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Case Control Study

Liver stiffness in pregnant women with intrahepatic cholestasis of pregnancy: a case control study

Liver stiffness and ICP

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Abstract

BACKGROUND

Intrahepatic cholestasis of pregnancy (ICP) is a rare but severe complication for both the mother and the unborn child. The diagnosis is primarily based on elevated serum levels of bile acids. In a large ICP cohort, we here study in detail liver stiffness (LS) using transient elastography, now widely used to non-invasively screen for liver cirrhosis within minutes.

AIM

The aim of the present study was to specifically explore LS in a large cohort of women with ICP compared to a control group with uncomplicated pregnancy.

METHODS

LS and hepatic steatosis marker Controlled Attenuation Parameter (CAP) were measured in 100 pregnant women with ICP using transient elastography (Fibroscan, Echosens, Paris, France) between 2010 and 2020. In 17 cases, LS could be measured postpartum. 450 women before and 38 women after delivery with uncomplicated pregnancy served as control group. Routine laboratory, levels of bile acids and apoptosis marker M30 were also measured.

RESULTS

Women with ICP had significantly elevated transaminases but normal gamma-glutamyl transferase (GGT). Mean LS was significantly increased at 7.3 ± 3.0 kPa compared to the control group at 6.2 ± 2.3 kPa ($p < 0.0001$). Postpartum LS decreased significantly in both groups but was still higher in ICP (5.8 ± 1.7 kPa *vs* 4.2 ± 0.9 kPa, $P < 0.0001$), respectively. In ICP, LS was highly significantly correlated with levels of bile acids and M30 but not transaminases. No correlation was seen with GGT that even increased significantly after delivery in the ICP group. Bile acids were mostly correlated with the liver apoptosis marker M30, LS and levels of ALT, AST, and bilirubin. In multivariate analysis, liver

stiffness remained the sole parameter that was independently associated with elevated bile acids.

CONCLUSION

In conclusion, LS is significantly elevated in ICP which is most likely due to toxic bile acid accumulation and hepatocyte apoptosis. In association with conventional laboratory markers, LS provides additional non-invasive information to rapidly identify women at risk for ICP.

Key Words: Intrahepatic cholestasis of pregnancy (ICP); transient elastography; bile acids; liver stiffness; high risk pregnancy

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Core Tip: Intrahepatic cholestasis of pregnancy (ICP) is a rare but severe complication for both the mother and the unborn child. In a large ICP cohort, we here study in detail liver stiffness (LS) using transient elastography, now widely used to non-invasively screen for liver cirrhosis within minutes. LS was significantly elevated in pregnancies with ICP which is most likely due to toxic bile acid accumulation and hepatocyte apoptosis. Interestingly, no correlation was seen with gamma-glutamyl transferase. In association with conventional laboratory markers, LS provides a novel non-invasive tool to rapidly identify women at risk for pregnancy complications.

INTRODUCTION

About 3% of pregnant women suffer from liver disorders which can cause severe problems for the mother and the unborn child *e.g.* liver failure, preterm labor and stillbirth ^[1-3]. Despite an intensive research on pregnancy-related liver complications in

the last decades, treatment options are still insufficient and no effective screening tests for early assessment have been established [4-6]. Intrahepatic cholestasis of pregnancy (ICP) with elevated serum bile acids levels higher than 20 $\mu\text{mol/L}$ is the most common pregnancy-specific liver disease. The etiology is complex consisting of genetic, endocrine (circulating estrogen and progesterone), and environmental factors (reduced vitamin D and selenium in winter). Severe forms with bile acids levels higher than >40 $\mu\text{mol/L}$ are associated with abnormal fetal echocardiography, meconium-stained amniotic fluid, spontaneous preterm labour and fetal asphyxia. Moreover, women with serum total bile acids of 100 $\mu\text{mol/L}$ have an increased risk of stillbirth. The incidence varies between 0.05 to 27.6% of all pregnancies [7-12].

