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The Non-Invasive Liver Biopsy

Determining Hepatic Function in Diffuse and Focal Liver
Disease

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Till Frida

”Pretending to know everything closes the door to learn more”

– Neil deGrasse Tyson

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ABSTRACT

The liver is one of the largest organs within the human body and it handles many vital tasks such as nutrient processing, toxin removal, and synthesis of important proteins. The number of people suffering from chronic liver disease is on the rise, likely due to the present 'western' lifestyle. As disease develops in the liver there are pathophysiological manifestations within the liver parenchyma that are both common and important to monitor. These manifestations include inflammation, fatty infiltration (steatosis), excessive scar tissue formation (fibrosis and cirrhosis), and iron loading. Importantly, as the disease progresses there is concurrent loss of liver function. Furthermore, postoperative liver function insufficiency is an important concern when planning surgical treatment of the liver, because it is associated with both morbidity and mortality. Liver function can also be hampered due to drug-induced injuries, an important aspect to consider in drug-development.

Currently, an invasive liver needle biopsy is required to determine the aetiology and to stage or grade the pathophysiological manifestations. There are important limitations with the biopsy, which include, risk of serious complications, mortality, morbidity, inter- and intra-observer variability, sampling error, and sampling variability. Clearly, it would be beneficial to be able investigate the pathophysiological manifestations accurately, non-invasively, and on regional level.

Current available laboratory liver function blood panels are typically insufficient and often only indicate damage at a late stage. Thus, it would be beneficial to have access to biomarkers that are both sensitive and responds to early changes in liver function in both clinical settings and for the pharmaceutical industry and regulatory agencies.

The main aim of this thesis was to develop and evaluate methods that can be used for a 'non-invasive liver biopsy' using magnetic resonance (MR). We also aimed to develop sensitive methods for measure liver function based on gadoxetate-enhanced MR imaging (MRI).

The presented work is primarily based on a prospective study on c. 100 patients suffering from chronic liver disease of varying aetiologies recruited due to elevated liver enzyme levels, without clear signs of decompensated cirrhosis.

Our results show that the commonly used liver fat cut-off for diagnosing steatosis should be lowered from 5% to 3% when using MR proton-density fat fraction (PDFF). We also show that MR elastography (MRE) is superior in staging fibrosis.

Finally we presented a framework for quantifying liver function based on gadoxetate-enhanced MRI. The method is based on clinical images and a clini-

cal approved contrast agent (gadoxetate). The framework consists of; state-of-the-art image reconstruction and correction methods, a mathematical model, and a precise model parametrization method. The model was developed and validated on healthy subjects. Thereafter the model was found applicable on the chronic liver disease cohort as well as validated using gadoxetate levels in biopsy samples and blood samples. The liver function parameters correlated with clinical markers for liver function and liver fibrosis (used as a surrogate marker for liver function).

In summary, it should be possible to perform a non-invasive liver biopsy using: MRI-PDFF for liver fat and iron loading, MRE for liver fibrosis and possibly also inflammation, and measure liver function using the presented framework for analysing gadoxetate-enhanced MRI. With the exception of an MRE-transducer no additional hardware is required on the MR scanner. The liver function method is likely to be useful both in a clinical setting and in pharmaceutical trials.

SVENSK SAMMANFATTNING

Levern är ett av de största organen i våra kroppar och levern utför många livsviktiga uppgifter så som processande av näringsämnen, avlägsnande av gifter samt syntes av viktiga proteiner. I vårt moderna samhälle ökar antalet personer som drabbas av kroniska leversjukdomar. När sjukdomarna utvecklas i levern är det flera patofysiologiska manifesteringar i leverparenkymet som är vanliga och viktiga att övervaka. Dessa manifesteringar inkluderar inflammation, fettinfiltration (steatos), kraftig ackumulering av ärrvävnad (fibros och cirrhos) samt järninlagring. I slutänden kommer de kroniska sjukdomarna att försämra leverfunktionen. Vidare är otillräcklig postoperativ leverfunktion en viktig aspekt när man planerar kirurgisk intervention i levern då detta tillstånd är associerat med både ökad morbiditet samt mortalitet. Leverfunktionen kan också försämrats på grund av läkemedelsinducerade skador, vilket är viktigt att ta hänsyn till i läkemedelsutveckling.

I nuvarande klinisk rutin krävs en invasiv nålbiopsi av levern för att diagnostisera sjukdomen samt för att gradera de patofysiologiska manifesteringarna. Det finns dock flera allvarliga begränsningar med en leverbiopsi vilka inkluderar: risk för allvarliga komplikationer, mortalitet, morbiditet, inter- och intra-observatörs variabilitet, samplingsfel, och samplingsvariabilitet. Det är uppenbart att det skulle vara fördelaktigt att kunna undersöka de patofysiologiska manifesteringarna kvantitativt, icke-invasivt, samt på en regional nivå, dvs. i de olika leversegmenten.

Nuvarande laboratorietester för leverfunktion, som är baserade på blodprover, är otillräckliga och påvisar ofta skada först i ett sent stadium av sjukdomen. Det vore fördelaktigt både för den kliniska vardagen men även för läkemedelsindustrin samt övervakande myndigheter att identifiera kliniskt användbara biomarkörer som är både känsliga och som påvisar tidiga förändringar i leverfunktion.

Målet med denna avhandling var att utveckla och utvärdera metoder som kan användas för en 'icke-invasiv leverbiopsi' med magnetisk resonanstomografi (MRT). Ett viktigt delmål var dessutom att utveckla känsliga metoder för att mäta leverfunktion baserat på gadoxetat-förstärkt MRT.

Detta arbete är primärt baserat på en prospektiv studie där patienter rekryterades från det kliniska flödet. Patienterna som rekryterades har drabbats av kronisk leversjukdom av varierande etiologi och de rekryterades på grund av förhöjda leverenzymnivåer, men utan tydliga tecken på dekompenserad cirrhos.

Våra resultat visar att det vanligen användas gränsvärdet för att diagnostisera fettlever bör sänkas från 5% till 3% när MR protondensitets fettfraktion

(PDFF) används. Vidare visar vi att MR elastografi (MRE) är överlägsen för att gradera leverfibros.

Slutligen presenterade vi ett ramverk för att kvantifiera leverfunktion baserat på gadoxetat-förstärkt MRT. Metoden är baserad på kliniska bilder med ett kliniskt godkänt kontrastmedel (gadoxetat). Detta ramverk består av toppmoderna bildrekonstruktions- och korrektionsmetoder, en matematisk modell, och en noggrann parametriseringsmetod. Modellen utvecklades och validerades på friska personer. Därefter visade vi att modellen var tillämpbar på kohorten av kroniskt leversjuka patienter. Vidare validerade vi modellen mot de gadoxetat-nivåer som uppmättes i biopsi- samt blodprov. Leverfunktionsparameterna korrelerade med kliniska markörer för leverfunktion och även leverfibros (som användes som surrogat för leverfunktion).

Sammanfattningsvis bör det vara möjligt att genomföra en icke-invasiv leverbiopsi med: MRT-PDFF för att mäta leverfett och järnhalt, MRE för leverfibros men potentiellt även inflammation samt att mäta leverfunktion med ramverket som presenteras i avhandlingen baserat på gadoxetat-förstärkt MRT. Med undantag för MRE krävs ingen extra hårdvara utöver en klinisk MR-kamera. Leverfunktionsmätningssmetoden är rimligen användbar både i den kliniska vardagen samt läkemedelsstudier.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals (I-V).

- I. Separation of Advanced from Mild Hepatic Fibrosis by Quantification of the Hepatobiliary uptake of Gd-EOB-DTPA**
Norén B, **Forsgren MF**, Dahlqvist Leinhard O, Dahlström N, Kihlberg J, Romu T, Kechagias S, and Lundberg P
European Radiology 2013 Jan;23:174-181
- II. Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data**
Forsgren MF, Dahlqvist Leinhard O, Dahlström N, Cedersund G, and Lundberg P
PLoS ONE 2014 April;9(4):e95700
- III. Using a 3% Proton Density Fat Fraction as a Cut-off Value Increases Sensitivity of Detection of Hepatic Steatosis, Based on Results from Histopathology Analysis**
Nasr P*, **Forsgren MF***, Ignatova S, Dahlström N, Cedersund G, Dahlqvist Leinhard O, Norén B, Ekstedt M, Lundberg P, and Kechagias S
Gastroenterology 2017 E-pub March 9
- IV. Quantitative Assessment of Liver Functions by Gadoxetate-Enhanced MRI: a Prospective Study of Chronic Liver Disease**
Forsgren MF, Karlsson M, Dahlqvist Leinhard O, Dahlström N, Norén B, Romu T, Ekstedt M, Kechagias S, Cedersund G, and Lundberg P.
In manuscript
- V. Prospective Comparison of Multimodal Magnetic Resonance, Blood Panels, and Transient Elastography for Staging Liver Fibrosis in a Clinical Setting**
Forsgren MF*, Nasr P*, Karlsson M, Dahlström N, Norén B, Ignatova S, Garteiser P, Sinkus R, Cedersund G, Dahlqvist Leinhard D, Ekstedt M, Kechagias S, and Lundberg P.
In manuscript

* Authors contributed equally

Other Related Publications Not Included in the Thesis

Peer Reviewed Full Length Articles

Gadoxetate DCE-MRI is a robust biomarker of liver function: multicenter preclinical imaging study in rats

Karageorgis A, Lenhard SC, Yerby B, **Forsgren MF**, Liachenko S, Johansson E, Piling MA, Peterson RA, Yang X, Williams DP, Ungersma SE, Morgan RE, Brouwer KLR, and Hockings PD

In manuscript

Positive Allosteric Modulator of GABA Lowers BOLD Responses in the Cingulate Cortex

Walter SA, **Forsgren MF**, Lundengård K, Simon R, Torkildsen Nilsson M, Söderfeldt B, Lundberg P, and Engström M

PLoS One 2016 Mar;11(3):e0148737

Comparing Hepatic 2D and 3D Magnetic Resonance Elastography Methods in a Clinical Setting – Initial Experiences

Forsgren MF, Norén B, Kihlberg J, Dahlqvist Leinhard O, Kechagias S, and Lundberg P

European Journal of Radiology Open 2015 April;2:66-70

Visual Assessment of Biliary Excretion of Gd-EOB-DTPA in Patients with Suspected Diffuse Liver Disease

Norén B, Dahlström N, **Forsgren MF**, Dahlqvist Leinhard O, Kechagias S, Almer S, Wirell S, Smedby Ö, and Lundberg P

European Journal of Radiology Open 2015 Jan;2:19-25

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Journal of Magnetic Resonance Imaging 2015 Aug;42(2):468-476

Decreased Muscle Concentrations of ATP and PCr in the Quadriceps Muscle of Fibromyalgia Patients – A ³¹P-MRS Study

Gerdle B, **Forsgren MF**, Bengtsson A, Dahlqvist Leinhard O, Sören B, Karlsson A, Brandejsky V, Lund E, and Lundberg P

European Journal of Pain 2013 Jan;17(8):1205-1215.

Peer Reviewed Conference Abstracts

Estimating Liver Function in Chronic Liver Disease Patients Using DCE-MRI and Whole-Body Pharmacokinetic Modeling

Karlsson M, **Forsgren MF**, Dahlqvist Leinhard O, Dahlström N, Norén B, Ekstedt M, Kechagias S, Cedersund G, and Lundberg P

ISMRM 25th Annual Meeting & Exhibition, Honolulu, USA 2017

A Multicenter in vivo Study to Evaluate Gadoxetate DCE-MRI as a Preclinical Biomarker of Liver Function

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ISMRM 25th Annual Meeting & Exhibition, Honolulu, USA 2017

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Nasr P, **Forsgren MF**, Ignatova S, Dahlqvist Leinhard O, Dahlström N, Ekstedt M, Lundberg P, and Kechagias S

EASL International Liver Congress (ILC), Barcelona, Spain 2016:S493

Kvantifiering av leversteatos: diagnostik utvärdering av protonmagnetresonansspektroskopi jämfört med histologiska metoder

Nasr P, **Forsgren MF**, Ignatova S, Dahlqvist Leinhard O, Dahlström N, Ekstedt M, Lundberg P, and Kechagias S

Gastrodagarna, Visby, Sweden 2016

A Robust, Non-Invasive, in vivo Technique Using DCE-MRI to Assess the Inhibition of Hepatic Transporters, Oatp1 and Mrp2, in Rats

Karageorgis A, Lenhard SC, Yerby B, **Forsgren MF**, Liachenko S, Johansson E, Peterson RA, Williams DP, Ungersma S, Morgan RE, Brouwer KLR, Jucker BM, and Hockings PD

46th Drug Metabolism Gordon Research Conference, Holderness, USA 2016

Prospective Evaluation of Liver Steatosis – Comparison of Stereological Point-Counting of Biopsies with ¹H MRS

Forsgren MF, Nasr P, Dahlström N, Dahlqvist Leinhard O, Smedby Ö, Ekstedt M, Kechagias S, and Lundberg P

ESMRMB 32nd Annual Scientific Meeting, Edinburgh, UK 2015;S25-S26

Hepatic MRE at Dual Field Strengths and Multiple Frequencies

Forsgren MF, Kinnunen N, Garteiser P, and Lundberg P

ESMRMB 32nd Annual Scientific Meeting, Edinburgh, UK 2015;S286-S287

Assessment of Hepatic Fibrosis: Mechanistic Mixed Effect Modelling of qDCE-MRI using Gd-EOB-DTPA

Karlsson M, **Forsgren MF**, Dahlström N, Norén B, Smedby Ö, Dahlqvist Leinhard O, Kechagias S, Lundberg P, and Cedersund G

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Forsgren MF, Ulloa JL, and Hockings PD

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Forsgren MF, Norén B, Kihlberg J, Dahlqvist Leinhard O, Kechagias S, and Lundberg P

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Röntgenveckan 2014, Karlstad, Sweden 2014

Whole Body Mechanistic Minimal Model for Gd-EOB-DTPA Contrast Agent Pharmacokinetics in Evaluation of Diffuse Liver Disease

Forsgren MF, Dahlström N, Karlsson M, Dahlqvist Leinhard O, Smedby Ö, Lundberg P, and Cedersund G

SAR 2014 Annual Meeting, Boca Raton, USA 2014

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Forsgren MF, Weber P, Janzén D, Dahlqvist Leinhard O, Lundberg P, Pena JM, Cedersund G

Ad hoc Constraints on Complex Liver DCE-MRI Models can Reduce Parameter Uncertainty

Forsgren MF, Dahlqvist Leinhard O, Cedersund G, and Lundberg P

ISMRM 20th Annual Meeting, Melbourne, Australia 2012;1977

³¹P MRS as a Potential Biomarker for Fibromyalgia

Forsgren MF, Bengtsson A, Dahlqvist Leinhard O, Sören B, Brandejsky V, Lund E, and Lundberg P

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Prospective Evaluation of Liver Steatosis Comparing Stereological Point-Counting Biopsy Analysis and ^1H MRS

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Norén B, **Forsgren MF**, Dahlqvist Leinhard O, Dahlström N, Kihlberg J, Romu T, Kechagias S, Almer S, Smedby Ö, and Lundberg P

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Andersson T, Romu T, Norén B, **Forsgren MF**, Smedby Ö, Kechagias S, Almer S, Lundberg P, Borga M, and Dahlqvist Leinhard O

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RSNA 98th Scientific Assembly and Annual Meeting, Chicago, USA 2012

The First Human Whole Body Pharmacokinetic Minimal Model for the Liver Specific Contrast Agent Gd-EOB-DTPA

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ISMRM 19th Annual Meeting, Montreal, Canada 2011;3016

Fat Water Classification of Symmetrically Sampled Two-Point Dixon Images Using Biased Partial Volume Effects

Romu T, Dahlqvist Leinhard O, **Forsgren MF**, Almer S, Dahlström N, Kechagias S, Nyström F, Smedby Ö, Lundberg P, and Borga M

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On the Evaluation of ^{31}P MRS Human Liver Protocols

Forsgren MF, Dahlqvist Leinhard O, Norén B, Kechagias S, Nyström FH, Smedby Ö, and Lundberg P

ISMRM-ESMRMB Joint Annual Meeting, Stockholm, Sweden 2010;2641

ABBREVIATIONS

^1H -MRS	Proton magnetic resonance spectroscopy
^{31}P -MRS	Phosphorous magnetic resonance spectroscopy
AAT	α 1-antitrypsin
AC	Anabolic charge
AIH	Autoimmune hepatitis
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
AMARES	Advanced methods for accurate, robust and efficient spectral fitting
ATP	Adenosine triphosphate
AUROC	Area under the receiver operator characteristics
ALD	Alcoholic liver disease
BMI	Body mass index
BW	Body weight
C	Concentration
CA	Contrast agent
DCE-MRI	Dynamic contrast-enhanced magnetic resonance imaging
DMMP	Dimethyl methyl phosphonate
F0	Fibrosis stage 0; no fibrosis
F1	Fibrosis stage 1; portal fibrosis
F2	Fibrosis stage 2; periportal fibrosis
F3	Fibrosis stage 3; septal fibrosis
F4	Fibrosis stage 4; probable or obvious cirrhosis
Gd-EOB-DTPA	Gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid; Gadoxetate; Primovist/Eovist
HCC	Hepatocellular carcinoma
EES	Extracellular extravascular space
FA	Flip angle
FID	Free induction decay
FOV	Field of view
HCV	Hepatitis C virus

IP	In-phase
IS	Intracellular space
jMRUI	Java-based magnetic resonance user interface
ICP-SFMS	Inductively coupled plasma-sector field mass spectrometry
ISIS	Image selected <i>in vivo</i> spectroscopy
LSC	Liver-to-spleen contrast ratio
LSC_10	Liver-to-spleen contrast ratio at 10 min post contrast injection
LSC_20	Liver-to-spleen contrast ratio at 20 min post contrast injection
LSC_N	Normalised liver-to-spleen contrast ratio
LSC_N10	Normalised liver-to-spleen contrast ratio at 10 min post contrast injection
LSC_N20	Normalised liver-to-spleen contrast ratio at 20 min post contrast injection
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NLME	Non-linear mixed effects
NTP	Nucleotide triphosphate
NTCP	Sodium-taurocholate co-transporting polypeptide
MP	Membrane protein
MeP	Methyl phosphate
MR	Magnetic resonance
MRE	Magnetic resonance elastography
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MRP2	Multidrug resistance-associated protein 2
MRP3	Multidrug resistance-associated protein 3
OATP	Organic anion transporting protein
OATP1B1	Organic anion transporting protein 1B1
OATP1B3	Organic anion transporting protein 1B3
ODE	Ordinary differential equations
OP	Opposing-phase
PBC	Primary biliary cholangitis
PCr	Phosphocreatine
PD	Pharmacodynamics
PDE	Phosphodiesterases

PDFF	Proton density fat-fraction
PK	Pharmacokinetics
PME	Phosphomonoesters
PopPK-PD	Population pharmacokinetics-pharmacodynamics
PRESS	Point resolved spectroscopy
PSC	Primary sclerosing cholangitis
QSP	Quantitative systems pharmacology
R_1	Longitudinal relaxation rate
R_2	Transversal relaxation rate
ROI	Region of interest
SI	Signal intensity
SE	Standard error of the mean
SNR	Signal-to-noise ratio
STEAM	Simulated echo acquisition mode
SPC	Stereological point-counting
T_1	Longitudinal relaxation time
T_2	Transversal relaxation time
T_E	Echo time
T_R	Relaxation time
TE	Transient elastography
VOI	Volume of interest

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1. INTRODUCTION

1.1 Liver Anatomy and Pathologies

The liver is one of the largest organs in the human body, and a typical liver weighs about 1.5 kg. The organ performs a wide range of crucial physiological functions such as nutrient processing, glucose homeostasis, detoxification, and protein assembly (1).

Figure 1. *A* Orientation image showing the approximate position of the liver in an adult. *B* The liver is positioned within the ribcage adjacent to organs such as the lungs, heart, and stomach. *C* The liver is divided into eight segments as shown by their roman numerals. Segment I is between segments VIII and IV. Segments II and III constitute the left liver lobe and the remaining segments constitute the right liver lobe.

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1.1.1 Anatomical Overview

The approximate position of the liver in an adult can be seen in Fig. 1A. More specifically, the liver is positioned adjacent to the lungs and is attached to the diaphragm within the ribcage (Fig. 1B). The liver has a dual blood supply, where about 20% of the blood is supplied via the hepatic artery and is highly oxygenated whilst the remaining 80% is low in oxygen and reaches the liver via the portal vein. The blood is mixed in the liver and leaves the organ in a single vein known as the inferior vena cava, which can be seen as the blue tube on top of the liver in Fig. 1C. The liver is also connected through bile ducts to the gallbladder which is positioned adjacent to the liver (barely visible as a green object beneath the liver in Fig. 1B-C). Anatomically the liver is divided into two lobes, the left and

the right lobe (Fig. 1C). These lobes are subdivided into eight unique segments which have independent vascular and biliary supplies. This segmental division is an important consideration when planning resective liver surgery (1).

The liver parenchyma is composed of a number of cells types which have unique functions and interact with each other. The main cell type is the hepatocytes, or the hepatic parenchymal cell. The hepatocytes account for about 60% of all cells in the organ, with a total of 80% of the volume in a healthy liver (1).

Figure 2 The primary functional unit in the liver parenchyma, the hepatic lobule. This hexagon-like structure is repeated throughout the entire parenchyma. Blood is provided by the portal vein and hepatic artery, and is then mixed in the sinusoid form in which the hepatocytes can interact with the constituents of the blood. The blood is thereafter drained through the central vein, and waste products and bile salts are eliminated through the bile ducts.

PV, portal vein; BD, bile duct; HA, hepatic artery; CV, central vein; PT, portal triad; HL, hepatic lobule.

The primary functional unit within the liver is the hepatic lobule, which is an in-plane hexagon-like structure (see Fig. 2). This structure is repeated throughout all segments of the liver parenchyma. At the edges of the hexagon, branches from the portal vein, hepatic artery, and the bile duct meet to form the 'portal triad' (see Fig. 2). From the portal triad, the blood (in the portal vein and hepatic artery) is mixed in blood vessels of a unique type, called the sinusoids (see Fig. 3). The sinusoids are fenestrated blood vessels, meaning that there are large gaps between the parenchymal cells that allow for rapid leakage into the space of Disse (the area between the sinusoid walls and hepatocytes). The blood in the sinusoids flows from the portal triad into the central vein (see Fig.3). As the blood flows, the hepatocytes are able to take up toxins, bile salts, and metabolites as well as excrete synthesised metabolites and proteins into the blood. The hepatic lobule is drained through branches of the central vein, which is positioned in the centre of the hepatic lobule (see Fig. 2 & 3). Waste and bile prod-

ucts, produced by the hepatocytes, are eliminated through the bile canaliculi, which drains into the bile ducts and ultimately reaches the gallbladder and intestine (1).

Figure 3 Detailed hepatic microvasculature. The inset shows how the microvasculature relates to the hepatic lobule. Blood enters the hepatic lobule from the portal vein and hepatic artery (two compartments of the portal triad), where it is mixed in the sinusoid. The sinusoid is a highly permeable blood vessel that allows leakage into the space of Disse, a process which allows the hepatocytes to interact with the constituents of the blood. The blood leaves the lobule and the liver via the central vein. The hepatocytes eliminate toxins, bile salts, and other waste products through the bile canaliculi. These waste products pour into the bile duct leading to the gallbladder and the intestine.

S, sinusoid; SD, space of Disse; BC, bile canaliculi; PV, portal vein; BD, bile duct; HA, hepatic artery; H, hepatocyte; CV, central vein.

1.1.2 Normal Functions

The hepatocytes produce and secrete bile. A significant part of the bile consists of bile acids which are produced from cholesterol. These acids aid the digestion of fat in the small intestine. Bilirubin, a toxic waste product from the degradation of red blood cells, is cleared from the blood by the hepatocytes the waste product is thereafter excreted into the bile. Enzymes in the hepatocytes metabolise drugs and toxins passing through the liver. On the cell membranes, specialized transporter proteins are present which actively take up these toxins from the passing blood. The hepatocytes also play a key role in the overall glucose

homeostasis in the body and are directly influenced by the pancreas in their effort to maintain a steady blood glucose level. The liver produces and secretes all major types of proteins circulating in the blood, with the exception of immunoglobulins (1).

1.1.3 Hepatic Transporting Proteins

The hepatocytes clear the blood stream of metabolites of toxic substances, bilirubin, bile salts, and other solutes which is thereafter excreted into the bile. This uptake and subsequent excretion from the cells is performed by a broad range of transporter proteins. In this thesis, the focus will be on a few select proteins, namely; sodium-taurocholate co-transporting polypeptide, NTCP; organic anion transporting proteins, OATP; multidrug resistance-associated protein 2, MRP2; multidrug resistance-associated protein 3, MRP3 (2).

Figure 4. Hepatobiliary transport systems in the liver. The arrows indicate the flow of metabolites (blue octagons) by the various transporting proteins (green ovals). The rounded rectangles indicate cotransport of ions for the passive transporters OATP1 and NTCP (both on the basolateral membrane). OATP1 (appears to) transfer one HCO_3^- ion out of the cell for each solute transferred into the cell. NTCP co-transfers two Na^+ ions for each solute molecule and has a high affinity for bile salts. The rectangles indicate the use of ATP to actively transfer a solute molecule against a steep gradient. MRP3 are positioned on the basolateral membrane of the hepatocyte, whereas MRP2 is on the canalicular membrane.

BS, bile salts; Na^+ , sodium ion; HCO_3^- , bicarbonate; OA, organic anions; NTCP, sodium-taurocholate co-transporting polypeptide; OATP1, organic anion transporting proteins; MRP2 & MRP3, multidrug resistance-associated protein 2 & 3; ATP, adenosine triphosphate; BC, bile canalculi; H, hepatocyte; SD, space of Disse; S, sinusoids.

1.1.3.1 NTCP

On the basolateral membrane of a hepatocyte NTCP can be found (meaning that NTCP is positioned on the side of the hepatocyte facing the sinusoids; see Fig. 4). NTCP is a primary carrier for sodium-dependent conjugate bile salt uptake from the portal blood into the hepatocytes. Bile salt uptake with NTCP is unidirectional and the transporter is an electrogenic sodium-solute cotransporter, i.e. the transfer of one solute molecule requires the cotransport of two Na^+ -ions.

The Na^+ transmembrane gradient is in turn maintained by Na^+/K^+ -ATPase, which is an active process requiring the oxidation of adenosine triphosphate (ATP) (2, 3). ATP is the primary energy carrier in the cells and most cellular functions need this energy source to for instance perform protein synthesis, genome transcription, and solutes transport.

Long-term regulators of NTCP transcription include substrates, cytokines, liver injury and hormones. There are many mechanisms that decreases NTCP transcription in pathological conditions, including inflammation, cholestatic liver disease, and toxins. Short-term regulation of NTCP activity is largely controlled by posttranscriptional mechanisms. The NTCP activity can be controlled within minutes by cyclic adenosine monophosphate which both increases the membrane retention of NTCP as well as the maximal transfer rate ($V_{\max}$)(3).

1.1.3.2 OATP

OATP, which can also be found on the basolateral membrane (see Fig. 4), is a family of multi-specific transporters responsible for the uptake of bile salts and a broad range of other organic anions and cations (2). Specifically the transporters OATP1B1 and OATP1B3 are of interest in this thesis. There is evidence that OATP1B1 and OATP1B3 function as bi-directional diffusion transporters, meaning that they may facilitate transfer both into as well as out of the hepatocytes (4). The passive transfer of solutes appears to be largely performed by exchange with intracellular anions HCO_3^- (bicarbonate) and/or GSH (glutathione), *i.e.* one anion is transfer out of the cell as one solute is taken up into the cell (2).

1.1.3.3 MRP2

On the canalicular membrane of the hepatocyte, the MRP2 transporter can be found (meaning that MRP2 is positioned on the side of the hepatocyte facing the bile canaliculi; see Fig. 4). It mediates the elimination of metabolites such as bilirubin and organic anions into the bile ductules through the use of ATP hydrolysis. The transport protein is a member of ATP-binding cassette (ABC) superfamily and capable of transporting solutes into the bile against steep concentration gradients, often in the range of 100 to 1000-fold (2). MRP2 is a major determinant of bile salt-independent bile flow (2, 5). It is the primary transporter in the biliary excretion of numerous drugs and their metabolites and is crucial for the understanding of hepatic drug elimination and toxicity (5, 6).

