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ORIGINAL ARTICLE

Metabolic liver diseases presenting with neonatal cholestasis: *at the crossroad between old and new paradigms*

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Abstract

Metabolic liver diseases (MLD) are an important group of disorders presenting with neonatal cholestasis (NC). The spectrum of liver involvement is wide and the presumptive diagnosis is traditionally based on clinical and laboratory findings. Recently, next-generation sequencing (NGS) panels have emerged as an appealing tool to diagnose neonatal/infantile cholestatic disorders. The aim of this study was to identify clinical phenotypes of liver injury and contribute to find a diagnostic methodology that integrates new molecular diagnostic tools. We retrospectively analyzed the clinical and biochemical features of 16 patients with MLD and NC. Patients were categorized into three groups: A—*NC with liver failure* ($N = 8$): tyrosinemia type I ($n = 2$), classic galactosemia ($n = 5$), mitochondrial DNA depletion syndrome ($n = 1$); B—*NC evolving with chronic liver disease* ($N = 5$): argininemia ($n = 2$); mitochondrial cytopathy ($n = 1$); congenital disorders of glycosylation type Ia ($n = 1$); Zellweger syndrome ($n = 1$); and C—*transient NC* ($N = 3$): Niemann-Pick type C ($n = 2$), citrullinemia type II ($n = 1$).

Conclusion: MLD presenting with NC can be categorized into three main clinical phenotypes of liver injury. We highlight transient NC as a clue for MLD that must be pursued. New molecular diagnostic tools can play a key role, but application criteria must be established to make them cost-effective.

What is Known:
- *Metabolic liver diseases are an important group of disorders presenting with neonatal cholestasis.*
- *The diagnostic approach is challenging and traditionally based on clinical and laboratory findings. Next-generation sequencing is a recent and rapidly developing tool in pediatric hepatology.*

What is New:
- *We provide a liver-targeted characterization of metabolic liver diseases presenting with neonatal cholestasis, categorizing them into three clinical phenotypes that may narrow the diagnostic possibilities.*
- *A clinical decision-making algorithm is proposed, in which the NGS technology is integrated.*

Keywords Neonatal cholestasis · Transient neonatal cholestasis · Liver failure · Metabolic liver diseases · Next-generation sequencing panels

Abbreviations

cB	Conjugated bilirubin
CDG	Congenital disorder of glycosylation
GGT	Gamma-glutamyltransferase
HE	Hepatic encephalopathy
IEM	Inborn errors of metabolism
INR	International normalized ratio
LF	Liver failure
MLD	Metabolic liver diseases
NBS	Newborn screening
NC	Neonatal cholestasis
NGS	Next-generation sequencing
NP-C	Niemann-Pick type C
OLT	Orthotopic liver transplant
PFIC	Progressive familiar intrahepatic cholestasis

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Introduction

Neonatal cholestasis (NC) is an infrequent pathological condition, affecting approximately 1 in 2500 live births [1, 22]. The diagnostic approach is challenging since the differential diagnosis is broad and can be divided into two main categories: biliary (e.g., biliary atresia) and hepatocellular (e.g., genetic and/or metabolic disorders) [26].

As the liver is a key metabolic organ, metabolic liver diseases (MLD) often involve disarrangements in enzymes and/or metabolic pathways highly expressed in the liver. Additionally, many multisystemic diseases such as mitochondrial disorders can primarily manifest as a cholestatic syndrome in infancy.

Newborn screening (NBS) programs using tandem mass spectrometry are used in neonatal screening for several inborn errors of metabolism (IEM) and have been widely adopted in Europe [3]. In Portugal, an expanded NBS program was implemented in 2005 for 24 treatable disorders [32]. However, most IEM presenting with NC are not covered in NBS. In addition, there are multiple rare types of genetic cholestasis presenting with a similar phenotype. Therefore, genetic testing may contribute to a precise diagnosis in this setting. The single gene approach (Sanger technique) is reliable for detecting single mutations, but it is expensive and time consuming. In contrast, next-generation sequencing (NGS) is a recent and rapidly developing tool in pediatric hepatology that may contribute to a prompt identification of patients with MLD [15, 25]. However, NGS should not be interpreted irrespective of the clinical phenotype.

Herein, we report the data (clinical presentation, laboratory profile, and outcome) of a cohort of 16 children with MLD presenting with NC, providing a workup approach and a clinically oriented overview of the differential diagnosis. We will also discuss the driving shift from the classical scenario, in which diagnostic hypotheses arise from clinical and biochemical data to multi-gene panel testing and its inclusion in diagnostic algorithms.

Methods

We retrospectively analyzed a cohort of 126 patients presenting with NC, referred to a tertiary university hospital in the northern region of Portugal, during a 30-year period (1987–2017). We identified a group of 13 patients with MLD. Additionally, three out of 582 patients with MLD followed at our institution in the same period were included as they were retrospectively identified as having NC. Patients with alpha-1-antitrypsin deficiency and cystic fibrosis were excluded. A final cohort of 16 patients was enrolled and analyzed.

