

Is a Leaky Gut Involved in the Pathogenesis of Intrahepatic Cholestasis of Pregnancy?

Humberto Reyes,^{1,2} Rodrigo Zapata,¹ Ismael Hernández,² Martín Gotteland,³ Lorena Sandoval,¹ María Isabel Jirón,¹ Joaquín Palma,¹ Ramón Almuna,⁴ and Juan Jorge Silva⁵

Increased gastrointestinal permeability has been demonstrated in several liver diseases. It may facilitate the absorption of gut-derived endotoxin-stimulating Kupffer cells to release proinflammatory cytokines or other potentially hepatotoxic compounds. We examined gastrointestinal permeability, plasma levels of anti-lipopolysaccharides (anti-LPS), and four proinflammatory cytokines in 20 patients with intrahepatic cholestasis of pregnancy (ICP) compared with 22 normal pregnant and 29 non-pregnant women. Urinary excretion of sucrose and the urinary lactulose/mannitol (L/M) ratio after a standard oral load were used to assess gastrointestinal permeability. Anti-LPS (IgA, IgM, and IgG) were measured in peripheral blood by Human EndoCAB test kit; TNF- α , IL-1 β , IL-6, and IL-10 by Quantikine HS human immunoassays. Sucrose urinary excretion was similar in the three groups, indicating normal gastric permeability. The urinary L/M ratio was significantly higher in ICP than in the other groups [median (interquartile range): 0.018% (0.011-0.023) in ICP, 0.012% (0.009-0.016) in normal pregnancies, and 0.009% (0.008-0.012) in non-pregnant women, $P < .01$]. No significant differences were found in anti-LPS or cytokines plasma levels except slightly higher levels of IL-6 in ICP patients than in non-pregnant women ($P < .05$). Four of five women with abnormal urinary L/M ratio during ICP continued to show abnormalities in tests up to 2 years after delivery. **In conclusion**, an increased intestinal permeability was detected in ICP patients during and after pregnancy. A “leaky gut” may participate in the pathogenesis of ICP by enhancing the absorption of bacterial endotoxin and the enterohepatic circulation of cholestatic metabolites of sex hormones and bile salts.

Intrahepatic cholestasis of pregnancy (ICP) is a rare disorder of unknown cause that may develop during the third or second trimester of pregnancy and resolves rapidly after delivery. The chief complaint is pruri-

Abbreviations: ICP, intrahepatic cholestasis of pregnancy; anti-LPS, anti-lipopolysaccharides; TNF, tumor necrosis factor; IL, interleukin; ALT, alanine aminotransferase; L/M ratio, lactulose/mannitol ratio; tBS, total bile salts.

From the ¹Departamento de Medicina Oriente, ²Instituto de Ciencias Biomédicas, ³Instituto de Nutrición y Tecnología de Alimentos (INTA), ⁴Departamento de Obstetricia y Ginecología Oriente, and the ⁵Centro de Epidemiología Clínica, Universidad de Chile, Santiago de Chile.

Supported by funds from FONDECYT, Chile, Grant 1010648.

This work was presented, in part, at the 2004 Meeting of the American Gastroenterological Association (Gastroenterology 2004; 126 (Suppl 2): A-715).

Address correspondence to: Dr. Humberto Reyes, Universidad de Chile, Facultad de Medicina, Departamento de Medicina Oriente, Casilla 16038, Santiago 9, Chile. E-mail: hreyes@med.uchile.cl; fax number: (56) 2-274-1628.

tus, and serum liver tests reveal a mild cholestasis with increased levels of bile salts and aminotransferases. ICP may cause fetal distress, with stillbirths or premature deliveries leading to increased perinatal morbidity and mortality.^{1,2} The pathogenesis of ICP appears to be multifactorial. Potential contributors include a genetic predisposition interacting with the effects of estrogen and progesterone metabolites on bile secretory mechanisms.³ The influence of environmental factors has been suggested by the observation of a seasonal variability in the incidence of ICP, with highest rates reported during winter, a recurrence rate of only 45% to 70% in subsequent pregnancies of multiparous women, and the decrease in the prevalence of ICP detected in Sweden and Chile during recent decades.⁴ Therefore, identifying factors that may explain these epidemiological changes appear to be important.

The gastrointestinal mucosal epithelium is an essential barrier that normally restricts the passage of harmful molecules into the mucosa and systemic circulation. An increased intestinal permeability has been observed in patients with enteric damage, such as in inflammatory

bowel disease and coeliac disease, in intestinal infections, during the intake of aspirin, nonsteroidal anti-inflammatory drugs, or alcohol, in malnourished individuals, after burns, during total parenteral nutrition, in critically ill patients, and also in various extraintestinal diseases.⁵⁻¹⁷ These conditions determine a “leaky gut syndrome” with increased portal uptake of inflammatory mediators, bacteria, antigens, and toxins, enhancing the systemic distribution of potentially injurious macromolecules.

Bacterial endotoxins (lipopolysaccharides, LPS) are components of the outer wall of gram-negative bacteria that may induce severe pathological effects, including lethal shock and multiple organ failure. LPS are normally removed from the circulation by liver macrophages (Kupffer cells), and during this process the cells are activated to produce chemical mediators including the proinflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, and IL-10, eicosanoids, and free radicals (superoxide and nitric oxide), which may in turn cause liver cell damage. Gut-derived LPS and proinflammatory cytokines have been implicated as cofactors in different forms of liver injury.¹⁸⁻²⁵ Some of these reports have also documented an increase in intestinal permeability in patients with liver diseases that are notoriously more severe than ICP.

