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Aspects of the Preoperative Assessment in Patients Undergoing Liver Surgery

Simon A.W.G. Dello

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Aspects of the Preoperative Assessment in Patients Undergoing Liver Surgery

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Chapter 1

General introduction

General introduction

Liver lesions consist of a large group of both benign and malignant tumors. Malignancies that have originated in the liver itself (primary liver cancer) are relatively rare in adults, with the exception of hepatocellular carcinoma. Metastases of colorectal cancer (CRC) are the most frequent malignant lesions in the liver. CRC is the second commonest cancer in the western world¹ and the incidence of CRC is high^{2,3}. Approximately 50% of all patients diagnosed with CRC will eventually develop colorectal liver metastases (CRLMs) at some stage of their disease⁴⁻⁶. At the time of initial diagnosis of CRC 20-25% of patients have clinically detectable liver metastases⁷. When left untreated, the presence of liver metastases is associated with a very poor prognosis^{8,9}. Liver resection is the only potentially curative treatment option for patients with CLRM, but the number of patients that are candidates for liver resection is limited; the resectability rate of liver metastases is only approximately 15-25%^{10,11}. Due to oxaliplatin or irinotecan-based neoadjuvant chemotherapy resectability rates can increase with an estimated 10-15%^{12,13}. For patients with CRLMs, liver resection offers a 5-year survival rate of 50-58% in selected patient groups^{14,15}. Furthermore an increasing number of patients can be offered long-term survival by liver resection due to a more aggressive approach such as two stage resections^{16,17} and repeat hepatic resections^{18,19}.

The first elective liver resection was performed in 1887 by Langenbuch²⁰. Even in the recent past, liver resection was still a dangerous surgical procedure with a high mortality of 20-30% in the seventies of the past century²¹. This was mainly due to excessive intra-operative bleeding. Over the subsequent decades, the procedure has become increasingly more safe due to improvements in surgical and anesthetic techniques^{22,23}. For example lowering the central venous pressure during liver surgery reduced blood loss and improved perioperative outcome considerably²⁴. At present, postoperative mortality rates are reported to be well below 5% also as a result of the creation of centers of excellence offering multidisciplinary expertise and technical innovation^{23,25-27}. Liver resection of even major parts of the liver (up to 70%) is feasible, because the liver has a remarkable capacity to regenerate²⁸. The residual liver will simply take over all vital liver functions such as clearing bacteria, urea synthesis, and synthesis of crucial proteins²⁹. The vast majority of liver regeneration occurs within the first week after major hepatectomy^{30,31}. Within six to eight weeks following 60-70% hepatectomy, the liver has regained nearly its original size and weight.

Preoperative assessment

Indications for liver resection are continuously being extended. Therefore hepatectomies are performed increasingly more often. In the past a number of exclusion criteria were applied strictly in the selection of patients for hepatectomy: tumour free margin of 1 cm³²,

unilobar localization, less than four metastases^{33,34} and a maximum tumour size of 5 cm^{34,35}. These criteria have been abandoned largely over time^{36,37}. This more aggressive approach will lead to more extensive liver surgery. Currently, the single most important cause of lethal outcome of this type of surgery is postoperative liver dysfunction as a consequence of a small functional remnant liver following major liver resection (3 or more segments)^{23,38,39}. Therefore maintaining sufficient liver function after liver resection has become a new challenge in liver surgery.

The risk for postoperative liver dysfunction is especially high if the underlying liver parenchyma is abnormal⁴⁰. In this context repeated liver resections, increasingly extensive resections and resections after aggressive chemotherapy are likely to lead to more patients with small (functional) remnant livers and hence more risk of postoperative liver failure. The risk of liver failure after liver resection is determined by two factors: the postoperative remnant liver volume and postoperative remnant function, and those have become the main determinants of resectability. For this reason, the goal of this thesis was to analyze different aspects of the preoperative assessment of patients undergoing liver resection with the ultimate goal to design a method to identify patients at risk of liver failure (and hence mortality) following liver resection.

Liver volumetry

The future remnant liver is determined by the anatomical positions of the lesions that are to be resected. A reliable volumetric assessment of the part of the liver to be resected as well as future residual liver volume should be a critical part of preoperative evaluation particularly in extended resections, in order to prevent postoperative liver failure after liver resection^{23,38,41,42}. Resections can only be performed safely in patients with normal liver parenchyma, if the functional remnant liver volume is at least 25-30%^{38,43}. In patients with impaired liver parenchyma due to pre-operative chemotherapy a remnant liver volume of 40% is needed⁴⁴.

Research has shown that pre- and postoperative liver volumes can be accurately calculated from CT or MRI scans^{38,45,46}. Liver volumetry is useful in patient selection and helps to reduce the incidence of complications due to insufficient residual liver volume. Unfortunately radiological image analysis software is typically linked to radiological hardware making it less accessible for non-radiologists. In addition, the intended operation should be known to the investigator to predict the remnant liver volume accurately. This requires the expertise of a liver surgeon. Therefore, CT-volumetry has hitherto been a multidisciplinary modality requiring the efforts of dedicated surgeons and radiologists. Advances in digitalization, the availability of broadband networks and the introduction of CT-scans on CD-ROM have enabled volumetry on a Personal Computer remote from radiological hardware (CT-scanners or MRIs). Advantages of stand-alone software are its applicability for tertiary referred patients, who already have a proper CT-

scan on CD-ROM from the referring institution and the possibility to perform liver volumetry independent of the input of a radiologist. However, commercially available stand-alone CT-volumetry software is expensive.

The development of freely available downloadable open-source image processing software packages such as ImageJ⁴⁷ and OsiriX[®] make it possible to perform a CT-volumetric analysis of the liver on a Personal Computer. These programs could bring liver volumetry within the surgeon's reach, independent of the support of a radiologist. Further development and validation of these freely available stand alone radiological software programs is crucial for the implementation of a reliable CT-volumetric analysis of the liver in the preoperative assessment for patients undergoing liver resection.

Liver function

Liver volume does not necessarily reflect liver function especially in patients with a compromised liver⁴⁸. Therefore preoperative evaluation of future remnant liver function is very important in patients undergoing liver surgery, not only in order to detect patients at risk of developing liver failure after liver resection but also for tailoring the appropriate extent of resection. Overestimation of the remnant liver function can on one hand lead to life-threatening postoperative liver failure, and on the other hand underestimation could lead to a lost opportunity for potentially curative surgery. Developing a dynamic liver function test can therefore be considered the 'holy grail' of liver surgery. This is challenging for several reasons. First, there is no good definition of liver function, and consequently a unanimous definition of postoperative liver failure until recently was also lacking⁴⁹. One of the problems in this context is that it is very hard to define the functional capacity of the liver. The term liver function is a crude denominator for a range of functions: conjugation, uptake, metabolism and excretion of endogenous and exogenous substances but also protein synthesis, immune and storage functions. Presently it is still unknown which liver function is mandatory and optimally reflects the functional reserve of the liver. Thus, the ideal function test that measures all components of liver function has not yet been invented.

Most conventional liver function tests are static. The tests currently in common use e.g. the ICG clearance test⁵⁰⁻⁵², the aminopyrine breath test^{53,54}, the galactose elimination test⁵⁵, and the combination of serum transaminases, bilirubin and clotting factors, do not demonstrate the liver's full functional capacity. Conventional liver functions become pathologic only late during disease progression. As a consequence, the early stages of liver dysfunction are often under-diagnosed. These tests are also not useful to predict future functional hepatic reserve in patients. For the latter a dynamic quantitative liver function test is probably the best solution (metabolism or time-based uptake of a substance). Most of the dynamic function tests that are presently studied are limited by the fact that they focus on very specific functions of the liver. If this specific function is impaired it remains

uncertain whether it reflects all components of liver function. This is illustrated by the fact that mortality occurred in patients with normal dynamic liver function test values, and survival has been noted in patients with predicted poor outcome.

One of the most important functions of the liver is its defense mechanism against many diverse forms of stress and intoxications. The intracellular content and synthesis of glutathione (GSH) plays a very important role in this⁵⁶⁻⁵⁸. GSH is the principal oxygen free radical scavenger in the liver. GSH is a tripeptide, which is synthesized from glutamate, glycine, and cysteine. GSH contains a sulfhydryl group which can reduce Reactive Oxygen Species (ROS) upon oxidation to glutathione disulfide (GSSG)⁵⁹. In absence of sufficient GSH, ROS can cause toxic protein modifications eventually causing cell death and tissue injury. It is generally accepted that GSH plays a key role in the protection of the liver against many forms of stress, ischemia and toxic compounds. During liver surgery, vascular inflow to the liver is often occluded temporarily to control bleeding. This induces ischemia and subsequent reperfusion depleting the GSH system. In the context of liver surgery for cancer, chemotherapy is often used and may additionally deplete liver GSH prior to surgery. Thus, the assessment of GSH content may be of major value.

Measuring GSH levels has not yet been implemented in the preoperative assessment of patients undergoing liver resection because there is currently no adequate test to assess hepatic GSH synthesis or its depletion *in vivo* in humans. The most important reason for this is auto-oxidation and instability of GSH. Recently, ophthamate (OPH), an endogenous tripeptide analogue of GSH, has been suggested as a biomarker for GSH homeostasis in animal studies, but its applicability in humans remains to be confirmed⁶⁰⁻⁶². In mice, OPH plasma levels rose inversely proportional to the GSH depletion in a paracetamol (PCM) induced hepatotoxicity model⁶⁰. This would suggest that plasma OPH levels could serve as a reciprocal read out for the main hepatic protection system against toxic insults: the GSH system.

Surgical aspects that may further affect the function of a reduced size liver: the Pringle manoeuvre

Despite improvements in surgical techniques, one of the main concerns of liver resection remains intra-operative bleeding. Intra-operative blood loss and red blood cell transfusions are associated with short- and long-term complications in liver surgery, such as operative mortality or major complications that require post-operative radiologic or surgical intervention^{42,63}. Intra-operative blood loss also predisposes patients to post-resectional liver failure⁶³.

In an attempt to reduce or eliminate operative bleeding during transection of the liver, vascular clamping techniques can be used. The simplest and most widely used method of liver vascular clamping is the Pringle manoeuvre, which was firstly described in 1908 by J. Hogarth Pringle⁶⁴. The hepato-duodenal ligament, which contains the portal vein and

hepatic artery, is clamped en masse in order to totally occlude hepatic inflow (Figure 1.1). Different clamping techniques can be applied, such as partial or complete hepatic inflow occlusion using either continuous or intermittent pedicle clamping⁶⁵. Generally, intermittent clamping is regarded as superior to continuous clamping as it results in a better preserved remnant liver function⁶⁶. As each period of reperfusion is associated with an increase in blood loss⁶⁶⁻⁶⁸, there is some evidence that prolonged ischemic intervals (for instance 30 minutes) might be preferable^{67,69}. However, the optimal duration of the ischemic intervals during intermittent Pringle manoeuvre is unknown and depends on the balance between ischemia-induced hepatocellular damage and blood loss. Investigating the optimal duration of the ischemic intervals and further unraveling of the pathophysiological mechanisms induced by the Pringle manoeuvre could play an important role in improving peri- and postoperative outcomes in these patients.

The intermittent Pringle manoeuvre may have a negative effect on outcome after liver resection as a consequence of liver injury due to ischemia reperfusion (I/R) damage^{70,71}. On the other hand, a short period of clamping of the portal triad is also used as a pre-conditioning method in order to protect the liver against ischemic damage before continuous clamping is performed^{72,73}. I/R damage of the liver as a consequence of the Pringle manoeuvre has been studied intensively. Although the effects of the Pringle manoeuvre in the liver have been elucidated to some extent, little is known about the effects of the Pringle manoeuvre on the gut. Clamping the hepatoduodenal ligament causes stasis in the portal vein and the superior and inferior mesenteric veins, thereby reducing splanchnic outflow⁷⁴. This in turn might lead to intestinal hypoperfusion which in turn may lead to enterocyte damage and gut barrier loss⁷⁵. The effect of splanchnic hypoperfusion on intestinal damage as a consequence of the Pringle manoeuvre has been shown in several animal studies⁷⁶⁻⁷⁸. However, data on the effect of the Pringle manoeuvre on the gut in human are scarce. Hypothetically, congestion of the gut might lead to gut barrier loss and bacterial translocation with subsequent systemic inflammatory response. This might put an additional burden on a liver that has been surgically reduced in volume and function. As most liver surgeons are well aware of the fact that liver failure occurs usually when an intercurrent infection supersedes an extended liver resection, reducing a systemic inflammatory response might be worthwhile. Only limited data on this subject are currently available. Although an acute major bleeding remains an indication for application of the Pringle manoeuvre during hepatic surgery, current evidence shows no benefit for the Pringle manoeuvre on outcome after liver resection⁷⁹. Therefore in the modern era of liver surgery systematic use of the Pringle manoeuvre has become more often a subject of debate. Good knowledge of all the benefits and drawbacks of the Pringle manoeuvre is a prerequisite for safe practice and to optimize both short and long term outcome in patients undergoing liver surgery.

Figure 1.1 The Pringle manoeuvre: by clamping or temporary ligation of the hepatoduodenal ligament, hepatic inflow can be occluded. Thus, blood flow through the liver is impaired, which diminishes blood loss during transection of the liver.

Preoperative chemotherapy and body composition

Some other factors influencing total liver volume and function were also analyzed in this thesis. Preoperative chemotherapy plays an important role in the treatment of patient undergoing liver surgery. In an attempt to increase resectability rates, a number of combined surgical and chemotherapy strategies are increasingly applied for patients with CRLM⁸⁰⁻⁸². As mentioned before, neo-adjuvant chemotherapy can change the surgical options and downstage patients who initially present with non-resectable disease¹². In patients with initially resectable disease secondary tumours can be downsized in order to achieve a radical margin with a smaller resection⁸³. Unfortunately, there is a clinical paradox, since preoperative chemotherapy may improve surgical options, but chemotherapy also has considerable hepatotoxic effects and consequently increases the risk of impaired liver regeneration and postoperative liver failure^{84,85}. The exact effect of preoperative chemotherapy on liver volume, function and regeneration after major liver resection in humans remains unknown and deserves further study.

Patients undergoing liver surgery often suffer from weight loss or even cachexia due to their malignancies and also as a consequence of chemotherapy toxicity. Central to the progress of cancer-related weight loss is the development of sarcopenia. Sarcopenia, defined as the depletion of muscle mass, has been proven to affect short-term outcome negatively after liver resection⁸⁶ and has e.g. also been associated with mortality in

patients with cirrhosis planned for liver transplantation⁸⁷. It was recently shown that sarcopenia is a strong indicator of a worse outcome for both disease-free and overall survival after liver resection⁸⁸. It is currently unknown whether sarcopenia is associated with a decrease of total hepatic functional mass. Investigating the relation between body composition and metabolic state of patients on the one hand, and liver volume/function on the other hand, could help to identify patients at increased risk of postoperative liver failure after liver resection.

Aim and outline of this thesis

The central theme of the studies underlying this thesis was the different aspects of the preoperative assessment of patients undergoing liver surgery.

The subject of the **first part** of this thesis is CT-volumetry of the liver. Validation of two freely available radiological software programs for liver CT-volumetry on a personal computer was studied. Also factors that might affect the function of the remnant liver volume were investigated: preoperative metabolic reserve (sarcopenia) and preoperative chemotherapy. In addition, it was analyzed whether having a decreased metabolic physiologic reserve could have an impact on the total functional liver volume preoperatively. Preoperative chemotherapy may improve surgical options, but also has considerable hepatotoxic effects. Therefore the effect of preoperative chemotherapy on postoperative liver regeneration measured with CT-volumetry was analyzed.

The **second part** of this thesis addresses aspects of the surgical procedure that affect the function of a reduced liver: the intermittent Pringle manoeuvre. The consequences of liver surgery and the Pringle manoeuvre on interleukin-6 (IL-6) levels were analyzed. Subsequently, the influence of the duration of ischemia during intermittent Pringle manoeuvre on hepatocellular damage was investigated. It was also investigated whether the intermittent Pringle manoeuvre leads to injury to the gut.

The **final part** of this thesis elaborates on the role ophthalmate and glutathione during liver surgery. The usefulness of OPH as a biomarker for oxidative stress and hepatic GSH homeostasis was reviewed. A new method was developed to measure OPH in human samples and several factors influencing OPH concentrations were studied. Finally, a human clinical model in which the hypothesis whether OPH is useful as a marker for hepatic glutathione depletion after PCM challenge during liver surgery was tested. This is followed by the general discussion with conclusions and recommendations following from this thesis.

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Chapter 2

Liver volumetry plug and play: do it yourself
with ImageJ

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Abstract

Background

A small remnant liver volume is an important risk factor for posthepatectomy liver failure and can be predicted accurately by CT-volumetry using radiologic image analysis software. Unfortunately, this software is expensive and usually requires support by a radiologist. ImageJ is a freely downloadable image analysis software package developed by the National Institute of Health (NIH), and brings liver volumetry to the surgeon's desktop. We aimed to assess the accuracy of ImageJ for hepatic CT-volumetry.

Methods

ImageJ was downloaded from <http://rsb.info.nih.gov/ij/>. Preoperative CT scans of 15 patients who underwent liver resection for colorectal cancer liver metastases were retrospectively analyzed. Scans were opened in ImageJ and the liver, all metastases and the intended parenchymal transection line were manually outlined on each slice. The area of each selected region, metastases, resection specimen and remnant liver, was multiplied by the slice thickness to calculate volume. Volumes of virtual liver resection specimens measured with ImageJ were compared with specimen weights and calculated volumes obtained during pathology examination after resection.

Results

There was an excellent correlation between the volumes calculated with ImageJ and the actual measured weights of the resection specimens ($r^2=0.98$, $p<0.0001$). The weight/volume ratio amounted to 0.88 ± 0.04 (standard error) and was in agreement with our earlier findings using CT-linked radiologic software.

Conclusion

ImageJ can be used for accurate hepatic CT-volumetry on a Personal Computer. This application brings CT-volumetry to the surgeon's desktop at no expense and is particularly useful in cases of tertiary referred patients, who already have a proper CT-scan on CD-ROM from the referring institution. Most likely the discrepancy between volume and weight results from exsanguination of the liver after resection.

Introduction

The incidence of colorectal cancer (CRC) is steadily increasing in the Western world ^{1,2}. Approximately 50 % of all patients diagnosed with CRC will develop liver metastases at some stage of their disease. For these patients liver resection is the only potentially curative treatment option. The number of candidates for liver resection is limited, however. One of the reasons is that liver dysfunction may occur when the extent of tumour involvement requires a major resection, leaving a small postoperative remnant liver volume ³⁻⁵.

As we ^{3,6} and others ⁷ have shown before, pre- and postoperative liver volumes can be accurately calculated from computed tomography (CT) or magnetic resonance imaging (MRI) scans. Liver volumetry is useful for patient selection and helps to reduce the incidence of complications due to insufficient residual liver volume ^{3,5,8,9}. Unfortunately radiologic image analysis software is typically linked to radiologic hardware making it less accessible for non-radiologists. In addition, the intended operation should be known to the investigator to predict the remnant liver volume accurately. This requires the expertise of a liver surgeon. Therefore, CT-volumetry has hitherto been a multidisciplinary modality requiring the efforts of dedicated surgeons and radiologists. Advances in digitalization, the availability of broadband networks and the introduction of CT-scans on CD-ROM have enabled volumetry on a Personal Computer remote from radiological hardware (CT-scanners or MRI's). Advantages of stand-alone software are its applicability for tertiary referred patients, who already have a proper CT-scan on CD-ROM from the referring institution and the possibility of performing liver volumetry independent of the input of a radiologist. However, commercially available stand-alone CT-volumetry software is often expensive. Recently an alternative approach using Adobe Photoshop was proposed to circumvent this problem, but this method is laborious ⁷. ImageJ is a freely downloadable image analysis software package developed at the National Institute of Health (NIH) to assist in clinical and scientific image analyses. The applicability of ImageJ for liver volumetry has not been addressed before, but it potentially brings liver volumetry to the surgeon's desktop ¹⁰. The objective of the present study was to establish the accuracy of ImageJ for CT-volumetric analysis of the liver on a Personal Computer in patients undergoing major liver resection for CRC metastases.

Materials and methods

Patients, CT-scans, liver weights

In 2000, a prospective database was created in which data on all patients undergoing liver resection in our institution were collected. From this database, all patients who underwent major liver resection (≥ 3 segments) for colorectal metastases ($n=70$) between

2000 and 2005 were selected. Subsequently, pathology files of these patients were checked by one of the authors (R.M.V.D.) to detect those cases in which liver specimens had been weighed by the pathologist. This yielded 15 patients (Table 2.1) who underwent major liver resection with a known specimen weight. Two of these patients underwent an extended procedure: one left hepatectomy + wedge resection and one right hepatectomy + segmentectomy. Patients underwent CT scanning in their routine preoperative assessment either in our hospital or in one of the surrounding university affiliated district general teaching hospitals. For volumetric analysis, four-phase CT scans were used that were provided on CD-ROM on four different viewers: eFilm Lite (eFilm Medical Inc, Toronto, Canada), SIENET MagicView 300 VA42D (Siemens, Erlangen, Germany), DICOM CDViewer 3.412 (Quazar Software GmbH, Hamburg, Germany) or DICOM LiteBox, version 2.02C (Rennes, France). The volumetric analyses were performed by two other investigators (S.A.W.G.D., J.J.G.S.) who were blinded to the weight of the resected specimens. Immediately following resection, the resection specimens were transferred to the pathology department by one of the surgeons to scrutinize resection margins. The specimens weighed immediately upon arrival at that department.

Table 2.1 Patient characteristics.

	Median (range)
Age (years)	60 (34-77)
Gender	M 7, F 8
Height (cm)	173 (160-186)
Body weight (kg)	75 (55-110)
Right hepatectomy	12
Left hepatectomy	3
Wedge-resection (VII)	1
Segmentectomy (III)	1
AST (IU/l)	71 (14-443)
ALT (IU/l)	69 (14-392)
LDH (IU/l)	554 (250-1617)
γ GT (IU/l)	43 (6-112)
ALP (IU/l)	109 (67-215)
Bilirubin (μ M)	18.4 (9.2-47)

AST aspartate aminotransferase, ALT alanine aminotransferase, LDH lactate dehydrogenase, γ GT Gamma-glutamyl transpeptidase, ALP alkaline phosphatase

Calculation of the liver volume with ImageJ

1. Downloading ImageJ

ImageJ (version 1.33) was downloaded from <http://rsb.info.nih.gov/ij/download.html> (accession date: 01-07-2005).

2. Making a stack

The portovenous phase of four phase contrast enhanced CT-scans was used to facilitate optimal identification of liver segments and the anatomic resection plane in the individual patients. Relevant CT-slices were searched in the original CT-viewer. Every CT-slice has a unique code or number, that can be found in the information menu of the CT viewer, matching a JPEG file. The JPEG files were retrieved with Windows Explorer and opened in ImageJ by dragging them to the ImageJ main window. Individual slices were transformed to a 'stack' using the function "Convert Images to Stack" in submenu "Stacks" in pulldown menu "Image". Figure 2.1.

Figure 2.1 ImageJ showing the "making a stack" pull down menus.

3. Adjusting scale

After opening DICOM images in ImageJ, the scaling of the images is corrected automatically and volumetric analysis can be continued. However in non-DICOM viewers the scale of the imported stack was adjusted by measuring the distance between two randomly chosen but clearly recognizable points on a slice in the original CT-viewer, using its measurement tool. Subsequently the line between these points was traced on the corresponding slice and its distance set in ImageJ, using the "Set scale" function in the "Analyze" submenu.

4. Creating a Region of Interest

On the CT-slices there were three Regions of Interest (ROI) relevant for the present study: total liver area, metastases area and resection specimen area. Before outlining the ROI on each slice the ROI manager in the pull down menu “Analyze”, submenu “Tools” was opened. The total liver, the metastases and resection specimen were manually outlined with the “Polygon selection tool”. This tool can create an irregularly shaped selection defined by a series of line segments. To create a ROI on the CT-slice, the mouse has to be clicked repeatedly to create line segments. When finished, one has to click in the small box at the starting point, and ImageJ automatically draws the last segment. The respective ROI of each slice was added to the ROI manager with the function “Add” in the ROI manager menu. The resection specimen was outlined according to transection planes as described in the operation notes. The gallbladder and the inferior caval vein were excluded from the ROI, intrahepatic biliary and vascular structures were included. In all patients with a hemihepatectomy the transection line of the real liver resection followed Cantlie’s line from the top of the gallbladder, paralleling the middle hepatic vein straight to the suprahepatic inferior caval vein. In all cases the middle hepatic vein stayed in situ with the liver remnant. Figure 2.2.

Figure 2.2 CT-scan opened in ImageJ showing the ROI pull down menus and a large tumour in the right hemi-liver, requiring right hepatectomy. The resection specimen is outlined on every slice as a region of interest (ROI) and added to the ROI manager (right lower part).

5. Calculating volume

In order to calculate the areas, all the ROIs must be selected in the ROI manager. The area of each ROI was calculated with the function “Measure” in the ROI manager menu. The calculated areas were then selected and copied (right click) to Microsoft Excel, where the areas were multiplied with slice thickness (ranging from 3 mm to 7 mm varying per viewer and/or patient). The last step was to add up these values per slice, to calculate the volume of each three-dimensional structure. The data are then presented in mm³. Subsequently these values per slice were added up, to calculate the volume of each three-dimensional structure.

Time required to perform CT-volumetry and effect of slice thicknesses

To assess the time required to perform liver volumetry with ImageJ, the time needed to outline the three ROIs (total liver, metastases and resection specimen) per slice was recorded in one liver (a liver with one large tumour that required a right hemihepatectomy). Time per slice was recorded and the average time per slice for the entire liver was calculated. In addition, we calculated liver volumes using various slice thicknesses in order to evaluate the maximum slice thickness at which volumetry was still accurate.

Statistics

Results are presented as median + range as indicated in the Results section. Correlation between the actual weights of the resection specimens (determined during pathology examination) and corresponding volumes calculated with ImageJ was evaluated with Pearson’s test. A value of $p < 0.05$ was considered to indicate statistical significance. Statistics were performed using Prism 4.0 for Windows (GraphPad software, San Diego, CA).

Results

Patients

Patient characteristics are shown in Table 2.1. The median age of the patients (n=15) was 60 years (range 34-77). None of the patients had any preoperative evidence of underlying liver disease and routine liver tests were uniformly normal in all but one patient who had slightly elevated plasma levels of liver enzymes. In total, 14 patients underwent a major anatomical resection. In 11 patients a right hepatectomy was performed, in one extended with a segmentectomy (segment III). In two patients a left hepatectomy was performed, in one extended with a metastasectomy in segment VII. One patient underwent a posterior

sectionectomy extended with a segmentectomy of segment III and one patient underwent a left lateral sectionectomy. There were no postoperative deaths.

Relation between resected liver weight and CT-measured liver volume

Median resected liver volume calculated with ImageJ was 887 ml (range 20-2173 ml). The median weight of the resection specimens measured during pathology examination was 732 g (range 23-1957 g). There was a highly significant correlation between resected liver volumes calculated with ImageJ and the actual measured weights of the resection specimens (Figure 2.3) ($r^2=0.98$, $p<0.0001$). The mean ratio between measured weight and calculated volume of the resection specimens was 0.88 ± 0.04 (SE) g/ml.

Figure 2.3 Correlation between volume of resection calculated with ImageJ and actual measured weights of the resection specimens (n=15, Pearson's test).

CT-measured total liver volume / weight

The calculated median total liver volume in our study population was 1610 ml (range 1185-2990 ml) and median total tumour volume 63 ml (range 4-1102 ml). Median functional liver volume (defined as total liver volume minus tumour volume, thus normal liver tissue containing volume) was 1606 ml (range 944-2851 ml).

Time required to perform CT-volumetry and effect of slice thickness

To assess the time required to perform volumetry with ImageJ, the time needed to outline the three relevant ROI's (total liver volume, metastases volume and volume of the resection specimen) was recorded for one liver with a total volume of 1680.1 ml and a metastases volume of 32.8 ml where a right hemihepatectomy was performed. There

were in total 34 slices and analysis took on average 50 (± 3) seconds per slice. Considering the average time needed per slice, it can be calculated that at a slice thickness of 5 mm a full volumetric analysis of a single liver requires on average 25-28 minutes. Increasing the slice thickness results in a decline of accuracy of the volumetric assessment (Figure 2.4). A maximum CT-slice thickness of 1.0 cm, reduces the number of slices needed to define ROI and only adds little error in weight/volume ratio, thus providing an optimal balance between accuracy and time-efficiency.

Figure 2.4 The effect of increasing slice thickness on the accuracy: It can be conceived clearly that a slice thickness of 2 cm or more leads to large deviations from actual liver volume.

Discussion

In this study we aimed to assess the accuracy of the freely downloadable image analysis software package ImageJ for hepatic CT-volumetry in patients undergoing major hepatectomy for colorectal cancer metastases. With the use of ImageJ, an accurate CT-volumetric analysis can be performed on a Personal Computer, bringing liver volumetry within the surgeon's reach, independent of the support of a radiologist. The results are in agreement with our previous experience using professional radiological software^{3,6}, supporting the accuracy and reproducibility of CT-volumetry with ImageJ. In accordance with the study by Wigmore *et al.*, we found a ratio between liver weight and CT-scan measured liver volume of 0.88 ± 0.04 (SE) g/ml⁶. The most likely explanation of the systematic overestimation of liver volume compared to resected weight is that the 3D reconstructions are based on a perfused liver, whereas the actual weight of the specimen was obtained directly after removal in a non-perfused state. Another explanation could be that the specific gravity of the liver is lower than 1 mg/ml. However the specific gravities

of the various cellular and tissue components in the liver vary between 0.9 g/ml (fat) and 1.10 mg/ml (hepatocytes)¹¹. A specific gravity significantly below 1 could only be explained by severe liver steatosis which was found in only one of the resected specimens. Finally aspiration of liver tissue by the Cavitron ultrasonic aspirator (CUSA) device may contribute.

At present, the limits of liver resection are being increasingly explored. Indications for liver resection are continuously being extended and former contraindications such as bilobar disease, number of metastases and even extrahepatic disease have been abandoned in selected cases¹²⁻¹⁴. In general, radical tumour clearance with a minimal resection margin of at least 1 mm is sufficient to achieve a significant survival benefit¹⁵. Consequently, postoperative remnant liver volume and function have become the most important determinants of resectability. Repeat liver resections, more extensive resections and resections after aggressive induction chemotherapy are likely to yield more patients with small remnant livers and more patients with post-resectional liver failure. The actual residual volume that limits resectability is still subject to debate and the suggested minimal required remnant liver volume varies from 25% to 30%^{3,4,16}. In keeping with this, it has been shown that if meticulous attention is paid to the remnant liver volume and function, mortality can be decreased^{5,8,9}. The variability in minimal required residual liver volume among individual patients is in all probability determined by the functional status of the parenchyma. It is common knowledge that major liver resection is less well tolerated in patients with cirrhosis¹⁷ or steatosis of the liver¹⁸. Also, post chemotherapy nodular regenerative hyperplasia is considered not to have the same functional capacity as normal liver tissue. Consequently plain CT-volumetry may not suffice to adequately select patients for liver resection in these subgroups. True dynamic functional liver tests may become of additional use in these patients. In this context, it is worthwhile to mention some recent reports using a combination of CT-volumetric analysis and functional tests such as indocyanine green (ICG) clearance tests¹⁹ or functional liver scintigraphy^{20,21}. Equally, new methods become available to assess the role of the hepatic reticuloendothelial system and nitrogen clearance against the background of liver volumetry^{22,23}. The clinical applicability of such methods, however, awaits further confirmation.

A substantial number of patients admitted to specialized hepatobiliary units are referred from district general hospitals²⁴. In our experience most of these patients already have a proper four phase CT-scan on CD-ROM from the referring institution. In the majority of these cases, however, it would not be possible to perform liver volumetry on these scans due to incompatibility between radiologic software packages. ImageJ eliminates the necessity to perform an additional CT-scan for the sole purpose of liver volumetry. Another advantage is that volumetry can be done by the surgeon without support from the radiology department. A potential disadvantage is the laboriousness of volumetry with ImageJ. Volumetric analyses takes on average 50 ($\pm$ 3) seconds per slice. At a slice thickness of 5 mm, a full volumetric analysis of a single liver requires on average

25-28 minutes for the entire liver. The time required to perform the measurement can be limited by increasing slice thickness and thereby reducing the number of CT-slices that have to be outlined. However an increase in slice thickness is accompanied by decreased accuracy of the measurements. The results show that a maximum CT-slice thickness of 1.0 cm provides a good balance between accuracy and workload (Figure 2.4). Time efficiency can be improved further by downloading a volume measurement “plug-in” that calculates the volume directly in the ROI manager. This eliminates the extra step of exporting calculated areas to Microsoft Excel. The plug-in can be downloaded freely from <https://list.nih.gov/cgi-bin/wa?A2=ind0202&L=IMAGEJ&P=R11200&I=-3>. The accuracy of ImageJ to predict postoperative residual liver volume prospectively depends on the adherence to the intended resection line during the actual operation. In this retrospective study the transection plane could be carefully reconstructed based on the operation notes and guidance by the surgeons who performed the resection. The present study suggests that, in view of the excellent correlation between the predicted and actually resected weight, a reliable estimate of postoperative residual liver volume can be generated. All hepatectomies in the present study were anatomical resections. In case of non-anatomical resections it may be more difficult to predict the definitive transection plane and to outline it on a preoperative CT-scan, but this problem would affect all software packages. Further studies are necessary to assess whether ImageJ provides the same volumetric accuracy for non-anatomical resections as for anatomical resections. In addition, evaluation of liver growth prior to repeated hepatectomy²⁵ or following other volume-enhancing procedures such as portal vein embolization would be interesting potential fields of application for ImageJ.

In conclusion, ImageJ can be used for CT-volumetric analysis of the liver on a Personal Computer. This application has the benefit that it is freely downloadable and brings CT-volumetry to the surgeon’s desktop independent of the support of a radiologist. ImageJ could be particularly useful in cases of tertiary referred patients who already have a proper CT-scan on CD-ROM from the referring institution.

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Chapter 3

Prospective volumetric assessment of the liver on
a personal computer by non-radiologists prior to
partial hepatectomy

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Abstract

Background

A small remnant liver volume is an important risk factor for posthepatectomy liver failure. ImageJ and OsiriX[®] are both free, open-source image processing software packages. The aim of the present study was to compare ImageJ and OsiriX[®] in performing prospective computed tomography (CT) volumetric analysis of the liver on a Personal Computer (PC) in patients undergoing major liver resection.

Methods

Patients scheduled for a right hemihepatectomy were eligible for inclusion. Two surgeons and one surgical trainee measured volumes of total liver, tumour and future resection specimen prospectively with ImageJ and OsiriX[®]. A radiologist also measured these volumes with CT scanner-linked Aquarius iNtuition[®] software. Resection volumes were compared with the actual weights of the liver specimens removed during surgery and differences between the measured liver volumes were analyzed.

Results

15 patients (8 males: 7 females) with a median age of 63 (48-79) years were included. There was a significant correlation between measured weights of resection specimens and the volumes calculated prospectively with ImageJ and OsiriX[®] ($r=0.89$; $r=0.83$, respectively). There was also a significant correlation between the volumes measured with radiological software iNtuition[®] and the volumes measured with ImageJ and OsiriX[®] ($r=0.93$; $r=0.95$, respectively). There were no major differences in total liver volumes, resection volumes, or tumour volumes for these three software packages.

Conclusions

Prospective hepatic CT-volumetry with ImageJ or OsiriX[®] is reliable and can be accurately used on a PC by non-radiologists. ImageJ and OsiriX[®] yield results comparable to the radiological software iNtuition[®].

Introduction

Hepatectomies are performed increasingly more often, mainly because indications for liver resection are continuously being extended. Whereas in the past a number of exclusion criteria were applied to the selection of patients for hepatectomy, they have now been largely abandoned and postoperative remnant liver volume and function have become the main determinants of resectability¹⁻³.