ICP typically presents in the third trimester with nocturnal pruritus of soles and palms [13]. ICP also increases the risk for gestational diabetes and pre-eclampsia [14]. Long-term consequences of ICP consist of higher risk for cancer of the liver and biliary tree, diabetes mellitus, thyroid disease, autoimmune (psoriasis, inflammatory polyarthropathies and Crohn's) and cardiovascular disease [15]. Treatment with ursodeoxycholic acid has been proven to significantly improve itching, blood levels and fetal outcome in numerous studies while termination of pregnancy remains the only causal therapy [2, 7]. A quick diagnosis of ICP is essential to protect mother and child from (long-term) complications [16]. Diagnosis is normally based on elevated serum levels of bile acids. Unfortunately, these blood tests usually take several hours, even at maximum care facilities, and they are not available at any time. So far, there are no screening test for liver disease in pregnancy besides serological testing for viral hepatitis in the third trimester.

Despite the scarcity of data on ICP, an enormous progress has been made in the molecular understanding of cholestatic liver diseases in the last decades [10]. Hepatocytes and cholangiocytes cooperatively produce bile which is a mixture of organic and inorganic compounds [17]. Cholestasis usually describes an impairment of

bile flow either caused by defects of the hepatocytes, which form and secrete bile, and/or caused by defects in the secretory machinery of cholangiocytes ^[17] . The detergent properties of bile render it highly toxic to cells and tissues ^[17]. In addition to drugs, inflammation, liver disease and hormones, a number of gene mutations have been discovered that can cause cholestasis and ICP ^{[8-10, 18-20][21]}.

Measurement of liver stiffness (LS) through elastographic techniques has become the novel gold standard for the non-invasive diagnosis of liver fibrosis and cirrhosis and it allows often to avoid invasive liver biopsies ^[22]. Transient elastography (TE, Fibroscan, Echosens, Paris, France) has been the first elastographic technique which is an ultrasound-based technique using a transducer probe creating an elastic shear wave ^[23]. It utilizes pulse-echo ultrasound to measure this shear wave velocity which is directly associated with liver stiffness expressed in kilopascals (kPa). TE takes only a few minutes, is highly accurate, has a lower sampling error compared to the biopsy and, thus, allows for repetitive measurements ^[24]. LS values below 6 kPa are considered as normal while generally accepted cut-off values for liver fibrosis (F3) and cirrhosis (F4) are 8 and 12.5 kPa ^[25]. However, LS is not only elevated by fibrosis stage but also other important confounding factors including physiological conditions such as food and alcohol intake or pathological confounders such as inflammation or pressure elevation ^[26]. Of note, all these confounders but also all artifacts will always increase LS but never decrease which is the most important reason while a normal LS has a very high negative predictive value in excluding liver pathologies ^[24].

For these reasons, liver elastography has been an ideal diagnostic tool to address hepatic complications during pregnancy. In the first elastography study in more than 500 pregnant women without liver disease, we could recently demonstrate that LS increased significantly in the third trimester and was an independent predictive factor for pre-eclampsia ^[27]. The data have been independently confirmed by a smaller study from Denmark ^[28]. Moreover, in pregnant women with manifest cirrhosis, LS predicts

hepatic decompensation after delivery [29]. ⁶ The aim of the present study was to specifically explore LS in a large cohort of women with ICP compared to a control group with uncomplicated pregnancy.

MATERIALS AND METHODS

Study design and patient cohort

The study protocol (435/2006 and S201/2015) of this observational, prospective, case-control study was approved by the Ethics Committee of the University of Heidelberg and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The study design is shown in Figure 1. Briefly, between February 2010 and March 2020, 652 women were recruited at the Department of Gynecology at the University of Heidelberg or at Salem Medical Center in Heidelberg during prenatal ultrasound or presented to the prenatal outpatient's department with prenatal complications or at the ward. Postpartum examination took place 24h after delivery. Inclusion criteria were age ≥ 18 years and an intact pregnancy at week 9 to 42 or status postpartum. The healthy control cohort was taken from our previous study [27]. ICP was diagnosed based on typical clinical symptoms such as pruritus and laboratory markers like elevated transaminase levels and serum bile acids levels $> 20 \mu\text{mol/L}$. Exclusion criteria were: No signed informed consent; other pregnancy complications such as preeclampsia or HELLP syndrome; no valid LS measurement.

