SGPL1 mutations cause primary adrenal insufficiency and steroid resistant nephrotic syndrome

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Abstract

Primary adrenal insufficiency is life-threatening and can present alone or in combination with other co-morbidities. We describe a novel syndrome of primary adrenal insufficiency and steroid resistant nephrotic syndrome caused by loss-of-function mutations in sphingosine-1-phosphate lyase (SGPL1). SGPL1 executes the final decisive step of the sphingolipid breakdown pathway, being the irreversible cleavage of the lipid signalling molecule sphingosine-1-phosphate (S1P). Mutations in other upstream components of the pathway lead to harmful accumulation of lysosomal sphingolipid species, which are associated with a series of conditions known as the sphingolipidoses. Four different homozygous mutations, c.665G>A (p.R222Q), c.1633_1635delTTC (p.F545del), c.261+1G>A (p.S65Rfs*6) and c.7dupA (p.S3Kfs*11), were identified in five families with the condition. In total eight patients were investigated, some of whom also manifested other features including ichthyosis, primary hypothyroidism, neurological symptoms and cryptorchidism. The Sgpl1−/− mouse recapitulated the main characteristics of the human disease with abnormal adrenal and renal morphology. Disruption of adrenocortical zonation and defective expression of steroidogenic enzymes were seen in the null together with renal histology in keeping with a glomerular phenotype. In summary we have identified the first SGPL1 mutations in humans perhaps representing a novel multi-systemic disorder of sphingolipid metabolism.
Introduction

Primary adrenal insufficiency (PAI) is most commonly congenital in children. Manifestations can include hyperpigmentation, failure to thrive, and a poor response to illness with hypoglycaemia and hypotension. Reduced life expectancy is described and the condition can be fatal if undetected. It is genetically heterogeneous with some gene defects causing syndromic disease. Mechanisms of disease include steroid biosynthetic defects, ACTH resistance, adrenal dysgenesis, cholesterol synthesis disorders, and metabolic disorders incorporating peroxisomal and mitochondrial defects [reviewed in (1, 2)]. Our group has previously identified a number of genes in adrenal insufficiency syndromes (3-8), however, within our patient cohort (n>350) the genetic cause is currently unknown for 38% of cases, rendering their prognosis uncertain.

We investigated one extended, consanguineous kindred with four affected individuals with PAI, two of whom also had steroid resistant nephrotic syndrome, described as focal segmental glomerulosclerosis (FSGS) (9) [Figure 1; Kindred 1]. Inheritance was suggestive of an autosomal recessive pattern and we used whole exome sequencing to investigate. Mutations identified in a novel candidate gene were then sought in the remainder of our cohort and in patients manifesting focal segmental glomerulosclerosis alone.
Results

Human genetic findings

The index case, patient 1, was diagnosed with an isolated glucocorticoid deficiency aged 8 months when he presented with genital hyperpigmentation. He subsequently developed steroid-resistant nephrotic syndrome with biopsy findings of focal segmental glomerulosclerosis (FSGS) at 2.5yr and received a kidney transplant aged 5yr (9). A younger sibling with similar clinical history (not sequenced) died aged 4yr whilst an older sibling, 8yr, and a cousin, 3yr, have only PAI (9) [Figure 1, Kindred 1; Table 1].

Affected individuals were mutation negative for the known genetic causes of PAI (3). Whole exome sequencing was carried out for two affected individuals in the pedigree (Patients 2 & 3; Figure 1). Single Nucleotide Polymorphisms, with a threshold coverage of at least 10 reads on the respective nucleotide, were assessed using Ingenuity Variant analysis (detailed description in methods). In brief, the number of variants was reduced by the following strategy; (i) identifying variants that were common to both individuals (ii) excluding variants that were heterozygous (iii) removing variants, annotated in SNP databases (dbSNP, release 85, ExAC database accessed July 2016; see URLs), with a minor allele frequency of >0.01 and (iv) evaluating non-synonymous coding variants, splice variants and indels only. Finally, candidate variants in 2 genes (chr10:69957117C>T in MYPN and Chr10:72628151G>A in SGPL1) were investigated for segregation with disease in the kindred by Sanger sequencing. Both variants segregated with disease in the family. MYPN encodes myopalladin, which is expressed in striated muscle and functions as a structural, signaling and gene expression regulatory molecule in response to muscle stress. Variants in MYPN have previously been implicated in dilated cardiomyopathy, not a clinical finding in our patients and, although rare (allele count 69/121208), there are 2 homozygotes noted in ExAC for this variant. Thus we demoted this variant in MYPN and focused on the variant in SGPL1. This variant, Chr10:72628151G>A c.665G>A; p.R222Q has a minor allele frequency of 1.656e-5, this represents 2/120744 alleles reported in ExAC browser,
both are heterozygotes; one is from the South Asian population and the other non-Finnish European. No homozygotes are annotated in any database (see URLs).

SGPL1 encodes sphingosine-1-phosphate lyase (SGPL1), an important endoplasmic reticulum (ER) enzyme in sphingolipid catabolism [reviewed in (10, 11)]. SGPL1 executes the final decisive step of the sphingolipid breakdown pathway, initiating irreversible cleavage of the lipid signalling molecule sphingosine-1-phosphate (S1P) (Figure 2A).