NC was defined as prolonged jaundice with conjugated bilirubin (cB) ≥ 1 mg/dL (in combination with a total bilirubin of < 5.0 mg/dL) or a cB fraction of > 20% of the total, detected either in a newborn or an infant up to 4 months old [24]. Transient cholestasis was retrospectively defined as the presence of cholestatic jaundice resolving in the first 6 months of life, in the absence of known risk factors for NC [7] and with complete and spontaneous normalization of liver function tests.

Liver failure (LF) was defined according to the Pediatric Acute Liver Failure Study Group [30] by the following: biochemical evidence of liver injury and coagulopathy [international normalized ratio (INR) ≥ 1.5], not correctable by vitamin K administration, in the presence of hepatic encephalopathy (HE) or an INR ≥ 2 regardless of presence or absence of HE.

Demographic data, family background, presenting symptoms, age at diagnosis, and laboratory investigations at admission were analyzed [full blood cell count, blood chemistry including direct and indirect bilirubin, serum aminotransferases (AST, ALT), gamma-glutamyltransferase (GGT), albumin, creatinine, urea]. Baseline metabolic workup included serum ammonia and lactate, plasma and urinary amino acids, urinary organic acids profiles, acyl-carnitine profile, plasma carbohydrate-deficient transferrin, and urinary reducing substances. Amino acids profile was determined by liquid ion-exchange chromatography. Organic acids were measured by gas chromatography-mass spectrometry. Other laboratory/metabolic investigations were carried out in a case-by-case approach. Diagnosis were confirmed by biochemical, enzymatic, and/or molecular testing, when available.

Results

During the study period, we analyzed 16 infants with NC and MLD with a mean age at presentation of 3.5 weeks (1–8 weeks) and a male preponderance (56.2%). Regarding the outcome, four patients died and three received orthotopic liver transplant (OLT)—Table 1. Retrospective analysis of clinical and laboratorial features allowed the categorization into three different groups: A - *NC with liver failure* ($N = 8$): tyrosinemia type I ($n = 2$), classic galactosemia ($n = 5$), mitochondrial DNA depletion syndrome ($n = 1$); B - *NC evolving with chronic liver disease* ($N = 5$): argininemia ($n = 2$); mitochondrial cytopathy ($n = 1$); congenital disorders of glycosylation type Ia ($n = 1$); Zellweger syndrome ($n = 1$); and C - *transient NC* ($N = 3$): Niemann-Pick type C ($n = 2$), citrullinemia type II ($n = 1$).

Group A—neonatal cholestasis with liver failure

Cases #1 and 2 These patients presented before the expanded NBS program.

Table 1 Clinical, biochemical, and molecular data

	Patient no.	Sex	Year of birth	Parental consanguinity	Week of cholestasis	TB/CB (mg/dl)	AST/ALT GGT (UI/L)	Hepatomegaly	Failure to thrive	Hypotonia/ development delay	Diagnosis	Molecular study	Cholestasis resolution	Outcome (liver disease/other)
Group A	1	M	1990	No	8	NA	NA	Yes	Yes	No	TYR I	ND	No	OLT/none
	2	M	1993	No	8	3.1/1.9	230/157 256	Yes	Yes	No	TYR I	ND	8 weeks	3rd OLT/none
	3	M	1989	Yes (first cousins)	2	26.0/3.8	254/258 150	Yes	Yes	No	GAL	ND	NA	None/none (lost from follow-up after 18 years old)
	4	F	1991	NA	4	8.7/7.4	119/103 45	Yes	Yes	No	GAL	ND	NA	None/ovarian failure, osteopenia
	5	M	1993	NA	2	17.0/7.0	245/NA 299	Yes	Yes	No	GAL	Homozygous Q188R mutation (GALT gene)	10 weeks	None/cognitive impairment, osteopenia
	6	M	1997	No	1	9.5/8.4	211/NA 103	Yes	Yes	No	GAL	Homozygous Q188R mutation (GALT gene)	4 weeks	None/osteopenia
	7	F	1998	No	1	16.5/10	134/124 NA	Yes	Yes	No	GAL	Homozygous Q188R mutation (GALT gene)	5 weeks	None/ovarian failure, osteopenia
	8	M	2015	No	4	3.3/1.34	101/51 1109	No	Yes	Yes	DGUOK	Compound heterozygous mutation (DGUOK gene)	No	Died at 8 months old
Group B	9	F	1992	No	8	10.8/9.4	800/530 80	Yes	No	No	ARG	Homozygous R21X mutation (ARG1 gene)	No	OLT/none
	10	F	2009	Yes (first cousins)	3	5.7/1.6	51/38 1295	No	No	No	ARG	Homozygous R21X mutation (ARG1 gene)	5 weeks	None/none
	11	M	2005	No	1	6.5/3.2	150/200 89	Yes	No	Yes	CDG Ia	ND	8 weeks	Transaminitis/severe neurological impairment
	12	M	2010	No	8	3.9/2.3	99/52 547	Yes	Yes	Yes	M CYT	Homozygous mutation (EARS2 gene)	6 months	Transaminitis/ neurological impairment
	13	F	2015	No	3	9.27/8.64	616/204 206	Yes	Yes	Yes	ZEL	Homozygous mutation (PEX12 gene)	No	Died at 9 months old
Group C	14	F	1983	No	1	7.2/3.6	NA	Yes	No	No	NP-C	ND	12 weeks	Died at 9 years old
	15	F	1983	No	1	8.3/3.8	NA	Yes	No	No	NP-C	ND	12 weeks	Died at 9 years old
	16	M	2007	No	1	12.7/1.8	80/93 131	Yes	Yes	No	CIT II	Compound heterozygous mutation (SLC25A13 gene)	5 months	None/none

