

Bile acid profiles by capillary electrophoresis in intrahepatic cholestasis of pregnancy

Gustavo CASTAÑO*†, Silvia LUCANGIOLI‡, Silvia SOOKOIAN*, Marcelo MESQUIDA*, Abraham LEMBERG§, Mirta DI SCALA||, Paula FRANCHI‡, Clyde CARDUCCI‡ and Valeria TRIPODI‡

*Gastroenterology Section, Department of Internal Medicine, Hospital J. M. Penna, Buenos Aires, Argentina, †Consejo de Investigación, Buenos Aires, Argentina, ‡Department of Analytical Chemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina, §Department of Physiopathology, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina, and ||Division of Obstetrics and Gynecology, Hospital J. M. Penna, Buenos Aires, Argentina

A B S T R A C T

ICP (intrahepatic cholestasis of pregnancy) is characterized by pruritus and biochemical cholestasis, including raised SBAs (serum bile acids) and, usually, elevated aminotransferases levels. However, AHP (asymptomatic hypercholanaemia of pregnancy) is defined as the presence of total SBA levels above the cut-off value ($11 \mu\text{M}$) in healthy pregnant women, thus elevation of total SBAs do not necessarily reflect an ICP condition. The aim of the present study was to describe clinical, obstetric, perinatal and biochemical findings, as well as the SBA profile, in pregnant women studied in the third trimester of pregnancy in order to define characteristic patterns of individual bile acids that enable women with ICP to be distinguished from AHP and healthy pregnancies. Free and conjugated ursodeoxycholic (UDCA), cholic (CA), lithocholic (LCA), deoxycholic (DCA) and chenodeoxycholic (CDCA) acids were evaluated by CE (capillary electrophoresis) in 41 patients (15 of them simultaneously by HPLC), in 30 healthy pregnant women and in 10 non-pregnant women. A highly significant correlation between CE and HPLC for total SBAs ($r = 0.990$) and for individual SBAs was found. Normal pregnant women had higher total SBA levels than non-pregnant women (due to an increase in taurine-conjugated dihydroxy SBAs). Women with ICP had higher levels of total SBAs, the free/conjugated ratio, LCA, CA, CDCA and DCA than normal pregnant women. Newborns from women with ICP had lower birth weight and gestational age. Women with AHP had higher levels of conjugated dihydroxy SBAs than normocholanaemic patients, without any evidence of a clinical difference. In conclusion, the present study has shown a clear difference in SBA profiles between ICP and normal pregnancies (including AHP), involving a shift towards a characteristic hydrophobic composition in women with ICP.

INTRODUCTION

ICP (intrahepatic cholestasis of pregnancy) is characterized by the presence of pruritus and biochemical cholestasis, appearing usually during the third trimester

of pregnancy, persisting until delivery and disappearing spontaneously after parturition. Although serious complications have been described, ICP is usually a benign disease in the mothers [1,2]; however, this disease may have serious consequences for the fetus, such

Key words: asymptomatic hypercholanaemia, bile acid, capillary electrophoresis, intrahepatic cholestasis, pregnancy.

Abbreviations: AHP, asymptomatic hypercholanaemia; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CA, cholic acid; CDCA, chenodeoxycholic acid; CE, capillary electrophoresis; DCA, deoxycholic acid; ICP, intrahepatic cholestasis of pregnancy; IU, international units; LCA, lithocholic acid; SBA, serum bile acid; UDCA, ursodeoxycholic acid.

Correspondence: Dr Valeria Tripodi (email vtripodi@ffyba.uba.ar).

as increased proportion of premature deliveries, fetal distress and perinatal mortality [3]. Therefore ICP should be considered a high-risk condition, and an early and accurate identification of high-risk pregnancies may improve fetal outcome [4,5].

