CLINICAL RESEARCH ARTICLE

Characterization and origin of heme precursors in amniotic fluid: lessons from normal and pathological pregnancies

Hana Manceau[1,2], Vincent Puy[3], Caroline M. Schmitt[2,4], Sophie Gil[5], Thibaud Lefebvre[2,4], Bichr Allaf[6], Jonathan Rosenblatt[7], Laurent Gouya[2,4], Hervé Puy[2,4], Francoise Muller[6] and Katell Peoc'h[1,5]

BACKGROUND: Heme is the prosthetic group of numerous proteins involved in vital processes such as oxygen transport, oxidative stress, and energetic mitochondrial metabolism. Free heme also plays a significant role at early stages of development and in cell differentiation processes. The metabolism of heme by the fetal placenta unit is not well-established in humans.
METHODS: In a retrospective study, we measured heme precursors in the amniotic fluid (AF) of 51 healthy women, and 10 AF samples from pregnancies with either upper or lower intestinal atresia or ileus were also analyzed.
RESULTS: We showed that the porphyrin precursors aminolevulinic acid, porphobilinogen, and protoporphyrin IX are present at the limit of detection in the AF. Total porphyrin levels decreased progressively from week 13 to week 33 ($p < 0.01$). Interestingly, uroporphyrin, initially detected as traces, increased with maturation, in contrast to coproporphyrin. Uro- and coproporphyrins were type I immature isomers (>90%), suggesting a lack of maturity in the fetal compartment of the heme pathway. Finally, the differential analysis of AF from normal and pathological pregnancies demonstrated the predominant hepatic origin of fetal porphyrins excreted in the AF.
CONCLUSION: This study gives the first insight into heme metabolism in the AF during normal and pathological pregnancies.

INTRODUCTION

Heme is a major component of numerous proteins involved in many vital processes. It corresponds to the prosthetic group of various hemoproteins bearing primary physiological functions such as oxygen transport and storage (hemoglobin and myoglobin), electron transfer (mitochondrial respiratory chain), and detoxification (cytochrome P450). Heme biosynthesis, which is mainly located in adults within the erythroid cells of the medulla and the liver, involves a succession of eight different steps, leading from the condensation of glycine and succinyl–CoA to the heme prosthetic group (Fig. 1a). Eight enzymes, either mitochondrial or cytosolic, ensure this biosynthesis, which is tightly regulated, noticeably by heme itself, and any mutations in those enzyme genes could lead to inherited metabolic disorders named porphyria. All of the naturally occurring porphyrins have side chains on the carbon atoms of the pyrrole rings. In humans, the three major porphyrins, namely uroporphyrin, coproporphyrin, and protoporphyrin, differ by the arrangement of this side-chain substituents. Although isomers I and III of uro- and coproporphyrin are observed, only isomer III is used in the subsequent steps of the heme biosynthetic pathway. When uroporphyrinogen III synthase (UROIIIS) is deficient or immature, uroporphyrinogen isomer I is formed spontaneously by chemical ring closure of hydroxymethylbilane. Recently, it has been demonstrated that free heme, per se, is an essential element for all life forms and plays a major role in cell differentiation, hypoxia-inducible

response, and transcription regulation[1], for review. Moreover, free heme catabolites have been implicated in placental and fetal functions, (Fig 1b)[2]. Heme oxygenase 1 activity leads noticeably to the formation of carbon oxide, iron, and bilirubin, and its induction using cobalt protoporphyrin leads to abortion in a mouse model.[3]

Porphyrias are inherited diseases, either dominant or recessive, that affect the heme biosynthesis pathway. Most of them present a low penetrance that is partially elucidated.[4]

They are associated with an accumulation of porphyrins and/or their precursors, aminolevulinic acid (ALA), and porphobilinogen (PBG) (Fig. 1a)[5,6] They are traditionally divided into two classes, namely the hepatic and the erythropoietic porphyria, according to the organ that is mainly implied in the physiopathology. Indeed, in the liver and in the bone marrow, the first enzyme acid delta amino levulinique synthase (ALAS) exist in two different forms, the ubiquitous ALAS1 and the erythroid-specific ALAS2, that are differently regulated.

