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## Genetic testing in steroid-resistant nephrotic syndrome: when and how?

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### ABSTRACT

Steroid-resistant nephrotic syndrome (SRNS) represents the second most frequent cause of chronic kidney disease in the first three decades of life. It manifests histologically as focal segmental glomerulosclerosis (FSGS) and carries a 33% risk of relapse in a renal transplant. No efficient treatment exists. Identification of single-gene (monogenic) causes of SRNS has moved the glomerular epithelial cell (podocyte) to the center of its pathogenesis. Recently, mutations in >30 recessive or dominant genes were identified as causing monogenic forms of SRNS, thereby revealing the encoded proteins as essential for glomerular function. These findings helped define protein interaction complexes and functional pathways that could be targeted for treatment of SRNS. Very recently, it was discovered that in the surprisingly high fraction of ~30% of all individuals who manifest with SRNS before 25 years of age, a causative mutation can be detected in one of the ~30 different SRNS-causing genes. These findings revealed that SRNS and FSGS are not single disease entities but rather are part of a spectrum of distinct diseases with an identifiable genetic etiology. Mutation analysis should be offered to all individuals who manifest with SRNS before the age of 25 years, because (i) it will provide the patient and families with an unequivocal cause-based diagnosis, (ii) it may uncover a form of SRNS that is amenable to treatment (e.g. coenzyme Q<sub>10</sub>), (iii) it may allow avoidance of a renal biopsy procedure, (iv) it will further unravel the puzzle of pathogenic pathways of SRNS

and (v) it will permit personalized treatment options for SRNS, based on genetic causation in way of 'precision medicine'.

**Keywords:** clinical genetic testing, molecular genetics, monogenic disease, pathogenesis of nephrotic syndrome, steroid-resistant nephrotic syndrome (SRNS)

### CLINICAL FEATURES OF STEROID-RESISTANT NEPHROTIC SYNDROME

Nephrotic syndrome (NS) is a chronic kidney disease (CKD) that is defined by significant proteinuria (>40 mg/m<sup>2</sup>/hr) with resulting hypoalbuminemia, which in turn causes edema [1, 2]. The annual incidence of NS in children in the USA is 2–6 per 100 000 children, with a cumulative prevalence of 16 per 100 000 children [3, 4]. NS is classified by response or lack of response to a standardized corticosteroid therapy into 'steroid-sensitive' (SSNS) versus 'steroid-resistant' nephrotic syndrome (SRNS), respectively. SRNS accounts for ~15% of childhood cases with NS and 40% of adult-onset cases with NS, and inevitably leads to CKD [3]. SRNS constitutes the second most frequent cause of CKD in children [5]. It carries a 33% risk of relapsing in a renal transplant, thereby causing recurrence of CKD [2]. No curative treatment is available. SRNS manifests histologically as focal segmental glomerulosclerosis (FSGS), a lesion characterized by sclerosis and podocyte foot process

effacement in a few capillary segments of a certain fraction of glomeruli [6].

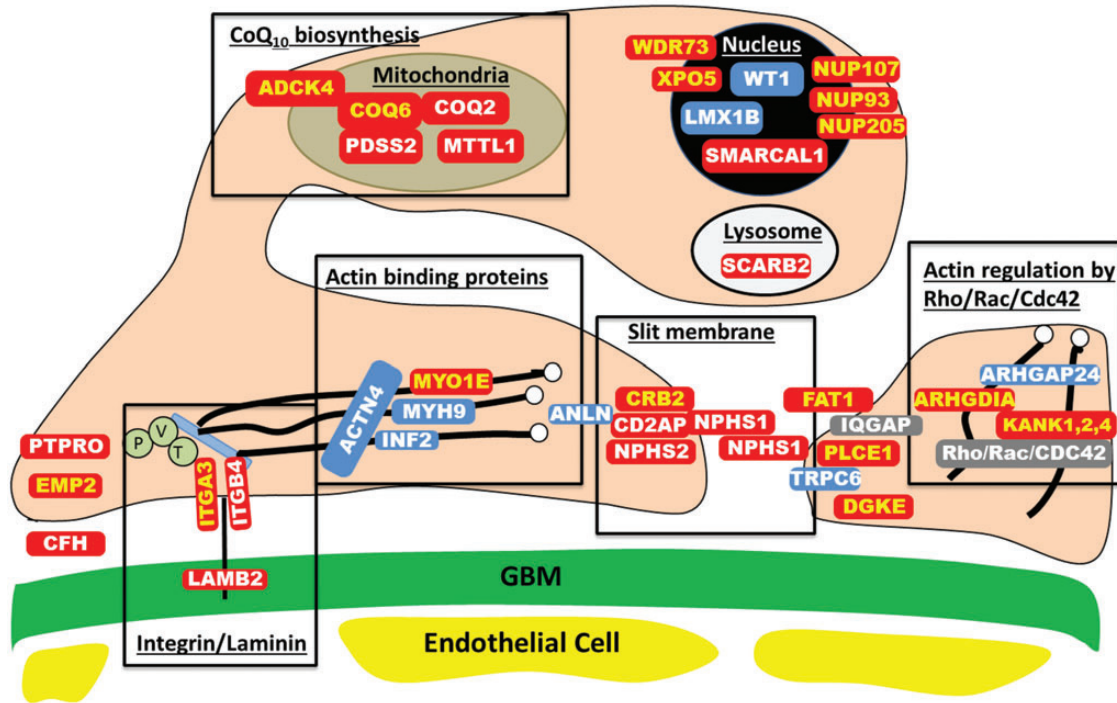
## MONOGENIC CAUSES OF SRNS ELUCIDATE THE PATHOGENESIS

The capillary tuft of the renal glomerular filtering apparatus consists of four major components: the fenestrated endothelial cell layer, the glomerular basement membrane (GBM), the epithelial podocyte layer, and the mesangial cells that help shape the glomerular tuft. In the last 15 years, >39 recessive or dominant genes have been discovered to cause SRNS in humans, if mutated (Table 1). Virtually all of the encoded proteins are localized in podocytes [43]. This discovery shifted the attention in the study of SRNS pathogenesis from mesangial cell dysfunction to podocyte dysfunction [1, 44]. It demonstrated that podocytes are essential for

normal glomerular function. This notion is confirmed by rodent models of inducible podocyte depletion, which demonstrate that podocyte damage is sufficient to cause FSGS [45, 46]. The podocyte is a neuron-like cell. It branches off cellular processes to cover the outside of the glomerular capillary, with primary, secondary and tertiary cellular processes, the latter called 'foot processes'. They interdigitate with foot processes from neighboring podocytes. The interdigitations form between them the glomerular slit membrane, which is critical for the filtering process and retention of protein in the blood stream (Figure 1). The integrity of the glomerular slit membrane is lost in NS. Thus, identification of single-gene causes of SRNS revealed dozens of proteins, each of which is an indispensable component of glomerular function, because loss of their function in a monogenic form of SRNS inescapably leads to proteinuria and FSGS. Thereby, the discovery of genes that if mutated cause monogenic forms of SRNS significantly increased our understanding of