A disruption of the intestinal barrier could be a link between pregnancy and cholestasis by favoring the absorption of bacterial endotoxin to initiate the liver inflammatory cascade. We could not identify reports addressing intestinal permeability in human pregnancy nor data on plasma levels of LPS and proinflammatory cytokines in this physiological condition.

The aim of the current study was to assess intestinal permeability in patients with ICP, correlating it with plasma levels of antiendotoxin antibodies and of four proinflammatory cytokines. Results were compared with normal pregnant women with similar gestational age. Reference values in non-pregnant healthy women were also obtained.

Patients and Methods

Patients With ICP. Twenty patients referred to the obstetric ward with a diagnosis of ICP were selected for this study because they fulfilled the following characteristics: (1) pruritus that appeared during the third or second trimester of a previously uneventful pregnancy, starting in the palms and soles and then extending to other body areas; (2) no visible skin lesions (other than excoriations secondary to scratching), diabetes, or other systemic diseases that could cause pruritus; (3) serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase

(AST) were greater than 40 or 35 IU/L, respectively, or fasting total bile salts $>12 \mu\text{mol/L}$, in two consecutive blood samples drawn within a week; (4) no urinary, intestinal, or other infections requiring antibiotics had been detected in the previous weeks, and they were receiving no medications except for vitamins or iron supplementation; (5). normal physical examination relative to gestational age.

A weekly medical and obstetrical follow-up was applied according to a standard protocol adopted by our hospital for the care of patients with ICP.²⁶ Pruritus and serum liver tests were measured weekly from the beginning of the study until their return to normality was documented postpartum. Decisions on whether deliveries should be induced or cesarean sections should be performed were taken by the attending obstetricians with total independence from our study.

Normal Pregnancies. Twenty-nine unrelated healthy women in their third trimester of pregnancy were included as controls. Besides a normal physical examination, their serum general biochemical and liver profiles were normal. Fifteen of them were multiparous and they had no history of pruritus in previous pregnancies. All were followed until postpartum.

Non-Pregnant Women. Twenty-two unrelated healthy non-pregnant women were recruited among hospital employees, health care personnel, and medical students, to be included as a second control group. Seven of them had one or two pregnancies before the current study, with no history of pruritus. None were taking medications during the last weeks before the study, particularly aspirin, other nonsteroidal anti-inflammatory drugs, hormones, antibiotics, or alcohol. They had no history of recent gastrointestinal complaints, fever, cough, urinary symptoms, or evidence of any other infection. There was no history of smoking, and none of them was taking oral contraceptives. All had normal serum general biochemical and liver profiles at the time of this study.

General Laboratory Determinations. In all subjects, a urine bacterial culture was performed immediately before the study. Serum general biochemical, lipid, and liver profiles were measured by standard techniques. Serum total bile salts were measured by an enzymatic test, using a commercial kit (Merckotest®, Merck KGaA, Darmstadt, Germany). The normal levels for liver tests in our laboratory are total bilirubin $< 1.2 \text{ mg/dL}$; ALT, 9-40 IU/L; AST, 10-35 IU/L; gamma-glutamyl transaminase (GGT), 17-32 U/L in women; total alkaline phosphatases, 64-300 U/L (in non pregnant individuals); and total bile salts (tBS), 1-10 $\mu\text{mol/L}$.

Evaluation of Intestinal and Gastric Permeability. Intestinal and gastric permeabilities were assessed by the

5-hour urinary excretion of sugar probes after standard oral doses. Lactulose and mannitol were used as probes for intestinal mucosal permeability. Both are water-soluble molecules that are not metabolized by the body and are excreted in the urine in proportion to the amount that has been absorbed through the intestinal mucosa. This test is considered a reproducible, reliable, and well-established noninvasive method for assessing intestinal passive permeability, and it has been used extensively to evaluate mucosal integrity in several disease states and in healthy individuals.^{13,15-17,25} Sucrose was added to the lactulose/mannitol solution as a permeability probe for absorption through the stomach and proximal duodenum.²⁷⁻³³

Permeability tests were carried out starting at 08:00 to 09:00 AM. After an overnight fast, each person was asked to empty her bladder completely and then to drink a 450-mL solution containing 40 g sucrose, 7.5 g lactulose, and 2 g mannitol. This solution gives a reasonable caloric intake (sucrose) to maintain fasting for the following 5 hours. Thereafter, urine was collected for a period of 5 hours in a plastic container with 10 mL 10% thymol in isopropanol to avoid bacterial overgrowth. The 5-hour urine volume was measured, and aliquots were frozen at -20°C until processed. After 3 hours of urine collection, the subjects were allowed to drink water *ad libitum*. No patient presented a positive urine bacterial culture when intestinal permeability was assessed.

