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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

We encourage authors to submit their manuscripts to *WJH*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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Diffusion weighted magnetic resonance imaging of liver: Principles, clinical applications and recent updates

Anuradha Shenoy-Bhangle, Vinit Baliyan, Hamed Kordbacheh, Alexander R Guimaraes, Avinash Kambadakone

Anuradha Shenoy-Bhangle, Beth Israel Deaconess Medical Center, Boston, MA 02215, United States

Vinit Baliyan, Hamed Kordbacheh, Avinash Kambadakone, Harvard Medical School, Abdominal Imaging and Interventional Radiology, Massachusetts General Hospital, Boston, MA 02114, United States

Alexander R Guimaraes, Oregon Health and Science University, Portland, OR 97239, United States

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Correspondence to: Avinash Kambadakone, MD, FRCR, Assistant Professor, Harvard Medical School, Abdominal Imaging and Interventional Radiology, Massachusetts General Hospital, White 270, 55 Fruit Street, Boston, MA 02114, United States. akambadakone@mgh.harvard.edu
Telephone: +1-617-6432009
Fax: +1-617-7264891

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Abstract

Diffusion-weighted imaging (DWI), a functional imaging technique exploiting the Brownian motion of water molecules, is increasingly shown to have value in various oncological and non-oncological applications. Factors such as the ease of acquisition and ability to obtain functional information in the absence of intravenous contrast, especially in patients with abnormal renal function, have contributed to the growing interest in exploring clinical applications of DWI. In the liver, DWI demonstrates a gamut of clinical applications ranging from detecting focal liver lesions to monitoring response in patients undergoing serial follow-up after loco-regional and systemic therapies. DWI is also being applied in the evaluation of diffuse liver diseases such as non-alcoholic fatty liver disease, hepatic fibrosis and cirrhosis. In this review, we intend to review the basic principles, technique, current clinical applications and future trends of DW-MRI in the liver.

Key words: Liver imaging; Diffusion weighted imaging; Magnetic resonance imaging; Focal liver lesion; Diffuse liver disease; Response assessment

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Core tip: This article reviews the current role of diffusion weighted imaging for various oncological and non-oncological applications in the liver.

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INTRODUCTION

Diffusion-weighted imaging (DWI) is a functional imaging technique, allowing qualitative and quantitative assessment of the diffusion properties of various types of tissues^[1,2]. Numerous studies over the past decade have validated the role of DWI in oncologic and non-oncologic applications in the body^[1,3-6]. Multiphase contrast enhanced MRI is an established technique for evaluation of a wide spectrum of liver diseases including focal lesions and diffuse parenchymal abnormalities. DWI compliments routine MRI of the liver by providing both qualitative and quantitative assessment for both focal and diffuse hepatic parenchymal processes. Factors such as the ease of acquisition and ability to obtain functional information in the absence of intravenous contrast, especially in patients with abnormal renal function, have contributed to the growing interest in exploring clinical applications of DWI. DWI improves sensitivity in detection of focal lesions, helps differentiate benign from malignant focal hepatic lesions, and also permits evaluation of treatment response to systemic and loco-regional therapies in primary and secondary hepatic malignancies. This review article focused on the basic principles, technique, current clinical applications and recent updates in DWI of the liver.

DWI: BASIC PRINCIPLES AND TECHNIQUE

DWI exploits the regional differences in the motion of water molecules within the extracellular/extravascular compartment of tissues. In highly cellular tissues (*e.g.*, lymphoma, carcinoma and abscess), the compact nature of the extracellular space causes increased impediment to motion of water molecules and the resultant water diffusion in such tissues is said to be "restricted". On the contrary, in tissues that are necrotic or fluid filled (*e.g.*, cysts), there is unrestricted motion of water molecules and water diffusion in such tissues, which is said to be "free". Therefore, the diffusion properties in different tissues provide information on tissue cellularity and the integrity of cellular membranes^[1,2]. DWI is basically a modified T2 weighted sequence where the signal intensity depicts the tissue diffusion characteristics.

Single-shot spin-echo (SE) echo-planar technique is the most commonly utilized technique to acquire DW-MRI in combination with fat suppression^[7]. To obviate the effect of motion, it can be acquired either using breath-hold or free breathing sequences with multiple signal acquisitions (in combination with respiratory and/or cardiac triggering). Free breathing sequences provide improved signal to noise ratios (SNR), thinner image sections, and higher number of b-values obtainable compared to breath-hold sequences. However, these take longer time (3-6 min) to acquire than breath hold sequences to evaluate the liver compared to

free breathing EPI which takes (40-60 s)^[8]. The free breathing technique has been shown to have better reproducibility of ADC values than other acquisition techniques like breath-hold, respiratory-triggered (RT), and navigator-triggered DWI^[9,10]. Although cardiac motion also impacts quantitative ADC measurements, cardiac triggering is not routinely used in clinical practice^[11].

Intravoxel incoherent motion (IVIM) imaging is a technique that has been introduced to quantitatively study the effects of tissue perfusion on the signal acquired with DWI and it resolves DWI measurements into true molecular-based (D) and perfusion-related (D^* , f) diffusion^[12].

In patients with renal failure, gadolinium is contraindicated due to risk for developing nephrogenic systemic fibrosis (NSF)^[13]. These patients also have a risk of worsening renal failure with iodinated CT contrast. MRI without contrast is a reasonable option for these patients but non-contrast protocols do not have a diagnostic accuracy comparable to multi-phase contrast MRI. DWI does not require administration of intravenous contrast, and because of its performance in oncological applications in general, it has generated much interest recently. The diagnostic performance of DWI has been tested in metastatic liver disease and HCC, and the results were comparable to contrast MRI^[14-16].

CLINICAL APPLICATIONS IN LIVER

Imaging of focal liver lesions

Lesion detection: Multiphase contrast enhanced-MRI is currently the state-of-the-art imaging method for liver lesion detection and characterization. DWI at high b-values ($\geq b100$) provides a low background signal from normal liver parenchyma and thereby results in increased contrast between the background liver and lesions, enhancing the detection of focal liver lesions^[17]. DWI is especially useful in detection of small lesions around vessels and in the periphery of liver which can be challenging to detect on routine T2 weighted images^[18,19]. The DW-MRI can be particularly valuable in oncologic patients with compromised renal function who cannot get intravenous gadolinium based contrast agents^[14,16]. DWI adds value in oncologic patients (Table 1)^[15,20-22] by depicting more metastatic liver lesions when combined with multiphase contrast enhanced-MRI protocols, and improves reader confidence in lesion detection^[22-25]. DW-MRI alone is less sensitive than gadoteric acid-enhanced MRI for detecting liver metastases, but increases the sensitivity of detection for liver metastases (90.6%-95.5%) when combined with multiphase contrast enhanced MRI^[25]. A major impact has been noted in the detection of metastases measuring ≤ 10 mm^[17,22,24-27] (Figure 1). DWI has been used in detection of metastatic liver lesions from colorectal, pancreatic and neuroendocrine primaries^[25,28,29].

DWI has also been found to be useful in detection

Table 1 Comparison of SSEPI diffusion-weighted magnetic resonance imaging *vs* conventional magnetic resonance sequences for detection of hepatic metastases^[15,20-22,27]

Ref.	b value (s/mm ²)	Compared with (Seq)	Sensitivity of DWI <i>vs</i> other sequences	Accuracy of DWI <i>vs</i> other sequences	Advantages of DWI
Bruegel <i>et al</i> ^[27]	50, 300, 600	5 different T2-TSE (Turbo Spin Echo) sequences	0.88-0.91 compared to 0.45-0.62	0.91-0.92 compared to 0.47-0.67	Better sensitivity and accuracy
Zech <i>et al</i> ^[21]	50	Fat suppressed T2WI	83% <i>vs</i> 61%	-	Better image quality Fewer artifacts Better sensitivity
Hardie <i>et al</i> ^[15]	0, 50, 500	Gadolinium enhanced T1WI	66.3% <i>vs</i> 73.5%	88.2% and 88.2% for DW-MRI, 90.2% and 92.2% for CE MRI, respectively, for observers 1 and 2	Not significantly different
Donati <i>et al</i> ^[20]	0, 150, 500	Combined (Gd-EOB-DTPA) enhanced MRI/DWI <i>vs</i> Gd-EOB-DTPA enhanced MRI and DWI alone	-	Gd- EOB-DTPA/DWI: 0.84 and 0.83 <i>vs</i> 0.73 and 0.72 for DWI alone	Increase in diagnostic confidence No significant increase in diagnostic accuracy
Colagranade <i>et al</i> ^[22]	0-500	Added value of DWI for lesion detection in unenhanced and Gd-EOB-DTPA enhanced MRI	-62.5% for unenhanced MRI w/o DWI -85.0% for unenhanced MRI+ DWI -95.6% for CE MRI -97.3% for CE MRI + DWI	-81.1% for unenhanced MRI w/o DWI -89% for unenhanced MRI + DWI -92.9% for CEMRI -95.5% for CE MRI + DWI	DWI improved all statistical parameters in the unenhanced examinations, as for nodules either smaller or greater than 1 cm. In EOB-enhanced examinations DWI increased specificity/negative predictive value

DWI: Diffusion-weighted imaging; MRI: Magnetic resonance imaging.

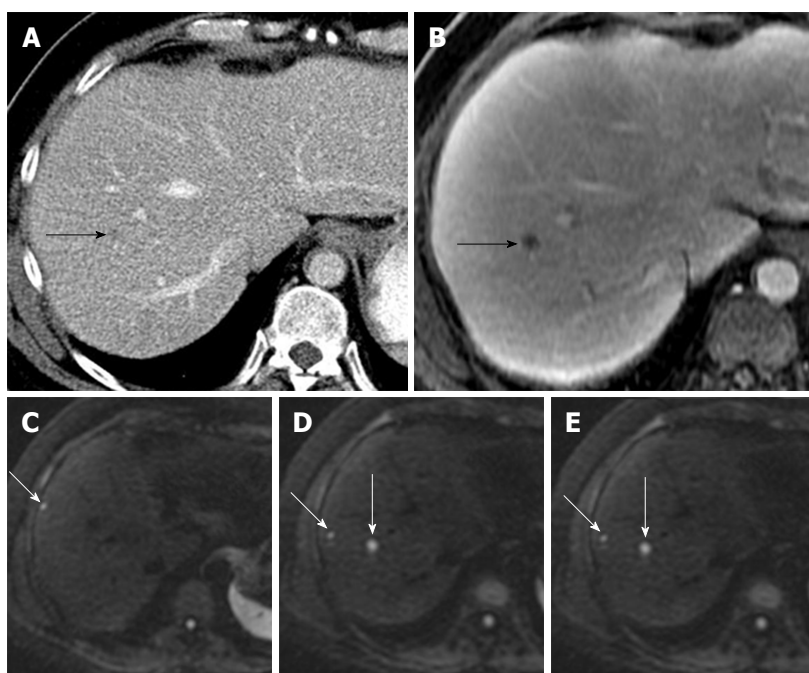


Figure 1 Value of diffusion-weighted magnetic resonance imaging in lesion detection in a 51-year-old male with metastatic leiomyosarcoma of the thigh. A: Axial contrast enhanced CT scan demonstrated a subtle hypodensity in the right lobe of liver (black arrow); B: Axial post gadolinium T1-weighted MR image demonstrates a single metastatic lesion (black arrow); C-E: DW-MR image at b-600 demonstrates additional lesions (white arrows). DW-MR: Diffusion-weighted magnetic resonance; CT: Computed tomography.

of primary hepatic malignancies such as hepatocellular carcinoma (HCC) and cholangiocarcinoma both in cirrhotic and non-cirrhotic livers (Figure 2). A combination of DW hyper-intensity and arterial hyper-enhancement results in increased sensitivity for diagnosis of HCC as compared to traditional criteria,

particularly for small HCC < 20 mm^[30,31].

