Association between gadoxetic acid–enhanced MR imaging, organic anion transporters, and farnesoid X receptor in benign focal liver lesions

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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>β-HCA</td>
<td>β-catenin mutated HCA</td>
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<tr>
<td>CRP</td>
<td>C reactive protein</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<td>FNH</td>
<td>Focal nodular hyperplasia</td>
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<td>FXR</td>
<td>Farnesoid X receptor</td>
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<tr>
<td>Gd-BOPTA</td>
<td>Gadobenate dimeglumine</td>
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<td>Gd-EOB-DTPA</td>
<td>Gadoxetic acid</td>
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<td>GS</td>
<td>Glutamine synthetase</td>
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<td>HCA</td>
<td>Hepatocellular adenoma</td>
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<td>H-HCA</td>
<td>HNF-1a-mutated HCA</td>
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<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
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<td>I-HCA</td>
<td>Inflammatory HCA</td>
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<tr>
<td>INHBE</td>
<td>Inhibit beta E chain</td>
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<tr>
<td>LFABP</td>
<td>Liver fatty acid–binding protein</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
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<tr>
<td>MRP</td>
<td>Multidrug-resistance associated proteins</td>
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<tr>
<td>NTCP</td>
<td>Sodium-taurocholate-cotransporting polypeptide</td>
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<tr>
<td>OATP</td>
<td>Organic anion transporting polypeptides</td>
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<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
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<tr>
<td>shHCA</td>
<td>Sonic hedgehog HCA</td>
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<tr>
<td>UBC</td>
<td>Ubiquitin C</td>
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<tr>
<td>U-HCA</td>
<td>Unclassified HCA</td>
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Abstract

The organic anion uptake and efflux transporters (OATP1B1, OATP1B3, MRP2 and MRP3) that mediate the transport of the hepatobiliary-specific contrast agent gadoxetate (Gd-EOB-DTPA), are direct or indirect targets of the farnesoid X receptor (FXR), a key regulator of bile acid and lipid homeostasis. In benign liver tumours, FXR expression and activation is not yet characterized. We investigated the expression and activation of FXR and its targets in hepatocellular adenoma (HCA) and focal nodular hyperplasia (FNH) and their correlation with Gd-EOB-DTPA-enhanced MRI. Gd-EOB-DTPA MRI pattern were assessed by an expert radiologist. The intensity of the lesions on the hepatobiliary phase was correlated to mRNA expression levels of OATP1B1, OATP1B3, MRP2, MRP3, FXR and small heterodimer partner (SHP) in fresh surgical specimens of patients with FNH or HCA subtypes. Normal and tumour sample pairs of 43 HCA and 14 FNH were included. All FNH (14/14) were hyperintense. Of the 34 HCA with available Gd-EOB-DTPA-enhanced MRI, six were hyperintense and 28 HCA were hypointense. OATP1B3 was downregulated in the hypointense tumours compared to normal surrounding liver tissue (2.77±3.59 vs 12.9±15.6, p<0.001). A significant positive correlation between FXR expression and activation and OATP1B3 expression level was found in the HCA cohort. SHP showed a trend towards downregulation in hypointense HCA. In conclusion, this study suggests that the MRI relative signal in HCA may reflect expression level and/or activity of SHP and FXR. Moreover, our data confirms the pivotal role of OATP1B3 in Gd-EOB-DTPA uptake in HCA.

Keywords: hepatocellular adenoma, focal nodular hyperplasia, gadolinium, magnetic resonance imaging, MRP, OATP
Significance statement

Farnesoid X receptor (FXR) represents a valuable target for the treatment of liver disease and metabolic syndrome. Currently, two molecules, ursodeoxycholate and obeticholate, are approved for the treatment of primary biliary cirrhosis and cholestasis, with several compounds in clinical trials for the treatment of metabolic dysfunction-associated fatty liver disease (MAFLD). Because FXR expression and activation is associated with gadoxetate accumulation in HCA, atypical gadoxetate-enhanced MRI pattern might arise in patients under FXR-targeted therapy, thereby complicating the differential diagnosis.
Introduction

Focal nodular hyperplasia (FNH) and hepatocellular adenoma (HCA) are two types of hepatic benign masses. FNH carries no risk of malignant transformation or bleeding and usually requires no treatment (van Aalten et al., 2011; Belghiti et al., 2014; 2016). Surgical resection of HCA may be required due to high risk of haemorrhage (20%) or of malignant transformation (4.2%) (Deneve et al., 2009; Stoot et al., 2010). An accurate differentiation between FNH and HCA is often possible by magnetic resonance imaging (MRI) with the hepatobiliary-specific contrast agents, gadobenate dimeglumine (Gd-BOPTA) or gadoxetate (Gd-EOB-DTPA) (Grazioli et al., 2012). Typically, FNH accumulates the contrast agent because the bile ducts of the lesion are not connected to the biliary tree and therefore contrast agents cannot be excreted (Pastor et al., 2014). Most HCA do not accumulate the contrast agent because of poorly functioning lesional hepatocytes and lack of bile ductules, resulting in hypointensity in the hepatobiliary phase (Figure 1).

