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CLINICAL REPORT

A novel *SPINK5* donor splice site variant in a child with Netherton syndrome

Dillon Mintoff[1] | **Isabella Borg**[2,3,4] | **Julia Vornweg**[5] | **Liam Mercieca**[1] | **Rijad Merdzanic**[6] | **Johannes Numrich**[6] | **Susan Aquilina**[1] | **Nikolai Paul Pace**[4] | **Judith Fischer**[5]

[1]Department of Dermatology, Mater Dei Hospital, Msida, Malta

[2]Department of Pathology, Faculty of Medicine and Surgery, University of Malta, Msida, Malta

[3]Medical Genetics Unit, Department of Pathology, Mater Dei Hospital, Msida, Malta

[4]Centre for Molecular Medicine and Biobanking, Faculty of Medicine and Surgery, University of Malta, Msida, Malta

[5]Institute of Human Genetics, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg im Breisgau, Germany

[6]CENTOGENE AG, Rostock, Germany

Correspondence
Dillon Mintoff, Dermatology OP, Outpatients Department, Level -1, Mater Dei Hospital, Triq id-Donaturi tad-Demm, MSD 2090 Msida, Malta.
Email address: dillon.mintoff@gov.mt

Abstract

Background: Netherton syndrome (NS) is a genodermatosis caused by loss-of-function mutations in *SPINK5*, resulting in aberrant LEKTI expression.

Method: Next-generation sequencing of *SPINK5* (NM_001127698.1) was carried out and functional studies were performed by immunofluorescence microscopy of a lesional skin biopsy using anti-LEKTI antibodies.

Results: We describe a novel *SPINK5* likely pathogenic donor splice site variant (NM_001127698.1:c.2015+5G>A) in a patient with NS and confirm its functional significance by demonstrating complete loss of LEKTI expression in lesional skin by immunofluorescence analysis.

Conclusion: The 2015+5G>A is a novel, likely pathogenic variant in NS. Herein we review and assimilate documented *SPINK5* pathogenic variants and discuss possible genotype–phenotype associations in NS.

KEYWORDS

LEKTI, Netherton syndrome, *SPINK5*, Splice donor site pathogenic variant

1 | INTRODUCTION

Netherton syndrome (NS) (OMIM #256500; ORPHA:634) is an autosomal recessive genodermatosis characterized by congenital ichthyosiform erythroderma, trichorrhexis invaginata (TI), and an atopic diathesis (Netherton, 1958), with an incidence estimated at 1/200,000 births. The condition is caused by pathogenic variants in *SPINK5* on chromosome 5q32 (Chavanas, Garner, et al., 2000) that code for the Lymphoepithelial Kazal Type Inhibitor (LEKTI) protease (Mägert et al., 1999). LEKTI is a 15-Domain Human Serine Protease Inhibitor (Serpine) (Mägert et al., 1999) that is strongly expressed in the granular and spinous layer of cutaneous epithelium (Bitoun et al., 2003; Hachem et al., 2006), as well as lymphatic tissue such as the thymus and tonsil, and other mucosal epithelia (Mägert et al., 1999). In NS, *SPINK5* mutations lead to the decay and/or complete proteolytic breakdown of LEKTI (Raghunath et al., 2004) resulting

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in the unabated activation of kallikreins and dysfunctional epidermal homeostasis. Ultrastructurally, the stratum corneum exhibits premature degradation of corneodesmosomes (Chao et al., 2005; Hachem et al., 2006). Disrupted epidermal homeostasis manifests cutaneously as ichthyosiform erythroderma, ichthyosis linearis circumflexa (ILC), and eczematous changes.

About 200 cases of NS have been described. A diagnosis of NS should be considered in infants presenting with recalcitrant eczema, especially if associated with short stature, lusterless hair, recurrent skin infections, and/or food allergies in the setting of hyperIgEemia (Komatsu et al., 2002).

In this report, we describe a novel *SPINK5* likely pathogenic variant in a NS patient and review the established pathological variants in *SPINK5*.

Key points

- Netherton syndrome is a genodermatosis caused by *SPINK5* mutations
- This article shows the functional proof for a novel homozygous *SPINK5* donor splice site variant and
- Expands and reviews the known *SPINK5* mutational spectrum and its potential phenotypic associations.

2 | CLINICAL PRESENTATION

The patient is the younger of two siblings born to nonconsanguineous parents of Maltese-Caucasian ethnicity. He was referred for a dermatological consultation at the age of 3 years for what was described as "generalized recalcitrant eczema" associated with hyperIgEemia, as well as IgE-proven allergies to egg-white albumen, soy, wheat, and nut mix. The patient did not have a personal or family history of asthma or allergic rhinitis.