Laboratory parameters

Routine blood parameters were measured at the general laboratory of the University Hospital Heidelberg and the Limbach laboratory in Heidelberg. In 100 patients, serological detection of caspase-cleaved (M30) and total (M65) cytokeratin 18 Levels as markers of liver apoptosis were measured as described previously by ELISA (Peviva, Bromma, Sweden) [30]. We also measured total bile acids not only in women suspected of ICP but also in 60 women of the control group for comparative purposes.

Liver Stiffness and controlled attenuation parameter (CAP)

LS and CAP were measured using transient elastography (Fibroscan, Echosens, Paris, France). The M or XL probes were used according to the manufacturer's specifications placing them on the right lobe of the liver, intercostal position as described previously [31]. The LS and CAP value is the calculated median of at least 10 consecutive measurements. In parallel to the liver elastography at the control group, a routine abdominal ultrasound was performed to exclude liver pathologies such as liver cirrhosis, liver congestion or liver tumors. In addition, the degree of liver steatosis was graded (0-3) and spleen size was determined. Cut-off values from a recent metaanalysis were applied [32]. Valid LS measurements could be obtained in all women.

Statistical methods

The statistical analysis was performed using SPSS Statistics version 23.0 (IBM, New York, USA), Excel 2016 (Microsoft, Redmont, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, USA). For group comparisons mean and standard deviations were calculated and the independent samples t-test were used. For conduction of a correlation analysis, the Spearman rank-order correlation coefficient was calculated. Univariate and multivariate binary logistic regression analysis was used to identify independent predictors for pregnancy complications and a receiver operator characteristic (ROC) analysis was performed.

RESULTS

Patient characteristics

For better comparison, Table 1 only presents patient characteristics of women (control and ICP) in the third trimester and after delivery. Almost all women with ICP (98 out of 100, 98%) were in the third trimester, which is consistent with the literature [33]. With regard to the control cohort, 228 out of 450 (50.7%) were in the third trimester. A smaller number of women could be followed up one day after delivery both for controls and ICP ($n = 38$ and $n = 17$). Suppl. Table 1 shows the patient characteristics of the total

cohort with all trimesters. Accordingly, the differences between controls and ICP remain. Women with ICP had a significantly younger age and, by definition, bile acids were significantly increased by a factor of ca. 6 ($p < 0.0001$). Women with ICP also had significantly elevated transaminases (predominantly alanine aminotransferase (ALT)) and bilirubin, but not elevated levels of alkaline phosphatase (AP) and GGT. Both caspase 3-cleaved CK18 Levels, the liver apoptosis marker M30, and the uncleaved CK18 Levels (M65) representative of liver necrosis, were significantly elevated. Of note, however, M65 showed a twofold increase in women with ICP, while M30 was only slightly higher in this group. Interestingly, although all liver-related parameters decreased after delivery, GGT was the sole marker with postpartum elevation as compared to controls.