Recently, classification of HCA based on molecular subtyping has raised concern regarding the diagnostic value of Gd-enhanced MRI within the different subtypes, especially in the presence of an inflammatory HCA (I-HCA) or β-catenin mutated HCA (β-HCA). I-HCA, the most common subtype, carries histomorphologic features of FNH (it was previously termed telangiectatic FNH), very much like FNH, might have a higher rate of hyper- or isointensity at the hepatobiliary phase of Gd-enhanced MRI (McInnes et al., 2015). Moreover, recent studies reported a strong correlation between the presence of a β-catenin mutation in HCA (β-HCA) and uptake of Gd-EOB-DTPA. Notably, the β-HCA has the highest risk of malignant transformation and warrant surgical resection (Reizine et al., 2021).
The varying hepatic accumulation of Gd-BOPTA/DTPA is considered dependent on the activity of organic anion uptake and efflux systems localized on the sinusoidal and canalicular membranes of hepatocytes. In vitro studies showed that Gd-BOPTA/DTPA are substrates of the organic anion transporting polypeptides (OATP)1B1 and OATP1B3, the multidrug-resistance associated proteins (MRP)2 and MRP3 and of the sodium-taurocholate-cotransporting polypeptide (NTCP) (Lorusso et al., 2002; Leonhardt et al., 2010; Jia et al., 2014) with the latter arguably not relevant in vivo (Tsuboyama et al., 2010; Ueno et al., 2014; Yoneda et al., 2016; Sciarra et al., 2019). Based on the localization and proposed transport mechanism of these transporters, it is likely that OATP1B1 and 1B3, expressed on the sinusoidal membrane of hepatocytes, mediate the uptake of Gd-BOPTA/DTPA into the hepatocytes, whereas MRP2 and MRP3, localized on the canalicular and on the sinusoidal membrane, respectively, pump the contrast agent into the canaliculus or back into the circulation (Pastor et al., 2014).

The expression level of these transporters is largely dependent on the expression and activation of the bile acid sensor farnesoid X receptor (FXR, NR1H4), which regulates bile acids de novo synthesis, and transport across epithelia, in tandem with other nuclear receptors such as the small heterodimer partner (SHP) and the hepatocyte nuclear factor 1α (HNF-1α) (Supratim Choudhuri, 2022). Expression of OATP1B1 was reported to be directly and indirectly modulated by the FXR-dependent repression of the HNF-1α and increased via direct upregulation by FXR (Stieger and Hagenbuch, 2014). Similarly, OATP1B3 transcription seems to be induced upon FXR activation (Jung et al., 2002). MRP2 and MRP3 have been shown to be upregulated by bile acids, although primarily in an FXR-independent manner (Zollner et al., 2003). Whether FXR maintains a key role in the regulation of organic anion transporters in hepatic benign tumors, hence in Gd-BOPTA/DTPA hepatic
accumulation, has not been studied. Besides its role as a metabolic sensor and transcriptional
regulator of organic anion transporters, FXR is also considered an oncosuppressor. FXR<sup>−/-</sup>
mice are characterized by spontaneous hepatocarcinogenesis (Kim et al., 2007; Yang et al.,
2007). In human hepatocellular carcinoma (HCC) specimens, expression of FXR is markedly
decreased in comparison with the level in normal liver (van den Esschert et al., 2011). A
recent immunohistochemical analysis on 17 primary HCC cases and 21 non-neoplastic
hepatic lesions (i.e., FNH, regenerative nodules, and dysplastic nodules) revealed that FXR
expression level was lower in HCC than in non-neoplastic lesions (Salama et al., 2023). The
expression and activation of FXR in HCA and its potential role in the malignant
transformation of HCA into HCC has not yet been investigated.