The diagnosis of ICP is based on pruritus with abnormal liver function tests in the absence of other pathological conditions. Biochemical characteristics of ICP usually include raised SBAs (serum bile acids) and mild or moderate levels of aminotransferases. Serum concentrations of ALP (alkaline phosphatase) and direct-reacting bilirubin are frequently high, and a minor number of patients may also present elevated levels of γ -glutamyl transpeptidase [6]. However, it is often difficult to diagnose ICP only by performing routine laboratory tests. In fact, the existence of subclinical cholestasis during pregnancy may also compromise the identification of the disease. Moreover, pruritus in pregnancy is a common symptom and it may be the only evidence in ICP, so it is necessary to discriminate women with ICP from those with the benign condition of pruritus gravidarum [5].

Up until now, the more specific biochemical parameter of ICP is the rise of total SBAs above the upper normal limit of $11 \mu\text{M}$ in late gestation [7]. Higher fetal complication rates have been associated with SBA levels $\geq 40 \mu\text{M}$ [8]. However, a subgroup of asymptomatic pregnant women with high levels of total SBAs and without pruritus and normal liver function tests has recently been described as women with AHP (asymptomatic hypercholanaemia of pregnancy) [9–11].

Some authors have observed differences in SBA profiles between ICP and normal pregnancies; nevertheless, their studies do not include patients with AHP and their distinctive profiles have not yet been clearly defined [4,5]. However, in a preliminary report [11], we have found a high incidence of AHP in our population.

Over the last few years, the increasing interest in the study of bile acid profiles in different diseases has led to improvements in the analytical methodology to determine SBA patterns. Although HPLC/MS and GC/MS represent the method of choice for bile acid measurements in biofluids, their usefulness is rather limited because of the complex instrumentation required for analysis. CE (capillary electrophoresis), with its relevant features of performance such as simplicity, very high resolution in a short time of analysis and low cost of operation, has become an alternative methodology in the analysis of SBAs [12–14].

We hypothesize that particular SBA profiles can distinguish ICP, normocholanaemic and hypercholanaemic normal pregnancies, and their characterization may help in the differential diagnosis of these conditions.

In the present study, we have determined SBA profiles using micellar electrokinetic chromatography, a type of CE methodology, and their relationship with biochemical, obstetric and perinatal characteristics in healthy

women in the third trimester of normal pregnancies, normocholanaemic pregnant women, women with AHP and women with ICP.

METHODS

Settings and study design

Between January 2004 and June 2005, we performed a cross-sectional study in normal pregnant women and women with ICP in the Hospital J. M. Penna, from the Government of the City of Buenos Aires, associated with the University of Buenos Aires. ICP incidence was calculated as 1.04% among 2596 deliveries occurring in 1 year.

SBAs were determined by CE and HPLC at the Department of Analytical Chemistry, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina.

This study was performed according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board and the Bioethical Committee of our Institution. Written consent was obtained from each subject in every case.

Patients

During the study period, 41 cases of women with ICP, 30 healthy pregnant women in the third trimester of pregnancy and ten non-pregnant women without history of oestrogen intake were studied.

Diagnosis of ICP was based on the presence of pruritus with elevated levels of aminotransferases and total SBAs during the second half of an otherwise uneventful pregnancy, and the absence of infection by hepatitis A, B or C viruses, autoimmune diseases, moderate-to-severe alcohol intake, HIV infection, skin diseases or biliary obstruction; followed by normalization of cholestasis after delivery.

Pruritus was measured arbitrarily: grade 1, intermittent, nocturnal and slight; grade 2, continuous, diurnal and nocturnal from slight to moderate; grade 3, severe; and grade 4, severe and also accompanied by insomnia or itching lesions. Complete medical, obstetric and perinatal data were recorded. Weight, Apgar score [15] and gestational age, evaluated by the method described by Capurro et al. [16], were recorded for the newborns. Small-for-gestational age was defined as birth weight for gestational age less than the specific 10th percentile cut-off of a published Argentinean fetal growth reference [17]. Additional clinical data of the patients are shown in Table 1.

Healthy pregnant women with total SBA levels above the usually accepted cut-off value of $11 \mu\text{M}$ in late pregnancy [7] were considered as having AHP. Healthy pregnant women with total SBA levels below the cut-off value were considered as normocholanaemic.

Liver function tests

Serum samples were obtained after a fasting period of 8 h, and aliquots were frozen at -20°C until bile acid determinations were performed. ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP and γ -glutamyl transpeptidase activities and total and conjugated bilirubin concentrations were carried out by routine automated techniques.

