nature publishing group

Relevance of C5b9 immunostaining in the diagnosis of neonatal hemochromatosis

Estelle Dubruc[1], Béatrice Nadaud[1], Eduardo Ruchelli[2], Sophie Heissat[3], Julien Baruteau[4], Pierre Broué[4], Dominique Debray[5], Marie-Pierre Cordier[6], Pierre Miossec[7,8], Pierre Russo[2] and Sophie Collardeau-Frachon[1,8,9]

BACKGROUND: Neonatal hemochromatosis caused by a gestational alloimmune mechanism or gestational alloimmune liver disease (GALD) is a rare perinatal disorder characterized by intra- and extrahepatic iron overload. It is believed to result from complement-mediated liver injury, in which the classical complement pathway is activated by maternal antibody/fetal antigen complexes, leading to hepatocyte lysis by the membrane attack complex C5b9. According to some authors, C5b9 expression in more than 75% of liver parenchyma is specific for GALD.

METHODS: We conducted a retrospective multicentric immunohistochemical study with anti-C5b9 in GALD cases ($n = 25$) and non-GALD cases with iron overload ($n = 36$) and without iron overload ($n = 18$).

RESULTS: C5b9 was expressed in 100% of GALD cases but involved more than 75% of the liver parenchyma in only 26% of the cases. C5b9 was detected in 26.75% of the non-GALD cases with more than 75% of positive parenchyma in maternal erythrocytic alloimmunization, herpes and enterovirus hepatitis, bile acid synthetic defect, DGUOK mutation, Gaucher disease, cystic fibrosis, and giant-cell hepatitis with autoimmune hemolytic anemia.

CONCLUSION: Diagnosis and therapeutic management of GALD cannot only be based on C5b9 expression in liver samples as it is not specific of this disease.

Neonatal hemochromatosis (NH) (OMIM 231100) is a rare perinatal disorder that occurs in fetuses and neonates. However, it is the most common cause of acute liver failure in newborns (1). It often causes unexpected intrauterine fetal death in the late-second and third trimester. NH is defined as the coexistence of severe liver injury and siderosis of hepatic and extrahepatic tissues in a distribution similar to that observed in hereditary hemochromatosis (2). However, several etiologies could lead to severe perinatal liver injury and NH is now recognized as a phenotype (3,4). Among the causes that can lead to such a phenotype, gestational alloimmune mechanism would be the most common cause (4,5). The alloimmune mechanism hypothesis, suggested by Whitington *et al.* in 2004 (6,7), was based on a high recurrence rate >80% similar to rhesus incompatibility and supported by intravenous immunoglobulin (IV-Ig) therapy efficiency. Indeed, administration of IgIV during pregnancy reduces the severity and the recurrence rate of the disease (8,10). In neonates, immunotherapy really improves their outcome (5,11–13).The liver allloimmune injury would result from complement activation by transplacental transfer of maternal IgG antibodies directed against a fetal antigen, presumably a fetal liver protein but currently still unknown (14). The complex of maternal antibody/fetal antigen is presumed to activate the complement classical pathway, leading to the formation of the membrane attack complex or terminal complement cascade or C5b9 on hepatocytes and cause hepatocytes lysis and liver parenchyma injury. Hepatic and extrahepatic iron overload would be secondary tissue damages resulting from complement mediated injury on hepatocytes (15). Complement mediated injury on hepatocytes could be demonstrated by immunohistochemical study using anti-C5b9. Whitington *et al.* conducted two retrospective studies with anti-C5b9 and showed that C5b9 was expressed in more than 75% of liver parenchyma in cases due to alloimmune mechanisms compared to control cases (14,16). According to them, the expression of C5b9 in liver parenchyma is specific for an alloimmune mechanism (4,5). Therefore, the name "gestational alloimmune liver disease" (GALD) should be applied for cases in which complement-mediated hepatocyte injury can be demonstrated by positive C5b-9 immunostaining on the hepatocytes (4,5).

We wanted to verify the specificity of C5b9 for the diagnosis of GALD. Indeed, immunotherapy by IVIg is very expensive and may have side effects (17). Indications for such treatment require a solid demonstration of GALD (18). Furthermore, the two studies conducted by Whitington lacked technical details about C5b9 immunostaining and included only few

[1]Department of pathology, Hôpital Femme-Mère-Enfant, CHU de Lyon, France; [2]Department of pathology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania; [3]Department of pediatrics, Hôpital Femme-Mère-Enfant, CHU de Lyon, France; [4]Pediatric Hepatology and Inborn Metabolic Diseases Unit, University Children's Hospital, Toulouse, France; [5]Department of Pediatric Surgery, Hepatology, and Transplantation, Necker Enfants Malades Hospital, Paris, France; [6]Department of genetics, Hôpital Femme-Mère-Enfant, CHU de Lyon, France; [7]Department of immunology, Hôpital Edouard Herriot, CHU de Lyon, France; [8]Université Claude Bernard Lyon 1, France; [9]SOFFOET, Société Française de Foetopathologie, Paris, France. Correspondence: Sophie Collardeau-Frachon (sophie.collardeau-frachon@chu-lyon.fr)

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cases of nonalloimmune NH (14,16). In addition, some studies reported positive C5b9 immunostaining in fulminant or acute hepatitis B, drug-induced hepatitis, and autoimmune hepatitis especially in giant cell hepatitis with autoimmune hemolytic anemia (19–21). Furthermore, some authors suggested that NH could have an autoimmune origin (22,23). To date, the diagnosis of alloimmune NH is difficult as there are no specific clinical or biological signs. It is often a diagnosis of exclusion after elimination of other fetal and neonatal liver diseases with a NH phenotype. If immunohistochemistry for C5b9 is highly specific for alloimmune NH cases, it could then represent a major tool to establish a definitive diagnosis of GALD. In order to evaluate the value of C5b9 immunostaining in the diagnosis of GALD, we conducted a retrospective multicentric immunohistochemical study with anti-C5b9 in the following four groups: GALD cases (group 1), nonalloimmune NH cases (group 2), neonatal liver disease without NH phenotype (group 3), and normal liver controls (group 4).

METHODS

Groups' Constitution

GALD cases (group 1) and non-GALD cases with NH phenotype (group 2) were collected between 2000 to 2015 from our department (local cases and consultation cases from France as a referral center for such cases) and the department of pathology of the Children's Hospital of Philadelphia. Some cases came from our previous retrospective study (23). Diagnosis of GALD had been made after medical consensus among our French national multidisciplinary committee of NH (24,25).

