

Is neonatal haemochromatosis related with maternal autoimmunity? Arguments from a retrospective histological and clinical french multicentric study of 33 cases.

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SUMMARY

Neonatal haemochromatosis (NH) is a rare disease causing fetal loss and neonatal death in the first weeks of life. This is one of the most commonly recognized causes of liver failure in the neonate. Diagnosis is mostly done retrospectively, on histopathological features, demonstrating severe liver fibrosis, associated with hepatic and extrahepatic siderosis. Several etiologies may lead to such a phenotype. Among them, a recent hypothesis of gestational alloimmune disease has been suggested. In this retrospective study of 51 cases with a neonatal haemochromatosis phenotype, we isolated 33 cases in whom NH might be imputed to an allo-immune mechanism. We describe clinical and histological characteristics of this group. Analysis of mothers' history revealed auto-immunity manifestations in 75%, which lead us to discuss new hypothesis to explain physiopathology of this disease.

KEY WORDS: Neonatal haemochromatosis, iron storage disorder, dysimmunity, allo-immunization, auto-antibodies, auto-immune diseases.

INTRODUCTION

Neonatal haemochromatosis (NH) is a polyvisceral iron storage disorder of prenatal onset. It usually causes severe fetal injury and often fetal loss. Liveborn infants present liver failure within hours of birth and die rapidly of progressive hepatic insufficiency and multivisceral failure. The recurrence rate is high around 80% in families with one affected child (1-11). Diagnosis is most often done retrospectively and based on demonstration of severe liver fibrosis, associated with hepatic and extrahepatic siderosis that spares reticuloendothelial system (10, 12-15). Recently, hypothesis of an alloimmune-mediated disease has been suggested by Whittington and al (1). This is supported by several arguments (1, 5, 16-19): (a) recurrence rate is similar to rhesus incompatibility and too high for inheritance explanation; (b) no mutations in genes of hereditary hemochromatosis have been detected; (c) NH also affects maternal half-siblings but no paternal half-siblings (verloes, Wh); (d) no parent of an affected child has been found to have NH disorder with subdued penetrance; (e) efficacy of intravenous immunoglobulin (IV-Ig) therapy during pregnancy in reducing severity and recurrence rate. But to date, the target antigen, supposed to be a liver fetal protein related to iron transport protein or its regulation, in the fetus or placenta (1), has not been identified.

IV-Ig therapy is very expensive and may induce maternal complications (20). Indications have to be limited. In this aim, we constituted a french expertise comity, composed of pediatricians, geneticians, pathologists and immunologists to decide which cases presenting with NH phenotype might be related to a possible allo-immune disease, in order to administrate or not IV-Ig during future gestations. We together defined criteria of inclusion and exclusion of cases, based on literature data (3, 4, 6, 10-15, 21-23). For each case, we collected mother and infant clinical data and reviewed all histological slides available. Between 2006 and 2009, 51 cases

with histological material were submitted to our comity because of suspicion of NH. Among them, diagnosis of NH imputed to an allo-immunization mechanism was retained for 33 (NH group). Eighteen were excluded because of finding other etiologies and/or uncharacteristic histological features. In this work, we presented clinical and pathological characteristics of NH group and first results of immunological study on sera of the mothers.

MATERIAL AND METHODS

Between 2006 and 2009, 51 cases with histological material were submitted to our comity because of acute liver failure with intra-hepatic siderosis.

1. Criteria of inclusion

Criteria of inclusion in possible allo-immune NH group were based on literature data (3, 4, 6, 10-15, 21-23) and are resumed on table 1. We defined a score on 7 points for fetus and 9 points for neonates. We also considered results of etiologic check up for main differential diagnosis (23,24): intrauterine infections (25,26), primary bile acid disorders (27-29), transaldolase deficiency (30,31), mitochondriopathies (32-37), but those were not always performed.