Determination of urinary sugar concentrations was carried out as previously described.³³ Control samples of urine with known amounts of added sucrose, lactulose, and mannitol were prepared and analyzed in parallel, using cellobiose and $\alpha\text{-CH}_3\text{-glucose}$ (Sigma Chemical Co., St. Louis, MO) as internal standards. Derivatized sugars were obtained after successive incubation of 10 mL urine sample with methoxyamine (Sigma) and N,O-bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane (Alltech, Deerfield, IL) in anhydrous pyridine. Two microliter samples dissolved in hexane were injected in the split mode on a AT1701 capillary column (Alltech) at a temperature of 200°C in a Varian 3600 gas chromatograph equipped with a split/splitless injector and a flame ionization detector (Varian Instruments, San Fernando, CA). Run-to-run variation of these measurements was $<10\%$. The results are expressed as the percentages of urinary recovery of sucrose, lactulose, and mannitol according to the 5-hour urine volume. The lactulose-to-mannitol ratio (L/M ratio) was calculated by dividing the percentage excretion of lactulose by the percentage excretion of mannitol.

Determination of Plasma Levels of Antiendotoxin Antibodies. Before starting the sugar absorption test, peripheral venous blood samples were taken into endotoxin-

free heparinized vacuum tubes to measure plasma levels of antibodies against endotoxin (anti-LPS) and fasting plasma levels of 4 cytokines: TNF- α , IL-1 β , IL-6, and IL-10. Aliquots of plasma were stored at -70°C until analysis. Anti-LPS were used as an evidence of previous exposure to endotoxin. Anti-LPS (IgA, IgM, and IgG) were quantified using an enzyme-linked immunosorbent assay (Human EndoCAB test kit from HyCult Biotechnology b.v., Uden, The Netherlands).³⁴ Results are expressed as standard median units per milliliter (MU/mL). Plasma levels of TNF- α , IL-1 β , IL-6, and IL-10 were measured using specific commercially available assays (Quantikine HS human immunoassays, R&D System, Minneapolis, MN). All measurements were performed in duplicate, and the intra-assay and inter-assay variability were below 10% for all assays.

Cytokines Concentration in Blood Mononuclear Cells After Stimulation In Vitro With LPS. Fasting blood samples were also used to measure in vitro LPS-stimulated peripheral-blood mononuclear cells production of TNF- α , IL-1 β , IL-6, and IL-10.³⁵ Quantitation of cytokines was performed in mononuclear cells incubated 24 to 48 hours with and without LPS as a stimulant factor.

Ethical Considerations. The study was approved by the Ethics and Clinical Research Committees of the University of Chile School of Medicine and the Hospital del Salvador, and an informed written consent was obtained from each participant.

Statistical Analysis. Normally distributed data are expressed as the mean \pm SD. Data that were not distributed normally are shown as mean and 95% confidence intervals or as the median and interquartile range. Student's *t* test for unpaired samples was used to compare demographic and laboratory characteristics between groups. Kruskal-Wallis one-way analysis of variance and Mann-Whitney rank sum test for unpaired data were used to analyze urinary sugar excretion, cytokines plasma levels, and *in vitro*-stimulated levels. Spearman rank order correlation test was used when pertinent. SigmaStat statistical package was used for data analysis. Statistical significance was established at $P \leq .05$.

Results

Subjects. Clinical and biochemical data are summarized in Table 1. The values shown were obtained in the date when intestinal permeability was assessed. Patients with ICP had significantly higher serum levels of aminotransferases, tBS, GGT, and alkaline phosphatases in comparison with normal pregnancies and non-pregnant women. An increased serum total bilirubin was observed in 5 of 20 ICP patients (25%), although clinically unde-

Table 1. Clinical and Biochemical Characteristics of Non-Pregnant and Normal Pregnant Women, and Patients With Intrahepatic Cholestasis of Pregnancy (ICP)

	Non-Pregnant (n = 22)	Normal Pregnant (n = 29)	ICP (n = 20)
Age (years)	26 ± 3 (20-34)	29 ± 4 (21-37)	28 ± 8 (17-42)
Pruritus in previous pregnancies	0/7	0/15	7/11
Onset of pruritus (week)	-	-	30.2 ± 4.4 (20-35)
Gestational age at study (week)	-	35.6 ± 1.9 (31-39)	34.2 ± 2.6 (28-38)
Pruritus score (0-4)	-	-	3.7 (3-4)
Bilirubin (mg/dL)	0.7 ± 0.4	0.4 ± 0.1	0.9 ± 0.3*
ALT (IU/L)	18 ± 7	13 ± 5	100 ± 127*
AST (IU/L)	22 ± 7	20 ± 5	85 ± 126*
GGT (IU/L)	18 ± 5	15 ± 7	47 ± 36*
Alk Phosph (U/L)	176 ± 60	385 ± 142†	825 ± 380*
Bile salts (μmol/L)	3.2 ± 2.9	6.7 ± 3.6	36.0 ± 32.8*

NOTE. Data shown as mean ± SD and, in parentheses, the range.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltranspeptidase; Alk Phosph, alkaline phosphatases.