Postoperative liver dysfunction may occur when the extent of tumour involvement requires major liver resection (3 or more segments), leaving a small postoperative remnant liver⁴⁻⁶. A reliable volumetric assessment of the part of the liver to be resected as well as future residual liver volume should be a critical part of preoperative evaluation particularly for extended resections, to prevent postoperative liver failure after liver resection. The safety of liver resection may increase if an estimate of minimal remnant liver volume is obtained via computed tomography (CT) volumetry^{4,6-9}. This is especially important if the underlying liver parenchyma is abnormal or has an impaired functional capacity. In this context repeated liver resections, increasingly extensive resections and resections after aggressive chemotherapy are likely to lead to more patients with small (functional) remnant livers and hence more risk of postoperative liver failure.

As we^{4,10-12} and others^{7,13,14} have shown before, liver volumes can be calculated accurately from CT or MRI scans. The liberal use of existing professional image-processing software is often limited by costs, access, flexibility or lack of radiology staff and specific hardware requirements. In addition, the intended operation and the potential surgical scenarios should be known to the investigator to predict the remnant liver volume accurately, which requires the expertise of a liver surgeon. The Digital Imaging and Communications in Medicine (DICOM) standard has enabled volumetry on a standalone or network attached Personal Computer remote from radiologic hardware (CT- or MRI scanner). In 2007 our group conducted a retrospective study and proposed a method to perform liver volumetry with ImageJ¹¹, which is a free open-source image analysis software package developed at the National Institutes of Health (NIH)⁸. In 2010 our group assessed the accuracy of OsiriX[®] for CT-volumetry of the liver¹². OsiriX[®] is a more user-friendly, freely downloadable open source image analysis software package for Apple Mac OS. The accuracy for CT-volumetry of the liver resection specimen prospectively prior to surgery has not yet been assessed for either of these software packages. Therefore the objective of the present study was to compare ImageJ and OsiriX[®] when performing prospective CT-volumetric analysis of the liver on a PC in patients undergoing major liver resection. In addition, the present research also aimed to compare the prospective measurements of ImageJ and OsiriX[®] to CT-volumetry performed by a radiologist using classical radiological software (iNtuition[®]) linked to a CT-scanner system.

Methods

Patients

Patients with primary or secondary liver tumours in otherwise normal livers (mostly colorectal cancer liver metastases) planned for a right hemihepatectomy at Maastricht University Medical Centre between January 2008 and April 2009 were eligible for inclusion in this study. All patients had a contrast enhanced CT-scan in their routine preoperative assessment either in our hospital or in one of the surrounding university affiliated district general teaching hospitals. Patients were admitted to the hospital one day preoperatively and routine blood tests were performed.

Surgical procedure

Liver resection was performed as detailed elsewhere¹⁵. In each patient, laparotomy was performed by bilateral subcostal incision, followed by intraoperative ultrasonographic assessment of the liver. Once resectability had been confirmed, appropriate mobilization of the liver was performed as a prelude to hepatic parenchymal transection, which was undertaken using a Cavitron Ultrasonic Surgical Aspirator (Force GSU System; Valleylab, Boulder, CO, USA). Argon beam coagulation (Erbe, Tübingen, Germany), clips and sutures were used for hemostasis. Immediately after liver resection, weights of resection specimens were recorded in the operating theatre with a Mettler ToledoTM scale.

CT-volumetry

For volumetric analysis, four-phase CT scans were used that were provided on CD-ROM on one of two different viewers: eFilm Lite (eFilm Medical, Toronto, Canada) or SIENET MagicView 300 VA42D (Siemens, Erlangen, Germany).

Calculation of the liver volume using ImageJ

ImageJ (version 1.33) was downloaded from <http://rsb.info.nih.gov/ij/download.html> (accession date: 01-01-08). The portovenous phase of all contrast enhanced CT-scans was used to facilitate optimal identification of liver segments and the anatomic resection plane. Details on how volumetry was performed with ImageJ have previously been described¹¹. Briefly, the relevant source images were individually copied (Ctrl+V) from the CT-viewer on the electronic patient care system and opened with ImageJ by dragging them to the ImageJ main window in the appropriate order. In cases of tertiary referred patients relevant CT-slices were searched in the original CT-viewer on the CD-Rom. CT-slices on a CD-rom were usually saved as compressed DICOM-files and these cannot be opened or viewed via Windows Explorer. Therefore the “tudor DICOM viewer plug-in” was downloaded for free from <http://imagejdocu.tudor.lu/> and installed in the ImageJ menu. This plug-in allowed us to preview the relevant CT-files and open them directly in ImageJ.

Total liver, tumour and resection specimen were manually outlined. The respective Region Of Interest (ROI) of each slice was added to the ROI manager. The area of each ROI was calculated and then multiplied with the slice thickness. Finally, all slice volumes were added up, to calculate the volume of each three-dimensional structure (i.e. total liver volume, resection specimen, tumour volume).

Prospective volumetric analysis ImageJ

Volumetric analysis using ImageJ was performed prospectively by two non-radiologists (S.A.W.G.D., J.H.M.B.S.) during the preoperative evaluation of the patient and was blinded to the operating surgeon. For definition of the anatomical segments Couinaud's classification was used. In all right hemihepatectomies, transection followed Cantlie's line from the top of the gallbladder, paralleling the middle hepatic vein straight to the supra hepatic inferior caval vein. In all patients the middle hepatic vein remained in situ with the liver remnant. Consequently during the venous phase the middle hepatic vein on CT was used to perform the volumetric measurement.

To assess the accuracy of volumetric measurements of the liver with ImageJ, volumes of resection specimens were compared with actual weights of the resection specimens. Immediately after liver resection, weights of resection specimens were recorded in the operating theatre. The actual weights of the resection specimens remained blinded to the investigators conducting CT-volumetry, until all patients had been included and analysed prospectively. The functional remnant liver volume (FRLV) percentage was calculated with the following formula: $\text{FRLV [\%]} = 100\% \cdot$

$$[\text{total liver volume} - \text{resected volume}] / [\text{total liver volume} - \text{tumour liver volume}]$$

Prospective volumetric analysis OsiriX®

The 32-bit OsiriX® version 3.3 was downloaded from: <http://www.osirix-viewer.com>. A 2.8-GHz Intel Core 2 Duo 24" iMac (Apple Inc., Cupertino, CA, USA) was used. Details on how volumetry was performed with OsiriX® have previously been described¹². Volumes calculated with ImageJ were compared to the volumes obtained by CT-volumetry using OsiriX® by one of the liver surgeons (RM van Dam).

Comparison of ImageJ and OsiriX® with iNtuition®

Volumes calculated with ImageJ and OsiriX® were compared to the volumes obtained by CT-volumetry performed by a specialized liver radiologist (RSA van Stiphout) using radiological image analysis software from TeraRecon® linked to an Aquarius iNtuition™ workstation. Volumetric analysis performed with ImageJ and OsiriX® was blinded to the radiologist. The volume of the liver resection specimen measured by the radiologist was used as a second standard of reference instead of the actual weight of the resection specimen.

Reliability ImageJ

For analysis of inter-user-variability of ImageJ, volume measurements of total liver volume, functional liver volume and tumour volume performed before surgery by two investigators (S.A.W.G.D., J.H.M.B.S.) were compared. The first investigator had three years experience and the second investigator had no experience with the applied software and received instructions and training before starting with the measurements. Bland Altman plots were used (see below, under Statistical Analysis) to analyze the reliability of the ImageJ method.

Time consumption ImageJ

To assess time consumption to perform one volumetric assessment of the liver with ImageJ including total liver, tumour and resection specimen, time was recorded for the individual measurements of the 15 livers. To assess time consumption, workload and influence of slice thickness, the time needed to outline these three regions of interest (total liver, tumour and resection specimen) was recorded per slice in 5 livers.

As in any volumetric measurement technique based on CT-imaging data sets, the accuracy of the volume measurements is dependent on the slice thickness used for the volume calculation. The slice thickness used for volume measurement is also crucial for the time needed to perform the analysis, because of manual outlining. Therefore, the influence of slice thickness on CT- based liver volume measurements using ImageJ was also investigated. Total liver volume (TLV) was analyzed based on different slice thicknesses in these patients undergoing a right hemihepatectomy. The relative change in TLV between volumetry performed with the standard of reference (0.5 cm) and volumetric measurements using thicker slices (1.0; 1.5 and 2.0 cm) was calculated with the following formulas: $(TLV_{1.0} - TLV_{0.5}) / TLV_{0.5} * 100\%$; $(TLV_{1.5} - TLV_{0.5}) / TLV_{0.5} * 100\%$; $(TLV_{2.0} - TLV_{0.5}) / TLV_{0.5} * 100\%$. Relative gain of time between volumetric measurement using different slice thicknesses was recorded and calculated in percentages as well.

Statistical analysis

Pearson's coefficient was used to quantify the association between the volume of the resection specimen and the actual weight of the resection specimen. A value of $p < 0.05$ was considered to indicate statistical significance. The resulting regression line was described as linear equation, and the correlation coefficient (r) was calculated. Changes of calculated volumes were tested against zero with the use of a Wilcoxon Signed Rank test with a theoretical mean of zero. Data are presented as the mean $\pm$ standard error of the mean (SEM).

Mean and standard deviation (SD) were calculated from the two volumetric results for each user using ImageJ. Next, the limits of agreement as proposed by Bland and Altman¹⁶ were calculated. This method is based on the mean and SD of the difference between two ratings of the same patient. The mean difference between two users indicates systematic

error. The SD of the difference between two users indicates random error. 95% limits of agreement as in Bland and Altman's plots result in both quantification of random error and systematic error ($1.96 * \text{sd}$) of the measurement method. Statistics were performed using Prism 5.0 for Windows (GraphPad software, San Diego, CA).

Results

Patients

Fifteen patients scheduled for right hemihepatectomy were included in the study. The median age of the patients was 63 (48-79) years. None of the patients had any pre-operative evidence of underlying liver disease and routine liver tests were uniformly normal (Table 3.1).

Table 3.1 Patients' characteristics.

Characteristics	Median (range)
Age (years)	63 (48-79)
Gender	M 8, F 7
Height (cm)	170 (155-191)
Weight (kg)	76 (55-127)
Body Mass Index	26 (19-43)
AST (IU/l)	22 (7-52)
ALT (IU/l)	30 (8-61)
LDH (IU/l)	372 (312-469)
γ GT (IU/l)	37 (29-258)
ALP (IU/l)	98 (57-168)
Bilirubin (μM)	12 (7-15)

AST aspartate aminotransferase, ALT alanine aminotransferase, LDH lactate dehydrogenase, γ GT Gamma-glutamyl transpeptidase, ALP alkaline phosphatase

Relation between prospective CT-measured liver volume and resected liver weight

The mean weight of the resection specimens measured in the operating theatre was 788.8 (± 53.7) g. The mean resected liver volumes calculated with ImageJ, OsiriX[®] and iNtuition[®] were 987.7 (± 64.0); 944.7 (± 64.1) and 905.0 (± 59.9) ml respectively. The mean ratios between measured weight and volume measured by ImageJ, OsiriX[®] and iNtuition[®] was 0.78 (± 0.03); 0.81 (± 0.04), and 0.83 (± 0.04) g/ml, respectively. Mean remnant liver volume percentages measured by ImageJ, OsiriX[®] and iNtuition[®] were 41.4 ($\pm 1.6\%$); 36.2 ($\pm 1.5\%$) and 37.8 ($\pm 1.6\%$), respectively (Table 3.2).

In one patient two surgical procedures were possible, either a right posterior sectionectomy or a right hemi hepatectomy. Both volumes were calculated prospectively and finally the volume of right posterior sectionectomy was included in the analysis. There was a significant correlation between resected liver volumes calculated with ImageJ,

OsiriX® and iNtution® prospectively and the actual measured weights of the resection specimens $r=0.89$, $p<0.001$; $r=0.83$, $p<0.001$; $r=0.85$, $p<0.001$; respectively (Figure 3.1).

Table 3.2 Mean results for CT-volumetry and intraoperative measurements.

n=15				
Mean (SEM)	ImageJ user 1	ImageJ user 2	OsiriX®	iNtution®
Total liver volume (ml)	1736.0 (± 104.0)	1785.0 (± 114.9)	1536.0 (± 98.6)	1540.0 (± 99.9)
Resected liver volume (ml)	987.7 (± 64.0)	1050 (± 78.6)	944.7 (± 64.1)	905.0 (± 59.9)
Tumour volume (ml)	14.0 (± 4.3)	18.9 (± 4.4)	12.0 (± 3.7)	12.4 (± 4.0)
Remnant liver volume (%)	41.4 (± 1.6)	39.0 (± 1.4)	36.2 (± 1.5)	37.8 (± 1.6)
Resection weight to volume ratio (g/ml)	0.78 (± 0.03)	0.77 (± 0.03)	0.81 (± 0.04)	0.83 (± 0.04)
Resection weight (g)	788.8 (± 53.7)

Figure 3.1 Correlation between volume measured prospectively with ImageJ, OsiriX® and iNtution® and resection weight measured in the operating theatre.

Inter-observer variability ImageJ

Liver volumetry measurements using ImageJ were compared using Bland-Altman plots. Mean difference of TLV measurements between both users was 49.4 ml (± 86.1 ml). The 95% limits of agreement were -218.2 to 119.4 ml (Figure 3.2A). Mean difference of the liver resection specimen measurements between both users was 53.0 ml (± 89.8 ml). The 95% limits of agreement were -228.9 to 122.9 ml (Figure 3.2B). Mean difference of the %FRLV measurements between both users was 2.3% ($\pm 4.6\%$). The 95% limits of agreement were -6.7 to 11.4% (Figure 3.2C).

Figure 3.2 A) Bland-Altman analysis for inter-observer variability of outlining the total liver volume with ImageJ. Horizontal solid line indicates mean average between the 2 users. Horizontal dashed lines indicate 95% limits of agreement (mean $\pm$ 1.96 SD).
 B) Bland-Altman analysis for inter-observer variability of outlining the resectional liver volume with ImageJ.
 C) Bland-Altman analysis for inter-observer variability of the %functional remnant liver volume (%FRLV) with ImageJ.

Time to perform measurement and effect of slice thickness

Time required for a single volumetric assessment of the liver with ImageJ including total liver, tumour and resection specimen, was on average 20 minutes (range 15-30). There was no statistical difference between the TLVs based on 0.5 cm versus 1.0 cm and 0.5 cm versus 1.5 cm and 0.5 cm versus 2.0 cm. The change in volume (calculated in percentages) was not significantly different for volumetric measurements with different slice thicknesses. For a slice thickness of 1.0 or 1.5 cm 80% of the measurements were within the 5 % limit of acceptance for the TLV change. When using the 2.0 cm slice thickness only 60% of the measurements were within this limit. The mean gain of time (calculated in percentages) by increasing slice thickness was 50% (0.5 cm vs. 1.0 cm), 64% (0.5 cm vs. 1.5 cm) and 72% (0.5 cm vs. 2.0 cm).

ImageJ versus OsiriX® and iNtuition®

There was a highly significant correlation between the volumes calculated with ImageJ and the volumetric analysis using OsiriX® ($r=0.95$, $p<0.001$). There was also a strong correlation between the volumes calculated with ImageJ and the volumetric analysis performed by a radiologist using radiologic image analysis software linked to radiologic hardware ($r=0.93$, $p<0.001$).

Discussion

The present study shows that prospective CT-volumetric analysis of the liver in patients undergoing right hemi-hepatectomy with ImageJ as well as OsiriX® on the surgeon's Personal Computer is valid and reliable. In this study we found a strong significant correlation between resection weight and resection volume measured prospectively with ImageJ and OsiriX®. Practical advantages of ImageJ and OsiriX® were described previously by our group^{11,12}. In our centre, the radiologists use the commercial image analysis software package iNtuition® for liver CT-volumetry. There was a significant correlation between the resection weight and the resection volume measured prospectively with iNtuition®. No significant differences in the mean volumes between the three software packages were found. There was a strong correlation between the volumes measured with radiologic software iNtuition® and the volumes measured with ImageJ and OsiriX®. This indicates that ImageJ and OsiriX® even if operated by non-radiologists are at least as appropriate for CT-volumetry of the liver as professional radiologic software. ImageJ and OsiriX® bring preoperative liver CT-volumetry within the surgeon's reach, independent of the support of a radiologist. Hepatopancreatobiliary (HPB) surgeons should routinely perform CT-volumetry for the preoperative assessment of patients undergoing a major liver resection. This is especially true for patients with a compromised liver function due to neo-adjuvant chemotherapy, cirrhosis or steatosis or patients scheduled for an extended liver resection.

As we¹¹ and others¹⁷ concluded before, time required to perform CT-volumetry of the liver can be reduced by increasing slice thickness and thereby diminishing the number of CT-slices that have to be outlined. Data from the current study show that in order to make a rough estimate of the FRLV (patients with a percentage FRLV of $\geq 30\%$) a slice thicknesses of 1,0 cm provides an optimal balance between accuracy and time-efficiency. However, in patients with a marginal functional liver remnant volume (20-30%) a greater accuracy is needed. In these patients it is better to use a smaller slice thickness in order to avoid over- or underestimating the volume. Time required to outline all ROIs is approximately 20 minutes with both ImageJ and OsiriX®. However, OsiriX® has a significant advantage because it has an integrated picture archiving and communication system (PACS-system) in which the data (CT-scans) of all examined patients are automatically stored.

Additionally, OsiriX® is more user-friendly and ROIs can be selected semi-automatically, which saves time and lessens the work-load¹².

Inter-observer variability for ImageJ between the two users for the volumetric assessment of the total and the resection liver volume was low. Bland-Altman plots revealed that there was one outperformer in the measurements of the total liver volume and the resection specimen volume. This resulted in relatively wide confidence intervals. However, it is mainly the %FRLV that is crucial. It has been shown elsewhere that to perform liver resection safely %FRLV has to be at least 26.6 % in patients with normal liver parenchyma⁴. In the present study the mean difference of the %FRLV measurements between both ImageJ users of 2.3% ($\pm 4.6\%$) was clinically acceptable because ImageJ user 1 and 2 predicted a %FRLV of 41.4 (± 1.6) and 39.0 (± 1.4) %, respectively. In other words the resection volume differences between the two ImageJ users should have had no clinical consequences. In patients in whom the preoperative liver volume assessment shows that the reported functional remnant liver volume percentage approaches 26.6% extra care is recommended.

A possible limitation of estimating the future remnant liver volume with CT-volumetry is the systematic overestimation of the liver volumes compared to resection specimens' weight. The physical density lower than 1.0 g/ml and the systematic overestimation of the liver volume in the present study was in accordance with previous studies^{7,10-12}. Inaccurate estimation of the resection specimen volume by CT-volumetry can occur because the liver on a CT-scan is in a perfused state and the postoperative resection specimen is in an unperfused state. This applies to every radiologic application and therefore some studies suggested the implementation of conversion factors to improve volumetric accuracy^{7,18,19}. These factors are mostly based on the intraoperative resection volume determined with the use of the Archimedes principle. Unfortunately, this approach does not determine the influence of the blood content of the resection specimen. It might be that these conversion factors can lead to a more precise volumetric analysis of the liver however their exact role remains to be determined. Patients with steatosis have more fat in the liver and the relative density of fat is 0.9 g/ml. Hence, the volume is larger and the weight less than that of a normal liver. Ultimately, this changes the volume/weight standard and can also contribute to this systemic overestimation.

In all right hemihepatectomies in the present study, virtual transection followed Cantlie's line and thus the middle hepatic vein (MHV) was used as a guide for prospective volume measurements. During surgery the demarcation line after selective inflow occlusion is used for transection and may leave liver parenchyma outside (i.e. to the right of) the MHV in some patients. As a consequence preoperative estimation of the future resection specimen volume may be overestimated in some individuals. In line with this, Karlo *et al.* showed in a retrospective study that despite a highly accurate definition of the transection line on preoperative CT- or MRI-scans using anatomic landmarks on postoperative MRI-scans, the resection specimen volume was still overestimated⁷. Systematic overestimation, however, does not affect the functional remnant liver volume

as a percentage of the total functional liver volume which is one of the most important indicators of the risk of postoperative liver failure.

In summary we conclude that user-friendly, easily accessible and free downloadable software packages like ImageJ and OsiriX[®] can be used reliably for prospective CT-volumetry of resected liver specimens. The performance of ImageJ and OsiriX[®] is comparable to radiologic software iNtuition[®]. OsiriX[®] is the most efficient and practical software package, but it can be used only at a Macintosh Personal Computer. For a Windows based Personal Computer ImageJ is a good alternative.

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Chapter 4

Sarcopenia negatively affects preoperative total
functional liver volume in patients undergoing
liver resection

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Abstract

Background

Sarcopenia may negatively affect short term outcomes after liver resection. The present study aimed at exploring whether total functional liver volume (fTLV) is related to sarcopenia in patients undergoing partial liver resection.

Methods

Volumetric analysis of total liver (TLV) and tumour volume (TV) and measurements of muscle surface were performed in patients undergoing liver resection using OsiriX® and preoperative CT-scans. fTLV-bodyweight ratio was calculated as: [fTLV (ml) / bodyweight (g)]*100%. The L3 muscle index (L3mi, cm²/m²) was then calculated by normalizing muscle areas (at third lumbar vertebral level) for height.

Results

Of 40 patients, 27 (67.5%) were classified as sarcopenic. There was a significant correlation between the L3 skeletal muscle index and the fTLV ($r=0.64$, $p<0.001$). The fTLV was significantly lower in the sarcopenia group than in the non-sarcopenia group (1396 (1129-2625) ml versus 1840 (867-2404) ml, respectively $p<0.05$). The fTLV-bodyweight ratio was significantly lower in the sarcopenia group compared with the non-sarcopenia group, 2.0 (1.4-2.5)% and 2.3 (1.5-2.5)% respectively ($p<0.05$).

Discussion

Sarcopenic patients had a disproportionally smaller preoperative fTLV compared with non-sarcopenic patients undergoing liver resection. The preoperative hepatic physiologic reserve may therefore be smaller in sarcopenic patients.

Introduction

Over recent decades, liver resections have become increasingly safe due to improvements in surgical techniques and peri-operative care. At present, mortality rates are reported to be well below 5%^{1,2}. However, post-operative liver failure remains the most important cause of lethal outcome after liver surgery. Patients with a small postoperative remnant liver volume (RLV) are at higher risk of developing post-resectional liver failure. The critical minimum RLV has been estimated to be approximately 25% after resection in normal functioning livers³. It is still unclear why some patients with smaller hepatic remnants do not develop liver failure or postoperative complications, whereas some with greater residual volumes do. In addition, postoperative morbidity remains a concern in these patients. Therefore it is of major importance to preoperatively identify patients who are at increased risk of postoperative liver failure and related morbidity. Unfortunately, at present there is no adequate method for assessing this risk in patients undergoing liver resection.

Information on body composition and the metabolic status of patients undergoing liver resection may help to identify patients at increased risk. Patients undergoing liver surgery often suffer from weight loss or even cachexia due to their malignancies or as a consequence of chemotherapy toxicity. Central to the progress of cancer related weight loss is the development of sarcopenia. Sarcopenia, defined as the depletion of muscle mass, is proven to affect short-term outcome negatively after liver resection⁴ and has also been associated with mortality in patients with cirrhosis scheduled for liver transplantation⁵. A recent study by van Vledder *et al.*⁶ showed that sarcopenia was a strong indicator of a worse prognostic outcome in terms of both disease-free and overall survival after liver resection. It is currently unknown whether sarcopenia is associated with a decrease of total hepatic functional mass. The present study hypothesized that sarcopenic patients may have impaired outcome after liver surgery, because their preoperative total functional liver volume (fTLV) is disproportionately small compared with non-sarcopenic patients. The present study was intended to explore whether fTLV is related to the level of sarcopenia in patients undergoing partial liver resection.

Methods

Ethics approval

The study was approved by the medical ethical committee of Maastricht University Medical Center and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). All patients had given written informed consent. For the current purpose, no new approval was needed as patients gave written informed consent on spin-off studies within 5 years after inclusion.

Patients

Patients with primary or secondary liver tumours (mostly colorectal cancer liver metastases) in otherwise normal livers scheduled for liver resection at Maastricht University Medical Centre between January 2008 and June 2009 were eligible for inclusion in this study. All patients had a contrast-enhanced computed tomography (CT)-scan within their routine preoperative assessment either at the present hospital or at one of the surrounding university affiliated district general teaching hospitals. Patients were admitted to the hospital one day preoperatively and were submitted to routine blood tests.

Surgical procedure

Liver resection was performed as detailed elsewhere⁷. In each patient, laparotomy was performed by bilateral subcostal incision, after which intraoperative ultrasonographic was used to assess the liver, which was then mobilized appropriately prior to hepatic parenchymal transection.

Image muscle area analysis using OsiriX[®]

All patients underwent a contrast enhanced CT-scan as part of routine preoperative assessment. Image analysis, was conducted using four-phase CT-scans provided on CD-Rom. The muscle and liver areas were outlined using the open source software programme OsiriX[®]. The 32-bit OsiriX[®] version 3.3 was downloaded from: <http://www.osirix-viewer.com>. A 2.8-GHz Intel Core 2 Duo 24" iMac (Apple Inc., Cupertino, CA, USA) was used. The muscle areas were measured on transverse slices of the CT-scans in a semi-automated fashion by two investigators (SAWG and TML). The "Grow Region (2D/3D Segmentation)" tool in the "ROI" dropdown menu enabled the automatic outlining of skeletal muscles. This modality of OsiriX[®] is based on the differences in Hounsfield Units (HU) among muscles, bony structures, and body fat. The threshold range for muscle was set between -30 and 110 Hounsfield Units (HU). If necessary, the automatically generated outlines were hand-adjusted with the "closed polygon selection" tool and "repulsor tool" to optimize the Region of Interest (ROI) (Figure 4.1).

Volumetric liver analysis using OsiriX[®]

Volumetric analysis using OsiriX[®] was performed by two investigators (SAWG and TML). Details on how volumetry was performed with OsiriX[®] have been described previously⁸. The total liver volume (TLV) and the tumour volume (TV) were manually outlined on the preoperative CT-scan. The total functional liver volume (fTLV) was calculated with the following formula: $TLV-TV = fTLV$.

Figure 4.1 CT-scan image at third lumbar vertebra level. The following skeletal muscles are outlined in red: rectus abdominis, oblique and lateral abdominal muscles, paraspinal muscles and psoas. This female sarcopenic patient had an L3 muscle index of $34.3 \text{ cm}^2/\text{m}^2$.

Calculations

Data are expressed as median and range. Skeletal muscle content was assessed by measuring the cross-sectional areas of the muscles at the level of the lumbar 3 vertebral landmark. Measurements were performed on the first image where both vertebral spinae were clearly visible and on the following image in the cranial direction. The areas of the following muscles were measured: psoas, paraspinal muscles, transversus abdominis, external and internal oblique abdominis and rectus abdominis. The mean of the skeletal muscle area was used for calculation. The measured skeletal muscle areas were then normalized for height as is conventional for other body composition measures, and the unit of expression of L3 skeletal muscle index (L3mi) was taken as cm^2/m^2 . fTLV-bodyweight ratio was calculated as: $[\text{fTLV (ml)} / \text{bodyweight (g)}] * 100\%$.

Total body fat-free mass was estimated by the method proposed by Prado *et al.*⁹ using the following formula: total body fat-free mass (kg) = $0.30 * (\text{skeletal muscle surface area at L3 in } \text{cm}^2) + 6.06$. These authors constructed this formula with regression equations relating muscle surface area in the lumbar region with total body fat-free mass. Body-surface area was calculated by using the Mosteller formula: body-surface area (m^2) = $[(\text{height (cm)} * \text{weight (kg)}) / 3600]^{1/2}$.

Sarcopenia

The definition of sarcopenia was based on a method which was validated in patients with cancer⁹. In this method, cutoff values associated with mortality were determined by optimal stratification. The L3 muscle index values associated with mortality determined in this way were 55.4 cm²/m² for men and 38.9 cm²/m² for women. Patients with an L3 muscle index lower than these values were considered sarcopenic⁹.

Statistics

Data are presented as median (range). Correlations were calculated using Pearson's test. A value of $p < 0.05$ was considered to indicate statistical significance. The resulting regression line was described as linear equation, and the correlation coefficient (r) was calculated. The Mann-Whitney U -test was applied for two group comparison for continuous data. Statistical analysis was performed using Prism 5.0 for Windows (Graphpad software, Inc., San Diego, CA) and the Statistical Package for the Social Sciences version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

Patients

A total of 40 patients were evaluated in the present study. Their median age of the patients was 62 (41-80) years and Body Mass Index (BMI) was 25.2 kg/m². Thirty-seven patients underwent liver resection because of colorectal liver metastases, 2 patients because of hepatocellular carcinoma and 1 patient because of a carcinoid metastasis. Patients were not infectious and did not have liver dysfunction. The median L3mi was 49.5 (35.5-70.8) in men and 38.4 (29.4-61.0) in women. Based on the predefined criteria 27 patients (67.5%) were sarcopenic. Sarcopenia was present in 17/24 men (70.8 %) and 10/16 women (62.5%) (Table 4.1).

Features associated with sarcopenia

TLV, fTLV and fat free body mass were significantly lower in the sarcopenia group compared with the non-sarcopenia group ($p < 0.05$). The fTLV-bodyweight ratio was also significantly lower in the sarcopenia group than in the non-sarcopenia group (2.0 (1.4-2.5)% versus 2.3 (1.5-2.5)% respectively $p < 0.05$) (Figure 4.2). Age, sex, weight and height did not significantly differ between the sarcopenia and non-sarcopenia group. The association between sarcopenia and BMI did not attain statistical significance ($p = 0.06$, Table 4.2). There was no significant correlation between age and L3mi ($r = 0.006$; $p = 0.97$).

Table 4.1 Patient characteristics at time of muscularity assessment.

Variables	Median (range)
Patient characteristics (n=40)	
Median age [range], y	62 (41-80)
Sex, number of male (%)	24 (60.0)
Weight (kg)	76 (49-127)
Height (cm)	172 (155-195)
BMI (kg/m ²)	25 (19.3-42.9)
Fat free body mass (kg)	48 (31-66)
Body surface area (m ²)	1.9 (1.47-2.46)
Preoperative laboratory testing	
Bilirubin (μmol/l) (normal <20)	11.8 (6.9-9.5)
ALT (U/l) (normal <50)	26 (7-19)
AST (U/l) (normal <38)	21 (7-16)
LDH (U/l) (normal 120-250)	367 (263-322)
γGT (U/l) (normal <55)	45 (13-258)
CRP (normal <10)	3 (1-88)
Creatinine (μmol/l) (normal 60-115)	87 (46-287)
Preoperative muscle status	
L3 muscle index (cm ² /m ²)	46.4 (29.4-70.8)
L3 muscle index in men	49.5 (35.5-70.8)
L3 muscle index in women	38.4 (29.4-61.0)
Sarcopenia, number of sarcopenic patients (%)	27 (62.5)

ALT alanine aminotransferase, AST aspartate aminotransferase, LDH lactate dehydrogenase, γGT Gamma-glutamyl transpeptidase, CRP C-reactive protein

Figure 4.2 The calculated functional total liver volume bodyweight ratio percentage # $p < 0.05$.

Relation body composition and total functional liver volume

The average time required to measure the L3 muscle area was 4 minutes per measurement per patient. There was a significant correlation between skeletal L3mi and the fTLV ($r=0.64$, $p<0.001$). There were also a significant correlations between fat free body mass, body surface area and fTLV ($r=0.74$, $p<0.001$ and $r=0.78$, $p<0.001$, respectively) (Figure 4.3A-C).

Table 4.2 Features associated with sarcopenia in patients.

Variables, median [range]	No sarcopenia (n=13)	Sarcopenia (n=27)	<i>p</i>
Median age [range] (y)	60 (43-80)	64 (41-79)	0.735
Sex, number of male (%)	7	17	0.733
Weight (kg)	85 (49-127)	74 (54-106)	0.363
Height (m)	1.7 (1.6-1.9)	1.7 (1.6-2.0)	0.108
BMI (kg/m ²)	29 (20-43)	25 (19-31)	0.06
Fat free body mass (kg)	57 (36-67)	48 (31-60)	<0.05
Body surface area (m ²)	2.0 (1.5-2.5)	1.9 (1.5-2.3)	0.709
L3 muscle index (cm ² /m ²)	59 (40-71)	42 (29-53)	<0.005
Total liver volume (ml)	1841 (919-2417)	1402 (1138-2630)	<0.05
Tumour volume (ml)	13 (0-112)	8 (1-37)	<0.05
Functional total volume (ml)	1840 (867-2404)	1396 (1129-2625)	<0.05

Figure 4.3 Correlation between the total functional liver volume and A) L3 muscle index B) fat free body mass and C) body surface area (BSA).

Discussion

The aim of the present study was to investigate whether total functional liver volume was influenced by the presence or absence of sarcopenia in patients undergoing liver surgery. There was a significant correlation between the L3mi and preoperative fTLV. The relationship between body composition and total functional liver volume was confirmed by a significant correlations between preoperative fTLV and both fat-free body mass and BSA. Sarcopenic patients had a significantly lower preoperative functional total liver volume compared with non-sarcopenic patients. As it is known that there is a relation between bodyweight and total liver volume, the fTLV-bodyweight ratio was subsequently calculated¹⁰. The fTLV-bodyweight ratio was significantly lower in the sarcopenia group than in the non-sarcopenia group providing evidence that liver weight is reduced to a disproportionally greater extent in sarcopenic patients.

The data from the present study provide novel background information that may help to explain the results reported by Peng *et al.*⁴ and van Vledder *et al.*⁶. Both these studies showed that sarcopenia has a negative impact on postoperative outcome after liver resection. However, the reason why sarcopenic patients have worse outcome compared with non-sarcopenic patients remains unclear. One explanation may be that the metabolic physiologic reserve in sarcopenic patients is decreased. The fact that CT-volumetric analysis of the liver in the present study revealed that sarcopenic patients have a smaller preoperative total functional liver volume compared to non-sarcopenic patients supports this hypothesis. It may very well be that patients with sarcopenia have worse outcome after liver surgery because their preoperative fTLV/hepatocyte mass is disproportionally small compared with that in non-sarcopenic patients. These findings underline the importance of the assessment of sarcopenia in addition to liver volumetry in patients scheduled for liver resection.

It has been suggested that in healthy individuals the level of sarcopenia is related to age¹¹. This was not found to be true in the patients enrolled in the present study since there was no significant difference in age between groups. There was also no significant correlation between age and L3 muscle mass index. This provides circumstantial evidence that the L3 muscle mass index reflects the extent of sarcopenia in patients with liver tumours and is not determined by age per se. Further, there was no significant correlation between tumour volume and level of sarcopenia.

The percentage future RLV is usually expressed as the ratio of RLV to TLV ($RLV/TLV \times 100\%$). The critical minimum RLV has been estimated to be approximately 25% after resection^{3,12-14}. Truant *et al.*¹⁰ showed that the ratio of RLV to body weight (RLV-BW) of the patient was more specific than RLV alone in predicting postoperative course after extended hepatectomy. These authors showed that a cut-off RLV-BW value of 0.5% can be used as a critical point associated with postoperative outcome. As the L3 muscle mass index is probably a more robust indicator for the physiologic reserves and cachexia than

bodyweight, perhaps an RLV-L3mi ratio would even be more accurate in predicting outcome after hepatectomies.

The extent of muscle waste/sarcopenia is often underestimated in cancer patients because a substantial proportion of patients with sarcopenia have a BMI within the normal range and some sarcopenic patients are even overweight⁹. For instance in the present study none of the sarcopenic patients were underweight (BMI<18.5 kg/m²) and one sarcopenic patient was overweight. Sarcopenic obese patients will probably be even more vulnerable to major surgery, because they have the health risks of both obesity and depleted lean body mass⁹. The L3 muscle mass index is a valuable additional tool for diagnosing frailty in patients with a normal or high BMI. Assessing muscle mass preoperatively in patients undergoing liver surgery, in order to estimate physiologic metabolic reserve, may soon become even more important as there are simultaneous incremental increases in the incidences of obesity and primary and secondary liver tumours in the Western world.

In conclusion, sarcopenic patients had a disproportionally small preoperative functional total liver volume compared with non-sarcopenic patients undergoing liver resection. The preoperative hepatic physiologic reserve may therefore be disproportionally low in sarcopenic patients compared with non-sarcopenic patients. The L3 muscle mass index is easily obtainable and may represent a valuable additional tool for preoperative risk assessment in patients undergoing partial liver resection.