Porphyria symptoms are mainly skin lesions and acute neurovisceral symptoms, which can coexist in certain porphyria. The symptoms are related to the accumulation of the precursors of heme, mainly ALA and PBG, and of porphyrins. The diagnosis is based on a multistep strategy. The first step is the measure of ALA and PBG in urines and total porphyrins in urines, feces, erythrocytes, and plasma (with the characterization of the plasma maximum fluorescence emission). The second step is the separation of different porphyrins in urine and feces by high-

[1]Biochimie Clinique, Hôpital Beaujon, APHP, HUPNVS, Clichy, France; [2]UMRs 1149, Centre de Recherche sur l'Inflammation, Institut National de la Santé et de la Recherche Médicale, F-75018 Paris, France; [3]Reproductive Medicine and Medical Cytogenetics Department, Regional University Hospital and School of Medicine, Amiens, France; [4]Centre Français des Porphyries, Hôpital Louis Mourier, APHP, HUPNVS, Colombes, France; [5]UMRs1139, UFR des Sciences Pharmaceutiques, Université Paris Descartes, Paris, France; [6]Biochimie-Hormonologie, Hôpital Robert Debré, APHP, Paris, France and [7]Gynécologie-Obstétrique, Hôpital Robert Debré, APHP, Paris, France
Correspondence: Katell Peoc'h (katell.peoch@aphp.fr)
These authors contributed equally: Hana Manceau, Vincent Puy.
These authors jointly supervised this work: Hervé Puy, Francoise Muller, Katell Peoc'h.

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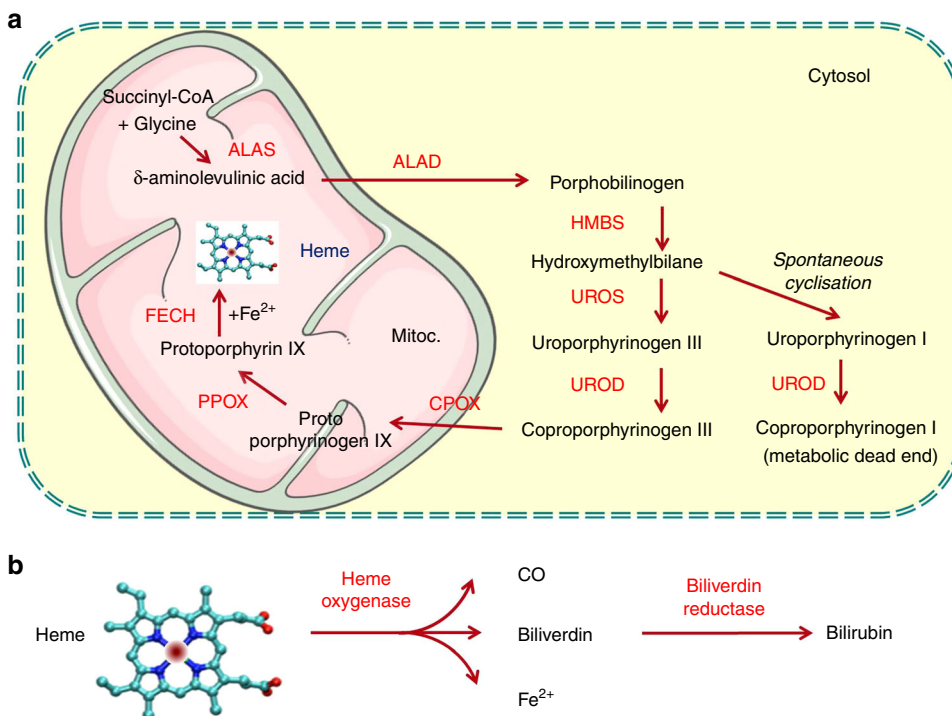H Manceau et al.

