Lysosomal storage diseases in non-immune hydrops fetalis pregnancies


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Available online 3 May 2006

Abstract

Background: At least 20 inborn errors of metabolism may cause hydrops fetalis. Most of these are lysosomal storage diseases. The study proposes a diagnostic flowchart for prenatal diagnosis of non-immune hydrops fetalis.

Methods: This study contains a series of 75 non-immune hydrops fetalis pregnancies. Mucopolysaccharides, oligosaccharides, neuraminic acid and 21 lysosomal enzymes were measured in amniotic fluid and cultured amniotic cells.

Results: The study gives reference values for mucopolysaccharides and neuraminic acid at various stages of gestation. Four definite and two probable lysosomal diagnoses were found among the 75 investigated cases (=5.3–8%). Fetal death was found to cause false positive values for mucopolysaccharides in amniotic fluid. In the galactosialidosis case, two novel mutations were found in the cathepsin A gene.

Conclusions: Reference values for mucopolysaccharides and neuraminic acid depend on gestational age. In a relatively high percentage of the hydrops foetalis pregnancies, a lysosomal aetiology is found. This study provides a strategy to diagnose lysosomal diseases in hydrops fetalis pregnancies. Awareness of lysosomal storage diseases causing hydrops fetalis is useful as it gives an opportunity for risk evaluation, genetic counseling to parents and targeted prenatal diagnostics for ensuing pregnancies.

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Keywords: Prenatal diagnosis; Galactosialidosis; Inborn errors of metabolism; Lysosomal storage diseases; Reference values; Mucopolysaccharides in amniotic fluid

1. Introduction

The diagnosis of hydrops fetalis (=HF), the presence of excessive fluid in more than one body cavity in the fetus, is being made increasingly and at an earlier stage during pregnancy owing to routine prenatal ultrasound screening. Estimates of the incidence of HF vary between one in 600 and 4000 pregnancies [1–3]. Estimates of mortality vary between 60% and 90% [3]. HF can have diverse and widely ranging causes due to disease processes in the cardiovascular or thoracic regions, fetal arrhythmia, monochorial twin pregnancies, fetal anaemia, chromosomal aberrations and genetic syndromes [4]. Traditionally, HF is subdivided in immunological and non-immunological HF (=NIHF). Inborn errors of metabolism are among the causes of NIHF and the group of lysosomal diseases is the most important subgroup. Prenatal diagnosis of a lysosomal disease in families at risk is well established in chorionic villi and in amniocytes. For the prenatal diagnosis of a
lyosomal disease in NIHF cases, the accumulating substrate of the defective enzyme and/or the enzymatic activity can be determined [5–8]. We have investigated a series of 75 pregnancies with NIHF at the metabolite level and at the enzyme level by measuring 21 lysosomal enzymes. Reference values for mucopolysaccharides (=MPS) and neuraminic acid are not available in literature. This paper gives gestational age-related reference ranges for MPS and neuraminic acid and gives examples of abnormal oligosaccharide profiles of amniotic fluid. Four definite and two probable cases of NIHF pregnancies due to lysosomal etiology are described.

2. Patients and methods

2.1. Patients

We have investigated a series of 75 pregnancies with NIHF. In all pregnancies, routine maternal antibody screening had excluded irregular antibodies. The classification of NIHF is based on the maternal blood group and the absence of irregular antibodies to red cell antigens Rhesus, c, E and Kell. Chromosomal abnormalities had been excluded in all cases. Investigations were carried out at the metabolite level and at the enzyme level to diagnose lysosomal diseases. In amniotic fluid these included the measurement of MPS, oligosaccharides and neuraminic acid. A panel of lysosomal enzyme determinations was performed in the cultured amniocytes. Forty control pregnancies were included in this study. Measurements on 75 NIHF pregnancies were performed as follows: in 42 pregnancies amniotic fluid was obtained and amniocytes were cultured; in 29 cases only amniotic fluid could be investigated and in four cases only cultured amniocytes were available. In our series four definite cases of lysosomal disease were found.

2.1.1. Case 1

In the second pregnancy of non-consanguineous parents (the first pregnancy had ended in miscarriage) ultrasound abnormalities were detected at 27 weeks of gestation. The male fetus had hydrops, polyhydramnion and ascites. Premature contractions started at 35 weeks and delivery was initiated. The baby died during delivery.