LS is significantly increased in women with ICP

As shown in Figure 2 and Table 1, LS was significantly higher in women with ICP both prior and after delivery. Moreover, in both groups, we observed an increase in LS as the pregnancy progressed. In the ICP group, mean LS was significantly increased at 7.3 ± 3.0 kPa compared to the control group at 6.2 ± 2.3 kPa ($p < 0.0001$). An increase in LS to above 6 kPa was observed in 24.9% of healthy pregnant woman and 44.2% of the ICP group, an elevated LS > 8 kPa was seen in 7.1% of the control and 15.6% of the ICP group. LS of > 12.5 kPa considered above the cut-off value for cirrhosis was measured both in 3.0% of the control and ICP group. In confirmation to our initial study [27], however, postpartum LS decreased significantly in both groups; down to 5.8 ± 1.7 kPa in the ICP group and down to 4.2 ± 0.9 kPa in the control group, respectively. On a final note, hepatic steatosis, as measured by CAP, was normal in most women. It slightly but significantly increased in the ICP group during delivery from 206 to 213 dB/m while no significant changes were observed in controls (see Table 1). In summary, although LS generally increases during pregnancy, livers are significantly stiffer in women with ICP prior and after delivery as compared to controls without hepatic complications.

Parameters associated with LS elevation

We next performed a Spearman Rho correlation analyzes with clinical and laboratory parameters to identify potential confounders of elevated LS. Table 2 shows the results for the ICP, control and total cohort. In the ICP cohort, only a few parameters were highly significantly correlated with LS, namely serum levels of bile acids and the liver apoptosis marker M30. Bilirubin levels hardly met the levels of significance while leukocyte count and Quick's test were negatively correlated. No association of LS with bile acids were seen in the control group and M30 Levels only weakly but significantly correlated with LS. In contrast and as described recently [27], LS was significantly correlated in the total cohort with the duration of pregnancy, the onset of gestational diabetes, body weight, mean arterial diastolic pressure (MAD), levels of AP and M65. Interestingly, levels of AST which are usually highly associated with LS in liver diseases [34], was only significantly correlated in the total cohort, but neither in the ICP and control group alone. In addition, no correlation was seen with GGT and ALT in the ICP cohort. Finally, no association with markers of hemolysis nor anemia was observed. In conclusion, in women with diagnosed ICP, bile acids are tightly associated with elevated LS and markers of liver apoptosis, but not with conventional liver function tests except bilirubin.

Liver stiffness is independently associated with elevated bile acids in ICP

Fig. 3 shows the levels of bile acids for both controls and women with ICP prior and after delivery. Bile acids which are a major criterium for the diagnosis of ICP were about 6 times elevated in the ICP cohort and promptly decreased after delivery. A slight and significant decrease was also observed in the control group suggesting that pregnancy generally causes some bile acid elevation. Notably, after delivery, bile acids were still markedly elevated in the ICP cohort. Suppl. Table 2 shows parameters associated with elevated bile acids. Bile acids were highly significantly associated with clinical features of pruritus and gestational diabetes. Within the laboratory parameters, bile acids were mostly correlated with the liver apoptosis marker M30, levels of ALT,

AST, and bilirubin. They were negatively associated with haptoglobin, leukocyte count and coagulation tests (Quick). No association was seen with liver steatosis (CAP), levels of M65, AP or spleen size. In the total cohort, bile acids were best associated with ALT, AST and LS in descending order. In multivariate analysis, liver stiffness remained the sole parameter that was independently associated with elevated bile acids (suppl. Table 3). In conclusion, in patients with ICP, bile acids are tightly associated with liver damage in the form of apoptosis and LS is independently associated with bile acid levels. In the 3rd trimester, a liver stiffness of 6.5 kPa significantly discriminates between ICP and controls ($P = 0.033$) although with a modest AUROC of 0.65 (0.58-0.72, $P = 0.033$).

DISCUSSION

In the present study, we non-invasively measured liver stiffness by TE and steatosis by CAP in a large cohort of pregnant women diagnosed with ICP primarily through elevated bile acids. Our data clearly show that LS is higher in women with ICP as compared to controls. Although LS decreased rapidly after delivery as described recently [27, 28], it remained significantly higher in women with ICP despite identical follow-up observation time. Women with ICP had predominantly elevated levels of ALT, followed by levels of AST and bilirubin. Besides levels of AP, no differences were seen with GGT. In fact, although all parameters including LS improved after delivery, GGT even increased significantly in the ICP cohort. In addition, hepatic fat content as measured by CAP, although in the normal range, was lower in patients with ICP as compared to controls. Finally, the liver apoptosis marker M30 showed the highest association with bile acid levels in univariate regression analysis while LS remained the strongest independent predictor of bile acid levels $>20 \mu\text{mol/L}$ in multivariate regression analysis.