Cases belonging to group 2 (non-GALD cases with NH phenotype) were ascertained by searching our databases for etiologies that have been reported in the literature to date: infections (parvovirus B19, CMV, HSV), mitochondrial cytopathies (*DGUOK* mutations, Gracile syndrome), alloimmunization anti-red blood cells, Down syndrome, Edward syndrome, Martinez-Frias, tricho-hepato-enteric syndrome, D4-oxosteroid reductase deficiency, Zellweger syndrome, Donohue syndrome, neonatal lupus (3,4), transaldolase deficiency (26), familial lymphohistiocytosis (27,28), and chromosome 16p duplication (29). The presence of hemosiderosis was checked by Perls staining on liver samples.

For non-GALD cases without NH phenotype (group 3), we looked for similar cases as studied by Whitington *et al.* (14), but we decided to study only those occurring in neonates less than 3 mo of age: biliary atresia, progressive familial intrahepatic cholestasis (PFIC) type 1 (PFIC1) and 2 (PFIC 2), total parenteral nutrition-associated cholestasis after bowel resection, Alagille syndrome, alpha-1-antitrypsin deficiency, tyrosinemia type 1, abetalipoproteinemia, glycogen storage disease type 1, and inspissated bile syndrome after cardiac surgery. We also added cases of giant cell hepatitis with autoimmune hemolytic anemia. The absence of hemosiderosis was demonstrated by Perls staining on liver samples.

Our group of control cases (group 4) included cases without hepatic disease or iron overload and without infectious or immune injury that can activate membrane attack complex. These were autopsy liver samples from fetal or stillborn at five different gestational ages without microscopic lesion: four fetuses of 14, 22, 25, and 27 gestational weeks (GW) and one newborn of 1 mo of age. None of placentas examined in these cases displayed intervillitis, villitis, or chorioamnionitis. There were no infectious or autoimmune maternal clinical background and no infectious injury in other tissue samples.

Immunohistochemical Study

Immunohistochemistry was performed using a Ventana automate (Ultra-XT Benchmark, Ventana Medical Systems SA, Roche, France) on liver formalin-fixed paraffin-embedded tissues.

For C5b9, we used the same antibody as Whitington *et al.* (12,14): clone A239 from QUIDEL (San Diego, CA) and tried different dilutions 1/50, 1/100, and 1/150. A 1/150 dilution gave optimal results.

We used as external positive controls, the expression of C5b9 inside myocytes in ischemic areas of myocardium (30). We considered as internal positive controls, the expression of C5b9 on endothelial cells lining hepatic central veins without or minimal background staining in liver sections.

Sections processed without primary antibody were considered controls for nonspecific reaction. To explore false-positive results due to iron overload and cross reaction with C5b9 antibody, we performed immunostaining with anti-epithelial membrane antigen (EMA) (clone E29 Dako, DakoCytomation, Glostrup, Denmark, dilution: 1/100), anti-CK AE1/AE3 (clone AE1/AE3 Dako, DakoCytomation, dilution: 1/400), and anti-synaptophysin (clone SP11Ventana Medical Systems, Tucson, AZ, prediluted). No cross-reaction with iron overload was observed with C5b9, EMA, CK AE1/AE3, and synaptophysin antibodies.

Heat antigen retrieval at 99° was used in Cell Conditioning Solution, pH = 8, Ethylene Diamine Tetraacetic Acid (EDTA), (VENTANA Medical System). The incubation with primary antibodies was performed at 37 °C for 32 min for C5b9 and synaptophysin, 20 min for EMA, and 24 min for AE1/AE3, and the presence of antigens was revealed with the UltraView Universal DAB Detection Kit (Ventana Medical Systems), which is a biotin-free system and comprises a cocktail of Horse Radish Peroxidase (HRP)-labeled antibodies (goat anti-mouse IgG, goat anti-mouse IgM, and goat anti-rabbit).

C5b9 Expression in the Liver

The following features were evaluated on liver sections stained for C5b9 separately by two pathologists:

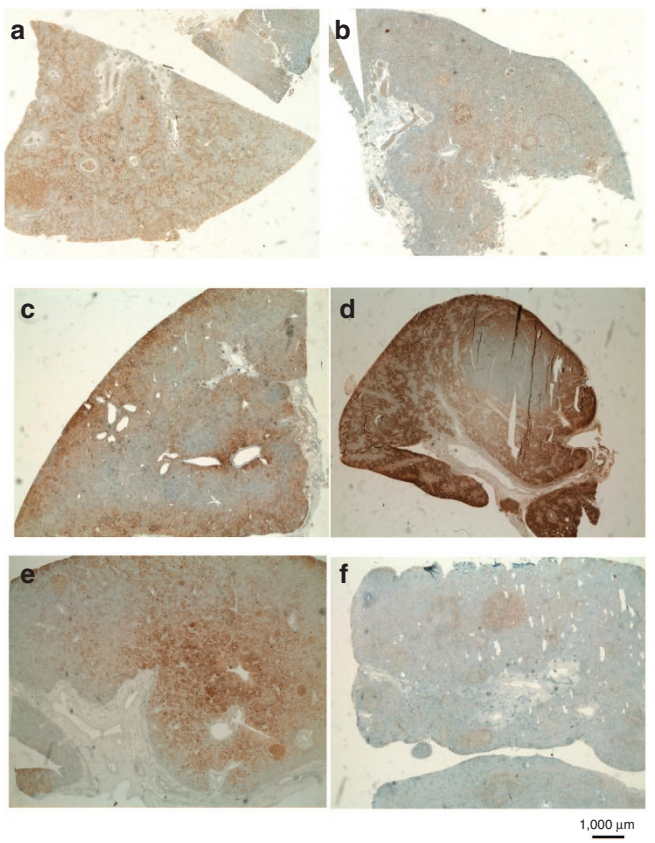

Figure 1. Topographic view of C5b9 immunostaining in autopsy GALD cases (group 1), magnification (×5). (**a**) Case 7 (fetus, 29GW+ 4d): 40% of liver parenchyma is stained. (**b**) Case 6 (fetus, 27 GW): 70% stained. (**c**) Case 24 (fetus, 32GW): 50% stained. (**d**) Case 13 (fetus, 26 GW): 75% stained. (**e**) case 24 (neonate, 34GW + 3 d): 50% stained. (**f**) Case 19 (neonate, 32GX+5d): 20% stained.

- Percentage of the stainable liver parenchyma at magnification ×5;
- Intensity at magnification ×40: (i) weak, moderate (ii), or strong (iii);
- Localization of the staining (type of cells: hepatocytes, bile ducts, macrophages, hematopoietic, or inflammatory cells) and subcellular localization of the staining (cytoplasm, nucleus, cytoplasmic membrane, nuclear membrane) at magnification ×200 and ×400.

For GALD cases, we also looked for factors that may influence the percentage of C5b9 expression in liver parenchyma: age of patient at time of sampling (fetus or neonate), Perls score (≤2 and ≥3), presence of massive necrosis (involving at least 90% of liver parenchyma), type of sample (autopsy or biopsy) and fixation (formalin or not, such as Bouin liquid).

