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ORIGINAL ARTICLE

A novel homozygous splicing mutation in *PSAP* gene causes metachromatic leukodystrophy in two Moroccan brothers

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Abstract Prosaposin (*PSAP*) gene mutations, affecting saposin B (Sap-B) domain, cause a rare metachromatic leukodystrophy (MLD) variant in which arylsulfatase A (ARSA) activity is normal. To date, only 10 different PSAP mutations have been associated with a total of 18 unrelated MLD patients worldwide. In this study, we report for the first time a family with Moroccan origins in which the proband, presenting with a late-infantile onset of neurological involvement and a brain MRI with the typical tigroid MLD pattern, showed normal values of ARSA activity in the presence of an abnormal pattern of urinary sulfatides. In view of these findings, *PSAP* gene was analyzed, identifying the newly genomic homozygous c.909 + 1G > A mutation occurring within the invariant GT dinucleotide of the intron 8 donor splice site. Reverse transcriptase-polymerase chain reaction (RT-PCR), showing the direct junction of exon 7 to exon 9, confirmed the skipping of the entire exon 8 (p.Gln260_Lys303) which normally contains two cysteine residues (Cys271 and Cys265) involved in disulfide bridges. Our report provides further evidence that phenotypes of patients with Sap-B deficiency vary widely depending on age of onset, type, and severity of symptoms. Awareness of this rare MLD variant is crucial to prevent delayed diagnosis or misdiagnosis and to promptly provide an accurate genetic counseling, including prenatal diagnosis, to families.

Keywords Splicing mutation · Saposin B · Prosaposin · Metachromatic leukodystrophy · Tigroid pattern · White matter disorder · Lysosomal disorder

Introduction

Metachromatic leukodystrophy (MLD) is an autosomal recessive lysosomal storage disease characterized by psychomotor regression, gait disturbances, ataxia, spastic paraparesis/tetraparesis, peripheral neuropathy, and visual disturbances leading to tonic spasms and decerebrate posturing. MLD is caused by defects in the degradation of cerebroside-3-sulfate (sulfatide), which is particularly abundant in the myelin of the nervous system. In vivo, the catabolism of sulfatides requires both the lysosomal enzyme arylsulfatase A (ARSA, E.C. 3.1.6.8) and the specific sphingolipid activator protein, saposin B (Sap-B) [15].

Sap-B protein is one of the four cleavage products (saposins A, B, C, and D) derived from a large precursor protein, prosaposin (PSAP) encoded by the *PSAP* gene (OMIM #176801) located on chromosome 10. Saposins are cofactors for lysosomal enzymes involved in the degradation of sphingolipids (SLs). Consequently, mutations in the *PSAP* gene can lead to the devastating neonatal condition with an acute generalized neurovisceral dystrophy associated with the multiple SL storage or conditions resembling either MLD (mutations in the Sap-B domain), or Gaucher disease (mutations in the Sap-C domain), or Krabbe disease (mutations in the Sap-A domain) [11].

MLD can result therefore from either ARSA deficiency (OMIM #250100) or Sap-B deficiency (OMIM #249900). Although the two distinct forms present with very similar

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clinical and neuroradiological features, Sap-B patients are clearly distinguishable from ARSA-deficient patients on the basis of laboratory findings: indeed, Sap-B patients show arylsulfatase A activity within the normal range values, but abnormal urine sulfatide excretion, totally comparable to that of patients with ARSA deficiency [15].

However, most MLD patients are due to mutations within the *ARSA* gene (OMIM #607574), while only 18 patients with normal enzyme levels and mutations in the *PSAP* gene, encoding for the Sap-B domain, have been described until now [2, 9]. To date, 10 different PSAP mutations, affecting the Sap-B domain, have been associated with MLD: among these, three were splicing mutations [12]. Here, we report the molecular and clinical features of two Moroccan brothers with late-infantile MLD owing to a novel homozygous splice site mutation in the *PSAP* gene.

Patients and methods

Patients The two siblings were born from healthy and first-degree-cousin Moroccan parents. Clinical, neuroradiologic, and laboratory investigations were performed.

Biochemical studies ARSA residual activity was determined with *p*-nitrocatechol sulfate in homogenates of leukocytes and fibroblast cell lines from both patients [3]. The Lowry assay was used for protein quantification in the samples.

Sulfatide excretion was evaluated in 24-h urine collections by thin layer chromatography [10].