A low cost abbreviated MRI (AMRI) protocol for HCC screening and surveillance has been proposed based on a simulation study using DWI and T1-weighted imaging obtained at the hepatobiliary phase (HBP) after gadoxetic acid injection^[32]. The AMRI

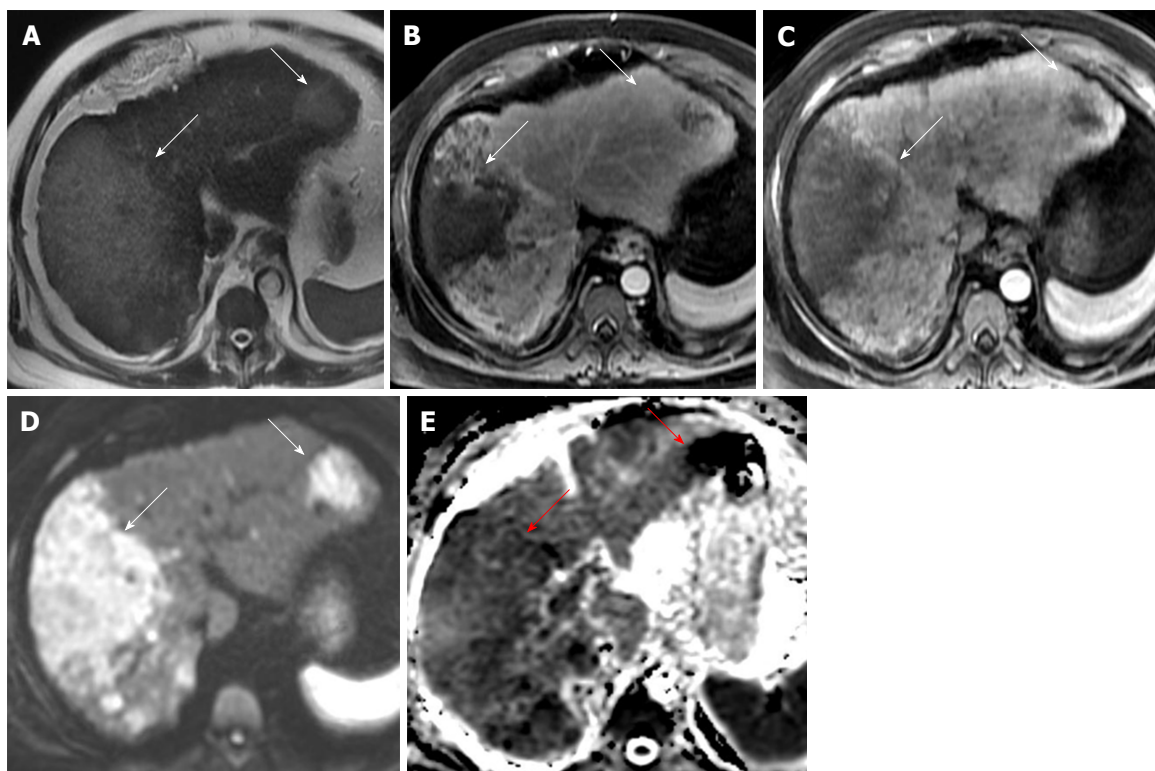


Figure 2 A 66-year-old lady with multifocal infiltrative hepatocellular carcinoma with improved detection on diffusion-weighted imaging. (A) Axial T2 weighted image demonstrates multifocal areas of T2 hyperintense masses (white arrows) which demonstrate heterogeneous arterial hyperenhancement on post gadolinium late arterial phase images (B) and washout appearance on portal venous phase images (C). (D) Axial DWI image at b-600 and (E) ADC image show that these masses demonstrate restricted diffusion and are better appreciated than the dynamic phase images. Serum Alpha feto-protein value of 1552. DWI: Diffusion-weighted imaging.

shows sensitivity and negative predictive values of 80.6% and 80% (for DWI + T1W HBP) compared to 90.3% and 94.9% for a full dynamic contrast enhanced data-set^[32].

Lesion characterization: Several studies have attempted characterization of liver lesions using DW-MRI^[33-38]. A general assumption is that ADC values are higher in benign lesions and lower in malignant liver lesions^[33-36]. In fact, studies have found statistically significant difference in ADC values between benign and malignant liver lesions^[3]. Different studies have reported variable success using various ADC cut-off values with high variability likely due to the difference in scanners and parameters used to obtain DW-MRI and ADC maps^[39-43]. Moreover, there is a high degree of overlap between solid benign and malignant lesions^[44,45]. Hence, the use of absolute ADC values or ADC value cut-off for characterization of focal hepatic lesions should be avoided and DWI should always be interpreted as a complimentary technique to conventional MR sequences^[42,46,47]. It is also important to note that solid benign lesions such as hemangioma, FNH and hepatocellular adenoma can also show diffusion restriction compared to normal liver parenchyma. ADC values for these lesions are intermediate, generally greater than solid malignant lesions but with a significant degree of overlap^[44,45]. Hepatic abscesses show lower ADC values than solid

malignant lesions, and restriction pattern may be different from malignant lesions^[42] (Table 2).

DWI has also been used to assist in differentiation of cirrhotic hepatocellular nodules^[48]. Lesion hyperintensity on DWI, especially in association with hypointensity on delayed hepatocellular phase images, and low lesion-to-liver ratios should raise the suspicion of HCC or high-grade dysplastic nodules^[49]. The HCCs have a tendency for angio-invasion and can present with filling defects in the portal or hepatic veins. Angio-invasion carries a high risk of distant metastasis and recurrence after transplantation. HCC invasion into the portal vein is considered as a contraindication for liver transplantation. It is important to distinguish tumor thrombus from a bland thrombus that is also common in chronic portal hypertension and has different clinical implications. In patients with locally advanced HCC, DW-MRI has been shown to be useful in characterization of the venous thrombus as bland vs tumor thrombus^[50]. The mean ADC ratio of tumor thrombus and HCC has been reported to be < 2 (0.998) as compared to bland thrombus (2.9)^[50].

Tumor grade and prognostication: Recently, there have been attempts to predict the histopathological grades of HCC using DWI. ADC values have been found to correlate with histopathological differentiation and microvascular invasion with poorly differentiated HCCs showing significantly lower ADC than well-differentiated

Table 2 Liver lesion characterization based on ADC values^[33,35,44,45,102]

Ref.	Lesion type	Mean ADC (10 ⁻³ mm ² /s)	Sample size	b-values	Conclusion
Parsai <i>et al</i> ^[44]	Cyst	2.66	2	100, 200, 500,	ADC cutoff value threshold of 1.6 × 10 ⁻³ mm ² /s yielded higher accuracy for differentiating benign from malignant lesions. DWI is not reliable to differentiate malignant from benign solid lesions
	HCC	1.07	26	750, and 1000	
	Metastases	1.04	39	mm ² /s	
Taouli <i>et al</i> ^[98]	Cyst	3.63	52	0, 500	Threshold ADC value of 1.5 × 10 ⁻³ mm ² /s to differentiate between benign and malignant lesions, but with a significant overlap between benign hepatocellular lesions and HCCs
	HCC	1.33			
	Metastases	0.94			
Parikh <i>et al</i> ^[35]	Cyst	2.54	211	0, 50, 500	Accuracy of 75.3% for differentiating benign from malignant, by using a threshold ADC of less than 1.60 × 10 ⁻³ mm ² /s. Equivalent performance of DW imaging and T2- weighted imaging for lesion characterization
	HCC	1.31			
	Metastases	1.5			
Bruegel <i>et al</i> ^[33]	Cyst	3.02	204	50, 300, 600	88% of lesions were correctly classified as benign or malignant using a threshold value of 1.63 × 10 ⁻³ mm ² /s. Measurements of the ADCs of focal liver lesions on the basis of a respiratory triggered DW-SS-EPI sequence may constitute a useful supplementary method for lesion characterization
	HCC	1.05			
	Metastases	1.22			
Gourtsoyianni <i>et al</i> ^[102]	Cyst	2.55	37	0, 50, 500, 1000	Sensitivity and specificity of 100% for differentiating benign from malignant lesions using a cutoff ADC value of 1.47 × 10 ⁻³ mm ² /s
	HCC	1.38			
	Metastases	0.99			

HCC: Hepatocellular carcinoma; DWI: Diffusion-weighted imaging.

and moderately differentiated HCCs^[51-54]. A cut-off value of 1.175 × 10⁻³ mm²/s has been recommended as a predictor of microvascular invasion^[52]. Additionally, the recurrence-free survival has been found to be significantly shorter in low-ADC group than in high-ADC group^[52].

The association of ADC and histopathological grades has shown conflicting results in few other studies^[55,56]. This might be a result of tumor necrosis, as it can result in reduced cellularity and increased ADC in high-grade lesions. Higher signal intensity on DWI has been reported to be associated with higher pathological grades despite insignificant correlation with ADC values^[54,56].

Diffuse liver diseases

Evaluation of NAFLD: Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder in western industrialized countries with a prevalence of 6%-35% worldwide^[57]. The severe form of this disease is steatohepatitis which can progress to cirrhosis in 15% of the patients^[58]. Currently, the diagnosis of NAFLD is established based on histopathological evaluation of liver biopsy specimens. Liver biopsy is invasive and has risks of complications and sampling error, and cannot be frequently repeated.

The feasibility of DWI and IVIM was first tested in animal models with early results showing that the IVIM diffusion parameters, in particular the "f" values, might be potential biomarkers of NAFLD^[59]. The correlation between histologic features of NAFLD and quantitative measures derived from IVIM-DWI was later tested in humans which showed that the true molecular diffusion was significantly decreased with steatosis^[60,61]. ADC was not found to be associated with any histological feature^[60]. Although these early results are promising, standardization of acquisition and post-processing

techniques of IVIM DW-MRI is needed.

Evaluation of liver fibrosis and cirrhosis: Aubé *et al*^[62] reported early benefits of DWI in the evaluation of diffuse liver diseases, particularly in the detection and quantification of hepatic fibrosis. Several authors thereafter have tried to find a simple, reliable and non-invasive method to detect and monitor hepatic fibrosis, thereby avoiding the existing gold standard involving liver biopsy and its complications^[63,64]. A recent meta-analysis suggests that DWI and IVIM parameters can reliably stage hepatic fibrosis^[65,66]. However, IVIM measurements and ADC values have been reported to be influenced by presence of fat or iron within the liver that can impact their accuracy for staging of fibrosis^[67-69] and ascites^[70]. Recent studies comparing MR elastography (MRE) and DWI in characterizing hepatic fibrosis demonstrate higher predictive ability of MRE in distinguishing stages of fibrosis compared to DWI^[71,72]. Gadoteric acid enhanced liver MRI is also more strongly correlated with fibrosis stage as compared to DWI^[73,74]. Considering the conflicting evidence, it can be concluded that at present, DWI cannot replace liver biopsy in liver fibrosis. Further investigations and analysis are needed to increase the reliability of the technique.

Monitoring treatment response

There has been a lot of interest in using DWI as an imaging biomarker for monitoring treatment response to various locoregional and systemic therapies in hepatic malignancies (Table 3)^[75-79]. In comparison to conventional morphological methods of monitoring response such as RECIST and WHO which rely on changes in tumor dimensions for quantitating tumor response, DW-MRI allows evaluation of treatment response to novel targeted therapies which cause

Table 3 Role of diffusion-weighted magnetic resonance in assessment of treatment response^[75-79]

Ref.	Treatment modality	Tumor type	DW-MR parameter evaluated	Study results/teaching point
Chapiro <i>et al</i> ^[79]	TACE	HCC	(3D) quantitative enhancement-based and DW volumetric MR	High accuracy and intermethod agreement of 3D quantitative techniques in the assessment of tumor necrosis after TACE is clinically relevant High diagnostic performance of qEASL criteria and qADC may help in triaging patients for repeat treatment after a TACE session
Mannelli <i>et al</i> ^[87]	TACE	HCC	ADC measured with DWI in treatment response	Pre TACE ADC obtained at 0, 50, 500 s/mm ² b values before and after treatment may be used to predict HCC response to TACE
Park <i>et al</i> ^[42]	Radiotherapy	HCC	DW MR <i>vs</i> conventional MR for treatment response	Improved detection of viable tumor when DW MR is added to conventional sequences
Yu <i>et al</i> ^[76]	Radiation therapy	HCC	DW MR	Change in ADC value before and after RT is related to local progression free survival. Hence ADC value and RECIST may substitute for mRECIST in patients who cannot receive contrast agents
Schraml <i>et al</i> ^[77]	Radiofrequency Ablation	<i>n</i> = 16 HCC, 1 = cholangiocarcinoma, and 37 = metastases (28 colorectal cancer, 3 melanoma, 3 breast cancer, 1 pancreatic cancer, 1 gastric cancer, esophageal cancer)	DW MR and mean ADC values	ADC-based evaluation of signal alterations adjacent to the ablation zone may contribute to the identification of local tumor progression and nontumoral post-treatment tissue changes

HCC: Hepatocellular carcinoma; DW MR: Diffusion-weighted magnetic resonance; TACE: Trans-arterial chemoembolization.

early changes in tumor physiology prior to change in tumor size. The increase in post-treatment ADC values precedes a decrease in size of tumor which has been the traditional method of measurement for post-treatment response, especially in systemic therapy^[80-82].

Percutaneous ablation: ADC-based evaluation of signal alterations adjacent to the ablation zone may contribute to the identification of local tumor progression and non-tumoral post-treatment tissue changes after radiofrequency ablation of hepatic primary tumors and metastases^[77]. Early post-ablation zone may show heterogeneous signal on non-enhanced T1 and T2 weighted images due to edema, hemorrhage and inflammatory reaction. These changes resolve within 4-6 mo after ablation leaving behind a characteristic homogenous high T1 signal and low T2 signal (coagulation necrosis). Nodular enhancing foci within the ablation zone are considered as a sign of local recurrence. Low ADC values at 1 mo ($< 1.145 \times 10^{-3} \text{ mm}^2/\text{s}$) after RFA have been shown to be associated with an early local recurrence of HCC^[83].

Intra-arterial therapies: The utility of DWI has been assessed in treatment response after trans-arterial chemoembolization (TACE) of HCC^[84-87]. DWI has been shown to perform equally^[78] or better than gadolinium-enhanced MRI in quantifying the area of tumor necrosis after chemoembolization^[78,86,88]. Increased ADC values in non-enhancing tumors show a high correlation to the degree of tumor necrosis at pathology^[86,88]. Mannelli *et al*^[78] showed excellent performance of ADC for prediction of complete tumor

necrosis after TACE (sensitivity of 75% and specificity of 88%) which was comparable to 100% sensitivity, and 58%-79% specificity for contrast-enhanced MRI.

Transarterial radioembolization (TARE) using yttrium-90 (⁹⁰Y)-loaded resin microspheres is a treatment option for various liver malignancies (including liver-dominant breast metastases). Early arterial blood flow stasis with consecutive incomplete dose administration may occur in 12%-25% of resin-based radioembolization procedures. The perfusion-sensitive IVIM parameter "*f*" may predict early blood flow stasis in patients undergoing TARE for liver-dominant breast metastases^[89].

Image-guided radiation therapy: Image-guided targeted external beam radiation therapy is emerging as an alternative option in the treatment of advanced unresectable HCC. Accurate post-radiation response assessment can be challenging due to the concomitant changes occurring in the radiation zone. MRI is the preferred modality for response assessment. Inclusion of DWI in the imaging protocol has been shown to significantly enhance the diagnostic accuracy (91%-97% vs 72%) for detection of viable tumors after radiation treatment with improved sensitivity, specificity, and negative predictive value as compared to routine MR sequences (90%-97%, 91%-97% and 91%-97% vs 41%-55%, 86%-97% and 67%-70%, respectively)^[75]. ADC values have also been shown to correlate with local progression-free survival^[76]. Another group demonstrated that ADC values correlate with local progression-free survival and proposed that ADC and RECIST criteria could be substituted for mRECIST in

post-radiation evaluation of patients not amenable to receiving contrast agents^[76].