*ICP vs. non-pregnant and vs. normal pregnant $P = .001$

†Normal pregnant vs non-pregnant $P < .001$.

tectable, with a highest value of 2 mg/dL. ALT was increased in 12 ICP patients (60%), AST in 15 patients (75%), and tBS in 17 patients (85%). ALT, AST, and tBS levels were simultaneously abnormal in 9 ICP patients (45%). Twelve ICP patients (60%) had GGT levels over our reference values in healthy pregnancies; however, the increase was mild (range, 38-140), and only three patients (15%) had values over 2 times the upper limit in normal pregnancies. Serum liver tests returned to normal within 3 weeks after delivery and, together with the abrupt fading of pruritus, they gave further support to the diagnosis of ICP. In all individuals serum urea nitrogen and creatinine were normal.

Gastric and Intestinal Permeability Studies. Table 2 shows the 5-hour urinary excretion of sucrose, lactulose, and mannitol in the three groups of individuals. Sucrose excretion in urine showed no significant differences among these groups. Lactulose excretion was significantly higher in ICP and in normal pregnancies than in non-pregnant women, but there was no difference between

patients with ICP and normal pregnant women. Mannitol excretion was significantly decreased in ICP patients, in comparison with normal pregnancies. The lactulose/mannitol ratio was similar in normal pregnancies and non-pregnant women, but it was significantly higher in patients with ICP. The increased L/M ratio in patients with ICP was due to the combined effect of an increase in urinary excretion of lactulose and a lower excretion of mannitol.

Figure 1 shows individual values of the ratio of lactulose/mannitol excretion in urine in the three groups studied. The broken horizontal line corresponds to a lactulose/mannitol ratio of 0.022 that was proposed in the literature as the upper limit in a large number of healthy adults.¹² Values in non-pregnant controls fell below this line or immediately over it (one case), whereas one normal pregnant and five patients with ICP gave values over the upper limit.

In ICP patients, there was no correlation between the urinary lactulose/mannitol ratio and serum bilirubin,

Table 2. Urinary Sugar Excretion in Non-Pregnant and Normal Pregnant Women, and in Patients With Intrahepatic Cholestasis of Pregnancy (ICP)

Urinary Excretion % Oral Dose	Non-Pregnant (n = 22)	Normal Pregnant (n = 29)	ICP (n = 20)
Sucrose	0.027 (0.023-0.035)	0.037 (0.025-0.055)	0.040 (0.023-0.073)
Lactulose	0.100 (0.071-0.145)	0.129* (0.101-0.187)	0.163† (0.119-0.201)
Mannitol	11.11 (7.57-13.43)	12.55 (10.54-15.60)	9.72‡ (7.67-11.10)
L/M ratio	0.009 (0.008-0.012)	0.012 (0.009-0.016)	0.018§ (0.011-0.023)

NOTE. Results are expressed as median values, with interquartile range in parentheses.

Abbreviation: L/M: lactulose/mannitol ratio (%).

Mann-Whitney Rank Sum Test:

*Normal pregnant vs. non-pregnant, $P = .038$.

†ICP vs. non-pregnant, $P = .005$.

‡ICP vs. normal pregnant, $P < 0.05$.

§ICP vs. normal pregnant and vs. non-pregnant, $P < .01$.

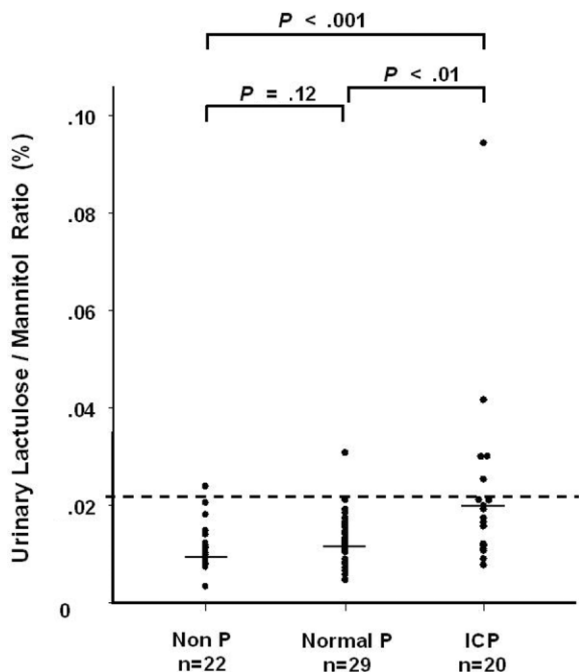


Fig. 1. Lactulose/mannitol ratio (%) in a 5-hour urine collection after a standard oral load of lactulose and mannitol, in non-pregnant women, in normal pregnancies (third trimester), and in patients with intrahepatic cholestasis of pregnancy (ICP). Short horizontal lines indicate group median values. The broken horizontal line corresponds to the upper limit in healthy adults (May et al.¹²).

aminotransferases, GGT, alkaline phosphatase, tBS or the time with pruritus (weeks) before the test was performed ($P > .05$ in all comparisons, Spearman rank order correlation).

Gastric and intestinal permeability tests could be repeated in the five ICP patients with abnormal L/M ratio, between 6 months and 2 years after delivery, when they were non-pregnant women with normal menstrual periods, normal physical examination results, and serum general biochemical and liver tests. Anti-endomysial and anti-transglutaminase antibodies were negative in them. In all these individuals urinary sucrose excretion was normal. In three women the L/M ratio was again abnormal, with values ranging from 0.023% to 0.078%. In another woman the L/M ratio was in the upper normal limit (0.022%), close to her previous value in pregnancy. In only one individual the L/M ratio had changed from 0.035% to 0.011%. The four individuals with abnormal (or borderline) L/M ratio during non-pregnant state were multiparous women with a history of recurrent ICP in almost all their pregnancies. The patient with the extreme L/M ratio of 0.094 % during ICP (Fig. 1) had the sugar absorption test repeated twice, giving values of 0.087% 6 months after delivery and 0.078% after 2 years.