Molecular studies Genomic DNA was extracted from cultured fibroblasts or peripheral blood leukocytes using QIAmp DNA Blood Mini Kit according to the manufacturer's protocol (Qiagen).

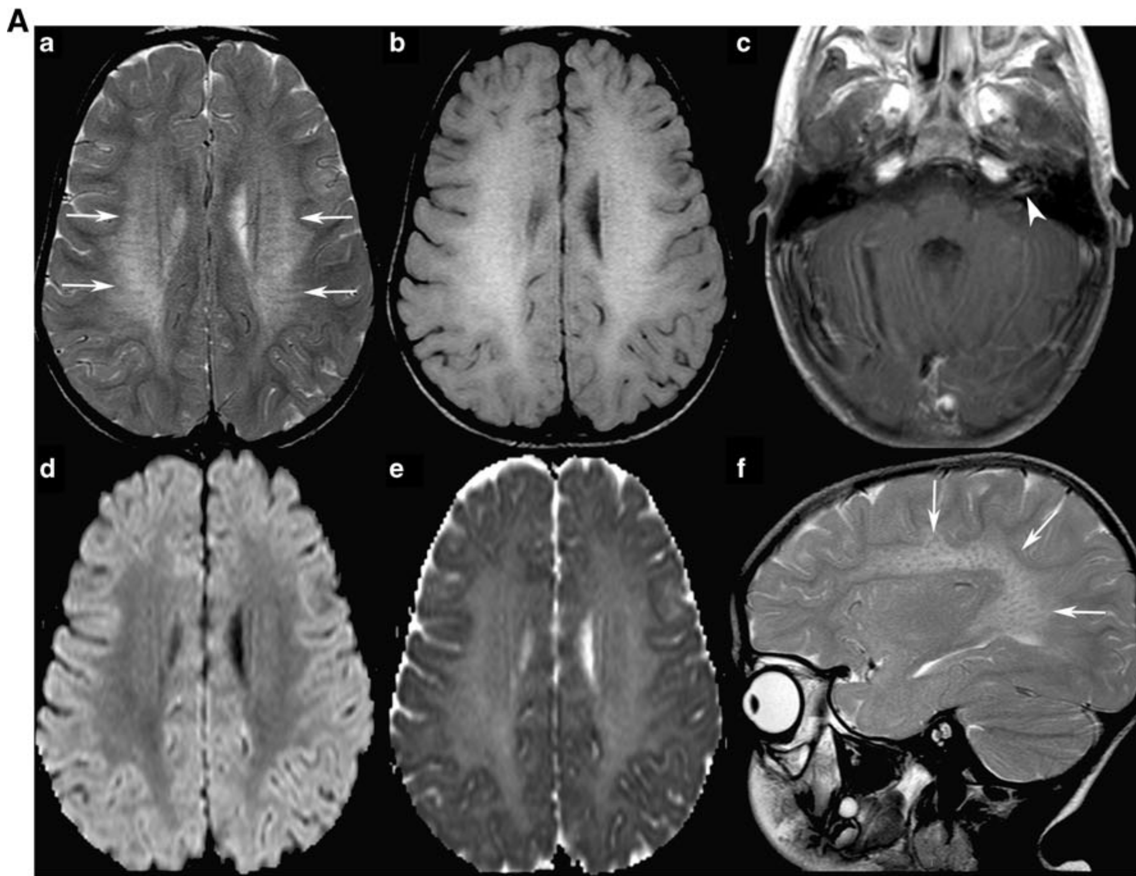
Total RNA was extracted from cell lines using RNeasy Mini Kit (Qiagen). First-strand cDNAs were synthesized by Advantage RT-for-PCR Kit (Clontech) using random hexamer primers. The products from PCR and/or reverse transcriptase-polymerase chain reaction (RT-PCR) were purified with Illustra Exonuclease I and Alkaline Phosphatase (Illustra ExoProStar 1-Step, GE Healthcare Life Sciences). The sequencing reaction was performed on the purified products using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Life Technologies). Electrophoresis of samples was performed on the ABI PRISM 377 DNA Sequencer, and for data analysis, the Sequencing Analysis Software 5.2 (Life Technologies) was used.

Mutation nomenclature Nucleotide numbers are derived from cDNA PSAP sequences (GenBank accession no. NM_002778.2). The mutation is described according to current mutation nomenclature guidelines (http://www.hgvs.org/mutnomen), ascribing the A of the first ATG translational initiation codon as nucleotide +1[4, 5].