2.1.2. Case 2

In the third pregnancy of consanguineous parents (one healthy child and one spontaneous abortion) ultrasound abnormalities were detected at 27 weeks of gestation. The male fetus had HF. In view of the infaust prognosis and after consultation of the parents the pregnancy was terminated in week 25. Increased intra- and extra-medullary haematopoiesis were documented. In the second pregnancy, hydrops, granulocytopenia and anaemia were noted at week 15. The parents have one healthy child.

2.1.3. Case 3

In the third pregnancy of consanguineous parents, intrauterine fetal death of a female fetus was established with hygroma colli and hydrops at 21 weeks of gestation. Electron microscopic investigation of the placenta revealed an increased number of strongly vacuolised Hofbauer cells in the villi. An earlier pregnancy had resulted in intrauterine fetal death with hygroma colli and hydrops feta
is for which no causative factor was found.

2.1.4. Case 4

The patient was a 16-week hydropic fetus from non-consanguineous parents of Dutch ancestry. Two previous pregnancies resulted in an affected hydropic fetus. In the first pregnancy, which ended after 34 weeks, hydrops was detected at week 25. Increased intra- and extra-medullary haematopoi
esis were documented. In the second pregnancy, hydrops, granulocytopenia and anaemia were noted at week 15. The parents have one healthy child.

2.2. Biochemical tests

MPS were quantified using the dimethylmethylene blue test in 180 mmol/l Tris buffer pH 8.8 essentially as described by the Jong et al. for urine samples [9]. The mixture of the colour reagent and the buffer is not stable and has to be prepared within 15 min of performing the assay. Under these conditions, the amniotic fluid samples do not require deproteinisation before measuring MPS. Bound plus free neuraminic acid (=sialic acid) was determined enzymatically using a spectrophotometric assay from Roche Mannheim Germany (reagent no. 784192) on a Cobas Fara analyzer (Hoffmann-LaRoche, Basel, Switzerland; absorbance 550 nm). The assay requires correction for the presence of pyruvate in the sample. The protein content of the samples was measured with a modified Folin–Lowry method.

Oligosaccharides were analysed using thin layer chromatography (=TLC) with modifications on the method described by Blom et al. [10]. Samples were deproteinised first on a 10-kDa filter (Sartorius, Goettingen Germany; reagent no. 13239E) and subsequently desalted with an Amberlite mixed bed resin ion exchanger (Sigma A-5710; 200 mg/ml). After concentrating the sample by a factor 20, 5 µl sample/cm was applied on silicagel 60 TLC plates (Merek, Darmstadt, Germany; no. 5553). TLC plates were developed in n-butanol–acetic acid–water (2:1:1, by volume). Oligosaccharides were visualised by orcinol staining (100 mg orcinol (BDH Laboratory Supplies, Poole, UK; no. 29418) in 100 ml aceton and 5 ml sulfuric acid). Plates were heated for 10 min at 90 °C.

For biochemical studies a panel of 21 lysosomal enzymes was measured in ~6 × 106 amniocytes, washed three times with saline. These included arylsulfatase A, β-galactosidase, α-galactosidase A, N-acetyl β-glucosaminidase (total and A-isoform), β-glucosidase, sphingomyelinase, galactocerebrosidase, α-iduronidase, iduronate 2-sulfatase, heparine sulfaminidase, N-acetyl α-glucosaminidase, α-glucosaminide N-acetyltransferase, N-acetylglucosamine 6-sulfatase, arylsulfatase B, β-galacturonidase, neuraminidase, α-fucosidase, α-mannosidase, β-mannosidase and α-glucosidase. Methods used for these assays are essentially
similar to the methods used for routine measurement of lysosomal enzymes in leucocytes.

3. Results

3.1. Reference ranges

No reference ranges are available for MPS and neuraminic acid in amniotic fluid. To establish these we have expressed both compounds per protein (MPS, Fig. 1A; neuraminic acid, Fig. 1B). Fig. 1 gives the individual values for the control samples and the NIHF cases. The figure shows a gradual increase in concentration of both parameters with gestational age. The increase is more pronounced for neuraminic acid.

