Dried Blood Spots Allow Targeted Screening to Diagnose Mucopolysaccharidosis and Mucopolipidosis

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Abstract *Background:* As patients with different types of mucopolysaccharidosis (MPS) and mucopolipidosis (ML) may present with overlapping clinical features – including coarse face, hepatosplenomegaly, bone dysplasia and claw-hand deformities, collectively also called ‘MPS-like phenotype’, enzymatic and/or molecular genetic analyses are indispensable for accurate diagnosis and applying specific therapy. In this prospective study, we screened patients with symptoms compatible with MPS for MPS I, II (males) and VI.

Methods: Dried blood spots/specimens (DBS) were collected from 200 patients with an MPS-like phenotype and analysed for activities of α -iduronidase (IDUA), iduronate-2-sulphatase (IDS), and arylsulphatase B (ARSB), the enzymes deficient in mucopolysaccharidosis (MPS) type I, II and VI, respectively. For the samples with pathologic enzyme activity, mutational analysis was carried out using the same DBS.

Results: Based on enzymatic analysis of 200 DBS samples, a total of 45 (22.5%) showed low activity; 17 for MPS I (8.5%), 11 for MPS II (5.5%) and 9 for MPS VI (4.5%). Enzyme activities were suggestive for ML II/III in

8 (4.0%) cases. For 41 (91.1%) samples, DNA could be extracted from the filter paper. Mutations were identified in 11 (64.7%), 11 (100%), 9 (100%) and 5 (62.5%) patients putatively diagnosed biochemically with MPS I, II, VI, and ML II/III, respectively.

Conclusions: DBS enzymatic analysis can be used to diagnose MPS/ML. Initial results should be confirmed by a second enzyme assay and/or by molecular genetic testing. Given the advantages of DBS over other sample types in terms of ease of collection, storage and transportation, DBS are particularly useful for screening patients with an MPS-like phenotype in regions lacking specialised laboratories. In order to ascertain the diagnosis in a large number of cases, patients should be assessed in parallel for at least MPS I, II and VI.

Introduction

Mucopolysaccharidoses (MPS) represent a group of rare inborn lysosomal storage disorders (LSDs), with an incidence of 0.05–1.89 per 100,000 live births (for recent review, see Muenzer 2011). To date, 7 different MPS types have been identified, and some of them have been subdivided into several subtypes. Each (sub)type is characterised by the deficiency of a specific lysosomal enzyme involved in the degradation of glycosaminoglycans (GAGs). Loss of enzyme activity results in accumulation of incompletely degraded GAGs in lysosomes and progressive cellular damage as well as dysfunction of multiple tissues and organs (Muenzer 2011). Patients typically appear normal at birth, but gradually develop a plethora of rather unspecific clinical manifestations, which vary according to the severity of the disease. All MPS types but type II, that is X-chromosomal, are inherited in an autosomal recessive manner. Despite the biochemical and

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genetic heterogeneity, there is a significant overlap in the clinical presentation among the different MPS types. For instance, MPS I, II and VI share many clinical features called ‘MPS-like’ phenotype. This phenotype includes, among others, coarse facial features, hepatosplenomegaly, joint and skeletal dysplasia (dysostosis multiplex, dwarfism, claw-like hand), cardiorespiratory problems, and vision and hearing impairment (Chamoles et al. 2001b; Muenzer 2011). In addition, patients with mucopolidosis (ML) II (I-cell disease) and III (pseudo-Hurler polydystrophy) (Van Hoof 1974) may also show a phenotype similar to that of patients with MPS. The latter disorders are characterised by the deficiency of one of the enzymes that play a role in generating the mannose-6-phosphate recognition marker on lysosomal enzymes. Consequently, missorting of multiple lysosomal enzymes leads to a general lysosomal dysfunction (Kollmann et al. 2010). Given these overlapping features between MPS and ML types, it is difficult to distinguish between them based on clinical findings alone.