RESULTS

Group 1: GALD Cases

Twenty-five cases were studied in this group: 19 came from our previous series. Liver blocks were no more available for 14 cases. There were 11 fetuses (gestational age from 21 to 40 GW) and 14 newborns (from 30GW wk to 45 d of life). Four cases came from two distinct siblings. One premature neonate (number 9) died at 4 d of life despite two injections of IVIg at 3 and 4 d of life.

Twenty-seven liver samples were available: two cases had subsequently a liver biopsy and then autopsy. Autopsy was performed in all cases except one in which only one postmortem liver biopsy was available. Twenty-one samples were fixed in formalin and six in Bouin liquid.

Liver injury was characterized by diffuse panlobular massive necrosis in six fetus cases. Perls score average was 2.4.

C5b9 was detected in all cases. Seven cases (26%) cases exhibited more than 75% of stainable liver parenchyma, five cases between 50 and 75% (18.5%), five cases between 25 and 50% (18.5%), and nine cases less than 25% (33.3%). The average of stainable liver parenchyma was 46.5% (47.3% if we exclude the case who received IVIg). The average intensity was 2. In most autopsy samples, the staining was patchy and not

Table 1. GALD cases

Case	Type of samples[b]	Fetus (F) neonate (N)	Age of lesions GW+ days	%C5b9	Intensity	Type of fixation	Perls score	Massive necrosis[c]
1	A	F	21GW+3d	5	1	F	4	Yes
2[a]	LB	N	39GW+3d	90	3	F	2	No
	A		39GW+30d	40	2	Bouin	1	No
3[a], S1	A	F	29GW+3d	5	1	F	3	Yes
4[a]	A	N	38GW+40d	50	2	F	1	No
5[a]	A	N	34GW +6d	20	2	Bouin	3	No
6	A	F	27GW	70	2	F	4	No
7[a]	A	F	29GW +4d	40	1	F	1	No
8[a]	A	N	40GW +5d	65	2	F	2	No
9	A	N	34 GW+5d	25	1	F	3	No
10[a], S2	A	N	33GW+6d	90	1	F	1	No
11[a]	A	F	32 GW	5	3	F	4	Yes
12[a]	A	N	32 GW + 6d	70	3	Bouin	3	No
13	A	F	26 GW	75	3	F	1	No
14[a]	A	N	34GW+24d	70	3	F	2	No
15[a]	A	F	40GW+5d	5	2	F	4	Yes
16[a]	A	F	33GW +5d	5	2	F	4	Yes
17[a]	A	N	38GW+45d	100	3	Bouin	1	No
18[a], S1	A	N	30GW+5d	80	2	F	3	No
19[a]	A	N	32GW+5d	20	2	F	2	No
20	A	F	32GW+4d	60	3	F	3	No
21[a], S2	LB	N	40 GW+18d	80	2	F	1	No
22[a]	A	F	27GW+3d	10	3	F	3	No
23[a]	LB	N	38 GW+2d	80	3	F	3	No
	A		38 GW+18d	40	2	Bouin	2	No
24[a]	A	N	34 GW+3d	50	2	Bouin	1	No
25	A	F	33GW	5	2	F	4	Yes

[a]Cases from our previous series (20). [b]Average surface of autopsy sample: 375 mm^2, average surface of biopsy sample: 15 mm^2. [c]Massive necrosis: necrosis involving at least 90% of liver parenchyma (337,5 mm^2 for autopsy samples and 13,5 mm^2 for biopsy samples).
A, autopsy; F: formalin; GW, gestational week; LB, liver biopsy; S, same siblings.

diffused without any specific localization (**Figure 1**). In 70% of cases, hepatocyte staining was observed throughout the cytoplasm and covered the nucleus with a "fried egg" appearance. In other samples, staining was only cytoplasmic. In addition to hepatocytes, macrophages, hematopoietic cells, and inflammatory cells often expressed C5b9 but with a mild intensity compared to hepatocytes.

Results of group 1 are summarized in **Table 1**.

The analysis of factors that may influence the C5b9 expression, such as the age, the Perls score, the type of fixation and the type of samples is resumed in **Table 2**. All samples with massive necrosis (six fetuses) exhibited 5% or less of stainable liver parenchyma. However, all remaining non-necrotic hepatocytes were stained. In the 21 cases without massive necrosis, the average of stainable liver parenchyma was 58.3% (60% if we exclude the case who received IVIg).

Perls score had no influence on C5b9 staining (**Figure 2**). Indeed C5b9 was less expressed in cases with Perls score ≥ 3 than in cases with Perls score ≤ 2, regardless massive necrosis or type of fixation.

The percentage of C5b9 expression, in cases formalin fixed without any massive necrosis, was higher in liver biopsy compared to autopsy samples (83.3 vs. 54.58%).

The percentage of C5b9 expression was higher in neonates than in fetuses, regardless massive necrosis or type of fixation: above 60% in neonates and 51% in fetuses without massive necrosis.

The percentage of C5b9 expression was higher in formalin fixed cases than in Bouin liquid fixed cases (57.14 vs. 53.3%), taking into account that all Bouin liquid fixed cases came from autopsy of neonates, the comparison was established with neonates autopsy fixed in formalin (71 vs. 53.3% if we exclude the case who received IVIg).

Table 2. Percentage of C5b9 expression in liver parenchyma depending of age, Perls score, type of fixation, and type of samples

		Number	% C5b9
All samples		**27 (26[a])**	**46.5 (47.3[a])**
Type of fixation	F	21 (20[a])	44.5 (45.5[a])
	Bouin	6	53.3
Fetus versus neonate	Fetus	11	25.9
	Neonate	14 (13[a])	60.6 (63[a])
Perls score	≤2	13	62.3
	≥3	14 (13[a])	33.8
Massive necrosis	yes	6	5
	no	21 (20[a])	58.3 (60[a])
Type of sample	LB	3	83.3
	A	24 (23[a])	41.8 (42.6[a])
Samples without massive necrosis		**21 (20[a])**	**58.3 (60[a])**
Type of fixation	F	15 (14[a])	60.3 (62.8[a])
	Bouin	6	53.3
Fetus versus neonate	Fetus	5	51
	Neonate	14 (13[a])	60.62 (63[a])
Perls score	≤2	13	62.3
	≥3	8 (7[a])	51.8 (55.7[a])
Type of sample	LB	3	83.3
	A	18 (17[a])	54.17 (55.8[a])
Formalin fixed samples without massive necrosis		**15 (14[a])**	**60.3 (62.8[a])**
Fetus versus neonate	Fetus	4	51
	Neonate	11 (10[a])	65 (69.4[a])
Perls score	≤2	9	64.4
	≥3	6 (5[a])	54.7 (60[a])
Type of sample	LB	3	83.3
	A	12 (11[a])	54.58 (57.2[a])
Neonates autopsy samples		**13 (12[a])**	**55.38 (57.9[a])**
Type of fixation	Bouin	6	53.3
	F	7 (6[a])	57.14(71%[a])

[a]Exclusion of case number 9 who received IVIg.