Tyr I, tyrosinemia type 1; *Gal*, galactosemia; *ARG*, argininemia; *CDG Ia*, congenital disorder of glycosylation type Ia; *M CYT*, mitochondrial cytopathy; *ZEL*, Zellweger syndrome; *NP-C*, Niemann-Pick disease type C; *CIT II*, citrullinemia type II; *OLT*, orthotopic liver transplantation; *NS*, neurosensorial; *NA*, non-available; *ND*, not done

Male patients with NC and liver dysfunction during the second month of life, in addition to craneotabes and renal tubulopathy. High urinary succinyl acetone suggested the diagnosis of *tyrosinemia type 1*, confirmed by enzymatic assay on fibroblasts. Patient 1 underwent OLT at the age of 11 months due to progression to liver failure, with no major complications. Patient 2, despite treatment with nitisinone since 3 months of age, received a first OLT at the age of 3 years due to a dysplastic nodule with suspected malignancy (later non-confirmed); complications related to OLT led to a second and then a third OLT.

Cases #3–7 Newborn infants with similar presentation of acute sepsis-like syndrome on the first 2 weeks of life, characterized by vomiting and hemodynamic instability (3 patients had *E. coli* sepsis). Laboratory workup revealed cholestatic liver dysfunction with coagulopathy and hypoalbuminemia. Physical examination showed hepatomegaly and congenital cataracts in patient #7. The diagnosis of *galactosemia* was confirmed by enzymatic assay in blood cells. Three patients had diagnostic confirmation by molecular study of GALT gene (Table 1). The liver disease resolved under galactose restriction.

Case #8 Male neonate with secondary biomarkers of liver disease (raised tyrosine and methionine) identified by the extended NBS program. On day 12, blood tests showed acidosis (pH 7.29), hyperlactacidemia (lactate 4.16 mmol/L), hypoglycemia, and coagulopathy. By the age of 2 months, he developed cholestatic liver injury (Table 1), severe hypotonia, rotational nystagmus, and cardiomyopathy. Genetic study confirmed the diagnosis of *mtDNA depletion syndrome* (compound heterozygous mutation c.677A>G (p.H226R) and c.749T>C (p.L250S) in DGUOK gene). He died at 8 months old due to an infection leading to acute-on-chronic liver failure.

Group B—neonatal cholestasis evolving with chronic liver disease

Cases #9 and 10 were previously described by our group [5, 14, 29].

Case #9 Two-month-old female with new onset of jaundice and hepatosplenomegaly. The elevation in plasmatic arginine to 1756 μmol/L (normal range 22–88) and ammonia led to the suspicion of *argininemia*, confirmed by the absence of arginase A1 activity in blood red cells and the molecular analysis of ARG1 gene (homozygous for R21X mutation). The patient had progressive biliary cirrhosis complicated with portal hypertension in the absence of neurological impairment. At the age of 7 years, she underwent successful OLT.

Case #10 Asymptomatic neonate, second child of first-degree consanguineous parents, diagnosed with *argininemia* through NBS (arginine level of 360 μM on day 5). At 21 days of age, she was found to have cholestatic jaundice and hyperammonemia. Plasmatic arginine was high (1600 μmol/L, $N < 140$) so as urinary orotic acid (5.3 μmol/mmol creat, $N = 0.1$). Homozygous R21X mutation on the ARG1 gene confirmed the diagnosis. Under proper diet and medical treatment, cholestasis resolved before 3 months old.

Case #11 Male newborn with cholestatic jaundice detected in the first week of life associated with severe feeding difficulties and failure to thrive. Coagulopathy was also predominant due to low prothrombin and antithrombin III. Physical examination revealed hepatomegaly and dysmorphic features (abnormal distribution of fat, inverted nipples, hypogonadism) that led to the suspicion of a congenital disorder of glycosylation (CDG), confirmed by isoelectric transferrin focusing (*CDG type 1a*).

Case #12 Male infant who had physiologic jaundice in the first week of life after which he developed a cholestatic pattern detected in the eighth week, in addition to episodes of hypoglycemia, metabolic acidosis, and hyperlactacidemia. He also had feeding difficulties and failure to thrive. On the physical exam, he had hepatomegaly in association to global hypotonia and hyperreflexia. Magnetic resonance imaging showed features of leukoencephalopathy involving the thalamus and brainstem. The genetic study revealed a homozygous mutation on the gene EARS2, confirming a *nuclear mitochondrial disorder*.

Case #13 Female patient presenting with NC during the first month of life. She had severe hypotonia and dysmorphic features (dolichocephaly, high forehead, large fontanelles). The very long-chain fatty acids in plasma were greatly increased. The PEX1 gene had no mutations. A diagnosis of *Zellweger syndrome* was confirmed by the identification of a homozygous mutation in the PEX12 gene from a NGS panel of genes associated with peroxisomal disorders. She died at 9 months of age.

