Implementation of an Affordable Method for MPS Diagnosis from Urine Screening to Enzymatic Confirmation: Results of a Pilot Study in Morocco


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SUMMARY

Background: Rapid and accurate diagnosis of mucopolysaccharidoses (MPS) is still a challenge due to poor access to screening and diagnostic methods and to their extensive clinical heterogeneity. The aim of this work is to perform laboratory biochemical testing for confirming the diagnosis of mucopolysaccharidosis (MPS) for the first time in Morocco.

Methods: Over a period of twelve months, 88 patients suspected of having Mucopolysaccharidosis (MPS) were referred to our laboratory. Quantitative and qualitative urine glycosaminoglycan (GAG) analyses were performed, and enzyme activity was assayed on dried blood spots (DBS) using fluorogenic substrates. Enzyme activity was measured as normal, low, or undetectable.

Results: Of the 88 patients studied, 26 were confirmed to have MPS; 19 MPS I (Hurler syndrome; OMIM #607014/Hurler-Scheie syndrome; OMIM #607015), 2 MPS II (Hunter syndrome; OMIM #309900), 2 MPS IIIA (Sanfilippo syndrome; OMIM #252900), 1 MPS IIIB (Sanfilippo syndrome; OMIM #252920) and 2 MPS VI (Maroteaux-Lamy syndrome; OMIM #253200). Parental consanguinity was present in 80.76% of cases. Quantitative urinary glycosaminoglycan (uGAGs) assays showed abnormal profiles in 31 cases, and further quantitative urinary GAG evaluation and Thin Layer Chromatography (TLC) provided important additional information about the likely MPS diagnosis. The final diagnosis was confirmed by specific enzyme activity analysis in the DBS samples.

Conclusions: The present study shows that the adoption of combined urinary substrate analysis and enzyme assays using dried blood spots can facilitate such diagnosis, offer an important tool for an appropriate supporting care, and a specific therapy, when available.


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List of abbreviations
4-MU - 4-methylumbelliferone
CPC - cetylpyridinium chloride
CS - chondroitine sulfatase
DBS - dried blood spot
DMB - dimethyl methylene blue
DS - dermatane sulfate
ENT - ear, nose and throat
ERT - enzyme replacement therapy
GAG - glycosaminoglycan
HS - heparane sulfate
HSCT - hematopoietic stem cell transplantation
KS - keratan sulfate
LSD - lysosomal storage disease
MPS - mucopolysaccharidosis
NBS - newborn screening
TLC - thin layer chromatography
UGAGs - urinary glycosaminoglycans

Introduction
High consanguinity, difficult access to accurate diagnostic tests, and costly therapies are all significant contributors to the lysosomal disease burden. The adoption of combined urinary procedure and enzyme assays using dried blood spot can facilitate earlier diagnosis and consequent earlier consideration of therapeutic options and earlier counseling to affected families. The Mucopolysaccharidosis (MPS) is a group of metabolic disorders characterized by a deficiency in one of the lysosomal enzymes necessary for the catabolism of glycosaminoglycans (GAGs), formerly known as mucopolysaccharides [1]. Deficiency of these enzymes leads to a widespread accumulation of undegraded or partially degraded GAGs in different tissues and to excessive excretion of these substances in urine [2]. The MPS subtypes have multi-systemic clinical manifestations of variable severity. In Morocco, obtaining an early clinical MPS diagnosis has historically been a challenge and patients have not been diagnosed until more advanced stages of the disease. Early diagnosis is of paramount importance for an optimum management of inborn errors and a better prognosis. There is no national protocol for newborn screening of inborn errors of metabolism in Morocco. Affordable, easy and accurate testing of suspected patients is therefore fundamental to diagnosis and management.