We subsequently identified further mutations in SGPL1 in 5 affected patients with a background of PAI from 4 additional families using whole exome or Sanger sequencing [Figure 1, Kindreds 2-5; Table 1]. In 4 out of these 5 patients PAI was also associated with a steroid resistant nephropathy. In all cases patients were homozygous for the change and unaffected parents were heterozygous. The c.665G>A (p.R222Q) mutation seen in kindred 1 was also identified in patient 4 (kindred 2) who presented with PAI at 18 months. Interestingly, the R222Q mutations in kindreds 1 and 2 have arisen independently, two synonymous exonic (rs827249 and rs865832) and two intronic (rs41315008 and rs923177) SNPs, within SGPL1 itself and surrounding the mutation, differ between patients in these kindreds (Supplementary Table 1). In Kindred 3, a female (patient 5) presented with PAI at 6 months, developing nephrotic syndrome on follow-up aged 5.5 yr. Patient 5 and her parents underwent WES and analysis through the GOSgene pipeline (12). Under a homozygous recessive model the only rare variant that segregated with the disease was an in-frame deletion c.1633_1635delTTC in SGPL1. Kindred 4 comprised two affected siblings (patients 6 & 7), manifesting PAI and nephrotic syndrome (<1yr for both), who had a canonical splice site change, c.261+1G>A; p.S65Rfs*6. Patient 8, presenting with nephropathy (<1yr) and later developing PAI, had a frameshift [c.7dupA; p.S3Kfs*11]. The mutations in kindreds 3, 4 and 5 were novel and have not been annotated in any database. Concurrently exome data from a cohort of 200 paediatric patients presenting with focal segmental glomerulosclerosis alone was examined for SGPL1 mutations but none were identified. The frameshift and splice site mutations, p.S3Kfs*11 and p.S65Rfs*6 respectively, are predicted to result in markedly truncated mRNA message and are likely...
to be destroyed by nonsense mediated mRNA decay suggesting the phenotype results from loss of enzyme activity and may represent a new defect of sphingolipid metabolism. The mutations p.R222Q and p.F545del are within highly conserved residues and are predicted to cause disruption to vital, highly conserved eukaryotic protein domains [Figure 2B]. To reveal whether the mutants possessed residual lyase activity, the mutated proteins were expressed in Sgpl1−/− mouse fibroblasts. The activity of both mutants was close to zero (Figure 2C). The mutations also appear to affect expression or possibly stability of the protein, with both the p.R222Q and p.545del detected at the expected size but in lower amounts than the wild-type on immunoblotting (Figure 2D).

In agreement with previous studies SGPL1 is ubiquitously expressed in human tissues, with moderate levels in the adrenal cortex and kidneys [Figure 2E], consistent with rat and mouse expression profiles (13-15). High levels were noted in the testes and thyroid [Figure 2E]. Mutations in other components of the sphingolipid breakdown pathway lead to harmful accumulation of lysosomal sphingolipid species, which is associated with a spectrum of conditions known as the sphingolipidoses (16). These include Niemann-Pick disease, Gaucher disease and Fabry disease amongst others, which are multi-systemic and often progressive. Whilst a renal phenotype is described in some of these conditions (17), adrenal disease has not been reported to date.

**Human Clinical Findings**

In our patients, PAI is common to all affected individuals presenting in infancy with the exception of patient 8 who presented aged 9 yr. For most this is an isolated glucocorticoid deficiency necessitating hydrocortisone replacement, two patients additionally have mineralocorticoid deficiency [Table 1]. The only post-pubertal patient within the cohort, patient 8, also has marked adrenal androgen deficiency [serum dehydroepiandrosterone sulphate level <3 mcg/dL (NR 26-460); androstenedione <1 ng/mL (NR 0.5-4.7)]. Adrenal imaging has been undertaken in some of the patients. Patient 5 has normal appearance of the adrenals on US and MRI (undertaken aged 0.5 yr
and 5.9 yr respectively). Patient 6 and patient 7 are both reported to have imaging suggestive of calcifications in the adrenals (imaging undertaken at age 1 yr and 0.6 yr respectively), with Patient 6 additionally reported to have bilateral enlarged adrenal glands.

The nephrotic syndrome is steroid resistant, present in 5 patients and manifested between the ages of 1.5 months to 5.5 yr [Figure 1, Table 1]. Biopsy findings were consistent with focal segmental glomerulosclerosis in all affected patients [Supplementary Table 2; further details for patients 1, 5-7]. Electron microscopy of the renal biopsy from Patient 5 showed cellular vacuolisations and partial effacement of podocytes [Supplementary Figure 1; Electron microscopy of renal biopsy for patient 5]. Patients 1, 6, 8 are post renal transplant whilst Patients 5 and 7 are currently awaiting transplantation. Patient 8, age 17.5 yr, redeveloped proteinuria at 12 yr following her initial transplantation at 5 yr, necessitating a second renal transplant, a graft biopsy showed graft failure from chronic rejection.

Extra-adrenal and -renal effects are described within our cohort. Generalised ichthyosis is present in most, though not all patients [Table 1, Supplementary Figure 2]. Skin biopsies conducted in patients 6 and 7 demonstrated a thinned epidermis with hyperkeratosis and decreased granular layer. Primary hypothyroidism is reported in 4 of the patients requiring treatment with L-thyroxine (Patients 5-8). Thyroid peroxidase antibodies are negative in all cases.

Neurodegenerative disease is seen in several of the sphingolipidoses associated with accumulating sphingolipid metabolites. Here, progressive motor and cognitive decline is described for three patients (5, 6 & 7), with ataxia and sensorineural hearing loss also reported (5 & 6) [Table 1]. Both Peruvian siblings (6 & 7) initially presented with development appropriate for age. However, during follow up, impaired acquisition of new skills is reported. Patient 6 has loss of speech and has developed progressive hypotonia and truncal ataxia, and aged 8.4 years is no longer walking. Following development of ataxia this year, cranial MRI showed contrast enhancement of cerebellar structures amongst other features. Patient 5 is reported to have had normal development as
assessed by Denver II developmental screening test aged 2.3 yr. However, assessment at 4.3 yr showed delayed gross motor, language and social skills. Currently aged 6 yr she has developed mild gait abnormalities associated with ataxia. Serial Cranial MRI highlights the progressive nature of her disease [Supplementary Figure 3]; at 4.3 yr, contrast enhancement was seen in the bilateral globus pallidus, medial thalamic nucleus and central pons (not observed in a previous scan at 8 months).