Group 2: Non-GALD Cases With NH Phenotype

Thirty-six cases were included in this group: 20 fetuses (gestational age from 17 to 36 GW) and 16 neonates (from 29GW days to 40GW+ 35 d). Autopsy was performed in 31 cases and liver biopsy in 5 cases. The following diagnoses were collected: 5 cases of materno-fetal erythrocytic incompatibility (MFEI) (two against rhesus, two against a private antigen, and one against Kell antigen), 3 cases of mitochondrial cytopathies (two cases of mitochondrial DNA depletion syndrome and 1 case with *DGUOK* gene mutation previously reported in reference 31), 15 cases of infection (5 parvovirus B19, 5 CMV, 2 HSV2, 2 HSV1, 1 enterovirus sepsis), 1 case of δ (4)-3-oxosteroid-5-β-reductase, 1 case of transaldolase deficiency (case previously reported in reference 26), 2 cases of trisomy 18, 1 case of trisomy 21 without acute megakaryocytic leukemia, 1 case of familial lymphohistiocytosis due to *XIAP* mutation, 1 case of Zellweger, 3 cases with congenital heart disease (1 tetralogy of Fallot and 2 left ventricular hypoplasia), 1 case of congenital dyserythropoietic anemia type 1 (*CDAN1* mutation), 1 case of congenital lymphoblastic leukemia in the setting of Bloom syndrome (chromosomal breakage syndrome), and 1 case of secondary liver hemosiderosis due to multiple transfusions in the setting of Blackfand Diamond anemia. We had no case of tricho-hepato-enteric syndrome, Donohue syndrome, 16p duplication and Martinez-Frias syndrome. Our cases of tyrosinemia type 1 and neonatal lupus did not exhibit iron overload and were not included in this group. IVIg have been administered in two cases of MFEI: case number 26 (anti-private Ag) at birth and case 29 (anti-Kell) during pregnancy.

Results of this group are summarized in **Table 3**.

C5b9 expression was found in 28 cases with an average of 32.5%.

More than 75% of liver parenchyma was stained in all cases of HSV infections (HSV1 and HSV2), all cases of MFEI who have not received IVIg, as well as in the cases of enterovirus hepatitis, *DGUOK* mutation, T21 and δ(4)-3-oxosteroid-5-β-reductase

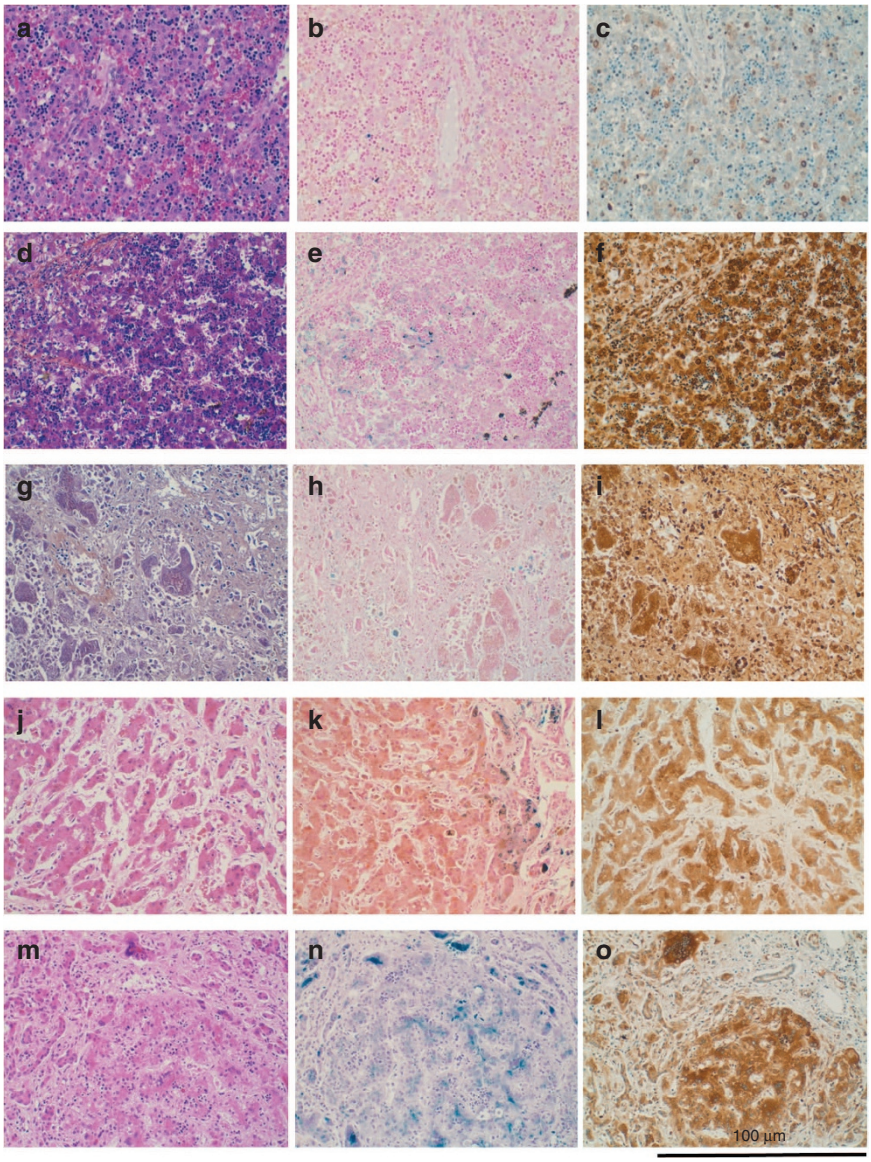

Figure 2. GALD cases (group 1): C5b9 staining compared to Perls score age and type of fixation, magnification (×40). (**a–c**) Control case of 27GW ×200 (A: HES, B: Perls score 0, C: C5b9 staining 0%). (**d–f**) GALD case 10 (neonate, 33GW+6d) ×200 (D: HES, E: Perls score 1, F: C5b9 90%), formalin fixation. (**g–i**) GALD case 2 (neonate, 34GW+24 d) ×200 (G: HES, H: Perls score 1, I: C5b9 40%), Bouin liquid fixation. (**j–l**) GALD case number 17 (neonate, 38GW+45 d) ×200 (J: HES, K: Perls score 1, L: C5b9 100%) Bouin liquid fixation. (**m–o**) GALD case number 20 (fetus, 32GW+4 d) ×200 (M: HES, N: Perls score 3, O: C5b9: 60%) formalin fixation.

deficiency (Figure 3a,d,e). C5b9 expression was absent or less than 5% in cases of CMV and parvovirus infections (Figure 3b,c), congenital cardiopathies, Zellweger syndrome, transfusions, T18, and 1 case of MFEI (anti-Kell) postadministration of IVIg. Immunostaining was between 20 and 40% in cases with mitochondrial DNA depletion syndrome (average 35%), transaldolase deficiency (Figure 3f) congenital dyserythropoietic anemia type 1, congenital proliferations (*XIAP* hereditary lymphohistiocytosis and leukemia) and 1 case of MFEI (anti-private antigen) postadministration of IVIg.