Materials and Methods
This study included 88 Moroccan patients suspected of MPS who were referred to the Biochemical Laboratory in the Marrakech Faculty of Medicine by the pediatric departments of Mohamed VI University Hospital. The patient ages ranged from 9 months to 15.6 years (median, 8 years). Most of the patients referred were from consanguineous families (74%). The first step in our laboratory diagnosis of these patients suspected of having an MPS was the quantitative and qualitative analysis of urinary glycosaminoglycans (uGAGs) [3,4]. An early morning urine sample (10 - 20 mL) was collected from each patient for the determination of GAGs using semi-quantitative, quantitative, and qualitative assays. Deficient enzyme activity testing was done using 60 - 70 µL of whole blood spotted on filter paper (Whatman-GE no. 903) and dried for 4 hours at room temperature. Filter papers were stored at 4°C in plastic containers. This algorithm for patient sample analysis is shown in Figure 1.

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 consent was obtained from patients and/or their parents or guardians.

Laboratory analysis of urinary glycosaminoglycans
The GAG assay was the usual first step in making our biochemical diagnosis [5,6]. uGAGs remain stable at room temperature for up to 10 days, so urine samples do not need to be frozen for transport to the laboratory [7]. Both semi-quantitative (the GAG test) and quantitative, using dimethyl methylene blue (DMB), assays were used.

Dimethyl methylene blue solution
DMB solution was prepared according to the method described by Andrade et al. [7]. In a volumetric flask of 1 L, 10.66 mg of DMB (research grade, purchased from Sigma-Aldrich, CAS: 931418-92-7) was added, with 3.33 mL of ethanol (purchased from Merck, CAS: 64-17-5), 1.33 g of sodium formate (purchased from Merck, CAS: 141-53-7), and 1.33 mL of formic acid (purchased from Sigma-Aldrich, CAS: 64-18-6). The pH value was adjusted to 3.75 by adding more concentrated acid or solid formate. The flask was filled up to 1 L with distilled water. This solution has proven to be stable for at least 10 months at room temperature. A chondroitin 6-sulfate solution was prepared at 100 mg/L in distilled water. The solution is stable for at least one year at 4°C, when stored in the dark.

Semi quantitative and quantitative urinary glycosaminoglycans determination
The GAG test was performed using a colorimetric method based on the color change produced by the complexes formed between DMB and GAGs [8,9]. The
DMB solution was transferred to transparent vials (2 mL), to which 50 µL of a centrifuged first morning urine was added. A high urine concentration of GAGs, which occurred in patients with MPS I, II and VI, was considered as a positive result (blue color changed to purple). A negative result was confirmed if the urinary GAG concentration was low (no color change). Finally, if the color of the GAG-tests changed to violet, it was considered as an inconclusive result.

The quantitative determination of urinary GAGs was performed on all patient samples, even if a negative reaction was observed in the first GAG test. It was performed using ultraviolet-visible spectrophotometry. For the calibration curve, we constructed a series of dilutions from a solution of chondroitin 6-sulphate: (100 mg/mL of water) by diluting 5, 10, 25, 50, and 100 µL in water to obtain a final volume of 500 µL. With the patient samples, 100 µL of urine were diluted in water to obtain a final volume of 500 µL. After the dilution, we added 2.5 mL of DMB. The readings were taken using the differences between the absorbance at 520 and 600 nm [7,9]. Spectrophotometric measurements were recorded on a UV-visible 1601 Shimadzu spectrophotometer (Shimadzu Co., Kyoto, Japan). If a particular urine sample was too diluted or concentrated, the volume of urine was increased or reduced, taking into account the appropriate dilution factor. Finally, the GAG concentration was calculated on the basis of creatinine values, determined by a automated chemistry analyzer using the Jaffe method. The final GAG results were expressed in mg/mmol creatinine. To avoid erroneous results in the concentration of GAGs, all urine samples with a creatinine concentration of less than 20 mg/dL or more than 200 mg/dL were recollected.

Since the method of measuring total GAGs in urine is incapable of distinguishing between the different types of GAGs excreted and considering that several studies have reported that patients with MPS III, IV, and VII may have comparatively lower levels of GAGs, resulting in a false-negative result [4,10,11], a qualitative analysis of urinary GAGs was needed for the group and in a false-negative result [4,10,11], a qualitative assay may have comparatively lower levels of GAGs, resulting in a false-negative result [4,10,11].