2- Mothers and infants' clinical data

For each case, we collected following data in hospitalization and autopsy reports:

- sexe, age at birth, ethny, consanguinity, position in the sibling, kind and age of death (fetal death, medical termination of pregnancy, neonatal death), circumstances of death, treatment administrated
- imagery findings: ultrasound and MRI
- laboratory results of liver tests, ferritin level and full blood count
- gross features of fetus and neonates at clinical examination or autopsy: intra uterine growth restriction, hydrops, ascite, other effusions, weight or volume of the liver, and all abnormalities found.
- macroscopic findings of placenta
- number and position in the sibship of died children
- pregnancy history
- mother's personal and familial backgrounds including index cases of fetal or child loss.

3- Histological study

All slides available were reviewed by 3 independant pathologists. All were stained with Hematoxylin Phloxin Saffron (HPS).

Following items were analyzed at liver microscopy: fibrosis (lobular, portal, incipient or severe), regenerative changes (giant cells, acinar formations, regenerative nodules) iron localization (hepatocytes alone, hepatocytes and Kupffer cells), intensity of iron overload, cholestasis (absent, mild, moderate, severe), necrosis, inflammation (absent, mild, moderate, severe), steatosis and oncocytes. Fibrosis was analyzed with two special stains: Masson's trichrome stain and rouge sirius. Intensity of liver siderosis was evaluated with Perls reaction. A semi-quantitative score was applied to evaluate intensity and localization of iron storage in liver, as follow: score 1 if less than 25% of liver samples were stainable, score 2 for 25 to 50%, 3 for 50 to 75%, and 4 for more than 75%. If several liver samples were available for a same case, score was established on the most severe areas.

Extra hepatic iron storage was systematically searched on all samples with Perls reaction. Cases issued of a same sibship were compared to each other for liver lesions and extrahepatic iron storage.

Liver lesions and extrahepatic iron storage were analyzed depending on age at lesions examined. We determined 3 groups in fetuses: (1) fetuses died before 31GW, (2) between 31 to 37GW, (3) after 37GW (31 GW corresponding to medium age of fetal death, and 37GW to prematurity). As well 4 groups were isolated among neonates: (1) premature infant died before 24 days of life, (2) premature infant died after 24 days of life, (3) term infant died before 24 days of life, (4) term infant died after 24 days of life (24 days corresponding to medium age of neonatal death).

4- Immunological study

Indirect immunofluorescence study was realized in 5 cases. Mothers's sera of affected infants was applied on frozen fetal liver of unaffected child. Six μm thick fetal liver frozen sections were washed in PBS for ten minutes, and then incubated with the mother's serum diluted at 1:20. Three washings of ten minutes each in PBS were carried out to remove unbound serum. The bound autoantibodies were labelled with fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulins. Incubation with the antisera for thirty minutes at 37° C was followed by three washings in PBS of ten minutes each. The sections were then mounted in a drop of buffered glycerol and viewed with the fluorescence microscope.

Sera were tested in immunology laboratory to characterize antibodies.

5. Ethics

All parents gave their consent for this study.

RESULTS

Diagnosis of possible allo-immune NH was retained in 33 cases, from 28 different siblings. Eighteen cases were excluded because of insufficient score correlated with insufficient clinical or histological data or other etiologies: no proof of extrahepatic siderosis because of no MRI or no autopsy (10 cases), no liver fibrosis and intrahepatic siderosis exclusively seen in macrophages (3 cases), mitochondriopathies (2 cases), possible familial lymphohistiocytosis (2 cases), and bacterial fulminans hepatitis due to E Coli (1case).

The 33 cases of NH group were composed of 9 fetus and 24 neonates. Sex ratio was 1.2 (18 males and 15 females). All were born to non consanguineous parents from European's origin in 87% and North African's one in 13%. In 4 cases (12%) the first infant was concerned by the disease. 2 cases of a same sibling were conceived by ovarian stimulation. In 2 families, death occurred in maternal half-siblings.