In cases of infections, the immunostaining was mainly localized to the infected cells: erythroblasts (parvovirus B19), bile duct and endothelial cells (CMV) infected and necrotic hepatocytes (HSV1 and 2), most often with a strong intensity (Figure 3a–c). In cases of MFEI, C5b9 was expressed in the cytoplasm of the hepatocytes, with a faint or moderate and speckling aspect. Macrophages (Kupffer cells) and hepatocytes were both stained in cases of mitochondrial cytopathies and congenital cardiopathies. Hematopoietic cells were mainly stained in congenital anemia and leukemia, and inflammatory elements in LFH and enterovirus hepatitis.

Group 3: Non-GALD Cases Without NH Phenotype (Neonatal Liver Disease Without Iron Overload)

Eighteen cases were included in this group: 15 liver biopsies and 3 autopsy samples, all from neonates aged from 30 GW to 3 mo, without any liver siderosis. There were three cases

Table 3. Non-GALD cases with NH phenotype (group 2) results

Case	Type of samples	Fetus (F) or neonate (N)	Age GW + days	Diagnosis	%C5b9	Intensity	Type of cells	Type of fixation	Perls score
26	A	N	29GW+3d	Anti-RBC alloimm (private Ag)	25	3	H	F	2
27	A	N	32GW +3d	Anti-RBC alloimm (private Ag)	80	2	H	F	2
28	A	N	38 GW +20d	Anti-RBC alloimm (rhesus)	90	1	H	F	3
29	A	F	35GW	Anti-RBC alloimm (Kell)	5	1	H	F	3
30	A	F	32GW	Anti-RBC alloimm (rhesus)	75	2	H	F	3
31	A	F	34GW+6d	Congenital dyserythropoietic anemia type 1	20	2	Hematop cells	F	4
32	A	F	22GW+2d	Leukemia (Bloom sd)	25	2	Hematop cells	F	2
33	A	F	20GW+5d	CMV	5	3	Infected bile duct and endothelial cells	F	1
34	A	F	20GW+4d	CMV	5	2	Infected bile duct and endothelial cells	F	3
35	LB	N	26GW+3d	CMV	0	0	0	AFA	1
36	A	F	31 GW	CMV	5	3	Infected bile duct and endothelial cells	F	2
37	A	F	26GW+3d	CMV	5	3	Infected bile duct and endothelial cells	AFA	2
38	LB	N	34GW+4d	mt DNA depletion sd	40	2	H> M	AFA	2
39	A	N	37GW+45d	mt DNA depletion sd	30	2	H>M	Bouin	1
40	A	N	35GW +90d	DGUOK	80	2	H>M	F	1
41	LB	N	38GW + 180d	Delta 4 -3 oxo defect	90	2	H	F	1
42	A	F	32GW	Enterovirus	90	2	H, IE	F	3
43	A	N	37 GW +45d	Cardiopathy	5	1	H & M	F	2
44	A	N	40GW+35	Cardiopathy	0	0	0	F	1
45	A	F	36GW+4d	Cardiopathy	5	1	H & M	F	2
46	LB	N	40 GW+10d	HSV1	90	3	Infected and necrotic H	F	1
47	A	F	25GW+2d	HSV1	90	3	Infected and necrotic H	F	1
48	A	N	37GW + 35d	HSV2	75	3	Infected and necrotic H	F	2
49	A	N	40GW+10d	HSV2	75	3	Infected and necrotic H	F	2
50	A	N	37GW	FLH (XIAP)	30	3	IE, M>>H	F	1
51	A	F	18GW+3d	Parvovirus	0	0	0	F	1
52	A	F	23GW	Parvovirus	0	0	0	F	1
53	A	F	25GW	Parvovirus	0	0	0	F	3
54	A	F	24GW	Parvovirus	5	1	Infected RBC	F	3
55	A	F	17GW	Parvovirus	5	1	Infected RBC	F	4
56	LB	N	40GW+ 30d	Transfusions	0	0	0	AFA	1
57	A	N	38 GW+5d	T18	5	1	M	F	3
58	A	F	28GW+3d	T18	0	0	0	F	1
59	A	N	32GW +60d	T21	90	2	H	F	3
60	A	F	28GW	Transaldolase	20	2	H	F	2
61	A	F	33GW +3d	Zellweger	5	2	H periportal	F	1

H, hepatocytes; IE, inflammatory elements; M, macrophages; mt, mitochondrial; RBC, red blood cells.

of biliary atresia, two cases of PFIC1, two cases of PFIC 2, two cases of Alagille syndrome, two cases of alpha-1-anti-trypsin deficiency, 1 tyrosinemia type 1, one case of giant cell hepatitis with autoimmune hemolytic anemia, one case of abetalipoproteinemia, one case of Gaucher disease, one case of Niemann-Pick C, one case of cystic fibrosis, and one case