Systemic chemotherapy: DWI can detect the effects of chemotherapy combined with antiangiogenetic treatment on liver metastases in patients with advanced colorectal cancer^[90]. An increase in ADC values following systemic chemotherapy can be a sign of tumor response with non-responders showing lower ADC values than responders^[91]. In addition to monitoring therapeutic response, DWI has also been found to be useful in prediction of response to chemotherapeutic agents^[92,93].

Limitations of DWI

Diffusion imaging has several limitations, mostly attributable to the EPI based nature of the sequence^[94,95]. SS EPI provides a limited image quality with low spatial resolution and poor SNR and is susceptible to several artifacts, including blurring, ghosting and distortions. Although modern scanners with multichannel coils, strong gradients, high magnetic fields and advanced software have been successful in reducing such effects to a great extent^[96]. In addition, parallel imaging techniques improve SNR by allowing a decrease in acquisition time (TE)^[97,98]. 3T MRI offers an advantage due to an inherent high SNR, but suffers from several limitations. Uniform fat suppression for liver DWI has always been a challenge with 3 Tesla magnets and susceptibility artifacts are also more pronounced at 3 Tesla scanners^[99].

The reproducibility of quantitative ADC values has also been questioned. ADC values have been reported to vary significantly depending on the hardware, human or biologic factors^[100]. There has been considerable effort, however, to "industrialize" this important biomarker across vendor platforms^[101].

CONCLUSION

DWI is useful for focal liver lesion detection and is a desirable tool in patients who cannot receive intravenous contrast. In patients receiving systemic and local therapies for hepatic malignancies, DWI acts as a clinical tool for monitoring treatment response and predicting prognosis. Its utility in the assessment of diffuse hepatic parenchymal diseases is still at a research level. Further investigation and analysis are needed to increase the reliability of the technique for these indications. DWI has certain limitations and remains an adjunct and not a replacement to conventional sequences.

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Risk of liver disease in methotrexate treated patients

Richard Conway, John J Carey

Richard Conway, Centre for Arthritis and Rheumatic Diseases, St. Vincent's University Hospital, Dublin 4, Ireland

Richard Conway, CARD Newman Research Fellow, University College Dublin, Belfield, Dublin 4, Ireland

John J Carey, Department of Rheumatology, Galway University Hospitals, Merlin Park, Galway H91 YR71, Ireland

John J Carey, Clinical Sciences Institute, National University of Ireland Galway, Galway H91 TK33, Ireland

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Correspondence to: Dr. Richard Conway, Centre for Arthritis and Rheumatic Diseases, St. Vincent's University Hospital, Elm Park, Dublin 4, Ireland. dr-richardconway@gmail.com
Telephone: +353-876097345
Fax: +353-12214170

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Abstract

Methotrexate is the first line drug treatment for a

number of rheumatic and non-rheumatic diseases. It is effective in controlling disease activity and preventing disease-related damage, and significantly cheaper than many alternatives. Use in rheumatoid arthritis infers a significant morbidity and mortality benefit. Methotrexate is generally well tolerated but can cause symptomatic adverse events. Multiple serious adverse events have been attributed to methotrexate, based largely on older reports using high or daily doses, and subsequent case reports and circumstantial evidence. The risk with modern dosing regimens: Lower doses, weekly schedules, and concomitant folic acid is less clear. Clarification and dissemination of the actual risk is crucial so appropriate judgements can be made for patients who may benefit from this treatment. Methotrexate has been associated with a range of liver related adverse events ranging from asymptomatic transaminase elevations to fibrosis and fatal hepatic necrosis. Concern over potential liver toxicity has resulted in treatment avoidance, cessation, or recommendations for investigations which may be costly, invasive and unwarranted. Modern laboratory monitoring of liver blood tests may also influence the risk of more serious complications. The majority of present day studies report an approximate doubling of the relative risk of elevated transaminases in methotrexate treated patients but no increased risk of symptomatic or severe liver related adverse events. In this article we will review the evidence around methotrexate and liver related adverse events.

Key words: Liver disease; Transaminases; Fibrosis; Cirrhosis; Methotrexate; Hepatic

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Core tip: Methotrexate is a highly effective treatment for many diseases. In rheumatoid arthritis it controls symptoms, prevents damage, and reduces mortality. The risks of methotrexate use are often over-estimated. Methotrexate may result in asymptomatic transaminase elevations. Historically methotrexate has been infrequently associated with more severe liver adverse

events. With modern monitoring and management of liver blood tests serious liver related adverse events related to methotrexate use appear to be avoidable.

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INTRODUCTION

Methotrexate, formerly known as amethopterin, is one of several folic acid antagonists originally utilised in children with acute leukaemia^[1]. Successful use in adults, and children with other tumours followed shortly thereafter^[2]. Use has increased dramatically since that time both in volume and in scope; methotrexate is now commonly used in the treatment of a wide range of malignant and non-malignant diseases^[3]. The importance of methotrexate as a treatment option is emphasised by its prominent place on the World Health Organisation's List of Essential Medicines, a list of those critical basic medications which should be available to every healthcare system^[4]. Indeed methotrexate is arguably one of pharmaceuticals greatest success stories, a medication which has found indications widely disparate from its original intention. Methotrexate is highly effective for a range of diseases which had been difficult to treat prior to its introduction, including rheumatoid arthritis, psoriasis, and Crohn's disease^[3]. Methotrexate has transformed the management of rheumatoid arthritis (RA), dramatically altering the disease course, patient's quality of life, and reducing RA-related mortality^[5,6]. Modern reviews and meta-analyses show methotrexate has similar or better efficacy to other available agents including biologic therapies^[7]. No treatment is superior to methotrexate monotherapy for inhibiting radiographic progression, but combination therapy is superior for methotrexate failures^[6].

Despite this success, the potential adverse events associated with methotrexate attract considerable attention. Several reasons account for this, mainly stemming from notable toxicity with early use employing daily or high-dose therapeutic regimes. The translation of the adverse events associated with oncological dosing to those of modern low-dose methotrexate regimes for the treatment of autoimmune disease should not be automatic. Recent studies suggest methotrexate carries a similar risk of adverse events and toxicity to other agents, but combination therapy may have a higher rate of infection and liver-related adverse events^[6-11].

METHOTREXATE RELATED ADVERSE EVENTS

The first papers on methotrexate use detailed the

acute toxicity associated with high dose therapy for cancer, and later long-term sequelae^[1,12]. Later studies in non-malignant disease showed similar problems with high dose therapy but not so with lower doses, weekly regimens and concomitant use of folic acid^[13,14]. The adverse events associated with methotrexate use can be divided into two broad subsets; those symptomatic but rarely life-threatening adverse events experienced by patients, and those rarely symptomatic (at least until the latter stages) but potentially life-threatening adverse events which require careful monitoring by physicians.

Symptomatic adverse events associated with methotrexate are reported relatively commonly. These include symptoms such as nausea, headaches, fatigue, mucositis, and hair loss^[3]. For the majority of patients they are an acceptable accompaniment to their treatment, and a minor inconvenience compared to their previously debilitating disease symptoms. These adverse events are common with many medications and also shared by many of the diseases which methotrexate is used to treat. This can make it difficult in many cases to know if methotrexate is definitively the cause of the symptoms the patient is experiencing. However, distinguishing the precise source of the symptoms is often not needed, provided they can be tolerated or managed with symptomatic interventions. The occurrence rate using modern dosing schedules appear similar to the placebo arm in some clinical trials yet the perception of toxicity persists, and often apportioned to methotrexate^[15]. More rarely patients will ultimately be unable to tolerate methotrexate due to intractable symptomatic adverse events leading to drug cessation.

The more serious and infrequent adverse events attributed to methotrexate use present more of a clinical dilemma. The evidence linking many of these events to methotrexate use is sometimes weak and circumstantial. Methotrexate has been utilised in clinical practice for a considerable period and the origin of the attribution of many of its associated adverse events lies in older studies which used daily dosing, or much higher doses for example 3 mg/kg per week, or 100 mg/wk^[1,2,12-14]. More recent reports of isolated cases, case series, and observational studies where established beliefs may bias the findings and/or conclusions are inconclusive. There is no doubt that very high doses are toxic to the marrow, gingiva, and long-term the liver, hence the introduction of lower dose, less frequent schedules^[1,12,13]. Examination of these potential risks using modern regimens requires thorough exploration in well designed and performed studies in order to establish robust evidence for what the risks are. Adverse events falling into this category include cytopenias, interstitial lung disease (or methotrexate pneumonitis), and indeed methotrexate related liver disease.

Methotrexate-induced lung disease is a good example, an entity widely believed to be common, serious and potentially fatal^[3]. Incorrectly apportioning

blame on methotrexate can result in two potential risks to the patient: (1) denying them an effective drug; and (2) delaying the appropriate investigation and treatment of the real cause of their symptoms. Recent studies show this is in fact a rare occurrence and may not exist at all^[8,9,11]. Furthermore, it appeared that any increased risk was likely due to a small increase in respiratory infections with methotrexate use, rather than interstitial lung disease^[8]. This knowledge has the potential to significantly change clinical practice as cessation of methotrexate frequently occurs as a knee jerk reaction to any cough or dyspnoea.

The nature of liver disease related to methotrexate is similarly complex. It is well established that patients treated with methotrexate may develop abnormal liver blood tests, but the long-term consequences of modern dosing regimens in people with normal renal function are unknown^[16]. Many such patients not treated with this agent can develop abnormal liver enzymes, potentially confounded by alcohol use, non-steroidal anti-inflammatory drugs, non-alcoholic fatty liver disease, and both related and unrelated de novo liver diseases^[16,17]. Patients prescribed methotrexate have liver blood tests performed at intervals far in excess of the general population so the significance of minor or transient abnormalities in these test results remain uncertain^[18]. This is of course a vital issue regarding what action, if any, should be taken when faced with an abnormal liver blood tests, as it clearly depends on what it signifies. Cessation of an effective drug due to a transient unrelated transaminase elevation is potentially harmful to patients, as is continuation of that agent in the face of a developing significant drug induced liver injury^[19].

EPIDEMIOLOGY OF METHOTREXATE RELATED LIVER DISEASE

Reported rates of liver blood abnormalities during methotrexate treatment vary. Initial reports of hepatic toxicity, and death from hepatic toxicity, as well as cumulative incidences of 48.9% for elevated transaminases and 16.8% for transaminases elevated more than twice the upper limit of normal have been reported^[12,16,20]. Hepatic toxicity is not universal with prolonged chemotherapeutic regimes and some demonstrated normal liver histology despite several months of therapy^[12]. The reported rates of hepatic toxicity appear to have decreased progressively over time, likely related to refinements in dosing and monitoring strategies^[13]. A 2009 systematic review of observational studies up to that time reported that elevated transaminases were found in 20% of patients treated with methotrexate for 1 year, with transaminases greater than twice the upper limit of normal in 13%^[21]. Present day monitoring strategies and treatment regimens appear to have significantly lower risks than those which have been historically

associated with methotrexate use. Two high quality recent studies reported elevated transaminases in 22% but with as little as 1% having transaminases greater than twice the upper limit of normal^[22,23]. A higher rate occurs when used in combination with other therapies^[6,7,17]. A number of other risk factors for hepatotoxicity have been identified including obesity and hypercholesterolaemia^[17].

In contrast to the frequently reported transaminase elevations in methotrexate treated patients, reports of serious adverse liver outcomes in appropriately treated patients are harder to find in more recent times. An estimated 5-year risk of 1/1000 patients is likely to be an over-estimate based on the limited histological information available^[19]. A study reported rates of mild liver fibrosis, severe fibrosis and cirrhosis based on liver biopsies performed before and after methotrexate use. Rates prior to methotrexate use were 9.1%, 0% and 0.3%. The corresponding results after methotrexate use were 15.3%, 1.3% and 0.5% respectively^[16]. A literature review on biopsy proven liver abnormalities found that 3% of methotrexate treated patients developed histological abnormalities after one year of treatment. Importantly however, when the results were confined to those controlled studies of patients with baseline biopsies prior to the introduction of methotrexate no biopsy proven histological abnormalities were identified after 4 years of treatment^[21].

A recent meta-analysis of clinical trials demonstrated a cumulative incidence of liver adverse events of 11.2% in methotrexate treated patients compared to 6.3% in patients on other treatments^[10]. Calculated incidence rates from this were 20/100 patient-years in methotrexate treated patients and 9/100 patient-years in patients on other treatments^[10]. The majority of these adverse events were low grade liver enzyme elevations with an incidence rate of 16/100 patient-years in methotrexate treated patients compared to 8/100 patient-years in others^[10]. The incidence rate of major liver enzyme elevations was 4/100 patient-years in methotrexate treated patients and 1/100 patient-years in other patients, which is concerning^[10]. Reassuringly more serious liver complications did not occur in any methotrexate treated patients in these studies^[10]. The short duration of clinical trials is universal, the mean duration of studies in this meta-analysis were 47 wk, therefore data from long-term registries with robust unbiased analyses are required.

PATHOGENESIS

Any discussion of the mechanisms of potential methotrexate toxicity must begin with an appreciation of our understanding of methotrexate's mode of action. It is here that we reach a major impediment, though perhaps an informative one. We simply do not fully understand how methotrexate, and in particular low dose methotrexate, achieves its clinical effects^[11].