**Thin layer chromatography GAG characterization**

The TLC conditions described by Humbel [13] were modified as follows: dried GAG pellets were dissolved in 20 µL phenol red solution 0.05% (purchased from Sigma-Aldrich, CAS: 143-74-8).

If the amount of precipitated GAGs was too elevated, the volume of 30 or 40 µL phenol red solution was added; 8 µL was applied on a TLC cellulose glass plates (20 x 20 cm, purchased from Merck 1.05716.0001). Starting with the origin, every 2 cm, six lines were drawn with a soft pencil. These indicated the borders for each solvent system; six TLC developing tanks were used. The samples were spotted on the TLC plate over a length of 1.5 cm, at 0.5 cm from each sample and approximately 2 cm from the lower edge and at least 1.3 cm from the sides. Standards were prepared: 10 µL of GAG standards, purchased from Sigma-Aldrich: Dermatan sulfate (DS, CAS: 54328-33-5), Heparan sulfate (HS, CAS: 57459-72-0), Chondroitin sulfate (CS4, CAS: 39455-18-0 and C6S, CAS: 9082-07-9), and Keratan sulfate (purchased from Glycofinechem, CAS: 9056-36-4) at 1 mg/mL in 0.05 M Sodium hydroxide (NaOH) and extracted GAGs were dissolved in 20 µL of red phenol solution (precipitated GAGs obtained as described above). Solvents were prepared just before the chromatography following the instructions given in Table 1.

The plate was subsequently placed into six different tanks, which contained decreasing amounts of ethanol. One complete run required approximately 7 hours. The plate was allowed to dry overnight. Chromatograms were stained with 0.1% toluidine blue (purchased from Sigma-Aldrich, CAS: 92-31-9) in 70% ethanol/acetic acid (95:5) and destained with 1% acetic acid solution (purchased from Sigma-Aldrich, CAS: 64-19-7). The plate was allowed to dry overnight.

**Enzyme activity assays**

Enzyme activity assays are the gold standard for diagnosis confirmation and determination of the MPS types and subtypes. The diagnosis is based on specific enzymatic assays performed in plasma, leukocytes or fibroblasts. Recently, DBS samples have been used for these assays [14]. This approach offers several advantages, including a simple and expedited sample collection, minimal invasiveness, reduced sample volume, easy sample handling, storage for extended periods of time.

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and the possibility of using DBS samples for assaying the activity of any lysosomal enzyme. Fluorometric methods which had already been validated [15,16], were completed for the evaluation of α-iduronidase (MPS I) and arylsulfatase B (MPS VI). To evaluate iduronate-2-sulfatase (MPS II), we adapted the method of Voznyi et al. [17] as follows:

**Iduronidase assay in DBS samples (MPS I)**

Twenty microliters of sodium formate buffer (50 mmol/L, pH 2.8) containing 0.3 µg/L of D-saccharic acid-1,4-lactone (purchased from Sigma-Aldrich, CAS: 61278-30-6) and 20 µL of water as elution liquid and 20 µL of 2 mmol/L 4-methylumbelliferyl α-L-idopyranosiduronic acid, sodium salt (purchased from Toronto Research Chemicals INC, CAS: 89157-94-8) in distilled water as the substrate, were added to microtiter plates, together with a 3 mm-diameter punched circle from DBS (3.6 µL of blood). The microtiter plates were incubated for 20 hours at 37°C using an agitator (Heidolph Instruments GmbH & Co. KG, Germany) at 600 rpm. The microtiter plates were allowed to stand for 30 minutes at room temperature, and 300 µL of ethylenediaminetetra-acetic acid 0.13 mol/L, pH 11.3 (purchased from Sigma-Aldrich, CAS: 107-15-3) was added to stop the reaction. Blanks were treated as described above but the substrate solution and stop solution were added after incubation. Finally, each sample was transferred into a cuvette and completed with a stop solution up to 2 mL. Fluorescence (excitation: 365 nm; emission: 450 nm, cuvette volume: 1 mL) of the enzyme product 4-MU was measured with a Jasco FP-750 spectrofluorometer. The fluorescence readings were corrected for blanks, and the results were compared with the fluorescence from a calibrator. A calibration curve was prepared using different dilutions of 4-MU standards (0 - 500 µmol/L). Enzymatic activity was expressed as micromoles of substrate hydrolyzed per liter of blood per hour (µmol/L*h).