The child was born at term by vaginal delivery. He did not demonstrate any skin pathology during the first days of life; however, 2 weeks postnatally, erythema and scaling of the skin were noted which subsequently progressed to involve approximately 75% of the body surface area. The patient also had required treatment with oral and intravenous antibiotics in view of recurrent cellulitis (mainly staphylococcal). No electrolyte disturbances were noted during in-patient care. The child maintained a weight-for-age development on the 25th centile, while his length-for-age growth hovered between the 2nd and 5th centiles. Apart from an older sister with Down syndrome, the patient's family history was unremarkable.

Clinical examination of the child at the age of 3 years revealed erythematous plaques with a double-edged scale characteristic of ILC involving 50% of his body surface area. ILC affected the trunk predominantly, and the limbs to a lesser extent (Figure 1). The hair was lusterless but the typical bamboo hairs TI were absent on trichoscopy. The nails and teeth were unremarkable. There were no concerns about his vision and hearing. The patient did not demonstrate any learning difficulties or neurological deficits. Prior to presentation at the dermatology clinic, various emollients, topical corticosteroids, and pimecrolimus cream had been prescribed for the treatment of his recalcitrant disease with only slight and temporary improvement. The parents recalled a significant improvement in the child's eczematous lesions during a tailing-down course of oral prednisolone, but he experienced a rebound a couple of days after the course of steroids was completed. The patient's symptoms were controlled by liberal application of bland emollients and the use of antihistamines as required. The parents were encouraged to bring their son for urgent review upon suspecting cellulitis. Early skin swabbing for bacterial culture and judicious antibiotic treatment diminished the frequency and severity of episodes of cellulitis.

A diagnosis of Netherton syndrome was suspected and genetic studies were subsequently carried out.

3 | MATERIAL AND METHODS

3.1 | Genetic analysis

Written consent for genetic analysis was obtained from the parents. Peripheral blood samples were collected from the proband, parents, and sibling.

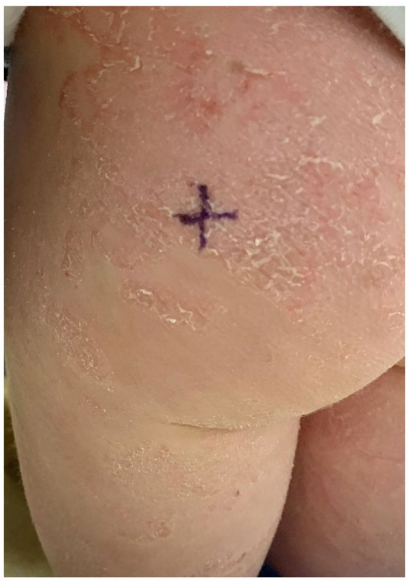

FIGURE 1 Ichthyosis linearis circumflexa. Typical double-edge scale in patients with NS. The cross indicates the biopsy site

Next-generation sequencing of *SPINK5* (NM_001127698.1) was carried out. Genomic DNA was enzymatically fragmented, and regions of interest enriched, using DNA capture probes targeted against the coding regions of *SPINK5*.

The captured library was subsequently sequenced on an Illumina® platform. A coverage depth of at least 50X was obtained for 100% of the targeted bases. Raw sequence data analysis, including base calling, demultiplexing, alignment to the hg19 human reference genome (Genome Reference Consortium GRCh37), and variant calling (single nucleotide variants, Indels, and copy number variations [CNVs]), was performed using validated in-house software. Analysis of the entire coding region of *SPINK5*, including 10 bp of flanking intronic sequences, was carried out. Segregation studies on the asymptomatic parents and sibling were performed using bidirectional Sanger sequencing of the identified *SPINK5* variant. Classification of the variant was according to established guidelines from the American College of Medical Genetics/Association for Molecular Pathology (ACMG/AMP) (Richards et al., 2015). All analyses were performed in concordance with the provisions of the German Gene Diagnostic Act (Gendiagnostikgesetz).

A homozygous intronic variant (chromosome 5, position 147494057 in hg19 reference assembly) NM_001127698.1:c.2015+5 G>A was identified in *SPINK5* (Figure 2). The variant is predicted to disrupt the highly conserved donor splice site of intron 21 and is absent from healthy cohorts in homozygous state. The frequency of this variant in heterozygotes is extremely low (gnomAD: 0.000004, ESP: 0.000082) and the variant had not been detected previously in Centogene's database (CentoMD). No other clinically relevant variant was identified.

In the relatives, the *SPINK5* variant NM_001127698.1:c.2015+5G>A was identified in the heterozygous carrier state (Figure 3). This result also confirmed homozygosity of the variant identified in the patient.