Group C—transient neonatal cholestasis

Cases #14 and 15 Twin females who presented at a neurology consultation at the age of 5 years because of marked cognitive impairment and ataxia. Upon physical examination, hepatosplenomegaly was noted. Retrospectively, they were found to have had cholestatic liver disease in the first week of life. Filipin staining of skin fibroblasts was positive, confirming a diagnosis of *Niemann-Pick type C* (NP-C).

Case #16 Male newborn presenting with cholestasis in the first week of life associated with poor weight gain. Developmental milestones and neurological exam were normal. The cholestasis resolved spontaneously by 5 months of age. A diagnosis of *citrullinemia type II* was suggested by hyperammonemia and raised citrulline and methionine. It was later confirmed by molecular study (a compound heterozygous mutation c.1056-1060 del A/ c.1231-1G>A in gene SLC25A13 gene).

Discussion

Recent advances in molecular genetics have led to NGS technology, resulting in a dramatic reduction in the time and cost required to perform DNA mutation analyses [2]. In contrast to Sanger sequencing, where genes are sequenced one at a time, in NGS, the entire sequence or a significant portion of the sequence of DNA is sequenced in a single procedure, improving the diagnostic efficiency particularly in entities with broad differential diagnoses, such as MLD. In clinical practice, NGS can be used to look for specific conditions (like Sanger sequencing) or as part of a standard panel [13, 15]. These panels are increasingly becoming an important procedure when the standard workup is unsuccessful, but no one has yet established guidelines for their use. Some centers have built NGS panels for NC, comprising a variable number of genes, designed to include not only IEM but also genetic cholestatic disorders [13, 31], irrespective of the clinical phenotype [25].

At first glance, the idea of NGS panels as a diagnostic tool for suspected MLD allowing the decentralization of the diagnosis of these patients is undoubtedly appealing. However, we believe that albeit exciting to have access to such a powerful instrument, NGS should be applied after a pre-test counseling to avoid a burden of interpretative challenges. In fact, NGS can detect variants of uncertain significance, not definitively linked to a disorder [16]. It is important to stress that the meaning of a gene mutation or polymorphism should be matched within the clinical context [9, 11], and in monogenic disorders where there is a predictable genotype-phenotype match [25], NGS panels have no advantage over the Sanger technique. In addition, it is critically important to state that it takes weeks to report the results of NGS; therefore, it is advisable not to depend on this instrument to diagnose urgent and treatable conditions. Finally, the cost-effectiveness of NGS needs to be assessed as it is still far more expensive than clinically oriented biochemical tests.

At our institution, infants with NC undergo a stepwise evaluation in which detailed clinical and analytical assessments are the main crossroads, fundamental to pinpointing the diagnosis [9, 34]. Additional and more specific tests are tailored according to the presenting features and suspected diagnosis (Table 2). Concerning the NGS technology, until the compilation of the last patient in this case series in 2015, we only had sub-panels for MLD subgroups, such as peroxisomal disorders (patient #13). It was only since mid-2017 that we had available a customized NGS panel comprising 54 genes related not only to IEM presenting with NC but also to genetic cholestatic disorders.

Our data provide a liver-targeted characterization of MLD presenting with NC, categorizing them into three clinical phenotypes that may narrow the diagnostic possibilities: *NC with liver failure*, *NC evolving with chronic liver disease*, and *transient NC*. Nine out of 16 cases had molecular studies (eight by Sanger sequencing), which was crucial to confirm the diagnosis in two (#8 and #12); only one patient was diagnosed by targeted NGS panel (#13). Among the remaining seven patients, five underwent invasive procedures that could have been avoided if genetic studies had been available in due time. Overall, NGS panel would have advantage over Sanger sequencing in 4 patients (#8, #12, #14, and #15).

It is true that in some instances, the inclusion of targeted NGS or a panel-based approach in diagnostic processes may prove useful. However, due to time and other logistic restraints, it is difficult to apply the NGS approach to individual patients with MLD irrespective of their clinical phenotype. The paradigmatic example is the category of patients with *NC with liver failure*, which represents a medical emergency. The differential diagnosis includes, among others, classic galactosemia [4], tyrosinemia type I [21], and mitochondrial disorders. In both galactosemia and tyrosinemia type I, the clinical assessment in combination with specific biochemical markers (Table 2) can guide towards the specific diagnosis. In these settings, establishing a timely and accurate diagnosis is fundamental to promptly institute a specific therapy. Later on, the Sanger technique may confirm diagnosis. Thus, a panel-based approach in such cases appears counter-wise. Nevertheless, in some other entities, the metabolite profile can be abnormal but not characteristic or, if present, the gold-standard investigations can be invasive. An example is the mitochondrial DNA depletion syndromes (e.g., DGUOK gene mutations) that have a severe course characterized by the onset in infancy of progressive LF and neurological abnormalities [12, 17, 18] (Table 1), as observed in patient #8. Respiratory chain disorders can also present with LF, but they often manifest with cholestatic jaundice in association with minor liver disease (e.g., transaminitis), as exemplified by patient #12. In both mitochondriopathies, multisystemic involvement (mainly neurological involvement) along with metabolic acidosis and hyperlactacidemia are important clues. However, the specific diagnosis is highly dependent on invasive diagnostic procedures (liver and/or muscle biopsies), and gene expression can be heterogeneous limiting the application of the traditional Sanger technique. In these cases, we believe that a targeted NGS or an NGS panel could be a valuable tool to overcome these diagnostic difficulties and at the same time determine the specific mutation, avoiding futile OLT and