In the current work, we correlated imaging patterns on MRI with Gd-EOB-DTPA with the
expression level of the main Gd-EOB-DTPA hepatic transporters and the FXR signalling
pathway in benign tumours and normal liver tissue.
Materials and Methods

Study design

All patients undergoing resection of FNH and HCA from 2010 to 2019 in the Amsterdam UMC, a tertiary referral centre, with sufficient material available for research purposes were prospectively included. Some of the tumour and/or normal liver tissue samples were previously used for unrelated studies (Visentin et al., 2017; El Saadany et al., 2019; Zanoni et al., 2022). The study adhered to the 1975 Declaration of Helsinki. The local medical ethics committee of the Amsterdam UMC, location AMC (W17_133#17.153) approved the protocol for this study before the enforcement of the General Data Protection Regulation (GDPR) law, waiving the need of informed consent because the study employed unused material collected from treatment-purpose surgery. The collected material consisted of spare tissue after diagnostic workup and was released coded. Collected baseline characteristics were age in years, BMI in kg/m$^2$, sex, and underlying liver disease.

Sample collection and gene expression analysis

Core biopsies (approximately 1 cm$^3$) were taken from the resection specimen tumour tissue. A sample of a similar size was collected outside the area macroscopically identified as tumor tissue and defined as normal tissue. Tumour percentage was determined preceding processing of the samples. Tumour percentage was >96% on average. The samples were snap-frozen in liquid nitrogen directly after the liver resection and stored at -80°C. Frozen tissue samples were directly thawed and incubated overnight at 4°C in Invitrogen™ TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA). After mechanical homogenisation of the tissue samples in TRIzol™, total RNA was isolated following the manufacturer's protocol. cDNA synthesis was performed from 2 µg of total RNA using random primers and Invitrogen™ MultiScribe™ Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA,
USA). The cDNA products were used as template for PCR amplification by Applied Biosystems™ Taqman™ Gene Expression Assay (Thermo Fisher Scientific, Waltham, MA, USA) analysis. A list of target genes is provided in Table S1. Based on a previous geNorm stability analysis within different liver diseases, ubiquitin C (UBC) gene was used in the current study as internal reference (Kim and Kim, 2003).

**Histological evaluation**

Tumours were diagnosed as FNH, HCA or uncertain HCA/HCC (borderline) according to the WHO-classification by an expert pathologist after the appropriate immunohistochemistry as recommended (Bosman et al., 2010) (Figure 1). FNH was defined as a lesion showing hepatocellular proliferation surrounded by fibrotic strands with aberrant arterial vessels and ductular reaction with inflammation. Additional immunohistochemical staining shows a characteristic map-like glutamine synthetase (GS) expression. Hepatocellular tumours without features indicative of malignancy, *i.e.*, cytoarchitectural atypia, loss of reticulin fibres and/or pseudoglandular formation were classified as HCA (Bioulac-Sage et al., 2009). Furthermore, ductular reaction, sinusoidal dilatation, polymorphous inflammatory infiltrates, areas of peliosis, thickened tortuous arteries, expression of serum amyloid A (SAA) protein and C reactive protein (CRP) defined an inflammatory HCA (I-HCA). Mutations of the hepatocyte nuclear factor 1a (HNF-1a) and loss of the liver fatty acid–binding protein (L-FABP) defined an HNF-1a-mutated HCA (H-HCA). Nuclear accumulation of β-catenin and/or strong diffuse staining for glutamine synthetase (GS) defined a β-catenin-mutated HCA (β-HCA). The absence of specific genetic and/or pathologic abnormalities defined an unclassified HCA (U-HCA). Sonic hedgehog HCA (shHCA) are characterized by focal deletions that fuse the promoter of inhibin beta E chain (INHBE) with GLI1, inducing the upregulation of GLI expression (Nault et al., 2018). In this study, GLI1 expression was determined in U-HCA by
RT-qPCR to identify shHCA, and compared to tumour samples of the other subtypes as internal reference. Tumours were classified as uncertain HCA/HCC if they had some features indicative of malignancy, not sufficiently convincing for the diagnosis of HCC, yet too atypical to be classified as HCA, and no expression of two out of three diagnostic HCC markers, namely glutamine synthase (GS), the 70-kDa heat shock protein (HSP70) and glypican-3 (GPC3). Steatosis of surrounding non-lesional liver tissue was scored as minimal or <5% steatosis (0), 5-33% steatosis (1), >33-66% steatosis (2) or >66% steatosis (3).