Micellar electrokinetic chromatography

Total SBAs, CA (cholic acid), CDCA (chenodeoxycholic acid), DCA (deoxycholic acid), LCA (lithocholic acid) and UDCA (ursodeoxycholic acid), in their free and glycine- and taurine-derivative forms, were assessed by micellar electrokinetic chromatography with UV detection. A detailed description of the analytical method has been described previously [12]. These determinations were carried out in all the patients and controls. Briefly, simultaneous determination of 15 SBAs was performed using an off-line C_{18} solid-phase extraction for sample clean-up and concentration. This step was followed by the complete separation of the bile acids in less than 12 min using cyclodextrin-modified micellar electrokinetic chromatography with UV detection (see Figure 1).

HPLC

Aliquots from 15 samples from women with ICP were separated and total SBAs, CA, CDCA, DCA, LCA and UDCA, in their free and glycine- and taurine-derivative forms, were evaluated by HPLC with UV detection following a standard protocol. These samples were also evaluated by CE simultaneously with HPLC. Details of the HPLC protocols have been described previously [13].

Statistical analysis

Results are expressed as means \pm S.E.M. Shapiro–Wilks' W of normality was performed. Differences between proportions were analysed by χ^2 test and Fisher's exact test. Differences between groups were analysed either by Student's t test or non-parametrical tests, according to the distribution. Levels of significance was established at $P < 0.05$. Pearson's r coefficient was calculated for correlations.

RESULTS

Women with ICP and normal pregnancies were comparable with respect to age, number of previous pregnancies, proportion of vaginal deliveries and proportion of patients born in other countries (Bolivia, Peru and Paraguay; Table 1).

ICP patients

Clinical and perinatal characteristics

Out of 41 ICP patients, ten had personal or familiar history of ICP. The onset of pruritus occurred before

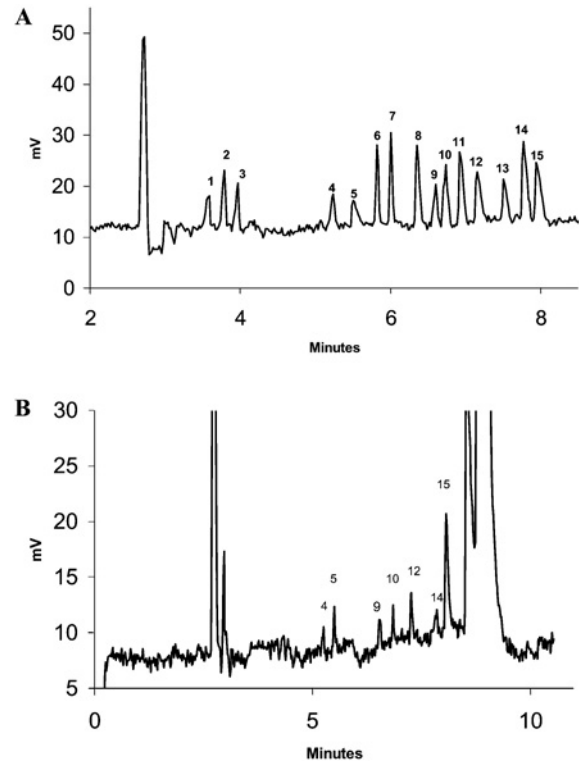


Figure 1 Separation of SBAs assessed by CE

(A) Electropherogram of a serum sample matrix spiked with 15 bile acid standards (6–10 μM for free bile acids and 4–6 μM for conjugated bile acids). (B) Electropherogram of a serum sample from a woman with ICP. 1, UDCA; 2, GUDCA (glycoursodeoxycholic acid); 3, TUDCA (tauroursodeoxycholic acid); 4, LCA; 5, CDCA; 6, GLCA (glycolithocholic acid); 7, TLCA (taurolithocholic acid); 8, GCDCA (glycochenodeoxycholic acid); 9, CA; 10, TCDC (taurochenodeoxycholic acid); 11, DCA; 12, GCA (glycocholic acid); 13, TCA (taurocholic acid); 14, GDCA (glycodeoxycholic acid); and 15, TDCA (taurodeoxycholic acid).

week 24 in eight patients. Five ICP patients had grade 1 or 2 pruritus and the others had grade 3 or 4. Labour at term was induced in seven patients. Additional data are shown in Table 1.