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Fig. 1 **a** Enzymes and intermediates of the heme biosynthetic pathway. The heme biosynthesis consists of eight successive steps that are localized either in the mitochondria or in the cytoplasm. The enzymes are indicated in red, whereas the substrates and products are indicated in blue. The first step is the condensation of succinyl–CoA and glycine. ALAS aminolevulinic acid synthase, ALAD aminolevulinic acid dehydrase, HMBS hydroxymethylbilane synthase, I, III or IX type isomers, UROIIIS uroporphyrinogen III synthase. This enzyme catalyzes the cyclization of four pyrrole rings into uroporphyrin isomer III. In the absence or limitation of this enzyme activity, hydroxymethylbilane is spontaneously converted into uroporphyrinogen I (UROI), without any isomerization. UROD urodecarboxylase, CPOX coproporphyrinogen oxidase, PPOX protoporphyrinogen oxidase, FECH ferrochelatase. **b** Heme catabolism. The bioavailability of heme is regulated by the induction of heme oxygenase 1 (HO-1) that leads to the biliverdin. The biliverdin is then oxidized into bilirubin, iron, and monoxide of carbon. HO-1 heme oxygenase 1, BVR biliverdin reductase, CO carbon monoxide

performance liquid chromatography (HPLC). Once the biochemical diagnosis of porphyria is certain, the measure of specific enzyme activity and the search of the genetic variants in the corresponding gene are performed.[7,8]

Prenatal diagnosis of porphyria was historically performed on the assay of porphyrins in the amniotic fluid (AF) due to a presumably pathological setting noticeably, in hepatoerythropoietic porphyria (OMIM #176100) and congenital erythropoietic porphyria (Günther's disease OMIM #263700). These diseases are associated with dark brown AF and increased porphyrins in the AF with a specific pattern of porphyrin and isomers.[9] Surprisingly, except in these rare pathological conditions, the metabolism of porphyrins and their precursors in the maternal–fetal compartment has never been described extensively.

The amniotic fluid is mainly composed of water, but also of fetal cells, fetal urine, fetal digestive secretions, and pulmonary secretions. Its composition is highly regulated.[10] This liquid is a part of the fetal compartment and undergoes frequent turnover. We previously demonstrated that digestive enzyme levels in AF during gestation reflect the different stages of development of the fetal intestinal tract[11,12] and allow to elucidate the origin of the different components of the AF. The first stage occurs at 7–8 weeks, with intestinal microvilli maturation, which leads to gastrointestinal secretions, followed at 9 weeks by the opening of the pharyngeal membrane and swallowing. Then, anal sphincter maturation occurs in several stages, the first being perforation of the anal membrane at 12 weeks, which coincides with the massive arrival of digestive enzymes in the AF. From 12 to 22 weeks, the digestive enzymes are present in the AF at high levels, peak at 16–17 weeks, and then progressively decrease to residual activities by 22–24 weeks. This phase corresponds to leakage of the

digestive enzymes into the AF. The second stage is the progressive development of the three anal sphincter muscles, leading to the gradual diminution of gastrointestinal secretion through the anal sphincter. In pathological situations, such as cystic fibrosis or anal atresia, the absence of digestive enzymes in the AF at 16–22 weeks confirms the relation between AF digestive enzyme levels and anal passage. Conversely, the abnormal presence of the digestive enzymes corresponding to bilious vomiting is observed in the upper gastrointestinal tract obstruction.[11]

To clarify the fetal origin of AF porphyrins, fetal bile, or fetal urinary, we studied AF porphyrin levels in three pathological situations, two limiting the bile excretion into AF by two independent mechanisms (anal atresia and slowing of intestinal transit up to the meconium ileus in cystic fibrosis), and one increasing the bile excretion into the AF (upper intestinal atresia associated with bile regurgitation and vomiting).