**Table 1. Recessive and dominant genes that cause monogenic SRNS, if mutated**

Gene	Protein	Accession number	Chromosome	Reference
<b>Autosomal recessive</b>				
ADCK4	AarF domain containing kinase 4	NM_024876.3	19	[7]
ARHGDI1A	Rho GDP dissociation inhibitor (GDI) alpha	NM_001185078.1	17	[8]
CD2AP	CD2-associated protein	NM_012120.2	6	[9]
CFH	Complement factor H	NM_000186.3	1	[10]
COQ2	Coenzyme Q2 4-hydroxybenzoate polyprenyltransferase	NM_015697.7	4	[11]
COQ6	Coenzyme Q6 mono-oxygenase	NM_182476.2	14	[12]
CRB2	Crumbs homolog 2	NM_173689.5	9	[13]
CUBN	Cubilin (intrinsic factor-cobalamin receptor)	NM_001081.3	10	[14]
DGKE	Diacylglycerol kinase, epsilon	NM_003647.2	17	[15]
EMP2	Epithelial membrane protein 2	NM_001424.4	16	[16]
FAT1	FAT tumor suppressor homolog 1	NM_005245.3	4	
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	NM_005501.2	17	[17]
ITGB4	Integrin, beta 4	NM_000213.3	17	[18]
KANK1	KN motif and ankyrin repeat domain containing protein 1	NM_001256876.1	9	[19]
KANK2	KN motif and ankyrin repeat domain containing protein 2	NM_015493.6	19	[19]
KANK4	KN motif and ankyrin repeat domain containing protein 4	NM_181712.4	1	[19]
LAMB2	Laminin, beta 2	NM_002292.3	3	[20]
MTTL1	Mitochondrially encoded tRNA leucine 1	NC_012920.1	Mito	[21]
MYO1E	Homo sapiens myosin IE (MYO1E)	NM_004998.3	15	[22]
NPHS1	Nephrin	NM_004646.3	19	[23]
NPHS2	Podocin	NM_014625.2	1	[24]
NUP93	Nucleoporin 93 kDa	NM_014669.3	16	
NUP107	Nucleoporin 107 kDa	NM_020401.2	12	[25]
NUP205	Nucleoporin 205 kDa	NM_015135.2	7	
PDSS2	Prenyl (decaprenyl) diphosphate synthase, subunit 2	NM_020381.3	6	[26]
PLCE1	Phospholipase C, epsilon 1	NM_016341.3	10	[27]
PTPRO	Protein tyrosine phosphatase, receptor type, O	NM_030667.2	12	[28]
SCARB2	Scavenger receptor class B, member 2	NM_005506.3	4	[29]
SMARCA11	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a- like 1	NM_014140.3	2	[30]
WDR73	WD repeat domain 73	NM_032856.2	15	[31–33]
XPO5	Exportin 5	NM_020750.2	6	
<b>Autosomal dominant</b>				
ACTN4	Actinin, alpha 4	NM_004924.4	19	[34]
ANLN	Anillin, actin binding protein	NM_018685.2	7	[35]
ARHGAP24	Rho GTPase-activating protein 24	NM_001025616.2	4	[36]
INF2	Inverted formin, FH2 and WH2 domain containing	NM_022489.3	14	[37]
LMX1B	LIM homeobox transcription factor 1, beta	NM_00117414.1	9	[38, 39]
MYH9	Myosin heavy chain 9	NM_002473.4	22	[40]
TRPC6	Transient receptor potential cation channel, subfamily C, member 6	NM_004621.5	11	[41]
WT1	Wilms tumor 1	NM_024426.4	11	[42]



**FIGURE 1:** Proteins involved in single-gene causes and pathogenic pathways of SRNS. Identification of single-gene (monogenic) causes of SRNS has revealed the renal glomerular epithelial cell, the podocyte, as the center of action in the pathogenesis of SRNS, because all of the related genes are highly expressed in podocytes. In this way, identification of genes that, if mutated, cause SRNS revealed certain proteins and functional pathways as essential for glomerular function, because a mutation in any single one of them is sufficient to cause SRNS. This figure depicts a simplified cross-section through two neighboring podocyte foot processes, which attach to the GBM via laminin/integrin receptors. Proteins that if mutated cause recessive monogenic forms of SRNS are in red, and proteins that if mutated cause dominant forms of SRNS are in blue. These SRNS-related proteins were found to be part of protein-protein interaction complexes that participate in defined structural components or signaling pathways of podocyte function (black frames). These proteins include: laminin/integrin receptors (focal adhesions), actin-binding proteins, glomerular slit membrane-associated components, actin-regulating small GTPases of the Rho/Rac/Cdc42 family, lysosomal proteins, nuclear transcription factors and proteins involved in coenzyme Q<sub>10</sub>(C<sub>10</sub>Q<sub>10</sub>) biosynthesis. Proteins that are encoded by recessive SRNS genes are marked in red: ADCK4, AarF domain containing kinase 4; ARHGAP24, Rho GDP dissociation inhibitor (GDI) alpha; CD2AP, CD2-associated protein; CFH, Complement factor H; COQ2, coenzyme Q2 4-hydroxybenzoate polyprenyltransferase; COQ6, coenzyme Q6 monooxygenase 6; CRB2, Crumbs family member 2; DGKE, Diacylglycerol kinase, epsilon; EMP2, epithelial membrane protein 2; GBM, glomerular basement membrane; ITGA3, integrin, alpha 3; ITGB4, integrin, beta 4; KANK, KN motif And Ankyrin Repeat Domains 1/2/4; LAMB2, laminin, beta 2; MTTL1, mitochondrial tRNA leucine 1; MYO1E, Homo sapiens myosin 1e; NPHS1, nephrin; NPHS2, podocin; NUP93, Nucleoporin 93 kDa; NUP107, Nucleoporin 107 kDa; NUP205, Nucleoporin 205 kDa; PDSS2, prenyl (deca-prenyl) diphosphate synthase, subunit 2; PLCE1, phospholipase C, epsilon 1; PTPRO, protein tyrosine phosphatase, receptor type, O; SCARB2, scavenger receptor class B, member 2; SMARCAL1, SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a-like 1; WDR73, WD repeat domain 73; XPO5, Exportin 5. Proteins that are encoded by dominant SRNS genes are marked in blue: ACTN4, actinin, alpha 4; ANLN, anillin; ARHGAP24, Rho GTPase-activating protein 24; INF2, inverted formin, FH2 and WH2 domain containing; LMX1B, LIM homeobox transcription factor 1-beta; MYH9, Myosin, heavy chain 9; TRPC6, transient receptor potential cation channel, subfamily C, member 6; WT1, Wilms tumor 1. IQGAP, IQ motif containing GTPase activating protein 1; P, Paxillin; V, Vinculin and T, Talin.

glomerular filtration barrier physiology and of pathogenic mechanisms of SRNS.