She has had a normal EMG. Ophthalmological findings in this patient include ‘salt and pepper’ retinopathy, cranial nerve III to IV synkinesia, ptosis and esotropia affecting the right eye. She has complex partial seizures. None of the above mentioned patients are reported to have autonomic dysfunction. The oldest patient in this cohort (patient 8 at 17.5 yr), however, has no intellectual disability and normal neurological findings including a normal brain MRI (conducted at 11 yr).

Fasting lipid profiling revealed raised total cholesterol and triglycerides in some of the patients (Table 1), though in all cases sampling was conducted with diagnosis of nephrotic syndrome. Mass spectrometric analysis of serum from Patient 5 (with p.F545del) revealed increased S1P and ceramide species in comparison to an age and sex matched control (Supplementary Figure 4), which would fit with our hypothesis that this disease alters sphingolipid metabolism.

Persistent lymphopenia is a reported feature in two of our patients (Table 1). This has been most extensively investigated in Patient 5 with lymphocyte subset analysis revealing low CD3+ and low naïve CD4+ and naïve CD8+ cells, but normal proliferative capacity [Supplementary Tables 3 & 4]. Neither of these patients has an increased frequency of infections.

Under-virilisation was reported in Patient 6 who has micropenis, right cryptorchidism and bilateral micro-orchidism, associated with low serum Anti-Müllerian hormone, suggesting partial gonadal dysfunction may be part of the clinical picture.

Sgpl1−/− mouse adrenal histology and renal histology; expression of SGPL1 in human adrenals
SGPL1 is highly conserved, with human SGPL1 sharing 84% identity and 92% similarity to mouse SGPL1 (14, 18). The mutations are predicted to be loss-of-function hence the Sgpl1<sup>−/−</sup> mouse could provide a model for the disorder. We investigated the phenotype of the Sgpl1<sup>−/−</sup> mouse, both our own findings together with previous reports in the literature, and compared this with the clinical findings in our patients [Table 2]. Sgpl1<sup>−/−</sup> mice are born normally, but fail to thrive around two weeks of age and most die within the first few weeks of weaning, the reason for this is unknown (19–21). Whilst Sgpl1<sup>−/−</sup> mice are reported to have impaired testicular and ovarian steroidogenesis and are infertile (22), an adrenal phenotype has not previously been investigated.

In our study of mouse adrenals, cortical zonation was found to be compromised in 10 day old Sgpl1<sup>−/−</sup> mice regardless of sex, with less definition between zona glomerulosa (ZG) and zona fasciculata (ZF), and between ZF and X-zone [Figure 3A]. Cells in the corticosterone-producing ZF were smaller, contained fewer lipid droplets and had a higher degree of eosinophilia. Compromised development of the X-zone is seen in other mouse models of adrenal insufficiency including those with Sf1 mutations (23) suggesting that SGPL1 may also have a role to play in the developing human adrenal.

Disruption in steroidogenesis was supported by the analysis of steroidogenic enzymes in the adrenal tissue from these mice. Sgpl1<sup>−/−</sup> adrenals showed lower expression of cytochrome P450 side chain cleavage (CYP11A1), the first enzyme in the steroidogenic cascade, whilst the classical pattern of subcapsular clusters of aldosterone synthase (CYP11B2) staining present in Sgpl1<sup>+/+</sup> mice was replaced by a more continuous pattern in adrenals from Sgpl1<sup>−/−</sup> mice [Figure 3B].

Adrenal glands from affected patients were not available for study but Western blotting confirmed the presence of SGPL1 in normal human adrenals; three immunoreactive bands were observed in lysates of adrenals and HEK293 overexpressing SGPL1, indicating the specificity of our antibody in detecting SGPL1 [Figure 3C]. SGPL1 was expressed in the human fetal adrenal at all stages analysed, namely Carnegie stage 19 (approximately 46<sup>th</sup> day) and Carnegie stage 22 (approximately 53<sup>rd</sup> day) (Figure 3D). At a later stage (18 weeks) when the fetal and definitive zone (which later constitutes
the ZG, ZF, ZR (zona reticularis)) are distinguishable, SGPL1 was found to be expressed in both
(Figure 3D). In the adult human adrenal, SGPL1 immunoreactivity was seen throughout the cortex,
with the highest signal in the ZR, responsible for adrenal androgen secretion, and very little
expression in the medulla and capsule [Figure 3E].

Consistent with previous reported findings in SGPL1 deficient mice (20, 24), we see mesangial
hypercellularity and proteinaceous casts in the tubules of Sgpl1<sup>−/−</sup> mice with overall histological
appearances supporting a glomerular phenotype [Figure 4A-H]. Moreover, a higher degree of
glomerular fibrosis was observed in kidneys from Sgpl1<sup>−/−</sup> mice using Masson’s trichrome stain
[Figure 4D &H].

Discussion

This is the first instance of SGPL1 deficiency in humans highlighting a novel biochemical player
involved in adrenal disease. The sphingolipids form one of the major classes of the mammalian
lipidome. They have multiple roles; some as structural components of cell membranes
(sphingomyelin, glycosphingolipid), and others as signaling molecules (ceramide, sphingosine,
sphingosine-1-phosphate (S1P)). S1P has autocrine, paracrine and distant effects, regulating cell
migration, differentiation, survival and other complex physiological processes by activating a
subgroup of G-protein coupled receptors referred to as S1P<sub>1</sub>-<sub>5</sub>. SGPL1, an intracellular ER enzyme,
carries out the final decisive step of the pathway with degradation of intracellular S1P, regulating
flow of the sphingolipid biochemical intermediates and therefore has a key role in cell metabolism
and determining cell fate [Figure 2A].