3.2. Amniotic fluid

Out of 75 NIHF pregnancies and 40 control pregnancies, 71 were investigated at the metabolite level (amniotic fluid obtained between 14 and 36 weeks of gestation). Standard investigations including quantitative analysis of MPS and neuraminic acid (bound + free), and oligosaccharide TLC revealed abnormal results in five samples (Figs. 1A, B and 2). Among these, two cases with highly increased MPS could not be followed up by additional investigations and remained without primary diagnosis. The protein concentration in these samples was normal. We consider these two cases as probable mucopolysaccharidoses (Fig. 1A: MPS unspecified). The other three cases could be further investigated on amniocytes.

Fig. 1. (A) Quantification in amniotic fluid of 40 control pregnancies (×) and 71 non-immune hydrops fetalis pregnancies (♦): MPS in mg/g protein. The amniotic fluid samples from case 3 (β-glucuronidase deficiency) and two further cases with MPS unspecified were clearly abnormal. (B) Quantification in amniotic fluid of 40 control pregnancies (×) and 71 non-immune hydrops fetalis pregnancies (♦): total neuraminic acid (=bound + free) in μmol/g protein. Case 1 (galactosialidosis) and a case of sialidosis (OMIM 256550; obtained as a gift from Dr. I. Maire (Lyon, France)) were clearly abnormal.
An increased MPS concentration was also found in two HF pregnancies with intrauterine fetal death (Fig. 1A). The protein content was high amounting to 15.5 and 14.3 g/l, respectively. The neuraminic acid concentration in both samples was normal.

### 3.3. Amniotic cells

Data obtained at the metabolite level on amniotic fluid were confirmed by enzyme determinations on cultured amniotic cells in 42 out of 71 NIHF pregnancies. The enzyme determinations confirmed galactosialidosis (OMIM 256540) in case 1, GM1-gangliosidosis (OMIM 230500) in case 2 and β-glucuronidase deficiency (OMIM 253220) in case 3. Additionally amniocytes from four other NIHF pregnancies (in which amniotic fluid was not available) were investigated revealing one additional case of β-glucuronidase deficiency. The enzyme assays did not reveal any deficiencies of lysosomal diseases that would not have shown up in the metabolite assays (for instance: arylsulfatase A, α-galactosidase A, β-glucosidase, sphingomyelinase, galactocerebrosidase). In the two fetal death cases, all mucopolysaccharidases enzymes in amniotic cells showed normal activity. This excludes a primary defect in the catabolism of MPS in these cases and indicates intrauterine fetal death as an independent cause for increased MPS in amniotic fluid.

### 3.4. Pregnancies with lysosomal diagnoses

#### 3.4.1. Case 1: Galactosialidosis

In amniotic fluid obtained at 27 weeks of gestation neuraminic acid was clearly increased (Fig. 1B: 142 μmol/g protein, reference value for this gestational age <90 μmol/g protein). TLC of oligosaccharides in the amniotic fluid showed the abnormal presence of several neuraminic acid containing oligosaccharides (Fig. 2, lane 4). β-D-Galactosidase and neuraminidase deficiency in cultured amniocytes suggested the diagnosis of Galactosialidosis. It was confirmed by demonstrating the primary cathepsin A defect (Table 1). The final diagnosis of galactosialidosis (OMIM 256540) was performed on fetal fibroblasts and leukocytes from umbilical cord blood. Molecular analysis on the PPGB gene (GenBank ID no. 5476) identified two novel mutations: 1. c.292C>T (in exon 3 leading to replacement of His98 by Tyr; 2. c.707T>G in exon 8 leading to Arg substitution of Leu236 (nomenclature including the signal peptide). In a later pregnancy specific enzymes were measured in chorionic villi and found normal. This pregnancy resulted in the delivery of a healthy child.

#### 3.4.2. Case 2: GM1-gangliosidosis

TLC showed abnormal oligosaccharide bands indicative for GM1-gangliosidosis (Fig. 2, lane 2). Cultured amniocytes revealed decreased β-D-galactosidase activity but normal neuraminidase activity (Table 1). After birth, the diagnosis GM1-gangliosidosis (OMIM 230500) was confirmed in fibroblasts and leukocytes. In the GLBI gene on chromosome 3p (GenBank ID no. 2720) encoding for β-D-galactosidase a novel homozygous mutation c.442C>T was found in exon 4 by direct sequencing of all exons of the genomic DNA. The mutation was confirmed with restriction enzyme analysis. It did not occur in 100 control alleles. At the protein level the mutation leads to a decrease in β-D-galactosidase activity.