Plasma Levels of Anti-LPS and Cytokines. Due to a temporary technical limitation, antibodies against LPS

and cytokines plasma levels could not be measured in the first individuals incorporated into this study, but they were measured in the subsequent 17 ICP patients, in 23 normal pregnancies, and in 18 non-pregnant women.

Antiendotoxin antibodies class IgA, IgM, and IgG were detectable in plasma samples from all individuals in whom these measurements were performed. In non-pregnant women, normal pregnant, and ICP patients, IgA EndoCAB values (mean, with 95% confidence intervals in parentheses) were 156 (89-222) MU/mL, 203 (126-280) MU/mL and 172 (23-320) MU/mL, respectively; IgM EndoCAB values were 255 (184-326) MU/mL, 236 (165-307) MU/mL, and 150 (111-188) MU/mL; and IgG EndoCAB values were 129 (82-175) MU/mL, 103 (77-130) MU/mL, and 280 (66-494) MU/mL. No significant differences were observed among the three groups examined ($P > .05$ for all comparisons) and in patients with ICP anti-LPS antibodies did not correlate with L/M ratio ($P > .05$).

Plasma levels of TNF- α , IL-1 β , IL-6, and IL-10 were slightly over the detection limit and in several samples they were undetectable, even using a high sensitive assay (Table 3). No significant differences were observed among ICP patients and controls. Plasma levels of IL-6 appeared slightly higher in normal pregnancies [3.0 (2.2-3.5) pg/mL], (median, with interquartile range in parentheses) and in ICP patients [3.4 (2.0-5.8) pg/mL] than in non-pregnant women [1.2 [0.5-1.9] pg/mL]; however, the difference was statistically significant only between ICP patients and non-pregnant women ($P = .011$).

In Vitro Secretion of Inflammatory Cytokines by LPS-Stimulated Monocyte Cells. *In vitro* production of TNF- α , IL-1 β , IL-6, and IL-10 by mononuclear cells stimulated with LPS was studied in 7 non-pregnant, in 14 normal pregnant women, and in 8 patients with ICP. None of the differences observed among the three groups reached statistical significance.

Outcome of Pregnancy. In normal pregnant women, all pregnancies were single, whereas among ICP patients one case had a twin pregnancy (dizygotic) and another

Table 3. Inflammatory Cytokine Plasma Levels in Non-Pregnant and Normal Pregnant Women, and in Patients With Intrahepatic Cholestasis of Pregnancy (ICP)

Cytokines pg/mL	Non-Pregnant (n = 18)	Normal Pregnant (n = 23)	ICP (n = 17)
TNF- α	0 (0-1.6)	0 (0-0)	0 (0-0)
IL-1 β	0 (0-0)	0 (0-4.5)	3.9 (0-4.4)
IL-6	1.2 (0.5-1.9)	3.0 (2.2-3.5)	3.4 (2.0-5.8)*
IL-10	3.4 (0-10.5)	0 (0-0)	0 (0-0)

NOTE. Results are expressed as median values with interquartile range in parentheses. Mann-Whitney Rank Sum Test: *ICP vs. non-pregnant; $P < .05$.

one had triplets. Deliveries at term (37-41 weeks of pregnancy) occurred in 97% of normal pregnancies and in 81% of patients with ICP and single pregnancies. All babies were born with weight adequate for gestational age and had an uneventful perinatal outcome.

Discussion

In the current study, we show a lactulose/mannitol ratio in urine significantly higher in ICP patients than in controls, identifying a "leaky gut" in 5 of 20 ICP patients in tests performed 2 to 10 weeks after the onset of pruritus. A mildly increased L/M ratio was also detected in one normal pregnant woman. The higher proportion of ICP patients with abnormal L/M ratio and the higher values observed in them suggest that the alteration is related to ICP and not only to pregnant state.

No differences were observed in the urinary excretion of sucrose among the three groups of individuals, indicating that gastric absorption of sucrose was unaffected by pregnancy or by ICP. Sucrose does not permeate to any appreciable extent the healthy gastric epithelium but is rapidly hydrolyzed in the small intestine by brush border enzymes. Absorption of intact sucrose and its excretion in urine reflects a damaged gastric or duodenal mucosa, as described by Meddings et al., using experimental models of gastric or duodenal lesions and confirmed in patients with gastric ulcers or severe gastritis.^{27,30} Other investigators have further validated this test as a marker of gastroduodenal mucosal injury in healthy volunteers after aspirin ingestion, or in patients with Crohn's disease, with gastric ulcer or cancer, with cirrhosis, or with Behcet's disease.^{32,36-40}

The L/M ratio is an index of integrity of the intestinal mucosa, under the postulate that the disaccharide lactulose crosses the epithelium through the paracellular pathway whereas the monosaccharide mannitol is preferentially absorbed by the transcellular pathway. Therefore, the area of absorption of mannitol is greater than that of lactulose, and more mannitol than lactulose is excreted in urine during the 5-hour collection test, giving an L/M ratio < 1.0% in healthy individuals. In pathological conditions, an increase in the L/M ratio is expected to result from increased absorption (and urinary excretion) of mannitol, whereas lactulose absorption (and urinary excretion) is less affected. However, contrasting results have been reported in several studies. Patients with untreated celiac disease have increased L/M ratio due to a higher lactulose permeability and a lower mannitol permeability, postulated as a result of the partial/total intestinal villous atrophy with a decreased area of absorption.⁴¹ Similar changes have also been observed in children with acute infectious diarrhea.⁴² Only in patients with celiac disease

have these functional changes been correlated with histological studies before and after treatment.