We evaluated the physiological and dynamic pattern of heme precursors in the AF during pregnancy in a cohort of 51 AF samples collected from weeks 13 to 33 during healthy human pregnancies. In addition, based on the differential analysis of ten pathological pregnancies, with fetuses suffering from lower digestive tract obstruction (anal atresia and cystic fibrosis) or upper intestinal atresia, we suggested that porphyrins present in the AF originate mainly from fetal bile and not from fetal urine.

PATIENTS AND METHODS
Samples
This study included two series of the AF samples. The first retrospective series consisted of 51 AF samples from unrelated pregnant women and healthy fetuses sampled in utero under

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ultrasound guidance using a 20-gauge needle. Visually bloody samples were excluded.

All samples were kept frozen at –40 °C following initial diagnostic work-up for a maximum time of 6 months. All AF samples were kept in a dark box or protected by aluminum paper to avoid any degradation by light.

Amniocentesis was a part of the routine diagnostic work-up and was performed in all cases for fetal karyotyping in singleton and caucasian non-smoker patients due to advanced maternal age (median maternal age: 38 years; range: 35–44 years).

Fetal karyotype was normal in all cases. Gestational age (weeks of amenorrhea) was determined by crown-rump length measurements at first-trimester ultrasound examination. The 51 samples were distributed between five periods of gestational age to allow the kinetic, quantitative, and qualitative study of porphyrins, and precursors in the AF, 12, 10, 12, 10, and 7 AF samples, were collected at 13–15 weeks, 16 weeks, 17–22 weeks, 23–26 weeks, and 29–33 weeks of gestation, respectively.

The second series consisted of a collection of ten AF samples from singleton and caucasian non-smoker pregnancies (median maternal age: 30 years; range: 25–35 years) with fetuses affected by, respectively, anal atresia ($n = 1$, gestational age at sampling: 16 weeks, surgery at birth), cystic fibrosis (homozygous for F508del *CFTR* gene mutation; rs113993960) ($n = 4$, gestational age at sampling: 16, 17, 24, and 24 weeks, termination of pregnancy for two early diagnosed cases and birth for two others), and upper intestinal atresia ($n = 5$, gestational age at sampling: 30–33 weeks, surgery at birth for all), associated with fetal bile vomiting in the AF.

By French law, consent for amniocentesis and laboratory testing was obtained from all women. All procedures were performed in accordance with the 1983 and 2008 revisions of the Declaration of Helsinki, and the study was approved by the Institutional Review Board and the Hospital Ethics Committee of the Bichat University Hospital.

Mass spectrometry LC-MS/MS analyses of ALA and PBG in the amniotic fluid

ALA and PBG levels were measured by an LC-MS/MS method (UPLC-Xevo TQMS, Waters, Guyancourt, France), as previously described. Briefly, ALA, PBG, and ALA-$^{13}C_5$, ^{15}N hydrochloride ($^{13}C_5$,^{15}N-ALA) were from Sigma-Aldrich (Saint Quentin Fallavier, France). Another set of ALA and PBG were from Frontier Scientific (Logan, UT). Two separate sets of 10 mM ALA and 2 mM PBG stock solutions were prepared in deionized H_2O for calibrators and quality control (QC) samples. The AFs were sonicated in the presence of 1 mM succinylacetone, and the samples were pretreated by solid-phase extraction using MCX columns (Waters, Guyancourt, France).

Calibrators were prepared by spiking ALA and PBG stock solutions in a solution pre-tested in charcoal-filtered AF that had no detectable ALA and PBG. The following ALA and PBG concentrations were added: 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 µM. All measures were assayed in duplicate.