The oft quoted explanation that methotrexate is a dihydrofolate reductase inhibitor, while of course true, does not fully explain either the clinical efficacy or potential toxicities which we see with this agent. Low dose methotrexate has a multitude of biochemical effects at the most basic level including influences on T-cell apoptosis, cell proliferation and cytokine production^[24]. Despite methotrexate's long historical use this remains an active area of research, in part due to a certain neglect of exploration of these pathways in the past, and in part due to the increasingly evident complexities of the effects of methotrexate.

A reduction in hepatic folate stores and toxicity due to a local folate deficiency is one possible toxic effect of methotrexate on the liver. A definitive relationship between folate depletion and hepatotoxicity has not been experimentally confirmed. However folic acid supplementation has been associated with a lower incidence of elevated transaminases^[25].

Early animal and clinical studies of high dose methotrexate demonstrated the development of liver fibrosis and cases of cirrhosis but subsequent low dose weekly regimens failed to demonstrate a similar effect^[13,26].

The histological appearance of the liver in methotrexate treated patients is generally graded according to the Roenigk classification system^[27]. The system progressively classifies changes from early fatty infiltration and pleomorphism, through inflammation and necrosis, varying degrees of fibrosis and ultimately cirrhosis. Importantly none of these findings are unique to methotrexate and can be seen in other disease processes.

LIVER ADVERSE EVENTS WITH ANALAGOUS MEDICATIONS

One of the key tenets of causation is specificity^[28]. If patients given alternative agents to methotrexate do not develop liver disease than this facet of evidence would strongly implicate methotrexate as a causative agent. If they do however, than this, while be no means definitive, raises a potential warning flag that we should reconsider our hypothesis. In inflammatory bowel disease thiopurines (azathioprine and 6-mercaptopurine) are the most commonly used alternatives to methotrexate. Hepatotoxicity due to thiopurines has been reported in 10%-17% of patients^[29,30]. Risk factors for thiopurine induced hepatotoxicity appear to be similar to methotrexate with age, obesity, and concomitant medications implicated^[29,30]. In randomised controlled trials comparing thiopurines with methotrexate hepatotoxicity appears to occur at a similar rate^[31-33]. Leflunomide is often used in rheumatoid arthritis as an alternative to methotrexate and has been associated with a variety of similar complications to methotrexate. Pulmonary disease in particular has been associated with both agents,

however our work has illustrated that leflunomide may not be causative in this regard^[34]. Leflunomide has also been associated with transaminase elevations with a similar frequency to methotrexate with elevations in 17% and elevations greater than twice the upper limit of normal in 1%-2%^[22]. Combining both agents appears to have additive effects with transaminase elevations seen in 31% and those greater than twice the upper limit of normal in 5%^[7,22]. Sulfasalazine, another agent used in similar settings also appears to show similar effects to leflunomide (and to methotrexate)^[7]. Anti-tumour necrosis factor alpha agents have been reported as a relatively frequent cause of mild transaminase elevations, however, as with methotrexate, significant elevations occur relatively infrequently and are reported in less than 1% of patients^[35]. Again similar to methotrexate, serious liver adverse events seen in association with these agents appear infrequent^[36].

All of this begs the question, what is the rate of transaminase elevations in a healthy population? Most laboratory tests define normality as lying within 2 standard deviations of the mean in a Gaussian distribution. In a normally distributed sample approximately 95% of values will lie within 2 standard deviations of the mean. Therefore 2.5% of the population will have transaminase levels above the normal range and 2.5% will have transaminase levels below the normal range. The importance of this is that the rate of "abnormality" is not zero and never can be if normality is defined in this manner. This must be born in mind in evaluating any reported rate of abnormalities. Since studies show a higher incidence of liver enzyme abnormalities and since there is well-documented hepatotoxic potential, understanding the relationship between the mild enzyme rises and long-term outcomes is necessary, but unclear at this time.

EFFICACY OF METHOTREXATE IN AUTOIMMUNE LIVER DISEASE

Methotrexate is a well-established treatment for a wide variety of autoimmune diseases^[3]. Given the concern over the association between liver adverse events and methotrexate use it is perhaps understandable that the evaluation of methotrexate efficacy in autoimmune liver diseases has proceeded more slowly than in other disciplines. However the treatment depends on the cause and early studies in malignancy showed dramatic improvements in hepatic manifestations, coupled with longer term toxicity in some cases^[1,12].

Primary biliary cholangitis (previously primary biliary cirrhosis) (PBC) is perhaps the liver disease with the best established evidence for an autoimmune basis. PBC is characterised by early lymphocytic infiltration and granulomatous inflammation progressing to chronic damage and scarring resulting in the ultimate clinical manifestations of the disease. PBC is more common

in a variety of rheumatic diseases including Sjogren's syndrome, rheumatoid arthritis, and a number of other connective tissue diseases^[37]. However, the full importance of autoimmunity in the disease pathogenesis has been questioned given the apparent lack of response of PBC to many traditional immunosuppressants^[38]. Ursodeoxycholic acid is recommended as the first-line treatment option in PBC, however even its benefits are at best modest and a substantial number of patients do not respond^[39-43]. There is therefore a significant unmet therapeutic need for safe and effective treatment options for PBC.

Given the suggested autoimmune basis of the disease and the proven efficacy of methotrexate in a number of the conditions associated with PBC it was perhaps a natural development to progress to studying this agent in PBC. The ultimate trigger for the initial use of methotrexate in PBC however was its apparent efficacy in early studies in primary sclerosing cholangitis (PSC)^[44]. Methotrexate has been demonstrated to improve liver blood tests and liver histology in a long-term open label study of PBC patients with an inadequate response to ursodeoxycholic acid^[45]. Despite these apparent benefits the more widespread use of methotrexate in these diseases is difficult to recommend given the lack of evidence for improvements in important outcomes such as mortality and progression to transplantation^[46]. Of even greater difficulty is the lack of convincing evidence for efficacy in the randomised controlled trials of methotrexate in PBC^[38,47]. The only commonality across the studies of methotrexate in PBC has been a lack of evidence of adverse events, including transaminase elevations^[38,45]. This picture is complicated by the inherent difficulties in studying treatment efficacy in PBC, a disease with widely variable outcomes, a prolonged course prior to the development of end-stage disease, and a lack of definitive surrogate markers of disease progression. It has been suggested that another aspect of the difficulty may be related to subsets of responders and non-responders among patients with PBC^[47]. While the use of methotrexate in PBC remains controversial, the lack of alternative treatment options and the good evidence regarding the drug's safety in this patient population may justify a therapeutic trial.

Primary sclerosing cholangitis (PSC) is in many ways even more challenging than PBC. In contrast to PBC, PSC is not a classical immune disease, lacking characteristic autoantibodies, but does certainly have an immune component, with evidence of T-lymphocyte driven inflammation^[48]. The use of immunosuppressants in PSC has not demonstrated convincing evidence of favourable responses^[49]. Methotrexate was first used in PSC in the 1980's with initial reports of good responses with early treatment initiation^[50,51]. Results from a subsequent randomised controlled trial and case series were not encouraging however with evidence of improvement only in alkaline phosphatase levels^[52,53].

The utility of methotrexate in cholestatic liver

diseases remains uncertain. Based on the clinical trials in these diseases however we can obtain some reassurance about the overall liver safety of methotrexate given the lack of evidence of significant adverse events in this group predisposed to liver adverse outcomes.

META-ANALYSIS

In view of the ongoing uncertainty over the risk of liver disease in methotrexate treated patients we recently performed a comprehensive meta-analysis of randomised controlled trials evaluating this issue^[10]. We choose to limit our assessment to double-blind randomised controlled trials in order to eliminate the potential bias, both overt and covert, inherent in any situation in which a physician knows that a patient is prescribed, or potentially prescribed methotrexate^[54]. Pre-existing perceptions among physicians regarding the liver toxicity of methotrexate are a major confounder in many of the previous assessments of methotrexate toxicities. An additional advantage of this methodology is that the very nature of a randomised controlled trial provides a large number of patients with similar clinical and demographic characteristics as a control group. Of course randomised controlled trials, and meta-analyses of such trials, have their own inherent limitations, including issues with generalizability to heterogeneous real world patient populations, and a limited period of follow-up^[55]. Hence it is important to interpret such studies in conjunction with other forms of evidence such as that from observational studies^[21].

In our meta-analysis we included randomised controlled trials in which patients were prescribed methotrexate for rheumatoid arthritis, psoriasis, psoriatic arthritis, or inflammatory bowel disease^[10]. A total of 32 studies with 13177 participants were included in the analysis, 6877 of these were prescribed methotrexate and 6300 comparator agents. The majority of included studies used active comparators to methotrexate, predominantly synthetic disease-modifying antirheumatic drugs (DMARDs) or biologic agents; there were also 5 studies with placebo comparators. The trial durations ranged from 24 to 104 wk with a mean duration of 47 wk. Liver adverse events were common in both cohorts, the cumulative incidence was 11.2% in methotrexate treated patients and 6.3% in the comparator group. This translated to an incidence rate of liver adverse events of 20/100 patient-years in methotrexate treated patients compared to 9/100 patient-years in the comparators giving an attributable risk of 11/100 patient years in methotrexate treated patients.

Our meta-analysis demonstrated that methotrexate use was associated with an increased relative risk (RR) of liver adverse events in this population of 2.19 (95%CI: 1.73-2.77). Additionally methotrexate use was associated with an increased risk of transaminase

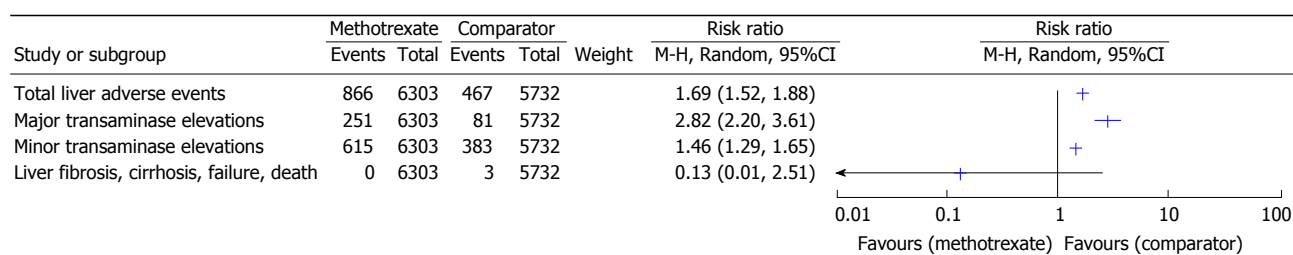


Figure 1 Risk of liver adverse events with methotrexate use.

elevation both less than or equal to three times the upper limit of normal, RR = 2.16 (95%CI: 1.67-2.79) and transaminases greater than three times the upper limit of normal, RR = 2.63 (95%CI: 1.90-3.64). The consistency in the increase risk across the various categories demonstrated by this portion of the meta-analysis was concerning, particularly given the utility of transaminases in predicting drug induced liver injury. We went on to analyse the hard endpoint of more serious liver outcomes, defined as hepatic failure, hepatic fibrosis, cirrhosis, or death due to liver disease. This was far more reassuring; methotrexate was not associated with any increased risk in these outcomes, RR = 0.12 (95%CI: 0.01-1.09). Indeed while not reaching statistical significance there was a strong trend towards less of these serious outcomes in methotrexate treated patients. The reasons why methotrexate could be associated with a possible reduction in serious outcomes but an increase in transaminase elevations are not immediately apparent. Methotrexate has shown potential efficacy in treating some autoimmune liver diseases^[45,49]. Methotrexate induced transaminase elevations frequently prompt further investigations, potentially identifying concomitant diseases at an earlier stage, allowing earlier treatment and thus less progression to the hard endpoints evaluated in this outcome. However caution is required as only having surrogate measures of hepatic toxicity (transaminase elevations) with very few serious events is another major limitation. The main findings of the meta-analysis are summarised in Figure 1.

MANAGEMENT OF ABNORMAL LIVER BLOOD TESTS IN METHOTREXATE TREATED PATIENTS

The management of abnormal liver blood tests in patients treated with methotrexate is a common clinical query. As with any management plan the key first step is in ensuring the correct diagnosis. Abnormal liver blood tests should never be presumed to be due to methotrexate. The available evidence indicates that methotrexate related liver adverse events are rarely serious, particularly in the short term, while many other causes of abnormal liver blood tests may be. An evaluation for other potential causes should follow identical pathways and similar rigor to that

applied to a patient who is not taking methotrexate. This investigative approach has been covered in detail elsewhere^[56].

If after exhaustive investigation no cause other than methotrexate is identifiable than the treatment approach recommended in guidelines depends on the degree of transaminase elevation. The baseline transaminase levels prior to methotrexate institution are also important; a previously elevated transaminase level that hasn't changed following institution of methotrexate is unlikely to need further intervention. The threshold for immediately interrupting methotrexate use differs by the respective guideline, however levels greater than 3 times the upper limit of normal are often used^[57]. Persistent lower grade elevations may also require intervention particularly if the trend is for a progressive increase in the transaminases^[18,57].

Widely differing recommendations regarding the indication for a liver biopsy in methotrexate treated patients exist^[58-60]. Increasingly a welcome move away from the routine performance of liver biopsies in methotrexate treated patients has accompanied a wider appreciation of the relative safety of this agent. Liver biopsy is the gold standard investigation as it allows direct assessment of liver histology, however it is imperfect and has a relatively high sampling error rate of 20%-30%^[61]. In addition it is an invasive procedure, and like any such procedure carries with it risks of morbidity and indeed mortality; therefore it should only be performed when the results will be clinically useful^[59]. In our practice a liver biopsy is infrequently clinically indicated and when it is performed is most commonly to investigate for another potential cause rather than investigation of suspected methotrexate induced hepatotoxicity.

