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# **Sphingolipidoses in Morocco: Chemical profiling for an affordable and rapid diagnosis strategy**

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## **Abstract**

Sphingolipidoses are a group of metabolic diseases in which lysosomal hydrolases dysfunction disrupt normal sphingolipids' metabolism, leading to excess accumulation in cellular compartments and excretion in urine. These pathologies represent a significant burden

among Moroccan population, for which an easy access to enzymatic assays and genetic tests is not guaranteed. Parallel analytical methods thus have to be developed for preliminary screening. In this study, 107 patients were addressed to the metabolic platform of the Marrakesh Faculty of Medicine for diagnosis confirmation. Thin-Layer Chromatography was used as a first step to perform chemical profiling of the patients' urinary lipids, allowing 36% of the patients to be efficiently oriented towards the adequate enzymatic assay. UPLC-MS/MS analyses of urinary sulfatides excreted in urines patient had been used to control the reliability of TLC analysis and to obtain more accurate information related to the sulfatides isoforms. This analytical process combining TLC with UPLC-MS/MS has enabled rapid and appropriate patient management in a reduced time and with reduced resources.

### **Keywords**

Sphingolipidoses, Thin-Layer Chromatography, Tandem mass spectrometry, Sulfatides, Urine screening

### **Introduction:**

Sphingolipidoses are metabolic diseases belonging to the general class of the lysosomal storage diseases (LSDs), of which over 70 forms have been described (Parenti, Andria and Ballabio, 2015; Platt *et al.*, 2018). These inherited pathologies are caused by a defect in sphingosine-containing lipids catabolism, which may result from the lack of a specific lysosomal enzyme, a protein cofactor, or a membrane transport protein. This altered protein functionality leads to an intralysosomal over-accumulation of unmetabolized sphingolipids (SPLs) in one or more organs (Kolter and Sandhoff, 1999; Pan *et al.*, 2023). SPLs, which are structural blocks of eukaryotic plasma membranes, have many physiological functions, including signal transduction, cell adhesion and differentiation, growth factor/hormone

receptor modulation, antigen recognition, protein trafficking or neuroplasticity (Dasgupta and Ray, 2017; (Olsen and Færgeman, 2017). Elevated levels of SPLs are notably found within brain, and central and peripheral nervous system (CNS and PNS). Defects in SPLs metabolic pathways, by affecting cellular and molecular processes, lead to CNS abnormal functioning and, consequently, to various organs disorders (Hussain *et al.*, 2019). Sphingolipidoses are clinically and pathologically highly variable, and exist from neonatal to adult forms varying from asymptomatic to lethal (Brown *et al.*, 2018); (De La Concepción Fournier Del Castillo *et al.*, 2020);(Meikle *et al.*, 1999). They mostly induce cerebral damage, even if neurological involvement is not systematically included in LSDs pathophysiology (Boustany, 2013). It is considered that onset, severity and progression of the disease may rely on the type, concentration and location of the stored lipids, and to the residual enzymatic activity of the impaired protein (Abed Rabbo *et al.*, 2021); (Futerman and Van Meer, 2004); (Kolter and Sandhoff, 2006); (Leal *et al.*, 2022); (Raas-Rothschild *et al.*, 2004).

Cerebral lipidoses are often suspected on clinical grounds, but the symptomatic heterogeneity of sphingolipidoses' presentation makes it difficult to diagnose accurately each pathology, potentially leading to years-long diagnostic processes (Meikle, Fietz and Hopwood, 2004). Diagnosis thus must rely on an interdisciplinary multi-step process. It includes patient history analysis, clinical expertise, biochemical analysis of stored undegraded materials in biofluids, specific enzyme and/or activator proteins assays, and eventually molecular analysis to search for genetic mutations (Filocamo and Morrone, 2011); (Kingma, Bodamer and Wijburg, 2015). The sphingolipids in body fluids are gold biomarkers to diagnose and monitor therapy efficiency (Ghosh and Nishtala, 2017). However, the availability of both diagnostic techniques and treatments is not equivalent in all countries.

In Morocco, access to biochemical assays is often tedious, expensive and time consuming. A case study on Gaucher disease highlighted that enzymatic dosage was only performed for less

than 50% of the patients (Essabar *et al.*, 2015). Moreover, if LSDs in general, and sphingolipidoses in particular, are rare diseases individually speaking, they represent important health problems as a group, and are often underdiagnosed (Bouayadi *et al.*, 2019). Among the factors linked to an elevated prevalence of such recessive autosomal diseases stand immigration dynamics, isolation, but also consanguineous marriages (Kadali *et al.*, 2014); (Kingma, Bodamer and Wijburg, 2015); (Özkara, 2004). This situation is frequently observed in Morocco, where an increased sensitization to these genetic pathologies is an essential need (Belhassan, Ouldin and Sefiani, 2016);(Fdil *et al.*, 2020);(Sabir *et al.*, 2020). Studies are therefore necessary to contribute to the implementation of preventive measures and early diagnosis. In this context, we developed a procedure based on the characterization of SPLs and phospholipids (*PLs*) using thin-layer chromatography (TLC) as a first step to orient the diagnosis process in a rapid, efficient and affordable manner (Deranieh *et al.*, 2013). Indeed, TLC is the earliest and widely established chromatographic method used for lipid assessment that continues to be employed today as reported by several researchers (Pati *et al.*, 2016); (Weerheim *et al.*, 2022); ( Fuchs *et al.*, 2022). However, the low resolving power and the lack of specificity limit the use of TLC as a confirmatory diagnostic assay, especially for quantification. Therefore, LC/MSMS, enzyme assays and molecular analysis are often combined with our first analytical diagnostic step to avoid diagnostic errors (Fdil *et al.*, 2020). Our objective was to improve existing analytical pipelines to reduce investment in time and resources, and prevent delayed diagnoses.