Therefore, next to clinical evaluation, laboratory tests are indispensable for an accurate and timely diagnosis. These diagnostic tests may include measurement of urinary GAG levels, enzyme activity assays and molecular genetic analysis (Muenzer 2011). Although urinary GAG analysis can be useful for screening, it is not sufficiently sensitive to rule out MPS in case of a negative test result. Consequently, measurement of enzyme activities – conventionally on leukocytes or cultured fibroblasts – is currently the gold standard for diagnosing a specific MPS disorder (Muenzer 2011; Muenzer et al. 2009; Scarpa et al. 2011; Wood et al. 2012). However, enzyme activities in leukocytes are very sensitive to prolonged shipping or unfavourable environmental conditions, while analysing cultured fibroblasts requires skin biopsy for sample collection and an expensive and lengthy procedure, taking a long time before results become available (for recent review see Wood et al. 2012). Therefore, dried blood spots/specimens (DBS) – obtained by drying a small blood volume on filter paper – represent a convenient alternative sample type, offering advantages in terms of ease of collection, storage and transportation (Reuser et al. 2011).

So far, several studies have shown the feasibility of the enzymatic diagnosis of MPS I, II or VI using DBS (Blanchard et al. 2008; Chamoles et al. 2001a; Chamoles et al. 2001b; Dean et al. 2006; Duffey et al. 2010a; Duffey et al. 2010b; Fuller et al. 2011; Wang et al. 2007; Wolfe et al. 2011). However, none of these studies had a prospective design, i.e. only patients with a confirmed diagnosis of MPS were included.

Enzymatic diagnosis should always be complemented by sequencing of the disease gene to confirm the diagnosis of MPS, especially if enzyme replacement therapy or haema-

topoietic stem cell transplantation is planned (Wang et al. 2011). DNA extracted from blood or saliva samples is most commonly used for this purpose (Wood et al. 2012). However, some recent studies have shown that genotyping is also feasible on DNA extracted from a DBS (Harahap et al. 2012; Hollegaard et al. 2011; Quraishi et al. 2012). Although this method has already been applied successfully to detect mutations involved in Fabry disease, an X-chromosomal LSD (Hagège et al. 2011; Tai et al. 2012), its usefulness remains to be shown for other LSDs such as MPS and ML II/III. Therefore, the current prospective study aimed additionally at testing whether DBS could be applied to ascertain the diagnosis of MPS in patients with an ‘MPS-like’ phenotype, using the same DBS for both enzymatic and molecular genetic analyses.

Methods

Dried Blood Spots

After obtaining informed consent, DBS were collected from patients with a suspicion of MPS based on clinical symptoms, mainly including coarse facial features, hepatosplenomegaly, corneal clouding, gibbus, developmental delay, short stature, frequent respiratory illness and/or umbilical hernia. In order to ascertain the diagnosis in a large number of patients with MPS, selection was based rather on the “overall” phenotypic impression than on the total number of symptoms. Many samples were submitted from patients with coarse face as the leading symptom. Patients were not selected on the bases of MPS-specific laboratory investigations and were sent for analysis to the Metabolic Laboratory (Hamburg University Medical Centre, Germany) by courier mail at room temperature between July 2011 and March 2012. The average age of the patient population was 9.3 a, while the median is 2.9 a. The age range of the population varies between 0 a (newborn) to 75.5 a. Approx. 60% were males. After reception, samples were stored with desiccant at -20°C to avoid degradation of enzymes until the analysis was completed. For this study consisting of enzymatic and molecular genetic assays, the first 200 samples were chosen without any bias.

Measurement of Enzyme Activity

To analyse the activity of the enzymes α -iduronidase, iduronate-2-sulphatase, arylsulphatase B and β -galactosidase in DBS, previously described methods were slightly modified (Civallero et al. 2006; Chamoles et al. 2001b). For each test, a 3 mm DBS was mixed with elution liquid and substrate in a 96-well plate (for details: see Table 1). For α -iduronidase and arylsulphatase B as well as β -galactosidase