Porphyrin separation and quantification in the amniotic fluid

All assays were performed according to the European Porphyria Network (EPNET) guidelines, and quality control schemes (www.porphyria-europe.org) were slightly adapted to AF. Total porphyrins, including uroporphyrin I, uroporphyrin III, coproporphyrin I, coproporphyrin III, and protoporphyrin IX (PPIX), were measured as follows: AF (300 µL) was diluted with 2.7 mL of 250 mmol/L phosphate buffer (pH 6.7) in a fluorometric microcuvette. After stirring, the fluorescence emission spectrum was recorded in a Shimadzu-RF 540 spectrofluorometer fitted with a red-sensitive photomultiplier (excitation at 405 nm, scan from 570 to 750 nm).

The separation of the different types of porphyrins (mainly protoporphyrin, coproporphyrin, and uroporphyrin) was determined by HPLC, in normal phase, after extraction. Porphyrins were extracted according to Lockwood et al.[13] and were analyzed by HPLC, according to Lim and Peters.[14] Briefly, porphyrins were extracted by vigorous vortex mixing of 0.5 mL of AF with 5 mL of ether-acetic acid (4:1 v/v). After centrifugation (10 min at $2000 \times g$), the supernatant was collected and vortex mixed with 3 mL of 2.7 M HCl. The lower acid layer was collected and methylated with methanol/sulfuric acid (95:5). After 12 h in the dark, the methyl ester porphyrins were extracted with chloroform and injected into the HPLC system.

The respective proportions of isomers I and III were analyzed by reverse-phase HPLC. HPLC separation of naturally occurring porphyrins was achieved on a Chromolith Reverse Phase-18 column with fluorimetric detection using a methanol/ammonium acetate gradient mobile phase. Eight porphyrins including protoporphyrin eluted within 20 min with a good resolution of each of the I and III positional isomer pairs for standards.[15]

Statistical analysis

Results are presented in the text as means and confidence interval (SD); they were compared using a two-tailed Wilcoxon rank-sum test (*$p < 0.05$; **$p < 0.01$) or Kruskal–Wallis test when appropriate. Nominal p values were considered significant when <0.05. Statistical analyses were performed using Prism 6 software (GraphPad, La Jolla, CA).

RESULTS

The goal of this study was to characterize in humans the heme precursor profile of AF. The sources of the AF samples are detailed in Table 1.

Changes in the amniotic fluid heme precursor levels during pregnancy

Porphyrin precursor levels in AF measured by LC-MS/MS were very low and did not vary significantly during gestation. PBG was below the quantification limit (0.5 µmol/L) and ALA was constant at 1.3 ± 0.1 µmol/L (detection limit of both parameters: 0.2 µmol/L).

The mean total porphyrins level in the AF from the 51 healthy pregnant women was 23.8 ± 17.0 SD (nmol/L; limit of quantification = 5 nmol/L; limit of detection = 3.5 nmol/L). Total porphyrins level in AF as a function of gestational age (Fig. 2a) tended to decrease during pregnancy, ranging from 140 to 6 nmol/L between weeks 13 and 33 (Fig. 2b; Kruskal–Wallis *$p = 0.0161$).

HPLC separation showed that coproporphyrin was overrepresented in AF (>90% of total porphyrins), the rest being uroporphyrin. Protoporphyrin IX was at the limit of detection (data not shown). Interestingly, the proportion of uroporphyrin progressively increased with gestational age (from 0.8% uroporphyrin at 13–14 weeks to 9.8% at 33 weeks), while coproporphyrin decreased (Fig. 3a, b). Analysis of copro- and uroporphyrin isomers I and III showed that isoform I predominated in AF (mean: 94.6% ± 0.03) and did not change during gestation.