Table 2 Suggested workup during evaluation of cholestatic infant with suspected MLD

	Clinical clues	Laboratory investigation	Molecular analysis (gene)
NC with liver failure			
Galactosemia (OMIM 230400)	Congenital cataracts Gram-negative septicemia	Urinary reducing substances Galactose-1-phosphatase uridyl transferase activity in red blood cells (↑)	GALT
Tyrosinemia type 1 (OMIM 276700)	Rickets Renal tubular dysfunction	Plasma and urine amino acids Plasma alpha-fetoprotein Urine organic acids—succinylacetone (↑)	FAH
Mitochondrial DNA depletion syndrome (OMIM 203700, 251880, 256810)	Multisystemic involvement (neurological—hypotonia, rotational nystagmus; cardiac—cardiomyopathy)	Metabolic acidosis Lactate, piruvate Biopsy (muscle/liver)	POLG DGUOK MPV17
Wolman disease (OMIM 278000)	Hepatosplenomegaly Adrenal calcification Diarrhea and malabsorption Failure to thrive	Lipid profile (type IIb dysliproteinemia) Peripheral blood smear (lymphocytes with cytoplasmic vacuolation) Lysosomal lipase acid (↓) in peripheral blood monuclear cell	LIPA
NC with transient cholestasis			
Neonatal intrahepatic cholestasis caused by citrin deficiency (OMIM 605814)	Feeding difficulties Failure to thrive Fatty liver	Plasma ammonia (↑) Urine organic acids Plasma amino acids (↑citrulline and methionine)	SLC25A13
Niemann-Pick type C (OMIM 257220)	Hepatosplenomegaly Vertical ophtalmoplegia and ataxia (later).	Plasma LDL and HDL (↓), triglycerides (↑) Plasma chitotriosidase (↑) Skin biopsy (filipin staining of skin fibroblasts)	NPC1 (> 95%) NPC2 (4%) Other genes (1%)
Peroxisomal disorders Infantile Refsum disease (OMIM 601539) Zellweger syndrome (OMIM 214100)	Craniofacial dysmorphism (dolichocephaly, esotropia, epicanthic folds, broad nasal bridge, high-arched palate, low-set ears, and anteverted nostrils) Severe axial hypotonia Pigmentar retinitis Neurosensorial hearing loss	Plasma very long-chain fatty acids (↑) Pattern of plasmalogens Urine organic acids (↑phytanic acid, pristanic acid)	PEX1 or PEX 6 (majority); PEX12, PEX26, PEX10, PEX2, PEX5, PEX13, PEX16, PEX3, PEX19, PEX14, PEX11β
NC with chronic liver disease			
Argininemia (OMIM 207800)	Feeding difficulties Vomiting	Plasma ammonia (↑) Amino acids profile Arginase A1 activity in blood red cells	ARG1
Congenital disorders of glycosylation type 1a (OMIM 212065)	Dysmorphic features (abnormal distribution of fat, inverted nipples, hypogonadism) Failure to thrive Neurological involvement	Plasma transferrin isoforms Triglycerides ↑ Coagulopathy (ATIII ↓, factor XI ↓, protein C and S ↓)	PMM2
Progressive familial intrahepatic cholestasis syndromes (PFIC) type 1 and 2 (OMIM 211600; 601847)	Pruritus	GGT (normal or ↓)	ATP8B1 (PFIC 1) ABCB11 (PFIC 2)
Defects in bile acid metabolism - Δ(4)-3-oxosteroid 5-β-reductase deficiency (OMIM 235555) - 3β-hydroxy-Δ5-C27-steroid dehydrogenase deficiency (OMIM 607765) - Oxysterol 7α-hydroxylase deficiency (OMIM 613812)	No pruritus	Normal serum bile acid levels Urinary bile acid analysis	AKR1D1 HSD3B7 CYP7B1

LDL, low-density lipoprotein; *HDL*, high-density lipoprotein

allowing genetic counseling. Figure 1 illustrates a proposed clinical-based diagnostic algorithm for MLD presenting with NC followed at our institution, and it additionally incorporates NGS.

In the other two clinical phenotypes with normal liver synthetic function, the differential diagnosis is broad and includes, among others, argininemia, CDG, and mitochondrial disorders—*NC evolving with chronic liver disease*—and citrin deficiency, peroxisomal biogenesis disorders (Refsum disease), and NP-C—*transient NC*. Argininemia is the second most common urea cycle disease in Portugal [14, 32]. Although moderate transaminitis may be observed in all urea cycle defects, NC as the first presentation of hyperargininemia is rare, but well-established by our group [14]. It is now diagnosed through the expanded NBS program, thus overcoming the initial diagnostic difficulties. CDG, similar to mitochondrial hepatopathies and peroxisomal biogenesis disorders, presents with multisystemic manifestations early in life [10, 19, 20] and can also have a wide spectrum of liver involvement, from mild disease (most common), as observed in patient #12, to liver failure (rarely). Chronic liver disease occurs in a minority of the reported CDG types (22%) [19] and the prognosis is poor, often precluding liver transplantation. Additional clinical and biochemical clues for the diagnosis are depicted in Table 2. However, the diagnosis may not be straightforward and these MLD may be candidates for an NGS-based approach due to extreme clinical and genotypic heterogeneity, complexity, and/or the invasiveness of traditional diagnostic approaches [13].