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Galactosialidosis (case 1)</th>
<th>GM1-gangliosidosis (case 2)</th>
<th>Reference range</th>
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<tr>
<td><strong>Amniocytes</strong></td>
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<td></td>
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<tr>
<td>β-D-Galactosidase</td>
<td>161</td>
<td>7</td>
<td>450–2050</td>
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<tr>
<td>Neuraminidase</td>
<td>7</td>
<td>11880</td>
<td>6500–21 500</td>
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<td>Cathepsin A</td>
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<td>na</td>
<td>142–292</td>
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<tr>
<td><strong>Fibroblasts</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>38</td>
<td>9</td>
<td>600–1500</td>
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<tr>
<td>Neuraminidase</td>
<td>150</td>
<td>na</td>
<td>10000–28000</td>
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<tr>
<td>Cathepsin A</td>
<td>4.6</td>
<td>na</td>
<td>191–478</td>
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<td><strong>Leucocytes from umbilical cord blood</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td>23</td>
<td>2</td>
<td>150–370</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>130</td>
<td>1675</td>
<td>800–2800</td>
</tr>
<tr>
<td><strong>Amniotic fluid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total neuraminic acid</td>
<td>142</td>
<td>na</td>
<td>&lt;90</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>Abnormal bands</td>
<td>Abnormal bands</td>
<td>Normal pattern</td>
</tr>
</tbody>
</table>

na: not analysed.
replacement of Arg by Cys (R148C). A mutation in the same codon leading to R148S has been described in infantile GM1-gangliosidosis [11]. Due to the position of the mutation both gene products β-D-galactosidase as well as elastin binding protein are likely to be affected. Both parents were heterozygous for the mutation. In the subsequent pregnancy, specific enzyme analysis was performed on amniocytes at 16 weeks of gestation. A deficiency of β-D-galactosidase was found indicating an affected fetus. This led to the parents’ decision to terminate the pregnancy.

3.4.3. Cases 3 and 4: Mucopolysaccharidoses

Amniotic fluid from case 3 showed an MPS increase with a factor 1.5 compared to controls (Fig. 1A). Further analysis with one-dimensional electrophoresis showed an increase in chondroitin sulfate and dermatan sulfate. A deficiency of β-glucuronidase was found in serum obtained by pericardial puncture of the fetus (6 nmol/h/mg protein; reference values 600–1200) and in fetal fibroblasts (6 nmol/h/mg protein; reference values 200–700). Based on these findings, mucopolysaccharidosis type VII (MPS VII; OMIM 253220) was diagnosed.

The same enzyme defect was found in case 4. The β-glucuronidase activity amounted to 7 in amniotic cells (reference 140–660). The deficiency was confirmed by enzyme analysis in leucocytes from cord blood (β-glucuronidase=10 nmol/h/mg protein; reference 600–1200). At the molecular genetic level, a 27-nucleotide deletion c.1069C > T). This heterozygous mutation also is in exon 7. This mutation was found by sequencing the genomic DNA (p.362_370del) at the protein level. A second pathogenic mutation in heterozygous form. It leads to the loss of nine amino acids (GenBank ID no. 2990). This mutation in exon 7 was present in amniocytes. As we have included 29 cases where we only found in the second trimester by enzyme testing in cultured amniocytes. As we have included 29 cases where we only found in the second trimester by enzyme testing in cultured amniocytes. As we have included 29 cases where we only found in the second trimester by enzyme testing in cultured amniocytes. As we have included 29 cases where we only found in the second trimester by enzyme testing in cultured amniocytes.