All the newborns, except one, had Apgar scores ≥ 7 at 1 min and ≥ 8 at 5 min. No neonatal deaths were observed. Newborns from women with ICP had lower weight and lower gestational age at birth, and a higher proportion of small-for-gestational age than those born from healthy women (Table 1). Four women with ICP presented pre-term deliveries because of meconium-stained amniotic fluid or Doppler sonography signs of chronic hypoxaemia to prevent neonatal death. Fetal complications (preterm deliveries and/or small-for-gestational-age newborns) were observed in 12 ICP cases compared with three in normal pregnant women. Fetal complications were not associated with total SBA levels.

Biochemical characteristics

Women with ICP had higher levels of total and direct bilirubin, aminotransferases and ALP than normal

Table 1 Clinical, obstetric, perinatal and biochemical characteristics of women with ICP and women with normal pregnancies

Results are expressed as means \pm S.E.M. For total bilirubin, the normal range is up to 18.8 $\mu\text{mol/l}$; for direct bilirubin, the normal range is up to 5.1 $\mu\text{mol/l}$. The normal ranges for AST, ALT and ALP are 12–31, 12–40 and 90–240 IU/l. * $P < 0.05$ and ** $P < 0.01$ compared with women with ICP.

	Women with	
	ICP	Normal pregnancies
<i>n</i>	41	30
Age (years)	27.3 \pm 1.0	26.1 \pm 1.4
Number of pregnancies (<i>n</i>)	2.8 \pm 0.3	2.8 \pm 0.5
History of ICP (<i>n</i>)	5	1
Familiar history of ICP (<i>n</i>)	5	0
Vaginal delivery (<i>n</i>)	22	12
Induced labour (<i>n</i>)	7	0*
Gestational age at delivery (weeks)	38.3 \pm 0.2	39.5 \pm 0.2**
Preterm delivery (< 37 weeks)	4	0
Birth weight (g)	2960 \pm 61	3303 \pm 71**
Small-for-gestational age (<i>n</i>)	9	3
Apgar score < 7 at min 1 (<i>n</i>)	1	0
Total bilirubin ($\mu\text{mol/l}$)	14.2 \pm 1.4	7.2 \pm 0.7**
Direct bilirubin ($\mu\text{mol/l}$)	6.8 \pm 0.9	1.7 \pm 0.3**
AST (IU/l)	96.0 \pm 14.2	21.0 \pm 1.5**
ALT (IU/l)	132 \pm 23	14.1 \pm 0.9**
ALP (IU/l)	662 \pm 53	321 \pm 27**

pregnant women (Table 1). Eleven women with ICP had γ -glutamyl transpeptidase levels above the upper normal limit of 32 IU (international units)/l.

Total SBAs and SBA profiles

Women with ICP had significantly higher ($P < 0.01$) levels of total SBAs than clinically healthy pregnant women (Table 2). Nonetheless, in some cases, total SBA values overlapped in both groups. We observed that CDCA, DCA and CA were increased moderately ($P <$

0.05), but LCA levels were increased 40-fold in women with ICP ($P < 0.01$). In addition, we observed that the percentage of LCA was higher than 5% of total SBAs in 31 out of 41 women with ICP, with a mean value of 25.2% (range, 0–97%).

With reference to the SBA conjugation pattern, no difference was found in conjugated SBAs between women with ICP and those with normal pregnancies (11.2 \pm 1.6 compared with 16.1 \pm 2.0 μM respectively). Nonetheless, a 6-fold increase in free SBAs was observed in women with ICP compared with women with normal pregnancies (13.4 \pm 2.2 compared with 1.9 \pm 0.7 μM respectively; $P < 0.01$), indicating that the free/conjugated bile acid ratio was higher in women with ICP ($P < 0.05$; Table 2).