Lipids including sphingolipids have been shown to control steroid hormone biosynthesis in adrenal
glands. Sphingolipid intermediates ceramide and sphingosine have been demonstrated to reduce
steroidogenesis in vitro (25-27). Sphingosine has been reported to interact directly with the orphan
nuclear receptor steroidogenic factor-1 (SF-1; NR5A1), which plays a role both in the acute phase of steroidogenesis and in adrenal and gonadal development (27). In binding to SF-1, sphingosine maintains the transcription factor in an inactive conformation. The sphingosine intermediate, S1P, however has been demonstrated to induce transcription of several steroidogenic factors (28). Thus, sphingolipid intermediates may have some role in modulating steroidogenesis and potentially adrenal development. We see increased plasma S1P and ceramide levels in patient 5 and we anticipate that SGPL1 deficiency would lead to cytosolic accumulation of sphingolipid intermediates. Studies of tissues from SGPL1 deficient mice demonstrate an accumulation of S1P (21, 24, 29). Whilst S1P is largely considered a pro-proliferative signaling molecule and ceramide pro-apoptotic in normal physiology, studies have demonstrated that pathological accumulation of S1P in the cytosol can induce apoptosis (30). In contrast to the mouse, SGPL1 deficiency is not lethal in humans, but in many aspects the mouse phenotype mirrors the human condition. In the mouse, imperfect adrenal gland zonation together with a reduction in expression of steroidogenic enzymes and loss of vacuolization in the ZF are consistent with the biochemical finding of adrenal hormone insufficiency in patients. Significant SGPL1 expression is observed in the kidney by analysis of β-galactosidase expression in organs dissected from male and female SGPL1 reporter mice, with specific patterns of expression in the cortex, medulla and papilla (13). Images from the human protein atlas (see URLs) show SGPL1 expression in the tubules and glomeruli in normal human adult kidney; within the glomerulus staining is apparent predominately in podocytes (see Supplementary Figure 5). Sgp1/− mice have previously been reported to have renal defects with increased blood urea levels (20) and a renal phenotype was confirmed in our studies, with histological changes in the kidney matching the human biopsy results. Inducible SGPL1 deficiency in mice, and pharmacological inhibition of SGPL1 in rats both cause podocyte-based kidney toxicity, with glomerular proteinuria (24). Increased mesangial matrix with obliteration of some capillary lumina is seen in the SGPL1 deficient mouse model with podocyte effacement on electron microscopy (24).
Additionally, ichthyosis, disordered lipid metabolism and lymphodepletion are shared in some patients with the mouse, whereas the neurological deficits and hypothyroidism have not been noted in mice. It is feasible that some of the clinical findings in patients are attributable to alternative gene defects given the high degree of consanguinity within the pedigrees.

Ceramide species constitute 50% of the lamellar membrane of the stratum corneum and play an important role in maintaining skin barrier function (31). Ichthyosis is also seen in Gaucher’s disease type 2, where substantial reductions in lysosomal β-glucocerebrosidase leads to excess glucosylceramides and decreased ceramides, imbalance of which is postulated to contribute to the barrier abnormality (32). In the skin of partially depleted SGPL1 mice a rise in S1P concentrations is seen with a reduction in ceramide concentrations which may in turn interfere with barrier function (24). The phenotype in these mice is one of acanthosis (epidermal hyperplasia) with orthokeratotic hyperkeratosis.

We observe high levels of SGPL1 in human thyroid tissue [Figure 2E] and four of the patients have primary hypothyroidism, however mild disruptions of thyroid function are a reported phenomenon with PAI, particularly in the context of untreated disease (33-35). Additionally, proteinuria, particularly in nephrotic syndrome, often results in the urinary loss of thyroid hormones with associated binding proteins resulting in a reduction in serum total thyroid hormone levels (36). This is generally equilibrated by increasing the free fraction of the hormones, though patients with a low thyroid reserve may develop hypothyroidism consequent to this urinary loss.

Progressive neurological disease is reported in three of our patients but has not been reported in Sgpl1−/− mice. Curiously, compared to other organs, SGPL1 expression is low in rat and mouse brain (13, 15). Within the brain, SGPL1 shows a distinct expression pattern, with highest activity in the olfactory bulb in mouse (15, 37). Other regions with higher expression are the hippocampus ([Van Veldhoven], unpublished observations) and Purkinje cells in cerebellum (13, 30). Neuroanatomical studies in the cerebellum demonstrate that neurons here are the first to degenerate in SGPL1
deficient mice (30). A calpain-mediated neurotoxic mechanism has been proposed relating to pathological accumulation of cytosolic S1P in SGPL1 deficiency (30).

Sgpl1−/− mice exhibit an extensive range of other phenotypes including skeletal (osteoporosis and dysfunctional osteoclasts), pulmonary (proteinaceous exudates in alveoli impairing gas exchange), cardiac (increased interstitial cellularity and myocardium vacuolation), urinary tract (urothelial cells affected by widespread ballooning vacuolation, degeneration and apoptosis), thymic (atrophy) and hepatic abnormalities (disruption of liver homeostasis with defective hepatic lipid metabolism) (21, 29, 38). Haematological abnormalities associated with lyase deficiency in the mouse model include platelet activation and lymphodepletion which occurs as a consequence of the disruption of the S1P gradient between lymphoid tissues and blood (normally high to low respectively). This leads to reduced T cell egress into the blood (21, 38). Similarly, platelet abnormalities and lymphopenia are reported in some of our patients (Table 1). Inhibition of SGPL1 in healthy subjects has also been demonstrated to cause a decrease in peripheral T cell numbers (21). Partial restoration of S1P lyase in human knock-in mice lines (expressing one or 2 normal human alleles and less than 10 and 20% of normal lyase activity respectively) confers full protection from lesions in the lung, bone, urinary tract and heart that develop in SGPL1 null mice (21). Lymphodepletion, however, still occurs in the partially rescued human knock in mouse line.