Imaging analysis

The MRI was performed using a 1.5-Tesla MRI scanner (Avanto, Siemens Healthcare). The full MRI protocol, including details on dosage and administration of Gd-EOB-DTPA are described in the publication of Bieze et al (Bieze et al., 2013). A radiologist with 15 years of experience in liver MRI reviewed available Gd-EOB-DTPA enhanced MRIs, while being blinded for the diagnosis and/or subtype based on histopathology. Each nodule was visually classified, based on their signal in the hepatobiliary phase (entry of contrast agent into hepatocytes), as being either hypointense or hyper- or isointense in comparison with the surrounding non-lesion liver parenchyma on the T1-weighted hepatobiliary series at 20 minutes after injection, hereinafter referred to as hypointense or hyperintense. Presence of a central scar, arterial enhancement, absence of signs of washout during portal phase, and hyperintense signal intensity of the lesion during the hepatobiliary phase were considered indicative of FNH. Arterial enhancement, with washout during portal phase; the presence of bleeding, fat, or glycogen, the absence of a central scar and hypointensity during the hepatobiliary phase were signs considered indicative of HCA (McInnes et al., 2015). To define sensitivity and specificity results of Gd-EOB-DTPA enhanced MRIs were compared to histopathological diagnosis.
Statistical analysis

GraphPad Prism version 8.0 for Windows (Graph Pad Software, San Diego, CA, USA) was used for statistical analyses and visual representation thereof. The statistical tests performed are indicated in the figure legend. Tumour mRNA data were expressed as fold-change of those in the respective normal liver tissue.
Results

Patient characteristics and diagnosis

Patients were included in the analysis if enough tumour and surrounding normal tissues were available for RNA extraction in addition to the sampling, needed for diagnostic purposes. Fifty-seven tumours and respective surrounding normal liver tissue were collected. Lesions with features diagnostic for hepatocellular carcinoma (HCC) were excluded from the study. The average age of 36 ± 8.5 years and the average BMI was 28 ± 6.0 kg/m². Underlying liver disease was present in 39% of the patients. Fourteen patients were diagnosed with FNH. Forty-three patients were diagnosed with HCA, mostly I-HCA 27 (63%), also three HCA/HCCs were identified, and one β-HCA. The latter four were analysed but excluded from the subsequent comparative analyses on HCA subtypes due to sample size. More details related to patients and diagnosis are listed in Table 1. Moreover, eight U-HCA were found based on histopathological examination, of which two expressed relatively high mRNA level of GLI1, a marker of shHCA (Figure S1).

Gd-EOB-DTPA-based imaging

Gd-EOB-DTPA-based MRI data of 48 out of 57 patients were available. Twenty-eight out of 48 lesions (all 28 were HCA) were defined as hypointense and twenty out of 48 lesions (fourteen FNH and six HCA) were defined as hyperintense. FNH was detected with a 100% sensitivity and 82% specificity. Overall accuracy was 88%. In the comparative analyses on HCA subtypes, Gd-EOB-DTPA-based MRI showed particularly poor performance in the detection of U-HCA, which were hyperintense in 50% of the cases. Two out of eight U-HCA showed a relatively high GLI1 expression level, which might be indicative of a sh-HCA (Nault et al., 2018). There appeared to be no correlation between intensity on Gd-EOB-DTPA-based MRI and GLI1 expression (Figure S1).
Association of mRNA expression of Gd-EOB-DTPA transporters with MRI pattern and HCA subtype

The expression of OATP1B1, OATPB3, MRP2 and MRP3 was analysed in 57 pairs of tumours, and the relative surrounding normal tissues samples. For 48 of these patients, Gd-EOB-DTPA-based imaging data were available. Figure 2A shows that the majority (23 out of 28) of the hypointense tumours (28 HCA) was characterized by a lower mRNA level of OATP1B3. The expression level of OATP1B3 in hypointense tumours was 5 times lower than that in the respective surrounding normal tissue (2.77±3.59 vs 12.9±15.6, P<0.001). Hyperintense tumours (14 FNH and 6 HCA) displayed OATP1B3 mRNA levels comparable to those of the respective surrounding normal tissue. When solely hyperintense HCA were considered, a pattern of upregulation emerged (n=6). This was not the case when solely (hyperintense) FNH were included in the analysis (Figure S2). In the comparative analyses on HCA subtypes, a significant change in the expression of organic anion transporters was found in the four H-HCA (Figure 3A and B). The H-HCA were characterized by a 21-fold reduction in OATP1B1 (0.60 ± 0.11 tumour vs 12.6 ± 1.83 normal, P=0.0010), an 8-fold reduction in OATP1B3 (2.09 ± 0.42 tumour vs 16.6 ± 3.00 normal, P=0.003) and a 36-fold reduction in MRP2 (0.17 ± 0.12 tumour vs 6.15 ± 0.46 normal, P=0.002) mRNA level, in comparison with the surrounding normal tissue. Notably, all H-HCA were hypointense on available Gd-EOB-DTPA-MRI (n=3). Twenty out of 22 I-HCA were hypointense and showed a significant downregulation of OATP1B3 (2.69 ± 3.36 tumour, 15.07 ± 17.10 normal, P=0.0008). Moreover, the two hyperintense I-HCA displayed among the highest upregulation of OATP1B3 (43-fold and 6-fold) relative to the surrounding normal tissues.
Correlation of mRNA expression of Gd-EOB-DTPA transporters with FXR expression level and activation and MRI pattern