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Chapter 5

Influence of preoperative chemotherapy on CT-volumetric liver regeneration following right hemihepatectomy

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Abstract

Background

An increasing number of patients undergo major liver resection following preoperative chemotherapy. Liver regeneration may be impaired in these patients, predisposing them to postoperative liver dysfunction. The aim of the present study was to evaluate the effects of preoperative chemotherapy on liver regeneration after partial liver resection.

Methods

Patients planned for right hepatectomy either with (group B) or without (group A) prior chemotherapy were identified retrospectively from a prospective multi-institutional database created in the conduct of a national randomised controlled trial (RCT). Prior chemotherapy was neither an inclusion nor an exclusion criterion of the trial. Future remnant liver volume (FRLV) was calculated by measuring total functional liver volume and resection specimen on preoperative CT-scans. Remnant liver volume after 7 days (VRLV7days) was measured on scheduled postoperative CT-scans. The early regeneration index 7 days after surgery (RI_{early}) was calculated as $[(VRLV7days - FRLV) / FRLV] * 100\%$. Data are expressed as median [interquartile range].

Results

A total of 72 patients were enrolled, 45 in group A and 27 in group B. In the whole group the liver remnant showed a 58% [39%] increase in volume at day 7 [1 day]. The RI_{early} was not significantly different between group A and B, 60% [36%] and 50% [43%], respectively ($p=0.47$). The RI_{early} was significantly lower in patients who had undergone more than 6 cycles of chemotherapy.

Conclusions

Preoperative chemotherapy does not seem to have a negative impact on early liver regeneration after partial liver resection.

Introduction

Liver resection is the only potentially curative treatment for patients with colorectal rectal liver metastases (CRLM). Unfortunately, due to local irresectability, extra-hepatic disease and/or co-morbidity only 15-20% of the patients with CRLM are eligible for liver resection. In an attempt to increase resectability rates, a number of combined surgical and chemotherapy strategies are increasingly applied for patients with CRLM¹⁻³. Neo-adjuvant chemotherapy can change the surgical options and downstage patients who initially present with non-resectable disease⁴. In patients with initially resectable disease secondary tumours can be downsized in order to achieve a radical margin with a smaller resection⁵. The most commonly applied chemotherapy is oxaliplatin or irinotecan combined with capecitabine or folinic acid (leucovorin)/fluorouracil (5-FU)^{6,7}. In some patients, targeted molecular therapy (bevacizumab or cetuximab) is added to this regimen to achieve higher clinical response rates^{8,9}.

Unfortunately, there is a clinical paradox, since preoperative chemotherapy may improve surgical options, chemotherapy also has considerable hepatotoxic effects^{10,11}. Oxaliplatin can cause vascular changes in the liver such as sinusoidal obstruction syndrome, which increases morbidity after liver resection^{11,12}. 5-FU and irinotecan regimens can lead to steatosis of the liver and consequently increase the risk of impaired liver regeneration and postoperative liver failure^{13,14}. However, the exact effect of preoperative chemotherapy on liver regeneration after major liver resection in humans is unknown. The vast majority of liver regeneration occurs within the first week after major liver resections^{15,16}. The aim of the present study was to investigate whether preoperative chemotherapy impairs liver regeneration in patients undergoing major liver resection. To that purpose CT-based liver volumetry was used before and 7 days after straight forward anatomical right hemihepatectomy.

Methods

Patients

All patients undergoing anatomical right hemihepatectomy were identified retrospectively from the prospective database of the FRESCO-trial. The FRESCO-trial was a multi center RCT on the efficacy of fibrin sealant in reducing resection surface-related complications after partial liver resections¹⁷. Patients were intraoperatively randomized to application of fibrin sealant or no application of fibrin sealant. Primary endpoint of the study was the incidence of resection surface-related complications after partial liver resection. The study included a CT-scan at day 7 after liver resection to evaluate resection surface-related complications. Patients were eligible to participate in this trial irrespective of whether

they had undergone preoperative chemotherapy, and this was not a factor in the randomization procedure.

To investigate the effect of preoperative chemotherapy on liver regeneration in the present study, all patients who had undergone a right hemihepatectomy only, and had had pre- and postoperative scans as per protocol in the FRESCO-trial were divided into two groups: patients who were not treated with preoperative chemotherapy before undergoing liver resection (group A, no chemotherapy group) and patients who were treated with chemotherapy, either in an adjuvant or neoadjuvant setting, before undergoing liver resection (group B, chemotherapy group). The following information was recorded: type of chemotherapy regime, number of cycles and the date of last chemotherapy administration. The influence of chemotherapy-surgery interval and the number of chemotherapy cycles were analyzed in different subanalysis.

Operative procedure

Liver resection was performed as detailed elsewhere¹⁸. In short, laparotomy was performed by bilateral subcostal incision, followed by intraoperative ultrasonographic assessment of the liver. Once resectability had been confirmed, mobilization of the liver was performed to prepare for hepatic parenchymal transection. In all these right hepatectomies, transection followed Cantlie's line from the top of the gallbladder, paralleling the middle hepatic vein straight to the suprahepatic inferior caval vein. In all patients the middle hepatic vein remained in situ with the liver remnant.

CT imaging details

All patients underwent a contrast-enhanced triphasic CT-scan in their routine preoperative assessment and a scheduled CT-scan 7 days after liver resection. The CT-scanning protocol was predefined and agreed between centres in the context of the FRESCO-trial.

Volume measurements

Two investigators (S.D.; P.K.) who were blinded to the different groups of patients, performed all measurements. This was supervised by a specialist consultant abdominal radiologist (E.J.v.d.J.) and a hepatobiliary surgeon (M.d.B.). After transferring imaging data, volume measurements were performed on a Siemens Syngo workstation (version CT 2007A) with the "Volume calculation" application. Scans were retrieved from the individual patients in various centres, transferred and then all measured in the University Medical Centre of Groningen.

Regions of interest (ROIs) were manually drawn in the axial view on 2-mm slices in the portal venous phase. ROIs were drawn in every other slice to minimize partial volume effects. For definition of the anatomical segments Couinaud's classification was used. The volume programme automatically interpolated between two pending ROIs. Each

interpolation was revised and corrected manually if necessary. Approximate volumes were calculated with automatic multiplication of the circumscribed areas by the CT slice thickness.

On the preoperative scan, total liver volume (TLV), the volume of the resection specimen ($V_{\text{resection}}$) and tumour volume (V_{tumour}) were measured. $V_{\text{resection}}$ was outlined according to Couinaud's classification and on the basis of hepatic (vascular) anatomy. Remnant liver volume after 7 days (V_{RLV7days}) was measured on the postoperative scan (Figure 5.1).

Figure 5.1 An outline of the remnant liver volume after 7 days (V_{RLV7days}) measured on the postoperative scan. Arrow shows the middle hepatic vein.

Calculations of volumes

Functional total liver volume (TLV_{func}) was calculated as: $TLV - V_{\text{tumour}}$. Future remnant liver volume (FRLV) was calculated as: $TLV - V_{\text{resection}}$. The early regeneration index 7 days after surgery (RI_{early}) was calculated as: $[(V_{\text{RLV7days}} - \text{FRLV}) / \text{FRLV}] * 100$. The functional resection percentage ($\text{Func}_{\text{resection}}\%$) was calculated as: $[(V_{\text{resection}} - V_{\text{tumour}}) / TLV_{\text{func}}] * 100$.

Volumetric analysis Syngo®

For assessment of the accuracy of volumetric measurements of the liver with Syngo®, volumes of resection specimens were compared with actual weights of the resection specimens. The resection weights were measured in only two of the seven participating medical centres as this was not part of the FRESCO-trial protocol. This was done immediately after liver resection: weights of resection specimens were recorded in the

operating theatre or at arrival on the pathology department. The actual weights of the resection specimens remained blinded to the investigators conducting CT-volumetry.

Postoperative complications

Postoperative complications were registered according to the Clavien-Dindo score in the prospective database¹⁹.

Liver function and liver cell damage markers

Patients were admitted to the hospital one day preoperatively and routine blood tests were performed by the clinical chemistry department in the individual hospital. Routine blood tests for liver function and liver cell damage were performed on preoperative day 1, on postoperative day 0, 1, 3, 5, 7 and on the day of discharge. The levels of alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), gamma-glutamyl transpeptidase (γGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), bilirubin total and prothrombin time (PT) were assessed.

Ethics

The study was approved by the medical ethical committee of the University Medical Center of Groningen and conducted according to the Declaration of Helsinki. All patients gave written informed consent.

Statistics

All data are expressed as median (interquartile range). To compare different subgroups the nonparametric Mann-Whitney *U*-test was applied. Dichotomous data were compared using Fisher's exact test. Multiple group comparisons for continuous data were conducted via Kruskal-Wallis test, with Dunn's post hoc test. A *p* value <0.05 was considered to indicate statistical significance. Statistical analysis was performed using Prism 4.0 for Windows (Graphpad software, Inc., San Diego, CA, USA).

Results

Patients

A total of 310 patients were included in the FRESCO-trial of whom 102 underwent a right hepatectomy. In 30 patients it was not possible to perform a complete CT-volumetric analysis (i.e. no adequate preoperative or postoperative CT-scan). This left 72 patients (35 male; 37 female) who underwent right hepatectomy for benign (n=7) or malignant liver tumours (n=65) for the final analysis. Of 72 patients, 27 (37.5%) had been treated

preoperatively with chemotherapy and 45 patients (62.5%) did not receive preoperative chemotherapy (group A) (Figure 5.2).

Figure 5.2 Flowchart of the study, *CT* computed tomography, *MRI* magnetic resonance imaging, *RFA* radiofrequency ablation.

There were no significant differences between groups in baseline characteristics (Table 5.1). The vast majority of patients in group B (59.3%) received preoperative chemotherapy consisting of oxaliplatin, capecitabine and bevacizumab. The other patients in group B (40.7%) received a variety of other chemotherapy regimens (Table 5.2). Of the patients in group B, 18.5% received adjuvant chemotherapy as part of treatment of the primary colorectal tumour and 81.5% of the patients in group B received neoadjuvant chemotherapy to treat the liver metastases. There was no significant difference in intraoperative blood loss between group A 750 ml [875 ml] and group B 800 ml [900 ml] ($p=0.57$). There was also no significant difference in operation time between group A and B: 310 min [185 min] and 320 min [202 min], respectively ($p=0.49$).

Table 5.1 Preoperative patient characteristics*.

	Group A No preoperative chemo (n=45)	Group B Preoperative chemo (n=27)	<i>p-value</i>
Age (years)	61 (16)	63 (11)	0.70
Sex	19 M, 26 F	16 M, 11 F	0.22
AST (IU/l)	28 (14)	26 (8)	0.60
ALT (IU/l)	26 (15)	28 (20)	0.35
LDH (IU/l)	206 (146)	226 (181)	0.37
γ GT (IU/l)	48 (60)	46 (66)	0.66
ALP (IU/l)	93 (50)	89 (29)	0.86
Bilirubin Total (μ M)	9 (3)	12 (8)	0.22
CRP (mg/l)	4 (6)	4 (5)	0.57
Leucocytes (10Eg/l)	7 (3)	7 (3)	0.13
Albumin (g/l)	45 (5)	44 (4)	0.30
INR	1 (0)	1 (0)	0.80
PT (seconds)	10.6 (0.6)	10.7 (0.6)	0.51

* Data are presented as median (interquartile range). *ALP* alkaline phosphatase, *ALT* alanine-aminotransferase, *AST* aspartate-aminotransferase, *CRP* C-reactive protein, *INR* international normalized ratio, *LDH* lactate dehydrogenase, *PT* prothrombin time, γ *GT* gamma-glutamyl transpeptidase

Table 5.2 Specification of the chemotherapeutic strategies.

Group B	n=27
Patients receiving 6 or more cycles	n=5
	Median (IQR range)
Number of cycles	6 (1)
Duration of chemotherapy (weeks)	15 (10)
Time between chemotherapy and surgery (months)	3 (3)
	Number (percentage of total)
Type of chemotherapy	
Oxaliplatin, Capecitabine, Bevacizumab	16 (59.3%)
Capecitabine	3 (11.1%)
Capecitabine, Bevacizumab	2 (7.4%)
5-FU, Leucovorin, Oxaliplatin, Bevacizumab	2 (7.4%)
5-FU, Leucovorin, Oxaliplatin	2 (7.4%)
5-FU, Leucovorin	1 (3.7%)

IQR interquartile range, *5-FU* fluorouracil

CT-measured liver volumes

The weight of the resection specimen as measured in 47 patients was 882 g [383 g]. A strong significant correlation was found between the resection weight and resection volume measured with Syngo® ($p < 0.0001$; $r = 0.92$) (Figure 5.3). In the total series (group A+B) the preoperatively calculated TLV, $V_{\text{resection}}$ and V_{tumour} were 1591 ml [368 ml]; 995 ml [322 ml] and 34 ml [80 ml], respectively. The TLV_{func} and FRLV were 1527 ml [390 ml] and 594 ml [233 ml], respectively. V_{tumour} was significantly larger in group A compared to group B: 38 ml [215 ml] vs. 19 ml [46 ml] ($p < 0.05$) (Table 5.3).

Figure 5.3 Correlation between volume measured with Syngo[®] and resection weight measured in the operating theatre.

Table 5.3 CT-volumetry of livers and tumours.

n=72	Group A No preoperative chemo (n=45)	Group B Preoperative chemo (n=27)	p-value
TLV (ml)	1595 (510)	1550 (336)	0.36
TLV _{Func}	1513 (404)	1570 (339)	0.57
V _{tumour} (ml)	38 (215)	19 (46)	0.04
V _{resection} (ml)	1000 (306)	967 (380)	0.36
Func _{Resection} %	59 (11)	63 (12)	0.10
FRLV (ml)	613 (263)	565 (225)	0.25
V _{RLV7days} (ml)	951 (306)	895 (318)	0.15
RI _{early} (%)	60 (36)	50 (43)	0.47

Data are presented as median (interquartile range). CT computed tomography, FRLV future remnant liver volume, Func_{Resection}% functional resection percentage, RI_{early} the early regeneration index 7 days after surgery, TLV total liver volume, TLV_{func} functional total liver volume, V_{resection} volume of the resection specimen, V_{RLV7days} remnant liver volume after 7 days, V_{tumour} tumour volume

Early liver regeneration

The RI_{early} at day 7 in the whole group of 72 patients was 58% [39%]. The RI_{early} was not significantly different between group A and B, 60 [36%] and 50 [43%] respectively; $p=0.47$) (Table 5.3, Figure 5.4A). When benign tumours were excluded in a subanalysis the difference in liver regeneration between the chemo and non-chemo group remained insignificant. In group B the time interval between chemotherapy and the liver resection was 3 months [3 months] (Table 5.2). There was no significant difference in liver regeneration between patients who underwent liver resection within 3 months after chemotherapy (n=17) and patients who underwent liver resection after 3 or more months (n=10), 49 [40%] and 58 [52%] respectively; $p=0.57$). There was also no significant difference in liver regeneration between patients who underwent liver resection within

3 months after chemotherapy (n=17) and patients who did not receive chemotherapy (n=45), 49 [40%] and 60 [36%] respectively; $p=0.38$).

Application of fibrin glue was equally distributed between groups. In the chemo group A 11 patients (40.7%) did not receive fibrin glue while 16 patients (59.3%) did. Equally, in the non-chemo group B, 21 patients (46.7%) did not receive fibrin glue while 24 patients (53.3%) did. The application of fibrin glue did not effect liver regeneration. There was no significant difference in liver regeneration between patients without fibrin glue (n=32) 57.1 [32.4%] and with fibrin (n=40) 58.0 [42.3%], $p=0.81$.

It can be argued that if the time interval between the last cycle of preoperative chemotherapy and liver surgery is 6 months or more the chance is small that chemotherapy has still an effect on liver regeneration. To analyse this, a subanalysis was performed in which patients with a chemotherapy-surgery interval of six months or more (n=5) were included in the group of patients who did not receive chemotherapy (n=45) and this group was then compared with the group of patients who received surgery within 6 months after chemotherapy (n=22). Again there was no significant difference in liver regeneration between these two groups: 59 [37%] and 51 [42%] respectively; $p=0.71$.

The RI_{early} was significantly lower in patients who had received more than 6 cycles of chemotherapy (n=5) than in patients who were treated with 6 or fewer cycles of chemotherapy (n=22) (37 [20%] and 62 [38%] respectively; $p<0.05$) (Figure 5.4B). The time interval between chemotherapy and liver resection was not significantly different between patients who had received more than 6 cycles of chemotherapy compared to patients who were treated with 6 or less cycles of chemotherapy, (5 months [14 months] and 3 months [2 months] respectively; $p=0.13$).

Some patients did not have a postoperative CT-scan at day 7, but at an earlier or later time point. This deviation from the original protocol was usually related to early functional recovery of the patient and early discharge from the hospital. There was no significant difference between groups in the number of days between the operation and the first postoperative CT-scan (group A: 7 [1 days] vs. group B: 7 [2 days], $p=0.24$). However, in order to exclude a potential difference in liver regeneration due to difference in time (surgery/CT-scan interval), the RI_{early} was also calculated and compared in patients who had a CT-scan on exactly postoperative day 7. In group A 26 patients and in group B 15 patients had a CT-scan on exactly postoperative day 7. In this sub-analysis the RI_{early} was also not significantly different between group A and B, (58 [55%] and 73 [47%] respectively; $p=0.84$). As mentioned RI_{early} in patients who received more than 6 cycles of chemotherapy was significantly lower than in patients receiving 6 or fewer cycles and this remained significantly different when only patients who had a CT-scan strictly on postoperative day 7 were analyzed.

In order to investigate the potential negative impact of bevacizumab on liver regeneration, the RI_{early} in patients treated with preoperative bevacizumab was compared with patients who had not received bevacizumab. The RI_{early} was not significantly different

between patients (n=20) who had received bevacizumab and patients (n=7) who had not (56 [42%] and 38 [61%] respectively; $p=0.18$).

Figure 5.4 A) R_{early} , as a marker of early regeneration in patients without preoperative chemotherapy (group A) and in patients treated with preoperative chemotherapy (group B). Data are medians and interquartile ranges.

B) R_{early} , as a marker of early regeneration in patients who were treated with more than 6 cycles of chemotherapy versus those who were treated with 6 or fewer cycles of chemotherapy (* $p<0.05$). R_{early} , the early regeneration index 7 days after surgery.

Postoperative complications

There was no significant difference between groups in postoperative complications, length of hospital stay or 30-day mortality (Table 5.4).

Table 5.4 Postoperative complications*.

	Group A n=45 No preoperative chemo	Group B n=27 Preoperative chemo	<i>p</i>
Minor Complications [#]			
Total	17	9	0.80
Grade I	7	1	0.24
Grade II	10	8	0.58
Major Complications [#]			
Total	10	7	0.78
Grade III a-b	7	3	0.73
Grade IV a-b	2	1	1.0
Grade V	1	3	0.15
Total	27	16	1.0
30-day mortality	1 (2.2% ⁵)	3 (11.1% ⁵)	0.15
Length of hospital stay	11 (9)	10 (8)	0.33
Length of intensive care stay	1 (1)	1 (0)	0.07

*Data are presented as median (interquartile range); [#] graded according to the Clavien–Dindo classification¹⁹;

⁵ Percentage (%) of the group

Liver function and liver cell damage markers

There were no significant differences in liver function and liver cell damage markers on the day before surgery between groups. Postoperatively, there were also no significant differences in liver function and liver cell damage markers between groups (Table 5.1, Figure S5.5A-D supplemental data, Figure S5.6A-C supplemental data).

Discussion

This study aimed to investigate whether preoperative chemotherapy impairs liver regeneration in patients undergoing major liver resection. To that purpose CT-based liver volumetry was used before, and 7 days after right anatomical hepatectomy. The results of the present study suggest that chemotherapy prior to major liver resection may have no negative impact on early liver regeneration. It could be that the number of chemotherapy cycles is pivotal, since patients who had received more than 6 cycles of chemotherapy had significantly less early liver regeneration than patients treated with 6 or fewer cycles of chemotherapy. However this should be studied in future larger studies.

There are only few studies that have focused on early liver regeneration. Most of these studies measure the early liver regeneration index in living donors for liver transplantation and the results in these studies vary, presenting different regeneration indices after one week^{15,16,20,21}. The data from the present study showed that approximately 58% of liver regeneration takes place in the first week after resection of the right lobe. These results are in accordance with results from Zappa *et al.*²⁰ who reported a regeneration index of 56% in the first week in patients undergoing right hepatectomy. Although several studies warn for the hepatotoxic effects of preoperative chemotherapy on liver regeneration in liver resection patients^{10-12,22}, data on the exact effect of preoperative chemotherapy on liver regeneration in humans are scarce. To our knowledge the present study is the first study that analyses the effect of preoperative chemotherapy on liver regeneration in a large group of patients after major liver resection. All patients underwent an anatomical right hemihepatectomy and there were no major differences in mobilization and transection of the liver. Therefore these patients were comparable and the protocol of this randomized controlled trial provided the opportunity to investigate early liver regeneration in an adequate and valid human study model. Since there is no reliable biochemical marker to assess liver regeneration in humans, CT- volumetry is an attractive approach. The CT-volumetric method with Syngo[®], a professional radiological software program used in the present study, was shown to be valid and reliable as evidenced by the excellent correlation between resected weight and volume. This method was comparable with other recently validated CT-volumetry methods^{23,24}. In order to conduct a better validation of the CT-volumetric method with Syngo[®] in future studies, it would be worthwhile to compare different liver volumetry measurements (TLV, FRLV etc.) with

different CT software programs or even with different modalities e.g. MRI or ultrasonography.

The type of chemotherapy and also the number of cycles of chemotherapy a patient receives often varies. One would expect that patients have worse liver regeneration with an increasing number of cycles of chemotherapy because their liver is longer exposed to hepatotoxic compounds. In line with this Karoui *et al.*²⁵ found that preoperative chemotherapy was an independent predictive factor of postoperative morbidity after liver surgery especially when an increasing number of cycles was given to patients. In the present study the hypothesis was confirmed that in patients who were treated with more than 6 cycles of chemotherapy a smaller RI_{early} was measured than in those who were treated with 6 or less cycles. Impaired early liver regeneration in these patients is probably also associated with an increased risk of complications after liver resection. However, only a small subgroup in this study had more than 6 cycles of chemotherapy. Future studies with larger groups of patients on the exact effect of chemotherapy and the length of chemotherapy on liver regeneration are necessary.

Contradictory results have been reported by several researchers regarding the potentially negative impact of bevacizumab on liver regeneration. Aussilhou *et al.*²⁶ have shown that bevacizumab impairs liver regeneration after preoperative portal vein embolization. On the other hand Gruenberger *et al.*²⁷ showed that neoadjuvant bevacizumab does not affect liver regeneration 3 months after resection. The same group of authors also showed in another study that when bevacizumab is combined with oxaliplatin, bevacizumab protects the liver against sinusoidal obstruction syndrome²⁸. Data from the present study showed that the preoperative administration of bevacizumab did not impair early liver regeneration after major liver resection.

The present study has several limitations. The most important limitation was that no power analysis was performed and that the sample size was relatively small. However, an important advantage was that the patients enrolled in this study were included in a multicentre prospective randomized clinical trial on a different subject, for which chemotherapy was not part of the selection criteria and therefore no selection bias took place. It was assumed that percentage early liver volume increase assessed with CT volumetry reflects liver regeneration and also liver function, however part of the liver volume increase may be caused by edema or hepatic swelling due to high portal pressure²⁹. The future liver remnant volume and postoperative true liver remnant ($V_{RLV7days}$) were measured differently, for future studies it would probably be better to conduct a direct measure of the left liver lobe pre- and postoperatively. There were no significant differences between group A and B in traditional liver function and liver cell damage markers preoperatively and postoperatively in the first week. Yet, it has to be taken into account that these are relatively crude markers³⁰.

In conclusion, this study shows that preoperative chemotherapy does not seem to have a negative impact on early liver regeneration after partial liver resection. However there was a significantly lower regeneration capacity in patients who were treated with

more than 6 cycles of chemotherapy. This study shows that strategies combining preoperative chemotherapy with liver surgery are safe, based on liver regeneration in the first week.

Figure S5.5 Postoperative plasma levels of A) ALAT, B) ASAT, C) γ GT and D) ALP as markers of liver cell damage. Group A # $p < 0.05$ compared to preoperative baseline level; group B * $p < 0.05$ compared to preoperative baseline level. No significant differences between groups. Data are medians and interquartile ranges.

Figure S5.6 Postoperative plasma levels of A) PT, B) bilirubin, and C) LDH as markers of liver function and liver cell damage, respectively. Group A # $p < 0.05$ compared to preoperative baseline level; group B * $p < 0.05$ compared to preoperative baseline level. No significant differences between groups. Data are medians and interquartile ranges. Supplemental data.

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Chapter 6

Gut and liver handling of interleukin-6 during liver resection in man

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Abstract

Background

Plasma interleukin-6 (IL-6) levels increase during liver resection. The source of this IL-6 is hitherto unclear. It has been demonstrated that the hepatosplanchnic area takes up IL-6 but the role of the gut and liver is unknown. The aim of the present study was to investigate the role of the gut and liver in IL-6 homeostasis during liver surgery.

Methods

IL-6 was measured in blood sampled from the radial artery, hepatic and portal vein before and after partial hepatectomy. Blood flow was measured to assess IL-6 fluxes (flow times AV-differences) across the gut, liver and hepatosplanchnic area.

Results

In 22 patients undergoing liver resection IL-6 release from the gut after transection was 90.9 (30.1) ng/min ($p < 0.001$), whereas net IL-6 uptake by the liver equalled 83.4 (41.7) ng/min ($p < 0.01$). Overall hepatosplanchnic flux was 7.3 (43.5) ng/min after transection and did not differ significantly from zero. Overall hepatosplanchnic flux was 87.8 (41.5) ng/min in the major resection group and -59.8 (67.5) ng/min in the minor resection group ($p < 0.05$).

Discussion

The gut releases IL-6 and the liver takes up IL-6 before and after liver resection. The loss of IL-6 uptake as a result of a small functional remnant liver could lead to higher IL-6 levels after surgery.

Introduction

Major abdominal surgery such as liver resection is followed by an inflammatory response, evidenced by increased systemic levels of pro- and anti-inflammatory cytokines¹⁻⁴. Interleukin-6 (IL-6) is one of the archetypical pro-inflammatory cytokines, which contributes to the acute phase response to injury and infection⁵⁻⁷.

Plasma IL-6 levels after liver resection are generally regarded to reflect the magnitude of the systemic inflammatory response and in fact elevated IL-6 levels during and after liver resection have been found repeatedly^{1,8-12}. Potential triggers for high IL-6 levels are ischemia-reperfusion injury, hepatocyte injury, intestinal manipulation and demand for liver regeneration^{8,9,11,13}. Prolonged and excessive release of IL-6 and other circulating cytokines after surgery is associated with systemic inflammatory response syndrome (SIRS) or sepsis^{9,10}. However, experimental animal studies have shown that the production of IL-6 is particularly important after partial hepatectomy and partial portal vein ligation, since it appears to be crucial in the onset of liver regeneration¹³⁻¹⁵. This shows that IL-6 has beneficial effects such as the onset of liver regeneration, but on the other hand high levels of IL-6 are also associated with postoperative sequelae such as SIRS or sepsis^{9,10}. Therefore a tight regulation of IL-6 levels postoperatively is probably needed.

Two studies performed in man showed that the hepatosplanchnic organs (comprising mainly the portal drained viscera and the liver) are responsible for uptake of endogenously produced IL-6^{16,17}. However the individual role of the gut and liver in determining systemic IL-6 levels was not analyzed in these previous studies. It has been hypothesized that IL-6 clearance could be a specific liver function¹⁶. Therefore, liver resection would affect the ability of the liver to take up IL-6 from the blood. Knowledge about the organs involved in IL-6 production and breakdown becomes pivotal not only for understanding the IL-6 response to liver surgery but also in the relation to the role of IL-6 in liver regeneration. The aim of this study was to investigate which of the hepatosplanchnic organs are responsible for release and uptake of IL-6. In addition, the present study also aimed to investigate IL-6 handling after major versus minor liver resection.

Methods

Patients

Patients scheduled for hepatectomy for primary or secondary malignant tumours in otherwise healthy livers at Maastricht University Medical Centre were eligible for inclusion. The study was approved by the medical ethical committee of the Maastricht University Medical Centre+ and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). All patients gave written informed consent.

Patients were admitted to the hospital one day preoperatively and routine blood tests were performed. Pre-operatively, all patients had radial artery and central venous catheters inserted to monitor arterial and central venous blood pressure as part of standard anaesthetic care.

Surgical procedures

Liver resection was performed as detailed elsewhere¹⁸. Laparotomy was performed by bilateral subcostal incision, followed by intraoperative ultrasonographic assessment of the liver. Once resectability had been confirmed, mobilization of the liver was performed to prepare for hepatic parenchymal transection, which was undertaken using a Cavitron Ultrasonic Surgical Aspirator (Valleylab, Tyco healthcare, Boulder, CO, USA). Argon beam coagulation (Force GSU System; Valleylab, Boulder, CO, USA), clips and sutures were used for hemostasis. If necessary hepatic inflow occlusion was applied by tightening a rubber tube around the entire hepatoduodenal ligament (total Pringle manoeuvre) or selectively around the left or right portal pedicle using an extra Glissonian approach as considered appropriate for the intended resection¹⁹. Cycles of 15-30 minutes occlusion alternated with 5 minutes reperfusion were applied. The choice of whether a Pringle manoeuvre was used or not as well as the type of inflow occlusion was made according to the surgeon's preference. Immediately after liver resection weights of resection specimens were recorded in the operating theatre.

Blood sampling and processing

Baseline samples were obtained from the arterial line preoperatively after induction of anaesthesia. Then, samples were obtained from the arterial line at predefined time points: after mobilisation of the liver, before and after transection of the liver and on the first postoperative day (POD). Finally, on the second and third postoperative day blood was drawn by venepuncture (the arterial line was routinely removed at day one). Blood was also drawn from the portal vein and hepatic vein (from the side of the liver with no tumour) before and after liver transection, to calculate arterio-venous (AV) differences of IL-6 across the gut, liver and hepatosplanchnic area (gut + liver). Blood samples were collected in pre-chilled EDTA containing vacuum tubes (BD vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium) and kept on ice. Hematocrit was determined using microcapillaries. Blood was centrifuged in a pre-chilled centrifuge at 4 C° at 2750 *g* for 15 minutes. Plasma was immediately stored at -80 C° until analysis. IL-6 levels were determined using a commercially available enzyme linked immunosorbent assay (ELISA) (Hycult Biotechnology, Uden, the Netherlands). The lower limit of detection for IL-6 was 16 ng/l.

Assessment of IL-6 flux across the gut and liver

Net organ fluxes (flow * arterial - venous concentration difference) were calculated across the gut, the liver and the hepatosplanchnic area. In order to calculate organ fluxes, plasma flows were calculated by correcting blood flow for hematocrit [plasma flow = blood flow * (1- hematocrit)]. Mean plasma flow in the portal vein and hepatic artery was obtained in a separate similar series of patients by our group in the recent past²⁰. These patients were comparable with patients in the present group with respect to baseline characteristics and surgical procedures.

Fluxes were calculated as $F_{\text{gut}} = \text{portal plasma flow} * ([\text{PV}] - [\text{A}])$, $F_{\text{splanchnic}} = \text{splanchnic plasma flow} * ([\text{HV}] - [\text{A}])$, $F_{\text{liver}} = F_{\text{splanchnic}} - F_{\text{gut}}$. Hepatosplanchnic plasma flow was calculated as portal flow plus hepatic artery flow. In these equations [PV], [A] and [HV] indicate portal venous, radial artery and hepatic venous plasma concentrations respectively, and $F_{\text{splanchnic}}$, F_{liver} , F_{gut} denote splanchnic flux, liver flux and gut flux, respectively. Positive fluxes indicate release, whereas negative fluxes indicate uptake.

Relation between remnant liver volume and systemic IL-6 level

Total liver volume and tumour volume were determined by CT-volumetry according to a previously described method²¹. In order to calculate volume of the resection specimen the actual, measured resection weight was multiplied by a factor of 1.22²². This conversion factor accounts for liver density (g/ml) and compensates the unperfused state of the resection specimen. The functional remnant liver volume (RLV) percentage was calculated with the following formula:

$$\text{RLV} [\%] = [\text{total liver volume} - (\text{resected weight} * 1.22)] / [\text{total liver volume} - \text{tumour volume}] * 100 \%$$

Statistics

All data are expressed as mean $\pm$ (SEM). Fluxes were tested versus a theoretical mean of zero using Wilcoxon Signed Rank test. The following outcome variables were calculated to analyse serial measurements and compare minor and major resections with respect to IL-6 handling²³: areas under the curve were calculated from baseline to IL-6 peak level and from IL-6 peak level to postoperative day 3, according to the trapezoid method. The slope of the rise in IL-6 levels was calculated by regressing the IL-6 levels at presurgery and the peak response (indicated as Slope_{rise}). The slope of the decline in IL-6 levels was calculated by regressing levels IL-6 levels from peak levels to postoperative day 3 (indicated as Slope_{decline}).

To compare different subgroups the nonparametric Mann-Whitney *U*-test was applied. Correlation was calculated with Spearman's test for nonparametric correlations. A multivariable regression analysis was conducted to explore whether there was an independent association of the use of the Pringle manoeuvre with peak IL-6 concentrations. A *p* value of <0.05 was considered to indicate statistical significance.

Statistical analysis was performed using Prism 4.0 for Windows (Graphpad software, Inc, San Diego, CA, USA).

Results

Patients

Twenty two patients scheduled for hepatectomy for primary (n=2) or secondary malignant liver tumours (n=20) were included. The median age of the patients was 65 years (30-81). Routine liver tests were within the normal range (Table 6.1). Patients underwent liver resection because of colorectal liver metastases except for 2 patients who underwent liver resection for hepatocellular carcinoma and carcinoid tumour, respectively. Ten patients underwent major liver resections (≥ 3 segments) and twelve a minor liver resection (< 3 segments). Time required for mobilisation of the liver was 60 (6) minutes. Time needed for transection of the liver was 114 (10) minutes. Plasma flows in the portal vein and hepatic artery were 320 (42) ml/min and 110 (23) ml/min, respectively. Splanchnic plasma flow was calculated to be 430 (47) ml/min²⁰. In 9 patients the liver resection was performed under total intermittent Pringle manoeuvre and in 5 patients the liver resection was performed under selective intermittent Pringle manoeuvre. The mean period of ischemia was 18 (2) minutes and the period of reperfusion was 5 minutes. In 8 patients no Pringle manoeuvre was used.

Table 6.1 Patient characteristics.

	Median (range)
Age (years)	65 (41-81)
Gender	M 12 F 10
Height (m)	1.74 (1.60-1.95)
Weight (kg)	80 (65-106)
Body Mass Index	26 (23-33)
Colorectal carcinoma	n=20
Hepatocellular carcinoma (HCC)	n=1
Carcinoid	n=1
Right hepatectomy	9
Central hepatectomy	2
Segmentectomy	11
AST (IU/L)	24 (13-46)
ALT (IU/L)	25 (12-55)
LDH (IU/L)	376 (294-529)
γ GT (IU/L)	44 (29-116)
ALP (IU/L)	91 (59-168)
Bilirubin (μ M)	13 (7-37)

AST aspartate aminotransferase, ALT alanine aminotransferase, LDH lactate dehydrogenase, γ GT Gamma-glutamyl transpeptidase, ALP alkaline phosphatase

Systemic IL-6 level

Arterial IL-6 levels were below the lower limit of detection preoperatively. Therefore the cut-off value of the ELISA (16.0 ng/l) was used. Arterial plasma IL-6 increased markedly during and after liver surgery. Before the start of liver transection arterial IL-6 levels were 73.3 (44.0) ng/l and increased progressively throughout transection to 528.3 (110.3) ng/l directly after transection. Eight hours after the start of surgery arterial IL-6 had increased further to 1125 (257.0) ng/l (Figure 6.1). On postoperative day 1, IL-6 levels had decreased to 529.4 (114.8) ng/l and a further decrease to 236.9 (40.4) ng/l and 66.4 (12.7) ng/l was observed on postoperative day 2 and day 3, respectively (Figure 6.1).

Figure 6.1 Interleukin-6 (IL-6) levels in patients (n=22) undergoing liver resection for primary or secondary tumours in an otherwise healthy liver, preoperatively, before and after transection, 8 hours after start surgery and on postoperative day 1,2 and 3. Data are means $\pm$ SEM. *Significant difference ($p < 0.05$) from baseline sample. POD, postoperative day.