In confirmation of earlier reports [27-29], the present study demonstrates that non-invasive assessment of LS by elastography is feasible and well accepted in pregnant

women. In contrast to reports from internal medicine departments [35], elastography could be performed in all women. Second, we show that LS is significantly elevated in women with ICP and higher as compared to controls. This is remarkable, as we and other showed that LS is generally and reversibly elevated in the third trimester [27, 28]. Consequently, and comparable to the previously reported LS elevation in women with preeclampsia, LS can be considered a feasible and non-invasive tool to screen, identify and follow-up women with pregnancy related liver complications.

What are the confounding factors of LS elevation in women with ICP? In contrast to initial believes, LS can be elevated due to many confounding factors including inflammation, arterial and venous pressure elevation but also physiological conditions such as meal intake [24, 26]. Mechanic cholestasis itself has been demonstrated to reversibly increase LS [36]. Of note, however, continued elevation of LS by these confounders has a negative impact on the liver and first long-term mortality data demonstrate that LS is one of the best long-term predictors of liver-related and even all-cause mortality [37]. Of course, LS measurement can also be used to identify and monitor those pregnant women with preexisting manifest liver cirrhosis and it predicts hepatic decompensation [29]. Already the first study in women with uncomplicated pregnancy showed that elevated LS is significantly correlated with AP, leukocytes, gestational age and an increase in body weight [27]. In our present study, in women with ICP, the confounders are totally different and LS is tightly associated with elevated serum levels of bile acids but also serum markers of liver apoptosis (M30). This is especially interesting with regard to the fact that in popular liver diseases such as alcohol liver disease or viral hepatitis, LS elevation is typically associated with transaminase levels, namely with AST but not ALT [34]. Although, we here show that LS is an independent predictor for elevated bile acids, performance of LS to predict ICP was only moderate and lower than in the previous smaller ICP cohort [27].

For the first time, our study shows an exceptional tight association of the established serum liver apoptosis marker (M30) with levels of bile acids in women with ICP and its association with elevated LS. In multivariate analysis, liver stiffness remained the sole parameter that was independently associated with elevated bile acids. Although, in patients with liver disease, the association of liver apoptosis and LS elevation has already been shown both at the histological level [38] and using serum markers such as M30 [30], the tight association of bile acids with LS and M30 Levels in pregnancy is new. Bile acids are synthesized in hepatocytes as essential part of bile formation [9, 17]. Due to their detergent nature, however, they are highly cytotoxic and can disrupt cellular membranes if not protected *e.g.* by phospholipids [9, 17, 19]. ¹ Specifically, serum cholic acid becomes the primary bile acid in ICP women in contrast to normal pregnant women and nonpregnant women, in whom its proportion is almost similar to chenodeoxycholic acid [39]. Typical examples are the so-called cholestatic liver diseases such as primary biliary cirrhosis that ultimately cause chronic bile duct inflammation and later cirrhosis and cancer. Even simple mechanic cholestasis through obstruction of the major bile ducts by biliary stones will cause severe tissue damage.

In the last three decades, many gene mutations have been discovered that can cause cholestasis through impairment of hepatocyte or cholangiocyte transport proteins relevant for bile formation [8-10, 18-20]. These discoveries have resulted ² in a group of diseases known as Progressive familial intrahepatic cholestasis (PFIC). The normal GGT level in our ICP cohort are a strong argument for the genetic cause. Hormonal changes/normalization after delivery with subsequent normalization of bile acid export through the hepatocellular apical membrane are considered an important argument for the role of sex hormones in ICP [9]. In line with this, we observed a postpartum increase of GGT in our ICP cohort suggesting a re-induction of GGT with the onset of bile flow.