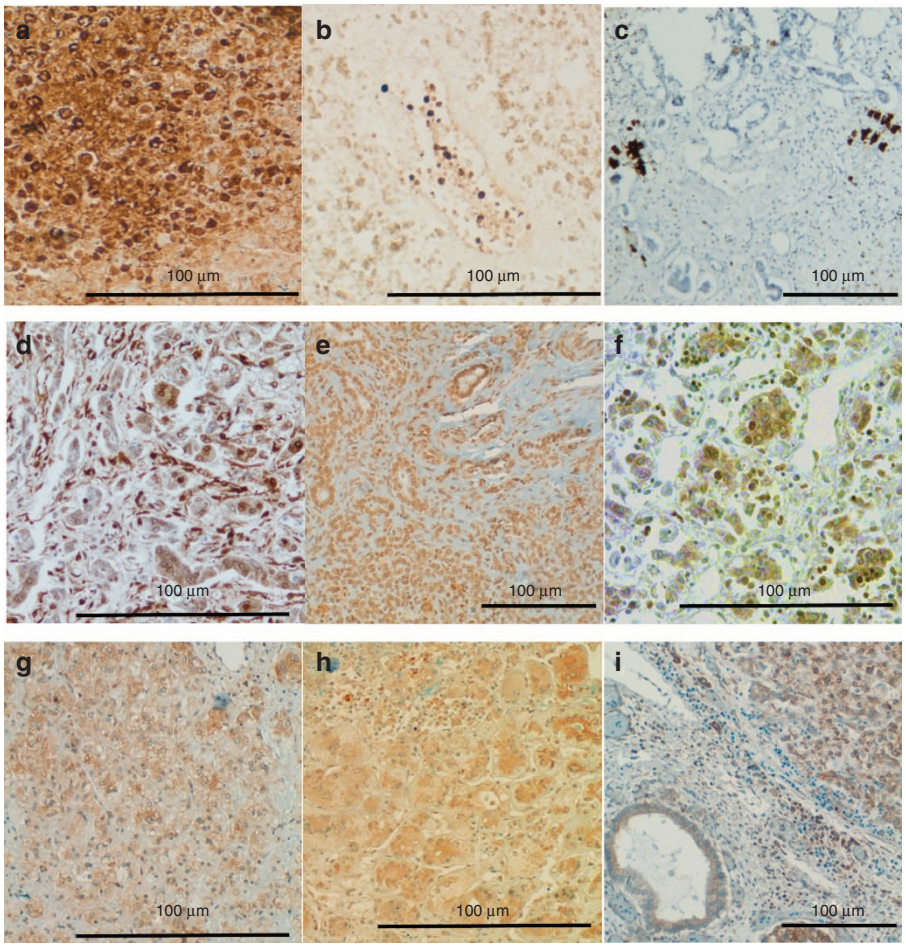

Figure 3. C5b9 immunostaining in non-GALD cases. (**a**) HSV2 (case 49): infected and necrotic hepatocytes are stained with a strong intensity, magnification (×200). (**b**) Parvovirus B19 (case 56): only infected erythroblasts are stained with a strong intensity, magnification (×200). (**c**) CMV (case 33): only infected bile duct and endothelial cells are stained with a strong intensity, magnification (×100). (**d**) DGUOK mutation (case 40): staining of hepatocytes and neoductular proliferation, mild to moderate intensity, magnification (×200). (**e**) Bile acid synthesis defect (case 41): staining of neoductular proliferation, mild to moderate intensity, magnification (×100). (**f**) Transaldolase deficiency (case 61): hepatocytes and giant cells are stained with a moderate intensity, magnification (×200). (**g**) Gaucher disease (case 73): hepatocytes are stained with a mild intensity, magnification (×200). (**h**) Giant cell hepatitis with autoimmune hemolytic anemia (case 76): hepatocytes are stained with a mild intensity, magnification (×200). (**i**) cystic fibrosis (case 75): hepatocytes, inflammatory elements and bile duct epithelium are stained, magnification (×100).

of neonatal lupus. We had no case of glycogen storage disease type 1, inspissated bile syndrome after cardiac surgery and total parenteral nutrition-associated cholestasis after bowel resection, in this group of age. Results of this group are summarized in **Table 4**.

C5b9 expression was found in nine cases with an average of 18%.

More than 75% of liver parenchyma was stained in three diseases: giant cell hepatitis with autoimmune hemolytic anemia, cystic fibrosis and Gaucher disease (**Figure 3g–i**). In these cases, staining was faint to moderate, freckling, located only in the cytoplasm of hepatocytes. In the case of cystic fibrosis, intense C5b9 staining was observed in canaliculi with mucus accumulation. In the case of Gaucher disease, we noted that Gaucher cells did not express C5b9.

In all other cases, the expression of C5b9 varied from 0 to 25%. The highest expression was observed in one case of PFIC1 (20%) and one case of PFIC2 (25%).

Group 4: Control Cases Without Liver Disease

Five autopsy cases were included in this group: four fetuses of 14, 22, 25, and 27 GW and one newborn of 1 mo of age. Death causes were respectively unexplained first trimester miscarriage, three terminations of pregnancy for osteochondrodysplasia, and one sudden infant death. No expression of C5b9 was observed in these cases.

DISCUSSION

Several diseases of the fetus and the newborn are characterized by a NH phenotype, defined by hepatic and extrahepatic epithelial iron overload. Until now, the diagnosis of GALD is most often done after exclusion of the differential diagnoses. The findings of Whitington *et al.* that GALD had extensive hepatocyte-associated C5b9 expression, lead to the conclusion that C5b9 immunostaining on hepatic sample is sufficient to make the diagnosis in cases in which extrahepatic siderosis could not be demonstrated (5,12,16). Taking into account the

Table 4. Non-GALD Cases Without NH Phenotype (group 3) results

Case	Type of samples	Age	Diagnosis	%C5b9	Intensity	Type of cells	Type of fixation
62	A	42 GW+3 d	Abetalipoproteinemia	0	0	0	F
63	LB	90 d	A1AT	<5	1	H periportal	F
64	LB	60 d	A1AT	<5	1	H periportal	AFA
65	LB	35 d	Alagille	0	0	0	F
66	LB	45 d	Alagille	0	0	0	F
67	LB	60 d	Biliary atresia	0	0	0	F
68	LB	70 d	Biliary atresia	0	0	0	F
69	LB	75 d	Biliary atresia	0	0	0	F
70	LB	19 d	Gaucher	80	1	H	F
71	A	35 GW	Niemann Pick C	5	1	H+M	F
72	A	30 GW	Cystic fibrosis	90	1	H+ bile duct epithelium +IE	F
73	LB	90 d	Giant cell hepatitis with autoimmune hemolytic anemia	90	1	H	AFA
74	A	90 d	Neonatal lupus	0	0	0	F
75	LB	60 d	PFIC1	20	1	H	F
76	LB	75 d	PFIC1	<5	1	IE	F
77	LB	65 d	PFIC2	0	0	0	AFA
78	LB	60 d	PFIC2	25	2	H	AFA
79	LB	60 d	Tyrosinemia	0	0	0	F

AFA, acidified formal alcohol; LB, liver biopsy; GW, gestational week.

economic issues and the potential side effects of immunotherapy with IV-IgG (9–11,17), we aimed to determine whether C5b9 staining was sensitive and specific for the diagnosis of GALD. For this purpose, we investigated a large collection of liver specimens of GALD and its differential diagnoses.

As Whitington *et al.* (14,16), we observed a C5b9 expression in hepatocytes in all cases of GALD (group 1), but with extensive staining of more than 75% of liver parenchyma in only 26% of our GALD cases, vs. 100% in their studies.