IL-6 organ fluxes

IL-6 release from the gut (F_{gut}) before transection was 10.5 (7.8) ng/min ($p < 0.01$), whereas net IL-6 uptake by the liver (F_{liver}) equalled 12.0 (8.4) ng/min ($p < 0.01$). Overall hepatosplanchnic flux ($F_{\text{splanchnic}}$) was -1.6 (1.2) ng/min before transection and did not differ significantly from zero.

IL-6 release from the gut (F_{gut}) after transection was 90.9 (30.1) ng/min ($p < 0.001$), whereas net IL-6 uptake by the liver (F_{liver}) equalled 83.4 (41.7) ng/min ($p < 0.01$). Overall hepatosplanchnic flux ($F_{\text{splanchnic}}$) was 7.3 (43.5) ng/min after transection and did not differ significantly from zero (Figure 6.2A and 6.2B).

Figure 6.2 Organ fluxes of IL-6 before A) and after B) transection (n=22). The gut releases IL-6 and the liver takes up IL-6. The hepatosplanchnic area flux is close to zero. Data are means $\pm$ SEM (* p <0.01, ** p <0.01, *** p <0.001, § p <0.01).

Effects of major versus minor liver resection on IL-6 handling

Patients who underwent major liver resection had significantly greater peak systemic IL-6 levels after resection than those undergoing minor liver resection. The area under the curve for the IL-6 response from baseline to IL-6 peak level and from IL-6 peak level to postoperative day 3 was significantly greater in the major than in the minor liver resection group. Slope_{rise} was also significantly higher in the major than in the minor liver resection group, whereas Slope_{decline} did not differ significantly (Table 6.2, Figure 6.3 and Figure 6.4A-C).

Table 6.2 IL-6 plasma differences between major and minor resection.

	Minor liver resection		Major liver resection		<i>p</i> between group
	Mean	SEM	Mean	SEM	
IL-6 Peak					
ng/l	707.7	146.9	1665.0	434.1	0.04
IL-6 AUC _{presurgery to peak}	3.2 *10 ³	1.1*10 ³	7.1 *10 ³	1.7*10 ³	0.04
IL-6 AUC _{peak to POD 3}					
ng/l*hour	15.4 *10 ³	2.7*10 ³	32.4*10 ³	8.0*10 ³	0.03
IL-6 Slope _{rise}					
ng/l/hour	62.5	12.8	203.4	59	0.02
IL-6 Slope _{decline}					
ng/l/hour	-10.6	3.0	-19.2	6.6	ns

Figure 6.3 IL-6 levels for minor (n=10) versus major (n=12) liver resections. POD, postoperative day.

Figure 6.4 A) Area under curve presurgery to peak, B) Area under curve peak to POD3 and C) Slope rise in patients who underwent major versus minor liver resection. Data are means $\pm$ SEM. *Significant difference ($p < 0.05$).

On multivariable analysis, the use of the Pringle manoeuvre was also an independent predictor of high peak IL-6 levels ($p=0.04$). The extent of the operation (minor versus major resection), when corrected for the Pringle manoeuvre, was also independently and significantly related (major versus minor, odds ratio=1020, confidence interval 233-1808, $p=0.01$) with peak IL-6.

IL-6 release from the gut (F_{gut}) after transection (151.2 (62.0) ng/min) was higher after major compared with minor liver resection (40.7 (9.4) ng/min) ($p=0.006$). IL-6 uptake by the liver (F_{liver}) was 63.4 (30.9) ng/min in the major resection group and 100.5 (73.7) ng/min in the minor liver resection group, which was not significantly different ($p=0.87$). Overall hepatosplanchnic flux ($F_{\text{splanchnic}}$) was 87.8 (41.5) ng/min in the major resection group and -59.8 (67.5) ng/min in the minor resection group ($p<0.05$) (Figure 6.5). This indicates net IL-6 release from the splanchnic area after major resection versus uptake in the minor resection group.

Figure 6.5 After transection IL-6 release from the gut is significantly higher in the major liver resection group. There is a net IL-6 release from the hepatosplanchnic area in the major liver resection group compared with IL-6 uptake in the minor resection group. Data are means $\pm$ SEM. *Significant difference ($p<0.05$).

Relation between remnant liver volume and systemic IL-6 level

Mean total liver volume in the whole group ($n=22$) was 1837 ± 101 ml and mean tumour volume was 23 ± 10 ml. Total functional liver volume (total liver volume – tumour volume) was 1814 ± 104 ml. Resection weight was 522 ± 69 g. After correction using the previously mentioned conversion factor the volume of the resection specimen weight was 637 ± 84 ml. The remnant liver volume was 1199 ± 133 ml. Mean functional remnant liver volume percentage was 65 ± 5 %. There was a significant correlation between the IL-6 AUC_{peak to POD3} and the remnant liver volume ($p<0.05$, $r = -0.46$) (Figure 6.6).

Figure 6.6 Spearman's correlation analysis of remnant liver volume with IL-6 AUC_{peak tot POD3}.

Discussion

The present study investigated the contribution of hepatosplanchnic organs to release and uptake of IL-6 in patients undergoing liver resection. To the best of our knowledge this study is the first in humans to demonstrate that the gut releases IL-6 and the liver takes up IL-6 before and after liver transection. The IL-6 flux across the hepatosplanchnic area was not significantly different from zero in the whole group of minor and major liver resections before and after liver resection. This suggests that this region does not in general contribute to systemic elevation of IL-6 levels. In the group undergoing major liver resection a net release of IL-6 from the splanchnic area was observed after liver resection.

Febbraio *et al.* used a human physical exercise model showing that in the physiological situation the hepatosplanchnic organs took up IL-6 while systemic levels were elevated¹⁶. The authors assumed that the intact human liver was probably responsible for this negative IL-6 flux across the hepatosplanchnic area. However, the previous study did not specify the individual role of the gut and liver in determining systemic IL-6 levels. The present study involved patients undergoing liver surgery allowing direct access to the portal and hepatic vein. Therefore, the role of the liver and gut in IL-6 metabolism could be studied. IL-6 release from the gut increased after liver resection. Conversely, the liver took up IL-6 to the same extent after liver resection and consequently the IL-6 inter-organ balance over the hepatosplanchnic area was unchanged and remained not different from zero in the whole group.

Comparison of the major and minor liver resection group revealed that the gut released significantly more IL-6 in the major liver resection group after resection. Because liver uptake of IL-6 did not entirely match gut IL-6 release, this led to a net IL-6 release from the hepatosplanchnic area after major hepatectomy. Eventually this caused significantly higher systemic IL-6 concentrations in the major liver resection group.

Although these data show that the gut is responsible for significant IL-6 production, it must be assumed, also based on the present data that extra-splanchnic production of IL-6 occurs as well. The difference in IL-6 handling between major and minor liver resections could most likely be a consequence of a smaller functional remnant liver volume diminishing IL-6 uptake capacity in the major liver resection group. In the present study a significant correlation between the remnant liver volume and area under the curve of IL-6 levels confirmed this association. However, this is only circumstantial evidence for the hypothesis that the IL-6 uptake capacity of the liver is related to the size of the remnant liver after liver resection.

The condition of a liver containing (metastatic) tumours can be different from a normal liver in a physiological condition. Blood was sampled from the non-tumorous side of the liver and the (metastatic) liver tumour was probably not producing IL-6 because IL-6 baseline samples were not above the cutoff value of the ELISA (16 ng/l) in the present patients. In addition, the data in the current paper regarding the splanchnic fluxes of IL-6 were comparable to the data given by Febbraio *et al.*¹⁶, measured in healthy volunteers, providing circumstantial evidence for the validity of the present data.

On postoperative days 1, 2 and 3, IL-6 levels were higher in the major liver resection group. The area under the curve for this period was significantly different between the two groups. However, the Slope_{decline} was not different between the two groups. This may have been because of the relatively small group size (type II error), but there are two other potential explanations. First, reduced IL-6 levels result from rapidly decreased production from the gut postoperatively. Second, it is known that in humans there is a very rapid increase in liver mass and function during the first 7 days after partial hepatectomy. Rapid restoration of liver mass may compensate any differences in diminished liver function between the major and minor liver resection group. It is highly likely that this will not be the case when postoperative liver failure occurs. The Pringle manoeuvre was also an independent predictor of high peak IL-6 levels. This can probably be explained by the fact that the Pringle manoeuvre leads to a temporary total or partial abrogation of IL-6 liver uptake ultimately leading to higher peak IL-6 levels in the Pringle group compared with the non-Pringle group. In accordance with the results in the present study, Castell *et al.* showed that intravenous injection of radio-actively labelled IL-6 into rats caused IL-6 to disappear from plasma. This radio labelled IL-6 was mostly localized on the surface of the parenchymal liver cells²⁴, indicating liver uptake. Although the nature of this process requires further clarification, an explanation for this process may be that IL-6 is necessary for liver regeneration^{14,25}. Cressman *et al.* showed that IL-6 plays a crucial role in the onset of liver regeneration and in promoting hepatocyte proliferation in mice after partial hepatectomy¹⁴. Most likely, specific binding of IL-6 to a membrane bound IL-6 receptor in the hepatocyte or via a soluble IL-6 receptor may play an important role in inducing liver regeneration²⁶.

Although there appears to be an obvious relation between IL-6 and liver regeneration, the absence of IL-6 merely delays restoration of liver mass and IL-6 does not appear to be

the only inducer of liver regeneration¹⁴. Blindenbacher *et al.* concluded that the major role of IL-6 after partial hepatectomy in mice is the induction of an adaptive response ensuring body homeostasis and survival²⁵. In line with this, administration of IL-6 can actually improve the regenerative response of the liver after acute injury in mice²⁷. In contrast, hyper-IL-6 (agonistic protein consisting of IL-6 linked the soluble IL-6 receptor) or chronically elevated IL-6 levels are associated with inhibition of liver regeneration after injury^{27,28}. Studies have shown that IL-6 acts as a pro-survival factor of apoptosis-mediated liver injury and that it is mainly the onset of liver regeneration that plays a role in the inhibition of hepatocyte apoptosis²⁹. Based on these observations and also based on the data of the present study it could be assumed that it is likely that the liver is not simply clearing IL-6 during liver resection. On the contrary, IL-6 might play an important role intracellularly in hepatocytes, not only to activate signal pathways that induce liver regeneration but also to act as a pro-survival factor of apoptosis-mediated liver injury.

In this study it was observed that the gut is responsible for IL-6 release during liver surgery. In the major liver resection group the gut released significantly more IL-6 after resection than in the minor resection group. These observations suggest that the gut plays a significant role in the inflammatory response after liver surgery. Little is known about the role of IL-6 in the intestinal response to liver surgery or other gut injury. The gut may release IL-6 as a result of major liver resection compromising the functional integrity of the gut barrier. Wang *et al.* showed in rats that bacterial translocation from the gut is caused by intestinal injury and increased gut capillary permeability after major hepatectomy³⁰. On the other hand apoptosis in gastrointestinal epithelium has been associated with liver surgery as well. Jin *et al.* showed in mice that IL-6 plays a crucial regulatory role in in vivo enterocyte homeostasis³¹. IL-6 appears to affect enterocyte apoptotic pathways, resulting in increased enterocyte resistance to apoptosis and oxidative injuries. The exact biological significance of IL-6 as a pro-survival factor in intestinal apoptosis and apoptosis-mediated liver injury after liver resection in humans remains unclear.

In conclusion in the present study it was observed that the gut releases IL-6 and that the liver takes up IL-6 in humans before and after liver resection. The loss of IL-6 uptake as a result of a small remnant functional liver volume could lead to higher IL-6 levels during and after surgery. However, the exact consequences of high IL-6 levels circulating after liver resection are unknown. The liver might take up IL-6 in order to regulate IL-6 levels and prevent excessively elevated systemic IL-6 levels. Equally this uptake may play a role in liver regeneration.

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Chapter 7

Randomized controlled trial analyzing the effect of 15 or
30 minutes intermittent Pringle manoeuvre on
hepatocellular damage during liver surgery

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Abstract

Introduction

Aminotransferases are commonly used to determine the optimal duration of ischemic intervals during intermittent Pringle manoeuvre (IPM). However, they might not be responsive enough to detect small differences in hepatocellular damage. Liver fatty acid-binding protein (L-FABP) has been suggested as a more sensitive marker. This randomized trial aimed to compare hepatocellular injury reflected by L-FABP in patients undergoing liver resection with IPM using 15 or 30 minutes ischemic intervals.

Patients and methods

Twenty patients undergoing liver surgery were randomly assigned to IPM with 15 (15IPM) or 30 (30IPM) minutes ischemic intervals. Ten patients not requiring IPM (noIPM) served as controls. Primary endpoint was hepatocellular injury during liver surgery reflected by systemic L-FABP plasma levels. Between group comparisons were performed using area under the curve and repeated measures two-way ANOVA.

Results

The IPM groups had similar characteristics. Aminotransferases did not differ significantly between 15IPM and 30IPM at any time point. L-FABP levels rose up to 1853 ± 708 ng/ml in the 15IPM and 3662 ± 1355 ng/ml in the 30IPM group after finishing liver transection and decreased rapidly thereafter. There were no significant differences between 15IPM and 30IPM in cumulative L-FABP level ($p=0.378$) or L-FABP level at any time point ($p=0.149$). Blood loss, remnant liver function and morbidity were comparable.

Conclusion

IPM with 15 or 30 minutes ischemic intervals induced similar hepatocellular injury measured by the sensitive marker L-FABP. The present study confirms the results of earlier trials, suggesting that IPM with 30 minutes ischemic intervals may be used.

Introduction

Hepatic inflow occlusion (Pringle manoeuvre) is used to minimize blood loss during liver surgery as excessive intra-operative blood loss and red blood cell transfusions adversely affect short- and long-term outcomes¹⁻³. Different clamping techniques can be applied, such as partial or complete hepatic inflow occlusion using either continuous or intermittent pedicle clamping⁴. Generally, intermittent clamping is regarded superior to continuous clamping as it results in a better preserved remnant liver function⁵. The optimal duration of the ischemic intervals during intermittent Pringle manoeuvre (IPM) is unknown and depends on the balance between ischemia-induced hepatocellular damage and blood loss. As each period of reperfusion is associated with increased blood loss⁵⁻⁷, prolonged ischemic intervals might be preferable. Indeed, two randomized trials showed that complete IPM using 30 minutes ischemic intervals resulted in similar remnant liver function and hepatocellular damage compared with IPM using 15 minutes ischemic intervals, while intra-operative blood loss was lower after 30 minutes ischemic intervals^{6,8}.

Hepatocellular damage after pedicle clamping is commonly evaluated using alanine and aspartate aminotransferase (ALAT and ASAT) levels on consecutive postoperative days⁷⁻⁹. However, it remains uncertain if the assay of aminotransferases is sufficiently sensitive enough to detect small differences in hepatocellular injury⁹. Due to the relatively large molecular mass (96 kDa) and long half-life of aminotransferases, their plasma levels react slowly to acute tissue damage. In addition, aminotransferase levels on the first postoperative days may not only reflect ischemia-induced hepatocellular damage but also effects of postoperative care such as drug-induced hepatotoxicity or blood transfusions. Furthermore, aminotransferases, and especially ASAT, have modest organ specificity¹⁰. More accurate markers for detection and monitoring of hepatocellular injury in man are available. One of these markers is liver fatty acid-binding protein (L-FABP)^{11,12}. L-FABP is a cytosolic protein that is abundantly present in liver tissue. Its biological function involves facilitation of intracellular fatty acid transport and participation in lipid metabolism¹². After hepatocyte damage, it diffuses quickly into the circulation because of its small mass ($\approx$ 13-14 kDa). Circulating L-FABP, released from damaged cells, is cleared by the kidneys with a half-life of 11 minutes and as a result, plasma levels rapidly return to normal¹³.

The present randomized controlled trial aimed to assess the effect of complete IPM using either 15 or 30 minutes ischemic intervals on hepatocellular injury reflected by L-FABP as opposed to the less sensitive damage markers ALAT and ASAT.

Materials and methods

Experimental design

Consecutive patients scheduled to undergo liver surgery at Maastricht University Medical Centre and willing to participate were enrolled in this prospective randomized controlled trial. Exclusion criteria were (1) presence of liver cirrhosis confirmed by biopsy, (2) concomitant extra-hepatic procedures or bilio-enteric anastomosis, (3) steroid hormone medication, (4) renal dysfunction defined as serum creatinine $>137 \mu\text{mol/l}$ in men and $>104 \mu\text{mol/l}$ in women¹⁴, and (5) laparoscopic liver resection.

Immediately after the surgeon decided complete IPM would be required during liver transection, patients were randomized in a 1:1 ratio to receive either IPM with 15 minutes ischemic intervals (15IPM) or 30 minutes ischemic intervals (30IPM), both followed by 5 minutes reperfusion. Randomization was performed in the operating theatre by a researcher using numbered, sealed, opaque envelopes. An independent researcher generated the randomization sequence. Patients were blinded to the allocated intervention. Patients who did not require IPM (noIPM) served as controls.

The study protocol was approved by the Medical Ethics Committee of Maastricht University Medical Centre and registered at ClinicalTrials.gov NCT01099475. The manuscript complies with the updated CONSORT guidelines¹⁵. Informed consent was obtained prior to surgery.

Surgical procedure

Patients were anaesthetized using isoflurane and propofol. They routinely had an epidural catheter, urinary catheter, two peripheral venous catheters and indwelling catheters in a jugular vein and radial artery. Body temperature was maintained using a Bair Hugger system (Arizant Healthcare Inc. Eden Prairie, MN).

The surgical procedure was performed using a subcostal bilateral incision and Olivier retractors to improve exposure¹⁶. After dissection of the teres hepatis ligament, the liver was mobilized. Thereafter, an intra-operative ultrasound was performed to define the position of the tumour in relation to vascular and biliary structures. As IPM was not routinely applied, a patient was randomized for 15IPM or 30IPM only after the surgeon had decided a complete Pringle manoeuvre would be required. During 15IPM or 30IPM, the complete portal triad was clamped using a rubber sling. The time of inflow occlusion was adapted to the need according to the randomization protocol. Occasionally, the left or right pedicle was ligated after protocolled IPM. Five minutes reperfusion intervals were applied during which transection was stopped and cut surfaces were gently compressed to ensure hemostasis. A Cavitron Ultrasonic Surgical Aspirator (CUSA system 200 macrodissector, Cavitron Surgical Systems, Stamford, CT) and Argon beam coagulation (Force GSU System, Valleylab, Boulder, CO) were used for liver transection. A stapler device or clamps were used for transection of the hepatic veins. Central venous pressure

was maintained below 5 cm H₂O during transection to reduce venous back-bleeding. After surgery, the weight of the resection specimen was recorded. Perioperative care was protocolled, as described earlier¹⁷.

Blood sampling

Arterial blood samples were drawn from the radial artery catheter according to a fixed protocol (Figure 7.1). Before and after parenchymal transection, blood was sampled from the portal vein and one of the hepatic veins from the non-tumorous side of the liver by direct puncture. Blood samples were transferred to prechilled EDTA-containing tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The tubes were centrifuged at 4°C at 3500 x g for 15 minutes to separate plasma and stored in aliquots at -80°C till batch analysis.

Figure 7.1 Study and sampling protocol. 15IPM, 15 minutes intermittent Pringle manoeuvre; 30IPM, 30 minutes intermittent Pringle manoeuvre; noIPM, no intermittent Pringle manoeuvre; HV, hepatic vein; PV, portal vein; POD, postoperative day; *only in 15IPM and noIPM group.

Outcome measures

The primary endpoint was hepatocellular damage reflected by systemic L-FABP plasma levels during and after liver surgery. Secondary endpoints were hepatocellular damage reflected by ALAT and ASAT, hepatic function reflected by total bilirubin level and prothrombin time, cumulative ischemia and reperfusion time, duration of operation, amount of intra-operative blood loss, blood loss per gram resected liver weight, need for red blood cell transfusion during surgery or within 2 days after the operation, and morbidity and 30-day mortality. Resections were divided into major (≥ 3 segments) or minor (< 3 segments or non-anatomical resections)¹⁸. The clinical course of the participants was studied prospectively. Postoperative complications were graded according to the Clavien-Dindo score¹⁹. Grades I and II were regarded as minor and grades III, IV and V as major morbidity. Post-resectional liver failure was defined according to the

“50-50 criteria” on postoperative day 5²⁰. Bile leakage was considered in case of (1) leakage of any quantity of bile via the abdominal wound or drain at least 48h postoperatively; (2) intra-abdominal collection of bile at the time of re-operation or percutaneous drainage; or (3) cholangiographic evidence of contrast leakage. Intra-abdominal abscess was present if there was (1) leakage of any quantity of purulent fluid via the abdominal drain or (2) intra-abdominal collection of pus at the time of re-operation or percutaneous drainage, both combined with a positive microbiological culture.

Determination of hepatocellular damage

L-FABP was used as a marker of liver tissue damage. L-FABP plasma levels were determined using a commercially available enzyme-linked immunosorbent assay (Hycult Biotechnology, Uden, the Netherlands). According to the manufacturer’s manual, L-FABP plasma levels in healthy individuals were approximately 12 ng/ml. ALAT and ASAT levels were determined by the clinical chemistry laboratory of Maastricht University Medical Centre. The upper limit of normal was 35 IU/l for ALAT and 30 IU/l for ASAT.

Source of L-FABP before and after IPM

L-FABP is highly expressed in the liver, intestine and kidneys¹². As a result, systemic L-FABP plasma levels during liver surgery can reflect either hepatic, intestinal or renal damage²¹. The source of systemic L-FABP plasma levels was therefore determined by sampling blood from the portal and hepatic vein simultaneously with an arterial sample and then by calculation of arteriovenous differences (ΔAV) and net organ fluxes (F ; flow $\times \Delta AV$) across the splanchnic area, portal drained viscera (PDV) and liver. Plasma flow was measured in a previous similar series of patients and amounted to 320 ± 42 ml/min in the portal vein and 110 ± 23 ml/min in the hepatic artery¹⁶. Splanchnic flow was calculated as portal vein plus hepatic artery plasma flow. Fluxes were calculated as $F_{PDV} = \text{portal plasma flow} \times ([\text{portal vein}] - [\text{artery}])$, $F_{\text{splanchnic}} = \text{splanchnic plasma flow} \times ([\text{hepatic vein}] - [\text{artery}])$ and $F_{\text{liver}} = F_{\text{splanchnic}} - F_{PDV}$ and corrected for body weight. Positive fluxes indicate release, whilst negative fluxes indicate uptake.

Determination of liver histology

The presence of underlying disease in the non-tumorous liver was assessed by an experienced HPB pathologist in the resection specimen using H&E staining. The presence of >30% hepatic steatosis, grade 1-3 fibrosis and nodular regenerative hyperplasia was evaluated in liver tissue distant from the tumour.

Statistical analysis

Based on previous observations, mean L-FABP plasma level after transection using IPM with 15 minutes ischemic and 5 minutes reperfusion intervals was $775 \pm 210 \text{ ng/ml}^{13}$. The present study was powered to detect a 100% relative difference in L-FABP levels between the groups with 15 or 30 minutes ischemic intervals, favouring the 15 minutes group. The 100% difference was chosen because it was regarded clinically relevant and precluded large influences of analytical variation. Assuming $\alpha=0.05$ and $\beta=0.2$ with two-sided testing, 10 patients would be required in each study arm.

Continuous data are expressed as mean and standard error if normally distributed or median and range if not normally distributed. Dichotomous data are presented as absolute numbers.

Differences in baseline, operative characteristics and postoperative outcomes between the 15IPM and 30IPM group were tested using a Mann-Whitney U test. Dichotomous data were compared using Pearson's chi-square test. Continuous variables with repeated measurements were summarized as area under the curve (AUC) from baseline to postoperative day 3²². AUCs of the 15IPM and 30IPM group were compared using the Student's t-test. Subsequently, the two IPM groups were compared with the noIPM group. As a third step, differences in hepatocellular damage markers at specific time points within and between the 15IPM and 30 IPM group were analysed using the repeated measures two-way ANOVA with Bonferroni correction if the *p*-value was below 0.05. L-FABP fluxes before and after liver transection were tested using a one-sample t-test with a theoretical mean of zero. A *p*-value <0.05 was considered statistically significant.

Results

Participant flow

One hundred and two patients were assessed between October 2007 and July 2009, of whom 60 were potentially eligible for inclusion. A number of patients who did meet the inclusion criteria were not eligible because of a simultaneous randomized trial on enhanced recovery after surgery. Out of these 60 patients, 30 patients were included in the present study consisting of 10 patients in the 15IPM group, 10 patients in the 30IPM group and 10 patients in the control group (Figure 7.2). All patients received the allocated intervention. Of the 20 IPM patients, 3 needed more than 2-times 15 minutes and 5 more than 1-time 30 minutes intermittent inflow occlusion. In the noIPM group, 5 patients underwent a liver resection without any form of inflow occlusion and 5 with pedicle ligation to either the left or right side. No unplanned complete hepatic inflow occlusion was required in this group. No adverse events related to the intervention were encountered.

Figure 7.2 CONSORT diagram of patient selection. n, number; RCT, randomized controlled trial; 15IPM, 15 minutes intermittent Pringle manoeuvre; 30IPM, 30 minutes intermittent Pringle manoeuvre; noIPM, no intermittent Pringle manoeuvre.

Patient characteristics

Table 7.1 shows the patients' demographics and operative characteristics. The indications for liver resection were colorectal liver metastases in 26 patients, primary liver cancer in non-cirrhotic liver in 2 patients and hepatic adenoma and liver metastases from a carcinoid tumour in 1 patient each. There were no differences in baseline or operative characteristics between the 15IPM and 30IPM group, except for baseline total bilirubin

level, which was significantly higher in the 15IPM group (Table 7.1). The cumulative ischemia time was

34 minutes (28-105) in the 15IPM and 51 minutes (30-65) in the 30IPM group ($p=0.380$). In the 15IPM group, median blood loss was 575 ml (100-2300 ml) compared with 450 ml (250-1000 ml) in the 30IPM group ($p=0.915$). Blood loss per gram resected liver weight was 1.3 (0.3-19.0) ml/gram in the 15IPM and 1.4 (0.3-9.8) ml/gram in the 30IPM group ($p=0.887$). Also transection times were comparable.

Table 7.1 Characteristics of patients included in the present randomized controlled trial.

	Control group noIPM (n=10)	Intervention groups 15IPM (n=10) 30IPM (n=10)		<i>p</i> -value*
Baseline characteristics				
Age (years)	60 (41-70)	64 (48-80)	62 (30-77)	0.631
Sex (male)	7	6	6	1.000
BMI (kg/m ²)	24 (22-31)	24 (20-31)	26 (20-33)	0.684
ASA classification				0.443
ASA I	2	0	2	
ASA II	8	8	5	
ASA III	0	2	3	
Preoperative laboratory tests				
Bilirubin (total) (μmol/l)	15 (7-37)	14 (11-17)	10 (8-16)	0.046
Prothrombin time (sec)	11 (10-11)	11 (10-14)	10 (10-11)	0.348
Creatinine (μmol/l)	90 (54-103)	89 (59-128)	83 (68-136)	0.617
Indication for resection				1.000
Benign lesions	0	0	1	
Malignant lesions	10	10	9	
Operative characteristics				
Type of resection				0.650
Major hepatectomy	3	5	3	
Minor hepatectomy	7	5	7	
Number of resected segments	2 (1-5)	2 (1-4)	2 (1-5)	0.964
Resected liver weight (grams)	224 (51-1065)	317 (95-934)	266 (41-1140)	0.764
Duration of surgery (min)	195 (150-345)	218 (120-315)	235 (165-330)	0.323
Mobilization time (min)	48 (20-85)	55 (40-130)	55 (25-105)	0.762
Transection time (min)	78 (55-160)	75 (40-155)	110 (31-146)	0.315
Cumulative ischemia time (min)	0	34 (28-105)	51 (30-65)	0.380
Cumulative release time (min)	0	6 (4-41)	5 (0-14)	0.113
Histology of non-tumorous liver				1.000
Normal	7	6	6	
Steatosis >30%	1	1	2	
NRH	2	1	1	
Fibrosis grade 1-3	0	2	1	
Intra-operative blood loss (ml)	500 (200-1200)	575 (100-2300)	450 (250-1000)	0.915

Numbers indicate median (range) or absolute number; noIPM, no intermittent Pringle manoeuvre; 15IPM, 15 minutes intermittent Pringle manoeuvre; 30IPM, 30 minutes intermittent Pringle manoeuvre; BMI, body mass index; ASA, American Society of Anaesthesiologists; NRH, nodular regenerative hyperplasia; **p*-value represents comparison between 15IPM and 30IPM group.

Hepatocellular damage

The degree of hepatocellular damage reflected by L-FABP, ALAT and ASAT in the 15IPM, 30IPM and noIPM group is depicted in Figures 7.3A, 7.3B and 7.3C. Already before parenchymal transection, systemic L-FABP levels were significantly increased compared with baseline levels in all groups. L-FABP levels reached their peak just after transection and rapidly returned to baseline levels after surgery.

Systemic ALAT and ASAT levels showed a different pattern. Aminotransferase levels increased significantly during mobilization of the liver and rose further after transection. ALAT and ASAT peaked 8 hours after the start of surgery and remained elevated during the first postoperative days.

Figure 7.3 Course of L-FABP (continuous line), ALAT (interrupted line) and ASAT (dotted line) in the A) 15IPM, B) 30IPM and C) noIPM group. Data are presented as mean and standard error. Data after cumulative 15' ischemia and 5' reperfusion lack in the 30IPM group; * $p < 0.050$ for L-FABP versus baseline calculated with repeated measures two-way ANOVA with post-hoc paired sample t-test. L-FABP, liver fatty acid-binding protein; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; IPM, intermittent Pringle maneuver; POD, post-operative day.

Source of L-FABP before and after IPM

Before liver transection, there was a significant release of L-FABP from the liver in the 15IPM and noIPM group (Figure 7.4A), indicating that mobilization of the liver induced liver cell damage. 7.

After transection, there was a remarkable difference between L-FABP flux in the groups with 15IPM and 30IPM versus the noIPM group, suggesting the induction of ischemic hepatocellular damage by the Pringle manoeuvre. There was no evidence for intestinal injury secondary to splanchnic vascular congestion during IPM as the PDV did not significantly release L-FABP after transection with IPM using either 15 or 30 minutes ischemic intervals (Figure 7.4B).

Figure 7.4 Fluxes of L-FABP A) before and B) after liver transection with and without intermittent Pringle manoeuvre. Data are presented as mean and standard error; * $p < 0.050$ and ** $p < 0.010$; PDV, portal drained viscera; IPM, intermittent Pringle manoeuvre.

Postoperative course

The postoperative course is depicted in Table 7.2. Seventeen patients experienced postoperative complications (57%), of whom 8 had only minor and nine a combination of minor and major complications. Two patients died within 30 days after liver surgery, accounting for a 30-day mortality rate of 7%. The reasons of death were sepsis with multi-organ failure and post-resectional liver failure in 1 patient each. The 30-day mortality in the total cohort of patients ($n=102$) who had undergone liver resection during the inclusion period of the present study was 4%. The complications in the two IPM groups were comparable. Four patients in the 15IPM group and two in the 30IPM group required a red blood cell transfusion ($p=0.628$).

Table 7.2 Postoperative course of patients undergoing liver surgery with or without intermittent Pringle manoeuvre.

	Control group noIPM (n=10)	Intervention groups 15IPM (n=10) 30IPM (n=10)		p-value*
Postoperative liver function				
Peak bilirubin (total) ($\mu\text{mol/l}$)	23 (16-101)	31 (22-194)	37 (14-84)	0.670
Peak prothrombin time (sec)	13 (11-21)	14 (11-62)	15 (11-21)	0.324
Minor complications [#]				
Pneumonia	1	3	1	0.582
Pleural effusion	1	2	1	1.000
Wound infection	1	1	0	1.000
Ileus	1	1	1	1.000
Red blood cell transfusion	1	4	2	0.628
Units	1	4 (2-7)	2 (1-2)	0.267
Major complications [#]				
Intra-abdominal abscess	0	3	2	1.000
Intra-abdominal haemorrhage	0	0	1	1.000
Bile leakage	0	4	1	0.303
Ascites	0	1	0	1.000
Sepsis	0	1	2	1.000
Post-resectional liver failure	1	2	1	1.000
30-day mortality	0	1	1	1.000
Postoperative stay				
Length of hospital stay (days)	8 (5-10)	11 (5-53)	8 (5-119)	0.955

Numbers indicate median (range) or absolute number; noIPM, no intermittent Pringle manoeuvre; 15IPM, 15 minutes intermittent Pringle manoeuvre; 30IPM, 30 minutes intermittent Pringle manoeuvre; [#]Graded according to the Clavien-Dindo classification [19]; *p-value represents comparison between 15IPM and 30IPM group

Discussion

The present randomized controlled trial was designed to compare the effect of intermittent hepatic inflow occlusion using either 15 or 30 minutes ischemic intervals on hepatocellular damage during liver surgery in man. In addition to the commonly used aminotransferases, the more sensitive marker L-FABP was studied as a read out for liver injury. We provide novel data that show no difference in hepatocellular damage reflected by L-FABP between patients undergoing liver resection with complete IPM using either 15 or 30 minutes ischemic intervals. These results corroborate results of earlier trials which used less sensitive markers for liver tissue damage. Intra-operative blood loss, red cell blood transfusions and remnant liver function were comparable. Moreover, complete IPM did not induce intestinal injury secondary to portovenous stasis.

Hepatic inflow occlusion is frequently applied during liver transection to prevent excessive blood loss^{4,23}. A drawback of the Pringle manoeuvre is the induction of ischemic hepatocellular injury resulting in postoperative morbidity^{24,25}. In order to limit this injury, techniques such as ischemic preconditioning and intermittent pedicle clamping are

effective^{5,7}. However, several trials suggested that IPM using 15 minutes ischemic intervals was associated with significantly higher blood loss during transection compared with continuous clamping, resulting from extensive bleeding during the 5 minutes reperfusion periods^{5,7}. Theoretically, ischemic intervals of 30 minutes are preferable as this time period allows the surgeon to control vessels at the cut surface of the liver meticulously whereas the liver can tolerate cumulative ischemia up to 120 minutes²⁶. Moreover, short ischemic intervals lead to repetitive reperfusion episodes, potentially resulting in more extensive hepatocellular damage. Two recent randomized trials did show that remnant liver function and hepatocellular damage after IPM using 15 or 30 minutes ischemic intervals were comparable, whereas blood loss was lower after 30IPM^{6,8}. In these studies, however, bilirubin or aminotransferase levels on different postoperative days were used as a read out for liver function and damage while it is questionable whether these levels are sufficiently sensitive to pick up slight differences between the two ischemic intervals¹⁰. Our trial confirms that hepatocellular damage, measured by the highly sensitive damage marker L-FABP, was similar between complete IPM with 15 or 30 minutes ischemic intervals. Also remnant liver function was comparable. However, no IPM resulted in significantly less hepatocellular damage in the control group of patients with similar characteristics. As a recent meta-analysis showed no definite advantage of any form of portal triad clamping in terms of postoperative outcomes, IPM should be preserved for individual patients undergoing complex resections with expected high blood loss²⁷. If a Pringle manoeuvre is necessary, 30 minutes ischemic intervals should be used as this led to decreased blood loss and a higher transection speed in previous studies with larger patient numbers^{6,8}.

Another concern of the Pringle manoeuvre is the induction of intestinal damage secondary to portovenous stasis²⁸. Theoretically, intestinal injury after hepatic inflow occlusion might result in intestinal barrier dysfunction and bacterial translocation. The clinical relevance of clamping induced intestinal injury in man is under debate^{28,29}. Kim *et al.* described an increase in portovenous interleukin-6 levels compared with arterial levels at the end of continuous inflow occlusion, suggesting interleukin-6 production in the mesenteric area²⁸. A small study by Huguet's group showed that, even though bacterial translocation to mesenteric lymph nodes occurred during liver resection under continuous inflow occlusion, no correlation between positive lymph nodes and postoperative infectious complications was present²⁹. Our data provide clear evidence that complete IPM using either 15 or 30 minutes inflow occlusion induced no significant intestinal damage, evidenced by minimal L-FABP fluxes over the portal drained viscera. L-FABP is abundantly present in intestinal villi and intestinal damage typically results in a rapid release of L-FABP²¹. Therefore, L-FABP plasma concentration is regarded a reliable marker for the detection of intestinal injury. The exact relation between L-FABP plasma level and gut barrier function has, to the best of our knowledge, not yet been elucidated. As a result, the effect of IPM with either 15 or 30 minutes inflow occlusion on intestinal barrier

function remains to be established. By measuring fluxes, an exclusive hepatic origin of systemic L-FABP plasma levels after liver transection could be confirmed.