Table 1. Amniotic fluid samples: source and gestational age when sampled

Gestational age (weeks)	All	13–15	16	17–22	23–26	29–33
Healthy cohort (*n*)	51	12	10	12	10	7
Affected fetus (*n*)	10					
- Cystic fibrosis	5		2	1	1	1
- Anal atresia	1			1		
- Upper intestinal atresia	4				1	3

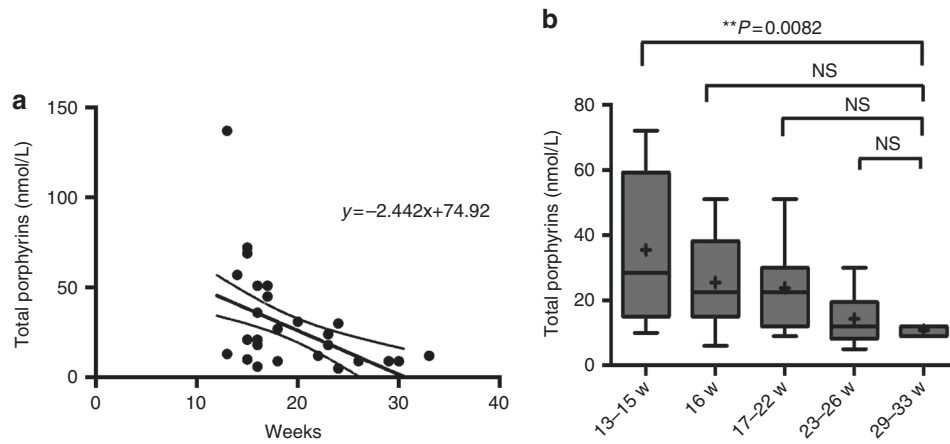Characterization and origin of heme precursors in amniotic fluid…
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Fig. 2 Porphyrin concentration in amniotic fluid as a function of gestational age. **a** Linear regression of total porphyrins (95% confidence interval are indicated). The equation is given. **b** Total porphyrin level in amniotic fluid during pregnancy. Mean levels of total porphyrins were plotted according to the week of gestation. Samples were as follow: 12 samples at 13–15 weeks, 10 samples at 16 weeks, the 12 samples at 17–22 weeks, 10 samples at 23–26 weeks, and 5 samples at 29–33 weeks
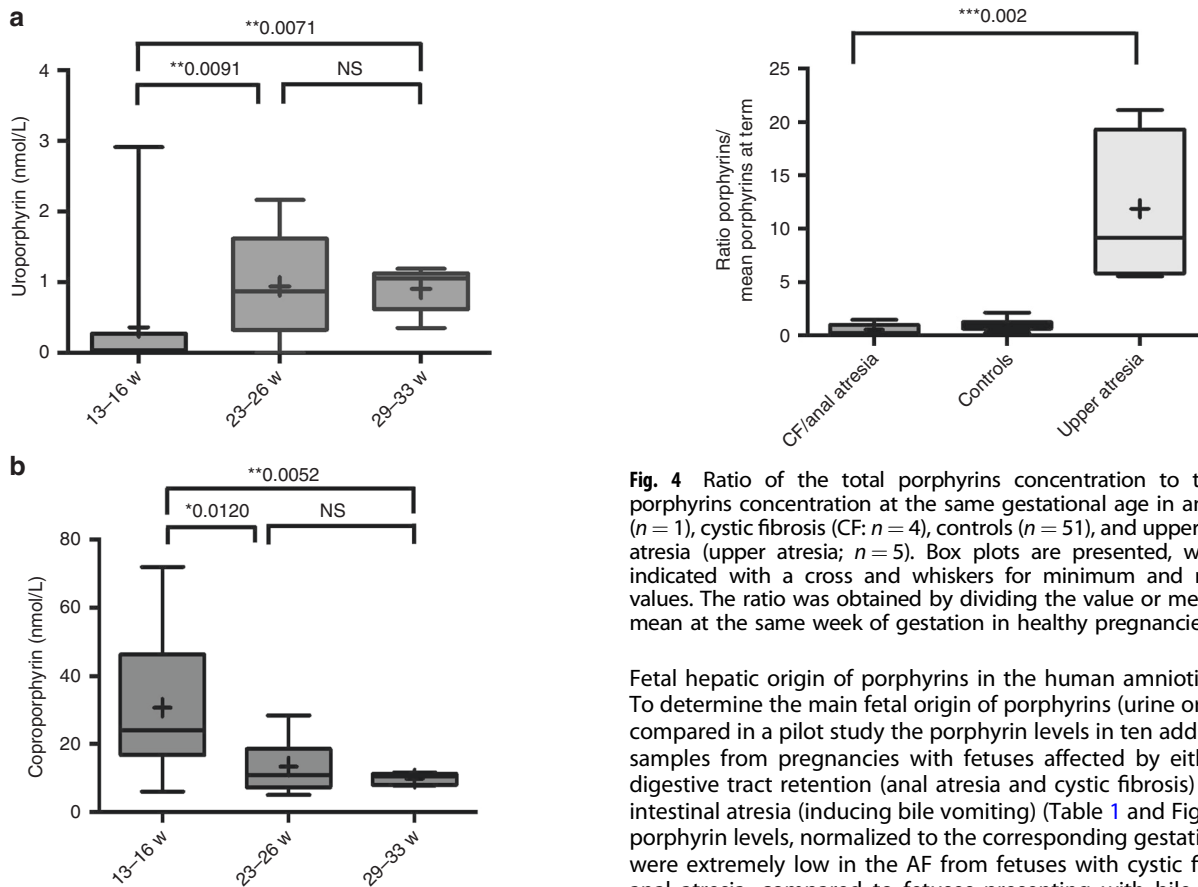