MRP2 expression and activity is regulated at multiple sites in the cell, mainly; (i) the endocytic retrieval and exocytic insertion can affect the number of proteins in the cell membrane within minutes, (ii) the translational regulation affects the number of proteins in the cell membrane within hours, (iii) the transcriptional regulation affects the protein expression within days, (iv) and the transfer rate or activity of individual proteins can be affected directly by modulating solutes (6). The control of MRP2 is a complex issue with multiple mechanisms, both endogenous and exogenous (5). In humans, MRP2 expression is highly individual in healthy subjects (7). In early cholestatic disease no change is

initially apparent but it has been shown that MRP2 expression levels are decreased in progressive disease (8). In a non-alcoholic fatty liver disease (NAFLD) rat model, MRP2 was found to be increased (9). In a review by Gerke and Vore the authors noted that the characterization of increased transcriptional expression of MRP2 is complex and that more studies are needed to understand the in vivo versus in vitro regulatory factors (6).

It is interesting that following a partial hepatectomy resulting in cholestasis (up 70% resection) with increase bile acid levels the regulation of MRP2 is maintained or even increased, whereas NTCP and OATP1 are down regulated (3).

1.1.3.4 MRP3

On the basolateral membrane of the hepatocyte MRP3 can also be found (see Fig. 4). This transporter protein transfers multiple organic solutes from the hepatocyte cytosol back into the sinusoid similar to MRP2 (2). It is weakly expressed in the normal liver but highly upregulated in hepatocytes in pathological conditions. MRP3 can for instance be upregulated in cholestatic liver injury (e.g. PBC and PSC) as well as chronic HCV infection. Moreover, intracellular bile acid overload due to loss of liver function can also lead to an increased MRP3 regulation (3).

1.1.4 Hepatic Manifestations of Chronic Liver Disease

As disease strikes and develops in the liver there are pathophysiological manifestations in the liver parenchyma that are common and important to monitor. These manifestations include inflammation, fatty infiltration (steatosis), fibrosis and cirrhosis, iron loading, and reduced liver function. In this thesis, the primary focus will be on steatosis, fibrosis and cirrhosis, as well as liver function.

1.1.4.1 Inflammation

Most liver injuries are associated with inflammation, which often drives the development of fibrosis (10) (see detailed discussion on fibrosis and specific disease below). The inflammation activity is typically graded as defined by the Batts-Ludwig system (11), on a 0 to 4 nominal scale.

1.1.4.2 Steatosis

Hepatic steatosis represents an excess accumulation of fat (triglycerides) in the hepatocytes, which is manifested as the accumulation of large (macrovesicular) or small (microvesicular) intracytoplasmic fat droplets in the hepatocytes. In macrovesicular steatosis, hepatocytes contain a single large vacuole of fat, which fills the cytoplasm and displaces the nucleus to the edge of the cytoplasm. In contrast, microvesicular steatosis includes numerous small lipid droplets which do not displace the nucleus. Traditionally a liver is deemed steatotic when the lipid content in the hepatocytes exceeds 5% by volume (12).

1.1.4.3 Fibrosis and Cirrhosis

Liver fibrosis is defined as the accumulation of extracellular matrix, or scar tissue, in the liver parenchyma. The accumulation is a response to acute or chronic liver injury. Ultimately fibrosis leads to cirrhosis, which can be considered the end-stage consequence of the fibrogenic processes (10, 13). The development of fibrosis and cirrhosis is commonly staged and classified according to the Batts-Ludwig system (see Table 1 and Fig. 5) or METAVIR (11, 14).

Table 1 Fibrosis staging terminology

Stage	Description	Criteria
F0	No fibrosis	Normal connective tissue
F1	Portal/mild fibrosis	Fibrous portal expansion
F2	Periportal/moderate/significant fibrosis	Periportal or rare portal-portal septa
F3	Septal/severe/advanced fibrosis	Fibrous septa with architectural distortion; no obvious cirrhosis
F4	Cirrhosis	Cirrhosis

Figure 5 Fibrosis staging. In portal fibrosis (F1; Panel A), the fibrous tissue is contained within the portal triad (represented by the grey oval). Panel B shows Periportal fibrosis (F2), where the fibrous tissue expands into the parenchyma as shown by the black lines. In panel C septal fibrosis (F3) is shown in which there is an increased spread of fibrosis as well as connections (septa) between the portal triads as well as central veins. In cirrhosis (F4; panel D) the fibrous tissue forms nodules where the parenchyma is completely surrounded by extensive fibrous tissue. Adopted from (11).

Liver injury is often followed by an inflammatory process, during which stellate cells become activated. Stellate cells are mobile star-shaped cells found in the space of Disse. The activated stellate cells promote the formation of scar tissue, and also have an autocrine effect in which they promote their own continued activation. As the disease progresses there is a great accumulation of extracellular matrix in the space of Disse, the hepatocytes lose their microvilli, and the sinusoids lose their fenestrae. All these effects leads to continued stellate activation, impaired hepatic function, and altered sinusoidal blood flow (10).

At all stages of the fibrogenesis there is an increased stress on the liver parenchyma due to the constant activation of the immune system, cytokines, and growth factors in the healing process. Ultimately this constant stimulation, including hepatocellular regeneration, could predispose the development of hepatocellular carcinoma (HCC). Also, as the fibrogenesis develops the excessive amount of scar tissue (see Fig 5) makes the liver parenchyma harder than a healthy individual's liver tissue (10, 13).

1.1.4.4 Iron Loading

In genetic diseases, such as hemochromatosis, there is an excessive amount of iron stored in the hepatic cells, which in turn leads to tissue damage and organ failure (15, 16). Iron overload is also a possible complication in other forms of advanced chronic liver disease (16).

1.1.4.5 Hepatic Function

At the end-stage of most liver diseases there is a significant loss of liver function. This function loss can be coupled to the reduced number of functional hepatocytes per unit volume, steric hindrance in the parenchyma, and reduced capacity of the hepatocytes. Knowledge about the hepatic functional reserve is of importance for planning treatment therapies and predicting post-surgical survival (17, 18).

As the liver performs a multitude of functions, the term 'liver function' is complex (19) and a detailed description of all possible interpretations is beyond the scope of this thesis. In this thesis the term 'liver function' will be primarily associated with the functions of the hepatic transporters OATP1 and MRP2. They will be viewed as surrogates of the overall liver function as they perform crucial tasks in for instance the detoxification processes.

1.1.5 Chronic Liver Disease

Chronic liver disease is one of the leading causes of public health burden in the Western world (20), and globally the burden has increased by 10.3% in the recent years (21). Alcoholic liver disease (ALD), hepatitis C virus (HCV), and NAFLD are thought to be the leading causes of chronic liver disease in the EU and the US (22). The overall prevalence of chronic liver disease in the US has risen from 12% in 1988 to 15% in 2008 (23). Other chronic liver diseases that will be touched upon in this thesis are primary sclerosing cholangitis (PSC), primary

biliary cirrhosis (PBC), autoimmune hepatitis (AIH), α_1 -antitrypsin deficiency (AAT deficiency), and drug-induced liver injury (DILI). All of which are briefly described in the following paragraphs.

1.1.5.1 Alcoholic Liver Disease (ALD)

In ALD the chronic alcohol abuse induces a wide range of morphological changes in the liver, primarily steatosis, fibrosis and cirrhosis, as well as inflammation. Hepatic steatosis is the earliest histopathological sign of hepatic manifestation and the continued heavy use of alcohol leads to inflammation and fibrosis progression to cirrhosis. In the US around half of the total numbers of deaths from chronic liver diseases are due to ALD. Advanced fibrosis is by itself a precursor to HCC, but evidence has also shown that alcohol itself can be a co-carcinogen (24).

1.1.5.2 Hepatitis C Virus (HCV) Infection

HCV was first reported in 1989. It is estimated that within 20 years of infection, about 20-30% of all patients develop cirrhosis. HCV is one of the leading causes of death from cirrhosis and HCC, and it is the most common indication for liver transplantation in the Western world. About 20-50% of all patients spontaneously recover from disease within the first six months; the remainder develop a chronic infection. The sustained inflammation leads to the development of fibrosis, which ultimately leads to cirrhosis, which in turn might induce HCC. In recent years, novel directing antiviral therapies have become available and vastly improving the outcome for chronically infected patients and curing >90% (depending on subtype) (25, 26).

1.1.5.3 Non-Alcoholic Fatty Liver Disease (NAFLD)

The obesity epidemic is an important factor in the rising prevalence of NAFLD. NAFLD represents the hepatic manifestation of the metabolic syndrome, the syndrome includes obesity, diabetes, hypertension, hyperglyceridaemia, and low high-density lipoprotein cholesterol (27). Globally, NAFLD has an estimated prevalence of 25% (28). The histopathological findings are similar to those of ALD, although they occur in patients without excessive alcohol abuse. The diagnosis is defined as the presence of fatty infiltration in the liver, and at later disease stages includes inflammation, fibrosis, cirrhosis and subsequently HCC. NAFLD encompasses a spectrum of histopathological conditions from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH is considered the more aggressive part of the NALFD spectrum, NASH includes steatosis, lobular inflammation and ballooning with or without fibrosis (29, 30).

1.1.5.4 Primary sclerosing Cholangitis (PSC)

PSC is a chronic inflammatory disease that is characterized by narrowing of the bile ducts. PSC is a progressive disease that ultimately leads to cirrhosis, and typically a liver transplant is required within 10 to 15 years after onset. In the

last few decades PSC has risen to become one of the leading indications for liver transplant in northern Europe and the US (31).

1.1.5.5 Primary Biliary Cirrhosis (PBC)

PBC is a fairly uncommon disease worldwide, with the highest prevalence in northern Europe and the US, and has been suggested to be related to certain environmental factors. PBC is an organ-specific autoimmune disease that occurs in genetically predisposed individuals. The autoimmune response primarily targets the biliary epithelial cells. The hepatic manifestations start with an inflammatory response in the bile ducts, which then spreads into the liver parenchyma, followed by fibrosis - spanning from the portal triads to the central vein. The final stage is cirrhosis with regenerative nodules, which can ultimately lead to liver failure (32).

1.1.5.6 Autoimmune Hepatitis (AIH)

AIH is a chronic inflammatory disease, in which the autoimmune response targets the hepatic tissue. AIH predominantly affects women of any age. The disease includes hepatic inflammation, and with advancing disease, bridging, panlobular, and multilobular necrosis, which leads to fibrosis and ultimately cirrhosis. AIH requires aggressive immunosuppressive treatment as untreated survival rates have been reported to be as low as 50% after five years and 10% after ten years. The concurrent development of HCC in AIH is rare and only occurs with long-standing cirrhosis (33).

1.1.5.7 Drug-Induced Liver Injury (DILI)

DILI is a result of a compounds toxic effect on the liver, and is a great concern for the pharmaceutical regulatory agencies and industries. It is also a leading cause of acute liver failure (34). As many as 28% of all drugs withdrawn from the marketplace after introduction between 1998 and 2008 were withdrawn due to liver toxicity (35). It is difficult to diagnose DILI as there are numerous potential causes of liver injury and it depends on a combination of the specific drug, the status of the patients, and the environment (*i.e.* factors such as other drugs and diet). The most common hepatic manifestation includes necrosis and apoptosis. Other manifestations include steatosis and inflammation. As there are different effects by different drugs, and the length of exposure varies, these manifestations also differ depending on the drug inducing the injury. Severe symptoms are often similar to those of acute viral hepatitis, although the majority of DILI-inducing drugs produce mild and asymptomatic hepatocellular injury (36).

1.1.5.8 α 1-Antitrypsin (AAT) Deficiency

AAT deficiency is an autosomal co-dominant disorder that leads to a protein being retained in the hepatic cell rather than being excreted into the blood stream. In a Swedish study 127 out of more than 200 000 new-borns were ho-

mozygotes, of which only nine developed significant liver disease. Hepatic manifestations include, inflammation, necrosis and carcinoma (37).

1.2 Liver Disease Diagnostics

There are a great many potential diagnostic procedures for different aspects of hepatic manifestations of chronic liver disease and in this section it is not my intention to provide a full description of all rather. Instead, I will focus on non-magnetic resonance (MR) procedures that are relevant in respect to the published articles.

1.2.1 Liver Biopsy

The current gold standard for diagnosing liver disease is the histologic evaluation of a liver biopsy. With the liver biopsy it is possible for a pathologist to qualitatively determine disease manifestations such as fibrosis, steatosis, inflammation, and iron loading (11, 14, 38, 39). By using special histochemical and immunohistochemically stains a variety of patterns can be investigated that aid in the diagnostic work-up (39). A typical liver biopsy is performed by puncturing the liver with a needle, which excises a small specimen of the liver parenchyma (about 1/50 000 of the liver). Often this is performed in combination with an abdominal ultrasound examination in order to maximise patient safety and specimen quality. The needle is typically inserted into the right liver lobe intercostally (38).

There are important limitations with the biopsy, which include; risk of serious complications, mortality, morbidity, inter- as well as intra-observer variability, sampling error, and sampling variability. Studies have also shown that the histopathological staging of fibrosis has a diagnostic accuracy as measured by the area under the receiver-operator characteristics curve (AUROC) of 0.82 (13, 40-45).

The recent advent of digital pathology and the use of computer software can assist with, automate, and perform quantitative assessment of these hepatic manifestations and possibly improve the quality and efficacy of histological evaluation (46).

1.2.2 Blood Panels

Serum markers or blood panels offer an alternative to the liver biopsy, as well as the ability to measure additional metabolites. There are five commonly used biochemical assays or biomarkers that are performed to screen people for the presence of liver disease. These biomarkers are; alanine aminotransferase, ALT; alkaline phosphatase, ALP; aspartate aminotransferase, AST; γ -glutamyl transferase, GGT; total bilirubin; prothrombin time, PK-INR (47). These biochemical biomarkers can be used to measure indirectly manifestations of chronic liver disease on the liver parenchyma. Multiple biomarkers are often combined to form scoring panels or algorithms that can be used to identify hepatic manifes-

tations such as potential loss of hepatic function, the fibrosis stage, and inflammation grade (13).

The benefit of serum biomarkers is that they are cost-effective, less invasive than a biopsy, and have practically no risk of complications, few or no sampling errors, and minute observer variability. The primary limitations are that they are indirect, depend on clearance rate (*i.e.* biliary and renal functions), and can be altered due to other extrahepatic pathologies (13).

1.2.3 Transient Elastography

Transient elastography (TE) is an ultrasound-based modality that performs elastographic measurement in the liver parenchyma. It allows for a 1D pencil beam assessment of the fibrotic load in close proximity to the skin surface, typically between 25 to 65 mm from the surface. Since a fibrotic liver is stiffer than a healthy liver the elastographic measurements from TE are used to stage the fibrotic load. TE is relatively inexpensive, can be used in the clinic, and is completely non-invasive. Important downsides include the high operator dependency, small region investigated (although significantly larger than a biopsy), susceptibility to fatty infiltration, and low technical success rates in obese populations (13, 48).

1.3 Magnetic Resonance

Magnetic resonance imaging (MRI) is the application of nuclear magnetic resonance (NMR) in order to produce images, and it is a relatively new imaging modality. The seminal papers by Lauterbur and Mansfield in 1973 marked the beginning of MRI, and the first whole-body MRI scanner was commissioned in the early 1980s. Today MRI is a common imaging modality in both clinical and research settings.

1.3.1 A Brief Description of (Nuclear) Magnetic Resonance

NMR is based on the fact that a nucleus with the quantum mechanical property of a half-integer angular momentum (or spin) precesses about an external magnetic field (B_0) at the Larmor frequency. Each nucleus with this property also has a magnetic moment. Metabolically interesting nuclei with this property are for instance proton (^1H), phosphor (^{31}P), carbon (^{13}C), and sodium ^{23}Na .

From an macroscopic point of view, as a body is placed in an external magnetic field all nuclei with half-integer spin (for simplicity I will refer to them as protons in this paragraph) will start to precess and slowly align along the direction of B_0 . As they align to B_0 , they can either align parallel (low-energy state) or anti-parallel (high-energy state), and as they do so their individual magnetization vectors will point in the same direction. If all protons align in perfect symmetry there will be no macroscopic net magnetization vector to detect. Luckily there will be a slight difference in the populations aligning parallel or anti-parallel which is proportional to the strength of B_0 . As an example, in a B_0 of 9.4

T and 310 K there would be abundance in the low-energy state of 0.0031%. As there are roughly 50×10^{24} protons per litre in a human there will be c. 1.6×10^{21} extra magnetization vectors pointing in the same direction as B_0 and therefore this would yield a net macroscopic magnetization vector (49, 50).

A static magnetization vector does not suffice to produce an interesting signal; it has to be tipped away from the external fields' direction (the z-direction in an x, y, z frame of reference) in order to initiate precessions which can be detected. An radiofrequency (RF) sent from a nearby transmit coil pulse at the Larmor frequency will flip the net magnetization vector away from B_0 into the x,y-plane. If the vector is fully flipped towards the x,y-plane, with respect to the z-axis, it is said that there is a flip angle (FA) of 90° (different angles are used in MR). As the magnetization vector precesses about B_0 , and returns to its equilibrium state there will be a detectable magnetization vector. This vector induces an electric current in nearby receive coils which is the detectable NMR signal. As the vector precesses about B_0 , the NMR signal oscillates since, during the precession, the net vector rotates around the z-axis and as it does so it will alternate in pointing towards and away from the receive coil. Only magnetization vector components in the x-y plane yield a signal; the magnetization in the z-plane yields no signal as it is then in plane with the receive coil and does not alternate. As the protons return to the equilibrium state the magnetization is fully aligned with B_0 (or the z-axis).

The time that it takes for the net magnetization vector to return to the equilibrium state is known as the 'spin-lattice' or 'longitudinal magnetic' relaxation time (T_1). The precessing nuclei can also fall out of alignment with the rest of the population, and their precessional frequencies are slightly altered as individual spins experience minute local differences in the external field due to interactions with their neighbours. Eventually this leads to the individual vectors being out of phase and the net magnetization vector in the x,y-plane is reduced. This dephasing is known as 'spin-spin' or 'transverse magnetic' relaxation (T_2). The combination of T_1 , T_2 , and the spin density (*i.e.* the concentration of the protons) are the principle factor controlling the NMR signal (49, 50).

If all nuclei of the same species resonated at the same frequency NMR would be of limited value. Fortunately the resonance frequency does not only depend on the external magnetic field and the gyromagnetic ratio, it is also highly sensitive to the local chemical environment of the nucleus. This local shift in frequency is known as chemical shift, and is caused by shielding of the nuclei from the external magnetic field by the electrons surrounding them. The distribution of electrons, and thus the shielding effect, differs depending on the molecule, which means that for instance T_1 varies depending on what the proton is attached to (*i.e.* water, fat) and where in the fat molecule the proton resides (49, 50).

1.3.2 Magnetic Resonance Imaging

As previously mentioned the precessional frequency of a nucleus is dependent on the external magnetic field, meaning that small changes in the applied field yield differences in the frequency of the signal. The essential concept of MRI is that the resonance frequency is made position-dependent by altering the magnetic field inside the bore of the magnet. Commonly, magnetic field gradients vary the amplitude of the magnetic field linearly with the position inside the bore. Typically three magnetic field gradients are found within the bore, X, Y, and Z. These are produced by so-called gradient coils. These gradient coils are positioned around the centre of the magnetic field; therefore, the field strengths of all gradients are zero at the magnet's isocenter. Images are computed by using Fourier transforms of the complex signals which are encoded with spatial information through manipulation of the magnetic field gradients and the RF pulses. Typical gradients are capable of producing gradients around 20-100 mT/m, so in a 1.5 T magnet this could mean the magnetic field is about 1.45 T at one end and 1.55 T at the other (49, 50).

As protons attached to different molecules have different chemical shifts and thus relaxation times (T_1 and T_2) one can modulate the signals to gain contrast in the final images, for instance water has T_1 of 663 ms at 3.0 T and 'fat' 236 ms (51). The relaxation time (T_R) and echo time (T_E) are two such modulating parameters that are used in MRI. These parameters control how long after the magnetization vector is flipped the image is acquired (T_E) and how long after each flip the next flip is performed (T_R); multiple flips are performed as the entire object is imaged.

1.3.2.1 Fat-Water Imaging

It is often of interest to only measure the water and/or fat signal separately. As previously mentioned protons in water and fat have different chemical shifts which can be exploited. Either the fat signal can be suppressed such that only water is present in the images, or chemical shift imaging can be performed. The principle of chemical shift imaging is that as the resonance frequency of the signals of the main fat peak and water peak will oscillate around B_0 with different periods. At 1.5 T this occurs at a period of 4.6 ms, meaning that at multiples of 4.6 ms the signal will be the sum of the main fat peak and water peak (in-phase (IP); see Fig. 6A) and at multiples of 2.3 ms the signal will be the difference of the main fat peak and water peak (opposing-phase (OP); see Fig. 6B). At 3 T this period is halved (52). By acquiring images at these time points (at least one IP and one OP image is required) it is possible to calculate separate water (see Fig. 6C) and fat (see Fig. 6D) images based on multi-echo or so-called Dixon images.

Figure 6 Example of a chemical shift or Dixon MRI-experiment. These transverse images are all in the same anatomical position, outside the torso sections of the subjects' arms are visible. In the abdomen the liver (L) and spleen (S) can be seen, surrounded by the musculature (in-between the liver and spleen the spine can also be seen; see panel C). Panel A shows the in-phase (IP) image where both water and the main fat peak are in phase and add to the image intensity. Panel B shows the opposing-phase (OP) image where the water and main fat peak are in OP and do both peaks do not add to the image intensity. Based on the IP and OP images it is possible to compute the pure water (panel C) and pure fat (panel D) images. In the fat image the subcutaneous fat is apparent and some visceral fat deposits can also be seen.

2-point Dixon MR images courtesy of Thobias Romu (AMRA AB).

1.3.2.2 Contrast Enhancement Agents

Tissues with short T_1 have a stronger signal in T_1 -weighted images than tissues with long T_1 . As an example, the T_1 of the main fat peak is about 35% of the water peak, thus fat has a stronger signal. The T_1 relaxation of a nucleus can be modulated, for instance by adding a paramagnetic atom to its local environment.

Paramagnetic atoms have an unpaired electron which leads to a permanent magnetic moment and a non-zero magnetic field. These magnetic moments are randomly distributed, and thus macroscopically there is a zero magnetic moment in the absence of a strong external magnetic field. Inside the bore of an MR scanner they align with the external magnetic field and produce a bulk magnetic moment that augments the T_1 relaxation of nearby nuclei. A commonly used paramagnetic atom in MR is gadolinium (Gd) (50).

Free gadolinium atoms are highly toxic, so in order to use them *in vivo* they are bound, or chelated, to an organic molecule so that the atom cannot bind to other molecules. These gadolinium-chelates are referred to as 'contrast agents'. Most contrast agents are injected intravenously into the blood stream, and stay within the blood pool until they are eliminated by the kidneys. As the contrast agents reside within the blood pool any tissue containing substantial amounts of blood, such as the heart and certain tumours, will have a stronger signal than the rest.

The increase in relaxation rate is in general directly proportional to the concentration of the contrast agent in the tissue:

$$R_1(t) = R_1(0) + r_1 C(t) \quad (\text{Eq. 1})$$

where $R_1(t)$ is the relaxation rate ($=1/T_1$) at a point in time after the contrast agent injection, $R_1(0)$ is the relaxation rate prior to the contrast injection, $C(t)$ is the concentration of the contrast agent at the tissue, and r_1 is the relaxivity of the contrast agent. Relaxivity depends on the structure of the contrast agent as well as the micro environment (50, 53).

1.3.2.3 Hepatobiliary Contrast Agents

As previously alluded to, most contrast agents primarily reside within the blood pool; whether the agent only resides in the vascular space or in for instance also in particular cells depends on the structure of the chelate. There are two chelates that are approved for clinical use that are also able to enter cells, namely gadoxetate (gadoteric acid; Gd-EOB-DTPA; Primovist®/Eovist®, Bayer HealthCare; see Fig. 7A) and gadobenate (gadobenic acid; Gd-BOPTA; Multi-Hance®, Bracco Imaging; see Fig. 7B). In addition to being cleared through the kidneys these two are accumulated and retained within the hepatocytes and thus also cleared through the bile. In a healthy human, c. 50% of a gadoxetate dose is eliminated through the bile whereas only c. 2-4% of a gadobenate dose follows the same path (54, 55). In humans with mild-to-moderate hepatic or renal impairment a compensatory effect occurs in the elimination of gadoxetate, where the non-impaired pathway increases the elimination of gadoxetate in relation to the impaired pathway (56).

This thesis focuses on gadoxetate and in the following paragraphs only the characteristics of this contrast agent will be discussed.

Figure 7 Structure of the hepatocyte-specific contrast agents (A) gadoxetate and (B) gadobenate. Note that there is one Gd^{3+} ion per chelate.

Image courtesy of: A) By LHcheM (Own work), CC BY-SA 3.0,

https://commons.wikimedia.org/wiki/File%3AStructure_of_Gadobenic_acid.png B) By Edgar181 (Own work), Public Domain, https://commons.wikimedia.org/wiki/File%3AGadoxetic_acid.png

1.3.2.4 The Bio-Distribution of Gadoxetate

As previously mentioned, the contrast agent gadoxetate is partially eliminated through the kidneys in a healthy human, accounting for c. 50% of the total dose. In the kidneys the major pathway for this renal elimination is glomerular filtra-

tion (54). The remaining dose of gadoxetate is accumulated in the hepatocytes. The hepatocytic uptake of gadoxetate (see Fig. 8) is performed by OATP1B1 and OATP1B3, gadoxetate is also a substrate for NTCP but this affinity is negligible compared to the affinity of OATP1B1 and OATP1B3 (57). The subsequent elimination from the hepatocytes into the bile canaliculi is performed by the MRP2 transporter (58). It is also possible that gadoxetate can be transported back into the blood stream in certain disease states, either by the OATPs' bi-directionality (4) or MRP3 up-regulation on the sinusoidal membrane (2). Also, there could be leakage in the tight junctions of the hepatocytes, thus allowing for leakage back from the bile canaliculi into the space of Disse (59).

Figure 8 Possible routes for the hepatobiliary transport of gadoxetate in the liver. Gadoxetate enters the sinusoids of the hepatic lobule and leaks through the fenestrae into the space of Disse. The arrows show the transfer of gadoxetate ('Gd'; blue octagons) by the various transporting proteins (green ovals). The rounded rectangles indicate cotransport of ions for the passive transporters OATP1 and NTCP (both on the basolateral membrane). OATP1 (appears to) transfer one HCO₃⁺ ion out of the cell for each solute transferred into the cell. NTCP co-transfers two Na⁺ ions for each solute molecule. OATP1B1 and OATP1B3 ('OATP1') are the primary transporters of gadoxetate into the hepatocytes, and can also facilitate efflux. NTCP has a very weak affinity for gadoxetate but is able to transfer minute levels of gadoxetate into the cell. The rectangles indicate the use of ATP to actively transfer a solute molecule against a steep gradient. MRP3 are positioned on the basolateral membrane of the hepatocyte, whereas MRP2 is on the canalicular membrane. MRP2 eliminates gadoxetate from the hepatocyte into the bile canaliculi, and the closely related transporter MRP3 can excrete gadoxetate back into the space of Disse in certain diseases where it is upregulated.

Gd, gadoxetate; NA, sodium; NTCP, sodium-taurocholate co-transporting polypeptide; OATP1, organic anion transporting proteins; MRP2 & MRP3, multidrug resistance-associated protein 2 & 3; BC, bile canaliculi; H, hepatocyte; SD, space of Disse; S, sinusoids.

A typical MR examination with gadoxetate is shown in Fig. 9 and starts with a bolus injection of gadoxetate in a vein. A bolus injection means that there is a rapid injection, lasting just a few seconds. This leads to a 'plug' of contrast agent that travels to the heart. As this plug travels through the blood it will disperse and after a few minutes there will be a homogenous spread of contrast throughout the entire blood pool. This also means that in the first 30-60 seconds there

will be an initial wash-in of gadoxetate in the liver as the arterial blood from the heart passes by. After some time the second wash-in occurs as the returning venous blood passes the liver on the way back to the heart and lungs (see Fig. 9B-C).

Figure 9 Enhancement of MR images following the injection of gadoxetate. The transverse MR images are reconstructed water-only images from a 2-point Dixon MR acquisition. Prior to the injection of contrast agent (panel A) all tissues have equal intensity. During the first wash-in (panel B) the aorta (A) is filled with contrast, also the highly vascularized spleen (S) has a stronger intensity. In the second wash-in phase (panel C) the aorta is still bright as well as most major vessels in the liver parenchyma (L). In three to five min post-injection (panels D & E) the liver, spleen and vessels are fairly similar in intensity. As the hepatocytes accumulate gadoxetate and the kidneys eliminate gadoxetate through the urine, the blood pool is diminished. This effect is apparent as time advances from 10 to 30 min post-injection (panels F-H). At 30 min after the injection the liver parenchyma is of significantly higher intensity than the remaining tissues.