Several works demonstrated that urine can be used as a promising sample for qualitative and quantitative determination of lipids for the detection of LSDs (Kelkel *et al.*, 2022); (Mirzaian, Kramer and Ben J.H.M. Poorthuis, 2015); (Natowicz *et al.*, 1996);(Whitfield *et al.*, 2001). The collection of urine samples from patients as well as healthy subjects is also simple and noninvasive. Moreover, urinary lipids are relatively more stable than in blood, which

facilitates their manipulation and analysis (González-Domínguez *et al.*, 2020); (Li *et al.*, 2021). In this context, TLC can stand as a simple, versatile, quite inexpensive and rapid procedure for lipid analysis, notably SPLs and PLs (Fuchs *et al.*, 2011). Preceded by an extraction step, followed by mass spectrometry analysis or using immunodetection, TLC, or its refined version HPTLC, was notably employed for lipid detection in urinary, plasma or cerebrospinal fluid samples from patients with LSDs (Berná *et al.*, 1999); (Colsch, Baumann and Ghandour, 2008); (Jarne *et al.*, 2021); (Kushi, Rokukawa and Handa, 1988). Mass spectrometry (MS), and tandem MS in particular, is nowadays a key method for lipid analysis and quantification (Piraud *et al.*, 2018). LC-MS or LC-MS/MS analysis has experienced a marked development in recent years, opening up new perspectives in the field of lipidomics (Cajka and Fiehn, 2014); (Merrill Jr *et al.*, 2005); (Pintado-Sierra *et al.*, 2017).

This article thus discusses our experience in demonstrating the relevance of detecting SPLs and PLs in patients' urine with symptoms of progressive neurologic dysfunction and/or with symptoms evoking a deficiency in PLs and/or SPLs metabolism using TLC, and the validation of a novel algorithm coupling chemical (TLC and complementary UPLC-MS/MS) and biochemical techniques for urinary lipidomic analysis in the diagnosis of sphingolipidoses.

## **2. Materials and methods**

### **2.1. Reagents and solvents**

TLC analysis and lipid extraction were performed using n-butanol (99.5% Loba Chemie PVT, Mumbai, India, CAS 71-36-3), chloroform ( $\geq 99.5\%$  CAS 67-66-3, Supelco, Sigma-Aldrich, Saint-Louis, MO, USA), methanol ( $\geq 99.5\%$  CAS 67-56-1, Supelco, Sigma-Aldrich, Saint-Louis, MO, USA) and distilled water (GFL 2004 Water Still for single distillation, Lauda, Burgwedel, Germany) for extract solubilization and as running solvents. TLC staining

solutions were prepared using orcinol (AppliChem GmbH, Darmstadt, Germany, CAS 6153-39-5), iodine (puriss, Honeywell Riedel-de-Haën, Charlotte, NC, USA, CAS 7553-56-2) and ninhydrin (Honeywell Riedel-de-Haën, Charlotte, NC, USA, CAS 485-47-2) as reagents. Sulfuric acid (H<sub>2</sub>SO<sub>4</sub> 98%, Loba Chemie PVT, Mumbai, India, CAS 7664-93-9), methanol (MeOH 99.9%, HPLC-grade, Sigma-Aldrich, Saint-Louis, MO, USA), and absolute ethanol (Biosmart, Merck, Darmstadt, Germany, CAS 64-17-5) were used as solvents, accordingly. For all analysis and extraction procedures, glacial acetic acid (CAS 64-19-7) was purchased from Loba Chemie PVT, Mumbai, India. Analytical standards for sphingomyelin (from chicken egg yolk, ≥95%, CAS 85187-10-6), sulfatides (porcine brain, CAS 383906-24-9), galactosylceramides (CAS 85305-88-0), and glucosylceramides (CAS 497155-61-0) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Water (CAS 7732-18-5) and acetonitrile (CAS 75-05-8) for UPLC-MS/MS analysis were of ULC/MS – CC/SFC grade and purchased from Biosolve (Dieuze, France). Ammonium formate for mass spectrometry (CAS 540-69-2) was obtained from Sigma-Aldrich (Saint-Louis, MO, USA). The extracts were prepared using chloroform (Chromasolv, Honeywell Riedel-de-Haën, Charlotte, NC, USA) and methanol (ULC/MS – CC/SFC, Biosolve, Dieuze, France).