**Iduronate sulfatase assay in DBS samples (MPS II)**

To 1.5 mL test tubes containing 3 mm diameter punched circle (3.6 µL of blood), we added 50 µL of bovine serum albumin (BSA, purchased from Sigma-Aldrich, CAS: 9048-46-8) 0.2% as the elution liquid. After gentle mixing for 30 minutes at 37°C, test tubes were centrifuged at 2,000 rpm for 10 minutes at room temperature. In microtiter plates, we introduced 10 µL of supernatant and we added 20 µL of 1.25 mmol/L 4-methylumbelliferyl α-L-idopyranosiduronic acid 2-sulfate disodium salt (purchased from Toronto Research Chemicals INC, CAS: 1045020-74-3) previously dissolved in sodium acetate buffer (purchased from LOBA Chemie, CAS: 127-09-3) (0.1 mol/L, pH 5.0). The microtiter plates were incubated for 24 hours at 37°C using an agitator at 600 rpm. After the incubation, we added 40 µL of phosphate citrate buffer (sodium phosphate dibasic anhydrous, purchased from Sigma-Aldrich, CAS: 7558-79-4, and citric acid monohydrate, purchased from Ridel- de Haen, CAS: 5949-29-1) (0.2 mol/L, pH 4.5) and 10 µL of purified α-iduronidase from bovine testis (LEBT M2 purchased from Moscerdam substrates). The microtiter plates were incubated a second time for 24 hours at 37°C using an agitator at 600 rpm. The microtiter plates were allowed to stand for 30 minutes at room temperature, and 200 µL of carbonate-bicarbonate buffer sodium (carbonate, purchased from Reacting, REF: 3565, and sodium bicarbonate, purchased from Sigma-Aldrich, CAS: 144-55-8) (0.5 mol/L, pH 10.5) were added to stop the reaction. Blanks were treated as described above but the substrate solution and stop solution were added at the end of second incubation. Finally, each sample was transferred into a cuvette and completed with a stop solution up to 2 mL. Fluorescence was read at excitation 360 nm and emission at 450 nm for the enzyme product 4-MU.

**Arylsulfatase B assay in DBS samples (MPS VI)**

A 2-mm diameter filter paper was punched (2 µL of blood) with a standard paper punch and 45 µL of distilled water and 30 µL of 15 mmol/L lead acetate in sodium acetate buffer (50 mmol/L, pH 5.0) were added as the elution liquid and 75 µL of 10 mmol/L 4-methylumbelliferyl-sulfate (purchased from Sigma-Aldrich, CAS: 15220-11-8) in sodium acetate buffer (0.05 M, pH 5.0) as the substrate. The microtiter plates were incubated for 6 hours at 37°C using an agitator at 600 rpm. The microtiter plates were allowed to stand for 30 minutes at room temperature and 150 µL of glycine-sodium hydroxide buffer (glycine, purchased from Sigma-Aldrich, CAS: 56-40-6 and sodium hydroxide, purchased from Panreac, CE: 215-185-5) (85 mmol/L, pH 10.5) was added to stop the reaction. Blanks were treated as described above but the substrate solution and stop solution were added after incubation. Finally, each sample was transferred into a cuvette and completed with a stop solution up to 2 mL. Fluorescence (excitation: 365 nm; emission: 450 nm, cuvette volume: 1 mL) of the enzyme product 4-MU was measured with a Jasco FP-750 spectrofluorometer.

As recommended, when arylsulfatase B or iduronate sulfatase activity was found to be low, a second sulfatase (iduronate sulfatase or arylsulfatase B) was measured to rule out multiple sulfatase deficiencies.