In conclusion we have identified a novel, potentially progressive, disorder incorporating PAI and nephrotic syndrome amongst other features. SGPL1 deficiency in humans has multi-systemic effects, although the precise impact on sphingolipid metabolism and consequent pathogenic mechanism are yet to be elucidated and are likely to be tissue-specific. Our findings highlight the importance of the sphingolipid metabolic pathway in adrenal function. A genetic diagnosis for patients with this form of PAI will be important for correct treatment, genetic counselling and screening for co-morbidities.

Since strategies to modify SGPL1 activity are being explored in preclinical and clinical trials of several conditions, including multiple sclerosis and rheumatoid arthritis (39, 40), further characterisation of
SGPL1 deficiency in human disease may provide important insights into the potential long-term effects of modulating this pathway in patients.
Methods

Whole exome sequencing

Whole exome sequencing (WES) using the Illumina HiSeq 2000 Sequencer was conducted on 2 affected individuals [Patients 2-3] (samples processed by Otogenetics Corporation, USA). WES samples were prepared as an Illumina sequencing library, and in the second step, the sequencing libraries were enriched using the Agilent V4 enrichment kit. The captured libraries were sequenced and downstream analysis conducted via DNAnexus and Ingenuity variant analysis (see URLs). Single Nucleotide Polymorphisms, with threshold coverage of at least 10 reads on the respective nucleotide, were assessed.

Confidence filter

For confidence, call quality was set to be at least 20, read depth at least 10 in any sample, only data outside 0.1% of most exonically variable 100 base windows in healthy public genomes and outside 0.1% most exonically variable genes in healthy public genomes (1000 genomes, EXAC) was included.

Common variants

Common variants were filtered out by excluding those observed with an allele frequency of at least 0.5% in any of the 1000 genomes, EXAC, and all of the NHLBI exomes.

Predicted deleterious changes

Were defined as those that are disease-associated according to computed ACMG guidelines classification being pathogenic or likely pathogenic OR are associated with loss of function of a gene being frameshift, in-frame indel, or start/stop codon change, missense or splice-site change up to 2 bases into the intron.

Genetic analysis

Variants were included if they were homozygous in both cases and excluded if they were homozygous, compound heterozygous, hemizygous, heterozygous, haploinsufficient or het-ambiguous. Variants were excluded if they were present in hetero- or homozygosity in 2 or more control subjects analysed on the same platform.
PCR and sequencing

Each exon of genes of interest including intronic boundaries was amplified by PCR using specific primers (primer sequences Supplementary Table 5). The reaction mixture contained 100 ng DNA template, 1 x PCR buffer, 200 µM each dNTP, 200 nM each primer and 1 U Taq DNA polymerase (Sigma-Aldrich). Cycling conditions were: 95°C for 5 mins (1 cycle); 95°C for 30s, 55°C for 30s, and 72°C for 30s (30 cycles); and 72°C for 5 mins. PCR products were visualized on 1% agarose gel and sequenced using the ABI Prism Big Dye sequencing kit and an ABI 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA), in accordance with the manufacturer’s instructions.

Quantitative real-time PCR

The expression of SGPL1 and GAPDH mRNA was investigated using a panel of cDNAs derived from 16 adult tissues (adrenal cortex, kidney, liver, testes, ovary, colon, small intestine, brain, thymus, adipose tissue, heart, lung, thyroid, skeletal muscle, bladder, placenta). Quantitative RT-PCR was set up in duplicate (per sample) on a Stratagene Mx3000P thermocycler using KAPA SYBR Fast qPCR master mix with 200 nM forward and reverse primers targeted to SGPL1 or GAPDH (primer sequences Supplementary Table 5); giving a total volume of 10µl. After an initial denaturation step of 3 min at 95°C, PCR cycling was performed for 40 cycles of 95°C for 3s, 55°C for 20s and 72°C for 1 sec, followed by 1 cycle of 1 min at 95°C, 55°C for 30s and 95°C for 30s. The $2^\Delta\Delta Ct$ algorithm was used for analysis, normalization to GAPDH.

Vector construction

Site directed mutagenesis

A commercially available SGPL1 (Myc-DDK-tagged)-Human sphingosine-1-phosphate-lyase 1 (SGPL1) expression vector (TruOrf Gold; Cambridge Bioscience, Cat. No. RC208705) was used. Site-directed mutagenesis was carried out to prepare the R222Q and F545del constructs (primer sequences
Supplementary Table 6) using the QuikChange II site-directed mutagenesis kit (Agilent), as per manufacturer’s instructions. The sequence of all constructs was verified by DNA sequencing.

**Lyase activity studies**

Mouse fibroblasts lacking SGPL1, obtained from Sgpl1<sup>-/-</sup> embryos (19) and immortalized by dilution culturing (41), were cultured in MEM-Eagle medium with 10% FBS, 2 mM ultraglutamine-1, and 0.2% Mycozap. Cells were transfected with SGPL1 vectors by electroporation (Neon Transfection System) using the parameters described for SV40-transformed MEF and a 10 µl microporator tip (42). Per plasmid tested, three aliquots of 10 µl transfected cells (~5x10<sup>5</sup> cells) were seeded in a 90 mm dish. Two days post transfection, cells were collected by trypsinisation, centrifuged and washed with PBS. The pelleted cells, stored at -20°C till use, were adjusted to ~250 µl with 0.25 M sucrose, 5 mM MOPS pH 7.2, 1 mM EDTA, 0.1% (v/v) ethanol and sonicated (Hielscher Ultrasonic tissue homogenizer UP50H; 3 pulses of 50 W) on ice. Lyase was measured as described before (43) with small modifications. The activity represents the amount of hexadecanal formed from sphinganine-1-phosphate and is corrected for endogenous levels, and values are given in pmol substrate converted per min per mg lysate protein (pmol/min.mg protein).