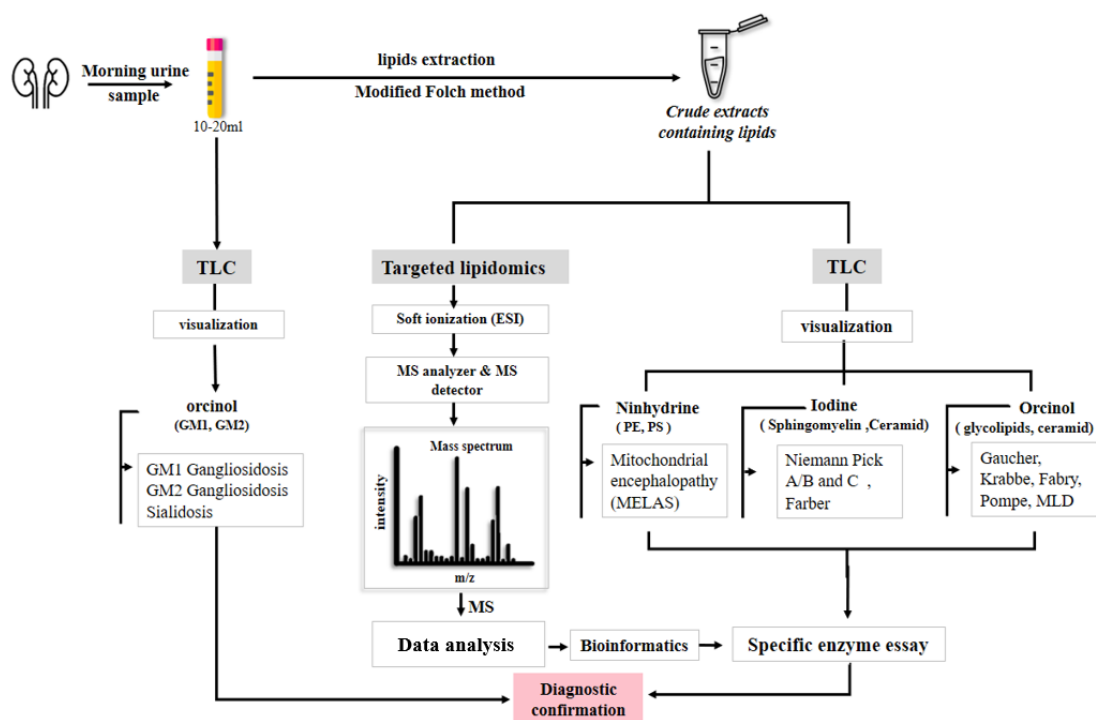
## **2.2. Patients**

Between 2020 and 2022, symptomatic individuals (n =107) clinically suspected with sphingolipidoses and/or presenting a psychomotor regression, and who applied to Mohammed VI University Hospital's Pediatric and Neurology departments, were referred to the metabolic platform of the Marrakesh Faculty of Medicine for a confirmatory diagnosis. The main inclusion criteria were psychomotor delay, visceral or neurological disorders, and/or any other symptom suggestive of sphingolipidoses (Raas-Rothschild *et al.*, 2004). The absence of an MRI was not an exclusion criterion. This study was conducted in compliance with the

recommendations of the Declaration of Helsinki and Good Clinical Practice (GCP) Guidelines. The study was approved by the Independent Ethics Committee of Marrakech Hospital University, and informed consent was obtained from patients and/or their parents or guardians.

### 2.3. Clinical samples

First-void morning urine samples (10-20 mL) were collected from each patient for the characterization of sphingolipids. The algorithm for patient sample analysis is shown in Figure 1. TLC (Thin Layer Chromatography) analysis was carried out on crude urine samples to allow for the detection of gangliosides, and urinary lipid extracts were concurrently analyzed. Biochemical analyses were confirmed using enzymatic assays depending on the suspected condition, and performed using 60-70  $\mu$ L of whole blood spotted on Whatman filter paper (Whatman-GE no. 903, Sigma-Aldrich, Saint-Louis, MO, USA) (Colsch, Baumann and Ghandour, 2008) ;(Whitfield *et al.*, 2001).





**Figure 1: Flow chart of the algorithm used for urinary lipid analysis for the diagnosis of sphingolipidoses**

**2.4. TLC analysis of untreated urine samples**

First-void morning urine was applied to glass silica gel coated TLC plates (60G F<sub>254</sub>, Sigma-Aldrich, Saint-Louis, MO, USA). The volume of urine used is calibrated to meet a 5- $\mu$ g creatinine content to enable different dilutions of urine to be compared (Sewell, 1979). The following formula was used for calculation:

$$\text{Volume of urine } (\mu\text{L}) = 5 / (0.113 \times \text{mmol creatinine} / \text{L})$$

After each sample deposit, the plate is dried under a stream of hot air, TLC plates were eluted with a solvent system composed of n-butanol/ glacial acetic acid/water (100:50:50, v/v/v). After two successive elutions (McLaren and Ng, 1979), the plates were stained with an 0.2% orcinol solution in a 98% sulfuric acid/methanol mixture (13:87, v/v) to detect oligosaccharides (Blau, Duran and Gibson, 2008). The TLC plate was incubated at 100°C during 10 min after spraying before observation. Urine samples from confirmed cases of GM1 and GM2 gangliosidosis were used for control/identification purposes.

**2.5 Extraction of urinary lipids**

The extraction method for non-polar urinary lipids including sulfatides, glycolipids and sphingomyelins, was adapted from Folch et al. (FOLCH, LEES and SLOANE STANLEY, 1957) and Rafi et al. (Rafi *et al.*, 2003) with slight modifications. Briefly, 15  $\mu$ L of glacial acetic acid were added to 10 mL of freshly collected morning urine and the sample was left overnight at 4 °C, since the acidification reduces the solubility of these lipids as reported by (Colsch *et al.*, 2008). After centrifugation during 10 min at 2500 rpm, the pellet was collected and extracted by 5 mL of 2:1 (v/v) chloroform/methanol mixture. After sonication (Branson®

sonicator, 6 x 30 s), 1 mL of distilled water was added, and the resulting solution submitted to magnetic stirring for 2 h and filtered through a sintered glass funnel of medium porosity. A 2-mL volume of distilled water was added to the filtrate and the mixture was stirred for 30 min at room temperature before centrifugation at 2000 rpm for 30 min. The lower phase, containing the lipid extract, was evaporated to dryness under a stream of nitrogen gas and stored at -20°C until use.