In silico analysis using splice site prediction tools was implemented (Table S1). The predictions from different algorithms, namely the Human Splicing Finder (HSF),

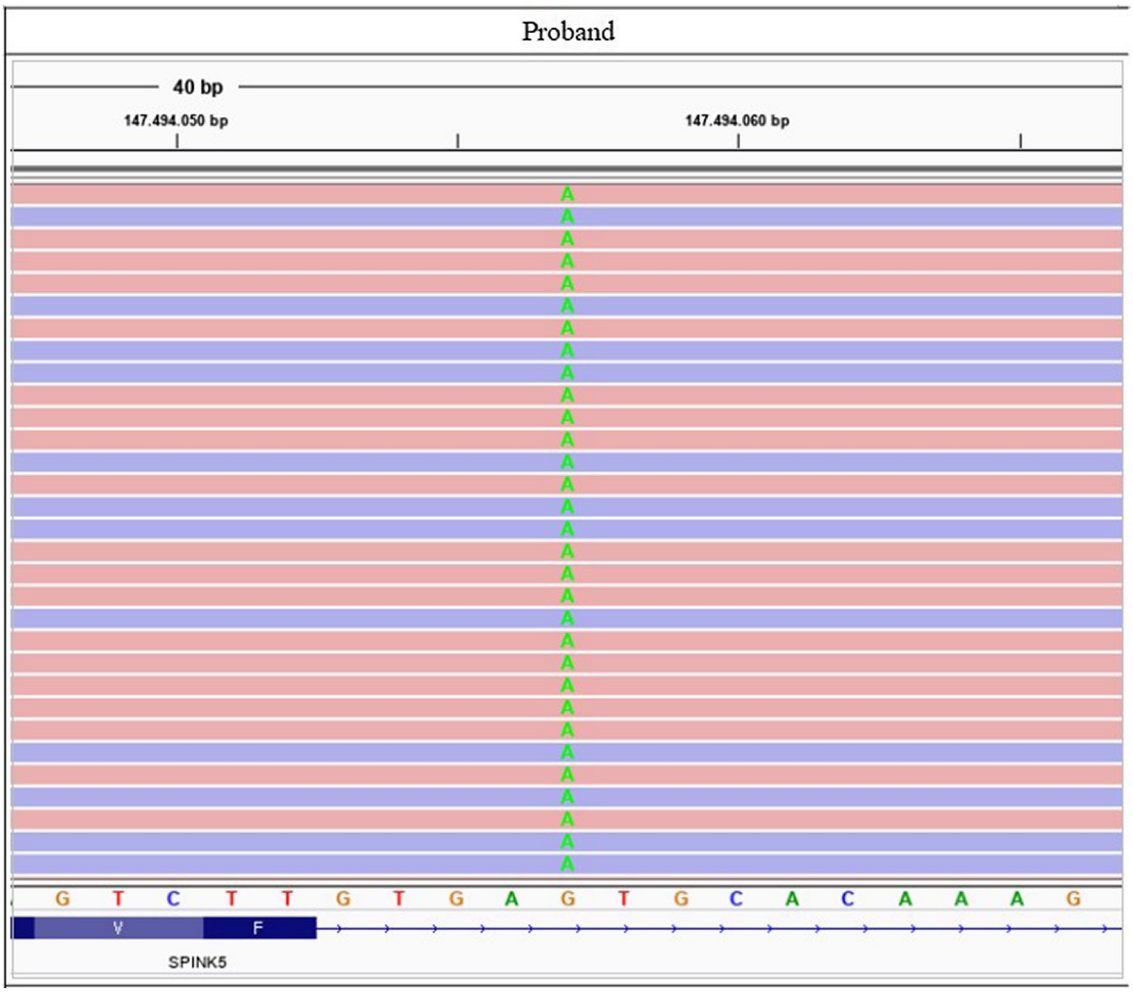

FIGURE 2 Excerpt from BAM file showing homozygous G>A base change at position c.2015+5(NM_001127698 *SPINK5* gene; Chr5(GRCh37):g.147494057G>A)