The specific enzyme essays for patients with Sanfilippo syndrome and for those suspected of Morquio syndrome were performed in referenced laboratories abroad since enzyme assays for these types are not available in our laboratory.

**RESULTS**

Analysis of 88 urine samples yielded 26 positive GAG test results, 5 inconclusive results, and 57 negative results. All patients with a negative GAG test showed quantitative GAG values within the normal range. Of
those with inconclusive GAG tests, 1 of 5 showed quantitative GAG values slightly elevated, whereas all of those with positive GAG tests had an increased GAG concentration in urine. TLC was carried out for all patients with total GAG levels strongly or slightly elevated, all those patients suspected of MPS IV, even with a low urinary excretion of GAG (3 cases), and those patients exhibiting symptoms consistent with mucopolysaccharidosis, including the microcephaly and several glycoprotein storage disorders.

All patients with increased GAG concentrations had a pathological profile on TLC corresponding to different GAGs excreted and were referred to a specific enzyme diagnosis. Measurement of enzyme activity in DBS was performed in those patients who had a specific pathologic chromatographic pattern (n = 26) and those who were suspected clinically to have MPS IV (n = 3) even with normal levels of total urinary GAGs and no specific pattern on TLC. A summary of quantitative GAG analysis in all patients confirmed to have an MPS and their subsequent enzyme confirmations are shown in Table 2. Twenty-six patients from nineteen families were diagnosed with MPS I, II, III, and VI. The percentage of consanguineous marriage among confirmed MPS cases was 80.76%. All patients were diagnosed after the age of 3 years except for two patients (P18, P25), for whom the diagnosis was made at 9 months and 17 months following the confirmation of a sister’s diagnosis. The common disease-related symptoms in patients diagnosed with MPS I, II, and VI were short stature, multiple joint contractures, inguinal/umbilical hernias, hepatomegaly, mild coarse facial features, and hypertrichosis. For patients clinically suspected of MPS IV, enzyme activity testing of N-acetylgalactosamine-6-sulfatase and β-galactosidase activities, essential in diagnosing MPS IVA and IVB, respectively, was performed to rule out this diagnosis, based initially on clinical symptoms.

In one case with GAG levels slightly elevated, TLC analysis showed strange spots not compatible with any of the MPS types (Figure 2, lane 5). As GAGs are present also in bones and cartilages, their levels can be elevated in other diseases affecting these tissues, such as mucolipidosis [18]. A diagnosis of mucolipidosis III was confirmed based on clinical and radiological findings, and elevated activity of lysosomal enzymes (measured on DBS sample) including α-iduronidase and iduronate-2-sulfatase. Among patients with inconclusive GAG test, three patients with GAG levels within normal ranges presented clinical similarities with diagnosed mucolipidosis III patient: short stature, multiple joint contractures, mild coarse facial features, and developmental delay. We performed a TLC analysis and found the same abnormal patterns (Figure 2, Lane 6). In the last infant with inconclusive GAG test, somatic features and GAG levels within normal ranges, a strong band was seen on TLC analysis, the position of which could not be confused with other MPS or mucolipidosis disorders (Figure 2, Lane 7). She is under investigation at the time of writing.

**DISCUSSION**

There is a growing body of evidence that early initiation of disease-specific therapy in MPS I, II, and VI leads to a substantial modification of the natural course of the disease. To achieve these goals, we are the first Moroccan/North African laboratory to invest in the diagnosis of mucopolysaccharidosis by enzymatic essays using dried blood spots. We proceeded by a simple, inexpensive, and practical first line combined procedure. This approach was derived from a combination of pre-existing methods such as GAG tests, quantitative urinary
Table 2. Clinical and biochemical characteristics of the Patients diagnosed with MPS.