## **2.6. TLC analysis of urinary lipid extracts**

The extract was dissolved in (0.1-0.2) mL of a chloroform: methanol 2:1 (v/v) mixture, and half of the solution was spotted on the Aluminium silica gel coated TLC plate (60G F<sub>254</sub>, Sigma-Aldrich, Saint-Louis, MO, USA) along with commercial standards sphingomyelin (SM), sulfatides (SGalCer, SUL), galactosylceramides (GalCer) and glucosylceramides (GlcCer). The running solvent was a mixture of chloroform/methanol/water (72:28:3.5, v/v/v). After drying, the lipids were revealed by spraying the following solutions, and then by heating: orcinol 0.2% in 98% sulfuric acid/methanol 13:87 (v/v) to detect glycolipids and sulfatides(Blau, Duran and Gibson, 2008), iodine to detect sphingomyelin (Boonla *et al.*, 2011). and ninhydrin 0.2% in absolute ethanol/glacial acetic acid 80:20 (v/v) to detect phosphatidylethanolamines(Kimura, Kawanami and Otsuka, 1967).

The Lipid extracts are separated by one-dimensional thin-layer chromatography (1D-TLC), 2-D TLC results in more sensitive separation; however, the number of samples is limited to one per plate. While 1D-TLC is somewhat less sensitive, multiple samples can be analyzed simultaneously on a single plate and in a shorter time than in 2-D TLC. This aligns more with our main objective: a rapid diagnosis orientation.

In order to capitalize on the information obtained by TLC and avoid errors, a preparative TLC was performed for each band visualized on our plates, at least once. In fact, once the bands

have been identified, they are removed by scraping the band with a spatula, the scraping powder obtained is transferred to a tube, the solvent is added and the adsorbent is separated by filtration. A determination of the relative structures is carried out by LC-MS/MS. Subsequently, a combination of all the data at our disposal: the retention factor ( $R_f$ ) of the biomarker (the band characterized by the preparative TLC), the patient's symptoms, and the pure  $R_f$  standards, allows us to associate a biomarker to a band revealed on our TLC plate

## **2.7. UHPLC-MS/MS analysis of urinary sulfatides**

UHPLC-HRMS profiling experiments were conducted with a Thermo system. The mass detector was an Orbitrap MS/MS FT Q-Exactive Focus mass spectrometer. The analyses were conducted in FullMS/data dependent  $MS^2$  mode. Analyses were performed in the positive ionization mode at the range of 700-1000 Da, in centroid mode. In FullMS, the resolution was set to 70000, and the AGC target was  $3 \times 10^6$ . In  $MS^2$ , the resolution was 17500, AGC target  $10^5$ , isolation window 0.4 Da, and stepped normalized collision energy 15/30/45 was used, with 6 s dynamic exclusion.

The UHPLC column was a Phenomenex Kinetex F5 150 mm  $\times$  2.1 mm, 1.7  $\mu$ m. The column temperature was set to 42 °C, and the flow rate was 0.5 mL.min<sup>-1</sup>. The solvent system was a mixture of water (solution A) with increasing proportions of water/acetonitrile 5/95 (solution B), both solvents modified with 5 mM ammonium formate. The gradient was as follows: 20% B 5 min before injection, then from 1 to 10 min, a shark fin gradient increase of B up to 100% (curve 3), followed by 100% B for 4 min. The flow was diverted (not injected into the mass spectrometer) before injection, up to 1 min after injection.

The urine extracts were dissolved in a mixture of chloroform ( $CHCl_3$ ) and methanol (MeOH) (30:70) to reach a concentration of the extract of 10 mg/mL in the  $CHCl_3$ : MeOH mixture, and this solution (100  $\mu$ L) was diluted with MeOH (900  $\mu$ L) to reach a final concentration of 1 mg/ml. The commercial standards were dissolved first in  $CHCl_3$ /MeOH 1/1 with the adequate volume to reach a concentration of 1 mg/ml, and the solution (10  $\mu$ L) was diluted

again with MeOH (990  $\mu\text{L}$ ) to obtain a concentration of 10  $\mu\text{g}/\text{mL}$ . In all cases, 5  $\mu\text{L}$  were injected for LC-MS analysis.