Alternative methods of assessing for liver toxicity including procollagen III aminopeptide, multibiomarker scores, and transient elastography in our opinion have potential but remain experimental and we do not recommend their use in routine clinical practice at the present time^[62]. A proposed approach to suspected methotrexate hepatotoxicity is outlined in Table 1. All of the suggestions in this table must be interpreted and modified in the light of the clinical scenario.

CONCLUSION

Methotrexate is a highly effective treatment for a

Table 1 Management of suspected methotrexate toxicity

Transaminase monitoring	Commencing Adjusting dose Stable dose	Every 2 wk Every 2 wk Every 12 wk
Elevated transaminases	New persistent elevation New elevation greater than 3 times upper limit normal	Reduce methotrexate, investigate Withdraw methotrexate, investigate, methotrexate may be restarted after normalisation
Liver biopsy	Indication	Investigation of other potential causes of elevated transaminases Very rarely for confirmation of methotrexate induced toxicity

broad range of diseases. Concern over potential adverse events has limited the use of methotrexate in certain populations. Robust evidence of the true risk of the majority of methotrexate associated adverse events with modern dosing regimens in patients with normal renal function have been lacking. Methotrexate use is associated with an increased risk of elevated transaminase levels; however the risk of an increased risk of serious liver adverse events with modern methotrexate monitoring protocols appears to be extremely low at present. Long-term follow-up studies of patients with mild transaminase elevations are needed. Large increases are rare, should be taken seriously, and the medication stopped. Physicians and patients should be comfortable using methotrexate where clinically indicated.

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Case Control Study

Regional differences in genetic susceptibility to non-alcoholic liver disease in two distinct Indian ethnicities

Govardhan Bale, Avanthi Urmila Steffie, Vishnubhotla Venkata Ravi Kanth, Padaki Nagaraja Rao, Mithun Sharma, Mitnala Sasikala, Duvvur Nageshwar Reddy

Govardhan Bale, Avanthi Urmila Steffie, Vishnubhotla Venkata Ravi Kanth, Mitnala Sasikala, Asian Healthcare Foundation, Hyderabad 500082, Telangana, India

Padaki Nagaraja Rao, Mithun Sharma, Duvvur Nageshwar Reddy, Asian Institute of Gastroenterology, Hyderabad 500082, India

ORCID number: Govardhan Bale (0000-0002-2027-1647); Avanthi Urmila Steffie (0000-0001-9086-4336); Vishnubhotla Venkata Ravi Kanth (0000-0001-6970-0169); Padaki Nagaraja Rao (0000-0003-2983-5768); Mithun Sharma (0000-0003-4497-9209); Mitnala Sasikala (0000-0002-3785-0530); Duvvur Nageshwar Reddy (0000-0001-7540-0496).

Author contributions: Bale G and Steffie AU performed research; Rao PN, Sharma M and Reddy DN recruited patients; Ravi Kanth VV, Sasikala M and Rao PN designed the research; Ravi Kanth VV monitored the study, performed statistical analyses, and drafted the manuscript.

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Correspondence to: Dr. Vishnubhotla Venkata Ravi Kanth, Group Leader-Genetics, Asian Healthcare Foundation, 6-3-661, Somajiguda, Hyderabad 500082, Telangana, India. drvavikanth@aigindia.net
Telephone: +91-40-23378888
Fax: +91-40-23324255

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Abstract**AIM**

To validate the association of variants in *PNPLA3* (rs2281135) and *TM6SF2* (rs58542926) genes with ultrasound detected non-alcoholic fatty liver disease (NAFLD).

METHODS

A total of 503 individuals with and without fatty infiltration were recruited. Fatty infiltration was confirmed based on ultrasound findings. Anthropometric data and blood samples were collected from the study group. DNA was isolated from peripheral blood, quality and quantity was assessed by gel electrophoresis and spectrophotometer respectively. Genotyping of the variants in *PNPLA3* and *TM6SF2* genes was carried out by employing taqman probes (C_15875080_10 for *PNPLA3* and C_8946351_10 for *TM6SF2* SNP) on real time PCR (StepOne-Lifetechnologies). Genotype data was tested for deviations from Hardy-Weinberg

equilibrium. χ^2 test was used to analyze the statistical significance of the difference in genotype distribution of the studied variants in patients and controls and the strength of association was expressed as odds ratio (95%CI). A two-tailed *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

The study group comprised of 503 individuals of which 256 had fatty infiltration and 247 without fatty infiltration and thus formed the patient and control groups respectively. As the patient group could be divided in to two distinct ethnicities (ancestral South Indians-ASI and North-East Indians-NEI), further recruitment of control cohort and association analyses was carried out based on ethnicities. Of the 256 with fatty infiltration 93 were ASI and 163 were NEI and of the 247 controls 138 were ASI and 109 were NEI. As expected, there were significant differences in the anthropometric and other clinical data between the control and the patient groups. However significant differences within the ethnicities were also noted. While rs2281135 in *PNPLA3* gene was significantly associated (*P* = 0.03) with higher risk (odds 1.9, 95%CI: 1.5-3.14, *P* = 0.03) of NAFLD in NEI ethnicity, rs58542926 in *TM6SF2* gene was significantly associated with NAFLD with a 2.7 fold higher risk (odds 2.7, 95%CI: 1.37-5.3, *P* = 0.0004) of the disease. There were significantly higher proportions of individuals with variants in both the genes in the patient group in both ASI (patients - 14/93 and controls - 7/138; *P* = 0.009) and NEI ethnicities (patients - 17/163 and controls - 7/109; *P* = 0.01).

CONCLUSION

Although the study identified distinct genetic susceptibility in the two ethnicities, transheterozygosity of the variants suggests higher risk of NAFLD in individuals with both the variants.

Key words: Transmembrane 6 superfamily 2; Patatin-like phospholipase domain-containing protein 3; Fatty infiltration; Genetic susceptibility; Ethnicity; Non-alcoholic fatty liver disease; Cirrhosis; Single nucleotide polymorphism

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Core tip: Non-alcoholic fatty liver disease has become the leading cause of liver damage contributing to considerable mortality. The spectrum spans from simple steatosis, through non alcoholic steatohepatitis, fibrosis, cirrhosis and finally to hepatocellular carcinoma. Genetic variants have now been recognized to contribute to a substantial extent to the onset of the disease. Reliable genetic markers that confer susceptibility to the disease have to be identified for better management of the disease. Identification of at risk individuals at a younger age by screening for genetic susceptibility will aid in better management by early interventions and lifestyle changes. This study identified regional differences and

ethnicity based genetic susceptibility for non-alcoholic liver disease.

Bale G, Steffie AU, Ravi Kanth VV, Rao PN, Sharma M, Sasikala M, Reddy DN. Regional differences in genetic susceptibility to non-alcoholic liver disease in two distinct Indian ethnicities. *World J Hepatol* 2017; 9(26): 1101-1107 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i26/1101.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i26.1101>

INTRODUCTION

Non-alcoholic liver disease (NAFLD) describes a range of liver conditions beginning with fatty liver (accumulation of fat in the liver) that progresses to non-alcoholic steatohepatitis (NASH; fat accumulation along with inflammation and scarring) and cirrhosis (scar tissue replaces hepatic cells)^[1], that may finally lead to hepatocellular carcinoma (HCC)^[2]. While conditions up to NASH are reversible^[3], progression beyond NASH to cirrhosis is irreversible^[4]. Therefore it is very important to identify individuals with genetic susceptibility to fat accumulation at an early stage so that appropriate interventions can be planned to curtail/avoid progression to higher stages. Environmental factors including intake of calories^[5], processed food^[6] and sedentary lifestyles^[7] have an impact on the predisposition of an individual to fatty liver and progression. Apart from environmental factors various studies have now confirmed the role of genetics in conferring susceptibility to the disease. Diseases with complex traits including NAFLD result from interactions between environment and polygenic genetic susceptibility made up of many independent modifiers^[8]. Family aggregation, studies on twins and differences in susceptibility and progression suggest a significant heritable component to NAFLD that may be classified under "common disease-common variant" hypothesis^[9].

The first Genome wide association study for NAFLD identified a SNP in *PNPLA3* gene (rs738409; c.444 C > G, p.I148M). Carrier of the minor allele and 148M was associated with a twofold increase in HTGC (Hepatic triglyceride content)^[10]. Subsequent to this, the SNP was replicated in almost all the ethnicities successfully^[8]. Further, two exome wide association studies^[11,12] carried out independently in African-American and Norwegian ethnicities identified that a variant rs58542926 (p.E167K) in *TM6SF2* gene was associated with susceptibility to NAFLD, influencing total cholesterol levels and enhanced risk of myocardial infarction. Subsequently, functional studies identified *TM6SF2* as a regulator of liver fat metabolism influencing secretion of triglycerides and lipid droplet content in the liver^[13]. A recent review suggested that male sex, *PNPLA3* I148M, *TM6SF2* E167K and low birth weight as important predictors of adult NAFLD^[14] reiterating the importance

of variants in both *PNPLA3* and *TM6SF2* genes.

Our earlier pilot study^[15] identified variants in *PNPLA3* (rs738409), *PARVB* (rs2073080), *SAMM50* (rs2143571) and *PZP* (rs6487679) genes to be associated with a higher risk of fatty infiltration in individuals of NEI ethnicity. In the present study we replicated variants namely rs58542926 in *TM6SF2* and rs2281135 in *PNPLA3* genes, identified earlier^[12] to confer susceptibility to NAFLD in two distinct ethnicities. While one ethnicity belonged to South India, the other belonged to the North-Eastern region of the country. An earlier study on South Indians has reported that the genomic affinity is proportionate to caste rank-the upper castes being most similar to Europeans, while the lower castes are more similar to Asians^[16]. However, the Northeast region's population results from ancient and continuous flows of migrations from Indo-Gangetic India, Tibet, the Himalayas, present day Bangladesh and Myanmar^[17].

MATERIALS AND METHODS

A total of 503 individuals were recruited for the present study from the Hepatology clinics of Asian Institute of Gastroenterology. Although liver biopsy is considered to be the gold standard for identifying NAFLD, risk of complications, costs involved and ethical concerns limit its use, hence, patients with fatty infiltration were recruited based on ultrasound findings. Ethnicity, age and sex matched healthy subjects who volunteered to be part of the study were recruited as controls based on the sole criteria of the absence of liver fat on ultrasonography with normal liver function tests and negative for other viral indications. Written informed consent was obtained from individuals and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review (Scientific) Board (AIG/AHF IRB: 16/2014). Demographic and anthropometric details (height, weight, BMI and waist circumference) were collected. Whole blood (3 mL) was collected in pre coated EDTA containers from the study group and stored at -20 °C until further analysis. Biochemical investigations like ALT, viral markers and lipid profiles were estimated as per standard methods.

Genotyping

DNA was isolated from blood using a commercial kit (Bioserve Biotechnologies, Hyderabad) following manufacturers protocol. DNA with high molecular weight on agarose gel and A260/280 ratios between 1.8-2.0 were included for genotyping analyses. All the samples were genotyped for SNPs namely rs2281135 in *PNPLA3* and rs58542926 in *TM6SF2* genes using Taqman single nucleotide genotyping assay (Life Technologies, United States) on the Realtime polymerase chain reaction (PCR) platform. PCR for genotyping consisted of 5 µL of 2 × Taqman

genotyping master mix, 0.5 µL of 1 × assay mix (C_15875080_10 for *PNPLA3* and C_8946351_10 for *TM6SF2* SNP) and 4.5 µL consisting of 8-10 ng of DNA in a final volume of 10 µL. PCR was performed on Step One Realtime PCR (Life technologies, United States) with the following cycling conditions: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min with fluorescence read after each cycle for a total of 40 cycles. Genotyping calls were made using the allelic discrimination software (Life Technologies, United States) and only auto calls made by the software were considered for further analysis. A known heterozygous and homozygous variant sample was replicated across all the plates and these known genotypes were verified manually during analysis in all the plates.

Statistical analysis

Data was entered in to MS-EXCEL and edited for consistency. Continuous variables were expressed as mean (95%CI) and categorical variables as proportions. Patient characteristics were compared using Student's *t* test for continuous variables and χ^2 test for categorical variables. χ^2 goodness-of-fit was used to confirm the agreement of the observed genotype frequencies with those of expected (Hardy-Weinberg equilibrium). χ^2 test was used to analyze the statistical significance of the difference in genotypic distribution of the studied SNPs in patients and controls. The association of the studied SNPs with the disease and various clinical parameters was expressed as odds ratio (95%CI). For transheterozygosity analysis chi-square test was applied to compare the number of variant carriers in both the genes between patients and controls. A two-tailed *P* value of ≤ 0.05 was considered statistically significant. The analyses were carried out using Med cal C package.

RESULTS

Although categorization of the study group based on the ultrasound findings yielded two groups, ethnicity was identified as a major confounder for further analysis. Samples were therefore sorted based on ancestry and classified in to Ancestral South Indians (ASI; *n* = 231; Controls-138 and patients-93) and North-East Indians (NEI; *n* = 272; controls-109 and patients-163). All the clinical characteristics as shown in Table 1 namely waist circumference, hip circumference, waist/hip ratio, BMI, ALT, AST, Triglycerides were significantly different between the cohorts from both the ethnicities. Further, there was significant difference in the HDL levels only in the NEI group but not in the ASI group.