Fig. 3 **a** Uroporphyrin level in the amniotic fluid as a function of gestational age. Results were pooled as follows: 12 samples at 13–15 weeks, 10 samples at 16 weeks, 10 samples at 23–26 weeks, and 5 samples at 29–33 weeks. Box plots are presented, with the mean indicated with a cross and whiskers for minimum and maximum values. **b** Coproporphyrin concentration in the amniotic fluid as a function of gestational age. Results were pooled as follows: 12 samples at 13–15 weeks, 10 samples at 16 weeks, 10 samples at 23–26 weeks, and 5 samples at 29–33 weeks. Box plots are presented, with mean indicated with a cross and whiskers for minimum and maximum values
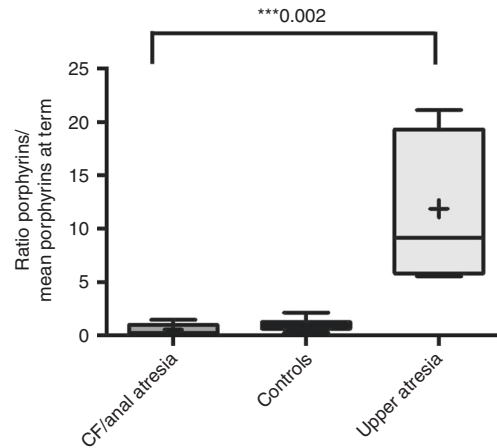

Fig. 4 Ratio of the total porphyrins concentration to the mean porphyrins concentration at the same gestational age in anal atresia ($n = 1$), cystic fibrosis (CF: $n = 4$), controls ($n = 51$), and upper intestinal atresia (upper atresia; $n = 5$). Box plots are presented, with mean indicated with a cross and whiskers for minimum and maximum values. The ratio was obtained by dividing the value or mean by the mean at the same week of gestation in healthy pregnancies

Fetal hepatic origin of porphyrins in the human amniotic fluid
To determine the main fetal origin of porphyrins (urine or bile), we compared in a pilot study the porphyrin levels in ten additional AF samples from pregnancies with fetuses affected by either distal digestive tract retention (anal atresia and cystic fibrosis) or upper intestinal atresia (inducing bile vomiting) (Table 1 and Fig. 4). Total porphyrin levels, normalized to the corresponding gestational age, were extremely low in the AF from fetuses with cystic fibrosis or anal atresia, compared to fetuses presenting with bile vomiting (Fig. 4; Kruska–Wallis ***$p = 0.002$).