Healthy pregnant women

Clinical and perinatal characteristics

Only one patient had a personal history of ICP. Spontaneous labour at term was observed in all vaginal deliveries.

No neonatal death was observed, and all the newborns had Apgar scores ≥ 7 at 1 min and ≥ 8 at 5 min. No preterm deliveries were observed, but three newborns were small-for-gestational age.

No difference was found in the outcome of newborns from normocholanaemic or hypercholanaemic women.

Biochemical characteristics

No difference was found either in aminotransferases or bilirubin levels between normocholanaemic or hypercholanaemic healthy pregnant women.

Total SBAs and SBA profiles

Normal pregnant women had higher total SBA levels than non-pregnant women ($P < 0.01$), due to an increment in taurine-conjugated dihydroxy SBA (mainly UDCA), without differences in LCA levels (Table 2). LCA in healthy pregnant women comprised up to 4% of total SBAs.

Table 2 Comparison of total SBA levels and SBA profiles in all of the groups studied

Results are expressed as means \pm S.E.M. Bile acids are expressed in their free, glycine and taurine forms.

	Non-pregnant women	Pregnant women with		
		Normocholanaemia	AHP	ICP
<i>n</i>	10	18	12	41
Total SBAs (μM)	3.2 \pm 0.7	6.6 \pm 0.8	21.9 \pm 3.2	29.5 \pm 3.3
LCA (μM)	0.05 \pm 0.06	0.3 \pm 0.2	0.10 \pm 0.03	8.2 \pm 1.7
CDCA (μM)	0.9 \pm 0.4	0.7 \pm 0.3	3.3 \pm 2.1	4.2 \pm 0.9
DCA (μM)	0.2 \pm 0.1	1.3 \pm 0.7	4.0 \pm 1.2	4.9 \pm 1.1
CA (μM)	0.7 \pm 0.4	0.7 \pm 0.1	1.1 \pm 0.7	3.6 \pm 0.8
UDCA (μM)	0.9 \pm 0.3	4.5 \pm 0.9	13.3 \pm 1.9	8.5 \pm 1.6
Free/conjugated ratio	1.1 \pm 0.3	0.05 \pm 0.02	0.3 \pm 0.1	1.3 \pm 0.3
Taurine/glycine ratio	5.0 \pm 0.2	5.6 \pm 0.2	6.7 \pm 0.2	5.7 \pm 0.1

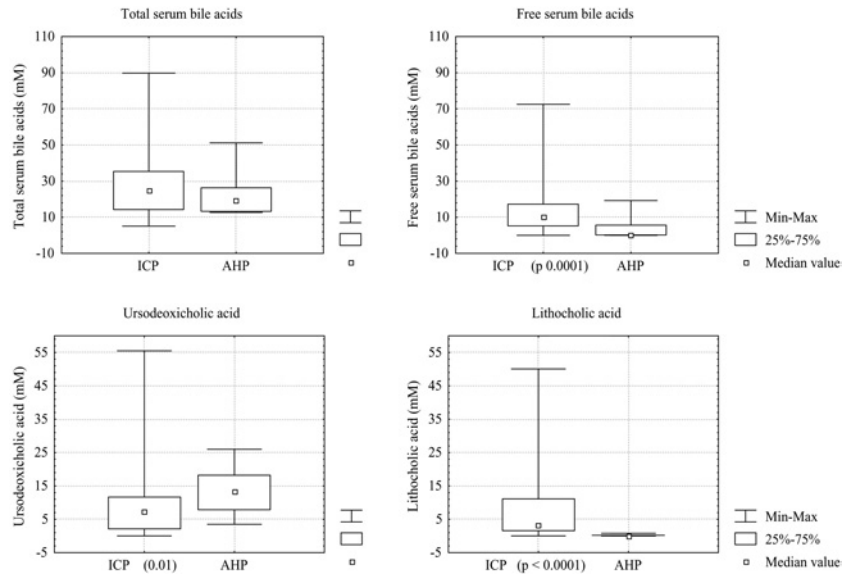


Figure 2 SBA profiles in women with ICP and AHP

Out of 30 normal pregnant women, 12 (40%) had total SBA levels above the cut-off value of $11 \mu\text{M}$ and were assigned to the AHP group. Women with AHP had significantly higher levels of total SBAs than normocholaemic pregnant women ($P < 0.001$; Table 2). This was due to an increase in the conjugated fraction (mainly taurine-conjugated forms) of UDCA (Table 2). No difference was found in LCA or free SBA levels between both groups of pregnant women.