### **3. Results and discussion**

#### **3.1 Patients' clinical features patients**

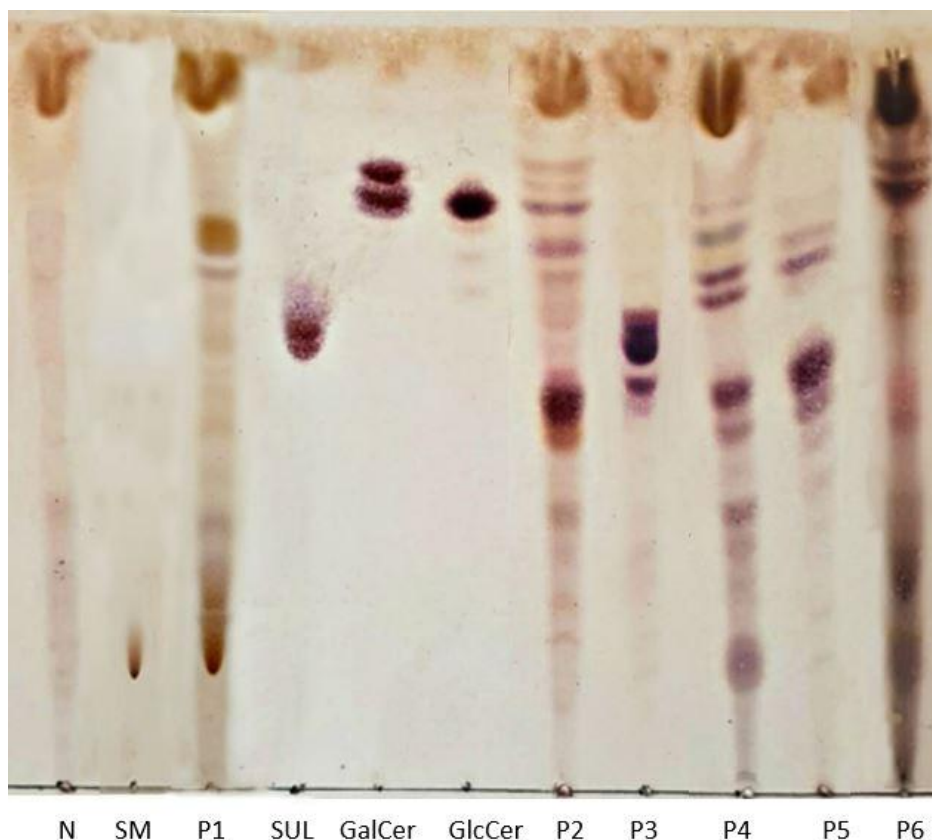
A total of 107 patients clinically suspected with sphingolipidoses were included in the study. The full dataset of the patients' characteristics and clinical features is available in Table S1. Three clinical forms were clearly distinguished for this set of patients (Table 1), in keeping with the features described for sphingolipid storage diseases (Abed Rabbo *et al.*, 2021);(Raas-Rothschild *et al.*, 2004)). The first group was characterized by a psychomotor regression and mainly central nervous system motor signs (pyramidal, cerebellar, seldom, dystonia) and a peripheral neuropathy. A total number of 73 patients (59%) was concerned with form I features, and were referred to the laboratory mainly for suspected metachromatic leukodystrophy (MLD). MRI were performed for 43 patients over 73, among those 43 patients, 32 (74%) had an MRI presenting pathological features, including white matter abnormalities. The second form was characterized in particular by visceral abnormalities (hepato-splenomegaly), and/or cholestasis (cholestatic jaundice, pigmentation disorders, facial dysmorphism), and/or hypotonia and cytopenia. Only one MRI was carried out for one patient. Overall, 27 patients with form II characteristics were referred to the platform for a suspicion of Gaucher disease and/or Niemann-Pick disease. The third form was mainly characterized by pain crises, and /or cardiac and /or renal failure, concerning 7 patients suspected with Fabry disease. Additionally, 67 patients over 107, i.e., 63% of the patients enrolled in the study, were born to first (69%), second (24%) or third-degree (7%) consanguineous parents (Table S1). These data are consistent with previous studies, thus highlighting the significant impact of Consanguinity and inbreeding in the occurrence and recurrence of LSDs (Fdil *et al.*, 2020);(Sabir *et al.*, 2020). Eventually, the 107 patients came from 98 different families, with 9

pairs of siblings included in the study (Table S1, patients in bold font). Four pairs of siblings were included in group I, three pairs in group II and two pairs in group III.

The patient's age ranged overall from 18 days to 59 years, with a median value of 4 years. These values correspond to the age at the time of the medical consultation, even if the onset was in some cases recorded by the clinician as observed earlier. Early infantile patients ( $\leq 2$  years) were the most represented (36%), and late infantile (3-6 years) to juvenile patients (7-15 years) accounted respectively for 27 and 25% of the addressed persons. Adult patients ( $\geq 16$  years) represented only 11% of the manifestations (Table 2). These data are consistent with previous studies performed in cohorts of patients with MLD or Niemann-Pick disease, indicating the high occurrence for infantile and juvenile forms of sphingolipidoses (Brown *et al.*, 2018); (Morton *et al.*, 2022) ; (Sévin *et al.*, 2007).

### **3.2. TLC analysis of urinary lipid extracts and untreated urine samples**

The extraction of lipids from biological samples is often the first step in lipidomic analyses, Prior to analysis, enrichment of urine lipids is often necessary due to their considerably low concentration compared to the abundance of salts and other charged compounds (Li *et al.*, 2021); (Tiphara and Thongboonkerd, 2016). In this study, we showed that the chloroform methanol extraction protocol is an appropriate extraction method to detect the main PLs/ SPLs in patients' urine samples using TLC, as showed in Figure 2 for some of our diagnosed patients. (Additional information are provided as Supplementary Material (Table S1)



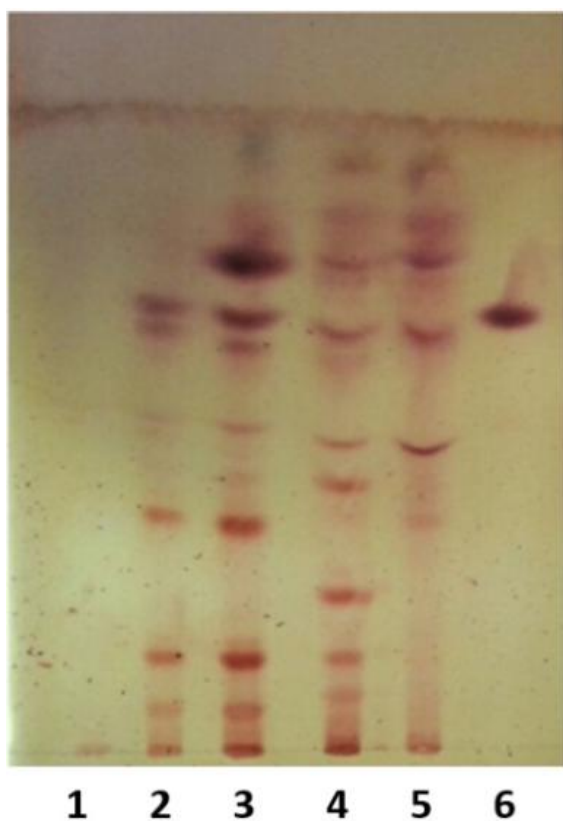
**Figure 2:** Thin layer chromatography (TLC) plates of urinary lipid extract showing the sphingolipids excretion from patients comparatively to commercial standards. Standards correspond to SM: sphingomyelin ( $R_f$  0.15); SUL: sulfatides (SGalCer,  $R_f$  0.59-0.62); GalCer: galactosylceramides ( $R_f$  0.79-0.82) and GlcCer: glucosylceramides ( $R_f$  0.77). Patients correspond to P1: patient with Niemann-Pick A/B disease; P2: patient with Gaucher disease type I; P3: patient with MLD; P4: patient with Gaucher disease type II, P5: patient with Fabry disease, P6: patient with Krabbe disease, N: healthy control. Compounds **1-3** corresponding to the bands in dotted boxes were attributed to **1**: Globotriaosylceramides (Gb3,  $R_f$  0.50), **2**: Sulfolactosylceramides (SLacCer,  $R_f$  0.53), and **3**: Lactosylceramides (LacCer;  $R_f$  0.64-0.67) according to the literature and LC-MS/MS validation. The amount of spotted extract was equivalent to 5-mg creatinine. Migration solvent: chloroform/methanol/water (72:28:3.5, v/v/v). Lipid fractions were visualized using orcinol.  $R_f$ : ratio to the front.