**Western blotting**

Human Embryonic Kidney (HEK)-293 cells were transfected with the SGPL1 vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 hours, cells were lysed in RIPA buffer (Sigma) supplemented with complete protease inhibitors (Roche), and lysates cleared by centrifugation at 13,000 g for 10 min, at 4 °C, and the supernatants were added to an equal volume of reducing Laemmli loading buffer 2X (Sigma). Normal adult adrenals (n=3) were placed in tubes with RIPA buffer supplemented with complete protease inhibitors and lysates obtained using a Precellys24 Tissue Homogenizer via bead beating technology. Lysates were then cleared and prepared as above. Samples were heated at 95-100°C for 5 mins and size-separated on
4-12% SDS-PAGE gels (NuPage, Thermo Scientific). Proteins were then transferred to nitrocellulose membrane (GE Healthcare Life Sciences) using a semi-dry transfer blot (Biorad) at 15V for 1 hr. Blots were blocked with 5% non-fat dry milk in PBS-Tween 0.1% (Blocking buffer) and immunolabeled overnight with a polyclonal anti-SGPL1 antibody (105183, Abcam, at 1:1000 in blocking buffer). The following day, the membrane was washed three times in PBS-Tween 0.1% and then incubated with a goat anti-rabbit IRDye 800 (926-6871, Li-cor) at a 1:5000 dilution, washed again and visualized using the Li-CoR Odyssey system.

For visualization of SGPL1 in transfected mouse fibroblasts, lysates were size-separated as above and membranes incubated with anti-FLAG antibodies (200472-21, Roche), followed by alkaline phosphatase-conjugated secondary antibody (A2429, Sigma). NBT/BCIP (Roche) was used for chromogenic detection.

**Generation and validation of mouse lines**

Heterozygous Sgpl1+/+ animals, obtained as described before (19, 41, 44) and bred to a C57Bl/6 background, were crossed to obtain Sgpl1−/− pups. Animals were housed in the animal facility of KULeuven (Belgium).

**Human and mouse histology**

Human adult adrenals, mouse adrenals and kidneys from Sgpl1+/+ and Sgpl1−/− were fixed in 4% paraformaldehyde (Sigma) and embedded in paraffin. Human Foetal Adrenals (HFAs) were obtained from the MRC-Wellcome Human Development Biology Resources (HDBR-Institute of Genetics Medicine, Newcastle, UK). Sections were obtained using a microtome (Microm HM 325, Thermo Fisher) at 6-μm thickness, deparaffinized, treated with 3% hydrogen peroxide in PBS for 30 min, and then, depending on the primary antibody (see below) treated or not with 10 mM Sodium Citrate pH 6.0 for 20 minutes at 95°C in water bath. Sections were then blocked with 5% normal goat serum (Sigma) in PBS 0.1% Triton X-100 (T-PBS) and incubated overnight with anti-CYP11B2 (a kind gift from Professor Celso Gomez-Sanchez, University of Mississippi, Oxford, Mississippi, USA, 1:200 in PBS, with previous antigen unmasking), anti-CYP11A1 (D8F4F, Cell Signaling, 1:200 in PBS, without...
previous antigen unmasking), anti-SGPL1 (105183, AbCam, 1:200 in PBS, without previous antigen unmasking). Sections were washed in T-PBS and incubated with a biotinylated goat anti-primary IgG secondary (BA-9200 and BA-1000, Vector Laboratories) diluted 1:500 in T-PBS for 2 h. Sections were washed, treated with avidin–biotin complex (ABC Elite kit, Vector Laboratories) according to the manufacturer's instructions, rinsed again and incubated with ImmPACT DAB peroxidase substrate kit (Vector Laboratories). The reaction was stopped with H₂O and slides were counterstained with Hematoxylin, dehydrated and coverslipped using Vecta Mount (Vector Laboratories).

Hematoxylin & Eosin staining was performed using standard procedures. For fibrotic changes in the kidney, Masson's trichrome staining kit (Sigma) was employed.

Images were acquired using a Leica DM5500B microscope (Leica, Nussloch, Germany), equipped with a DCF295 camera (Leica) and DCViewer software (Leica), and then processed with Adobe Photoshop CS6 and Adobe Illustrator CS6.

Statistics

In studies in which statistical analyses were performed, a 2-tailed Student’s t test was used to generate P values. P values less than or equal to 0.05 were considered significant. Data are presented as mean ± SD in all figure parts in which error bars are shown.

Study approval

This study was approved by the local hospital ethics committees, and all parents (and children, when possible) gave written informed consent. Approval was obtained from the Outer North East London Research Ethics Committee, reference number 09/H0701/12, the local ethics committee in Istanbul, reference number B.30.2.MAR.0.01.02/AEK/108, the Vall d’Hebron Hospital Ethics Committee (Paediatric Endocrinology Collection at Vall d’Hebron Biobank). All the experiments involving mice were approved by the ethical committee for animal research of KULeuven.
**URLs**


466 Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: [http://exac.broadinstitute.org](http://exac.broadinstitute.org)) [July 2016 accessed].