Results

The first child (patient #1) was born at term after an uneventful pregnancy and normal delivery. His psychomotor development was reported to be normal until the age of 18 months. The patient was referred to our hospital for an acute respiratory infection at 27 months. At that age, the patient was unable to walk without support and showed frequent falls and gait instability, ataxia, and speech disturbances. Neurological examination showed inability to sit without support, truncal titubation, increased muscle tone at the lower limbs, dysarthria, strabismus, nystagmus, and dysphagia for liquids. Having observed this severe neurologic picture, we performed brain MRI. It showed diffused and symmetrical areas of high signal intensity on T2-weighted and fluid-attenuated inversion recovery (FLAIR) images in the periventricular white matter with a posteroanterior gradient, with a "tigroid pattern," as well as in the corpus callosum (Fig. 1a). Magnetic resonance spectroscopy showed a reduction in *N*-acetyl aspartate and elevated coline and lactate levels. To confirm the diagnostic suspicion of MLD, we assessed arylsulfatase A activity that was in the normal value range (nv) in both white blood cells (155.9 nmol/mg/h; nv=120±30) and in the cultured fibroblasts (481.6 nmol/mg/h; nv=520±149). Thus, urinary sulfatides were investigated and found to be abnormally increased (data not shown). In view of these findings, the *PSAP* gene was analyzed. The molecular analysis, performed as previously described [6], identified the newly genomic

Fig. 1 Brain MRI of the two patients. **a** Brain MRI of patient #1 at 2 years and 3 months: *A* Axial T2-weighted image shows abnormally hyperintense white matter in the centrum semiovale bilaterally, with a radial arrangement of hypointense stripes consistent with the so-called tigroid pattern (*arrows*). The subcortical white matter is spared. *B* Axial FLAIR image shows diffusely hyperintense white matter in the centrum semiovale. The "tigroid pattern" is not apparent in this image. *C* Contrast-enhanced axial T1-weighted image at the level of the posterior cranial fossa shows enhancing left cranial nerves VII–VIII consistent with peripheral neuropathy. *D* Axial diffusion-weighted image. *E* Corresponding apparent diffusion coefficient (ADC) map shows signal consistent with increased water diffusivity throughout the supratentorial white matter. *F* Sagittal T2-weighted image shows the abnormally hyperintense white matter of the centrum semiovale interspersed with multiple hypointense dots (*arrows*) corresponding to the tigroid radial stripes seen on image *A*. **b** Brain MRI of patient #2 at 14 months: *A* Axial T2-weighted image. *B* Coronal FLAIR. *C* Coronal T2-weighted image shows tenuous white matter hyperintensity in the posterior centrum semiovale bilaterally (*arrows*). The "tigroid pattern" is not visible. *D* Axial diffusion-weighted image and *E* corresponding ADC map are normal. *F* Sagittal T2-weighted image confirms mild hyperintensity of the deep parietal white matter. Myelination of the subcortical white matter is physiologically incomplete at this age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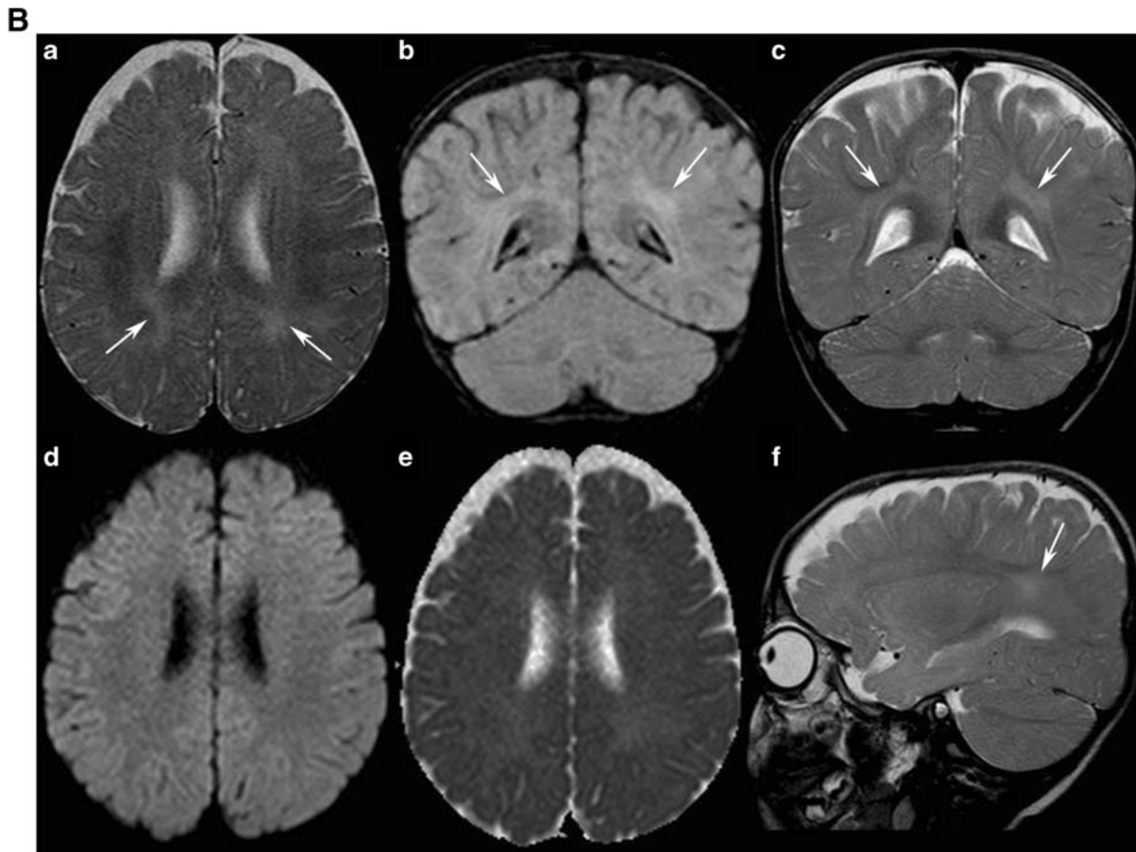
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homozygous c.909 + 1G > A transition occurring within the invariant GT dinucleotide of the intron 8 donor splice site. RT-PCR analysis was carried out on PSAP mRNA extracted from the patient's fibroblast cell line since this mutation abolished the canonical splice site and might potentially alter mRNA processing. The abnormally spliced short transcript was amplified by the RT-PCR analysis and sequenced. The findings showed a direct junction of exon 7 to exon 9, confirming the abrogation of the donor splice site leading to the skipping of the entire exon 8 (p.Gln260_Lys303) (Fig. 2).