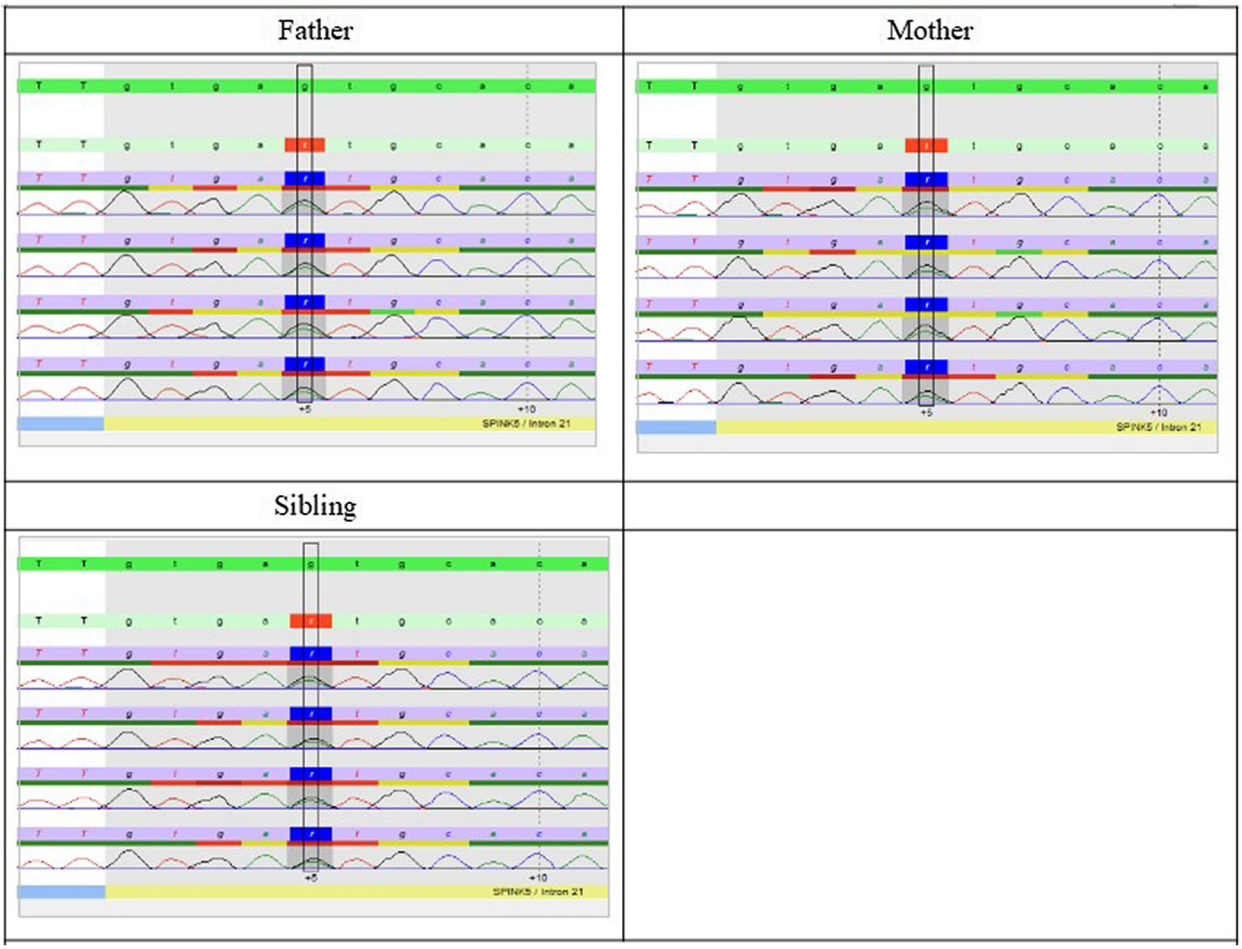

FIGURE 3 Chromatograms of Sanger sequencing. Sequences show heterozygous G>A base change at position c.2015+5 (NM_001127698 *SPINK5* gene)

MaxEntScan (MES), NNSplice, and NetGene2, showed that the substitution has deleterious predictions and is likely to alter the splicing mechanism through either exon skipping or the use of cryptic splice sites.

3.2 | Histology and Immunofluorescence

A 6 mm punch biopsy of lesional skin was obtained from the patient's left buttock (Figure 1). Histological examination of hematoxylin and eosin (H&E)-stained formalin-fixed paraffin-embedded (FFPE) sections revealed hyperkeratosis and epidermal thickening as well as reduction of keratohyalin granules in the stratum granulosum of the patient's skin (Figure 4).

LEKTI expression was subsequently evaluated by immunofluorescence microscopy using commercially available monoclonal antibodies (Primary antibodies: rabbit anti-LEKTI 1:200, HPA011351 by Sigma; rabbit anti-loricrin 1:500, ab24722 by Abcam) and secondary antibody (anti-rabbit IgG Fab2 Alexa Fluor R594 1:500). Immunofluorescence analysis demonstrated complete loss of LEKTI protein, which is encoded by *SPINK5* (Figure 4). In view of a low 4',6-diamidino-2-phenylindole (DAPI) signal, a false negative result was excluded by positive loricrin staining (Figure S1).

The absence of immunoreactivity for LEKTI in the lesional skin supports the diagnosis of NS in the patient. Based on these findings, the NM_001127698.1:c.2015+5G>A substitution is classified as a likely pathogenic variant according to the ACMG, applying the criteria: PS3 (functional studies provide evidence for damaging effect on protein), PM2 (present at extremely low frequency in controls), PP3 (multiple lines of computational evidence support a deleterious effect on the gene or gene product—splicing impact), and PP4 (patient's phenotype or family history is highly specific for a disease with a single genetic etiology) (Richards et al., 2015).