We found no reports assessing gastric and intestinal handling of sugars during pregnancy. Most pre-mucosal factors can reasonably assumed to be equal in pregnant and non-pregnant women. Nevertheless, small bowel and colonic transit has been shown to be prolonged during pregnancy.⁴³ In our study, differences in intestinal transit time between subjects were controlled by the simultaneous administration of a large and a small sugar and expressing the results as a ratio between the probe molecules. Post-mucosal factors also should be similar in pregnant and non-pregnant individuals, although a different volume of distribution for most absorbed solutes can be expected in late pregnancy. The fact that sucrose was handled similarly in healthy pregnant and non-pregnant women makes it difficult to expect that changes in urinary excretion of lactulose or mannitol in normal pregnancies and patients with ICP were attributable to a different volume of distribution. Therefore, the abnormal L/M ratio in 5 patients with ICP reflects an increased intestinal permeability in them. Whether this is a consequence of cholestasis or a preexisting condition is difficult to clarify. In the 5 patients with L/M ratio over 0.022%, the values appeared to be independent from the severity of biochemical parameters of cholestasis and also from the previous duration of the disease, estimated by the time with pruritus. However, the abnormal L/M ratio detected in four of these individuals in tests repeated long-term after delivery suggests that the "leaky gut" is a permanent abnormality in them and not a transient change during the cholestatic episode.

The finding of a "leaky gut" in some patients with ICP opens the possibility that it can lead to a pathological absorption of bacterial endotoxin from the gut lumen. Direct measurement of LPS was discarded because previous experiences by others have shown variable results in peripheral blood samples. Thus, we chose to measure antibodies against LPS as a reflection of previous exposure to bacterial endotoxin. A tendency to lower levels of anti-LPS class IgM was observed in ICP patients more than in controls, whereas anti-LPS class IgG appeared higher in ICP patients, but the differences were not statistically significant ($P = .052$ for IgG). No definite explanation can be anticipated for this borderline difference. Other authors have shown that Anti-LPS EndoCAB levels in peripheral blood may not correlate with L/M ratio in patients with abnormal sugar absorption tests or with increased orocecal transit time.^{14,44}

An altered intestinal barrier function could cause an increase in plasma levels of proinflammatory cytokines, released after exposure of Kupffer and other reticuloendo-

thelial cells to endotoxin delivered by the portal vein. However, among four cytokines measured in peripheral blood, only a mild (not significant) increase in IL-6 was observed in ICP patients and also in normal pregnant women. By measuring the same cytokines in mononuclear cells stimulated *in vitro* with LPS, we could have detected an exaggerated response in individuals who had recently been exposed to an abnormal absorption of LPS,³⁵ but we did not find significant differences among the three groups of individuals.

Studies in patients with acute pancreatitis, alcoholism associated with liver disease, and in patients with extensive burns documented a relationship between abnormal urinary L/M ratio and increased plasma levels of antiendotoxin antibodies and proinflammatory cytokines.^{17,25,29} Those clinical situations are clearly more severe than the mild biochemical cholestasis in our patients, who also had a less prominent increase in L/M ratio.

It has been shown that the sensitivity to endotoxin *in vivo* is increased in female rats and during pregnancy, when estrogen levels are high.^{45,46} Moreover, estradiol increases portal endotoxemia by increasing gut permeability, probably through vasodilatation and proliferation of gram-negative bacteria in the gut.⁴⁷ These animal models open the possibility that higher plasma endotoxin levels may lead to more extensive Kupffer cell activation in pregnant women.

In the current study, cytokine concentrations were measured in peripheral blood samples, a site that may give a weak prediction of a phenomenon expected to occur in a greater magnitude in the portal and sinusoidal circulation. Previous reports have shown that the concentrations of TNF- α and other proinflammatory cytokines in peripheral blood are low.^{48,49} Measurements are highly dependent on the sensitivity of the assays used, and some samples may fall below the limit of detection, mainly in healthy controls, as observed in our individuals.

In conclusion, an increased intestinal permeability—"leaky gut"—was detected in some patients with intrahepatic cholestasis of pregnancy, illustrating that it is a more complex and multifactorial disease than has hitherto been considered. We propose that an altered intestinal barrier function may facilitate the absorption of bacterial endotoxin and also increase the enterohepatic circulation of cholestatic metabolites of sex hormones and bile salts, influencing the pathogenesis of this disease.