Few months later, a further deterioration was observed with initial blindness, progressive spastic quadriparesis with hyperreflexia, and mental regression. Tonic seizures were documented and treated with valproate. The electroencephalogram disclosed high-voltage slow waves and burst of spikes. The ophthalmological examination showed optic atrophy. Percutaneous gastrostomy was performed for feeding purposes. Nerve conduction velocity was not performed. At the age of 3.5 years, he presented features of tonic spasms and decerebrate posturing. The child died at 4 years because of worsening respiratory failure.

The youngest brother (patient #2) is a 2-year-old boy born at term after an uneventful pregnancy and normal delivery. At 5 months, he was visited for an upper respiratory infection; the neurological examination showed mild axial hypotonia, brisk tendon reflexes at lower limbs, and partial head control. At 10 months, he acquired autonomous sitting position, and at 14 months of age, he started to walk with support and frequent falls. At this age, on the basis of the clinical picture and of the diagnosis obtained in the brother, brain MRI was performed, showing white matter involvement as described in Fig. 1b. The electroencephalogram disclosed posterior slow waves. The ophthalmological examination showed optic subatrophy. We therefore proceeded directly with molecular analysis, demonstrating the same homozygous c.909 + 1G > A mutation as in the brother, thus indicating that he was also affected. At last follow-up, at age 24 months, neurological examination showed muscle axial hypotonia, increased muscle tone at the lower limbs, brisk tendon reflexes, ankle clonus, bilateral Babinski sign, and speech regression.

The analysis of the genomic DNA samples of both parents confirmed their heterozygous carrier status of the mutation c.909 + 1G > A.