<table>
<thead>
<tr>
<th>Family/patient</th>
<th>Diagnostic age (years)</th>
<th>Gender</th>
<th>Consanguinity</th>
<th>Pheno-type</th>
<th>Urinary GAG (mg/mmol creatinine)</th>
<th>Urinary GAG normal ranges (mg/mmol creatinine</th>
<th>Enzyme deficiency</th>
<th>Enzymatic Activity</th>
<th>Enzyme activities normal ranges in DBS</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1/ P1</td>
<td>11.2</td>
<td>F</td>
<td>+</td>
<td>HS</td>
<td>90.90</td>
<td>3.3 - 13.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F1/ P2</td>
<td>12.3</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>75.80</td>
<td>3.3 - 13.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F2/ P3</td>
<td>3.1</td>
<td>M</td>
<td>+</td>
<td>IH</td>
<td>117.83</td>
<td>9.5 - 25.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.0 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F2/ P4</td>
<td>7.4</td>
<td>F</td>
<td>+</td>
<td>IH</td>
<td>117.12</td>
<td>6.7 - 15.5</td>
<td>Alpha-L-iduronidase</td>
<td>0.0 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F3/ P5</td>
<td>15.5</td>
<td>F</td>
<td>+</td>
<td>IH</td>
<td>102.68</td>
<td>3.3 - 13.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS I</td>
</tr>
<tr>
<td>F4/ P6</td>
<td>8.3</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>75.80</td>
<td>6.7 - 15.5</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS I</td>
</tr>
<tr>
<td>F5/ P6</td>
<td>5.8</td>
<td>F</td>
<td>+</td>
<td>HS</td>
<td>96.91</td>
<td>7.9 - 16.2</td>
<td>Alpha-L-iduronidase</td>
<td>0.2 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F6/ P6</td>
<td>7.1</td>
<td>F</td>
<td>-</td>
<td>HS</td>
<td>95.68</td>
<td>6.7 - 15.5</td>
<td>Alpha-L-iduronidase</td>
<td>0.0 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F6/ P6</td>
<td>10.8</td>
<td>M</td>
<td>-</td>
<td>HS</td>
<td>95.05</td>
<td>3.3 - 13.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.0 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F7/ P10</td>
<td>8.7</td>
<td>F</td>
<td>+</td>
<td>HS</td>
<td>56.70</td>
<td>6.7 - 15.5</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS I</td>
</tr>
<tr>
<td>F5/ P11</td>
<td>9.3</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>88.94</td>
<td>6.7 - 15.5</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F8/ P12</td>
<td>7.6</td>
<td>M</td>
<td>-</td>
<td>HS</td>
<td>75.88</td>
<td>6.7 - 15.5</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F9/ P13</td>
<td>4.9</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>68.33</td>
<td>7.9 - 16.2</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F10/ P14</td>
<td>7.1</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>60.51</td>
<td>6.7 - 15.5</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
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<tr>
<td>F11/ P15</td>
<td>10.8</td>
<td>M</td>
<td>-</td>
<td>HS</td>
<td>59.15</td>
<td>3.3 - 13.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
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<tr>
<td>F12/ P16</td>
<td>10.9</td>
<td>F</td>
<td>-</td>
<td>HS</td>
<td>71.89</td>
<td>3.3 - 13.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
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<tr>
<td>F13/ P17</td>
<td>6.9</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>162.05</td>
<td>6.7 - 15.5</td>
<td>Iduronate-2-sulfatase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-II</td>
</tr>
<tr>
<td>F14/ P18</td>
<td>0.9</td>
<td>F</td>
<td>+</td>
<td>HS</td>
<td>115.86</td>
<td>13.3 - 36.3</td>
<td>Heparan-N-sulfatase</td>
<td>0.2 (nmol/mg/17h)</td>
<td>-</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F14/ P19</td>
<td>8.6</td>
<td>F</td>
<td>+</td>
<td>HS</td>
<td>69.05</td>
<td>6.7 - 15.5</td>
<td>Heparan-N-sulfatase</td>
<td>0.4 (nmol/mg/17h)</td>
<td>-</td>
<td>MPS-IIA</td>
</tr>
<tr>
<td>F15/ P20</td>
<td>4.9</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>79.95</td>
<td>7.9 - 16.2</td>
<td>N-acetylglucosami</td>
<td>0.0 (nmol/mg/17h)</td>
<td>-</td>
<td>MPS-IIIB</td>
</tr>
<tr>
<td>F16/ P21</td>
<td>15.5</td>
<td>F</td>
<td>+</td>
<td>HS</td>
<td>72.08</td>
<td>3.3 - 13.7</td>
<td>Arylsulfatase B</td>
<td>1.82 (µmol/L*h)</td>
<td>12 - 30</td>
<td>MPS-VI</td>
</tr>
<tr>
<td>F17/ P22</td>
<td>9.3</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>96.83</td>
<td>6.7 - 15.5</td>
<td>Arylsulfatase B</td>
<td>0.46 (µmol/L*h)</td>
<td>12 - 30</td>
<td>MPS-VI</td>
</tr>
<tr>
<td>F18/ P23</td>
<td>14.8</td>
<td>F</td>
<td>+</td>
<td>IH</td>
<td>77.50</td>
<td>3.3 - 13.7</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F18/ P24</td>
<td>5.7</td>
<td>F</td>
<td>+</td>
<td>IH</td>
<td>111.90</td>
<td>7.9 - 16.2</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F18/ P25</td>
<td>1.5</td>
<td>M</td>
<td>+</td>
<td>IH</td>
<td>155.80</td>
<td>8.1 - 35.</td>
<td>Alpha-L-iduronidase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-I</td>
</tr>
<tr>
<td>F19/ P26</td>
<td>8.4</td>
<td>M</td>
<td>+</td>
<td>HS</td>
<td>78.00</td>
<td>6.7 - 15.5</td>
<td>Iduronate-2-sulfatase</td>
<td>0.1 (µmol/L*h)</td>
<td>2.1 - 11.7</td>
<td>MPS-II</td>
</tr>
</tbody>
</table>