Table 1 Reagents used for analysis of enzyme activity

Enzyme	Elution liquid	Substrate buffer
α -L-iduronidase	20 μ L 10 mM D-saccharic acid 1,4-lactone (Sigma-Aldrich, Taufkirchen, Germany) (inhibitor) 110 μ L 0.05 M sodium phosphate buffer pH 3.5 (0.1 M citric acid and 0.2 M Na ₂ HPO ₄ dihydrate (Merck, Darmstadt, Germany))	20 μ L 4-methylumbelliferyl- α -L-iduronidase (Toronto Research Chemicals, North York, Canada) in 0.05 M sodium phosphate buffer pH 3.5
Iduronate 2-sulphatase	40 μ L 0.1 M sodium acetate/0.1 M acetic acid buffer pH 5.0	20 μ L 1.25 mM methylumbelliferyl- α -L-iduronide-2-sulphate (Moscerdam, Rotterdam, Netherlands) in 0.1 M sodium acetate/0.1 M acetic acid buffer pH 5.0 (Merck, Darmstadt, Germany)
Arylsulphatase B	30 μ L dH ₂ O water +20 μ L 15 mM lead acetate in sodium acetate buffer pH 5.0 (Merck, Darmstadt, Germany)	50 μ L 0.01 M 4-methylumbelliferone sulphate (Sigma-Aldrich, Taufkirchen, Germany) in 0.05 M sodium acetate buffer pH 5.0
β -galactosidase	100 μ L 0.1 M citric acid buffer	100 μ L 3.4 mg 4-methylumbelliferyl- β -D-galactoside (Sigma Aldrich, Taufkirchen, Germany) in 5 mL 0.1 M citric acid buffer pH 4.4 (Merck, Darmstadt, Germany)

dH₂O demineralised water, EC Enzyme Commission

Table 2 Statistical data for the fluorometric MPS tests using dried blood specimens

Test	Controls (nmol/punch*21 h)			Patients (nmol/punch*21 h)		
	Median (1st/99th percentile)	Range	<i>n</i>	Median	Range	<i>n</i> ^a
MPS I	0.08 (0.05/0.16)	0.04–0.26	106	0	0–0.01	15
MPS II	0.06 (0.03/0.1)	0.02–0.25	112	0	0–0.01	15
MPS VI	0.53 (0.3/0.86)	0.2–1.41	104	0.03	0–0.1	12

^a Confirmed patients

duplicates were run, while for iduronate-2-sulphatase, we ran samples in single and repeated the analysis of samples with low enzyme activity in duplicate because of the higher cost of that particular assay. In total, 9–14 DBS punches (3 mm) are necessary for analysis of the full MPS panel. Enzyme activities were compared with laboratory-specific reference ranges determined using unaffected controls (around 100 newborns and 20–30 older individuals). After shaking the plate for 45 min, all reactions were incubated for 21 h at 37°C. For α -iduronidase, arylsulphatase B and β -galactosidase, the enzymatic reaction was stopped by adding 200 μ L 0.17 M glycine carbonate buffer (12.8 g glycine and 18 g sodium carbonate in 200 mL demineralised water – Merck, Darmstadt, Germany). For iduronate-2-sulphatase, the reaction was stopped by adding 40 μ L Pi/Ci stop buffer (0.4 M sodium phosphate/0.2 M citric acid pH 4.5 (Merck, Darmstadt, Germany)) and 10 μ L of lysosomal enzyme solution (Moscerdam, Rotterdam, Netherlands), followed by a second incubation for 24 h at 37°C, which was stopped with 200 μ L 0.17 M glycine carbonate buffer. For all samples in all assays, a blank was run in parallel to correct for fluorescence quenching by haemoglobin. For these

blanks, the DBS was only added to the well after stopping the incubation, followed by 30 min shaking at room temperature. All plates also contained a positive and a negative control (i.e. samples from a healthy volunteer and from patients with confirmed diagnosis, respectively). For all enzyme assays, calculation of results was based on a standard curve with different dilutions of 0.05 M 4-methylumbelliferone (Sigma-Aldrich, Taufkirchen, Germany) in 0.17 M glycine carbonate buffer. Fluorescence was read on a Victor D instrument (Wallace Oy, Turku, Finland). Enzyme activities were expressed as nmol per 21 h and per punch and compared with laboratory-specific reference values (Müller et al. 2010; Wood et al. 2012). Details on the individual ranges are shown in Table 2.