The mRNA level of FXR and SHP, a bona fide target gene of FXR employed as a surrogate of FXR signalling pathways activation, were assessed in tumours and the surrounding normal tissue. The role of bile acids and the bile acid sensor FXR in the regulation of OATPs and MRPs has been extensively studied in vitro (Zollner et al., 2003; Yang et al., 2007; Stieger and Hagenbuch, 2014). Table 2 summarizes the Person’s correlation factors between the mRNA level of FXR or SHP, and the mRNA level of the Gd-EOB-DTPA transporters. Considering the critical role of OATP1B3 in Gd-EOB-DTPA accumulation in HCA, the significant positive correlation between FXR expression and activation and OATP1B3 expression level in HCA is noteworthy, suggesting that the MRI pattern could provide information on the FXR and SHP expression level. Indeed, SHP showed a trend towards downregulation in hypointense HCA (Figure 4A). However, no changes were observed in the hyperintense (14 FNH and 6 HCA) and FXR was not downregulated. Comparative analysis of HCA subtypes in figure 4B shows that FXR expression level was comparable between normal tissue and tumour samples, regardless of the HCA subtype. Conversely, SHP expression level was markedly reduced, albeit not significant, in the H-HCA, suggesting the FXR signalling pathway might be inhibited or inactive in this subtype.
Discussion

This study tested the hypothesis that Gd-EOB-DTPA MRI may yield information on the FXR-OATP axis in focal liver lesions. Our data support the concept that Gd-EOB-DTPA MRI provides information on activation state of the FXR signalling pathway in HCA (Figure 5). FXR is considered a major driver of hepatic carcinogenesis, as it upregulates the production of FGF15/19, which in hepatocytes binds to the FGF receptor 4. Activation of FGFR4 may also lead to HCC (Huang et al., 2015; Piglionica et al., 2018). Here, low mRNA levels of OATP1B3 were found in hypointense HCA and conversely, high levels of OATP1B3 were found in hyperintense HCA. In HCA, the mRNA level of FXR and SHP, a bona fide target gene of FXR, positively correlated with OATP1B3 expression level (Jung et al., 2002). SHP showed a trend towards downregulation in hypointense HCA (Figure 4). Yet, notably, FXR and SHP expression was seemingly unchanged between HCA and the respective surrounding normal tissues. The main pharmacological ramification stemming from our data is that drugs that directly or indirectly modulate FXR expression level and/or activation, such as obeticholic acid (Neuschwander-Tetri et al., 2015) and ursodeoxycholic acid (Mueller et al., 2015), might alter the MRI imaging pattern.

A deeper molecular characterization of HCA is important for a better interpretation of gadolinium-based MRI. One interesting finding of the present study is that U-HCA showed an overall higher, albeit not significant, FXR mRNA level as compared with the surrounding normal tissue. A further analysis on a larger cohort of U-HCA is needed to assess FXR as an informative marker for further stratification of U-HCA. Additionally, in H-HCA, SHP but not FXR mRNA level was markedly lower than in the normal liver (Figure 4). This finding suggests that HNF-1α-mediated induction of FXR promoter activity shown in vitro might not be relevant in vivo (Lou et al., 2007; Purushotham et al., 2012). Conversely, our data are in
line with a previous report showing that HNF1a suppression in rat hepatocytes markedly decreased the SHP-1 level, and a positive correlation between the mRNA level of HNF1a and SHP-1 in the liver from patients with liver cirrhosis (Qian et al., 2015). Similarly, it has been shown that SHP expression was reduced in mouse Hnf-1a\(^{-/-}\) pancreatic islets via Hnf-4a (Miyachi et al., 2022). Our data suggest that SHP might also be involved in the development of HCA lacking HNF-1a.