* - in MPSI disorder, ^ - reference values are age-dependent, † - performed at outside laboratory, enzymatic essays were performed in leucocytes, normal ranges are 10 - 45 nmol/mL/h, uGAGs - urinary Glycosaminoglycan, MPS - Mucopolysaccharidosis, + - Present, - - Absent, M - Male, F - Female.
Biochemical and Molecular Characterization of Patients with Mucopolysaccharidosis in Morocco

Figure 1. Flow-chart for our laboratory diagnosis of MPS.

DBS - Dried blood Spot, DMB - Dimethyl methylene blue, GAGs - Glycosaminoglycans, LSDs - Lysosomal storage diseases, TLC - Thin layer chromatography.

Figure 2. Thin layer chromatography of standards GAGs and GAGs isolated from MPS diagnosed patients and healthy control.

Urinary GAGs were isolated and chromatographed as described. Lane 1: QC, quality control (DS - dermatan sulfate, HS - heparan sulfate, C4S - chondroitin 4 sulfate, C6S - chondroitin 6 sulfate, and KS - keratan sulfate. Lane 2: patient with Hurler syndrome (MPSI). Lane 3: patient with Maroteaux-Lamy syndrome (MPS VI). Lane 4: patient with Hunter syndrome (MPSII). Lane 5: patient with Sanfilippo syndrome (MPS IIIB). Lane 6: healthy control. Lane 7: patient with moliclipidosis III. Lane 8: patient on diagnosis with somatic features.
GAG evaluation, and TLC characterization modified and simplified to lead us to a specific enzyme activity assay using DBS samples. The use of GAG tests was useful for a quick screening of MPS among those patients suspected of suffering from MPS (III/HS, II, III, or VI). The test enabled us to make a quick detection, confirmation, or exclusion of an MPS diagnosis, thus reducing the number of individuals requiring future testing. This proved to be very helpful especially in rural areas of the country where consanguineous marriage is frequent.