Recently, doubts have arisen concerning the contribution of ischemia- reperfusion to total hepatocellular damage caused by liver surgery. Several experimental studies confirm our observation that mobilization of the liver results in profound liver tissue damage, even before inflow occlusion is applied^{13,30}. It is hypothesized that liver manipulation results in a disturbed hepatic microcirculation leading to hypoxia and Kupffer cell activation which causes additional hepatic injury³¹. Therefore, interventions aiming at minimization of hepatocellular damage should be started early during liver surgery.

Our study has some limitations. First, the relation between hepatocellular damage reflected by L-FABP or aminotransferases and postoperative outcome is indistinct. The current trial was powered to detect a 100% difference in L-FABP level between the 15IPM and 30IPM group. In order to study differences in postoperative morbidity, a considerably larger number of patients should have been included^{27,32}. Furthermore, our results may not apply to patients with underlying liver disease. Insights in the effect of IPM on hepatocellular damage in these patients were reported recently⁹. After liver surgery with inflow occlusion, patients with liver cirrhosis had a lower difference between preoperative and maximum postoperative ASAT and ALAT levels compared with patients with normal liver, while their postoperative remnant liver function was worse. This further underlines the idea that aminotransferases, measured during hospital stay, may not accurately reflect the course of events secondary to hepatic inflow occlusion. Finally, a relation between prolonged pedicle clamping and accelerated outgrowth of residual liver disease was demonstrated in a murine model³³. Clinical data on this topic in man are contradictory^{34,35}. Long-term results of a randomized controlled study that compared liver surgery with or without IPM show no difference in disease-free or overall survival between the two patient groups³⁶.

To conclude, complete IPM using 30 minutes ischemic intervals resulted in similar hepatocellular damage, reflected by the highly sensitive damage marker L-FABP, compared with complete IPM using 15 minutes ischemic intervals, without induction of intestinal damage or loss of remnant liver function. If inflow occlusion during liver transection is necessary, intermittent pedicle clamping using 30 minutes ischemic intervals may be used.

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Chapter 8

Total intermittent Pringle manoeuvre during liver resection can induce intestinal epithelial cell damage and endotoxemia

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Abstract

Objectives

The intermittent Pringle manoeuvre (IPM) is frequently applied to minimize blood loss during liver transection. Clamping the hepatoduodenal ligament blocks the hepatic inflow, which leads to a non circulating (hepato)splanchnic outflow. Also, IPM blocks the mesenteric venous drainage (as well as the splenic drainage) with raising pressure in the microvascular network of the intestinal structures. It is unknown whether the IPM is harmful to the gut. The aim was to investigate intestinal epithelial cell damage reflected by circulating intestinal fatty acid binding protein levels (I-FABP) in patients undergoing liver resection with IPM.

Methods

Patients who underwent liver surgery received total IPM (total-IPM) or selective IPM (sel-IPM). A selective IPM was performed by selectively clamping the right portal pedicle. Patients without IPM served as controls (no-IPM). Arterial blood samples were taken immediately after incision, ischemia and reperfusion of the liver, transection, 8 hours after start of surgery and on the first post-operative day.

Results

24 patients (13 males) were included. 7 patients received cycles of 15 minutes and 5 patients received cycles of 30 minutes of hepatic inflow occlusion. 6 patients received cycles of 15 minutes selective hepatic occlusion and 6 patients underwent surgery without inflow occlusion. Application of total-IPM resulted in a significant increase in I-FABP 8 hours after start of surgery compared to baseline ($p < 0.005$). In the no-IPM group and sel-IPM group no significant increase in I-FABP at any time point compared to baseline was observed.

Conclusion

Total-IPM in patients undergoing liver resection is associated with a substantial increase in arterial I-FABP, pointing to intestinal epithelial injury during liver surgery.

Introduction

Intra-operative blood loss and red blood cell transfusions are associated with short- and long-term complications in liver surgery, such as operative mortality or major complications that require post-operative radiologic or surgical intervention^{1,2}. Intra-operative blood loss also predisposes patients to post-resectional liver failure². In an attempt to avoid blood loss, the intermittent Pringle manoeuvre (IPM) is frequently applied in patients undergoing liver surgery. This implies intermittent clamping of the hepatoduodenal ligament, thereby occluding hepatic inflow³.

IPM may have a negative effect on outcome after liver resection as a consequence of liver injury due to ischemia reperfusion (I/R) damage⁴. On the other hand, a short period of clamping of the portal triad is also used as a pre-conditioning method in order to protect the liver against ischemia damage when continuous clamping is performed^{5,6}. I/R damage of the liver as a consequence of IPM has been well studied, however little is known about the effects of IPM on the gut. Clamping the hepatoduodenal ligament causes stasis in the portal vein and the superior and inferior mesenteric veins, thereby reducing splanchnic outflow⁷. Intestinal hypoperfusion leads to enterocyte damage and gut barrier loss⁸. The effect of splanchnic hypoperfusion on intestinal damage as a consequence of IPM has been proven in several animal studies⁹⁻¹¹. Sheen-Chen *et al.*¹² showed recently in rats that occlusion of the hepatoduodenal ligament significantly increased jejunal apoptosis. However, data on the effect of IPM on the gut in man are scarce. Loss of intestinal epithelial integrity is clinically important, as it is associated with the development of sepsis and multiple organ failure (MOF) following major surgery, trauma and shock^{13,14}. This might be especially important in patients with small for size liver remnant volume and/or parenchymal dysfunction due to neo-adjuvant chemotherapy, cholestasis and/or cirrhosis¹⁵⁻¹⁷.

Intestinal fatty acid binding protein (I-FABP) is a small, water-soluble cytosolic protein that is easily released into the circulation upon enterocyte membrane integrity loss. I-FABP is solely present in mature epithelial cells of the small intestine and to a lesser extent in the large intestine¹⁸. We^{8,19} and others^{18,20,21} have shown that I-FABP is an accurate marker for intestinal epithelial cell damage.

The principal aim of this study was to investigate whether IPM causes intestinal epithelial cell damage and barrier loss in patients undergoing liver resection.

Methods

Patients

Patients who underwent liver surgery at Maastricht University Medical Center were eligible for inclusion in this prospective trial. The study was approved by the medical

ethical committee of Maastricht University Medical Center and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). All patients gave written informed consent.

Pre-operatively it was decided whether a Pringle manoeuvre was required according to the surgeon's preference. If a Pringle manoeuvre was required, patients were randomly assigned to IPM with 15 (15-IPM) or 30 minutes (30-IPM) ischemic intervals. Patients who did not require IPM served as controls (no-IPM). These patients were investigated in an RCT on a different topic by our group in the recent past²². The current study uses samples obtained from this previous RCT titled 'Randomized controlled trial analyzing the effect of 15 or 30 min intermittent Pringle manoeuvre on hepatocellular damage during liver surgery'. The trial is registered at ClinicalTrials.gov - registration number: NCT01099475. No new randomization was carried out for this study. A subgroup of 6 selective intermittent Pringle manoeuvre patients (15 minutes ischemic intervals, sel-IPM) was added to the present study. The addition of these patients was approved by the above-mentioned IRB, and informed consent was obtained from each. The 15-IPM and 30-IPM groups were later pooled in a subanalysis to compare total-IPM and no-IPM.

Operative procedure

Pre-operatively, all patients had radial artery and central venous catheters inserted to monitor arterial and central venous pressure as part of standard anesthetic care. Liver resection was performed as detailed elsewhere²³. Resections were classified as major (≥ 3 segments) or minor (< 3 segments or non-anatomical) resections. Laparotomy was performed by bilateral subcostal incision, followed by intraoperative ultrasonographic assessment of the liver. Once resectability had been confirmed, mobilization of the liver was performed to prepare for hepatic parenchymal transection, which was undertaken using a Cavitron Ultrasonic Surgical Aspirator (Force GSU System; Valleylab, Boulder, CO). Argon beam coagulation (Erbe, Germany, Tübingen), clips and sutures were used for hemostasis. Central venous pressure was maintained below 5 cm H₂O during transection to reduce venous back-bleeding. The Pringle manoeuvre was performed by tightening a rubber tube around the entire hepatoduodenal ligament (total Pringle manoeuvre) or selectively around the left or right portal pedicle using an extra Glissonian approach²⁴. Cycles of 15 or 30 minutes of occlusion were alternated with 5 minutes of reperfusion. The control and Pringle manoeuvre patients were operated by the same surgeons.

Blood sampling and processing

Arterial blood samples were obtained from the radial artery line at predefined time points (Figure 8.1). Blood samples were collected in pre-chilled EDTA containing vacuum tubes (BD vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium) and kept on ice. Blood was centrifuged in a pre-chilled centrifuge at 4°C (3500 rotations per minute, 15 minutes). Plasma was immediately stored at -80°C until batch analysis. All analyses were performed

by one person after completion of patient inclusion. Patients were admitted to the hospital one day pre-operatively and routine blood tests were performed by the clinical chemistry department.

Figure 8.1 Timeline of sample collection. I= 15 or 30 minutes of ischemia, R= 5 minutes reperfusion, POD1= postoperative day one.

I-FABP measurement

I-FABP is a highly sensitive and specific marker of intestinal epithelial cell damage¹⁹. I-FABP plasma levels were determined using an in-house enzyme-linked immunosorbent assay (ELISA) that selectively detects human I-FABP (range: 10-1280 pg/ml). Due to its small molecular weight, plasma I-FABP passes the glomerular filter (fractional renal excretion: 28%, half-life time: 11 minutes)²⁵.

Source and fate of I-FABP

In order to measure the source and fate of I-FABP, blood was also drawn from the portal vein and hepatic vein after liver transection in the no-IPM and total-IPM group (T=4, Figure 8.1). Arterio-venous (AV) differences of I-FABP across the gut and the hepatosplanchnic area (gut + liver) were calculated. AV-differences of I-FABP were calculated using the following formulae:

AV-difference gut = portal venous [I-FABP] – arterial [I-FABP]

Hepatosplanchnic AV-difference = hepatovenous [I-FABP] – arterial [I-FABP].

EndoCAb measurement

IgG Endotoxin Core Antibodies were used to quantify endotoxemia. A drop from pre-operative values to post-operative values was interpreted as consumption of antibodies to endotoxin by systemic release of endotoxin. IgG EndoCAb was measured using a commercially available enzyme-linked immunosorbent assay (ELISA), kindly provided by

Hycult Biotechnology, Uden, the Netherlands (range: 0.13-8.00 GMU/ml). EndoCAb data are expressed in General Median Units (GMU)/ml. General Median Units of IgG are arbitrary and are based on medians of healthy adults, with 100 GMU/ml being the median.

Statistics

Mann Whitney *U* test was applied for two group comparison for continuous data. Wilcoxon signed rank test was applied for pairwise comparison for continuous data. Dichotomous data were compared using Fisher exact test. Multiple group comparisons for continuous data were done by Kruskal-Wallis test, with Dunn's post hoc test. All data are expressed as median and range. A *p*-value <0.05 was considered statistically significant. Statistical analysis was performed using Prism 5.0 for Windows (Graphpad software, Inc, San Diego, CA).

Results

Patients

Twenty-four patients (11 females; 13 males) scheduled for hepatectomy for primary (n=2) or secondary malignant liver tumours (n=22) were included. Thirteen patients underwent major liver resections (≥3 segments) and eleven a minor liver resection (<3 segments). The 15-IPM group received 15 minutes of ischemia (n=7), median 2 (2-5) cycles and a cumulative total ischemia time of 33 (30-75) minutes. The 30-IPM group received 30 minutes of ischemia (n=5), median 1 (1-2) cycle and a cumulative total ischemia time of 30 (30-56) minutes.

The sel-IPM group received selective clamping of the right portal pedicle (n=6), and controls (no-IPM) received no vascular clamping (n=6). There were no relevant significant differences between groups neither in baseline characteristics (Table 8.1) nor in operation time, intra-operative blood loss, extent of resection and post-operative creatinin levels (Table 8.2). There were no significant differences in pre- and post-operative creatinin levels (Table 8.1, 8.2).

Plasma baseline I-FABP values

Baseline (T=1) arterial I-FABP levels did not differ significantly between groups (15-IPM, 532 pg/ml [353–1,078]; 30-IPM, 565 pg/ml [500–1,156]; sel-IPM, 874 pg/ml [478–1,198]; no-IPM, 502 pg/ml [161–966] *p*=0.26).

Table 8.1 Patient characteristics.

	15 min total-IPM (n=7)	30 min total-IPM (n=5)	No-IPM (n=6)	Sel-IPM (n=6)	<i>p</i>
Age (years)	60.8 (48.3-79.9)	67.3 (60.3-77.4)	60.5 (59.6-70.1)	64.6 (42.9-70.1)	0.64
Gender	3 F; 4 M	2 F; 3 M	1 F; 5 M	5 F; 1 M	0.14
Height (cm)	1.77 (1.55-1.92)	1.70 (1.63-1.75)	1.76 (1.72-1.95)	1.65 (1.60-1.86)	0.11
Weight (kg)	72 (56-100)	74 (54-83)	75 (68-90)	71 (55-88)	0.83
Body Mass Index	23.2 (22.6-27.4)	24.2 (20.3-28.7)	23.4 (23.0-25.7)	25.1 (19.3-30.8)	0.70
Aspartate-aminotransferase (IU/l)	33 (13-52)	16 (7-25)	21 (10-32)	19 (11-26)	0.13
Alanine-aminotransferase (IU/l)	36 (11-51)	26 (8-41)	26 (7-55)	23 (21-29)	0.75
Lactate dehydrogenase (IU/l)	399 (305-595)	356 (299-432)	319 (291-557)	389 (316-514)	0.55
Gamma-glutamyl transpeptidase (IU/l)	56 (22-204)	37 (32-169)	34 (18-83)	33 (29-63)	0.61
Alkaline phosphatase (IU/l)	134 (55-256)	90 (66-124)	80 (58-128)	115 (57-126)	0.52
Bilirubin (μM)	13.8 (11.3-14.2)	10.6 (8.3-13.0)	14.0 (6.9-16.5)	11.3 (7.8-12.9)	0.15
Pre-operative creatinin (μmol/l)	78 (59-125)	92 (85-137)	76 (54-96)	80.5 (46-287)	0.40

Data are presented as median (range)

Table 8.2 Characteristics of surgical procedures.

	15 min total-IPM (n=7)	30 min total-IPM (n=5)	No-IPM (n=6)	Sel-IPM (n=6)	<i>p</i>
Operation time (hours: minutes)	3:15 h (2:10-6:30)	4:15 h (3:09-4:45)	3:24 h (2:20-4:10)	3:45 h (2:27-4:30)	0.62
Blood loss (ml)	850 (250-3900)	1000 (250-2500)	750 (200-2600)	1050 (400-2500)	0.93
Number of resected segments	3 (2-3)	3 (1-3)	2 (1-3)	3 (2-3)	0.48
Post-operative creatinin (μmol/l) day 0/1	71 (55-114)	110 (101-115)	88 (52-93)	76 (44-248)	0.17

Data are presented as median (range)

Intestinal epithelial injury in 15 min vs. 30 min total IPM

No significant differences between 15-IPM and 30-IPM groups in median plasma I-FABP values were found at any time point. In the 15 minutes IPM group a significant increase in I-FABP was observed from baseline (T=1) to after transection (T=4) (15-IPM: 532 pg/ml [353–1,078] to 891 pg/ml [392–3,053] $p<0.05$) and from baseline (T=1) to 8 hours after start of surgery (T=5) (15-IPM: 532 pg/ml [353–1,078] to 1,478 pg/ml [627–2,000] $p<0.05$). Application of 30 minutes cycles of inflow occlusion did not significantly increase the release of I-FABP (30-IPM: 597 pg/ml [500–1,156] to 1,077 pg/ml [560–1,664], $p=0.19$).

Intestinal epithelial injury in total IPM vs. control

The 15-IPM and 30-IPM groups were subsequently pooled and compared with controls (no-IPM) since there was no significant difference in intestinal epithelial cell damage between 15-IPM and 30-IPM. In the total-IPM group, plasma I-FABP levels increased significantly from baseline to 8 hours after start of surgery (549 pg/ml [353–1,156] to 1,279 pg/ml [560–2,000], $p<0.005$). In the no-IPM group, no significant differences were observed in I-FABP concentrations between the different time points. Consequently, plasma I-FABP levels 8 hours after start of surgery were significantly higher in the total-IPM group compared to the no-IPM group (respectively, 1,279 pg/ml [560–2,000] and 413 pg/ml [245–1,388], $p<0.01$) (Figure 8.2A).

Figure 8.2 A) Time course of I-FABP plasma levels and interorgan arterio-venous concentration differences. For visual purposes data were plotted as mean and SEM. * $P<0.005$ compared to baseline of total-IPM ($T=0$), $P<0.01$ compared to no-IPM on $T=5$. B) Mean (SEM) arterio-venous concentration gradients of I-FABP across the gut (portal venous minus arterial) and the hepatosplanchnic area (hepatic venous minus arterial). I-FABP was specifically released from the gut (* $p<0.0001$ vs. zero) and this resulted in a net I-FABP release from the hepatosplanchnic area ($^{\#}p<0.005$ vs. zero).

Intestinal epithelial injury in sel-IPM

In patients who received selective clamping of the right portal pedicle (sel-IPM), plasma I-FABP levels did not increase significantly from baseline to 8 hours after start of surgery (806 pg/ml [478–1,198] to 924 pg/ml [248–2,823], $p=0.31$). Moreover, there were no significant differences at any time point between sel-IPM and no-IPM.

Organ specific I-FABP release

In order to prove that I-FABP is specifically released from the gut we performed an organ balance analysis to reveal the origin of circulating I-FABP. The data show that I-FABP was

specifically released from the gut after liver transection. This resulted in a net release of I-FABP from the hepatosplanchnic area (Figure 8.2B).

Determination of endotoxemia

Plasma levels of natural IgG against endotoxin were significantly decreased in the total-IPM group on post-operative day 1 (POD1) compared to baseline (baseline, 52.9 GMU/ml [10.0–112.1], POD1, 33.2 GMU/ml [10.0–89.1] $p<0.005$). In the no-IPM and sel-IPM group, no significant decrease from baseline to POD1 was observed (Figure 8.3A-C).

Figure 8.3 Values are median (range), pairwise comparisons of EndoCAB at baseline (T=0) and on post-operative day one (T=6). A) Total-IPM: two patients were excluded from this analysis because of missing samples on post-operative day 1. EndoCAB levels of one patient were undetectable and therefore the lower detection limit of the EndoCAB ELISA kit, corrected for dilution factor was used (12.5 GMU/ml). B) No-IPM. C) Sel-IPM: 1 patient was excluded from analysis because of missing samples on post-operative day 1.

Discussion

This study aimed to investigate whether IPM causes loss of intestinal epithelial cell integrity and leads to endotoxemia in patients undergoing liver resection. The results of the present study show that the use of total IPM is associated with intestinal epithelial cell damage and subsequent endotoxemia. In the total-IPM group (15 min and 30 min total IPM combined), plasma I-FABP levels were significantly increased 8 hours after start of surgery compared to baseline, while in the no-IPM group and sel-IPM group no significant differences in I-FABP levels were observed between different time points. By measuring concentration differences across the gut and the hepatosplanchnic area, we were able to show that there was a net I-FABP release from the hepatosplanchnic area in the total-IPM group, explaining the high levels of I-FABP in this group.

In the total-IPM group IgG EndoCAB decreased significantly on POD1 compared to baseline while this effect was not observed in the no-IPM and sel-IPM group. Consumption of IgG EndoCAB in the total IPM group suggests that total IPM resulted in translocation of gut derived endotoxins, possibly by intestinal barrier dysfunction due to epithelial cell damage.

The results of the present study are in line with several animal studies. Ochiai *et al.*⁹ showed that IPM caused intestinal epithelial cell damage and increased small intestinal permeability in rats. In two other rat studies, increased bacterial translocation¹⁰ and endotoxemia^{10,11} were demonstrated after IPM. Data on the effect of IPM on the gut in man are scarce. It remains unknown whether it is only the impaired microcirculation in the intestine during portal clamping which causes cell damage or that the temporal acute rise of venous pressure in the mesenteric system in itself also plays a role. King *et al.*²⁶ showed that when splanchnic venous outflow was occluded in patients, both intestinal oxygen extraction ratio and portal venous lactate increased. An acute rise of venous pressure in the microcirculation in itself will probably also impair perfusion and consequently cause tissue hypoxia. It is most likely a combination of the two mechanisms that explains the high levels of I-FABP in these patients. It would be worthwhile to study the microcirculation in vivo during the total IPM in order to further explore the pathophysiological mechanism behind the intestinal cell damage in these patients. In this context Ferri *et al.*²⁷ showed that bacterial translocation to mesenteric lymph nodes occurred during liver resection in patients under continuous inflow occlusion, but there was no correlation between positive lymph nodes and post-operative infectious complications.

Patients undergoing liver resection are more susceptible to development of a systemic inflammatory response due to endotoxemia, as hepatic endotoxin clearance is compromised due to a reduction of Kupffer cells²⁸. This is caused by reduction of the functional hepatic liver mass and by ischemia reperfusion damage to the functional liver parenchyma when IPM is applied. Translocation of intraluminal intestinal toxins as a consequence of intestinal epithelial cell damage due to total-IPM could further contribute

to systemic endotoxemia. It may therefore play an important role in the pathogenesis of the systemic inflammatory response syndrome (SIRS) and sepsis in patients undergoing liver resection. In line with this, infective complications are reported to negatively affect both short- and long-term outcome after liver resection^{29,30}. This might be especially important in patients with underlying liver disease and cholestasis. In the latter, most often infected areas of non-optimally drained liver segments are present during surgery³¹. To prove these assumptions it would be worthwhile to investigate in future studies whether there is a relation between infective complications and the application of the total-IPM.

The present study sheds new light on the question whether performing IPM is favorable in patients undergoing liver surgery. Hepatectomies without IPM can be performed safely due to advances in liver surgery such as the development of modern hemostatic devices and improvements in anesthesiological management³². Acute major bleeding remains an indication for IPM, but current evidence shows no benefit for IPM on outcome after liver resection³³. Therefore in the modern era of liver surgery systematic use of IPM has become more often a subject of debate. Our data show that total-IPM is associated with intestinal epithelial cell damage and endotoxemia. This could play a role in the pathophysiology of infective postoperative complications. It is clinically relevant that the role of gut integrity loss induced by ischemia/reperfusion of the liver during liver resections and liver transplantations is further investigated in future studies. This pathophysiologic mechanism is probably underestimated in these patients. Unravelling this mechanism could help to (preoperatively) identify patients with a higher risk of infective postoperative complications.

A possibly more safe approach than total IPM is selective IPM, which is performed by selectively excluding the right or left hemi liver from the circulation. Selectively clamping the right or left portal pedicle is safe and feasible for patients with normal liver parenchyma and especially in cirrhotic patients the selective IPM induces less ischemic liver injury compared to total IPM³⁴. One would expect that also the intestinal epithelial cell integrity is less compromised in these patients because splanchnic outflow is only partly reduced. This hypothesis was confirmed in the present study as no significant increase was observed in arterial I-FABP levels in 6 patients undergoing selective IPM. However, there were no significant differences on any time point between the sel-IPM group and the total-IPM group. This is probably due to the relatively small group size (type II error), as only one patient showed a substantial increase in I-FABP.

The present study shows that the use of the total intermittent Pringle manoeuvre causes intestinal epithelial cell damage and endotoxemia during liver surgery in man. IPM can therefore potentially increase the risks of liver resections despite a possible reduction of intraoperative blood loss. Intestinal epithelial cell damage and endotoxemia induced by IPM could negatively affect the patient's condition and post-operative recovery. Whether gut damage as a consequence of the total-IPM is causally related to systemic inflammation remains to be established.

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Chapter 9

Systematic review of ophthalmate as a novel biomarker of hepatic glutathione depletion

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Abstract

Background

Sustainability of hepatic glutathione (GSH) homeostasis is an important cellular defense against oxidative stress. Therefore, knowledge of liver GSH status is important. However, measurement of plasma GSH and tissue is difficult due to its instability. Alternatively, ophthalmate (OPH), an endogenous tripeptide analog of GSH, has been suggested as a potential indicator to assess GSH depletion.

Aim

To provide an overview of present knowledge with respect to the usefulness of OPH as a biomarker for oxidative stress and hepatic GSH homeostasis.

Methods

A systematic, computerized search combined with a cross-reference search of the literature described in PubMed (January 1975 to January 2012) was conducted, key words: 'ophthalmate' and 'ophthalmic acid'.

Results

Twenty-two articles were included. Hepatic OPH levels increase inversely proportional to a drop in hepatic GSH in mice with paracetamol (PCM) induced hepatotoxicity. Little is known about the stability of OPH in human plasma. To measure the very low physiological concentrations of plasma OPH, liquid chromatography-mass spectrometry techniques can be employed. OPH synthesis can be measured in humans, using stable isotope labeling with a deuterated water ($^2\text{H}_2\text{O}$) load.

Conclusion

OPH may be a promising biomarker to indicate hepatic glutathione depletion, but the suggested biological pathways need further unravelling.

Introduction

The anti-oxidant glutathione (GSH) is the main hepatic protection system against redox imbalances and many forms of oxidative stress and intoxications¹⁻³. Unfortunately, thus far assessment of hepatic GSH homeostasis *in vivo* in humans has remained to be difficult, yet the availability of an adequate test to measure hepatic GSH depletion would be of great clinical importance. In order to estimate the GSH pool of the liver a liver biopsy would be required, which necessitates an invasive procedure. Even when such a liver biopsy can be obtained, measurement of the GSH content of a liver biopsy alone does not provide information on the capacity of the liver for *de novo* GSH synthesis. Attempts have been made to measure hepatic GSH depletion *in vivo* in human plasma, but this proved to be difficult because of instability of GSH due to auto-oxidation.

Recent research has shown that ophthalmate (OPH), which is an endogenous analog of GSH, can be used as a potential marker for GSH depletion⁴⁻⁶. OPH is synthesized when GSH is depleted and when the hepatic availability of cysteine is limited. OPH is tri-peptide very similar to GSH (glutamate-glycine-cysteine) but in OPH the cysteine moiety is replaced by the non-protein amino acid 2-aminobutyrate. OPH is synthesized through the same enzymatic machinery as GSH, but OPH lacks the reducing cysteine moiety and therefore it is far more stable than GSH⁶. Since OPH is released into the blood stream, a rise in plasma OPH concentration may thus indicate hepatic GSH depletion. In this context, plasma OPH has been suggested to be a reciprocal read out of hepatic intracellular GSH content. This review gives an overview on the state of the art knowledge on the potential of OPH to be used as a biomarker for oxidative stress and hepatic intracellular glutathione depletion.

Methods

Literature search strategy

A systematic, computerized search of the literature described in PubMed (January 1975 to January 2012) was undertaken independently by two investigators (SD and EN) using a combination of the key words 'ophthalmate' and 'ophthalmic acid'. All obtained abstracts were read and relevant studies were selected using three levels of screening. The first exclusion criterion was language other than English. The second exclusion criterion involved absence of relevance to hepatic metabolism, such as ophthalmology. Finally the full text of every study was considered. If there was a discrepancy in inclusion between the two investigators, a decision was reached by a third reviewer (CD). Finally, papers obtained via cross-referencing were also included.

Results

Forty papers were identified from PubMed. In total 26 papers were excluded for review: in 2 papers the language was other than English and 24 papers involved OPH in relation to other topics mostly ophthalmology and were not relevant to hepatic metabolism (Figure 9.1). Of these forty papers, fourteen papers fell within the scope of this review. Three papers described OPH as a marker for glutathione depletion⁵⁻⁷ and 2 papers described technique of measuring OPH^{8,9}. Two papers investigated the relation between the GSH/OPH redox buffer system in primary and secondary liver tumours^{4,10}. Three papers studied the breakdown of OPH and its physiological function¹¹⁻¹³. Finally 4 papers investigated the synthesis of OPH and GSH¹⁴⁻¹⁷. Eight additional papers were identified by manual cross-referencing and were included: 5 general papers on GSH metabolism¹⁸⁻²², 1 paper on paracetamol (PCM) hepatotoxicity¹ and 2 papers on OPH synthesis^{23,24}. Thus, 22 papers were considered appropriate for inclusion for review.

Figure 9.1 Selection of articles for review.

Glutathione

Many forms of stress, sepsis, ischemia and toxic compounds are associated with severe redox imbalance in cells. Glutathione (GSH) is the main intracellular antioxidant and thus protects cells against oxidative stress caused by radicals from reactive oxygen species (ROS). These radicals are produced continuously through intracellular processes in mitochondria, by enzyme actions of cytochrome P-450 (CYP450) and exposure to environmental factors such as ultraviolet-radiation.

The CYP450 system is one of the main metabolic pathways of generation of ROS¹⁸. Radicals damage DNA, lead to protein oxidation and cell dysfunction and ultimately cell death¹⁹. Therefore scavenging radicals is important for maintaining cell integrity and

function. Apart from its antioxidant capacities, GSH also seems to play a role in modulation of cell proliferation, immune responses and cell signaling²⁰. As the main site of drug transformation, the liver is particularly exposed to radicals.

The antioxidant properties of GSH are dependent on a sulfide group in the cysteine moiety. GSH can scavenge radicals in two different ways: (1) by forming a dimer through a disulfide bridge, whilst capturing a radical in this bond and forming the oxidized GSSG (glutathione disulfide), and (2) by conjugation to reactive molecules. Glutathione peroxidase (GP) catalyses the formation of GSSG. The dimer can be reduced to the original GSH state by GSSG reductase, using NADPH as a reductant agent. Glutathione S-transferase (GST) catalyses the conjugation of GSH to the reactive molecules.

Synthesis of glutathione

The liver is the major GSH storage organ and the major source of plasma GSH. GSH is a tripeptide consisting of the amino acids glutamate, cysteine and glycine. It is synthesized by the sequential actions of γ -glutamylcysteinyl synthetase (GCS) and GSH synthetase (GS). First, GCS binds cysteine to γ -glutamate, producing γ -glutamylcysteine. Then GS binds glycine to γ -glutamylcysteine producing γ Glu-Cys-Gly (glutathione). GSH exerts a negative feedback on GCS, limiting its own synthesis if sufficient GSH is present. The rate-limiting enzyme in this cascade is GCS while GS is probably not a rate-limiting step²¹. GSH synthesis depends on the availability of cysteine (Figure 9.2). Cysteine is synthesized in the liver from methionine through homocysteine via the transsulfuration pathway. Therefore, the liver is not only the major GSH storage organ, but also the major source of plasma GSH. GSH is released across both the canalicular membrane into bile and across the sinusoidal membrane into blood for delivery to other tissues²².

Glutathione and its role in xenobiotic metabolism

Hepatic metabolism, the hepatic GSH pool and de novo GSH synthesis, play an important role in the detoxification of many drugs and toxic compounds. For some drugs (e.g. PCM) an overdose leads to a severe redox imbalance in hepatocytes which consequently leads to hepatotoxicity and even acute liver failure (ALF)¹. At therapeutic doses, over 90% of PCM entering the body is metabolized by conjugation. Specifically, two thirds are metabolized through glucuronidation and one third through sulfation¹. Approximately 5–9% undergoes oxidative conversion by Cytochrome P450 (CYP 450) into the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI)¹. At therapeutic doses this reactive metabolite is detoxified by GSH. Overdoses of PCM lead to saturation of the glucuronidation and sulfation pathways and consequently more PCM will be metabolized through the CYP-450 system¹. The increased metabolism of PCM by the CYP-450 system increases the production of NAPQI. GSH stored within the liver is limited and will be depleted in an attempt to scavenge the increased NAPQI concentration and so will its most important precursor

cysteine. Consequently, the surplus of NAPQI cannot be removed adequately and accumulates, leading to hepatotoxicity.

OPH versus GSH

In vivo measurements of hepatic GSH synthesis in humans have shown to be complex and attempts to use stable isotopes in order to solve these difficulties have led to data that are difficult to interpret and not easy to reproduce. Therefore metabolites of GSH which are more stable than GSH itself have been suggested as alternatives to solve this problem. An interesting analog of GSH is ophthalmate (OPH). In 1956 OPH, also known as ophthalmic acid, was discovered in a calf lens²³. It was noted that both GSH and OPH were formed via a similar pathway of synthesis catalyzed by the same two enzymes: γ -glutamyl-cysteine-synthetase (GCS) and glutathione-synthetase (GS) and that the structure of OPH closely resembled that of GSH^{14,15,24}. In 2006 Soga *et al.*⁶ showed that tissue and plasma OPH levels can be used as an 'alternative read-out' for hepatic glutathione synthesis in mice with paracetamol-induced hepatotoxicity. These authors also showed that OPH indeed is an analog of GSH and has the same pathway of synthesis as GSH. The only difference between OPH and GSH is that in OPH cysteine is replaced by 2-aminobutyrate.

Synthesis of OPH

The first step in the synthesis of OPH is linking of 2-aminobutyrate to glutamate, a step catalyzed by GCS. Subsequently, glycine is linked to this dipeptide via GS. Importantly, and as mentioned before, glutathione has a negative feedback on GCS. However, in a situation of oxidative stress (for example PCM-induced hepatotoxicity) this negative feedback mechanism disappears and due to cysteine depletion by oxidation, activation of the alternative pathway will take place, leading to generation of OPH. However, since OPH lacks a thiol group and is not an antioxidant, it can not conjugate with free radicals and is therefore more stable than GSH. The increase of OPH is proportionally related to the decrease of GSH and can thus be used as reciprocal "read out"⁶ (Figure 9.2A-B).

Soga *et al.*⁶ demonstrated previously that the rise in OPH was inversely proportional to the decrease in hepatic GSH content after PCM induced hepatotoxicity in mice. The hepatic GSH content was lowered by pre-treatment with diethylmaleate (DEM) or buthionine sulfoximine (BSO). DEM leads to oxidation of the thiol group in GSH, while BSO inhibits GCS. The levels of GSH as well as γ -Glu-2AB and OPH were very low in both the liver and in serum of the GCS-inhibited mice, while in the DEM-treated mice a marked increase in γ -Glu-2AB and OPH levels was seen. These authors therefore concluded that OPH was synthesized using the same pathway as GSH and that GSH depletion by oxidative compounds such as PCM and DEM resulted in GCS activation, which in turn induced OPH synthesis. In different research models the usefulness of OPH was investigated. In several studies OPH appeared to be a reciprocal read out for GSH depletion^{4,6,10}. Paradoxically

Geenen *et al.*⁸ showed that hepatotoxicity induced with methapyrilene (compound which induces oxidative stress) in rats did not cause increased OPH levels (Table 9.1).

Figure 9.2 Schematic overview of the glutathione (GSH) and ophthalmate (OPH) synthesis pathway: (A) Normal situation (B) During paracetamol overdose GSH and cysteine will be consumed, and the feedback inhibition on the enzyme glutamyl-cysteine-synthetase (GCS) will be lost. In the absence of precursor cysteine, 2-aminobutyrate (2AB) will be linked to glutamate. Consequently linking of glycine via enzyme glutathione-synthetase (GS) will lead to the formation of OPH. The magnitude of circles and arrows is proportional to concentrations.

Table 9.1 Overview of relation between GSH and OPH in normal/control conditions and during oxidative stress conditions in different studies.