MLD causing transient cholestasis (i.e., citrin deficiency, Refsum disease, and NP-C) pose a diagnostic challenge as they can be misinterpreted as prolonged physiologic jaundice and the diagnosis can be missed early in the disease course. NP-C is the paradigmatic example of this heterogeneous group of disorders, with manifestations occurring along a continuum in the disease course. The neonatal-onset NP-C has a more aggressive clinical course [33] and remains the most challenging. Neonatal cholestasis, the first phase of the disease (and sometimes the only presenting symptom), is usually self-limiting, with resolution at 2–4 months of age [23]. Additionally, other NP-C manifestations are not specific and only appear later in the disease course, e.g., hepatosplenomegaly and central nervous system involvement as occurred in our twin patients. In these cases, when there are no clear pointers to the diagnosis, NGS panels or even whole exome sequencing can be considered to improve the diagnostic yield (Fig. 1). However, it should be ascertained whether it is cost-effective to routinely apply an NGS panel to all transient NC.

Finally, we highlight other MLD causing cholestatic liver disease in infancy not present in our cohort: Wolman disease [6, 27], progressive familiar intrahepatic cholestasis (PFIC) [28], and bile acid synthesis defects [8] (Table 2). These disorders are being increasingly recognized thanks to the application of NGS panels reducing the percentage of idiopathic cases [25]. In our cohort of patients with NC, some patients deceased without a definitive diagnosis. Therefore, we believe that this may be a subgroup of patients in which NGS

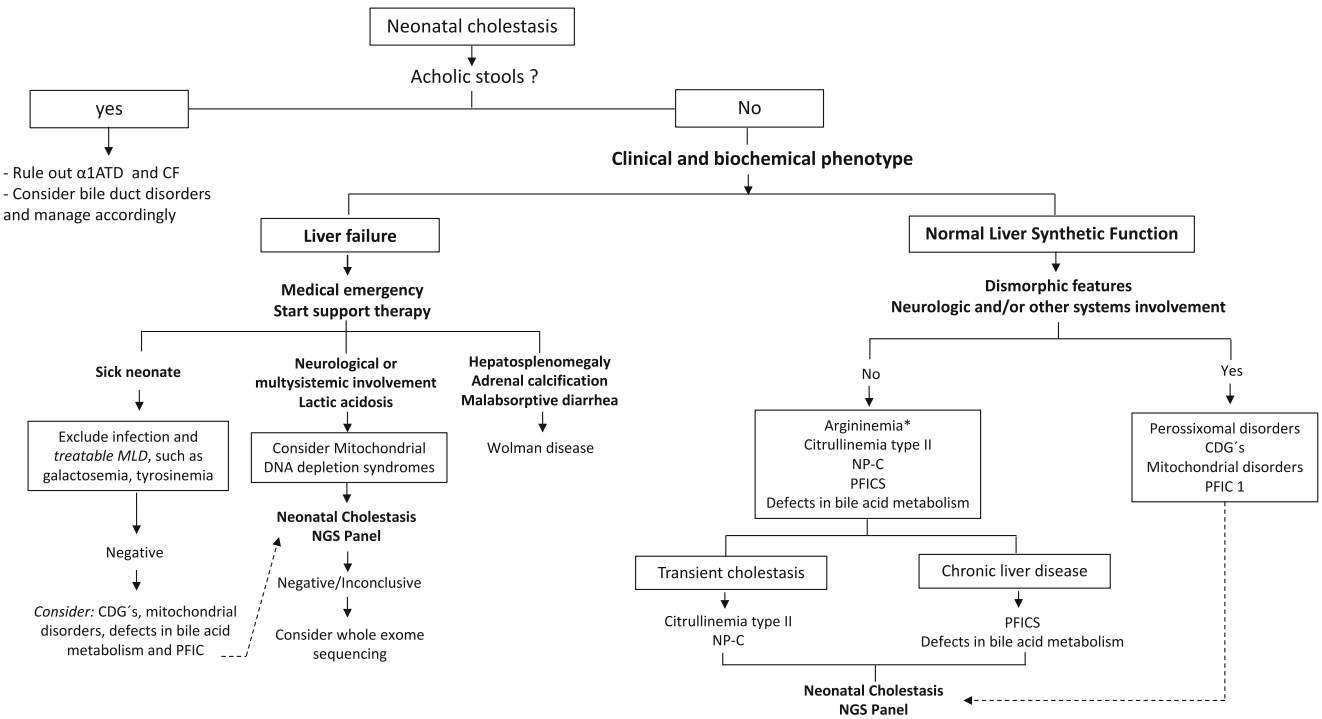

Fig. 1 Diagnostic algorithm to identify MLD in neonatal cholestasis. An asterisk indicates that it is identified through the NBS program

technology could have an added value, allowing to increase the diagnostic efficiency, provided that there are stored samples.