This discovery started with the genes encoding the slit membrane proteins nephrin (*NPHS1*) [23] and podocin (*NPHS2*) [24]. In the meantime, acceleration of next-generation sequencing has permitted identification of >27 monogenic causes of SRNS, and this number is rapidly increasing (see references in Table 1) (Figure 1). Fascinatingly, the encoded proteins begin to map back onto distinct structural protein complexes and signaling pathways that reveal what is essential for glomerular function (Figure 1). These functional complexes include besides glomerular slit membrane components, laminin/integrin signaling components, actin-binding proteins, actin-regulating small GTPases, lysosomal proteins, transcription factors and proteins of coenzyme Q<sub>10</sub> biosynthesis.

### A SURPRISINGLY HIGH FRACTION OF EARLY-ONSET SRNS IS CAUSED BY MONOGENIC MUTATIONS

We have previously shown that 85% of SRNS cases with onset by 3 months of age and 66% of cases with onset by 1 year of age can be explained by recessive mutations in one of four genes only (*NPHS1*, *NPHS2*, *LAMB2* or *WT1*) [47]. However, a significant proportion of cases with later-onset SRNS were still molecularly unsolved [47].

When examining 48 individuals with early-onset SRNS for monogenic mutations in 16 recessive and 5 dominant SRNS genes, we detected causative mutations in 16 of the 21 genes in 33% of the individuals [48]. More recently, we examined a

**Table 2. International cohort of 526 of the 1783 families, in whom a single-gene cause of SRNS was detected in 1 of 21 monogenic SRNS genes [49]**

Gene causing SRNS	Mode of inheritance	Total SRNS families with molecular diagnosis
NPHS2	AR	177 (9.93)
NPHS1	AR	131 (7.34)
WT1	AD	85 (4.77)
PLCE1	AR	37 (2.17)
LAMB2	AR	20 (1.12)
SMARCAL1	AR	16 (0.89)
INF2	AD	9 (0.5)
TRPC6	AD	9 (0.53)
COQ6	AR	8 (0.45)
ITGA3	AR	5 (0.28)
MYO1E	AR	5 (0.28)
CUBN	AR	5 (0.28)
COQ2	AR	4 (0.22)
LMX1B	AD	4 (0.22)
ADCK4	AR	3 (0.17)
DGKE	AR	2 (0.11)
PDSS2	AR	2 (0.11)
ARHGAP24	AD	1 (0.06)
ARHGDI1	AR	1 (0.06)
CFH	AR	1 (0.06)
ITGB4	AR	1 (0.06)
Total		526 (29.5)

AR, autosomal recessive; AD, autosomal dominant.

large worldwide cohort of 1783 individuals from different families with SRNS manifesting before 25 years of age. We performed exon sequencing in all 27 genes known at the time to cause monogenic SRNS, if mutated [49]. We employed a strategy of high-throughput/low-cost multiplex PCR that we developed [50, 51]. It permits rapid sequencing of all ~600 exons of the 27 genes, barcoding PCR products by patient using the Fluidigm™ platform with consecutive next-generation sequencing. Twenty-one of the genes were recessive and six were dominant. We detected a single-gene cause of SRNS in the surprisingly high fraction of 29.5% (526 of 1783) of the individuals from different families with SRNS worldwide, who manifested before 25 years of age (Table 2) [49]. We applied stringent criteria for calling mutations ‘disease causing’ (see Boxes 1 and 2). Causative mutations were found in 21 of the 27 genes examined (Table 2). Mutations were most frequently found in *NPHS2*, *NPHS1* and *WT1* (Table 2). This surprisingly high fraction of individuals with SRNS (manifesting before 25 years), in whom a causative mutation may be detected, was recently confirmed by three other groups [52–54].

We found that the fraction of individuals in whom a single-gene cause was identified inversely correlated with age at manifestation (Figure 2). This fraction was related to age of onset as follows: onset in the first 3 months of life (69.4%), from 4 to 12 months (49.7%), from 1 to 6 years (25.3%), from 7 to 12 years (17.8%) and from 13 to 18 years (10.8%). For the *PLCE1* gene, specific mutations correlated with age of onset [49]. The frequencies in which mutations of SRNS genes were found were also dependent on the fraction of consanguineous marriages in the cohorts examined, most likely due to the increased risk for recessive disease in offspring of consanguineous unions. Specifically, in non-consanguineous families, the detection

### Box 1. Assignment of autosomal recessive mutations as being disease causing

- Include allele as disease causing if:
  - Truncating mutation (stop, abrogation of start or stop, obligatory splice, frame shift) in an expressed gene (well-annotated mRNA, sequence conservation, protein expression) or
  - Missense mutation if:
    - Continuously conserved at least up to *Danio rerio* (zebrafish) and
    - Loss of function in human allele is supported by functional data.
- Exclude allele as disease causing if:
  - Heterozygous allele frequency >1% (in EVS server: 13 000 control chromosomes) or single homozygous reported
  - Non-segregation (e.g. ‘compound heterozygous’ *in cis*; affected family member is *without* the variant; unaffected parent is *with* homozygous variant).

Baseline assumptions: (i) Full penetrance (age related). (ii) Defined clinical phenotype. (iii) ‘Mutation’ implies that an allele changes the phenotype. (iv) Known genes with similar phenotype have been excluded.