Molecular Genetic Analysis

Molecular genetic analysis was carried out on the DNA extracted from DBS used previously for enzymatic analysis. DNA-samples were isolated with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Mutation analysis was performed by exon-specific PCR amplification of individual

Fig. 1 Geographic distribution of all analysed dried blood spots and of those samples that were putatively positive for mucopolysaccharidosis (MPS) I, II, VI or potential mucopolidoses II/III. Turkey, Saudi Arabia, Kuwait, United Arab Emirates, Syria and Israel were

considered as countries in the Middle East, while Romania, Russia, Kazakhstan, Croatia, Armenia, Bulgaria, Lithuania, Latvia, Serbia and Slovenia were considered as Eastern/Southern European countries

exons together with short flanking intronic sequences, followed by conventional Sanger sequencing (using GeneAnalyzer 3500/Applied Biosystems).

Results

Demographics

In total, 200 DBS were analysed. About 50.0% originated from countries in the Middle East, mainly Turkey (18.0%) and Saudi Arabia (16.0%), while about 25% originated from Eastern and Southern European countries and another quarter from Mexico (Fig. 1).

Enzymatic and Molecular Genetic Analyses on Dried Blood Spots

All DBS were analysed for activities of the enzymes α -iduronidase, iduronate-2-sulphatase and arylsulphatase B. In addition, activity of a reference lysosomal enzyme, β -galactosidase, was measured to confirm sample integrity (Gasparotto et al. 2009). If α -iduronidase, iduronate-2-sulphatase or arylsulphatase B activity was below the normal reference range and with a value consistent with enzyme activities of patients with established diagnosis, samples were considered as putatively positive for MPS I, MPS II or MPS VI, respectively. If activities of at least two of the three enzymes tested were elevated or in the upper reference range, while β -galactosidase activity was within the normal range, patients were considered potentially having ML II/III (Gusina and Tsukerman 1988; Wood et al. 2012). To rule out multiple sulphatase deficiency, activities of both sulphatases (and possibly other sulphatases) were determined in parallel (Scarpa et al. 2011; Wood et al. 2012).

In total, 45 DBS (22.5%) tested putatively positive: 17 (8.5%) for MPS I, 11 (5.5%) for MPS II, 9 (4.5%) for MPS VI and 8 (4.0%) for ML (Tables 3, 4, 5, and 6). For the positive samples, mutation analysis of the corresponding disease gene was carried out.

MPS I

For 17 DBS, α -iduronidase activity was well below the reference range, while β -galactosidase activity was normal (Table 3). The samples belonged to 12 males and 5 females, with a median age of 2.0 years at the time of DBS collection. The geographic origin of the patients was similar to that of the total group of patients (Fig. 1).

Genotyping of the α -L-iduronidase gene (*IDUA*) was performed for 13 DBS (76.5%), whereas there was no material left over after enzymatic analysis of 4 samples to do a molecular genetic test (Table 3). For 11 patients (84.6%), a total of 10 different mutations of the *IDUA* gene were detected: eight patients were homozygous, two patients carried two different heterozygous mutations each, and in one patient, only one heterozygous mutation was detected. Remarkably, only four of the mutations identified have been previously reported (Table 3). Of the six novel mutations, the two intronic splice site mutations, the nonsense mutation, and the 52 base pair insertion are very probably disease causing, whereas the pathogenic significance of the two missense mutations needs to be proven in the future. For two patients (15.4%), no mutations were found in the *IDUA* gene.

MPS II

Enzymatic analysis revealed a lower-than-normal activity of iduronate-2-sulphatase for 11 DBS (all from male patients, Table 4). Normal β -galactosidase and arylsulphatase B

Table 3 Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots of 17 patients diagnosed with mucopolysaccharidosis type I

