin. Peptides were quantified after digestion.

Peptides were pooled in groups according to the different type of samples collected (**pool PRE** = samples of recurrent SRNS before plasmapheresis; **pool POST** = samples of recurrent SRNS after plasmapheresis, pool **GENETIC** = samples of genetic SRNS).

Peptides were separated by Ultra Performance Liquid Chromatography (UPLC) and analyzed by High Definition–Ion Mobility Separation-MSE (HDMSE). Experiments were replicated 3 times. Data were analyzed by PLGS v 3.0.3 in order to identify the proteins contained in the samples and to analyze the differential expression in the 3 groups (Expression Analysis).

RESULTS

A total of 5 patients with recurring SRNS undergoing plasmapheresis and 3 patients with genetic SRNS was identified. For each patients with recurring SRNS a sample pre- and post-plasmapheresis was collected. A total of 13 samples were analysed. 5 samples of recurring SRNS before plasmapheresis (group PRE), 5 samples of recurring SRNS after plasmapheresis (group POST), 3 samples from genetic SRNS (group GENETIC).

Figure 1 shows the Venn diagram summarizing the protein content of three groups.

Fig. 1. Venn diagram showing the different protein profile of each group.
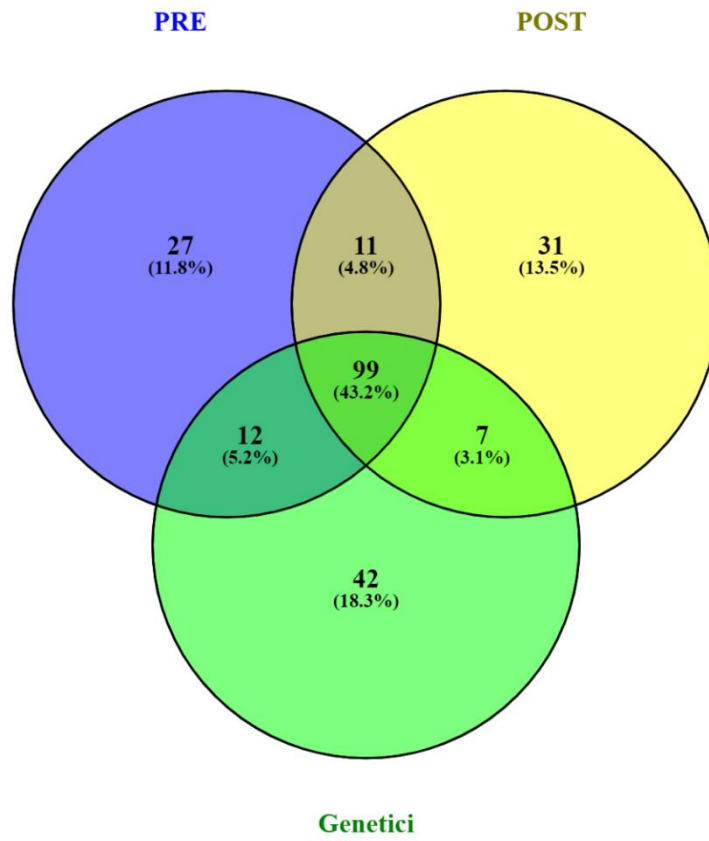
149 proteins were identified in the group PRE, 148 in patients from group POST and 160 in the GENETIC group. 99 proteins are shared by all groups, 11 proteins are shared by PRE e POST, 12 proteins can be identified in the groups PRE and GENETIC, 7 proteins are shared by POST and GENETIC.

In contrast and more interestingly, 27 proteins are only present in PRE samples, 31 in the POST samples and 42 in the GENETIC samples.

The protein-protein interactions between identified proteins was analyzed by (Search Tool for the Retrieval of Interacting Genes/Proteins) STRING analysis.

Figure 2A, 2B and 2C show the different protein pattern in the 3 groups. As shown in the figure, each group show different protein-protein interactions, with different metabolic profile.

Fig. 2. Protein to protein interaction in the 3 different groups according to STRING analysis. A, pre-plasmapheresis, B post-plasmapheresis, C genetic SRNS
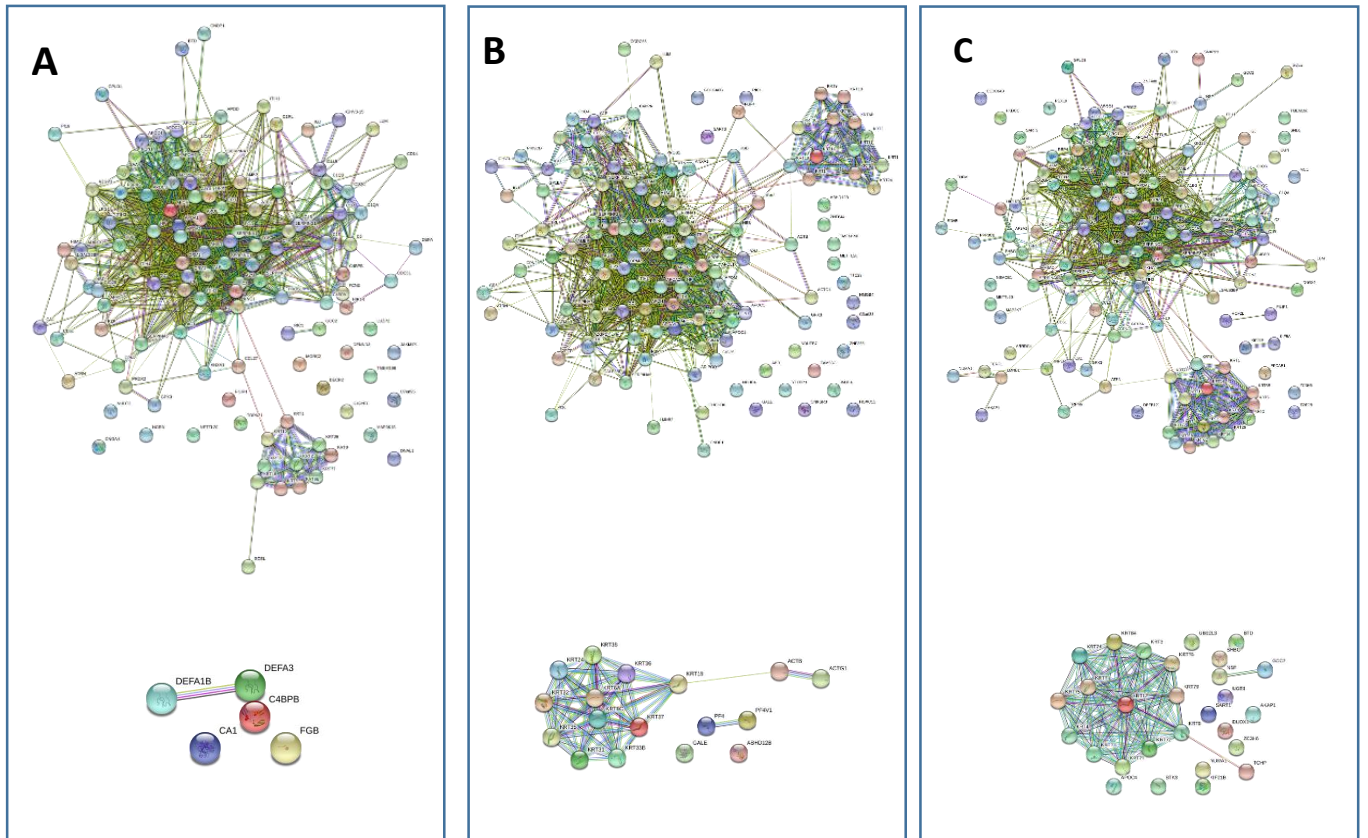
The functional analysis of identified protein content performed through

Kyoto Enciclopedia of Genes and Genomes (KEGG) database is shown in

figure 3. The Heat Map of protein profiles shows the involvement of
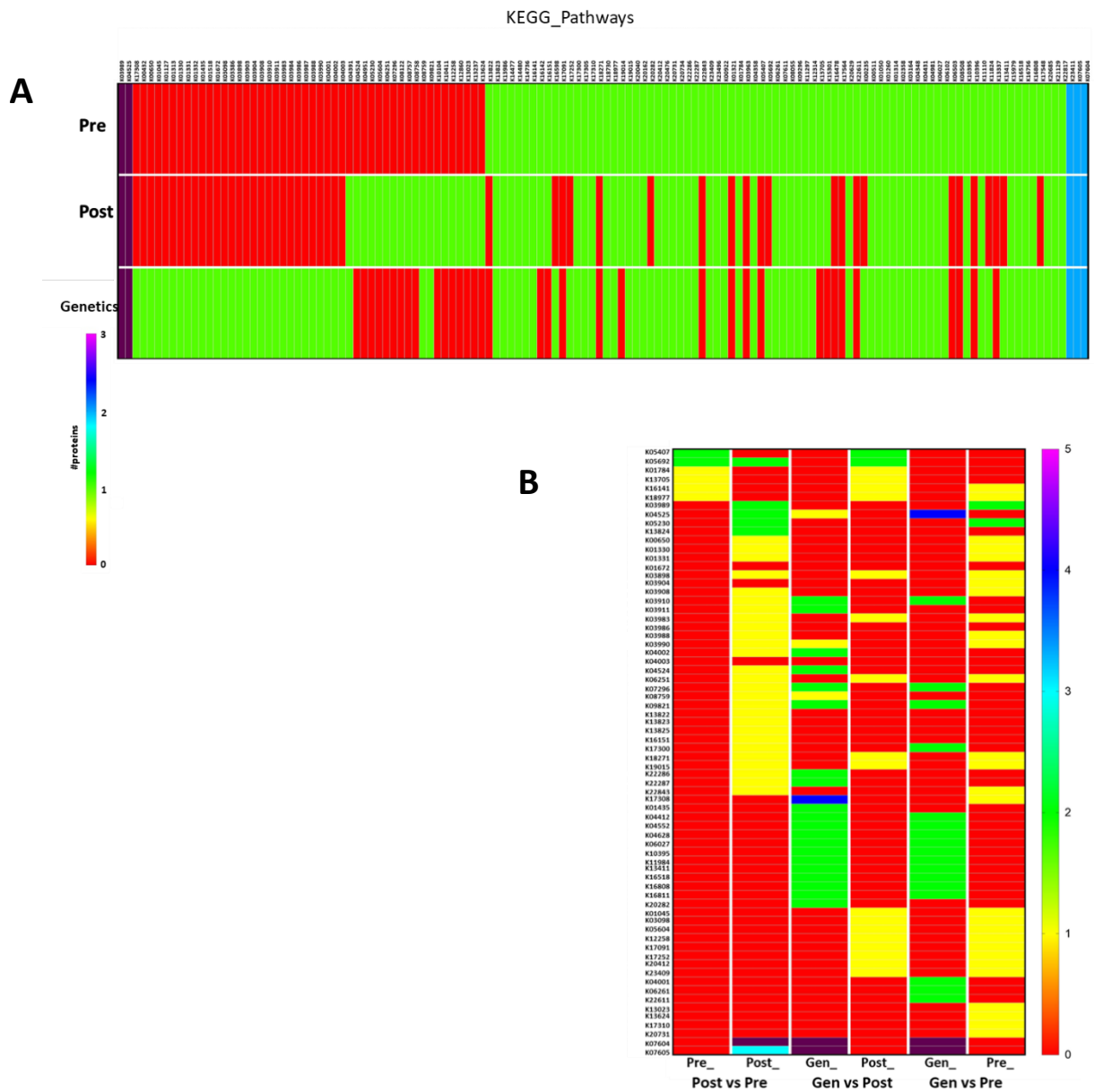
different biological pathways for each group.