In humans the hepatocyte retains gadoxetate for a prolonged time; the accumulation peaks at about 30-60 min after contrast injection (53, 56), see Fig. 9H. This prolonged retention points to the fact that the rate-limiting step in the extra-renal elimination pathway is the function of MRP2. This is in striking contrast to rats where it just takes a few minutes (60). This difference could possibly be due to the protein concentrations of MRP2 where in a human liver there is 0.4 pmol/mg protein compared to 2.1 pmol/mg protein in the rat liver (61).

1.3.2.5 Quantification of the Gadoxetate Concentrations in situ

As previously alluded to, the increased relaxation rate in contrast-enhanced MR images is directly proportional to the contrast agent concentrations. It is thus possible to calculate time series of the *in situ* concentrations in various tissues.

The procedure can be summarized in the following steps: First the signal intensity (SI) is extracted from the images. This extraction can for instance be

performed by drawing regions of interest (ROI) in the images to extract data from selected portions of for instance the liver and spleen. Second, each individual ROIs SI is divided with the corresponding unenhanced SI (SI(o)). This removes constant background effects from differences in image sensitivity, proton density, transverse relaxation, and fatty infiltration (53):

$$S(t) = \frac{SI(t)}{SI(o)} \quad (\text{Eq. 2})$$

Third, the relaxation rate for each ROI and time point is calculated using:

$$\left\{ \begin{aligned} R1(t) &= -\frac{1}{T_R} \left(\ln \left[S(t) \frac{1-E(0)}{1-\cos(\alpha)E(0)} - 1 \right] - \ln \left[\cos(\alpha) S(t) \frac{1-E(0)}{1-\cos(\alpha)E(0)} - 1 \right] \right) \\ E(t) &= e^{-\frac{T_R}{T_1(t)}} \end{aligned} \right. \quad (\text{Eq. 3})$$

where α is the FA (53). In Eq. 4 it is also required to know the intrinsic unenhanced T_1 values for the tissues, either by measuring them or by using literature values. After these calculations it is possible to use Eq. 1 to calculate the *in situ* concentrations of gadoxetate.

1.3.3 Magnetic Resonance Spectroscopy

In MRI field gradients and RF pulses are used to manipulate the precessional frequencies of nuclei in order to gain spatial information, whereby all nuclei at a specific position add to the total intensity (regardless of the compound). In magnetic resonance spectroscopy (MRS) the aim is to identify and quantify the concentrations of different compounds in the sample. In a successful identification of the compounds, the precessional frequencies, or chemical shifts, are crucial as they give direct information about the chemical environment of a nucleus.

The chemical shifts depend on the applied magnetic field; this means that they have different precessional frequencies depending on the applied magnetic field. When chemical shifts, or the precessional frequencies, are presented in Hertz one must take into account the external magnetic field strength. In order to express these chemical shifts without also mentioning the external magnetic field strength, they are expressed in terms of ppm (δ):

$$\delta = \frac{\nu - \nu_{ref}}{\nu_{ref}} \times 10^6 \quad (\text{Eq. 4})$$

where ν and ν_{ref} are the precessional frequencies of the compound of interest and a reference compound. In ^3P -MRS, phosphocreatine (PCr) is often assigned as this reference and set to 0 (49).

The acquired signal in the receive coils following an RF pulse which flips the magnetization vector is called the free induction decay (FID). The FID is the time-dependent signal which is transformed into the frequency domain using a Fourier transform, as it is in the frequency domain that compounds can be iden-

tified (see Fig. 10). Typically, multiple FIDs are acquired and averaged in order to enhance the signal-to-noise ratio (SNR).

Figure 10 Example of 3 T ^{31}P -MRS pulse acquire experiment in a human quadriceps muscle. The signal is the average of 16 fully relaxed acquisitions ($T_R = 15$ s). Panel **A** shows the time dependent signal, or the free induction decay (FID). As can be seen, the FID oscillates around the zero crossing as the magnetization vector precesses about the external magnetic field. The spectra in panel **B** is the Fourier transform (FT) of the FID. The individual resonances can now be assigned based on prior knowledge: Pi, organic phosphate; PDE, phosphodiester; PCr, phosphocreatine; α, β, γ -NTP, α, β, γ -nucleoside triphosphate (mainly ATP).

In principle the area under the resonances is directly proportional to the concentration of the compounds; however, as there is no reference scaling it is not possible to directly scale the integrals. A solution can be to use an internal reference standard, such as water, which can be assumed to be constant, and scale the integrals with the known concentration. It is also possible to calculate ratios of various compounds, or use an external reference standard whereby a known mixture of compounds is placed next to the receive coil and included in the spectral acquisitions (see Fig. 11).

Figure 11 Principle for using an external reference for absolute quantification of resonances. Panel A shows the fully relaxed 3 T ^{31}P -MRS pulse acquire spectra (16 averages; $T_R = 15$ s; $T_E = 0.15$ ms) with resonances from a human quadriceps muscle as well as two external references. In this experiment a large cylinder filled with 85 mM DMMP is attached to the coil, and the spectra is acquired both in the cylinder and in the muscle simultaneously. The circular transmit/receive ^{31}P coil is shown in panel B. Inside the coil is also a small capsule of MeP. By using this setup it is possible to measure the concentrations simultaneously in vivo and in the external reference and thus scale the compounds concentrations.

DMMP, di-methyl methyl phosphonate; MeP, methyl phosphonate; Pi, organic phosphate; PDE, phosphodiester; PME, phosphomonoester; PCr, phosphocreatine; α, β, γ -NTP, α, β, γ -nucleoside triphosphate (mainly ATP).

When analysing MR spectra there are additional features that aid in the identification and spectral assignments. In high-resolution MRS one can observe splitting of resonances into several smaller resonances. This phenomenon is known as ‘scalar coupling’, ‘J-coupling’, or ‘spin-spin coupling’. J-coupling originates from the fact that nuclei with magnetic moment can influence each other through electrons in the chemical bounds. The splitting of resonances yields unique fingerprints of the compound as the splits are determined by the local environment (49). *In vivo* these J-couplings are less apparent as the resonances are broadened due to effects such as field inhomogeneities, coil sensitivities, and imperfections in the excitation pulses. As an example, protons in water do not experience any J-coupling and the resonance is thus a single peak (see Fig. 12), whereas the three phosphor nuclei in the complex compound adenosine triphosphate (ATP) split into a range of different unique resonances (see for instance the triplet β -NTP in Fig. 13B). Thus the combinations of the chemical shifts as well as any splitting of the resonances due to J-couplings are the fingerprints that identify specific compounds in MR spectra.

1.3.3.1 Volume Selection in MRS

As the reader might have noted in the figure headings for Fig. 10 and Fig. 11 there is a note about a ‘pulse acquire’ experiment. This means that the spectra have been acquired without volume selection. A pulse acquire experiment is performed with a single excitation pulse and the receive coil thereafter acquires

all signals within its sensitivity region. The coil used in the examples (Fig. 11B) has a sensitivity that can be described as a semi-sphere originating from the coil and outwards, the sphere's radius is about the square root of the diameter of the coil. Outside this sensitivity sphere hardly any resonances add to the spectra. This is all fine when looking at large muscles as there is no significant contamination, but it can be a substantial problem when, for instance, the liver or the brain are analysed as they are surrounded by tissue that distorts the signals (and the compounds of interest are in low concentration compared to the potential contaminants). Currently a wide range of methods to select volumes of interest (VOI) for a localised spectroscopic acquisition are available. Just like in MRI, there is a combination of magnetic field gradients and RF pulses that modify the magnetization vectors such that only the signals in the desired VOI are acquired. However, there is the issue of not distorting the frequencies as the chemical shifts are crucial in spectral assignments. So instead of modifying the precessional frequencies, they typically flip the vectors such that unwanted regions give a negative signal or no signal.

Image selected *in vivo* spectroscopy (ISIS) is a method that is popular in ^{31}P -MRS, and is also used in this thesis for acquiring hepatic ^{31}P -MR spectra. It typically requires a total of eight scans to acquire a single spectrum with 3D localisation. The spectrum is achieved by using a combination of 90° and 180° excitation pulses and field gradients that produce eight unique spectra around the VOI. The eight acquisitions all are combined (by subtraction and addition) to form a final single spectrum, which only contains resonances from the wanted VOI (49). In ^{31}P -MRS a typical T_R might be 5 or 7 s, which means that a single spectrum takes between 40 and 56 s to acquire and a few averages are typically desirable, so ISIS acquisition takes a significant time. It is also possible to select two VOIs in a double-ISIS experiment. The double-ISIS experiment is suitable when for instance it is desirable to measure simultaneously in an external reference (Fig. 11B) and *in vivo* (62). The double-ISIS takes twice the time to acquire as it still requires eight spectra per VOI in order to calculate a single localised spectrum.

Point resolved spectroscopy (PRESS) as well as simulated echo acquisition mode (STEAM) are commonly used in ^1H MRS and have served as gold standards for liver fat quantification in clinical studies (63). In this thesis, PRESS is used. PRESS is a so-called double spin-echo method, meaning that a chain of two echoes is used to gain spectral localisation. The localisation is performed by using a combination of focusing and 180° refocusing pulses. After the first echo, a column is selected by the intersection of two orthogonal planes (*e.g.* x and y); this is followed by a second excitation and refocusing in which the intersection of all three planes results in spectral localisation. Any signal outside this intersection is either not excited or not refocused, thus they are rapidly dephased by crusher gradients (49).

1.3.3.2 Proton (^1H) MRS

^1H -MRS is commonly used in clinical and research neurological applications, for instance in the analysis of tumours (64). Another important application is the quantification of liver fat content.

Two hepatic ^1H -MRS spectra are shown in Fig. 12 with spectral assignments as in (65). As previously mentioned each nucleus has specific chemical shifts according to its chemical environment so each resonance has to be taken into account when assessing the fat content in the liver. To complicate matters there are trace levels of protons from the triglycerides masked by the water resonance, these masked protons has to be taken into account when quantifying the liver fat content (66). A preferred method to report and quantify the fat content in the liver is to calculate the ratio of the signal from protons from fat (or triglycerides) and the sum of water and fat. If all confounding factors have been addressed or mitigated (see discussion in chapter 1.3.3.4) this ratio is referred to as the proton density fat-fraction (PDFF) (67). In Fig. 12 the two subjects have elevated PDFF levels. The subject in panel B has 4.8% which is close to the commonly used cut-off for abnormal hepatic fat content of 5.00 or 5.56% (68, 69).

Figure 12 Two hepatic 1.5 T ^1H -MR spectra with one steatotic liver (panel A; 20.3% proton density fat fraction; PDFF) and one borderline steatotic liver (panel B; 4.8% PDFF). Spectral assignments for panel A: 1), $-\text{CH}=\text{CH}-$ and $-\text{CH}-\text{O}-\text{CO}-$; 2) $-\text{CH}_2-\text{O}-\text{CO}-$, and one resonance is only really seen in vivo at 3 T: 3) $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$. Three resonances were observed in vivo at 1.5 T: 4) $-\text{CO}-\text{CH}_2-\text{CH}_2-$ and $-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-$; 5) $-\text{CO}-\text{CH}_2-\text{CH}_2-$ and $-(\text{CH}_2)_n-$; 6) $-(\text{CH}_2)_n-\text{CH}_3$. The spectra are the average of two repeated acquisitions (PRESS $30\times30\times30\text{ mm}^3$ volume selection; NSA = 8; $T_R=1.5\text{ s}$; $T_E=35\text{ ms}$).

1.3.3.3 Phosphorous (^{31}P) MRS

Phosphorus is the second most commonly used MRS nucleus in humans. In relation to the concentrations of ^1H ^{31}P has a concentration of c. 7%, with a 100% natural abundance of ^{31}P . As the concentrations are lower and the interesting compounds have long T_1 it takes a longer time and more averages to acquire sufficient SNR compared to ^1H -MRS (remember that the signal is proportional to

the concentration). An interesting feature with ^{31}P -MRS is that the relative chemical shifts of certain resonances can be used to measure the intracellular pH.

Commonly, ^{31}P -MRS is applied in musculoskeletal applications, such as fibromyalgia syndrome (62), chronic obstructive pulmonary disease (70), and to measure the mitochondrial capacity (71). ^{31}P -MRS has also been applied in hepatic applications (72, 73) where the ratio of phosphomonoesters (PME) and phosphodiester (PDE), which reside in the cell membranes, are thought to be indicative of the regenerative processes of the hepatocytes in the damaged liver parenchyma (72). A variant of this is the PME/PDE ratio is the anabolic charge (AC; $\text{AC} = \text{PME}/(\text{PME} + \text{PDE})$), which has been shown to be affected in the fibrotic liver (74).

Two examples of ^{31}P spectra are shown in Fig. 13, where Fig. 13A is a muscle spectrum from the quadriceps muscle and Fig. 13B is a hepatic spectrum. As can be seen, there is a high SNR in the muscle spectra, in part because phosphocreatine (PCr) has a very high concentration in muscles, but also since there is a much larger volume measured than in the hepatic spectra as well as a higher B_0 . An important difference is that PCr should not exist in the hepatic spectra, there are however minute traces in this examples which are due to contamination from surrounding musculature (49).

Figure 13 $3\text{ T }^{31}\text{P}$ -MRS human spectra examples with spectral assignments. Panel **A** shows a fully relaxed pulse acquire acquisition (16 averages, $T_R = 15\text{ s}$) in a human quadriceps muscle. Panel **B** shows a partially relaxed 1.5 T ISIS acquisition (192 averages, $T_R = 5\text{ s}$) in a human liver. Note that there is less SNR in the liver as the volume selection method samples a small volume $60 \times 60 \times 60\text{ mm}^3$ whereas the pulse acquire experiment acquires all available signals (limited by the coil sensitivity). Also the liver spectra should have no PCr (at 0 ppm). As can be seen there is a resonance in panel **B**, which is not uncommon as the liver moves every time the subject breathes. Also there are higher levels of PDE and PME in liver spectra.

Pi, organic phosphate; PDE, phosphodiester; PME, phosphomonoester; PCr, phosphocreatine; α, β, γ -NTP, α, β, γ -nucleoside triphosphate (mainly ATP).

1.3.3.4 Quantifying MRS

The quantification of the area under the resonances can either be performed in the frequency domain (such as in the commercial software package LCModel) or in the time-domain by using tools such as jMRUI (75). In this thesis, jMRUI is used together with the AMARES algorithm for spectral quantification (76).

The quantification of MR spectra using jMRUI with AMARES requires a set of ‘prior knowledge’ on how the data was generated. It is up to the user to define the spectral assignments for each resonance, such as the type of shape (Gaussian or Lorentzian), the chemical shift, and the phase of the resonance. After this prior knowledge or signal model has been defined, the AMARES algorithm is used to optimize the parameters such that the residual (the difference between the model and data) is minimized.

In Fig. 14 an example of such a prior knowledge or signal model composition is shown for a hepatic ^1H -MRS acquisition. Each panel in Fig. 14 shows the spectral models for each resonance. In the last panel the sum of all modelled resonances is presented.

Figure 14 Spectral quantification with jMRUI, the thin blue line represents the average of spectra of two repeated acquisitions (1.5 T; PRESS 30x30x30 mm³ volume selection; eight averages; $T_R = 1.5$ s; $T_E = 35$ ms), the thick orange line represents the signal model. Panel A shows the sum of a Gaussian and Lorentzian fit of the water resonance. Panel B is the Gaussian estimate of $-\text{CH}=\text{CH}-$ and $-\text{CH}-\text{O}-\text{CO}-$. Panel C is the Gaussian estimate of $-\text{CO}-\text{CH}_2-\text{CH}_2-$ and $-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-$. Panel D is the Gaussian estimate of $-\text{CO}-\text{CH}_2-\text{CH}_2-$ and $-(\text{CH}_2)_n-$. Panel E is the Gaussian estimate of $-(\text{CH}_2)_n-\text{CH}_3$. Finally, in panel F the total signal model is shown, which successfully explains the spectra.

As previously mentioned, care has to be taken in order to perform an absolute quantification of the resonances. After the area under the resonances has been quantified the data has to be correct for the MRS acquisition parameters, such as T_R and T_E . These acquisition parameters modulate the signal because different resonances have different T_1 and T_2 , and the relaxation rates can also be further modulated by contrast modifying agents such as excessive iron loading in the liver parenchyma. One method to correct for the signal modulation is to acquire multiple spectra using multiple T_R and/or T_E . With this approach it is possible to estimate the intrinsic T_1 and/or T_2 values and the correct concentrations. This allows using shorter T_R (*i.e.* not fully relaxed spectra) but in turn it requires more time to acquire the sought after T_R and T_E combinations. It is feasible in ^1H -MRS but takes much time in ^{31}P -MRS. Another possible solution is to acquire fully relaxed spectra and adjust with literature T_1 and T_2 values. Regardless of the approach used, the correction is defined using the following equation for the magnetization decay in the x,y-plane:

$$M_{xy} = M_0(1 - e^{-T_R/T_1})e^{-T_E/T_2} \quad (\text{Eq. 6})$$

where M_{xy} is the magnetization vector in the x,y-plane and M_0 is the initial state. As can be seen, the magnetization (and thus the signal) depends on T_1 , T_2 , T_R , and T_E .

After the spectral correction for T_1 and T_2 , it is time to perform the data calibration either to an external or internal reference. This reference has to be a stable compound of known concentration. In ^{31}P -MRS, di-methyl methyl phosphonate (DMMP) is such a suitable external reference as it is situated far away from any natural compounds *in vivo* (see Fig. 11A). As previously mentioned, in ^1H -MRS water is useful unless it masks the compounds and thus needs to be removed by special spectral editing methods. However, if ratios are used there is no need for data calibration (as it is removed in the division). In this thesis, two ratios are used namely; PDFF (=fat/(water+fat)) for quantifying the fat content in the liver using ^1H -MRS, and AC (=PME/(PDE+PME)) for quantifying the anabolic charge in the liver using ^{31}P -MRS.

1.3.4 Magnetic Resonance Elastography

Magnetic resonance elastography (MRE) has in the last decade emerged as a useful method for quantitative imaging of mechanical properties (stiffness or elasticity and viscosity) in soft tissues *in vivo*. It has even started to be used as a clinical tool in the evaluation of chronic liver disease in certain clinics such as the Mayo clinic in the US. Other uses for MRE than hepatic investigations have also been explored such as in neural disease, cardiac disease, and mammography.

Recall that the liver in a patient suffering from fibrosis and cirrhosis has an altered physical structure due to the increased amount of scar tissue or collagen

fibres. As a result the fibrotic liver is less elastic (stiffer) than a healthy liver, and a cirrhotic liver is stiffer still.

There are variants of MRE hardware and methodology but the main principles of the MRE methodology can be summarized in three steps: First, apply a force or motion (either external or internal) that deforms the tissue. Second, image the tissue response to the motion by phase contrast MRI. Third, compute images (elastograms) that correspond to the mechanical properties (77).

Of the variants of MRE hardware and methodology there is one setup for hepatic MRE that has reached the stage of a commercial product (made by Resoundant). In this thesis a research system built by Philips is primarily used, but at the institution we also have the Resoundant installation which was used in (78) when we compared the two variants. As there are interesting differences between the two, both will be described briefly in the following paragraphs, and the discussion will only focus on MRE applied in hepatic investigation.

1.3.5.1 Inducing Mechanical Motion in the Liver

In order to investigate the mechanical properties of the liver, motion has to be induced in the liver. This is not that different from traditional palpation of the tissue by a physician (the stiff cirrhotic liver can be detected by palpation). The motion can be induced either externally or internally (*i.e.* the motion induced by the beating heart) (77). Externally induced motion is performed by strapping a transducer against the subject (see Fig. 15 F). This transducer can be acoustic, electromagnetic, or pneumatic (48). In the research system from Philips used in this thesis an active electromagnetic transducer is used (see Fig. 15B-C). The electromagnetic transducer receives a signal from a wave form generator (see Fig. 15A), which generates a sinus wave at a predefined frequency. Inside the bore of the MRI the transducer utilizes the external magnetic field to produce mechanical motion of a piston, according to the specified wave form. The frequency of the wave form is dependent on the pulse sequence and also in turn on the field strength. Typically at 1.5 T frequencies between 50-60 Hz are used. In the Resoundant systems a passive acoustic transducer (see Fig. 15E) is used, which has a (drum-like) membrane attached to a tube. The force or motion is created by an apparatus (see Fig. 15D) which produces air pressure waves that propagate inside the tube until it reaches the drum.

When comparing these two, the acoustic transducer is much easier to use as it is less cumbersome; however a purer wave form is generated by an electromagnetic transducer as the air pressure waves can be distorted as they travel along the length of the hose.

Figure 15 Hardware for hepatic MRE. Panel **A** shows the signal generator in the research system from Philips. In the oscilloscope the electrical wave form that is sent to the transducer can be seen (typically operating at 56 Hz at 1.5 T). In panels **B-C** two versions of the active electromagnetic transducers used together with the generator in **A** are shown. The older version in panel **B** was used to acquire the MRE data in this thesis. Panel **D** shows the signal generator from Resoundant, which produces acoustic waves (typically operating at 50 Hz at 1.5T). Panel **E** shows the passive acoustic transducer used with the generator in **D**. Panel **F** shows the positioning of a transducer in a typical hepatic MRE experiment where the transducer is placed against the ribs adjacent to the right liver lobe such that the mechanical waves can penetrate the entire width of the liver.

Anatomical graphics in panel **F** reproduced and modified, with permission, from the Wolfram Language. © 2017 Wolfram Research Inc.

1.3.5.2 Imaging the Motion and Computing the Elastograms

As the mechanical waves are induced in the liver parenchyma the motion is recorded using 2D or 3D special phase contrast MRI sequences. The motion induced by the transducers is synchronized with the pulse sequence of the MR imaging and the pulse sequences are modified to include additional motion-encoding gradients in order to fully capture the motion (77). As an example, in 3D MRE the motion is recorded in all three directions (x,y,z). A complete description of this can be found in the excellent review by Glaser *et al.* (77). In the research system from Philips a 3D imaging method is used ('3D MRE'), and in the Resoundant system available at our institution a 2D imaging method is used ('2D MRE').

The use of either 2D or 3D imaging dictates how complex the post-processing algorithms that calculate the mechanical properties can be. The use of 3D imaging allows for the calculation of the shear modulus, storage modulus

(representing elasticity), and loss modulus (representing viscosity), whereas 2D imaging allows calculation of the shear stiffness (which consists of both elasticity and viscosity components). An important implicit assumption with 2D MRE is that the waves only propagate in the imaging plane, whereas in 3D MRE the algorithm calculates possible motion in all three directions.

In Fig. 16 an example of two such elastograms from 3D MRE is shown. As can be seen the elasticity is higher in the fibrotic (Fig. 16B) than the non-fibrotic liver (Fig 16C).

Figure 16 Example of elastograms for a non-fibrotic (F0; panel A) and a subject with severe fibrosis (F3; panel B). The elastograms are acquired using an active electromagnetic transducer operating at 56 Hz and 3D imaging. Histograms from ROIs (the orange outline in panels A-B) are shown in panels C-D. As can be seen the fibrotic liver is significantly stiffer than the non-fibrotic one. Also note the heterogenic appearance in the elastogram of the fibrotic liver.

1.4 Mathematical Modelling

Biological systems such as a human have complex interactions of biochemical reactions. In biochemistry it is often the case that isolated transporters such as MRP2 are well understood on their own; that is, they have been analysed *in vitro*. However this does not reflect their true function *in vivo* due the numerous interactions with other proteins, compounds, and physical situations. When observing biological systems the observed variable is thus the combination of this intricate interaction rather than the isolated function. In order to understand these complex interactions for purposes such as the development of novel drugs or for describing drug actions as well as drug-drug interaction mathematical descriptions or models have been developed. In the pharmaceutical industry these have been used for decades and the methodologies are known as pharmacokinetics (PK), pharmacodynamics (PD), and the combination in the form of pharmacokinetic-pharmacodynamics models (PK/PD). In these methods, small mathematical models describe the macroscopic distribution of drugs in the blood (PK) and the qualitative dynamics of treatment effect (PD) as a function of time (79). PK/PD models are thus not a complete mechanistic description of the drug distribution and action.

Recently, systems biology has become an established way to understand the mechanisms in biological systems. A key hallmark of systems biology is that the data should reflect the processes of an intact biological system rather than an isolated situation. In systems biology the data analysis is entirely focused on mechanistic, biologically relevant mathematical models (80), in other words these models are formalised hypothesis on how the data has been generated. The mathematical models consist of elements that describe properties of the biological system under investigation, such as variables describing the concentration of a compound. As the model cannot, and should not, describe all other compounds and reactions in the complete biological system these other compounds and reactions are neglected. For instance, describing the function of the eye when the liver is investigated is somewhat overambitious and unnecessarily complex as the eye probably has a negligible effect on the liver, of course depending on the function being investigated. It is to some extent both an art and a science to construct the models so that disregarded aspects do not compromise the basic behaviour of the system (79, 81).

The systems biology approach to analysing biology has several advantages; the implementation of models requires the modeller to clarify the verbal hypotheses, and highlights gaps in knowledge and understanding of the biological system (81). Models also assist in developing novel experiments based on a competing hypothesis, whereby the modeller can identify unique behaviours of different hypotheses that can be tested experimentally to drive the knowledge forward (82). The models or hypotheses not able to describe these new experiments lead to rejected hypotheses, and the non-rejected hypotheses (or models; see Fig. 17) can be used in the identification of mechanistic parameters and non-

directly measurable properties of the complex systems. This iterative development concept is often used when developing mathematical models in systems biology, and also in this thesis. With the concepts and tools available to the modeller in systems biology it is also possible to develop models that not only cover the level of organ interactions, the biochemistry of cells, or even the molecular binding in a receptor, but span the 'entire' scale of the biological system (83). This means that it is possible to merge different hypotheses in a single mathematical model in order to 'completely' describe multiple levels in a hierarchical manner, as well as to couple macroscopic observations with *in vitro* data.

Quantitative systems pharmacology (QSP) is an emerging field where the theories of systems biology are specifically applied in pharmacological applications. It can be viewed as the merger of systems biology and pharmacokinetics and pharmacodynamics. A recent white paper from the National Institute of Health work group on QSP stated that systems pharmacology will "[...] create the knowledge needed to change complex cellular networks in a specified way with mono or combination therapy, alter the pathophysiology of disease so as to maximize therapeutic benefit and minimize toxicity and implement a 'precision medicine' approach to improving the health of individual patients." (84).

In this thesis, the theories and methods of systems biology and pharmacology form the basis for the development of mathematical models and the analysis of their properties. In the following paragraphs key systems biology methods used in this thesis are discussed.

Figure 17 Outline of the iterative systems biology strategy for testing and developing new hypothesis (models). Here a model is formalised and tested against data and prior knowledge. If the model is rejected a new model is constructed. Non-rejected models are investigated for predictions that can be tested further.

This figure was originally published in the *Journal of Biological Chemistry*. Brännmark C, Palmér, R, Glad, ST, et al. (2010) Mass and Information Feedbacks through Receptor Endocytosis Govern Insulin Signalling as Revealed Using a Parameter-free Modelling Framework. *JBC*;285(26):20171-20179 © the American Society for Biochemistry and Molecular Biology.

1.4.1 Mathematical Formalism

The mathematical models are typically formalised as systems of ordinary differential equations (ODE), where properties such as concentrations, numbers, and reaction rates depend on time. There are other forms of mathematical models. Some can include spatial information and are known as systems of partial differential equations. There can also be stochastic elements and Boolean or probability information in the mathematical formalism. In this thesis, and in most systems biology and systems pharmacology applications, systems of ODE are the primary mathematical description. As such this introduction will only discuss models in terms of ODEs.

Systems of ODE can be described with the following notation:

$$\begin{cases} \dot{x} = f(x, p_x, u) \\ x(0) = x_0 \\ \hat{y} = g(x, p_y) \end{cases} \quad (\text{Eq. 5})$$

where x denotes the states, or state variables. States are the mathematical representation of an entity, such as concentration, for which the differential equations define how the state variables change as a function of time and each other. $\dot{x}$ refers to the time-derivate of x , which is defined by f which are smooth non-linear or linear continuous functions that depend on x , p_x , and u . p_x represents the parameters which are quantities with a given value that are constant within

the scope of the model. u is an input to the system, for instance the administration of a drug. x_0 is the initial state of the system, that is the numerical values of all states at time zero. $\hat{y}$ is the model output of the system, often corresponding to an experimentally measurable quantity that the model is to be compared with or other variables that are of interest regarding the use of the model. The output is defined by g , which are smooth nonlinear or linear continuous functions dependent on x and the parameters p_j . Note that all symbols are vectors and the time-dependency of x , $\hat{y}$, and u is usually dropped to simplify the notations.