No	Origin	Age (years)	Gender	α -Iduronidase (nmol/21 h*punch)	β -Galactosidase (nmol/21 h*punch)	Mutation <i>IDUA</i> gene ^a	
						Mutation 1	Mutation 2
1	Armenia	6	M	0	0.74	p.Met1? ^{b,1} (c.1A>C)	p.Met1? ^{b,1} (c.1 A>C)
2	Kuwait	1.5	M	0	0.54	p.Arg628* (c.1882C>T)	p.Arg628* ^{b,2} (c.1882C>T)
3	Latvia	2	M	0	0.89	p.Ser633Trp (c.1898C>G)	p.Gln70* ^{b,3} (c.208C>T)
4	Mexico	4	F	0	0.85	p.Ser633Trp (c.1898C>G)	p.Ser633Trp (c.1898C>G)
5	Mexico	59	M	0	0.58	–	–
6	Mexico	2	M	0	0.72	p.Ser633Trp (c.1898C>G)	p.Ser633Trp (c.1898C>G)
7	Mexico	5	M	0	0.5	NA	NA
8	Mexico	2	M	0.01	1.35	NA	NA
9	Portugal	54	F	0	0.53	–	–
10	Russia	21	M	0.01	1.09	NA	NA
11	Saudi Arabia	4	M	0	1.0	c.1525-1G>C	c.1525-1G>C
12	Saudi Arabia	0.5	M	0	0.5	p.Leu623Pro (c.1868T>C)	p.Leu623Pro (c.1868T>C)
13	Saudi Arabia	5	F	0.01	1.04	p.Trp402* ^{b,4} (c.1206G>A)	–
14	Saudi Arabia	1	F	0.01	2.2	c.1598_1599ins52	c.1598_1599ins52
15	Serbia	1	M	0.01	0.7	p.Pro22Ser (c.64C>T)	c.1829-1G>A
16	Turkey	0.5	F	0	1.08	p.Trp68* (c.203G>A)	p.Trp68* (c.203G>A)
17	Turkey	2	M	0	1.12	NA	NA
Reference range				0.04–0.26	0.5–3.2		

NA not assessed due to insufficient material, – mutation not found

^a Nomenclature: <http://www.hgvs.org/mutnomen/>

^b Mutations already reported

¹ Lee-Chen and Wang (1997), ² Beesley et al. (2001), ³ Clarke and Scott (1993), ⁴ Scott et al. (1992)

activities (assessed for all DBS with sufficient material) excluded multiple sulphatase deficiency (MSD; data not shown). Consequently, these samples were considered positive for MPS II. Their median age was 4.0 years at the time of DBS collection. Remarkably, more than 50% of these patients originated from Mexico (Fig. 1).

A (probably) disease-causing mutation of the iduronate 2-sulphatase gene (*IDS*) was identified in all 11 DBS. Most of these mutations have already been reported in MPS II patients (Table 4). Of the six patients originating from Mexico, two carried the same mutation, i.e. p.Arg468Gln. Of the four novel mutations, three are very probably pathogenic, whereas further studies will be needed to assess the missense variant (p.Ser349Arg).

MPS VI

Nine patients showed arylsulphatase B activities far below the reference range and β -galactosidase activity within normal limits, compatible with MPS VI (Table 5). Wherever possible, iduronate-2-sulphatase activity was also determined to exclude MSD (data not shown). Of

the nine MPS VI patients, five were males and four were females, with a median age of 2.0 years at the time of DBS collection. The majority of these patients (seven patients; 77.8%) came from countries in the Middle East (Fig. 1).

Molecular genetic analysis of the DBS revealed mutations in the arylsulphatase B gene (*ARSB*) in all nine patients: seven were homozygous, while two patients carried two different heterozygous mutations each. Remarkably, 7 out of 14 disease alleles (50%) of patients from the Middle East carried the p.Tyr251* mutation (three homozygous, one heterozygous). Only three of the mutations found by us have previously been reported (Table 5). Of the five novel mutations, two are very probably disease causing, whereas future studies are needed concerning the pathogenic significance of the three novel missense mutations.

Mucopolidoses

For eight DBS, belonging to four males and four females with a median age of 2.0 years, enzymatic analysis

Table 4 Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots of 11 patients diagnosed with mucopolysaccharidosis type II

No	Origin	Age (years)	Gender	Iduronate-2-sulphatase (nmol/21 h*punch)	β -Galactosidase (nmol/21 h*punch)	Mutation <i>IDS</i> gene ^a
1	Armenia	19	M	0	0.5	p.Ala346Val ^{b,1} (c.1037C>T)
2	Armenia	23	M	0	0.5	p.Ala346Val ^{b,1} (c.1037C>T)
3	Lithuania	1.5	M	0	0.79	p.Arg468Gln ^{b,2} (c.1403G>A)
4	Mexico	7	M	0	0.68	c.1463delT
5	Mexico	5	M	0	0.9	p.Gly340Asp ^{b,3} (c.1019G>A)
6	Mexico	2	M	0	0.57	p.Arg468Gln ^{b,2} (c.1403G>A)
7	Mexico	1	M	0	0.54	p.Gln75* ^{b,4} (c.223C>T)
8	Mexico	2.5	M	0	0.67	p.Ser349Arg (c.1047C>A)
9	Mexico	12	M	0	0.6	p.Arg468Gln ^{b,2} (c.1403G>A)
10	Saudi Arabia	4	M	0.01	0.78	Rearrangement ^c
11	Turkey	2	M	0	0.62	c.213_215del13 ^d
Reference range				0.02–0.25	0.5–3.2	