Fig. 3. Heat maps of KEGG identifiers. A, differential analysis of each group. B, pairwise comparison

DISCUSSION AND PRELIMARY CONCLUSION

The mechanisms underlying non-genetic SRNS are poorly understood.

Evidence suggests the presence of a circulating permeability factor, never

identified. Here we performed a preliminary proteomic analysis of sera

from SRNS patients relapsed after renal transplantation. Sera were

selected through an innovative GFB model and showed the ability to

induce albumin permeability on a three-layer device reproducing GFB

(group PRE). Proteomic analysis of these samples were compared with

those of same patients, after treatment with plasmapheresis (group POST)

and with those of genetic patients (group GENETIC). Both POST and

GENETIC samples tested negative on the 3LD and had no effect on

albumin permeability. We were able to demonstrate that protein content

is different in the three groups with specific proteins profiles for each

group. Through STRING and KEGG analysis we were also able to show the

activation of different protein-protein interactions and metabolic

patterns in the 3 groups.

Those preliminary results indicated the possibility to identify, through a

proteomic analysis, proteins and pathway involved in the recurrence of

SRNS after transplantation that can be removed with plasmapheresis and that are not involved in the genetic forms of SRNS.

The specific identification of those pathway will shed light on the pathogenesis of non-genetic SRNS.

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CHAPTER 5

MIF PLASMA LEVEL AS A POSSIBLE TOOL TO PREDICT STEROID RESPONSIVENESS IN CHILDREN WITH IDIOPATHIC NEPHROTIC SYNDROME

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ABSTRACT

Purpose Idiopathic nephrotic syndrome (INS) is the most frequent form of childhood nephrotic syndrome. Steroids represent the best therapeutic option; however, inter-individual differences in their efficacy and side effects have been reported. To date, there is no way to predict patients' resistance and/or dependence. Alterations in the cytokine profile of INS patients might contribute to proteinuria and glomerular damage and affect drug sensitivity. Methods The cytokine plasma levels were measured in 21 INS children at diagnosis to investigate the association among cytokines pattern and clinical response. Patients were selected on the basis of their clinical response: 7 steroid sensitive (SS), 7 dependent (SD), and 7 resistant (SR). Significant results were then analyzed in 41 additional pediatric INS patients. Results Within the 48 cytokines analyzed, macrophage migration inhibitory factor (MIF) was a good predictor of steroid response. Indeed, SR patients showed significantly higher MIF plasma levels compared with all others (p = 0.022; OR = 4.3, 95%CI = 1.2–25.4): a cutoff concentration of MIF > 501 pg/ml significantly

discriminated SR patients (sensitivity = 85.7%, specificity = 71.4%). On the contrary, SD patients showed lower MIF plasma levels compared with others (p = 0.010; OR = 0.12, 95%CI = $9.2 \times 10^{-3} - 6.7 \times 10^{-1}$). Significant results were confirmed in the entire cohort. Conclusions Our comprehensive cytokine analysis indicates that assessing MIF plasma levels at diagnosis could predict response to glucocorticoids in children with INS.

Keywords: Idiopathic nephrotic syndrome · Glucocorticoid response · Cytokines · Pediatrics

INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most common pediatric primary glomerular disease, affecting 16–17 per 100,000 children between the ages of 2 and 8 years, with a peak of incidence between 3 and 5 years. INS is characterized by an increase in permeability of the capillary walls of glomeruli, leading to proteinuria.

Glucocorticoids (GCs) are the first-choice drugs and in- duce remission in 85–90% of children with this disease; how- ever, 10–15% of patients are steroid resistant (SR) [1–3]. Moreover, despite initial complete remission, almost 50% of patients show recurrence of the proteinuria and are classified as steroid dependent (SD): these patients, after a prolonged steroid therapy, with the possibility of severe adverse effects, often need to switch to other immunomodulating or immunosuppressive drugs. Responsiveness to steroids is the most important prognostic factor and patients that do not respond to therapy are subjected to aggressive treatments and often develop several complications and side effects. The mechanisms involved in GC dependence and resistance are scarcely

under- stood and studies considering GC pharmacodynamics and pharmacogenetics have been performed without conclusive results [4–6].

Various studies have demonstrated the involvement of cytokines in the occurrence of proteinuria that characterizes INS [4, 7, 8]. Relapses are quite frequent in this disease, and are often triggered by viral infections, which result in the release of cytokines, causing immunoregulatory imbalances [9]. Cytokines levels and other markers of immune activation have been used in the diagnosis of different diseases, also for prognostic purposes [1, 10]. However, in INS patients, changes in various plasma cytokine profiles prior to and after steroid treatment have not been extensively examined.

In the present study, we have investigated the plasma levels of a panel of cytokines in patients with INS undergoing steroid treatment, in order to elucidate whether there is any specific cytokine that could serve as biomarker to predict treatment efficacy.

METHODS

Study design and population

One hundred eighty-four pediatric patients were enrolled be- tween August 2011 and February 2014 in the prospective multicenter trial for INS treatment from the Italian pediatric nephrology network NEFROKID (ClinicalTrials.gov identifier: NCT01386957). Approval was obtained from the ethics committees of all the participating centers and parents gave written informed consent before enrollment in the study. One hundred twenty two patients were excluded because of the following reasons: non-adherence to the protocol (5 patients), written informed consent for the biological part of the study could not be obtained (1 patient), onset of the disease at weekends or holidays and blood samples could not be sent to the collecting center in Trieste (52 patients), the sample volumes were insufficient or not correctly shipped (32 patients), other reasons (32 patients). Therefore, 62 patients were enrolled in the present study; this group of patients was representative of the whole cohort with regard to demographical and clinical characteristics [11].

All patients were treated with a common therapeutic protocol consisting of prednisone at a dose of 60 mg/m2/ day for either 4 or 6 weeks, depending on whether time to remission was < or ≥ 10 days, respectively, and tapering of steroids over 16 weeks. Remission was defined as the disappearance of proteinuria for at least 3 consecutive days. Total prednisone dosage was 2828 mg/m2 in patients who went into remission within 10 days and 3668 mg/m2 in the others. Patients were divided into 3 groups, defined as in the therapeutic protocol of the clinical trial NCT01386957, according to their clinical response: absence of remission despite steroid therapy (steroid resistant SR), steroid dependent (SD) patients, presenting two relapses during treatment or within 14 days of discontinuation of therapy or presenting two or more relapses within 6 months of initial response or four or more relapses in any 12-month period and steroid sensitive (SS), with less than two relapses within 6 months of initial response.

The first 7 consecutive patients for each group of steroid response (SR, SD and SS) were characterized for their plasma cytokine levels. Significant results were then investigated in the entire cohort of 62 patients and

analyzed also in a sub- group of healthy pediatric subjects without any acute or chronic infectious disease, any clinically significant disorder, and any medication with known influence on immunological factors.

Sample and cytokine measurements

Peripheral venous blood, anticoagulated with EDTA, was collected at the onset of the disease and sent refrigerated within 24 h to the Department of Life Sciences at the University of Trieste; plasma was separated as described [11] and stored at

− 80 °C for measurement of 48 cytokines. Each sample (20 µl) was analyzed by magnetic beads suspension array using the Bio-Plex Pro Human Cytokine 21- and 27-plex panels (Bio- Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The 21-plex panel and 27-plex panels are described in the Supplementary Materials. A Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA, USA), with Bio-Plex Manager 6.0 software (Bio-Rad Laboratories, Hercules, CA, USA) was used. Detection ranges for each cytokine, with the lowest concentration of analyte that can be detected (LOD), and the uppermost and lowest

quantifiable concentration (respectively ULOQ and LLOQ) are reported in the Supplementary materials. The method was validated in the laboratory and tested for recovery.

Genetic analysis

For the most significant cytokine associated in this study with steroid response, MIF, a common genetic variant G-173C (rs755622) was examined. Total genomic DNA was isolated from peripheral blood by using a commercial kit (Gene Elute Blood Genomic DNA kit, Sigma Aldrich, Milan, Italy) and the MIF polymorphism was determined by TaqMan® SNP genotyping technologies (Applied Biosystems, Bedford, UK) on an ABI7900 HT sequence detection system device.

Statistical analysis

For statistical purposes, for cytokines with concentration out of the detection ranges (Supplementary Table 1), an arbitrary value was defined, corresponding to half of the minimum or double of the maximum

detectable concentration, according to whether the samples were below or above the sensitivity range of the assay. Initially, 7 patients for each response group were analyzed, selected on the basis of sample availability for resistant patients, and each resistant case was matched with an SS and SD patient. Statistical comparisons were done considering each response group against the other two grouped together, to increase statistical power. This approach provided a statistical power sufficient to identify an effect of large magnitude (non- overlap between the two distributions of at least 65%) with a p value threshold of 0.05 and a power of 0.80 [12]. Univariate analysis was performed by logistic regression models testing any possible association between cytokine levels, MIF polymorphism and clinical response. Multivariate analyses were performed by logistic regression models combining independent variables significant in the univariate analysis. The best cutoff value to determinate patients' response using cytokine concentration was identified by receiver operating characteristic (ROC) curves. Sensitivity and specificity of the selected cutoffs were analyzed. Fisher's exact test was applied to support the significance of these cutoff values. Statistical analyses were

performed using the software R version 3.2.4. P values lower than 0.05 were considered statistically significant. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated for all the analyses.

RESULTS

Patients

Demographical characteristics of the 62 enrolled patients are reported in Table 1.

Table 1 Demographic characteristic of the 62 patients. SR, steroid resistant; SD, steroid dependent; SS steroid sensitive

All patients	SR ($n=9$)	SD ($n=24$)	SS ($n=29$)
Male, n (%)	6 (66)	14 (58)	21 (72)
Age in years, median (range)	8.5 (2–17)	3.2 (1–13)	4.3 (2–11)

A statistically significant difference in age was observed between SR and SS+SD patients, the former group being older (univariate logistic regression, p value = 0.024, OR = 1.33, 95% CI = 1.03–1.92) while SD patients and SS were not different compared with other patients; gen- der distribution was not different among patients' groups. The first analyses

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were performed in a group consisting of 21 patients that were the first 7 consecutively enrolled subjects for each group of steroid response (SR, SD and SS); this group was representative of the entire group of patients in terms of age and sex distribution.

Plasma cytokine measurements

Using the Bioplex assays, in 21 patients (7 SS, 7 SD and 7 SR), we measured the concentration of 48 soluble plasma immune mediators at baseline, before initiation of steroid treatment. Cytokine concentrations are presented in Supplementary Table 1. No measurable value for MCP-3, IL-15, IL-12p40, IL-3, IL-1α, TNF-β, and MCP-1 was obtained; therefore, these cytokines were excluded from data analyses.

Baseline plasma cytokines in SR patients

Given the clinical interest in recognizing SR patients, the association between cytokine levels and clinical response was analyzed by comparing SR with the other patients. Univariate logistic regression analysis showed significantly elevated con- centration of MIF (mean concentration 759.7

pg/ml in SR vs 414.1 pg/ml in SD + SS, p = 0.022, OR = 4.3, 95% CI = 1.2–25.4)

and SCGF-β (mean 33.5 pg/ml in SR vs 21.2 pg/ml in SD + SS, p = 0.034, OR

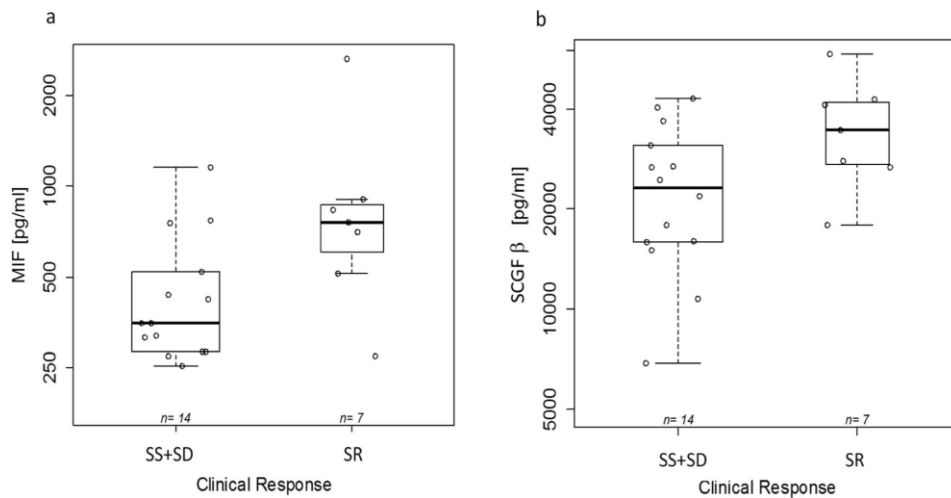= 5.5, 95% CI = 1.1–56.4) (Fig. 1).

Fig. 1 Boxplot comparing cytokine concentrations and clinical response between SR and SS+SD. Cytokine concentrations are plotted in logarithmic scale. The bold horizontal line represents the distribution median. Statistical significance was assessed by logistic regression analysis. A significant association was found for a MIF (p = 0.022, OR = 4.3, 95% CI = 1.2–25.4) and for b SCGF-β (p = 0.034, OR = 5.5, 95% CI = 1.1–56.4)

However, when multivariate logistic regression analysis was performed

considering all significant variables (MIF, SCGF-β and age), only MIF (p =

0.022) and age at diagnosis (p = 0.025) were able to distinguish the two

groups.

Baseline plasma cytokines to identify SD patients

Treatment of SD patients is a serious challenge for clinicians; therefore, we analyzed the differences in cytokine levels be- tween SD and all other patients. Univariate logistic regression analysis identified 5 cytokines as differentially expressed be- tween SD and all other patients (SR+SS; Fig. 2).
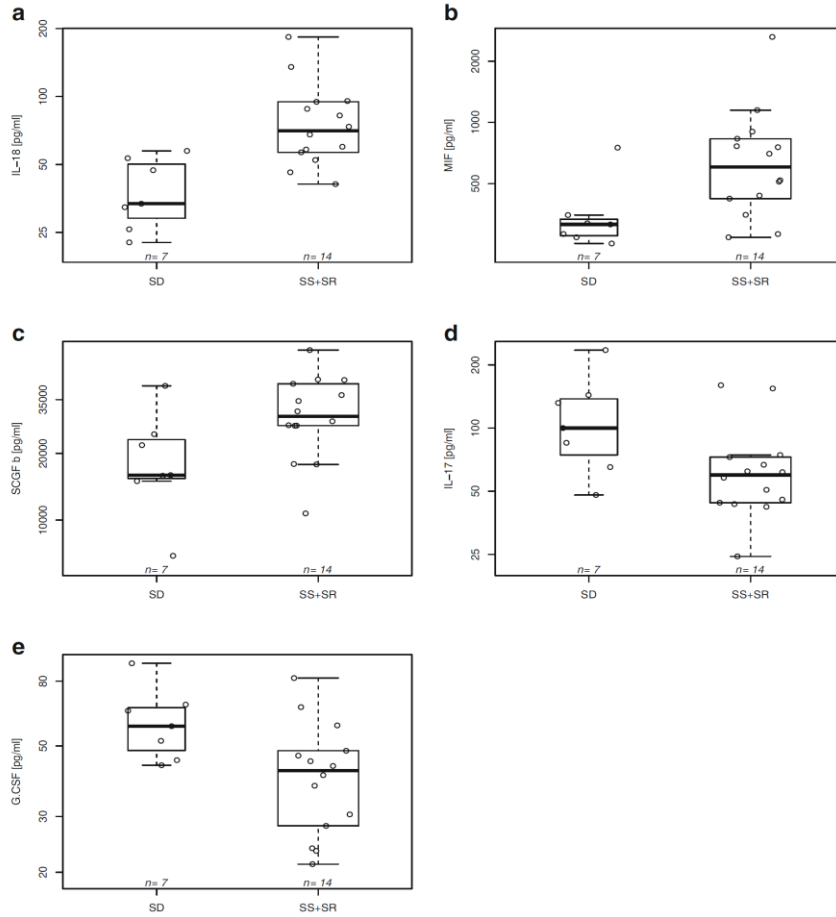
Fig. 2 Boxplot comparing cytokine concentrations and clinical response between SD and SS+SR. Cytokine concentrations are plotted in logarithmic scale. The bold horizontal line represents the distribution median. Statistical significance was assessed by logistic regression analysis. A significant association was found for *a* IL-18 (p = 0.0003 OR = 0.01, 95% CI = 4.5 × 10−5–2.4 × 10−1), b MIF (p = 0.010, OR =0.12, 95% CI = 9.2 × 10−3–6.7 × 10−1), c SCGF-β (p = 0.030, OR = 0.21, 95% CI = 2.9 × 10−2–8.8 × 10−1),

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Significantly lower concentrations of IL-18 (mean concentration 36.8 pg/ml in SD vs 74.4 pg/ml in SR+SS, p = 0.0003, OR = 0.01, 95% CI = 4.5×10^{-5}–2.4×10^{-1}, Fig. 2a), MIF (mean concentration 339.6 pg/ml in SD vs 619.4 pg/ml in SR + SS, p = 0.010, OR = 0.12, 95% CI = 9.2×10^{-3}–6.7×10^{-1}, Fig. 2b), and SCGF-β (mean concentration 17.8 pg/ml in SD vs 29.1 pg/ml in SR+SS, p = 0.030, OR = 0.21, 95% CI = 2.9×10^{-2}–8.8×10^{-1}, Fig. 2c) and significantly higher concentrations of IL-17 (mean concentration 102.5 pg/ml in SD vs 60.7 pg/ml in SR+SS, p = 0.031, OR = 4.1, 95% CI = 1.1– , Fig. 2d) and G-CSF (mean concentration 58.4 pg/ml in SD vs 39.3 pg/ml in SR+SS, p = 0.019, OR = 11.2, 95% CI = 1.4–255.6, Fig. 2e) When multivariate analysis was performed, only MIF, IL- 18, and SCGF-β were able to significantly distinguish the two groups (p = 0.028, p = 0.00033 and p = 0.0056, respectively).

Confirmation of results in the entire cohort: IL-18, SCGF-β, and MIF

To confirm the results obtained, we investigated the cytokines significantly associated with clinical response (IL-18, MIF and SCGF-β) in the entire cohort of patients. Considering all 62 patients, univariate logistic regression models showed a significant difference only for MIF plasma levels between SR patients and SD+SS ones (mean concentration 683 pg/ml in SR vs 436.8 pg/ml in SD + SS, p = 0.039; OR = 0.4, 95% CI = 0.2–0.9) and between SD patients and SR+SS (mean concentration 375.5 pg/ml in SD vs 534.3 pg/ml in SD+SS, p = 0.014; OR = 2.3, 95% CI = 1.2–5.5).

Identification of a MIF cutoff to distinguish SR patients

Since after the multivariate analysis MIF was the only cytokine able to distinguish SR patients, we performed ROC curves to identify cutoff values for MIF levels significantly associated with steroid resistance: a unique cutoff of 501 pg/ ml was found. Area under the ROC curve (AUC) was 76.0% (Fig. 3). The test had high sensitivity (85.7%) and specificity (71.4%). Fisher's exact test confirmed a higher proportion of SR patients among those who reached the optimal cutoff point (p value = 0.024, OR = 7.8×10^{-2}, 95% CI = 1.3×10^{-3}–9.4×10^{-1}) in comparison to those who did not. For SD patients,

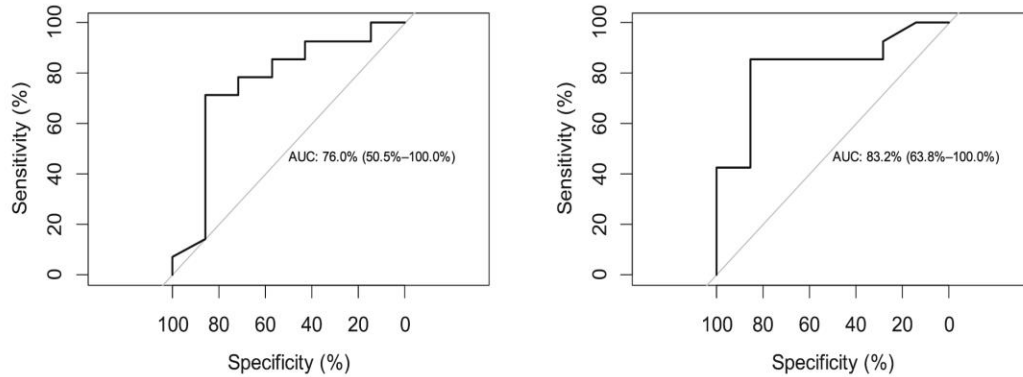a cutoff of 355 pg/ml was found (AUC = 83.2%, sensitivity = 85.7%, specificity = 78.6%; Fig. 3).

Fig. 3 Areas under the ROC curves of MIF plasma level in SR (panel **a**) or SD (panel **b**) vs all other patients among 7 SS, 7 SD, and 7 SR patients. ROC, receiver operating characteristic. Optimal cutoff value for SR patients was 501 pg/ml (sensitivity 85.7%, specificity 71.4%, panel **a**, while for SD patients was 355 pg/ml was found (sensitivity = 85.7%, specificity = 78.6%; panel **b**

Fisher's exact test confirmed higher proportion of SD patients among those who did not reach the cutoff point (p = 0.016, OR = 18.1, 95% CI = 1.4–1.1 × 10^3) in comparison with those who reach it. The cutoff value found was investigated in the extended cohort of 62 patients. Fisher's exact test analysis confirmed a higher proportion of SR patients among those who reached the optimal cutoff point (MIF > 501 pg/ml; p = 0.02; OR = 0.14, 95% CI = 0.01–0.8) in comparison with those who did not. For SD patients, the cutoff value of 355 pg/ml, found in the discovery cohort, was not confirmed

in the entire cohort of patients. Finally, we evaluated the differences in MIF plasma levels between our cohort of INS patients and 11 pediatric controls; control subjects were similar in terms of age compared with our cohort (median 6.0; range 1–11 years). Univariate logistic regression model shows a significantly higher concentration of MIF in patients with INS, in comparison with healthy controls (MIF mean concentration 466.1 pg/ml in patients with INS vs 124.5 pg/ml in healthy controls, $p < 0.0001$; Fig. 4).
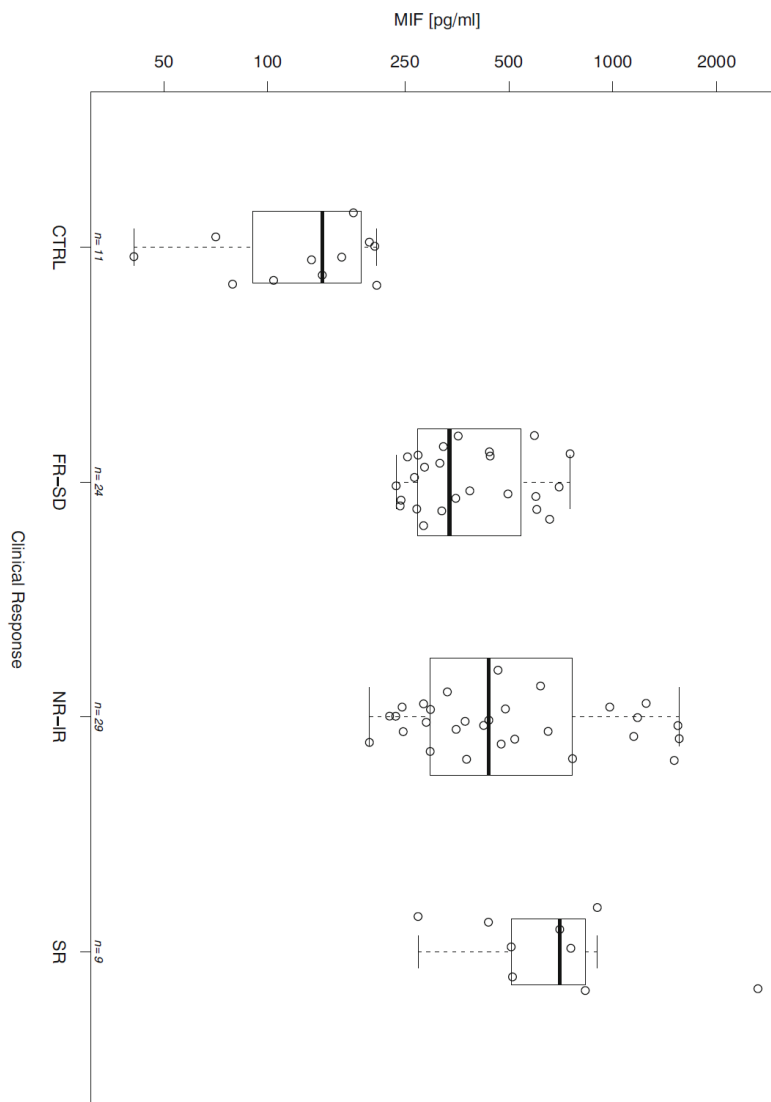
Fig. 4 Boxplot comparing MIF plasma levels between INS patients and healthy subjects (CTRL). Cytokine concentrations are plotted in logarithmic. The bold horizontal line represents the distribution median. Statistical significance was assessed by logistic regression analysis ($p < 0.0001$)

MIF level correlation with MIF G-173C polymorphism

Elevated circulating serum MIF levels have been related to *MIF* gene polymorphisms, although with controversial results [6]. The 62 patients involved in the study were therefore genotyped for *MIF* polymorphism G-173C (rs755622). Genetic results were available for 59 patients and the genotype distribution was in Hardy-Weinberg equilibrium (p = 0.28); 44 patients were wild type (74.6%; 22 SS, 15 SD and 7 SR), 15 were heterozygous (25.4%; 5 SS, 8 SD and 2 SR), while none of the patients showed a mutated genotype. We evaluated the possible correlation be- tween *MIF* G-173C polymorphism and MIF plasma levels with- out finding any significant association (Supplementary Fig. 1). Moreover, there was no genotype effect on clinical response.

DISCUSSION

In this study, we evaluated the plasma concentration of 48 cytokines in patients at the first episode of INS who underwent steroid treatment, with the final aim of finding a biomarker useful to predict their clinical response. The study was at first conducted in a group of 21 patients. Patients were selected from an Italian cohort of pediatric patients,

clinically characterized and treated with a common protocol. Significant results were then considered including additional 41 patients treated with the same therapeutic protocol. INS is the most frequent primary glomerular disease in children [3, 13]. The physiopathologic mechanisms of the disease are still not clear; however, the disease is triggered by an increase in glomerular permeability caused by an abnormal immunologic response, resulting in an alteration of the capillary structure and of the integrity of the glomerular mem- brane [3].

Since the 1950s, steroid treatment is the first line therapy for INS [14], but response to these drugs is variable. Steroids are potent inhibitors of cytokines production in immune and non- immune cells and are able to induce remission in about 85–90% of subjects; however, variable degrees of steroid responsiveness and different patterns of disease relapse have been observed [15]. Response to steroid treatment is a key index of outcome; indeed, patients with steroid resistant disease represent a difficult therapeutic challenge for clinicians; moreover, to date, ap- proximately 40–50% of responding patients presents frequent relapses or steroid dependence when therapy is discontinued; these patients are at

high risk of treatment related side effects [3, 16]. There is still no way of predicting this pattern of ineffective therapy and steroid dependence.

In this study, we showed that, within the 48 cytokines analyzed, MIF is the best predictor of steroid response before any treatment in children with INS. Indeed, patients non- responsive to GCs show significantly higher MIF plasma levels compared with steroid sensitive ones. These results are supported also by Wang et al. [17] in patients with system- ic lupus erythematosus; these authors demonstrated that MIF serum levels were correlated with steroid resistance. In the present study, for the first time, a cutoff value for MIF plasma level could be identified at 501 pg/ml to distinguish resistant and sensitive patients, with a high sensitivity and specificity (respectively 85.7% and 71.4%). Considering patients achieving this cutoff, almost all (7/9) of SR patients could be identified. This finding could be useful for the early identification of patients who will not respond to steroids, avoiding ineffective treatments. Furthermore, MIF plasma levels were also able to distinguish INS patients from healthy subjects that show lower levels of the cytokine.

Moreover, in this study we focused our attention also on SD patients, who show very low plasma MIF secretion as compared with all other patients. This is the first study, to our knowledge, which investigates cytokine plasma levels in patients who show steroid dependence. These patients represent almost 40–50% of INS patients and may require a more aggressive treatment with increased risk of adverse events and disease related complications. Plasma MIF level in these patients was shown to be lower than in all other patients; however, a cutoff value able to distinguish SD patients could not be confirmed in our extended cohort of patients. This finding of low plasma MIF levels in SD patients was quite unexpected, even if previous studies have shown that these patients do not have a pharmacological phenotype intermediate between SS and SR [11]; more studies are needed to shed light on the contribution of MIF plasma levels as a biomarker for this clinically challenging group of patients.

MIF is a pleiotropic cytokine with pro-inflammatory activity, which appears to be due to effects on macrophages and T cells. Steroids reduce the production of inflammatory molecules; however, they increase the release of MIF from macro- phages [18] and T cells [19]. In turn, MIF

counter-regulates the inhibitory activity of steroids on pro-inflammatory cytokines [18, 20]. It is not clear yet how MIF can exert this effect; it has been suggested that, in inflammatory conditions, MIF interferes with GC activity on cytokine transcription, mediated by nuclear factor κB (NFκB) [21]. Under basal conditions, NFκB, complexed with IκBα (inhibitor of NFκB) is located in the cytosol. Inflammatory stimuli activate IκBα kinase (IκK) that phosphorylates and degrades IκBα. NFκB can therefore enter into the nucleus, inducing transcription. GCs induce the synthesis of IκBα, that binds to NFκB in the cytosol, preventing its nuclear localization. MIF, on the contrary reduces IκBα cytosolic levels, increasing NFκB migration in the nucleus and transcription and counteracts GC effects [21] (Fig. 5).

Fig. 5 Pro-inflammatory mechanism of action of MIF [24]. GC, glucocorticoid; GR, glucocorticoid receptor; PLA₂, phospholipase A2; IkBα, inhibitor of NFκB; NFkB, nuclear factor κB; ERK1/2, extracellular-signal-regulated kinase 1 and 2; AA, arachidonic acid
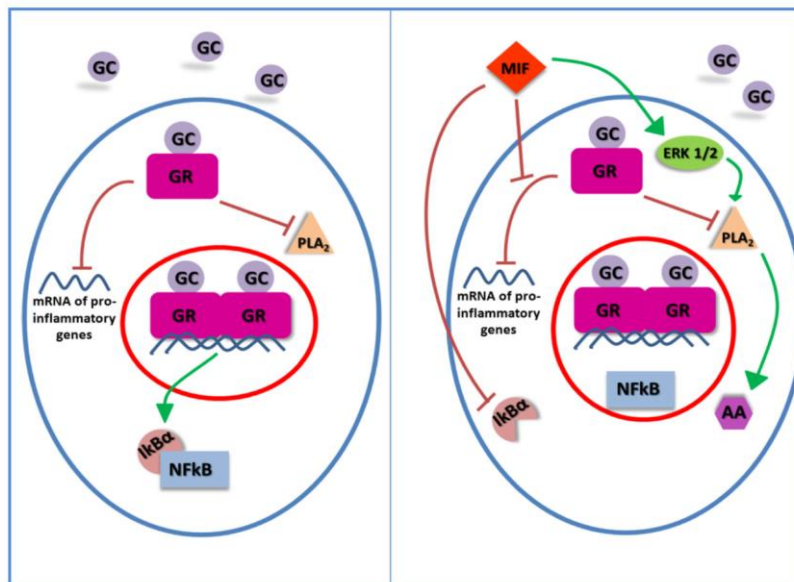
Moreover, MIF strongly stimulates the extracellular- signal-regulated kinase (ERK)-1 and ERK-2 pathways; the cytoplasmic isoform of phospholipase A2 (PLA2) is activated and arachidonic acid is released [22]. GCs are well-known inhibitors of PLA2 activation, and this effect is counteracted by MIF. In addition to repressing the transcription of immune genes, GCs are also able to increase the degradation of mRNAs of pro-inflammatory genes [23]; also, this phenome- non has been shown to be related to MIF inhibitory effect on GC activity [24](Fig. 5). Although insufficient to explain all the pro-inflammatory activities of MIF, the

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mechanisms de- scribed can add to explain its antagonism on GC-mediated immunosuppression.

MIF has been already investigated and proved to be involved in diseases such as systemic lupus erythematous [25], rheumatoid arthritis [26], and chronic kidney disease [27]; however, to our knowledge, this is the first work that found a cutoff value for MIF plasma level able to distinguish different clinical response.

Several studies in INS patients have shown that specific MIF polymorphisms are associated with GC response. The most studied and correlated polymorphism in these patients is the MIF G-173C (rs755622), although literature studies show controversial results [6]. Berdeli et al. [28] showed a correlation of the MIF–173C allele with INS and steroid resistance in Turkish children and Vivarelli et al. [29] obtained similar results in an Italian population. However, these findings are not in agreement with Choi's study that could not find an association between the polymorphism and steroid responsiveness [30]. In our study, MIF G-173C did not affect MIF plasma level measured in the patients

(Supplementary Fig. 1), as recently demonstrated also by Ramayani et al. [31]; more- over, no significant correlation was found between this SNP and clinical response.

This study has some limitations, in particular the number of patients enrolled is small and there is a significant difference in age between GC resistant and dependent / sensitive patients. Therefore, it would be important to extend similar studies to larger patients' groups.

In conclusion, the results of the present study suggest that, within the 48 cytokines considered, increased MIF plasma levels could be used to clinically identify patients at high risk of steroid resistance at diagnosis; if these results are confirmed in a larger cohort of patients, MIF plasma levels could be considered for alternative treatments avoiding useless steroid administration and subsequent side effects.

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CHAPTER 6

Urinary Extracellular vesicles profile discriminates different clinical subgroups of children with Idiopathic Nephrotic Syndrome

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Manuscript in preparation

ABSTRACT

There is growing interest regarding the use of minimally invasive "liquid biopsies" to identify new biomarkers. Urinary extracellular vesicles (UEv) are nanovesicles released into urine by cells facing the urinary space. Their molecular composition depends upon the type of the cell of origin, providing a fingerprint, capable to monitor its status. Their presence in urine makes them readily accessible, giving the possibility to investigate pathological conditions especially related to kidney.

While exosome research has flourished, few studies have specifically targeted the role of UEv in Idiopathic Nephrotic Syndrome (INS) in children the most frequent childhood glomerular disease. The pathogenesis of INS is still unknown and response to initial treatment with corticosteroids is the major indicator of long-term prognosis, as resistant patients often progressive to end-stage renal disease.

The aim of the study is to verify the feasibility to use UEv of INS patients as a source of predictive markers of response to corticosteroid treatment, and/or for clarifying the disease etiopathogenesis and/or the pharmacoresistance. Thus, we investigated the UEv protein content of a paediatric cohort of 34 patients, classified in three clinical classes, according to the corticosteroids response: steroid-Dependent, steroid-Sensitive and steroid-Resistant.

Firstly, a characteristic SDS-PAGE protein profile emerged to be associated with each class, which preserves its peculiarity also when compared to UEv protein content of non-INS patients (orthostatic proteinuria, hereditary tubulopathies) and healthy age-matched controls. Secondly,

we also pinpointed different levels of specific glomerular membrane proteins (Podocin, P-glycoprotein, Neprylisin, Nephrin and Transient receptor potential cation channel subfamily C member 6), described as involved in the INS development.

These evidences confirmed the feasibility to use a UEv approach to intercept the pathophysiological differences underlying response to therapy.

INTRODUCTION

Idiopathic Nephrotic Syndrome (INS) is the most common glomerular disease in children and it is characterized by proteinuria, hypoalbuminaemia, and oedema. The pathogenesis is poorly understood [1]. Evidence suggests an involvement of immunological mechanisms, with B- and T-cell dysfunction [2] and a possible role of Epstein-Barr Virus (EBV) infection [3]. A yet unidentified permeability factor is believed to be involved at least in patients with INS relapsing after renal transplantation [4]. Underlying mutations in genes encoding podocyte associated proteins, resulting in structural or functional disruption of the glomerular filtration barrier, can be found in 30% of cases refractory to treatment [5]. The mainstay of therapy are steroids, and INS is usually classified according to the response to treatment, as steroid-sensitive, SSNS (S) or steroid-resistant, SRNS (R). At least 50% of SSNS will require long-term steroid or immunosuppressive treatment to maintain remission and will be further classified as steroid-dependent, SDNS (D). The prognosis is extremely different according to the subgroup, with SRNS resistant to second-line treatments progressing into end stage renal disease in virtually 100% of cases [1]. Unfortunately, established and reliable biomarkers of response are lacking and children may be exposed to unnecessary and toxic immunosuppressive therapies.

In this contest, the proteomic study of the urinary extracellular vesicles (UEv) represents a valid approach. UEv are nanometer-sized vesicles (50-200 nm), that can originate from endothelia and glomerular cells,

podocytes or tubular epithelial cells. UEv act as a vehicle by which cells communicate, delivering their functional content, with biological, physiological and pathological functions [6] . Enriched in renal proteins, UEv proteome contains less than 3% of total urine proteins (>3000 species), depleting it from the most abundant urinary proteins, reducing in that way the complexity of the urine proteome. Their molecular composition depends upon the type, and even status, of the origin cell. As such, they provide an easily accessible window to monitor the status of renal tissue. For all these reasons, they can be considerate a sort of liquid biopsy, able to provide potential pathophysiological biomarkers and possible protagonists of the disease's pathogenesis.

Few studies have specifically targeted the role of UEv in INS, maybe due to the high proteinuria typical of these patients. In fact, large amount of proteins, in particular the highly abundant ones, influences negatively UEv isolation and the following proteomic analysis

Aware of the technical challenges related to the study of UEv in case of proteinuria, we approached this issue from another point of view, focusing on UEv isolated from the urine samples of patients under treatment in remission. Here we aimed at investigating the role of UEv in differentiating patients according to the response to treatment, comparing S, R and D patients, in order to find possible implications in the pathogenesis of the disease, other than predicting the evolution of therapy response.

Materials and Methods

Clinical data and study design

We performed a pilot study of UEv in pediatric patients with INS. Urine samples were prospectively collected from all INS children, attending the Pediatric Nephrology Dialysis and Transplant unit of Milan from July 2018 and July 2019 and stored at −80°C until the analysis. Patients were classified according to the response to the initial steroid therapy and the need for further immunosuppressive treatment into SSNS, SDNS, SRNS, according to international consensus. In order to prevent interferences related to the presence of serum protein in urine, patients with significant proteinuria were excluded (uPr/uCr > 1 mg/mg). A total 74 samples were collected, of which 41 were later excluded for the presence of significant proteinuria. Therefore, 33 samples were analyzed (9 S, 17 D, 6 R). Table 1 summarize the main clinical characteristics of patients included.

Patient	Group	Sex	Age at collection	uPr/uCr	Ongoing Therapy			
					Pred	Cycl	MMF	Tacr
5	D	M	5	0.24	X		X	X
6	D	M	10	0.14			X	
7	D	M	12	0.60	X		X	
8	D	M	5	0.23			X	X
10	D	M	18	0.08			X	
13	D	F	4	0.19			X	
14	D	F	8	0.18			X	
18	D	M	8	0.15			X	
30	D	F	6	0.16			X	
32	D	F	8	0.15	X		X	
38	D	M	15	0.27	X		X	
53	D	M	10	0.84			X	
55	D	M	5	0.74		X	X	
58	D	M	12	0.15		X		
61	D	M	7	0.25			X	
66	D	F	6	0.14			X	
69	D	F	15	0.15			X	
2	S	M	7	0.14	X			
15	S	F	2	1.03	X			
16	S	M	5	0.13	X			
24	S	M	4	0.27	X			
43	S	M	17	0.08				
44	S	F	16	0.13				
67	S	F	11	0.16				
73	S	M	10	0.12	X			
74	S	M	11	0.12	X			
1	R	M	10	0.35	X	X	X	
3	R	M	4	0.18	X		X	X
11	R	F	14	0.10		X	X	
12	R	F	8	0.11		X		
39	R	F	12	0.14		X		
70	R	F	11	0.13				
4	OP	M	13	1.08				

Legend: **PRED**. Prednisone; **CYCL**. Cyclosporin; **MMF**. Mycophenolate mofetil; **TACR** Tacrolimus; OP. Orthostatic Proteinuria; uPr. urinary proteins; uCr. urinary creatinine

Urine collection

Table 1. Clinical characteristics of enrolled patients

Urine samples, (mean volume = 20 ml) were centrifuged for sediment removal (10 min at 1'000 xg, 4°C) within 4 hours from the collection. The supernatant was supplemented with protease inhibitors (Complete, Roche) and stored at −80°C until exosome isolation. From an aliquot of each sample (500 µl out of about 20 ml), urinary proteins were precipitated by nine volumes of cold 90% ethanol and pelleted at 3'500 xg for 30 minutes [8]. After drying, proteins were dissolved in bidistilled water, and protein concentration was assessed by BCA assay (SIGMA Chemical Co), using BSA as standard.

Before exosomes isolation, the remaining stored urine samples were thawed, thoroughly vortexed while thawing and adjusted to pH 7.4, if needed.

UEv isolation

UEv were prepared by ultracentrifugation [9] according to HKUPP (http: www.hkupp.org), with minor modifications. All steps were performed at 20°C. Briefly, urine were added with $ZnSO_4$ 10 mM, incubated at RT for 1 hour and then centrifuged for 30 min at 3'000 *xg* at 20°C, to eliminate THP [10] (data not shown). Supernatants were then subjected to further centrifugation for 15 min at 17'000 *xg*. Supernatants were finally submitted to ultracentrifugation for 70 minutes at 200'000 *xg*: crude UEv

pellets were washed and suspended in PBS, in the presence of protease inhibitors. The UEv samples were stored at -80°C until use.

UEv characterization by Nanoparticle tracking analysis

UEv size and concentration were measured by Nanoparticle Tracking Analysis (NTA) using a NanoSight NS300 (Malvern Instrument Inc., Malvern, UK) equipped with a 488 nm laser and a syringe pump system. Before injection, UEv were diluted in sterile PBS. The camera operated at 30 frames per second (fps), the threshold for the tracking system was set at 5 and the sample analysis was performed around 1 minute for 3 times. After three technical replicates, the resulting tracking graphs were analysed by NTA 3.2 software (dev build 3.2.16).

Electrophoresis and Western Blotting

Protein separation was performed with the NuPAGE® electrophoresis system, using 4-12% NuPAGE and MOPS SDS buffer, as described [9]. Proteins were stained by SYPRO™ Ruby Protein Gel Stain to evaluate and compare the protein profiles, or were transferred to nitrocellulose membranes using a "tank" electrophoretic transfer apparatus (Hoefer), to detect typical exosome markers (TSG-101, flotillin 1) and glomerular proteins (Nephrin, podocin, TRPC6. P-glycoprotein, CD10). The blots were developed as described [9]. Densitometric analysis was performed by ImageQuant TL software (GE Healthcare).

Experimental Section

Milli-Q water was used for all solutions. PBS (EuroClone), Bovine serum Albumin (BSA), BCA protein assay, methanol, CAPS / SYPRO Orange Protein Gel Stain and ZnSO4 were from SIGMA Chemical Co. (St. Louis, MO, USA); Hybond-ECL nitrocellulose membrane was from GE (Little Chalfont, Buckinghamshire, UK). NuPAGE® SDS-PAGE Gel Electrophoresis System components (mini gels, running and loading buffers, molecular weight markers and coomassie blue staining) were supplied by Life Technologies (Paisley, Renfrewshire, UK). Anti-protease inhibitor cocktail (Complete) was from Roche (Monza, Italy).

anti-Tumor Susceptibility Gene 101 (TSG101) polyclonal antibody (pAb) from Abcam (Cambridge, UK) CD9 come markers e CD10 come differenziale. Species-specific secondary peroxidase conjugated antibodies and ECL reagents were from Thermo Scientific (USA).

RESULTS

UEv characterization: NTA and marker enrichment

We isolated UEv from all samples by an optimized protocol for small volume of urine. NTA shows that the size distribution is typical of UEv [11] and not significantly different among the three groups (Figure 1), while UEv concentration shows different values according to the subgroup.
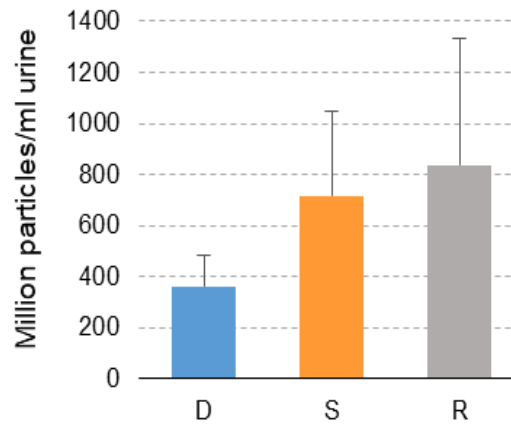
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a)
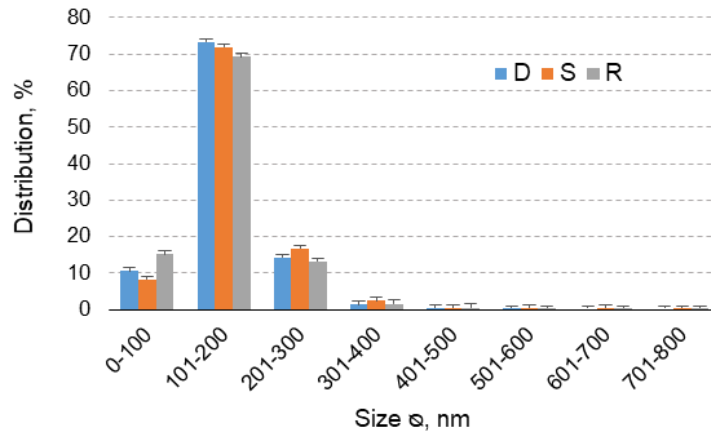
b)

Figure 1. NTA: a) Percentage distribution of UEv isolated from the urine of ISN patients. Mean ± SE; a) UEv concentration. Mean n of 3 samples for each group.

UEv purity was checked by evaluating two commonly used UEv markers, TSG101 and CD9 [12]. Results show that markers are reproducibly enriched in the vesicle fraction, in all the three patient groups (Figure 2),

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although with some inter-individual variability. The assessment of UE protein markers was extended to UEv isolated from all cases and demonstrated that their purity was comparable in all the preparations.
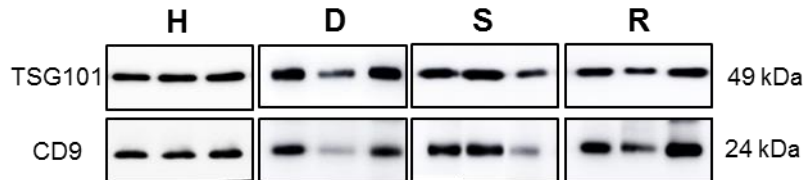

Figure 2: TSG101 e CD9 marker enrichment. Western blotting with anti-TSG101 and anti-CD9. Three representative cases for each group are shown. Lanes were loaded with equal protein amounts.
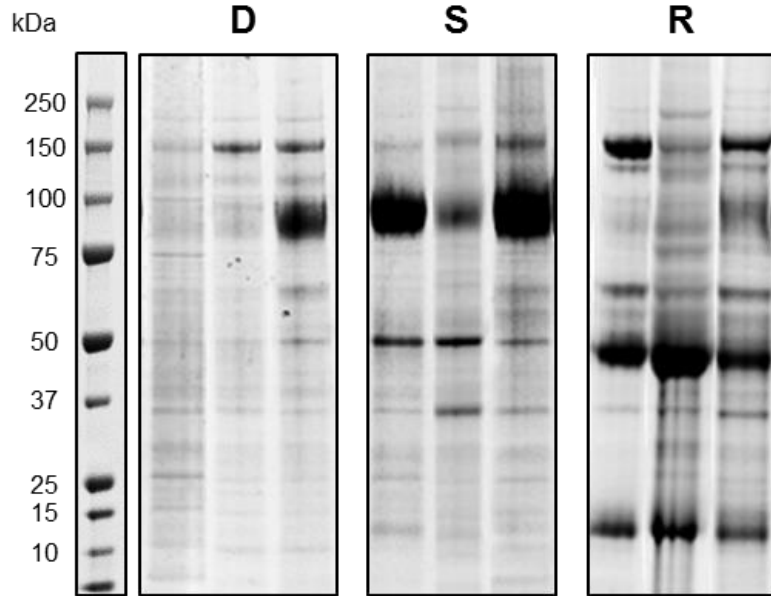
UEv protein profiling

The protein pattern of the isolated vesicles was analysed by NuPAGE, followed by SYPRO™ Ruby Protein Gel Stain (Figure 3). UEv isolated from the patients belonging to the three groups showed a peculiar protein pattern: apart from THP band (visible at 80-100 kDa) which has a highly variable level of expression in each patient, regardless of whether they belong to any of the groups, some other bands are rather specifically associated to the profile of each group (Figure 3a).

In order to highlight the specificity of the INS SDS-PAGE pattern, a panel of UEv protein profiles obtained from healthy children and paedriatic patients affected by hereditary tubulopathies (Figure 3c) are shown for comparison. Results show that each INS gel profile preserves its

peculiarity and substantially differs to UEv protein content of not-INS patients and healthy subjects.
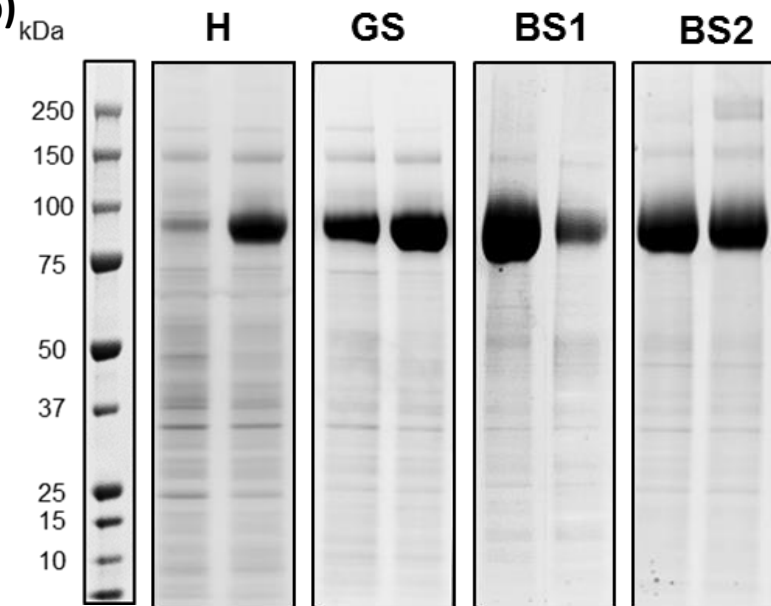
a)

b)

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Figure 3: UEv protein profile. NuPAGE 4-12% electrophoresis and Sypro Ruby protein gel staining. a) Patients affected by INS: corticosteroid-dependent (D), corticosteroid-sensitive (S), corticosteroid-resistant (R); three representative cases for each patient group are shown. b) Healthy subjects (H), patients affected by hereditary tubulopathies: Gitelman syndrome (GS), Bartter syndrome type 1 (BS1) and type 2 (BS2); two representative cases for each patient group are shown [13]. UEv protein profiles correspond to 3 ml of starting urine.

Moreover, we were able to collect a follow-up sample for UEv isolation and protein profile analysis, in a subgroup of six patients (Table 2). At the 2nd access, the patients were mainly in the same condition as before regarding pharmacological response. The results of the profile comparison show that the 2nd profile is very similar to the first, considering some variability effects, confirming the reproducibility of UEv protein profile (Figure 4a). In two cases (41-45; 33-71), the initial response to drug changed, and the patients needed to be re-classified. Interestingly, the first profiles of these cases (41 and 33) were somehow different from that typical of their group (Figure 4b). This finding would further confirm the specificity of UEv protein signature related to patient state, as if there were UEv profile characteristics, indicating the future modification of clinical course (S to D or R to D). In fact, among the R group, some patients presented UEv 1D profile similar each other (Figure 4c- R' subgroup), but different from the typical pattern shown in figure 3a. Although these are preliminary data, since the patients 3 and 41 changed their response to therapy, becoming D, it is possible that this type of UEv profile could be predictable of this behaviour.
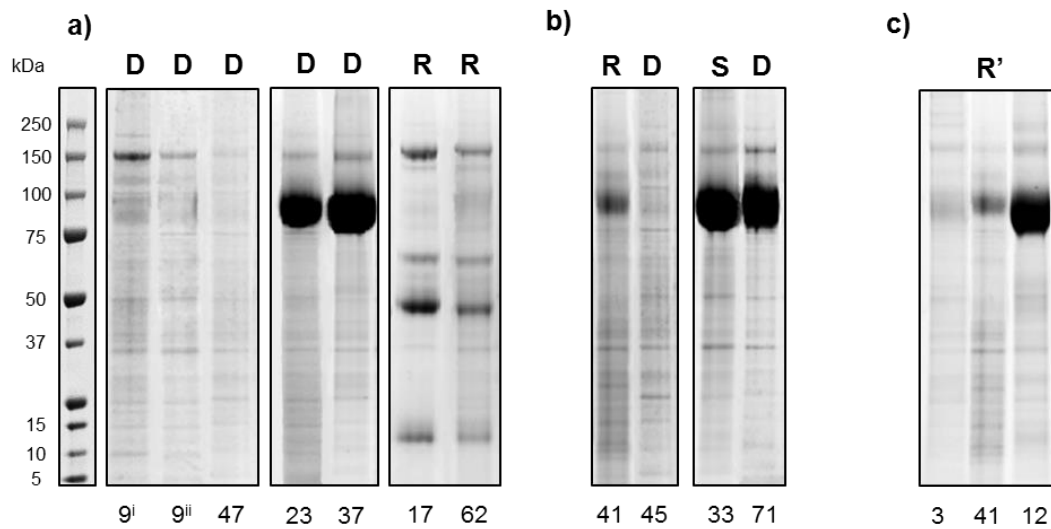
Figure 4: Reproducibility and atypical UEv protein profile. NuPAGE 4-12% electrophoresis and Sypro Ruby protein gel staining. a) UEv protein profile reproducibility: UEv isolated from patients at different time of collection; b) UEv protein profile of patients that changed their response to therapy; c) atypical UEv protein profile of R patients (R'). D, corticosteroid-dependent; S, corticosteroid-sensitive; R, corticosteroid-resistant; UEv protein profiles correspond to 3 ml of starting urine (see Table 2).

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Patient	Group	Sex	Interval (months)	uPr/uCr	Ongoing Therapy			
					Pred	Cycl	MMF	Tacr
9i	D			0.18			X	
9ii	D	M	4	0.18			X	
47	D		10	0.18			X	
17	R	M	8	0.44	X			X
62	R			0.32				X
20	D	M	10	0.17			X	
72	D			0.13			X	
41	R	F	2	0.20				X
45	D			0,14				X
23	D	F	5	0.34			X	
37	D			0.30			X	
33	S	M	4	0.24	X			
71	D			0.20			X	

Table 2. List of patients with a follow-up samples

Legend: D, corticosteroid-dependent; S, corticosteroid-sensitive; R, corticosteroid-resistant; PRED, Prednisone; CYCL, Cyclosporin; MMF, Mycophenolate mofetil; TACR, Tacrolimus; uPr, urinary proteins; uCr, urinary creatinine

Evaluation of potential INS markers by Western blotting

To validate the differential proteomic profiles of UEv from INS patient's different group, according to their pharmacological response, we examined some specific protein levels in UEv. Protein selection was mainly based on their potential roles to INS pathogenesis, according to literature [14]–[17] and on their tissue expression, typically glomerular. Figure 5a shows the preliminary results for 5 glomerular proteins, nephrin, P-glycoprotein (P-gp), neprylisin (CD10), transient receptor potential cation channel subfamily C member 6 (TRPC6) and podocin, whose UEv differential content was investigates by Western blot analysis. After densitometric quantification of band intensity, results were expressed as OD/mg of UEv proteins in figure 5 b.
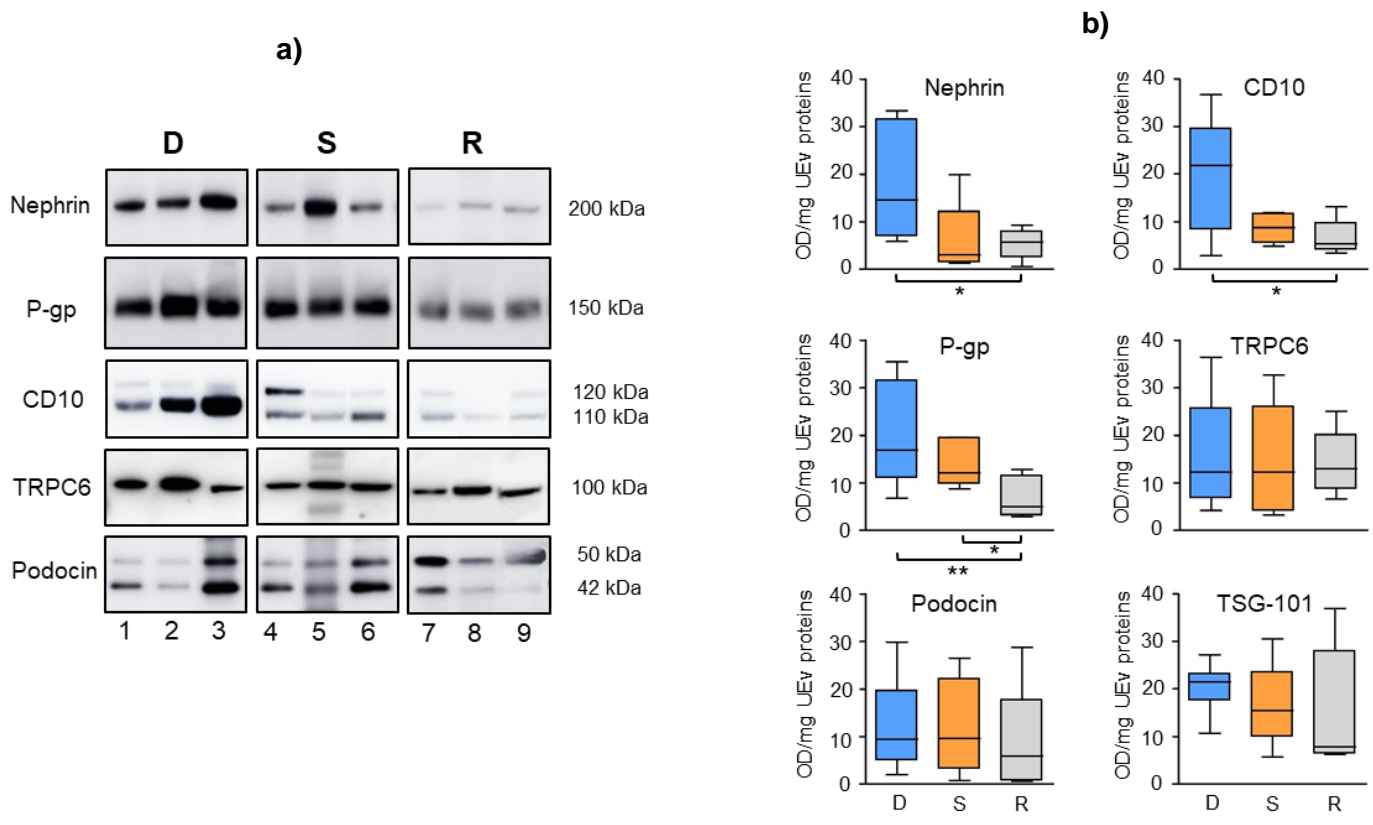
Figure 5. Glomerular protein level in INS UEv. a) Western blotting analysis; 3 representative cases are shown; b) densitometric analysis of bands (Optic Density, OD) normalized by UEv protein content (mg). Mann Whitney *t*-test was performed: * = *p* < 0.05, ** = p < 0.005.

Statistical analysis by Mann Whitney test shows that CD10, nephrin and P-gp UEv levels are significantly different among D and R patients, with a higher content in UEv of D patients. Moreover, P-gp seems to differentiate also S and R patients. In

general, it seems to be a trend of decrease of these protein levels from D to S and to R group, suggesting the possibility of a bias in the analysis. However, TRPC6, podocin and the exosomal marker TSG-101 do not show the same comportment: these proteins result unchanged, supporting the hypothesis that the different levels of CD10, nephrin and P-gp is a specific signature of the different therapy response of INS patients.

DISCUSSION AND CONCLUSION

Given the potential offered by UEv, we explored their role as biomarkers source in children with INS. Nowadays, the nucleic acids present in the UEv of children affected by INS were mainly investigated. Chen et al., for example, focused on exosomal microRNA (miR), showing the alteration of specific miR (miR-194-5p and miR-23b-3p) in response to treatment in children INS affected. This finding revealed that miR could be promising biomarkers for predicting and monitoring patients undergoing severe complications [18].

While genomic investigation through miRNAs analysis has attracted most attention and received major efforts, few proteomics studies have specifically targeted the role of UEv in the INS. After a period in which the EUv *proteomic potential* for the study of INS was considered but not investigated, recently two works proposed Wilms tumor 1 (WT1) transcriptor factor, a well-known marker for differentiated podocytes, as a non-invasive biomarker for the detection of podocyte injury, predicting

either therapy responsiveness or monitoring progression in patients with NS (FSGS and SSNS) [19].

In addition, Rood et al. proposed a method based on ultracentrifugation and size exchange chromatography to overcome the problem, allowing detecting of lower abundant UEv proteins [7]. However, the protocol was laborious, and did not have a follow-on, at least with regard to INS.

Since proteinuria is a negative interferer for UEv purification, we decided to investigate only patients with uPr/uCr ratio < 1mg/mg. In particular, we focused the attention to the response to initial treatment with corticosteroids, the main indicator of long-term prognosis, as steroid-resistant patients often progress to end-stage renal disease [20]. Patients were classified according to the response to the initial steroid therapy and the need for further immunosuppressive treatment into SSNS, SDNS, SRNS. This allowed us to obtain good quality UEv preparation. In fact, the isolated UEv presented the typical size distribution of urinary exosomes and the enrichment of the exosomal markers (TSG101 and CD9) [12]. The SDS-PAGE protein profile of UEv is usually characteristic and different from the total urine one: it was confirmed also for our samples. Moreover, it was clear that each patient group UEv had a peculiar protein band pattern, considering the biological variability. Furthermore, these protein profiles resulted specific for INS diseases, since they was different from the UE protein content of non-INS patients (hereditary tubulopathies) and healthy age-matched controls [13]. In addition, we confirmed that the protein profile remained constant over time, indicating a good

reproducibility, as shown for the UEv isolated from urine collected from the same patients several months later. From a careful observation of the bands, the group R presented some abundant bands that correspond to the molecular weights typical of the heavy and light chains of the immunoglobulins. This could be expected since is a peculiarity of this type of patients due to the disease. However, not all the R patients showed the enrichment of the same signals: we noticed that among the 7 patients enrolled as clinically R at the time of urine collection, 3 had a different protein pattern. Interestingly, two of these patients changed their response to the therapy, becoming drug-dependent (D). Although the low number of cases, it was an intriguing finding, that gave us confidence in the usefulness of UEv in the investigation of INS.

The analysis of specific proteins also enforced this results. We checked the UEv content of some glomerular protein, known as involved in INS and present in extracellular vesicles [21]: P-glycoprotein (P-gp), nephrin, neprylisin (CD10), transient receptor potential cation channel subfamily C member 6 (TRPC6) and podocin. These proteins were analysed with the intent to prove the specificity of INS UEv protein content, without any aim of functional or molecular explanation, that will be explored in future. However, P-gp, CD10 and nephrin seem to differentiate D from R patients, while TRPC6 and podocin show unchanged levels, further indicating a specific UEv signature of the different therapy response of INS patients.

Although preliminary, these evidences confirmed that the proteomic UEv approach is promising in the study of INS to identify physiopathological

differences underlying the response to therapy. To reach this goal, we need digging deeper the proteome of the INS UEv, performing MS analysis to uncover the emerged differences in the UEv proteome of the pilot cohort. Simultaneously, the sample size will have to be enlarged in order to develop a robust classification model (in collaboration with the statistics unit), able to use UEv as predictive parameter of long term prognosis to treatment response.

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CONCLUSIONS AND FUTURE PERSPECTIVES

This project aimed to clarify molecular mechanisms involved in INS in children, focusing on the role of genetic mutations in relapse, the identification of patients whose sera contain the circulating permeability factor able to induce dysfunction of GFB, and looking for innovative biomarkers for the disease.

We performed a retrospective, multicentre, national cohort study to address the long-term prognosis of renal graft, the risk factors for recurrence and the predictors for response to therapy following recurrence in paediatric patients undergoing renal transplantation because of a SRNS (CHAPTER 2). We were able to demonstrate that genetic forms of NS do not relapse after renal transplantation. We were also able to exclude any protective effect of prolonged dialysis before transplantation on the risk of recurrence and to demonstrate the feasibility of a second transplantation, even after a relapse.

At the bench side, in order to detect sera of patients with SRNS containing the so-called permeability factor, we identify patients with different forms of SRNS, collected their sera and tested the samples by means of a novel method which assess the permeability to bovine serum albumin (BSA) through a three-layer device (3LD) (Chapter 3). We were able to demonstrate that sera from patients recurring after transplantation can

induce albumin permeability on the GFB model. This effect is lost after plasmapheresis. Secondary and genetic SRNS do not have any effect on albumin permeability.

Following the obtained in Chapter 3, we have decided to perform a preliminary proteomic analysis of sera from SRNS children relapsed after renal transplantation and tested through the GFB in order to identify the proteins involved in the pathogenesis of disease and recurrence (CHAPTER 4). We were able to identify different protein profiles and protein-protein interaction in patients recurring on transplant when compared to genetic forms of SRNS. The profile is also different in samples collected after plasmapheresis.

In chapter 5, INS children with different response to the initial steroid treatment were screened for cytokine plasma level at the diagnosis. We were able to demonstrate that macrophage migration inhibitory factor (MIF) was a good predictor of steroid response.

Moreover, in Chapter 6, protein content of urinary extracellular vesicles in INS children with different response to the available treatment was screened in order to provide biomarker of response and prognosis and to shed light on molecular mechanism of the disease.

This additional information will contribute to the understanding of molecular pathogenesis of INS in children. In particular, if the results of our GFB model will be replicated in larger scale, this method could become an effective tool for disease monitoring and to assess the

prognosis at disease onset and before transplantation. Moreover, the final results of the proteomic analysis will shed light on the proteins and pathway involved in the pathogenesis of the disease, on the hunt for the so-called circulating permeability factor.

SCIENTIFIC PUBLICATIONS

"Post-transplant Recurrence of Steroid Resistant Nephrotic Syndrome in Children: The Italian experience" - Journal of Nephrology - doi: 10.1007/s40620-019-00660-9.

Assessment of increased glomerular permeability associated with recurrent focal segmental glomerulosclerosis using an in vitro model of the glomerular filtration barrier. - Journal of Nephrology - doi: 10.1007/s40620-019-00683-2.

MIF plasma level as a possible tool to predict steroid responsiveness in children with idiopathic nephrotic syndrome" European Journal of Clinical Pharmacology, DOI 10.1007/s00228-019-02749-3

"Updated Italian recommendations for the diagnosis, treatment and follow-up of the first febrile urinary tract infection in young children." - Acta Paediatrica, doi: 10.1111/apa.14988.

"Antibiotic Prophylaxis for Urinary Tract Infection-Related Renal Scarring: A Systematic Review." Pediatrics - doi: 10.1542/peds.2016-3145.