A simple example of such a system of ODE is the enzymatic catalyzation of a substrate into a product via Michaelis-Menten kinetics. Michaelis-Menten kinetics can be used to describe the kinetic properties of many enzymes, such as the breakdown of alcohol to acetaldehyde (the first step in the metabolism of alcohol in the liver) (85). In practice almost all reactions in biological systems are reversible but for the sake of simplicity this example will be of an irreversible kind. The system is defined as:

$$\begin{aligned}\dot{x} &= \begin{cases} -\frac{V_{Max}x_1}{K_M+x_1} \\ \frac{V_{Max}x_1}{K_M+x_1} \end{cases} \\ x_0 &= \begin{cases} 1 \\ 0 \end{cases} \\ \hat{y} &= \begin{cases} x_1 \\ x_2 \end{cases}\end{aligned}\tag{Eq. 6}$$

where

$$p_x = \begin{cases} V_{Max} = 1/2 \\ K_M = 1 \end{cases}\tag{Eq. 7}$$

Solving these systems manually is cumbersome, especially in systems larger than the above example, therefore these models are typically solved (and simulated) using numerical ODE solvers in software packages such as Mathematica, MATLAB, or R. Such a solution to the system in Eq. 6-7 is shown in Fig. 18.

Figure 18 A numerical solution to a system of ODEs with two states, where the substrate is transformed into a product by Michaelis-Menten kinetics.

1.4.2 Model Parametrization and Goodness-of-Fit

A mathematical model of an electrical motor is easily parametrized as one typically knows which parts were used when the motor was constructed and thus model parametrization is straight-forward. This is not the case in biological systems. In models of biological systems many model parameters have to be identified by using experimental data. Typically a model parametrization or optimization algorithm is used for this task. During a model parametrization the model output ($\hat{y}$) is compared to some experimental observations or system outputs (y) by quantifying the residual (e):

$$e = \hat{y} - y \quad (\text{Eq. 8})$$

If these residuals are normalised by the standard deviation of the measurement uncertainty (σ) the following ‘cost function’ measures the overall agreement (V) between the model and experimental data:

$$V = \sum \frac{(\hat{y} - y)^2}{\sigma} \in \chi^2(df) \quad (\text{Eq. 9})$$

where all symbols ($\hat{y}$, y , σ) are vectors and time-dependent, and thus the summation is over both these dimensions. The $\chi^2(df)$ symbol is described in relation to the statistical tests and will be discussed later in this paragraph. In the model parametrization algorithms the parameter space is searched with the objective of minimizing Eq. 9, i.e.:

$$\hat{p}_x := \arg_{p_x} \min(V) \quad (\text{Eq. 10})$$

The interested reader can find a detailed discussion on the mathematical formalism for model evaluation and parametrization in the review by Cedersund and Roll (80).

Deferent optimization algorithms have been developed, where some merely investigate the parameter space in close proximity to some initial parameter

guesses (local optimization) while in other algorithms which can be described as global, the aim is to investigate the ‘entire’ parameter space. It is of course not feasible to investigate the entire parameter space as the precisions can span an arbitrary number of digits, thus the search would continue infinitely. In practice the modeller sets reasonable limits for the parameter space as well as limits to the number of digits sought. Often these global algorithms start with or include random guesses, whereby the algorithm randomly picks numbers in the parameter space and investigates them further. Eventually a global minimum of Eq. 9 will be found. Controlling and choosing the right optimization algorithm is by no means a trivial task.

When an ‘optimal’ set of parameters has been identified it is prudent to assess the goodness-of-fit in a statistical framework. A common null hypothesis when evaluating goodness-of-fit is that each term in the residual sum of squares (Eq. 9) should be normality distributed, thus Eq. 9 should follow a χ^2 distribution (80). The degrees of freedom (df) are chosen depending on the available data. If there is independent validation data available then df is equal to the number of data points in the validation set. If however, as is commonly the case, there is no additional validation data then df should be the number of data points reduced by the number of identifiable parameters (see discussion on identifiability in the next section) (80). The value of the cost function is compared with the inverse cumulative χ^2 -distribution for some α -level, and if the cost function has a value lower than the inverse cumulative χ^2 -distribution it is not possible to reject the hypothesis that the model (in combination with the parameters) cannot describe the data.

1.4.3 Parameter Identifiability and Core Predictions

As previously mentioned, not all compounds in a biological system can be observed and measured directly. It is thus not guaranteed that model parameters can be estimated unambiguously given the limited experimental data. This can lead to model parameters being non-identifiable, and identifiable parameters can only be determined within confidence intervals given the data, which include the true parameter values. Importantly, if the model parameters are not well determined then the model predictions are also not unambiguously determined (86, 87).

1.4.3.1 Parameter Confidence Intervals

Parameter confidence intervals can be estimated by different methods. A commonly used form is asymptotic confidence intervals. These confidence intervals are good approximations of the actual uncertainty if the amount of experimental data is large in relation to the number of parameters and the measurement noise is small. They do however rely on the assumption that the observed data depends linearly on the estimated parameters, which in practice is never the case (86). A superior method is the use of finite simple or likelihood-based confidence intervals where the data through the χ^2 test (Eq. 9) defines the confi-

dence interval. In practice, these confidence intervals can be computed by profile likelihood (86). The principle of profile likelihood is that each parameter is evaluated individually, whereby starting from the ‘optimal’ parameter value a small step is taken in either direction and all other parameters are re-fitted given the data. Steps are continuously taken until the cost has risen to the equivalent of $\chi^2(df = 1)$ which is the upper/lower confidence limit given the data and α -level.

1.4.3.2 Identifiability

The problem of identifying non-identifiability can be solved by using profile likelihood to estimate parameter intervals. In this approach, identifiable parameters will have finite parameter intervals and non-identifiable parameters will not. Importantly non-identifiable parameters can be subdivided into structural and practical non-identifiability. Due to the mathematical structure of the model, structurally non-identifiable parameters can never be determined uniquely, for instance a ratio of two parameters. Practically non-identifiable parameters are in theory identifiable but cannot be unambiguously determined with the available data. As an example, consider a two-parameter space, where the z-axis is the measure of model agreement to data (Eq. 9). If there is structural non-identifiability the confidence interval will extend into infinity in both axes (see Fig. 19A), whereas practical non-identifiability will have a lower or upper limit going to infinity (see Fig. 19B). Finally, a fully identifiable situation is shown in Fig. 19C where a neat circular confidence region is shown (86, 87).

Figure 19 Contour plots of a two dimensional parameter space. The shading from black to white corresponds to low to high values of χ^2 (in other words high to low model agreement). The white line represents the likelihood-based confidence regions and white stars represents optimal parameter values. In panel A the case of structural non-identifiability is illustrated in which the confidence interval continues to infinity. In panel B the case of practical non-identifiability is illustrated in which only a lower bound is found. In panel C both a fully indefinable system I shown with finite confidence intervals.

This figure was originally published in Bioinformatics. Raue A, Kreutz C, Maiwald T, et al. (2009) Structural and practical identifiability analysis of partially observed dynamical models by exploiting the profile likelihood. *Bioinformatics*;25(15):1923-1929 © the Authors.

1.4.3.3 Core Predictions

As it is common in biological systems that models due to the available data suffer from ambiguously determined parameters values the conclusions can be limited. Recently, the concept of core predictions was introduced as a way to draw strong conclusions and predictions despite uncertainty, and it has been shown to be as strong in a statistical sense as model rejections (87). A core prediction is a unique model property that must be fulfilled if the model is to explain the data despite the fact that the parameter values are not unambiguously determined. Core predictions can in practice be identified by modifying the optimization algorithm such that all sets of parameters values that result in the cost (Eq.9) being lower than the threshold (*i.e.* sufficient goodness-of-fit) are stored. Thereafter, all these parameter vectors are simulated and model predictions are analysed. If there exist uniquely defined properties of the system for all parameter sets, that is a core prediction (87). This concept has successfully been applied in the papers by Bränmark *et al.* as well as Nyman *et al.* (82, 83) where they used the core prediction methodology to reject hypotheses and identify novel experiments. The interested reader can find detailed definitions and discussions on core predictions in Cedersund 2012 (87).

1.4.4 Non-Linear Mixed Effect Modelling

In the pharmaceutical industry, since the early 1980s population pharmacokinetic-pharmacodynamics (PopPK-PD) has combined the study of drug dynamics and kinetics in populations. Today PopPK-PD is an intricate part of regulatory applications for drug approval. The key methodological development was the introduction of non-linear mixed effect (NLME) models. The goal of NLME is to combine a mathematical model with independent variables for the populations such as individual age, weight, and gender. A goal of NLME is to partition the total variability into the different sources in a population, such as between-subject, within-subject, and inter-occasion. Importantly it allows estimation of population estimates of parameter values as well as individual estimates (79).

An advantage of NLME is that the data does not need to be rich, *i.e.* sparse data can still be analysed and well-defined parameter values can be identified. Recently it has also been demonstrated that NLME is usable in systems biology applications in situations where the data is either too sparse, noisy, or weakly stimulated (88).

The principle of model parametrization using NLME can be described as follows: first an *a priori* distribution is defined for each parameter, which defines how this parameter is distributed in the population. Individuals' parameter values belong to the population parameter distribution, so during model parametrization the algorithm iteratively fits the population parameter estimates and the individual parameter estimates. This coupling of individual parameter values with the population distribution allows for information sharing, which should improve parameter estimates (79, 88). Commonly, systems biology models and other mathematical models are fitted to each individual or to the

mean of the population. By using NLME instead it is possible to produce better individual estimates than by parametrizing the model to each individual. NLME should be used when the data for an individual is not informative enough to accurately parametrize the model; in other cases the conventional methodology will suffice (88).

2. AIMS

The aim of my PhD project was to develop and investigate MR based techniques and analysis methods that can be combined in a single clinical MR examination in order to achieve a comprehensive understanding of the disease status in the liver. With this single MR examination, the idea is to gather as information dense data as possible so that there is no need for a needle biopsy of the liver. In order to achieve this goal the single MR examination should be able to accurately stage fibrosis in the liver as well as determine liver function and grade steatosis. This MR examination should also include techniques aimed to grade inflammation, measure iron content, energy levels (ATP; anabolic charge, AC), and conventional MR images for morphological investigation. In other words a non-invasive liver biopsy. Such a non-invasive liver biopsy would likely prove valuable both in detecting and monitoring disease progression in chronic liver disease, and aid in effective therapeutic planning. It would also likely prove valuable for the pharmaceutical industry as a tool to detected drug-induced liver injury.

2.1 The Specific Aims in Each Contribution

- I. The aim of paper I was to characterise and investigate the late-phase gadoxetate enhancement patterns in subjects with elevated liver enzymes, but without any clinical signs of hepatic decompensation. Primarily we wished to analyse if gadoxetate-enhanced MRI showed merit for identifying advanced fibrosis.
- II. The primary aim of this work was to develop and explore more precise methods for quantifying and exploring the underlying mechanisms behind the distinctly altered gadoxetate enhancement patterns observed in paper I. In addition we wished to develop an approach that is compatible with routine clinical protocols, allowing for morphological and regional evaluation of liver status.
- III. We aimed to assess whether steatosis can be reliably detected noninvasively using ^1H -MRS PDFF in a cohort of subjects with chronic liver disease, irrespective of the suspected aetiology. Moreover, we aimed to determine at which PDFF concentration subjects were diagnosed with steatosis, that is how PDFF related to the histopathological semi-quantitative '5% liver fat level'.
- IV. Our goal was to prospectively apply robust image reconstruction and adjustment methods with the model developed in paper II in order to determine liver function quantitatively (primary the activity of OATP1 and

MRP2) in a cohort of subjects with chronic liver disease by analysing dynamic gadoxetate-enhanced MRI. We also aimed to validate the model versus actual gadoxetate concentrations in liver biopsy samples as well as in blood samples, in addition to other clinical biomarkers of liver disease. Furthermore, an important aspect was also to explore if advanced parameterisation methods could reduce the required temporal resolution.

- V. The aim of this study was to stage fibrosis in a non-invasive manner in a prospective cohort of subjects with chronic liver disease in wide range of aetiologies. We also sought to assess the diagnostic performance of elastography (TE and MRE), MR spectroscopy, gadoxetate-enhanced MRI, and blood panel methods.

3. SUBJECTS AND METHODS

In this section, the subjects, data collection methods, analysis and processing methods and the development strategy of the mathematical model are presented. The first section briefly describes the two groups of data used in this thesis. Then the study protocols are described for the chronic liver disease cohort followed by a brief summary of the two published papers from which data is used in the development of the mathematical model. The analysis methods are divided into three parts; (i) analysis and processing, (ii) development of the mathematical model, and (iii) statistical tests. The reason for separating out the methods concerning the development and analysis of the mathematical model is that the development and analysis is an iterative process where the methods are tightly coupled with the results (see Fig. 17).

3.1 Subjects

In this thesis, essentially two set of data have been used; (i) a prospective study on a chronic liver disease cohort and (ii) a retrospective set of data on healthy subjects.

The prospective chronic liver disease cohort is used in papers I, III, IV, and V. Paper I is an early evaluation of the cohort which includes about one-third of the final total cohort. In the remaining papers (III-V) the full cohort is used, although there are slight differences in the number of subjects used in each paper due data availability and quality.

The development of the mathematical model in paper II used already existing data from a study on healthy volunteers at our institutions well as a previously published data set.

3.1.1 Prospective Diffuse Liver Disease Cohort

Between 2007 and 2014, 100 patients were recruited as they were referred to the Department of Gastroenterology and Hepatology at Linköping University Hospital for chronically (> 6 months) elevated levels of alanine aminotransferase, and/or aspartate aminotransferase, and/or serum alkaline phosphate. At the time of enrolment an extensive diagnostic work-up was performed and patients who, on clinical indication, needed a liver biopsy for final diagnosis were asked to participate in this study.

Exclusion criteria were contraindications for MR examination and liver biopsy. Contraindication for MRI included the presence of pacing devices, implants with ferromagnetic properties, pregnancy, and claustrophobia. Contraindications for liver biopsy were the presence of primary or secondary coagulative

disorder, prothrombin time > 1.5 INR, platelet count < 50 x 10⁹ platelets/l, hepatic malignancy, and clinical signs of decompensated cirrhosis.

The sample size was decided upon following power calculation on the separation of mild from advanced hepatic fibrosis in patients suffering from chronic liver disease using ³¹P-MRS (74). The study was approved by the regional ethics committee (Reference No. M72-07 T5-08). All patients gave their informed consent to participate before inclusion in this study.

In paper I 38 subjects were used. After the complete cohort was recruited in 2014, the work on analysing the data resulted in papers III-V. As previously mentioned, there are slight differences in the number of subjects in each paper (III-V) which is due to data availability and in some cases poor data quality. The data availability was reduced primarily during or prior to the MR examination due to: claustrophobia, no-specific reason given by the subject, and one subject failed beforehand to report metal objects inside the subjects' body. As some of these aborted MR examinations had already been ongoing for some time there was in some cases enough data to perform sub-analyses. This is the reason for the slight difference in data availability. Fig. 20 summarizes the number of subjects in papers I, III, IV, and V.

Figure 20 Number of subjects in the main analysis for papers I, III, IV, and V presented with the main modality used in the paper.

An additional sub-analysis in paper V was performed where because of technical problems with the multinuclear hardware not all ³¹P-MRS acquisitions were of sufficient quality. As a result 74 ³¹P-MRS acquisitions were used in the final

analysis. MRE was available at our institution in 2012; thus 36 subjects were investigated with MRE. TE was only acquired in 69 subjects.

3.1.2 Retrospective Data for the Development of a Mathematical Model

The development of the mathematical model was performed using retrospective estimation data acquired at our institution from ten healthy volunteers and presented in Dahlqvist Leinhard *et al.* (53). The model was validated using independent validation data from eighteen healthy volunteers that was extracted from the paper by Schuhmann-Giampieri *et al.* (54). Table 2 describes the demographics of these two data sets.

Table 2 Demographics of data sets used in developing the mathematical model. Descriptive data presented as mean and range.

	Estimation data	Validation data
# subjects	10	18 (6 per dose group)
Age (years)	25 (22-27)	30 (20-40)
Weight (kg)	73 (62-84)	83 (69-87)
Gender	5 males, 5 females	18 males

3.2 Data Collection

The timeline for the data collection in the chronic liver disease cohort is shown in Fig. 21. Typically all MR examinations were performed within two months of the enrolment, and the biopsies were performed within an hour of the MR examinations.

Figure 21 Timeline for the data acquisition in the chronic liver disease cohort used in papers I, III, IV, and V.

The following paragraphs briefly describe each data acquisition method used in papers I, III, IV, and V.

3.2.1 Enrolment

At the time of enrolment, a hepatologist recorded each subject's medical history, including pharmacological treatments. Blood pressure, waist circumference,

body weight, and height were measured. The subjects were also interviewed to establish their alcohol consumption.

Blood samples were taken from all subjects for analysis of routine biochemical parameters. Additional blood samples were obtained for detection of hepatitis B surface antigen, anti-HCV antibodies, HCV-RNA (in the case of the presence of anti-HCV antibodies), and autoantibodies. For subjects with elevated serum transferrin saturation, genomic DNA was isolated for the identification of C282Y, H63D, and S65C mutations in the human hemochromatosis protein (HFE) gene. For subjects with low serum levels of α_1 -antitrypsin, the Pi gene was analysed for identification of the Z and S mutations.

3.2.1.1 Transient Elastography

TE was performed using FibroScan (Echosens, Paris, France) as a part of the clinical work-up at the time of enrolment. The subjects were asked to fast ≥ 3 h before the examination. The procedure was performed in a supine position with the right arm abducted and placed under the head. The probe, operating at 50 Hz, was applied over the area of the right liver lobe and a minimum of 10 measurements were made to obtain the median valid liver stiffness in kilopascal (kPa) and interquartile range.

3.2.2 Magnetic Resonance Examination

The MR examination was performed within two months of the enrolment. MR protocols relevant for this thesis and papers I, III, IV, and V included (see Fig. 22): ^{31}P -MR spectroscopy, MR elastography, ^1H -MR spectroscopy, T_2 relaxometry, gadoxetate-enhanced MRI (or dynamic contrast-enhanced (DCE) MRI). In all MR examinations a 1.5 T Philips Achieva MR system was used, and the subject was lying in a supine position.

Figure 22 Order of the MR examination protocols that are relevant for this thesis.

3.2.2.1 ³¹P Magnetic Resonance Spectroscopy

³¹P-MRS was performed using a flat non-flexible 12 cm circular single tuned transmit/receive coil. The coil was placed on the anterior chest wall to the right of the xiphoid process of the subject. The acquisition was performed during free breathing. The localised spectra were acquired using ISIS volume selection. Briefly the MR parameters were; VOI = 60 x 60 x 40 mm³, averages = 192, spectral bandwidth = 5 kHz, data points = 1024, T_R = 7 s, T_E = 95.6 μs.

3.2.2.2 Magnetic Resonance Elastography

MRE was performed using an active electromagnetic transducer operating at 56Hz (see Fig. 15A-B). The transducer was placed on the anterior chest wall to the right of the xiphoid process of the subject. The images were acquired in four breath-holds using a phased array body coil (Sense TorsoXL) was used. Briefly, the 3D gradient echo MR parameters were; field of view (FOV) = 320 x 256 mm, matrix = 80 x 38, slice thickness = 4 mm, #slices = 9, FA = 9 °, T_R = 112 ms, T_E = 9.21 ms, SENSE = 2. The shear waves were obtained by applying the curl operator and using the Voigt rheological model to obtain shear elasticity amps. Details of the elasticity and viscosity map calculations can be found in (89, 90).

The MRE equipment was available at our institution in 2012, thus only subjects recruited in 2012 and onwards were investigated using MRE.

3.2.2.3 ¹H Magnetic Resonance Spectroscopy

¹H-MRS was performed using a single detection element of a four-element SENSE body coil. Two localised spectra were acquired in two breath-holds using PRESS volume selection. Briefly the MR parameters were; VOI = 30 x 30 x 30 mm³, averages = 8, dummy excitations = 2, spectral bandwidth = 1 kHz, data points = 512, T_R = 1.5 s, T_E = 35 ms.

3.2.2.4 T₂ Relaxometry

In order to correct the attenuation of the ¹H-MRS due to possible presence of excess hepatic iron deposition, T₂ relaxometry was performed on the water protons on the liver. The data was acquired using an axial single-slice fat-saturated multi-echo turbo gradient spin-echo sequence during a single breath-hold. The FOV and acquisition matrix were adjusted, if necessary, to accommodate subject size. Briefly the MR parameters were; typical FOV = 295 x 10 x 375 mm³, typical matrix = 168 x 168, T_R = 190 ms, T_E = 6 x n ms (n = 1, 2, 3, ..., 16), FA = 90 °.

3.2.2.5 Gadoxetate-Enhanced MRI

The gadoxetate-enhanced MRI (or DCE-MRI) was acquired using a phase-array body coil. Single breath-hold symmetrically sampled two-point Dixon 3D images were acquired using SENSE. All subjects received a bolus injection of gadoxetate administered intravenously at a rate of 1 mL/s using a power injection followed by a 30 mL saline flush. Gadoxetate was administered using a clinical dose of approximately 0.025 mmol/kg, the exact dose in tenths of a mL was

noted for each subject. Gadoxetate comes in vials of 10 mL and if subjects weighed just over 100 kg no new vial was opened. Images were acquired prior to (non-enhanced) and directly following the injection of gadoxetate. The post-injection time series corresponded to arterial (30 s) and venous portal phase (1 min), 3, 10, 20, and 30 min post-injection. Additional acquisitions between 3 and 30 min post-injection were added in the subject recruited from 2012 and onwards, corresponding to 5, 8, 13, 15, 18, 23, 25, and 28 min post-injection.

The FOV and acquisition matrix were adjusted, if necessary, to accommodate subject size. The non-enhanced and post-injection images were acquired using the following MR parameters; $T_R = 6.5$ ms, $T_E = 2.3$ and 4.6 ms, $FA = 13^\circ$, typical matrix = 168×168 , typical FOV = $261 \times 200 \times 342$ mm³, slice thickness = 4 mm, slice gap = -2 mm, reconstructed slice thickness = 2mm.

3.2.2.6 Blood Sample for Elemental Analysis

In the MR preparation room, immediately following the MR examination, venous blood was drawn from each subject for elemental analysis. These samples were immediately frozen and stored at -80°C .

3.2.3 Biopsy

Two ultrasonographically guided liver biopsy procedures were performed on an outpatient basis within an hour following the completion of MR examination. The liver biopsy samples were obtained percutaneously using a 1.6 mm needle in either the left or right liver lobe depending on which location offered a combination of successful biopsy and maximum subject safety. No biopsy-related complications occurred. The median (range) biopsy length was 19 mm (12–32 mm). The first biopsy was processed according to clinical guidelines, and the second was weighed and directly frozen and stored at -80°C .

3.3 Summary of the Retrospective Data

As previously mentioned, no new data was acquired for the development of the mathematical model (paper II). The estimation or training data was obtained from a previously published study at our institution (53) and the validation data was obtained from a previously published study (54). The chapters below briefly summarise the acquisition and post-processing of the data as described in the two papers (53, 54).

3.3.1 Estimation Data

The gadoxetate-enhanced MRI was acquired using a phase-array body coil. Single breath-hold axial fat-saturated T_1 -weighted 3D GRE were acquired without SENSE. All subjects received a bolus injection of gadoxetate administered intravenously at a rate of 1 mL/s using a power injection followed by a 30 mL saline flush. Gadoxetate was administered using the clinical dose of approximately 0.025 mmol/kg. One pre-injection and six enhanced images stacks were ac-

quired (arterial, portal venous, 10, 20, 30, and 40 min post-injection) (53). The images were acquired using the following MR parameters; $T_R = 5.2$ ms, $T_E = 2.5$, $FA = 10^\circ$, typical matrix = 256×164 mm², slice thickness = 3.3 mm, reconstructed voxel size = $1.6 \times 1.6 \times 1.6$ mm³ (53).

SI curves were obtained using the averages from seven ROIs (see Fig 23A-G) in the liver, three ROIs in the spleen, and one each in the portal and splenic veins. The liver ROIs were distributed in both the left and right lobes, and placed in parenchymal areas without any large vessels, of focal lesions (53). The induced increase in relaxation rate (directly proportional to the gadoxetate concentration) was calculated on the basis of the SIs as described in the introduction of this thesis (chapter 1.3.2), see Fig 23H.

3.3.2 Validation Data

The validation data was acquired from Tables 1, 2, and 4 in (54). Briefly the eighteen healthy subjects were divided into three dose groups: 0.2 mmol/kg, 0.35 mmol/kg, and 0.5 mmol/kg. Gadoxetate was administered in a 10 min infusion, and blood samples were taken up to 36 h after the infusion. During the first hour, 10 samples were taken. Faecal and urine samples were also acquired at fixed intervals to measure the elimination of gadoxetate. The gadoxetate levels were measured using inductively coupled plasma mass-spectroscopy (ICP-MS) (54).

Figure 23 A-G Example of regions of interest placed in the liver (red arrow). H shows the induced changes in relaxivity which are directly proportional to the contrast agent concentration.

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

3.3 Data Processing and Analysis

This section covers the processing and analysis methods used with the chronic liver disease cohort (papers I, III, IV, and V). The analysis of the gadoxetate-enhanced imaging is described in this section to the point of the induced change in relaxivity time series calculations. The analysis of these times series are discussed in the next chapter.

3.3.1 Biopsy and Blood Samples

3.3.1.1 Histopathological Examination of Biopsy Samples in the Interim Analysis

For the interim analysis (paper I) biopsy samples were graded and classified according to the Batts and Ludwig system (11) (see the introduction), *i.e.* fibrosis was staged as no fibrosis (F0), portal fibrosis (F1), periportal fibrosis (F2), septal fibrosis (F3), and probable or obvious cirrhosis (F4). All biopsy samples were examined by the same liver pathologist, who was blinded to patient details and the MR examination results.

3.3.1.2 Comprehensive Histopathological Examination of Biopsy Samples

The biopsy samples were all re-examined using a comprehensive research protocol after the inclusion of the last subjects (for papers III-V). All biopsy samples were examined by the same hepatopathologist who was blinded to patient details and MR results, as defined by Kleiner *et al.* (41). The following manifestations of liver disease were investigated by the hepatopathologist:

- The degree of steatosis was graded as 0-3, corresponding to fat deposition in < 5%, 5-33%, 34-66%, and > 66% of the hepatocytes, respectively.
- Fibrosis was staged on a 5-point scale: stage 0 = no fibrosis, stage 1 = zone 3 perisinusoidal/perivenular fibrosis, stage 2 = zone 3 and periportal fibrosis, stage 3 = septal/bridging fibrosis, stage 4 = cirrhosis.
- Lobular inflammation was graded 0-3. Grades 0-3 correspond to none, < 2 foci, 2-4 foci, and > 4 foci per 200x field, respectively.
- Hepatocellular ballooning was graded 0-2. Grades 0-2 correspond to none, few balloon cells, and many cells/prominent ballooning, respectively.
- Iron stains of liver biopsy samples were performed with Pearls' iron stain according to Turlin *et al.* (91). Hepatic iron content was scored at x400 magnification on a three-point scale (0-2) as none, mild, or more than mild.

3.3.1.3 Stereological Point-Counting

Stereological point-counting (SPC) was performed as previously described by Franzén *et al.* (92). Briefly, a Nikon Eclipse E800 microscope with a Nikon DS-Ri1 digital camera was used to capture the biopsy images using the software

package NIS-Elements BR v 4.0 (Nikon Corporation, Tokyo, Japan), see Fig. 24A. A point grid consisting of 221 crosses, 35 μm apart, was superimposed upon each image (Fig. 24B). The number of fat vacuoles in the hepatocytes was counted manually. Ballooned hepatocytes could in most cases be distinguished from macrovesicular steatotic cells on the basis of the centrally placed nuclei and fragmented membranes or cell organelles in the cytoplasm of the ballooned cells. If distinguishing was impossible they were omitted. Crosses overlying damaged tissue and larger areas with fibrosis were excluded. The number of hits on fat vacuoles divided by the total number of hits on liver tissue represented the ratio of steatotic area within the section (expressed in %).

Figure 24 Biopsy samples at two different magnifications; **A** 200x and **B** 400x magnification. Hepatocytes with large fat vacuoles are clearly visible in both images (the white round objects). In panel B the superimposed crosses for the SPC are visible.

3.3.1.4 Elemental Analysis of Biopsy and Blood Samples

The frozen blood samples were sent in batches to an external laboratory (ALS Scandinavia AB, Luleå, Sweden) for elemental analysis using inductively coupled plasma-sector field mass spectrometry (ICP-SFMS). The elemental analysis procedure was performed as follows: In preparation for the analysis, 0.20 mL of each thawed blood sample was mixed with 1.00 mL 'super pure' HNO_3 (pure with respect to traces of metal) and subsequently digested in a 600 W microwave oven operating at 75% for 30 min. Thereafter, the sub-samples were diluted up to 10.00 mL with MilliQ. The elemental analyses were performed using ICP-SFMS. This method had a detection limit for gadolinium of 0.05 $\mu\text{g/L}$ solution.