	Normal		Oxidative stress conditions	
	Healthy volunteers		Patients with PCM intoxication	
	GSH	OPH	GSH	OPH
Humans ⁵				
plasma	d	d	↓	unknown
liver	d	unknown	↓	unknown
	Controls		After PCM induced hepatotoxicity	
Mice ⁶	GSH	OPH	GSH	OPH
plasma	ND	d	ND	↑
liver	d	d	↓	↑
	Controls		After methapyrilene induced hepatotoxicity	
Rats ^{5,8}	GSH	OPH	GSH	OPH
plasma	d	d	↓	↓
	Controls		After implantation of secondary liver tumours	
Rabbits ⁴	GSH	OPH	GSH	OPH
plasma	d	d	↓	↑
	Controls		Hepatitis virus and hepatocellular carcinoma	
Woodchucks ¹⁰	GSH	OPH	GSH	OPH
plasma	d	d	↓	↑
red blood cells	d	d	↑	↑

d=detectable; ND=non-detectable; ↓ increase in concentration; ↑ decrease in concentration; ↓ no difference in concentration vs. controls

In a more recent study Geenen *et al.*⁷ investigated whether OPH was a robust biomarker for GSH depletion in a kinetic mathematical model for GSH and its detoxification pathway for PCM. The liver has the unique ability to produce cysteine from methionine through transsulfuration. The correlation of OPH with GSH concentration was tested, while varying the methionine concentration in their model. The authors showed that OPH was dependent on the methionine status of the cell. Therefore, in this mathematical model OPH alone was not a reliable biomarker for intracellular GSH depletion induced by PCM.

Breakdown of GSH and OPH

GSH breakdown is catalyzed by the enzyme γ -glutamyl transpeptidase, which leads to γ -glutamyl-glycine formation¹¹. γ -glutamyl-glycine is then converted to mercapturic acid for excretion via the kidneys^{11,17,19}. Plasma levels of GSH are maintained primarily by a balance between secretion from the liver and degradation in the kidney. It is unknown whether OPH has the same enzymatic degradation pathway as GSH.

Physiological function of OPH

Literature on the physiological function of OPH is scarce and therefore little is known about the exact function of the compound. In 1979 Offord *et al.*¹⁶ reported that OPH inhibited the degradation of insulin in rat adipose tissue. Ballatori and Dutczak *et al.*¹² showed in 1994 that OPH has an effect on GSH transport across rat canalicular liver plasma membranes. GSH is released through a carrier across the canalicular and sinusoidal membrane into bile and blood, respectively, for delivery to other tissues²². Ballatori and Dutczak *et al.*¹² noted that OPH inhibited the GSH uptake across the membrane with only a small effect on the V_{max} , indicating competitive inhibition. It trans-stimulated (counter transport) GSH uptake. OPH may therefore be a substrate of the GSH carrier⁵. Circu *et al.*¹³ showed in an in vitro study that apoptosis and GSH efflux in colonic epithelial cells induced by staurosporine was reduced after pre-treating them with exogenous OPH.

OPH in human plasma

In 2008 Kombu *et al.*⁵ were the first to study the usefulness of OPH as an index of GSH depletion in humans. These authors proposed an LC-MS-MS assay of the ^2H labeling of free glutathione (GSH) and bound glutathione (GSSR) and OPH labeled from ^2H -enriched body water in both rats and humans. Healthy individuals ingested $^2\text{H}_2\text{O}$ in amounts calculated to enrich body fluids by 0.5%. This made it possible to measure ^2H concentrations and labeling of the plasma glutathione-ophthalmate system 72 hours after ingestion. Geenen *et al.*⁸ also investigated OPH levels in control human plasma samples, however these authors did not detect endogenous concentrations of OPH. Contrary to Kombu *et al.*⁵, they suggested that there might be a difference between human and rat metabolism of OPH.

OPH as a marker for tumour growth

Malignant liver tumour growth could disturb the GSH buffer system because it is associated with oxidative stress and redox imbalance of cells. Two recent papers investigated whether OPH can be used as a surrogate for early tumour growth of secondary liver tumours⁴ and hepatocellular carcinoma¹⁰. OPH may turn out to be useful as a surrogate of early tumour growth because plasma and red blood cell concentration of OPH significantly increased in animals after liver tumour implantation.

OPH mass spectrometry assay

The measurement of OPH and GSH has hitherto been fraught with difficulty. Both OPH and GSH are hydrophilic and have a polar nature. Therefore achieving good retention, resolution, and peak shape with chromatography is difficult. In an attempt to amplify the small differences between OPH and GSH several forms of chromatography have been tried (straight, reversed phase or HILIC) to maximize their separation⁹. Several chromatographic columns have been tested for suitability of OPH, GSH and GSSG analysis. However, the necessity for separation has become less important with the advent of liquid chromatography tandem mass spectrometry (LC-MS-MS) technology. With LC-MS-MS, sensitive and selective analysis of OPH can be performed in different matrices (plasma, liver, cell culture media)^{5,8,9}. It has been shown that when measuring OPH levels with an LC-MS-MS assay, a lower limit of quantification of OPH for cell culture medium of approximately 0.003 $\mu\text{mol/l}$ and for rat plasma of 0.1 $\mu\text{mol/l}$, can be achieved⁸.

OPH Stability

A key issue of the use of OPH as alternative “read out” for GSH is that OPH has no thiol-group and thus has no antioxidant capabilities. Auto-oxidation of OPH will therefore not occur. Its concentration is hypothesized to be stable in plasma and it could therefore be a better index for GSH depletion than GSH plasma levels itself. Therefore it is crucial that this stability is thoroughly investigated. Geenen *et al.*⁸ performed several OPH stability experiments in both cell growth medium and rat plasma samples. Cell culture media and rat plasma samples were spiked and tested for bench top stability (21°C), freezer stability and freeze-thaw stability. No significant decreases in concentrations for OPH were measured under these conditions.

Discussion

In several experimental studies OPH appears to be a reciprocal readout for GSH depletion. OPH is, in contrast to GSH, stable in animals and very low concentrations of OPH can be accurately measured with an LC-MS-MS assay in plasma, red blood cells and liver biopsies.

However, most publications on OPH are hampered by certain weaknesses. Up till now there are no studies that validate OPH as a biomarker for GSH depletion in patients with liver disease (e.g. liver resection/liver transplantation patients, PCM auto-intoxication patients, acute liver failure patients). There are also some paradoxical results in studies^{4,6-8,10}, which underline the necessity for a proof of principle study for the validation of OPH as a potential biomarker for GSH depletion in a human research model.

In future studies the validity of OPH as a biomarker for GSH depletion and oxidative stress can probably be tested best in PCM intoxication patients. As described before, PCM overdose can induce hepatic GSH depletion and consequently severe hepatotoxicity, which can eventually lead to acute liver failure (ALF)¹. In these patients it can be investigated whether plasma OPH levels are negatively correlated with plasma GSH levels, and whether OPH levels are also correlated with dosage of PCM. Quantifying the depletion and biosynthesis of GSH by measuring OPH levels may also be a very useful and innovative method to measure early liver toxicity and in titrating therapy in patients with PCM overdose.

Kombu *et al.*⁵ investigated OPH synthesis in healthy volunteers, using stable isotope labeling with a deuterated water ($^2\text{H}_2\text{O}$) load, followed by measurement of the label incorporation in GSH species and OPH. The authors showed that a rise in OPH levels was a consequence of enhanced synthesis. The proposed method gives the unique opportunity for further unravelling of the GSH-OPH system in patients with various liver pathologies. However, this complex and expensive method might be less applicable in the diseased patient due to the prolonged time required for the method and because of possible side effects (vertigo, nausea) of the ^2H enrichment of body water⁵.

At this moment some key issues have to be investigated before the biomarker OPH is valid for use in clinical practice. For example the stability of OPH in human plasma is unknown. This should be investigated thoroughly because there could be important differences in OPH metabolism between humans and animals. Also the optimal pre-storage treatment of OPH has to be investigated in future studies. For instance it is unknown whether plasma or serum is the ideal matrix for OPH measurement. The potential influence of temperature, acidity and enzymatic breakdown on OPH is unknown. Whether OPH has the same enzymatic breakdown pathway as GSH, needs to be verified in future studies. The magnitude of the OPH pool in human liver and its proportional size compared to GSH:GSSG during physiological and pathophysiological circumstances has not been investigated. Whether OPH is actively transported to the plasma or if the physiological function of OPH is to decrease cellular GSH efflux¹² and preserve cellular integrity is uncertain.

OPH concentration has been shown to be lower in the plasma compartment than in red blood cells¹⁰. OPH measured in plasma can only reflect the hepatic OPH and GSH synthesis of the liver, when samples are treated very carefully and hemolysis is avoided⁵. Although the relation between erythrocyte OPH synthesis and the exchange between plasma/liver OPH and the red blood cells has not been well studied yet, this has been

investigated meticulously for GSH. Because OPH is an analog of GSH, it is conceivable that OPH has a similar kinetic mechanism as GSH. Kombu *et al.*⁵ proved with a $^2\text{H}_2\text{O}$ isotope labelling technique of the OPH-GSH system that in rats most of plasma GSH is synthesized in the liver. There was a rapid labelling of liver GSH, reflected by rapid turnover of the large GSH/GSSR pool in rats. Interestingly, it was also shown that the labelling profile of plasma GSH was comparable to that of liver GSH. The labelling of the GSH/GSSR pool in red blood cells was shown to be much slower compared to the liver. This is probably due to the low rate of GSH synthesis in red blood cells and/or due to the low import of plasma GSH into red blood cells. If hemolysis can be avoided, plasma OPH levels may therefore accurately reflect hepatic OPH synthesis.

The most important limitation of OPH as marker for GSH depletion was recently presented by Geenen *et al.*⁷. The authors showed in a kinetic mathematical model for GSH and its detoxification pathway for PCM that OPH concentration was influenced by the methionine concentration in the liver. This would reduce the reliability and usefulness of OPH as a biomarker for intracellular GSH depletion. However, this study was based on a mathematical model for which many parameter values were uncertain. In line with this Geenen *et al.*⁸ showed in another recent publication that hepatic methapyrilene toxicity in rats did not cause an increase in OPH levels. Methapyrilene causes oxidative stress and this was expected to have an effect on the GSH-OPH system. Apart from this study most of the research on the validity of OPH is merely focused on PCM induced hepatotoxicity. This is important because PCM induced hepatotoxicity is the most common cause of ALF in adults in the UK²⁵. Even administration of PCM in doses that are thought traditionally to be safe might lead to raised transaminases, suggesting some degree of hepatocellular damage²⁶. However redox-imbalance and GSH depletion occur in many other conditions amongst which sepsis and SIRS are the most important. The value of OPH should also be investigated in other oxidative stress associated diseases.

The advantage of OPH as a biomarker would be that alterations in the liver GSH buffer system and consequently liver dysfunction can be measured directly in the plasma compartment and liver biopsies are no longer required. Several animal studies have shown data that OPH might also be used as a biomarker to evaluate the liver oxidoreductive status in order to detect or evaluate tumour growth^{4,10}. The exact value of OPH as a biomarker for early tumour development in patients with chronic liver disease and primary liver tumours or in patients with secondary liver tumours remains to be determined.

Conclusion

Based on this overview of the currently available literature, we can conclude that OPH may be a promising marker to assess oxidative stress and hepatic GSH metabolism. This systematic review has identified strengths and limitations of the assessment of GSH depletion with the use of OPH. Unfortunately human studies are scarce and the proof of principle: whether there is a correlation between an increase of OPH and a decrease of

GSH, was not tested in a human research model. The suggested biological mechanisms need further unravelling.

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Chapter 10

Ophthalmate detection in human plasma
with LC-MS-MS

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Abstract

Background

Based on animal experimentations, ophthalmate (OPH) has recently been suggested as a potential plasma biomarker to probe hepatic GSH homeostasis. Up until now, the inability to accurately determine OPH concentrations in human plasma prohibited further studies of OPH metabolism in humans. This study therefore aimed to study the influence of delayed sample preparation on OPH concentrations using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS).

Methods

Venous plasma samples from 5 healthy human volunteers were incubated for varying times (5, 30, 60 and 120 min.) at temperatures of 4°C and 37°C to investigate potential enzymatic degradation. At 37°C, the decrease in OPH reached significance after 120 min. (74.6% (range: 56.2-100.0%; $p < 0.0001$)). At 4°C, the same trend was observed but did not reach significance. These findings indicate ongoing enzymatic activity, stressing the need for immediate sample deproteinization to obtain reliable plasma concentrations.

Results

To investigate the feasibility of the here developed method, baseline arterial plasma values of 21 patients scheduled for partial liver resection were determined to be 0.06 ± 0.03 $\mu\text{mol/l}$ (mean $\pm$ s.d.). In addition, in pooled samples from 3 patients, an OPH calibration curve was spiked to arterial plasma, arterial whole blood and liver biopsy material, resulting in a linear calibration curve in all cases. Individual measurements of baseline samples revealed that both arterial whole blood and liver biopsy material contained significant levels of endogenous OPH, namely 16.1 (11.8-16.4) $\mu\text{mol/l}$ and 80.0 (191.8-349.2) $\mu\text{mol/kg}$, respectively.

Conclusions

In conclusion, the present LC-MS/MS assay enables the accurate measurement of OPH in human plasma, whole blood and liver biopsies. Freshly prepared samples and immediate deproteinization are mandatory to block enzymatic degradation.

Introduction

The intracellular content and hepatic synthesis of glutathione (GSH) is considered to be the main hepatic protection system against redox imbalances and many forms of oxidative stress and intoxications¹⁻³. Unfortunately, thus far assessment of hepatic GSH homeostasis *in vivo* in humans is difficult and the availability of an adequate test to measure hepatic GSH depletion would be of great clinical importance. Estimation of hepatic GSH content would require a liver biopsy, which necessitates an invasive procedure. Although taking a liver biopsy is not an insurmountable issue, measurement of the GSH content of a liver biopsy alone does still not provide information on the capacity of the liver for *de novo* GSH synthesis.

Recently, ophthalmate (OPH, Figure 10.1), an endogenous tripeptide analog of GSH, has been suggested as a potential biomarker for GSH homeostasis as its production was found to be reciprocally promoted when GSH becomes depleted⁴⁻⁶. Kombu *et al.*⁵ suggested that when the hepatic availability of the amino acid cysteine, providing the reducing moiety of GSH, becomes limiting, the amino acid 2-aminobutyrate is incorporated instead, resulting in the formation of OPH⁵. Although the physiological background for this switch is still not elaborated, it has been suggested that OPH makes use of the same transporter system as GSH and in this way OPH would minimize cellular GSH efflux to preserve cell integrity⁷. Since OPH is released into the blood stream following its synthesis in the liver, a reciprocal rise in plasma OPH concentration may thus indicate hepatic GSH depletion.

Figure 10.1 The structure formula of ophthalmate (OPH), also known as ophthalmic acid.

Although several reports have been published so far describing the analysis of OPH^{4,5,8,9}, none of them were able to determine the physiological concentrations of OPH in plasma of healthy human subjects. Surprisingly, we obtained plasma OPH levels well above the level of detection (LOD) found before⁹. However, this report focused on the better chemical stability of OPH compared to GSH due to its lack of the thiol moiety, but its resistance to enzymatic degradation was not investigated. Hahn *et al.*⁸ suggested that OPH is degraded by the same enzymatic machinery as GSH, which is known to rapidly degrade excreted GSH in the bloodstream. Therefore, we postulated that a rapid enzymatic breakdown might explain the inability to measure human OPH levels.

Consequently we aimed to investigate the effects of delayed sample deproteinization and temperature on OPH concentrations. To guarantee optimal selectivity and sensitivity, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed to enable quantitative analysis of OPH in human plasma, whole blood and liver biopsy material.

The here presented protocol meets these requirements, facilitating future studies to investigate the usefulness of OPH as a marker of hepatic GSH depletion in humans.

Methods

Patients and healthy volunteers

Baseline blood samples were obtained from five healthy volunteers. 21 patients, scheduled for liver surgery were also included in the present study. In 3 patients also whole blood and a liver biopsy was collected. This study was approved by the medical ethical committee of the Maastricht University Medical Centre+ and conducted according to the revised version of the declaration of Helsinki (October 2008, Seoul). All subjects and patients gave written informed consent.

Materials and reagents

OPH was obtained from Bachem (Torrance, CA, USA) and dissolved in Milli-Q water (Waters, Etten-Leur, Netherlands) to a standard of 10.0 mmol/l. The standard was divided into 1.0 ml aliquots and stored at -80°C, guaranteeing its stability⁹. All reagents and solvents used were of analytical grade or better (Sigma-Aldrich, Zwijndrecht, Netherlands).

Sample collection and preparation

Five milliliter blood samples were collected in pre-chilled heparinised vacuum tubes (BD vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium) and kept on ice. In healthy volunteers, blood samples were collected by venepuncture of an antecubital vein. In 21 patients scheduled for liver surgery, a baseline blood sample was taken from an indwelling catheter in the radial artery. Plasma was prepared by centrifugation in a pre-chilled centrifuge at 4°C at 3000 *g* for 10 min. and 100 µl aliquots were immediately deproteinized by the addition of 100 µl of 5% solid 5-sulfosalicylic acid solution (5-SSA: British Drug Houses, Amsterdam, The Netherlands) in water and stored at -80°C until analysis.

From 3 patients, also whole blood and liver biopsy samples were collected. 100 µl aliquots of whole blood were also immediately deproteinized by the addition of 100 µl of 5% 5-SSA and stored at -80°C until analysis. After collection, liver biopsies were snap-frozen in liquid nitrogen. Next, 10 mg liver biopsies were homogenized in 100 µl 5% ice-

cold SSA solution in water, using a mini beat beater (Biospec Products, Applikon, Amsterdam, Netherlands) set to 2 min. at full speed. Homogenates were snap frozen in liquid nitrogen and stored at -80°C until analysis.

Prior to analysis all samples were centrifuged for 10 min. at 50,000 *g* at 4°C and the clear supernatants were transferred to 200 µl glass inserts spring-loaded in 4 ml WISP style (Waters, Etten-Leur, Netherlands) and stored in the chilled sample compartment of the sample processor systems. The analysis was initiated with the injection of 20 µl sample or standard.

Calibration and validation

As all biological samples investigated contain endogenous OPH, the limit of detection (LOD) and lower limit of quantification (LOQ) cannot be determined in these samples. Instead, the limit of detection (LOD) and limit of quantification (LOQ) were determined by diluting aqueous standards. LOD was defined as the concentration corresponding to a signal to noise ratio of 3, while LOQ was defined as the concentration corresponding with a signal to noise ratio of 5 according to¹³.

For each set of samples and each sample matrix, a calibration curve was prepared. For this, a 10.0 µmol/l aqueous OPH solution was prepared by diluting a freshly thawed 10.0 mmol/l stock solution in Milli-Q water. Calibration curves were prepared by adding 20 µl of pre-diluted OPH-standards to 80 µl of water, plasma supernatant, whole blood supernatant or liver homogenate supernatant. In water and plasma, 9 point calibration curves were constructed to obtain the following concentrations above baseline: 0.0 µmol/l, 0.1 µmol/l, 0.25 µmol/l, 0.5 µmol/l, 0.75 µmol/l, 1.0 µmol/l, 1.5 µmol/l, 2.0 µmol/l and 2.5 µmol/l. Because in whole blood and liver supernatant higher baseline concentrations of OPH were expected, 7 point calibration curves were constructed to obtain the following concentrations above baseline: 0.0 µmol/l, 1.0 µmol/l, 1.5 µmol/l, 2.0 µmol/l, 2.5 µmol/l, 5.0 µmol/l and 10.0 µmol/l. Pearson coefficient was used to determine the linearity of the method. The resulting regression line of these 9 and 7 point standard curves was described as linear equation, and the correlation coefficient (r^2) was calculated.

For each set of samples measured, a control plasma spiked with OPH to raise baseline concentrations with 1 µmol/l was measured in triplicate at the beginning, middle and end of the sample sequence.

The injection carry over was determined by measuring the peak area in 5 blank samples following analysis of samples spiked at a concentration which was the highest concentration of the calibration curve (2.5 µmol/l for plasma and 10.0 µmol/l for whole blood and liver tissue).

We initially aimed at applying an internal standard throughout all experiments, but as the production of the standard ($[^{13}\text{C}_2, ^2\text{H}_2]$ -Ophthalmic acid hydrochloride, AlsaChim, Illkirch, France) took too long, we could only apply it for the analysis of the unknown patient plasma samples. In addition, we also constructed a calibration curve in water in

which we varied the concentration of unlabelled OPH between 0 and 20 $\mu\text{mol/l}$, while we kept the concentration of the tracer constant at 10 $\mu\text{mol/l}$.

Instrumentation

The HPLC system consisted of a Gilson Model 233 XL sample processor, equipped with Peltier-chilled sample and reagent trays (4°C) and a Rheodyne 6 way high-pressure valve (Meyvis, Bergen op Zoom, The Netherlands) equipped with a 20 μl sample loop. A high-pressure gradient was generated using two Model PU 2085 pumps connected through a T-piece (Jasco Benelux, Maarssen, The Netherlands). The separation was performed on a 150 x 3mm (i.d.) Allsphere ODS-2 3 μm column (Grace, Breda, The Netherlands) set to 20°C. The bench top ion-trap mass spectrometer used was a model LTQ XL (Thermo Electron Veenendaal, The Netherlands), equipped with an ion-max electrospray (ESI) probe. The system was operated in positive mode. To obtain maximal analytical sensitivity, the liquid-chromatographic-mass spectrometry approach described by Geenen *et al.* [9] was modified. Maximal sensitivity was obtained by applying Selective Reactant Monitoring (SRM) to the precursor ion of OPH at m/z 290 and the product ion at m/z 215, using 35% (arbitrary value) normalized collision energy. The heated capillary was set to 220°C and sheath and auxiliary gas were set at 99 and 35 arbitrary system units respectively. The tube lens voltage was 20V and the spray-voltage was 4.5kV. Solvent A was a 0.3% formic acid (v/v) solution in Milli-Q-water (pH=2.4). Solvent B was a mixture of acetonitrile/water (95/5; v/v). Flow was 0.3 ml/min throughout the gradient. From T=0 min to T=1 min, gradient was kept at 100% solvent A, after which solvent B was increased to 50% in 6 min. Next, the column was cleaned by flushing at 100% B for 2 min, after which initial conditions were restored to 100% A in 1 min. The column was next re-equilibrated for 10 min. prior to the next analysis. Data were processed using Perkin Elmer TotalChrom version 6.2.0.

Stability determination of OPH in deproteinized plasma

To determine OPH stability in deproteinized plasma and water during analysis, aliquots of deproteinized aqueous standards and deproteinized plasma were stored in the sample compartment of the Gilson sample processor set at 4°C and analysed consecutively during an 8 hour period.

Enzymatic activity of plasma on OPH

In order to investigate OPH stability and to quantify enzymatic activity in plasma leading to conversion of OPH, plasma aliquots from the same five volunteers were spiked with OPH (to guarantee measurable concentrations). Next, samples were incubated at 4°C or 37°C for 5, 30, 60 and 120 minutes. Enzymatic activity was terminated by the addition of 5% SSA solution and subsequent thorough mixing before snap freezing in liquid nitrogen.

Statistics

Mann Whitney *U* test was applied for two group comparison for continuous data. Wilcoxon signed rank test was applied for pairwise comparison for continuous data. All data are expressed as median and range. A *p*-value <0.05 was considered statistically significant. Statistical analysis was performed using Prism 5.0 for Windows (Graphpad software, Inc, San Diego, CA).

Results

Calibration and validation

The limit of detection, determined according to ¹³ at a signal to noise ratio of 3 was found to be 4.5 nmol/l. The lower limit of quantification, determined at a signal to noise ratio of 5, was 6.5 nmol/l.

Using the present LC-MS/MS method, the retention time of OPH was about 6.27 min, with a total run time of 17.4 min. Representative chromatograms of aqueous OPH standard, baseline plasma, whole blood and liver samples are shown in Figure 10.2, panels A-D. Calibration curves in water and plasma, whole blood and liver were linear (Figure 10.3), with the following respective equations: $r^2=0.99$, $y=15.4 \times 10^3 (\pm 274.8)x + 477.0 (\pm 347.5)$; $r^2=0.99$, $y=7.0 \times 10^3 (\pm 620.6)x - 336.3 (\pm 784.5)$; $r^2=0.97$, $y=6.7 \times 10^3 (\pm 508.3)x + 6.5 \times 10^4 (\pm 2.3 \times 10^3)$ and $r^2=0.94$, $y=3.4 \times 10^3 (\pm 387.9)x + 25.0 \times 10^4 (\pm 7 \times 10^3)$.

Figure 10.2 Chromatographic separation and detection of OPH in the LC-MS/MS chromatogram of A) spiked 1.5 μ M H₂O SSA, (area=24736), B) blank human plasma (area=292), C) blank human whole blood (area=106624), D) blank human liver (area=139743).

Figure 10.3 Calibration curves of OPH in different matrices: A) H₂O SSA; B) plasma; C) whole blood; D) liver. Calibration curves of liver and whole blood did not pass through zero because of relatively high endogenous OPH concentration in these matrices.

A control plasma, prepared from pooled plasma of the healthy volunteers and spiked to raise baseline concentrations with 1.0 $\mu\text{mol/l}$ was analyzed in triplicate, namely at the beginning, middle and end of each sample set. Mean spike recoveries, calculated on 4 consecutive days of analysis varied from 103.4 to 106.2% with a standard deviation, varying between 0.39% and 2.35%.

Injection carry-over for plasma after a sample injection of a 2.5 $\mu\text{mol/l}$ OPH standard was not detectable. Injection carry-over for whole blood and liver tissue after injection of a 10.0 $\mu\text{mol/l}$ OPH standard was negligible with 0.13% and 0.45% respectively.

The internal standard stability was investigated by the construction of a calibration curve in which we varied the unlabeled standard between 0 and 20 $\mu\text{mol/l}$ and kept the labeled internal standard ($[\text{}^{13}\text{C}_2, \text{}^2\text{H}_2]$ -ophthalmic acid) constant at 10 $\mu\text{mol/l}$. In Figure 10.4 the results of this experiment are presented, indicating a linear increase in the unlabeled standard, while maintaining a stable area for the internal standard. In addition, we also added 20 μl of a 1 $\mu\text{mol/l}$ internal standard solution to 80 μl of the unknown patient plasma supernatant samples resulting in a final concentration of 0.2 $\mu\text{mol/l}$. We obtained a mean concentration for the standard of 0.21 μmol , indicating 102.5% recovery. The standard deviation was 0.018 $\mu\text{mol/l}$, resulting in a coefficient of variation of 8.5% at this low level indicating a good applicability of this approach.

Figure 10.4 OPH tracer stability and normal OPH standard.

Baseline OPH levels

The developed method enabled the measurement of endogenous OPH levels in baseline venous control plasma of 5 healthy volunteers at a mean level as low as 0.02 $\mu\text{mol/l}$ (range: 0.01-0.03 $\mu\text{mol/l}$). Mean OPH levels in arterial blood plasma of 21 patients scheduled for liver surgery were 0.06 $\mu\text{mol/L}$ (with an S.D. of 0.03 $\mu\text{mol/l}$). Mean OPH levels in whole blood and liver biopsy samples of patients undergoing liver resection were 16.1 $\mu\text{mol/l}$ (range: 11.8-16.4 $\mu\text{mol/l}$) and 80.0 $\mu\text{mol/kg}$ (range: 19.1-349.2 $\mu\text{mol/kg}$) respectively.

Stability of OPH

The replicate analysis in time of a spiked deproteinized plasma sample stored in the chilled sample compartment of the sample processor, showed no significant decrease in sample response during an eight hour period, indicating samples can be run safely during this period. The intra-assay precision during this period was 3.5% with an accuracy of 1.9% (Figure 10.5).

Stability experiments in spiked human plasma revealed that no significant decrease in exogenous OPH levels occurred at any time point during incubation at 4°C. However, 120 minutes incubation at 4°C resulted in a non-significant tendency towards a decrease of OPH levels in spiked human plasma of 9.7% (6.4- 21.0%). Interestingly, when spiked plasma was kept at 37°C, exogenous OPH levels showed a significant decrease of 74.6% (56.2- 100.0%) at 120 minutes compared to baseline ($p < 0.0001$) (Figure 10.6, panels A and B).

Figure 10.5 Stability of OPH in water and deproteinized plasma in the auto sampler for 8 hours at 4°C (n=5).

Figure 10.6 OPH levels at different time points in untreated plasma that was incubated at A) 4°C B) 37°C (n=5), error bars represent standard error of the mean.

Discussion

The aim of the present study was to develop and validate a protocol to enable the quantitative analysis of OPH in human plasma, thus enabling future research on the value of OPH as a marker for intra-cellular GSH depletion. The here described protocol indeed enabled this, resulting in baseline OPH levels in venous plasma of healthy human volunteers (10-30 nmol/l) as well as in arterial plasma of patients scheduled for (partial) liver resection (20-140 nmol/l). In human whole blood and liver biopsy homogenates baseline OPH concentrations were markedly higher ($\mu\text{mol/l}$ range) as compared to plasma. It was also shown that immediate deproteinization of plasma with SSA at 4°C and finally storage at -80°C was optimal to prevent OPH degradation in collected plasma samples. OPH concentrations in untreated plasma of healthy volunteers did not significantly decrease within 2 hours if kept at 4°C.

An increasing number of studies have suggested that OPH could be a marker of hepatic GSH depletion^{4-6,9-11}. However, most of these data are derived from animal experiments and human OPH data are scarce. Kombu *et al.*⁶ investigated the GSH-OPH system by labeling free GSH, bound glutathione (GSSG) and OPH following intravenous infusion of ²H-enriched water and subsequently used an LC-MS-MS assay to determine enrichments. This method provided the opportunity to measure the synthesis of these components in rat plasma, erythrocytes and liver. However, the pool size of OPH in human liver is unknown. Our data show, for the first time, the presence of relatively high levels of OPH in human liver biopsies. From a translational research point of view this observation is crucial support for the value of OPH as a biomarker for hepatic GSH synthesis/depletion. An explanation for these relatively high liver OPH levels may be the fact that the patients enrolled in the present study had colorectal liver metastases. Although liver biopsies were taken remote from the hepatic tumours to be removed, it is known that malignant liver tumour growth, both primary and secondary, can disturb the GSH buffer system and may therefore also be associated with high OPH levels^{4,11}.

The results of the present study are largely in concordance with results published recently by Geenen *et al.*⁹ who showed that OPH could be measured accurately with LCMS in rat plasma and cell culture media. Since endogenous OPH levels could not be detected in human plasma in their study⁹, they speculated on a potential difference between rat OPH metabolism and human OPH metabolism. In contrast to this assumption we postulated that although a chemical degradation had been excluded in this paper, an enzymatic degradation had not been ruled out and this was the key target for this study to investigate. In addition we modified their LC-MS approach, now incorporating an MS-MS transition, as described elsewhere⁶ to enhance the level of sensitivity and accuracy even more. This resulted in sensitive and selective method allowing detection of very low endogenous baseline OPH levels in plasma of both healthy volunteers and patients undergoing liver resection.

It is suggested that OPH is subject for the same pathway for enzymatic degradation as GSH, catalyzed by γ -glutamyl transpeptidase^{7,8}. Therefore, in this study, we focused on optimization of the sample preparation procedure. Indeed, we were able to show that OPH levels in untreated plasma samples decreased at a temperature and time dependent rate. Especially, when untreated plasma samples spiked with OPH were kept at 37°C, a significant amount (75%) of the OPH was degraded within 1 hour. When kept at 4°C the degradation process was evidently diminished. These experiments confirmed the necessity to deproteinize collected plasma samples as soon as possible in order to obtain accurate results. Whether OPH follows the same enzymatic breakdown pathway as GSH, still needs to be verified in future studies.

As it was clear that even within the period required to prepare plasma still a 5-10% decrease of OPH levels could occur, we tried to further reduce sample preparation time by also measuring whole blood OPH concentrations. As erythrocytes contain a relatively high level of GSH, we found, not unexpectedly, that whole blood contained a 10-20 times

higher level of OPH (~12-16 $\mu\text{mol/l}$) than plasma. This observation could present a potential problem when using OPH as a biomarker of GSH depletion, for instance in case plasma is haemolytic. Although the relation between erythrocyte OPH synthesis and the exchange between plasma/liver OPH and the erythrocytes has not been well studied yet, for GSH it has been investigated thoroughly. Research has previously shown that most of plasma GSH is formed in the liver¹². Kombu *et al.*⁶ confirmed this by using a stable isotope labelling technique with a $^2\text{H}_2\text{O}$ load, followed by measurement of the label incorporation in GSH moieties. The authors showed that there was a rapid labelling of liver GSH, reflected by a rapid turnover of the large GSH/GSSG (the ratio reduced versus oxidized GSH) pool in rats. Interestingly, they also showed that the labelling profile of plasma GSH was comparable to that of liver GSH. The labelling of the GSH/GSSG pool in erythrocytes was shown to be much slower compared to the liver. This is probably due to the low rate of GSH synthesis in erythrocytes and/or due to the low import of plasma GSH into erythrocytes. Since OPH is an analog of GSH, it is conceivable that OPH follows the same kinetic mechanisms as GSH. This would mean that if haemolysis can be avoided, plasma OPH levels may accurately reflect hepatic OPH synthesis. In order to guarantee accurate measurement of plasma OPH and to correctly interpret OPH levels in the future it might be necessary to determine the level of haemolysis, for instance by determining free haemoglobin in plasma.

Whether OPH levels can be used reliably as a reciprocal readout of GSH depletion should be investigated in a future proof of principle study, preferably in a human translational research model. If validated thoroughly, OPH could be a candidate dynamic liver function biomarker. This proposed biomarker could for instance be used to preoperatively predict liver function and could also allow early diagnosis of hepatic dysfunction in patients undergoing liver resection. In addition, OPH as a biomarker for GSH depletion could help to titrate the treatment of patients with paracetamol (PCM) intoxication. It could also be used to measure susceptibility to hepatotoxicity to develop individualized pharmacotherapeutic regimens, in order to optimize drug efficacy in the treatment of patients.

Conclusion

The developed LC-MS/MS protocol enables the measurement of OPH in plasma of healthy volunteers as well as in patients with colorectal liver metastases undergoing liver resection. Accurate data can only be collected if freshly prepared plasma samples are immediately deproteinized, kept on ice and haemolysis is avoided. With use of this LC-MS/MS assay and the here described pre-storage treatment protocol, it is now possible to validate OPH as a biomarker for hepatic GSH depletion in a future human proof of principle study. Provided the precautions shown in the present study the applicability of OPH as a biomarker for liver function is promising.

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Chapter 11

Ophthalmate as a read out for hepatic glutathione
metabolism in humans in vivo

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Abstract

Background

Ophthamate (OPH) has been hypothesized to be a biomarker for hepatic glutathione (GSH) homeostasis, which is one of the most important indicators of liver function. According to this hypothesis, OPH increases when GSH decreases upon a challenge. This study investigated whether OPH can be used in humans as a read out for GSH homeostasis after paracetamol (PCM) challenge during liver resection.

Methods

Nineteen patients scheduled for liver resection (n=12) or major non-hepatic gastrointestinal surgery (control group) (n=7) were included in this study. Intravenous PCM-challenges (1000 mg) took place before transection (first challenge), 6 hours after the first challenge and 6 hours after the second challenge. Arterial, hepatic and portal venous blood samples and liver biopsies were taken at regular intervals to quantify both OPH and GSH. Also, thyl radical formation in the liver was measured by electron spin resonance (ESR) spectroscopy. The OPH/GSH ratio was calculated.

Results

In the liver resection group an expected decrease in arterial plasma GSH levels was seen six hours after the first PCM-challenge compared to baseline GSH [0.30 (0.05) $\mu\text{mol/l}$ versus baseline 1.0 (0.20) $\mu\text{mol/l}$ ($p=0.005$)]. In the control group there was a tendency towards a decrease [0.36 (0.11) $\mu\text{mol/l}$ versus baseline 1.0 (0.23) $\mu\text{mol/l}$ ($p=0.06$)]. Unexpectedly, OPH levels did not increase but showed a significant decrease at 6 hours after the first PCM-challenge: 0.05 (0.009) $\mu\text{mol/l}$ versus baseline 0.11 (0.02) $\mu\text{mol/l}$ ($p=0.03$). In the liver resection group the OPH/GSH ratio was significantly increased 6 hours postoperatively after the third PCM-challenge compared to baseline, [24.9 (3.4) % versus baseline 13.4 (2.2) % ($p=0.03$)]. No differences in GSH, thyl radicals or OPH levels were found in liver biopsies.

Conclusion

In the present study, no inversely proportional increase in OPH was observed, related to the GSH drop after PCM-challenge in both groups. However, in the liver resection group, there was a late increase in the OPH/GSH ratio, which could indicate increased production of OPH.