When we compared the subgroup of normal pregnancies with AHP and women with ICP, no significant difference in total SBA levels was observed. However, women with ICP had significantly higher levels of LCA ($P < 0.0001$; Table 2) and free SBA (13.4 ± 2.1 compared with $3.2 \pm 1.7 \mu\text{M}$; $P < 0.001$) and lower levels of UDCA (Table 2) than patients with AHP (Figure 2).

Comparison between micellar electrokinetic chromatography and HPLC

When serum samples of 15 women with ICP were processed simultaneously by micellar electrokinetic chromatography and HPLC, a very high correlation coefficient between both methods for total SBA ($r = 0.990$) was found. Likewise, a good correlation was obtained for most of the individual SBAs analysed by the two methods (Figures 3 and 4).

DISCUSSION

In the present study, we describe the clinical, biochemical and obstetric characteristics, the perinatal outcomes and their relationship with the bile acid profiles assessed by CE in women with ICP and normal pregnant women in a hospital in the metropolitan area of Buenos Aires.

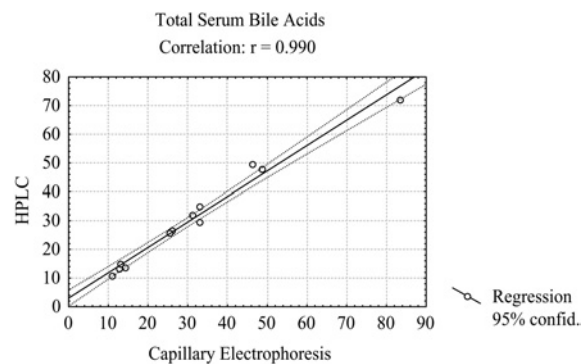


Figure 3 Correlation of total SBA measurements between CE and HPLC

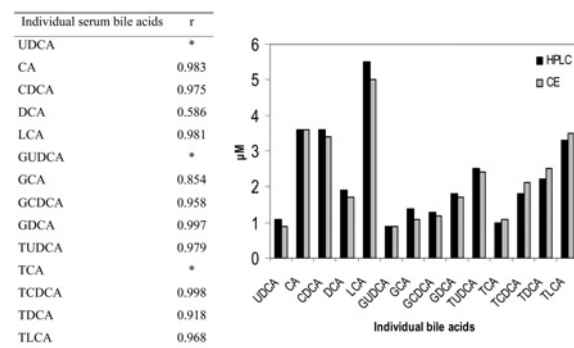


Figure 4 Comparison of the means and correlation coefficients for individual SBA measurements determined by CE and HPLC

Abbreviations of the individual SBAs are as indicated in the legend to Figure 1.

*Correlation cannot be calculated with less than three valid cases.

Micellar electrokinetic chromatography with UV detection allowed the quantification of CA, CDCA, DCA, LCA and UDCA in their free and conjugated forms. The complete separation of these bile acids, not easily feasible simultaneously with other methods, was performed not only with precision and accuracy, but also with more technical simplicity and lower costs of operation than other techniques [12]. Although GC/MS and HPLC/MS methods are the most suitable procedures for bile acid analysis, the high resolution achievable in a short time of analysis and simplicity with lower costs of operation makes micellar electrokinetic chromatography an excellent method of choice. Even though the CE method used in the present study has been validated following international guidelines ([12] and <http://www.fda.gov/cder/guidance/4252fnl.htm>), we compared our results by applying this methodology and HPLC with UV detection [13]. We found significant correlation between both methods for total and individual SBAs.