The second liver biopsy sample was freeze dried, and the dry weight was weighed prior to being submitted to our external analysis partner (ALS Scandinavia AB) for elemental analysis. The dried liver biopsy sample were digested by adding 2.50 mL 'super pure' HNO_3 and 0.25 mL H_2O_2 followed by a 30-min treatment at 170 $^\circ\text{C}$ in a microwave oven. The samples were then diluted up to

5.00 mL using MilliQ. The elemental analysis was performed in the same manner as the blood samples (vide supra).

3.3.2 T_2 Relaxometry and ^1H Magnetic Resonance Spectroscopy

3.3.2.1 T_2 Relaxometry

The T_2 relaxation times of the water protons in the liver were quantified by fitting a mono-exponential decaying function (Eq. 12) to the mean signal intensities of the multi-echo image series.

$$SI(T_E) = M_0 e^{-T_E/T_2} \quad (\text{Eq. 12})$$

where M_0 is the initial signal directly following the RF pulse, T_E is the echo time and the equation is solved for T_2 as a function of multiple T_E 's. The mean SIs were derived from ROIs placed in approximately the same anatomical region as the ^1H -MRS VOIs. The ROIs were placed and the equation was solved using a custom tool implemented in Mathematica (10.0.2, Wolfram Research Inc. Champaign, US). As the tool loaded the image series the spectroscopy VOIs were read from the meta data files and the VOI was presented to the user in the images (if they were in the FOV).

3.3.2.2 ^1H -Magnetic Resonance Spectroscopy Proton Density Fat Fraction

The software package jMRUI (75) was used for time-domain analysis of the ^1H -MRS data as described in the introduction. The amplitudes of the water resonances (Gaussian; 6.0-3.3 ppm) and the lipid resonances (three separate Gaussian resonances; 2.7-0.4 ppm) were quantified using the AMARES algorithm within jMRUI (65, 76), see Fig. 12 and 14. Finally, the quantified resonances were corrected for ^1H -MRS sequence parameters (T_E and T_R) as well as water and lipid proton relaxation times (T_1 and T_2), see Eq. 6. The T_1 relaxation times were assumed to be 663 and 236 ms for the water and lipid protons, respectively (51). The T_2 relaxation time was assumed to be 58.5 ms for lipid protons (93), and the water proton T_2 relaxation time was measured in vivo as described above.

The lipid content (PDFF), correcting for lipids masked by water proton resonances was quantified as previously described by Longo *et al.* (66), and reported as the mean of the repeated spectroscopic acquisitions.

3.3.3 Gadoxetate-Enhanced Magnetic Resonance Imaging

3.3.3.1 Quantification of the Gadoxetate Concentrations in the Interim Analysis

The acquired in- and opposite-phase images were reconstructed into water and fat images using the inverse gradient method (94). In order to correct for intensity heterogeneity in the water and fat Dixon images, for the purpose of reference scaling throughout the time series, the intensities of voxels identified as pure adipose tissue were used as an internal reference (95).

Data were acquired from seven ROIs placed in the liver and three in the spleen (see Fig. 25). The ROIs were placed in the same anatomical location throughout the times series by an experience radiologist, avoiding focal lesions, large vessels and bile ducts without the intention of strictly following the segmental division as described by Couinaud (96). The radiologist was blinded to the results of the histopathological findings. The induced change in relaxivity was calculated as described in the introduction according to Dahlqvist Leinhard *et al.* (53).

Figure 25 Example of placement of seven ROIs in a subject within the left (three) and the right (four) liver lobes as well as three in the spleen. These are reconstructed water-only images acquired at 20 min post-injection of gadoxetate.

Reproduced from: Norén B, Forsgren MF, Dahlqvist Leinhard O, *et al.* (2013) Separation of advanced form mild hepatic fibrosis by quantification of the hepatobiliary uptake of Gd-EOB-DTPA. *Eur Radiol* 23:174-81

3.3.3.2 Quantification of the Gadoxetate Concentrations

Following the final data acquisition, the data was reconstructed and ROIs were placed as described in the sub-chapter above. However, the tool used in paper I was no longer operational at this point. A new and also a more user-friendly tool

was implanted in Mathematica, which also allowed for reading the old ROIs placed in paper I. Examples of ROIs placed in the new tool are shown in Fig. 26. The induced change in relaxivity was calculated based on the extracted mean SI as described in the previous sub-chapter.

The lower limit of uncertainty (or noise) in the extracted gadoxetate time series was estimated by calculating the average standard deviation (σ) in the normalised SIs (Eq.13).

$$\sigma S = |S| \sqrt{\left(\frac{\sigma SI(t)}{SI(t)}\right)^2 + \left(\frac{\sigma SI(0)}{SI(0)}\right)^2} \quad (\text{Eq. 13})$$

where t refers to a point in time following the gadoxetate injection, and 0 refers to pre-gadoxetate injection. The uncertainty was averaged over the entire study cohort. The estimated mean uncertainty of the cohort was used as a lower limit of uncertainty, meaning that unless the standard error of the mean across the splenic or liver ROIs exceeded the lower limit it was used as the standard deviation in the statistical tests.

3.3.3.3 Quality Assurance of the Final Gadoxetate Concentrations

Quality assurance was performed as follows. The image data were inspected visually for quality issues and potential data exclusion. As the two radiologists independently placed ROIs in the images they took particular notice of cases of poor image quality (such as artefacts due to breathing or post-processing failure). Then both radiologists together reviewed these cases of potential poor quality for data quality and decided in consensus whether to exclude the images, to return the images for manual image reconstruction, or to accept the images as they were. After the radiologists were satisfied, the data analyst continued to search for any significant outliers in the extracted time series. The radiologists were then instructed to review these latter cases. Importantly, they were not informed of why a specific case was to be reviewed. If they were still satisfied with the placement of ROIs as well as with the image quality, nothing was corrected.

Figure 26 Example of seven ROIs at different phases of a gadoxetate-enhanced magnetic resonance experiment placed with the new tool in papers IV and V. The images are reconstructed water-only images based on a 2-point Dixon imaging protocol.

Reproduced from: Forsgren MF, Karlsson M, Dahlqvist Leinhard O, et al. (2017) Quantitative Assessment of Liver Detoxification and Drug Transporter Functions Using Gadoxetate-Enhanced MRI – A Liver Biopsy and Blood Sample Validated Prospective Study on Chronic Liver Disease. In Manuscript

3.3.4 ^{31}P -Magnetic Resonance Spectroscopy

The ^{31}P -MRS data was post-processed and the time-domain analysis of the data was performed as described in the Introduction using the software package jMRUI (75). The amplitudes of PME (two separate Lorentzian resonances; [6.3; 6.7] ppm), Pi (Lorentzian; 4.0 ppm), PDE (two separate Lorentzian resonances; [2.7; 3.4] ppm), MP (2.3 ppm), PCr from the surrounding muscle tissue (Lorentzian, 0.0 ppm), γ -NTP (two separate Lorentzian resonances; [-2.3; -2.9] ppm), α -NTP (two separate Lorentzian resonances; [-7.5; -7.9] ppm), β -NTP (three separate Lorentzian resonances; [-15.7; -17.1] ppm), and NAD(H) (Lo-

rentzian, -8.5 ppm) were quantified using the AMARES algorithm within jMRUI (73, 74, 76), see Fig. 13A.

The MRS data was analysed by calculating the anabolic charge ($AC = PME / (PME + PDE \text{ incl. MP})$) as described previously (74).

3.3.5 Magnetic Resonance Elastography

Elastograms were calculated using custom software provided by Ralph Sinkus *et al.* (King's College, UK). In the elastograms a ROI was placed manually to cover over three slices such that the total cranio-caudal coverage of the ROIs was 12 mm. The ROI was drawn as the largest possible ROI avoiding focal lesions, large vessels and bile ducts. Thereafter, the mean and standard deviation elasticity (unit of kPa) was calculated.

3.4 Analysis of Gadoxetate Fluxes and the Development and Analysis of the Mathematical Model

This chapter is divided into three parts; (i) a brief description of previously developed methods to interpret the gadoxetate signal enhancement in the liver, (ii) the iterative development process of the mathematical model using healthy subjects, and (iii) the application of the developed mathematical model to the chronic liver disease cohort.

3.4.1 Liver-to-Spleen Contrast Ratios and Contrast Agent Uptake

On the basis of the normalised SI (see chapter 1.3.2) the normalised liver-to-spleen contrast ratios (LSC_N) were calculated at 10 min (LSC_N10) and 20 min (LSC_N20) post-contrast injection as described by Dahlqvist Leinhard *et al.* (53). These ratios are simply the mean of the liver divided by the mean of the spleen values. The contrast agent uptake rate (K_{Hep}) was also calculated as follows:

$$F_{Hep} = 0.39 \left(\frac{\Delta R_{1,Liver}}{\Delta R_{1,Spleen}} - 0.77 \right) \quad (\text{Eq. 14})$$

$$F_{Hep} = K_{Hep} \times t$$

where F_{Hep} is the fraction of contrast in liver residing in the hepatocytes. The values 0.39 and 0.77 are dependent on the assumed sizes of the vascular compartments in the liver and the spleen. The increase of F_{Hep} is assumed to be linear in the first 20 min after the injection, and K_{Hep} is obtained by fitting a slope dependent on time (53). For comparison the live-to-spleen contrast ratios (LSC) were also determined at 10 and 20 min post-contrast injection according to Motosugi *et al.* (97).

3.4.2 The Development of a Mechanistic Mathematical Model for Gadoxetate Fluxes

In paper IV, ten hypotheses concerning the gadoxetate fluxes in a human, formalised into mathematical models were analysed. This section covers the development and testing of these models

3.4.2.1 Model Description

All ten model structures were analysed in detail, even though only a single model was ultimately suggested. The suggested model structure is denoted M0 (see Fig. 27), and the additionally proposed model structures are denoted M1-M9 (see Fig. 28).

Figure 27 Diagram of model M0. ‘CA injection’ refers to the injection of the contrast agent gadoxetate.

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

The proposed model structure depicted in Fig. 27 contains four rounded-edge rectangles, which correspond to the four major compartments in the model: ‘Blood Plasma’, ‘Extracellular Extravascular Space’ (EES), ‘Splenic Intracellular Space’ (Splenic IS), and ‘Hepatocytes’; corresponding to the concentrations C_p , C_e , C_s , and C_h respectively. The two circles, ‘Bile’ and ‘Urine’, correspond to sinks in the model, meaning that there is no flow going from these compartments to other parts of the model. The shaded areas correspond to the three measurement signals. The leftmost signal in Fig. 27 is the MRI ‘Liver Signal’, which is composed of contributions from the Hepatocytes, Blood Plasma, and EES. Simi-

larly, the MRI ‘Spleen signal’ has contributions from Splenic IS, Blood Plasma, and EES; and the MRI ‘Plasma Signal’ only has contributions from the Blood Plasma compartment. The arrows correspond to fluxes of gadoxetate between different compartments. The different model versions differ from each other in the characterization of these fluxes.

Figure 28 Diagram of the rejected models M1-M9. Omitted here is the signal model, which is identical in all model configurations. The dashed arrow represents the use of Michaelis-Menten kinetics instead of linear mass-action of diffusion-like kinetics.

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

3.4.2.2 Transport Equations in the Models

The first transport step is the injection of gadoxetate. This is modelled as a step function:

$$\begin{cases} u = k_{syr} C_{EOB} & 0 \leq t \leq \tau \\ u = 0 & t > \tau \end{cases} \quad (\text{Eq. 15})$$

where k_{syr} corresponds to the gadoxetate injection rate, C_{EOB} corresponds to the gadoxetate concentration in the syringe, and τ corresponds to the time point where the injection is terminated. This input is the same for all different models,

and in the case of the using the model with the validation data, these parameters were updated in order to match the experimental setup.

There is no transport of gadoxetate between Splenic IS and the other compartments; this is the same for all the different models.

There is a bi-directional flow between the blood plasma and EES. This flow is caused by passive diffusion, where the diffusion constant is the same in both directions:

$$\begin{aligned} v_1 &= k_{diff}(C_p - C_{SA}) \\ v_2 &= k_{diff}C_e \end{aligned} \quad (\text{Eq. 16})$$

where v_1 and v_2 correspond to the flow to and from EES, respectively; where k_{Diff} is the diffusion rate constant; and where C_{SA} is the concentration of gadoxetate bound to serum albumin (54). C_{SA} is given by $C_{SA} = 0.1C_p$. Note that C_{SA} is located in the Plasma compartment; therefore it is a part of the Plasma gadoxetate (*i.e.* contributes to C_p); in other words, C_p consists of both free and bound gadoxetate. Eq. 16 describes the flow between Plasma and EES for all model structures, with the exception of M7.

As mentioned in the introduction (chapter 1.3.2.4) the uptake of gadoxetate into the hepatocytes is mainly governed by OATP1B1, OATP1B3 and to some extent NTCP (57). Gadoxetate can potentially run to the blood plasma from hepatocyte via three different routes; (i) due to the OATP1's bi-directionality (4), (ii) upregulation of MRP3 (2), and (iii) leakage of tight junctions (59). The fluxes between Plasma and Hepatocyte compartments were, due to the multiple transporters, modelled in a phenomenological manner: as linear mass-action transports in Mo-M4, M6, and M7, alternatively as Michaelis-Menten expressions in M8 and M9 (Fig. 28). Furthermore, omitted in M5 and in some model structures (M2, M4, and M9) the flow was modelled as irreversible, *i.e.* where there is no flow from the hepatocytes to the blood. In other words, for Mo, the flows are given by:

$$\begin{aligned} v_3 &= k_{ph}(C_p - C_{SA}) \\ v_4 &= k_{hp}C_h \end{aligned} \quad (\text{Eq. 17})$$

where v_3 and v_4 correspond to the flow to and from the hepatocyte, respectively, k_{ph} and k_{hp} are the kinetic parameters for the two rate equations.

As discussed in the introduction (chapter 1.3.2) blood plasma is also cleared of contrast agent by the kidneys. This clearance primarily consists of glomerular filtration, which results in an apparent clearance of approximately 118 mL of blood per minute (54, 56, 98), which means that the renal clearance can be modelled as:

$$v_5 = CLr(C_p - C_{SA}) \quad (\text{Eq. 18})$$

where CLr is the kinetic parameter.

The final flux in the model is the elimination from the hepatocytes to the bile, which is mediated via MRP2 (58). This transport is generally believed to be rate limiting, displays Michaelis-Menten kinetics, and is an ATP-driven strictly unidirectional process (99-101). For these reasons, the flow is in most models described by Michaelis-Menten kinetics (M1, M3-M4, M7-M9), but in model Mo it was shown possible to use a linear expression instead:

$$v_6 = k_{hb}C_h \quad (\text{Eq. 19})$$

where k_{hb} is the kinetic parameter for gadoxetate elimination from the Hepatocytes to the Bile. Also note that in M6, this flux (v_6) is omitted (Fig. 28).

By including these flows, the differential equations become:

$$\begin{aligned} \dot{C}_h &= v_3 - v_4 - v_6 \\ \dot{C}_p &= \frac{(-v_3 + v_4)V_l\gamma_h - v_5 + (v_2 - v_1)V_e + u}{V_p} \\ \dot{C}_e &= v_1 - v_2 \end{aligned} \quad (\text{Eq. 20})$$

These ODEs are the same for all model structures, even though some flows v_i may always be zero in some models.

3.4.2.3 Signal Model

The signal model depended on whether comparison was made with estimation (gadoxetate-enhanced MRI) or validation data (blood samples). For the validation data, the measurement equation was simply:

$$g(p_x, x) = \xi C_p \quad (\text{Eq. 21})$$

where ξ is an arbitrary scaling constant. For the estimation data, the conversion of contrast agent concentrations in the model to relative change in relaxivity rests on the following relationship (53) (chapter 1.3.2):

$$\Delta R_1 = Cr_1 \quad (\text{Eq. 22})$$

each ROI is an observation of the combined signal from multiple compartments, *e.g.* the MRI Liver signal contains contributions from EES, Blood Plasma and the Hepatocytes. In order to derive a value comparable to the ROIs placed in the tissue, the following equation was used, based on Eq. 9:

$$g_i(p_x, p_y, x) = \Delta R_1 = \xi \sum_{j=1}^n C_j \gamma_{i,j} r_{1,j} \quad (\text{Eq. 23})$$

Where $i = 1, 2, 3$ for the three measurements in the liver, spleen and veins, respectively; where C_j is the gadoxetate concentration in compartment j , for the four compartments (Hepatocyte, EES, Splenic IS, or Blood Plasma); where $\gamma_{i,j}$ is the fraction of the volume in ROI i consisting of compartment j and $r_{1,j}$ is the *in situ* relaxivity in compartment j . The *in situ* compartment specific relaxivity val-

ues at 1.5 T and 37 °C were assumed to be: EES 6.9 mmol⁻¹s⁻¹, Blood Plasma 7.3 mmol⁻¹s⁻¹, and Hepatocytes 10.7 mmol⁻¹s⁻¹ (102, 103).

3.4.2.4 Literature Model Parametrization

Some of the parameters were estimated using optimization, as described in the following chapter, and parameters were obtained from literature. In Table 3, the individual fractions of each ROI in the signal model (Eq. 23) are presented. These fractions are based on a 70 kg, 20% fat ‘standard human’ (104). Note that the fractions for the splenic ROIs do not add up to zero. The reason is that the cells of the spleen were not modelled because they do not take up any observable amounts of gadoxetate.

Table 3 Fraction parameters defining the contents of each region of interest in the gadoxetate-enhanced magnetic resonance signal model.

Compartment	Fraction of Liver ROI	Fraction of Spleen ROI	Fraction of Vessel ROI
Blood Plasma	0.12	0.35	1.00
EES	0.20	0.20	
Hepatocyte	0.68		

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The volumes were also assumed to be known, and they were also obtained from the ‘standard human’: $V_{liver} = 1.43$ L, $V_{blood} = 4.51$ L, and $V_{EES} = 14.77$ L (104). Since gadoxetate does not enter the erythrocytes the blood plasma volume is also needed. Assuming a haematocrit of 0.43, the blood plasma volume in an average subject is 1.94 L.

Once the final model structure was identified a sensitivity analysis was performed to assess the sensitivity of the model predictions according to literature parameters. In the sensitivity analysis the model was simulated while varying each volume parameter ± 20 %.

3.4.2.5 Identification of Model Parameters

All model structures were parametrized using the estimation data by calculating the mean values from the ten healthy subjects. The estimation data included six time-points with observations from the liver, spleen, and blood vessels (a total of 6x3 data points). During the model parametrization the model predictions were compared to the estimation data by evaluating the size of the residuals, as described in the introduction (chapter 1.4.2). A global optimization algorithm, Simulated Annealing (105), modified to return not only the optimal parameters but also all parameters that passed the χ^2 test was used for the model parametrizations.

3.4.2.6 Model Analysis

The theory of the methods used here is described in the introduction, primarily in chapters 1.4.2 and 1.4.3.

The parameters identified for each model structure were analysed in three ways. First, the goodness-of-fit was analysed. This was performed by using the χ^2 test as described in the introduction. In the χ^2 test the degrees of freedom were chosen as the number of data points (18 for the estimation data, and nine for each set of validation data) minus the number of identifiable parameters (four for the estimation data, and 0 for the validation data) (80). Non-rejected models were passed on to the remaining two steps in the model analysis.

The second test concerns predictions. In this step, the core prediction methodology (87) was used by translating the uncertainty in the parameters into an uncertainty in the predictions. The basic idea was to determine a representative subset of all acceptable parameters (here obtained in the profile likelihood analysis), and then observe the combined predictions of the entire parameter set. Two different aspects of the model were evaluated using the core prediction methodology: (i) the amount of gadoxetate eliminated via the bile and urine, and (ii) the amount of gadoxetate residing within the blood plasma.

The third test concerns parameter identifiability. As previously mentioned, a parameter is (practically) identifiable if its value can be determined from data with a reasonably small uncertainty. This identifiability analysis was carried out using the profile likelihood method (86), as described in the introduction.

3.4.3 Applying the Mechanistic Model to the Chronic Liver Disease Cohort

The mechanistic model for gadoxetate transports in a human as described in the previous chapter (see Fig. 27) was applied to each subject in the chronic liver disease cohort. The methods for the model parametrization and analysis are described in this chapter.

3.4.3.1 Model Parametrization

The model was parametrized to the data in two different ways, by using: (i) the standard-two-stage (STS) method and (ii) the NLME method (chapter 1.4.4). Briefly, the basic principles of STS consist of parameterizing a model for each single subject independently of each other, and the population parameter distributions are derived by summarizing the results (see Fig. 29). The NLME methodology (see Fig. 29) can be summarized in the following two steps. First data from all the subjects in the population are used to determine the population distributions of the model parameters. Thereafter the individual subject data and the population parameters are used to determine individual patient parameter values (88).

Figure 29 Summary of the methods used to parametrize the model to the chronic liver disease cohort. In the **left panel** the ‘standard-two-stage’ (STS) method is depicted: the model is parameterized to each data element separately, thereafter the parameter values are combined in order to derive population parameter distributions. In the **right panel** the ‘non-linear mixed effect’ (NLME) method: first population parameter distributions are postulated, thereafter the distributions are parametrized to the entire dataset, finally the model is parametrized to each data element while following the population parameter distributions using a joint likelihood function.

Reproduced from: Forsgren MF, Karlsson M, Dahlqvist Leinhard O, et al. (2017) Quantitative Assessment of Liver Detoxification and Drug Transporter Functions Using Gadodotate-Enhanced MRI – A Liver Biopsy and Blood Sample Validated Prospective Study on Chronic Liver Disease. In Manuscript

3.4.3.1 NLME Model Definitions

In the NLME method the parameter distributions need to be defined and initialized. In this analysis the population distributions in the NLME model parametrization were defined as a normal distribution for the scale parameter (ξ) and lognormal distributions for the four rate parameters (k_{diff} , k_{ph} , k_{hp} , and k_{hb}). The distributions were *a priori* parametrized from the results of parametrizing the model to healthy human subjects (Table 3 in paper II), where the expectation values were $\xi = 1.6$, $k_{diff} = 1.7 \text{ ms}^{-1}$, $k_{ph} = 4.7 \text{ ms}^{-1}$, $k_{hp} = 28 \text{ ms}^{-1}$, and $k_{hb} = 38 \text{ ms}^{-1}$. The *a priori* standard deviations were chosen such that the optimization algorithm would not be unnecessarily limited ($\xi = 1$, $k_{diff} = 0.1 \text{ ms}^{-1}$, $k_{ph} = 0.1 \text{ ms}^{-1}$, $k_{hp} = 0.01 \text{ ms}^{-1}$, and $k_{hb} = 0.01 \text{ ms}^{-1}$).

3.4.3.3 NLME versus STS

In order to assess if the NLME model parameterization method outperforms the STS method (and also to assess if it is possible to reduce the examination time down to 10 min post gadodotate injection), the dataset for each subject was divided into estimation and validation parts. The estimation data included all data points up to 10 min post gadodotate injection and the validation data included the remaining points. Both the NLME and STS methods were fitted to the esti-

mation data and thereafter the model predictions were compared with the validation data. In order to assess the NLME method with a full database, a leave-one-out regime was used, in which for each subject the NLME method was used on the full datasets for all but the subject that was to be parametrized, which only had the reduced estimation data.

3.4.3.2 Distribution Volumes

The distribution volumes for gadoxetate in the subjects were estimated and defined as the sum of the intracellular space in the liver, the EES and the blood plasma volume. The liver volume was determined using the software package SmartPaint (106) on the 20 min post-contrast images (for the strongest separation between the liver and the surrounding tissues), by an experienced radiologist. The EES volumes and blood volumes were also estimated using the weight, height and gender of the subjects as previously published in (107-109). The blood volumes were adjusted for individual haematocrit levels by measuring the haemoglobin concentrations in the blood samples.

3.5 Statistical Tests

In all papers a p-value less than 0.05 was considered statistically significant. A α -level of 0.05 was used in all tests, unless otherwise stated.

3.5.1 Interim Analysis of Gadoxetate Fluxes in Chronic Liver Disease

Group differences were compared using unpaired Wilcoxon test. Receiver-operator characteristics (ROC) analysis was performed and the AUROC was calculated for each parameter to assess overall diagnostic accuracy.

3.5.2 Development of the Mathematical Model for Gadoxetate Fluxes

The statistical analyses used in the construction of the mathematical models in paper II are described in detail in the previous section. Briefly, model goodness-of-fit analysis was performed using a χ^2 test on the size of the residuals, parameter identifiability was investigated using profile likelihood, core prediction methodologies were applied to investigate the model predictions, and the model was validated using external data.

3.5.3 Quantification of Steatosis in Chronic Liver Disease

Pearson's correlation coefficient and linear regression were used when comparing the correlation between continuous variables. Cohen's kappa was used when comparing the agreement between categorical and stratified continuous variables. Spearman's rank correlation coefficient was used when comparing categorical and continuous variables. For continuous variables, the differences between the two groups were evaluated using a Student's t-test when the data were normally distributed and with a Mann-Whitney U-test when the assumption of

normality was not met. For dichotomous variables, differences were tested using the χ^2 test corrected for continuity.

AUROC was used to assess the overall diagnostic accuracy and to identify the optimal cut-off values.

The existence of subpopulations within the cohort in terms of PDFF was assessed by fitting a single lognormal (= no subpopulations) and a mixture of two lognormal distributions (= two subpopulations) to the ^1H -MRS PDFF. The Cramér-von Mises and Pearson's χ^2 test were used to test the hypotheses of no subpopulations and two subpopulations, respectively.

3.5.4 Applying the Mathematical Model for Gadoxetate Fluxes to the Chronic Liver Disease Cohort

The goodness-of-fit of the mechanistic model to data was investigated on a subject basis using a χ^2 test (see Introduction), with the degrees of freedom equal to the number of observations in the gadoxetate-enhanced MRI time series.

Group differences were investigated using an unpaired two-tailed Mann-Whitney U-test. A paired two-tailed Mann-Whitney U-test was used when comparing the parameter estimates from the two model parametrization methods. Linear regression and Lin's concordance correlation were used to investigate correlation between variables that measure or describe similar entities.

An analysis of variance (ANOVA) with Tukey's post-test was used to investigate sources of variation and biomarker performance between stages of fibrosis ($\alpha = 0.001, 0.01, 0.05$). In the variance calculations the following sources of variation were explored; liver fat content, gender, diagnosis, inflammation, fibrosis stage, BMI, and in the case of comparison with model prediction and biopsy as well as blood samples, the laboratory analysis batch was used.

3.5.5 Multimodal Identification of Liver Fibrosis in Chronic Liver Disease

The χ^2 test, Student's t-test, and Mann-Whitney U-test were used to investigate group differences where applicable, the latter two depending on whether the criterion for normality and equal variance were fulfilled. AUROC was used to investigate the diagnostic performance of the evaluated methods and to calculate optimal cut-off values.

An ANOVA was used to investigate potential confounders and the ability of the biomarkers to distinguish between distinct stages of fibrosis. In the ANOVA analysis, inflammation and the aetiology of liver disease were investigated as potential confounding factors.

4. RESULTS

4.1 Gadoxetate-Enhancement is Strongly Influenced in Subjects with Advanced Fibrosis

In the interim analysis of the chronic liver disease cohort, the late-phase gadoxetate-enhancement was investigated in terms of how the disease processes affects the enhancement pattern. Histopathological fibrosis staging was used as a measure of disease severity.

4.1.1. Study Population

In this interim analysis a total of 38 subjects were investigated. The final diagnosis and the histopathological fibrosis stage for the subjects are presented in Table 4.

Table 4 Final diagnosis and fibrosis scoring for the subject investigated in the interim analysis. Detail of the diseases can found in chapter 1.1.5.

Final diagnosis	F0	F1	F2	F3	F4
Normal	2				
NAFLD	5		3		2
PSC		2	2	4	
HCV		4	2	1	
AIH	1	3	2	1	1
PBC			1		1
AAT-deficiency				1	
Total no. of subjects	8	9	10	7	4

Reproduced from: Norén B, Forsgren MF, Dahlqvist Leinhard O, et al. (2013) Separation of advanced from mild hepatic fibrosis by quantification of the hepatobiliary uptake of Gd-EOB-DTPA. *Eur Radiol* 23:174-81

4.1.2 The Late-Phase Gadoxetate-Enhancement Pattern is Significantly Altered in Subjects with Advanced Fibrosis

The enhancement patterns in the liver and spleen are significantly affected by the fibrosis stage as is shown in Fig. 30. The mean normalised signal intensities are presented for the entire cohort stratified by fibrosis stage. A trend in which the amount of gadoxetate in the liver is reduced in subjects with advanced fibrosis can be observed, and the cirrhotic group is also clearly separated.