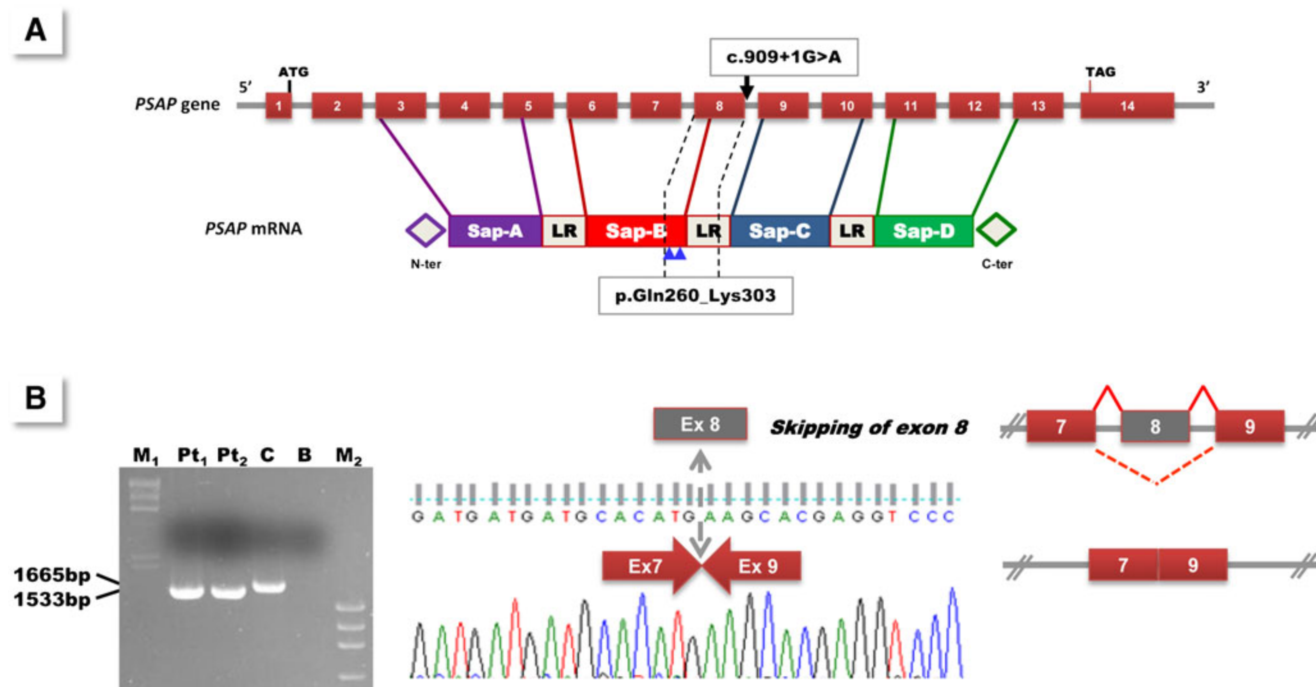

Fig. 2 The graphical representation of the impact of c.909 + 1G > A mutation upon mRNA processing. **a** the *PSAP* gene contains 14 exons encoding for four central domains delimited by three linker regions (LR), and for two peripheral domains, N-terminus (N-ter) and C-terminus (C-ter). Subsequent to PSAP transport into the lumen of the lysosomes, the linker regions of the central domains are proteolytically cleaved to liberate the four active saposin A, B, C, and D domains. The novel intron 8 donor splice site mutation (c.909 + 1G > A) abolished normal RNA splicing and resulted in the skipping of exon 8 predicting to remove 43 amino acids (p.Gln260_Lys303) comprising two cysteines (*blue triangles*) involved in the formation of disulfide bridges (Cys198–Cys271 and Cys201–Cys265). **b** *From the left*, RT-PCR analysis, performed on the RNA sample by a set of primers encompassing the entire cDNA, revealed the presence of an abnormally shorter PSAP transcript (1,533 bp) in addition to the normally sized product (1,665 bp). Sequence analysis of the RT-PCR shorter product from the two patients, demonstrating the direct junction exons 7–9, confirmed that the intronic mutation c.909 + 1G > A caused the skipping of the exon 8. Note that only the involved exonic regions are graphically shown (*on the right*). *Ex* exon, M_1 λ DNA *HindIII*-digested molecular weight marker, M_2 ΦX DNA *HaeIII*-digested molecular weight marker, *Pt* patient, *C* normal control, *B* no template internal PCR control

Discussion

PSAP gene mutations involving the coding region of the Sap-B domain are known to cause sulfatide accumulation leading to a rare MLD variant in which arylsulfatase A activity is normal. To the best of our knowledge, only 18 families for a total of 26 patients from different ancestries have been reported until now [[2] and related references; [9]] and the Human Gene Mutation Database barely lists 10 different PSAP mutations [12].