Acknowledgment: The authors thank Professors Guillermo Figueroa and Carlos Muñoz (INTA, Chile) for discussing the protocol for this study. Marcelo López and Miriam Troncoso provided expert technical assistance in

laboratory determinations. Helpful comments by the anonymous reviewers and Marco Arrese, MD (Catholic University of Chile) allowed us to improve this manuscript.

References

1. Lammert F, Marschall H-U, Glantz A, Matern S. Intrahepatic cholestasis of pregnancy: molecular pathogenesis, diagnosis and management. *J Hepatol* 2000;33:1012-1021.
2. Riely CA, Bacq Y. Intrahepatic cholestasis of pregnancy. *Clin Liver Dis* 2004;8:167-176.
3. Reyes H, Sjövall J. Bile acids and progesterone metabolites in intrahepatic cholestasis of pregnancy. *Ann Med* 2000;32:94-106.
4. Reyes H, Báez ME, González MC, Hernández I, Palma J, Ribalta J, et al. Selenium, zinc and copper plasma levels in intrahepatic cholestasis of pregnancy, in normal pregnancies and in healthy individuals, in Chile. *J Hepatol* 2000;32:542-549.
5. Bjarnason I, Ward K, Peters TJ. The leaky gut of alcoholism: possible route of entry for toxic compounds. *Lancet* 1984;i:179-182.
6. Ukabam SO, Cooper BT. Small intestinal permeability to mannitol, lactulose, and polyethylene glycol 400 in celiac disease. *Dig Dis Sci* 1984;29:809-816.
7. Bjarnason I, Peters TJ. Intestinal permeability, non-steroidal anti-inflammatory drug enteropathy and inflammatory bowel disease: an overview. *Gut* 1989;30:22-28.
8. Deitz EA. Intestinal permeability is increased in burn patients shortly after injury. *Surgery* 1990;102:411-412.
9. Teahon K, Smethurst P, Levi AJ, Menzies IS, Bjarnason I. Intestinal permeability in patients with Crohn's disease and their first degree relatives. *Gut* 1992;33:320-323.
10. Harris CE, Griffiths RD, Freestone N, Billington D, Atherton ST, MacMillan RR. Intestinal permeability in the critically ill. *Intensive Care Med* 1992;18:38-41.
11. Van Elburg RM, Uil JJ, Mulder CJ, Heyman HS. Intestinal permeability in patients with celiac disease and relatives of patients with celiac disease. *Gut* 1993;34:354-357.
12. May GR, Sutherland LR, Meddings JB. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? *Gastroenterology* 1993;104:1627-1632.
13. Bjarnason I, MacPherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 1995;108:1566-1581.
14. Welsh FKS, Farmery SM, MacLennan K, Sheridan MB, Barclay GR, Guillou PJ, et al. Gut barrier function in malnourished patients. *Gut* 1998;42:396-401.
15. Hollander D. Intestinal permeability, leaky gut, and intestinal disorders. *Curr Gastroenterol Rep* 1999;1:410-416.
16. Kesharvazian A, Holmes EW, Patel M, Iber F, Fields JZ, Pethkar S. Leaky gut in alcoholic cirrhosis: a possible mechanism for alcohol-induced liver damage. *Am J Gastroenterol* 1999;94:200-207.
17. Penalva JC, Martínez J, Laveda R, Esteban A, Muñoz C, Sáez J, et al. A study of intestinal permeability in relation to the inflammatory response and plasma Endocab IgM levels in patients with acute pancreatitis. *J Clin Gastroenterol* 2004;38:512-517.
18. Nolan JP. Endotoxin, reticuloendothelial function, and liver injury. *HEPATOLOGY* 1981;1:458-465.
19. Nolan JP. Intestinal endotoxins as mediators of hepatic injury—an idea whose time has come again. *HEPATOLOGY* 1989;10:887-891.
20. Bird GLA, Sheron N, Goka AKL, Alexander GJ, Williams RS. Increased plasma tumor necrosis factor in severe alcoholic hepatitis. *Ann Intern Med* 1990;112:917-920.
21. Gershwin ME, Ansari AA, MacKay IR, Nakahuma Y, Nishio A, Rowley MJ, et al. Primary biliary cirrhosis: an orchestrated immune response against epithelial cells. *Immunol Rev* 2000;174:210-225.
22. Neuman M, Angulo P, Malkiewicz J, Jorgensen R, Shear N, Dickson ER, et al. Tumor necrosis factor-alpha and transforming growth factor-beta