Although several sphingolipids are excreted simultaneously in some patients' urinary extracts, we focused on the main biomarkers detected in TLC (Bobillo Lobato, Jiménez Hidalgo and Jiménez Jiménez, 2016); (Mak and Cowan, 2021) and the patients' symptoms, to orient towards the enzymatic assay to be performed for the confirmation of the diagnosis. Indeed, Niemann-Pick A/B disease, characterized by a deficiency in the enzyme acid sphingomyelinase (ASM), was suspected for patients with urinary lipid extract containing sphingomyelin (Carsana *et al.*, 2022); (Mauhin *et al.*, 2022). The presence of glucosylceramides (GlcCer) led to conducting a  $\beta$ -glucocerebrosidase (GBA) assay to confirm Gaucher disease (Kushi, Rokukawa and Handa, 1988); (Mirzaian *et al.*, 2015); (Murugesan *et al.*, 2016). Interestingly, and as illustrated in Figure 2 for patients P2 and P4, the urinary lipid extracts obtained from the patients can display different profiles, notably in terms of chemical variability of Glycosylceramide compounds (Ishibashi, Kohyama-Koganeya and Hirabayashi, 2013). In the case of the patient P4, the excretion of the lactosylceramides (LacCer) was detected. Both Gaucher patients' TLC profiles also showed the presence of globotriaosylceramide (Gb3), considered as the major accumulated substrate in the cases of Fabry disease. However, both Gb3 and its lyso-form (Globotriaosylsphingosine, lysoGb3) were detected in patients suffering from Gaucher disease as reported by (Beasley *et al.*, 2020); (Kushi, Rokukawa and Handa, 1988). The identification of these latter compounds was done according to the order of elution of reference sphingolipids described in the literature (Berná *et al.*, 1999); (Hůlková *et al.*, 2005); (Kushi, Rokukawa and Handa, 1988); (Li *et al.*, 2011) and concurrent LC-MS/MS analysis of our patient's lipid extracts. Sulfatides (SGalCer) were key biomarkers for lipid extracts orienting to MLD, thus indicating the arylsulfatase A (ASA) assay (Berná *et al.*, 1999); (Colsch *et al.*, 2008). This class of compounds was clearly visible for patient P3 when compared to the commercial standard. TLC analysis performed in other studies indicated variations of the sulfatides level in urinary extract, but with no

correlation with the clinical form observed (Colsch *et al.*, 2008). In the case of patient P3, the sulfolactosylceramides (SLacCer) was also detected, this was previously highlighted by (Berná *et al.*, 1999). Concerning Fabry disease ( $\alpha$ -galactosidase A deficiency), the presence of Gb3 was used to guide the enzymatic assay (Jarne *et al.*, 2021);(Shiga *et al.*, 2021). Eventually, the realization of galactocerebrosidase (GALC) assay to confirm a Krabbe disease was performed when galactosylceramides (GalCer) were visible in TLC analysis, as observed in P6 patient profile and reported by (Bobillo Lobato, Jiménez Hidalgo and Jiménez Jiménez, 2016);(Wenger *et al.*, 2000).

In parallel, gangliosides, i.e., glycosphingolipids containing sialic acid, are a lipidic group that generally requires specialized extraction methods, and therefore constitutes a significant obstacle to the goal of obtaining a truly comprehensive analysis of all SPLs species present in a biological sample (Svennerholm and Fredman, 1980). To remedy this issue, we carried out a TLC characterization directly on the untreated urine sample. An illustration of the results obtained using TLC analysis is presented in Figure 3. In this case, the orientation through the enzyme assay to be performed is acquired based on the classification of the patients according to the obtained TLC profiles.





**Figure 3:** Thin layer chromatography (TLC) plates of urine samples. All the urine samples were deposited directly on TLC without pre-treatment and the chromatography was carried out with double migration. The amount of spotted extract was equivalent to 5-mg creatinine. Migration solvent: n-butanol/ glacial acetic acid/water (100:50:50, v/v/v). Lipid fractions were visualized using orcinol. Urine samples from known cases of GM1 and GM2 gangliosidoses (confirmation with enzymatic assays) were used for control/identification purposes. Lane 1: normal control; Lanes 2 and 3: patients with sialidosis type II; Lane 4: patient with GM1 gangliosidosis; Lane 5: patient with mucopolipidosis type III; Lane 6: lactose.