Figure 30 Normalised signal intensities for the liver and spleen ROIs stratified by histopathologically determined fibrosis stage. The data is presented as the mean and the error bars represent one standard deviation.

Reproduced from: Norén B, Forsgren MF, Dahlqvist Leinhard O, et al. (2013) Separation of advanced from mild hepatic fibrosis by quantification of the hepatobiliary uptake of Gd-EOB-DTPA. *Eur Radiol* 23:174-81

4.1.3 The Diagnostic Accuracy of the Identification of Advanced Fibrosis Using the Liver-to-Spleen Contrast Ratios and Hepatocyte Uptake Rate

The normalised liver-to-spleen contrast ratios (LSC_N) and the hepatocyte uptake rate (K_{Hep}) showed the highest diagnostic accuracy (Table 5). The chronic liver disease subjects were stratified into two groups by the histopathologically determined fibrosis stages; none to moderate fibrosis (F0-2, $n = 27$) and advanced fibrosis (F3-4, $n = 11$). The mean and standard deviation of LSC_N , K_{Hep} , and LSC are presented in Table 5. The highest diagnostic accuracy for the identification of advanced fibrosis was found for the normalised ratios, LSC_N_{10} and LSC_N_{20} , followed by the hepatic uptake rate, K_{Hep} . No significant differences were found for LSC_{10} and LSC_{20} .

Table 5 Diagnostic accuracy of gadoxetate-enhanced MRI parameters.

	F0-2	F3-4	p	AUROC	95% CI
<i>K_{Hep}</i>	0.44 ± 0.26 ^a	0.29 ± 0.25 ^c	0.050*	0.71	0.50-0.91
LSC_N10	1.41 ± 0.12 ^a	1.24 ± 0.14 ^c	0.004**	0.80	0.64-0.97
LSC_N20	1.56 ± 0.16 ^b	1.38 ± 0.16 ^d	0.010*	0.78	0.62-0.94
LSC_10	1.65 ± 0.20 ^a	1.50 ± 0.25 ^c	0.099	0.68	0.48-0.88
LSC_20	1.82 ± 0.27 ^b	1.66 ± 0.29 ^d	0.223	0.64	0.42-0.85

^a n = 27, ^b n = 26, ^c n = 11, ^d n = 10

Reproduced from: Norén B, Forsgren MF, Dahlqvist Leinhard O, et al. (2013) Separation of advanced form mild hepatic fibrosis by quantification of the hepatobiliary uptake of Gd-EOB-DTPA. *Eur Radiol* 23:174-81

4.2 A Minimal Mathematical Model for Gadoxetate Fluxes in Humans

Following the finding that the gadoxetate levels in the human liver were significantly lower in subjects with advanced hepatic fibrosis, we aimed to gain a deeper understanding of the mechanisms governing the gadoxetate fluxes. These mechanisms were therefore formalised into mathematical models. In this section the analysis of the different proposed model structures and the selection of a minimal mathematical model describing these fluxes in a human are presented. This chapter follows the analysis procedure outlined in chapter 3.4.2, followed by the model selection and finally a sensitivity analysis is performed for the literature derived constants.

4.2.1 Model Analysis

The model analysis discussed in this section are summarised in Table 6.

Table 6 Model selection and testing process summary

Model	Fits the estimation data?	Lowest cost for estimation data ^a	Passes the <i>ad hoc</i> constraint?	No of fitted parameters	No. of identifiable parameters ^b	Fits the validation data
M0	Yes	16.2 (20.3)	Yes	5	5	Yes
M1	Yes	16.2 (21.9)	Yes	6	3	Yes
M2	Yes	16.2	No	4	-	-
M3	Yes	20.5	No	6	-	-
M4	Yes	16.2	No	5	-	-
M5	No	332.0	-	4	-	-
M6	No	28.6	-	4	-	-
M7	No	63.1	-	5	-	-
M8	Yes	16.2 (21.9)	Yes	8	3	Yes
M9	Yes	16.2	No	6	-	-

^aThe cut-off for the chi-square test was 23.684. The value in the parenthesis is the lowest cost passing the *ad hoc* constraint. ^bAs defined by the profile likelihood-analysis.

Reproduced and modified from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

4.2.1.1 Step One – Can the Models Describe the Estimation Data?

An extensive global model parameterization was performed in order to evaluate if the model structures could describe the estimation data. Of the ten models three (M5-7; see Fig. 28) were rejected as they were not able to fit the estimation data (Table 6). The predictions of two of the evaluated model structures are presented in Fig. 31; panels A-C shows the model predictions for the final model (M0) and panels D-F shows the predictions of a rejected model (M7).

Figure 31 Model predictions of the gadoxetate-enhanced signal in the liver, spleen and vein after fitting the proposed model (M0, panels A-C) and a rejected model (M7, panels D-F) to the estimation data. The solid line represents the optimal values and the dashed lines represents the range of predictions found within the 95% parameter confidence interval as defined by profile likelihood analysis.

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4.2.1.2 Step Two – Core Predictions and ad hoc constraints

When the parameter vectors and model structures that passed the χ^2 test (i.e. could describe the estimation data) were investigated certain parameter sets and model structures were found to predict dose-normalised plasma concentrations that were not realistic. In previous studies, it has been shown that 3 h following the gadoxetate injection at least 1% of the administered dose resides within the blood plasma (54, 56). This is true for healthy subjects and in disease states there is even higher concentrations in the blood plasma (56). Concentrations below this limit are not physiologically realistic which suggests that even more parameters and models should be rejected. We therefore added an additional condition, an *ad hoc* constraint, which stated that the dose-normalised serum concentration at 3 h must be > 1%. As can be seen in Table 6 only three model structures were able to pass this condition (M0, M1, and

M8). In the following test only the parameter vectors resulting in predictions that passed the *ad hoc* constraint were investigated.

4.2.1.3 Step Three – Identifiability

An important goal was to achieve a model structure which was fully identifiable given the data, meaning that it should be possible to determine the parameter values with low uncertainty using the available experimental data (86). This identifiability criterion was analysed using profile likelihood. An example of a profile likelihood analysis is shown in Fig. 32 for model M0. The analysis of all remaining model structures (after step two) showed that only one single model was fully identifiable. A summary of this analysis can be found in Table 6 where the number of estimated model parameter is shown together with the number of identifiable parameters.

Figure 32 Profile likelihood parameter uncertainties for model M0. The solid lines show the profile likelihood versus parameter values given the estimation data. The intersections with the dashed line show the 95% confidence interval.

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

4.2.1.4 Step Four – Model Validation

All models that passed the second step were further evaluated using independent validation data. The validation data set included blood plasma samples obtained following the administration of gadoxetate with doses reaching up to 20 times higher amounts than those used in clinical practice (and in the estimation data). The param-

eter vectors used in the model validation were derived from step one (*i.e.* estimated using the estimation data), only the arbitrary scaling constant was re-calculated for the validation data, but kept equal for all three doses. The input function in the models was modified to mimic the experimental setup of the study from which the validation data was acquired. In Fig. 33 the agreement of the model predictions of blood plasma gadoxetate concentrations with the validation data is presented for model Mo. As can be seen by visual inspection, the model agrees reasonably well with the validation data. Moreover a χ^2 test confirmed this agreement statistically. All three remaining model structures (Mo, M1, and M8) were able to describe the validation data (Table 6).

Figure 33 The M0 model predictions of the blood plasma gadoxetate concentrations versus validation data. Panels A-C corresponds to gadoxetate doses 8, 14, and 20 times higher than the clinical dose. The blue lines are model predictions obtained using the same set of parameters in all three cases derived from the estimation data.

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

The model predictions of gadoxetate elimination (via the renal or biliary pathways) were also investigated using literature data for comparison. As can be seen in Table 7 there is good agreement with between the model and the literature. The increased certainty in the predicted elimination fractions due to the *ad hoc* constraint on the blood plasma concentrations is clearly shown in Table 7.

Table 7 Predictions of renal and biliary elimination

Route	Estimation data			Reference values	
	Predictions form the initial model fit	Predictions after applying the ad hoc constraint	Prediction based on the profile likelihood analysis		
Bile	38.4 - 75.4%	43.2 - 44.5%	38.4 - 63.5%	$31 \pm 17\%$ (56)	$36.8 \pm 8.5\%$ (54)
Urine	23.0 - 40.6%	39.0 - 39.7%	30.6-40.6%	$48 \pm 5\%$ (56)	$43.6 \pm 8.6\%$ (54)

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

4.2.2 Choosing a Minimal Model

As can be seen in Table 6 only the model structure designated Mo was able to fulfil all of the requirements. An important property of a useful model is that it is minimal given the estimation data and requirements. The different model structures that were explored corresponded to simplifications such as excluding certain fluxes (in M2–8), or by incorporating nonlinearities such as Michaelis-Menten expressions for describing some of the fluxes (M1 and M9) in an appropriate kinetic meaningful fashion (see Fig. 28). The analysis showed that a majority of the simplified or extended models were not able to describe the data in a fully identifiable or statistically acceptable manner (as shown in Fig. 31C–F for M7). Furthermore, the models that had nonlinear characteristics had at least three unidentifiable parameters (Table 6).

Thus Mo was chosen as the final model, the estimated parameter values and confidence intervals are presented in Table 8 for the healthy subjects.

Table 8 Estimated parameter values for the human gadoxetate flux model using ten healthy subjects

Parameter	Estimated value	Confidence interval ^a		Unit	Description
ξ	1.604	1.431	1.801	-	Scaling
k_{hb}	3.852e-4	2.108e-4	6.657e-4	s ⁻¹	Excretion rate
k_{diff}	1.731e-3	9.626e-4	2.915e-3	s ⁻¹	Diffusion rate
k_{ph}	4.776e-3	3.265e-3	6.682e-3	s ⁻¹	Hepatocyte uptake rate
k_{hp}	2.857e-4	4.593e-6	6.754e-4	s ⁻¹	Hepatocyte efflux rate

^aAs defined by the profile likelihood-analysis.

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

4.2.3 Sensitivity of the Liver Signal Predictions

Since literature values were used in the model to define the compartment volumes it is prudent to investigate what impact any alterations to these values would have on the model predictions. Thus a sensitivity analysis was performed in order to investigate how much the model predictions were affected by altering each volume constants by $\pm 20\%$. The results are presented in Fig. 34 and it is obvious that the final

model (Mo) was primarily sensitive to changes in the whole liver and EES volume constants.

Figure 34 Sensitivity analysis, the solid line indicates the nominal prediction and the dashed line corresponds to altering the volume parameter by $\pm 20\%$.

Reproduced from: Forsgren MF, Dahlqvist Leinhard O, Dahlström, N et al. (2014) Physiologically Realistic and Validated Mathematical Liver Model Reveals [corrected] Hepatobiliary Transfer Rates for Gd-EOB-DTPA Using Human DCE-MRI Data. *PLoS One* 9(4):e95700

4.3 A Proton Density Fat Fraction Cut-off of 3% Should Be Used for Detecting Hepatic Steatosis

For the purpose of investigating whether steatosis can reliably be detected non-invasively the entire chronic liver disease cohort was used. We measured quantitative liver fat using both ^1H -MRS PDFF and SPC of the biopsy samples and compared these findings with semi-quantitative histopathological steatosis grades.

4.3.1 Study Population

The suspected and final diagnoses of the study subjects are shown in Fig. 35.

Figure 35 Suspected and final diagnoses of study subjects with division into analysed subgroups.

Reproduced from: Nasr P, Forsgren MF, Ignatova, S et al. (2017) Using a 3% Proton Density Fat Fraction as a Cut-off Value Increases Sensitivity of Detection of Hepatic Steatosis, Based on Results from Histopathology Analysis. *Gastroenterology E-pub* March 9

4.3.2 ^1H -MRS PDFF and SPC has a Fair Agreement with the Semi-quantitative Histopathological Steatosis Grades

All biopsy samples were semi-quantitatively graded histopathologically from 0 to 3, where grade 0 corresponds to fat deposition in < 5% of the hepatocytes, which is per the conventional clinical definition absence of steatosis. In Fig. 36 the liver fat content quantified with ^1H -MRS PDFF and SPC is presented stratified by the histopathological steatosis grades. As can be seen in Fig. 36 histopathological steatosis grade 1, which is generally considered to include 33% fat in the biopsy samples, only corresponds to about 10% fat as estimated using ^1H -MRS PDFF and SPC.

Figure 36 A) Distribution of PDFF as quantified by ^1H -MRS and B) the steatotic area of the liver biopsy sample as measured with SPC in different histopathological steatosis grades.

Reproduced from: Nasr P, Forsgren MF, Ignatova, S et al. (2017) Using a 3% Proton Density Fat Fraction as a Cut-off Value Increases Sensitivity of Detection of Hepatic Steatosis, Based on Results from Histopathology Analysis. *Gastroenterology E-pub* March 9

4.3.3 Liver Fat Estimated with ^1H -MRS PDFF and SPC Strongly Correlates

Liver fat content measured with ^1H -MRS PDFF and SPC were then compared to each other (see Fig. 37). The Pearson correlation between the two different methods were found to be excellent ($r = 0.92$; $p < 0.001$).

In Fig. 37 the semi-quantitative histopathological grading is also shown, where the blue circles correspond to the absence of steatosis (grade 0). As can be seen, all of the non-steatotic livers are found to have less than 3% liver fat. It is also apparent that there is a slight overestimation of liver fat content using SPC as compared to ^1H -MRS PDFF, a linear regression was found to have a slope of 1.20.

Figure 37 Correlation between PDFF (%) as quantified with ^1H -MRS and the steatotic area of liver biopsy sample (%) as determined using SPC. For values <5% the correlation is displayed with a higher resolution in the inset.

Reproduced from: Nasr P, Forsgren MF, Ignatova, S et al. (2017) Using a 3% Proton Density Fat Fraction as a Cut-off Value Increases Sensitivity of Detection of Hepatic Steatosis, Based on Results from Histopathology Analysis. *Gastroenterology E-pub* March 9

4.3.4 Determination of ^1H -MRS and SPC Cut-off Values for the Diagnosis of Hepatic Steatosis

Next, we investigated diagnostic performance (AUROC) for ^1H -MRS PDFF and SPC for the detection of hepatic steatosis as diagnosed by a pathologist. Histopathology was used to define absence (grade 0) and presence (grade 1-3) of steatosis. The AUROC values for the methods were similar; 0.97 (0.93-1.00) for ^1H -MRS PDFF, and 0.98 (0.96-1.00) for SPC.

The optimal quantitative cut-off values for steatosis were investigated by evaluating the sensitivity and specificity for a range of plausible values (Table 9). In other words, we investigated how much liver fat is present when the pathologist diagnoses a subject as steatotic, which corresponded to a semi-quantitative fat content of 5%. The conventional cut-off for steatosis is 5.0%, by using this cut-off all subjects with SPC $\geq 5.0\%$ ($n = 31$) were also identified by the histopathologist as having steatosis (100% specificity). Of the 63 subjects with SPC < 5% 16 were diagnosed with steatosis (64% sensitivity). All subjects with ^1H -MRS PDFF $\geq 5.0\%$ ($n = 25$) had steatosis, according to the histopathologist (100% specificity). Of the 69 subjects with ^1H -MRS PDFF < 5.0% 22 were diagnosed with steatosis by the histopathologist (53% sensitivity). The sensitivity and specificity for lower cut-off values for ^1H -MRS PDFF and SPC are shown in Table 9. As can be seen a 100% specificity is found using a cut-off of 3.0% (both methods), this lower cut-off drastically increases the sensitivity to about 80%.

Table 9 Diagnostic accuracy of ¹H-MRS PDFF and SPC for the absence vs. presence of histopathological steatosis.

Cut-off value	¹ H-MRS PDFF		SPC	
	Sensitivity	Specificity	Sensitivity	Specificity
5.0%	53%	100%	64%	100%
3.0%	79%	100%	81%	100%
2.0%	87%	94%	87%	94%
1.4%	98%	89%	96%	81%

Reproduced from: Nasr P, Forsgren MF, Ignatova, S et al. (2017) Using a 3% Proton Density Fat Fraction as a Cut-off Value Increases Sensitivity of Detection of Hepatic Steatosis, Based on Results from Histopathology Analysis. *Gastroenterology E-pub* March 9

4.4 The Mathematical Model for Gadoxetate Fluxes is Applicable on a Chronic Liver Disease Cohort

The findings that the gadoxetate-enhancement pattern is significantly lower in subjects with chronic liver disease (as determined by advanced fibrosis; chapter 4.1) led to the development of the mathematical model that describes gadoxetate fluxes in healthy subjects (chapter 4.2). The next step was to apply the model and the gadoxetate quantification methods to the complete chronic liver disease cohort. We also used a new parametrization method (NLME) to enhance model parametrization. In this section, the term model framework refers to the complete package of (i) the gadoxetate quantification methods, (ii) the gadoxetate flux model, and (iii) the model parametrization approach using NLME.

4.4.1 Study population

A total of 91 of 100 recruited subjects were used in this study; a few subjects were rejected due to data quality issues and subjects not willing to participate further in the MR examination. The demographic characteristics and clinical diagnosis of the final study population are presented in Table 10. Importantly none of the subjects included showed signs of hepatic decompensation. Only one was classified as Child-Pugh class B (seven points), all other were Child-Pugh class A (median five points).

Table 10 Demographics and clinical description of the final study population

Clinical diagnosis ¹	di-	Sex (no of subjects)		Age (years)	BMI ² (kg/m ²)	Bilirubin (μM)	AST ³ (μkat/L)	ALT ⁴ (μkat/L)	ALP ⁵ (μkat/L)	Fibrosis stage (no of subjects)				
		Male	Female							F0	F1	F2	F3	F4
Normal		5	3	50 (33-65)	27.6 (20.9-33.6)	11 (5-12)	0.78 (0.44-1.50)	1.25 (0.38-2.6)	2.15 (0.78-3.60)	7	1	-	-	-
NAFLD		26	9	62 (23-81)	27.4 (20.3-35.0)	10 (5-31)	0.65 (0.29-2.40)	0.91 (0.35-3.30)	0.97 (0.51-5.50)	16	5	7	4	3
HCV		4	4	49 (39-71)	24.8 (16.9-32.6)	12 (5-17)	1.01 (0.32-3.50)	1.07 (0.48-5.80)	0.96 (0.64-1.50)	-	3	4	-	1
PSC		8	5	39 (20-78)	24.5 (21.9-34.9)	10 (7-19)	0.95 (0.39-4.50)	1.60 (0.19-8.40)	3.20 (1.50-10.00)	2	2	5	4	-
PBC		1	3	59 (48-76)	26.9 (24.2-31.5)	8 (4-29)	1.33 (0.34-2.90)	1.05 (0.38-3.30)	3.80 (1.40-9.70)	-	-	3	-	1
AIH		3	9	45 (22-67)	23.8 (20.3-29.7)	15 (5-27)	0.75 (0.34-2.90)	1.25 (0.32-9.10)	0.89 (0.74-1.90)	2	3	4	3	-
AIH-PBC overlap		-	1	72	19.6	10	2.20	4.50	7.00	-	-	-	1	-
AIH-PSC overlap		1	1	29, 28	20.7, 29.4	19, 48	1.40, 2.40	3.80, 1.80	2.50, 6.60	-	-	-	1	1
AAT deficiency		1	1	66, 25	20.2, 26.3	12, 5	0.56, 0.49	0.31, 1.20	0.43, 1.40	-	1	-	1	-
Hemo-chromatosis		1	-	61	22.4	9	0.42	0.62	1.10	1	-	-	-	-
DILI		-	2	62, 28	20.4, 21.5	8, 8	0.61, 0.68	0.78, 1.20	0.79, 1.60	-	-	2	-	-
Wilson's disease		-	1	45	21.1	6	0.42	0.93	1.40	-	1	-	-	-
Alcoholic liver disease		-	2	61, 64	27.0, 24.0	25, 4	2.20, 0.98	2.70, 0.59	2.00, 1.80	1	-	-	-	1
All diagnoses		50	41	53 (20-81)	26.4 (16.9-35.0)	11 (4-48)	0.75 (0.29-4.50)	1.10 (0.19-9.10)	1.20 (0.43-10.00)	29	16	25	14	7

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4.4.2 The Model Framework is Applicable to Chronic Liver Disease

First, we tested if the model framework was applicable on a cohort of subjects suffering from low grade chronic liver disease. As in the model development, a χ^2 test was used to test if the model was able to describe the data. All but one subject in the cohort could be described using the model framework. The test statistics are shown in (see Fig. 38A). The different χ^2 -values is a consequence of a varying number of data points, for instance additional time points were added later in study, and in some cases also that subjects aborted the examination prematurely. In Fig. 38 the model predictions and MRI data for five subjects are shown with varying stages of liver fibrosis. As can be seen the amount of gadoxetate in liver parenchyma is lower in the presence of liver fibrosis.

Figure 38 In panel A, a summary of the statistical test is shown, with one line per subjects. As is demonstrated the model failed to describe a single subject accurately (which is shown in panel B). In panels C-G model predictions and data are shown for increasing fibrosis stage (F0-F4), where G shows data from a subject suffering from cirrhosis.

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4.4.3 Validation of the Model Framework using Blood Samples and Biopsy Samples

Both blood samples and biopsy samples were obtained following the MR examinations and analysed chemically for gadolinium content. In total eighty-eight blood samples ($n = 88$) and eighty ($n = 80$) biopsy samples were analysed.

The concentrations (both measured and predicted) were corrected for systematic differences in gadoteric acid doses and distribution volumes. The adjustment factor for the model predictions were 0.37, the median factor in all subjects were 0.36 (range 0.31 to 0.47). The adjustment factors were normalised to the model prediction adjustment factor such that the model predictions were multiplied with 1 and the median subject adjustment factor was 0.96 (range 0.82 to 1.27).

Following this, ANOVA models were used to investigate the differences between measured blood and biopsy concentrations of gadoteric acid, and those predicted by the model framework. Seven possible confounders were included in the analysis: fat content, laboratory analysis batch number (there were three separate batches), gender,

diagnosis, inflammation stage, fibrosis stage, and BMI. Only the analysis batch number was found to be a significant source of variation in both the blood samples (p -value = 0.002), as for the liver biopsy sample both the laboratory batch number (p -value = 0.004) as well as the fibrosis stage (p -value = 0.030) was significant. In the following analysis, the data was also corrected for the laboratory batch differences by using the group means for the batch numbers in the ANOVA models.

The comparison between the measured and predicted values is shown in Fig. 39. There was a non-significant difference between the model predictions and measured blood (Mann-Whitney U-test; p = 0.24) as well as biopsy (p = 0.76) levels of gadoxetate. Lin's concordance correlation, which penalizes divergence from the line of identity, was found to be high for the blood levels (r_c = 0.62) low for the biopsy levels (r_c = 0.31).

Figure 39 Blood samples and biopsy samples validate the model predictions. The agreement between the measured and prediction blood levels of gadolinium is shown in panels A-B, and the biopsy agreement is shown in panels C-D

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4.4.4 The Hepatic Accumulation of Gadoxetate is Significantly Affected in Subjects with Fibrosis

Similar to what was found in the interim analysis (chapter 4.1) the full cohort had significant changes in the gadoxetate enhancement pattern in the liver as can be seen in Fig. 40. Noteworthy is that in this full cohort the advanced fibrosis stage F3 also clear stands out from the rest.

An ANOVA analysis including six possible sources of variation was used to investigate the model predicted hepatocyte uptake rate of gadoxetate (k_{ph}). These included; liver fat content gender, diagnosis inflammation stage, fibrosis stage, and BMI. The result of the analysis was that only fibrosis had a significant affect ($p = 0.003$).

The model parameters ability to distinguish between subjects with probable reduced liver function associated with the disease processes driving their fibrogenesis were investigated using ANOVA analysis with Tukey's post-test. As can be seen in Fig. 40 the hepatocyte uptake rate (k_{ph}) was sensitive enough to differentiate between subjects due to their small reduction of their liver (OATP1) function associated with the fibrosis stage Fo/1 and F3/4 ($p = 0.05$) and between Fo and F4 ($p = 0.01$). The hepatocyte excretion (k_{hb}) was only affected in more severe disease stages, allowing for a separation between F 2/3 and F4 ($p = 0.01$) and Fo/1 and F4 ($p = 0.001$).

Figure 40 In panels A-B the gadoxetate enhancement pattern is shown for the choric liver disease cohort stratified by fibrosis stage. In panels C-D the model parameters for OATP1 and MRP2 hepatocyte function is shown, stratified by fibrosis stage. The vertical lines indicate significant differences in an ANOVA (Tukey's post-test with p-values: * = 0.05; ** = 0.01; *** = 0.001). In panel E, the less detailed model for hepatocyte uptake is shown. Panels F-G show the data for simple ratio based methods, which are strongly affect by structural changes (rather than purely loss of function).

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4.4.5 The Model Parameters Correlates with Clinical Variables

The model parameters correlation to clinical markers of hepatic disease was investigated by performing Pearson correlation with the serum markers bilirubin, albumin, and the AST/ALT ratio (see Fig. 41). Bilirubin is taken up into the hepatocytes by the same transporters as gadoxetate (OATP1). Albumin is reduced in chronic liver disease (a level below 35 g/L is considered low). Elevated AST/ALT ratios are indicative of liver disease.

The model parameter for hepatocyte uptake (k_{ph} ; OATP1 function) had a low negative correlation ($r = -0.22$; $p = 0.04$) with bilirubin, thus a high serum bilirubin correlated with low gadoxetate uptake. The hepatocyte excretion rate (k_{hb} ; MRP2 function) also had a low but positive correlation ($r = 0.21$; $p = 0.05$) with bilirubin, thus high bilirubin levels correlated with increased gadoxetate excretion into the bile.

The albumin levels had a high positive correlation with the hepatocyte uptake rate ($r = 0.52$; $p < 0.001$), thus low serum albumin levels correlated with low gadoxetate uptake. There was a low and borderline non-significant negative correlation with the hepatocyte excretion rate ($r = -0.19$; $p = 0.06$).

Interestingly, the AST/ALT ratio was only moderately negatively correlated with the hepatocyte uptake rate ($r = -0.36$; $p < 0.001$), thus a high ratio correlated to a reduced accumulation of gadoxetate. There was no correlation between the AST/ALT ratio and the hepatocyte elimination ($r = 0.00$; $p = 0.94$).

Figure 41 Correlation of the model parameters for hepatocyte uptake of gadoxetate (panels A-C) and hepatocyte elimination of gadoxetate (panels D-F) with clinical variables.

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4.4.6 Shorter Examinations Times is Possible using NLME

As a 30 or 40 min long gadoxetate-examinations are too expensive in a clinical situation, we wished to explore if it was possible to reduce this time. Moreover, we wished to investigate the value of NLME as compared to the traditional STS methods with sparse data. How much does NLME improve analysis of sparse data? The experiments were carried out by first parametrizing the model to each subject's estimation data (the first 10 min; see materials and subjects) and thereafter we assessed the goodness-of-fit of the estimation, as well as of the validation data (the remaining late gadoxetate phase data). Due to insufficient late-phase data only eighty-seven subjects were used in this sub-analysis.

Both the NLME and STS model parametrization methods produced model predictions that passed the χ^2 test for the estimation data in all subjects. The NLME method produced model predictions that were able to accurately predicted the data in eighty-one subjects (93%) in the validation dataset, whereas the STS method produced predictions that were able to predict the data from only thirty-seven (43%) subjects in the validation dataset.

When the NLME parameter values for the reduced set with the full data set were analysed, no significant group difference of the parameter values was observed for hepatocyte uptake (k_{ph} ; Mann-Whitney U-test; $p = 0.82$) and biliary excretion (k_{hb} ; $p = 0.70$). Lin's concordance correlation was high for the hepatocyte uptake ($r_c = 0.98$) and low for the elimination ($r_c = 0.09$). The efflux from the hepatocytes back to the blood plasma was significantly different ($p < 0.001$), although in both cases (full and estimation data set) it was so low that there was virtually no flow of gadoxetate via this route.

4.5 Identification of Liver Fibrosis

Fibrosis is an important aspect of liver disease, and we found that gadoxetate-enhanced MRI (chapter 4.1 and 4.4) can be used to identify such loss of function associated with liver disease. In this concluding study, we evaluated a range of potential non-invasive methods to stage liver fibrosis including gadoxetate-enhanced MRI, serum fibrosis algorithms, MRE, ^{31}P -MRS, and TE. The histopathological fibrosis staging was used as a 'golden standard', albeit imperfect such.

4.5.1 Study Population

In this analysis the entire chronic liver disease cohort was used. However, as not all subjects were successfully examined with all methods a total of ninety subjects were at the most used (see Fig. 42). These ninety subjects were selected as they had both full gadoxetate-enhanced MR data as well as serum fibrosis algorithms. MRE was added in the year 2012, TE was not acquired in all subjects, and in 2009 there were technical issues with the multinuclear hardware that reduced the total number of subjects successfully analysed with ^{31}P -MRS.