Fetuses were aged of 27 to 40 GA (medium 31GA). Five of them resulted from fetal deaths and 4 from medical termination of pregnancy because of recurrence (2 cases) or severe anamnios with IUGR (2 cases). Infants were born between 30 to 42 GA (medium 36 GA). Half of them were premature. All neonates presented physical impairment immediately after birth. This consisted in liver deficiency or multivisceral failure. Hemorrhagic manifestations with decreased coagulation's factor, anemia and thrombopenia were frequently misinterpreted as manifestations of disseminated intravascular coagulation. Except one case who is still alive and healthy after 12 years of follow up, all neonates died between 3 to 90 days after birth (medium 24 days). Therapy consisting in iron chelation and cocktail of antioxidants was administrated in 7 cases and exsanguinotransfusion was performed in 1 case.

Pregnancy history

Ultrasound detected in utero abnormalities in 68%. Most of them were seen at the end of second trimester (range 24 to 32 GW): IUGR in 7 cases, oligo or anamnios in 15, hydrops in 3, ascites in 4, pleural or pericardial effusions in 2 and placental hypertrophy in 3. No significant other ultrasound abnormalities were noted. Pregnancy was marked by gestational thrombopenia in one case, cytopenia in 2 cases, gestational diabetes in 2 cases, toxemia in 2 cases. One mother presented seizures resulting of a cerebrovascular accident in a previous pregnancy. To prevent recurrence of seizures, anticoagulant therapy (molecular weight heparin and aspirin) was administered during pregnancy of the 2nd case reviewing in this sibship. In one case, seroconversion against toxoplasma was detected at 19 weeks of gestation but PCR on infant sera and frozen liver biopsy were both negative for this agent genomic sequences. Cesarean delivery was performed in half of cases.

Morphological findings

Autopsy was performed in 9 fetus and 14 infants, immediate post-mortem liver biopsy in 10 infants.

Liver analysis

Among autopsy cases, liver weight revealed hypotrophy in most cases (74%) (range 27 to 41GW). Liver hypertrophy was noted in 4 autopsy cases and in 6 cases of 10 non autopsy cases at abdominal ultrasound performed in the first days of life (range 29 to 40GW).

At microscopic examination (table 2), severe lobular liver fibrosis was constant (inclusion criteria). It was less pronounced in fetus than neonates, with an incipient feature in 5 of the 9 fetus (figure1). In 2 cases, 1 fetus and 1 neonate, portal spaces were enlarged and fibrotic without portal septa. Because of autolytic lesions in fetal death's cases, fibrosis was only identified after special staining. Regenerative changes were present in almost all cases (94%) with regenerative nodule formation in 61%, hepatocellular giant cell transformation in 73%, and acinar formations in 76%. Siderosis was exclusively seen in hepatocytes in 18 cases. In 15 cases it was also present in some Kupffer cells. Perls score was of 1 in 10/33 (30%), 2 in 9/33 (27%), 3 in 12/33 (36%) and 4 in 2/33 (6%). Perls score ≥ 3 was predominantly seen in fetus (7/9), whereas in neonates it was predominantly ≤ 2 (17/24). Perls score was highly correlated with intensity of liver fibrosis: in cases of incipient fibrosis, it was always ≥ 3 . It decreased with fibrosis progression. Cholestasis was minimal or absent in fetus and premature neonates who died before 24 days of life. In other cases, numerous bile plugs were observed in acinar formations. Inflammatory infiltrate with increased hematopoiesis in fetus or abnormal persistence of hematopoiesis in neonates, was mild and decreased with fibrosis progression. Focal steatosis involving less than 10 % of liver samples was observed in 5 cases, macrovacuolar in 3 cases, microvacuolar in 2 cases. Oncocytic changes suggestive of mitochondriopathy was never seen.

Liver lesions were different among cases issued of a same sibling (table 3).