4 | DISCUSSION

In this report, we describe a novel homozygous *SPINK5* donor splice site variant in intron 21 leading to absent LEKTI expression in the epidermis.

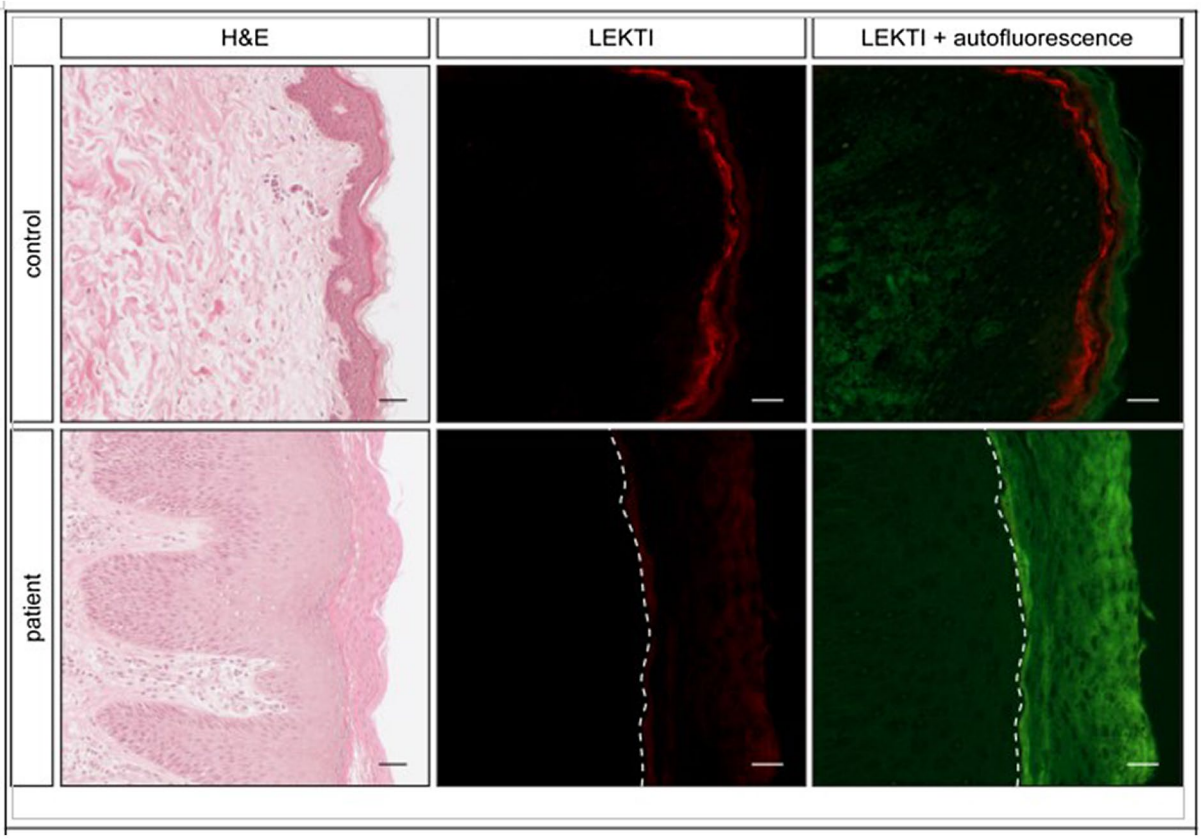

FIGURE 4 Histopathological and immunofluorescence analysis of control and patient's skin. Hematoxylin and eosin (H&E) staining of patient's skin shows hyperkeratosis, epidermal thickening, and reduction of the basophilic keratohyalin granules (40 μm). Immunofluorescence revealed no LEKTI signal in the individual carrying homozygous *SPINK5* c.2015+5G>A variant (20 μm)

Many pathogenic exonic and intronic *SPINK5* variants have been described, the majority being loss-of-function, nonsense, or frameshift variants. These variants are summarized in Table 1. Complete *SPINK5* deletions have also been described (Hachem et al., 2006). Most *SPINK5* pathogenic variants in NS patients from the Mediterranean region are exonic, including the commonest mutation; c.891C>T (p.Cys297Cys) in exon 11 (Lacroix et al., 2012). This synonymous mutation induces incomplete exon skipping, resulting in residual LEKTI production and a relatively mild phenotype (Fortugno et al., 2012). The *SPINK5* variant c.2015+5G>A described in this report is intronic and the patient's nonconsanguineous parents were both heterozygous carriers. Additionally, this variant was not detected in an in-house Maltese genome dataset.