Our study confirms that OATP1B3 expression level is arguably the most important molecular determinant of the uptake and accumulation of Gd-EOB-DPTA in HCA but not in FNH. OATP1B3 was consistently downregulated in all hypointense HCA and upregulated in most hyperintense HCA. Interestingly, the hyper- and isointense HCA are mainly U-HCA (hyper- or isointense in 4 out of 8 cases). Conversely, no trend of expression could be associated to the MRI pattern of FNH, suggesting the hyperintense signal that characterized FNH in our study and in previous studies (Vilgrain, 2006; Grieser et al., 2013) might be primarily related to the altered architecture of the biliary tree in FNH resulting in impaired excretion of the contrast agent (Pastor et al., 2014). Our findings are in line with previous studies addressing the role of Gd-EOB-DPTA transporters in imaging. Taken together, our gene expression data confirm some of the findings of a recent study, in which the expression of Gd-EOB-DTPA transporters in FNH and HCA was assessed by immunohistochemistry. Although in that study OATP1B1 and OATP1B3 expression level could not be assessed separately, it was found that the expression level of OATP1B1/B3 and MRP3 was critical in Gd-EOB-DTPA accumulation in HCA, whereas no significant changes in transporter expression were detected in FNH (Sciarra et al., 2019). Our data strongly suggest that the expression level of OATP1B3 determines cellular intensity in liver Gd-EOB-DPTA MRI \textit{in vivo}. Retrospective analyses of patients with HCC who underwent preoperative Gd-EOB-DTPA-enhanced MRI showed that
the uptake of Gd-EOB-DTPA correlated with the expression level of OATP1B3 (Narita et al., 2009; Zhou et al., 2021) but not with the expression of OATP1B1 (Yoneda et al., 2016). In vitro kinetic analysis revealed comparable intrinsic clearances for Gd-EOB-DPTA transport by OATP1B1 and 1B3 (assuming comparable expression levels in the in vitro system used) (Leonhardt et al., 2010). Notably, a recent study associated the expression of OATP1B3 to gadoxetate-enhanced MRI pattern in localized and metastatic prostate cancers (Lochrin et al., 2021). The uptake as pace-setting step of Gd-EOB-DTPA accumulation is confirmed by the lack of significant changes in the expression level of the efflux pumps MRP2 and MRP3.

Our study also has some limitations. Only resection specimens were included, not biopsies, for the sake of having enough tissue to analyze and of collecting representative normal tissue at proper distance from the tumor. FNH has no risk of malignant transformation, thus the main reason to perform surgery in patients with FNH is uncertain diagnosis at MRI. This may have introduced a selection bias. Moreover, the nearly absence of males was expected. Consequently, almost exclusively female patients were included, challenging the transferability of these findings to male patients, in whom β-catenin mutations and malignant transformation of HCA are more prevalent. This prevents us to speculate on the possible role of FXR in malignant transformation of HCA (van Rosmalen et al., 2021). Similarly, the relatively low percentage of H-HCA and high percentage of U-HCA in this cohort makes the results more difficult to extrapolate to the general population. Moreover, at least two of the eight U-HCA were presumably shHCA, introducing heterogeneity. In summary, despite these limitations, the present study (i) shows that Gd-EOB-DTPA accumulation, hence the MRI relative signal, may reflect the FXR and SHP expression level in HCA, (ii) suggests that HNF-1α may be a regulator of SHP in an FXR-independent manner in vivo, (iii) confirms the central role for OATP1B3 in Gd-EOB-DTPA uptake in HCA, but not in FNH, and (iv)
suggests that Gd-EOB-Imaging might be a tool for in vivo investigating potential alterations of treatments with FXR (Katafuchi and Makishima, 2022) or FGFR4 antagonists (Lang and Teng, 2019).
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Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request. Due to the nature of the research, due to privacy/ethical reason, some supporting data is not available.
Authorship contributions

Participated in research design: van Rosmalen, Visentin, Stieger

Conducted experiments: van Rosmalen, Visentin

Performed data analysis: van Rosmalen, Visentin, Furumaya, van Delden, Kazemier, van Gulik, Verheij, Stieger

Wrote or contributed to the writing of the manuscript: van Rosmalen, Visentin, Furumaya, van Delden, Kazemier, van Gulik, Verheij, Stieger
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Footnotes

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Figure legends

Figure 1. Histological and radiological features of FNH and HCA, and schematic representation of uptake and efflux of Gd-EOB-DTPA. (A1) Typical hematoxylin and eosin staining pattern of FNH with fibrous area (1) and ductular reaction (2). (A2) Glutamine synthetase immunostaining of an FNH. (B1) Typical histopathological image of an I-HCA, hematoxylin and eosin stain. (B2) C-reactive protein (CRP) of an I-HCA. (C1) Typical Gd-EOB-DTPA–enhanced Magnetic Resonance Imaging (MRI) of an FNH. (C2) Typical Gd-EOB-DTPA–enhanced Magnetic Resonance Imaging (MRI) of an HCA.