Extrahepatic siderosis analysis

Iron storage was rarely seen with HPS, but only after Perls staining. In autopsy cases, intracellular iron overload was identified in pancreatic acini (74%), thyroid follicles (65%), renal tubes (26%), and others epithelial cells of different organs (35%)

such tracheal glands, digestive glands (stomach and duodenum) or thymus. Localization of extrahepatic iron overload varies for a same age, from age to age (table 4) and among cases of a same sibship (table 5). In some cases, it was only seen in few organs, for example thymus and tracheal glands, and sometimes with mild intensity. In fetus, extrahepatic iron overload was more frequent in thyroid than pancreas (67% versus 45%), whereas in neonates, it was more frequent and nearly constant in pancreas (93% of cases) than in thyroid (64%). In the youngest fetus of this group aged of 27 GW + 3 days, no extrahepatic iron overload was detected, but diagnosis of NH was highly suggestive because of typical liver lesions and autopsy findings in their sibling. Oral mucosal sample was available in 5 cases and showed stainable iron in the submucosal salivary glands in only one of these 5 cases, whereas iron overload was detected in other organs when autopsy was performed (pancreas 2 cases, thyroid 1 case).

Others lesions

Gross examination revealed IUGR in 48%, hydrops in 30%, ascites in 36%. Splenomegaly imputed to portal hyper pressure was found in half of cases. Dismorphic facies suggesting oligoamnios sequence was noted in 4 cases. One case presented facial dysmorphism of Down syndrome, which was confirmed on karyotype. Autopsy samples examination of this case of Down syndrome did not show megakaryocytic leukemia. In one case, we observed myofibroma in lungs and heart, suggesting myofibromatosis. One case presented a cardiac juvenile xanthogranuloma, without macrophagocytosis activation syndrome. In 30% of necropsy cases, we observed renal tubular dysgenesis. Among them, it occurred 3 times in the same sibship. Renal dysgenesis was associated with hypocalvaria in 2 cases of 2 different sibships, and microcephalia in 2 cases of a same sibship. In 22%, lesions of renal ischemia were also observed (glomerular flocculus retractions) and these lesions were always associated with cerebral ischemia when brain was available for examination. Placenta was available for examination in all fetus and 7 infants. Placental hydrops was noted in 81%. Microscopic placental examination showed fetal thrombotic vasculopathy in 5 cases and chronic intervillitis in 2 cases. Multivisceral extrahepatic hematopoiesis was present in 8 of the autopsy cases including placenta. Thymic hypoplasia was observed in 43% as usual in chronic distress.

Biological data and etiologic check up

Ferritin level was available in 16 cases, range 560 to 5195 μ g/l (median 1884 μ g/l). Fifteen cases underwent MRI. T2 signal intensity of liver was decreased in 7/15. Pancreatic MRI exploration was realized in 9 cases and showed a T2 hyposignal in only 2 cases in which no autopsy was performed. Among the seven others, 4 underwent autopsy which showed pancreatic siderosis at microscopic examination in 2 cases whereas it was undetected on MRI. Except the case of Down syndrome, karyotype was always normal. Results of viral serology were available in 19 cases and were negative for CMV, and parvovirus. Histopathology never showed hemorrhagic necrosis which are associated with most viral infections and no viral inclusion was seen. Transaldolase deficiency was excluded in 4 cases by absence of increased urinary polyols. Furthermore, absence of consanguinity and dysmorphic features like hypertrichosis or genital abnormalities were against transaldolase deficiency. Mass spectroscopy of urinary bile acids was performed in 15 cases and excluded an inborn error of bile-acid synthesis. Mitochondrial respiratory chain

complexes were explored in 14 cases and were never suggestive of mitochondriopathy. Furthermore, neither oncocytes nor diffuse steatosis was observed in liver.

Mothers

The twenty eight mothers were 21 to 38 years old (median 29,5) at time of the first infant's death. Index cases of child or fetal loss occurred in 14 mothers. Eighteen mothers loosed at least two children. Recurrence rate was 81%.