The heterogeneous NS phenotype reported in the literature is due to variable expressivity of *SPINK5* variants, which would explain why robust genotype–phenotype associations have been difficult to establish (Bitoun et al., 2002; Komatsu et al., 2008; Lacroix et al., 2012). Pathophysiologically, the severity of cutaneous findings can be related to the extent of epidermal serpine activity. In patients exhibiting moderate to severe NS phenotypes, serpine activity extends across the superficial and deep epidermis and includes degradation of Desmoglein 1 (Dsg1) and (to a lesser extent) Desmocollin 1 (Dsc1). Patients with a milder NS phenotype have serpine activity which is limited to the epidermis with preservation of Dsg1 and Dsc1 (Hachem et al., 2006). Homozygous frameshift and splicing pathogenic variants resulting in early truncation of LEKTI are more likely to be associated with a severe NS phenotype and potential lethality (Sprecher et al., 2001). Patients harboring pathogenic variants that generate transcripts allowing residual LEKTI synthesis exhibit a milder phenotype (Fortugno et al., 2012; Lacroix et al., 2012). For example, compound heterozygotes for the *SPINK5* p.Arg371Ter variant as well as compound heterozygotes for the c.375-376delAT *SPINK5* variant demonstrate milder NS phenotypes when compared to patients harboring these variants in their homozygous state. The latter may potentially result in lethal forms of the disease (Diociaiuti et al., 2013; Itoh et al., 2015). Similarly, a NS patient demonstrating heterozygosity for the *SPINK5* variants c.1431-12G>A and c.1816_1820+21delinsCT was described as having a severe clinical phenotype (erythroderma, hypotonia, hypernatremia, sepsis, and respiratory failure) (Śmigiel et al., 2016), while three patients (born to consanguineous parents) who were homozygous for the

TABLE 1 Published *SPINK5* pathogenic variants according to their location on the gene and the corresponding protein effect

Location	Pathogenic variant	Protein effect	First Reported
Exon			
1	c.20C>A	p.Ser7Ter	Bellon et al. (2020)
3	c.153delT	p.Gln52LysfsTer6	Bellon et al. (2020)
3	c.136C>T	p.Gln46Ter	Sprecher et al. (2001)
3	c.153delT	p.Gln52LysfsTer6	Chavanas, Bodemer, et al. (2000)
3	c.184A>T	p.Lys62Ter	Bellon et al. (2020)
4	c.238dupG	p.Ala80glyfsTer19	Chavanas, Bodemer, et al. (2000)
4	c.286_269insT	p.Thr90IlefsTer9	Descargues et al. (2006)
5	c.301A>T	p.Lys101Ter	Diociaiuti et al. (2016)
5	c.316_317delGA	p.Asp106TrpfsTer7	Kogut et al. (2015)
5	c.318G>A	p.Asp106Ter	Xi-Bao et al. (2012)
5	c.354_357delTTGT	p.Cys119fs	Roedl et al. (2011)
5	c.355_357delTGinsGC	p.Cys119Ala	Renner et al. (2009)
5	c.378T>G	p.Tyr126Ter	Komatsu et al. (2002)
5	c.377_378delAT	p.Tyr126Ter	Bitoun et al. (2002)
5	c.389_392dupCTGC	p.Leu132CysfsTer5	Sprecher et al. (2001)
5	c.399_400delTG	p.Ala134Ter	Raghunath et al. (2004)
5	c.307G>T	p.Gly103Ter	Sprecher et al. (2001)
6	c.474G>A	p.Gln158=	Numata et al. (2016)
7	c.581_582delGT	p.Cys194fsTer4	Kilic et al. (2006)
8	c.628C>T	p.Arg210Ter	Bitoun et al. (2002)
8	c.649 C>T	p.Arg217Ter	Bitoun et al. (2002)
8	c.652C>T	p.Arg218Ter	Chavanas, Bodemer, et al. (2000)
9	c.691delC	p.Gln231LysfsTer2	Sprecher et al. (2004)
9	c.715dupT	p.Cys239fs	Raghunath et al. (2004)
9	c.720_721InsT	p.Arg241fs	Chavanas, Bodemer, et al. (2000)
9	c.724G>T	p.Glu242Ter	*LOVD Database*
10	c.803G>A	p.Arg268Cys	Lin et al. (2007)
11	c.891C>T	p.Cys297=	Lacroix et al. (2012)
11	c.900T>G	p.Tyr300Ter	Roedl et al. (2011)
11	c.957_960dupTGGT	p.Pro321TrpfsTer23	Alpigiani et al. (2012)
11	c.966_967insC	p.Gly323fs	Mizuno et al. (2006)
11	c.995delT	p.Met332fs	Nevet et al. (2017)
11	c.997C>T	p.Gln333Ter	Fong et al. (2011)
12	c.1024ins5	p.Lys344fs	Sprecher et al. (2001)
12	c.1036insG$(A)_4$	p.Lys346ArgfsTer4	Bitoun et al. (2002)
12	c.1048C>T	p.Arg350Ter	Macknet et al. (2008)
12	c.1086delAT	p.Tyr363CysfsTer6	Chavanas, Bodemer, et al. (2000)
13	c.1111C>T	p.Arg371Ter	Bitoun et al. (2002)
15	c.1258G>A	p.Glu420Lys	Ilias et al. (2015)
15	c.1320C>G	p.Tyr440Ter	Bellon et al. (2020)
15	c.1346_1352insT	p.Cys451LeufsTer5	Renner et al. (2009)
16	c.1432C>T	p.Gln478Ter	*LOVD Database*
16	c.1476delA	p.Arg899Ter	Nijman et al. (2014)