469 National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project (ESP), [https://www.nhlbi.nih.gov/](https://www.nhlbi.nih.gov/)


472 DNAnexus, [https://www.dnanexus.com/](https://www.dnanexus.com/)


Author contributions

LAM, RP designed the study. NR, TG, DB, IB, EB, HC, MC, NC, F-C, UD, RK, BH, ES, EY, RP, HS recruited and clinically characterized patients. RP, LAM, HL, FB, JA, TG, AB analysed WES data. RP, AM, LAM conducted Sanger sequencing and analysis of data. AM, EM, and RP generated mutant constructs and PPVV conducted lyase activity experiments. PPVV generated and validated mouse lines. IH, LG, JH, MS conducted mouse/human adrenal and kidney histology. RP, LAM prepared the draft manuscript. All authors contributed to the discussion of results and edited and approved the final manuscript.
Acknowledgements

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Figure 1. Pedigrees of Kindreds 1 to 5 where all affected individuals manifested PAI, with or without SRNS, and are positive for mutations in SGPL1. Black-filled symbols indicate individuals with primary adrenal insufficiency alone, half-filled in green those who additionally have steroid resistant nephrotic syndrome and green filled indicate those with SRNS alone. All affected individuals were homozygous for the indicated mutations (patients sequenced have been numbered from 1-8) and parents were heterozygous (those sequenced denoted by grey-filled symbols). Mutations for patients 2, 3 and 5 were identified by whole exome sequencing and the remainder by Sanger sequencing of SGPL1.
Figure 2. p.R222Q and p.F545del mutations affect highly conserved areas in SGPL1 and are loss-of-function, resulting in proteins with reduced lyase activity (A) SGPL1 regulates flow of the sphingolipid biochemical intermediates (in green) and carries out the final degradation step in the pathway. (B) Partial alignment of SGPL1 protein sequences, generated by Clustal Omega (45), showing conservation of Arginine (R) at position 222 and Phenylalanine (F) at position 545, highlighted in yellow, numbering relative to human sequence. For all but the most distant organisms these amino acids are conserved. Alignment source accession numbers from ENSEMBL are H. sapiens, Human, ENSP00000362298; M. musculus, mouse, ENSMUSP00000112975; R. norvegicus, rat, ENSRNOP00000070983; T. nigroviridis, pufferfish, ENSTNIP00000016065; X. tropicalis, clawed frog, ENSXETP00000017960; C. intestinalis, sea squirt, ENSCINP00000002369; D. melanogaster, fruitfly, FBpp00086158; C. elegans, nematode, B0222.4 and S. cerevisiae, yeast, YDR294C. Sequence conservation is beneath the alignment, * = total conservation, : = partial conservation. (C) SGPL1 activities were measured in lysates of Sgpl1−/− mouse fibroblasts, ** = P
<0.01, in 2-tailed Student’s t test (n=3). (D) Lysates of Sgp1−/− mouse fibroblasts, expressing wild-type or the mutant SGPL1 (25 μg protein/lane) were analysed by immunoblotting for the presence of the FLAG-tagged protein (representative image, n=3). (E) SGPL1 mRNA expression in a human tissue panel, analysis using the 2^(-deltadeltaCT) algorithm (n=3).
Adrenals from Sgpl1^−/− mice show histological abnormalities and SGPL1 is expressed in human adrenals. (A-B) Adrenals from Sgpl1^−/− mice show histological abnormalities. (A) H&E staining of Sgpl1^+/+ and Sgpl1^−/− adrenals. Note the less defined morphological zonation in the Sgpl1^−/− adrenals compared to that from Sgpl1^+/+ mice. Moreover, the characteristic lipid droplets found in the ZF (arrowheads in top-right panel) and visible as large areas in the cytoplasm devoid of eosin staining (as lipids are extracted during the paraffin-embedding procedure) are strongly reduced in Sgpl1^−/− adrenals (n=3). (B) CYP11A1 and CYP11B2 expression in Sgpl1^+/+ and Sgpl1^−/− adrenals. CYP11A1 staining in Sgpl1^−/− adrenals is less prominent compared to Sgpl1^+/+ while the characteristic patchy expression of aldosterone synthase (CYP11B2) is lost in Sgpl1^−/− adrenals (n=3). (C-E) Expression of SGPL1 in human adrenals. (C) Western Blotting of lysates from human adrenal,
HEK293 cells and HEK293 cells overexpressing SGPL1, probed with anti-SGPL1 antibody (representative image of n=3). (D) SGPL1 expression in the human fetal adrenal at 19 and 22 Carnegie (Carn) stage, as well as 18 weeks showing widespread expression (n=1 each). FZ= foetal zone; DZ= definitive zone. (E) SGPL1 expression in the human adult adrenal (n=3). Note the stronger expression of SGPL1 in the ZR compared to ZG and ZF, while the capsule (Cap) and medulla (M) are negative.
Figure 4 Histological features of the glomeruli. (A-C) H&E staining of Sgpl1^+/+ kidney showing normal cortical histology (A) and glomeruli with open capillary loops and normal cellularity (B, C, yellow arrowhead). The kidneys from Sgpl1^-/- mice (E-G) have mild mesangial hypercellularity with glomerular hypertrophy (F, G, yellow arrowhead) and large protein casts in the tubules (white arrows). (D and E) Masson’s trichrome stain. Kidneys from Sgpl1^-/- mice (H) show increased glomerular fibrosis (red stain) compared to Sgpl1^+/+ (D), n=3 in all cases.
Table 1: Clinical phenotype of the patients included in the study with associated mutations in SGPL1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Ethnicity</th>
<th>SGPL1 mutation</th>
<th>MAF in ExAC</th>
<th>Age at last review (yr)</th>
<th>PAI (age at diagnosis in yr)</th>
<th>Presenting complaint</th>
<th>ACTH (pg/mL); Cortisol (µg/dL) at diagnosis</th>
<th>SRNS (age at diagnosis in yr)</th>
<th>Presence of ichthyosis</th>
<th>Neurological features</th>
<th>Primary hypothyroidism (age at diagnosis in yr)</th>
<th>Additional Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1^</td>
<td>Pakistani</td>
<td>c.665G&gt;A; p.R222Q</td>
<td>1.7e-5</td>
<td>5^</td>
<td>0.6^</td>
<td>Hyperpigmentation^a</td>
<td>In keeping with IGD^a</td>
<td>2.5^a</td>
<td>-</td>
<td>Normal neurology^a</td>
<td>N/A^</td>
<td></td>
</tr>
<tr>
<td>2^</td>
<td>Pakistani</td>
<td>c.665G&gt;A; p.R222Q</td>
<td>1.7e-5</td>
<td>8^</td>
<td>&lt;1^</td>
<td>Hyperpigmentation^a</td>
<td>In keeping with IGD^a</td>
<td>N/A</td>
<td>-</td>
<td>Normal neurology^a</td>
<td>N/A^</td>
<td></td>
</tr>
<tr>
<td>3^</td>
<td>Pakistani</td>
<td>c.665G&gt;A; p.R222Q</td>
<td>1.7e-5</td>
<td>3^</td>
<td>&lt;1^</td>
<td>Hyperpigmentation^a</td>
<td>In keeping with IGD^a</td>
<td>N/A</td>
<td>-</td>
<td>Normal neurology^a</td>
<td>N/A^</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Saudi</td>
<td>c.665G&gt;A; p.R222Q</td>
<td>1.7e-5</td>
<td>3.6</td>
<td>1.5^</td>
<td>Hyperpigmentation</td>
<td>&gt;1250; undetectable</td>
<td>N/A</td>
<td>No</td>
<td>Normal neurology</td>
<td>N/A</td>
<td>Lymphopenia</td>
</tr>
<tr>
<td>5</td>
<td>Turkish</td>
<td>c.1633_1635delITTC;p.S45del</td>
<td>Novol</td>
<td>5.9</td>
<td>0.5^</td>
<td>Failure to thrive, vomiting, convulsion</td>
<td>&gt;1250; 3.9</td>
<td>5.5</td>
<td>Yes</td>
<td>Neurodevelopmental delay on follow up^a, regression, mild ataxia, sensorineural deafness</td>
<td>0.5 (with goitre)</td>
<td>Lymphopenia^a</td>
</tr>
<tr>
<td>6</td>
<td>Peruvian</td>
<td>c.261+1G&gt;A; p.S65fs*6^a</td>
<td>Novol</td>
<td>8.4</td>
<td>0.9</td>
<td>Hyperpigmentation, convulsions secondary to hypocalcaemia, nephrotic syndrome diagnosed concurrently</td>
<td>&gt;1250; 11.4</td>
<td>0.9^a</td>
<td>Yes</td>
<td>Neurodevelopmental delay on follow up^a, ataxia, left sensorineural deafness</td>
<td>1.0</td>
<td>Cryptorchidism and bilateral micro-orchidism, tertiary hyperparathyroidism, raised serum cholesterol and triglycerides, anaemia secondary to chronic renal failure, few episodes of haemorrhage^a and low white cell count</td>
</tr>
<tr>
<td>7</td>
<td>Peruvian</td>
<td>c.261+1G&gt;A; p.S65fs*6^a</td>
<td>Novol</td>
<td>2.4</td>
<td>0.3^a</td>
<td>Screened for nephrotic syndrome, Hyperpigmentation, adrenal crisis during illness</td>
<td>&gt;1250; 1.9</td>
<td>0.1</td>
<td>Yes</td>
<td>Neurodevelopmental delay on follow up^a</td>
<td>0.3</td>
<td>Bilateral cataracts, Anaemia secondary to chronic renal failure, raised serum cholesterol and triglycerides</td>
</tr>
<tr>
<td>8</td>
<td>Spanish</td>
<td>c.7dupA; p.S3Kfs*11</td>
<td>Novol</td>
<td>17.5</td>
<td>9</td>
<td>Hypoglycaemia and hypotension during illness</td>
<td>&gt;1500; 2.7</td>
<td>0.8^a</td>
<td>Yes</td>
<td>Normal neurology and normal MRI brain</td>
<td>12</td>
<td>Raised serum triglycerides</td>
</tr>
</tbody>
</table>