^a Nomenclature: <http://www.hgvs.org/mutnomen/>^b Mutations already described^c Complex rearrangement between gene and pseudogene^d c.213_225delCCTCCTCTTCCAG¹ Li et al. (1995), ² Whitley et al. (1993), ³ Karsten et al. (1998), ⁴ Kato et al. (2005)**Table 5** Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots in nine patients diagnosed with mucopolysaccharidosis type VI

No	Origin	Age (years)	Gender	Arylsulphatase B (nmol/21 h*punch)	β -Galactosidase (nmol/21 h*punch)	Mutation <i>ARSB</i> gene ^a	
						Mutation 1	Mutation 2
1	Kuwait	1	M	0.03	0.61	p.Tyr251* (c.753C>G)	p.Tyr251* (c.753C>G)
2	Russia	8	F	0.04	0.7	p.Thr92Lys ^{b,1} (c.275C>A)	p.Leu98Pro ^{b,2} (c.293T>C)
3	Russia	2	F	0.04	0.52	c.691-1G>A	c.691-1G>A
4	Saudi Arabia	1	M	0.03	0.7	p.Tyr251* (c.753C>G)	p.Tyr251* (c.753C>G)
5	Saudi Arabia	1.5	M	0.04	0.84	p.Tyr251* (c.753C>G)	p.Tyr251* (c.753C>G)
6	Saudi Arabia	3	M	0.06	0.78	p.Gly56Asp (c.167G>A)	p.Gly56Asp (c.167G>A)
7	Syria	4	M	0.02	0.55	p.Tyr251* (c.753C>G)	p.Gly308Glu (c.923G>A)
8	Turkey	1	F	0.03	0.5	p.Leu321Pro ^{b,3} (c.962T>C)	p.Leu321Pro ^{b,3} (c.962T>C)
9	Turkey	14	F	0.03	0.54	p.Val376Glu (c.1127T>A)	p.Val376Glu (c.1127T>A)
Reference range				0.2–1.41	0.5–3.2		

^a Nomenclature: <http://www.hgvs.org/mutnomen/>^b Mutations already reported (www.hgmd.cf.ac.uk)¹ Karageorgos et al. (2007), ² Voskoboeva et al. (2000), ³ Isbrandt et al. (1994)

suggested ML II/III: activities of α -iduronidase, iduronate-2-sulphatase and/or arylsulphatase B were in the upper reference range or above, while β -galactosidase activity fell within the normal range (Table 6). The majority (75.0%) of these samples originated from the Middle East (Fig. 1).

Genotyping of *GNPTAB*, one of the genes implicated in ML II/III revealed probably disease-causing mutations in

five of the samples. All these patients carried different alterations, all in apparently homozygous state. None of these mutations have previously been associated with ML II/III (Table 6). Of the five novel mutations, three are very probably pathogenic. As to the two novel missense variants, one changes arginine to cysteine whereas the other one cysteine to arginine.

Table 6 Demographic data and results of enzymatic and molecular genetic analyses on dried blood spots in eight patients with the possible diagnosis of mucopolidoses II/III