(Continues)

TABLE 1 (Continued)

Location	Pathogenic variant	Protein effect	First Reported
17	c.1530C>A	p.Cys510Ter	Skoczen et al. (2020) Zelieskova et al. (2020)
18	c.1621G>T	p.Glu541Ter	Komatsu et al. (2002)
19	c.1772delT	p.Leu591GlnfsTer124	Hannula-Jouppi et al. (2016)
19	x.1732C>T	p.Arg578Ter	Sprecher et al. (2001)
20	c.1887G>C	p.Lys629Asn	Patel et al. (2020)
21	c.1913delT	p.Leu639CysfsTer76	Roedl et al. (2011)
22	c.2039_2049del	p.Lys680ArgfsTer26	Goujon et al. (2010)
22	c.2041delA	p.Arg681GlyfsTer34	Bitoun et al. (2002)
22	c.2098G>T	p.Gly700Ter	Renner et al. (2009)
23	c.2137C>T	p.Gln713Ter	Shimomura et al. (2005)
24	c.2258dupG	p.Asn755fs	Chavanas, Bodemer, et al. (2000)
24	c.2260A>T	p.Lys754Ter	Chao et al. (2005)
24	c.2264dupA	p.Asn755LysfsTer2	Sprecher et al. (2001)
24	c.2313G>A	p.Lys771=	Chavanas, Bodemer, et al. (2000)
25	c.2423C>T	p.Thr808Ile	Lin et al. (2007)
25	c.2368 C>T	p.Arg790Ter	Chavanas, Bodemer, et al. (2000)
26	c.2459_2468delA	p.Lys823ArgfsTer100	Renner et al. (2009)
26	c.2468delA	p.Lys823ArgfsTer119	Bitoun et al. (2002)
26	c.2468dupA	p.Lys824fs	Chavanas, Bodemer, et al. (2000)
26	c.2471_2474delAAGA	p.Lys824ArgfsTer99	Bellon et al. (2020)
26	c.2471_2475delAAGAG	p.Lys824ArgfsTer2	Goujon et al. (2010)
26	c.2473-4delGA	p.Glu825GlyfsTer1	Renner et al. (2009)
26	c.2487_2490dupGAGC	p.Asn831GlufsTer15	Nijman et al. (2014)
27	c.2557C>T	p.Arg853Ter	Lacroix et al. (2012)
27	c.2579_2587delAGCTTATCT	p.Lys860_Cys863delinsSer	Sprecher et al. (2001)
28	c.2677delG	p.Ala893LeufsTer49	Patel et al. (2020)
Intron	***Intronic Variants Causing Splicing Defects***		
2	c.81+5G>A		Bitoun et al. (2002)
2	c.81+2T>A		Sprecher et al. (2001)
4	c.283-12T>A		Lacroix et al. (2012)
4	c.283–2 A > T		Chavanas, Bodemer, et al. (2000)
4	c.410+1G>A		Renner et al. (2009)
6	c.475-2 G>A		Sprecher et al. (2001)
8	c.603+1G>A		Renner et al. (2009)
9	c.794+6T>C		*LOVD database*
11	c.1010+7A>G		Bellon et al. (2020)
12	c.1092+5G>A		Bellon et al. (2020)
14	c.1302+4A>T		Hachem et al. (2006)
15	c.1430+2T>G		Zhao et al. (2007)
15	c.1431-12G>A		Renner et al. (2009)
15	c.1432-13G>A		Raghunath et al. (2004)
17	c.1607+2T>C		Diociaiuti et al. (2016)

(Continues)

TABLE 1 (Continued)