Genotyping and association with clinical traits

While rs58542926 in *TM6SF2* gene was significantly associated (*P* = 0.0004) with a 2.7 fold higher risk of fatty infiltration in ASI ethnicity, rs2281135 in *PNPLA3*

Table 1 General characteristics of the study

Characteristics	Ancestral South Indians			North-East Indians		
	Controls	Patients	<i>P</i> value ¹	Controls	Patients	<i>P</i> value ¹
	(<i>n</i> = 138) (mean ± SD)	(<i>n</i> = 93) (mean ± SD)		(<i>n</i> = 109) (mean ± SD)	(<i>n</i> = 163) (mean ± SD)	
Age (yr)	34.2 ± 11.9	35.3 ± 8.0	0.43	38.5 ± 12.7	36.5 ± 9.2	0.13
Gender male/female (<i>n</i>)	95/43	87/6	0.64	72/37	150/13	0.84
Waist circumference (cm)	83.3 ± 9.4	94.7 ± 10.2	0.01	81.1 ± 10.7	93.8 ± 10.1	0.01
Hip circumference (cm)	93.0 ± 7.1	100.5 ± 8.6	0.01	91.2 ± 6.9	95.1 ± 8.5	0.01
Waist/hip ratio	0.89 ± 0.06	0.95 ± 0.13	0.01	0.89 ± 0.07	0.99 ± 0.12	0.01
BMI (kg/m ²)	23.2 ± 4.0	27.7 ± 4.1	0.01	22.1 ± 3.5	25.7 ± 4.0	0.01
ALT (IU/L)	19.8 ± 7.6	88.1 ± 49.5	0.01	24.6 ± 7.9	119.3 ± 68.3	0.01
AST (IU/L)	21.2 ± 5.4	55.3 ± 25.6	0.01	24.6 ± 6.9	72.3 ± 39.8	0.01
Triglycerides (mg/dL)	134.8 ± 72.6	169.7 ± 82.1	0.01	131.4 ± 60.8	180.3 ± 93.7	0.05
HDL (mg/dL)	38.7 ± 8.5	36.3 ± 6.9	0.09	47.9 ± 28.7	40.5 ± 13.8	0.02

¹Unpaired students *t* test (two tailed). BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL: High density lipoprotein.

Table 2 Genotype distribution of *Tm6SF2* and *PNPLA3* variants in the two ethnicities studies

	Ancestral South Indians						North-East Indians					
	TM6SF2 rs58542926						TM6SF2 rs58542926					
	Controls (<i>n</i>)	Patients (<i>n</i>)	Odds	95%CI	χ^2	<i>P</i> value ¹	Controls (<i>n</i>)	Patients (<i>n</i>)	Odds	95%CI	χ^2	<i>P</i> value ¹
Wild (CC)	110	61	2.7	1.37-5.3	15.28	0.0004	80	110	1.51	0.86-2.66	2.29	0.31
Heterozygous (CT)	18	22					22	44				
Homozygous (TT)	0	7					2	6				
<i>PNPLA3</i> rs2281135												
Wild (GG)	79	49	1.34	0.78-2.33	2.12	0.34	63	71	1.9	1.5-3.14	6.48	0.03
Heterozygous (GA)	45	35					32	71				
Homozygous (AA)	4	6					9	17				

¹ χ^2 test. Odds: Odds ratio.

gene was associated with 1.9 fold higher risk in the NEI ethnicity (Table 2). rs58542926 in *TM6SF2* gene was associated with higher ALT, AST levels in the ASI ethnicity and higher BMI in NEI ethnicity. rs2281135 in *PNPLA3* gene was associated with ALT, AST levels in the NE ethnicity (Table 3).

Transheterozygosity analysis

On transheterozygosity analysis (χ^2 test), it was seen that there was a significant difference in individuals who carried variants in both the genes in the patient group as compared to control group in ASI ethnicity (*P* = 0.009), but not NEI ethnicity (*P* = 0.26) and increased the risk of the disease by 3 fold (OR = 3.11, 95%CI: 1.20-8.04) in the ASI ethnicity. Further, there were significantly higher proportion of individuals with variants in both the genes in the patient group in ASI (patients - 14/93 and controls - 7/138; Z proportion test *P* = 0.009) and NEI ethnicities (patients - 17/163 and controls - 7/109; Z proportion test *P* = 0.06).

Comparison of controls and patients within the ethnicities

There were significant differences in BMI (higher) AST, ALT and HDL levels (lower levels) in ASI controls as compared to NEI controls. While patients of ASI ethnicity had higher hip circumference, BMI and lower

HDL levels patients of NEI ethnicity had higher waist-hip ratios, ALT and AST levels. Likewise, there were significant differences in hip circumference, BMI (higher levels in ASI as compared to NEI patients), waist-hip ratio, ALT, AST levels (higher levels in NEI patients as compared to ASI patients). It was also interesting to note that the HDL levels were significantly lower in the ASI patients (Table 4).

DISCUSSION

In a cohort of 503 individuals comprising individuals with and without NAFLD belonging to two distinct Indian ethnicities, we show here that rs58542926 in *TM6SF2* in South Indian and rs2281135 in *PNPLA3* in North-East Indian ethnicities confer higher susceptibility to ultrasound measured NAFLD. Further, there were a significant proportion of individuals with variants in both the genes in the patient group as compared to controls, in both the ethnicities, suggesting that although individually the variants may not confer susceptibility in the ethnicity, however carrying an additional variant might compound the risk of the disease. Our earlier pooled genetic association study in a predominantly North-East Indian ethnicity identified that rs738409 in *PNPLA3* gene was associated with higher risk of NAFLD apart from variants in *PARVB*, *SAMM50* and *PZP*

Table 3 Association of variants with clinical data

	Ancestral South Indians								North-East Indians							
	CC	CT	TT	GG	GA	AA	χ^2	P value ¹	CC	CT	TT	GG	GA	AA	χ^2	P value ¹
<i>TM6SF2</i> rs58542926																
BMI																
< 22.9	49	12	2				0.03	0.98	75	18	5				8.24	0.01
> 22.9	110	28	4						94	47	2					
<i>PNPLA3</i> rs2281135																
BMI																
< 22.9				38	23	2	0.59	0.74				48	37	10	0.077	0.96
> 22.9				82	52	8						71	58	14		
<i>TM6SF2</i> rs58542926																
ALT																
< 30	89	17	1				6.52	0.038	65	20	1				2.45	0.29
> 30	56	19	5						108	42	7					
<i>PNPLA3</i> rs2281135																
ALT																
< 30				64	39	4	1.3	0.52				54	28	4	10.27	0.005
> 30				42	33	5						66	71	19		
<i>TM6SF2</i> rs58542926																
AST																
< 30	90	18	0				10.19	0.006	68	20	3				0.96	0.61
> 30	55	18	6						105	42	5					
<i>PNPLA3</i> rs2281135																
AST																
< 30				65	40	3	2.89	0.23				54	30	7	5.55	0.06
> 30				41	32	6						66	69	16		
<i>TM6SF2</i> rs58542926																
TG																
< 150	54	13	4				1.64	0.43	58	25	6				3.89	0.14
> 150	33	12	1						60	29	1					
<i>PNPLA3</i> rs2281135																
TG																
< 150				42	25	8	0.29	0.86				49	32	8	2.49	0.28
> 150				29	14	3						39	40	11		
<i>TM6SF2</i> rs58542926																
HDL																
> 40	26	5	0				2.03	0.36	49	22	3				0.05	0.97
< 40	57	18	3						59	27	3					
<i>PNPLA3</i> rs2281135																
HDL																
> 40				20	11	0	2.52	0.28				39	28	7	0.5	0.77
< 40				46	26	6						71	58	14		

¹ χ^2 test. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglycerides; HDL: High density lipoprotein.

Table 4 Comparison of clinical data within the ethnicities

Characteristics	Controls			Patients		
	ASI controls (n = 138) (mean \pm SD)	NEI controls (n = 109) (mean \pm SD)	P value ¹	ASI patients (n = 93) (mean \pm SD)	NEI patients (n = 163) (mean \pm SD)	P value
Age (yr)	34.2 \pm 11.9	38.5 \pm 12.7	0.006	35.3 \pm 8.0	36.5 \pm 9.2	0.29
Gender male/female (n)	95/43	72/37	-	87/6	150/13	-
Waist circumference (cm)	83.3 \pm 9.4	81.1 \pm 10.7	0.27	94.7 \pm 10.2	93.8 \pm 10.1	0.54
Hip circumference (cm)	93.0 \pm 7.1	91.2 \pm 6.9	0.23	100.5 \pm 8.6	95.1 \pm 8.5	0.01
Waist/hip ratio	0.89 \pm 0.06	0.89 \pm 0.07	1.0	0.95 \pm 0.13	0.99 \pm 0.12	0.03
BMI (kg/m ²)	23.2 \pm 4.0	22.1 \pm 3.5	0.02	27.7 \pm 4.1	25.7 \pm 4.0	0.003
ALT (IU/L)	19.8 \pm 7.6	24.6 \pm 7.9	0.01	88.1 \pm 49.5	119.3 \pm 68.3	0.01
AST (IU/L)	21.2 \pm 5.4	24.6 \pm 6.9	0.01	55.3 \pm 25.6	72.3 \pm 39.8	0.01
Triglycerides (mg/dL)	134.8 \pm 72.6	131.4 \pm 60.8	0.79	169.7 \pm 82.1	180.3 \pm 93.7	0.44
HDL (mg/dL)	38.7 \pm 8.5	47.9 \pm 28.7	0.02	36.3 \pm 6.9	40.5 \pm 13.8	0.01

¹Unpaired students *t* test (two tailed). ASI: Ancestral South Indians; NEI: North-East Indians; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HDL: High density lipoprotein.

genes^[15].

The first Genome wide association study for NAFLD

identified rs738409 in *PNPLA3* gene conferring susceptibility to NAFLD^[10]. Subsequent to this, the variant

was found to be associated with the disease in various ethnicities across the world including our own and other studies from India^[8,15,18]. *PNPLA3* is a 481-residue protein, exhibiting lipase activity against triglycerides in hepatocytes and a missense variant (I148M; rs738409-C>G) results in loss of function promoting hepatic steatosis by limiting triglyceride hydrolysis^[19]. Further, another variant (rs2281135) in *PNPLA3* gene was identified, that conferred higher risk for NAFLD^[11]. rs2281135 is an intronic variant and is known to be in tight linkage disequilibrium with rs738409 in ethnicities including African, Caucasian, Mexican Americans and East African (HapMap data). Apart from variants in *PNPLA3*, recent research has identified rs58542926 in *TM6SF2* gene to be associated with NAFLD. Recombinant protein expression in cultured hepatocytes confirmed that 50% less Glu167Lys TM6SF2 protein was produced relative to wild-type TM6SF2^[11]. Further a study identified that TM6SF2 regulates liver fat metabolism and influences triglyceride secretion and lipid droplet content^[13]. There is compelling evidence by now that variants in *PNPLA3* and *TM6SF2* genes are associated with progressive fatty infiltration (steatosis and cirrhosis) and further have a higher risk of progressing to HCC. It is therefore very important to understand the genetic susceptibility an ethnicity carries, so that appropriate lifestyle interventions can be planned to minimize the risk of progression, more so in the absence of reversing the genetic defect.

The intronic SNP (rs2281135) in *PNPLA3* gene was associated with a higher risk of fatty infiltration only in NEI ethnicity but not ASI. In an earlier study with a predominant NEI ethnicity we identified that rs738409 in *PNPLA3* conferring a higher susceptibility to fatty infiltration. It is known in literature that rs2281135, an intronic variant and rs738409 a functional variant are in tight LD in ethnicities including African, Caucasian, Mexican Americans and East African (HapMap data).

Although the general characteristics between patients and controls were significantly different as expected, it was interesting to note ethnicity based differences in the patient cohorts that could be predictive of higher susceptibility to NAFLD. While, higher hip circumference, BMI, and lower HDL levels could be predictive of a higher risk for NAFLD in the SI ethnicity, higher Waist-Hip ratio could be predictive in NE ethnicity. Further, higher BMI and lower HDL levels were seen in the controls of SI ethnicity and higher AST and ALT levels were seen in the controls of NE ethnicity suggesting cohort based differences and cutoffs in the clinical characteristics. Further, interestingly there were higher ALT and AST levels in the NEI ethnicity as compare to ASI ethnicity both between control and patient cohorts suggesting a higher necroinflammatory state in the patients of NEI ethnicity. Earlier genome wide studies have ascribed higher levels to genetic predisposition apart from other influencing factors including demographic such as age, sex, ethnicity, anthropometric features (waist circumference, BMI)

and diurnal variation^[20].

The genotype data in general did not deviate from Hardy-Weinberg equilibrium. However, it was interesting to note that there was a significant difference ($P = 0.02$) in the observed and expected genotype frequencies from the patient cohort of ASI ethnicity. Although the samples were represented in sufficient numbers, genotypes visually checked and manually re-scored, non-random mating and population structure excluded, the deviation persisted suggesting that the variant may contribute to disease risk in this ethnicity.

The genotyping data from this study suggests that while *TM6SF2* variant was significantly associated with susceptibility to fatty infiltration in the ASI ethnicity, *PNPLA3* variant was associated in the NEI ethnicity. However, it was interesting to see that there were a higher proportion of individuals in the patient group who were transheterozygous for *PNPLA3* and *TM6SF2* variants as compared to the control group suggesting that although there might be individual susceptibility in the two ethnicities, it is important to genotype the individuals for both the variants as there might be additive risk in the presence of the other risk allele. A recent study from Chinese ethnicity corroborated the same^[21].

In conclusion, our study has identified distinct genetic susceptibility for ultrasound detected NAFLD in the two ethnicities. However, it is suggested that both the variants have to be genotyped for assessing the risk of the disease, as transheterozygosity of the studied variants seems to confer a higher risk in the population.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) with an incidence of 25%-30% is an epidemic that is on the rise globally. There are significant differences in the prevalence, severity and outcome of the disease in various ethnicities that suggests a genetic background to it. Approximately 26%-35% of NAFLD may be contributed by genetic susceptibility according to a study. Therefore it is important to identify genetic susceptibility an individual carries for better management of the disease.