^aClinical phenotype as published by Ram et al, 2012 (9). ^bPatients required renal transplantation, patient 7 currently has continuous peritoneal dialysis, patient 8 required a re-transplant. ^cClinical symptoms from 1 yr. ^dAdditional mineralocorticoid treatment ^eNormal neurology and development noted on initial assessment. ^fT cell subsets and T cell proliferation response in Supplementary Tables 3 and 4. ^gPredicted result if exon s skipped. ^hPlatelet dysfunction with normal platelet count. ^iBone marrow biopsy demonstrated decreased erythroid series, otherwise normal. MAF; minor allele frequency, ExAC; Exome Aggregation Consortium, ACTH; Adrenocorticotropic hormone (Normal range 10-60 pg/mL), IGD; Isolated glucocorticoid deficiency.
Table 2. Comparison of SGPL1 deficient mouse phenotype with clinical findings in the patients

<table>
<thead>
<tr>
<th></th>
<th>Adrenal pathology</th>
<th>Nephrotic disease</th>
<th>Ichthyosis</th>
<th>Primary Hypothyroidism</th>
<th>Abnormal Neurology</th>
<th>Lymphopenia</th>
<th>Dyslipidaemia</th>
</tr>
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<tbody>
<tr>
<td>Patient 1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patient 2&lt;sup&gt;A&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Patient 3&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patient 4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Patient 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Patient 6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Patient 7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Patient 8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(not reported)</td>
<td>(not reported)</td>
<td>+</td>
</tr>
<tr>
<td>SGPL1 deficient mice&lt;sup&gt;B&lt;/sup&gt; (with associated references)</td>
<td>+ (see Results)</td>
<td>+ (see Results; (24))</td>
<td>+ (24)</td>
<td>(not reported)</td>
<td>(not reported)</td>
<td>+ (21, 38)</td>
<td>+ (29)</td>
</tr>
</tbody>
</table>

<sup>A</sup>Clinical phenotype as published by Ram et al, 2012 (9). <sup>B</sup>As compared with clinical findings in our patients, full extent of SGPL1 deficient mouse phenotype is not included in table, see Discussion. +, feature reported; -, feature not reported.