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Title of the joint PhD thesis:
Pharmacological advances in liver transplantation and resection

Subtitle:
Evidences on the role of anti-T-cell immunoglobulins and roxadustat in liver surgery

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Foreword

This work is a tribute to the study of the liver. This cryptic organ, in Mediterranean cultures, catalyses anger and love, and designates no less than life, in Germanic languages.
The Promethean cycles of liver decay and regeneration passionately lure intellectuals, from the times of the Etruscan haruspices to contemporary scientists.

To my love who blesses my life, here and beyond,
To my mom who struggled to reach Heaven,
To my dad who is my father twice.

Summary

Abstract

This joint PhD thesis has been carried out through clinical works, and, then, an experimental research, with the overall purpose of exploring the way pharmaceutics might contribute to liver surgery.

The first part consisted in a prospective randomised controlled phase-three trial in adult liver transplantation to assess whether a depleting-antibody induction is superior to placebo in obtaining stable liver function without rejection on maintenance tacrolimus monotherapy. Depleting antibodies could streamline the minimisation of maintenance immunosuppression and the associated cohort of chronic side effects but the role of this substantial induction strategy is still inconclusive. We then compared a single, intraoperative, high-dose of polyclonal rabbit anti-human-T-lymphocyte antibodies followed by tacrolimus-based maintenance therapy with a control group who received only a tacrolimus-based maintenance. The protocol notably included Banff-scored liver biopsies on postoperative day seven. The primary endpoint consisted of immunosuppression minimisation to monotherapy. Secondary endpoints were biopsy-proven rejection, clinical rejection, and patient and graft survival. The primary endpoint was not met. Despite significantly fewer day-seven moderate-to-severe T-cell-mediated acute rejections in the study group, the cumulative proportion of treatment-requiring rejection episodes was comparable. At any rate, the study group exhibited more life-threatening complications perioperatively. We surmised that an induction based on depleting antibodies does not significantly affect the immunosuppressive load within the first year, or the incidence of treatment-requiring rejection, despite an effective mitigation of histological day-seven rejection. Five-year results are awaited to assess the effects on tolerance.

Liver transplantation has a revolutionary potential and that its indications are considerably expanding. While organ shortage commonly dooms patients on waiting list, novel indications exacerbate the gap between supply and demand of

deceased-donor livers. In this context, there is hardly an alternative to living-donor liver transplantation. However, the left-to-right liver segmentation imposes a respective volume distribution of one third-two thirds. In this regard, a left-lobe graft is often inadequate to ensure the hepatostat in an adult recipient, whereas the procurement of a right lobe would jeopardise the donor's life. Ultimately, the handling of hepatic remnants that are too small for the physiological requirements remains a challenge. The ensuing small-for-size syndrome is a complex process resulting primarily from ischemia–reperfusion injury and portal hyperflow associated with size mismatch between graft and recipient. In the early period following liver resection or liver transplantation, molecular events related to apoptosis, necrosis, proliferation and regeneration appear in specific patterns. The recent amount of evidence pinpointing hypoxia as a crucial field player for the success of liver resection in the early postoperative phase informed the last part of this thesis. Post-hepatectomy hypoxia-driven pathways induce the formation of new vessels and reduce the shift between hepatocellular and vascular proliferation by slowing the former and furthering the latter. While hypoxia cannot be translated as such into clinical practice, a hypoxia-induced signature can still be elicited by stabilising its downstream transducers, the hypoxia-inducible factors. Roxadustat, a small molecule that belongs to the isoquinolines family, selectively inhibits the degradation of these transcription factors. In a rat model of standard 70% hepatectomy, we assessed the effect of roxadustat on the evolution of histological changes and liver function, by means of cutting-edge magnetic-resonance-based functional imaging. Despite the absence of an overt improvement in liver function or regeneration, the drug alleviated hepatocellular steatosis and ballooning, which are known markers of cellular suffering after liver resection. RNA sequencing confirmed a surprising increase in lipid breakdown and cellular respiration. It is thus worth exploring these enthralling metabolic effects in models of more extended liver resection and fatty liver disease.

Résumé

Cette thèse de doctorat en cotutelle a été conduite en développant, d'une part, des travaux cliniques et, d'autre part, une recherche expérimentale, avec pour objectif général d'explorer la manière dans laquelle des composés pharmaceutiques pourraient offrir une contribution à la chirurgie du foie.

En premier, nous avons mené un essai prospectif randomisé contrôlé de phase trois en transplantation de foie adulte pour évaluer si une induction à base d'anticorps déplétants était supérieure au placebo pour obtenir une fonction hépatique stable sans rejet en monothérapie de maintien au tacrolimus. Les anticorps déplétants pourraient faciliter la minimisation de l'immunosuppression de maintien et de la cohorte d'effets secondaires chroniques associés, mais le rôle de cette substantielle induction n'est toujours pas concluant. De ce fait, nous avons comparé une forte dose d'anticorps polyclonaux de lapin anti-lymphocytes T humains, unique et peropératoire, suivie d'une thérapie de maintien à base de tacrolimus, avec un groupe de contrôle qui ne recevait que le traitement de maintien à base de tacrolimus. Le protocole prévoyait notamment des biopsies hépatiques classifiées selon le score de Banff au septième jour postopératoire. Le principal critère d'évaluation consistait en l'obtention d'une immunosuppression minimisée à base de tacrolimus administré en monothérapie. Les critères d'évaluation secondaires étaient le rejet confirmé par biopsie, le rejet clinique, et la survie du patient et du greffon. Le critère d'évaluation primaire n'a pas été atteint. Le nombre de rejets aigus cellulaires de modérés à sévères au septième jour postopératoire était significativement inférieur dans le groupe d'étude. Cependant la proportion cumulée d'épisodes de rejet nécessitant un traitement était comparable. Quoi qu'il en soit, le groupe d'étude a présenté davantage de dangereuses complications péri-opératoires. Nous avons conclu qu'une induction à base d'anticorps déplétants n'affecte de manière significative ni la charge immunosuppressive au cours de la

première année ni l'incidence des rejets nécessitant un traitement, et ce malgré une atténuation efficace du rejet histologique au septième jour. On attend tout de même les résultats à cinq ans pour évaluer les effets sur la tolérance.

La transplantation du foie a un potentiel révolutionnaire et que ses indications sont en cours de croissance considérable. Alors que la pénurie d'organes grève généralement les patients sur liste d'attente, les nouvelles indications augmentent l'écart entre l'offre et la demande de foies de donneurs décédés. Dans ce contexte, il n'existe guère d'alternative à la transplantation de foie de donneur vivant. Cependant, la segmentation du foie de gauche à droite impose une distribution respective de volume d'un tiers-deux tiers. À cet égard, un lobe gauche est souvent insuffisant pour assurer l'hépatostat chez un receveur adulte, alors que le prélèvement d'un lobe droit pourrait mettre en danger la vie du donneur. En fin de compte, la gestion des foies trop petits pour les besoins physiologiques du patient reste un défi. Le syndrome subséquent du foie trop petit pour la taille du patient, connu en tant que « small-for-size syndrome », est un processus complexe résultant de la lésion d'ischémie-reperfusion et de l'hyperdébit portal associés à la différence de tailles entre le greffon et le receveur. Dans la première période postopératoire qui suit la résection ou la transplantation hépatique, les événements moléculaires liés à l'apoptose, à la nécrose, à la prolifération et à la régénération apparaissent selon des schémas spécifiques. La dernière partie de cette thèse s'appuie sur les évidences récentes qui montrent que l'hypoxie est un facteur crucial pour le succès de la résection hépatique en postopératoire précoce. La réponse à l'hypoxie post-hépatectomie induit la formation de nouveaux vaisseaux et réduit le déséquilibre entre la prolifération hépatocellulaire et vasculaire en ralentissant la première et en favorisant la seconde. Bien que l'hypoxie ne puisse pas être traduite en tant que telle dans la pratique clinique, une signature génique induite par l'hypoxie peut toujours être obtenue en stabilisant ses transducteurs en aval, c'est-à-dire les facteurs induits par l'hypoxie. Le roxadustat, une petite molécule qui appartient à la famille des isoquinoléines, inhibe sélectivement la dégradation de ces facteurs de

transcription. Dans un modèle standard d'hépatotomie de 70% chez le rat, nous avons évalué l'effet du roxadustat sur l'évolution des changements histologiques et de la fonction hépatique au moyen d'une imagerie fonctionnelle de pointe basée sur la résonance magnétique. Malgré l'absence d'amélioration manifeste de la fonction ou de la régénération hépatique, le médicament a atténué la stéatose hépatocellulaire et le ballonnement, marqueurs connus de souffrance cellulaire après une résection hépatique. Le séquençage de l'ARN a confirmé une augmentation surprenante de la dégradation des lipides et de la respiration cellulaire. Il est donc intéressant d'explorer ces captivants effets métaboliques dans des modèles de résection hépatique plus étendue et dans des cadres d'infiltration graisseuse du foie.

Sintesi

Questa tesi di dottorato in cotutela è stata condotta attraverso una fase di ricerca clinica e una di laboratorio, con lo scopo generale di esplorare gli apporti della farmacologia alla chirurgia epatica.

Abbiamo, in primo luogo, condotto uno studio prospettico randomizzato controllato di fase tre nel trapianto di fegato adulto per valutare se un'induzione a base di anticorpi depletivi sia superiore al placebo nell'ottenere una funzione epatica stabile senza rigetto in monoterapia di mantenimento con tacrolimus. L'uso di anticorpi depletivi potrebbe semplificare la minimizzazione dell'immunosoppressione di mantenimento e, così, ridurre la coorte associata di effetti collaterali cronici, ma il ruolo di questa strategia d'induzione massiccia non è ancora chiaro. Abbiamo, quindi, confrontato una dose singola, intraoperatoria, elevata di anticorpi policlonali di coniglio anti-linfociti T umani, seguita da una terapia di mantenimento a base di tacrolimus, con un gruppo di controllo che ha ricevuto solo un mantenimento a base di tacrolimus. Il protocollo includeva, in particolare, delle biopsie epatiche sistematiche, in settima giornata postoperatoria, classificate secondo lo score di Banff. L'endpoint primario consisteva nel raggiungimento della monoterapia. Gli endpoint secondari erano il rigetto istologico, il rigetto clinico e la sopravvivenza del paziente e dell'organo. L'endpoint primario non è stato raggiunto. Nonostante un numero significativamente inferiore di rigetti cellulari acuti moderati-gravi in settima giornata nel gruppo di studio, la proporzione cumulativa di episodi di rigetto che hanno richiesto il trattamento è risultata comparabile. In ogni caso, il gruppo di studio ha mostrato più complicazioni perioperatorie severe. Abbiamo ipotizzato che un'induzione basata su anticorpi depletivi non influisca significativamente sul carico immunosoppressivo entro il primo anno, o sull'incidenza del rigetto clinicamente rilevante, malgrado un'efficace attenuazione del rigetto istologico in

settima giornata. Si attendono i risultati a cinque anni per valutare gli effetti sulla tolleranza.

Il trapianto di fegato ha un potenziale rivoluzionario e le sue indicazioni si stanno espandendo notevolmente. Mentre la carenza d'organi grava sui pazienti in lista d'attesa, le nuove indicazioni accrescono il divario tra l'offerta e la domanda di fegati da donatore deceduto. In questo contesto, l'unica strategia alternativa appare il trapianto di fegato da donatore vivente. Tuttavia, la segmentazione del fegato da sinistra a destra comporta una rispettiva distribuzione volumetrica di un terzo-due terzi. A questo proposito, un lobo sinistro è spesso inadeguato a garantire l'epatostato in un ricevente adulto, mentre il prelievo di un lobo destro metterebbe in pericolo la vita del donatore. Rimane, in definitiva, una sfida la gestione dei pazienti con residuo epatico di dimensioni troppo ridotte rispetto alle esigenze fisiologiche. La conseguente sindrome da "fegato troppo piccolo", nota come "small-for-size syndrome", è un processo complesso che deriva principalmente dal danno d'ischemia-riperfusione e dall'iperafflusso portale associato alla differenza di dimensioni tra organo e ricevente. Nel primo periodo successivo alla resezione o al trapianto di fegato, gli eventi molecolari legati all'apoptosi, alla necrosi, alla proliferazione e alla rigenerazione si susseguono secondo schemi specifici. Le recenti evidenze, che indicano come l'ipossia sia cruciale per il successo della resezione epatica nella prima fase postoperatoria, hanno dato forma all'ultima parte di questa tesi. Le vie di segnalazione suscitate dall'ipossia dopo una resezione epatica inducono la formazione di nuovi vasi e riducono il disaccoppiamento tra proliferazione epatocellulare e vascolare, rallentando la prima e velocizzando la seconda. Benché l'ipossia non possa essere trasferita come tale in pratica clinica, una risposta di tipo ipossico può essere sollecitata stabilizzando i suoi trasduttori a valle, cioè i fattori inducibili dall'ipossia. Il roxadustat, una piccola molecola che appartiene alla famiglia delle isochinoline, inibisce selettivamente la degradazione di questi fattori di trascrizione. In un modello di ratto di epatectomia standard al 70%, abbiamo valutato l'effetto del roxadustat sull'evoluzione dei cambiamenti

istologici e della funzione epatica, per mezzo di tecniche d'immagine funzionale all'avanguardia basate sulla risonanza magnetica. Nonostante l'assenza di un miglioramento evidente della funzione epatica o della rigenerazione, il farmaco ha alleviato la steatosi e il rigonfiamento epatocellulare, che sono noti marcatori di sofferenza cellulare post-epatectomia. Il sequenziamento dell'RNA ha confermato un sorprendente aumento del catabolismo lipidico e della respirazione cellulare. Questi affascinanti quanto inattesi effetti metabolici meriterebbero uno studio ulteriore in modelli di resezione epatica più estesa e di quadri da infiltrazione lipidica epatica.

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Abbreviations

ALPPS, associating liver partition and portal vein ligation for two-stage hepatectomy;
ALT, alanine transaminase;
AMR, antibody-mediated rejection;
APC, antigen-presenting cell;
AST, aspartate transaminase;
ATG, anti-thymocyte immunoglobulins;
ATLG, anti-T-lymphocyte immunoglobulins;
AUC, area under the curve;
BMI, body-mass index;
BP, biological process (Gene Ontology);
CC, cellular component (Gene Ontology);
CD, cluster of differentiation;
CMV, cytomegalovirus;
CNI, calcineurin inhibitor;
CUSL, Cliniques Universitaires Saint-Luc, Brussels, Belgium;
CVP, central venous pressure;
DBD, donor after brain death;
DCD, donor after cardiac death;
(DD)LT, (deceased-donor) liver transplantation;
DMOG, dimethyloxalylglycine;
DMSO, dimethyl sulfoxide;
DSA, donor-specific antibodies;
EDHB, ethyl-3,4-dihydroxybenzoate;
EdU, 5-ethynyl-2'-deoxyuridine;
eGFR, estimated glomerular filtration rate;
EMA, European Medicine Agency;

FDA, Food and Drug Administration;

FKBP12, FK506 binding protein 12;

FRL, future remnant liver;

Gd-EOB-DTPA, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid;

γGT, γ-glutamyltransferase;

GO, Gene Ontology;

GSEA, gene set enrichment analysis;

HABR, hepatic arterial buffer response;

HAV, hepatitis A virus;

HBV, hepatitis B virus;

HCC, hepatocellular carcinoma;

HCV, hepatitis C virus;

HDV, hepatitis D virus;

H&E, haematoxylin-eosin;

HEV, hepatitis E virus;

HIF, hypoxia-inducible factor;

HLA, human leukocyte antigen;

HSC, hepatic stellate cell;

HSV, herpes simplex virus;

HVPG, hepatic venous pressure gradient;

Hx, hepatectomy;

HZV, herpes zoster virus;

FOXP3, forkhead box P3;

IDO, indoleamine 2,3-dioxygenase;

IL, interleukin;

IQR, interquartile range;

IRI, ischemia-reperfusion injury;

ITBL, ischemic-type biliary lesion;

KEGG, Kyoto encyclopaedia of genes and genomes;

(LD)LT, (living-donor) liver transplantation;

MAPK, mitogen-activated protein kinase;

MELD, model for end-stage liver disease;

MF, molecular function (Gene Ontology);

MHC, major histocompatibility complex;

MMF, mycophenolate mofetil;

MRI, magnetic resonance imaging;

mTOR(i), mechanistic target of rapamycin (inhibitor);

mTORC1/-2, mTOR complex-1/-2;

OATP, organic anion-transporting polypeptide;

ORA, over-representation analysis;

NAFLD, non-alcoholic fatty liver disease;

NASH, non-alcoholic steatohepatitis;

NFAT, nuclear factor of activated T cell cytoplasm;

NK, natural killer cell;

PD1, programmed cell death protein 1;

PHLF, post-hepatectomy liver failure;

PHD, prolyl hydroxylase domain protein;

PBS, phosphate-buffered saline;

PVF, portal vein flow;

PVP, portal vein pressure;

RCT, randomised controlled trial;

RLBWR, remnant-liver-to-body-weight ratio;

RNAseq, RNA sequencing;

ROI, region of interest;

ROS, reactive oxygen species;

RT-qPCR, real-time quantitative polymerase chain reaction;

RXD, roxadustat;

SFSS, small-for-size syndrome;

TAC, tacrolimus monotherapy control group;

TCMR-A/-C, T-cell-mediated acute rejection (acute/chronic);

TCR, T-cell receptor;

Tc, cytotoxic T cell;

Texh, exhausted T cell;

Th, helper T cell;

TIPSS, trans-jugular intrahepatic portosystemic shunt;

Treg, regulatory T cell;

VEGF, vascular endothelial growth factor.

Introduction to the first part

Human liver transplantation (LT) was first attempted by Thomas E. Starzl on March 26, 1963, in Denver, Colorado.[1] Intraoperative bleeding and liver dysfunction eventually led to the recipient's death. After other eight equally unsuccessful attempts, Starzl observed a self-imposed moratorium until 1967 when his team performed a tenth LT, successfully this time.[2] Those initial failures highlighted the insufficient knowledge about organ procurement, preservation, surgical technique, and perioperative care at the time. The following two decades were dedicated to improve the safety of this difficult surgical procedure. The release, at the beginning of the 1980s, of ciclosporin, a selective immunosuppressant, was a breakthrough in the field. Rising from less than 50% during the period 1976–1979, one-year survival rate surpassed 75% during the following five years. Between March 1963 and June 1983, the procedure was performed more than five hundred times, in two American (Denver, Pittsburgh) and three European (Groningen, Hannover, and Cambridge) centres, so that the National Institutes of Health Consensus Development Conference on LT concluded that LT was "a promising alternative to current therapy in the management of late phase of several forms of serious liver diseases" and that it had the potential to become a "clinical service" as opposed to an experimental procedure.[3] This potential was acknowledged only on the condition that the procedure be restricted to strictly selected patients (Table 1). As rightly predicted by Starzl six years later when he stated that "the conceptual appeal of LT [was] so great that the procedure [might] come to mind as a last resort for virtually every patient with lethal hepatic disease"[4], LT is now the main cure in respect of more than fifty different liver diseases (Table 2).[5] All but one - i.e. active sepsis outside the hepatobiliary system - of the contraindications to LT laid down by the Consensus Conference have been progressively waived over the last forty years.[5]

Table 1. Indications and contraindications for liver transplantation in 1983

Conclusions from the National Institutes of Health Consensus Development Conference on LT.[3]

Indications	**Contraindications**	
	Absolute	**Relative**
1. Young patient <50 years	1. Age >55 years	1. Age >50 years
2. No viral infection	2. HBsAg–HBeAg-positive state	2. HBsAg-positive state
3. No alcohol and drug abuse	3. Active alcoholism	3. Intrahepatic or biliary sepsis
4. Ability to accept procedure / understand its nature	4. Inability to accept procedure or understand its nature or costs	4. Advanced alcoholic liver disease in abstinent alcoholic
5. Ability to accept costs	5. Sepsis outside hepatobiliary system	5. Prior abdominal surgery [a]
6. Normal vessel state	6. Portal vein thrombosis	6. Portal hypertension surgery
7. No cardiopulmonary or renal disease	7. Advanced cardiopulmonary or renal disease	
8. No prior abdominal surgery	8. Severe hypoxemia (right to left shunts)	
9. No infection	9. Metastatic hepatobiliary malignancy	
10. No (advanced) malignancy	10. Primary malignant disease outside the hepatobiliary system	

Chronic parenchymal diseases

Alcohol-related liver disease

While LT represents the best therapy for alcohol-related liver disease - which is the first most common indication throughout Europe and the USA for LT -, the six-month abstinence rule, considered as a "safety belt" in many centres, has proven to be an unreliable selection criterion. Alcohol consumption after LT remains a concern, all the more since its exact incidence is poorly documented,[6] and, with the best positive prognostic factor lying with a nurturing familial, professional, and social environment, only a structured and tight follow-up after LT appears to guarantee long-term abstinence. The Lille group's recent suggestion to perform LT in case of severe acute alcoholic hepatitis irresponsive to medical therapy has revived the debate surrounding this matter.[7,8]

Non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

The "non-alcoholic fatty liver disease" was first described in the 1980s as a progressive fat accumulation within hepatocytes, associated with obesity and metabolic syndrome, in the absence of alcohol consumption.[9] The spectrum spans from steatosis to steatohepatitis, the latter leading to fibrosis and cirrhosis due to ongoing inflammation. Cirrhosis related to non-alcoholic steatohepatitis is not only, given the current obesity epidemic, the fastest growing indication but also the second leading cause for LT and the second aetiology for HCC requiring LT nowadays in the USA.[10] Despite long-term survival rates similar to other indications, these recipients have a higher postoperative mortality due to cardiovascular events or sepsis. Besides, immunosuppression based on corticosteroids and calcineurin inhibitors (CNIs) exacerbates the metabolic comorbidities already affecting these patients, such as obesity, insulin resistance and diabetes. As recurrent graft steatosis has become a standard post-operative

follow-up, only lifestyle changes, tailored immunosuppression and bariatric surgery[11] are viable options in the absence of approved drugs.

Viral liver diseases

HBV-related cirrhosis

Despite aggressive vaccination campaigns and the exceptional evolution of antiviral treatments over the years, HBV infection remains the leading cause of cirrhosis and HCC in Far Eastern countries. Where prophylactic antiviral therapy is administered, LT for HBV-related cirrhosis has achieved five-year recipient survival rates exceeding 80%. Prophylaxis has freed patients from early postoperative cholestatic fibrosis. Initially, only expensive IV-specific immunoglobulins were available. The introduction of nucleos(t)ide analogues produced excellent results in case of active viral replication by the time of LT.[12] Due to IV-specific immunoglobulins high costs and low graft recurrence after the third post-transplant year, alternative strategies have been successfully explored, e.g. self-administered subcutaneous immunoglobulins and the switch to oral nucleoside analogue monotherapy.[13]

HCV-related cirrhosis

HCV-related cirrhosis is one the most common indications for LT, especially in the Mediterranean Basin. Before direct-acting antiviral agents (DAAs) were used, one recipient out of three was suffering HCV graft reinfection and rapid cirrhosis[14], which condemned him or her to near certain death and required retransplantation as a last resort. By offering effective pangenotypic approaches, DAAs have proven to be game-changers, resulting in the number of primary HCV-infected patients and candidates for retransplantation on waiting lists to drop and the possibility for HCV-positive and otherwise healthy subjects to join the scarce donor pool.[15]

Autoimmune liver disease

First reported in 1950, "lupoid hepatitis" was labelled "autoimmune hepatitis" (AIH) fifteen years later. Hepatocyte-directed T cell activity induces interface hepatitis, fibrosis, and ultimately cirrhosis. AIH is an archetype autoimmune condition, with female preponderance, hypergammaglobulinemia, serum autoantibodies, response to corticosteroids, and association with other autoimmune features in 40% of patients.[16] The heterogeneous serology and genetics accounts for the variability in disease expression with regard to ethnicity, geographical distribution, and genetic predisposition.[17] Despite the increased risk of developing infectious complications in the early post-LT period, long-term results are excellent. Recurrent graft disease, observed in 12–46% of recipients, is treatable in most cases by means of corticosteroids. Progress to cirrhosis and graft failure is rather uncommon.[18] Furthermore, while occurring increasingly frequently, de novo - thus alloimmune - hepatitis after LT, which is plausibly part of the chronic rejection spectrum, has yet to be fully understood.[19] The reporting on both conditions is influenced by local histological follow-up practice (per-protocol vs. per-cause biopsies).[20]

Cholestatic Diseases

Primary Biliary Cholangitis

This autoimmune liver disease is characterised by typical antimitochondrial antibodies and lymphocytic cholangitis. The chronic destruction of small-to-medium-calibre intrahepatic biliary ducts leads to cholestasis, fibrosis, and cirrhosis, an evolution that ursodeoxycholic acid can dampen.[21] Recently, obeticholic acid, an extremely promising farnesoid X receptor agonist, has been released for first-line resistant patients.[22] Although increasingly rarer, accepted indications for LT are Mayo Risk Score ≥7.8,[23] MELD ≥15, cirrhosis complicated with ascites, hepatic encephalopathy, and/or variceal haemorrhage, and intractable

pruritus.[24] Five-year patient survival rates reach 85%.[25] Chronic rejection and repeated relapse are frequent and overlapping, thus requiring careful immunosuppression and histological follow-up since specific antibodies and normal liver tests are no longer diagnostic criteria after LT. Fatigue and bone disease are typical and should be treated aggressively.[24]

Primary Sclerosing Cholangitis

This cholestatic autoimmune inflammatory disease, in respect of which no specific therapy exists, involves the macroscopic, intra- and extrahepatic, biliary tract. It may naturally evolve towards decompensated cirrhosis and biliary cancer. An alternation of multiple biliary strictures and dilatations appearing on imaging are its distinctive feature.[26]. Up to 70% of patients suffer from comorbidities such as inflammatory bowel disease, vitiligo, and sacro-ileitis. Aggressive screening for cholangiocarcinoma and colorectal cancer is warranted. While the best selection criteria and timing for LT remain unclear, standard MELD allocation is widely accepted, except for intractable pruritus or recurrent cholangitis.[27] Despite excellent early survival rates right after LT, frequent relapse detrimentally affects late survival rates. A specific technical issue is biliary reconstruction. Although Roux-en-Y hepaticojejunostomy is the preferred option,[28] where the recipient bile duct appears normal macroscopically, the easier duct-to-duct anastomosis and even choledochoduodenostomy are valid alternatives[29] if a troubled abdomen due to prior surgery has to be treated.

Secondary Biliary Cirrhosis

A number of biliary injuries can evolve into chronic biliary inflammation and liver scarring. These include biliary atresia, laparoscopic cholecystectomy or surgery for cystic echinococcosis, graft-versus-host disease, cystic fibrosis, and ischemic biliary tract lesions due to severe hemodynamic shock. Unfortunately, these patients are often belatedly transferred to a referral centre, i.e. with advanced disease and when the biliary tree harbours multi-drug resistant pathogens.[30,31]

Hepatobiliary Oncology

Primary Tumours

Hepatocellular carcinoma in cirrhotic or fibrotic liver

HCC is the fifth most common malignant tumour worldwide and the third leading cause of cancer-related death. Ninety per cent of HCC occur in a diseased liver. LT is the gold standard for it simultaneously removes tumour and underlying disease. The introduction of the Milan criteria (i.e. one lesion smaller than 5 cm or up to three lesions smaller than 3 cm, without extrahepatic manifestations or vascular invasion) and of neoadjuvant locoregional treatments resulted in five-year disease-free survival rates reaching 85%.[32] Although the Milan criteria appear to be too restrictive, their extension remains based on local practice. The integration of "dynamic" imaging and biological criteria (e.g. response to local treatments and evolution of tumour markers, like α-fetoprotein and des-γ-carboxyprothrombin) are needed to refine the therapeutic algorithm.[33]

Hepatocellular carcinoma in non-cirrhotic non-fibrotic liver

Ten per cent of HCC result in non-cirrhotic non-fibrotic liver. Selection criteria for LT differ in this context, not including dimensions. Liver resection is the therapeutic gold standard for these patients even though reported five-year recurrence rates, ranging from 40% to 70 %, remain high. A large European study including cases with initially non-resectable HCC showed that acceptable five-year disease-free survival rates of 60% and 48% can be obtained after primary and salvage LT for intrahepatic recurrence.[34] Alpha-fetoprotein, nodal status and number of lesions are major prognostic factors.

Cholangiocarcinoma

This aggressive primary neoplasm originating from biliary epithelium has long been considered an absolute contraindication to LT. The Mayo Clinic recently has recently pioneered a strategy of neoadjuvant chemoradiation followed by LT and

adjuvant chemotherapy for very selected patients with unresectable hilar cholangiocarcinoma. Despite the complex algorithm, the high dropout and the very strict patient selection, five-year recurrence-free survival rates reached 68%.[35] It is clear that timing is crucial and a planned surgical approach, made possible by living-donor LT (LDLT), is key to succeed.[36] While intrahepatic cholangiocarcinoma is not a standard indication for LT in a non-experimental context, lesions ≤2 cm with no nodal involvement and no vascular invasion were reported as showing good results. More evidence about the use of Y-radioembolization as neoadjuvant therapy in this context is needed. Conversely, overall results of LT for mixed hepatocellular and cholangiocellular carcinoma appear comparable to those of HCC,[37] which questions the necessity of pre-LT tumour biopsies in case of atypical imaging features.[36]

Vascular liver tumours

LT is an key tool in the therapy of hepatic epithelioid haemangioendothelioma, even in the presence of limited extrahepatic spread.[38] Macrovascular invasion, lymph node involvement, waiting time exceeding 120 days are unfavourable prognostic factors whereas extrahepatic disease is not. Ten-year disease-free survival rates of around 80% can be achieved.[39] On the contrary, haemangiosarcoma is an absolute contraindication to LT due to invariably grim results.[40]

Secondary liver tumours

Neuroendocrine tumour liver metastases

LT for unresectable metastases is yet another evolving area in transplantation oncology. LT can cure up to 85% of patients with unresectable metastases provided that strict inclusion criteria are met - i.e. low proliferation index (Ki67<5%), delay between R0 resection of the primary tumour and LT ≥6 months, tumour location in the portal venous drainage system, and absence of disease progression under

neoadjuvant therapies.[41] Excellent survival is then the more frequent prospect.[42] Major or multivisceral resection in addition to LT hepatomegaly, and age >45 years are additional poor prognostic factors.[42] It was very recently shown that the survival benefit offered by LT compared to a non-transplant approach increases over time and reaches a very significant difference after 10 years (88.2% vs. 22.4%).[43] As for other oncological indications, LDLT is the way forward.

Colorectal carcinoma liver metastases

Showing a disappointing 19% five-year patient survival rate, colorectal carcinoma unresectable metastases were an absolute contraindication for LT still in 2010. At the time, nearly half of the reported patients had died of non-oncological causes and two thirds of them were on heavy immunosuppression.[44] Improved staging, selection, chemotherapy, and immunosuppressive management resulted in better LT's outcomes for this indication as the five-year survival rate reached 83%.[45] Even in case of relapse, since the lungs are the site of recurrence in most cases, one third of the patients can be rendered disease-free after pulmonary resection.[46] Such compelling improvements have driven two randomised controlled trials (RCTs) comparing LT against standard care in unresectable colorectal metastases patients. The first one is the TRASMET study, for which completion of the recruitment phase is scheduled for February 2022 and whose stringent selection criteria include absence of BRAF mutations and elsewhere recurrence, response to chemotherapy and CEA levels <80 µg/l.[47] The completion of the recruitment phase for the SECAIII study is scheduled for January 2025.[48] These advancements are shifting the therapeutic paradigm by making the case for LDLT and, in so doing, are putting pressure on regulatory agencies to consider such an indication.[49,50]

Metabolic diseases

Hereditary haemochromatosis

Involving disproportionate iron absorption despite body iron levels, this protean genetic disorder, which induces cardiac failure, diabetes, cirrhosis and HCC, is a rather uncommon indication for LT. However, LT helps restore the secretion of hepcidin, the inhibitor of iron transporters, and prevents the recurrence of hepatic iron overload.[51] These recipients have a higher morbidity and mortality due to cardiac complications, diabetes, infections, and cancer recurrence.

Wilson's Disease

Responsible for systemic copper accumulation, this autosomal recessive disease of copper metabolism specifically damages liver, brain, cornea and kidneys.[52] An adequate long-term therapy with copper-chelating agents or zinc salts curbs the progression of systemic complications and hepatic involvement. However, disease-related acute liver failure, subacute liver disease, and end-stage liver disease, with or without neuropsychiatric manifestation, invariably require LT. Outcomes of LT are better in chronic liver disease than in the acute setting. While copper metabolism always normalises after LT,[53] neuropsychiatric disorders do not, and some patients may even experience a severe degradation of their condition.[54] Living donation is an excellent option for heterozygote carrier relatives.[55]

Haemophilia

Haemophilia refers to a family of X-linked coagulation disorders most commonly caused by the deficit of factor VIII (F VIII, haemophilia A) or factor IX (F IX, haemophilia B). Over time, plasma derivatives, and recently recombinant factors, have dramatically improved patients' life expectancy. Historically human derivatives might transmit HCV and HIV infections.[56] When these patients develop end-stage liver disease requiring LT, coagulopathy is usually rapidly corrected by the graft.[57]

Familial amyloid polyneuropathy

This slow, eventually fatal autosomal dominant disorder, which presents a widely variable clinical course and entails the accumulation of amyloidogenic transthyretin, may be caused by several mutations.[58] The most frequent mutation is the Val30Met, which induces an early onset (between 25 and 35 years), a relatively more benign course as well as a cardiac involvement in the form of rhythmic disturbances. Novel disease-modifying anti-amyloid treatments have recently been released as "breakthrough therapy". Tafamidis, a transthyretin stabiliser, has proven to reduce mortality and cardiovascular-related hospital admissions in a substantial absence of adverse events.[59] Patisiran, the first FDA-approved small interfering RNA, has been shown to stop disease progression.[60] This progress is probably going to change the known scenario in which LT was the only curative treatment to remove the source of the variant molecule. A distinctive feature of LT in this context is the possible transfer of the recipient's liver to another recipient. This procedure, known as sequential (or domino) LT, which bears a low risk of disease transmission with the graft, can be a last resort for marginal candidates who are otherwise denied LT.[61]

Primary hyperoxaluria

This group of autosomal recessive disorders of endogenous oxalate overproduction typically develop in childhood. While the type 1 is caused by a hepatocellular alanine–glyoxylate aminotransferase deficiency and is the most aggressive form, the milder type 2 is related to glyoxylate reductase/hydroxypyruvate reductase deficiency. Deposition of calcium oxalate crystals in the kidney, nephrocalcinosis, and progressive renal failure are the main clinical manifestations. First used in 1984, the combined kidney–liver transplantation is the preferred treatment for it tackles both the cause of the disease and the main target organ.[62] Unfortunately, systemic oxalosis, which can cause kidney graft loss, undermines the outcomes.[63]

Tyrosinemia type 1

This autosomal recessive metabolic disorder is caused by a deficiency of fumarylacetoacetate hydrolase, an enzyme involved in the final catabolism of tyrosine and phenylalanine. Toxic metabolites drive the apoptosis of hepatocytes and kidney tubular epithelial cells, which increases the risk for HCC. This disorder can take two forms, acute or chronic. The acute form occurs within the first months of life and leads to acute liver failure during the first year. Failure to thrive, hepatomegaly and chronic liver disease, renal tubular dysfunction, rickets, cardiomyopathy, and porphyria-like neurological syndrome are specific to the chronic form.[64] The backbone treatments are a tyrosine-/phenylalanine-free diet and nitisinone, which blocks the second step of tyrosine catabolism.[65] Indications for LT include unresponsive acute liver failure, end-stage liver disease, HCC, and even dysplastic liver nodules due to their high risk of progression. Heterozygotes do not develop the disease, and healthy relatives can be considered as living donors. LT does not correct renal deficiency, but nitisinone's renal-sparing properties have curbed the need for combined transplantations.[66]

Glycogen storage disease type 1

This autosomal recessive inborn error of carbohydrate metabolism is caused by defects in the glucose-6-phosphate transporter/glucose-6-phosphatase complex. Clinical complications include hepato- and nephromegaly, hypoglycaemia, hyperlipidaemia, hyperuricemia, lactic acidosis, and growth retardation.[67] LT resolves metabolic derangement, obviates the need for dietary restrictions and reduces the risk of malignancy, yet not always that of renal failure so that simultaneous or sequential liver and kidney transplantation (KT) might be required.[68,69] The timing of LT is open to debate since a prophylactic approach might prevent irreversible systemic damages but LT bears a non-negligible mortality risk for young patients.[69] Nonetheless, LT is indicated in the presence of

HCC and even adenoma, poor metabolic control, growth retardation, and renal failure. LDLT is also an excellent option.

Acute liver failure

Characterised by markers of liver damage (elevated serum transaminases) and compromised liver function (hyperbilirubinemia and impaired coagulation), acute liver injury is caused by massive necrosis of a previously normal liver. It sometimes precedes clinical encephalopathy, whose occurrence indicates the acute liver failure.[70] The concepts of hyperacute, acute, subacute, and late-onset failure refer to the time range between jaundice and encephalopathy (i.e. less than 2 weeks, from two to eight weeks, and more than 8 weeks).[71] The main cause is drug-induced toxicity, upon deliberate ingestion of paracetamol in most cases. Other drugs are less likely to cause acute liver failure but, when this is the case, the course is usually more aggressive. Identified viruses (in descending order of proportion: HBV, HAV, HEV, HSV1 and -2, VZV, CMV, EBV) account for 7-37% of cases in Western countries, with an additional 17-43% of unknown aetiology. Autoimmune hepatitis, Budd-Chiari syndrome, Wilson's disease, ingestion of *Amanita phalloides*, HELLP syndrome, and acute fatty liver related to pregnancy are other less common causes. Acute liver failure advances rapidly and has a high mortality rate. Specialised intensive care and emergency LT are the therapeutic pillars. In general, prognosis is worse in patients with more severe liver injury, extrahepatic organ failure and subacute presentations. Although the traditional King's College[72] or Clichy[73] criteria have shown poor accuracy, declining over time following improvements in medical therapy, LT should still be considered in respect of patients fulfilling the aforementioned criteria.[70] Much attention has been focussed over the recent past to mechanical or biological artificial liver devices, purported to provide an effective "bridge" pending LT or recovery of the liver function. However, despite some positive effect on encephalopathy, none of them has proven to reduce mortality in an RCT. Auxiliary

LT is another option for these patients, who could be weaned from immunosuppression upon recovery of the native liver.[74,75] However, this is technically complex, and inappropriate in case of severe encephalopathy and high risk of brain death.[70] Poorly considered in both Europe and the USA in such a setting, LDLT is an option that achieves similar results as deceased-donor transplantation.[76]

Conclusions

In essence, this condensed but updated list of current indications of LT was meant to confirm that the dream of Starzl has truly become reality, over time, and that LT is *the* treatment for an ever-increasing number of liver or liver-based conditions. The questions about the scarcity of the donor pool remain unanswered instead.

Table 2. Indications for liver transplantation in 2022

Acute liver failure	Acute viral infection (HAV, HBV, HBV-HDV, HEV and other viruses) Acetaminophen Drug-induced liver injury Acute alcoholic hepatitis Reye syndrome Postoperative Post-traumatic Wilson's disease Budd–Chiari syndrome Autoimmune hepatitis Cryptogenic Fatty liver diseases spectrum Acute fatty liver of pregnancy
Chronic liver failure	Chronic viral infection (HBV, HBV-HDV, HCV, HEV and other viruses) Drug-induced cirrhosis Alcoholic cirrhosis Autoimmune hepatitis Cryptogenic cirrhosis Fatty liver diseases spectrum
Cholestatic liver diseases	Primary biliary cholangitis Secondary biliary cirrhosis Primary sclerosing cholangitis Caroli disease Graft-versus-host disease Congenital biliary fibrosis Extrahepatic biliary atresia Alagille syndrome Byler disease
Vascular liver diseases	Budd–Chiari syndrome Hereditary haemorrhagic telangiectasia Veno-occlusive disease Nodular regenerative hyperplasia

Metabolic liver-based cirrhotic and non-cirrhotic diseases	Wilson's disease Hereditary hemochromatosis Alpha-1 antitrypsin deficiency Tyrosinemia Crigler–Najjar syndrome Cystic fibrosis Galactosemia Glycogen storage disease I and IV Familial amyloid polyneuropathy Familial homozygous hypercholesterolemia Primary hyperoxaluria type I Protoporphyria and other types of porphyria Factor VIII, IX, and V deficiency Thrombophilia (e.g., protein C and S deficiency)
Benign tumours	Degenerated hepatic adenoma Adenomatosis Giant haemangioma Polycystic liver disease Alveolar echinococcosis Cystic echinococcosis
Malignant tumours	Primary hepatobiliary tumours 1. Hepatocellular carcinoma (HCC) 2. Cholangiocellular carcinoma 3. Epithelioid haemangioendothelioma 4. Hepatoblastoma Secondary liver tumours 1. Neuroendocrine liver metastases 2. Colorectal metastases
Miscellaneous	Hepatic trauma Schistosomiasis Sarcoidosis

Immunology in liver transplantation

Essential for survival in end-stage organ failure, transplanted organs can be sensed as dangerous foreign bodies and regrettably attacked by the recipient's immune system. The essential information concerning human immunology and LT is exposed below in order to help the reader familiarise with current immunosuppression strategies in LT.

The immunogenicity of the graft

Jan Van Rood, the founder of Eurotransplant, highlighted first the role of the human leukocyte antigen (HLA) system in allogeneic transplantation.[77] This system is behind the development of a host immune and of graft rejection. Traditionally, four consecutive stages are described.[78]

Alloantigen recognition

The recognition of allogeneic HLA takes place via a direct, an indirect or a semidirect pathway, and consists of an interaction between the major histocompatibility complex (MHC) of an antigen presenting cell (APC) and the T-cell receptor (TCR) of an inactive T cell. The mismatch between donor and recipient's HLAs tends to result in hyperactivation of these pathways. As immunosuppressive agents, anti-lymphocyte antibodies exert their effects at this stage.

Co-stimulation

Costimulatory molecules exposed on APCs, the cluster of differentiation (CD)80 and CD86, meet the T-cell receptor CD28. This double interaction is followed by the activation of the calcineurin pathway, resulting in major interleukin (IL-)2 transcription. Calcineurin inhibitors (ciclosporin and tacrolimus) inhibit this stage of immune activation.

Clonal expansion

Through paracrine and autocrine stimulation, these interleukins activate the mTOR pathway resulting in cell proliferation. $CD4^+$ T cells or T helper cells (Th) are also prompted to differentiate in several subtypes and to release cytokines accordingly. This milieu recruits more lymphocytes and partner cells (macrophages, B cells, etc.). Conversely, $CD8^+$ T cells or cytotoxic T cells (Tc) are prompted to release cytotoxins and to express FAS ligand to induce apoptosis of target cells.

Graft inflammation

The pattern of Th cytokine production steers the reaction, in which either cell-mediated or antibody-mediated immunity prevail. Th1 lymphocytes, associated with IFN-γ, promote a cell-mediated response. Th2 lymphocytes, linked to IL-4, -5, and -6, foster antibody production via B cell recruitment, the so-called humoral response, are referred to as Th2 and are associated with the generation of IL-4, -5, and -6. Th17 population, characterised by IL-17, promotes neutrophil infiltration. The inflammatory environment created by the release of toxic and vasoactive mediators boosts the killing efficacy of macrophages and Tc, resulting in graft cytolysis.

Phenotypes of graft rejection

Consensus exists over two distinct, but overlapping, phenotypes of rejection: (1) the T-cell–mediated (TCMR) and (2) the antibody-mediated rejection (AMR). TCMR manifests as $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T cell infiltrates accompanied by fewer $CD20^+$ B cells, macrophages, natural killer (NK) cells, eosinophils, plasma cells, neutrophils, and mast cells (9). TCMR severity is graded based on: (1) inflammation intensity and distribution; (2) tissue damage extent; and (3) direct or indirect signs of vascular/ischemic injury. Qualifying descriptors include “early” or “acute” and “late” or “chronic.”

T-cell-mediated acute rejection

Recent literature properly refers to acute cellular rejection as T-cell-mediated acute rejection (TCMR-A), whose incidence fluctuates from one study to another, according to local per-cause or systematic biopsy protocols. However, it is esteemed that about 30% of recipients experience at least one episode, the large majority of them within the first year.[79] There is no stringent cut-off to distinguish early and late TCMR. Anyway, it is generally believed that early (<6 months) TCMR-A is consequent to direct alloantigen presentation (from donor's APCs), while indirect presentation (from recipient's APCs) elicits late (>6 months) or chronic TCMR. TCMR-A entails more prevalent inflammatory bile duct damage, pleomorphic portal inflammation (lymphocytes, macrophages, eosinophils, etc.), and minor necro-inflammatory interface activity than late TCMR.[80]

TCMR-A in LT seems to bring little to no impact on mortality after LT and it appears detrimental only in HCV-positive recipients. Some evidence exists that TCMR-A could even promote immune tolerance. Indeed, a single episode within the first six weeks after LT appears to be associated with an improvement in long-term patient and graft survival rates.[81,82]

Clinically, acute rejection is heralded by hyperthermia, asthenia, hepatomegaly and, occasionally, by jaundice and ascites. However, the sensitivity and specificity of these signs are poor.

A common blood workup usually shows an increase in liver enzymes, hyperleukocytosis and decreased prothrombin levels, which are not specific to the TCMR-A. Thus, no cut-off value has been found predictive of rejection.[83] Rodríguez-Perálvarez et al. reported that the likelihood of TCMR-A is quadrupled in the presence of serum bilirubin >4 mg/dl, rising bilirubin within four days before liver biopsy, and blood eosinophils count $>0.1\times10^9$/l.[84] Specific plasma miRNAs, in particular miR-146a, may reflect cellular rejection.[85]

Histology shows that the peak of TCMR-A occurs on the seventh postoperative day.[86] Therefore, a biopsy is most discriminative at this time point. TCMR-A

displays an attack on three elements: the portal space, the bile ducts, the portal and hepatic venules. The Banff score ranks the severity of acute rejection by adding points (0-3) attributed to each component of the triad. The total score is used to determine the severity of the rejection: indeterminate (0-3), mild (4-5), moderate (6-7), severe (8-9).[80] At any rate, caution is required with regard to overlapping conditions that may conceal or lead to overestimate TCMR-A severity. In HCV-positive patients, the main histological differential diagnosis is HCV infection recurrence, because both entities show mononuclear portal inflammation, endotheliitis and ductular damage. Interface hepatitis and necro-inflammatory lobular activity are more peculiar to viral recurrence. Bile duct stenoses, characterised by centrilobular cholestasis, ductular reaction, portal neutro-lymphocytic infiltration, and damage to biliary epithelium, should also be ruled out.[87]

In the absence of a uniform practice concerning postoperative biopsies, and considering that overimmunosuppression is not obtained without consequences,[88] the criteria for the decision of when and how TCMR-A is to be treated are disparate. At the *Cliniques Universitaires Saint-Luc* (CUSL), Brussels, Belgium, per-protocol day-seven liver biopsy is a standard in stable patients. In this frame, only moderate (Banff score 6-7) and severe (8-9) histological rejections are taken into consideration for a possible treatment. The decision to treat or not a histological TCMR-A is based on a locally developed index, which has been labelled Seven-up score. In this frame, a single point is attributed in case of increase in eosinophils and total bilirubin for two following days, decrease in platelets for two consecutive days, and of absolute eosinophilia >600/mm^3. The treatment is established if this biochemical score amounts to ≥2.[89] In the same centre, standard treatment consists of 3 to 5 oral boluses of 200 mg of methylprednisolone. In 5 to 15% of cases, the treatment fails. TCMR-A resistant to one gram of methylprednisolone is accordingly labelled corticosteroid-resistant

rejection and is approached with 5 mg/kg of intravenous rabbit anti-human-T-lymphocyte immunoglobulins for seven to ten days.

Antibody-mediated rejection

The presence of alloantigen-specific antibodies, or alloantibodies, secreted by plasma cells, can trigger an antibody-mediated rejection (AMR). Pre-existing (e.g. for blood transfusions, repeated pregnancies, previous organ transplantation) donor-specific antibodies (DSAs) origin the most severe and early form of rejection. When the donor endothelium comes into contact with the recipient's blood, DSAs react with the complement, which damages the endothelium of the graft. Vascular breaches summon platelets that aggregate en masse and obliterate microvessels, thus depriving the graft of its blood supply. More frequently, a B cell response, primed by activated effector Th, determines DSA production after transplantation. Intraluminal pooling and margination of various leukocytes (monocytes, macrophages, lymphocytes, neutrophils, and eosinophils) in dilated and irregularly shaped capillaries are the hallmarks of this kind of AMR. Still, the extension of the vascular bed and its immunological characteristics make the liver relatively resistant to both forms of humoral rejection. ABO compatibility and recipient’s pre- and regular post-transplant screening prevent and help identify AMR.[78,80]

Chronic rejection

Chronic TCMR (TCMR-C) display more uniform infiltrates of plasma cell and histiocytes, less lymphocytic cholangitis, and low-grade interface and perivenular necro-inflammatory activity, features shared with late TCMR.[80] This condition is defined as an immunological lesion evolving from a severe or persistent TCMR-A, which leads to irreversible biliary, arterial and venous lesions, and whose typical features are fibrosis, obliterative arteriopathy, and ductular rarefaction.[90] Hence comes the alternative name "vanishing-bile-duct syndrome". It presents significant overlap with chronic AMR. Under effective immunosuppression, five-year

prevalence of chronic rejection does not exceed 3-5% of patients.[91] It should be considered in differential diagnosis for patients who have experienced acute rejection and develop progressive cholestasis. Histology shows ductopenia in more than 50% of the portal spaces, cholestasis, periportal and perivenular fibrosis.

Fibrosis

The common final feature in parenchymal disorders, fibrosis is a key finding in liver pathology. Atypical fibrosis patterns without previous overt rejection might be the only sign of chronic antibody-mediated injury or mixed TCMR and chronic AMR.[92-95] The paradigm for quantification of liver fibrosis is the METAVIR score since 1994. Introduced for staging fibrosis in HCV-positive patients, it is used now for any liver disease. It consists of five stages: F0) absence of fibrosis, F1) minimal portal fibrosis without septa, F2) portal fibrosis with few septa, F3) numerous septa with marked bridging fibrosis, and F4) cirrhosis.[96] In 2012, Venturi et al. proposed another staging system of liver fibrosis paediatric recipients, with the merit of distinguishing compartments, i.e. portal spaces, sinusoids and centrilobular veins.[97] Fibrosis is thus ranked from 0 to 3 for each compartment, offering a more informative representation of the hepatic acinus. Different immunological reactions originate different patterns of fibrosis in specific compartments. The concept of irreversible fibrosis has evolved to give way to a dynamic and reversible representation of hepatic fibrosis. It follows from this evidences that the use of a score distinguishing compartments is should be preferred.[80,98]

Table 3. Quantitative scoring of T-cell-mediated acute rejection

Rejection activity index (RAI) score criteria:[80]

A) Portal inflammation:
1) Mostly lymphocytic inflammation involving, but not noticeably expanding, a minority of the triads.
2) Expansion of most or all of the triads, by a mixed infiltrate containing lymphocytes with occasional blasts, neutrophils, and eosinophils. If eosinophils are conspicuous and accompanied by oedema and microvascular endothelial cell hypertrophy is prominent, acute antibody-mediated rejection (AMR) should be considered.
3) Marked expansion of most or all of the triads by a mixed infiltrate containing blasts and eosinophils with inflammatory spillover into the periportal parenchyma.

B) Bile duct inflammation damage:
1) A minority of the ducts are cuffed and infiltrated by inflammatory cells and show only mild reactive changes such as increased nuclear-to-cytoplasm ratio of the epithelial cells.
2) Most or all of the ducts infiltrated by inflammatory cells. More than an occasional duct shows degenerative changes such as nuclear pleomorphism, disordered polarity, and cytoplasmic vacuolization of the epithelium.
3) As above for 2, with most or all of the ducts showing degenerative changes or focal luminal disruption.

C) Venous endothelial inflammation:
1) Subendothelial lymphocytic infiltration involving some, but not a majority of the portal and/or hepatic venules.
2) Subendothelial infiltration involving most or all of the portal and/or hepatic venules with or without confluent hepatocyte necrosis/dropout involving a minority of perivenular regions.
3) As above for 2, with moderate or severe perivenular inflammation that extends into the perivenular parenchyma and is associated with perivenular hepatocyte necrosis involving a majority of perivenular regions.

Immunosuppression

Introduction

The type, amount and combination of immunosuppressants are actual concerns for the transplant team. Provided that immunosuppression is key the engraftment of an allogeneic organ, immunosuppressive tailoring is needed because immunosuppressants carries significant side effects and each recipient is unique in terms of performance status, oncological precedents and serological viral status. The death of recipient in the presence of a functioning graft is usually related to infectious, oncological and cardiovascular complications, all of which are influenced by immunosuppression. Clinical "operational tolerance", defined as stable and acceptable graft function without immunosuppression, has thus become a target in LT. Some estimates put the percentage of recipients who can be safely withdrawn immunosuppression at 20%, which can rise to 40% in subpopulations selected for the absence of autoimmune liver disease, rejection, and abnormal graft histology.[99,100]

Apart from the treatment of overt rejection episodes, pre-emptive immunosuppressive therapy is divided into two phases: induction and maintenance. Induction therapy, usually intense, is administered within the first days after LT. Maintenance therapy is started in the perioperative period and is maintained, though gradually tapered, over time.[99,101]

The question of the best immunosuppressive regimen is a very sensitive issue in LT and, as such, there is no unanimity within the transplantation experts' community on any particular immunosuppressive regimen. There is however a general consensus that patients should be weaned from corticosteroids within three months from LT, and that stable recipients are candidates for CNI monotherapy, after three months, if the previous period has been uneventful and that tacrolimus is the drug of choice.[102] Extensive experience has been gained over time with tacrolimus monotherapy associated with immediate perioperative hydrocortisone,

at the CUSL.[89,103,104] One gram overall of hydrocortisone is administered from before revascularisation and within the first 72 hours postoperatively, and tacrolimus monotherapy is started within the first 12 hours after LT.

Maintenance therapy

Glucocorticoids

Corticosteroids exert manifold effects on the immune system, from a general anti-inflammatory action on eicosanoids release, to the inhibition of the antigen-presenting activity of APCs as well as the downregulation of interleukin (IL-2, in particular) transcription in T cells. This valuable modulation unfortunately comes with a cohort of worrisome side effects, especially regarding metabolism and infections. Though essential in the early decades of transplantation as inducing and maintenance agents, corticosteroids have been progressively limited to the early post-LT period, to specific LT indications (i.e. autoimmune hepatitis) and to the treatment of rejection episodes. In 2009, a meta-analysis of 21 RCTs confirmed that a substantially corticosteroid-free maintenance immunosuppression is associated with similar rejection rates, patient and graft survival. In addition, a decrease in metabolic and infectious complications of corticosteroids was observed.[105] Despite this growing body of evidence, still in 2016 about 60% of LT recipients in the US are reportedly on glucocorticoids one year after LT.[106]

In steroid-based regimens, corticosteroids can be discontinued or replaced by other immunosuppressive drugs either within the first post-LT period or at a later stage.[102,107]

Calcineurin inhibitors

CNIs act by inhibiting the calcineurin pathway, which results in inhibition of IL-2 transcription. Ciclosporin and tacrolimus constituted a major revolution in the field of immunosuppression in the eighties and in the nineties, respectively. They curbed rejection rates, improved survival and raised recipients' quality of life, compared to

earlier immunosuppression schemes heavily based on corticosteroids. Tacrolimus came to be favoured for better patient and graft survival, and fewer acute rejections and corticosteroid-resistant rejections, as shown in a meta-analysis carried out by Haddad et al. in 2006.[108]

As for their mechanism of action, ciclosporin binds to a cytosolic immunophilin, the cyclophilin. This ciclosporin-cyclophilin complex blocks the phosphatase activity of calcineurin. In standard conditions, upon T cell activation calcineurin dephosphorylates the nuclear factor of activated T cell cytoplasm (NFAT), a transcription factor responsible for the transcription of IL-2.[109] Tacrolimus binds instead to the FK506 binding protein 12 (FKBP12), another immunophilin, and this complex exerts the same effect on calcineurin as ciclosporin.[110]

Unfortunately, CNIs are known to produce side effects of particular concern in LT, such as diabetes, hypertension, and, particularly, nephrotoxicity. Nephrotoxicity can occur in acute or chronic form, and is often but not always dependent on the dose and the duration of administration. Its most dismal features are arterial-wall hyalinosis, thrombotic microangiopathy in arterioles and glomerular tufts, and striped interstitial fibrosis with associated tubular atrophy.[111] It is estimated that 22% of patients on CNIs is bound to develop some chronic kidney disease within five years from LT, which is why a strategy for CNI minimisation is warranted in LT. Tacrolimus monotherapy with progressive tapering is indeed possible.[104] Besides, powerful antibody-based induction enables a delay in the introduction and a reduction in the dose of CNIs.[102,112-115]

mTOR inhibitors

Previously identified as mammalian target of rapamycin, the mechanistic target of rapamycin (mTOR) is a ubiquitous serine/threonine kinase that regulates cell metabolism, growth, proliferation and survival.[116] This enzyme was initially identified as the molecular target of rapamycin (or sirolimus), a lactone macrolide produced by *Streptomyces hygroscopicus*,[117] and constitutes the core component of

two distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), watchdogs of different but overlapping cellular functions.[118] In T cells, mTOR is an important trigger for cell replication cycle upon activation by APCs. As such, mTOR inhibitors (mTORis) dampen antigen-induced lymphocytes proliferation.[119] Additionally, this class of molecules is appealing for their antiproliferative potential, which is of use for some oncological indications. Yet mTORis have not shown an undisputed benefit in terms of disease-free survival and overall survival in patients transplanted for HCC.[120-122] Sirolimus has never been approved by the Food and Drug Administration (FDA) or the European Medicine Agency (EMA) for LT, because of some episodes of hepatic artery thrombosis and graft loss.[123,124] Nonetheless, a cognate molecule exists, labelled as everolimus and approved for liver, kidney and heart transplantation.[125,126] Though extensively promoted in single or variably combined regimens for its renal-sparing properties, the use of everolimus is fraught with a number of frequent adverse events, to name a few stomatitis, infections, diarrhoea, peripheral oedemas, hypercholesterolemia, myelosuppression, diabetes, interstitial pneumonia, proteinuria, and incisional hernia.[127]

Antimetabolites

Often used in combination with glucocorticoids, with CNIs, or with the two, antimetabolites too have a massive inhibitory effect on lymphocyte proliferation and cell-mediated immune responses. Their use in LT is reserved to patients at high immunological risk or in need for low-dose CNIs, in order to help handle metabolic syndrome and declining kidney function.[102]

Azathioprine is the prodrug of 6-mercaptopurine, a purine analogue that disrupts DNA synthesis. It is known to induce non-melanoma skin cancers in solid organ recipients but was a major game changer in transplantation when it was introduced.[128]

Mycophenolic acid is a non-competitive inhibitor of inosine 5'-monophosphate dehydrogenase and blocks de novo synthesis of guanosine nucleotides. Thus it essentially targets lymphocytes, cells dependent on *de novo* synthesis, while other cells can rely on salvage pathways.[129]

Induction therapy

The use of induction agents has intensified over the last two decades. Despite the lack of international consensus, by 2016, more than 30% of LT recipients received antibody induction, in the form of either T-cell depleting antibodies or non-depleting IL-2 receptor antagonists (IL2-RA), essentially basiliximab (Simulect, Novartis, Basel, Switzerland).[106] The general use of these agents is well established in KT,[130] while their indication in LT is defined in patients at high immunological risk (ABO-incompatible organs, retransplantation for rejection, positive crossmatch). Another common use of antibody induction is in the frame of a CNI belated introduction or reduced exposure, and for early glucocorticoids weaning.[102,131,132]

Two retrospective analyses found that induction is associated with an improvement in long-term patient and graft survival in kidney, liver and lung transplantation.[133,134] These agents seemed associated with a modest gain in estimated glomerular filtration rate (eGFR) and with a reduced rate of acute rejection in the first year post-LT.[134,135] Antibody induction could theoretically facilitate infections and post-transplant lymphoproliferative disease (PTLD). Yet no association has been established between induction and the appearance of de novo cancers.[136] There was instead some evidence that depleting antibodies might reduce the rate of HCV reinfection after transplantation.[137] However, in the 2014 Cochrane systematic review by Penninga et al. (1341 retrieved articles dealing with induction for LT, only 10 retained for analysis), antibody induction appeared to reduce diabetes mellitus and cytomegalovirus infection, compared to corticosteroid induction, without any other clear benefits or harms. In particular, no significant

differences were found in patient or graft survival, infections or acute rejection rates. Serum creatinine appeared higher in patients who received non-depleting antibody induction. This review contained several problems: it was based on heterogeneous papers and mixed all different types of inducing antibodies, form non-depleting (nine studies) to depleting agents (one study).[138]

Non-depleting antibodies

Daclizumab (Zenapax, Hoffmann-La Roche, Basel, Switzeland), a humanized monoclonal antibody directed against the α chain (CD25) of the IL-2 receptor, was originally introduced in 1997 and withdrawn in 2017 for fatal immune adverse events.[139,140] Basiliximab, a chimeric mouse-human monoclonal antibody to the same antigen, was released in 1998 and is still approved for kidney transplantation, so that the use in LT is off-label.[141,142] The full two-shot course for adult recipients costs about 1,600€.[143]

In 2009, Adam et al. acknowledged that there was no evidence to support the use of basiliximab over depleting agents in LT, though they recognised that the use of IL2-RA was more common.[144] Again in 2016, basiliximab represented the most frequent alternative to glucocorticoids in induction.[106] A very recent network meta-analysis has remarkably found out that basiliximab alone halves the number of fatal events and graft losses compared with standard induction with glucocorticoids in LT, at maximal follow-up, although this evidence is based on small and potentially biased studies.[145] Conversely, the effect on graft and patient survival of depleting agents was inconclusive. With respect to graft rejection, the level of uncertainty was high for any regimen, so that valid conclusion cannot yet be drawn.

Depleting antibodies

T-cell depleting antibodies are derived from the immunisation of animals with human white blood cells in order to induce the production of polyclonal immunoglobulins directed against human leukocytes. Atgam (Pfizer, New York,

NY-US) has been developed through the immunisation of horses. Grafalon (originally Fresenius, Bad Homburg, Germany, and now Neovii Biotech, Rapperswil-Jona, Switzerland), obtained by inoculation of Jurkat immortalised $CD4^+$ T cells, and Thymoglobulin (Sanofi Genzyme, Cambridge, MA-US), obtained by injection of human thymocytes, originate from rabbit immunisation. For adult recipients of about 70 kg, the full course of nine mg/kg of Grafalon roughly costs 1,800€ while the standard course of five mg/kg of Thymoglobulin about 2,400€.[146]

Depleting antibodies bind to membrane proteins and induce leukocyte destruction via the complement-dependent lysis, in peripheral blood, and via the antibody-dependent cell-mediated cytotoxicity and Fas-dependent apoptosis, in lymphoid organs.[147] Altogether, these biological agents exert a profound and not entirely elucidated immunomodulation, which explains the variability of their side effects.[148] Very few studies have compared the use of these agents. Though approved only for the prophylaxis and treatment of acute rejection in patients receiving a kidney transplant, the off-label experience with antithymocyte immunoglobulin (ATG, i.e. Thymoglobulin) in LT is extensive.[149] The experience with Grafalon in LT is more limited because it has not yet been approved by the FDA and, in Europe, it is agreed only for the conditioning in view of stem-cell transplant and for graft-versus-host disease.[150]

Depleting antibodies are currently used also for the prevention of graft-versus-host disease in haematology and are of particular interest in patients with highly aggressive T-cell tumours.[151] In solid organ transplantation, antilymphocyte globulin is used as a treatment for acute corticosteroid-resistant rejection and as prophylaxis for rejection in induction protocol.[102,131] These compounds are supposed not to be nephrotoxic, and therefore also have their place in recipients with acute kidney injury or chronic kidney disease. However, the multitude of epitopes targeted by depleting antibodies can cause a cytokine release syndrome, a form of severe vasoplegia pursuant to massive cell lysis. Their use should be

carefully pondered in patients with cardiovascular instability, as hypotension and coagulation disorders may deteriorate.[152-154]

The difficult immunological handling in kidney transplantation has encouraged the practice of induction with depleting antibodies for a long time. In 1999, Brennan et al showed that, in recipients at high immunological risk, an ATG-based induction compared to Atgam reduces incidence and severity of acute rejection and increases graft survival, without influencing delayed graft survival. ATG appeared beneficial due to a sustained depletion of immunocompetent cell populations.[155] In 2013, Cicora et al. found no significant differences between ATLG and ATG. Indeed, the preferential use of one of the two products does not influence rejection rate, delayed graft function, infections or malignancies compared to the other.[156] The ELITE-Symphony study set as a new standard a quadruple therapy in the form of IL2-RA-based induction with mycophenolate, corticosteroids and low-dose tacrolimus.[157] Nevertheless, the Harmony study confirmed that induction based on IL2-RA or on ATG are equipotent in low-immunological-risk population while in high-immunological-risk recipients, ATG is superior.[158]

Thymoglobulin

In 2001, Eason et al. demonstrated in an RCT that an induction with ATG without steroids is superior to steroid-based induction in terms of rejection, metabolic complications, HCV recurrence, and CMV infection. In particular, rejection episodes responded more easily to simple tacrolimus increase in the ATG group. All patients received a double maintenance including tacrolimus and mycophenolic acid. Significantly, 78% of patients in the study group were successfully weaned off mycophenolate after three months while tacrolimus monotherapy with stable function was attained only in 63% of the glucocorticoid group.[159]

In 2005, Bogetti et al. reported the results at three months of a small RCT (22 cases) exhibiting similar rejection rates, patient and graft survival between patients receiving ATG as induction or not.[160]

In 2007, Soliman et al. published a retrospective comparison of patients who received immediate ciclosporin and patients who received an induction with ATG and ciclosporin delayed for three days. The former group exhibited a better renal function after one year. In addition, the rate of rejection within the first year was halved in the ATG group compared to the control group.[161]

In 2009, Boillot et al. published an RCT that compared an ATG induction (44 subjects) with no induction (49 subjects). The maintenance regimen included tacrolimus, mycophenolate and glucocorticoids. Apart from excess leukopenia rate, ATG did not induce differences in terms of survival, number of acute rejection episodes, or liver function, within five years after LT.[162]

In 2011, Uemura et al. authored a retrospective paper that compared different induction regimens: ATG alone, ATG and steroids, daclizumab alone, or steroid alone. ATG appeared overall detrimental for HCV recipients in terms of renal function, and patient and graft survival but yielded comparable results in the HCV-negative population.[163] However, the literature is inconclusive on this score. In a paper authored by Horton et al. in 2005, ATG induction had no influence on graft and patient survival in subjects who underwent LT for HCV.[164] In 2006, Nair et al. assessed the severity of recurrent HCV infection in a subgroup of patients who were included in an RCT comparing ATG-rapid steroid withdrawal with standard steroid-containing immunosuppression. The authors showed that the strategy in study did not exert any negative influence on HCV recurrence in hepatitis C patients after LT.[165,166] In 2007, in a non-randomised study by Humar et al., early steroid withdrawal seemed to reduce HCV recurrence and de novo diabetes.[167] In 2014, Garcia-Saenz-de-Sicilia et al. proved in an RCT that HCV recipients exhibited a comparable patient and graft survival after ATG or no induction while ATG reduced HCV recurrence from 73.9% to 26.9% (p=0.001). Rejection, infections and malignancies were not significantly impacted.[137]

More recently, Mangus et al. compared, in a retrospective study published in 2012, three induction regimens: ATG with the first dose during surgery (n=166), ATG

with the first dose delayed until 48 hours post-LT (n=259), and ATG with the first dose delayed until 48 hours post-LT and one-shot basiliximab on the third postoperative day (n=588). Maintenance immunosuppression consisted of tacrolimus monotherapy. Results were comparable in terms of one- and five-year patient and graft survival, acute rejection, de novo malignancies and infections.[168]
In 2015, Halldorson et al. showed that an ATG-based induction was associated with a reduced risk of fibrosis and biliary strictures compared to basiliximab-based induction in patients who received a graft from a donor after cardiac death (DCD). Lymphocyte depletion may interfere with the inflammation and fibrosis response following warm ischaemia, resulting in a decrease in biliary complications.[169] In the same year, Yoo et al. published a prospective uncontrolled study including 500 patients who received ATG induction and minimised immunosuppression without corticosteroids. The work appeared to confirm the fair results that can be obtained on such a regimen in terms of patient and graft survival, rejection rate, renal function, de novo diabetes and HCV-related liver disease recurrence.[170]
Interestingly, in the quest for operational tolerance, Donckier et al. attempted immunosuppression withdrawal in a pilot uncontrolled study published in 2013. Their strategy included an ATG-based induction and a maintenance treatment based on sirolimus to be weaned off by the fourth month in ten LT recipients. The protocol failed the objective and this was correlated to the expansion of releasing memory $CD8^+$ T cells and an increased release of IFN-γ and IL-17.[171]

Grafalon

Experimental evidences suggested that the use of anti-T-lymphocyte immunoglobulin (ATLG) comes with multiple perks in the context of allogeneic transplantation. In addition to $CD3^+$ $CD4^+$ T cell depletion, ATLG modifies the immune response at several levels. ATLG suppresses the activity of T and NK cells. It increases the production of indoleamine 2,3-dioxygenase (IDO) mRNA in dendritic cells, allowing the catabolism of tryptophan. This effect inhibits T-cell

activation to an extent comparable to that of ciclosporin.[148,172] IDO fosters $CD4^+$ $CD25^+$ regulatory T cells (Tregs) and myeloid-derived suppressor cells, favouring a tolerogenic environment.[173] ATLG stimulates the crucial transcription regulator called scurfin or forkhead box P3 (FOXP3), which determines a down-regulation in inflammatory cytokine release and switches naïve T cells to Tregs,[174] although the duration of this effect is unclear.[175] ATLG is thus assumed to stimulate host tolerance to allograft in animal models.[176]

To date 27,362 human studies and 330 reviews concerning depleting antibodies have been published, all disciplines combined. Of these, 1,249 articles reported on LT, 6,598 on KT, and 480 on combined liver and KT. Upon restriction for LT and controlled trials, 101 studies have been published, ten of which included ATLG.[177] Three studies are not helpful to the purpose of this short review because they compared glucocorticoid withdrawal with standard administration, and all subjects received either ATG or ATLG without further details.[178,179] Two articles report on the same RCT comparing an IL2-RA with ATLG. The authors found out that the patients who received IL2-RA experienced fewer episodes of acute rejection and of early complications, but patient and graft survival were comparable over three years.[180,181] Four articles, by the same Berliner group, report the results over 12 years of an RCT assessing a study group receiving tacrolimus and steroids against a control group receiving their standard quadruple regimen (induction with ATLG and maintenance with ciclosporin, steroids and azathioprine). Although the results were roughly comparable up to five years, later on the arm on tacrolimus-steroids showed a decreased rate of graft loss. The authors noticed extra deaths for de novo tumours in the control group and attributed the difference to the use of ATLG. Nonetheless, it should be noted that the control group was treated according an old-fashioned heavy protocol, which limits the current applicability of those assumptions.[182-185]

Several other uncontrolled or nonrandomised reports are available. In 2000, Fischer et al. investigated the use of ATLG in combination with tacrolimus and

corticosteroids in only 14 patients, with a follow-up of one year, documenting the absence of acute rejection episodes, fatalities or graft losses.[186] In 2002, Oertel et al. compared, in a nonrandomised controlled trial, a quadruple immunosuppressive regimen including prednisone, ciclosporin, azathioprine and an ATLG-based induction (5 mg/kg, n=20) to standard prednisone, and ciclosporin, (n=15). No significant differences were found in terms of biopsy-proven acute rejection (indeed frequent: 46% of the whole sample), severe infections or patient survival. The study confirmed a sustained decrease in $CD4^+$ T cells in the ATLG group, for at least two years.[187]

More recently, Benítez et al. published in 2010 the only RCT about induction with ATLG (9 mg/kg) followed by a low-dose tacrolimus monotherapy. The protocol included few patients (intention-to-treat analysis ATLG n=21, control n=16, post-hoc analysis ATLG n=12, control n=13) and called for rapid immunosuppression withdrawal, starting as early as three months after LT. The definition of rejection unfortunately included biopsy-proven and -unproven episodes. Acute rejection rate within three months appeared higher in the study arm than in the control group (52.4% vs. 25%, p=0.09) and was overtly greater after three months (61.9% vs. 6.2%, p=0.001). The study was aborted. Each patient who received ATLG and was weaned off immunosuppression developed acute rejection. Although ATLG allowed for lower tacrolimus doses, lower tacrolimus trough levels, and lower accumulated doses of steroids, the authors did not detect clinical benefits in terms of immunosuppression adverse effects and no patient was able to achieve the main goal of the study: tacrolemia <5ng/mL without rejection by one year. The authors postulated that ATLG promoted the generation and expansion of alloantigen-specific $CD4^+$ $FOXP3^+$ Tregs but failed in suppressing memory T cells, obstinate rejection-drivers. They surmised that operational tolerance, defined as stable organ function without immunosuppression, or *prope* tolerance, defined as stable organ function on infratherapeutic immunosuppression, is more of a grey-scale

phenomenon and that a steep withdrawal of maintenance immunosuppression cannot be carried out without major immunological risks.[188]

In conclusion, this concise review of the literature points to the absence of RCTs carried out on a large sample about the topic of induction based on depleting agents. As a result, there is currently no consensus on the use of induction therapy in LT. Many questions about ATLG remain unanswered, including the risk-benefit balance, the correct dosage, the time to introduce and, possibly, withdraw maintenance immunosuppression, and the best combinations of immunosuppressive drugs.

The aim of the first part of the thesis

The general aim of this study was to define whether the induction, by means of a single intraoperative high-dose of ATLG (9 mg/kg), favours clinical operational tolerance or *prope* tolerance in LT.

The first endpoint was the proportion of patients on minimised immunosuppression one year after LT. A previous experience from the same working group showed that stable liver function without rejection can be obtained on tacrolimus monotherapy in about 90% of adult recipients after one year from LT.[103] We hypothesised that a substantial induction with depleting antibodies can raise this proportion to 99% of LT recipients. We meant to accept a 5% chance of incurring in Type I error and a 20% chance of Type II error, when rejecting the null hypothesis under the alternative hypothesis. We thus calculated that we needed 200 patients, evenly distributed between the study and the control group.[189]

The secondary endpoints were the proportion of histological TCMR-A diagnosed at day-seven biopsy, the incidence and severity of TCMR-A requiring steroid treatment and of TCMR-A resistant to steroid treatment, one-year graft and patient survival, and the evolution of kidney function.

First work: Tacrolimus and single intraoperative high-dose of anti-T-lymphocyte globulins versus tacrolimus monotherapy in adult liver transplantation: one-year results of an investigator-driven randomized controlled trial

Tacrolimus and Single Intraoperative High-dose of Anti-T-lymphocyte Globulins Versus Tacrolimus Monotherapy in Adult Liver Transplantation

One-year Results of an Investigator-driven Randomized Controlled Trial

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Objective: The aim of the study is to evaluate whether intra-operative induction with anti-lymphocytic serum (ALS) is superior to no induction in adult liver transplantation (LT).

Background: The efficacy of ALS induction remains inconclusive in LT, because of poorly designed trials.

Methods: A randomized controlled trial was conducted, including 206 adults (>15 years) and comparing tacrolimus monotherapy (TAC, n = 109) and tacrolimus plus a single, intraoperative, high-dose (9 mg/kg), rabbit anti-T-lymphocyte globulins (ATLG; n = 97). All patients had similar follow-up, including Banff-scored biopsies. Rejection was considered clinically relevant and treated if pathologic and biochemical changes were concordant. The primary endpoint was immunosuppression minimization to monotherapy; secondary endpoints were biopsy-proven rejection, clinical rejection, patient (PS) and graft (GS) survival.

Results: At 1 year, 79/81 (96.3%) ATLG and 101/102 (99.0%) TAC patients were steroid-free ($P = 0.585$); 28 (34.6%) ATLG, and 31 (30.4%) TAC patients were on double-drug immunosuppression ($P = 0.633$). One-year PS and GS of ATLG and TAC patients were 84% and 92% ($P = 0.260$) and 76% and 90% ($P = 0.054$).

Despite significantly a fewer day-7 moderate-to-severe acute cellular rejections (ACR) in ATLG group (10.0% vs 24.0% in TAC group, $P = 0.019$), cumulative proportion of patients experiencing steroid-sensitive (11.3% ATLG vs 14.7% TAC, $P = 0.539$), steroid-resistant (2.1% ATLG vs 3.7% TAC, $P = 0.686$) and chronic rejection (1.0% ATLG vs 0.9% TAC, $P = 1.000$) were similar. ATLG administration brought about greater hemodynamic instability and blood products use ($P = 0.001$).

Conclusions: At 1 year from LT, ATLG induction did not significantly affect immunosuppressive load, treated rejection, patient, and graft survival. The observed adverse events justify a modification of dosing and timing of ATLG infusion. Long-term results are required to judge the ATLG possible benefits on immunosuppressive load and tolerance induction.

Keywords: anti-T-lymphocyte globulins, graft survival, immunosuppression, induction, liver transplantation, patient survival, rejection, tacrolimus

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Major progress in surgery, perioperative care and immunosuppressive therapy has raised 1- and 5-year survival after liver transplantation (LT) to 90% and 75%.[1,2] Initial immunosuppression consisted of a combination of steroids and azathioprine. From 1967 on, anti-lymphocytic serum (ALS) and antibodies have been added as steroid- and renal-sparing agents.[1] The concept of induction immunosuppression was challenged by the introduction of the efficient calcineurin inhibitors (CNIs).[2] The 2014 Cochrane meta-analysis concluded that neither prophylactic nor therapeutic use of ALS or antibodies altered graft survival.[3] The results of the large (>1000 patients), uncontrolled, Indianapolis and Memphis LT series, obtained with ALS-based immunosuppression, and the discovery of chimerism, renewed the interest for induction.[4–9] Randomized controlled trials (RCT) in LT assessing ALS-based induction are rare.[3] This investigator-driven RCT comparing tacrolimus-based immunosuppression, with and without administration of a single, high-dose, rabbit anti-T-lymphocyte globulins (ATLG, Grafalon, Neovii Biotech, Gräfelfing, DE) was designed to evaluate immunosuppressive load, incidence of rejection, patient and graft survival, in a large adult LT cohort. The 1-year results are reported here.

MATERIALS AND METHODS

Trial Design, Randomization, Study Setting

This investigator-driven, single-centre, prospective, phase-III clinical RCT was registered in 2006 under EudraCT number 2006–004830–34. The study was single-blinded. The allocation was hidden from participants because the study drug was administered intraoperatively. Double blinding was not considered due to the production costs of an ATLG-alike solution.

Our group showed that tacrolimus monotherapy at 12 months is feasible in 90% of recipients.[10] We postulated that an induction with high-dose ATLG helps raise this proportion without endangering graft survival. Accordingly, 200 patients (100/arm) were required to detect a 9% increase in the percentage of patients on tacrolimus monotherapy at 1 year after LT, accepting a power of 80% and 5% level of α error. The final sample size was increased by 5% to allow for possible dropouts.[11]

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During the period October 1, 2007 to October 31, 2016, the adults (>15 years) undergoing a primary LT were assessed for enrollment before surgery. Recipients were 1:1 randomized, independently of cross-match, hepatitis B viral (HBV) status, sickness status and presence of artificial organ support at moment of LT, using serial concealed envelopes. A computerized random number generator produced the sequence of randomization, taking into account hepatitis C viral (HCV) status.

Exclusion criteria comprised previous or multi-organ transplantation, re-transplantation, advanced (stage IV–AJCC-UICC) hepatocarcinoma, ABO incompatibility, steroid-treated autoimmune hepatitis, HIV infection, leucocyte count $<1500/mm^3$, and inclusion in another trial.

The protocol included also lymphocytic subpopulations typing and cytokines profiling, at baseline and 7, 28, 180, and 365 days (data not shown because analysis is underway).

Events and results were classified following the European Liver Transplantation Registry criteria as "early" (≤3) and "late" (>3 months).

The ethics committee of the Université Catholique de Louvain approved the protocol.

Participants and Interventions

Eighty of 352 patients were not eligible for enrollment and 23 declined participation. Thirty-seven recipients were not included because of enrollment interruption, from September 12, 2014 to January 05, 2015, because of logistic reasons making immunological profiling impossible. Altogether, 212 participants were randomized to tacrolimus monotherapy (TAC group, n = 107) or to tacrolimus plus induction with a single, intraoperatively administered, high dose (9 mg/kg) of ATLG (ATLG group, n = 105) (Supplementary Figure 1; http://links.lww.com/SLA/B487). All patients received 400 mg hydrocortisone to reduce ischemia-reperfusion injury as well as acetaminophen and dexchlorpheniramine to prevent reactions to ATLG. Half the dose was given at the beginning of anaesthesia, the other half after allograft revascularization. Supplementary hydrocortisone (200 mg/day for 3 days) was administered postoperatively. Initial maintenance immunosuppression consisted of tacrolimus monotherapy (0.025 mg/kg/day, Prograft, Astellas Pharma, Tokyo, Japan), from 12 hours after surgery. Later, the daily dose was adapted by targeting trough levels of 4 to 6 ng/mL. In all patients, subsequent steroid use was restricted to rejection treatment.

After enrollment, 3 ATLG and 1 TAC patients, not complying with inclusion criteria, and 2 ATLG patients, not transplanted, were excluded from analysis. Three cases presenting a severe skin-test allergic reaction were relocated to TAC group. Overall, 109 TAC and 97 ATLG patients were analyzed.

As previously described, all patients received similar perioperative anti-infectious prophylaxis and follow-up.[10] All patients signed an informed consent after extensive and repetitive information. The minimum follow-up was 1 year for all patients.

Endpoints

The primary endpoint was the attainment of stable liver function (defined as up to 1.5 normal liver test values) with tacrolimus monotherapy, in absence of rejection. Secondary endpoints were incidence of biopsy-proven acute cellular rejection (ACR), based on per-cause and per-protocol day-7, 180, and 365 biopsies, clinical (ie, requiring treatment) rejection, PS and GS. Histologic specimens were blindly scored, following Banff criteria, by an experienced pathologist (MK).[12] Moderate and severe ACR equaled a score of ≥6 and ≥8. A biochemical score, based on the combination of progressive rise in total bilirubin and eosinophilia, platelets reduction, and absolute eosinophilia count $> 600/mm^3$, during postoperative days 5 to 7, was taken into account.[10,13,14] To avoid interferences with biochemical parameters, no blood products or other medications were administered within the first week. Rejection was considered clinically relevant and treated only if biochemical (>2) and histologic (≥6) scores were concurrently present.[10,13,14] ACR treatment consisted of administration of steroid pulses and, if required, antibodies. A rejection was defined as steroid-sensitive in case of response to 3-to-5 oral or IV boluses of 200 mg methylprednisolone. Steroid-resistant ACR was treated with a 10-day course of muromonab-CD3 (Orthoclone OKT3, Janssen Pharmaceutica, Beerse, Belgium) or ATLG (5 mg/kg). Further immunosuppression handling was blindly adapted to clinical evolution, as observed at the outpatient clinic.

Survival rates were separately analyzed for the whole patient group, the group without artificial organ support at LT and the HCV-positive group. Participation in the study was discontinued after graft loss.

Safety endpoints included hemodynamic instability at first half-dose administration, coagulopathy, blood-product requirement, serum sickness, renal function, glucose tolerance, cholesterolemia, arterial hypertension, body mass index, biliary complications, infections, and de novo tumor formation within 12 months.

Statistical Methods

All variables were tested for normality with the Kolmogorov-Smirnov test. As only a few variables resulted normally distributed, continuous data were reported as medians and interquartile ranges and tested with the Mann-Whitney *U* test. Binomial variables were reported as percentages and tested with Fisher exact test. Time to clinical rejection, PS and GS were analyzed with the Kaplan-Meier method and compared with the log-rank test. The significance of statistical tests was taken at a $P < 0.05$. Analyses were run using SPSS (version 23.0; IBM Corp., Armonk, NY).

RESULTS

Patients' Characteristics

The randomization process generated 2 generally homogeneous groups (Supplemental Digital Content, http://links.lww.com/SLA/B456).

Immunosuppression

Immunosuppression was recorded at 3, 6 (data not shown), and 12 months. At 1 year, 53/81 (65.4%) ATLG and 71/102 (69.6%) TAC patients had a stable graft function on single-drug immunosuppression ($P = 0.633$). Seventy-nine of 81 (96.3%) ATLG patients were steroid-free, compared with 101/102 (99.0%) TAC patients ($P = 0.585$). These results were obtained under progressive tacrolemia lowering (Table 1). In 28/81 (34.6%) ATLG and 31/102 (30.4%) TAC patients, immunosuppression required adaptation during the first post-LT year to their clinical evolution. Different double-drug immunosuppressive combinations ($P = 0.633$) were chosen to handle renal failure (20 vs 14), oncologic progression (0 vs 6), noncompliance (2 vs 2), neurotoxicity (2 vs 2), de novo autoimmune hepatitis (1 vs 2), diabetes (0 vs 1), recto-colitis (0 vs 1), acute (2 vs 2), and chronic rejection (1 vs 1).

Histologic and Clinical Rejection

Day-7 biopsies showed a significantly lower incidence of moderate-to-severe ACR in the ATLG group [8/80 (10.0%) vs 25/104 (24.0%) in TAC group, $P = 0.019$]. During the first three months, incidences of steroid-sensitive [10/97 (10.3%) ATLG vs 13/109 (11.9%) TAC patients, $P = 0.826$] and of steroid-resistant rejection [2/97 (2.1%) ATLG vs 3/109 (2.8%) TAC

TABLE 1. Immunosuppressive Load at 3, 6, and 12 Months After Liver Transplantation

	TAC (n = 109)	ATLG (n = 97)	
	Med (IQR) or n (%)		*P*
First week			
Tacrolimus trough levels, ng/mL	6.0 (4.4–7.6)	5.8 (4.1–7.3)	0.645
3 months			
Tacrolimus per day, mg/kg	0.05 (0.03–0.07)	0.04 (0.03–0.07)	0.868
Tacrolimus trough level, ng/mL	5.9 (4.0–7.5)	5.8 (3.4–8.2)	0.801
Infratherapeutic tacrolemia (<6 ng/mL)	54/104 (51.9)	44/82 (53.7)	0.883
Tacrolimus alone	73/104 (70.2)	50/84 (59.5)	0.165
Tacrolimus and MMF	26/104 (25)	28/84 (33.3)	0.256
Tacrolimus and azathioprine	0/104 (0.0)	3/84 (3.6)	0.087
Tacrolimus and steroids	1/104 (1.0)	1/84 (1.2)	1.000
Tacrolimus and sirolimus	0/104 (0.0)	1/84 (1.2)	0.447
MMF and sirolimus	2/104 (1.9)	0/84 (0.0)	0.503
Tacrolimus, steroids, MMF	2/104 (1.9)	1/84 (1.2)	1.000
Single-drug immunosuppression	73/104 (70.2)	50/84 (59.5)	0.165
Multiple-drug immunosuppression	31//104 (29.8)	34/84 (40.5)	
12 months			
Tacrolimus per day, mg/kg	0.03 (0.01–0.05)	0.03 (0.02–0.05)	0.264
Tacrolimus trough level, ng/mL	4.8 (3.4–6.3)	4.4 (3.3–6.3)	0.904
Infratherapeutic tacrolemia (<6 ng/mL)	72/102 (70.6)	57/81 (70.4)	1.000
Tacrolimus alone	68/102 (66.7)	53/81 (65.4)	0.876
Sirolimus alone	1/102 (0.9)	0/81 (0.0)	1.000
Everolimus alone	1/102 (0.9)	0/81 (0.0)	1.000
MMF alone	1/102 (0.9)	0/81 (0.0)	1.000
Tacrolimus and MMF	23/102 (22.5)	23/81 (28.4)	0.394
Tacrolimus and azathioprine	2/102 (1.9)	2/81 (2.5)	1.000
Tacrolimus and steroids	1/102 (1.0)	2/81 (2.5)	0.584
Tacrolimus and sirolimus	4/102 (3.9)	0/81 (0.0)	0.131
Tacrolimus and everolimus	1/102 (1.0)	0/81 (0.0)	1.000
MMF and sirolimus	0/102 (0.0)	1/81 (1.2)	0.443
Single-drug immunosuppression	71/102 (69.6)	53/81 (65.4)	0.633
Multiple-drug immunosuppression	31/102 (30.4)	28/81 (34.6)	

IQR indicates interquartile range; MMF, mycophenolate mofetil.

patients, $P = 1.000$] were similar. At 1 year, the incidence of steroid-sensitive rejection was 11.3% (11/97) in ATLG versus 14.7% (16/109) in TAC group, $P = 0.539$, whereas steroid-resistant rejections occurred in 2.1% (2/97) ATLG versus 3.7% (4/109) TAC patients, $P = 0.686$ (Supplementary Table 1; http://links.lww.com/SLA/B487 and Fig. 1), according to the practice of treating histologic ACR (Banff score ≥6) only in case of concurrent major biochemical alterations (Bio-score >2).[13,15] During the first year, we detected 2 cases of chronic rejection, because of noncompliance, one per group.

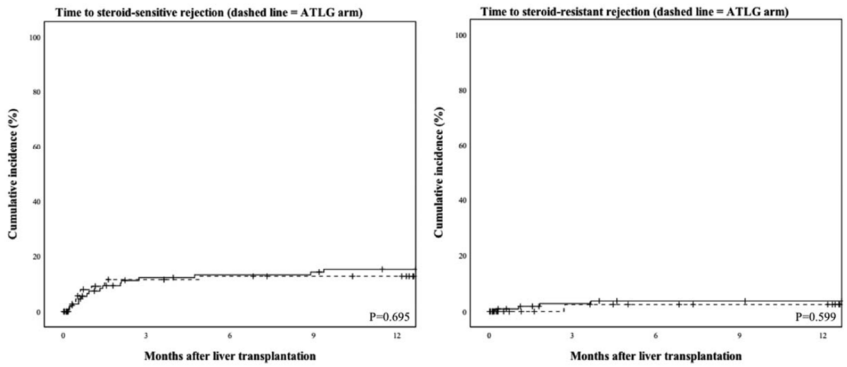


FIGURE 1. Time to steroid-sensitive and steroid-resistant rejection.

Graft and Patient Survival

At three and 12 months, PS rates in ATLG and TAC groups were 87.6% (85/97) and 83.5% (81/97) and 95.4% (104/109) and 91.7% (100/109), $P = 0.269$. Early ATLG mortality (≤3 months) was 12.4% (12/97) and 4.5% (5/109) in TAC group, $P = 0.073$; late mortality (3–12 months) 4.7% (4/85) and 3.8% (4/104), $P = 1.000$. Causes of early death were, in the ATLG group, surgical bleeding (n = 2), coagulopathy (n = 2), sepsis (n = 2), bleeding due to hereditary telangiectasia (n = 1), pneumonia (n = 1), gastric perforation (n = 1), post-ATLG cardiocirculatory failure (n = 2), and, in the TAC group, were urosepsis (n = 1), constrictive pericarditis (n = 1), pulmonary aspiration (n = 1), humoral rejection (n = 1) and OKT3-induced cytokine-release syndrome (n = 1). Late ATLG deaths followed, once each, postbleeding multiorgan failure, posterior reversible encephalopathy syndrome, cerebral abscess, liver abscess and fungal sepsis; in the TAC group, late mortality resulted from liver abscess (n = 2), tumor recurrence (n = 1), and sepsis (n = 1) (Fig. 2).

Three and 12-month GS rates in ATLG and TAC groups were 82.4% (80/97) and 76.3% (74/97) and 93.5% (102/109) and 89.9% (98/109) ($P = 0.054$). Early ATLG graft loss was 17.5% (17/97) and 6.4% (7/109) in TAC group, $P = 0.016$; late graft loss was 7.5% (6/80) and 3.9% (4/102), $P = 0.339$. Causes of early ATLG graft loss were death (n = 12), arterial dissection caused by procurement lesion (n = 1), small-for-size syndrome (n = 1), hepatic artery thrombosis (n = 1), and biliary tract necrosis (n = 1). In TAC group, early graft loss was caused by death (n = 5), hepatic artery thrombosis (n = 1), and pseudo-aneurysm (n = 1). Three late graft losses for ischemic-type biliary lesions (ITBL) occurred in ATLG group. A subanalysis for cardiac- and brain-dead donors did not show differences in ITBL distribution between groups.

In patients not on life support at LT or transplanted for HCV-related cirrhosis, PS and GS proved comparable with the whole population (data not shown).

One-year death-censored GS was lower in the ATLG group (89%) compared with the TAC group (98%), $P = 0.011$.

ATLG Safety

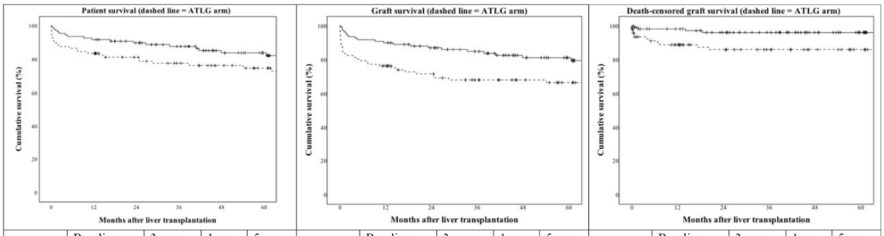
Four (4.1%) of 97 ATLG patients had a minor skin-test reaction. ATLG patients had a significantly greater need for intraoperative blood products ($P = 0.001$), ventilatory support ($P = 0.028$) and intensive care unit (ICU) stay ($P = 0.003$) (Supplementary Table 1; http://links.lww.com/SLA/B487). ATLG impacted ischemia-reperfusion injury, with significantly higher first-week total bilirubin ($P < 0.001$), AST ($P < 0.001$), peak AST ($P = 0.007$), and γGT ($P < 0.001$) levels. Eighty (82.5%) ATLG patients had accurate hemodynamic data recording after administration of the first half dose. Six (7.5%) presented an arrhythmia and 14 (17.5%) a 50% blood pressure drop. Two (2.1%) ATLG recipients presented, after an uneventful procedure, major hemodynamic instability leading to perioperative death. Two patients (2.1%) developed serum sickness and both rapidly recovered after steroids administration.

DISCUSSION

Potent immunosuppressive drug combinations, improved surgery and standardized perioperative care contributed to the major improvement of early results after LT.[6] However, long-term results remain compromised by the (small but unresolved) problem of chronic allograft rejection and, chiefly, by the high morbidity and mortality linked to the chronic immunosuppression.[2,6,12] Many recipients still die with functioning grafts, because of metabolic (40%), cardiovascular (20%), renal (15%), infectious, and oncologic complications.[16,17]

It has been recently observed that rejection and tolerance represent stages of a same continuum and that extended organ engraftment under conventional immunosuppression can be interpreted as a manifestation of partial tolerance. These observations have shed a different light on the immunosuppressive handling of liver recipients.[5,6,18,19] Accordingly, it has been postulated that the combination of the principles of pretreatment or induction with a minimized immunosuppression could bring the recipient to a complete state of tolerance of donor tissues, based on the clonal exhaustion-deletion process.[4–6,12,20]

Several ALS and antibodies, initially introduced as renal- and steroid-sparing agents, were progressively used not only as prophylactic and therapeutic anti-rejection treatments, but also as tolerance-inducing drugs. Starzl first used induction immunosuppression, aiming to reduce lymphocytic load and to promote the clonal exhaustion-deletion process. A further strategy is the immunosuppression minimization from LT,[4–6] allowing a vigorous clonal exhaustion-deletion process under a "soft-immunosuppression umbrella."[4,18] In contrast to complex immunologic manipulations, both approaches are easy to apply in tolerance induction protocols.[19,21,22] The excellent results with CNI-based immunosuppression, along with the implementation of hundreds of (mostly industry-driven) studies, unfortunately reduced the interest for



	Baseline	3 mos.	1 yr.	5 yrs.
Patient survival (%)				
ATLG	100	88	84	76
TAC	100	95	92	84
No of patients exposed to risk of death				
ATLG	97	97	97	49
TAC	109	109	109	55
P=0.269				

	Baseline	3 mos.	1 yr.	5 yrs.
Graft survival (%)				
ATLG	100	82	76	66
TAC	100	94	90	81
No of patients exposed to risk of graft loss				
ATLG	97	97	96	43
TAC	109	109	109	54
P=0.054				

	Baseline	3 mos.	1 yr.	5 yrs.
Death-censored graft survival (%)				
ATLG	100	93	89	86
TAC	100	98	98	96
No of patients exposed to risk of graft loss				
ATLG	97	92	91	43
TAC	109	107	105	54
P=0.011				

FIGURE 2. Patient and graft survival of the whole study population.

induction strategies and led to inconsistencies in immunosuppression handling and disregard for long-term outcomes.[15,20,23] Extensive literature review revealed that well-designed, investigator-driven RCTs, dealing with induction immunosuppression in LT, are scarce and often contain small cohorts.[3,4,7,8,24–27] Investigator-driven studies better adhere to daily clinical practice and overcome the restrictions on inclusion (for the selection of the best recipients and donors, and their pairing) and on handling of the investigated immunosuppressant (with the obliged adhesion to narrow therapeutic ranges).[10,15,20,23,28–31]

The excellent results of tacrolimus monotherapy suggested the idea of merging immunosuppressive minimization and induction, in order to raise the proportion of patients with low immunosuppressive load and, eventually, to favor clinical operational tolerance in the long run.[4–6,10,29,32] Induction can be performed by administering either a full 3-to-10-day course or a short course of ALS. Commonly, the polyclonal anti-thymocyte (Thymoglobuline, Genzyme, Naarden, the Netherlands) and anti-lymphocyte globulins are used. Both are strong lymphocyte-depleting agents, which allow immune reconstitution and stimulation of regulatory T-cells.[24,33–35] They reportedly attenuate ischemia-reperfusion injury and protect the bile duct epithelium.[32,35–41]

Thus, we chose a single-dose induction with ATLG, based on their higher impact on activated T-cells, and reduced promotion of CMV infection and de novo tumors (including lymphoproliferative disease) and considering large, favorable, previous experiences in kidney transplantation.[41,42] The different ATLG dose-dependent activity accounts for the higher dose (9 mg/kg) required to equal 4.5 to 5 mg/kg of anti-thymocyte globulins.[43–49]

ATLG induction did not affect one-year immunosuppressive load or clinical rejection rate. While about 30% of patients in both groups were on double immunosuppression, 1 year after LT, only a minority of them (10%) needed a second immunosuppressant because of immunologic reasons (previous severe rejection or histologic features of chronic rejection at per-protocol biopsy).

Although ATLG induction significantly reduced day-7 Banff score, the incidences of treated steroid-sensitive, steroid-resistant and chronic rejection were similar. Indeed, our strict approach towards ACR ensured that pharmacological treatment was envisaged only in case of simultaneous biochemical and pathologic disturbances. This attitude is at variance with the design of many industry-driven trials, commonly meant to prove immunologic superiority of a drug in terms of rejection, based either on liver biopsy findings or clinical suspicion, only. Clearly, superior properties of an immunosuppressant can be adequately appraised only by combining biopsy-proven acute and chronic rejection and long-term GS and PS.[13,15,30]

This study was all-inclusive and enrolled consecutive patients, independently of their sickness state. Regardless of organ support, patient and graft survival were not significantly lower in the ATLG arm, whereas death-censored GS was significantly worse after induction. PS and GS in HCV-positive recipients were not roughly affected.

ALS induction has been reported to cause cardiopulmonary problems (hemodynamic instability, cardiac arrhythmia, oxygen desaturation, and respiratory distress syndrome), gastro-intestinal disorders (nausea, vomiting), fever, anaphylactic shock (to carbohydrate excipient), renal failure, coagulopathy (fibrinolysis, bleeding), hematological disorders (leucocyte and thrombocyte depletion, hemolysis), and serum sickness.[4,50–53] Most of these side effects occurred in this study, along with an exacerbated ischemia-reperfusion injury. ATLG patients experienced a greater need for blood products and early relaparotomy for bleeding (events behind prolonged ventilation, ICU stay and ischemia/reperfusion injury). Two recipients died possibly for a cytokine-release syndrome and the subsequent irreversible hemodynamic failure, in the absence of bleeding. After perioperative complications, early graft loss was more frequent in the study group.

The abovementioned adverse events are clearly underreported in the literature, probably because the authors focus on rejection rates, PS and GS. Only Kyllonen[43] and De Pietri[52] described in detail the ALS perioperative side-effects, in 53 renal and 16 liver recipients. Our own observations and the scarce relevant literature should prompt a modification in ALS dosing (towards lower doses) and timing (with earlier administration and longer infusion time), in the safer ICU environment both before and after implantation of the allograft, whereas severe hyperthermia and cardiovascular dysfunction should lead to its interruption.[43,50–52,54]

The early results of this study show that a single-dose short-course induction does not confer advantages in relation to immunosuppressive load, clinical rejection, patient and graft survival, during the first posttransplant year, and that there are concerns about safety. However, these early "negative" results might be counteracted by (what should be considered as the real advantage of induction IS) long-term immunologic benefits, in terms of lower immunosuppressive load, more frequent operational tolerance, and less graft losses for chronic rejection. To confirm this hypothesis, long-term (5-year) follow-up, including liver biopsies, is required.

CONCLUSIONS

This investigator-driven RCT in adult LT, comparing tacrolimus monotherapy and a high-dose, intraoperatively administered, ATLG induction plus tacrolimus, could not demonstrate a benefit in relation to immunosuppressive load nor clinically relevant rejection during the first postoperative year, although the Banff scores of day-7 protocol biopsies were significantly lower. Treatment of ACR was restricted to patients having concordant biochemical and pathologic findings. Patient and graft survival rates were, although not significantly, lower in the ATLG group. The presence of serious adverse events should foster a modification in timing and dosing of ATLG administration. Five-year follow-up, including histology, is required to evaluate the possible impact of induction on long-term immunosuppressive load and operational tolerance.

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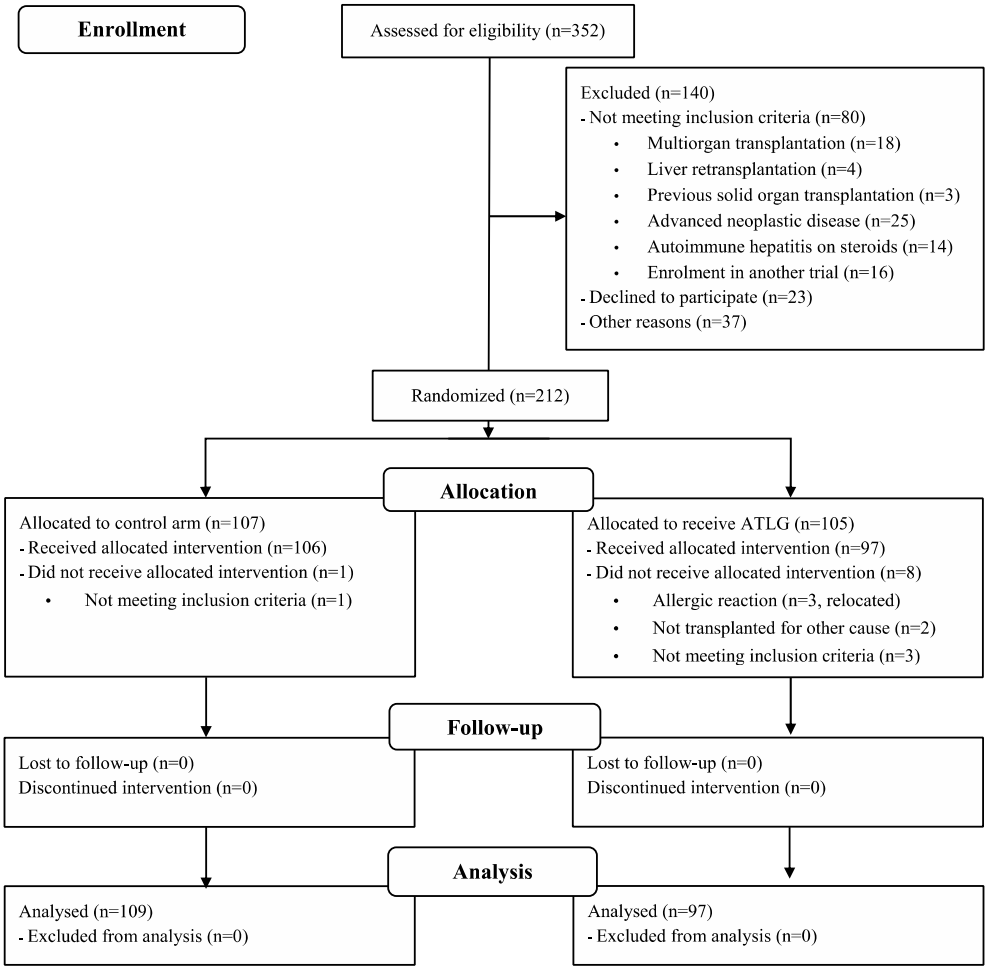
Supplementary Figure 1: Flow diagram of enrollment in the RCT comparing ATLG plus tacrolimus with tacrolimus.



Supplementary Table 1: Baseline characteristics of ATLG and TAC cohorts.

Variables	TAC (n=109)	ATLG (n=97)	P
	Med (IQR) or n (%)		
Recipient gender:			
- Male	81 (74.3)	69 (71.1)	0.640
- Female	28 (25.7)	28 (28.9)	
Recipient age (years)	56.6 (46.6-63.6)	57.7 (49.9-63.5)	0.674
Recipient ethnicity:			
- Afro-European	2 (1.8)	6 (6.2)	0.152
- Other	107 (98.2)	91 (93.8)	
Recipient BMI	26.6 (23.6-30.3)	25.8 (23.3-30.5)	0.476
Indication for LT:			
- HCV-associated liver disease	21 (19.3)	22 (22.7)	0.608
- HBV-associated liver disease	5 (4.6)	4 (4.1)	1.000
- Alcoholic liver disease	49 (45.0)	41 (42.3)	0.779
- Non-alcoholic steato-hepatitis	3 (2.8)	4 (4.1)	0.709
- Cryptogenic cirrhosis	3 (2.8)	3 (3.1)	1.000
- Cholestatic liver disease	7 (6.4)	8 (8.2)	0.789
- Autoimmune hepatitis	1 (0.9)	3 (3.1)	0.344
- Metabolic liver disease	9 (8.3)	5 (5.2)	0.419
- Acute liver failure	4 (3.7)	3 (3.1)	1.000
- Hepatocellular cancer	54 (49.5)	48 (49.5)	1.000
- Malignancy (other than HCC)	6 (5.5)	5 (5.2)	1.000
- Benign tumour	11 (10.1)	6 (6.2)	0.448
Donor CMV +/Recipient CMV -	16 (14.7)	16 (16.5)	0.848
Child-Turcotte-Pugh classification			
- A	33/87 (37.9)	36/83 (43.4)	0.533
- B	28/87 (32.2)	19/83 (22.9)	0.230
- C	26/87 (29.9)	28/83 (33.7)	0.624
MELD	13.4 (9.6-18.6)	13.5 (8.7-22.6)	0.940
Transplantation technique:			
- Standard technique	100 (91.7)	84 (86.6)	0.264
- Right split liver	2 (1.8)	1 (1.0)	1.000
- Left split liver	0 (0.0)	1 (1.0)	0.471
- Whole-graft domino	1 (0.9)	1 (1.0)	1.000
- Live-donor right liver	3 (2.8)	4 (4.1)	0.709
- Live-donor left liver	3 (2.8)	6 (6.2)	0.311
Previous locoregional chemotherapy	50 (45.9)	42 (43.3)	0.779

Variables	TAC (n=109)	ATLG (n=97)	P
Previous upper abdominal surgery	23 (21.1)	14 (14.4)	0.275
Splanchnic venous thrombosis	13 (11.9)	11 (11.3)	1.000
TIPSS	9 (8.3)	5 (5.2)	0.419
Type-2 diabetes mellitus	33 (30.3)	24 (24.7)	0.436
Serum Creatinine (mg/dl)	0.88 (0.70-1.20)	0.90 (0.71-1.15)	0.869
eGFR (ml/min/1.73 m^2)	85 (63-118)	84 (62-111)	0.855
Life support at the moment of LT:	7 (6.4)	7 (7.2)	1.000
- Renal replacement therapy	5 (4.6)	3 (3.1)	0.725
- Extracorporeal liver support-albumin dialysis†	3 (2.8)	3 (3.1)	1.000
- Invasive ventilatory support	6 (5.5)	5 (5.2)	1.000
Positive cross-match	10 (9.3)	14 (14.6)	0.281
HLA mismatch	5 (4-5)	5 (4-6)	0.568
Ischemia time (min):			
- Cold ischemia time	517 (405-663)	511 (370-618)	0.660
- Warm ischemia time	42 (36-49)	41 (36-50)	0.590
Donor gender:			
- Male	67 (61.5)	53 (54.6)	0.327
- Female	42 (38.5)	44 (45.4)	
Donor age (years)	51.7 (37.7-61.9)	49.8 (40.7-60.7)	0.850
Donor after cardiocirculatory death	18 (16.5)	17 (17.5)	0.855
Follow-up (months)	54.7 (29.6-80.5)	54.9 (13.5-79.5)	0.289

† Molecular Adsorbent Recirculating System (MARS). Abbreviations: ATLG, anti-T-lymphocyte globulins; BMI, body-mass index; CMV, cytomegalovirus; eGFR, estimated glomerular filtration rate (according to the MDRD formula by Levey AS *et al.* Ann Intern Med. 1999 Mar 16;130(6):461-70.); HCC, hepatocellular cancer ; HLA, human leukocyte antigen; IQR, interquartile range; LT, liver transplantation; MELD, model for end-stage liver disease; TAC, tacrolimus monotherapy control group; TIPSS, transjugular intrahepatic portosystemic shunt.

Supplementary Table 2: Secondary outcomes immediately after liver transplantation and at 3 and 12 months.

	TAC (n=109)	ATLG (n=97)	P
	Med (IQR) or n (%)		
Perioperative outcomes			
IO PRBCs transfusion (ml)	644 (246-1305)	1055 (458-2556)	0.001
IO Prothrombin complex concentrate (ml)	1975 (1009-2800)	2200 (1500-2800)	0.149
IO Fresh frozen plasma (ml)	0 (0-0)	0 (0-1000)	0.001
IO Platelet transfusion (ml)	0 (0-0)	0 (0-24)	0.010
ICU stay (days)	2 (1-4)	3 (2-6)	0.003
Hospital stay (days)	16 (13-28)	20 (15-32)	0.359
Post-LT life support:	38/109 (34.9)	47/97 (48.5)	0.065
- Renal replacement therapy	25/109 (22.9)	30/97 (30.9)	0.210
- Liver support-albumin dialysis	0/109 (0.0)	2/97 (2.1)	0.221
- Prolonged invasive ventilatory support†	29/109 (26.6)	40/97 (41.2)	0.028
Post-LT invasive ventilatory support (hours)	2.0 (0.0-8.0)	9.5 (2.5-48.0)	<0.001
Bleeding requiring transfusion	25/109 (22.9)	39/97 (40.2)	0.010
Bleeding requiring relaparotomy	5/109 (4.6)	14/97 (14.4)	0.017
Day-1-to-10 platelets (*10^3/mmc)	75 (49-122)	44 (27-89)	<0.001
First-week total bilirubin (mg/dl)	2.9 (1.7-5.4)	6.3 (3.4-11.4)	<0.001
First-week AST (IU/l)	114 (66-196)	160 (113-277)	<0.001
First-week AST peak (IU/l)	836 (427-2238)	1183 (611-2281)	0.007
First-week ALT (IU/l)	347 (178-599)	327 (239-650)	0.194
First-week ALT peak (IU/l)	847 (469-1764)	808 (409-1577)	0.863
First-week γGT (IU/l)	189 (114-331)	121 (63-223)	<0.001
First-week eGFR (ml/min/1.73 m^2)	77 (42-120)	60 (32-104)	0.032
Day-7 biopsy-proven ACR (Banff score)	4.5 (3.0-5.3)	3.0 (3.0-5.0)	0.002
• Banff 6-7	25/104 (24.0)	7/80 (8.8)	0.010
• Banff 8-9	0/104 (0.0)	1/80 (1.3)	0.435
• Banff 6-9	25/104 (24.0)	8/80 (10.0)	0.019
Day-7 steroid-sensitive	2/109 (1.8)	4/97 (4.1)	0.423

	TAC (n=109)	ATLG (n=97)	P
rejection			
Day-7 steroid-resistant rejection	1/109 (0.9)	0/97 (0.0)	1.000
3 months			
Total bilirubin (mg/dl)	0.7 (0.5-1.1)	0.9 (0.6-1.6)	0.057
ALT (IU/l)	40 (25-77)	34 (22-83)	0.942
γGT (IU/l)	63 (29-144)	99 (36-165)	0.085
eGFR (ml/min/1.73 m^2)	72 (57-93)	68 (52-88)	0.237
New-onset diabetes*	2/74 (2.7)	3/69 (4.3)	0.672
BMI (kg/m^2)	24.2 (21.0-26.2)	23.4 (20.9-25.9)	0.624
Infections	41/109 (37.6)	49/97 (50.5)	0.069
Viral	4/109 (3.7)	5/97 (5.2)	0.737
• HSV, HZV	2/109 (1.8)	2/97 (2.1)	1.000
• CMV disease	3/109 (2.8)	3/97 (3.1)	1.000
Fungal	4/109 (3.7)	2/97 (2.1)	0.686
• Systemic	2/109 (1.8)	1/97 (1.0)	1.000
• Oral/oesophageal	2/109 (1.8)	1/97 (1.0)	1.000
Bacterial	40/109 (36.7)	47/97 (48.5)	0.092
• Systemic	15/109 (13.8)	19/97 (19.6)	0.267
• Pneumonia	12/109 (11.0)	8/97 (8.2)	0.639
• Urinary tract	9/109 (8.3)	10/97 (10.3)	0.637
• Cholangitis	2/109 (1.8)	5/97 (5.2)	0.258
• Surgical site	9/109 (8.3)	14/97 (14.4)	0.187
Biliary complications§	14/109 (12.8)	22/97 (22.7)	0.069
• Biliary leak	2/109 (1.8)	8/97 (8.2)	0.049
• Ischemic-type biliary tract lesions	2/109 (1.8)	2/97 (2.1)	1.000
• Anastomotic biliary stricture	9/109 (8.3)	10/97(10.3)	0.637
Overall steroid-sensitive rejection	13/109 (11.9)	10/97 (10.3)	0.826
Overall steroid-resistant rejection	3/109 (2.8)	2/97 (2.1)	1.000
Overall chronic rejection	0/109 (0.0)	1/97 (1.0)	0.471
12 months			
Total bilirubin (mg/dl)	0.8 (0.6-1.1)	0.8 (0.6-1.2)	0.733
ALT (IU/l)	28 (19-53)	36 (20-54)	0.391
γGT (IU/l)	62 (29-124)	54 (31-134)	0.810
eGFR (ml/min/1.73 m^2)	74 (60-92)	67 (52-82)	0.018
New-onset diabetes*	2/73 (2.7)	2/65 (3.1)	1.000

	TAC (n=109)	ATLG (n=97)	P
BMI (kg/m^2)	25.9 (22.4-28.7)	25.4 (22.6-28.7)	0.833
Infections	8/102 (7.8)	10/83 (12.0)	0.455
Viral	1/102 (1.0)	4/83 (4.8)	0.175
• HSV, HZV	0/102 (0.0)	3/83 (3.6)	0.088
• CMV disease	0/102 (0.0)	0/83 (0.0)	-
Fungal	2/102 (2.0)	1/83 (1.2)	1.000
• Systemic	0/102 (0.0)	1/83 (1.2)	0.449
• Oral/oesophageal	2/102 (2.0)	0/83 (0.0)	0.503
Bacterial	6/102 (5.9)	5/83 (6.0)	1.000
• Systemic	2/102 (2.0)	3/83 (3.6)	0.658
• Pneumonia	2/102 (2.0)	0/83 (0.0)	0.503
• UTI	2/102 (2.0)	4/83 (4.8)	0.411
• Cholangitis	0/102 (0.0	0/83 (0.0)	-
• Surgical site	0/102 (0.0	0/83 (0.0)	-
Biliary complications[§]	13/102 (12.7)	5/83 (6.0)	0.142
• Lithiasis	2/102 (2.0)	0/83 (0.0)	0.503
• Biliary leak	1/102 (1.0)	0/83 (0.0)	1.000
• Ischemic-type biliary tract lesions	4/102 (3.9)	0/83 (0.0)	0.129
• Anastomotic biliary stricture	6/102 (5.9)	5/83 (6.0)	1.000
Overall ITBL	7/109 (6.4)	3/97 (3.1)	0.340
• DBD recipients	6/91 (6.6)	1/80 (1.3)	0.123
• DCD recipients	1/18 (5.6)	2/17 (11.8)	0.603
Tumours	7/109 (6.4)	2/97 (2.1)	0.177
• Recurrence	3/109 (2.8)	2/97 (2.1)	1.000
• De novo	4/109 (3.7)	0/97 (0.0)	0.124
Karnofsky performance status	100 (90-100)	100 (90-100)	0.415
Biopsy-proven ACR (Banff score)	2.0 (1.0-3.0)	2.0 (1.0-3.0)	0.909
• Banff 6-7	1/98 (1.0)	3/68 (4.4)	0.306
• Banff 8-9	0/98 (0.0)	0/68 (0.0)	-
• Banff 6-9	1/98 (1.0)	3/68 (4.4)	0.306
Overall steroid-sensitive rejection	16/109 (14.7)	11/97 (11.3)	0.539
Overall steroid-resistant rejection	4/109 (3.7)	2/97 (2.1)	0.686
Overall chronic rejection	1/109 (0.9)	1/97 (1.0)	1.000
Biopsy-proven ACR during the	5.0 (4.0-6.0)	4.0 (3.0-5.0)	0.027

	TAC (n=109)	ATLG (n=97)	P
first 12 months			
• Banff 6-7	26/107 (24.3)	13/86 (15.1)	0.149
• Banff 8-9	1/107 (0.9)	1/86 (1.2)	1.000
• Banff 6-9	27/107 (25.2)	14/86 (16.3)	0.158

† >24-hour-long intubation. § Including cholangitis. * Excluding patients with pre-LT diabetes mellitus. Abbreviations: ACR, acute cellular rejection; ATLG, anti-T-lymphocyte globulins; BMI, body-mass index; CMV, cytomegalovirus; eGFR, estimated glomerular filtration rate (according to the MDRD formula); HSV, herpes simplex virus; HZV, herpes zoster virus; ICU, intensive care unit; IQR, interquartile range; LT, liver transplantation; TAC, tacrolimus monotherapy control group.

Summary of the main findings of the first work

This all-inclusive, investigator-driven, prospective, randomised clinical trial evaluated the combination of the principles of induction and minimised immunosuppression and portrayed daily real-world clinical practice during the first year after enrolment. Our main findings are wrapped up below:

1. ATLG induction did not have an impact on one-year immunosuppressive load.
2. ATLG induction significantly reduced the number of TCMR episodes as assessed at per-protocol day-seven liver biopsy.
3. The number of episodes of clinically relevant steroid-sensitive acute rejection, steroid-resistant acute rejection and chronic rejection was not affected. We recall that, in this protocol, TCMR was considered clinically relevant only in case of simultaneous biochemical and histological disturbances.
4. Patient and graft survival were comparable between the two groups, whereas death-censored graft survival was significantly worse after induction with ATLG. Induction with ATLG was followed in some cases by potentially drug-related life-threatening adverse events. This explains the excess in early death-censored graft losses in the study group.

Introduction to the second part

The *weight* of living-donor liver transplantation in organ shortage

The misalignment between organ availability and demand is dramatic. The Eurotransplant International Foundation coordinates organ allocation in an area inhabited by 137 million people in Europe. In this area, every year around 20% of patients registered for LT leave the waiting list because they die or the disease progresses rendering the candidate unfit for the procedure (Figure 1).[190] We can only imagine the consequences on this gloomy and stable dynamic of the introduction of novel indications, such as secondary liver malignancies.

Organ shortage cannot be overcome without living liver donation. Through living donation, demonstrated to be feasible and associated with low risk for the donor, optimal organs can be offered to recipients in the best circumstances, i.e. early after wait listing or when oncological condition is stable.

Living-donor liver transplantation (LDLT) certainly requires high ethical, medical and surgical standards, and a solid backbone of multidisciplinary collaboration in order to be successful for the donor and the recipient.[191-193] Nonetheless, since the first description of the procedure in paediatric,[194] and then in adult recipients thirty years ago,[195] LDLT has become an essential tool in the treatment of liver diseases at large.

Living donation must abide by the general rules of hepatobiliary surgery. The first requirements concern the volume and the quality of the organ. A liver surgeon should leave in the donor should no less that 30-35% of a healthy liver. This proportions commonly refers to right-liver donation, which is problematic per se. It has been clearly shown that the rate of overall and biliary complications is significantly higher among donors of right lobe than it is in case of left-lobe donation and that right hepatectomy is an independent risk factor for

complications.[196] Moreover, left-lobe donors have a larger remnant liver volume compared with right-lobe donors. Altogether, these data advocate for the use of left lobes, as opposed to right grafts, in order to maximise the sacred donor safety. Left grafts are often used with apprehension because liver segmentation imposes a distribution of volume from left to right in ratios of one third against two thirds or, worse, one fourth against three fourths. A left graft is then bound to have a smaller size and this might result in post-hepatectomy liver failure (PHLF) in the recipient. In the context of LT, this condition takes the name of small-for-size syndrome (SFSS). The pathophysiology is the same for both conditions, as shown in pathological and hemodynamic studies, and they are thus considered as the same entity.

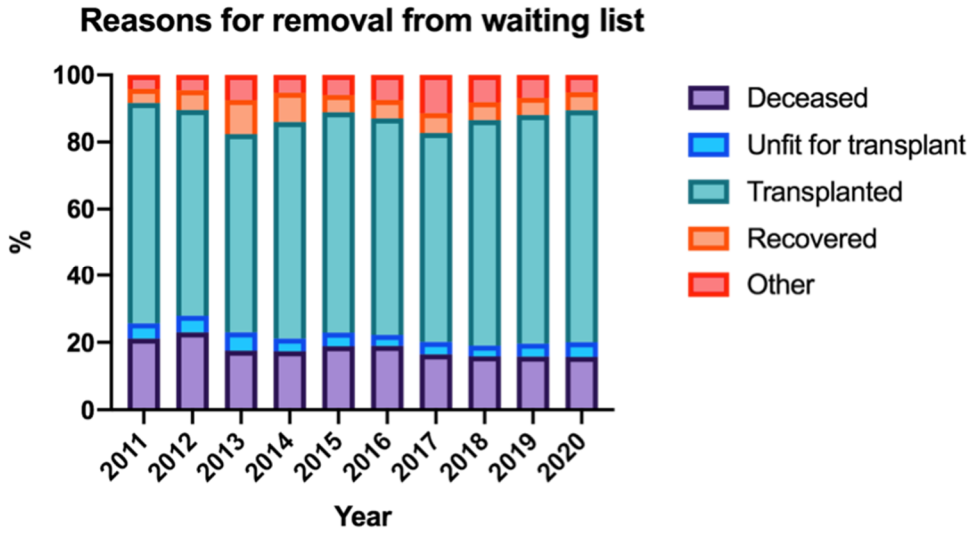


Figure 1. Evolution over ten years of the reasons for the removal of candidates from the waiting list in the Eurotransplant area.[190]

The aim of the second part of the thesis

Reappraised lessons from the UCLouvain experience in adult living-donor transplantation

What are the constraints that refrain Western medical personnel from proposing LDLT? After all, live donors account for the large majority of donor in Asia, where, for cultural, ethical and logistical reasons, LDLT is full blown. Strong familiar bonds prompt family members to engage when someone is sick in the clan and that deceased-donor organ procurement is seen as a violation of body integrity, all of which naturally foster live donation in Asia. On the other hand, the presence of a strongly organised state-run procurement, allocation and transplantation networks favours in Western countries an effective DDLT activity.[197]

A successful LDLT programme requires technical skills of a very high standard. Surgical skills are quintessential to the donor and the recipient operations to manage the arterial, the double venous and the biliary structures. The reconstruction of small-calibre arteries is the most demanding anastomosis during implantation. A sound microsurgical technique and repeated Doppler ultrasound scans are of paramount importance in curbing complications because the snowball consequences of arterial thrombosis or stenosis are biliary strictures, graft loss and even death of the recipient.[198] Portal flow is crucial for liver function and portal vein thrombosis is a major hurdle in LT.[199-201] Furthermore, baseline portal and splanchnic abnormalities hinder graft implantation and require creative solutions to assure proper portal vein reperfusion. A full array of technical solutions has been described, which depend on specific anatomy, and are highly customised.[202,203] The venous drainage of the graft is as critical as hepatic inflow. A series of seminal works has detailed the caveats and characterised the cartography of hepatic veins and their respective parenchymal districts, showing that hepatic vein reconstruction entails multiple reassembling with correct angles and studied rheology, in order to

prevent outflow obstruction and ensuing graft failure.[204,205] The biliary reconstruction is a significant source of morbidity that can be tackled by minimising hilar plate dissection, during the donor's procedure, and by means of ductoplasty and microsurgical anastomoses, during the recipient's procedure. While the adoption of a standard algorithm for biliary reconstruction can remarkably reduce biliary complications from 40 to 10%, advanced endoscopic techniques have progressively become the preferred way of treatment of biliary complications after transplantation and also resection.[206]

More than thirty years of technical progress have morphed a risky procedure into a highly rewarding option. Nonetheless, the expansion of this promising option needs pressure on volume limits and a watchful internal audit. A reassessment of previous experience is useful to get insights into the intricate interactions of graft volume, inflow and outflow required to meet the metabolic demand of the recipient. The model of epicyclic gearbox has been convincingly proposed to describe the multiple kinematic combinations of relevant parameters and the interdependency of graft size, inflow, and outflow in shaping LDLT outcomes.[205]

In this sense, we have carried out a second study with the aim of analysing our experience in adult LDLT, understanding weak areas, improving practices, and escalating the activity.

Second work: Adult-to-adult living-donor liver transplantation: The experience of the Université catholique de Louvain

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Adult-to-adult living-donor liver transplantation: The experience of the Université catholique de Louvain

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ABSTRACT

Background: Liver transplantation is the treatment for end-stage liver diseases and well-selected malignancies. The allograft shortage may be alleviated with living donation. The initial UCLouvain experience of adult living-donor liver transplantation (LDLT) is presented.

Methods: A retrospective analysis of 64 adult-to-adult LDLTs performed at our institution between 1998 and 2016 was conducted. The median age of 29 (45.3%) females and 35 (54.7%) males was 50.2 years (interquartile range, IQR 32.9–57.5). Twenty-two (34.4%) recipients had no portal hypertension. Three (4.7%) patients had a benign and 33 (51.6%) a malignant tumor [19 (29.7%) hepatocellular cancer, 11 (17.2%) secondary cancer and one (1.6%) each hemangioendothelioma, hepatoblastoma and embryonal liver sarcoma]. Median donor and recipient follow-ups were 93 months (IQR 41–159) and 39 months (22–91), respectively.

Results: Right and left hemi-livers were implanted in 39 (60.9%) and 25 (39.1%) cases, respectively. Median weights of right- and left-liver were 810 g (IQR 730–940) and 454 g (IQR 394–534), respectively. Graft-to-recipient weight ratios (GRWRs) were 1.17% (right, IQR 0.98%-1.4%) and 0.77% (left, 0.59%-0.95%). One- and five-year patient survivals were 85% and 71% (right) vs. 84% and 58% (left), respectively. One- and five-year graft survivals were 74% and 61% (right) vs. 76% and 53% (left), respectively. The patient and graft survival of right and left grafts and of very small (<0.6%), small (0.6%–0.79%) and large (≥0.8%) GRWR were similar. Survival of very small grafts was 86% and 86% at 3- and 12-month. No donor died while five (7.8%) developed a Clavien–Dindo complication IIIa, IIIb or IV. Recipient morbidity consisted mainly of biliary and vascular complications; three (4.7%) recipients developed a small-for-size syndrome according to the Kyushu criteria.

Conclusions: Adult-to-adult LDLT is a demanding procedure that widens therapeutic possibilities of many hepatobiliary diseases. The donor procedure can be done safely with low morbidity. The recipient operation carries a major morbidity indicating an important learning curve. Shifting the risk from the donor to the recipient, by moving from the larger right-liver to the smaller left-liver grafts, should be further explored as this policy makes donor hepatectomy safer and may stimulate the development of transplant oncology.

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Introduction

Introduced in clinical practice in 1963 by Starzl, it took two decades before liver transplantation (LT) became recognized as a life-saving treatment for many acute and chronic end-stage liver diseases. Since then, surgical techniques, immunosuppressive therapies, perioperative and long-term medical care have been continuously improving. Nowadays, 1- and 5-year patient survival rates reach 90% and 70%, respectively. The discrepancy between numbers of available liver allografts and potential recipients is still responsible for 20%–30% waiting list mortality, in our center, during the last decade. Indeed, the initial favorable situation "more livers than recipients" rapidly inverted, despite continuous efforts to enlarge the liver allograft pool, and led to the development of surgical strategies, such as domino LT (limited due to the rarity of some well identified metabolic diseases), split LT (limited due to the reduced number of "good quality" organs) and cardiac-death donor LT (limited due to difficult logistic, legal and reduced organ quality). However, the only technical alternative that allows a substantial increase in LT is living-donor LT (LDLT). Unfortunately, the Western LT community, in contrast to the Eastern one, poorly embraces this approach (due to ethical and technical constraints), in spite of the increasing Western "popularity" of living-donor kidney transplantation.

The transplantation center of the Université catholique de Louvain (UCLouvain) embarked in 1994 on a pediatric LDLT programme. Four years later, the adult-to-adult LDLT (A2ALDLT) program was launched after several preparatory study visits to Japan and China. This paper presents a detailed analysis of the A2ALDLT UCLouvain experience. Lessons taken from this small single-center experience are discussed.

Methods

During the period of January 1998 to October 2016, 64 A2ALDLTs were performed at the Saint-Luc University hospitals in Brussels, Belgium. A detailed follow-up report is presented here. All events and results in both donors and recipients were classified following the European Liver Transplantation Registry criteria as early if occurring within three months and late if occurring later. Donor and recipient complications were classified according to the Clavien–Dindo classification [1]. Small-for-size syndrome was defined taking into consideration the Kyushu criteria (total bilirubin >20 mg/dL for seven consecutive days after post-LT day seven in absence of technical and immunologic factors [2–4] and the Hernandez-Alejandro's criteria (prolonged ascites, hyperbilirubinemia, INR or encephalopathy in absence of ischemia) [5]. The median follow-up of donor and recipient cohorts was 93 months (interquartile range, IQR 41–159) and 39 months (IQR 22–91), respectively.

Donor characteristics and procedure (Table 1)

One hundred twenty-two donor-recipient pairs were screened, in accordance to the requirements prescribed by the Institutional Review Board of the UCLouvain Faculty of medicine. The assessment included a mandatory evaluation by the deputy heads of the Departments of Internal Medicine and Psychiatry, both serving as "donor's advocates". Donor candidacy was rejected for medical reasons (obesity, diabetes and cardiovascular diseases) and/or insufficient graft-to-recipient weight ratio (GRWR) determined at ≤0.8% in patients presenting portal hypertension. Finally 64 selected donor-recipient pairs were selected. There were 34 (53.1%) female and 30 (46.9%) male donors; their median age was 34.8 years (IQR 27.6–46.2). The offspring composed the majority of donors (29 cases; 45.3%). ABO-incompatible LDLT was performed twice (3.1%). Thirty-seven (57.8%) LDLT was gender identical; 16 (25.0%) donor-recipient pairs were female-to-male and 11 (17.2%) male-to-female. Median donor body mass index (BMI) was 24.2 kg/m^2 (IQR 21.3–26.4). Pre-transplant liver biopsies were done in 19 (29.7%) patients in order to rule out steatosis. Eight specimens presented minor macro-steatotic changes and one a 30% macro-steatosis; living donation was performed after dietetic care.

During the first decade of our experience, anatomy and volumetry were determined using thin-slice angio-CT scan and cholangio-MRI; afterwards all 36 donors (56.3%) were worked-up using the MeVis software (MeVis GmbH, Bremen, DE).

All donor hepatectomies were performed or assisted by the leading surgeon (Lerut J), under combined general and epidural anesthesia. The incision consisted of a right subcostal incision extended to the right border of the right rectus muscle and to the xiphoid process. The decision to proceed with a right or left hepatectomy was based on the preservation of a minimal residual donor liver volume of ≥30% and to reach a GRWR of 0.8%. In the absence of recipient portal hypertension, the GRWR was deliberately lowered to 0.5%–0.6%. Very small and small-for-size graft were defined as those resulting in a GRWR of <0.6% and <0.8% (used in 7 patients each). For right-liver grafts, the hepatic venous allograft outflow was initially assured by including the middle hepatic vein in the graft and later on by draining ≥5 mm large segment V and/or VIII veins, using free vascular arterial or venous grafts from the post-mortem donor vessel bank.

In order to maximize donor safety and recovery, growing experience led to a progressive shift from the larger right (segments V to VIII) to the smaller left hemi-liver (segments I to IV) graft [6–8]. This shift was favored by the gradual increase of transplantation for primary and secondary liver malignancies.

The parenchymal transection was done combining bipolar water jet-coagulation and ultrasonic dissection combined with intermittent hilar clamping. Intraoperative cholangiography was performed three times: at the beginning of surgery (in order to detect eventual unknown anomalies), just before cutting the bile duct(s) (in order to optimize bile duct division) and after bile duct division (in order to verify integrity of the remaining biliary tree and to comply with possible medico-legal issues). The biliary transection plane was determined using a double metal wire identification method using two fine wires used in maxillofacial surgery sutured at the proposed transection plane. One infra-hepatic closed silicone drain was left in place for five days. Back-table work consisted of anterograde and retrograde rinsing of portal and hepatic veins as well as of the biliary tree using UW solution.

Because of security reasons, all donors stayed one day in the intensive care unit. Doppler ultrasound was done daily during the first five days in order to check the patency of the liver vasculature. Length of hospital stay was kept as short as possible in order to minimize infection risk. All donors had a MRI at six and twelve months in order to verify anatomy and regeneration of the residual liver. Donors were followed up yearly at outpatient clinic or contacted by telephone in order to document their physical and psychological evolution.

Recipient characteristics and procedure (Table 2)

Twenty-nine (45.3%) female and 35 (54.7%) male patients with a median age of 50.2 years (IQR 32.9–57.5) were transplanted. Thirty-six (56.3%) patients had a liver tumor: three (4.7%) had a benign tumor (alveolar echinococcosis, hemangiomatosis, and polyadenomatosis) and 33 (51.6%) a malignant tumor [19 (29.7%) hepatocellular cancer, 11 (17.2%) a secondary, bi-lobar and irresectable liver tumor (9 neuroendocrine and 2 colorectal metastases), and one each (1.6%) epithelioid hemangioendothelioma, hepatoblastoma and primary embryonal liver sarcoma]. Median

recipient BMI was 24.5 kg/m^2 (IQR 20.4–26.8). Median Child-Turcotte-Pugh and MELD scores were 7.5 (6.0–9.0) and 11 (IQR 7–16), respectively. Twenty-seven (42.2%) patients had a MELD score greater than 14; 22 (34.4%) recipients had no underlying primary parenchymal liver disease, and, consequently, no portal hypertension.

All transplant procedures included a vena cava sparing technique without use of veno-venous bypass. LDLT implantation was adapted to the optimal hepatic venous outflow and arterial inflow. In secondary liver tumors a coelio-mesenteric lymphadenectomy was performed. Venous outflow reconstruction was done by anastomosing donor and recipient hepatic veins in an end-to-end fashion. In left hemi-liver LDLT the hepatic veins of the graft are anastomosed to the widened cuff of the middle and left hepatic veins. In right hemi-liver LDLT a widening plasty was mostly added on the recipient inferior vena cava. Drainage of the anterior right allograft sector was restored depending on the volume of this sector and in case of GRWR <0.8%, by using free vascular grafts (see above). Hepatic artery reconstruction was done using magnifying loupes; in some cases the microscope was used. Graft inflow modulation, done either using splenic artery ligation (13/64, 20.3%) or embolization (2/64, 3.1%), was decided depending on the real allograft weight and the result of the intraoperative transit time electromagnetic flow measurement (>3 mL/g of liver tissue), done with adapted VeriQ flow probes (Medistim, Oslo, NO) [9]. Biliary reconstruction and drainage were adapted to diameter, number of bile ducts and judgment of the implantation surgeon.

All recipients had a similar postoperative infectious and tacrolimus-based minimization immunosuppressive treatment [10]. MRI and hepato-IDA scintigraphy were done at postoperative day 7 in order to document perfusion anomalies as well as function (excretion of the tracer) of the graft and (asymptomatic) biliary collections. Anti-thrombotic treatment consisted of low-molecular-weight heparin was used for the first postoperative month. Later on, patients received salicylic acid for six months.

Outpatient follow-up consisted of regular blood testing, Doppler ultrasound and systematic percutaneous or endoscopic control of the biliary tract, six months after LT and when clinically indicated. Cancer patients had three- to six-monthly thoraco-abdominal CT scan, bone scintigraphy and determination of tumor markers (CEA, DCP, CA19-9); in neuroendocrine patients six-monthly chromogranin A and DOTATOC PET/CT scan were added. In order to document biliary complications, endoscopic retrograde or percutaneous anterograde cholangiography were performed depending on the type of biliary reconstruction.

Statistical analysis

Continuous data were reported as median and IQR and tested with the Mann–Whitney *U* test, where appropriate. Binomial variables were reported as percentage and tested with Fisher's exact test, where appropriate. The time to events was analysed with the Kaplan-Meier method and compared with the log-rank test. The significance of statistical tests was taken at a *P* value <0.05. Analyses were run using SPSS (version 25.0; IBM Corp., Armonk, NY, USA).

Results

The donor procedure

The different features in relation to the donation of left- or right-liver grafts are displayed in Table 1. Over time, the team leant towards a more frequent use of left-liver grafts. During the first decade (1997–2006), 18 LDLTs were performed; 17 (94.4%) were right grafts and only one (5.6%) a left graft. During the second decade (2007–2016), 46 LDLTs were performed; 22 (47.8%) were right- and 24 (52.2%) left-livers. Accordingly, the follow-up after right hepatectomy was longer after right donation (116 months, IQR 64–189) than after left donation (53 months, IQR 31–95, $P < 0.001$).

The foremost difference between the left- and right-liver donation groups lies in the estimated remnant volumes. Right hepatectomy (including segments V to VIII) was performed in 39 (60.9%) and left hepatectomy (including segments I to IV) in 25 (39.1%) donors. In 17 (26.6%) donors, the middle hepatic vein was included in the right hemi-liver. Six (9.4%) and two (3.1%) grafts had a double arterial and portal supply and 15 (23.4%) and one (1.6%) grafts had two and three bile ducts, respectively.

The median predicted graft weight was 726 g (IQR 496–933) and the real median graft weight was similar (725 g, IQR 466–848, $P = 0.469$). The median percentage of the remnant liver in the donor was 35% (IQR 31%–61%). The median estimated remnant-to-body-weight ratio was 0.69% (IQR 0.56%–1.15%).

Median operative time was 475 min (IQR 420–510). No donor required allotransfusion; 53 (82.8%) donors received intraoperative autotransfusion (median 300 mL, IQR 218–558) using the CellSaver® (Haemonetics, Braintree, USA). The median lengths of intensive care unit and hospital stays were 1 day (IQR 1–1) and 10 days (IQR 9–12).

Early donor morbidity following Clavien–Dindo was as follows: 16 (25.0%) patients had a grade I complication, 7 (10.9%) a grade II complication; one a grade IIIa (drainage for pleural effusion) and two (3.1%) a grade IIIb complication (reoperation for biliary leak from the cut surface and from an aberrant missed right duct. Two (3.1%) donors experienced significant temporary elevation of bilirubin level (IVa).

Accordingly, the total bilirubin peak was higher after right donation (2.6 mg/dL, IQR 1.7–4.2) than after left donation (1.6 mg/dL, IQR 1.2–2.0, $P = 0.003$). Likewise, the INR peak amounted to 1.49 (IQR 1.32–1.64) after right hepatectomy, and to 1.27 (IQR 1.20–1.38) after left hepatectomy ($P < 0.001$).

In one case, elevation of total bilirubin up to 21 mg/dL (in the absence of encephalopathy, ascites and coagulation disturbances) can be explained by a low estimated remnant volume (24%), even though preoperative MeVis imaging estimated the remnant-to-body-weight ratio at 0.40%.

Two (3.1%) patients needed repair of a midline incisional hernia 32 and 75 months after donation, respectively. During the entire follow-up, all donors remained in accordance with their initial decision to donate and none regretted donation. Only the patient who experienced severe liver dysfunction still has some psychological difficulties interfering with his daily life ("no drive anymore").

The recipient procedure

The different features of LDLT in relation to the type of graft are displayed in Table 2. The main difference between the two groups obviously is in graft weights and ratios. The predicted GRWR was 1.10% (IQR 0.81%–1.33%) while the real GRWR was 1.05% (IQR 0.82%–1.27%, $P = 0.682$). The median graft weight, for left-liver recipients, was 454 g (IQR 394–534) and their median GRWR 0.77% (IQR 0.59%–0.95%), for right-liver recipients, the weight amounted to 810 g (IQR 730–940, $P < 0.001$) and the GRWR to 1.17% (IQR 0.98%–1.40%, $P < 0.001$). Thus, 21.9% (14/64) of recipients received a small-for-size graft. The actual GRWRs were less than 0.6% (very small graft) and between 0.6% and 0.79% (small graft) in 7 (10.9%) patients each and ≥0.8% (standard graft) in 50 (78.1%) recipients. Twenty-two (34.4%) patients did not present portal hypertension. Median operative time was 543 min (IQR 450–720). Cold ischemia time and warm ischemia time were 78 min (IQR 53–133)

Table 1
Data concerning living-donors.

Graft type	All grafts ($n=64$)	Left graft ($n=25$)	Right graft ($n=39$)	P value
Age (yr)	34.8 (27.6–46.2)	34.4 (27.0–50.4)	35.0 (27.9–42.8)	0.752
BMI (kg/m^2)	24.2 (21.3–26.4)	24.6 (22.9–26.9)	23.9 (21.2–26.0)	0.274
Donor-recipient relationship				
Related	48 (75.0%)	18 (72.0%)	30 (76.9%)	0.770
Child to parent	29 (45.3%)	11 (44.0%)	18 (46.2%)	1.000
Parent to child	6 (9.4%)	3 (12.0%)	3 (7.7%)	0.671
Sibling to sibling	10 (15.6%)	4 (16.0%)	6 (15.4%)	1.000
Other	3 (4.7%)	0	3 (7.7%)	0.275
Unrelated	16 (25.0%)	7 (28.0%)	9 (23.1%)	0.770
Spouse to spouse	4 (6.3%)	1 (4.0%)	3 (7.7%)	1.000
Friend to friend	3 (4.7%)	2 (8.0%)	1 (2.6%)	0.555
Families-in-law	8 (12.5%)	3 (12.0%)	5 (12.8%)	1.000
Other	1 (1.6%)	1 (4.0%)	0	0.391
Estimated remnant/liver volume proportion (%)	35 (31–61)	67 (58–71)	32 (30–35)	**<0.001**
Estimated remnant-to-body-weight ratio (%)	0.69 (0.56–1.15)	1.19 (1.07–1.36)	0.60 (0.51–0.69)	**<0.001**
Operative time (min)	475 (420–510)	475 (383–518)	470 (420–510)	0.725
Intraoperative blood loss (mL)[a]	300 (218–558)	300 (100–481)	330 (236–650)	0.255
Length of hospital stay (d)	10 (9–12)	10 (9–12)	10 (9–12)	0.873
Length of ICU stay (d)	1 (1–1)	1 (1–1)	1 (1–1)	0.311
Total bilirubin peak (mg/dL)[b]	2.0 (1.3–3.3)	1.6 (1.2–2.0)	2.6 (1.7–4.2)	**0.003**
ALT peak (IU/L)[b]	244 (199–334)	229 (199–367)	249 (198–328)	0.741
INR peak[b]	1.37 (1.23–1.54)	1.27 (1.20–1.38)	1.49 (1.32–1.64)	**<0.001**
Complications (Clavien–Dindo score)[c]				
I	16 (25.0%)	8 (32.0%)	8 (20.5%)	0.379
II	7 (10.9%)	2 (8.0%)	5 (12.8%)	0.696
IIIa	1 (1.6%)	0	1 (2.6%)	1.000
IIIb	2 (3.1%)	1 (4.0%)	1 (2.6%)	1.000
IVa	2 (3.1%)	0	2 (5.1%)	0.516
Follow-up (mon)	93 (41–159)	53 (31–95)	116 (64–189)	**<0.001**

ALT: alanine aminotransferase; BMI: body mass index; ICU: intensive care unit; INR: international normalised ratio.
[a] CellSaver® recovery.
[b] During the first postoperative month.
[c] Until discharge.

and 37 min (IQR 31–53), respectively. Forty-four (68.8%) patients needed transfusion (median 267 mL, IQR 0–1176).

Since the left graft is always procured with the middle hepatic vein, the necessity of venous outflow reconstruction prevailed in case of right-graft LT (64.1% vs. 24.0%, $P=0.002$). Venous outflow was reconstructed in 31 (48.4%) by means of vena cava plasty ($n=4$) or the use of a venous ($n=14$), or arterial ($n=12$) free vessel or a polytetrafluoroethylene graft ($n=1$). Graft inflow modulation was done in 15 (23.4%) recipients using splenic artery modulation because of excessive portal graft flow (>3 mL/g liver tissue) and small-for-size graft (7 cases). End-procedure hepatic arterial flow was 105 mL/min (IQR 59–148) for left grafts, and 150 mL/min (IQR 125–254, $P=0.003$) for right grafts. The difference in end-procedure portal vein flow [left: 505 mL/min (IQR 344–848) vs. right: 770 mL/min (IQR 600–1257), $P=0.012$] was annihilated when considering the weight of the graft (Table 2).

Biliary reconstruction consisted of duct-to-duct anastomosis (37 patients, 57.8%), Roux-Y hepatico-jejunostomy (26, 40.6%) and combined duct-to-duct and hepatico-jejunostomy (1, 1.6%). Biliary duct plasty was done in nine (14.1%) patients and three months long, internal biliary drainage was done in 44 patients (68.8%).

The median duration of intensive care unit and hospital stays were 3 days (IQR 2–10) and 20 days (IQR 16–31). Recipient morbidity recorded following the Clavien–Dindo classification was as follows: grade I in 7 (10.9%), grade II in 16 (25.0%), grade IIIa in 2 (3.1%), grade IIIb in 7 (10.9%), grade IVa in 8 (12.5%) and grade IVb in 15 (23.4%) patients. According to the Kyushu and Hernandez-Alejandro definitions of small-for-size syndrome, 3 (4.7%) and 15 (23.4%) recipients experienced liver insufficiency, respectively. Nine patients (14.1%) died (grade V) during the hospitalization of sepsis ($n=6$), perioperative cardiac arrest ($n=2$) and coeliac trunk dissection ($n=1$) following interventional radiology done the day before discharge to embolize a splenic artery aneurysm. Eleven recipients died later (>3 months) after LT of recurrent hepatocellular cancer ($n=4$), HCV ($n=2$) and alcoholic ($n=1$) allograft diseases, development of *de novo* tumor ($n=3$) and suicide ($n=1$).

Seven (10.9%) patients required early re-transplantation (re-LT) due to hepatic artery thrombosis ($n=2$), portal vein thrombosis ($n=2$) and one each due to coeliac trunk dissection, ruptured mycotic arterial pseudoaneurysm and graft dysfunction. Late re-LT was required six times due to intrahepatic biliary tract lesions ($n=4$), recurrent primary sclerosing cholangitis ($n=1$) and chronic rejection related to non-compliance ($n=1$).

Endoscopic or percutaneous biliary imaging was systematically done in all patients. Thirty (46.9%) recipients exhibited at least one biliary complication; 14 (21.9%) as early and 16 (25.0%) as late occurring events. Eight (61.5%) recipients, out of the 13 patients who presented multiple bile ducts and who survived the early postoperative period, developed biliary complications. Twelve (18.8%) patients experienced a biliary leak requiring surgical ($n=6$) and/or radiologic ($n=6$) and/or endoscopic ($n=3$) interventions. Twenty-two (34.4%) patients developed an anastomotic biliary stricture, requiring interventional endoscopy ($n=14$) and/or radiology ($n=16$); three times surgical correction became necessary. Ten (15.6%) recipients developed non-anastomotic biliary strictures; 5 patients (7.8%) finally required re-LT after several radiologic interventions.

Arterial complications were diagnosed in 10 (15.6%) patients. Two stenoses were balloon dilated. Early hepatic artery thrombosis was diagnosed in five patients; in three of them the quality of the artery was seriously compromised due to pre-LT long-standing steroid therapy, locoregional arterial chemo- and radio-embolisation. One recipient had a successful surgical redo, one interventional radiology, one medical treatment, while two patients actually needed re-LT. Two patients developed a hepatic artery pseudoaneurysm and underwent interventional radiology. One patient presenting a ruptured mycotic aneurysm in the

Table 2
Data concerning recipients.

Graft type	All grafts ($n=64$)	Left graft ($n=25$)	Right graft ($n=39$)	P value
Age (yr)	50.2 (32.9–57.5)	43.6 (26.7–52.9)	51.9 (35.3–60.4)	0.122
BMI (kg/m^2)	24.5 (20.4–26.8)	22.3 (18.8–25.7)	25.5 (22.4–27.7)	**0.005**
Indication for LT				
HCV-cirrhosis	9 (14.1%)	3 (12.0%)	6 (15.4%)	1.000
HBV-cirrhosis	3 (4.7%)	0	3 (7.7%)	0.275
Alcoholic cirrhosis	9 (14.1%)	4 (16.0%)	5 (12.8%)	0.728
Non-alcoholic steato-hepatitis	3 (4.7%)	0	3 (7.7%)	0.275
Cholestatic liver disease	12 (18.8%)	6 (24.0%)	6 (15.4%)	0.514
Autoimmune hepatitis	4 (6.3%)	2 (8.0%)	2 (5.1%)	0.640
Metabolic disease	4 (6.3%)	1 (4.0%)	3 (7.7%)	1.000
Budd–Chiari syndrome	2 (3.1%)	0	2 (5.1%)	0.516
Benign tumors	3 (4.7%)	0	3 (7.7%)	0.149
Primary tumors	22 (34.4%)	8 (32.0%)	14 (35.9%)	1.000
Hepatocellular cancer	19 (29.7%)	6 (24.0%)	13 (33.3%)	0.577
Primary undifferentiated embryonal liver sarcoma	1 (1.6%)	1 (4.0%)	0	0.391
Hepatoblastoma	1 (1.6%)	1 (4.0%)	0	0.391
Epithelioid haemangioendothelioma	1 (1.6%)	0	1 (2.6%)	1.000
Secondary liver malignancies	11 (17.2%)	7 (28.0%)	4 (10.3%)	0.092
Neuroendocrine tumor	9 (14.1%)	6 (24.0%)	3 (7.7%)	0.137
Colorectal carcinoma	2 (3.1%)	1 (4.0%)	1 (2.6%)	1.000
Child-Turcotte-Pugh	7.5 (6.0–9.0)	8.0 (6.5–10.5)	7.0 (6.0–9.0)	0.591
Class A	12 (18.8%)	3 (12.0%)	9 (23.1%)	0.338
Class B	20 (31.3%)	6 (24.0%)	14 (35.9%)	0.411
Class C	10 (15.6%)	4 (16.0%)	6 (15.4%)	1.000
Non-parenchymal liver disease	22 (34.4%)	12 (48.0%)	10 (25.6%)	0.105
MELD	11 (7–16)	10 (7–16)	12 (7–17)	0.431
MELD ≥14	27 (42.2%)	10 (40.0%)	17 (43.6%)	0.801
Estimated graft weight (g)	726 (496–933)	473 (399–526)	880 (750–993)	**<0.001**
Actual graft weight (g)	725 (466–848)	454 (394–534)	810 (730–940)	**<0.001**
Estimated GRWR (%)	1.10 (0.81–1.33)	0.73 (0.63–1.07)	1.18 (1.02–1.40)	**<0.001**
Actual GRWR (%)	1.05 (0.82-1.27)	0.77 (0.59-0.95)	1.17 (0.98-1.40)	**<0.001**
<0.6	7 (10.9%)	7 (28.0%)	0/39	**0.001**
0.6–0.79	7 (10.9%)	6 (24.0%)	1 (2.6%)	**0.012**
≥0.8	50 (78.1%)	12 (48.0%)	38 (97.4%)	**<0.001**
ABO-incompatibility	2 (3.1%)	1 (4.0%)	1 (2.6%)	1.000
Middle hepatic vein in graft	27 (42.2%)	23 (92.0%)	4 (10.3%)	**<0.001**
Outflow venous reconstruction	31 (48.4%)	6 (24.0%)	25 (64.1%)	**0.002**
Graft inflow modulation	15 (23.4%)	9 (36.0%)	6 (15.4%)	0.074
Splenic artery ligation	13 (20.3%)	8 (32.0%)	5 (12.8%)	0.109
Splenic artery embolization	2 (3.1%)	1 (4.0%)	1 (2.6%)	1.000
Multiple hepatic arteries	6 (9.4%)	4 (16.0%)	2 (5.1%)	0.199
Multiple bile ducts	16 (25.0%)	1 (4.0%)	15 (38.5%)	1.000
Bile duct anastomosis				
Duct-to-duct	37 (57.8%)	13 (52.0%)	24 (61.5%)	0.605
Multiple ducts	5/37 (13.5%)	0	5/24 (20.8%)	0.147
Roux-Y hepaticojejunal	26 (40.6%)	12 (48.0%)	14 (35.9%)	0.436
Combined	1 (1.6%)	0	1 (2.6%)	1.000
T-tube	20 (31.3%)	7 (28.0%)	13 (33.3%)	0.784
Hepatic arterial flow before modulation (mL/min)	75 (36–167)	95 (30–95)	123 (55–123)	1.000
End-procedure hepatic arterial flow (mL/min)	119 (104–173)	105 (59–148)	150 (125–254)	**0.003**
End-procedure portal vein flow (mL/min)	540 (430–1072)	505 (344–848)	770 (600–1257)	**0.012**
Portal veinflow per 100 g graft weight (mL/min/100 g)	123 (80–166)	121 (83–174)	102 (72–158)	0.321
Cold ischemia time (min)	78 (53–133)	66 (49–126)	87 (54–133)	0.245
Warm ischemia time (min)	37 (31–53)	37 (31–57)	38 (31–53)	0.710
Intraoperative transfusions	44 (68.8%)	16 (64.0%)	28 (71.8%)	0.585
Intraoperative transfusion (mL)	267 (0–1176)	241 (0–721)	473 (0–1322)	0.330
Operative time (min)	543 (450–720)	555 (480–740)	510 (440–696)	0.432
Length of hospital stay (d)	20 (16–31)	20 (16–33)	18 (16–28)	0.411
Length of ICU stay (d)	3 (2–10)	4 (2–10)	3 (2–10)	0.248
SFSS (Kyushu)	3 (4.7%)	2 (8.0%)	1 (2.6%)	0.555
SFSS (Hernandez-Alejandro)	15 (23.4%)	7 (28.0%)	8 (20.5%)	0.553
Complications (Clavien–Dindo score)[a]				
I	7 (10.9%)	2 (8.0%)	5 (12.8%)	0.696
II	16 (25.0%)	4 (16.0%)	12 (30.8%)	0.243
IIIa	2 (3.1%)	2 (8.0%)	0	0.149
IIIb	7 (10.9%)	5 (20.0%)	2 (5.1%)	0.516
IVa	8 (12.5%)	3 (12.0%)	5 (12.8%)	1.000
IVb	15 (23.4%)	5 (20.0%)	10 (25.6%)	0.765
V	9 (14.1%)	4 (16.0%)	5 (12.8%)	0.728
Follow-up (mon)	39 (22–91)	31 (23–53)	71 (21–134)	0.085

BMI: body mass index; GRWR: graft-to-recipient weight ratio; ICU: intensive care unit; IQR: interquartile range; LT: liver transplantation, MELD: model for end-stage liver disease; SFSS: small-for-size syndrome.
[a] Until discharge.

context of a small biliary leak needed urgent arterial ligature followed by re-LT. Unfortunately, he died 15 months later due to the development of an aggressive Castleman disease.

Early portal vein thrombosis was diagnosed in 4 (6.3%) patients. One of them occurred in a patient who underwent graft inflow modulation and whose graft had two portal veins, so that the event was caused by a technical shortcoming. This patient died of pulmonary embolism just before further surgery. Two others were retransplanted and one patient underwent thrombolysis.

Hepatic vein stenosis was diagnosed in four (6.3%) patients: in three cases, as early complication and, in one instance, as a late complication. In three cases, widening of right hepatic vein was erroneously judged unnecessary due to the large diameter of the right hepatic vein. All three were treated by interventional radiologic stenting. The fourth one transplanted for Budd–Chiari syndrome with vena cava involvement, reconstructed with a peritoneal patch, developed allograft dysfunction in the context of a re-thrombosis of vena cava and hepatic veins; he died of sepsis after re-LT.

The 1- and 5-year actuarial patient and graft survival rates were 84% and 68%, and 75% and 60%, respectively (Fig. 1). Outcomes were comparable for right and left LDLT (Fig. 2) as well as for the different thresholds of GRWR (<0.6%, 0.6%–0.79%, and ≥0.8%) (Table 2 and Fig. 3). Survival of very small grafts was 86% and 86% at 3- and 12-month. Overall survival rates were similar to those obtained after deceased-donor LT done during the same time period (86% and 75% for 1- and 5-year patient survival, $P=0.534$); graft survival was somewhat better, although not significantly, in deceased-donor LT (83% and 70% for 1- and 5-year graft survival, $P=0.109$).

Discussion

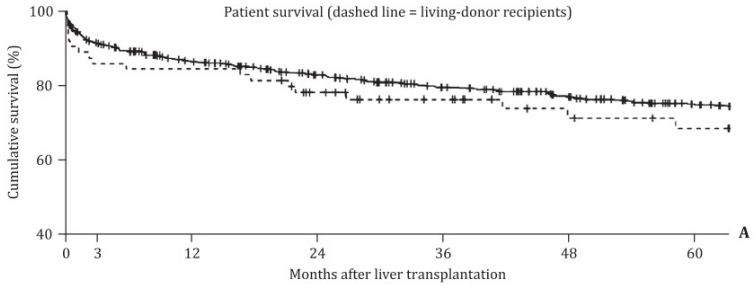
The first A2ALDLT using a left-liver (weighing 434 g) was performed by Makuuchi in 1993 at Shinshu University, Japan, in a female patient suffering from end-stage primary biliary cirrhosis [11]. This procedure was the start for a LT "tsunami" in the Eastern world. However, the enthusiasm for this procedure rapidly lost sympathy within the Western liver transplant community, as a result of a too high morbidity and even mortality in both donor and recipient surgeries. Today, very few Western centers perform A2ALDLT on a regular base [12,13]. Perhaps, many centers embarked on a LDLT program without sufficient knowledge of all the knacks and pitfalls of the complex, surgical and medical care of both donors and recipients. To avoid this, our center embarked on an A2ALDLT program following several tutorials and study visits in leading Asian centers. Our "real-world" experience shows that shortage of deceased-donor grafts and low-volume LDLT practice leads to a probably too rapid extension of indications, despite anatomical and clinical complexity in both donors and recipients.

The larger experience in hepatobiliary surgery without doubt explains the minimal risk of donor hepatectomy in Asian centers. Good outcomes are secured by precise preoperative knowledge of liver segmentation, of frequent vascular and biliary variations and of morphologic and functional graft (for the safety of recipients) and residual liver volumes (for the safety of donors) [3,14–17]. Every donor procedure should aim to procure the graft safely, leaving a sufficient residual liver volume in the donor. The residual liver volume should be more than 30% of the original volume; in older (>50 years) or steatotic donors this proportion should be raised to 35%–40% in order to cope with the reduced regeneration capacity [15]. Nowadays, quantification of steatosis can be calculated very precisely using mass-spectroscopy CT. If unavailable, a liver biopsy is advocated in donors with a BMI over 28 kg/m^2 [14,18,19]. In case of severe steatosis, dietary counselling, and physical exercise during 2 to 4 weeks help reduce the fat content and allow to proceed with donation [20]. Our experience confirmed that with good preoperative planning, donor hepatectomy can be done with minimal morbidity and no mortality.

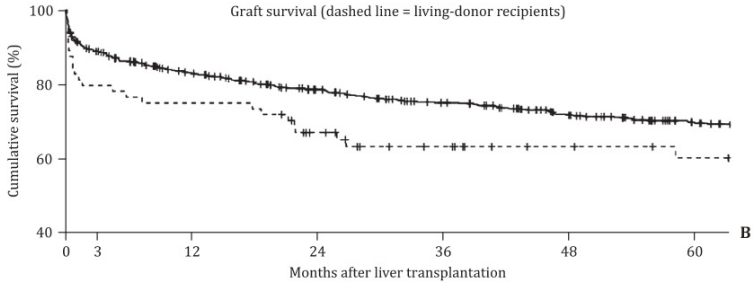
Besides disease severity and patient frailty, a successful A2ALDLT is highly dependent on the following four conditions: adequate graft volume, proper allograft outflow and inflow, and adequate biliary anastomosis [15]. The Kyoto group rapidly experienced that adequate liver volume is of utmost importance for a good outcome [21]. GRWR less than 0.8% [or correspondingly graft weight to standard liver volume (GW/SLV) less than 0.40] markedly reduced patient and graft survival, consequence of small-for-size syndrome. This condition is caused by intra-graft shear stress, a force associated with portal "overflow", which triggers arterial buffer response, in form of vasoconstriction, ultimately leading to arterial hypo-perfusion. These disturbances lead to the potentially unfavorable sequence of sinusoidal injury, excessive ineffective regeneration, severe cholestasis, impaired synthetic capacity and refractory ascites [2,22]. This chain of events is responsible for increased morbidity, prolonged hospital stay and higher costs, due to the intensive medical care including administration of large quantities of albumin and somatostatin [23].

In order to counteract small-for-size syndrome, several technical modifications of the allograft implantation, aiming to reduce portal vein flow and pressure, have been developed during the last two decades. The Kyoto group repeatedly reported that lowering portal vein pressure beneath 15 mmHg markedly improves outcome. If higher, graft inflow modulation, using proximal splenic artery obliteration and partial porto-systemic shunting, has been proposed. A more radical solution, consisting of interruption of venous collaterals along with splenectomy, has been advocated by the Kyushu group [24]. The excellent outcomes, obtained by these two approaches, have led the Kyoto and Kyushu groups to the more frequent (and successful) use of left-liver with GRWR as low as 0.6%. Their remarkable results triggered a shift from right (corresponding to a retrieval of 60% to 70% of liver mass) to left (corresponding to a retrieval of 30% to 40% of the liver mass) liver allografts. This policy allows not only to expand the donor availability but also to shift the risk from the donor (keeping a higher residual liver mass) to the recipient (receiving a lower liver mass) [3,6–8,24,25]. The implantation of a smaller liver graft mass in the presence of portal hypertension is riskier and reasonable only if both graft inflow and outflow are optimized, conditions that have to be assured based on intraoperative hemodynamic flow and pressure measurements. The modulation of portal and arterial inflow is the mainstay of graft flow control. The necessity for graft inflow modulation should be carefully evaluated, based on intraoperative flow and pressure measurements. Additionally, ligation of large portosystemic collaterals can improve portal vein flow [26]. Likewise, adequate venous outflow is of importance to avoid graft congestion, especially in right grafts. Optimization of hepatic outflow can be pursued by widening the anastomosis between hepatic veins and vena cava and/or by guaranteeing decongestion of the right anterior sector of the allograft by draining any segment V/VIII vein having a diameter larger than 5 mm. These veins can be connected to the vena cava or the cuff of middle and left hepatic veins using free venous or arterial grafts [15,27].

Biliary complications are the Achilles' heel of LDLT, with a reported incidence ranging from 10% to 67% [28–30]. The incidence of complications rises with the number of bile ducts to reconstruct. Likewise, our small series suffered from a high incidence of biliary complications. The high incidence of biliary complications reported in these series may be partly explained by the fact that our center developed a policy, based on an extensive experience in deceased-donor LT, to perform direct biliary imaging in every recipient, even in case of normal liver tests. Technical refinements are crucial elements to reduce the incidence of biliary complications. The three



Patient survival (%), $P = 0.534$					
DDLT	100	91	86		75
LDLT	100	86	84		68
Number of patients exposed to the risk of death					
DDLT	631	626	523		344
LDLT	64	64	54		26



Graft survival (%), $P = 0.109$					
DDLT	100	89	83		70
LDLT	100	80	75		60
Number of patients exposed to the risk of graft loss					
DDLT	631	626	501		324
LDLT	64	64	48		21

Fig. 1. Patient (**A**) and graft (**B**) survival rates after deceased-donor liver transplantation (DDLT) (full line) and living-donor liver transplantation (LDLT) (dashed line).

keys are minimal dissection during the donor procedure, in order to preserve the blood supply of bile ducts, microsurgical reconstruction and routine use of biliary drainage, during the recipient surgery [15,16,30,31]. The Seoul National University Hospital reported excellent results using the telescopic technique [32]. The use of biliary drainage remains a matter of debate. The worldwide most experienced center, Asan Medical Center in Seoul, strongly advocates routine *in situ* and internal-external biliary drainage for several months, in order to reduce biliary leakage. This approach also has the advantage to regularly control the biliary tree [15].

Our small experience confirms the impact of vascular complications on graft survival. Early arterial complications are reported in around 4% of adult LDLTs [33]. Results of arterial reconstruction can be improved using microsurgical techniques routinely, as shown by the Kaohsiung team [30]. Moreover, better handling of all different anatomic variations and modifications such as those caused by locoregional oncologic treatments (and unfortunately experienced in our series) is important to secure allograft arterialisation. Multiple surgical adaptations are "graft savers" in these situations and include extra-anatomical reconstructions using other arteries, instead of unusable hepatic arteries. Among these, we mention the recipients' right gastroepiploic (first choice), gastroduodenal, splenic, ileocolic and inferior mesenteric arteries, [31,32,34–43]. The incidences of portal and hepatic vein complications are similarly reported to be around 4%. In case of portal vein anomalies, several technical adaptations such as portal vein plasty and the Y-graft interposition have proven very successful [15,44–46].

In case of direct anastomosis, a wide plasty of hepatic vein and vena cava, eventually extended with a quilt venoplasty, is necessary to avoid (right) hepatic vein stenosis and thrombosis. Multiple hepatic veins are preferentially transformed to a common opening by using fresh or cryopreserved arterial or venous allografts or autologous saphenous vein [15,40,47]. These technical modifications will also counteract eventual stretching or compression of the anastomotic site by the regenerating liver [15,48].

The here mentioned, detailed, review of the complications encountered in the recipient as well as their management, involving

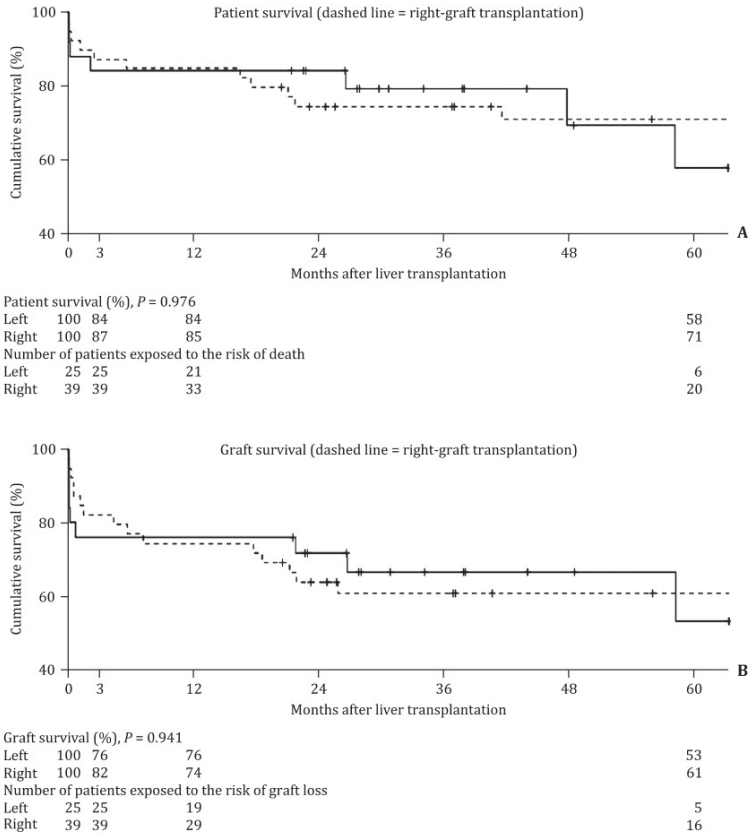
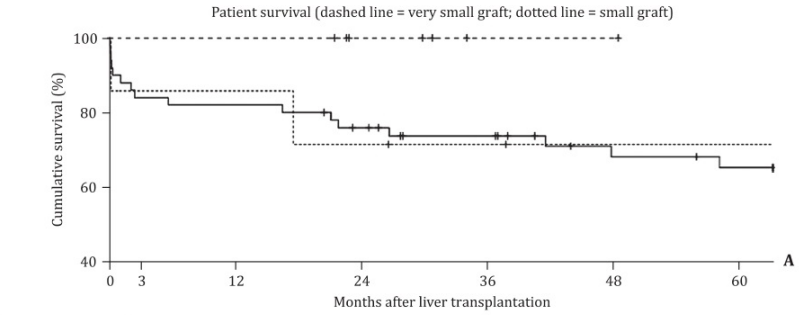


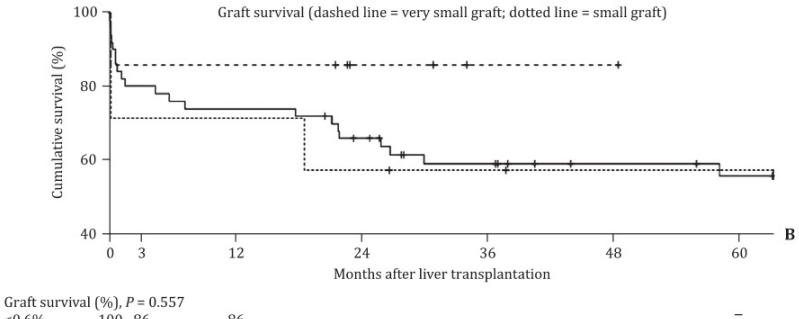
Fig. 2. Comparison of patient (**A**) and graft (**B**) survival rates after left-graft (continuous line) and right-graft (dashed line) living-donor liver transplantation.

surgeons, interventional radiologists and endoscopists, clearly indicate that the learning curve of A2ALDLT is long and complex. Open and repeated discussion of all encountered complications together with a perfect planning of both donor and recipient operations are the keys to progress and make the program successful: "there should be no surprises during the surgeries" [34].

At our center, LDLT is selectively offered to adults with low to no chance to be timely transplanted, as a consequence of the Eurotransplant organ allocation system. This applies in particular to patients suffering from autoimmune liver diseases [they have less chances to be transplanted because of their, usually, low body weight and preserved synthetic liver function resulting in low(er) MELD scores] and patients presenting advanced primary hepatobiliary and secondary liver cancers (not yet considered valid indications for LT). Therefore, LDLT represents for these patients (almost) the only chance to get access to a potential curative LT [49–52]. As such, our small experience underlines the value of A2ALDLT in the field of autoimmune diseases and transplant oncology. Indeed, 25% of patients were transplanted for autoimmune disorders and 56% for primary and secondary liver tumors. Liver metastases were the indication for LDLT in 17.2% of cases. In this setting, A2ALDLT has several advantages: (i) small left-liver grafts, with GRWR around 0.6%, can be used, in the absence of portal hypertension; (ii) interference with the scarce deceased-donor allograft pool is avoided for not yet validated indications for LT, avoiding ethical discussions about the justification of LT in such diseases; (iii) patients benefit from minimized and tailored immunosuppression; and (iv) basic oncological principles can be followed, implementing neo and adjuvant treatment protocols [53]. In the future, the choice of A2ALDLT is expected to be applied more frequently in the treatment of Milan-out hepatocellular cancer, cholangiocellular cancer as well as secondary liver tumors [50,54]. Additionally, LDLT will offer the opportunity to further explore, in a controlled way, the boundaries of inclusion criteria of cirrhotic patients harboring hepatobiliary tumors, as this approach controls both factors "tumor" and "time". The Eastern LDLT experience clearly showed that the Milan criteria are too restrictive [55–57]. Tumor morphology (number and diameter) and biology (tumor markers AFP, DCP or PIVKA-II and PET uptake), along with dynamic tumor behaviour (response to neoadjuvant



Patient survival (%), $P = 0.335$				
<0.6%	100	100	100	–
0.6% - 0.79%	100	86	86	71
≥0.8%	100	84	82	65
Number of patients exposed to the risk of death				
<0.6%	7	7	6	0
0.6% - 0.79%	7	7	7	3
≥0.8%	50	50	41	23



Graft survival (%), $P = 0.557$				
<0.6%	100	86	86	–
0.6% - 0.79%	100	71	71	57
≥0.8%	100	80	74	58
Number of patients exposed to the risk of graft loss				
<0.6%	7	7	6	0
0.6% - 0.79%	7	7	5	2
≥0.8%	50	50	37	19

Fig. 3. Patient (**A**) and graft (**B**) survival analysis per graft-to-recipient weight ratio (GRWR). Very small graft was defined as GRWR <0.6%, small graft was defined as GRWR between 0.6% and 0.79%.

locoregional therapies), are decisive for the fine-tuning of LT indication in hepatobiliary oncology. However, when LDLT is envisaged, the aggressive application of pre-transplant locoregional therapies (such as transarterial chemo- and radio-embolization) should be advocated with caution, because the arterial vessels risk severe damage so that the outcome of the transplant procedure can be compromised, a feature that was encountered in our series [43]. Recently, the Western transplant world has shown a renewed interest in LT for secondary liver tumors. Indeed, it has been shown that patients with non-resectable liver metastases from neuroendocrine and colorectal neoplasms can benefit from transplantation, when strict selection criteria are respected [58,59]. This indication is particularly important, in the context of LDLT, because these patients still have no access to deceased-donor LT and because the absence of portal hypertension permits the use of smaller left allografts safely.

In conclusion, A2ALDLT represents a major surgical and medical endeavor. Our small experience shows that living-donor hepatectomy can be done safely. The recipient operation still presents important morbidity, linked to biliary and vascular complications. Continuous technical refinements are necessary to reduce as much as possible recipient morbidity and mortality, in order to increase LDLT applicability, especially in the Western world. LDLT is a promising additive tool to the therapeutic armamentarium of the transplant surgeon and is worth a place, especially, in the treatment of primary hepatobiliary and secondary unresectable liver

tumors. The progressive shift, in our experience, from right- to left-liver grafting has to be considered in this context. The more frequent combination of the smaller (left) liver graft (up to 0.6% GRWR) use and both graft inflow and outflow modulation are required to optimize results and to make this procedure safe in both donor and recipient. By doing so, LDLT will avoid interference with the use of scarce deceased-donor allograft pool and will represent a boost to transplant oncology.

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Contributors

IS and LJ designed the study and drafted the article. IS, INME, RJJM, RC and LJ collected and analyzed the data. IS, CO, BRE, CL, GP and LJ critically revised the manuscript. All authors involved in medical care and approved the final version. LJ is the guarantor.

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Ethical approval

This study was approved by the Institutional Review Board of the UCLouvain Faculty of medicine.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Summary of the main findings of the second work

This retrospective case-series gave a real-world picture of a Western single-centre experience with LDLT. Our main findings are wrapped up below:

1. Shortage of deceased-donor grafts and the shift towards the use of low-volume live-donor grafts push for a rapid extension of indications, while anatomical and clinical complexity in both donors and recipients must be met with extreme care.
2. Donor hepatectomy can be carried out with minimal morbidity and no mortality provided a meticulous pre-operative planning.
3. The success of the recipient procedure is contingent on, generally speaking, disease severity and patient frailty, and, specifically, graft volume, proper graft inflow, ample graft outflow, and adequate biliary reconstruction. As a matter of fact, the occurrence of small-for-size syndrome, arterial thrombosis, arterial stenosis, portal vein thrombosis, hepatic vein stenosis and biliary complications hamper graft survival, though the unusually high incidence of biliary abnormalities we reported is partly consequence of per-protocol imaging screening. The management of all these complications requires well-trained surgeons, interventional radiologists, and endoscopists. In summary, the technical learning curve of adult LDLT is lengthy and demanding.
4. LDLT might be a chance for patients affected by autoimmune liver diseases, who generally show lower MELD scores, and by primary and secondary cancers because of several inherent advantages: a) small left-liver grafts are relatively safe in the absence of portal hypertension; b) selection can be modulated and does not have to abide by the rules of priority of deceased-donor waiting list; c) timing can be chosen in keeping with neo-adjuvant protocols.

Introduction to the third part

Liver structure

The liver performs crucial metabolic, synthetic and detoxification functions. To name a few, the liver metabolises and parcels glucose and lipids, synthetises plasma proteins and bile acids, degrades ammonia, and deactivates and excretes xenobiotics.[207] These intertwined tasks are carried out thanks to a hierarchical arrangement of the organ, whose periodic unit is alternatively portrayed as lobule or acinus, depending on which physiological property is studied.[208] The most common description of microscopic functional liver unit is the lobule, a hexagonal structure, delimitated by six portal triads and hinged upon a central vein.[209] The portal triad contains a branch of the hepatic artery, a branch of the portal vein, and a bile duct ramification. The blood from both the supplying vascular trees mingles in fenestrated liver capillaries, the sinusoids, which filter substrates for the metabolic activity of surrounding hepatocytes. From sinusoids, blood is conveyed to central veins, ending in hepatic veins.[210]

This structure contains endothelial cells, epithelial cells, i.e. hepatocytes, which constitute two thirds of total liver mass, and cholangiocytes, and non-epithelial cells. Hepatocytes are polarised and exhibit three plasma membrane domains.[211] The basolateral domain faces the sinusoids and is in contact with the space of Disse, the interspace between hepatocytes and the fenestrated sinusoidal endothelium. At the lateral domain, tight junctions link adjacent hepatocytes. The apical domain is the secretory pole, set with transporters for bile acids and anions. Two or three juxtaposed biliary domains of adjacent hepatocytes form an intercellular space, called the bile canaliculus, which drains the primary bile. Bile then flows out through the bile ducts, vessels paved cuboidal epithelial cells, the cholangiocytes, which modulate the composition of bile by means of absorption and secretion of ions, solutes and water.[212] The canals of Hering, midway between

canaliculi and bile ducts, host differentiated hepatocytes, cholangiocytes, and progenitor cells.[213,214]

Among non-epithelial cells, the most relevant types are the Kupffer cells, and the Ito cells or hepatic stellate cells (HSCs). The Kupffer cells, resident macrophages, are located within the sinusoidal vascular space, where they phagocyte cell debris and secrete inflammatory factors upon stimulation, promote tissue repair but also contribute to the progression of liver diseases.[215] The perisinusoidal space of Disse harbours HSCs, vitamin-A-storing cells that secrete extracellular matrix and collagen scar tissue upon activation.[216]

Liver haemodynamics

The liver accounts for 20% of total body oxygen consumption at rest. Accordingly, total hepatic blood flow is approximately 1.5 l/min or 25% of the cardiac output, via a dual blood supply.[217,218] Hepatic artery accounts for about 20-25% of the flow and portal vein for about 75-80% of the total hepatic inflow. Given that portal blood shows significantly higher oxygen saturation – around 85% – compared to standard vein blood,[219] hepatic artery and portal vein provide 50% each of the oxygen supply.[220]

The hepatic arterial pressure is comparable to systemic blood pressure, while normal portal pressure is between 5 and 10 mmHg. Variations in portal pressure depend essentially on two elements: the degree of dilation of mesenteric arterioles and intrahepatic resistance.[221]

Portal hypertension is defined as portal pressure greater than 12-15 mmHg or as pressure gradient between portal vein and inferior vena cava greater than 5-10 mmHg.[222]

Portal flow and pressure are mutually dependent according to Ohm's law: $P = FR$, where P is the pressure gradient through the portal venous system, F is the volume of blood flowing through the system, and R is the resistance to flow.[223,224] The complex relationship between splanchnic haemodynamic parameters is not yet

fully understood. The healthy liver exhibits a significant compliance and buffers an increase in portal flow by a reduction in vascular resistances, thus maintaining a stable pressure. Two states alter this balance: chronic portal hypertension and portal hyperperfusion syndrome. In chronic portal hypertension, periportal fibrosis initially raises intrahepatic resistances. Then, splanchnic vasodilatation ensues leading to splanchnic hyperflow.[225-228] Conversely, the portal hyperperfusion syndrome or SFSS is caused by an abrupt reduction in the hepatic vascular bed after surgical liver resection, which acutely generates a mismatch between portal blood flow and the drainage capacity of the liver.[229,230] While portal hypertension is a chronic condition that develops over several months or years,[226,228] SFSS is an acute phenomenon that occurs mainly after partial liver transplantation or after extended hepatectomy.[229,231,232] However, both conditions can coexist in a large spectrum of combinations. One is partial liver transplantation in cirrhotic patients with basal portal hyperflow, or major hepatectomy in patients with compensated cirrhosis, in whom liver compliance is compromised and incapable of accommodating an increased portal flow. At the other end of the scale there are very small-graft transplantation and extreme liver resection in patients with normal portal circulation.

Portal flow also regulates arterial inflow in an intimate dynamic adaptive relationship.[233] Arterial flow decreases when portal flow increases, while it increases when portal flow decreases, so that total hepatic blood inflow is relatively constant. This non-reciprocal relationship is termed hepatic arterial buffer response (HABR).[233,234] This specific arterial response exists because the liver has no active role in regulating portal inflow and is a passive recipient of changing PVF.[235-237] Remarkably since liver receives oxygen in excess, fluctuating oxygen concentration in inflowing blood does not trigger HABR.[238] The regulation of HABR is mainly humoral and is mediated by portal adenosine washout.[239] This molecule is constantly released in the space of Mall, the interstitial compartment surrounding the portal triad, and is responsible for arterial vasodilation.[240]

Adenosine regulates arterial contractility directly, by relaxing smooth muscle fibres, and indirectly, by stimulating nitric oxide and prostacyclin release and inhibiting thromboxane and endothelin-1 secretion.[239] An increased portal flow washes out adenosine, decreasing adenosine concentration in the space of Disse, and favouring hepatic arterial vasoconstriction.[239] Conversely, a decreased portal flow is less efficient in clearing adenosine, which mediates hepatic arterial dilation and increased flow rate. The role of the nervous system in mediating HABR has not been completely elucidated because HABR is lost upon brain death but undeniably maintained after selective liver denervation, e.g. in case of liver transplantation.[241-244] In vivo, the surge in portal flow following extreme hepatectomy or partial-graft transplantation results in a strongly decreased arterial flow. This explains some histological features of SFSS, such as ischaemic cholangitis or parenchymal infarctions.[229,234] A slow arterial flow can also predispose to severe and long-lasting complications such as hepatic artery thrombosis,[245] and ischaemic-type biliary stenoses.[246]

Liver regeneration and splanchnic haemodynamic changes

The whole mammalian gastrointestinal system metabolises and detoxifies foodborne, waterborne and microbiota-generated toxic compounds. These protective functions come with collateral damage, which causes cell loss through physical attrition, chemical injury and immune destruction. To prevent tissue loss and dysfunction and maintain homeostasis, liver parenchymal cells and intestinal epithelial cells show strong regenerative capacity throughout life.[247] The unique regenerative potential of the liver is the premise for major hepatectomies and partial liver transplantation. In baseline conditions, the liver is quiescent and liver cells have negligible proliferative and apoptotic activity. However, in case of significant parenchymal loss that exceeds two thirds of the initial volume, hepatocytes, before, and, then, the other parenchymal cells start proliferating rapidly. [248,249] This compensatory hyperplasia leads to the recovery of an adequate

volume for homeostasis, the so-called "hepatostat",[250] within 7-10 days. The peak of regenerative activity varies between mammalian species but is mostly within the third postoperative day.[251]

Although a number of cytokines, growth factors, and signalling pathways are clearly involved in liver regeneration,[252,253] it is less understood how they interact and integrate. Besides, the splanchnic haemodynamic variations following hepatectomy and transplantation appear to be closely related to liver regeneration. While a disproportionate increase in portal flow and pressure impairs regeneration,[254,255] excessive diversion of portal flow compromises regeneration.[256-258] The effects of splanchnic haemodynamic variations on regeneration are the consequence of 1) physical shear stress,[259-267] 2) fluctuations in the concentration of signalling molecules from the digestive tract,[268-272] and 3) release of growth factors from coresident cells and remodelling extracellular space.[273]

Postoperative liver failure stems from an insufficient or an ineffective regenerative process or from a combination of the two. The orchestration of regeneration of the several parenchymal cells is a particularly critical issue to ensure homeostasis from the growing liver. The imbalance between hepatocyte and non-hepatocyte proliferation causes architectural disruption and impaired function.[274-276] An excessive portal flow triggers an excessive growth of hepatocytes while the rest of the cell populations (in particular, endothelial cells and cholangiocytes) lag behind, leading to the proliferation of non-functional avascular and relatively hypoxic hepatocytes islands.[276,277]

Failed regeneration: SFSS and PHLF

A definition for SFSS

SFSS implies the occurrence of a set of symptoms during the first week after liver transplantation of a small, usually partial, graft, after elimination of other causes of liver dysfunction, such as acute rejection, ischaemia, sepsis and technical vascular

or biliary problems. Symptoms include jaundice, coagulopathy, ascites and encephalopathy.[278,279] Similarly, PHLF occurring after liver resection has been defined as a "postoperative acquired deterioration in the ability of the liver to maintain its synthetic, excretory and detoxifying functions, which is characterized by an increased INR and concomitant hyperbilirubinaemia on or after the 5th postoperative day".[280] The severity of this syndrome has been graded according to the necessity of treatment, with a frightening correlation with increasing mortality rates.

Although the mechanisms behind liver failure are complex and still debated, the syndrome is typically explained by a mismatch between portal inflow and downstream vascular bed. Portal flow is increased independently from, as in case of pre-existing portal hypertension, or relatively to a reduced liver mass. Hyperflow results in greater portal pressure, which in turn is responsible for shear stress to sinusoidal endothelium and hepatocyte injury.[229,281] Histologically, this process results in denudation of sinusoidal endothelium and parenchymal haemorrhage, microvascular thrombosis, necrosis, and, lately, arterial vasospasm with ischaemic cholangitis.[243]

A small-for-size or a small-for-flow syndrome?

Many transplant centres follow the arbitrary criterion that recommends a graft-to-recipient-body-weight ratio (GRBWR) larger than or equal to 0.8%. Indeed, a very recent comprehensive meta-analysis integrating also our experience and reporting on 4001 patients of different ethnic groups has been published on this topic. The authors concluded that a low ratio, defined as a GRBWR<0.8%, is significantly associated with inferior 1- and 3-year survival rates and SFSS.[282] However, there is evidence that SFSS can occur even when the aforesaid criterion is fulfilled whereas a strict control of graft inflow can effectively prevent PHLF even with small grafts.[283-285]

The unclear limits, the role of portal vein pressure (PVP), and the feature of an early histological damage have progressively clarified that difference between liver mass and portal flow that is similarly responsible for the damage after small-graft transplantation or extended hepatectomy, and that portal hyperperfusion is crucial in the development of SFSS. Consequently, in 2013 another term, maybe more appropriate, was coined for this condition: the small-for-flow syndrome (SFFS, Table 4).[286]

Intraoperative portal vein flow (PVF) and PVP measurements predict SFSS with thresholds set at PVP 20 mmHg and PVF 250 ml/min/100 g.[230,287] An elevation in PVP is necessary to trigger liver regeneration.[287] Nevertheless, an excessive PVP produces sinusoidal endothelial cell injury, haemorrhage, oedema, architectural disruption, and impaired liver function. Additionally, an elevated PVF reduces the hepatic artery blood flow, possibly leading to ischaemia and biliary damage. The large capacity of the splanchnic venous system endures fluctuations in PVF with minimal effects on PVP,[288-291] but, once inelastic veins are fully distended, pressure quickly rises with increased PVF.[292] After resection or graft revascularisation, the total blood flow that the liver has to accommodate is increased because the vascular bed is amputated, and this situation is exacerbated in case of a pre-existing portal hypertension. Higher PVPs actually correlate with lower graft weights.[293]

Table 4. Experimental studies analysing SFSS and their used strategies to prevent it.[294]

Author	Year	Animal	Model	Focus	Prevention strategy
Nagano et al.[295]	2002	Rats	90% hepatectomy	Size	Portal vein ligation
Smyrniotis et al.[296]	2003	Pigs	20% liver graft	Size	Mesocaval shunt
Kelly et al.[297]	2009	Pigs	20% liver graft	Size	Adenosine administration
Ladurner et al.[298]	2009	Pigs	75% hepatectomy	Size	Portocaval shunt
Di Domenico et al.[299]	2011	Rats	80% hepatectomy	Flow	Splenectomy or splenic transposition
Wang et al.[257]	2014	Pigs	85–90% hepatectomy	Flow	Mesocaval shunt
Wang et al.[300]	2015	Pigs	85–90% hepatectomy	Flow	Extracorporeal continuous portal diversion
Mohkam et al.[301]	2016	Pigs	70 and 90% hepatectomy	Flow	Somatostatin administration
Carrapita et al.[302]	2016	Rats	85% hepatectomy	Flow	Splenic artery ligation
Xiang et al.[303]	2016	Pigs	80, 85, and 90% hepatectomies	Flow	-
Asencio et al.[304]	2017	Pigs	90% hepatectomy	Flow	Portal vein embolization
Bucur et al.[305]	2017	Pigs	75% hepatectomy	Flow	Adjustable portal ring
Athanasiou et al.[306]	2017	Pigs	75–80% hepatectomy	Flow	Splenectomy
Song et al.[307]	2018	Mice	IRI + 70% or 80% hepatectomy, and 30% graft	Size	Melatonin administration
Kohler et al.[308]	2019	Pigs	70% hepatectomy	Flow	70% reduction of PVF

Tackling SFSS

The role of ischemia-reperfusion injury

SFSS is often associated with sepsis and ischemia-reperfusion injury (IRI), conditions that shrink the adaptive capacity and the regenerative potential of the remnant liver. Consequently, along with sound infection prevention and control, specific hepatoprotective measures are employed to counteract IRI, among them: intermittent portal clamping during resections and hypothermic liver preservation before graft implantation.[309]

Inflow modulation

On the side of haemodynamic stress, the mainstay of surgical management lies in liver inflow modulation. The aim of inflow modulation is to improve liver haemodynamic through a reduction of excessive inflow without weakening liver function and hampering regeneration by excessive shunting.[256,287,310]

Three indicators help tailor inflow modulation, namely PVF, PVP and hepatic arterial flow.[263,293,311-314] The reduction in portal flow contains shear stress and yields a concomitant improvement in hepatic artery flow.[312] The putative threshold is four times the flow measured in healthy subjects, i.e. 360 ml/min/100 g of liver weight, and the target is placed between twice the perfusion observed in full-size grafts (260 ml/min/100 g) and as twice the baseline flows detected in healthy patients (180 ml/min/100 g).[263,314-318] A portal pressure threshold is undecided between 15 and 20 mmHg.[293,313,319] Some authors recommend not to consider just portal pressure but to combine the information with central venous pressure (CVP) to obtain the hepatic venous pressure gradient (HVPG), where HVPG=PVP-CVP. The gradient takes into account both the upward and downward forces exerted on liver, because 60-90% of CVP fluctuations are transmitted to portal pressure, and because CVP regulates the relationship between portal flow and pressure according

to the formula PVF=PVP-CVP/resistance, where the intrahepatic resistance pertains to the quality of liver parenchyma.[263,320] In this case, the threshold of HVPG to suggest modulation is set at 15 mmHg. Arterial flow is considered adequate if greater than or equal to 100 ml/min. The integration if the three parameters stems from the evidence that the correlation between portal flow and pressure is poor,[263,321,322] and that the liver should be protected against portal and arterial hypoperfusion as well.[263] This algorithm and the relative gimmicks for inflow modulation are wrapped up in Figure 2 and consist of splenic artery obliteration and portosystemic shunts.

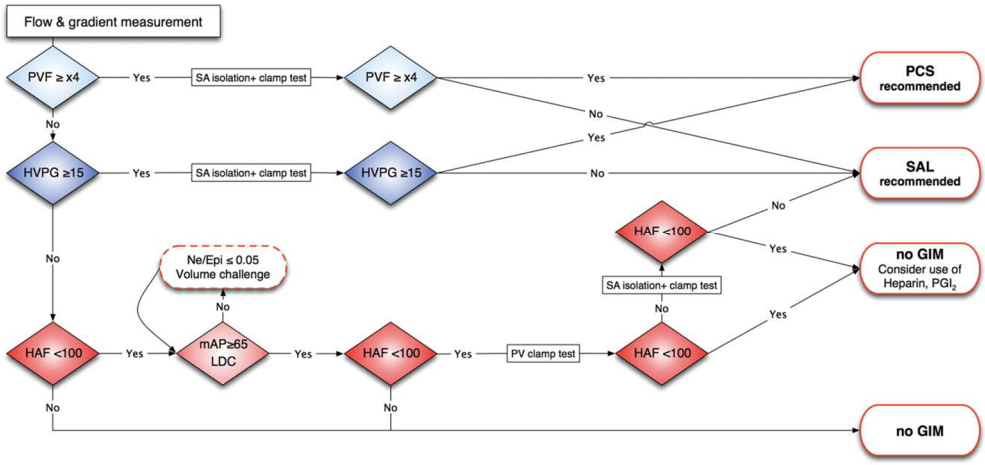

Figure 2. Algorithm for inflow modulation according to flows, gradients and systemic haemodynamics. The measurements can be relied upon provided stable haemodynamic conditions, absence of active bleeding, optimal outflow, exclusion of technical problems with the artery. Low dose catecholamine (LDC) is ≤0.05 g/kg/minute.[263]

Portosystemic shunts

Different types of surgical portosystemic shunts have been described to prevent SFSS after partial liver transplantation. The first type of shunt was described by Boillot et al. in 2002 and consisted of a meso-caval shunt with downstream ligation of the superior mesenteric vein.[323] Consequently, the portal flow directed to the liver comes from the duodeno-pancreatic and gastric region, while the entire intestinal flow is diverted into the inferior vena cava. The shunt resulted in a 27% decrease in portal pressure.[324] Sato et al. reported on a second type of shunt, consisting of a termino-lateral meso-renal shunt between the inferior mesenteric vein and the left renal vein. This shunt decreased portal pressure by 20%.[325] Other porto-systemic shunts were subsequently described but the most common one remains the partial porto-caval shunt, which reduces portal pressure by around 30%.[314,326] Porto-systemic shunts help lower the limit of liver weight/total body weight ratio from 0.8% to 0.65% without increasing mortality.[327] However, these shunts can cause portal vascular steal resulting in postoperative hepatic encephalopathy and liver failure.[314,324,328] To counterbalance this issue, some have proposed secondary closure of the shunt after sufficient liver regeneration.[329]

Splenic flow modulation

Splenic flow modulation aims to limit the flow of the portal vein while avoiding bypassing the portal flow into the systemic circulation. Splenectomy was first proposed to limit portal hyperperfusion after partial liver transplantation.[330] However, the technique is fraught with major morbidity and asplenia increases the risk of overwhelming bacterial infections.[331,332]

Splenic artery ligation is a simple means of limiting splenic venous return and thus decreasing portal flow, without the drawbacks of splenectomy.[263,313,333] Ito et al. compared the results of living-donor liver transplants with and without splenic artery ligation.[293] Ligation significantly decreased portal pressure and improved

graft survival. In addition, splenic artery ligation has been shown to increase hepatic arterial flow, which in turn is decreased in SFSS. The mechanism behind the effectiveness of splenic artery ligation is debated but may consist of the inhibition of the hepatic arterial buffering effect or the prevention of splenic artery steal syndrome.[234,334]

Given the effectiveness of splenic artery occlusion, arterial embolization has been proposed. The interruption of splenic arterial flow can be scheduled before or after transplantation. Umeda et al. showed that prophylactic embolization of the splenic artery prior to living-donor liver transplantation improved regeneration and decreased the risk of postoperative ascites, hyperbilirubinaemia, and SFSS.[335] Conversely, splenic artery embolization during the first week after transplant has been shown to treat SFSS.[336,337]

Pharmacological modulation

A number of molecules have been used to modulate portal flow, most of which are not yet validated in clinical practice. Somatostatin and its analogues are the most widely investigated molecules for this indication. In preclinical studies, in SFS transplantation, somatostatin has proved to reduce portal flow and protect sinusoidal endothelial cells in the critical postreperfusion period.[338] Interestingly, the group from Barcelona showed that somatostatin protects and prevents stellate cells activation independently of changes in portal flow, in a model of SFS transplantation and liver resection. This change might result in less liver fibrogenesis even later after surgery.[339] In clinical studies, Busani et al. reported the effectiveness of octreotide in decreasing portal pressure after left liver transplantation. This effect was increased when the infusion was combined with the administration of esmolol, a cardioselective beta-blocker.[340] Ozden et al. reported a case of SFSS after transplantation of right liver without the middle hepatic vein, which was successfully treated with combined somatostatin and propranolol infusion.[341] Feng et al. compared 20 living-donor liver transplants that

received postoperative somatostatin infusion with 20 controls: the authors reported better recovery of function and a more rapid decrease in post-transplant bilirubin levels in recipients who received somatostatin infusion.[342] Troisi et al. reported the efficacy of somatostatin as liver inflow modulator even after whole liver transplantation.[343]

Preoperative optimisation: methods of liver volume augmentation

In healthy patients, at least 20% remaining healthy liver tissue, or 0.5% liver to body weight ratio,[344] is required after liver resection. Up to 30% of liver is needed after extended chemotherapy. In case of primary impairment of the liver, e.g. steatosis, cholestasis or cirrhosis, the preservation of at least 40% of the liver is required.[345,346] For these patients, it is also important not to rely only on determination of future remnant liver (FRL), but also to perform a functional liver examination. However, if the relationship between haemodynamic parameters, volume and function is so stringent, a brilliant solution to increase operability while respecting the requirements for a minimum FRL volume is to preoperatively increase the remnant volume.

Maakuchi first described the concept of preoperative portal vein embolization for perihilar cholangiocarcinoma, a technique that stimulates hypertrophy of the contralateral hemiliver.[347] The increase in volume and function of the FRL effectively mitigates the risk of post-hepatectomy liver failure.[348,350-353] It is indicated when the volumetric and functional criteria predict an inacceptable risk of post-hepatectomy liver failure. It increases the eligibility of patients for hepatectomy in about 20% of cases,[354] while in about 5% of cases it results in insufficient liver growth.[352] While severe complications of the procedure are rare (2.5%),[352] the main pitfall of portal embolization is that it may promote progression of neoplastic disease (observed in about 66% of cases) while awaiting hypertrophy and because of the increased arterial flow in the embolised liver.[355] In this context, the optimal interval for adequate hypertrophy with a limited risk of progression

would span two to three weeks between portal embolization and hepatectomy.[355,356] In order to limit the risk of neoplastic progression, portal embolization is accompanied by neoadjuvant chemotherapy in the case of colorectal liver metastases or preceded by transarterial chemoembolization in the case of hepatocellular carcinoma.[357,358] Portal vein embolization and ligation are comparable in terms of efficacy and safety,[359] though the occlusion of the segment-IV branch is more easily obtained via embolization than surgical ligation.[359]

The game-changing procedure that associates liver partition and portal vein ligation for staged hepatectomy (ALPPS) was conceived to increase the feasibility of two-stage hepatectomies and first reported in 2012.[360] As suggested by the acronym, this technique combines portal branch ligation and parenchymal transection during the first step, so that all intrahepatic venous collaterals are severed. The first stage is followed by a completion hepatectomy once sufficient liver mass is restored. The extent and pace of liver regeneration induced by this technique outstrip portal embolization. The first stage brings about a stunning hypertrophy of the FRL, up to 80%, and cuts the time between the two stages from four-six to two weeks.[361]

ALPPS is suggested for patients with insufficient hypertrophy of the FRL after portal embolization,[362,363] but it is burdened with a very high morbidity (28%) and mortality (9%), which warrant strict yet not well-defined patient selection criteria.[362] In patients who develop severe complication after the first stage, usually biliary leaks and sepsis, the second stage should be delayed or avoided.[364] Significant underlying fibrosis contraindicates ALPPS.[365] The hypertrophy ensuing the first stage of ALPPS overestimates the effective functional capacity of the FRL. In this context, functional imaging, like liver scintigraphy, has an important role to play.[366]

Why ALPPS is effective in fostering regeneration?

During the past decade the scientific community has made a considerable effort to elucidate the mechanisms of the success of ALPPS. In particular, a number of animal models of ALPPS have been created to investigate how the first stage is that successful in accelerating the recovery of hepatic mass and function, compared to other techniques of portal vein occlusion.[367-369] It is largely undisputed that portal vein ligation and parenchymal transection push hepatocytes into cell cycle in an accelerated rate and to a greater extent than conventional portal vein obliteration techniques. This faster regeneration brings about significantly increased mass recovery and survival after completion hepatectomy.[370] From the viewpoint of haemodynamics, portal vein obliteration rapidly raises portal pressure while portal flow to the FRL intuitively triplicates. Nonetheless, adding or omitting parenchymal split does not imply substantial portal flow variations.[371] Altogether, these evidences suggest that parenchymal split mediates a pro-regenerative action by means of liver growth factor release rather than portal flow dynamic changes.[367] Moreover, it is known that a compensatory HABR accompanies the steep increase in portal inflow after portal obliteration but this HABR results in distinct tissue hypoxia in ALPPS models and mild hypoxia after portal vein ligation.[372] Thus, while low tissue oxygenation is traditionally considered to prompt liver failure after extreme hepatectomy, hypoxia and hypoxia-induced signalling are ostensibly associated with accelerated regeneration and better survival in ALPPS.[373]

Dili et al. in a series of seminal works investigated the role of hypoxia in rat model of ALPPS.[370,373] First, compared to partial hepatectomy, ALPPS first stage induced hypoxic signalling, in terms of nuclear concentration of hypoxia-inducible factor (HIF)□1α and □2α early after surgery, and an angiogenic transcription profile, later on the first postoperative day. Not only at a molecular level, ALPPS first stage rescued liver sinusoidal morphology, measured as physical size and reduction of collapsed sinusoids. The authors reproduced the hypoxic effects of the ALPPS first

stage by associating liver resection and hepatic artery ligation or the administration of dimethyloxalylglycine (DMOG), a hypoxia simulator, and remarkably obtained comparable results with regards to the angiogenic transcription profile, sinusoidal morphology, hepatocytes proliferation, and increased survival. The authors sensibly concluded that the hypoxic signal response protects from SFSS in this model of ALPPS.[373]

Hypoxia in liver regeneration

As a general assumption, cell regeneration requires great amounts of oxygen because of an increased metabolic demand.[374] A small FRL causes an intense proliferative stimulus. Mitochondrial oxidative phosphorylation consumes about 90% of the cellular O_2 to produce ATP. Hepatocyte proliferation occurs before efficient angiogenesis causing transient microcirculatory disturbances, inadequate oxygen and substrates delivery, alteration of the mitochondrial redox state, and reduced ATP production.[374,375] In line with these concepts, hypoxia might sensibly prove detrimental for liver regeneration. Yet, the role of hypoxia and the response to hypoxia during liver regeneration are not entirely clear. Oxygen tension is distributed in a decreasing gradient across the hepatic lobule, from the periportal to perivenous hepatocytes. Hepatocytes bear with and respond to low oxygen according to their location. Hypoxia worsens the intracellular redox state, which may result in the production of reactive oxygen species (ROS). ROS also work as signalling molecules and activate mitogen pathways, such as the mitogen-activated protein kinase (MAPK) cascade (ERK1/2, c-jun), crucially involved in liver regeneration.[376] Moreover, it is known that cellular metabolic rate declines by 50% every 10°C drop in temperature.[377] Coherently, hypoxia evokes a regulated hypothermia, in an attempt to reduce energy expenditure by curbing oxygen consumption and by increasing oxygen affinity of haemoglobin.[378] The same as for cold storage in organ transplantation, hypothermia saves ATP by reducing

hepatocyte metabolic rate, mitigates acidosis and its associated metabolic dysregulation, decreases IRI, and prevents cell death.[377]

Hypoxia sensing

Intracellular oxygen sensing mechanisms rapidly reacts to low oxygen by stabilising HIFs. HIF-1α and -2α are two cytosolic heterodimers that act as transcription factors when translocate to the nucleus, partner with the constitutively expressed β subunit to form the active complex, bind with specific sequences, the hypoxia-responsive elements (HRE), and regulate the expression of target genes. In conditions of standard oxygen concentration, propyl-hydroxylase domain (PHD) proteins constantly hydroxylate the α subunit. Hydroxylated α subunits are recognised by the Von Hippel–Lindau tumour suppressor gene (VHL) protein of the E3 ubiquitin ligase complex, and rapidly degraded via the ubiquitination/proteasome pathway.[379] PDH hydroxylase activity requires as cofactors oxygen, α-ketoglutarate, iron, and ascorbate.[379] As a consequence, PHDs qualify as the true oxygen sensors. Hypoxia, precisely the lack of oxygen as PHD cofactor, makes HIF-α subunits escape hydroxylation and, thus, degradation.

HIFs in liver regeneration

HIF stabilisation may favour liver regeneration in at least three ways. First, HIF-induced pathways favour glycolysis to the detriment of mitochondrial respiration.[380-382] Matsuo et al. observed high glycogen concentrations in regenerating hepatocytes after ALPPS first stage.[383] Increased cell glycogen is a HIF-1α-dependent metabolic adaptation to endure glucose deficiency.[381,384] PHD knockout mice show ischaemia-resistant hepatocytes compared to wild-type animals. PDH-1 loss reduces the oxidative stress, reduces oxygen need, soothes hepatocyte swelling and, consequently, sinusoids compression, improving local microcirculation.[385,386] Secondly, pharmacological HIF stabilisation increases the

expression of cell cycle-promoting cyclins after liver resection.[387] Thirdly, HIFs activate angiogenic genes that increase vascular permeability, endothelial cell proliferation and new vessel development.[380] Activation of HIF pathways results in mobilisation and engraftment of bone marrow progenitors of liver sinusoidal endothelial cells into the regenerating liver, via VEGF and stromal cell-derived factor-1 signalling.[388-390] These progenitors are responsible for angiogenesis and production of hepatocyte growth factors.[388-392]

During the early phase after liver resection, hypoxia sensors are activated and this has been observed in plentiful settings.[370,373,393] These pathways are activated because of the early hypoxic environment after liver resection, a condition induced by the imbalance between the increased parenchymal oxygen requirements the excess of oxygen-poor portal blood inflow, and the reduced arterial inflow. However, this response is highly conserved across species and hepatectomy models, and it is, thus, probably necessary to trigger the early phase of liver regeneration.[393] Remarkably, these specific pathways are rapidly suppressed after two-third hepatectomy. Consequently, hypoxia tunes early regeneration, as a master regulator of angiogenesis. On the contrary, prolonged hypoxia-associated signalling dampens cell proliferation, by preventing G1/S transition through regulation of p27 expression.[394] In the very early phase after liver resection, lobular architecture is transitorily disorganised, with proliferating hepatocytes forming avascular clusters. The extent of liver resection drives the extent of hepatocyte proliferation. Subsequently, postoperative liver dysfunction might stem from this transient structural disorder, in a hypothetically predictable relationship with the magnitude of hepatectomy[274,276,395,396] Measures that activate HIF-related pathways, i.e. ALPPS, pharmacological stabilisers, etc., might fill the gap between the proliferation of avascular hepatocyte islands and neo-vessels rearrangement and, therefore, prevent liver failure and associated mortality.

Simulation of hypoxia in liver surgery

Pharmacological developments in hypoxia sensors stabilisation

As aforementioned, Dili et al. have recently demonstrated that ALPPS is effective in reducing mortality after extensive liver resection and that local hypoxia in the FRL is responsible for this beneficial effect.[370,373] Among possible explanations, hypoxia would induce new vessel formation and, at least, partly settle the mismatch between sinusoidal and hepatocellular proliferation. Still, it is not yet clear the impact of hypoxia on the different cell types, whether hypoxia improves or supports liver function and whether this strategy can be transferred to other liver surgery models, such as partial liver transplantation, in which there is also an IRI component.

A major question is whether this state of hypoxia can be mimicked and whether a putative simulated hypoxia confers the same advantages to the regenerating liver in vivo. This is particularly relevant because strategies that induce straightforward hypoxia, such as asphyxiation or hepatic artery ligation, cannot be proposed *in vivo*. If nothing else, human bile ducts are very sensitive to ischaemia. Indeed, pharmacological inhibition of oxygen-sensing prolyl hydroxylase domain (PHD) proteins, which degrade HIFs in vivo and thus confer oxygen sensitivity on the HIF pathway, has a therapeutic potential.[397] This inhibition does not require the induction of a real hypoxia. DMOG and ethyl-3,4-dihydroxybenzoate (EDHB) are used at times for this purpose,[373,387] but the former is not approved for human use and both inhibit dioxygenases at large. Deferoxamine, an old iron-chelating agent, shows similar activity in stabilising HIF by interfering with iron metabolism.[398] In summary, none of these molecules is selective and this lack of selectivity poses a significant burden on the interpretation of the results. DMOG and EDHB compete with α-ketoglutarate. Hundreds of α-ketoglutarate-dependent dioxygenases have been described, which influence the more diverse reactions, including hydroxylation, dealkylation, desaturation, epoxidation, epimerisation,

halogenation, cyclisation, peroxide formation, and ring expansion/contraction.[399] With regards to iron chelation, it is estimated that 6.5% of all human enzymes are iron-dependent.[400]

It has been recently developed a new set of isoquinolines with a significant potential in the approach to anaemia secondary to renal failure through increased endogenous production of erythropoietin, improved iron absorption and mobilisation, and reduction of hepcidin.[401] Among these, i.e. roxadustat, desidustat, daprodustat, molidustat, and vadadustat, the first is already approved in Chile, China, European Union, Japan, South Korea, for the treatment of anaemia in chronic kidney disease. Moreover, roxadustat has already been shown to reduce ischaemia-reperfusion in a DCD (donor after cardiac death) rat liver model preserved on a perfusion machine.[402]

The administration of such substances for the prevention of SFSS and their effects on function and morphology of a regenerating liver have never been explored.

Measures of successful regeneration

A large body of literature has pointed out that a primary prevention strategy for SFSS or PHLF might be the promotion of an angiogenic switch to produce an early effective regeneration. Nonetheless, it is less clear how to measure the degree of effectiveness and its translational potential to humans. In this sense, successful regeneration is not entirely reflected by mere liver growth because volume and weight might just be the result of interstitial oedema or cell swelling.[403] Similarly, the results in terms of mortality extrapolated from murine models, although reproducible, can hardly be translated to humans. The clinical patterns observed in small animals are different from what is described in patients. Hypoglycaemia is often a later event, biochemical profiles are irrelevant to man and surgical models are often irreversible.[404]

The study of hepatocellular function in liver regeneration is crucial for the translational potential of this research domain. These issues call for a more

mechanistic insight of liver working during regeneration to interpret whether a proposed treatment might be successful in humans.

The aim of the third part of the thesis

Based on the large body of evidences summarised herein, we postulated that hypoxia is a key mechanism for liver regeneration after resection, and that the HIF pathways mediate the beneficial effects exerted by hypoxia. It is known that intracellular HIF are stabilised by reducing the activity of PHD1-2-3, where there is no selectivity possible because the catalytic site of the three PHD isoforms are highly conserved. However, the isoquinolines, recently developed to act as PHD inhibitors, are highly selective for this catalytic site. The use of selective PHD inhibitors in hepatobiliary surgery for the prevention of human SFSS or to foster enhanced regeneration has never been explored so far.

In this exploratory project, we meant to test roxadustat, a new selective PHD inhibitor, versus placebo, as a hypoxia simulating treatment after non-lethal hepatectomy, i.e. 70% hepatectomy, and to evaluate in detail their effects: 1) on the regeneration process of hepatic parenchyma, and 2) on the overall hepatocellular function during the course of regeneration.

Third work: Selective HIF stabilization alleviates hepatocellular steatosis and ballooning in a rodent model of 70% liver resection

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Research Article

Selective HIF stabilization alleviates hepatocellular steatosis and ballooning in a rodent model of 70% liver resection

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Background: Small-for-size syndrome (SFSS) looms over patients needing liver resection or living-donor transplantation. Hypoxia has been shown to be crucial for the successful outcome of liver resection in the very early postoperative phase. While poorly acceptable as such in real-world clinical practice, hypoxia responses can still be simulated by pharmacologically raising levels of its transducers, the hypoxia-inducible factors (HIFs).
We aimed to assess the potential role of a selective inhibitor of HIF degradation in 70% hepatectomy (70%Hx).
Methods: In a pilot study, we tested the required dose of roxadustat to stabilize liver HIF1α. We then performed 70%Hx in 8-week-old male Lewis rats and administered 25 mg/kg of roxadustat (RXD25) at the end of the procedure. Regeneration was assessed: ki67 and 5-ethynyl-2′-deoxyuridine (EdU) immunofluorescent labeling, and histological parameters. We also assessed liver function via a blood panel and functional gadoxetate-enhanced magnetic resonance imaging (MRI), up to 47 h after the procedure. Metabolic results were analyzed by means of RNA sequencing (RNAseq).
Results: Roxadustat effectively increased early HIF1α transactivity. Liver function did not appear to be improved nor liver regeneration to be accelerated by the experimental compound. However, treated livers showed a mitigation in hepatocellular steatosis and ballooning, known markers of cellular stress after liver resection. RNAseq confirmed that roxadustat unexpectedly increases lipid breakdown and cellular respiration.
Conclusions: Selective HIF stabilization did not result in an enhanced liver function after standard liver resection, but it induced interesting metabolic changes that are worth studying for their possible role in extended liver resections and fatty liver diseases.

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Introduction

In liver surgery, the management of liver resection in cirrhotic patients or small-size hepatic remnants even in healthy livers still represents a challenge. The postoperative conundrum alternatively called post-hepatectomy liver failure (PHLF) or small-for-size syndrome (SFSS) is the major cause of death after liver resection [1].

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In recent years, selective liver hypoxia has been shown to be beneficial after extended hepatectomy. In rat models of human associating liver partition and portal vein ligation technique for two-stage hepatectomy (ALPPS), parenchymal hypoxia, resulting from a reduced arterial flow, has been associated with accelerated liver regeneration after portal vein ligation and parenchymal transection [2], and evidence showed that ALPPS curbs mortality after extended liver resection [3,4]. Indeed, post-hepatectomy hypoxia-driven response induces new vessels formation and reduces the shift between hepatocellular and vessel proliferation by slowing down the former and accelerating the latter [4,5]. Tissue hypoxia activates the α subunits of hypoxia-inducible factor (HIF) transcription factors, HIF1α and HIF2α, which mediate a wide array of responses to parenchymal low oxygen pressure. Accordingly, increased HIF-α transactivity leads to new blood vessel formation in response to hypoxic injury [6]. In view of the translational relevance of these findings, introducing hypoxia in hepatobiliary surgery is an appealing perspective, though apparently unrealistic because it is known that systemic hypoxia impairs hepatic regeneration [7], and that the human biliary tract is exquisitely sensitive to hypoxia [8]. The inhibition of prolyl hydroxylase domain (PHD) proteins effectively increases HIF-α activity. PHDs require oxygen, iron, and α-ketoglutarate, to hydroxylate HIFs and steer them towards degradation. In this regard, PHDs are considered intracellular oxygen sensors. Several molecules inhibit PHD function, such as deferoxamine, dimethyloxalylglycine (DMOG), ethyl-3,4-dihydroxybenzoate (EDHB), and selective PHD inhibitors [6,9–11]. Indeed, deferoxamine protects from oxidative stress and ischemia/reperfusion injury (IRI) in animal models of liver resection [12–19]. DMOG increases sinusoidal endothelium density [11], preserves sinusoidal diameter, and significantly improves survival after 87% hepatectomy in rats [4]. EDHB acts as a preconditioning agent and improves liver regeneration after ALPPS-like procedure, by inducing cell cycle-promoting cyclins [9]. Because of their mechanism of action, the cited PHD inhibitors lack selectivity [20]. DMOG and EDHB compete with ketoglutaric acid and inhibit other α-ketoglutarate-dependent dioxygenases [21,22]. Deferoxamine sequesters iron, which is required for PHD activity, but it affects any iron-requiring enzymes. Recently, a new class of selective PHD inhibitors has been released: the isoquinolines, collectively known as HIF stabilizers because they selectively compete for the active site of PHDs. Among them, roxadustat (FibroGen, San Francisco, CA, U.S.A.), a small (352.34 Da) orally active molecule, appears particularly promising and has been already licensed in the European Union and China to treat anemia in end-stage renal disease as erythropoietin release is controlled by HIF-α [23,24]. Besides, the effects of this entire class of molecules have not yet been reported in the framework of liver resection. Roxadustat reported half-life in Sprague–Dawley rats is 3.4–5.6 h and is not dose-dependent. To obtain a steady hematocrit increase, it is chronically administered to rats three times a week [25]. Chronic administration of roxadustat > 15 mg/kg yields polycythemia and associated adverse events in rats, while doses up to 10 mg/kg are active and do not induce significant mortality [25–27]. The intraperitoneal injection of roxadustat is known to effectively target the liver and stabilize HIF-α with a peak at 6 h after administration in mice [24], while residual HIF1α stabilization is detectable after 30 h in an *ex-situ* liver graft perfusion model [26].

Thus, we hypothesized that the beneficial effects elicited by hypoxia after liver resection are linked to HIF-associated response pathways. We chose a standard rat model of 70% hepatectomy (70%Hx), known to induce intense liver regeneration without mortality [28]. We aimed to assess whether a selective pharmacological HIF stabilization, obtained by the administration of roxadustat, affects liver function and regeneration postoperatively.

Methods

Animals

Male Lewis rats came from Janvier Labs (Le Genest-Saint-Isle, France). At the time of the procedure, they were 8 weeks old and weighed 244 g (interquartile range, IQR = 234–247). The animals had been housed for at least 6 days in the local animal facility (Laboratory of Experimental Surgery and Transplantation, Harvey Tower, 4th floor, 55 Avenue Hippocrate, 1200 Brussels, Belgium) before the experimental procedures, which took place in the same laboratory. The animals were kept in standard cages, in 12-h light–dark cycle, and received standard chow and drinking water *ad libitum*. Our institutional review board approved this protocol number 2019/UCL/MD/043 on 17 December 2019.

Pharmacological pilot study

Dosage, timing of injection, and effectiveness of intraperitoneally administered roxadustat in inducing liver HIF stabilization was assessed through a pharmacological study that entailed three groups of three rats each: the first receiving roxadustat 10 mg/kg (the RXD10 group), the second receiving RXD 25 mg/kg (the RXD25 group), and the third undergoing hepatic artery ligature at the hilum (the HAL group). Animals were euthanized at three time points: baseline, 60 min, and 6 h. Toxicity was assessed via a biochemical panel and death rate. Effectiveness was assessed through HIF1α measurement in cytosolic and nuclear liver extracts, and the evaluation of expression of HIF-regulated genes.

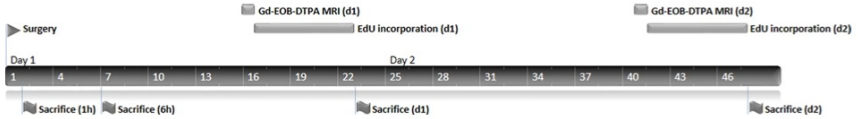

Figure 1. Timeline

Surgery

General anesthesia was obtained with isoflurane in oxygen at 2 l/min: induction 4%, maintenance 2% (IsoFlo, Zoetis, Parsippany, NJ, U.S.A.). Pre- and post-operative analgesia entailed subcutaneously injected buprenorphine 0.01 mg/kg (Temgesic, Schering-Plough, Kenilworth, NJ, U.S.A.), and ketoprofen 2 mg/kg (Ketofen, Zoetis).

70%Hx was performed by removing the median and the left lateral lobes after median laparotomy, according to the principles described by Higgins and Anderson [29]. Since the ligation at the basis of the median lobe might constrict the lumen of the inferior vena cava, we ligated the vascular pedicles individually, we divided the median lobe into its right and left segments, finally the lobes were ligated and severed one by one, according to Kubota et al. [30].

In the sham procedure, rats underwent median laparotomy and dissection of liver ligaments. The liver was wrapped in humid gauze for the duration of the procedure.

All the procedures were carried out during the afternoon (between 3:30 p.m. and 10:00 p.m.) to ensure that animals had a spontaneously empty foregut.

Study groups and postoperative follow-up

At the end of surgery, a single intraperitoneal dose of roxadustat 25 mg/kg was injected. The drug was dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, U.S.A.) and then diluted in pH 7.4 phosphate-buffered saline (PBS) to a 2% concentration of DMSO. The placebo consisted of 2% DMSO-PBS, in the same volume as the experimental drug. Postoperatively, all rats received a single subcutaneous shot of 4 ml/kg of 2.1% $NaHCO_3$ and had free access to food and 20% glucose as drinking water to avoid post-resection hypoglycemia [28].

Our study included three groups of animals (hepatectomy and roxadustat [70%Hx-RXD25], hepatectomy and placebo [70%Hx-placebo], and sham procedure and placebo [sham-placebo]), which underwent blood sampling and euthanasia after 1 (1 h), 6 (6 h), 22 (day 1, d1), and 47 h (day 2, d2) from the end of surgery and drug administration. At 15 and 40 h after surgery, rats whose euthanasia was planned on d1 and d2 underwent contrast-enhanced magnetic resonance imaging (MRI). The animals then received an intraperitoneal injection of 50 mg/kg of 5-ethynyl-2′-deoxyuridine (EdU, Carbosynth, Compton, U.K.) and were euthanized 6 h later (Figure 1).

Tissue assays

Liver nuclear and cytosolic protein extracts were obtained with the Nuclear Extract Kit (#40010, Active Motif, Carlsbad, CA, U.S.A.) and concentrations were measured via a bicinchoninic acid assay. HIF-1α and -2α were quantified in subcellular extracts using an enzyme-linked immunosorbent assay (#LS-F11633 and #LS-F9117, LSBio, Seattle, WA, U.S.A.), as per manufacturer's instructions. Total liver lipids were extracted with water, methanol, and chloroform, and quantified by the vanillin-phosphoric acid reaction.

Assessment of the hepatic function with MRI

MRI experiments were performed on a Bruker Biospec 11.7 Tesla (Bruker Biospin GmbH, Ettlingen, Germany) equipped with a volume coil (inner diameter; 74 mm). Animals were anesthetized during imaging session with isoflurane mixed with air at 3.5% for induction and 2% for maintenance (duration approx. 1 h). Estimation of global hepatocellular function was obtained through gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA, Primovist, Bayer, Leverkusen, Germany)-enhanced MRI using a dynamic contrast-enhanced sequence [31,32]. The contrast agent was injected intravenously via a catheter in the tail vein, at a dose of 0.025 mmol/kg for 30 s after the beginning of the acquisition [33–35]. Acquisition parameters were as follows: TE: 1.2 ms, TR: 9.62 ms, NA: 3, number of repetitions: 500, TA: 33 min, FA: 15°, FOV: 60 × 60 mm^2, Mat: 128 × 128, slice thickness: 1 mm. The resulting temporal resolution was 4 s. In post-processing, we selected the regions of interest (ROIs) in the right lobe from which we averaged the signal. The hepatic function was estimated with a homemade algorithm on Matlab (MathWorks, Natick, MA, U.S.A.) by fitting the signal from the ROI [36]. We obtained the following parameters from the curve: time to peak, i.e. the delay between the arrival time (t_0) of the contrast agent and the maximum signal in

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the ROI, time to 30% signal decay, i.e. the delay between the maximum signal and its reduction in 30%, and the areas under the curve (AUC) between t_0 and t_0+60 s (AUC60), and between t_0 and t_0+90 s (AUC90) [37]. We calculated the AUC between t_0 and the time to peak and the whole AUC.

Biochemistry

Complete blood cell count was done with the MS-9/3 (Melet Schloesing, La Chaux-de-Fonds, Switzerland). Lactate and pH were measured with the i-STAT (Abbot, Chicago, IL, U.S.A.). Serum analytes were measured with the DRI-CHEM NX500i (Fujifilm, Tokyo, Japan): albumin, bilirubin, NH_3, AST, ALT, creatinine. Plasma factor V was measured via an enzyme-linked immunosorbent assay (ELISA, #DL-F5-Ra, DlDevelop, Jiangsu, China). Peripheral and portal plasma triglycerides, total cholesterol, and HDL were measured with the DRI-CHEM NX500i (Fujifilm, Tokyo, Japan). Plasma erythropoietin levels were measured with the rat erythropoietin ELISA Kit, #E-EL-R0007 (Elabscience, Houston, TX, U.S.A.) following the manufacturer's instructions.

Histology

An experienced liver pathologist evaluated Hematoxylin–Eosin (H&E)-stained whole-liver sections following these parameters: presence of mitoses, inflammation, endothelial denudation, microvascular thrombosis, parenchymal hemorrhage, hepatocellular ballooning, steatosis, and necrosis. Endothelial denudation was defined as enlargement of endothelial nuclei or endothelial dislocation or extravasation of red blood cells in the Disse space. Hepatocellular ballooning was defined as clear-cell change with doubling in size of hepatocytes. Steatosis was further classified based on its location [38], its proportion (<5, 5–33, 33–66, >66%), and the type of lipid droplets (micro- or macrovesicular). Steatosis involving ≥5% of hepatocytes was considered relevant.

Fluorescence immunostaining, whole-slide imaging, and quantitative evaluation of immunostaining

Paraffin-embedded sections were deparaffinized. Endogenous peroxidases were inhibited with 3% hydrogen peroxide in methanol for 20 min. Antigens were retrieved in pH 5.7 10 mM citrate buffer. Aspecific antigen binding sites were blocked in tris-buffered saline (TBS) + 5% bovine serum albumine + 0.1% Tween20. Slides were then submitted to Ki67 staining or EdU revelation. Anti-Ki67 primary antibody (rabbit, clone SP6, #ab16667, 1/100 dilution, Abcam, Cambridge, U.K.) was incubated for 90 min at room temperature (RT). Slides were then incubated with anti-rabbit secondary antibodies (#K4003, Dako, Glostrup, Denmark) for 60 min at RT. This reaction was visualized using Alexa555-conjugated tyramide (#B40955, 1/200 dilution, Thermo Fisher, Waltham, MA, U.S.A.). For EdU revelation, after a washing step of 10 min in TBS/Tween 0.1%, slides were incubated for 30 min at RT with a TBS solution containing 4 mM $CuSO_4$, 8 mM sulfo-Cy3 azide, and 100 mM sodium ascorbate. After a washing step in PBS, nuclei were stained with Hoechst 33342 (Abcam, Cambridge, U.K.) and slides were mounted with Dako Fluorescent Mounting medium.

Fluorescence immunostained liver sections were digitalized using a Pannoramic 250 FlashIII scanner (3DHistech, Budapest, Hungary) at ×20 magnification. Scanned slides were then analyzed using the image analysis tool Author version 2017.2 (Visiopharm, Hørsholm, Denmark). On each slide, tissue sections were automatically surrounded at low magnification. Delineations were visually checked and manually corrected if required. Cells were then detected at high resolution (×20) with a nucleus-based cell classification relying on Hoechst labeling. Following segmentation, post-processing steps were applied to separate Ki67+/− and EdU+/− cells. The parameters were constant for all slides. Results were expressed as permillage of stained cells. We assumed that hepatocellular nuclei appear as vague spheres and, on histological sections, as circles. Thus, $V = 4/3\ \pi\ r^3$, where V is the volume of the sphere, π is the Archimedes' constant, r is the radius, and $A = \pi\ r^2$, where A is the area of the circle. Therefore, $A = \pi\ (3/4\ V/\pi)^{2/3}$ where V is the mean volume of non-neoplastic hepatocytes (287 μm^3) reported by Jack et al. [39]. The average nuclear area of rat hepatocytes is consequently 50–53 μm^2 [39,40]. Nuclei larger than 40 μm^2 were considered to pertain to hepatocytes. This threshold was chosen to accommodate for the different levels at which nuclei might have been cut.

RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction

Total RNA was extracted from homogenized rat liver with the RNeasy Mini kit (Qiagen, Hilden, Germany) including on-column DNaseI treatment. RNA purity and quantity were measured by NanoDrop spectrophotometry, and 1 μg total RNA (A260/280 ratio of 2.03 ± 0.04) used to synthesize cDNA in the presence of 2 μl Random Primers using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher) as per manufacturer's instructions. cDNA

samples were diluted 1:10 and 2 μl used in 25 μl real-time quantitative polymerase chain reaction (RT-qPCR) together with 12.5 μl SYBR Select Master Mix (Thermo Fisher), 1 μl of sequence-specific forward primer and 1 μl of sequence-specific reverse primer (Invitrogen, Carlsbad, CA, U.S.A.), and 8.5 μl sterile water. The primers are detailed below. Ribosomal Protein L19 (RPL19), i.e. the internal reference gene: forward CAAGCGGATTCTCATGGAACA, #128363, R4653 (A03), reverse TGGTCAGCCAGGAGCTTCTT, #128363, R4653 (A04); vascular endothelial growth factor-α (VEGFα): forward ATAGCAGATGTGAATGCAGACCA, #154160, R7216 (B09), reverse TCACAGTGAACGTCCAGGA, #154160, R7216 (B10); platelet-derived growth factor-β (PDGFβ): forward GGTGAGAAAGATCGAATTGT, #011376, R7603 (B07), reverse GAGTTTGAGGTGTCTTGGCT, #011376, R7603 (B08); angiopoietin-2 (AGPT2): forward GCTGGGCAACGAGTTTGTCT, #011376, R7603 (F01), reverse CAGTCCTTCAGCTGGATCTTCA, #011376, R7603 (F02). RT-qPCR assays were run in duplicates on a Rotor-Gene Q (Qiagen, Hilden, Germany) in 72-well plates over 45 cycles of 95°C for 15 s and 60°C for 60 s in a two-step thermal cycle preceded by an initiation step of 95°C for 10 min. RT-qPCR C_t values were acquired with the Rotor-Gene Q Series Software (Qiagen, Hilden, Germany) and relative gene expression calculated by the $2^{-\Delta C_t}$ method. The results were normalized for baseline expression in control rats.

RNA sequencing

Sequence libraries were prepared from the liver RNA extracts with the Lexogen QuantSeq 3′ mRNA-Seq library prep kit according to the manufacturer's protocol. Samples were indexed to allow for multiplexing. Library quality and size range was assessed using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.) with the DNA 1000 kit (Agilent Technologies). Libraries were subsequently sequenced on an Illumina HiSeq4000 instrument. Single-end reads of 50-bp length were produced with a minimum of 1 M reads per sample. Quality control of raw reads was performed with FastQC 0.11.7 [41]. Adapters were filtered with ea-utils fastq-mcf v1.05 [42,43]. Splice-aware alignment was performed with HiSAT2 against the *Rattus norvegicus* reference genome (genome build = Rnor6.0). Reads mapping to multiple loci in the reference genome were discarded. Resulting BAM files were handled with Samtools v1.5 [44]. Quantification of reads per gene was performed with HT-seq Count v2.7.14. Count-based differential expression analysis was done with R-based Bioconductor package DESeq2 [45]. Reported *P*-values were adjusted for multiple testing with the Benjamini–Hochberg procedure, which controls false discovery rate (FDR). The raw RNA sequencing (RNAseq) data are available from http://www.ncbi.nlm.nih.gov/bioproject/705692.

Results from the DESeq2 package were used to perform Over-Representation Analysis (ORA) and Gene Set Enrichment Analysis (GSEA) with the WebGestaltR package [46]. These analyses were made on six reference sets including *Biological Process* (BP), *Cellular Component* (CC), and *Molecular Function* (MF), from *Gene Ontology* (GO) [47,48], the *Kyoto Encyclopedia of Genes and Genomes* (KEGG) [49], the *Reactome* [50], and from the *Panther* databases [51].

Statistical analysis

Continuous variables were reported as medians and interquartile ranges (IQRs). Differences in medians were tested with the Mann–Whitney U test. Overall differences in distributions between groups were tested with the Kruskal–Wallis omnibus test. Post-hoc tests were run to identify single differences by adjusting for multiple testing (Dunn's test). Dichotomous variables were explored using the maximum likelihood χ^2 test or the Fisher's exact test as appropriate. Significance was retained at $P<0.05$. Analyses were run with SPSS 25.0 (IBM Corp., Armonk, NY, U.S.A.) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, U.S.A.).

Results

Pharmacological pilot study

Roxadustat was well tolerated. HIF1α nucleus-cytoplasm ratio was higher, 1 h after injection, only in RXD25 compared with baseline. VEGF gene expression was higher in RXD25 than in RXD10 or in HAL, 1 h after injection. Likewise it was higher than in HAL 6 h after injection. At 6 h, PDGFβ gene expression was higher in RXD25 than in HAL, the expression of AGPT2 was higher in RXD25 than in RXD10 (Figure 2). As roxadustat stabilized liver HIF1α and drove an HIF-related gene signature only at 25 mg/kg, this dosage was chosen to proceed further.

Surgery

Operations took 40 (IQR = 38–45) min to be completed, with no differences between groups (Supplementary Information S1). The hepatectomy entailed a similar removal of 6.2 g of mass (IQR = 6.0–6.8, P=0.229). The remnant liver, estimated from a pool of rats of comparable age and body weight, amounted to 2.7 g (IQR = 2.6–2.9). The absolute

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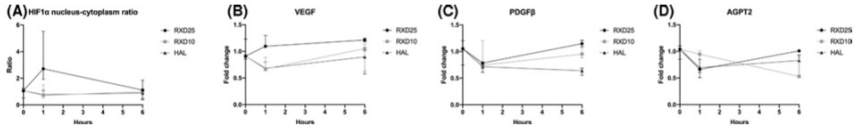

Figure 2. Preliminary evaluation of HIF-signaling pathway activation

(**A**) HIF1α nucleus-cytoplasm ratio, at 1 h, in RXD25 vs. baseline (P=0.042). (**B**) VEGF gene expression, at 1 h, in RXD25 vs. RXD10 (P=0.047), and vs. HAL (P=0.047). VEGF gene expression, at 6 h, in RXD25 vs. HAL (P=0.037). (**C**) PDGFβ gene expression, at 6 h, in RXD25 vs. HAL (P=0.044). (**D**) AGPT2 gene expression, at 6 h, in RXD25 vs. RXD10 (P=0.044). Given the exploratory nature of this phase, results are not corrected for multiple testing. Data shown as medians and IQR.

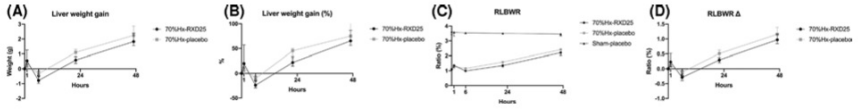

Figure 3. Evolution of remnant-liver-to-body-weight ratio and liver weight over time

(**A**) Resected rats exhibited a similar liver weight gain, at all time points but d1 (70%Hx-RXD25 vs. 70%Hx-placebo P=0.025). (**B**) Resected rats exhibited a similar percentage of liver weight gain, at all time points but d1 (70%Hx-RXD25 vs. 70%Hx-placebo P=0.025). (**C**) Resected rats exhibited a similar RLBWR, at all time points but d1 (70%Hx-RXD25 vs. 70%Hx-placebo P=0.016). (**D**) Resected rats exhibited a similar difference in RLBWR rise, at all time points but d1 (70%Hx-RXD25 vs. 70%Hx-placebo P=0.013). Data shown as medians and IQR. Abbreviation: RLBWR, remnant-liver-to-body-weight ratio.

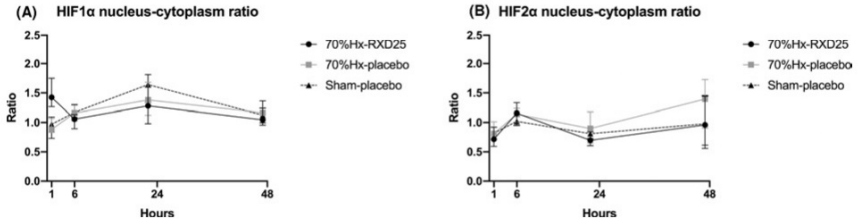

Figure 4. Post-operative evolution of HIF1α and HIF2α in the liver

(**A**) HIF1α nucleus-cytoplasm ratio in 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.022) at 1 h after the end of surgery and drug administration. (**B**) HIF1α nucleus-cytoplasm ratio comparable among groups at all time points. Data shown as medians and IQR.

and relative growth of the liver remnant was comparable between the two operated groups at all time points except for d1, when 70%Hx-RXD25 rats showed a reduced liver weight gain compared with 70%Hx-placebo (Figure 3).

HIF1α and HIF2α stabilization

The HIF1α nucleus-cytoplasm ratio was greater in 70%Hx-RXD25 livers compared with 70%Hx-placebo at 1 h (P=0.022). After this first time point, no differences between groups were detected. At no time point, the HIF2α nucleus-cytoplasm ratio was significantly different among the three groups (Figure 4).

Complete blood count

The red blood cell count showed that, only in 70%Hx-RXD25, there was a surge in red blood cell count on d2 compared with d1 (P<0.001), and with 6 h (P=0.003). Concordantly, hemoglobin concentration was greater on d2 compared with d1 (P<0.001), and almost significantly greater if compared with 6 h (P=0.050), hematocrit was greater on d2 compared with d1 (P<0.001), and to 6 h (P=0.003) Platelet count was greater in 70%Hx-RXD25 compared with 70%Hx-placebo and sham-placebo on d1. Peripheral white blood cells count was higher in 70%Hx-RXD25 compared

with 70%Hx-placebo on d2. This divergence is accounted for by differences in lymphocytes and monocytes (Figure 5).

Plasma analytes

Resected rats exhibited a similar rise in transaminases compared with sham-placebo, with a peak on d1 and a partial decline on d2 (Figure 6). While albumin levels are overall stable after liver resection, resected rats showed lower levels on d1 compared with sham-placebo. We observed an increase in factor V concentration in resected rats on d1, with no difference between 70%Hx-RXD25 and 70%Hx-placebo. Ammonia levels were higher in 70%Hx-RXD25 than in sham-placebo at 1, 22, and 47 h after hepatectomy. On d1 and d2, lactate levels were higher in 70%Hx-RXD25 than in sham-placebo. Similarly, total bilirubin in 70%Hx-RXD25 showed a modest but significant increase compared with sham-placebo on d1 and d2, and with 70%Hx-placebo only on d1. On d1, pH was higher in 70%Hx-RXD25 than in sham-placebo. Creatinine was within normal range at all time points.

Gd-EOB-DTPA MRI

On d1, the time to peak was similar among groups. AUC60 was significantly reduced in 70%Hx-RXD25 compared with 70%Hx-placebo and with sham-placebo. AUC90 data followed a similar trend (not shown). Accordingly, the AUC from t0 to peak, which accounts for contrast uptake, was reduced in 70%Hx-RXD25 compared with sham-placebo. The total AUC, which accounts for total contrast exposure, was one-third in 70%Hx-RXD25 compared with 70%Hx-placebo and sham-placebo, though the time for 30% decrease was similar among groups. On d2, except for the time to peak that was longer in 70%Hx-RXD25 than in 70%Hx-placebo, no parameter showed significant differences (Figure 7).

Survival

We recorded comparable survival rates, with only 4 events out of 34 animals in the 70%Hx-RXD25. All the events happened during or soon after d1 MRI (Supplementary Information S2).

Immunofluorescence

While unaffected on d1, the proportion of Ki67-stained cells, increased significantly on d2 in regenerating lobes, with no differences between 70%Hx-RXD25 and 70%Hx-placebo. Hepatocytes, i.e., liver cells with large round nucleus, were the main contributor to this increase. A similar behavior was observed at EdU immunofluorescence, labeling cells in S-phase of cell cycle (Figures 8 and 9).

Histology

Mitoses were not detected on d1 and were extensively displayed on d2 in resected rats without differences between 70%Hx-RXD25 and 70%Hx-placebo. On d1, inflammation, endothelial denudation, microvascular thrombosis, parenchymal hemorrhage, and necrosis were virtually absent from all sections. Hepatocellular ballooning was detected in all 70%Hx-placebo samples but only in 2/13 70%Hx-RXD25 and in 1/8 sham-placebo. Steatosis occurred in all but one 70%Hx-placebo samples, mainly as microvesicular and midzonal. In contrast, fatty infiltration was detected in only 5/13 of liver remnants in the 70%Hx-RXD25 group. When steatosis was present, its degree was less severe in 70%Hx-RXD25 than in 70%Hx-placebo (Figure 10).

On d2, inflammation, endothelial denudation, parenchymal hemorrhage, and necrosis were virtually absent from all sections. Only one instance of microvascular thrombosis was detected in the 70%Hx-placebo group. Hepatocellular ballooning was milder and clearly detectable only in one case of 70%Hx-RXD25. Steatosis lost zonation and was generally diffuse, appearing in all cases of resected rats without differences in proportion or in degree (Table 1).

RNAseq

The relief from hepatocellular ballooning and steatosis, the only significant effect of roxadustat as opposed to placebo in the context of 70%Hx, was identified after 22 h from roxadustat administration. Liver samples coming from the three experimental groups underwent analysis of global transcriptome changes with an RNAseq approach. The analysis is reported in Supplementary Information S3. We detected 2521 genes whose expression was significantly different in 70%Hx-RXD25 compared with 70%Hx-placebo. The principal component analysis separated the samples according to the treatment. We employed ORA to determine if the significantly modulated genes likely originated from any known gene pathways, while GSEA was performed to assess the enrichment of up- or down-regulated genes in these pathways. We identified nine clusters of cellular functions that were recurrently enriched in 70%Hx-RXD25 compared

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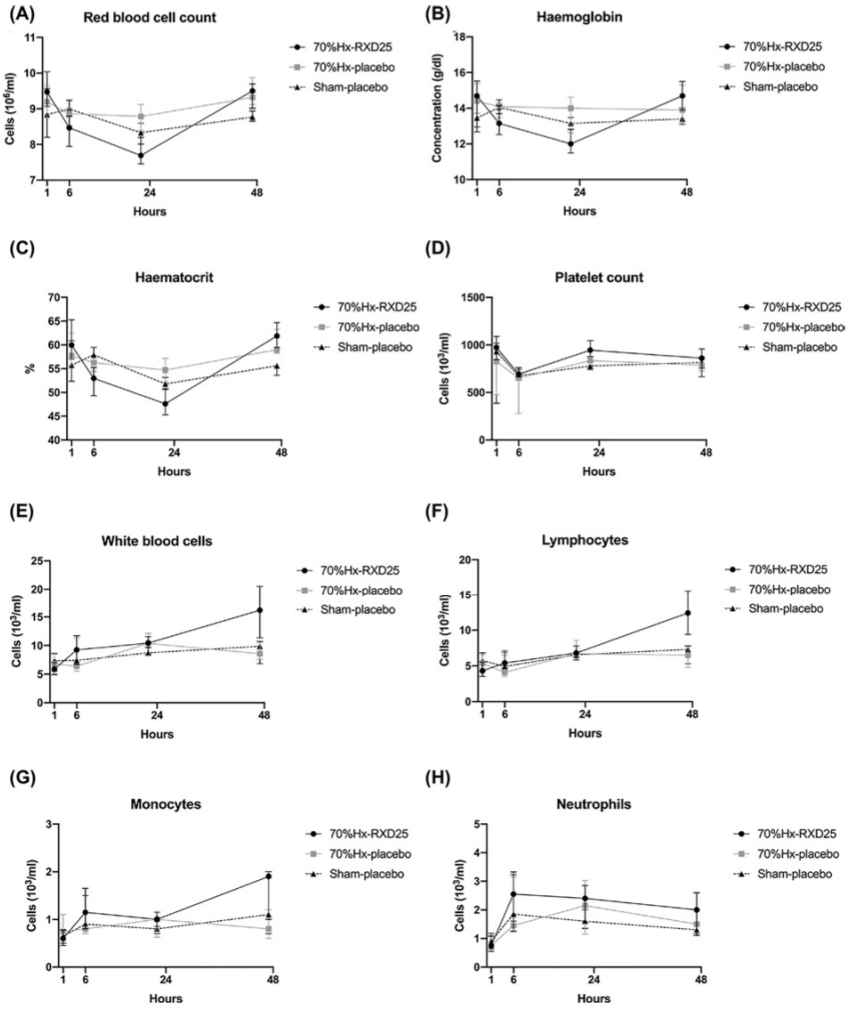

Figure 5. Complete blood count

(**A**) Erythrocytes. On d1, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.012). On d2, 70%Hx-RXD25 vs. sham-placebo (P=0.012). (**B**) Hemoglobin. On d1, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.005). On d2, 70%Hx-RXD25 vs. sham-placebo (P=0.030). (**C**) Hematocrit. At 6 h, 70%Hx-RXD25 vs. sham-placebo (P=0.019). On d1, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.013). On d2, 70%Hx-RXD25 vs. sham-placebo (P<0.001). (**D**) Platelets. On d1, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.007), and vs. sham–placebo (P<0.001). (**E**) Leukocytes. On d2, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.012). (**F**) Lymphocytes. On d2, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.004) and vs. sham-placebo (P=0.031). (**G**) Monocytes. On d2, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.031). (**H**) Neutrophils. No statistically significant differences. Data shown as medians and IQR.

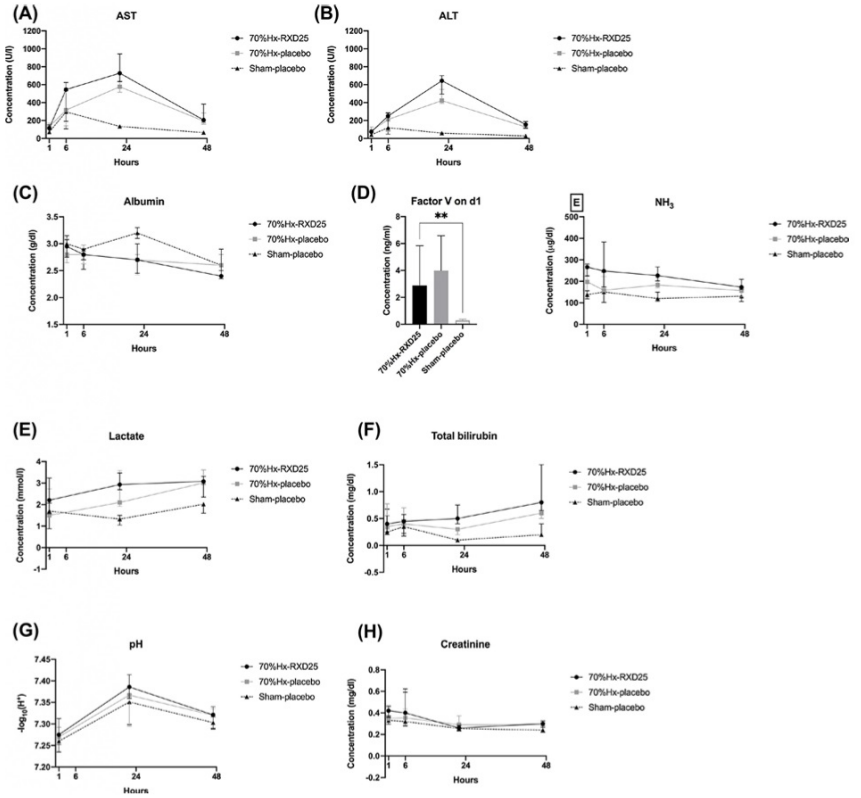

Figure 6. Evolution of plasma analytes over time

(**A**) AST on d1, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.279), and vs. sham-placebo (P<0.001); on d2, 70%Hx-RXD25 vs. 70%Hx–placebo (P=0.923), and vs. sham-placebo (P<0.001). (**B**) ALT on d1, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.308), and vs. sham–placebo (P<0.001); on d2, 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.922), and vs. sham-placebo (P<0.001). (**C**) Albumin on d1, 70%Hx-RXD25 vs. 70%Hx-placebo (P=1.000), and vs. sham-placebo (P=0.002). (**D**) Factor V on d1, 70%Hx-RXD25 vs. 70%Hx–placebo (P=1.000), and vs. sham-placebo (P=0.003). (**E**) Ammonia in 70%Hx-RXD25 vs. sham-placebo after 1 (P=0.009), 22 (P<0.001), and 47 h (P=0.012) after surgery. (**F**) Lactate in 70%Hx-RXD25 vs. sham-placebo on d1 (P<0.001) and on d2 (P=0.006). (**G**) Total bilirubin in 70%Hx-RXD25 vs. 70%Hx-placebo on d1 (P=0.025) and vs. sham-placebo on d1 (P<0.001) and on d2 (P=0.002). (**H**) pH in 70%Hx-RXD25 vs. sham-placebo on d1 (P=0.035). (**I**) Creatinine within normal range. Data shown as medians and IQR.

with 70%Hx-placebo. Three of those pathway clusters appeared generally up-regulated: HIF signaling, energy production via oxidative phosphorylation, and lipid metabolism, while six pathway clusters were down-regulated: cell growth and death, oxidative stress, xenobiotic metabolism, transmembrane transporters, inflammation, endocrine signaling (Supplementary Information S4). The comparison between the 70%Hx-RXD25 group and the sham-placebo group showed that the former exhibits an up-regulation of cell cycle genes and the HIF1α pathway. The comparison between the 70%Hx-placebo group and the sham-placebo group showed that the former exhibits an up-regulation of

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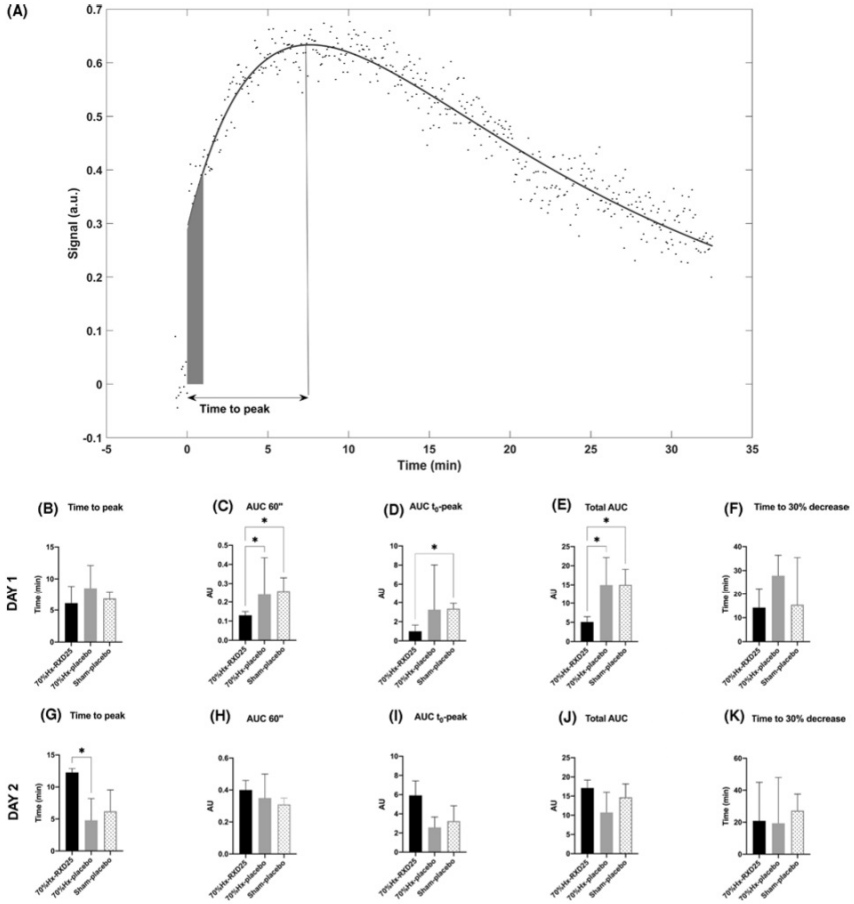

Figure 7. Results of the Gd-EOB-DTPA MRI

(**A**) Example of MRI normalized signal analysis. The area in gray marks the AUC60′. (**B**) d1 time to peak similar among groups (Kruskal–Wallis P=0.594). (**C**) d1 AUC60 in 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.013), and vs. sham-placebo (P=0.035). (**D**) d1 AUC from t_0 to peak in 70%Hx-RXD25 compared with sham-placebo (P=0.035). (**E**) d1 total AUC in 70%Hx-RXD25 vs. 70%Hx–placebo (P=0.012) and vs. sham (P=0.012). (**F**) d1 time to 30% signal decrease (Kruskal–Wallis P=0.300). (**G**) d2 time to peak in 70%Hx-RXD25 vs. 70%Hx-placebo (P=0.027). (**H**) d2 AUC60 (P=0.813). (**I**) d2 AUC from t_0 to peak (Kruskal–Wallis P=0.085). J: d2 total AUC (Kruskal–Wallis P=0.291). (**K**) d2 time to 30% signal decrease (Kruskal–Wallis P=0.910). *$P<0.050$. Data shown as medians and IQR.

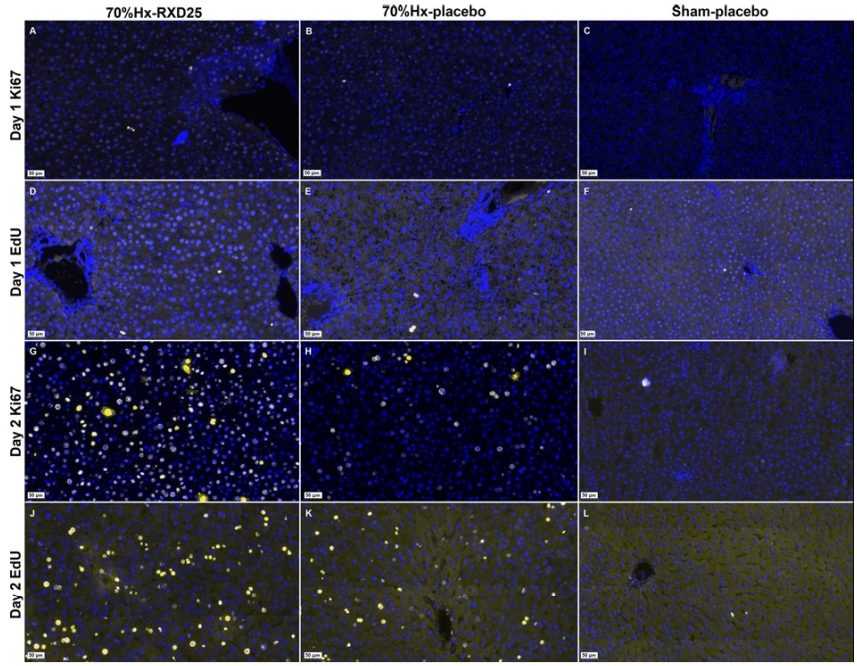

Figure 8. Immunofluorescence for Ki67 and EdU (×20)

Positive nuclei are labeled in yellow, filter SP Gold, 555 nm. (**A**) d1 70%Hx-RXD25 Ki67-positive cells. (**B**) d1 70%Hx-placebo Ki67-positive cells. (**C**) d1 sham-placebo Ki67-positive cells. (**D**) d1 70%Hx-RXD25 EdU-positive cells. (**E**) d1 70%Hx-placebo EdU-positive cells. (**F**) d1 sham-placebo EdU-positive cells. (**G**) d2 70%Hx-RXD25 Ki67-positive cells. (**H**) d2 70%Hx-placebo Ki67-positive cells. (**I**) d2 sham-placebo Ki67-positive cells. J: d2 70%Hx-RXD25 EdU-positive cells. (**K**) d2 70%Hx-placebo EdU–positive cells. (**L**) d2 sham-placebo EdU-positive cells.

cell cycle genes. Both resected groups showed a down-regulation in lipid metabolism genes as opposed to the sham group (Supplementary Information S5 and Figures 11-12).

Discussion

Liver resection causes a rapid induction of HIF1α-associated main intracellular signaling pathways in a murine model of both standard (68%) and extended liver resection (86%) [52]. Activation of HIF-driven pathways could be a consequence of the immediate post-resection hypoxic conditions, caused by the overflow of oxygen-poor portal blood in a partially resected liver and the consequent buffering reduction in oxygen-rich arterial supply. This ischemic phase is probably required to trigger the early phase of liver regeneration [5]. Interestingly, no later than 32 h after standard two-third hepatectomy, hypoxia-induced pathways are rapidly down-regulated. Hypoxia may thus be an important fine-tuning mechanism to induce liver regeneration [52], which acts in the very first days after liver resection. This might explain why even extreme resections performed in the setting of a reinforced hypoxia evolve favorably [4].

This is the first reported experience with roxadustat, a selective HIF stabilizer, in partial liver resection. In terms of safety, roxadustat tolerance was unexplored in this kind of hepatobiliary surgery. Data in the literature report that healthy adult rats should well tolerate a single dose of Roxadustat 25 mg/kg [25]. A few roxadustat-treated rats, but

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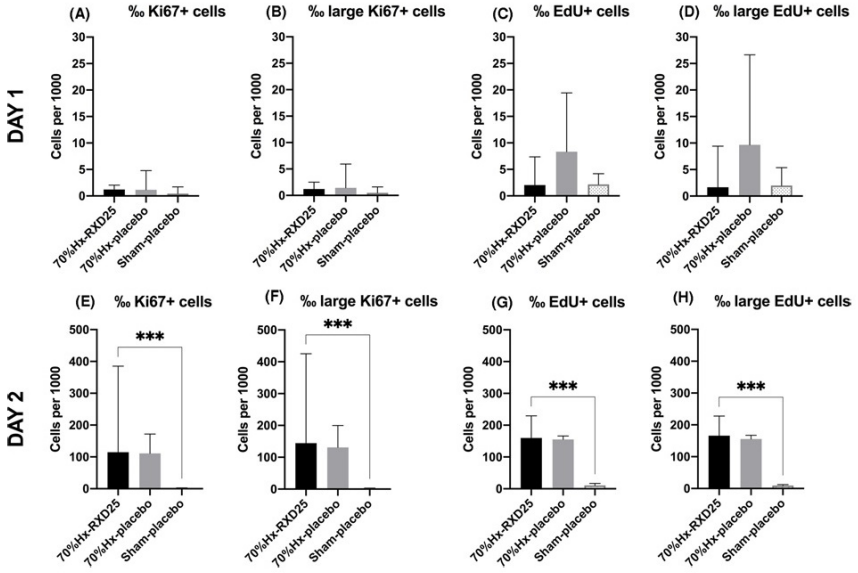

Figure 9. Results of immunofluorescence for Ki67 and EdU

(**A**) d1 Ki67-positive cells (Kruskal–Wallis P=0.391). (**B**) d1 large Ki67-positive cells (Kruskal–Wallis P=0.483). (**C**) d1 EdU-positive cells (Kruskal–Wallis P=0.129). (**D**) d1 large EdU-positive cells (Kruskal–Wallis P=0.107. (**E**) d2 Ki67-positive cells in 70%Hx-RXD25 vs. sham-placebo (Dunn's P<0.001). (**F**) d2 large Ki67-positive in 70%Hx-RXD25 vs. sham-placebo (Dunn's P<0.001). (**G**) d2 EdU-positive cells in 70%Hx-RXD25 vs. sham-placebo (Dunn's P<0.001). (**H**) d2 large EdU-positive cells in 70%Hx-RXD25 vs. sham-placebo (Dunn's P<0.001). ***P<0.001. Data shown as medians and IQR.

none of the placebo-treated, died on the first day after liver resection. Remarkably, death occurred during of immediately after MRI imaging, suggesting that roxadustat might result from a reduced tolerance to prolonged anesthesia after liver resection. Besides, we cannot fully exclude an unexpected toxicity from the association between roxadustat and Gd-EOB-DTPA.

While liver HIF1α stabilization vanishes by the sixth hour from roxadustat administration, RNAseq confirmed that the roxadustat-induced up-regulation of HIF1α-associated pathways is detectable at least until 22 h later. Similarly, the rise in the erythroid lineage on d2 along with the increased platelet release on d1 [53] support the evidence for an efficient HIF1α stabilization. Roxadustat has been indeed developed to raise the levels of erythropoietin and, upon roxadustat administration, we registered a blood cell response. Moreover, erythropoietin *per se* exerts an influence on liver regeneration and metabolism, by inducing hepatic proliferation and decreasing lipid accumulation in the liver [54,55]. Then we additionally measured the plasma levels of erythropoietin to investigate whether our findings could be partly explained by a raise in erythropoietin release but the results did not confirm this hypothesis (Supplementary Information S6).

At variance with data concerning genetic PHD1 silencing in a murine model of liver ischamia [56], we could not detect a roxadustat-induced differential HIF2α stabilization. Other less-selective PHD inhibitors prompted a response similar to ours: nucleus pulposus cells, once exposed to DMOG, showed an accumulation of HIF1α and an up-regulation of its target genes, but not of HIF2α [57]. These data, the evidence that both HIF1α and HIF2α are stabilized after hepatectomy [4], along with our experience suggest that proteasomal degradation of HIF2α is only partly or not primarily mediated by classical oxygen-dependent PHD pathway after liver resection.

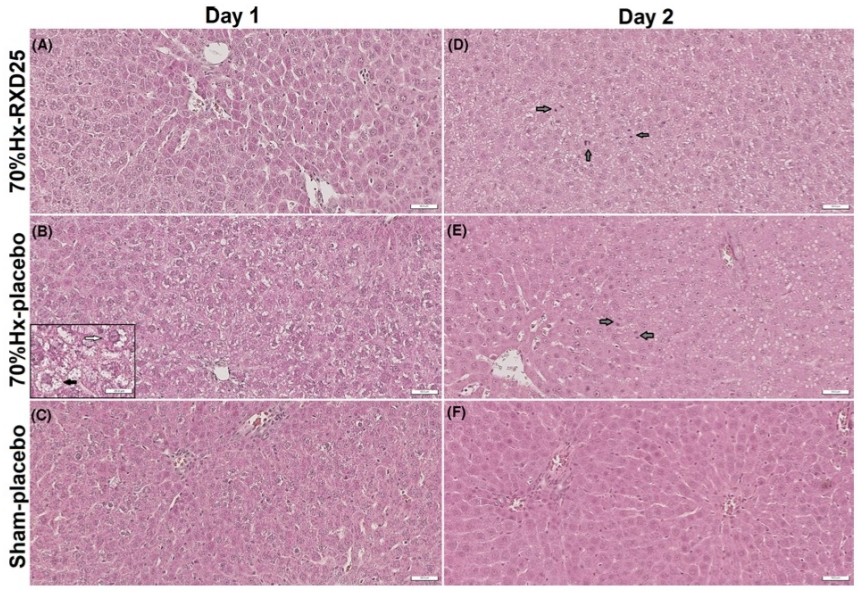

Figure 10. H&E-stained whole-liver sections (×20)

(**A**) d1 70%Hx-RXD25 slide showing minor steatosis and no ballooning. (**B**) d1 70%Hx-placebo slide. To the bottom right, a X40 magnified section showing an example of hepatocellular ballooning (black-filled arrow) and microvesicular steatosis (white-filled arrow). (**C**) d1 sham-placebo slide appearing essentially normal. (**D**) d2 70%Hx-RXD25 slide showing mild diffuse microvesicular steatosis and a number of mitotic figures (some examples are pointed out by grey-filled arrows). (**E**) d2 70%Hx-placebo slide showing mild diffuse microvesicular steatosis. (**F**) d2 sham-placebo slide appearing essentially normal.

Overall, hypoxia is supposed to coordinate murine liver regeneration by coupling parenchymal growth to vascular expansion [4,5]. However, because fatalities occur during the first 48 h after liver resection in murine models and hypoxia-driven response rapidly fades out after 32 h, the putative protective effect of HIF stabilization should entail very early functional adjustments consequent to changes in hepatocellular energetic status [58]. Therefore, beside morphological regeneration, we evaluated function, essential to claim that a treatment has an effect whatever on post-operative liver failure [59]. More than crude mortality rate, liver function is a reproducible and meaningful endpoint because it reflects the pathophysiology of human hepatocellular dysfunction in terms of synthesis, detoxification, inflammation, necrosis, and collateral damage [60].

Gd-EOB-DTPA uptake appeared generally hindered in roxadustat-treated resected rats as assessed by the reduction in AUC60 and AUC90 on d1, and the delay of the time to peak on d2 compared with 70%Hx-placebo and sham-placebo. Gd-EOB-DTPA employs organic anion-transporting polypeptides (OATPs) to enter hepatocytes following the electrochemical gradient across plasma membrane [61–63]. Thus, decreased AUC could correspond to a decreased uptake. Correspondingly, RNAseq confirmed that roxadustat specifically inhibited the expression of Slco1a1, the gene encoding OATP1, in the context of liver resection (Supplementary Information S3). Inversely, we did not detect a significant effect on Gd-EOB-DTPA biliary excretion, which relies on the ATP-dependent canalicular membrane multidrug resistance proteins [61–63]. We did not gather evidence that roxadustat improved hepatocellular function postoperatively. Yet, to our knowledge, this is the first established rat model of standard hepatectomy, in which hepatocellular function was detailed through contrast-enhanced MRI and which showed a substantial invariability in MRI parameters between sham-operated and 70%-liver resected rats. Previously, in a rat model of 70 and 90% liver resection, the vascular clearance of Gd-EOB-DTPA was utilized to identify liver function differences

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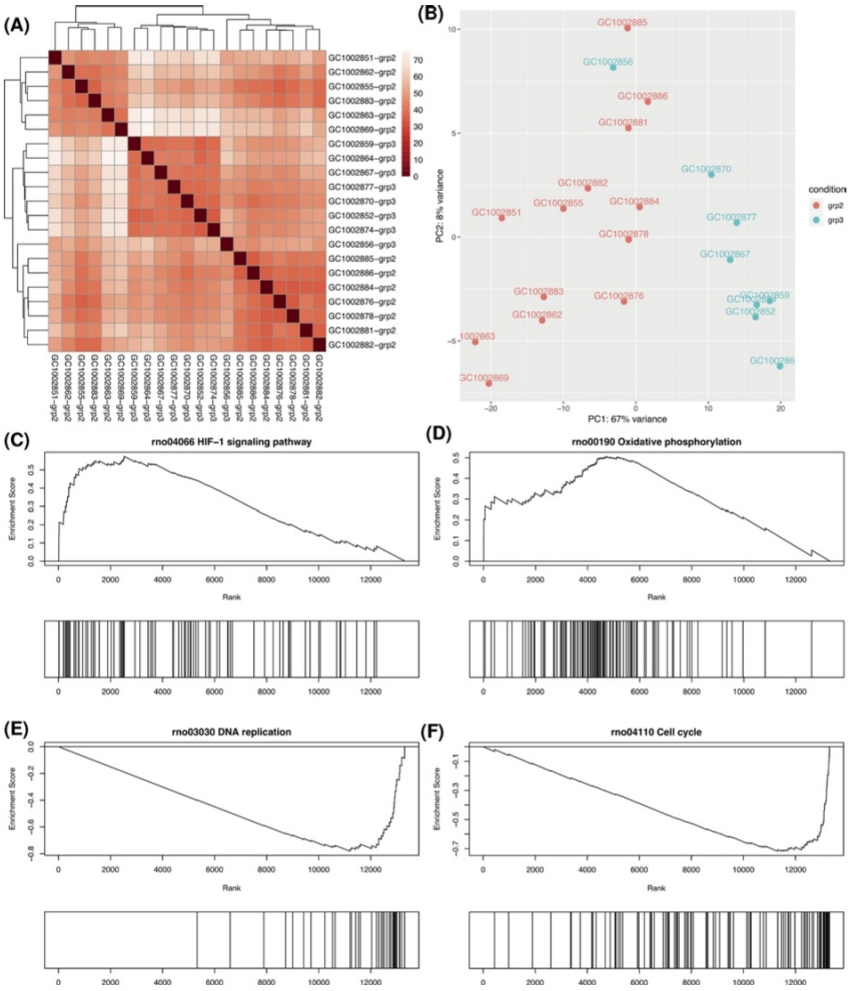

Figure 11. RNAseq

(**A**) Heatmap of the sample-to-sample distances. Sample clustering. (**B**) Principal component analysis. The plot shows the samples spanned in the 2D plane and grouped by their first two principal components, in order to visualize biases and batch effects. Grp2 = 70%Hx-RXD25, grp3 = 70%Hx-placebo. (**C–F**) Preranked enriched plots [81]. Excerpts from GSEA based on the KEGG database. (C) HIF1α-signaling pathway up-regulated in 70%Hx-RXD25 compared with 70%Hx-placebo. (D) Oxidative phosphorylation gene set up-regulated in 70%Hx-RXD25 compared with 70%Hx-placebo. (E) DNA replication gene set down-regulated in 70%Hx-RXD25 compared with 70%Hx-placebo. (F) Cell cycle gene set down-regulated in 70%Hx-RXD25 compared with 70%Hx-placebo.

Table 1 Histology report

	70%Hx-RXD25 (N (%))	70%Hx-placebo (N (%))	Sham-placebo (N (%))	*P*
Day 1				
Mitoses	0/13 (0.00)	0/8 (0.00)	0/8 (0.00)	-
Inflammation	0/13 (0.00)	0/8 (0.00)	0/8 (0.00)	-
Endothelial denudation	0/13 (0.00)	0/8 (0.00)	0/8 (0.00)	-
Microvascular thrombosis	0/13 (0.00)	0/8 (0.00)	0/8 (0.00)	-
Parenchymal hemorrhage	0/13 (0.00)	0/8 (0.00)	0/8 (0.00)	-
Necrosis	0/11 (0.00)	0/8 (0.00)	0/7 (0.00)	-
Hepatocellular ballooning	2/13 (15.38)	7/8 (87.50)[1]	1/8 (12.50)	0.001[2]
Steatosis (≥5%)	5/13 (38.46)	7/8 (87.50)[3]	1/8 (12.50)	0.005[2]
Degree of steatosis				
Undetectable (steatosis 0%)	3/13 (23.08)	1/8 (12.50)		0.028[4]
Healthy hepatocytes (steatosis < 5%)	5/13 (38.46)	0/8 (0.00)		
Mild (steatosis 5–33%)	5/13 (38.46)	4/8 (50.00)		
Moderate (steatosis 33–66%)	0/13 (0.00)	3/8 (37.50)		
Severe (steatosis > 66%)	0/13 (0.00)	0/8 (0.00)		
Day 2				
Mitoses	9/9 (100.00)	9/9 (100.00)[5]	1/7 (14.29)	<0.001[2]
Inflammation	0/9 (0.00)	0/9 (0.00)	0/7 (0.00)	-
Endothelial denudation	0/9 (0.00)	0/9 (0.00)	0/7 (0.00)	-
Microvascular thrombosis	0/9 (0.00)	1/9 (11.11)	0/7 (0.00)	0.347[2]
Parenchymal hemorrhage	0/9 (0.00)	0/9 (0.00)	0/7 (0.00)	-
Necrosis	0/9 (0.00)	0/8 (0.00)	0/7 (0.00)	-
Hepatocellular ballooning	1/7 (14.29)	0/9 (0.00)	0/7 (0.00)	0.289[2]
Steatosis (≥5%)	9/9 (100.00)	9/9 (100.00)[6]	1/7 (14.29)	<0.001[2]
Degree of steatosis				
Undetectable (steatosis 0%)	0/9 (0.00)	0/9 (0.00)		0.229[4]
Healthy hepatocytes (steatosis < 5%)	0/9 (0.00)	0/9 (0.00)		
Mild (steatosis 5–33%)	0/9 (0.00)	1/9 (11.11)		
Moderate (steatosis 33–66%)	9/9 (100.00)	8/9 (88.88)		
Severe (steatosis > 66%)	0/9 (0.00)	0/9 (0.00)		

[1]70%Hx-RXD25 vs. 70%Hx-placebo: Fisher's *P*=0.002.
[2]Maximum likelihood chi square test.
[3]70%Hx-RXD25 vs. 70%Hx-placebo: Fisher's *P*=0.067.
[4]Mantel–Haenszel test of trend.
[5]70%Hx-RXD25 vs. 70%Hx-placebo: Fisher's *P*=1.000.
[6]70%Hx-RXD25 vs. 70%Hx-placebo: Fisher's *P*=1.000.

in a plasma pharmacokinetic study [64]. In our experience, other measures of liver function did not appear to be influenced by roxadustat compared with 70%Hx-placebo. In particular, synthesis of albumin or factor V, clearance of lactate, ammonia or bilirubin, and transaminases showed no differences.

The current literature concerning the models of liver regeneration after hepatectomy almost invariably emphasizes results in terms of death rate, immunohistological measures of cell proliferation, and increase in remnant size [56], as proxies for improved liver regeneration or reduced SFSS [4,56]. However, while death is an inhumane endpoint [65], evidence coming from SFSS-associated rodent mortality can hardly be translated to humans [66]. Moreover, graft size is influenced by inflammation and edema, and it does not necessarily correlate with function. Finally, the fine-tuned study of liver function, much advocated in order to produce meaningful insights into the physiology of liver regeneration, should be routinely reported [67].

After extreme resections, liver regains mass for up to 7 days and the difference between livers exposed to hypoxia or not peaks on postoperative day 3 [4]. In the standard model of liver resection we used, there is little room for improvement because the hepatostat is reached within 3 days after 70%Hx [68]. Liver growth, as we assessed by size measurement, proved actually similar between the treatment groups, in keeping with the evidence coming from ki67 staining, EdU labeling, and mitoses count. Our results are not in line with previous research with PHD inhibitors.

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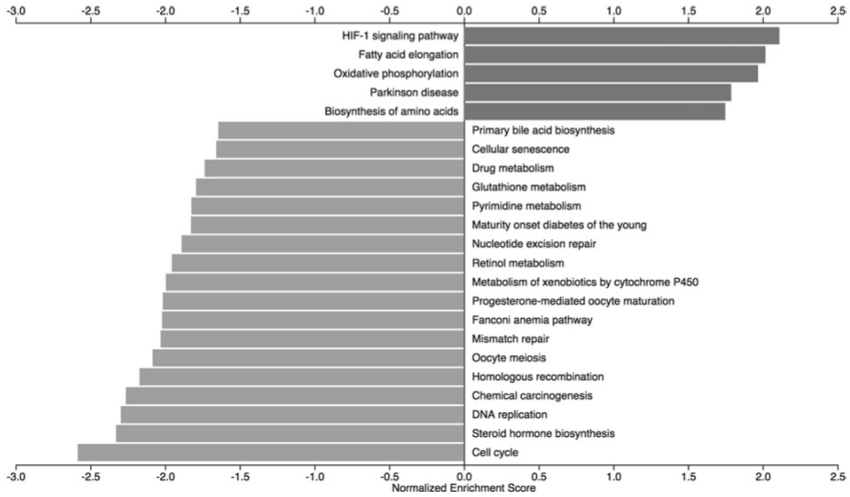

Figure 12. GSEA based on the KEGG database, showing only gene sets with FDR $P<0.05$

Harnoss et al. observed that EDHB improved hepatocellular proliferation, measured on d2 after 75% hepatectomy [9]. Correspondingly, EDHB induced cyclin D1 and cyclin D2 up-regulation after liver resection. These effects are HIF2α-dependent [69], and, while the authors did not show the differential profile of HIF1α–HIF2α stabilization in their experience, it is safe to assume that the growth-promoting effects of EDHB are mediated by an increase transactivity of HIF2α via a non-selective inhibition of its degradation. Whether this HIF2α stabilization is obtained via the inhibition of PHDs or other dioxygenases or a mix of the two remains unclear, because in our experience roxadustat, which effectively and selectively inhibits PHDs, stabilized only HIF1α and did not promote liver regeneration after hepatectomy. Conversely, the data coming from RNAseq unanimously underline that the HIF1α-stabilizing treatment dampens cell cycle progression.

The most relevant effect observed upon the administration of roxadustat in 70%Hx was a marked improvement in hepatocellular ballooning and steatosis, known hallmarks of hepatocellular stress, on d1. