81. Kambham N, Tanji N, Seigle RL *et al.* Congenital focal segmental glomerulosclerosis associated with beta4 integrin mutation and epidermolysis bullosa. *Am J Kidney Dis* 2000; 36: 190–196
82. Dreyer SD, Zhou G, Baldini A *et al.* Mutations in LMX1B cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. *Nat Genet* 1998; 19: 47–50
83. Mele C, Iatropoulos P, Donadelli R *et al.* MYO1E mutations and childhood familial focal segmental glomerulosclerosis. *N Engl J Med* 2011; 365: 295–306
84. Kestila M, Lenkkeri U, Mannikko M *et al.* Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol Cell* 1998; 1: 575–582
85. Lopez LC, Schuelke M, Quinzii CM *et al.* Leigh syndrome with nephropathy and CoQ10 deficiency due to decaprenyl diphosphate synthase subunit 2 (PDSS2) mutations. *Am J Hum Genet* 2006; 79: 1125–1129
86. Hinkes B, Wiggins RC, Gbadegesin R *et al.* Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat Genet* 2006; 38: 1397–1405
87. Ozaltin F, Ibsirlioglu T, Taskiran EZ *et al.* Disruption of PTPRO causes childhood-onset nephrotic syndrome. *Am J Hum Genet* 2011; 89: 139–147
88. Winn MP, Conlon PJ, Lynn KL *et al.* A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* 2005; 308: 1801–1804
89. Jeanpierre C, Denamur E, Henry I *et al.* Identification of constitutional WT1 mutations, in patients with isolated diffuse mesangial sclerosis, and analysis of genotype/phenotype correlations by use of a computerized mutation database. *Am J Hum Genet* 1998; 62: 824–833
90. Wang Y, Dang X, He Q *et al.* Mutation spectrum of genes associated with steroid-resistant nephrotic syndrome in Chinese children. *Gene* 2017; 625: 15–20
91. Goicoechea de Jorge E, Harris CL, Esparza-Gordillo J *et al.* Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc Natl Acad Sci USA* 2007; 104: 240–245
92. Zipfel PF, Edey M, Heinen S *et al.* Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. *PLoS Genet* 2007; 3: e41
93. Lovric S, Ashraf S, Tan W *et al.* Genetic testing in steroid-resistant nephrotic syndrome: when and how? *Nephrol Dial Transplant* 2016; 31: 1802–1813
94. Povey S, Al Aqeel AI, Cambon-Thomsen A *et al.* Practical guidelines addressing ethical issues pertaining to the curation of human locus-specific variation databases (LSDBs). *Hum Mutat* 2010; 31: 1179–1184

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Genetic variants in the *LAMA5* gene in pediatric nephrotic syndrome

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ABSTRACT

Background. Nephrotic syndrome (NS), a chronic kidney disease, is characterized by significant loss of protein in the urine causing hypoalbuminemia and edema. In general, ~15% of childhood-onset cases do not respond to steroid therapy and are classified as steroid-resistant NS (SRNS). In ~30% of cases with SRNS, a causative mutation can be detected in one of 44 monogenic SRNS genes. The gene *LAMA5* encodes laminin- α 5, an essential component of the glomerular basement membrane.

Mice with a hypomorphic mutation in the orthologous gene *Lama5* develop proteinuria and hematuria.

Methods. To identify additional monogenic causes of NS, we performed whole exome sequencing in 300 families with pediatric NS. In consanguineous families we applied homozygosity mapping to identify genomic candidate loci for the underlying recessive mutation.

Results. In three families, in whom mutations in known NS genes were excluded, but in whom a recessive, monogenic cause of NS

was strongly suspected based on pedigree information, we identified homozygous variants of unknown significance (VUS) in the gene *LAMA5*. While all affected individuals had nonsyndromic NS with an early onset of disease, their clinical outcome and response to immunosuppressive therapy differed notably.

Conclusion. We here identify recessive VUS in the gene *LAMA5* in patients with partially treatment-responsive NS. More data will be needed to determine the impact of these VUS in disease management. However, familial occurrence of disease, data from genetic mapping and a mouse model that recapitulates the NS phenotypes suggest that these genetic variants may be inherited factors that contribute to the development of NS in pediatric patients.

Keywords: genetic variants, inherited diseases, nephrotic syndrome, treatment response in pediatric nephrotic syndrome, whole exome sequencing

INTRODUCTION

Nephrotic syndrome (NS), a chronic kidney disease, is characterized by proteinuria (>40 mg/m²/h) that causes hypoalbuminemia, edema and hyperlipidemia. While most affected children and young adults show a clinical response to steroid therapy, ~10–15% of cases exhibit steroid-resistant NS (SRNS; defined as persistent proteinuria after 6 weeks of oral steroid treatment) [1, 2]. Patients with steroid-sensitive NS (SSNS) can have a single episode of NS, responding promptly to therapy, or show a complicated clinical course with frequent relapses of NS after termination of steroid therapy. Some patients who are resistant to steroid therapy may still respond to a more intensified drug regimen with different immunosuppressive drugs, whereas others are completely resistant to therapy [3]. This observation suggests that in some cases SSNS and SRNS can represent a continuum of the same disease rather than being two distinct disease entities. Early response to therapy is a strong predictor of long-term renal outcome [4], and patients with SRNS progress almost inevitably to end-stage renal disease (ESRD) [2]. In patients with SRNS, the most frequently observed histological diagnosis on renal biopsy is focal segmental glomerulosclerosis (FSGS) [5]. Patients displaying minimal change nephropathy (MCN) on renal biopsy when first diagnosed tend to have better outcomes [4]. However, frequently MCN evolves into FSGS over time [5]. Despite intensive research, no effective treatment for FSGS/SRNS exists and FSGS constitutes the third most frequent cause of ESRD in childhood and adolescence [6].

The discovery of monogenic causes of SRNS has identified glomerular podocytes as the primary site of damage in this disease, because many genes that, if mutated, cause SRNS are predominantly expressed in this cell type [7, 8]. Mutations in four genes, namely *NPHS1*, *NPHS2*, *LAMB2* and *WT1*, are most frequently observed, and together explain up to 66% of cases with an onset of SRNS within the first year of life [9]. Advances in next-generation sequencing have facilitated the identification of more than 44 genes that cause SRNS if mutated [10, 11]. It was recently shown in a worldwide study in 1873 families that in 29.5% of cases with an onset of SRNS before age 25 years, a causative single-gene mutation could be identified [12]. This

finding was confirmed in two additional studies that demonstrated monogenic disease causation in 26.2% and 32.3% of patients with SRNS, respectively [13, 14].

The glomerular filter, which is functionally impaired in NS, consists of podocytes, fenestrated endothelial cells, mesangial cells and the glomerular basement membrane (GBM). One essential component of the GBM is laminin, which forms obligate heterotrimers composed of α , β and γ chains [15]. Laminins and integrin receptors are important for cross talk between podocytes and the GBM [16]. Laminin- $\alpha 5$, the predominant α -chain in the mature GBM [17], is encoded by the gene *LAMA5*. Knockout of *Lama5* in mice causes embryonic lethality and severe defects of glomerular development [18]. In contrast, mice with a hypomorphic *Lama5* allele in the homozygous state demonstrated proteinuria, cystic kidney disease and death from progressive renal failure at 3–4 weeks of age [19]. Interestingly, *Lama5* mutations in mice have a strictly recessive mode of inheritance and heterozygous mice were indistinguishable from wild-type litter mates [18]. Two previous publications suggested an association between heterozygous mutations of *LAMA5* and adult-onset FSGS [20, 21].

We here performed whole exome sequencing (WES) in 335 individuals of 300 families with NS. After excluding likely disease-causing mutations in known monogenic causes of SRNS, we used homozygosity mapping in consanguineous families and identified recessive mutations in the gene *LAMA5* in three unrelated families with childhood-onset NS that responded to immunosuppressive therapy.

MATERIALS AND METHODS

Study participants

Following informed consent, we obtained pedigree information, clinical data and blood samples from individuals with proteinuria or NS (onset <25 years of age). Study approval was obtained from the institutional review boards of the University of Michigan and Boston Children's Hospital. Patients were enrolled between 1998 and 2016. We performed WES in 335 individuals from 300 families. Patients were recruited in collaborating centers worldwide and were ethnically diverse. Individuals of consanguineous kindred were preferentially sent for WES.

Treatment response to immunosuppressive therapy in NS was defined as follows: complete response—remission of proteinuria, with a urine protein:creatinine ratio of <0.2 mg/mg, normalization of albumin; partial response—a decrease in proteinuria by 50%; resistance—persistent, fixed proteinuria.

In cases with complete response, the absence of proteinuria was confirmed in laboratory values of negative proteinuria either in a 24-h urine specimen or in the first morning urine specimen.

A total of 258 patients with renal ciliopathies based on increased echogenicity and/or cysts on renal ultrasound who did not have proteinuria were used as a control cohort. Informed consent, sample processing and WES evaluation were performed as for NS samples.

WES

Genomic material deoxyribonucleic acid (DNA) for WES was extracted from saliva or blood lymphocytes. Following

library preparation and exome capture using the Agilent SureSelect human exome capture arrays (Life Technologies, Carlsbad, CA, USA), next-generation sequencing was performed on a HighSeq platform (Illumina, San Diego, CA, USA). Sequence reads were aligned to the human reference genome (National Center for Biotechnology Information build 37/hg19) using CLC Genomics Workbench (version 6.5.2) (CLC bio, Aarhus, Denmark). Subsequent bioinformatics analysis excluded all variants with a minor allele frequency >1% in the Short Genetic Variations database (dbSNP, version 142) or in the 1000 Genomes Project database (1094 subjects of various ethnicities; May 2011 data release). Noncoding variants outside canonical splice regions as well as synonymous variants were excluded, and variants were annotated using the Exome Aggregation Consortium (ExAC) database. Variants that were reported more than 10 times in the homozygous state in the ExAC database were excluded as likely nonpathogenic polymorphisms. Remaining variants were ranked based on established criteria [10, 11, 22] and were evaluated for likely pathogenicity under consideration of phenotypic as well as pedigree information.

Homozygosity mapping

Homozygosity mapping was calculated based on WES data. In brief, aligned Binary Alignment Map files were processed using Picard and SAMtools4 as described by other groups [23]. Single-nucleotide variant calling was performed using Genome Analysis Tool Kit [24]. The resulting variant call format files were used to generate homozygosity mapping data and visual outputs using the program HomozygosityMapper [25].

Screening for mutations in known monogenic causes of SRNS

We concatenated the genomic loci of 34 genes that are known to cause SRNS if mutated (Supplementary data, Table S1). Aligned reads from WES data for all 335 individuals were first filtered using this file and evaluated for potentially disease-causing variants in these known monogenic genes of SRNS. Further analysis seeking potential novel candidate genes was only performed if no likely pathogenic mutation was identified.

Variant filtering to identify novel monogenic causes of NS

We used homozygosity mapping data in consanguineous families (>100Mb of cumulative homozygosity according to mapping) to identify genomic candidate loci for the underlying recessive mutation. Within these areas we restricted our analysis to homozygous mutations. Remaining calls were ranked for their predicted pathogenicity based on the following criteria [10, 11, 22]: (i) protein truncating or obligatory splice site mutation versus missense mutation or in-frame deletion/insertion, (ii) evolutionary conservation, (iii) minor allele frequency in control databases [Genome Aggregation Database (gnomAD), Exome Variant Server (EVS)], (iv) chemical difference between wild-type and altered amino acid residue and (v) web-based mutation analysis prediction tools [Sorting Intolerant From Tolerant (SIFT), PolyPhen-2, MutationTaster].

Implementation of structural data and evolutionary conservation for variant evaluation

Protein domain structure cartoons and evaluation were based on the uniprot (Universal Protein Resource) database. Orthologue proteins used to evaluate evolutionary conservation were obtained from the Ensemble Genome Browser and were aligned using the Clustal Omega multiple sequence alignment tool [European Molecular Biology Laboratory (EMBL) - European Bioinformatics Institute (EBI)] [26].

Web resources

The following web resources were used: UCSC Genome Browser, <http://genome.ucsc.edu/cgi-bin/hgGateway>; 1000 Genomes Browser, <http://browser.1000genomes.org>; Clustal Omega, <https://www.ebi.ac.uk/Tools/msa/clustalo/>; Ensembl Genome Browser, <http://www.ensembl.org>; Uniprot Consortium, <http://www.uniprot.org/>; Exome Variant Server, <http://evs.gs.washington.edu/EVS> ExAC, <http://exac.broadinstitute.org>; gnomAD, <http://gnomad.broadinstitute.org>; HGMD Professional 2016.3, <https://portal.biobase-international.com/hgmd>; Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>; Polyphen2, <http://genetics.bwh.harvard.edu/pph2>; SIFT, <http://sift.jcvi.org>; MutationTaster, <http://www.mutationtaster.org> and HomozygosityMapper, <http://www.homozygositymapper.org/>.

All websites were last accessed on 2 February 2018.

RESULTS

Recessive variants of LAMA5 in patients with NS

We performed WES in 335 individuals of 300 families with NS. As a first step, we excluded likely disease-causing mutations in 34 genes that are known to cause monogenic NS if mutated (Supplementary data, Table S1). We subsequently used homozygosity mapping in consanguineous families to identify candidate loci for the underlying recessive mutation. In three unrelated families with NS that shared a homozygous peak region on chromosome 20q13 (Figure 1), we identified three different homozygous variants of unknown significance (VUS) in the gene LAMA5 (NM_005560.4) encoding laminin- α 5. In family A4389 from Turkey we detected the variant c.2239 C>T, p.Arg747Trp, conserved to *Drosophila melanogaster*; in family B150 from Egypt we detected the variant c.3002 A>G, p.Glu1001Gly, conserved to *Ciona intestinalis*; and in family B1284 from Saudi Arabia we identified the variant c.8842 G>A, p.Gly2948Ser, conserved to *D. melanogaster* (Table 1, Figure 2).

Each family had two affected children with NS, strongly suggesting an inherited cause of disease. The parents, who were heterozygous carriers of the VUS, were phenotypically healthy and did not have proteinuria. In family B1284, a DNA sample for the younger sibling was not available. In the other two families (A4389 and B150) we demonstrated the presence of the homozygous VUS in both affected children. Segregation analysis in families B150 and B1284 showed that, as expected in a recessive mode of inheritance, the parents were healthy heterozygous carriers of the VUS (Figure 2D). All three VUS were missense alleles affecting amino acid residues of strong

FIGURE 1: Homozygosity mapping in families with NS identifies recessive candidate loci. Homozygosity mapping was calculated from WES data using HomozygosityMapper. A homozygous peak on chromosome 20q13 (arrow head) that includes the *LAMA5* locus was present in all three families. (A, B) Homozygosity mapping for the two affected siblings (-21 and -22) of family A4389. (C, D) The two affected siblings (-21 and -22) of family B150. (E) Homozygosity mapping data of patient B1284-21.

evolutionary conservation among orthologue proteins of laminin- $\alpha 5$ (Figure 2C, Table 1). Bioinformatics scoring programs (SIFT, PolyPhen-2 and MutationTaster) predicted the impact of the identified variants as likely pathogenic (Table 1). Furthermore, none of the three variants has been reported in the homozygous state in databases of healthy control populations (EVS and gnomAD [27]) (Table 1). The protein laminin- $\alpha 5$ carries an N-terminal LN (laminin N-terminal) domain, followed by multiple epidermal growth factor (EGF) domains and 5 C-terminal laminin G-like (LamG) domains. Interestingly, two of the variants affected amino acid residues that are located within one of the functional domains of the protein, namely the ninth EGF-like domain (p.Arg747Trp, A4389) and the second laminin G-like domain (p.Gly2948Ser, B1284) (Figure 2B, Table 1).

Because individuals of Middle Eastern descent are poorly represented in publicly available control databases, we used 258 individuals with renal ciliopathies, a different entity of inherited kidney disease in which proteinuria is characteristically absent,

as a control cohort. However, we did not identify recessive mutations in the *LAMA5* gene in these individuals.

Treatment response and renal outcome differ in three families with VUS in *LAMA5* and NS

All five patients in whom we detected homozygous VUS in *LAMA5* shared the phenotype of NS with onset before 4 years of age. Extrarenal manifestations or syndromic features were not present in any of the affected children. However, the five children differed notably in their response to immunosuppressive treatment, their clinical presentation and regarding progression to ESRD.

In family A4389, the two children presented with NS at ages 22 months and 3 years, respectively. Both siblings responded promptly to steroids but had frequent relapses within the first year of diagnosis. Subsequently, stable remission was achieved and they have been maintained off steroids without further relapse for an interval of 3 years (at the time of this publication). The younger sibling received cyclophosphamide due to steroid dependency. No

Table 1. Genetic variants of LAMA5 in three families with nephrotic syndrome.

Family-individual	Nucleotide change	Amino acid change	protein domain	Exon (zygosity, segregation)	PPH2 score	SIFT	Mut	Amino acid conservation to species	gnomAD allele frequencies (hom/het/wild-type)	EVS allele frequencies (hom/het/wild-type)	Gender	Ethnic origin	Parental consanguinity	Age of onset (ESRD)	Extrarenal manifestations	Biopsy (at age)	Therapy and response
A4389	c.2239C>T	p.Arg747Trp		18	1.0	del	DC	<i>Drosophila melanogaster</i> ^b	0/34/275,534	0/1/4,297		Turkey	Yes		None		SSNS
-21		EGF-like domain 9		(hom, ND)					E: 0/4/125,468 A: 0/9/23,894 SA: 0/20/30,752 EA: 0/0/18,826		Male			3 yrs (no)		n.p.	3 relapses (no IS beyond steroids)
-22				(hom, ND)							Male			22 mo (no)		n.p.	4 relapses (IS with steroids and cyclophosphamide) Normal renal function in both siblings (11 and 9 yrs.)
B150	c.3002A>G	p.Glu1001Gly		24	0.92	del	DC	<i>Ciona intestinalis</i>	Not reported	Not reported		Egypt	Yes		None		
-21		n/a		(hom)							Male			4 yrs (no)		FSGS (2 yrs)	SRNS
-22				(het, m; het, p)							Female			1.5 yrs (6 yrs)		FSGS (2 yrs)	SRNS (no response to CSA)
B1284 ³	c.8842G>A	p.Gly2948Ser		65	1.0	del	DC	<i>D. melanogaster</i>	0/33/195,366	Not reported		Arabic	Yes		None		SSNS/SDNS with freq. relapses (IS with Tac, MMF)
-21		Laminin G-like domain 2		(hom)					E: 0/0/85,398 A: 0/0/17,686 SA: 0/1/24,934 EA: 0/31/12,864		Male			4 yrs (no)		n.p.	

LAMA5, NM_005560.4.

^aAllele frequencies in European Americans.^bChemically conserved as arginine or lysine.^cYounger brother presented with SSNS at age 4 years, frequent relapses of NS, no DNA sample available.

A, African (allele frequencies, gnomAD); DC, predicted to be disease-causing (SIFT); del, predicted to be deleterious (MutationTaster); E, European (allele frequencies, gnomAD); EA, East Asian (allele frequencies, gnomAD); het, heterozygous; hom, homozygous; IS, immunosuppressive therapy; m, maternal; mo, months; n/a, not applicable; ND, no data; n.p., no biopsy performed; p, paternal; PPH2 score humvar PolyPhen2 prediction score; SA, South Asian (allele frequencies, gnomAD); SDNS, steroid-dependent nephrotic syndrome; Tac, tacrolimus; tol, tolerated; yrs, years.

FIGURE 2: WES identifies recessive variants in the gene *LAMA5* in three families with nephrotic syndrome. **(A)** Exon structure of *LAMA5* cDNA (NM_005560.4). The positions of start codons, stop codons and mutated nucleotides are indicated. **(B)** Domain structure of the encoded protein laminin- $\alpha 5$. Arrows indicate the positions of the altered amino acid residues in families A4389, B150 and B1284. **(C)** Evolutionary conservation among orthologous proteins of laminin- $\alpha 5$. Altered amino acid residues in families A4389, B150 and B1284 are indicated with arrowheads and a yellow box. **(D)** Sanger sequencing of the respective regions of *LAMA5* in genomic DNA of affected patients, parental DNA and control samples as indicated. aa, amino acid; bp, base pairs; *C. elegans*, *Caenorhabditis elegans*; *D. melanogaster*, *Drosophila melanogaster*; EGF, laminin EGF-like domain; het, heterozygous; Hom, homozygous; LAM G, laminin G-like domain; LAM IV-A, laminin IV type A; LAM NT, laminin N-terminal domain.

further immunosuppressive therapy was required and both siblings showed normal renal function and persistent remission of NS at ages 11 and 9 years, respectively (Table 1).

The two siblings of family B150 had their first episode of NS at ages 1.5 and 4 years, respectively. Both siblings developed SRNS. However, while the older sibling (B150-21) initially responded to steroids and only later developed SRNS, the younger sibling (B150-22) had SRNS at her initial

presentation. The renal histology in both siblings demonstrated FSGS and the younger sister progressed to ESRD by the age of 6 years. Unfortunately, this patient died prior to renal transplantation so data on disease recurrence after transplantation are not available.

Individual B1284-21 first presented with NS at 4 years of age. He initially responded to steroid therapy but had frequent relapses and ultimately developed steroid-dependent NS,

requiring additional immunosuppressive agents to achieve remission. He has a younger brother with a similar clinical presentation. However, his genotype is not known because a DNA sample for genetic analysis could not be obtained.

DISCUSSION

We here identify recessive VUS in the gene *LAMA5* in three unrelated families with two children each with childhood-onset NS. The familial occurrence and presence of homozygosity in all three families strongly suggested a recessive cause of disease. We excluded mutations in 34 genes that are known to cause NS, if mutated. By homozygosity mapping, we identified the *LAMA5* locus as a candidate region common to all three families. We then used WES and identified recessive VUS in the *LAMA5* gene in all three families. However, because experimental evidence is lacking, the impact of these genetic variants on protein function and disease pathogenesis cannot be judged with certainty. Therefore, more data are needed to evaluate the functional relevance of these VUS in NS and to determine their potential impact on prognosis and disease management.

While ~80% of pediatric patients with NS promptly respond to therapy with steroids, inherited forms of the disease typically present with the phenotype of SRNS. We and others have previously shown that, depending on age of onset, consanguinity and familial occurrence of disease, in ~30% of SRNS cases a causative single-gene mutation can be identified [12–14]. Having a conclusive, monogenic diagnosis provides affected patients and their families with an unequivocal diagnosis, can help guide therapy and predicts a lower risk for disease recurrence after renal transplantation [5]. Therefore, genetic testing should be implemented as the standard of care in all patients with SRNS [10]. However, considering the growing number of known monogenic causes of SRNS and better availability of genetic testing, it becomes increasingly important to determine standardized criteria that help distinguish polymorphisms from true pathogenic variants [28]. This is particularly important if functional data for the identified variants are lacking and if ethnically matched control populations are unrepresented in available large datasets [27].

Familial cases of SSNS have been described [29] and genetic causation may not be limited to patients with NS that is strictly resistant to drug treatment. This was first observed for the gene *EMP2*, which, if mutated, caused a steroid-sensitive form of NS in some patients [30]. Subsequently, recessive mutations of *TNS2*, *DLC1*, *CDK20*, *ITSN1* and *ITSN2* were found to cause partially treatment-sensitive NS [31]. Our findings suggest that VUS of *LAMA5* may contribute to the development of a form of NS that in some patients responds to drug treatment. Traditionally, SSNS and SRNS are regarded as two distinct disease entities, and it has been proposed that the pathogenesis of SSNS is likely immune mediated [32]. Interestingly, the three families with VUS in *LAMA5* differed significantly in their response to drug therapy, ranging from SSNS to SRNS, including one family with an intermediate phenotype (B1284, Table 1). The observation that the same molecular defect, an alteration of the laminin- $\alpha 5$ protein, can act as a contributing factor in the pathogenesis of both phenotypes (SSNS and

SRNS) may suggest that steroid resistance and steroid sensitivity in NS occupy a phenotypic spectrum.

Despite their long-term therapeutic use, the exact mode of action of steroids is unknown. Growing evidence suggests that in addition to their immunoregulatory function, immunosuppressive drugs also act directly on podocytes [33, 34]. Via direct binding to the mineralocorticoid receptor, which has been shown to be expressed in podocytes, steroid therapy induced changes in the podocyte transcriptome [35]. In addition, it has been shown that cyclosporine A (CsA) acts directly on the podocyte actin cytoskeleton, thereby influencing actin dynamics [36]. While inherited forms of NS are characteristically regarded as non-responsive to immunosuppressive therapy, there are also some reports demonstrating that CsA can be effective in patients with Alport syndrome [37]. Alport syndrome is a form of inherited glomerulopathy caused by mutations in genes that encode collagens of the GBM. Based on these observations, one could speculate that steroids act directly or indirectly on components of the GBM by changing the gene expression profile of podocytes.

Heterozygous mutations in the gene *LAMA5* have been previously reported by two other groups as being associated with adult-onset FSGS in an autosomal dominant manner [20, 21]. However, the pathogenicity of the heterozygous mutations described in *LAMA5* remains controversial [21]. In addition, a genetic mouse model of *Lama5*, in which heterozygous mice are phenotypically indistinguishable from wild-type littermates, demonstrates a strictly recessive mode of inheritance [18]. In accordance with these findings, heterozygous parents in our study did not manifest with NS. However, it is possible that, as described for other genes, such as *COL4A3* and *COL4A4* [38], heterozygous carriers may manifest with adult-onset disease of variable penetrance, whereas recessive mutations cause a fully penetrant, early-onset disease manifestation. Further studies will be required to determine the pathogenicity of heterozygous mutations of *LAMA5*.

The mouse orthologue *Lama5* encodes Laminin- $\alpha 5$, an essential component of basement membranes. A complete genetic knockout of *Lama5* in mice caused various developmental defects and was embryonic lethal at e14–e17 [39]. A targeted deletion of *Lama5* in the kidney resulted in severely disrupted glomerulogenesis, defects in branching of the ureteric bud and sporadic renal agenesis [18]. Both findings suggest an important function of Laminin- $\alpha 5$ during renal development and glomerular maturation. Interestingly, a hypomorphic *Lama5* allele (*Lama5^{neo}*) allowed normal formation of glomerular architecture but caused delayed onset of proteinuria with thickening of the GBM and secondary glomerulosclerosis [15, 19]. This finding is consistent with the phenotype that we observed in three families with recessive, hypomorphic VUS of *LAMA5*. Furthermore, *Lama5* hypomorphic mice developed polycystic kidney disease [19]. In contrast, the renal ultrasound in all five patients with VUS in *LAMA5* was unremarkable. In particular, no cystic lesions were detected and there were no radiological signs of congenital malformations of the kidney or urinary tract.

In summary, we here identify recessive VUS in the *LAMA5* gene as genetic variants that may contribute to the development

of NS in pediatric patients. Interestingly, some of the described patients responded partially to steroids or other immunosuppressive drug regimens, whereas others were resistant to therapy. Additional data are required to determine the impact of genetic variants in the *LAMA5* gene on the pathogenesis, prognosis and potentially on disease management in pediatric patients with NS.

SUPPLEMENTARY DATA

Supplementary data are available at [ndt online](http://ndt.online).

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AUTHORS' CONTRIBUTIONS

D.A.B., J.K.W., S.A., S.S., W.T., A.D., R.S., T.H., T.J.S., E.W., A.J.M., M.N., J.M.S., C.A.H., H.H., J.R., S.M., R.P.L. and F.H. generated total genome linkage data, performed exome capture with massively parallel sequencing and performed WES and mutation analysis. D.A.B., J.K.W., S.A., S.A.B., J.A.K., S.E.D., G.D. and F.H. recruited patients and gathered detailed clinical information for the study. All authors critically reviewed the article. F.H. conceived of and directed the project. D.A.B., J.K.W. and F.H. contributed by writing the article.

CONFLICT OF INTEREST STATEMENT

F.H. is a cofounder of Goldfinch Biopharma and receives royalties from Claritas Genomics. The other authors declare that they have no competing financial interests.

REFERENCES

- Kim JS, Bellew CA, Silverstein DM *et al*. High incidence of initial and late steroid resistance in childhood nephrotic syndrome. *Kidney Int* 2005; 68: 1275–1281
- Benoit G, Machuca E, Antignac C. Hereditary nephrotic syndrome: a systematic approach for genetic testing and a review of associated podocyte gene mutations. *Pediatr Nephrol* 2010; 25: 1621–1632
- Ehrich JH, Geerlings C, Zivicnjak M *et al*. Steroid-resistant idiopathic childhood nephrosis: overdiagnosed and undertreated. *Nephrol Dial Transplant* 2007; 22: 2183–2193
- Trautmann A, Schnaidt S, Lipska-Zietkiewicz BS *et al*. Long-term outcome of steroid-resistant nephrotic syndrome in children. *J Am Soc Nephrol* 2017; 28: 3055–3065
- Trautmann A, Bodria M, Ozaltin F *et al*. Spectrum of steroid-resistant and congenital nephrotic syndrome in children: the PodoNet registry cohort. *Clin J Am Soc Nephrol* 2015; 10: 592–600
- NAPRTCS 2014 Annual Transplant Report 2014.
- Somlo S, Mundel P. Getting a foothold in nephrotic syndrome. *Nat Genet* 2000; 24: 333–335
- Zenker M, Machuca E, Antignac C. Genetics of nephrotic syndrome: new insights into molecules acting at the glomerular filtration barrier. *J Mol Med* 2009; 87: 849–857
- Hinkes BG, Mucha B, Vlangos CN *et al*. Nephrotic syndrome in the first year of life: two thirds of cases are caused by mutations in 4 genes (NPHS1, NPHS2, WT1, and LAMB2). *Pediatrics* 2007; 119: e907–e919
- Lovric S, Ashraf S, Tan W *et al*. Genetic testing in steroid-resistant nephrotic syndrome: when and how? *Nephrol Dial Transplant* 2016; 31: 1802–1813
- Vivante A, Hildebrandt F. Exploring the genetic basis of early-onset chronic kidney disease. *Nat Rev Nephrol* 2016; 12: 133–146
- Sadowski CE, Lovric S, Ashraf S *et al*. A single-gene cause in 29.5% of cases of steroid-resistant nephrotic syndrome. *J Am Soc Nephrol* 2015; 26: 1279–1289
- Bierzynska A, McCarthy HJ, Soderquest K *et al*. Genomic and clinical profiling of a national nephrotic syndrome cohort advocates a precision medicine approach to disease management. *Kidney Int* 2017; 91: 937–947
- Giglio S, Provenzano A, Mazzinghi B *et al*. Heterogeneous genetic alterations in sporadic nephrotic syndrome associate with resistance to immunosuppression. *J Am Soc Nephrol* 2015; 26: 230–236
- Goldberg S, Adair-Kirk TL, Senior RM *et al*. Maintenance of glomerular filtration barrier integrity requires laminin alpha5. *J Am Soc Nephrol* 2010; 21: 579–586
- Sachs N, Sonnenberg A. Cell-matrix adhesion of podocytes in physiology and disease. *Nat Rev Nephrol* 2013; 9: 200–210
- Miner JH. Glomerular basement membrane composition and the filtration barrier. *Pediatr Nephrol* 2011; 26: 1413–1417
- Miner JH, Li C. Defective glomerulogenesis in the absence of laminin alpha5 demonstrates a developmental role for the kidney glomerular basement membrane. *Dev Biol* 2000; 217: 278–289
- Shannon MB, Patton BL, Harvey SJ *et al*. A hypomorphic mutation in the mouse laminin alpha5 gene causes polycystic kidney disease. *J Am Soc Nephrol* 2006; 17: 1913–1922
- Chatterjee R, Hoffman M, Cliften P *et al*. Targeted exome sequencing integrated with clinicopathological information reveals novel and rare mutations in atypical, suspected and unknown cases of Alport syndrome or proteinuria. *PLoS One* 2013; 8: e76360
- Gast C, Pengelly RJ, Lyon M *et al*. Collagen (COL4A) mutations are the most frequent mutations underlying adult focal segmental glomerulosclerosis. *Nephrol Dial Transplant* 2016; 31: 961–970
- MacArthur DG, Manolio TA, Dimmock DP *et al*. Guidelines for investigating causality of sequence variants in human disease. *Nature* 2014; 508: 469–476
- Li H, Handsaker B, Wysoker A *et al*. The sequence alignment/map format and SAMtools. *Bioinformatics* 2009; 25: 2078–2079
- Van der Auwera GA, Carneiro MO, Hartl C *et al*. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinform* 2013; 43: 11.0.1–33.
- Seelow D, Schuelke M, Hildebrandt F *et al*. HomozygosityMapper—an interactive approach to homozygosity mapping. *Nucleic Acids Res* 2009; 37: W593–W599
- Sievers F, Wilm A, Dineen D *et al*. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 2011; 7: 539
- Lek M, Karczewski KJ, Minikel EV *et al*. Analysis of protein-coding genetic variation in 60 706 humans. *Nature* 2016; 536: 285–291
- Richards S, Aziz N, Bale S *et al*. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015; 17: 405–424

29. Dorval G, Gribouval O, Martinez-Barquero V *et al.* Clinical and genetic heterogeneity in familial steroid-sensitive nephrotic syndrome. *Pediatr Nephrol* 2018; 33: 473–483
30. Gee HY, Ashraf S, Wan X *et al.* Mutations in EMP2 cause childhood-onset nephrotic syndrome. *Am J Hum Genet* 2014; 94: 884–890
31. Ashraf S, Rao J, Lawson JA *et al.* Recessive mutations in 5 novel genes of interaction partners elucidate steroid sensitivity in nephrotic syndrome [abstract 6]. In: Assady S, Skorecki K eds. Abstracts. 11th International Podocyte Conference: Podocyte Biology in Glomerular Health and Disease. A Global Scientific Perspective. 3–6 April 2016. Haifa and Jerusalem, Israel: Abstracts. *Nephron* 2016; 132: 258
32. Bierzynska A, Saleem M. Recent advances in understanding and treating nephrotic syndrome. *F1000Res* 2017; 6: 121
33. Jiang L, Hindmarch CC, Rogers M *et al.* RNA sequencing analysis of human podocytes reveals glucocorticoid regulated gene networks targeting non-immune pathways. *Sci Rep* 2016; 6: 35671
34. Schonenberger E, Ehrich JH, Haller H *et al.* The podocyte as a direct target of immunosuppressive agents. *Nephrol Dial Transplant* 2011; 26: 18–24
35. McCaffrey JC, Webb NJ, Poolman TM *et al.* Glucocorticoid therapy regulates podocyte motility by inhibition of Rac1. *Sci Rep* 2017; 7: 6725
36. Faul C, Donnelly M, Merscher-Gomez S *et al.* The actin cytoskeleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A. *Nat Med* 2008; 14: 931–938
37. Charbit M, Gubler MC, Dechaux M *et al.* Cyclosporin therapy in patients with Alport syndrome. *Pediatr Nephrol* 2007; 22: 57–63
38. Buzza M, Wang YY, Dagher H *et al.* COL4A4 mutation in thin basement membrane disease previously described in Alport syndrome. *Kidney Int* 2001; 60: 480–483
39. Miner JH, Cunningham J, Sanes JR. Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. *J Cell Biol* 1998; 143: 1713–1723

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The impact of outpatient acute kidney injury on mortality and chronic kidney disease: a retrospective cohort study

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ABSTRACT

Background. Acute kidney injury (AKI) has been extensively studied in hospital settings. Limited data exist regarding outcomes for patients with outpatient AKI who are not subsequently admitted. We investigated whether outpatient AKI, defined by a 50% increase in creatinine (Cr), is associated with increased mortality and renal events.

Methods. In this retrospective study, outpatient serum Cr values from adults receiving primary care at a health system during an 18-month exposure period were used to categorize patients into one of five groups (no outpatient AKI, outpatient AKI with recovery, outpatient AKI without recovery, outpatient AKI without repeat Cr and no Cr). Principal outcomes of all-cause mortality and renal events (50% decline in estimated glomerular filtration rate to <30 mL/min/1.73 m²) were examined using Cox proportional hazards models.

Results. Among 384 869 eligible patients, 51% had at least one Cr measured during the exposure period. Outpatient AKI occurred in 1.4% of patients while hospital AKI occurred in only 0.3% of patients. The average follow-up was 5.3 years. Outpatient AKI was associated with an increased risk of all-cause mortality {adjusted hazard ratio [aHR] 1.90 [95% confidence interval (CI) 1.76–2.06]} and results were consistent across all AKI groups. Outpatient AKI was also associated with an increased risk of renal events [aHR 1.33 (95% CI 1.11–1.59)], even among those who recovered.

Conclusions. Outpatient AKI is more prevalent than inpatient AKI and is a risk factor for all-cause mortality and renal events, even among those who recover kidney function. Further research is necessary to determine risk factors and identify strategies for preventing outpatient AKI.

Keywords: acute kidney injury, albuminuria, chronic kidney disease, epidemiology, mortality