No	Origin	Age (years)	Gender	α -Iduro-nidase (nmol/21 h*punch)	Iduronate-2-sulphatase (nmol/21 h*punch)	Arylsulphatase B (nmol/21 h*punch)	β -Galactosidase (nmol/21 h*punch)	Mutation <i>GNPTAB</i> gene ^a	
								Mutation 1	Mutation 2
1	Mexico	1	F	0.45	NA	1.69	0.59	c.616_619delACAG	c.616_619delACAG
2	Mexico	2	M	0.45	1.37	1.55	0.53	–	–
3	Saudi Arabia	1	F	0.21	0.93	1.06	0.70	p.Arg46* (c.136C>T)	p.Arg46* (c.136C>T)
4	Saudi Arabia	5	M	0.5	1.15	2.06	0.44	–	–
5	Turkey	0.5	F	0.29	0.25	1.77	0.75	–	–
6	Turkey	3	F	0.33	NA	1.29	0.50	p.Glu279* (c.835G>T)	p.Glu279* (c.835G>T)
7	Turkey	4	M	0.23	1.47	1.28	0.64	p.Arg986Cys (c.2956C>T)	p.Arg986Cys (c.2956C>T)
8	UAE	2	M	0.74	NA	1.54	0.51	p.Cys523Arg (c.1567T>C)	p.Cys523Arg (c.1567T>C)
Reference range				0.04–0.26	0.02–0.25	0.2–1.41	0.5–3.2		

NA not assessed due to insufficient material on dried blood spot, UAE United Arab Emirates, – mutation not found

^a Nomenclature: <http://www.hgvs.org/mutnomen/>

Discussion

This study shows that DBS are very useful samples for diagnosing many forms of MPS, because they can be used for both enzymatic and molecular genetic analyses and generally facilitate sampling, shipping and handling of the samples.

The advantage of using DBS for enzymatic diagnosis of MPS and ML II/III has been demonstrated (Chamoles et al. 2001b; Chamoles et al. 2001a). In the past years, fluorometric methods, immunocapture enzyme assays and even high-throughput tandem mass spectrometry assays have been developed and optimised to allow (large-scale) screening for LSDs (Blanchard et al. 2008; Dean et al. 2006; Duffey et al. 2010a; Duffey et al. 2010b; Fuller et al. 2011; Reuser et al. 2011; Wang et al. 2007; Wolfe et al. 2011). However, all studies using DBS were feasibility studies, including only patients with a confirmed diagnosis of MPS. In contrast, in our study, DBS of 200 patients with a vague clinical suspicion of MPS, i.e. an ‘MPS-like phenotype’, were analysed for the first time in a prospective way. Patients were screened for α -iduronidase activity and also for iduronate-2-sulphatase and arylsulphatase B activities because there is a significant overlap in clinical manifestations between the corresponding MPS types. Indeed, of the 200 patients, 8.5% were tested positive for MPS I, 5.5% for MPS II and 4.5% for MPS VI. Remarkably, eight patients were classified as potentially having ML II or III, as the latter disorders are characterised by elevated activities of acid hydrolases in plasma – and therefore potentially also in DBS. Indeed, in ML II or III, the mannose-6-phosphate tag on the lysosomal enzymes – which ensures sorting to the lysosome – is missing, leading to the excretion of the enzymes into the plasma. This yields elevated in vitro plasma enzyme activities, which can also be found in DBS (Gusina and Tsukerman 1988; Fuller et al. 2011; Wood et al. 2012).

Based on the data of this study, we recommend that patients with an ‘MPS-like phenotype’ should be tested for at least MPS I, II and VI. As soon as it will be possible to reliably perform DBS testing for MPS IVA, this disease should also be included. Leading clinical features of MPS III are neurological symptoms whereas coarse face has rarely been described in this group of patients. Therefore we think that it is justified not to include MPS III in the targeted screening. In contrast, MPS VII may present with a Hurler-like phenotype. However, given the rarity of this condition, we have decided not to include it in our program.

Our strategy may allow a timely diagnosis of three MPS types – and potentially even ML II/III and multiple sulphatase deficiency – at a relatively low additional cost. Urinary GAG analysis (quantitative and qualitative) as the first tier testing followed by enzyme determination may

currently cost in our laboratory approximately 120 Euros, whereas the immediate analysis of the three enzymes on DBS costs approximately 80 Euros.

Genotyping of DNA extracted from filter paper has already been successfully performed in a number of diseases, including Fabry disease (Hagège et al. 2011; Tai et al. 2012), an X-chromosomal LSD. Our study also demonstrates that DBS can also be used for molecular genetic analysis of the genes involved in MPS I, II, VI and ML II/III.