Figure 2. Gene expression of hepatic transporters and association with magnetic resonance imaging pattern. (A) Heat-map of mRNA profiling of indicated target genes in tumour samples relative to the respective normal samples. The relative expression values of each target gene were measured in the tumour and in the matched healthy tissue, normalized by the expression of the housekeeping UBC gene and then expressed as tumour: normal ratio (ΔΔCT). Data are reported in logarithmic scale and clustered according to the Gd-EOB-DTPA–enhanced Magnetic Resonance Imaging (MRI) pattern, where each column represents one patient, n=48. Blue and red colours indicate downregulation and upregulation, respectively. (B) Relative expression of the indicated target genes in tumour and normal liver tissues. mRNA values of the genes were normalized by the expression of the housekeeping gene ubiquitin C (UBC), and visually displayed in Log10 scale. Analysis was performed on non-transformed data, using a two-sided paired sample T-test. Significant differences are shown in a scatter plot of the means ± S.D., where each dot represents one patient.

Figure 3. Gene expression of hepatic transporters and association with tumour histotype. (A) Colour-code representation of MRI pattern and relative heat-map of mRNA profiling of indicated target genes in tumour samples relative to the respective normal
samples, where each column represents one patient. The relative expression values of each
target gene were measured in the tumour and in the matched healthy tissue, normalized by the
expression of the housekeeping UBC gene and then expressed as tumour: normal ratio
(ΔΔCT). Data are reported in logarithmic scale and clustered according to the histotype, n=48.
Blue and red colours indicate downregulation and upregulation, respectively. (B) Relative
expression of the indicated genes in tumour and normal liver tissues. mRNA values of the
genes were normalized by the expression of the housekeeping gene ubiquitin C (UBC), and
visually displayed in Log10 scale. Analysis was performed on non-transformed data, using a
two-sided paired sample T-test. Significant differences are shown in a scatter plot of the
means ± S.D., where each dot represents one patient.

**Figure 4.** mRNA expression level of FXR and SHP and association with magnetic
resonance imaging pattern and association with tumour histotype. (A) Relative
expression of FXR and SHP in hypo and hyperintense tumours. mRNA values of the genes
were normalized by the expression of the housekeeping gene ubiquitin C (UBC), and visually
displayed in Log10 scale. Analysis was performed on non-transformed data, using a two-
sided paired sample T-test. Significant differences are shown in a scatter plot of the means ±
S.D., where each dot represents one patient. (B) Relative expression of FXR and SHP in
tumour and normal liver tissues of I-HCA, H-HCA, U-HCA and FNH. mRNA values of the
genes were normalized by the expression of the housekeeping gene ubiquitin C (UBC), and
visually displayed in Log10 scale. Analysis was performed on non-transformed data, using a
two-sided paired sample T-test. Significant differences are shown in a scatter plot of the
means ± S.D., where each dot represents one patient.

**Figure 5.** Schematic representation of the role of the FXR-OATP1B3 axis in Gd-based
MRI of HCA. The movement of the contrast agent from the sinusoidal blood into the
hepatocytes is facilitated by the organic anion transporting polypeptides (OATP) 1B1 and
1B3, anion exchangers expressed at the sinusoidal membrane of hepatocytes. The intracellular contrast agent is pumped out of the hepatocytes into the bile canaliculus by the multidrug resistance-associated protein 2 (MRP2), expressed on the canalicular membrane, and back into the blood stream by MRP3, expressed on the sinusoidal membrane. OATP1B3 expression level is the main molecular determinant of Gd-EOB-DTPA accumulation in HCA. When FXR activation is reduced, OATP1B3 expression and activity is repressed, resulting in a hypointense-imaging pattern of the HCA. Conversely, a greater activation of FXR results in a higher expression of OATP1B3 and a hyperintense-imaging pattern of the HCA.
### Table 1. Patient clinical characteristics