Introduction

Prediction of remnant liver function is becoming increasingly important to identify patients at risk of postresectional liver failure^{1,2}. Unfortunately, most liver function tests presently available cannot predict future remnant hepatic functional reserve. In the present study one the most important functions of the liver, its glutathione (GSH) based defense mechanism against many diverse forms of stress and intoxications was exploited³⁻⁵. During conditions of oxidative stress, the liver responds with a cellular efflux of GSH. After reaction, most of the oxidized GSH is broken down into its constituent amino acids, which are taken up again by the cells and re-synthesized into GSH. However, if the balance between efflux and re-synthesis cannot be maintained, the intracellular levels of GSH become depleted and cell damage occurs⁶.

Soga *et al.*⁷ showed that ophthalmate (OPH) was synthesized at an enhanced level in a paracetamol (PCM) induced hepatotoxicity mice model. OPH is an endogenous tri-peptide analogue of GSH, in which the limiting amino acid cysteine has been replaced by 2-aminobutyric acid, a cysteine analog which lacks the thiol moiety⁷⁻¹⁰. OPH synthesis is enhanced under circumstances of limited cysteine availability, such as following a PCM challenge. Consequently, a rise in plasma OPH concentration may be a reciprocal read out of hepatic GSH depletion. At nontoxic doses PCM is detoxified by sulfation or glucoronidation followed by excretion in the urine¹¹. However, in high doses saturation of this system occurs and PCM is also metabolized by P450 cytochromes generating the reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI)¹¹⁻¹³. NAPQI binds to GSH, and is then removed from the liver and excreted via urine which results in a sudden drop in GSH levels.

Patients undergoing resectional liver surgery often receive PCM pre- and postoperatively for analgesia to enhance recovery¹⁴. These patients have frequently a reduced liver function because a considerable part of their liver is removed. In the presence of surgical stress the administration of (normal dose) PCM might lead to a faster depletion of GSH since the hepatic GSH storage can be reduced by enhancing oxidative stress¹⁵. Moreover, patients undergoing liver surgery often receive preoperative chemotherapy, which can also cause hepatotoxicity of the non-tumour bearing liver¹⁶⁻¹⁸. The exact effect of the different types of preoperative chemotherapy on hepatic GSH metabolism is unknown. Platinating agents are detoxified by GSH through adduct formation¹⁹ and therefore one could anticipate that the administration of these agents is associated with lower intra- and extracellular levels of GSH.

In the present study, the hepatic GSH-system was repeatedly challenged intra- and postoperatively with PCM to investigate whether a change in plasma or tissue OPH may be indicative for changes in hepatic GSH metabolism. In addition, thyl radicals were measured with electron spin resonance spectroscopy (ESR) to quantify oxidative stress on the level of GSH.

Methods

Patients

Patients scheduled for liver surgery or pancreatic surgery at Maastricht University Medical Center were eligible for inclusion in this observational study. The major non-hepatic gastrointestinal surgery group (control group) consisted of non-jaundiced patients who underwent pancreatic surgery. These patients were included in the control group for several reasons. Firstly, they experience comparable surgical stress compared to patient undergoing liver resection, but their functional liver capacity remains intact. Secondly, there are no major differences in anesthetics during liver surgery and pancreatic surgery. Finally, during pancreatic surgery blood can be drawn more easily from the portal and hepatic vein than during other types of major abdominal surgery.

The study was approved by the medical ethical committee of Maastricht University Medical Center and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). All patients gave written informed consent. Patients were admitted to the hospital one day preoperatively and routine blood tests were analysed by the clinical chemistry department. It was recorded whether patients had received preoperative chemotherapy. It was also monitored which type of chemotherapy regime and the number of cycles patients had received.

Operative procedure

Patients were anaesthetized using isoflurane and propofol. They routinely had an epidural catheter, urinary catheter, two peripheral venous catheters and indwelling catheters in a jugular vein and radial artery. Body temperature was maintained using a Bair Hugger system (Arizant Healthcare Inc. Eden Prairie, MN).

Liver resection group

Liver resections were classified as major (≥ 3 Couinaud segments) or minor (< 3 segments or non-anatomical) resections. Liver resection was performed as detailed elsewhere²⁰. In short, laparotomy was performed by bilateral subcostal incision, followed by intraoperative ultrasonography of the liver. Once resectability had been confirmed, mobilization of the liver was performed to prepare for hepatic parenchymal transection, which was undertaken using a Cavitron Ultrasonic Surgical Aspirator (Force GSU System; Valleylab, Boulder, CO). Central venous pressure was maintained below 5 cm H₂O during transection to reduce venous back-bleeding.

Non-hepatic gastrointestinal surgery group (control group)

Pylorus preserving pancreaticoduodenectomy (modified Whipple procedure) was performed via a bilateral subcostal incision. The Kocher maneuver was performed by

identifying the inferior vena cava (IVC) at the level of the proximal portion of the vertical part of the duodenum. The duodenum and pancreatic head were then mobilized off the IVC. When the duodenum was transected the pylorus was preserved. The jejunum was transected approximately 10 cm distal to the ligament of Treitz. The bile duct was transected at the hepatic hilus. The pancreas was transected at the neck of the pancreas. An end-to-side, one layer running duct-to-mucosa pancreaticojejunostomy was performed followed by a one-layer, end-to-side hepaticojejunostomy (orthotopic). Finally a duodenojejunostomy was performed.

Intravenous PCM-challenge model and sample collection

Baseline blood samples were obtained from the arterial line after induction of anaesthesia. PCM-challenges of 1000 milligrams paracetamol [Perfalgan® solution for intravenous infusion, 10 mg/ml (100 ml), Bristol-Myers Squibb Pharmaceuticals Ltd] took place intra-operatively directly after mobilisation of the liver but before transection (first challenge), 6 hours after the first challenge (second challenge) and 6 hours after the second challenge (third challenge) (Figure 11.1). PCM was administered in less than 5 minutes. Blood samples were obtained from the arterial line at predefined time points: after incision, before the first PCM-challenge, 1 hour after the first PCM-challenge and after transection of the liver/pancreas. Postoperatively arterial blood samples were taken 6 hours after the first PCM-challenge, 6 hours after the second PCM-challenge and 6 hours after the third PCM-challenge. During surgery a total of three liver biopsies were taken in both groups on specific time points: before the first PCM-challenge, 1 hour after the first PCM-challenge and after transection of the liver/pancreas (Figure 11.1).

Sample preparation

Blood samples were collected in pre-chilled heparinised vacuum tubes (6 ml) [BD vacutainer, Becton Dickinson Diagnostics, Aalst, Belgium] and kept on ice. In the liver resection group the biopsies were taken from a non-tumour bearing side. Blood was immediately centrifuged at 4°C at 3500 rates per minute for 10 minutes in the operating theatre. Only the separated plasma samples used for OPH/GSH measurements were “deproteinized” by the addition of an equal volume of a freshly prepared 5% (w/v) 5-sulfosalicylic acid solution and 0.1% (w/v) Vitamin C (5-SSA, Vit C: British Drug Houses, Amsterdam, The Netherlands). Liver samples were homogenized by microbeating 10 mg of liver tissue in 100µL of the SSA/Vitamine C solution. Vitamin C was added to the samples to prevent oxidation of GSH. Samples were stored at -80°C until analysis. Prior to analysis all samples were centrifuged for 10 minutes at 50.000 *g* at 4°C. The clear supernatants were used for analysis.

Figure 11.1 Timeline of sample collection and PCM-challenges. Transection = transection of the liver or pancreas; PCM = 1000 mg paracetamol intravenously; Bx = liver biopsy; Arterial = blood sample from radial artery, PV = blood sample from portal vein, HV = blood sample from one of the hepatic veins.

OPH and GSH measurements

OPH and GSH were measured in plasma and homogenized liver samples with a newly developed method based on liquid chromatography-tandem mass spectrometry²¹.

OPH/GSH ratio

The ratio between arterial OPH and arterial GSH levels was calculated on every time point for both groups and expressed as a percentage using the following formula: [arterial OPH/arterial GSH]*100%.

Source and fate of OPH

In order to measure the source and fate of OPH, blood was drawn from both the portal and one of the hepatic veins (from the non-tumorous side of the liver) before, one hour after the first PCM-challenge and after transection. Arterio-venous (AV) differences of OPH across the gut and the hepatosplanchnic area (gut + liver) were calculated. The OPH balance of the liver was expressed by the OPH concentration in hepatic venous blood minus the OPH concentration of hepatic inflow (which is considered to consist of 30%

arterial blood and 70% portal blood). AV-differences were calculated using the following formulae:

Gut AV-difference = portal venous [OPH] – arterial [OPH]

Liver inflow = 0.30 * arterial [OPH] + 0.70 * portal venous [OPH]

Liver AV-difference: hepatovenous [OPH] – liver inflow

Hepatosplanchnic AV-difference = hepatovenous [OPH] – arterial [OPH]

ESR detection of Thiyl

GSH protects cells by reducing the phenoxyl radical of PCM²². In this reaction a thiyl radical is formed. These thiyl radicals can be detected with electron spin resonance (ESR) spectroscopy²³. In short, frozen human liver biopsies were placed in liquid nitrogen in a quartz liquid finger dewar at the center of the 1273 ER4119HS high sensitivity cavity. ESR spectra were recorded on an X-band spectrometer (Bruker EMX 1273, Biospin, Rheinstetten, Germany) operating at 9.50 GHz. Instrumental conditions for the recorded spectra were: magnetic field: 3325 G; scan range: 150 G; modulation frequency 100 kHz modulation amplitude: 5 G; receiver gain: 1×10^5 ; power: 20 mW; time constant: 20.84 ms; scan time: 40.96 ms; number of scans: 20. Thiyl radicals were quantified by peak surface measurements using the WIN-EPR spectrum manipulation program (Version 2.11, Bruker, Rheinstetten, Germany).

Statistics

All data are expressed as mean (SEM). Means were compared with Wilcoxon matched pairs tests. To compare the different groups the Mann-Whitney *U* test was applied. Dichotomous data were compared using the chi-squared test. AV-gradients were tested versus a theoretical mean of zero using the one sample t-test. A *p*-value <0.05 was considered statistically significant. Statistical analysis was performed using Prism 5.0 for Windows (Graphpad software, Inc, San Diego, CA) and the Statistical Package for the Social Sciences (SPSS) version 19.0 (IBM, New York).

Results

Patients

Nineteen patients (7 females; 12 males) scheduled for either a liver resection or a pancreaticoduodenectomy, were included. Twelve patients underwent a liver resection for primary (n=1) or secondary malignant liver disease (n=11). Three patients underwent major liver resection (≥ 3 Couinaud segments) and 9 patients underwent minor liver resection (<3 segments). Seven patients received pancreatic surgery of whom 4 patients

underwent a pancreaticoduodenectomy and 3 patients received a palliative double bypass. There were no clinically relevant differences between routine blood tests in both groups (Table 11.1).

Table 11.1 Patient characteristics.

	Liver resection group (n=12)	Whipple / double bypass group (n=7)
Age (years)	65 (40-72)	67 (60-73)
Sex (female:male)	3:9	4:3
Height (cm)	174 (160-190)	173 (160-180)
Weight (kg)	80 (69-105)	73 (61-103)
Body Mass Index	26.5 (22.8-35.8)	24.4 (21.1-31.8)
Aspartate-aminotransferase (IU/l)	24 (8-85)	19 (17-24)
Alanine-aminotransferase (IU/l)	34 (12-140)	29 (24-41)
Lactate dehydrogenase (IU/l)	117 (125-455)	138 (122-154)
Gamma-glutamyl transpeptidase (IU/l)	66 (28-308)	75 (74-128)
Alkaline phosphatase (IU/l)	112 (68-522)	138 (125-138)
Bilirubin (μ M)	21 (18-27)	13 (6-54)
Pre-operative creatinin (μ mol/l)	77 (49-108)	65 (43-108)

Data are presented as median (range). All data are preoperative values

Preoperative chemotherapy

None of the patients of the control group received preoperative chemotherapy. In the liver resection group 6 patients (50%) had received chemotherapy. All 6 patients received capecitabine. In 4 of the 6 patients this was combined with oxaliplatin, 3 patients also received bevacizumab and 1 patient received irinotecan. The cycles of chemotherapy were on average 4.5 (3-8) weeks. None of the patients showed intraoperative signs of the “blue liver syndrome” caused by oxaliplatin¹⁸. Postoperative histology of the non-tumour bearing side of the liver revealed that 4 patients of the control group had normal livers, 1 patient suffered from severe steatosis. In 2 patients of the control group no histological examination of the liver took place. In the liver resection group one patient showed nodular regenerative hyperplasia and one patient showed minimal sinusoidal dilatation. Histological examination showed that 5 patients had mild/moderate steatosis and 3 patients had signs of severe steatosis. One patient had congestion of the liver parenchyma and one patient’s liver showed signs of iron overload. None of the patients had significant fibrosis or cirrhosis of the liver.

In the liver resection group there were no significant differences in GSH and OPH plasma levels at baseline between patients who had received preoperative chemotherapy (n=6) and patients who did not (n=6): GSH 0.42 μ mol/l (0.06) versus 0.62 μ mol/L (0.19) ($p=0.87$); OPH 0.06 μ mol/l (0.01) versus 0.06 μ mol/l (0.01) ($p=0.94$), respectively. There were no significant differences in GSH and OPH plasma levels between groups at any time point.

In the total group (liver resection group and control group) there were also no significant differences in GSH and OPH levels at baseline between patients who had received preoperative chemotherapy (n=6) and patients who did not (n=13): GSH 0.42 $\mu\text{mol/l}$ (0.06) versus 0.56 $\mu\text{mol/l}$ (0.11) ($p=0.79$); OPH 0.06 $\mu\text{mol/L}$ (0.01) versus 0.06 $\mu\text{mol/l}$ (0.01) ($p=0.93$), respectively. This remained insignificant for both markers throughout the whole study protocol.

Systemic GSH and OPH levels

Control group

GSH and OPH arterial plasma levels measured directly after anaesthesia were not significantly different from GSH and OPH levels measured directly after incision and before transection (baseline) in the control group (data not shown).

As expected there was a significant decrease in the control group in arterial GSH concentration 1 hour after the first PCM-challenge compared to baseline: 0.62 (0.19) $\mu\text{mol/l}$ versus 1.0 (0.23) $\mu\text{mol/l}$ ($p=0.05$). GSH levels showed a strong tendency towards a further decrease six hours after first PCM-challenge 0.36 (0.11) $\mu\text{mol/l}$ versus baseline 1.0 (0.23) $\mu\text{mol/l}$ ($p=0.06$) (Figure 11.2A). Plasma GSH levels at six hours after the second and third PCM-challenge were not significantly different from baseline.

Unexpectedly, arterial OPH levels followed a similar pattern as GSH levels. There was a tendency towards a decrease in OPH level 6 hours after the first PCM-challenge compared to baseline, 0.06 (0.02) $\mu\text{mol/L}$ versus 0.13 (0.03) $\mu\text{mol/l}$ ($p=0.06$). OPH levels measured 6 hours after the second and third PCM-challenge postoperatively were not significantly different from baseline (Figure 11.2B).

Liver resection group

Plasma GSH levels measured directly after anaesthesia were not significantly different from GSH levels measured directly after incision and before transection (baseline) in the liver resection group (data not shown). GSH levels did not differ significantly 1 hour after the first PCM-challenge compared to baseline. However there was a significant decrease in arterial GSH concentration 6 hours after the first PCM-challenge compared to baseline: 0.30 (0.05) $\mu\text{mol/l}$ versus 1.0 (0.20) $\mu\text{mol/l}$ ($p=0.005$). GSH remained significantly decreased at 6 hours after the second and third PCM-challenge compared to baseline: 0.38 (0.06) $\mu\text{mol/l}$ versus 1.0 (0.20) $\mu\text{mol/l}$ ($p=0.004$) and 0.56 (0.20) $\mu\text{mol/l}$ versus 1.0 (0.20) $\mu\text{mol/l}$ ($p=0.003$) (Figure 11.2A).

Unexpectedly, OPH levels in the liver resection group levels seemed to follow a similar pattern as GSH levels. Six hours after the first PCM-challenge OPH levels were significantly decreased in comparison to baseline: 0.05 (0.009) $\mu\text{mol/l}$ versus 0.11 (0.02) $\mu\text{mol/l}$ ($p=0.03$). No significant difference in OPH levels was seen at 6 hours after the second and third PCM-challenge in comparison to baseline (Figure 11.2B).

Figure 11.2 Time course of A) OPH and B) GSH plasma levels in both groups. Data were plotted as mean and SEM. * $p < 0.05$.

OPH/GSH ratio

The OPH/GSH ratio plasma in the control group was significantly increased 1 hour after first PCM-challenge compared to baseline, 21.0 (3.9) % versus 12.7 (2.0) % ($p=0.05$) (Figure 11.3A). In the liver resection group the OPH/GSH ratio was significantly increased 6 hours postoperatively after third PCM-challenge compared to baseline, 24.9 (4.9) % versus 13.1 (2.2) % ($p=0.03$) (Figure 11.3B). At other time points there were no significant differences compared to baseline in both groups.

Figure 11.3 Time course of OPH/GSH ratio in A) control group and B) the liver resection group. Data were plotted as mean and SEM. * $p \leq 0.05$.

OPH organ AV-gradients

In order to investigate OPH AV-gradients both groups were analyzed as one total group.

Baseline (before transection)

At baseline there was no OPH uptake or release by the gut: -0.018 (0.016) $\mu\text{mol/l}$ ($p=0.5$), whereas net OPH release from the liver equalled 0.12 (0.04) $\mu\text{mol/l}$ ($p=0.004$). Overall hepatosplanchnic OPH AV-gradient was 0.11 (0.04) $\mu\text{mol/l}$ ($p=0.008$).

1h after 1st PCM-challenge

One hour after first PCM-challenge there was no OPH uptake or release by the gut: -0.009 (0.008) $\mu\text{mol/l}$ ($p=0.12$), whereas net OPH release by the liver equalled 0.08 (0.05) $\mu\text{mol/l}$ ($p=0.04$). Overall hepatosplanchnic OPH AV-gradient was not significantly different from zero: 0.08 (0.05) $\mu\text{mol/l}$ ($p=0.06$).

After transection

Liver transection resulted in a significant uptake of OPH by the gut -0.034 (0.013) $\mu\text{mol/l}$ ($p=0.01$). The OPH AV-gradient from the liver was not significantly different from zero: 0.08 (0.04) $\mu\text{mol/l}$ ($p=0.06$). Overall hepatosplanchnic OPH AV-gradient was also not significantly different from zero 0.06 (0.04) $\mu\text{mol/l}$ ($p=0.29$) (Figure 11.4).

Figure 11.4 Mean (SEM) arterio-venous concentration gradients of OPH across the gut (portal venous minus arterial) and the hepatosplanchnic area (hepatic venous minus arterial). OPH was specifically released from the liver (vs. zero) and this resulted in a net OPH release from the hepatosplanchnic area (vs. zero). * $p < 0.05$

GSH and OPH levels in liver

Baseline hepatic GSH levels in the control group and liver resection group were 1288 (196) $\mu\text{mol/kg}$ and 1323 (93) $\mu\text{mol/kg}$ ($p=0.74$), respectively. In both the control and the liver resection group GSH levels measured in liver tissue did not change significantly intra-operatively and after transection following PCM administration compared to baseline.

Baseline hepatic OPH levels in the control group and liver resection group were; 76 (17) $\mu\text{mol/kg}$ and 81 (16) $\mu\text{mol/kg}$ ($p=0.9$), respectively. Also OPH levels remained equal in both groups at each time point. In addition there were no significant differences in OPH or GSH hepatic levels between groups (Figure 11.5A-B).

Thiyl radicals

There were no differences in thiyl radical levels measured with ESR in liver biopsies between groups at baseline. In both groups there were no significant changes in thiyl radicals at any time point after PCM-challenge (figure 11.6A-B).

Figure 11.5 Levels of A) GSH and B) OPH measured in homogenized liver biopsies in the liver resection and control group.

Figure 11.6 A) Typical ESR spectrum of a thiyl radical observed as broad signals (45G) with $g = 2.02$ B) thiyl radicals measured in liver biopsies.

Discussion

This study aimed to investigate whether plasma OPH is useful as a read out for GSH metabolism in humans. Data of the present study showed that intraoperative and postoperative intravenous administration of PCM (1000 mg) did not lead to GSH depletion in both plasma and liver in either of the groups. There was an expected significant decrease in systemic GSH levels at 6 hours after an intra-operative PCM in the liver resection group. In contrast to what was expected there was no reciprocal increase of OPH in plasma or liver when GSH was decreased in the plasma at any time point. In fact, OPH seemed to follow the same pattern as GSH which was evidenced by a significant drop

in OPH plasma level 6 hours after the intraoperative PCM-challenge in the liver resection group. A smaller functional liver volume after liver resection did not lead to significantly lower levels of GSH and higher levels of OPH in the liver resection group, compared to the control group.

Our data suggest that a decrease in plasma GSH concentration in patients with a normal intracellular hepatic GSH pool does not lead to an increase in OPH production by the liver. However, the OPH/GSH ratio was postoperatively increased in the liver resection group, which might give circumstantial evidence that OPH production could be increased in the liver. This was supported by the fact that AV-gradients in the total group indicated OPH release from the liver, but this occurred at other (intraoperative) time points. The change in OPH/GSH ratio could be an important observation. Since OPH and GSH are synthesized through the same enzymatic machinery⁷, is it likely that there is competition for synthesis between OPH and GSH. An increased OPH/GSH ratio could be an earlier and more sensitive sign for increased OPH production than higher OPH liver or plasma levels. However, the effect of this competition of OPH on GSH synthesis rate and vice versa remains to be investigated.

The administration of preoperative chemotherapy did not seem to have an effect on the GSH and OPH plasma levels in these patients. However, this needs to be investigated in a future study with a larger group of patients, to correct for potential confounding factors, such as length of chemotherapy and different chemotherapy agents.

This study is the first translational *in vivo* human study for the biomarker OPH. It is to the best of our knowledge the first time that the effect of PCM on GSH and OPH levels was studied in human liver samples and blood samples from the hepatic and portal vein. This gave important data and new insights on the value of OPH as a read out for GSH homeostasis. However, the results of this study are to some extent in contrast with data of animal and experimental OPH studies in the past^{7,8}. A possible explanation for this is that the present study is hampered by some limitations. Thus, it was unclear whether the PCM-challenge was “powerfull” enough to induce important differences in GSH metabolism. The applied PCM doses in this study were equal to doses used in standard postoperative care and herewith comparable with normal clinical practice (4 grams a day), but relatively low in comparison to levels created in mouse models and *in vitro* studies^{7,8}. This was confirmed by the lack of increase in thiyl radicals after PCM administration. This might indicate that there is no significant increase in phenoxy radicals of PCM, which need to be reduced by GSH. It could be that the dosages of PCM in the present study were too low to exert the same effect as seen before in mice. However, increasing PCM dosages for patients undergoing liver surgery would not appear feasible because of concerns of postoperative liver failure. Perhaps it would be possible in the future to investigate the effect of higher dosage PCM on OPH levels in a healthy volunteer study.

Another explanation could be that hepatectomy itself increases GSH biosynthesis immediately, which could compensate for any GSH consumption²⁴. In this study only 3 patients underwent a major hepatectomy. If more patients were included that

underwent major or extended hepatectomy perhaps the PCM-challenges would have had more effect on the GSH stores in the liver. Finally, it may be that OPH synthesis is dependent on the bioavailability of other (yet unknown) intracellular substances. This was suggested by Geenen *et al.*²⁵ who used a kinetic mathematical model for GSH and its detoxification pathway for PCM to investigate whether OPH was a robust biomarker for GSH depletion. Since the liver has the ability to produce cysteine from methionine, the methionine concentration was varied in this model while the correlation of OPH with GSH was tested. It was shown that OPH was dependent on the methionine status. Therefore, in this mathematical model OPH alone was not a reliable biomarker for intracellular GSH depletion induced by PCM²⁵. The same group of authors investigated OPH production in an *in vitro* hepatic cell line study while methionine and PCM concentrations were varied²⁶. Results showed elevated intracellular and extracellular concentrations of OPH following PCM treatment while methionine concentrations were low. The authors also showed that OPH flux remained stable until the GSH concentration decreased under a certain level, after which OPH production increased sharply with further decreases of GSH concentration. Based on the above mentioned studies it can be concluded that OPH may be a good marker for GSH depletion only under very specific conditions.

Since it appears that increased OPH production only occurs when GSH is depleted, it would be worthwhile to investigate the dynamics of OPH and GSH in patients with GSH depletion, for instance septic patients, jaundiced patients with chronic liver failure, patients with PCM intoxication or patients suffering from (postoperative) liver failure. In addition, the model could be tested in patients with methionine depletion (for instance malnutrition)^{25,26}.

The fact that PCM administration during liver surgery did not lead to GSH depletion or high OPH levels granted valuable information about the safety of administration of PCM used after liver surgery in a standard postoperative care program. It has been argued that PCM administration is unsafe after liver resection but the present study provides arguments against this belief. In this study group PCM is thus considered to be safe with regard to GSH consumption. However no general statements can be made on the basis of these experiments, since GSH homeostasis and susceptibility to xenobiotic toxicity are influenced by numerous factors. These include genetic polymorphism in glutamate cysteine ligase²⁷ altered levels of the expression of genes encoding the c-glutamyltranspeptidase enzyme, GS deficiency and changes in methionine metabolic pathway (i.e. in cirrhotic patients and in patients with homocystinemia)²⁸. Moreover, administration of PCM has proven to cause hepatocellular damage even in healthy adults in the past²⁹. Therefore, caution is still demanded with PCM as a postoperative analgesic following liver resection.

Conclusion

This is the first human study in which the usefulness of OPH as a read out for hepatic GSH metabolism was studied. The results show that plasma OPH was not inversely proportionally related to the expected GSH plasma level drop after PCM-challenge. Both GSH and OPH levels remained unchanged in the liver after PCM-challenge. However an increased OPH/GSH ratio gave circumstantial evidence for an increased production of OPH which was not evidenced by increased OPH plasma levels. Future studies are needed in order to validate plasma OPH as a biomarker for hepatic GSH depletion.

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Chapter 12

General discussion

General discussion/future perspective

Liver surgery is a relatively young type of surgery but has evolved quickly over the years. The post-operative outcome has improved considerably over time due to increased knowledge of liver anatomy and liver function, novel operating techniques and advances in anesthesia. As a consequence of this every year more (extended) major liver resections are performed. The extent of liver resections will be mainly limited by the risk of postoperative liver failure. From this thesis, it appears that liver failure in these patients is determined by size of the postoperative remnant liver or more importantly the functional quality of the remnant liver. Preventing liver failure and designing a method to identify patients at risk of liver failure (and hence mortality) following liver resection will be the ultimate goal for the future. The major findings of the studies of this thesis will be discussed in the context of our current knowledge and suggestions for further studies and research will be given.

Liver volumetry

In order to perform liver resections safely the percentage functional remnant liver volume has to be at least 26.6% in patients with normal liver parenchyma^{1,2}. It is mandatory to estimate the remaining liver size in major liver resections to avoid insufficient functional reserve of the liver remnant after resection. A relatively small inaccuracy could have major consequences for these patients. Radiologic assessment of liver size is preferable over formulas that estimate liver size. It was shown in the present thesis that two open source image programs (ImageJ and Osirix[®]) were accurate and applicable. However, there is still room for improvement in accuracy and applicability of these programs. For the future a few hurdles have to be taken before liver volumetry will be applied world wide in a standard protocol preoperatively in every patient undergoing major liver surgery.

New promising developments in the era of liver volumetry have been made. Both ImageJ and OsiriX[®] involve manual tracing on 2-D CT-scan images, which takes time and effort. It takes 25–40 minutes on average to assess liver volume for one patient and there is a risk of intra-observer and inter-observer variability. One of the possible solutions for this is automated computerized liver volumetry, which may completely replace manual liver volumetry in the future.

An important improvement in order to reduce laboriousness would be “auto segmentation” of the liver. In fact computerized liver extraction schemes for CT have been developed and analyzed in several studies³⁻⁵. Suzuki *et al.*⁶ showed that automated volumetry is almost as accurate as manual volumetry for measuring liver volume with CT, but with the advance that automated volumetry is substantially more efficient. A possible downside of automated liver extraction can be occasional false-positive and false-negative extractions that still require manual correction.

Detailed analysis such as individual vessel territory is very difficult on 2D images. A possible solution for this is three dimensional (3D)-volumetry of the liver⁷. Due to the

technical advances in software full 3D reconstructions of the liver can be made. With 3D-volumetry it is for example possible to reconstruct the vascular tree of the liver which gives the opportunity to extract each vessel territory in the liver. This in turn gives the opportunity to better predict non-anatomic liver resection planes in the future. The additional information on vascular anatomy of the liver of an individual patient acquired with 3D-volumetry prior to surgery can also minimize intra-operative blood loss. 3D-volumetry can therefore open the door for virtual resection planning and computer-assisted surgery. Preoperative optimizing and visualization of the functional liver remnant incubates not only mere liver volume but also sufficient perfusion and drainage, as well as vessels at risk during an operation. These afore mentioned factors can be interpreted more accurately on 3D-volumetry data than on 2D CT/MRI data and can therefore help to find the optimal resection plane, adjust the functional remnant liver and eventually even augment resectability in patients to a substantial degree. Further validating of 3D-volumetry software seems mandatory for the future.

For liver surgery, CT-volumetry is currently the gold standard method to decide on resectability. However, in some cases liver volume is not equal to liver function, for instance in patients with steatohepatitis, steatosis or cirrhosis especially after neoadjuvant chemotherapy. When CT-volumetry is used as a prognostic tool a significant overestimation of the functional remnant liver function can be made. Therefore in the future the ideal liver volumetry method would give information on the postoperative liver remnant volume and on future remnant liver function. Recent research in the field of hepatobiliary scintigraphy has yielded interesting and promising results. The principle is based on the fact that ^{99m}Tc -mebrofenin has the same hepatic uptake, biliary excretion and transport mechanism as various exogenous and endogenous substances in the liver^{8,9}. Therefore ^{99m}Tc -mebrofenin hepatic uptake could reflect liver function. As a result of technical advances it recently became possible to combine ^{99m}Tc -mebrofenin scintigraphy with single photon emission computed tomography (SPECT). This gives the opportunity to combine the functional data from the ^{99m}Tc -mebrofenin SPECT with the anatomical information from the CT-scan, enabling 3D measurement of segmental liver function and liver functional volume. De Graaf *et al.*¹⁰ showed that combination of SPECT with the dynamic uptake data from planar hepatobiliary scintigraphy allowed accurate prediction of postoperative remnant liver function. In addition, the ^{99m}Tc -mebrofenin SPECT with low-dose CT-scan method allowed image overlays, which provided valuable visual information on liver function distribution. Although ^{99m}Tc -mebrofenin scintigraphy can be combined with imaging this functional/imaging test did not find its way into the clinic yet. It is unknown whether hepatic uptake of ^{99m}Tc -mebrofenin really reflects specific liver function because of the functional complexity of the liver. One of the problems is that ^{99m}Tc -mebrofenin can be hindered by hypoalbuminemia (which is not rare in patients with liver malignancies). The ^{99m}Tc -mebrofenin is also less applicable in patients with prolonged cholestasis (e.g. hilar cholangio carcinomas). Prolonged cholestasis produces hepatocellular injury and fibrosis which impairs the uptake of ^{99m}Tc -mebrofenin.

In line with this, there are also new developments on magnetic resonance imaging (MRI) of the liver. Nilsson *et al.*^{11,12} performed several studies in which a dynamic hepatocyte-specific contrast-enhanced MRI was studied to evaluate liver volume and function. Gd-EOB-DTPA is a contrast agent developed for MRI that is extracted by the liver for 50%. Therefore, the differential hepatic parenchymal enhancement can be measured from Gd-EOB-DTPA-enhanced liver MRI and interpreted to assess hepatic function. This method is in particular appropriate for patients with locally varying hepatic parenchymal abnormalities. However, the technique is still evolving and requires more development and validation to be standardized and accepted in routine clinical practice.

A method to assess liver function which uses an endogenous biomarker for liver function (like creatinin is for renal function) is probably more accurate and useful than using the elimination of pharmacons (exogenous substances like Gd-EOB-DTPA or ^{99m}Tc-mebrofenin). It is known that there is considerable diversity between individuals in metabolising/transporting administered pharmacons (so called high or low metabolizers). This diversity is due to different polymorphisms of the genes responsible for the metabolism or uptake of these pharmacons. Up till now there is no imaging modality that combines imaging with a quantitative liver function test of an endogenous biomarker.

During liver resection the transection plane of the liver is often altered, based on the findings of intraoperative ultrasonography (US) of the liver. Due to technical advances it is now possible to perform 3D US of the liver. These US images can also be combined with other modalities such as CT-scan images. Data on intraoperative 3D US of the liver are still scarce and attempts by our group to validate 3D US (data not shown in this thesis) have failed due to several factors including the complex manual outlining of the liver segments. However, 3D-ultrasonography remains a promising technique and if technical advances are made in the future it may allow assessment of the liver volume during the operation.

Liver function

As mentioned before, not only a reliable volumetric assessment of the liver is needed in order to identify patients with increased risk for liver failure after liver resection but also a quantitative liver function test. Unfortunately an accurate liver function test is not yet available. This is why part of this thesis focused on hepatic metabolism of glutathione (GSH). GSH is generally assumed to be the main hepatic protection system against many forms of stress, including ischemia and subsequent reperfusion and various toxic compounds¹³⁻¹⁶.

It was studied in a newly developed human proof of principle model, whether the decrease of hepatic GSH is reciprocally related to the increase of tissue and plasma ophthalmate (OPH) and can therefore be used as an alternative “read out” for hepatic GSH content. The results showed that plasma OPH was not inversely proportionally related to the expected GSH plasma level drop after repeated PCM-challenges. Both GSH and OPH levels remained unchanged in the liver after PCM-challenge. However, an increased OPH/GSH ratio gave circumstantial evidence for an increased production of

OPH. The change in OPH/GSH ratio could be a very important observation. Since OPH and GSH are synthesized through the same enzymatic machinery¹⁷, it is likely that there is competition for synthesis between OPH and GSH. An increased OPH/GSH ratio could be an earlier and more sensitive sign for OPH production than increased OPH plasma levels. However, the effect of this competition of OPH versus GSH synthesis rate should be studied in future studies.

From the results of this thesis it can be concluded that there are more pieces to the puzzle in the OPH-GSH pathway. The results of the human clinical study were in contrast with data of animal and experimental OPH studies in the past^{17,18}. There are several explanations why the data were not reproduced in this human study. The PCM-challenges in this study were probably not “powerfull” enough to induce important differences in GSH metabolism and consequently differences in OPH production were not found. The applied PCM doses in this study were equal to doses used in standard postoperative care and herewith comparable with normal clinical practice, but relatively low in comparison to levels created in mouse models and *in vitro* studies.

OPH synthesis may be strongly dependent on the bioavailability of other (unknown) intracellular substances. This has been suggested by Geenen *et al.*¹⁹ who used a kinetic mathematical model for GSH and its detoxification pathway for PCM to investigate whether OPH was a robust biomarker for GSH depletion. Since the liver has the ability to produce cysteine from methionine, the methionine concentration was varied in this model while the correlation of OPH with GSH was tested. It was shown that OPH was dependent on the methionine status. Therefore, in this mathematical model OPH alone was not a reliable biomarker for intracellular GSH depletion induced by PCM¹⁹. The same group of authors investigated OPH production in an *in vitro* hepatic cell line study while methionine and PCM concentrations were varied²⁰. Results showed elevated intracellular and extracellular concentrations of OPH following PCM treatment while methionine concentrations were low. Based on the above mentioned studies it may be concluded that OPH could be a good marker for GSH depletion but only under very specific conditions. It will be challenging to discover what these conditions are and to further explore whether OPH can be used as a biomarker of general liver function.

The PCM challenge model in its current form was not suitable to challenge the liver and show its full functional capacity. OPH plasma levels were not an appropriate read out for GSH consumption. Therefore OPH can not yet be used as a biomarker for GSH consumption and to predict liver function. Further unravelling of the OPH-GSH pathway is necessary and perhaps the validation of OPH/GSH ratio as a marker of increased GSH consumption could play an important role in this. In the future also numerous other factors which can perturb cellular GSH homeostasis should be studied. These include altered levels of expression of genes encoding enzymes of the GSH pathway, changes in metabolic pathways (methionine cycle) and environmental factors that influence GSH synthesis (for example, nutrition, drugs and amino acid uptake). The OPH-GSH pathway could also play an important role in the development of antioxidant strategies to prevent

liver cell damage due to preoperative chemotherapy and hepatic reperfusion injury during liver resection¹⁶. However these antioxidant strategies need to be further analyzed and developed.