In summary, our study highlights the critical role of clinical and biochemical evaluations for decision-making in the setting of MLD. The proposed "phenotypic" categorization can be achieved in a tertiary center with high level of competence. NGS is an emerging and appealing tool that in our opinion should be judiciously applied and not used as a first-line approach nor in a decentralized model of care. Although we believe that NGS will never replace clinical and biochemical assessments in the management of MLD, incorporating NGS into the diagnostic algorithm of MLD may improve the accuracy of diagnosis. However, it may be advisable to centralize NGS technology in a few hospital centers (tertiary centers) with clinical and laboratorial expertise to overcome some of its restraints. Further work is required to formally assess the cost-effectiveness of NGS and explore the optimal approach to the timing of NGS in the diagnosis of MLD.

Authors' Contributions Helena Moreira Silva: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript.

Inês Maio: acquisition of data, analysis and interpretation of data.

Anabela Bandeira and Esmeralda Martins: patient diagnosis and follow-up, analysis and interpretation of data; critical revision of the manuscript.

Ermelinda Santos Silva: patient diagnosis and follow-up, study concept and design; study supervision; critical revision of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by our institutional scientific and ethics committee [Study N/REF.ª 2016. 081 (069-DEFI/066-CES)].

Clinical trial registration Not applicable.

Informed consent Informed consent was obtained from all individual participants included in the study.

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References

1. Balistreri WF (1985) Neonatal cholestasis. J Pediatr 106(2):171–184
2. Behjati S, Tarpey PS (2013) What is next generation sequencing? Arch Dis Child Educ Pract Ed 98(6):236–238
3. Bodamer OA, Hoffmann GF, Lindner M (2007) Expanded newborn screening in Europe 2007. J Inherit Metab Dis 30:439–444
4. Bosch AM (2006) Classical galactosemia revisited. J Inherit Metab Dis 29:516–525
5. Braga AC, Vilarinho L, Ferreira E, Rocha H (1997) Hyperargininemia presenting as persistent neonatal jaundice and hepatic cirrhosis. J Pediatr Gastroenterol Nutr 24(2):218–221
6. Burton BK, Balwani M, Feillet F et al (2015) A phase 3 trial of sebelipase alfa in lysosomal acid lipase deficiency. N Engl J Med 373(11):1010–1020
7. Champion V, Carbajal R, Lozar J, Girard I, Mitanchez D (2012) Risk factors for developing transient neonatal cholestasis. J Pediatr Gastroenterol Nutr 55(5):592–598
8. Clayton PT (2011) Disorders of bile acid synthesis. J Inherit Metab Dis 34(3):593–604
9. Coene KLM, Kluijtmans LAJ, van der Heeft E, Engelke UFH, de Boer S, Hoegen B, Kwast HJT, van de Vorst M, Huigen MCDG, Keularts IMLW, Schreuder MF, van Karnebeek CDM, Wortmann SB, de Vries MC, Janssen MCH, Gilissen C, Engel J, Wevers RA (2018) Next-generation metabolic screening: targeted and untargeted metabolomics for the diagnosis of inborn errors of metabolism in individual patients. J Inherit Metab Dis 41(3):337–353
10. Damen G, de Klerk H, Huijmans J, den Hollander J, Sinaasappel M (2004) Gastrointestinal and other clinical manifestations in 17 children with congenital disorders of glycosylation type Ia, Ib, and Ic. J Pediatr Gastroenterol Nutr 38(3):282–287
11. Fawaz R, Baumann U, Ekong U, Fischler B, Hadzic N, Mack CL, McLin VA, Molleston JP, Neimark E, Ng VL, Karpen SJ (2017) Guideline for the evaluation of cholestatic jaundice in infants: joint recommendations of the North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition and the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition. J Pediatr Gastroenterol Nutr 64:154–168
12. Fellman V, Kotarsky H (2011) Mitochondrial hepatopathies in the newborn period. Semin Fetal Neonatal Med 16(4):222–228
13. Ghosh A, Schlecht H, Heptinstall LE (2017) Diagnosing childhood-onset inborn errors of metabolism by next-generation sequencing. Arch Dis Child 102(11):1019–1029
14. Gomes Martins E, Santos Silva E, Vilarinho S, Saudubray JM, Vilarinho L (2010) Neonatal cholestasis: an uncommon presentation of hyperargininemia. J Inherit Metab Dis 33(Suppl 3):S503–S506
15. Herbst SM, Schirmer S, Posovszky C, Jochum F, Rödl T, Schroeder JA, Barth TF, Hehr U, Melter M, Vermehren J (2015) Taking the next step forward - diagnosing inherited infantile cholestatic disorders with next generation sequencing. Mol Cell Probes 29(5):291–298
16. Kuhlenbäumer G, Hullmann J, Appenzeller S (2011) Novel genomic techniques open new avenues in the analysis of monogenic disorders. Hum Mutat 32:144–151
17. Mancuso M, Ferraris S, Pancrudo J, Feigenbaum A, Raiman J, Christodoulou J, Thorburn DR, DiMauro S (2005) New DGK gene mutations in the hepatocerebral form of mitochondrial DNA depletion syndrome. Arch Neurol 62:745–747
18. Mandel H, Szargel R, Labay V, Elpeleg O, Saada A, Shalata A, Anbinder Y, Berkowitz D, Hartman C, Barak M, Eriksson S, Cohen N (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nature Genet 29:337–341
19. Marques-da-Silva D, dos Reis Ferreira V, Monticelli M, Janeiro P, Videira PA, Witters P, Jaeken J, Cassiman D (2017) Liver involvement in congenital disorders of glycosylation (CDG): a systematic review of the literature. J Inherit Metab Dis 40:195–207
20. Matthijs G, Schollen E, Pardon E, Veiga-Da-Cunha M, Jaeken J, Cassiman JJ, Van Schaftingen E (1997) Mutations in PMM2, a phosphomannomutase gene on chromosome 16p13, in carbohydrate-deficient glycoprotein type I syndrome (Jaeken syndrome). Nature Genet 16:88–92
21. Mayorandan S, Meyer U, Gokcay G, Segarra NG, de Baulny HO, van Spronsen F, Zeman J, de Laet C, Spiekerkoetter U, Thimm E,