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<thead>
<tr>
<th>Clinical Parameters</th>
<th>Category</th>
<th>N (%)</th>
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<tr>
<td>Age at diagnosis</td>
<td>N.A.</td>
<td>57 (100)</td>
</tr>
<tr>
<td></td>
<td>Mean = 36</td>
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<tr>
<td></td>
<td>SD ± 8.5</td>
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<tr>
<td>Sex</td>
<td>Male</td>
<td>2 (3.5)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>55 (96)</td>
</tr>
<tr>
<td>Body Mass Index (BMI)</td>
<td>18.5 – 25 (normal)</td>
<td>11 (19)</td>
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<tr>
<td></td>
<td>25 tot 27 (mild obesity)</td>
<td>4 (7.0)</td>
</tr>
<tr>
<td></td>
<td>27 tot 30 (moderate obesity)</td>
<td>4 (7.0)</td>
</tr>
<tr>
<td></td>
<td>30 tot 40 (morbid obesity)</td>
<td>12 (21)</td>
</tr>
<tr>
<td></td>
<td>Not reported</td>
<td>26 (46)</td>
</tr>
<tr>
<td>Underlying liver disease</td>
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<td>22 (39)</td>
</tr>
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<td>No</td>
<td>35 (61)</td>
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<td>Histopathological diagnosis</td>
<td>HCA</td>
<td>43 (74)</td>
</tr>
<tr>
<td></td>
<td>- I-HCA</td>
<td>27 (63)</td>
</tr>
<tr>
<td></td>
<td>- H-HCA</td>
<td>4 (9)</td>
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<tr>
<td></td>
<td>- U-HCA†</td>
<td>8 (19)</td>
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<tr>
<td></td>
<td>- β-HCA</td>
<td>1 (2)</td>
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<td></td>
<td>- HCA/HCC</td>
<td>3 (7)</td>
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<tr>
<td></td>
<td>FNH</td>
<td>14 (25)</td>
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<tr>
<td>Gd-EOB-DTPA-enhanced MRI</td>
<td>Hypointense</td>
<td>28 (49)</td>
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<tr>
<td></td>
<td>Hyperintense</td>
<td>20 (35)</td>
</tr>
<tr>
<td></td>
<td>Not performed</td>
<td>9 (16)</td>
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<tr>
<td>Histological assessment of steatosis</td>
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<td>15 (26)</td>
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<tr>
<td></td>
<td>1 (5-33%)</td>
<td>5 (8.8)</td>
</tr>
<tr>
<td></td>
<td>2 (&gt;33 - 66%)</td>
<td>8 (14)</td>
</tr>
<tr>
<td></td>
<td>3 (&gt;66%)</td>
<td>3 (5.3)</td>
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<tr>
<td></td>
<td>Not reported</td>
<td>26 (46)</td>
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</table>

Gd-EOB-DTPA, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid; MRI, magnetic resonance imaging

† Based on histology, 8 HCA were classified as U-HCA. After RT qPCR, two potential sh-HCA were identified within this group.
Table 2. Pearson correlation of the expression of Gd-EOB-DTPA transporters with expression and activation of FXR.

<table>
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<th></th>
<th>FXR</th>
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<th>SHP</th>
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<tr>
<td></td>
<td>r</td>
<td>P-value</td>
<td>r</td>
<td>P-value</td>
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<tr>
<td>Normal (n=57)</td>
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<tr>
<td>OATP1B1</td>
<td>0.45</td>
<td>0.0005</td>
<td>0.46</td>
<td>0.0002</td>
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<tr>
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<td>0.17</td>
<td>0.19</td>
<td>0.14</td>
<td>0.27</td>
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<tr>
<td>MRP2</td>
<td>0.20</td>
<td>0.13</td>
<td>0.15</td>
<td>0.25</td>
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<tr>
<td>MRP3</td>
<td>0.34</td>
<td>0.01</td>
<td>0.38</td>
<td>0.004</td>
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<td>HCA (n=43)</td>
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<tr>
<td>OATP1B1</td>
<td>0.43</td>
<td>0.004</td>
<td>0.72</td>
<td>0.0001</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>0.37</td>
<td>0.01</td>
<td>0.48</td>
<td>0.001</td>
</tr>
<tr>
<td>MRP2</td>
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<td>0.02</td>
<td>0.60</td>
<td>0.0001</td>
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<tr>
<td>MRP3</td>
<td>0.25</td>
<td>0.10</td>
<td>0.22</td>
<td>0.15</td>
</tr>
<tr>
<td>FNH (n=14)</td>
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<tr>
<td>OATP1B1</td>
<td>0.78</td>
<td>0.001</td>
<td>0.47</td>
<td>0.09</td>
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<tr>
<td>OATP1B3</td>
<td>0.02</td>
<td>0.93</td>
<td>-0.23</td>
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<tr>
<td>MRP2</td>
<td>0.33</td>
<td>0.25</td>
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<td>0.79</td>
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<td>MRP3</td>
<td>0.50</td>
<td>0.07</td>
<td>0.27</td>
<td>0.34</td>
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</table>

FNH, focal nodular hyperplasia; FXR, farnesoid x receptor; HCA, hepatocellular carcinoma; OATP, organic anion transporting polypeptide; MRP, multidrug resistance associated protein; SHP, small heterodimer partner.
Figure 2
Figure 3
Figure 4