For the future it would be worthwhile to investigate OPH and GSH levels when higher doses of PCM are used. Of course this is not possible in patients undergoing major abdominal surgery. However, higher doses of PCM have been administered safely in healthy volunteers in the past. Administration of PCM has proven to cause hepatocellular damage even in healthy adults²¹. At higher doses, the GSH metabolism is more challenged and higher OPH production can be expected.

Since it appears that increased OPH production merely occurs when GSH is completely depleted, it would be worthwhile to investigate the dynamics of OPH and GSH in patients with GSH depletion: septic patients, jaundiced patients with chronic liver failure or patients suffering from (postoperative) liver failure. In addition, the model could also be tested in patients with methionine depletion (for instance malnutrition)^{19,20}. In future studies the validity of OPH as a biomarker for GSH depletion and oxidative stress can probably be tested best in PCM intoxication patients. PCM overdose can induce hepatic GSH depletion and consequently severe hepatotoxicity, which can eventually lead to acute liver failure (ALF)¹⁴. In these patients it can be investigated whether OPH is reciprocally related to GSH, and whether OPH levels are also correlated with dosage of PCM. Quantifying the depletion and biosynthesis of GSH by measuring OPH levels could also be a very useful and innovative method to measure early liver toxicity and in titrating therapy in patients with PCM overdose.

Surgical aspects that may further affect the function of a reduced size liver: the Pringle manoeuvre

The second aim of this thesis was to evaluate the effect of surgical techniques on the future remnant liver by inducing hepatocellular damage. Hepatic inflow occlusion (Pringle manoeuvre) is often used to minimize blood loss during liver surgery as excessive intra-operative blood loss and red blood cell transfusions adversely affect short- and long-term outcomes²²⁻²⁴. Studies in the past showed advantages of 30 minutes ischemic intervals instead of 15 minutes, because in the 30 minutes ischemia group hepatocellular damage was comparable while intra-operative blood loss was lower^{25,26}. However, only crude markers of liver cell damage were used in these studies, probably not capable to detect small differences in liver cell damage. Therefore a randomised controlled trial was performed using liver fatty acid-binding protein (L-FABP), which is a more sensitive marker for liver cell damage. L-FABP is a cytosolic protein with a low molecular mass, which is involved in intracellular fatty acid transport and lipoprotein metabolism²⁷. It was shown in patients undergoing liver resection that intermittent Pringle manoeuvre (IPM) using 30 minutes ischemic intervals resulted in similar hepatocellular damage, as reflected by L-FABP, compared to IPM using 15 minutes ischemic intervals. With longer ischemic intervals a smaller transection time per area of liver transected can be achieved and

therefore it was concluded that when it is necessary to use the IPM, 30 minutes ischemic intervals are preferred for safe liver surgery. Another important conclusion was that when no IPM was used this led to significantly less hepatocellular damage. Using IPM could therefore reduce the remnant liver function and even worsen the postoperative outcome after liver resection. However up till now there is no robust evidence in the literature that this cell damage simply reflects ischaemia-reperfusion injury.

In a follow up study by our group it was investigated by measuring intestinal fatty acid binding protein (I-FABP)(marker for intestinal epithelial cell damage^{28,29}) and IgG Endotoxin Core Antibodies (Endocab) (marker for endotoxaemia) whether the IPM led to induction of intestinal damage secondary to inflow occlusion and portovenous stasis. It was shown that the use of total IPM was associated with intestinal epithelial cell damage and subsequent endotoxemia. Intestinal epithelial cell damage and endotoxemia induced by IPM could negatively affect the patient's condition and post-operative (bowel movement) recovery. This could also play a role in the pathophysiology of infective postoperative complications, however this should be studied in a larger future study.

The results of the two studies from this thesis underline that the rationale for the use of the Pringle manoeuvre should be under discussion since it can lead to more hepatocellular damage and can even cause intestinal epithelial cell damage. More importantly, there is little evidence that applying IPM during routine liver resection is useful. A recent randomised controlled trial showed that the IPM did not reduce blood loss, but was associated with raised levels of postoperative liver parenchymal enzymes and more complications³⁰. On the other hand, it is not surprising that the Pringle manoeuvre did not reduce the blood loss significantly, because due to modern effective haemostatic dissecting devices and improved technical skills, median blood loss has decreased already significantly without IPM (~2 litres to ~300 ml). An even further decrease by the application of the IPM would be unlikely and it would be of little clinical relevance.

The exact impact of IPM on routine liver resection warrants further evaluation on the issues of IPM as discussed above. Until these issues are addressed, it should not be believed that clamping represents failure and no clamping a success³¹.

Preoperative chemotherapy

In an attempt to increase resectability rates in liver surgery, a number of combined surgical and chemotherapy strategies are increasingly applied for patients with CRLM³²⁻³⁴. Neo-adjuvant chemotherapy can change the surgical options and downstage patients who initially present with non-resectable disease³⁵. Unfortunately, there is a clinical paradox, since preoperative chemotherapy may improve surgical options but chemotherapy also has considerable hepatotoxic effects³⁶⁻⁴⁰. In order to investigate the effect of chemotherapy on liver regeneration a CT-based liver volumetry study was performed to investigate early liver regeneration 7 days after right anatomical hepatectomy. The results suggested that chemotherapy prior to major liver resection did not have a negative impact

on early liver regeneration. This study showed that strategies combining preoperative chemotherapy with liver surgery are safe, based on liver regeneration in the first week. Takeda *et al.*⁴¹ showed in a comparable study that liver regeneration was also the same in normal livers, tumour bearing livers and post-chemotherapy tumour-bearing livers.

It could be that the number of chemotherapy cycles is pivotal, since in our study patients who had received more than 6 cycles of chemotherapy had significantly less early liver regeneration than patients treated with 6 or fewer cycles of chemotherapy. However, only a small subgroup in this study had more than 6 cycles of chemotherapy. Moreover, Millet *et al.*⁴² showed that liver regeneration measured one month postoperatively was not influenced by the number of cycles of chemotherapy. Future studies with larger groups of patients on the exact effect of chemotherapy and the duration of chemotherapy on liver regeneration are necessary.

The underlying etiology of hepatic toxicity for various agents remains unexplained. Further analysing the exact effect of the different types of chemotherapy on liver regeneration and also the postoperative liver remnant in human studies will be crucial for tailoring the risk assessment for these patients, because more patients are treated with preoperative chemotherapy and more extended and complex liver surgery is performed.

Body composition

As primary or secondary liver tumours often are accompanied by weight loss and cachexia, disturbances in body composition and metabolic state are suggested to be risk factors for the development of major postoperative morbidity and post-resectional liver failure⁴³. It was shown by our group that sarcopenia negatively influences total functional liver volume in patients undergoing liver surgery. The mechanism for this phenomenon is still unknown. Studies have shown that when depletion of muscle mass (i.e. sarcopenia) occurs, the risk of developing major complications after liver resection increases strongly from 8 to 22%⁴³. Sarcopenia also negatively affects disease free and overall survival⁴⁴. The preoperative hepatic physiologic reserve may be disproportionately small in sarcopenic patients compared with non-sarcopenic patients. Sarcopenia seems to play an important role and should be studied more thoroughly in the future, for instance by analysing the effect of sarcopenia on hepatocellular damage and liver function.

Over the last decades the prevalence of obesity has increased in the western world and also more patients undergoing liver surgery suffer from obesity. Although these patients are overweight, they can still suffer from sarcopenia. Sarcopenic-obesity seems to have an adverse impact on outcome after major surgery^{45,46}. It would be worthwhile to investigate in future studies the influence of body composition (sarcopenia, obesity and sarcopenic obesity) on the functional remnant volume and short- and long-term outcome after liver resection. Body composition analysis could become an important part of routine preoperative assessment.

Conclusion

Preoperative volumetric and functional assessment of the liver is the cornerstones in the pursuit of safe resectional liver surgery. CT-volumetry can be performed accurately with ImageJ and OsiriX®. However important improvements are necessary and especially 3D-volumetry is promising. Combining volumetry and assessment of the liver function in one method for example scintigraphy/SPECT or MRI are promising. GSH is the main protection system of the liver and assessing the glutathione store, by challenging the liver, should be an important part of a future dynamic liver function test. OPH or the OPH/GSH ratio could be a measure for GSH consumption, however this was not confirmed beyond reasonable doubt in the preliminary results of the proof of principle study in this thesis. It was shown that preoperative chemotherapy did not have an effect on early liver regeneration after liver resection. However, the exact effect of preoperative chemotherapy on hepatotoxicity and liver regeneration is unknown and should be studied in the future. Finally, sarcopenia seems to have a negative impact on the functional total liver volume. Body composition analysis could become an important part of routine preoperative assessment.

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Summary

Summary

Chapter 1 presents the introduction, the hypothesis and outline of this thesis. The risk of liver failure after liver resection is determined by two factors: the postoperative remnant liver volume and postoperative remnant function, and those have become the main determinants of resectability. The aim of this thesis was to analyze the different aspects of the preoperative assessment in patients undergoing liver resection with the ultimate goal to design a method to identify patients at risk of liver failure (and hence mortality) following liver resection. Both preoperative and peroperative factors that might affect the function of the remnant liver volume were investigated: preoperative metabolic reserve (body composition), preoperative chemotherapy and the intermittent Pringle manoeuvre.

Liver volumetry

In **chapter 2** data are presented that show that ImageJ can be used for CT-volumetric analysis of the liver on a personal computer. This application has the benefit that it is freely downloadable and brings CT-volumetry to the surgeon's desktop independent of the support of a radiologist.

In **chapter 3** it was shown that free downloadable software packages such as ImageJ and OsiriX[®] can be used reliably for prospective CT-volumetry of resected liver specimens. The performance of ImageJ and OsiriX[®] was comparable to that of professional radiology software iNtuition. OsiriX[®] was the most efficient and practical software package, but it can only be used on a Macintosh PC. For a Windows-based PC, ImageJ was a good alternative.

In **chapter 4** sarcopenia, defined as the depletion of muscle mass, is proven to affect short-term outcome negatively after liver resection. It was hypothesized that the preoperative hepatic physiologic reserve might be disproportionally low in sarcopenic patients. By measuring skeletal muscle areas at the level of the third lumbar vertebral landmark on a CT-scan, the L3 muscle mass index was calculated. This index is a known measure for sarcopenia, and this index was related to CT-volumetry liver data. Data of this study showed that sarcopenic patients had a disproportionally small preoperative functional total liver volume compared with non-sarcopenic patients undergoing liver resection. The L3 muscle mass index is easily obtainable and can therefore represent a valuable additional tool for preoperative risk assessment in patients undergoing partial liver resection.

One of the factors that may further affect the function of the future remnant liver is preoperative chemotherapy. There is a clinical paradox, since preoperative chemotherapy may improve surgical options but chemotherapy also has considerable hepatotoxic effects. The effect of preoperative chemotherapy on early liver regeneration is presented in **chapter 5**. Data of this study give evidence that preoperative chemotherapy does not have a negative impact on early liver regeneration after partial liver resection. This study

shows that strategies combining preoperative chemotherapy with liver surgery are safe, based on liver regeneration in the first week.

Surgical aspects that may further affect the function of a reduced size liver

The second part of this thesis handles aspects of the surgical procedure that affect the function of a reduced liver: the intermittent Pringle manoeuvre and inflammation. **Chapter 6** elaborates on the inflammatory response after liver resection which is evidenced by increased systemic levels of pro- and anti-inflammatory cytokines. Interleukin-6 (IL-6) is one of the archetypical pro-inflammatory cytokines, which contributes to the acute phase response to cell injury and infection. IL-6 plays also an important role in the liver regeneration. It was hypothesized in the literature that hepatosplanchnic organs (comprising mainly the portal drained viscera and the liver) are responsible for uptake of endogenously produced IL-6. In this chapter data show that the gut releases IL-6 and that the liver takes up IL-6 in humans before and after liver resection. The liver might take up IL-6 in order to regulate IL-6 levels and prevent excessively elevated systemic IL-6 levels. Equally this uptake may play a role in liver regeneration.

In an attempt to reduce or eliminate operative bleeding during transection of the liver, the Pringle manoeuvre can be applied (liver vascular clamping). The influence of the duration of ischemia during intermittent Pringle manoeuvre (IPM) on hepatocellular damage was investigated in **chapter 7**. Complete IPM using 30 minutes ischemic intervals resulted in similar hepatocellular damage, reflected by the highly sensitive damage marker L-FABP, compared with complete IPM using 15 minutes ischemic intervals. If inflow occlusion during liver transection is necessary, intermittent pedicle clamping using 30 minutes ischemic intervals may be used.

Finally, it was investigated whether the IPM leads to injury to the gut in **Chapter 8**. The use of the total IPM causes intestinal epithelial cell damage and endotoxemia during liver surgery in man. IPM can therefore potentially increase the risks of liver resections despite a possible reduction of intraoperative blood loss. Intestinal epithelial cell damage and endotoxemia induced by IPM could negatively affect the patient's condition and post-operative recovery. Whether gut damage as a consequence of the total-IPM is causally related to systemic inflammation remains to be established.

Liver function

The final part of this thesis elaborates on the role of ophthalmate (OPH) and glutathione (GSH) during liver surgery. **Chapter 9** presents a systematic review that was undertaken to give an overview on currently available literature on OPH. In this review it was concluded that OPH may be a promising marker to assess oxidative stress and hepatic GSH metabolism. This systematic review identified strengths and limitations of the assessment of GSH depletion with the use of OPH.

In **chapter 10** it was shown how a LC–MS/MS protocol was developed which enabled the measurement of OPH in plasma of healthy volunteers as well as in patients with colorectal liver metastases undergoing liver resection. This LC–MS/MS assay and the developed prestorage treatment protocol made it possible to validate OPH as a biomarker for hepatic GSH depletion in a future human proof of principle study.

Chapter 11 reports on the data of the first human study in which the usefulness of OPH as a read out for hepatic GSH metabolism was tested. The results show that plasma OPH was not inversely proportionally related to the expected GSH plasma level drop after paracetamol(PCM)-challenge. Both GSH and OPH levels remained unchanged in the liver after PCM-challenge. However an increased OPH/GSH ratio gave circumstantial evidence for an increased production of OPH which was not evidenced by increased OPH plasma levels. Future studies are needed in order to validate plasma OPH as a biomarker for hepatic GSH depletion.

Chapter 12 provides the general discussion with conclusions and recommendations following from this thesis.

Samenvatting

Samenvatting

In **hoofdstuk 1** worden de introductie, de hypothese en de hoofdlijnen van de thesis gepresenteerd. Het risico op leverfalen na leverresectie wordt bepaald door twee factoren: het volume van de postoperatieve rest lever en de postoperatieve rest leverfunctie. Deze twee factoren zijn de belangrijkste determinanten van de resectabiliteit. Het doel van de thesis is om verschillende aspecten van de preoperatieve screening te analyseren in patiënten die een leverresectie ondergaan, met het ultieme doel om uiteindelijk patiënten te detecteren die een verhoogd risico hebben op leverfalen (en dus ook mortaliteit) na een leverresectie. Zowel preoperatieve als peroperatieve factoren die invloed kunnen hebben op de toekomstige rest leverfunctie zijn onderzocht, zoals de preoperatieve metabole reserve (lichaamssamenstelling), het effect van preoperatieve chemotherapie en de intermitterende Pringle manoeuvre.

Lever volumetrie

In **hoofdstuk 2** worden data gepresenteerd die laten zien dat ImageJ gebruikt kan worden voor CT-volumetrie analyse van de lever op een personal computer. Voordeel van deze applicatie is dat het CT-volumetrie naar de desktop van de chirurg heeft gebracht, onafhankelijk van de ondersteuning van een radioloog en dat het bovendien gratis te downloaden is.

In **hoofdstuk 3** wordt aangetoond dat de gratis te downloaden software pakketten ImageJ en Osirix[®] betrouwbaar zijn om prospectief CT-volumetrie uit te voeren van het gedeelte van de lever dat verwijderd zal worden. De prestaties van ImageJ en Osirix[®] waren vergelijkbaar met het professioneel radiologisch software programma iNtution. Osirix[®] was het meest efficiënte en praktische software pakket, maar kan alleen worden gebruikt op een Macintosh personal computer. Voor een computer met Windows is ImageJ een goed alternatief.

In **hoofdstuk 4** wordt aangetoond dat sarcopenie, wat wordt gedefinieerd als het verlies van spiermassa, een negatief effect heeft op de korte termijn uitkomst na leverresectie. De hypothese werd gesteld dat de preoperatieve hepatische fysiologische reserve disproportioneel lager is in sarcopene patiënten. De L3 muscle mass index werd berekend door het meten van de oppervlakte van skeletspieren op het niveau van de derde lumbale ruggenwervel op een CT-scan. Dit is een bekende index voor sarcopenie en deze index werd gerelateerd aan de CT-volumetrie lever data. Data van deze studie laten zien dat sarcopene patiënten een disproportioneel kleiner preoperatief functioneel totaal levervolume hadden, vergeleken met niet-sarcopene patiënten die een leverresectie ondergingen. De L3 muscle mass index kan makkelijk worden gemeten en kan daarom een waardevol additioneel middel zijn in de preoperatieve risico screening voor patiënten die een partiële leverresectie ondergaan.

Een van de factoren die de toekomstige restleverfunctie kan beïnvloeden, is preoperatieve chemotherapie. Er is helaas een klinische paradox omdat preoperatieve

chemotherapie aan de ene kant de chirurgische opties kan verbeteren maar aan de andere kant ook aanzienlijke hepatotoxische effecten heeft. De effecten van preoperatieve chemotherapie op vroege leverregeneratie worden gepresenteerd in **hoofdstuk 5**. De data van deze studie bewijzen dat preoperatieve chemotherapie geen negatieve impact heeft op vroege leverregeneratie na partiële leverresectie. De studie laat zien dat strategieën die preoperatieve leverchemotherapie combineren met leverchirurgie veilig zijn, gebaseerd op de leverregeneratie in de eerste week.

Chirurgische aspecten die effect hebben op de restleverfunctie

Het tweede gedeelte van de thesis heeft betrekking op aspecten van de chirurgische procedure die effect hebben op de functie van de restlever: ontsteking en de intermitterende Pringle manoeuvre (IPM). **Hoofdstuk 6** gaat over de inflammatoire respons na leverresectie die gepaard gaat met verhoogde systemische levels van zowel pro- als anti-inflammatoire cytokinen. Interleukine-6 (IL-6) is één van de meest belangrijke pro-inflammatoire cytokinen en draagt bij aan de acute fase respons op celschade en infectie. IL-6 speelt ook een belangrijke rol in de leverregeneratie. In de literatuur is de hypothese gesteld dat de hepatosplanchnische organen (organen die hun afvoer hebben via de venaportae en de lever) verantwoordelijk zijn voor de opname van endogeen geproduceerd IL-6. In dit hoofdstuk laten data zien dat in de mens de darm IL-6 afgeeft en dat de lever IL-6 opneemt voor en na leverresectie. Het zou het kunnen zijn dat de lever IL-6 opneemt om hoge systemische IL-6 levels te voorkomen en te reguleren. Tegelijkertijd zou deze opname ook een hele belangrijke rol kunnen spelen in leverregeneratie na resectie.

In een poging om een operatieve bloeding te voorkomen of te stoppen tijdens de transsectie van de lever kan de Pringle manoeuvre worden toegepast (afklemmen van de lever vasculatuur aan inflow zijde). In **hoofdstuk 7** wordt de invloed van de duur van ischemie gedurende de IPM op hepatocellulaire schade onderzocht. Complete IPM waarbij dertig minuten continue ischemie werd gebruikt, resulteerde in hepatocellulaire schade, gereflecteerd door de zeer sensitieve marker L-FABP, vergelijkbaar met de hepatocellulaire schade bij complete IPM waarin vijftien minuten ischemie intervallen werden gebruikt. Als het afklemmen van de vasculatuur nodig is tijdens de levertranssectie, kan de porta pedikel het beste met intervallen van dertig minuten intermitterend worden afgeklemd.

Tot slot wordt in **hoofdstuk 8** onderzocht of de IPM tot darmschade leidt. Het gebruik van de totale IPM veroorzaakt intestinale epitheliale celschade en endotoxemie tijdens leverchirurgie in de mens. IPM kan daarom potentieel het risico verhogen van leverresecties ondanks een mogelijke reductie van het inter-operatieve bloedverlies. Intestinale epitheliale celschade en endotoxemie geïnduceerd door IPM kan een negatief effect hebben op de conditie van de patiënt en het post-operatieve herstel. Of darmschade als een consequentie van de totale IPM causaal gerelateerd is aan systemische inflammatie moet nog worden onderzocht.

Leverfunctie

Het laatste gedeelte van deze thesis gaat over de rol van ophthalmaat (OPH) en glutathion (GSH) tijdens leverchirurgie. **Hoofdstuk 9** is een systematische review van de huidige literatuur over OPH. In dit review wordt geconcludeerd dat OPH een veelbelovende marker is om oxidatieve stress en hepatische GSH metabolisme te bepalen. Het review identificeert sterke punten en beperkingen van de bepaling van GSH depletie met behulp van OPH.

In **hoofdstuk 10** wordt de ontwikkeling van een LC-MS/MS protocol gepresenteerd waarmee OPH gemeten kan worden in plasma van zowel gezonde vrijwilligers als van patiënten met colorectale levermetastasen die een leverresectie ondergingen. Deze LC-MS/MS techniek en het ontwikkelde pre-opslag protocol maakten het mogelijk om OPH als biomarker voor hepatische GSH depletie in een toekomstig humane proof of principal studie te valideren.

Hoofdstuk 11 laat de data zien van de eerste humane studie waarin de bruikbaarheid van OPH als marker voor hepatische GSH metabolisme wordt getest. Uit de resultaten blijkt dat plasma OPH niet omgekeerd proportioneel gerelateerd is aan de verwachte GSH plasma level daling na een paracetamol (PCM) challenge. Zowel GSH als OPH levels blijven onveranderd in de lever na een PCM challenge. Daarentegen werd een verhoogde OPH/GSH ratio gezien hetgeen mogelijk een bewijs is voor een toegenomen OPH productie die zich niet uitte in verhoogde OPH plasma levels. Toekomstige studies zijn nodig om plasma OPH als biomarker voor hepatische GSH depletie te valideren. **Hoofdstuk 12** bevat de algemene discussie met de conclusies en aanbevelingen die uit deze thesis volgen.

Valorization

Valorization

This chapter is intended to take a brief look upon the return of investment prospects for society from the knowledge gathered during this PhD-trajectory. Every (medical) researcher knows that one of the crucial parts of developing a new technique or treatment is validating experimental data in a human translational model or in the clinical setting. However, when a certain technique or treatment is validated properly this should not be the end of the story. It is important that researchers challenge themselves to translate the gained academic wisdom to societal or economic benefit. This is particularly the case because often taxpayers, charities or foundations have funded the research. The possibilities for future implementation and also the economic and societal value of the findings of this thesis will be discussed.

Liver volumetry

The results from this thesis have contributed to the implementation of liver volumetry in the preoperative assessment of patients undergoing liver surgery. In the Netherlands every year more than 1000 liver resections are performed. Unfortunately, liver volumetric analysis is currently not standard in the preoperative assessment in patients undergoing major liver resection. Collaboration with the (liver) radiologist is key and will become increasingly more important in the future. Radiology will continue to play a central component in the surgical treatment of malignant/benign liver tumours. Radiologists and HPB surgeons should work together in the form of an interdisciplinary interface. At the present time, radiology is faced with the challenge of visualizing more discrete aspects of the liver tumour than size and location, such as tumour necrosis and altered tumour perfusion. In MRI scans newly developed contrast agents are used that are extracted by the liver and which give the opportunity to assess liver function. These developments in radiology will contribute actively to a better understanding of the genesis of malignant disease of the liver and to the improvement of existing surgical therapy regimes. It is all about identifying patients at increased risk of postoperative liver failure after liver resection. Assessing the future remnant liver volume is critical in this context. It was shown by our group that sarcopenia negatively influences total functional liver volume in patients undergoing liver surgery. Sarcopenia could therefore be one of the decisive factors in the risk analysis for postoperative liver failure. This should be studied more thoroughly in the future. For instance it could be studied whether sarcopenia has an effect on hepatocellular damage and liver function. If patients with sarcopenia are identified preoperatively, this gives the chance to adapt the intended operation. In these frail patients with sarcopenia it would for example be possible to leave more residual liver volume, or increase preoperatively the functional liver volume by portal vein embolisation or perform a two-stage hepatectomy. Perhaps treating malnutrition preoperatively in sarcopenic patients could also reduce the risks of liver surgery. If patients with sarcopenia are treated better and have a more “tailor made” approach, this will eventually lead to

less complications, shortening in length of hospital stay and better survival and therefore this may result in considerable cost-reduction. Collaboration and continuing to improve the aspects of preoperative assessment in patients undergoing liver resection will improve the quality of surgical care.

It is important that in the future every HPB-surgeon should have access to a Picture Archiving and Communication System (PACS) workstation and it would be valuable if HPB surgeons were able to use OsiriX[®] software. The validation of OsiriX[®] in this thesis made it possible to accelerate the development of liver volumetry in general on a personal computer but in the future this may perhaps also be applicable on mobile radiological wireless communication devices. Currently, not only in every day life but also in science the tablet computer and the smartphone are replacing the personal desktop computer step by step. The development of applications for mobile devices has exploded the last couple of years and the possibilities seem to be endless. It is already possible to run OsiriX[®] on a tablet computer or even on a smartphone. Developments like this can help to better visualize and apprehend the anatomy of the liver pre- and intraoperatively. It can also be useful for training, teaching and performing safer surgical procedures.

There are still shortcomings in the currently freely downloadable volumetry software programs. In order to improve these programs we believe that medical scientists should work together with technical engineers. Medical doctors know best what the clinical problems are and engineers are best at finding the technical answer for these problems. Entrepreneurship will also be important for further implementation of liver volumetry. However, commercial software programmes do not often find their way into the clinic because they are expensive. High costs remain a problem because of reduced budgets in hospitals in the Netherlands as well as elsewhere in Europe. In the near future this will be an even bigger problem because of new cost-cutting political programs in healthcare. Another problem is that with the current infrastructure in science and business we have learned from the past that it will take at least six to seven years to distribute a new product (developed in a research project) on the market. This process can be enhanced and shortened through open innovation. Medical doctors, technicians and entrepreneurs should work together without barriers and such collaboration should be facilitated and promoted. Medical products like for instance liver volumetry software programmes should not be subject to copyright protection and should be in the public domain. ImageJ is a good example of a product like this. Its user community can, once the programme is in the public domain, further develop the software and solve any problems they encounter while using the software. When liver volumetry is further implemented in the workup of patients undergoing liver surgery, more patients can be operated and more complex liver resections can be performed safely. From a societal/economic point of view this is very important because in the Western world every year there are more and more patients with colorectal liver metastases in need of liver resection.

Ischemia-reperfusion injury

Ischemia-reperfusion injury is a pathophysiological phenomenon in a variety of diseases. Ischemia-reperfusion injury of the liver occurs frequently during liver resection when blood flow is occluded temporarily during liver resection to reduce blood loss [the so-called intermittent Pringle manoeuvre (IPM)]. To find new targets for therapy and characterize pathophysiological events during ischemia of the liver, the effect of the IPM was investigated. The results of the two studies on the IPM reported in this thesis underline that the rationale for the use of the IPM should be under discussion since it can lead to more hepatocellular damage and can even cause intestinal epithelial cell damage. More importantly, there is little evidence that applying IPM during routine liver resection is useful. The exact impact of IPM on routine liver resection warrants further evaluation. Some of the issues revealed in the studies in this thesis will help to understand the potential harmful effects of the IPM better. As a consequence it will be easier to make the right choice when to apply the IPM during liver surgery and when not. Optimizing surgical techniques like the IPM will eventually not only improve short- and long-term patients' outcome but also improve cost effectiveness. Optimizing these techniques will for example reduce costs of theatre time, hospital stay, and intensive care unit usage. Such cost-reduction is of great economical relevance in times of cut-backs on healthcare. Funding for research that focuses on the improvement of liver surgery techniques could translate into considerable savings to hospitals and health care programmes and this is therefore important for society.

Paracetamol and ophthalmate

The data of the thesis considering the relation between paracetamol (PCM), ophthalmate (OPH) and glutathione (GSH) have initiated a line of thought towards the development of a PCM tolerance test. This test would imply administering PCM and using OPH as an alternative readout for hepatic GSH to help predict liver failure post liver resection. A patent possibility was explored, but it was felt to be non-patentable by the University of Maastricht patentability agency. From a business point of view this test would be interesting since PCM is not an expensive drug and every year there will be more and more patients who could benefit from such a test. A PCM-test would be very useful for patients undergoing liver resection for benign or malignant tumours. These may be patients with an otherwise normal liver, or patients with underlying cirrhosis of the liver. Equally, it may be a helpful tool to test liver function in patients subjected to chemotherapy for cancer, because the chemotherapy may have serious adverse effects on the liver. Along the same lines, it may be a valuable test in patients undergoing liver transplantation, particularly for those involved in a living-related liver donation programme. Finally, it may be effective in titrating therapy in patients with PCM overdose, either accidentally or intentionally.

From a valorisation point of view this test was considered of interest since PCM is already FDA (Food and Drug Administration) approved and PCM is often used in standard care medicine. Therefore clinical trials would not be delayed by phase 1 and 2 studies and a potential PCM-test could be brought on the market faster compared to completely new substances. The described study in this thesis in which the biomarker OPH was tested after a PCM-challenge during liver surgery was innovative. In this study no inversely proportional increase in OPH was observed, related to the GSH drop after PCM-challenge. The data and set-up of this study are likely to be useful for future studies and experiments to eventually develop a PCM-test, which can demonstrate liver function capacity. We developed a method for pre-storage treatment of OPH samples and proposed also a LC-MS-MS method for accurately measuring OPH levels in human samples. This knowledge opens the door for new studies on the GSH-OPH pathway and creates opportunities. The developments described in this thesis can be important to perform liver surgery more safely in the future and will have a positive impact, in the first place for patients undergoing liver surgery, but also on society and on economy.

Scientific output

Scientific output

List of publications

1. Lodewick TM, van Nijnatten TJ, van Dam RM, van Mierlo K, **Dello SA**, Neumann UP, Olde Damink SW, Dejong CH. Are sarcopenia, obesity and sarcopenic obesity predictive of outcome in patients with colorectal liver metastases? *HPB (Oxford)*. 2014 Dec; *Epub ahead of print*.
2. **Dello SA**, Kele PG, Porte RJ, van Dam RM, Klaase JM, Verhoef C, van Gulik T, Molenaar Q, Bosscha K, van der Jagt EJ, Dejong CH, de Boer MT. Influence of preoperative chemotherapy on ct volumetric liver regeneration following right hemihepatectomy. *World J Surg*. 2014 Feb; 38(2): 497-504.
3. van den Broek MA, Vreuls CP, Winstanley A, Jansen RL, van Bijnen AA, **Dello SA**, Bemelmans MH, Dejong CH, Driessen A, Olde Damink SW. Hyaluronic acid as a marker of hepatic sinusoidal obstruction syndrome secondary to oxaliplatin-based chemotherapy in patients with colorectal liver metastases. *Ann Surg Oncol*. 2013 May;20(5): 1462-9.
4. **Dello SA**, Neis EP, de Jong MC, van Eijk HM, Kicken CH, Olde Damink SW, Dejong CH. Systematic review of ophthalmate as a novel biomarker of hepatic glutathione depletion. *Clin Nutr*. 2013 Jun; 32(3): 325-30.
5. **Dello SA**, Lodewick TM, van Dam RM, Reisinger KW, van den Broek MA, von Meyenfeldt MF, Bemelmans MH, Olde Damink SW, Dejong CH. Sarcopenia negatively affects preoperative total functional liver volume in patients undergoing liver resection. *HPB (Oxford)*. 2013 Mar;15 (3): 165-9.
6. **Dello SA**, van Eijk HM, Neis EP, de Jong MC, Olde Damink SW, Dejong CH. Ophthalmate detection in human plasma with LC-MS-MS. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2012 Aug 15; 903:1-6.
7. **Dello SA**, Reisinger KW, van Dam RM, Bemelmans MH, van Kuppevelt TH, van den Broek MA, Olde Damink SW, Poeze M, Buurman WA, Dejong CH. Total intermittent Pringle maneuver during liver resection can induce intestinal epithelial cell damage and endotoxemia. *PLoS One*. 2012; 7(1):e30539.
8. **Dello SA**, Bloemen JG, van de Poll MC, van Dam RM, Stoot JH, van den Broek MA, Buurman WA, Bemelmans MH, Olde Damink SW, Dejong CH. Gut and liver handling of interleukin-6 during liver resection in man. *HPB (Oxford)*. 2011 May; 13(5): 324-31.
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10. **Dello SA**, Stoot JH, van Stiphout RS, Bloemen JG, Wigmore SJ, Dejong CH, van Dam RM. Prospective volumetric assessment of the liver on a personal computer by nonradiologists prior to partial hepatectomy. *World J Surg*. 2011 Feb;35(2):386-92.

11. **Dello SA**, van Dam RM, Slangen JJ, van de Poll MC, Bemelmans MH, Greve JW, Beets-Tan RG, Wigmore SJ, Dejong CH. Liver volumetry plug and play: do it yourself with ImageJ. *World J Surg*. 2007 Nov; 31(11): 2215-21.

Submitted manuscripts

12. **Dello SA**, van Mierlo KM, de Jong MC, van Eijk HM, de Kok TM, Briedé JJ, Olde Damink SW, Dejong CH. Ophthalmate as a read out for hepatic glutathione metabolism in humans in vivo. *Submitted*.

List of publications not related to the thesis

13. **Dello SA**, Alings M. Reply to letter from J. Finsterer and S. Zarrouk-Mahjoub. *Neth Heart J*. 2014 Jan 8.
14. **Dello SA**, Kievit C, Dunselman PH, Alings M. Loss of consciousness and convulsion induced by a ventricular tachycardia mimicking epilepsy in a patient with noncompaction cardiomyopathy: a case report. *Neth Heart J*. 2013 Jul 5.
15. Schellekens DH, Grootjans J, **Dello SA**, van Bijnen AA, van Dam RM, Dejong CH, Derikx JP, Buurman WA. Plasma intestinal fatty acid-binding protein levels correlate with morphologic epithelial intestinal damage in a human translational ischemia-reperfusion model. *J Clin Gastroenterol*. 2014 Mar;48(3):253-60.

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Dankwoord

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Curriculum Vitae

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Simon Alphons Wilhelmus Gerardus Dello was born in Geleen, the Netherlands, on December 4th 1984. After finishing gymnasium at the Stella Maris College in Meerssen in 2003, he attended Medical School at the University of Maastricht. In August 2009 he obtained his MD degree.

During his clerkships which were part of the Onderzoeks-Traject Geneeskunde (OTG) in 2006 he started his research eventually leading to his first publication as a fourth year medical student in 2007. This research led to the start of his PhD program in September 2009 supervised by Prof. dr. C.H.C. Dejong at the department of Surgery. On the basis of the research he conducted as a student, he managed to obtain a Kootstra Talent fellowship that enabled him to do the work in this PhD.

In March 2010 he continued his work at the department of Surgery in Maastricht, since he obtained funding from Maastricht University. The topic of his research was the preoperative assessment of patients undergoing a liver resection. The first part of his thesis was about CT-volumetry of the liver and the second part of the thesis focused on developing a new quantitative liver function biomarker called ophthalmate. The results of the work in this thesis were presented in public at several national and international conferences. In November 2010 he received funding from the Nederlandse Vereniging Gastro-enterologie (Gastrostart) to support the work on this new liver function biomarker ophthalmate.

In April 2012 the work for his thesis was finished and he started working as a resident - not in training- at the department of Cardiology, Amphia hospital Breda. In March 2013 he started as a resident at the department of Cardiology of the Catharina Hospital in Eindhoven and from April 2014 he is in training to become a cardiologist (under the supervision of dr. J.M. van Dantzig and Prof. dr. N.H.J. Pijls). He happily lives together with Carlijn Simons in Breda.