Subjects evaluated because of
chronically elevated levels of serum
aminotransferases and/or alkaline
phosphatase

Subjects with incomplete gadoxetate-
enhanced MRI data removed from
the analysis

Figure 42 Available data

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Demographic, clinical, biochemical, and histological data are presented in Table 11. The diagnosis ‘normal’ is used where the biopsy showed normal histological features. None of the subjects included showed signs of hepatic decompensation. Only one subject had Child-Pugh class B (seven points), and all other subjects had Child-Pugh class A (median five points).

4.5.2 ³¹P-MRS and Modelling of Late-Phase Gadoxetate-Enhanced MRI were Not Strongly Correlated with Methods Directly Measuring Fibrosis (in Mild Fibrosis)

In order to investigate if the different aetiologies and inflammation grades affected the fibrosis staging ANOVA was applied to all evaluated methods. Only the gadoxetate-enhanced methods K_{Hep} , LSC₁₀, and the serum fibrosis biomarker NAFLD fibrosis score was significantly affected by both diagnosis as well as fibrosis. All other methods were only slightly affected by fibrosis stage.

Since the different methods evaluated in this study measure fundamentally different aspects of the liver (see Fig. 43) we wished to explore how these related to each other by using correlation (see Fig. 44).

Table 11 Demographics, laboratory characteristics, and diagnosis of the cohort.

Variable	Entire cohort (n = 90)	None to mild fibrosis (F0-2; n = 69)	Advanced fibrosis (F3-4; n = 21)	p
<i>Demographics</i>				
Gender (n; % males)	Female = 41, Male = 49 (54%)	Female = 28, Male = 41 (59%)	Female = 13, Male = 8 (38%)	0.142 ^a
Age (year)	52.5 (20-81)	52 (20-81)	62 (22-81)	0.280 ^b
BMI (kg/m²)	26.4 (19.6-35.9)	26.3 (20.1-35.5)	27.8 (19.6-35.9)	0.989 ^b
Diabetes/IGT (n; %)	18 (20%)	13 (19%)	5 (24%)	0.852 ^a
<i>Laboratory characteristics</i>				
Platelet count (10⁹/L)	244 (60-441)	256 (60-400)	185 (76-441)	0.048^b
Prothrombin time (INR)	1.0 (0.8-1.3)	1.0 (0.8-1.2)	1.1 (0.9-1.3)	<0.001^c
Bilirubin (mg/dL)	0.6 (0.2-2.8)	0.6 (0.2-1.7)	0.8 (0.4-2.8)	0.007^c
ALP (U/L)	72 (26-600)	66 (31-582)	120 (26-600)	0.077 ^c
AST (U/L)	45 (17-270)	39 (17-270)	78 (21-210)	0.001^c
ALT (U/L)	66 (11-546)	56 (11-546)	84 (19-348)	0.106 ^c
Child-Pugh class	A = 89, B = 1	A = 69, B = 0	A = 20, B = 1	
<i>Histological features</i>				
Fibrosis stage (n; %)	F0 = 28 (31%) F1 = 16 (18%) F2 = 25 (28%) F3 = 14 (16%) F4 = 7 (8%)			
Moderate/pronounced inflammation (n; %)	38 (43%)	25 (37%)	13 (62%)	
<i>Diagnosis</i>				
Normal (n; %)	8 (9%)	8 (12%)	-	
NAFLD (n; %)	34 (38%)	27 (39%)	7 (33%)	
HCV (n; %)	8 (9%)	7 (10%)	1 (5%)	
PSC (n; %)	13 (14%)	9 (13%)	4 (19%)	
AIH (n; %)	12 (13%)	9 (13%)	3 (14%)	
PBC (n; %)	4 (4%)	3 (4%)	1 (5%)	
AIH-PBC overlap (n; %)	1 (1%)	-	1 (5%)	
AIH-PSC overlap (n; %)	2 (2%)	-	2 (10%)	
AAT deficiency (n; %)	2 (2%)	1 (1%)	1 (5%)	
Hemochromatosis (n; %)	1 (1%)	1 (1%)	-	
DILI (n; %)	2 (2%)	2 (3%)	-	
Wilson's disease (n; %)	1 (1%)	1 (1%)	-	
Alcoholic liver disease (n; %)	2 (2%)	1 (1%)	1 (5%)	

^a χ^2 test, ^b Student t-test, ^c Mann-Whitney U-test.

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Figure 43 Summary of the evaluated method for staging fibrosis. (A) The gold standard – liver biopsy – uses a needle to remove a specimen of liver tissue which is analysed by a pathologist. (B) Elastographic methods apply mechanical waves which propagate into the liver. A soft and healthy liver dampens the waves more efficiently than a stiff fibrotic liver. The elasticity is calculated by analysing the wave propagation. (C) ^{31}P -MRS allows for measuring PDE and PME in the cell membrane, which reflects cell turnover, as well as ATP and Pi in the cells. (D) Gadoxetate (Gd) is taken up into the hepatocytes by OATP1 and to some extent NTCP. Gadoxetate is subsequently excreted into the bile canaliculi by MRP2. These transporters play crucial physiological roles. Gadoxetate increases the signal intensity in MR images. Series of late enhancement phase images can be used to estimate the function of OATP1 and MRP2. There are also methods that quantify signal intensity ratios. (E) Antibodies, enzymes, and other metabolites can be measured in blood samples. The measurements can be combined in serum fibrosis algorithms which directly couple to the fibrotic processes.

S, sinusoid; SD, space of Disse; ECM, extracellular matrix; SC, Stellate cells; BC, bile canaliculi; H, Hepatocyte.

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Figure 44 Correlation between the methods analysed. Each element in the matrix represented the correlation coefficient between the two methods. The coefficients are grouped into: very high $\pm[0.9; 1.0]$, high $\pm[0.7; 0.9]$, moderate $\pm[0.5; 0.7]$, low $\pm[0.3; 0.5]$, negligible $\pm[0; 0.3]$, and non-significant correlations.

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Noteworthy there was a trend between TE and MRE ($p = 0.06$; $\rho = 0.34$), likely due to limited number of subjects with advanced fibrosis in the cohort analysed with both MRE and TE. This is important as TE has a low dynamic range for advanced fibrosis (see Fig. 45). The correlation analysis showed that AC (determined using ^{31}P -MRS and subjects mainly with mild fibrosis) was uncorrelated with gadoxetate transfer, elastographic, and serum fibrosis algorithms. The model based function measurements from late-phase gadoxetate-enhanced MRI (OATP1 and MRP2 function) and K_{Hep} was negligible to low correlated with the direct fibrosis markers. However, there was one exception; the uptake of gadoxetate (OATP1) was highly correlated with MRE.

4.5.3 Discrimination of Advanced Fibrosis (F0-2 vs. F3-4)

All evaluated methods are shown in Fig. 45 and Fig. 46; they were stratified by fibrosis stage. In Table 12 the diagnostic accuracy and cut-off values for detecting advanced fibrosis are presented.

Subjects with advanced fibrosis could be identified with all of the gadoxetate-enhanced MRI methods with AUROC values in the range of 0.68-0.81, even though their liver function was not strongly reduced (none of the subjects suffered from decompensated fibrosis and all but one had Child-Pugh class A). The gadoxetate-enhancement ratio LSCN_10 had the highest of AUROC for fibrosis. The sensitivities were 71% for all gadoxetate-enhanced MR biomarkers, whereas specificities varied (LSC_N10 had the highest specificity of 80%, closely followed by the hepatocyte uptake of gadoxetate (OATP1 function; k_{ph}) with 75%).

Both elastography methods showed the highest AUROC values of all evaluated methods, with 0.93 for MRE and 0.84 for TE. MRE had the highest sensitivity and specificity of all evaluated methods (83% and 90%), closely followed by TE (86% and 84%). MRE could also uniquely distinguish between fibrosis stages 2 and 3. Phosphorous spectroscopy was not able to identify advanced fibrosis (N.B., there were few stage 4). All serum fibrosis algorithms presented significant AUROC values (0.67-0.82), with APRI, GUCI, and King's generating values > 0.80 . With the exception of Forn's score (sensitivity of 65%) all blood panels had a sensitivity of 71%. Forn's score, King's score, and APRI all presented with a specificity of 78%.

Table 12 Receiver-operator characteristics and cut-off values for each fibrosis biomarker. AUROC values presented with 95% confidence interval. Optimal cut-off values are unit less with the exception of MRE (kPa), TE (kPa), k_{ph} (ms^{-1}), k_{ph} (ms^{-1}), and K_{Hep} (s^{-1}).

Detection of advanced fibrosis (F0-2 vs. F3-4)					
Fibrosis biomarker	AUROC	<i>p</i>	Cut-off	Sens	Spec
<i>Elastography</i>					
MRE	0.93 (0.84-1.00)	0.001	2.598	83%	90%
TE	0.84 (0.71-0.97)	<0.001	10.150	86%	84%
³¹ P MRS					
AC	0.54 (0.37-0.71)	0.624	-	-	-
<i>Gadoxetate-enhanced MRI</i>					
k_{ph} (OATP1)	0.76 (0.65-0.88)	<0.001	1.956	71%	75%
k_{hb} (MRP2)	0.68 (0.56-0.80)	0.012	0.143	71%	49%
K_{Hep}	0.72 (0.61-0.84)	0.001	0.205	71%	62%
LSC_N10	0.81 (0.69-0.93)	<0.001	1.276	71%	80%
LSC10	0.77 (0.65-0.90)	<0.001	1.497	71%	74%
<i>Serum fibrosis algorithms</i>					
GUCI	0.82 (0.72-0.92)	<0.001	0.631	71%	75%
King's score	0.81 (0.70-0.91)	<0.001	12.315	71%	78%
APRI	0.80 (0.70-0.90)	<0.001	0.702	71%	78%
FIB-4	0.75 (0.62-0.89)	<0.001	1.420	71%	73%
Lok Index	0.75 (0.60-0.90)	0.001	0.264	71%	71%
NIKEI	0.75 (0.62-0.89)	<0.001	-0.987	71%	73%
NCRS	0.75 (0.62-0.88)	0.001	0.303	71%	64%
Forn's score	0.73 (0.60-0.86)	0.002	5.700	65%	78%
NFS	0.67 (0.52-0.82)	0.018	-2.513	71%	49%

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Figure 45 Fibrosis biomarkers stratified by the stage of fibrosis (F0-4). The asterisks represent Tukey's post-test at $\alpha = 0.05^*$, 0.01^{**} , 0.001^{***} . (**A**, **B**) shows the ultrasound and MR based elastography biomarkers. The ^{31}P -MRS based AC is shown in (**C**). (**D**, **E**) shows the parameter representing the function of OATP1 and MRP2, derived from a model-based analysis of gadoxetate-enhanced MRI. (**H**) shows the hepatic accumulation rate and (**F**, **G**) shows the ratios between the liver and spleen at 10 min, all of which are also based on gadoxetate-enhanced MRI.

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Figure 46 Fibrosis biomarkers stratified by the stage of fibrosis (F0-4). The asterisks represents Tukey's post-test at $\alpha = 0.05^*$, 0.01^{**} , 0.001^{***} . (A-I) shows nine commonly used blood panel scores.

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5. DISCUSSION

This discussion begins with my thoughts on a MR based non-invasive liver biopsy followed by a discussion on the results from papers I-V listed by application (*i.e.* function, steatosis, and fibrosis). Finally, some limitations on the papers and methods are discussed.

5.1 A Non-Invasive Liver Biopsy

There are pathophysiological manifestations in the liver parenchyma that are common in different chronic liver diseases and they are important to monitor. The combination and extent of the manifestations depends on the aetiology and severity of the disease. These manifestations include inflammation, fat inflation, and fibrosis. As the disease develops there is also concurrent loss of liver function (see Introduction). Currently, an invasive liver needle biopsy is required to determine the etiology and to stage and grade the pathophysiological manifestations. However, recent guidelines have endorsed the use of non-invasive methods for screening purposes (110). As discussed in the Introduction there are several disadvantages with a liver biopsy, for instance only a minute part of the liver is evaluated and for many reasons continues monitoring by liver biopsy is not feasible. Hence, there is a need for comprehensive non-invasive methods to accurately grade and stage the pathophysiological manifestations of liver disease.

Ideally a ‘non-invasive liver biopsy’ should be able to accurately grade the inflammation, grade steatosis, stage fibrosis and cirrhosis, measure the iron loading in the liver parenchyma, as well as quantify liver function. For these purposes, MR is a uniquely suitable imaging modality. MR can image a multitude of functional aspects of soft tissues as well as image morphology. The following sub-chapters briefly discuss the MR methods that I currently believe are the strongest candidates for such a non-invasive liver biopsy. It is not within the scope of this discussion and has not been my intent to give a thorough review of all possible MR methods; this is a summary of the methods that I think are most suitable.

5.1.1 Grading Inflammation

Currently no established method exists for grading of hepatic inflammation non-invasively using MR. There are, however for this purpose, promising reports from ongoing efforts into exploiting the various visco-elastic parameters determined using multi-frequency MRE. In contrast to a typical MRE examination, where the liver is vibrated with a single frequency, multi-frequency MRE includes at least two additional excitation frequencies. By investigating the visco-elastic properties at more frequencies it is possible to calculate the frequency

response, *i.e.* the slope for the visco-elasticity vs. excitation frequency. The early studies have indicated that this response, or wave scattering coefficient, has the potential to grade liver inflammation independently from the underlying fibrosis. The diagnostic accuracy (AUROC) for identifying mild to severe inflammation with multi-frequency MRE was reported to be as high as 0.84 (111-113).

5.1.2 Grading Steatosis

PDFF either by MR imaging or spectroscopy is by far the most suitable biomarker for grading steatosis non-invasively using MR. PDFF closely correlates with the liver triglyceride concentrations measured with biochemical assays. Moreover, when used correctly, PDFF represents a fundamental property of tissue that reflects the concentration of fat in the tissue. PDFF is also independent of acquisition parameters, platform, manufacturer, centre, and field strength (67). A benefit with using MRI based PDFF is that the entire liver can be investigated. On the other hand, ^1H -MRS PDFF is more accurate for low fat concentrations.

5.1.3 Staging Fibrosis

There is ample evidence that MRE is a superior method for non-invasive fibrosis staging, as reported in for instance (77, 113-115). Of the different MRE methods available (see Introduction) 3D MRE allows for assessing the full spectra of visco-elastic properties; including the shear modulus, storage modulus (representing elasticity), and loss modulus (representing viscosity). Studies have shown that the fibrosis stage mainly correlates with tissues elasticity, the wave scattering of the shear modulus mainly correlates with inflammation grade (requiring multi-frequency MRE as discussed above), and portal hypertension with viscosity (113). Multi-frequency 3D MRE could also allow for standardizing and platform independent reporting of MRE (111, 116).

In order to be able to accurately calculate the visco-elastic properties it is also important that the transducers induce motion in a correct manner (see Introduction). Specifically, it is important that the transducers produce pure waveforms without off-resonance harmonics, do not induced image artefacts, and operate with constant amplitude for reasonable frequency ranges. The common pneumatic and electromagnetic transducer designs more or less suffer from some of these limitations (117). A novel 'gravitational' MRE transducer has recently been developed and the first results looks very promising (117, 118); this could potentially prove to be a superior transducer design.

5.1.4 Iron Loading

As discussed in the Introduction hepatic iron content increase T_2^* , which can be measured with for instance MR relaxometry. However, because T_2^* is part of the signal models used when calculating MRI-PDFF based on multi-echo Dixon imaging (119-121) it is possible to calculate T_2^* at the same. As such if multi-echo Dixon imaging is acquired for the purpose of evaluating liver steatosis a meas-

urement of T_2^* , closely relating to liver iron content, can be made available ‘for free’.

5.1.5 Liver Function

There are many potential causes of a reduction in liver function, including advanced chronic liver disease and acute drug-induced liver injury. This means that the accurate measurement of liver function is important both in a clinical setting as well as in drug trials (*e.g.* assess drug toxicity). Liver function, relating to the hepatocytes ability to transport *e.g.* toxins and bile salts out of the blood and into the bile can be evaluated by analysing gadoxetate-enhanced MRI.

When analysing gadoxetate-enhanced MRI it is important to ensure that the data is consistent in both time and space such that changes due pathology can be detected (95). Therefore I think that the methods used in this thesis to ensure such consistency are essential, *i.e.* using Dixon imaging with suitable reconstruction and correction methods (94, 95).

The late gadoxetate-enhancement phases are needed in order to be able to quantify both the uptake of gadoxetate from the blood by OATP1 as well as excretion into the bile by MRP2. Different methods for analysing the transport of gadoxetate by the hepatocytes have been proposed, but since there are no direct comparisons between them it is not possible to state which is superior. Together with Karageorgis *et al.* we showed that one such method to quantify the hepatocytes uptake and excretion functions was robust in a four centre trial (122). This finding indicates that the quantitative analysis of gadoxetate-enhanced MRI can be made platform independent.

I strongly believe that a mathematical description, which takes into account all major transfer routes within the time-frame of the images, is crucial in order accurately measure the important functions of the liver, such as drug clearance and bile salt excretion. Ideally methods will be developed that allows for automated regional whole liver investigation of liver function. Until these methods are mature ROI based analysis has to be used, which might be sufficient in most applications.

5.1.6 A Non-Invasive Liver Biopsy

It is my opinion that a non-invasive liver biopsy is possible with only three main MR methods. (i) The use of multi-point Dixon imaging allows for the assessment of both liver fat by MRI PDFF and iron loading by T_2^* . (ii) The visco-elastic properties obtained with multi-frequency 3D MRE allows for staging fibrosis, and potentially also grade inflammation and investigate portal hypertension. (iii) Late-phase gadoxetate-enhanced MRI could be used to investigate the liver function (in terms of the hepatocyte functions performed by OATP1 and MRP2). Additionally an investigation of the whole body fat and muscle composition can be added, which might be proven useful in personalizing treatments.

In summary three techniques could allow for a comprehensive whole-liver investigation. With the exception of an MRE-transducer, no additional hardware is required on the MR scanner.

5.2 Quantifying Liver Function

Throughout this thesis, the term ‘liver function’ has been used synonymously with the functions of the hepatocytes carried out primarily by OATP1 and MRP2, *e.g.* detoxification and bile salt excretion. The following chapter discusses the results from the papers directly involved in measuring liver function (papers I, II, and IV) in chronological order. This is followed by a discussion on a selection of other related methods and how the model presented in this thesis relates to these methods as well as clinical parameters. Finally, some possible future endeavours are discussed.

5.2.1 The Gadoxetate-Enhancement Pattern is Strongly Influenced in Subjects with Advanced Fibrosis

The main finding in paper I was that subjects with advanced liver fibrosis had strongly affected gadoxetate enhancement patterns. In the paper we used ratios, such as LSC and LSC_N, to quantify the enhancement as well as a measurement of the hepatocyte uptake rate of gadoxetate by a previously developed approach.

The findings in paper I are in line with earlier gadoxetate-enhancement studies. Tsuda *et al.* showed that it was possible to assess the progress of liver fibrosis in a rats with NASH by evaluating the gadoxetate SI time-series (123). Three other studies reported relationships with indocyanine green retention rate (a dye which is also eliminated by the hepatocytes from the blood) and simple measurements of gadoxetate-enhancement (97, 124, 125). Lee *et al.* evaluated SI changes in late-phase gadoxetate-enhancement (20 min) and reported significant differences between subjects with liver cirrhosis or chronic hepatitis and healthy controls (126). In addition, Watanabe *et al.* demonstrated that a gadoxetate enhancement index significantly correlated with fibrosis stage (127).

The findings in paper I motivated us to explore and develop more precise methods to quantify and understand the mechanisms behind the altered gadoxetate enhancement patterns in subjects with advanced chronic liver disease. It is important to keep in mind that simple ratios are highly sensitive to perfusion changes as well as structural changes of the parenchyma in addition to the hepatocyte uptake and elimination functions. In other words, the ratios are affected by a combination of multiple functions as well structural changes such as fibrosis. Thus, conclusions concerning the underlying pathophysiological mechanisms are limited when ratios are used.

5.2.2 A Minimal Mathematical Model for Gadoxetate Fluxes in Humans

Motivated by the findings in paper I we summarized different mechanistic hypothesis concerning the fluxes of gadoxetate in humans and tested them rigor-

ously. The main aim was to develop a minimal and physiologically relevant mathematical model that describes the late-phase gadoxetate enhancement in humans. In the model, we describe the hepatocyte gadoxetate uptake and excretion via the bile as well as extrahepatic elimination via the kidneys. The hepatocyte uptake and excretion, which is discussed at length in the Introduction of this thesis, primarily uses the physiologically important transporters OATP1 (uptake) and MRP2 (excretion). Moreover, the model also allows for upregulation of MRP3 (efflux back into the blood from the hepatocytes). The model was developed using images with sufficiently high spatial resolution so that they can be visually interpreted by radiologists, meaning that we can use clinical data.

The model was developed and tested using principles derived from systems biology and pharmacology. An important implication of mechanistic models is that they allow for the coupling of different models to each other. Moreover, such models can potentially be used for the identification and quantification of non-measurable, but real, properties of the system.

From a model point of view it is important to note that the model is minimal, identifiable, and provides physiologically realistic predictions. The practical identifiability was investigated using a novel method, profile likelihood (86). Profile likelihood is more correct and generally applicable than the more commonly used approaches based on the Hessian of the cost function (128). Since the model is identifiable the model it is also observable, *i.e.* all model predictions will have both well-defined and small uncertainties.

A few final comments regarding the strength of the model is that the same model parameters were able to describe both the estimation and validation data. In the validation data, the administered gadoxetate dose was up to twenty times higher than what was used in the estimation data and in clinical practice. Furthermore, the gadoxetate was administered using a ten minute infusion instead of a short seven second bolus injection (as used in the estimation data). This indicates a remarkable robustness of the model that is useful in future work. Since the model is physiologically realistic and identifiable it is also expandable. This means that new information can be added into the model framework such as other relevant observations, *e.g.* renal clearance, volumetric MRI, and gadoxetate quantification in the bile. The added information will improve the predictions made by the model, as illustrated by the inclusion of the *ad hoc* constraint on the blood plasma. Here we put constraints on the blood plasma concentrations which in turn led to more well defined elimination fractions (a different property than was used in the constraint). This *ad hoc* constraint should also be useful in the case of hepatic and/or renal impairment, as shown in (56). It is also possible to extend the model with detailed sub-models, *e.g.* renal function or perhaps some drug metabolism mechanism in drug-drug-interaction studies.

5.2.3 The Mathematical Model is Applicable to Chronic Liver Disease

In paper IV we presented a complete and novel framework for quantifying liver function based on late-phase gadoxetate-enhanced MRI. In this framework, we

combined a novel and robust reconstruction and correction methods of gadoxetate-enhanced MRI time series (94, 95), the systems pharmacology model developed in paper II, and improved model parametrisation with NLME. This framework was successfully applied on data from a cohort suffering from a wide range of chronic liver diseases. Importantly, all but one subject had Child-Pugh class A, and none of the subjects had decompensated cirrhosis. This means that the subjects did not have a severe reduction in their hepatic function.

The model was in paper IV validated using concentrations of gadoxetate measured with mass spectroscopy in liver biopsy samples and blood samples. These samples were taken directly following the MR examination. The model predictions of the gadoxetate concentrations in the blood were strongly correlated with the measured values. The model predictions of the gadoxetate concentration in the liver biopsy samples were moderately correlated with the measured values; although on a group level there was no significant difference. The moderate correlation can have multiple reasons such as contamination of the biopsy sample from gadoxetate in the bile ducts. Moreover, the properties of the biopsy samples may not fully represent the whole-organ properties.

The model parameters representing the hepatocytes uptake (OATP1) and excretion (MRP2) of gadoxetate were significantly altered in subjects with increased severity of the underlying liver disease. This was investigated by comparing the parameters with conventional clinical serum biomarkers for liver function and histopathologically determined fibrosis stage. Fibrosis stages were used as a surrogate marker for reduced hepatic function. The variation of the model parameters in the cohort were neither associated with a specific diagnosis, fat content, BMI, or inflammation, when fibrosis was included in the ANOVA model. These findings support the notion that this model framework is widely applicable in a clinical setting.

As in paper II, the images used in this framework had both sufficient quality and spatial resolution to be useful in a clinical setting for radiological inspection. The use of the advanced image reconstruction and correction procedures (94, 95) allowed us to correct for image inhomogeneities in a dual manner. The images were corrected both with respect to intensity variations across individual images, and within the entire gadoxetate-enhanced image time series. This correction ensures homogenous images and removes the effects introduced by breathing and system drift (95). Moreover, the extracted signal intensities from the image series were also corrected with respect to the pre-contrast injection images, meaning that each subject had its own baseline correction.

By using this framework, it is not only possible to investigate liver function in a clinical setting, but also to monitor drug-induced liver injury in pre-clinical drug trails. Importantly, the framework could allow for separate characterization of the impact of drugs on OATP1 and MRP2 respectively as well as drug-drug-interactions as indicated in pre-clinical rat studies (60, 122, 129). Such a detailed separation between the function of OATP1 and MRP2 is not possible

using signal intensity ratios and qualitative description of gadoxetate enhancement.

5.2.4 The Mathematical Model in Relation to Other Methods

This chapter begins with a discussion on different types of imaging approaches and related methods for the analysis of gadoxetate-enhanced MRI. The discussion is continued with how the estimated transport rates of gadoxetate via OATP1 and MRP2 compare to clinical data and other studies. Finally, a brief conclusion on the methods is made.

5.2.4.1 A Brief Introduction and Comparison with Related Methods

In this thesis, a range of methods aimed to analyse gadoxetate-enhanced MRI has been discussed. The designs of these methods often depend on the imaging approach and if the early or late gadoxetate phases are imaged. The early-phase typically includes data up to the first 3 to 5 min and primarily contains information pertaining to perfusion and early uptake. In contrast, the late-phase typically includes data up to 20 to 40 min after injection and contains information pertaining to uptake as well as excretion.

The simplest analysis methods are the signal intensity ratios. They are typically estimated at a single point in time, such as 10 min after the injection of gadoxetate. As discussed in chapter 5.2.1 these straightforward approaches are susceptible to a number of confounding factors and suffer from the lack of functional interpretation which is required in order to understand the pathophysiological processes underlying the biomarker.

A more sophisticated method is to acquire data with high temporal resolution, which results in low spatial resolution. Nilsson *et al.* used a model-free perfusion-based approach to analyse such data, and showed that a description of gadoxetate extraction can be derived in such a manner (130). In contrast, a quantitative and mechanistic description of the hepatocyte uptake and liver perfusion is possible by combining a mathematical model with a perfusion-based approach. Such a method was developed by Sourbron *et al.*, and the analysis of the early-phase gadoxetate enhancement allows for investigating both liver perfusion properties as well as the hepatocyte gadoxetate uptake by OATP1 (131). More recently Georgiou *et al.* presented a perfusion-based model which also included the subsequent excretion into the bile in healthy volunteers using late-phase images (132).

In contrast to data with high temporal resolution, it is also possible to acquire data with high spatial resolution, which by necessity is associated with low temporal resolution. Importantly, these images can be acquired such that they are suitable for radiological interpretation and with full liver coverage. This is not possible with perfusion-style images as they need a high temporal resolution to properly capture the rapid perfusion properties. Moreover, by using data with full liver coverage it is also possible to estimate liver function on regional level in

the entire organ. It was for this type of clinically applicable data that the mechanistic model framework presented in this thesis was developed.

The use of late-phase gadoxetate enhancement is also needed in order to assess the function of the hepatocyte transporter MRP2 by the biliary excretion of gadoxetate, in addition to the function of the hepatocyte transporter OATP1 by the uptake of gadoxetate. The biliary excretion is a much slower process than uptake in humans, which can be appreciated in the prolonged enhancement. In humans, the gadoxetate liver enhancement peaks at around 30 min after injection whereas in rats the enhancement peak at about 5 min. This stark difference could in part be due to that healthy humans has about 20% of the MRP2 amount in the cell membrane compared to rats (61).

The mathematical model framework presented in this thesis is extensively discussed in chapters 5.2.2 and 5.2.3. A similar model framework developed for the use in rats has been used for investigating DILI in pre-clinical trials by Ulloa *et al.* (60). Recently we showed that the rat model could be simplified, similar as in the model presented in this thesis with linear kinetics for MRP2 (129). I also contributed to the work involving the simplified rat model together with Kara-georgis *et al.* in a multi-centre study. In this study we showed that the study effects identified by the model was robust and independent to scanner and study centre (122).