For two DBS with pathologically decreased activity of α -iduronidase (MPS I), no *IDUA* mutation was found (Table 3, patients 5 and 9). This might be due to the fact that certain mutations – e.g. exon-spanning inversions, duplications, or deep intronic mutations – cannot be detected by conventional sequencing. This might also be the case in the patient with putative MPS I, in which only a heterozygous mutation was found (Table 3, patient 13). Alternatively, the lack of identifiable mutations may be due to false positive results in the initial enzymatic analysis. Indeed, based on our experience, α -iduronidase appears to be less stable in DBS than the other enzymes tested. As such, the enzyme may be more sensitive to unfavourable preanalytical conditions (during sampling, shipping, etc.), leading to diminished α -iduronidase activity while the activities of the other enzymes remain within the normal range, as might be the case in the two patients. Consequently, confirmatory testing of an independently obtained second DBS is important to validate the initial results of the enzymatic analysis (Wood et al. 2013). As no second DBS has been received for the two putative MPS I patients without identifiable mutations in the *IDUA* gene, the results of their enzymatic tests should be considered tentative.

Our experience of performing both enzymatic and molecular genetic analysis on DBS for diagnosing MPS or ML II/III has important practical consequences because DBS offer many advantages over other sample types, such as leukocytes, cultured fibroblasts, whole blood or saliva. Indeed, sample collection is easy and minimally invasive, requiring only a small blood volume (Reuser et al. 2011). Furthermore, we show that a single specimen can be used for both biochemical and molecular genetic testing. However, results of a single DBS enzyme assay are not sufficient for diagnosis and should always be accompanied by further confirmatory tests, which can be either a second enzyme test or molecular genetic studies. If protected from humidity, DBS can be stored easily for long time with only minimal losses in enzyme activity (Reuser et al. 2011) or stability of genomic DNA (Chaisomchit et al. 2005; Hollegaard et al. 2011). In addition, DBS can be shipped via regular mail at room temperature – and thus at a low cost – and pose little biohazard risk to the handlers. Consequently, DBS may be especially useful for diagnosing

MPS or ML II/III in regions of the world that lack specialised laboratories. In such regions, it may not be feasible to transport whole blood or other tissue samples, in order to confirm the DBS-based diagnosis. In the latter case, a second independently collected DBS would be needed to confirm the diagnosis (Wood et al. 2013). To elucidate the diagnostic efficacy of the DBS method to detect affected individuals, a study for MPS I, MPS II, MPS VI and ML II/III is necessary with patients of confirmed diagnosis, especially as the sensitivity of our screening assay remains unknown because of the small group of patients diagnosed.

Conclusions

Our prospective study shows that DBS can be used successfully for both enzymatic and molecular genetic diagnosis of MPS I, II, and VI, and ML II/III in patients with a clinical suspicion of MPS. Given the advantages of DBS over other sample types in terms of ease of collection, storage and transportation, DBS are particularly useful for screening patients in regions lacking specialised laboratories. We suggested that patients should always be screened in parallel for at least α -iduronidase, iduronate-2-sulphatase and arylsulphatase B (MPS I, II, and VI), i.e. by a multitasking assay that also provides a potential for diagnosing ML II/III and MSD.

Take-Home Message

In patients with suspicion of mucopolysaccharidosis (MPS) based on clinical features, enzymatic analysis on dried blood spots (DBS) may allow diagnosis of MPS I, II, VI and mucopolipidosis II/III and it is suggested to corroborate the initial findings by molecular genetic testing from the same DBS or by another enzyme measurement.

Authors' Contributions

Paulina Nieves Cobos was involved in designing the study, carried out the biochemical measurements and revised the manuscript.

Cordula Steglich carried out the genetic analysis.

René Santer was involved in the design of the study, review of the data and revised the manuscript.

Andreas Gal was responsible for the molecular genetic testing. He was involved in drafting the manuscript and revising it critically for important intellectual content.

Zoltan Lukacs was involved in designing the study and collecting, analysing and interpreting the data. He was

involved in drafting the manuscript and revising it critically for important intellectual content. Zoltan Lukacs accepts full responsibility for the work and the conduct of the study, had access to all data and controlled the decision to publish.

All authors have given final approval of this version to be published.

Competing Interests

Paulina Nieves Cobos has no competing interests.

Cordula Steglich has no competing interests.

René Santer has no competing interests.

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Ethical Approval

Ethical approval was not required for this study.

Patient Consent Statement

All patients included in the study or their parents/caretakers gave informed consent.

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