Intron	*Intronic Variants Causing Splicing Defects*	
17	c.1608-1 G>A	Bitoun et al. (2002)
19	c.1816-1820+21delinsCT	Śmigiel et al. (2016)
19	c.1820+53G>A	Lacroix et al. (2012)
20	c.1888-1G>A	Chavanas, Bodemer, et al. (2000)
21	c.2015+5 G>A	*Present*
22	c.2112+2T>A	Özyurt et al. (2019)
22	c.2108_2112+4delCTCAGGTGA	Sprecher et al. (2001)
22	c.2212+1G>A	Sprecher et al. (2001)
23	c.2240+1 G>A	Sprecher et al. (2001)
23	c.2240+5G>A	Israeli et al. (2014)
25	c.2441+3delGAGT	Tüysüz et al. (2010)

c.1431-12G>A *SPINK5* variant exhibited a lethal form of disease (Capri et al., 2011). It is important to note that these inferences are non-dogmatic, especially since NS patients, who are homozygous for more upstream nonsense mutations than the aforementioned (such as c.997C>T), exhibit a milder, non-lethal form of NS (Fong et al., 2011). With regard to LEKTI expression, some intronic *SPINK5* variants, such as c.1820+53G>A and c.283-12T>A, are described as "leaky" as they do not abolish normal splicing in its entirety thereby allowing for a low-level production of the protein (Lacroix et al., 2012).

In a study of five Japanese NS patients, early correlations have been claimed between genotype and cutaneous severity, growth retardation, and frequency of infections. However, such association could not be made for the occurrence of allergic disease, TI, sweat secretion, and temperature homeostasis (Komatsu et al., 2008). Similarly, conflicting results were obtained in a larger study of 21 NS families where no genotype–phenotype associations were established (Bitoun et al., 2002).

The absence of epidermal LEKTI expression demonstrated in this study should be correlated with evidence from in vitro assays, including *SPINK5* mRNA expression studies to identify nonsense-mediated decay and analysis of LEKTI activity by in situ zymography. Potentially, additional insight into variant pathogenicity can be obtained through targeted editing of cell lines with the use of CRISPR-Cas9 techniques. Recently, *SPINK5*-knockout keratinocyte clones were successfully made to re-express *SPINK5* through treatment with lentiviral vectors of the gene, reverting NS skin to normal skin phenotype both in vivo and in vitro (Gálvez et al., 2020).

Apart from cutaneous and hair manifestations, patients with NS exhibit atopic and allergic tendencies. Several loci influencing serum IgE levels lie in close proximity to *SPINK5* on 5q31.3 (Meyers et al., 1994). The common c.1258G>A missense variant has been associated with atopy and atopic dermatitis in NS families (Walley et al., 2001). In the perinatal period, infants with NS are also susceptible to growth failure and electrolyte disturbances. The latter is partly due to lamellar barrier dysfunction as a result of the proteolytic degradation of extracellular hydrolases such as β-glucocerebrosidase and acidic sphingomyelinase (Hachem et al., 2006).

Despite the severe skin barrier dysfunction, most NS patients survive to adulthood, with compensatory mechanisms such as the upregulation of Desmoglein (Dsg3), Desmocollin 3 (Dsc3), and lamellar body secretion coming into play, protecting against water loss and skin fragility (Hachem et al., 2006).

Due to the varied phenotypic manifestations in patients with NS, severity is difficult to score objectively. Attempts at creating a NS-severity scoring system have been made whereby patients are scored according to the area of skin involvement, severity of hair shaft abnormalities, serum IgE levels, and other associated features of NS (Sprecher et al., 2001). This scoring system has not been reproduced in other studies.

In summary, none of the few attempts made at identifying genotype–phenotype associations in NS have been conclusive or replicated. This can be partly explained by the lack of a universally accepted NS severity classification as well as the variable expressivity of *SPINK5* mutations and the extent of the compensatory molecular mechanisms at play.

5 | CONCLUSION

We have identified a novel *SPINK5* c.2015+5G>A likely pathogenic variant in a Maltese family thus expanding the *SPINK5* mutational spectrum. The characterization of this c.2015+5G>A likely pathogenic variant in a Mediterranean

island population where a high propensity for founder effects exists warrants further investigation. The functional evaluation of *SPINK5* variants of unknown significance is often necessary and may be obtained through immunohistochemical analysis which helps elucidate pathogenicity.

AUTHOR CONTRIBUTIONS

DM, IB, NPP conceived the design and wrote the paper. JV, RM, JN and JF contribued analysis tools. All authors contributed data and revised the manuscript.

INFORMED CONSENT

The patient's legal guardians (parents) have given explicit, informed consent for the publication of the case details, for which we thank them.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the text and supplementary material of this article.

ORCID

Dillon Mintoff https://orcid.org/0000-0003-3705-0119
Isabella Borg https://orcid.org/0000-0003-0639-3055
Julia Vornweg https://orcid.org/0000-0001-9808-4983
Nikolai Paul Pace https://orcid.org/0000-0002-7332-874X
Judith Fischer https://orcid.org/0000-0002-8580-8118

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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