### **3.3. Efficiency of TLC analysis for diagnosis proposal**

In our hands, TLC analysis led to 38 abnormal profiles (36% of patients included in the study) for which characteristic biomarkers were identified, including the analysis of both urinary lipid extracts and untreated urine samples, and that allowed the proposition of an appropriate

enzymatic assay. For the remaining 64% of patients, further investigations are considered in consultation with clinicians and geneticists to acquire a confirmatory diagnosis (Table S1, Table 3).

The detailed information concerning the patients with TLC profiles displaying the lipidic species known to accumulate for each disorder, and cited in the literature as diagnosis markers, is presented in Table 4, as a subset of the data available in Table S1. Based on the major metabolite excreted in the patient's urine, a specific enzyme/protein assay was performed, and almost one-third of the total number of patients addressed to the laboratory thus benefited from this direct proposal of an oriented assay to diagnose their pathology. TLC profiles including biomarkers represented 27% of the analyses of group I patients (20 patients over 73); originally addressed to the laboratory to confirm a MLD diagnosis. These 20 patients were successfully diagnosed with sphingolipidoses, including 9 MLD cases, 1 Krabbe disease case, 2 sialidosis type II cases, and 3 GM1, and 5 GM2 gangliosidosis cases (3 with Tay saches disease and 2 with Sandhoff disease). Among the 27 patients with form II characteristics and which referred to the laboratory for confirmation of Gaucher disease and/or Niemann-Pick disease, 14 TLC profiles with biomarkers were obtained, thus allowing for a rapid diagnosis of 52% of the patients. Eventually, 5 patients were readily confirmed with Niemann-Pick A/B disease, and 9 with Gaucher disease. Among 7 patients with form III clinical features, 4 were rapidly oriented towards an  $\alpha$ -galactosidase enzymatic assay, and confirmed with Fabry disease.

Thus, the detection of different sphingolipidoses biomarkers is performed concomitantly within 24 hours in our laboratory making it possible to orient towards the adequate enzymatic assay to be carried out with high certainty, allowing us to rapidly start a treatment if available, and/or to benefit genetic counseling. In this study, the diagnosis was confirmed after a single

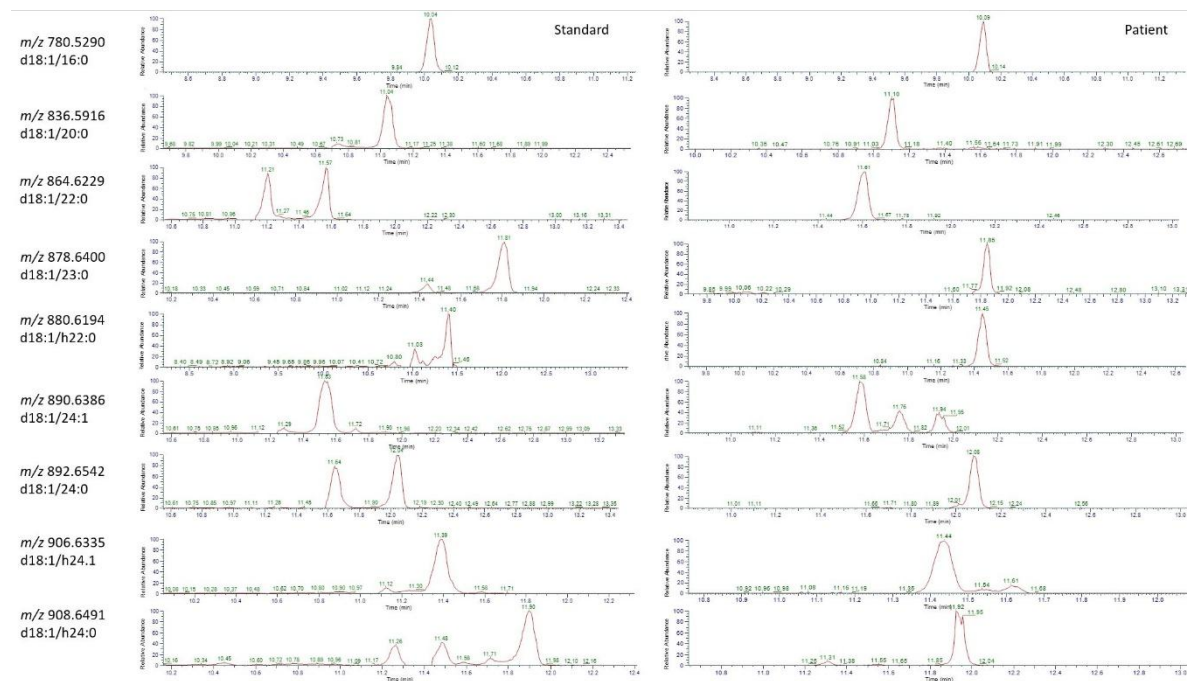
(35 cases) or a maximum of 2 (2 cases) enzymatic assay for overall 36% of the addressed patients.

### 3.4. Complementary UPLC-MS/MS analysis for the exploration of metabolic patterns

The main objective of this study was to implement an affordable laboratory strategy using TLC as a first step for the rapid diagnosis of sphingolipidoses. However, TLC can also be used to guide the setup of UPLC-MS/MS protocols and the data analysis for urinary lipids extracts, thus minimizing both cost and time dedicated to analysis, as this data treatment may be complex and time-consuming (Xuan *et al.*, 2018). The second objective was to have more precise information, making it possible to explore correlations between the diversity of the phenotypes presented by patients suffering from the same disease, and the diversity of sphingolipids excreted in their urine.