Standard criteria for how genetic variants are filtered to exclude benign changes or single-nucleotide polymorphisms when applying mutation analysis to monogenic disease genes. Alleles included for recessive gene analysis are then confirmed via Sanger sequencing and by segregation via parental or other affected family member DNA.

rate was ~25% of cases, whereas it was ~50% in consanguineous families (Figure 3) [49]. We also detected founder mutations that had a higher occurrence rate in certain regions of the world [49]. For instance, the R138Q mutation of *NPHS2* occurred frequently in Western Europe and the USA. These findings will be important for the establishment of genotype–phenotype correlations in clinical settings at distinct locations around the world. There was no gender difference for the likelihood of detecting the disease-causing mutation.

## GENOTYPE–PHENOTYPE CORRELATIONS IN MONOGENIC SRNS

### Steroid-sensitive nephrotic syndrome

It was shown early on that individuals with monogenic mutations will hardly ever exhibit steroid sensitivity of their NS [55, 56]. Very recently, however, we discovered mutations in the *EMP2* gene as a rare cause of SSNS [16, 57].

### Recessive versus dominant disease

One of the most important genotype–phenotype correlations in SRNS is the distinction between recessive versus dominant SRNS genes (Table 1). In recessive mutations, family

### Box 2. Assignment of autosomal dominant mutations as being disease causing

- Include allele as disease causing if:
  - Truncating mutation (stop, abrogation of start or stop, obligatory splice, frame shift) in an expressed gene (well-annotated mRNA, sequence conservation, protein expression) and
    - Continuously conserved to at least up to *Danio rerio* (zebrafish) or
  - Missense mutation if:
    - Continuously conserved to *Danio rerio*, and
    - Human allele is supported by functional data, and
    - Full segregation exists ( $\geq 11$  affected), and
    - Known genes with similar phenotype have been excluded.
- Exclude allele as disease causing if:
  - Heterozygous allele frequency  $>0.1\%$
  - Non-segregation—i.e. *affected* family member is *without* the allele
  - Caveat regarding non-segregation: if an *unaffected* family member is *with* the allele, consider incomplete penetrance and variable expressivity.

Standard criteria for how genetic variants are filtered to exclude benign changes or single-nucleotide polymorphisms when applying mutation analysis to monogenic disease genes. Alleles included for dominant gene analysis are then confirmed via Sanger sequencing and by segregation via parental or other affected family member DNA.

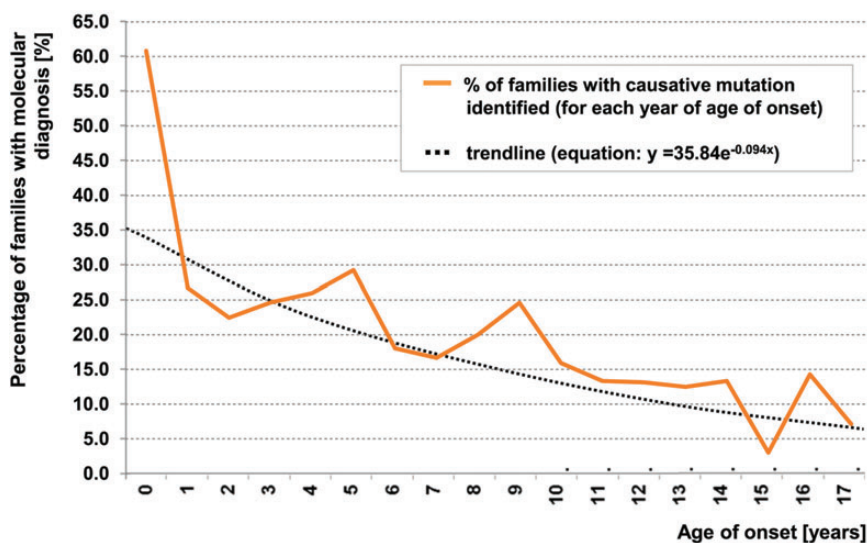
history is most likely negative, because parents of individuals with recessive mutations will be healthy heterozygous carriers, and no one in the ancestry will have had disease (because, if there is any inherited mutation, it will be heterozygous only). In contrast, in dominant disease, one of the parents of an affected individual will most likely be affected, and the disease may have been handed down through multiple generations (except for situations of *de novo* mutations, or incomplete penetrance, which can occur in autosomal dominant genes). Thereby, the detection of dominant mutations has important clinical implications, e.g. in situations of a planned living-related donor kidney transplantation. Here, it will be important to exclude the presence of the disease-causing mutation in the related donor, in whom SRNS may not yet have manifested.

#### Gene-specific phenotypes

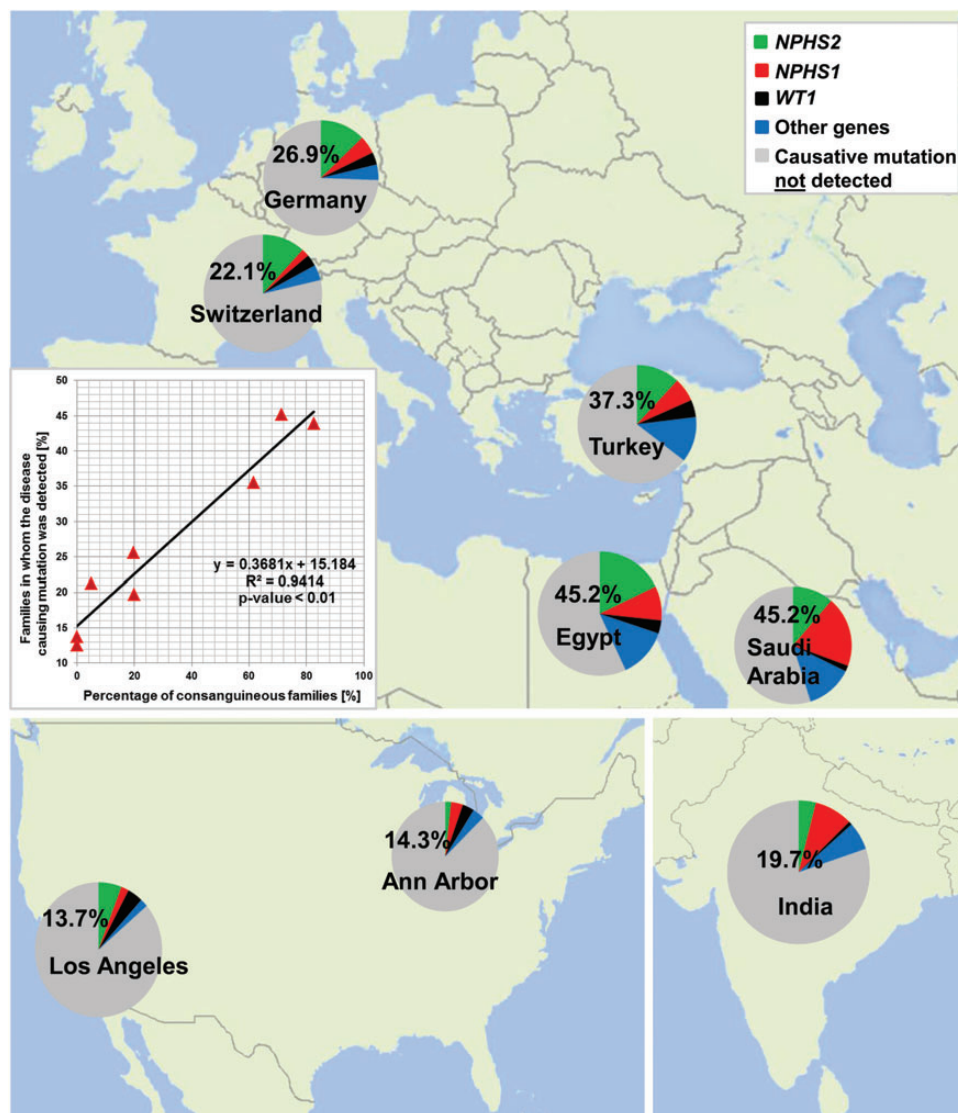
Specific SRNS genes or specific mutations (alleles) in the same SRNS gene may cause characteristic phenotypes. This may pertain to age of onset of disease [49, 58]. For instance, mutations in the recessive genes *NPHS1*, *LAMB2* or *PLCE1* lead to onset of SRNS in early childhood, whereas mutations in other recessive genes, such as *NPHS2*, lead to onset in later childhood. As a rule, mutations in dominant SRNS genes (*ACTN4*, *TRPC6*, *INF2*, *ANLN* and *ARHGAP24*) cause adult-onset SRNS, with the exception of *WT1* [49].

#### Allele-specific phenotypes

Within the same gene, specific mutations may determine a range for age of onset of SRNS that is dependent on the specific mutation. The phenomenon is known as ‘multiple allelism’. For instance, in *NPHS2* mutations, the mutation R138Q causes onset in early childhood [56, 59], whereas the mutation R229Q in compound heterozygosity with specific second



**FIGURE 2:** Age of onset distribution (in years) for 1589 of 1783 examined families with SRNS [49]. A total of 1589 individuals from different families manifested with SRNS before 18 years of age. Graph indicates percentage of solved families per year of age of onset. Black dotted line represents a binomial fit of age-related percent of families with causative mutation (in families with more than one affected family member, the mean age of onset from all affected individuals was used).



**FIGURE 3:** Percentage of genetic findings in SRNS families from the eight largest contributing centers. We obtained samples from 1783 SRNS families worldwide and detected the disease-causing mutation in 506 families (28.4%). For eight centers, we detected the disease-causing mutations in the following fractions (families in whom we detected the causative mutation/total families examined from this center): Saudi-Arabia (45.2%, 28/62), Egypt (43.8%, 64/146), Turkey (35.5%, 60/169), Germany (25.6%, 117/457), Switzerland (21.3%, 20/94), India (19.7%, 25/127), Ann Arbor (12.5%, 7/56) and Los Angeles (13.7%, 7/51). Inset: the detection rate of the disease-causing mutations strongly correlates with the rate of consanguinity between the different centers ( $R^2 = 0.9414$ ) [49].

mutations causes adult-onset SRNS [60]. Whereas currently only ‘strong’ mutations are called disease causing (Boxes 1 and 2), it is very likely that a high percentage of adult-onset SRNS is caused by ‘weak’ recessive alleles (such as R229Q of *NPHS2*), which have not yet been revealed as deleterious. One of the most important tasks in the future of renal genetics is to define deleteriousness of ‘weak’ recessive mutations that are present in the population using cell-based and animal model systems.

#### Allele-specific clinical and syndromic features

Specific SRNS genes, if mutated, may cause distinct clinical phenotypes in a gene-specific and/or allele-specific way [58]. This is especially apparent for *WT1*, where mutations in the KTS domain cause Frasier syndrome, whereas missense mutations can cause Denys–Drash syndrome or isolated NS [61]. *LAMB2*

mutations are usually associated with neuronal or retinal involvement [20, 62]. *INF2* mutations can lead either to isolated NS or can be present in individuals with Charcot–Marie–Tooth disease [63]. Individuals with *LMX1B* mutations usually present with Nail-Patella syndrome. However, specific *LMX1B* mutations have been found in individuals with isolated SRNS [64]. It is important that individuals with SRNS and certain clinical phenotypes obtain early clinical genetic testing, because this may have important consequences for clinical management, as in *WT1* where there is a risk for developing gonadoblastoma [61].

#### Correlation between mutated gene and renal biopsy pattern

For specific genes, and for specific mutations within the same mutated gene, there can be a correlation between genotype and renal histologic pattern. Individuals with congenital

onset NS (i.e. onset within the first 90 days of life) and with the renal histology of diffuse mesangial sclerosis (DMS) usually have mutation in one out of the four following genes: *LAMB2*, *WT1*, *NPHS1* or *PLCE1*. In contrast, we did not detect any specific genotypes for the histologic pattern of FSGS in SRNS [49].

### An allelic spectrum for DMS versus FSGS

Data from human genetics and from mouse models of SRNS/FSGS show that the renal histologic patterns of DMS and FSGS lie at different ends of a spectrum of a shared pathogenesis. This means that ‘severe’ recessive mutations (protein truncating mutations) of *NPHS2* cause a fetal-onset renal ‘developmental’ phenotype of immature glomeruli (i.e. DMS), whereas ‘mild’ mutations (missense mutations) in the same gene cause the renal ‘degenerative’ phenotype of FSGS [58].

## CLINICAL CONSEQUENCES FROM MUTATION ANALYSIS

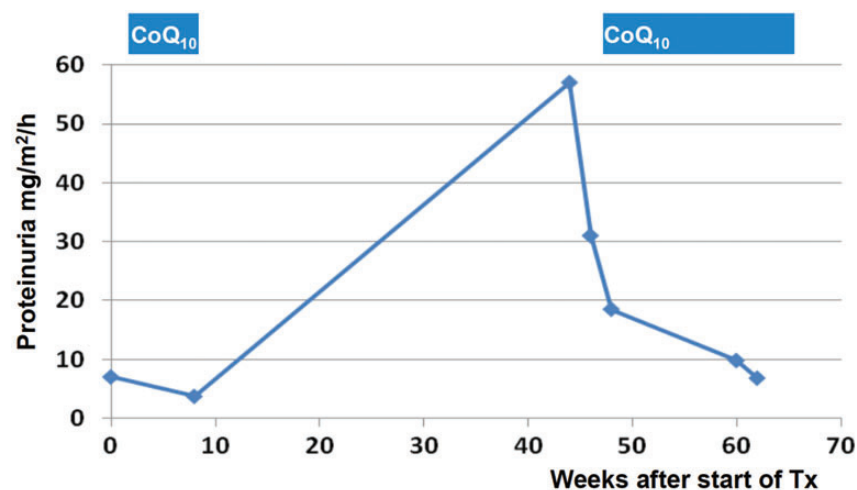
Besides the genotype–phenotype correlations discussed above, single-gene mutations in SRNS genes may have therapeutic consequences in some cases. For instance, most individuals with a single-gene cause of SRNS will not respond to steroid treatment [55, 56]. Furthermore, identification of the causative mutation may reveal that a potential therapy is available for some rare single-gene causes of SRNS. For example, if a mutation in a gene encoding enzymes of the coenzyme Q<sub>10</sub> biosynthesis is detected (*COQ2*, *COQ6*, *ADCK4* or *PDSS2*), experimental treatment with coenzyme Q<sub>10</sub> may be warranted [12, 65], because a partial response to treatment with coenzyme Q<sub>10</sub> has been described in individuals with SRNS and mutations in *COQ2* [65], *COQ6* [12] and *ADCK4* [7] (Figure 4). Likewise, a patient with recessive mutations in *PLCE1* responded fully to treatment with steroids

or cyclosporine A [27]. Finally, individuals with mutations of *CUBN* may be amenable to treatment with vitamin B<sub>12</sub>, and individuals with *ARHGDI1* may theoretically be responsive to the eplerenone treatment [66]. The detection of *WT1* mutations often has clinical consequences as, for instance, KTS+ mutations depending on karyotype may confer a risk for gonodblastoma [61]. *TRPC6* mutations may potentially be amenable to treatment with calcineurin inhibitors [67].

In summary, the benefit of identifying single-gene causes of SRNS lies in the fact that it provides an unequivocal molecular genetic diagnosis for the patients and families involved; it helps define important genotype–phenotype correlations. Furthermore, discovery of specific mutations can reveal rare monogenic causes of SRNS that may be amenable to treatment such as mutations in the coenzyme Q<sub>10</sub> biosynthesis pathway.

## WHEN TO INITIATE MUTATION ANALYSIS?

Because of the high likelihood of finding a causative monogenic mutation in SRNS with onset before 25 years of age, and because of the many important implications for disease management, it is advisable to suggest clinical genetic testing to all individuals with FSGS or with persistent proteinuria that manifests before age 25 years. For practicability and cost containment, the entire panel of ~39 monogenic genes that are known to cause SRNS if mutated can now be examined (see below) [51]. Because the likelihood of detecting a causative mutation in SRNS is inversely related to age of onset (Figure 2) [49, 59], initiation of genetic testing should be considered especially in childhood onset SRNS. However, even in young adults, the likelihood of detecting a monogenic cause of SRNS is still substantial, being >10% [49]. Mutations in recessive disease genes are found more frequently in early-onset disease, whereas



**FIGURE 4:** Response of SRNS to oral coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) in monogenic SRNS due to a mutation in an enzyme of the coenzyme Q<sub>10</sub> biosynthesis pathway. In a 5-year-old girl with SRNS and a causative homozygous mutation in the *COQ6* gene, treatment with coenzyme Q<sub>10</sub> was commenced during remission. Following inadvertent interruption of coenzyme Q<sub>10</sub> administration, proteinuria rose into the nephrotic range. Following reinstitution of therapy, proteinuria normalized [12].

mutations in dominant genes more frequently cause adult-onset disease [49]. In addition, the likelihood of finding a causative (recessive) mutation is very high in individuals with SRNS from consanguineous marriages (Figure 3).

## HOW TO INITIATE MUTATION ANALYSIS?

### Clinical testing versus research-based testing

‘Clinical genetic testing’ is the term for mutation analysis directed at finding the causative mutation(s) in a monogenic disease. It is usually performed by a certified clinical genetic testing laboratory (e.g. Clinical Laboratory Improvement Amendments, CLIA certification). Due to the rarity of some disease-causing genes, and due to the dynamic process by which thousands of novel monogenic disease genes are still discovered daily by research laboratories around the world, many diseases still require mutation analysis that is run by a research lab (‘research-based testing’). The research lab makes a decision on whether the genetic variant identified explains the disease phenotype, i.e. whether the variant can be viewed as ‘disease causing’. However, no clinical decision-making can be based on research-based findings. Therefore, the findings from research-based testing, if related ‘informed consent’ from the patient is in place, can then be utilized by the patient with the help of their physician to obtain a CLIA certified report through ‘specific mutation confirmation’ for the mutation. In this process, the physician will refer the patient to genetic counseling, during which the patient may confirm that he/she requests clinical genetic testing. A certified clinical genetic testing lab can then contact the research lab on behalf of the patient with the question, which specific gene and exon to examine for independent ‘specific mutation confirmation (SMC)’. The certified clinical testing lab can then generate a mutation analysis report for the patient. This report can be a basis for clinical decision-making.

## METHODS OF CLINICAL GENETIC TESTING IN SRNS

### Recent advances in high-throughput sequencing

Before considering screening for a causative mutation in NS, the clinician may want to be aware of the different methods of sequencing patient DNA. Recent advances in high-throughput sequencing, and the continuous reduction in cost for whole-exome sequencing (WES), have made mutation analysis less time and cost intensive. Sanger sequencing for detection of single-gene cause of SRNS has been replaced by indication-driven gene panel analysis, i.e. instead of testing for one gene at a time, >30 genes can be tested simultaneously by using high-throughput PCR amplification and sequencing approaches [49–51, 68].

Multiple commercial laboratories offer clinical genetic testing in SRNS. An internet search for ‘clinical genetic testing for nephrotic syndrome’ will reveal some of these sources. Cost ranges widely, e.g. from \$600 to \$3500 for examination of 26–30 SRNS genes. Cost may also vary depending on the

institution from which the sample is sent, as individual rates can sometimes be negotiated. As more genes are being discovered mutation analysis will be performed using whole exome sequencing, therefore the cost for mutation analysis will further decrease.

### Gene panels

Gene panel analysis is currently considered the most cost-effective approach to indication-driven mutation analysis [48–51, 68]. In this approach, all protein-coding exons of a panel of ~30 genes that are assumed to bear causative mutations in patients with a certain phenotype (e.g. proteinuria or SRNS) are sequenced. The analysis is usually based on classic Sanger sequencing within a multiplex-PCR format (i.e. amplifying multiple exons in the same PCR). For this purpose, microfluidic systems can be employed that allow thousands of reactions to occur simultaneously in nanoliter volumes [48–51, 68]. Once amplified, the samples are individually given a unique barcode for identification and then pooled for sequencing using a next-generation sequencer. This technique allows targeted sequencing at ~5% of what a traditional Sanger sequence would cost [51]. However, the number of genes that can be examined using this technique is limited to ~30 genes.

### Whole exome sequencing

In contrast to gene panel sequencing, WES allows sequencing of all ~330 000 exons in the human genome (i.e. the ‘exome’). Exon-containing fragments of DNA are first enriched from the patient’s DNA sample, using solid-phase/array-based hybridization of the patient’s DNA fragments with bait probes that represent the sequences of all exons to capture the entire exome [69]. It is currently assumed that WES offers a theoretical likelihood of 86% of detecting the disease-causing mutation in a recessive disease [70, 71]. Besides its use to detect mutations in an established list of known disease-causing genes, WES was also very successfully applied to detecting novel disease-causing genes (*ADCK4*, *KANK2*, *EMP2*, *CRB2* and *CEP164*).

Following exome capture, all exon fragments are sequenced using a high-throughput next-generation sequencing platform [72]. The sequences are then compared with the human reference genome ([www.genome.ucsc.edu](http://www.genome.ucsc.edu)) for genetic sequence variants that differ between the patient and a normal reference sequence. Any given two individuals differ by ~2000 genetic variants [51, 73]. However, in a monogenic disease, only one or two variants in a single gene represent the causative mutation. Finding the relevant causative genetic mutation requires an elaborate *a priori* reduction process, either by genetic mapping [74], by application of stringent genetic criteria (see Boxes 1 and 2) or by applying algorithms on ‘deleteriousness’ of genetic variants (see below ‘Calling genetic variants mutations’).

### Whole genome sequencing

Whole genome sequencing (WGS) uses massively parallel DNA enrichment technology to sequence the entire genome of an individual, including the 99% of the genome that is non-coding and highly variable. WGS is the most inclusive method



of sequencing a DNA sample of an individual for genetic variants, but it is still the most costly method. Currently, there is no significant advantage of its use over WES in monogenic diseases, as all mutations that lie outside exons or exon splice sites are difficult to implicate as causative for disease, due to their very indirect role for protein function. This situation may change within the next 10–15 years, as more informed algorithms for allele calling may evolve, together with deeper sources of WGS sequence data from worldwide human populations.

### Sanger sequencing

The classic chain-termination method of DNA sequencing ('Sanger sequencing') still plays a very important role for confirmation of genetic variants that result from high-throughput sequencing techniques such as WES. It also plays a role in SMC following research-based genetic testing (see above).

## CALLING GENETIC VARIANTS MUTATIONS

The term 'genetic variant' is used for any difference in the DNA sequence between two individuals, e.g. a patient and a 'normal reference individual'. In contrast, the term 'mutation' is used only if there is a strong level of certainty that a genetic variant alters the phenotype of an individual, i.e. causes disease. In a potentially monogenic disease such as SRNS, very stringent genetic and biological criteria should be followed before a mutation is called disease causing. The criteria that we follow for mutation calling in recessive and dominant renal diseases are summarized in [Boxes 1 and 2](#).

To ensure identification of disease-causing variants, there is a process of quality control that minimizes the technical errors in sequencing and gene annotation that have been introduced throughout the process of DNA sequencing. In addition, there is an elaborate process for reduction of variants from the many genetic variants that result when comparing exonic sequences from a patient with a normal reference sequence. Clinical exome sequencing studies have been published that feature a tiered pipeline to sort allelic variants into different categories of disease based on an *a priori* gene list generated from the clinical phenotype [75]. Variants are then assigned different designations to reflect likelihood of pathogenicity ranging from 'probable' to 'unknown significance'. Between clinical genetic diagnostic programs and research laboratories, there is little difference in the actual variant reduction process, once the variant list is generated [51, 69, 70, 73, 75–77].

Genetic criteria that we use for reduction of genetic variants and identification of the unique disease-causing mutation(s) are listed separately for recessive versus dominant genes in [Boxes 1 and 2](#). In general, the decision that a genetic variant can be called a disease-causing mutation is made if a genetic variant truncates the encoded protein or represents an obligatory splice site altering variant. In missense mutations (non-synonymous variants), the degree of evolutionary conservation of the related amino acid residue is evaluated. The frequency of the variant is also compared with population databases of healthy control individuals ('1000 genomes', [\[genomes.org\]\(http://genomes.org\); 'Exome Variant Server', \[evs.gs.washington.edu\]\(http://evs.gs.washington.edu\); and the ExAC Browser, \[exac.broadinstitute.org\]\(http://exac.broadinstitute.org\)\). Variants in dominant disease genes should be absent from these databases even in the heterozygous state, except when incomplete penetrance is expected for a given gene. In recessive disease genes, heterozygous variants may be present in healthy control populations. A minor allele frequency of <1% is usually expected when judging a recessive variant as potentially disease causing. It is also reviewed if a certain variant has been published as disease causing before \(e.g. HGMD Database, \[www.hgdm.cf.ac.uk\]\(http://www.hgdm.cf.ac.uk\)\). Finally, it is judged if loss of function for a variant has been tested in a cell-based or animal model system, which usually requires a sizable investment of work by a research laboratory. Electronic prediction programs that integrate the above criteria are also consulted \(e.g. PolyPhen-2 database; \[genetics.bwh.harvard.edu/pph2\]\(http://genetics.bwh.harvard.edu/pph2\)\).](http://www.1000</a></p>
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Variants called as potentially disease causing will then have to be confirmed by Sanger sequencing. Segregation analysis in parental DNA samples will show that the two compound heterozygous mutations in a recessive gene come from one parent each and are not inherited from the same parent. If the latter was the case, this, which would exclude at least one of the variants as not disease causing if both parents are healthy, is usually the case in a recessive disease.

## HOW TO INTERPRET RESULTS FROM MUTATION ANALYSIS

### Variants of unknown significance

It is very important to understand that a finding of 'variants of unknown significance (VUS)' resulting from clinical genetic testing has absolutely no heuristic bearing on the diagnostic test and should be considered inconclusive. Often these findings are misinterpreted in the way that there is 'a little bit of an abnormal finding', where the correct interpretation would be that those VUS are meaningless biologically and clinically, because the hypothesis that these variants could explain disease had to be rejected according to *a priori* interpretation criteria.

### Recessive versus dominant

Similarly, when interpreting the meaning of results from clinical genetic testing, it is very important to have a clear understanding of the action of recessive versus dominant disease genes. In dominant diseases, a heterozygous mutation alone is sufficient to cause disease (with the exception that there can be incomplete disease penetrance in a relative or ancestor of the patient). In contrast, in a recessive disease gene, both gene copies need to be mutated, either in a homozygous way (i.e. the same mutation is inherited from father and mother) or in a compound heterozygous way (i.e. two different mutations in the same gene are inherited from each parent).

### Challenges to clinical genetic testing

Because gene panel sequencing and WES generate a problem of 'multiple testing', it is very important to assure, when interpreting clinical genetic testing results, that the interpreting lab follows strict genetic interpretation criteria (see [Boxes 1 and 2](#))

in order to avoid false-positive results, which may lead to unwarranted actions in the clinical management of the patient. Even if the sequencing process correctly annotates the variant, the reference sequence in itself contains built-in errors, including reference errors such as pseudogenes annotated as coding regions.

## FUTURE DIRECTIONS

The recent identification of single-gene causes of SRNS in a surprisingly high fraction of individuals worldwide has shown that SRNS and FSGS are not single disease entities but rather represent a spectrum of distinct diseases with clearly defined etiology. Gene identification will offer many advantages for future management of SRNS. With the available sequencing technology and the continuous reduction in sequencing cost, panel genetic testing should be offered to every patient with persistent proteinuria occurring before 25 years of age if the patient consents for clinical genetic testing for the following reasons:

- (i) Gene panel sequencing became technically feasible only very recently and therefore represents a modern approach to the diagnostics of SRNS.
- (ii) It was discovered very recently that in SRNS with onset before 25 years of age, there is a very high likelihood to successfully reveal a causative mutation [49].
- (iii) Mutation analysis will provide the patient and families with an unequivocal cause-based (etiologic) diagnosis, potentially enabling etiology-based ‘personalized’ clinical management of SRNS.
- (iv) Mutation analysis may allow avoidance of a renal biopsy procedure.
- (v) Mutation analysis may reveal a form of SRNS that is amenable to treatment, as exemplified by rare genetic forms of SRNS that can be treated with coenzyme Q<sub>10</sub> (Figure 4).
- (vi) Clinical, epidemiologic, and treatment studies should always attempt to reveal the etiologic type of SRNS by identifying any causative mutation in a monogenic gene, in order to stratify those cohorts according to ‘etiologic’ criteria. Study data should not be obscured by generating an unspecific ‘genetic burden analysis’ that does not take the action of Mendelian (monogenic) genes and mutations into account.
- (vii) Clear definition of the ‘monogenic landscape’ in large cohorts of patients with SRNS will help further unravel the puzzle of pathogenic pathways of SRNS.
- (viii) Specific disease-causing mutations from patients with SRNS may be studied in cell-based and animal model systems to develop ‘personalized’ treatment approaches.

In the near future we should expect personalized treatment options for SRNS (‘precision medicine’) based on genetic causation, as exemplified by mutation in genes of the coenzyme Q<sub>10</sub> biosynthesis pathway (*COQ2*, *COQ6*, *ADCK4* or

*PDSS2*) where treatment with coenzyme Q<sub>10</sub> can be attempted [7, 12].

Recently, a single-gene cause of SSNS has been discovered by revealing mutation in *EMP2*. It is expected that more SSNS genes will be discovered and that the discovery of these genes may offer inroads into understanding the therapeutic actions of glucocorticoids in SSNS.

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## Innate immunity in CKD-associated vascular diseases

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### ABSTRACT

Chronic kidney disease (CKD) is associated with an increased risk for cardiovascular events. Therefore, the activation of the innate immune system plays an important role. In contrast to the adaptive immunity, unspecific recognition of conserved endogenous and exogenous structures by pattern recognition receptors (PRRs) represents a key feature of the innate immunity. Of these PRRs, Toll-like receptors (TLRs) as well as the inflammasome complex have been documented to be involved in the pathogenesis of cardiovascular diseases (CVDs). They are not only expressed in leukocytes but also in a variety of cell types such as endothelial cells or fibroblasts. While activation of TLRs on the cell surface leads to nuclear factor  $\kappa$ B-dependent expression of pro-inflammatory mediators, the inflammasome is a cytosolic multimeric protein complex, which cleaves cytokines such as interleukin-1 $\beta$  into their biologically active forms. Several endogenous ligands for these PRRs have been identified as contributing to the development of a CKD-specific pro-inflammatory microenvironment. Notably, activation of TLRs as well as the inflammasome is associated with arterial hypertension, formation of atherosclerotic vascular lesions and vascular calcification. However, detailed molecular mechanisms on how the innate immune system contributes to CKD-associated CVDs are as yet poorly understood. Currently, several agents modulating the activation of the innate immune system are the focus of cardiovascular research. Large clinical studies will provide further information on the therapeutic applicability

of these substances to reduce cardiovascular morbidity and mortality in the general population. Further trials including patients with CKD will be necessary to assess their effects on CKD-associated CVD.

**Keywords:** cardiovascular disease, chronic kidney disease, inflammasome, innate immunity, Toll-like receptors

### INTRODUCTION

Chronic kidney disease (CKD) is frequent in Western populations. The incidence of patients with end-stage renal disease receiving dialysis therapy or kidney transplantation is 200 cases per million per year in many European countries [1]. Especially patients with end-stage renal disease, but also patients with slightly reduced kidney function exhibit a high cardiovascular burden leading to an increased risk of death, cardiovascular events and hospitalization [2]. Therefore, it is not surprising that recent studies identified CKD as a potent and independent risk factor for cardiovascular disease (CVD) in addition to traditional risk factors, e.g. arterial hypertension, smoking, obesity, dyslipidemia and diabetes [3]. Hereby, traditional risk factors as well as non-traditional risk factors, such as inflammation, contribute to the high cardiovascular burden in CKD patients [4].

Inflammation is mediated by an interaction of multiple components of the innate and adaptive immune systems including complement factors, white blood cells and cytokines. Moreover,