We would finally mention that, to our surprise, hepatic steatosis as measured by CAP which is now widely explored in patient with fatty liver [40], did not show any

conclusive data both in women with normal pregnancy nor with ICP. The reason for it remains unclear to us since we had expected that at least some women will present with steatosis which can be a severe complication during pregnancy. We also want to briefly discuss some limitations of our study that are mostly due to the challenging setting of performing clinical studies in late-pregnancy, especially in women with suspected complications. Not in all women, serum was available to allow the subsequent measurement of markers such as M30 and M65. In addition, we only managed in a few cases to measure LS sequentially prior and after delivery in the same person. Another limitation with regard to the postpartum follow-up measurements is that we only included women 24 h after delivery. This rather short time may explain why some parameters did not reach levels of significance.

CONCLUSION

In conclusion, we here show in a large cohort that women with ICP show a significantly elevated LS in comparison to women with uncomplicated pregnancy. In contrast to a large body of evidence in the liver literature, elevated LS in ICP is primarily correlated with the accumulation of bile acids known to be highly toxic to hepatocytes, and liver apoptosis as measured by M30 Levels, but not with transaminases, bilirubin or GGT. We also show for the first time, that the typically low GGT levels in ICP increase after delivery. Consequently, we believe that screening for LS in pregnancy is not “another diagnostic tool” to further complicate the already intensive surveillance during pregnancy, but could provide a novel non-invasive strategy to early identify women at risk for complications.

ARTICLE HIGHLIGHTS

Research background

Intrahepatic cholestasis of pregnancy (ICP) is a rare but severe hepatic complication for both mother and unborn child. Diagnosis is normally based on elevated serum levels of bile acids. Unfortunately, these blood tests usually take several hours, even at

maximum care facilities. So far, there are no screening test for liver disease in pregnancy besides serological testing for viral hepatitis in the third trimester.

Research motivation

Measurement of liver stiffness through elastographic techniques has become the novel gold standard for the non-invasive diagnosis of liver cirrhosis often avoiding invasive liver biopsies. Liver stiffness is not only highly correlated to the hepatic fibrosis stage but can also be elevated due to other important confounding factors such as inflammation, congestion or cholestasis. For these reasons, liver elastography could be an ideal diagnostic tool to address hepatic complications during pregnancy.

Research objectives

The aim of the present study was to specifically explore liver stiffness in a large cohort of women with ICP before and after delivery compared to a control group with uncomplicated pregnancy.

Research methods

Liver stiffness and the hepatic steatosis marker Controlled Attenuation Parameter (CAP) were measured in 100 pregnant women with ICP using transient elastography (Fibroscan, Echosens, Paris, France). In 17 cases, liver stiffness could be measured after delivery. A large cohort of women with uncomplicated pregnancy served as control group. Routine laboratory, levels of bile acids and the apoptosis marker M30 were also measured.

Research results

In the third trimester, women with ICP show a significantly increased liver stiffness at 7.3 ± 3.0 kPa compared to controls (6.2 ± 2.3 kPa, $P < 0.0001$). Liver stiffness decreases significantly 24 h after deliver and remains higher in ICP (5.8 ± 1.7 kPa *vs* 4.2 ± 0.9 kPa, $P < 0.0001$). In ICP, liver stiffness is mainly correlated with levels of bile acids and the

apoptosis marker M30. No correlation was seen with GGT and GGT even increased after delivery in women with ICP.

Research conclusions

In conclusion, liver stiffness is significantly elevated in ICP which is most likely due to toxic bile acid accumulation and hepatocyte apoptosis. In association with conventional laboratory markers, LS provides additional non-invasive information to rapidly identify women at risk for ICP.

Research perspectives

In the future, elastography should be further validated in order to early identify women at risk for complications. Moreover, elastography studies should be combined with genetic risk assessment, as several mutations of bile transport proteins are involved in the development of ICP.

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