We used simultaneous quantitative and qualitative methods to reduce the chances of a false-negative result. The quantitative data were also used to normalize GAGs on TLC plates by adjusting for individual variation in excretion. This ensured uniform chromatographic behavior and facilitated estimation of the relative abundance of specific GAGs. The TLC analysis method allows the identification of an abnormal GAG pattern in all MPS and mucolipidosi patients and can help in the diagnosis of several other oligosaccharidoses based on abnormal patterns detected. It was not possible to distinguish MPS I from MPS II, on the basis of urinary GAG. Measuring both the iduronidase and iduronate-2-sulfatase enzyme activities was therefore required to make a proper diagnosis. Since MPS I is the more common type in the North African populations [19,20], the iduronidase was assayed first. Maroteaux-Lamy syndrome (MPS VI) patients had a TLC pattern easily distinguished from MPS I and II with excretion of a large excess of DS and presence or absence of trace amounts of HS. In MPS III patients, a straightforward banding pattern was observed corresponding to HS, and two bands were observed in mucolipidosis III. Furthermore, it should also be emphasized that the TLC pattern provided a diagnostic orientation of the different types of MPS and/or mucolipidosis III/II, which directed us to focus on patients having symptoms consistent with mucopolysaccharidosis diseases and exhibiting characteristics of oligosaccharidosis abnormal patterns on TLC analysis.

Definitive diagnosis of inherited lysosomal storage diseases is based on specific enzymatic assays performed on plasma, leukocytes, fibroblasts, and lately, dried blood spot samples. We confirmed the diagnosis of patients affected by several mucopolysaccharidosis types using DBS samples for appropriate enzyme assays. This method offers several advantages, including a simple and expedited sample collection, minimal invasiveness, reduced sample volume and easy sample handling and storage for extended periods. DBS samples can be used for assaying the activity of other lysosomal enzymes to reach a diagnosis.

Clinical signs of different mucopolysaccharidosis types are variable from one disorder to another, but also in terms of the severity between patients suffering from the same MPS disorder. The total GAG levels in urine seem to be proportional to the severity of the symptoms. Indeed, among MPS I patients, three patients (P4, P12, and P14) who shared facial dysmorphism, joint stiffness, hepatomegaly, umbilical hernia symptoms, and ear, nose, and throat (ENT) infections, presented different clinical symptoms that could be related to the levels of GAG. Patient P4 (GAG 117.12) had severe central and peripheral nervous system involvement, cardiac symptomatology, and hearing loss. Patient P12 (GAG 75, 88) had mild cardiac symptomatology, and patient P14 (GAG 60, 51) showed less severe symptoms. We performed enzyme activity assays on 3 patients clinically suspected with MPS IV, even though uGAG levels were normal, in order to avoid false-negatives. These three patients exhibited normal cognitive function and presented with chest deformity, short stature, and growth retardation. Both N-acetylgalactosamine-6-sulfatase and B-galactosidase were within normal ranges, and the diagnosis was excluded. These findings taught us to rely on the clinical presentation that should generate clinical suspicion of MPS IV like joint hypermobility (of the wrist in particular) as it is unique to MPS IV, kyphosis/gibbus, genu valgum, and shortness of breath [21].

In this study, we have used a targeted screening program for children suspected of having MPS in our university hospital. Our pilot study results allow us to submit proposals to implement the first national newborn screening (NBS) for MPS I. The key factors in recommending MPS I for inclusion in NBS are the strongly improved efficacy of early-onset therapy with HSCT/ERT, or ERT, making early diagnosis beneficial for the patient and society.

Several limitations, however, should be acknowledged. First, the patients diagnosed in this study do not represent the distribution of MPS in the Moroccan population. Second, DMB-based spectrophotometry can give rise to false negatives, especially in MPS III and IV [4]. In addition, in some cases; high levels of GAG may be due to other pathologies, such as diabetes, lupus, arthritis, or mucolipidosis [18]. Finally, the method requires an investment of time (at least 4 days of handling are required to obtain a final confirmation) and an experienced manipulator.

**CONCLUSION**

An early and accurate diagnosis of MPS is critical not only in permitting disease-specific genetic counseling but also in guiding appropriate national supportive care and disease-specific therapy. This study is the first work diagnosing MPS in Morocco based on enzymatic activity assays in DBS specimens. This method, which is efficient in diagnosing MPS subtypes, will offer several advantages to our laboratory in achieving its goal of becoming a reference center for the diagnosis of MPS in North Africa.
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