In a retrospective study using early-phase gadoxetate enhancement Saito *et al.* (133) used the perfusion-based model framework developed by Sourbron *et al.* (131) to determine the hepatocyte gadoxetate uptake rate based on clinical image data (*i.e.*, limited temporal resolution). They showed that it was possible to use a perfusion-based model framework in combination with clinical MR images similar to those used in this thesis. Such a perfusion-based framework requires both arterial and venous input functions, which have to be extracted from data. However, using the perfusion approach on data obtained with low temporal sampling rate is susceptible to misrepresenting input functions (133), potentially distorting hepatic function estimates. Perfusion-based analysis strongly benefits from high temporal resolution in order to capture and describe the early arterial and venous wash-in phases which occurs in matter of minutes following the injection of gadoxetate.

In contrast to perfusion methods, the mathematical model presented in this thesis takes into account all significant aspects of gadoxetate fluxes in a human. This includes the total blood pool which in a way corresponds to the perfusion model's arterial and venous input functions. A major benefit of such an extensive modelling is that any noise in the data can be taken into account in a robust statistical sense, and noisy input data does not necessarily render the model predictions distorted. In other words, the model framework presented in this thesis is parametrized to all data points (both blood and liver). In contrast, the perfusion methods and the method used in Ulloa *et al.* where the blood enhancement is used directly as an input to the model. Another important difference is that in our model framework the spleen is used as a surrogate approach

for obtaining direct measurements in the blood vessels. Such an approach circumvents the potential artefacts that may arise as a consequence of the challenges that one faces when attempting to position reliable ROIs in blood vessels. Moreover, the SI from blood vessel ROIs might also be distorted by flow or SENSE artefacts and partial volume effects. The use of the spleen as a surrogate blood marker is advisable since the spleen only contains gadoxetate residing in the blood pool. Obviously, such an approach is only possible in patients in whom the spleen has not been surgically removed. In such cases one has to revert to the use ROIs placed in blood vessels instead. Our proposed model framework is independent of the source of available data in the model parametrization as the model parametrization does not require a particular input function; therefore, any type of data can be used such as blood vessels instead of the spleen. Limited amount of observations will of course impact the identifiability and observability properties.

5.2.4.3 Gadoxetate Uptake via OATP1

In paper IV the hepatocyte uptake of gadoxetate via the OATP1 transporters was found to decrease significantly in subjects with increasing amounts of fibrotic tissue. This is not a surprise since fibrosis a factor which is expected to contribute to a restricted hepatic function (10). In the chronic disease cohort, we found that the highest OATP1 uptake rate was 5.6 ms^{-1} which is in line with the findings in a group of healthy subjects (paper II), where the OATP1 uptake rate confidence interval was 3.3 to 6.7 ms^{-1} . In the chronic liver disease cohort, the lowest uptake rate was 0.5 ms^{-1} .

The model parameter corresponding to the hepatocyte uptake was in paper IV correlated with bilirubin, which is also taken up into the hepatocytes by OATP1. The analysis showed that a high serum concentration of bilirubin corresponded to a low hepatocyte uptake of gadoxetate. The commonly use liver-function test AST/ALT was also shown to correlate with the uptake of gadoxetate. Low ratios corresponded to low hepatocyte uptake. Also, albumin which is reduced in severe liver disease was strongly correlated with the gadoxetate uptake. The correlation showed that low albumin levels corresponded to low hepatocyte uptake rates of gadoxetate. Even though the cohort did not consist of subjects with severe liver disease and liver function impairment the methods were sufficiently sensitive to pick up the minor loss in function associated with the compensated fibrosis. These findings and the correlations with serum markers indicate that the model parameters estimated from late-phase gadoxetate enhancement are coupled with reduced hepatic function.

The observations in paper IV of reduced gadoxetate uptake agrees with those of Saito *et al.* (133) as well as Juluru *et al.* (134), both whom used a perfusion style model framework to assess the hepatic uptake rates of gadoxetate in humans. The reduction of the OATP1 function could for instance be due to restricted access of gadoxetate to the hepatocytes, reduction in the number of functional hepatocytes per unit volume, or competitive inhibition.

5.2.4.4 Gadoxetate Excretion via MRP2

It was interesting to observe that the hepatocyte excretion through MRP2 was increased in the cirrhotic subjects. In paper IV the highest MRP2 excretion rate was 0.8 ms^{-1} which is slightly elevated compared with a group of healthy subjects (paper II) where the confidence interval for MRP2 excretion was 0.2 to 0.7 ms^{-1} . In paper IV the lowest uptake rate found in this cohort was $40 \text{ }\mu\text{s}^{-1}$. The correlation analysis of the gadoxetate excretion showed non-significant correlations with AST/ALT ratio as well as albumin. There was a low correlation with bilirubin, where high bilirubin levels corresponded to high elimination. Possibly this could be linked to an upregulation of the MRP2 transporter.

We have previously reported that there was no significant difference in the visual appearance of gadoxetate in the common bile ducts in subjects with advanced fibrosis compared to non-fibrotic livers (135). This finding supports the notion that the hepatocyte excretion rate could be increased, possibly due to upregulation of MRP2, as a consequence of for instance increased levels of anions, bile salts, and bilirubin in the cytoplasm. As noted in the review by Gerk and Vore the upregulation is a complex issue and the regulation and activity of the hepatocyte transporters can be modified within minutes, hours, and days (6). The complex web of regulation and control can be attributed to many mechanisms such as disease process and toxins. An observation that agrees with this finding is that the rat homologue *mrp2* is upregulated in dedicated cirrhotic rat model systems (136-138). In contrast, in humans Bonin *et al.* did not find a significant difference in MRP2 expression (139), whereas Wang *et al.* found some increased MRP2 expression in cirrhotic patients (140).

The findings indicate that in our chronic liver disease cohort there are both slightly higher excretion rates as well as drastically reduced excretion rates compared to healthy subjects. This is likely associated with the specific aetiologies, but since there were a limited number of subjects per aetiology no detailed sub-analyses could be performed. As this type of analysis of MRP2 function has not previously been performed in a clinical setting there are no published rate parameters to compare with.

5.2.4.5 Liver Function by the Analysis of Gadoxetate-enhanced MRI

There is a growing body of evidence that gadoxetate-enhanced MRI is suitable for measuring altered liver function, in both clinical and pre-clinical situations. As the methodologies develop further we predict that the most useful approach would be to use a model framework (such as has been demonstrated in this thesis, and by other groups (131-134)), as well as image reconstruction methods to ensure reliable data (94, 95), and robust model parametrization. Such approach makes mechanistic and physically meaningful clinical interpretations possible, in contrast to other phenomenological and ratio based approaches. More work is however still required to ascertain that the mathematical models produce robust and reliable estimates of OATP1, MRP2, and likely also MRP3 function.

5.2.5 Future Work

A multi-centre study should be performed to explore the robustness measuring liver function based on gadoxetate-enhanced MRI in a clinical setting. A model structure and gadoxetate quantification method closely related to the one presented in this thesis has recently been investigated in a multi-centre study of rats with induced DILI (122). The framework was robust across the four study sites for the quantification of the function of Oatp1 and Mrp2, and was suitable for the use in pre-clinical drug trials to identify DILI (122). Recently, an alternative model for analysing gadoxetate-enhanced MRI has recently been investigated in healthy humans, demonstrating reproducible estimation of gadoxetate uptake in repeated measurements, whereas estimation of efflux was less accurate (132). These study studies indicates that this type of analysis can be made robustly.

Another future improvement would be removal of the need for a radiologist to identify ROIs in the initial model parametrization – this process is time consuming and may introduce operator bias. Additionally, uptake rates differs across the parenchyma (134) so it seems preferable to investigate the liver on a regional level. A suitable additional approach might be to implement a sophisticated multi-atlas-based segmentation method (perhaps similar to one that has been used for segmentation of muscle and fat volumes in whole-body MRI (141)) to enable quantification of function throughout the liver – nor just in selected ROIs, but in a range of Couinaud segments. A particularly useful application of this method would be to plan surgical resection. The clinical potential of our method could eventually be realized by a procedure in which a set of MR images, acquired following injection of gadoxetate, is transferred from the clinic to an external service provider for image re-construction, ensuring consistent image quality and homogeneity. Segmentation of the entire liver with a multi-atlas-based approach would enable application of the model estimate liver function to each image particle by NLME parametrization in conjunction with extensive *a priori* data. The clinic or study centre would then obtain parametric 3D maps of the liver functions of OATP1 and MRP2, from which a clinical decision could be made.

In the model the renal clearance of gadoxetate is assumed to be constant. As discussed in this thesis the presented model framework is extendable and modifiable. It would therefore be interesting to explore if adding a detailed description of kidney function improves the accuracy of the liver function estimates. Eikefjord *et al.* recently presented a method to measure kidney function with another gadolinium-based MRI contrast-enhancing agent. They used perfusion-based methods to quantify the renal clearance of the contrast agent (142). Since the model framework presented in this thesis does not use the first 3 min after gadoxetate injection this 3 min window could be used to acquire data of sufficient high temporal resolution to enable such a perfusion-based analysis of kidney function. After these 3 min the image acquisition methods outlined in this thesis would be used as a basis for measuring liver function. Finally, the model

framework would include the kidney function information from the perfusion analysis in the quantification of liver function.

5.3 Non-Invasive Steatosis Grading

In paper III, the correlation between the liver fat content according to semi-quantitative histopathological grading and SPC as well as ^1H -MRS PDFF was low. These results are in line with previous reports that showed that the histopathological semi-quantitative estimates greatly overemphasize the true density of fat in the liver tissue (92, 143, 144).

We found an excellent correlation ($r = 0.92$) between ^1H -MRS PDFF and SPC. This finding confirms the results from previous studies, which reported correlation coefficients from 0.82 to 0.93 between a near-continuous steatosis score and PDFF (145-149). Importantly, in contrast with other studies, there was no time bias in the present study because the liver biopsy was performed within a few hours after the completion of the MR examination.

When evaluating hepatic steatosis using different methods, it is important to note that histopathological grading, SPC, and PDFF measure fundamentally different tissue properties (67). Histopathological grading of steatosis is based on a visual estimation of the percentage of hepatocytes that contain intracellular fat droplets. SPC measures the steatotic area of the liver biopsy sample, which corresponds to volume-%. PDFF is defined as the ratio of density of mobile protons from fat and the total density of protons from mobile fat and water (67).

Recently published guidelines recommend screening for NAFLD amongst patients with obesity or metabolic syndrome by the use of non-invasive methods (110). If the widely used threshold of 5.0% had been used to define hepatic steatosis with PDFF in paper III, 32% of the subjects with elevated liver enzyme levels due to NAFLD would have been incorrectly considered to not have steatosis. Thus we show that the currently used cut-off for diagnosing liver steatosis is too high and should be lowered. A possible cut-off at 3.0% PDFF had a sensitivity of 100% and 84% of the subjects were correctly identified with steatosis. Our results are also in accordance with those of Rehm *et al.* (150) who recently reported that a lower cut-off (3.5%) constitute an optimal PDFF threshold for predicting the metabolic syndrome.

5.4 Non-Invasive Fibrosis Staging

In paper V we evaluated the diagnostic performance of a range of MRI methods, TE, and commonly-used serum fibrosis algorithms for the identification of fibrosis in a clinical setting. Overall, the elastographic methods (MRE and TE) generated the highest diagnostic accuracy.

Late-phase gadoxetate-enhanced MRI ratios were as good as certain serum fibrosis algorithms in detecting subjects advanced fibrosis. Late-phase gadox-

etate-enhanced MRI also had merit in dichotomising stages of fibrosis, where the top serum fibrosis algorithms were only able to in the advanced stages. As previously eluded to the subjects in the chronic liver disease cohort did not have a severe reduction in their hepatic function (as indicated by the fact that none of the subjects had decompensated cirrhosis and all but one had Child-Pugh class A). Nevertheless, the late-phase gadoxetate-enhancement methods were able to detect the low alteration in hepatocyte function (OATP1 and MRP2 transport rates) associated with the disease processes, with severity indicated by the fibrosis stages.

Notably, the best-performing serum fibrosis algorithms (APRI, King's Score, and GUCI) which were originally developed for specific liver diseases, were able to identify fibrosis successfully in this cohort in terms of liver disease with various aetiologies.

5.4.1 Elastographic Methods

The AUROCs for the elastographic methods were constantly high in terms of detecting increasing degrees of fibrosis. The insignificant correlation between MRE and TE was probably due to that there were few subjects with advanced fibrosis evaluated with both methods. This is important to keep in mind since TE has a limited dynamic range in the low to moderate fibrosis stages. Moreover, MRE had higher specificity than TE in terms of discriminating advanced fibrosis. MRE was also able to dichotomise between, intermediate levels of fibrosis. The AUROC values for MRE (0.93) were consistent with previous studies which reported AUROC in the range of 0.90 to 0.99, and meta-analysis findings of 0.92 (151-154). Our AUROC for detecting advanced fibrosis using TE (0.84) was also in agreement with previous studies where 0.77 was found in a mixed cohort (153) and by meta-analysis 0.89 (155).

MRE has several advantages over TE. Firstly, it allows full liver coverage whereas TE only assesses a 1D beam which penetrates about 25 to 65 mm into the subject, from the skin meaning that heterogeneities might affect the results. Moreover, TE is difficult to perform in obese subjects, which MRE is not, as long as the subjects can physically fit into the MR bore. Also, increased fatty infiltration of the liver parenchyma affects the measurement acquired by TE. In contrast, studies have shown that MRE is insensitive to simple steatosis and ascites. Finally, the success rate of TE is also operator dependent, which is not as important in MRE (48, 84, 114).

An important disadvantage of MRE is cost, as it requires an MRI scanner. TE, on the other hand, is much less costly and is easy to use in the clinical setting. Moreover, MRE is not feasible in patients with contraindications to MR, such as claustrophobia or metallic implants.

An important benefit of MRE is that the examination can be coupled with other imaging methods which measure properties such as fat distribution and iron loading, and which can image the morphological properties of the liver.

MRE is preferable for many reasons, although as a rapid and in-expensive first assessment, TE has clear merits.

5.4.2 Phosphorous MR Spectroscopy

The AC measured with ^3P -MRS showed significant differences between non-fibrotic and cirrhotic livers, which is in line with previous studies (72, 74). The AC is closely related to the PME/PDE ratio ($\text{AC} = \text{PME}/[\text{PME}+\text{PDE}]$). Godfrey *et al.* did not find the PME/PDE ratio to be better than chance for the detection of fibrosis (151). A difference between Godfrey's study and ours is that the former did not use localised acquisition which could lead to distortions in the concentrations due to metabolites in the muscles. However, in the intermediate stages of fibrosis the AC AUROC values were not better than chance in paper V, in agreement with Godfrey *et al.* (151).

As can be seen from the correlation analysis AC was uncorrelated with the other methods evaluated which indicate that AC does not contain the same information. Moreover, AC is probably not suitable for distinguish between stages of fibrosis in this cohort of subjects which did not have decompensated cirrhosis. It is useful for investigating other aspects of liver disease processes in relation to the enhanced regenerative processes, or possibly alterations in ATP patterns. Hepatic ^3P -MRS is of interest in scientific explorations, though currently there are more suitable methods if the main goal is assessing fibrosis in a clinical setting.

5.4.3 Analysis of Gadoxetate Enhancement Patterns

Liver function as quantified by analysing the late-phase gadoxetate enhancement patterns was also successful in the identification of advanced fibrosis. In paper V we evaluated both ratio-based and model-based approaches. The specifics concerning the model parameter values and parameters relation to other studies are discussed at length in chapter 5.2.4. In this chapter I will discuss the diagnostic performance in detecting advanced fibrosis.

Of the two evaluated ratios LSC_N10 was superior (AUROC of 0.81). There are not many studies using LSC_N10 to compare the results in paper V with. A pilot study reported that subjects with no or mild manifestations of hepatobiliary disease processes had a value of 1.33, and subjects with hepatobiliary diseases which probably affect hepatobiliary function but which have no major biliary obstruction (such as severe cirrhosis, thromboses, and PSC) had a value of 1.05 (53). These values are in line with the optimal LSC_N10 cut-off of 1.28 for advanced fibrosis, which was found in paper V. As previously discussed the ratio based methods are affected by multiple mechanisms such as gadoxetate enhancement and structural changes associated with fibrosis. It is possible that this aspect is seen in the low to high negative correlation with both elastographic and serum fibrosis algorithms.

In the pilot study discussed previously, K_{Hep} was reported with a value of 0.24 s^{-1} in subjects with mild or no hepatobiliary disease processes and subjects

with hepatic or biliary disease, which probably affects hepatobiliary function, had a value of 0.09 s^{-1} (53). This is in line with our study where 0.21 was found to be an optimal cut-off value for advanced fibrosis.

The more advanced quantification of hepatocyte transport of gadoxetate presented a mixed view. It was possible to dichotomise between intermediate stages of fibrosis using the hepatocyte uptake rate (via OATP1). The biliary excretion rate (via MRP2) was unaffected in moderate stages of fibrosis, and increased in cirrhosis; see the discussion in chapter 5.2.2. The AUROC for hepatocyte uptake were 0.76 which is lower than in a recently published study (0.84) by Juluru *et al.*, where the uptake rate was quantified using a perfusion-style approach (134).

The function of OATP1 and MRP2 is not directly involved in fibrogenesis, and therefore these methods may not be best suited for identifying fibrosis, as there is limited loss in the low stages of fibrosis. There is a strong correlation between hepatocyte uptake (OATP1 function) and hepatic stiffness (MRE), but not with the other methods. The quantified functions of K_{Hep} and hepatocyte excretion (MRP2) do not appear to contain the same information as the elastographic or serum fibrosis biomarker methods, as indicated by the negligible to low correlation. Nevertheless, these functional measurements were sufficiently sensitive to measure low function loss in this low Child-Pugh class chronic liver disease cohort.

5.4.4 Serum Fibrosis Algorithms

In paper V nine serum fibrosis algorithms were included. Three of them were developed using NAFLD cohorts (NAFLD fibrosis score, NIKEI, and NASH-CRN regression score) and six in cohorts with HCV infection (APRI, FIB-4, GUCI, Lok Index, Forn's score, and King's score). Although only 9% of our cohort consisted of subjects with HCV infection, the three similar algorithms King's score, GUCI, and APRI showed adequate accuracy in diagnosing advanced fibrosis in our cohort (AUROC values around 0.8). In contrast, even though 38% of our cohort had NAFLD, NAFLD fibrosis score, NIKEI and NASH-CRN regression score did not generate the same precision. These findings are in line with a recent study which showed that FIB-4 and King's score were superior in diagnosing advanced fibrosis in a pure NAFLD cohort (156). This recent study and ours indicates that King's score or similar algorithms (GUCI and APRI) might be suitable in cohorts with liver disease of various aetiologies for the detection of advanced fibrosis.

The benefit of serum fibrosis algorithms is that they are cost-effective, less invasive than biopsy, are not associated with a risk of complications, exhibit little or no sampling errors and have low observer variability (13). The disadvantage of serum fibrosis algorithms is that they are indirect measures, depend on clearance rates (*i.e.* biliary and renal function), and can be elevated due to extrahepatic pathologies (13).

5.5 Limitations

This sub-chapter discusses the limitations and in papers in I-V in order.

A common limitation in papers III-V is that the data acquisition period was extended (6 years), which in some manner may have affected the MR data quality, although the same scanner was used throughout the work. Also, predominantly relating to paper IV and V, the cohort is biased towards low grade fibrosis and liver disease, although this clearly reflects the cross-section on patients referred to our hospital and therefore the prospective nature of our study.

5.5.1 Limitations in Paper I Concerning the Separation of Advanced Fibrosis with Gadoxetate-enhanced MRI

The main limitation with the interim analysis in the first paper is that there were a limited number of subjects with advanced fibrosis.

5.5.2 Assumptions and Limitations in Paper II Concerning the Mathematical Model for Gadoxetate Fluxes in Humans

When interpreting the model and the results in paper II, there are a few underlying assumptions and limitations that should be kept in mind.

First, due the bolus injection of gadoxetate there is an initial wash-in phase where gadoxetate is not evenly distributed in the blood vessels. This initial wash-in is not accurately described by our model, because of the formulation of ODEs which imply instantaneously mixed compartments. Typically, it takes about 1-2 min for gadoxetate to become evenly spread in the blood pool. However, the hepatocyte accumulation is a slow process and peaks around 20-30 min post-injection so there is no need to describe this initial phase if only OATP1 and MRP2 are of interest and not the perfusion patterns.

Second, we have used some parameter describing tissue volumes and composition from the literature.

Third, it would be beneficial to include measurements of the common bile as it would likely improve the model parametrization and predictions. However, at the time we were not able to obtain sufficiently reliable gadoxetate quantifications in the common bile duct.

Fourth, the *ad hoc* constraint could not be implemented directly in the profile likelihood analysis. The constraint was included as a new data-point which leads to a larger parameter uncertainty than would otherwise be expected, should the constraint be applied strictly.

Fifth, when comparing the elimination fractions to the reference values, it should be kept in mind that there are differences in; gender distribution, temporal differences (6 days in the reference data, 3 h in the simulations), slight differences in doses.

Sixth, we did not implement enterohepatic recirculation of gadoxetate from the intestine in the model. Studies on rats have indicated that this possibility of

reabsorption into the blood in the intestine can be excluded which we assumed was a reasonable assumption in humans too (157). Moreover, if such reabsorption would exist in humans the effect on the gadoxetate concentrations (*e.g.* in the pool vessels) would be negligible within the time frame of the gadoxetate-enhanced examinations.

5.5.3 Limitations in Paper III Concerning the Detection of Steatosis

There are some limitations paper III that should also be taken into account for the observed differences between the ^1H -MRS PDFFF, SPC, and histopathological grading.

First, ^1H -MRS voxels and the biopsy locations were not co-localised, which may have introduced a significant degree of variability in the analysis. Bannas *et al.* recently showed that multiple samples from a single liver segment can vary in fat content (158).

Second, during the post-processing of the ^1H -MRS spectra the quantified resonances were corrected for the effects of T_1 and T_2 because the T_R was not sufficient for a complete recovery of the magnetization vector. In the correction we used previously reported values for T_1 (representing the water and fat resonances) and T_2 (representing the fat resonances), which might have introduced a potential bias. The correcting procedures could be improved by using multi-echo MRS, which allows for direct resonance-specific corrections and estimates of T_1 and T_2 -values. In this study, measurement of water T_2 allowed us to at least correct for excessive hepatic iron accumulation in each individual.

Third, there is also a possible minor limitation in the use of PRESS due to the modulating effect of J-couplings (63). However, this bias is probably minimal at low fat fractions, and therefore it is not likely to have affected the overall results of this work.

5.5.4 Limitations in Paper IV Concerning the Application of the Mathematical Model in Chronic Liver Disease

When interpreting the results in paper IV where the model was applied on the chronic liver disease cohort there is, in addition to those mentioned previously, also a potential limitation the assumption of a constant renal function. Renal function can deviate in subjects due to pathology. However, in our study no subjects suffered from renal impairment. As the model structure is easily modified the renal function either be modified by a more advance description and fitted to data or simply include the subjects measured GFR rate as input to the model.

5.5.5 Limitations in Paper V Concerning Identification of Advanced Fibrosis

When interpreting the results in paper V concerning the identification of advanced fibrosis the following possible limitations should be kept in mind.

First, as hepatocyte transporters, such as MRP2, have altered expression levels in different diseases, such as PSC (8), it would have been beneficial to have sufficient number of subjects in each diagnosis to allow for a detailed sub-

analysis in various aetiologies. Although, in the analysis no significant differences were found between groups of aetiologies.

Second, as MRE was added later in the study not all subjects were investigated with MRE. This hampered the direct comparisons against the other biomarkers in this study.

Third, there was no co-localization for the biopsy with either of the other imaging or spectroscopy methods which could potentially have affected the results.

6. CONCLUSIONS

The conclusions are listed in the same manner as the discussion, *i.e.* first the papers directly concerning liver function are presented (paper I, II, and IV) followed by non-invasive grading of liver steatosis (paper III) and fibrosis staging (paper V).

6.1 Quantification of Liver Function

6.1.1 Gadoxetate-Enhancement was Significantly Altered in Subject with Advanced Fibrosis

We demonstrated that the late-phase gadoxetate-enhancement pattern was significantly altered in subjects with advanced fibrosis. The analysis of the enhancement patterns by the use of ratios and a procedure for calculating the hepatocyte-specific contrast agent uptake rate was successful in identifying subjects with advanced fibrosis.

6.1.2 A Mathematical Model for Late-Phase Gadoxetate Fluxes in Humans

A mathematical model to analyse the fluxes of gadoxetate in humans from MRI examinations was developed and implemented. The primary model parameters describe the uptake and excretion rates of gadoxetate in the hepatocytes. These rates are coupled with the functions of physiologically important transporter proteins in the hepatocytes. The uptake is primarily associated with the transporting function of OATP1, and the excretion by MRP2. We showed that the model could accurately describe both estimation and validation gadoxetate enhancement patterns in healthy subjects. The mechanistic model was found to be minimal, physiologically correct, and fully identifiable. Since the model is mechanistic it is also possible to link the model to other data sources (such as bile ducts) and other models. An important aspect is that the model is applicable on conventional data suitable for radiological viewing.

6.1.3 The Mathematical Model for Late-Phase Gadoxetate Fluxes is Applicable on a Chronic Liver Disease Cohort

To quantify liver function non-invasively we have developed an extensive and novel framework of quantitative MR image-processing of a late-phase gadoxetate-enhanced time series, in combination with a systems pharmacology model to describe liver functions, and NLME algorithm to ensure robust liver function estimates. This framework was successfully applied in a clinical setting to prospective data obtained from a diverse cohort of patients with low-grade CLD, to quantitatively characterize liver function. Importantly, this represents the first

quantitative assessment of liver function in a clinical setting based on both OATP1 and MRP2 simultaneously, using gadoxetate-enhanced MRI with sufficient spatial resolution for full liver coverage and suitable for use in conventional radiological reading

The liver function parameters were correlated with clinically biomarkers for liver injury and the framework was validated with biopsy and blood samples. As our study included subjects suffering from a wide range of liver diseases and the model parameters were not affected by specific diagnoses, fat content, BMI, or inflammation, we propose that this framework is widely applicable in clinical settings.

6.1.4 Quantifying Liver Function

In summary, we have developed a model framework that can be used to analyse late-phase gadoxetate enhancement patterns. The primary model parameters estimated from the data describe the rate of uptake and excretion of gadoxetate through the hepatocytes, and potentially new imaging biomarkers for liver function. These rates are associated with the physiologically important functions of OATP1 and MRP2. The model was in this thesis applied to late-phase gadoxetate-enhanced MRI, both from healthy subjects and subjects with low grade chronic liver disease with a wide range of aetiologies. The model predictions were validated using blood samples with up to twenty times higher doses, elimination fractions, biopsy and blood samples after the MR examination. From a clinical point of view the model parameters describing the function of OATP1 and MRP2 were found to correlate in a meaningful fashion with bilirubin, albumin, and AST/ALT ratio. The method was also sufficiently sensitive such that it was possible to detect the small alteration in hepatocyte function associated with the disease processes in subjects with compensated advanced fibrosis, regardless of aetiology.

In conclusion, this framework is a potential approach for robustly measuring liver function non-invasively, ensuring robust estimates for clinical decision making. Quantification of biomarkers by imaging will potentially increase the effectiveness of surgical liver resection, add important diagnostic information about both early and late liver diseases, and be highly useful as a monitoring tool for pathophysiological developments. Finally, we predict that it will also be of great value for pharmaceutical companies and agencies in drug safety evaluations.

6.2 Non-Invasive Steatosis Grading

We showed that the currently used cut-off for diagnosing liver steatosis by PDFFF is much too high. In the study, up to one third of all subjects with histopathological NAFLD were not identified using the current definition (5%). Instead a PDFFF cut-off of 3% should be used for detecting hepatic steatosis non-

invasively. We also showed that SPC and PDFF strongly correlates and produce comparable results.

Future studies on epidemiology and pathophysiology of NAFLD will more often rely on non-invasive techniques, and it is therefore vital that we have valid cut-off values for the diagnosis of NAFLD. In caring for patients, a correct diagnosis of liver steatosis will impact screening and treatment strategies.

6.3 Non-invasive Fibrosis Staging

In conclusion, the serum fibrosis algorithms developed for specific chronic liver disease are applicable in a clinical setting, and perform as well as late-phase gadoxetate-enhanced MRI in detecting liver fibrosis. It is interesting that the late-phase gadoxetate methods which measure hepatocyte function (not directly coupled with fibrosis) perform as well as serum algorithms developed to detect the histopathologically-determined stage of fibrosis. This indicates that these methods are sufficiently sensitive to detect the low function loss in this cohort. Gadaxetate-enhanced MRI is able to dichotomise stages of fibrosis, and phosphorus MRS may be able to distinguish between healthy and fibrotic, as well as cirrhotic livers, although ^{31}P MRS is unlikely to be a suitable clinical biomarker for fibrosis.

In line with numerous other publications the most prominent option for diagnosing advanced hepatic fibrosis involves the elastographic methods, and MRE is the highest-scoring biomarker.

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