Research frontiers

Understanding and identifying ethnicity based variants that confer higher risk of disease will aid in imparting lifestyle and nutrient based recommendations to an individual with fatty infiltration for better management of the disease.

Innovations and breakthroughs

The authors have identified distinct genetic susceptibility for NAFLD in the two ethnicities that were studied. However, it was interesting to note that transheterozygosity of both the variants conferred a higher risk of the disease irrespective of ethnicity.

Applications

Individuals can be screened for these variants to assess their risk of developing NAFLD. Further, life style based modifications can be suggested to delay the onset/progression of the disease.

Terminology

NAFLD describes a range of liver conditions that begins with accumulation

of fat in the liver (fatty liver) and progresses to fat accumulation along with inflammation and scarring non-alcoholic steatohepatitis, hepatic cells replaced by scar tissue (cirrhosis) finally leading to hepatocellular carcinoma.

Peer-review

The present work deals with a human study in which genetic susceptibility to NAFLD in two Indian ethnicities is evaluated. This study constitutes an interesting work as the identification of population at risk is always desirable.

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Retrospective Study

Conjugated hyperbilirubinemia presenting in first fourteen days in term neonates

Fang Kuan Chiou, Christina Ong, Kong Boo Phua, Fares Chedid, Ajmal Kader

Fang Kuan Chiou, Christina Ong, Kong Boo Phua, Gastroenterology Service, Paediatric Medicine, KK Women's and Children's Hospital, Singapore 229899, Singapore

Fares Chedid, Neonatal Medicine, Al Jalila Children's Specialty Hospital, Dubai, United Arab Emirates

Ajmal Kader, Pediatric Gastroenterology, Dubai Hospital, Dubai, United Arab Emirates

Author contributions: Chiou FK and Kader A contributed equally to this work; Chiou FK contributed to study design, collected and analyzed the data, and drafted and revised the manuscript; Kader A is the principal investigator who designed and supervised the study, provided direction and guidance in data analysis, and reviewed and revised the manuscript; Ong C and Phua KB contributed to study design and reviewed the manuscript for intellectual content; Chedid F contributed to data analysis and provided expertise in statistical analysis; all authors have read and approved the final version of the manuscript.

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Informed consent statement: Singhealth Centralised Institutional Review Board has approved waiver of informed consent based on ethical considerations, that the study involved only a retrospective review of medical records, did not require any additional visit, procedure or intervention for study patients, involved minimal risk to study patients, and no risk of breach in patient confidentiality as all data were anonymized with no patient identifier.

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Data sharing statement: Dataset is available from the corresponding author at ajmalkader@dha.gov.ae. Consent for data sharing from study participants was not obtained as presented data are anonymized and risk of identification is low.

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Correspondence to: Ajmal Kader, MBBS, MD, FRCPCH, Consultant, Pediatric Gastroenterology, Dubai Hospital, Al Khaleeja Street, PO Box 7272, Dubai, United Arab Emirates. ajmalkader@dha.gov.ae
Telephone: +97-15-59886975

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Abstract

AIM

To describe the etiology and characteristics of early-onset conjugated hyperbilirubinemia (ECHB) presenting within 14 d of life in term neonates.

METHODS

Retrospective review was performed of term infants up to 28-d-old who presented with conjugated hyperbilirubinemia (CHB) at a tertiary center over a 5-year period from January 2010 to December 2014. CHB is defined as conjugated bilirubin (CB) fraction greater than 15% of total bilirubin and CB greater or equal to 25 $\mu\text{mol/L}$. ECHB is defined as CHB detected within 14 d of life. "Late-onset" CHB (LCHB) is detected at 15-28 d of life and served as the comparison group.

RESULTS

Total of 117 patients were recruited: 65 had ECHB, 52

had LCHB. Neonates with ECHB were more likely to be clinically unwell (80.0% *vs* 42.3%, $P < 0.001$) and associated with non-hepatic causes (73.8% *vs* 44.2%, $P = 0.001$) compared to LCHB. Multifactorial liver injury (75.0%) and sepsis (17.3%) were the most common causes of ECHB in clinically unwell infants, majority (87.5%) had resolution of CHB with no progression to chronic liver disease. Inborn errors of metabolism were rare (5.8%) but associated with high mortality (100%) in our series. In the subgroup of clinically well infants ($n = 13$) with ECHB, biliary atresia (BA) was the most common diagnosis (61.5%), all presented initially with normal stools and decline in total bilirubin but with persistent CHB.

CONCLUSION

Secondary hepatic injury is the most common reason for ECHB. BA presents with ECHB in well infants without classical symptoms of pale stools and deep jaundice.

Key words: Conjugated hyperbilirubinemia; Biliary atresia; Cholestasis; Direct hyperbilirubinemia; Neonatal jaundice

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Core tip: Conjugated hyperbilirubinemia (CHB) is not routinely checked before 14-21 d of life, hence incidence and etiology of early-onset CHB (ECHB) before 14 d are not well-documented. Nearly three-quarters of ECHB have non-hepatic cause and are expected to recover with supportive treatment, while biliary atresia and metabolic disorders are important etiologies associated with significant morbidity. In our study, BA presenting before 14 d were detected solely from low levels of CHB without pale stools or worsening jaundice. Further studies are needed to determine if CHB screening before 14 d would lead to improved detection and outcome in neonatal liver disorders.

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INTRODUCTION

Conjugated hyperbilirubinemia (CHB) in a neonate signifies an underlying hepatobiliary dysfunction. A significant proportion of neonates with CHB do not have a primary liver disease^[1,2]. According to current recommendations, serum conjugated bilirubin (CB) is checked when neonatal jaundice is prolonged beyond 14-21 d, prior to that only total bilirubin (TB) is checked^[3,4]. The detection of CHB presenting before 14 d of life is usually triggered by specific clinical

situations, therefore the real incidence and etiology of CHB in neonates below 14 d are unknown.

Even with well-established guidelines for the screening of neonatal CHB, actual referral for evaluation of CHB is frequently delayed to beyond 45 to 60 d of age^[5-7]. Substantial observational evidence show that earlier diagnosis and surgical repair of biliary atresia (BA) result in better outcomes^[8-11]. Early diagnosis of many of the other cholestatic conditions may also lead to improved outcomes^[4]. Studies on infants with liver diseases including BA have shown that CB is often elevated in the first week of life^[12-14]. Researchers have also found that CB level performed during the early newborn period is a useful "screening tool" for liver disorders especially biliary atresia^[15].

We studied term newborns with CHB within 14 d of life, aiming to describe the etiology, clinical features and outcome in this poorly studied group, and to find out how they compare to those presenting with CHB between 15 to 28 d of life. To date, our study is the first to address CHB in full-term infants aged below 14 d.

MATERIALS AND METHODS

Retrospective data was collected from consecutive term infants with CHB below 28 d of age within a 5-year period from January 2010 to December 2014. Study was conducted at KK Women's and Children's Hospital which is the largest tertiary pediatric and neonatal facility in Singapore. The study was approved by Singhealth Centralised Institutional Review Board.

CHB is defined as CB fraction greater than 15% of TB, and $CB \geq 25 \mu\text{mol/L}$ ^[16-18]. We define "early-onset" as detection of CHB within 14 d of life (ECHB). Cases were identified through a search in the laboratory database using the inclusion criteria "conjugated bilirubin $\geq 25 \mu\text{mol/L}$ ", "conjugated bilirubin/total bilirubin $> 15\%$ ", "test performed at patient age ≤ 14 d." Infants born at less than 36 wk gestation were excluded.

Consecutive term neonates presenting with CHB aged 15-28 d within the same period served as the comparison group. For the purpose of this study, this group presenting after 14 d of life is referred to as "late-onset" CHB (LCHB).

CB was measured using an automated diazo dye reaction method from venous blood obtained by venipuncture in all patients. Blood samples were delivered immediately to the laboratory in covered specimen tubes to minimize the effect of light on the samples. Blood samples underwent an automated estimation of the hemolysis index, and samples that were found to be hemolysed based on established laboratory criteria were rejected, and repeat samples were taken.

Infants with CHB underwent a variety of investigations that included liver enzyme measurements, hepatobiliary ultrasonography, hepatobiliary iminodiacetic acid (HIDA) scan, liver biopsy, tests for inborn errors of metabolism (IEM), thyroid functions,

Table 1 Baseline clinical characteristics and biochemical indices at onset of conjugated hyperbilirubinemia

Baseline characteristics	ECHB (<i>n</i> = 65, %)	LCHB (<i>n</i> = 52, %)	<i>P</i> value
Ethnic origin			
Chinese	34 (52.3)	27 (51.9)	0.547
Malay	15 (23.1)	18 (34.6)	
Indian	8 (12.3)	4 (7.7)	
Others	8 (12.3)	3 (5.8)	
Male gender	38 (58.5)	40 (76.9)	0.035
Gestational age (wk)	38 (37-39)	38 (37-39)	0.303
Birth weight (g)	2918 (2570-3245)	3068 (2753-3416)	0.114
Apgar			
At 1 min	9 (6-9)	9 (9-10)	0.217
At 5 min	9 (8-9)	9 (9-10)	0.134
Cesarean section	25 (38.5)	14 (26.9)	0.190
Clinically ill status on presentation	52 (80.0)	22 (42.3)	< 0.001
LFT (at diagnosis)			
Total bilirubin (μmol/L)	147 (100-201)	120 (91-163)	0.033
Conjugated bilirubin (μmol/L)	46 (32-65)	38 (30-74)	0.310
Conjugated fraction (%)	35.7 (24.0-51.4)	37.4 (26.3-61.5)	0.159
ALP (IU/L)	160 (119-261)	322 (238-418)	< 0.001
ALT (IU/L)	20 (13-42)	23 (16-32)	0.377
AST (IU/L)	35 (26-75)	35 (25-52)	0.512
GGT (IU/L)	142 (74-334)	199 (131-273)	0.045

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; LFT: Liver function test; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transferase.

bacterial cultures and viral serologies depending on the judgement of the treating physician. Surgical conditions such as BA and choledochal cysts were diagnosed from biochemical tests, radiologic findings and intra-operative cholangiography. IEM were diagnosed if confirmed report of an abnormality was found on appropriate testing. Multifactorial liver injury (MLI) was defined in our study as secondary hepatic insult in an unwell neonate with any combination of the following: Severe cardiorespiratory instability, hepatotoxic medications and parenteral nutrition. Sepsis was defined as infection in which a viral or bacterial agent was isolated, and the infection was the primary cause of illness in the child. CHB was categorized as idiopathic if no cause was identified.

Data on patient demography, clinical history, comorbid conditions, drug history, clinical status at time of detection of CHB, laboratory parameters, radiologic investigations and histologic studies, final diagnoses as well as outcome were retrospectively obtained from medical records. An infant was classified as clinically unwell when the admitting physician documented that the infant appeared unwell.

Data analysis was performed using IBM SPSS Statistics for Windows, version 19 (IBM Corp, Armonk, NY, United States). Continuous variables were expressed as mean \pm SD or median (25%-75% interquartile range). Categorical variables were expressed as number (proportion). Comparisons were performed using two sample *t*-test in normally distributed data with equal variance or Mann-Whitney *U* test when the assumptions of two sample *t*-test were not met. χ^2 test or Fisher's exact test was used to compare categorical variables.

Statistical significance was set at $P < 0.05$.

RESULTS

Total of 117 neonates with CHB were included in the study. Sixty-five had ECHB, and 52 LCHB. Baseline characteristics and liver function tests at presentation are summarized in Table 1. There was a significant male preponderance in both groups, and higher proportion of clinically unwell neonates in ECHB.

Etiology of CHB was identified in about 93% and 60% of cases in ECHB and LCHB groups respectively, rest were classified as idiopathic. Non-hepatic cause for CHB was 73.8% vs 44.2% ($P = 0.001$) in ECHB and LCHB respectively. MLI was an attributable cause of ECHB in 60%, followed by primary sepsis (13.8%) and BA (12.3%) (Table 2). In contrast, the most common cause found in LCHB was idiopathic (40.4%), followed by MLI (34.6%) and BA (9.6%). Factors associated with MLI in both ECHB and LCHB groups are summarized in Table 3.

There was a significantly higher proportion of unwell infants in ECHB group, 80.0% vs 42.3% in LCHB group ($P < 0.001$) (Tables 1 and 4). In the subgroup of patients who were clinically well within the ECHB group, BA was the most common diagnosis (61.5%), the remaining were idiopathic. The most common etiology/association found in well infants in the LCHB group was idiopathic (70.0%), followed by surgical causes (23.4%). No patient with BA was clinically unwell.

Out of the 65 patients with ECHB, 47 (72.3%) resolved within a mean period of 1.9 ± 1.4 mo with eventual normalization of liver tests, 8 (12.3%) had

Table 2 Comparison of causes between early-onset conjugated hyperbilirubinemia and "late-onset" conjugated hyperbilirubinemia groups *n* (%)

Etiology	ECHB (<i>n</i> = 65)	LCHB (<i>n</i> = 52)	Total (<i>n</i> = 117)	<i>P</i> value
Non-surgical causes	56 (86.2)	45 (86.5)	101 (86.3)	0.962
Multifactorial liver injury	39 (60.0)	18 (34.6)	57 (48.7)	0.007
Sepsis	9 (13.8)	3 (5.8)	12 (10.3)	0.154
Inborn errors of metabolism	3 (4.6)	1 (1.9)	4 (3.4)	0.428
CMV infection	0 (0)	2 (3.8)	2 (1.7)	0.112
Idiopathic	5 (7.7)	21 (40.4)	26 (22.2)	< 0.001
Surgical causes	9 (13.8)	7 (13.5)	16 (13.7)	0.952
Biliary Atresia	8 (12.3)	5 (9.6)	13 (11.1)	0.647
Choledochal cyst	1 (1.5)	2 (3.8)	3 (2.6)	0.435
Non-hepatic causes	48 (73.8)	23 (44.2)	71 (61)	0.001

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; CMV: Cytomegalovirus.