In this study, we report for the first time a family with Moroccan origins. The clinical and neuroradiologic features, namely, the so-called tigroid pattern, found in the proband, raised the diagnostic suspicion of MLD. As arylsulfatase A activity was found to be in the normal value range in the presence of an abnormal urine sulfatide excretion, our experimental investigations were direct to the *PSAP* gene demonstrating that a novel homozygous splicing mutation (c.909 + 1G > A) underlies the rare variant of MLD variant (Sap-B deficiency).

The c.909 + 1G > A mutation occurred in the evolutionarily conserved donor splice site of *PSAP* gene intron 8. Hence, the disruption of the normal PSAP splicing process was expected. The functional relevance of the intronic mutation was therefore assessed by RT-PCR analysis on the patients' mRNA, clearly confirming the presence of a transcript bearing the skipping of the entire exon 8. This in-frame mutation resulted in the removal of 132 bases normally encoding the final 14 amino acids of the Sap-B domain and 29 amino acids of the linker region between the Sap-B and Sap-C domains (p.Gln260_Lys303). While the functional relevance of this linker region is still unknown, the lack of the terminal portion of Sap-B is expected to be very detrimental, as it leads to the removal of two cysteines (Cys271 and Cys265) (Fig. 2a), predicted to be involved in the formation of two out of the three canonical disulfide bridges Cys198–Cys271, Cys201–Cys265, and Cys230–Cys241, respectively. The localization of the disulfide bridges has been well established, the first two cysteine residues being disulfide-linked to the last two (Cys198–Cys271 and Cys201–Cys265) and two cysteine residues centrally placed (Cys230–Cys241) being linked to each other [1, 14]. It is well known that the disulfide bonds, by interacting between distant parts of the saposin domains, play a major role in stabilizing protein conformation. The importance of the disulfide arrangement for the Sap-B domain function has been also experimentally confirmed in Sap-B-deficient mice created by a knock-in mutation of a cysteine corresponding to human Cys241[13]. Indeed, no Sap-B protein was detected in the homozygous (Sap-B −/−) mice, resembling human phenotype, whereas saposins A, C, and D were at normal levels [13].

To date, only three mutations occurring in intronic regions of the *PSAP* gene have been reported and all resulted in splice site abnormalities: (a) A homozygous transversion (C-to-A) in intron 4, leading to a longer splice variant owing to the retention of 33 intronic nucleotides, was found in a young woman who showed slowly progressive neurological impairment and died at 22 years of age [7, 16]; (b) another homozygous transversion (G-to-T) in intron 5, resulting in two distinct mutant transcripts with partial or complete in-frame skipping of exon 6, was identified in a patient with peripheral neuropathy and slight psychological delay at age 25 months, bone marrow transplanted at 3 years due to the mild CNS involvement [8]; and (c) the compound heterozygous mutation A-to-G in intron 5 (in association with c.828-829delGA, located in exon 8), resulting in two in-frame transcripts, was found in a patient who presented clinical, neurophysiological, and MRI findings consistent with late-infantile MLD, although he presented with a mild spastic hemiparesis due to middle cerebral artery infarction at age 9 months [9].

To conclude, our report of a novel splice site mutation of the *PSAP* gene has provided further evidence that phenotypes of patients with Sap-B deficiency vary widely depending on age of onset, type, and severity of symptoms, and that this is not closely correlated with the type of mutation. Indeed, in the presence of splice site abnormalities, patients showed a range of different phenotypes from late infantile to adult onset of MLD [7–9]. It is therefore likely that the severe phenotype of our patients results from the failure to form the two canonical disulfide bridges (Cys198–Cys271, Cys201–Cys265) and from the resultant disruption of the normal three-dimensional structure which makes the mutant Sap-B unstable.

Awareness of this rare MLD variant is crucial to avoid delayed diagnosis or even misdiagnosis and to properly give genetic counseling, including prenatal diagnosis, to families.

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Conflict of interest No conflict of interest to declare

Ethical standards Following ethical guidelines, cell line and nucleic acid samples were obtained for analysis and storage in the Biobank with the patients' (and/or a family member's) written informed consent. The consent was sought using a form approved by the local Ethics Committee.

The experiments of the present study comply with the current Italian laws and in accordance with the Declaration of Helsinki.

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