Maiorana A, Dionisi-Vici C, Moeslinger D, Brunner-Krainz M, Lotz-Havla AS, Cocho de Juan JA, Couce Pico ML, Santer R, Scholl-Bürgi S, Mandel H, Bliksrud YT, Freisinger P, Aldamiz-Echevarria LJ, Hochuli M, Gautschi M, Endig J, Jordan J, McKiernan P, Ernst S, Morlot S, Vogel A, Sander J, Das AM (2014) Cross-sectional study of 168 patients with hepatorenal tyrosinaemia and implications for clinical practice. Orphanet J Rare Dis 9(1):107

22. McKiernan PJ (2002) Neonatal cholestasis. SeminNeonatol 7(2): 153–165
23. Mengel E, Klünemann HH, Lourenço CM, Hendriksz CJ, Sedel F, Walterfang M, Kolb SA (2013) Niemann-Pick disease type C symptomatology: an expert-based clinical description. Orphanet J Rare Dis 8:166
24. Moyer V, Freese DK, Whitington PF, Olson AD, Brewer F, Colletti RB, Heyman MB (2004) Guideline for the evaluation of cholestatic jaundice in infants: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. J Pediatr Gastroenterol Nutr 39(2):115–128
25. Nicastro E, D'Antiga L (2018) Next generation sequencing in pediatric hepatology and liver transplantation. Liver Transpl 24(2): 282–293
26. Santos Silva E, Moreira Silva H, Azevedo Lijnzaat L, Melo C, Costa E, Martins E, Lopes AI (2017) Clinical practices among healthcare professionals concerning neonatal jaundice and pale stools. Eur J Pediatr 176(3):361–369
27. Santos Silva E, Klaudel-Dreszler M, Bakuła A, Oliva T, Sousa T, Fernandes PC, Tylki-Szymańska A, Kamenets E, Martins E, Socha P (2018) Early onset lysosomal acid lipase deficiency presenting as secondary hemophagocytic lymphohistiocytosis: two infants treated with sebelipase alfa. Clin Res Hepatol Gastrenterol 42(5):e77–e82
28. Schukfeh N, Metzelder ML, Petersen C, Reismann M, Pfister ED, Ure BM, Kuebler JF (2012) Normalization of serum bile acids after partial external biliary diversion indicates an excellent long-term outcome in children with progressive familial intrahepatic cholestasis. J Pediatr Surg 47(3):501–505
29. Silva ES, Cardoso ML, Vilarinho L, Medina M, Barbot C, Martins E (2013) Liver transplantation prevents progressive neurological impairment in argininemia. JIMD Rep 11:25–30
30. Squires RH, Shneider BL, Bucuvalas J, Alonso E, Sokol RJ, Narkewicz MR, Dhawan A, Rosenthal P, Rodriguez-Baez N, Murray KF, Horslen S, Martin MG, Lopez MJ, Soriano H, McGuire BM, Jonas MM, Yazigi N, Shepherd RW, Schwarz K, Lobritto S, Thomas DW, Lavine JE, Karpen S, Ng V, Kelly D, Simonds N, Hynan LS (2006) Acute liver failure in children: the first 348 patients in the pediatric acute liver failure study group. J Pediatr 148(5):652–658
31. Togawa T, Sugiura T, Ito K, Endo T, Aoyama K, Ohashi K, Negishi Y, Kudo T, Ito R, Kikuchi A, Arai-Ichinoi N, Kure S, Saitoh S (2016) Molecular genetic dissection and neonatal/infantile intrahepatic cholestasis using targeted next-generation sequencing. J Pediatr 171:171–7.e1–4
32. Vilarinho L, Rocha H, Sousa C, Marcão A, Fonseca H, Bogas M, Osório RV (2010) Four years of expanded newborn screening in Portugal with tandem mass spectrometry. J Inherit Metab Dis 33(Suppl 3):S133–S138
33. Yerushalmi B, Sokol RJ, Narkewicz MR, Smith D, Ashmead JW, Wenger DA (2002) Niemann-Pick disease type C in neonatal cholestasis at a North American center. J Pediatr Gastroenterol Nutr 35(1):44–50
34. Yubero D, Brandi N, Ormazabal A, Garcia-Cazorla À, Pérez-Dueñas B, Campistol J, Ribes A, Palau F, Artuch R, Armstrong J (2016) Targeted next generation sequencing in patients with inborn errors of metabolism. PLoS One 11(5):e0156359