Table 3 Factors associated with multifactorial liver injury *n* (%)

Factors associated with multifactorial liver injury	ECHB (<i>n</i> = 39)	LCHB (<i>n</i> = 18)	Total (<i>n</i> = 57)
Antibiotics	38 (97.4)	18 (100.0)	56 (98.2)
Parenteral nutrition	35 (89.7)	16 (88.9)	51 (89.5)
Sedatives/opioid	29 (74.4)	14 (77.8)	43 (75.4)
Mechanical ventilation	26 (66.7)	12 (66.7)	38 (66.7)
Inotropic support	23 (59.0)	9 (50.0)	32 (56.1)
Recent surgery	20 (51.3)	12 (66.7)	32 (56.1)
PPHN	19 (48.7)	4 (22.2)	23 (40.4)
Intestinal obstruction	13 (33.3)	7 (38.9)	20 (35.1)
Congenital heart disease	12 (30.8)	4 (22.2)	16 (28.1)
HFOV	11 (28.2)	3 (16.7)	14 (24.6)
Pneumothorax	8 (20.5)	1 (5.6)	9 (15.8)
CDH	5 (12.8)	4 (22.2)	9 (15.8)
MAS	6 (15.4)	2 (11.1)	8 (14.0)
Renal impairment	6 (15.4)	2 (11.1)	8 (14.0)
Seizures/anti-epileptic	4 (10.3)	2 (11.8)	6 (10.7)
Perinatal asphyxia	3 (7.7)	3 (16.7)	6 (10.5)
Intracranial haemorrhage	2 (5.1)	1 (5.6)	3 (5.3)
Trisomy 21	2 (5.1)	1 (5.6)	3 (5.3)
ECMO	2 (5.1)	1 (5.6)	3 (5.3)
Turner's syndrome	1 (2.6)	0	1 (1.8)
Trisomy 18	1 (2.6)	0	1 (1.8)

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; PPHN: Persistent pulmonary hypertension of the newborn; HFOV: High frequency oscillatory ventilation; CDH: Congenital diaphragmatic hernia; MAS: Meconium aspiration syndrome; ECMO: Extra-corporeal membrane oxygenation.

Table 4 Comparison of causes of early-onset conjugated hyperbilirubinemia and "late-onset" conjugated hyperbilirubinemia between subgroups of clinically well and unwell infants *n* (%)

	ECHB (<i>n</i> = 65)		LCHB (<i>n</i> = 52)	
	Unwell (<i>n</i> = 52)	Well (<i>n</i> = 13)	Unwell (<i>n</i> = 22)	Well (<i>n</i> = 30)
Non-surgical causes				
Multifactorial liver injury	39 (75.0)	0 (0)	18 (81.8)	0 (0)
Sepsis	9 (17.3)	0 (0)	3 (13.6)	0 (0)
Inborn errors of metabolism	3 (5.8)	0 (0)	1 (4.5)	0 (0)
CMV infection	0 (0)	0 (0)	0 (0)	2 (6.7)
Idiopathic	0 (0)	5 (38.5)	0 (0)	21 (70.0)
Surgical causes				
Biliary atresia	0 (0)	8 (61.5)	0 (0)	5 (16.7)
Choledochal cyst	1 (1.9)	0 (0)	0 (0)	2 (6.7)

ECHB: Early-onset conjugated hyperbilirubinemia; LCHB: "Late-onset" conjugated hyperbilirubinemia; CMV: Cytomegalovirus.

surgery for BA and 8 (12.3%) died. Five deaths were due to multi-organ failure and three due to IEM. In the

subgroup of patients with ECHB due to non-hepatic causes (*n* = 48), 42 (87.5%) achieved complete

resolution of CHB without progression to chronic liver disease. In comparison, in the LCHB group overall ($n = 52$), 41 (78.8%) had complete resolution, 7 (13.5%) underwent surgery for BA and choledochal cyst, 2 (3.8%) patients died, one due to IEM and the other died with multi-organ failure. Two patients from each group, ECHB and LCHB, were lost to follow-up. Death occurred in all 4 patients with IEM, three of them in the ECHB group (two mitochondrial disorders and one organic aciduria) and one in LCHB group with urea cycle defect. In both ECHB and LCHB groups, all patients with MLI who survived and all those with idiopathic CHB had complete resolution of liver dysfunction on follow-up.

The reasons for measuring serum CB in the well-looking ECHB cases were atypical "bronze" appearance of skin (38.5%), screening at physician's discretion (30.8%), antenatally detected hepatobiliary anomalies (15.4%) and non-specific symptoms such as vomiting, abdominal distension, respiratory distress and hypoglycemia (15.4%). In eight infants with biliary atresia who presented with ECHB, four had atypical "bronze" appearance, two had antenatally detected hepatobiliary anomalies, and two were screened on physicians' discretion. None of these BA infants had acholic stools at presentation. They also had an initial declining trend of TB, reaching below 50% of initial values in 5 of them, while their CB remained persistently elevated.

DISCUSSION

CHB is often detected when infants are investigated for prolonged neonatal jaundice beyond 14–21 d of life^[4]. Although less routinely encountered, neonatal CHB presenting within 14 d of life can pose considerable diagnostic and management challenges. In one study, the most common etiology of CHB (mean age 10 d) admitted to neonatal intensive care unit (NICU) was culture-proven sepsis (35.5%) and 30 out of 42 (71%) had non-hepatic cause^[1]. In our study, the proportion of neonates with non-hepatic cause for CHB was similar (61%). However, the incidence of sepsis was much lower (10.3%), this difference is because 36% of neonates in that study were preterm requiring NICU care who were more likely to be predisposed to sepsis. Reported etiology of CHB differed depending upon age distribution, geographical region, type of study center and diagnostic approach^[19]. We excluded preterm infants and focused on CHB in term neonates, including those who did not require hospitalization. Most studies on infantile cholestasis focus on BA but we did not find any study looking specifically into the clinical course of neonates with CHB aged below 14 d.

Similar to several other studies, MLI was an important etiology in our series and accounted for almost fifty percent^[2,19–22]. Neonates are predisposed to MLI and cholestasis due to the relative immaturity of the hepatobiliary system, exacerbated by a wide variety of neonatal events such as hypoxia, prolonged fasting,

parenteral nutrition, drug toxicity and sepsis^[2–3,22–25]. Liver injury in such cases is part of multi-organ involvement. The severity and persistence of liver dysfunction depend on underlying disorders, and the dysfunction is usually reversible after resolution of the primary problem^[21–23]. Standard intensive care management of the sick infant and close monitoring of liver function are the mainstays of treatment in these cases. In our study, CHB resolved without any long term liver complications in all the surviving infants with MLI and sepsis, majority of them (91%) recovered within 3 mo.

A significantly higher proportion of newborns who presented before 14 d were clinically unwell compared to those presenting later (80% vs 42%), (Table 4). As per guidelines, healthy infants below 14 d with jaundice are rarely tested for CB, potentially missing CHB in healthy patients and over-estimating the proportion of unwell patients. We observed that about three-quarters of clinically unwell CHB patients presenting within 14 d had non-hepatic cause for CHB. Importantly, no clinically unwell patient had BA (Table 4). The presence of IEM was an important risk factor for mortality. IEM have been reported to account for about 20% of all cases of neonatal cholestasis^[16,19]. It is therefore recommended to maintain a high level of suspicion for IEM in unwell infants with CHB^[26].

Excluding clinically unwell infants, the most common cause of ECHB is BA (61.5%). Notably all infants with BA had pigmented stools at this early stage. Prognosis of BA is dependent on timely diagnosis and surgical intervention. Despite data from BA case series suggesting presence of jaundice before 14 d^[27,28], a significant proportion of cases are referred after 6 to 8 wk of life^[5], and the age at which the Kasai operation is performed has not decreased over the years^[8–11].

In our study, all patients with BA in the ECHB group had a significant initial decline of TB, and in 5 out of 8, TB fell by over 50% from presentation levels, reaching clinically undetectable levels (below 70 $\mu\text{mol/L}$). It can be argued that BA cases may initially have unconjugated hyperbilirubinemia, and CHB develops later. In our study the subset of infants with ECHB who were diagnosed to have BA continued to have persistently raised CB, and this observation was also seen in other studies^[15,28]. Measuring CB in all patients with neonatal jaundice regardless of age, and investigating those with CHB could potentially discover BA at an earlier stage. A recent study examined the potential utility of newborn direct bilirubin measurements performed prior to 60 h of life when infants are still in the hospital as a screen for BA. Authors predicted sensitivity of 100%, based on 35 subjects with BA and predicted specificity of 98.2% based on 9102 subjects without BA^[15].

A few indications to measure CB in well looking neonates below 14 d are antenatally detected hepatobiliary anomalies, pale stools, dark urine and bronze baby syndrome^[29]. In our study approximately

one-third developed bronze baby syndrome, 15% had antenatally detected hepatobiliary anomalies, while none had pale stools or dark urine. This highlights that even with good antenatal ultrasonogram and careful clinical evaluation, a significant proportion of ECHB can be missed.

Delayed detection of neonatal CHB and BA in particular is unlikely to be confined to lack of training and awareness of guidelines among healthcare providers, as despite having guidelines for over 2 decades, cases continue to be missed and treatment delayed^[7]. This is likely to be due to subjectivity in assessment of jaundice. Firstly, it is difficult for parents and physicians to detect minimal jaundice. In addition, as shown in our study, the initial decline of TB may give a false reassurance and the well-looking infant may not be followed-up with blood tests^[5]. Parents may also avoid clinic visits if the infant appears to be improving, this may be for economic reasons or to protect infants from the discomfort of venipuncture.

Hypothetically, if CB is checked with TB measurement during neonatal jaundice screening, or within 60 h of life in all infants^[15], we believe liver disorders and BA can be detected earlier. However, there is no data on the cost-effectiveness of such an approach. It is worthwhile to study the increased economic and logistic burden that arises from over-investigating the self-resolving cases and weigh it against the benefits of earlier detection of CHB. We acknowledge that this approach may not be applicable in centers relying on transcutaneous bilirubin (TcB) or in areas where BA prevalence is low. Hussein *et al.*^[6] discussed screening for CHB and suggested checking urine for conjugated bilirubin, its usefulness as an adjunctive test could be explored in scenarios where blood testing is deemed unnecessary and/or in units relying on TcB.

The main limitation of this study is the single-center retrospective data that could result in selection bias, particularly over-representation of unwell infants with ECHB and under-representation of untested well-looking infants with BA. Another limitation is the non-availability of liver biopsy data in all the cases which could potentially influence the accuracy of diagnosis. This study serves as a primer for prospective studies to evaluate the role of routine measurement of CB in neonatal jaundice and its impact on the outcomes of CHB.

In conclusion, non-hepatic etiology is the most common reason for ECHB in term neonates aged below 14 d. In clinically unwell neonates who do not have IEM, CHB is expected to resolve with supportive management.

BA is an important cause of ECHB in well-looking, jaundiced term infants; it is also an unlikely diagnosis in clinically unwell neonates. Low level of CHB is present in all cases of BA who had CHB tested prior to 14 d of life; large population-based studies may be able to provide the answer whether routine

measurement of conjugated bilirubin in all neonates with jaundice regardless of age, may potentially lead to earlier detection of biliary atresia and other neonatal liver disorders.

COMMENTS

Background

Conjugated hyperbilirubinemia (CHB) in a neonate may be indicative of serious hepatobiliary pathology, such as biliary atresia (BA) or inborn errors of metabolism (IEM). Based on current guidelines, conjugated bilirubin (CB) is screened when neonatal jaundice persists beyond 14-21 d. Hence, incidence and etiology of neonatal CHB before 14 d are not well-defined. Published data suggest that diagnosis of neonatal liver diseases including BA is frequently delayed, and earlier detection can lead to improved outcomes for these infants.

Research frontiers

Early-onset CHB (ECHB) presenting in the first 14 d of life in neonates remain poorly-defined. At the time of writing, there is no other study looking specifically into the clinical course of term neonates presenting with ECHB. The results of this study may contribute to understanding the etiologies of ECHB in term infants and earlier detection/diagnosis of neonatal liver disorders.

Innovations and breakthroughs

This study shows that non-hepatic etiology is the most common reason for ECHB in term neonates aged below 14 d, particularly in the subgroup who are clinically unwell. IEM are rare but associated with high mortality. On the other hand, BA is an important cause of ECHB in well-looking, jaundiced term infants who may not exhibit classical symptoms and signs at this early stage, making the diagnosis of BA difficult if current guidelines are followed. Low level of CHB was found to be present in all cases of BA who had CHB tested prior to 14 d of life.

Applications

In clinically unwell infants with ECHB, if rare IEM are excluded early, majority of cases with non-hepatic causes are expected to resolve with supportive management without progression to chronic liver disease. However, BA should be suspected in well infants presenting with ECHB, even in the absence of pale stools or deep jaundice. This study serves as a primer for larger population-based studies to evaluate the cost-effectiveness of earlier screening for conjugated bilirubin before 14 d in term infants, and its impact on the outcome of neonatal liver disorders including BA.

Terminology

CHB is defined as conjugated bilirubin CB fraction greater than 15% of TB, and $CB \geq 25 \mu\text{mol/L}$. ECHB is defined as CHB detected within 14 d of life.

Peer-review

This retrospective single-center study may contribute to early detection of the cause of conjugated hyperbilirubinemia in term infants.

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