We report here as an example the LC MS-MS analysis performed on the urine extract of patient P3 with MLD based on his TLC profile (Figure 2). The search for sulfatides was performed based on the comparison with an analytical standard and its composition provided by the supplier as well as literature data. Nine compounds (Fig. 4 and Table 5) were annotated both in the standard and in the patient's extract, while analysing MS<sup>2</sup> spectra according to the fragmentation pattern described by (Pintado-Sierra *et al.*, 2017). In particular, MS<sup>2</sup> spectra obtained in positive mode displayed the characteristic peak at  $m/z$  264, indicating the presence of the 4-sphingenine (d18:1 sphingosine base) moiety (Blomqvist *et al.*, 2017); (Saville *et al.*, 2017). (Supplementary Material Fig. S1-S36). C16:0 ( $m/z$  [M+H]<sup>+</sup> 780) to C24:0 ( $m/z$  [M+H]<sup>+</sup> 892) sulfatides (according to the length of the fatty acyl chain) were annotated, as well as some hydroxylated species (C22-OH,  $m/z$  [M+H]<sup>+</sup> 880, C24:1-OH,  $m/z$  [M+H]<sup>+</sup> 906, and C24-OH,  $m/z$  [M+H]<sup>+</sup> 908). Seven compounds were characterized by a saturated acyl chain, whereas two species (C24-1 and C24:1-OH) had a double bond on the

fatty acid moiety. These species, based on a d18:1 sphingosine moiety, are common among mammals (Blomqvist *et al.*, 2021).



**Figure 4:** Extracted ion chromatograms of individual sulfatide compounds identified in the analytical standard (porcine brain, left) and in the lipid extract of a patient diagnosed with MLD (right). All sulfatides are annotated with the calculated parent  $[M+H]^+$  ion mass.

Interestingly, the isomeric profile varied for the sulfatides species between the standard and the patient's extract, revealing several sphingolipids isoforms (Fig. 5) that could be explored through UHPLC/MS/MS analysis, alongside with other sulfatides species. Thus, a thorough analysis of a large dataset of extracts would be useful to progress towards a better description of the chemical diversity of sulfatides among MLD patients (Piraud *et al.*, 2018). Indeed, most studies have been dedicated to a quantitative assessment of sulfatides in biological fluids, as a valuable tool for MLD diagnosis (Barcenas *et al.*, 2014); (Hong *et al.*, 2021); (Kubaski *et al.*,

2022);(Kuchař *et al.*, 2013);(Mirzaian, Kramer and Ben J H M Poorthuis, 2015);(Whitfield *et al.*, 2001). In terms of sulfatides diversity, up to thirty-seven species, with various chain length in the fatty acid moiety and different unsaturation as well as hydroxylation degrees, have indeed been annotated in mouse brain myelin samples using LC/MS/MS techniques, (Moyano *et al.*, 2014);(Pintado-Sierra *et al.*, 2017). However, to our knowledge, a maximum of 19 species was identified up to date in biological samples obtained from MLD patients, with inhomogeneous degrees of precision in terms of structural analysis ((Cao *et al.*, 2020); Saville *et al.*, 2017; Spacil *et al.*, 2016). In parallel, quantitative assays were also used to study correlations between the amount of sulfatides in biological samples and the phenotype of the disease (Colsch *et al.*, 2008);(Spacil *et al.*, 2016). Interestingly, urinary sulfatides can also be useful to monitor MLD patients, such as demonstrated by(Saville *et al.*, 2017). To our knowledge, no study had explored the correlation between the sulfatides chemodiversity and the disease phenotypes. Confirming this proposal could have an impact on the management of patients with MLD

## **Conclusion**

This study allowed us to develop a rapid diagnosis method based on the combination of the clinical symptoms and the detection of known biomarkers for several sphingolipidoses using TLC analysis of crude urine samples and urinary lipid extracts. From the 107 patients included in the study, 38 patients were thus directly oriented towards the adequate enzymatic assay, and benefited from a rapid diagnosis. The most represented pathologies were Gaucher disease, MLD, and Niemann-Pick disease. The use of TLC was also highlighted as a valuable tool allowing to faster and orient the selection of a specific UPLC-MS/MS method for lipids characterization. Altogether, the proposed pipeline proved to be useful in preventing invasive samplings from patients, reducing samples volumes as well as analysis cost and time. Moreover, the combined TLC – UPLC-MS/MS method could be of interest to progress

toward the diagnosis of other LSDs among the Moroccan population. This study thus underlines the importance of early and accurate diagnoses for appropriate preventive measures, including genetic counselling, and the need to explore chemical diversity and excretion metabolic patterns in sphingolipidoses.

## **Declarations**

**Availability of data and materials:** Not applicable

**Competing interests:** The authors declare that there are no competing interests associated with the manuscript.

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## **Author's contribution:**

M. Hammoud & N. Fdil designed and conceived the study, and contributed to the acquisition, analysis, and interpretation of data.

M. Hammoud, E. Houël and N. Fdil wrote the manuscript.

M. Hammoud, A. Rodrigues, E. Houël, D. Stien, N. Fdil, E. Sabir, I. Assiri, K. Lafhal & S. Najeh contributed to acquisition, analysis, and interpretation of data.

N. Imad, A. Bourrahouat, I. Ait Sab, R. Elqadiry, H. Nassih, A. Outzourit, M. Elamiri, F. Maoulainine, N. Slitine, F. Bennaoui, M. Bourouss, A. Rodrigues, D. Stien, S. Mrhar, L. Essaadouni, N. Rada, M. Bouskraoui contributed to the interpretation of data and critically revised the manuscript.

All authors read and approved the manuscript.

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