4. Discussion

Lysosomal storage diseases (LSD) are extremely rare. However, the estimated combined birth incidence for all lysosomal diseases is 14 per 100,000 live births [14]. Deficiency of a lysosomal enzyme (nearly always) results in decreased haematopoiesis, hypoalbuminaemia, visceralomegaly, damage of the myocardium, inhibited venous drainage of the heart and ascites due to portal hypertension. It is believed that these changes lead to the development of HF that represents the severe end of the wide spectrum of LSD phenotypes [1,13]. Table 2 lists LSD and non-lysosomal diseases found in association with HF. Machin [13] and Jauniaux et al. [15] reported that 1.0–1.4% of NIHF is due to LSD. In the present study we have found four definite and two probable LSD diagnoses among 75 NIHF pregnancies (5.3–8%). Our figures on the prevalence of LSD are in line with three more recent studies presenting estimates ranging from 5.9% to 15% [7,16,17]. The highest estimate is from Burin et al. [16]. When chromosomal abnormalities were first excluded in this series of 33 NIHF cases the estimate becomes even higher (21%). Burin et al indicate that their numbers probably are an overestimation as they are a national reference centre for lysosomal diseases. This is likely to cause a selection bias in their series which is illustrated by the fact that 3 of the 5 positive cases had a suggestive family history for a lysosomal disease. Comparing the various studies, the gestational age must be taken into account. We have analyzed NIHF pregnancies between 14 and 36 weeks of gestation, while the study of Piraud mainly included third trimester samples [7]. The low urinary volume production of the fetus in the first two trimesters may imply that LSD cannot be diagnosed at the metabolite level before a certain gestational age. These diagnoses will be missed if investigations are performed at the metabolite level only. Of course, these would also be found in the second trimester by enzymed testing in cultured amniocytes. As we have included 29 cases where we only tested at the metabolite level, this may explain partly why we found a somewhat lower percentage of lysosomal diagnoses than the French study by Piraud et al. [7]. The six diagnoses in our study all were found in the period between weeks 21 and 36 of gestation. This study describes a case of NIHF due to β-glucuronidase deficiency in the 21st week (case 3). This could be shown also at the metabolite level, suggesting that the urine production at this stage of the pregnancy is sufficient to find lysosomal diagnoses. However, it is uncertain whether all

<table>
<thead>
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<th>Table 2</th>
<th>Hereditary metabolic diseases found in association with HF</th>
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<tr>
<td>Lysosomal storage diseases:</td>
<td>Non-lysosomal diseases:</td>
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<tr>
<td>Mucopolysaccharidoses:</td>
<td>Glycogenoses:</td>
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<tr>
<td>Mucopolysaccharidosis I (Hurler) [18]</td>
<td>Glycogenosis type IV (Anderson disease) [20]</td>
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<td>Mucopolysaccharidosis IVA (Morquio A) [6]</td>
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<tr>
<td>Mucopolysaccharidosis VII (β-glucuronidase deficiency) [7]</td>
<td>Fatty acid oxidation defects:</td>
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<td>Oligosaccharidoses:</td>
<td>Long-chain hydroxacyl CoA dehydrogenase deficiency [21]</td>
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<td>Galactosialidosis [2]</td>
<td>Cholesterol biosynthesis defects:</td>
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<td>Sialidosis [27]</td>
<td>Smith–Lemli–Opitz syndrome [22]</td>
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<td>Congenital disorders of glycosylation:</td>
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<td>Niemann-Pick C [2]</td>
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<tr>
<td>Lipogranulomatosis (Farber) [2]</td>
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<td>Wolman [18]</td>
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<td>Mucolipidosis II (I-cell disease) [2]</td>
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<tr>
<td>Others:</td>
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<tr>
<td>Multiple sulfatase deficiency</td>
<td></td>
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Jauniaux et al. [15] reported that 1.0–1.4% of NIHF is due to LSD. In the present study we have found four definite and two probable LSD diagnoses among 75 NIHF pregnancies (5.3–8%). Our figures on the prevalence of LSD are in line with three more recent studies presenting estimates ranging from 5.9% to 15% [7,16,17]. The highest estimate is from Burin et al. [16]. When chromosomal abnormalities were first excluded in this series of 33 NIHF cases the estimate becomes even higher (21%). Burin et al indicate that their numbers probably are an overestimation as they are a national reference centre for lysosomal diseases. This is likely to cause a selection bias in their series which is illustrated by the fact that 3 of the 5 positive cases had a suggestive family history for a lysosomal disease. Comparing the various studies, the gestational age must be taken into account. We have analyzed NIHF pregnancies between 14 and 36 weeks of gestation, while the study of Piraud mainly included third trimester samples [7]. The low urinary volume production of the fetus in the first two trimesters may imply that LSD cannot be diagnosed at the metabolite level before a certain gestational age. These diagnoses will be missed if investigations are performed at the metabolite level only. Of course, these would also be found in the second trimester by enzyme testing in cultured amniocytes. As we have included 29 cases where we only tested at the metabolite level, this may explain partly why we found a somewhat lower percentage of lysosomal diagnoses than the French study by Piraud et al. [7]. The six diagnoses in our study all were found in the period between weeks 21 and 36 of gestation. This study describes a case of NIHF due to β-glucuronidase deficiency in the 21st week (case 3). This could be shown also at the metabolite level, suggesting that the urine production at this stage of the pregnancy is sufficient to find lysosomal diagnoses. However, it is uncertain whether all
cases will be diagnosed at the metabolite level in these early stages.