- reflect severity of liver damage in primary biliary cirrhosis. *J Gastroenterol Hepatol* 2002;17:196-202.
23. Neuman MG. Cytokines—central factors in alcoholic liver disease. *Alcohol Res Health* 2003;27:307-316.
 24. McClain CJ, Song Z, Barve SS, Hill DB, Deaciuc I. Recent advances in alcoholic liver disease. IV. Dysregulated cytokine metabolism in alcoholic liver disease. *Am J Physiol (Gastrointest Liver Physiol)* 2004;287:G497-G502.
 25. Rao RK, Seth A, Sheth P. Recent advances in alcoholic liver disease: role of intestinal permeability and endotoxemia in alcoholic liver disease I. *Am J Physiol (Gastrointest Liver Physiol)* 2004;286:G881-G884.
 26. Zapata R, Sandoval L, Palma J, Hernández I, Ribalta J, Reyes H, et al. Ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy: a 12-year experience. *Liver Int* 2005;25:548-554.
 27. Meddings JB, Sutherland LR, Byles NI, Wallace JL. Sucrose: a novel permeability marker for gastroduodenal disease. *Gastroenterology* 1993;104:1619-1626.
 28. Gotteland M, Cruchet S, Frau V, Wegner ME, López R, Herrera T, et al. Effect of acute smoking, alone or with alcohol, on gastric permeability to sucrose on healthy volunteers. *Dig Liver Dis* 2002;34:702-706.
 29. Olgún F, Araya M, Hirsch S, Brunser O, Ayala V, Rivera R, et al. Prebiotic ingestion does not improve gastrointestinal barrier function in burn patients. *Burns* 2005;31:482-488.
 30. Sutherland LR, Verhoef M, Wallace JL, Van Rosendaal G, Crutcher R, Meddings JB. A simple, non-invasive marker of gastric damage: sucrose permeability. *Lancet* 1994;343:998-1000.
 31. Smecuol E, Bai JC, Vazquez H, Kogan Z, Cabanne A, Niveloni S, et al. Gastrointestinal permeability in celiac disease. *Gastroenterology* 1997;112:1129-1136.
 32. Wyatt J, Oberhuber G, Pongratz S, Puspok A, Moser G, Novacek G, et al. Increased gastric and intestinal permeability in patients with Crohn's disease. *Am J Gastroenterol* 1997;92:1891-1896.
 33. Gotteland M, Araya M, Pizarro F, Olivares M. Effect of acute copper exposure on gastrointestinal permeability in healthy volunteers. *Dig Dis Sci* 2001;46:1909-1914.
 34. Barclay GR. Endogenous endotoxin-core antibody (EndoCAB) as a marker of endotoxin exposure and a prognostic indicator: a review. *Prog Clin Biol Res* 1995;392:263-272.
 35. Murata H, Shimizu Y, Okada K, Higuchi K, Watanabe A. Detection and analysis of intracytoplasmic cytokines in peripheral blood mononuclear cells in patients with drug-induced liver injury. *J Hepatol* 2003;38:573-582.
 36. Rabassa AA, Goodgame R, Sutton FM, Ou CN, Rognerud C, Graham DY. Effects of aspirin and *Helicobacter pylori* on the gastroduodenal mucosal permeability to sucrose. *Gut* 1996;39:159-163.
 37. Puspok A, Oberhuber G, Wyatt J, Maier-Dobersberger T, Hammer J, Pfeffel F, et al. Gastroduodenal permeability in Crohn's disease. *Eur J Clin Invest* 1998;28:67-71.
 38. Kawabata H, Meddings JB, Uchida Y, Matsuda K, Sasahara K, Nishioka M. Sucrose permeability as a means of detecting diseases of the upper digestive tract. *J Gastroenterol Hepatol* 1998;13:1002-1006.
 39. Di Leo V, Venturi C, Baragiotta A, Martines D, Floreani A. Gastroduodenal and intestinal permeability in primary biliary cirrhosis. *Eur J Gastroenterol Hepatol* 2003;15:967-973.
 40. Koc B, Aymelek S, Sonmez A, Yilmaz MI, Kocar H. Increased sucrose permeability in Behcet's disease. *Rheumatol Int* 2004;24:347-350.
 41. Johnston SD, Smye M, Watson RP. Intestinal permeability tests in coeliac disease. *Clin Lab* 2001;47:143-150.
 42. Isolauri E, Juntunen M, Wiren S, Vuorinen P, Koivula T. Intestinal permeability changes in acute gastroenteritis: effects of clinical factors and nutritional management. *J Pediatr Gastroenterol Nutr* 1989;8:466-473.
 43. Baron TH, Ramírez B, Richter JE. Gastrointestinal motility disorders during pregnancy. *Ann Intern Med* 1993;118:366-375.
 44. Soza A, Riquelme A, González R, Alvarez M, Pérez-Ayuso RM, Glasinovic JC, et al. Increased orocecal transit time in patients with nonalcoholic fatty liver disease. *Dig Dis Sci* 2005;50:1136-1140.
 45. Ikejima K, Enomoto N, Iimuro Y, Ikejima A, Fang D, Xu J, et al. Estrogen increases sensitivity of hepatic Kupffer cells to endotoxin. *Am J Physiol (Gastrointest Liver Physiol)* 1998;274:G669-G676.
 46. Nanji AA, Jokelainen K, Fotouhinia M, Rahemtulla A, Thomas P, Tipoe GL, et al. Increased severity of alcoholic liver injury in female rats: role of oxidative stress, endotoxin, and chemokines. *Am J Physiol (Gastrointest Liver Physiol)* 2001;281:G1348-G1356.
 47. Farhat MY, Lavigne MC, Ramwell PW. The vascular protective effects of estrogen. *FASEB J* 1996;10:615-624.
 48. Mendall MA, Patel P, Asante M, Ballam L, Morris J, Strachan DP, et al. Relation of serum cytokine concentrations to cardiovascular risk factors and coronary artery disease. *Heart* 1997;78:273-277.
 49. Loos BG, Craandijk J, Hoek FJ, Wertheim-Van Dillen PME, Van Der Velden U. Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. *J Periodontol* 2000;71:1528-1534.