Auto-immune manifestations were found in 20 mothers in personal and/or her familial backgrounds (table 6). One had systemic erythematous lupus (SLE) which was not treated during pregnancy. Thyroid diseases were present in 3 cases associated with uveitis and dysovulation in one case, peripheral vascular disease in one other case. One had history of auto-immune hepatitis. No patient received corticoid or other immune suppressive therapy during pregnancy. Pregnancy was marked by gestational thrombopenia in one case, epilepsy imputed to cerebrovascular accident in one case, cytolysis in 2 cases, gestational diabetes in 2 cases, toxemia in 2 cases. Familial history revealed that one had a sister with SLE, one had a grand-mother with dermatomyositis, and one had a mother with insulin dependent diabetes mellitus (IDDM). In 9 mothers without clinical auto immune manifestations, antinuclear auto-antibodies (ANAs) were found in sera during pregnancy. Auto antibodies against mitochondria were also found in 2 cases and against cell membrane in 2 cases. To date, 8 mothers received IV-Ig therapy during ulterior pregnancy and gave birth to unaffected child, with transient mild liver test abnormalities in 4 cases.

Indirect immunofluorescence study showed nuclear spot on hepatocytes and endothelial cells (figure 2).

DISCUSSION

Some elements of our study are similar to literature data :

(a) we found arguments which reinforce that NH begins in utero (**1- 6, 10, 13-15, 22**): half of fetuses had an atrophic liver with severe dissecant fibrosis at microscopic examination. The youngest fetus of this group presented ultrasound abnormalities (IUGR, anamnios and pericardial effusion) at 24 GW.

(b) NH phenotype is characterized by a great intra and interfamilial heterogeneity (**38**): extrahepatic siderosis localization and severity of liver lesions were variable inside a same sibship and for a same age.

(c) Fetal liver seems to be the first target of the disease and liver lesions precede extrahepatic siderosis (**1, 5 38-41**). Extrahepatic siderosis would traduce incapacity of deficient liver in iron storage, which is uptake by other tissues. Iron overload in hepatocytes lead to cell lysis with inflammation. Hepatocytes are then replaced by fibrosis and regenerative changes. Iron uptake by other tissues is the end of the process. We noted that Perls score and inflammation decreased with fibrosis progression and no extrahepatic iron storage was seen in the 2 youngest fetuses. Furthermore, the pattern of iron deposition is similar to that of the hereditary haemochromatosis (**42**). It is interesting to note that thyroid iron storage was most frequent in fetuses than in pancreas. This could be explained by fetal circulation system in which thyroid is placed before pancreas (**43**). The absence of stainable extrahepatic iron in the youngest fetus of our series, aged of 27 GW, make us wonder about rejecting NH diagnosis in cases with typical clinical and histological liver lesions.

(d) As previous reports, our group contained one case of Down syndrome (**44-46**) and one case of myofibromatosis (**47**). To date, we could not explain these associations with NH. To our knowledge, xanthogranuloma was never report with NH before. This benign histiocytic tumor may induce hemophagocytic syndrome, which was not observed in our case (**48**). We also found 7 cases of renal dysgenesis associated with hypocalvaria or microcephaly. These associations were previously described and imputed to hypoperfusion mechanism (**49-51**). Indeed final stage of renal development is dependant upon liver function. In some cases, we observed other ischemic lesions in kidneys and brain. Both renal and cerebral ischemic lesions were constantly associated in our study which reinforces hypoperfusion physiopathological mechanism.

Our work emphasizes importance of autopsy with Perls staining in all samples for NH diagnosis. Combination of ferritin level and liver failure is not sufficient to detect NH (**52-54**). Autopsy allows the detection of extrahepatic iron overload which is not limited to pancreas and oral mucosal salivary glands (**23, 55-57**). In our series, iron overload in salivary glands was only seen in 1/5 and T2 sequence MRI showed pancreatic hyposignal in 2/9. in half of autopsy cases, pancreatic MRI was negative. But this may be imputed to the timing, if MRI is realized to early in the process of the disease. As well autopsy and Perls staining allow to see iron storage in less usual localization such as tracheal or duodenal glands.