In conclusion, our data suggest that the prevalence of LSD is significantly higher than the estimate of 1.0–1.4% in previous studies. Therefore, we recommend the inclusion of metabolic analyses of amniotic fluid and amniocytes in the routine diagnostic work-up of NIHF.

Techniques described in this paper will facilitate establishing a diagnosis in cases that would have previously been considered idiopathic. Finding the primary cause of HF will not only provide better understanding of the mechanism, but will also enable more accurate risk estimates and genetic counseling in future pregnancies. The present study applied a new diagnostic strategy using a protocol of prenatal diagnostic procedures. Fig. 3 presents a flowchart illustrating the strategy to detect LSD in HF pregnancies. At the metabolite level, the protocol relies on the measurement of MPS and neuraminic acid and on TLC of oligosaccharides. The cases with LSD gave unequivocal abnormal results that were well above the established reference ranges. Fetal death may cause increased MPS concentration in amniotic fluid and therefore complicates the interpretation of the laboratory result. The protein concentration of the amniotic fluid was clearly increased in the fetal death cases in our study while a normal value for protein was found in the β-glucuronidase deficient cases. At the enzyme level this study worked with a panel of 21 lysosomal enzymes. Obviously for routine diagnostic purposes this would not be feasible in most centers. The laboratory workload can be diminished by measuring only those lysosomal enzymes that are frequently involved in the etiology of HF (such as β-glucuronidase, β-glucosidase). Before week 18 of gestation, urine production of the fetus is limited, bringing the risk that a lysosomal diagnosis would be missed at the metabolite level. Therefore, it may be considered to add β-galactosidase to the enzyme panel to pick up cases with GM1-gangliosidosis and galactosialidosis is such cases. This combination of measurements at the metabolite and the enzyme levels will allow a diagnostic laboratory to pick up the most frequent LSD known to be associated with NIHF. However, defects that do not result in an increase of MPS, neuraminic acid or oligosaccharides in amniotic fluid will be missed with this strategy. Niemann-Pick types A and C, Wolman, Farber, Mucolipidosis II and multiple sulfatase deficiency are among the diseases that will not be picked up. Diagnostic centres may want to include a selection of the enzymes involved in these diseases in their NIHF protocol.

In NIHF pregnancies that have not been investigated prenatally, postnatal tests for LSD should always be performed. This became even more important through the availability of enzyme replacement therapy for an increasing number of LSD [26].

Vacuolization in the placenta or in fetal cells may be further clues in the direction of a lysosomal etiology [1,2]. A systematic approach of HF as proposed in this paper will contribute to our understanding of HF in individual cases, improve genetic counseling and provide chances for family planning in families at risk.

Fig. 3. Proposed flowchart for prenatal diagnosis of non-immune hydrops fetalis.
Acknowledgements

We thank Dr. M. Verjaal, clinical geneticist and Dr. C.M. Bilardo, gynaecologist of Academic Medical Centre Amsterdam for their comments in 3 cases and Dr. W. Kleijer, clinical biochemical geneticist Erasmus MC Rotterdam for measuring cathepsin A. The authors thank Dr. W. Lissens (Brussels, Belgium), Dr. E. Paschke (Graz, Austria) and Prof. A. D’Azzo (Memphis, USA) for molecular genetic analysis of the cases with β-glucuronidase, β-D-galactosidase and cathepsin A deficiency respectively. We are grateful to Dr. I. Maire (Lyon, France) for her gift of amniotic fluid of a case of sialidosis. The authors thank Jenne den Hartog (Nijmegen) for her studies in the initial phase of this project.

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