DISCUSSION
In this study, we aimed to determine for the first time the pattern of excretion of porphyrin precursors, ALA and PBG, and porphyrins in the AF during pregnancy. In adults, heme biosynthesis is supposed to be ubiquitous, although two principal organs (bone marrow and liver) support 95% of its synthesis, the kidneys being involved at a low level. Heme biosynthesis during fetal life is not

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well characterized. To date, the physiological excretion of porphyrin precursors and porphyrins in human AF during the second and third trimesters of pregnancy is unknown.

In AF, ALA and PBG levels were at the limit of the detection range, and we showed that total porphyrin levels in the AF were in a similar range to those usually found in healthy adult sera (15–20 nmol/L).[8] Interestingly, we found that total porphyrins decrease during gestation and are mainly coproporphyrin. Uroporphyrin represented less than 1% of total porphyrins up to 23–26 weeks and then increased slowly. We also showed that copro- and uroporphyrins were type I isomers, with a tiny proportion of type III isomers. This observation suggested an immaturity of UROIIIS, the only enzyme that catalyzes the formation of the physiological type III isomers by dehydration and cyclization of the hydroxymethylbilane into uroporphyrinogen III.

To date, there are very few studies on porphyrins in the AF. First found in AF in 1967 by Fikenstcher[16], coproporphyrin was then studied in a large cohort of 207 samples by Goodlin and Schwarz in 1962.[17] However, no precise information regarding gestational age at sampling was available. Low levels (<3 μg/100 mL) were noted in 36 samples from healthy pregnancies, whereas high values in 13 abnormal samples were associated with erythroblastosis ($n = 1$) or with fetal death ($n = 12$), in which recessive porphyrias were not excluded.

Wolkoff et al.[18] reported a higher ratio of coproporphyrin I/III in six AF samples obtained during abortion, when compared with urine from adults or infants. They suggested that this predominance of type I isomer could be related to developmental immaturity of one or more enzymes of the biosynthetic pathway including UROIIIS.

The digestive origin of coproporphyrin in the AF was suggested by Gourey et al.,[19] who found a relationship between the leakage of meconium into the AF and the increase of coproporphyrins, leading to the use of coproporphyrin as a marker of fetal meconium inhalation[19,20]

To clarify the fetal origin of AF porphyrins, bile, or renal origin, we studied AF porphyrin levels in three pathological situations, two limiting bile excretion into AF by two independent mechanisms (anal atresia and slowing of intestinal transit up to the meconium ileus in cystic fibrosis), and one increasing bile excretion into the AF (upper intestinal atresia associated with bile regurgitation and vomiting). The results of these three independent investigations were consistent. Porphyrin levels were very low in anal atresia and cystic fibrosis, but high in upper intestinal atresia, compared to the normal values in AF. Moreover, the porphyrin profile found in the bile vomiting cases was qualitatively similar to that observed in normal AF. Altogether, these findings strongly suggest that fetal bile is the main source of AF porphyrins.

In conclusion, we showed that ALA, PBG, and PPIX were barely detectable in human AF. Total porphyrins followed the same pattern as digestive enzymes, with a high value up to 22 weeks and then a progressive decrease. Most of these porphyrins were coproporphyrin type I isomer, suggesting either immaturity of UROIIIS associated with decreased enzymatic activity or selectivity in the excretion of isomers.[21] During gestation, coproporphyrin gradually decreased and uroporphyrin increased. Finally, we concluded that porphyrins derive mainly from the fetal liver. Although pregnancy is usually uncomplicated in acute porphyria, heme therapy has been shown to be safe in pregnant women with acute attacks of porphyria.[22] However, the transplacental passage of porphyrins and heme remains undocumented. Our observations shed light on heme metabolism during the fetal life and indicate probable immaturity of the heme biosynthetic pathway during development. Further investigation of the transplacental passage of heme and its precursors is needed.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

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