In the present study, we found that healthy pregnant women in the third trimester of pregnancy had higher total SBA levels than non-pregnant women. Moreover, we also observed that this increase in SBA levels was a result of a rise in dihydroxy SBA, predominantly UDCA. Additionally, an important increase in conjugation with taurine was observed in pregnant women. This finding is in agreement with previous studies that have shown that conjugated bile acid levels are higher in late pregnancy than in non-pregnant women [7,19,20]. This result could be associated with the concept of subclinical or physiological cholestasis of pregnancy [9,10]. It is important to emphasize that no difference was found in LCA levels, the most hydrophobic and toxic bile acid.

On the basis of our present results, it is important to highlight several aspects. First, from an epidemiological point of view, we found a relatively high incidence of ICP (1%), similar to those reported in Portugal or Finland [21,22]. Nonetheless, this incidence was lower than those previously found in neighbouring countries such as Chile or Bolivia [23,24]. In concordance with the literature, women with ICP had a history of personal or familial episodes of obstetric cholestasis more frequently than healthy pregnant women [22].

Secondly, from a clinical perspective, newborns from women with ICP had a higher incidence of fetal complications than those from normal pregnant women. A higher proportion of small-for-gestational-age newborns and preterm deliveries and lower gestational age and birth weight was associated with ICP. Nevertheless, no neonatal death was observed in our study.

Finally, with regard to the analysis of SBAs in normal pregnancy, we found a large proportion of healthy women with AHP in Argentina, with total SBA levels above the usually accepted cut-off value [9,10]. With the exception of a preliminary study by Pascual et al. [10], to our knowledge this is the first report on the SBA profiles

in AHP. First, women with AHP did not differ from normocholanaemic women in any clinical, biochemical or perinatal characteristic. In accordance with Pascual et al. [10], our present study of SBA profiles showed that no difference was found in CDCA and LCA levels in AHP compared with normocholanaemic pregnant women, whereas an increase in UDCA was observed (especially in its taurine forms). However, in contrast with Pascual et al. [10], no significant increase in CA levels in women with AHP was observed. Consequently, we can infer that AHP is not a clinical entity, but a subgroup of healthy pregnancies with high levels of conjugated dihydroxy SBA.

By contrast, the SBA pattern in women with ICP was rather different. Consistent with the literature, we observed higher total SBA levels in women with ICP than in women with normal pregnancies [7,25–27]. Nevertheless, total SBA levels overlapped with those of healthy pregnant women, making it difficult to obtain a differential diagnosis on the basis of the SBA measurements alone. In women with ICP, SBA profiles reported by other authors [7,10,26,27] had a prevalence of CA (up to 70% of total SBAs) with a smaller increase in CDCA. Likewise, in other cholestatic liver diseases, such as primary biliary cirrhosis, drug-induced cholestasis and alcoholic cholestasis, the most common finding reported is the rise in primary conjugated bile acids, especially the taurine-conjugated forms, although secondary bile acids are not increased significantly [28–32]. Although we also observed an increase in CA and CDCA, the highest increases were found in LCA and the free/conjugated ratio. Taking into account that our present results differ from other studies, probably due to differences in geography and, therefore, in genetic backgrounds of our population, a reference HPLC method [13] performed in 15 women with ICP allowed us to confirm the results obtained by CE.

Consequently, in our present study, the SBA composition from women with ICP revealed a shift towards a hydrophobic pattern, with higher levels of LCA and free SBAs, indicating that both parameters are better than total SBAs in discriminating and diagnosing AHP. It is assumed that these hydrophobic compounds may have a pathological role in the clinical and biochemical symptoms of cholestasis.

In conclusion, in our present study, we have observed that a high proportion of normal pregnancies had total SBA levels above the cut-off of 11 μ M, suggesting that AHP is a relatively common condition in Argentina. AHP does not appear to be a clinical entity itself, but a subgroup of normal pregnancies with higher levels of conjugated dihydroxy SBAs. Moreover, the present study has also shown a clear difference in the SBA profiles between ICP and normal pregnancies, even in the cases of AHP within the group of normal pregnant women. These differences involve a shift towards a hydrophobic

composition with higher levels of LCA and free bile acids in women with ICP. Finally, we propose that determining free SBAs, especially the measurement of LCA, is a useful parameter in the differential diagnosis of ICP and AHP.

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