Staining was present throughout the hepatocyte cytoplasm and covered the nucleus with a "fried eggs" appearance and a moderate intensity in the majority of our cases. The average of stainable liver parenchyma was around 47% in our series. However, this average percentage varied if we took into account factors that might influence the fixation. Indeed, we noticed that the percentage of positive parenchyma depended on age (60% in neonates vs. 51% in fetuses), the type of fixation (% higher in formalin than in nonformalin-fixed tissue), the surface of the sample examined (% higher in liver biopsy), the presence of massive necrosis (5%) or not (60%) and whether IVIg have been administered or not before sampling. However, the percentage of C5b9 expression was independent of the intensity of iron overload. In the studies of Whitington (14,16), C5b9 staining was described as diffuse, covering the cytoplasm, without details about intensity of the staining and its relationships with the Perl's score. In similar condition of cases reported by Whitington (14), the average of stainable liver parenchyma among neonates GALD cases in formalin-fixed autopsy samples was 71%.

Our findings highlight the heterogeneity of C5b9 expression among GALD patients. The expression of C5b9 can be focal, patchy, and therefore difficult to interpret. This heterogeneity could reflect the range of severity in liver injury observed among patients. It may be explained by variations in the maternal IgG adaptive immune response to sensitization and individual differences in the capacity of fetal complement to promote and sustain injury. Indeed, individual sensitivity is illustrated by the fact that twins may differ significantly in the clinical severity of liver disease (32). It is important to note that all our samples with massive necrosis (six fetuses) exhibited 5% or less of stainable liver parenchyma. In non-GALD cases with NH phenotype (group2), we observed diffuse C5b9 positivity in more than 75% of parenchyma in five diagnoses: MFEI, HSV hepatitis, *DGUOK* mutation, enterovirus hepatitis, and bile acid defect due to δ(4)-3-oxosteroid-5-β-reductase deficiency. HSV and enterovirus hepatitis and δ(4)-3-oxosteroid-5-β-reductase deficiency were included in the studies of Whitington *et al.* and did not exhibit this percentage of expression: it was less than 25% (14,16). The other cases were not included. Nevertheless, in practice, these five phenotypes rarely challenge differential diagnosis with GALD because they have clinical, biological, and histological characteristics. However, they are an important differential diagnosis and should prompt specific investigations in order to make the correct diagnosis. DGUOK can present with liver manifestation alone and steatosis might be focal and therefore be absent on a liver biopsy sample. To our knowledge, NH case

due to MFEI against private antigen has never been reported to date and is not systematically investigated.

Among neonatal liver disease without NH phenotype (group 3), we observed C5b9 positivity in more than 75% of liver parenchyma in cystic fibrosis, Gaucher disease, and giant cells hepatitis Coombs +. These cases were not included in the series of Whitington *et al.* (14). Intriguingly, their cases with the highest percentage of C5b9 expression were Alagille syndrome, tyrosinemia type 1, biliary atresia, PFIC, and tricho-hepato-enteric syndrome. However the staining interested less than 45% of liver parenchyma. We did not observe these findings with our cases of tyrosinemia type 1, Alagille syndrome, PFIC and biliary atresia. Consistent with our findings, a recent study reported C5b9 intense staining in six cases of giant cells hepatitis Coombs+ (21).

C5b9 expression in other etiologies than GALD is not surprising. It reflects complement activation that especially affects hepatocytes particularly exposed to blood due to the fenestrated sinusoids and the lack of an organized basement membrane (33,34). Many components may induce final involvement of membrane attack complex via activation of one of the three complement pathways (classical, alternative, or lectin pathways): this includes infectious agents, hypoxia, stress, and metabolites (20). Complement-mediated membrane attack can be a beneficial mechanism for removing infected or damaged cells (35), or be the result of hepatocytes injury following metabolic overload, hepatocytes necrosis, and consequently marked inflammation (30,36–38). That could explain C5b9 positivity in some metabolic or cholestasis diseases that should not imply a complement-mediated mechanism. Similarly, in GALD, C5b9 could also be activated by iron overload, in the way to remove damaged hepatocytes, instead of being the first event of the disease that lead to secondarily hepatic and extrahepatic iron overload. Also, the increased permeability of hepatocytes secondary to iron overload could expose intracellular metabolites and induce activation of complement. The special "fried egg " appearance of C5b9 staining we observed in the hepatocytes of GALD cases could reflect this increased permeability of hepatocytes and the severity of liver injury in GALD-NH. This could represent an advanced stage of injury regardless of the mechanism involved in the origin of the destruction of hepatocytes (iron overload, antibodies, and inflammatory mechanisms). The characterization of the targeted antigen involved in GALD would really be helpful for understanding the mechanism involved in this disease.

Besides, complement regulatory proteins, such as CD59and CD46 expression, have never been studied in GALD cases. Indeed CD59 is broadly distributed in the fetal liver and could prevent complement-mediated damage arising from maternal Ig (33). These proteins could be deficient in GALD leading to excessive C5b9 deposition.

In conclusion, positive immunostaining of C5b9 in hepatocytes is obviously not specific of GALD, as it can be present in non-GALD cases. Furthermore, its expression in GALD cases can be focal and weak and difficult to interpret. C5b9 immunohistochemistry interpretation requires integration of clinical and paraclinical data and a careful histological analysis to rule out differential diagnoses. The diagnosis of GALD and its therapeutic management cannot only be based on C5b9 expression in liver samples. It relies on correlations of clinical, biological, radiological, and pathological findings.

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AUTHOR CONTRIBUTIONS

E.D., B.N., and S.C-F.: Performed the study with immunohistochemical analysis. E.D., E.R., P.R., and S.C-F.: Wrote the article. E.R. and P.R.: Gave their data. Performed the diagnosis of the cases: French group of neonatal hemochromatosis: S.H., J.B., P.B., D.D., M.P.C., and S.C-F. E.R. and P.R. for the American cases. P.M.: Gave his opinion as an immunologist.

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REFERENCES

1. Dhawan A, Mieli-Vergani G. Acute liver failure in neonates. Early Hum Dev 2005;81:1005–10.
2. Knisely AS, Mieli-Vergani G, Whitington PF. Neonatal hemochromatosis. Gastroenterol Clin North Am 2003;32:877–89, vi–vii.
3. Sergi C, Himbert U, Weinhardt F, et al. Hepatic failure with neonatal tissue siderosis of hemochromatotic type in an infant presenting with meconium ileus. Case report and differential diagnosis of the perinatal iron storage disorders. Pathol Res Pract 2001;197:699–709; discussion 711–3.
4. Feldman AG, Whitington PF. Neonatal hemochromatosis. J Clin Exp Hepatol 2013;3:313–20.
5. Lopriore E, Mearin ML, Oepkes D, Devlieger R, Whitington PF. Neonatal hemochromatosis: management, outcome, and prevention. Prenat Diagn 2013;33:1221–5.
6. Whitington PF, Malladi P. Neonatal hemochromatosis: is it an alloimmune disease? J Pediatr Gastroenterol Nutr 2005;40:544–9.
7. Whitington PF. Neonatal hemochromatosis: a congenital alloimmune hepatitis. Semin Liver Dis 2007;27:243–50.
8. Whitington PF, Hibbard JU. High-dose immunoglobulin during pregnancy for recurrent neonatal haemochromatosis. Lancet 2004;364:1690–8.
9. Whitington PF, Kelly S. Outcome of pregnancies at risk for neonatal hemochromatosis is improved by treatment with high-dose intravenous immunoglobulin. Pediatrics 2008;121:e1615–21.
10. Baruteau J, Heissat S, Broué P, et al. Transient neonatal liver disease after maternal antenatal intravenous Ig infusions in gestational alloimmune liver disease associated with neonatal haemochromatosis. J Pediatr Gastroenterol Nutr 2014;59:629–35.
11. Rand EB, Karpen SJ, Kelly S, et al. Treatment of neonatal hemochromatosis with exchange transfusion and intravenous immunoglobulin. J Pediatr 2009;155:566–71.
12. Whitington PF. Gestational alloimmune liver disease and neonatal hemochromatosis. Semin Liver Dis 2012;32:325–32.
13. Jimenez-Rivera C, Gupta A, Feberova J, de Nanassy JA, Boland MP. Successful treatment of neonatal hemochromatosis as gestational alloimmune liver disease with intravenous immunoglobulin. J Neonatal Perinatal Med 2014;7:301–4.
14. Pan X, Kelly S, Melin-Aldana H, Malladi P, Whitington PF. Novel mechanism of fetal hepatocyte injury in congenital alloimmune hepatitis involves the terminal complement cascade. Hepatology 2010;51:2061–8.
15. Bonilla S, Prozialeck JD, Malladi P, et al. Neonatal iron overload and tissue siderosis due to gestational alloimmune liver disease. J Hepatol 2012;56:1351–5.

16. Whitington PF, Pan X, Kelly S, Melin-Aldana H, Malladi P. Gestational alloimmune liver disease in cases of fetal death. J Pediatr 2011;159:612–6.
17. Pierce LR, Jain N. Risks associated with the use of intravenous immunoglobulin. Transfus Med Rev 2003;17:241–51.
18. Tsunoda T, Inui A, Kawamoto M, et al. Neonatal liver failure owing to gestational alloimmune liver disease without iron overload. Hepatol Res 2015;45:601–5.
19. Smyk DS, Mytilinaiou MG, Grammatikopoulos T, et al. Primary biliary cirrhosis-specific antimitochondrial antibodies in neonatal haemochromatosis. Clin Dev Immunol 2013;2013:642643.
20. Pham BN, Mosnier JF, Durand F, et al. Immunostaining for membrane attack complex of complement is related to cell necrosis in fulminant and acute hepatitis. Gastroenterology 1995;108:495–504.
21. Whitington PF, Vos MB, Bass LM, et al. Humoral immune mechanism of liver injury in giant cell hepatitis with autoimmune hemolytic anemia. J Pediatr Gastroenterol Nutr 2014;58:74–80.
22. Knisely AS, Vergani D. "Neonatal hemochromatosis": a re-vision. Hepatology 2010;51:1888–90.
23. Collardeau-Frachon S, Heissat S, Bouvier R, et al. French retrospective multicentric study of neonatal hemochromatosis: importance of autopsy and autoimmune maternal manifestations. Pediatr Dev Pathol 2012;15:450–70.
24. Baruteau J, Heissat S, Collardeau-Frachon S, Debray D, Broué P, Guigonis V. Nouveaux concepts dans l'hémochromatose périnatale. Arch Pediatr 2012; 19:755–761.
25. Heissat S, Collardeau-Frachon S, Baruteau J, et al. Neonatal hemochromatosis: diagnostic work-up based on a series of 56 cases of fetal death and neonatal liver failure. J Pediatr 2015;166:66–73.
26. Valayannopoulos V, Verhoeven NM, Mention K, et al. Transaldolase deficiency: a new cause of hydrops fetalis and neonatal multi-organ disease. J Pediatr 2006;149:713–7.
27. Parizhskaya M, Reyes J, Jaffe R. Hemophagocytic syndrome presenting as acute hepatic failure in two infants: clinical overlap with neonatal hemochromatosis. Pediatr Dev Pathol 1999;2:360–6.
28. Senger C, Gonzalez-Crussi F. Histiocytic-phagocytic infiltrates in the liver of an infant: a case clinically simulating perinatal hemochromatosis. J Pediatr Gastroenterol Nutr 1999;29:215–20.
29. Schwaibold EM, Bartels I, Küster H, et al. De novo duplication of chromosome 16p in a female infant with signs of neonatal hemochromatosis. Mol Cytogenet 2014;7:7.
30. Schäfer H, Mathey D, Hugo F, Bhakdi S. Deposition of the terminal C5b-9 complement complex in infarcted areas of human myocardium. J Immunol 1986;137:1945–9.
31. Labarthe F, Dobbelaere D, Devisme L, et al. Clinical, biochemical and morphological features of hepatocerebral syndrome with mitochondrial DNA depletion due to deoxyguanosine kinase deficiency. J Hepatol 2005;43:333–41.
32. Ekong UD, Kelly S, Whitington PF. Disparate clinical presentation of neonatal hemochromatosis in twins. Pediatrics 2005;116:e880–4.
33. Halme J, Sachse M, Vogel H, Giese T, Klar E, Kirschfink M. Primary human hepatocytes are protected against complement by multiple regulators. Mol Immunol 2009;46:2284–9.
34. Koch CA, Kanazawa A, Nishitai R, et al. Intrinsic resistance of hepatocytes to complement-mediated injury. J Immunol 2005;174:7302–9.
35. Rogers J, Cooper NR, Webster S, et al. Complement activation by beta-amyloid in Alzheimer disease. Proc Natl Acad Sci USA 1992;89: 10016–20.
36. Storrs SB, Kolb WP, Pinckard RN, Olson MS. Characterization of the binding of purified human C1q to heart mitochondrial membranes. J Biol Chem 1981;256:10924–9.
37. Pinckard RN, Olson MS, Giclas PC, Terry R, Boyer JT, O'Rourke RA. Consumption of classical complement components by heart subcellular membranes *in vitro* and in patients after acute myocardial infarction. J Clin Invest 1975;56:740–50.
38. Giclas PC, Pinckard RN, Olson MS. *In vitro* activation of complement by isolated human heart subcellular membranes. J Immunol 1979;122: 146–51.