In this work, we found arguments for an immune mechanism: (a) recurrence rate was 81%.; (b) in 2 different sibships, infants were issued from a same mother but different father; (c) clinical and histological manifestations of the disease appeared previously at the end of the 2nd gestational trimester (24GW), so after the passage of maternal IgG through the placenta (**58**); (d) efficacy of IV-Ig therapy in the 8 mothers treated to date; (e) and placental lesions which are classically described in dysimmunity diseases such as chronic intervillitis and fetal thrombotic vasculopathy (**59,60**). But we also found dysimmunity manifestations in most of the mothers. If we combined mother's personal and familial histories, placental lesions, and first results of immunological study, dysimmunity manifestations were present in 75 % of mothers (21/28). These results lead us to discuss auto-immune versus allo-immune mechanism in the pathogenesis of this disease. Only few previous reports mentioned NH cases issued of mothers with auto-immunity disease. Schoenlebe and al described NH in an infant of a mother with Sjogren's syndrome and high anti-Ro/SSa and anti R-La/SSB antibody titers (**61**). In their series of NH, Kelly and al reported 2 cases of the 8 mothers tested with anti-Ro/SSa and anti R-La/SSB antibody and one mother had SLE but received no treatment during pregnancy (**5**). But all infants issued from mothers affected by SLE or Sjogren's syndrome treated or not do not present NH phenotype. It is interesting to note that some authors found auto-antibodies in mothers of children presenting neonatal cholestasis, with a statistical difference with the control group. None of these mothers presented clinical manifestations (**62**). The authors suggested that fetal hepatopathy could stimulate maternal antibodies synthesis or that a maternal auto-immunity background could predispose to fetal liver disease. In allo-immune hypothesis, NH is supposed to result from maternal antibodies which pass through placental barrier and would link to a fetal liver antigen (**1**). To date, this antigen, maybe a 32 KDa protein has not yet been characterized. Allo-immune mechanism implies that this antigen previously passes into maternal blood and that it is not recognized by maternal immune system (**63-69**). In our series, mothers have serum auto-antibodies during pregnancy or at

distance. We wonder if the presence of these auto-antibodies in mother's sera is a consequence of NH. Indeed auto immune manifestations often appear after pregnancy, and some of our cases have a familial predisposing background. Second hypothesis is that auto-antibodies are directly responsible of NH lesions. But is there a specific fetal antigen? Different auto-antibodies were found in mothers' sera, sometimes associated together, directed against nuclear and/or mitochondria and/or cell membrane. We have to keep in mind that after placenta, liver is the first organ exposed to maternal blood on fetal circulation (**43, 70**); so maybe nuclear and/or mitochondria and/or cell membrane factors lead to hepatocytes lysis which seems to be the primary injury in this disease. Others lesions seem to be the consequences of this lysis. Severity and heterogeneity of NH phenotype may be due to level of auto-antibodies circulating in fetal blood. Absence or minimal level of auto-antibodies could explain why all infants issued from mothers affected by auto-immune diseases do not develop NH. No mother of our series received corticoid or other immune suppressive therapy during pregnancy. We noted also that 2 mothers of our series with ANAs presented toxemia during pregnancy. To date hypothesis of auto-immune mechanism is suggested in the pathophysiology of preeclampsia (**71, 72**) : pre-eclamptic mothers develop agonistic autoantibodies against the angiotensin AT₁ receptor, which contribute to many features of the disease. These auto antibodies induce chronic placental hypoperfusion and perhaps might have a role in renal dysgenesis. In our series, the 2 children issued of pre-eclamptic mothers demonstrated ischemic renal features without complete characteristics of renal dysgenesis.

Response to IV-Ig is not a discriminate criteria between allo and auto-immune mechanisms. Ig-IV therapy reduce the maternal immune response to fetal antigens flooding the maternal IgG transport mechanisms with non-reactive antibodies, or by promoting non-specific antibody binding, which limits the binding of reactive alloantibodies to target antigens (**41**). Perhaps corticoid alone may prevent NH.

CONCLUSION

In conclusion, our study brings news data in physiopathological mechanisms of NH. We now have to consider mother's personal and family history of auto-immune disease and systematically search auto-antibodies on sera during pregnancy and at distance. This may have a strong impact in positive and differentiate diagnosis of NH phenotype. Furthers studies are necessary to understand role of maternal auto antibodies in this disease.

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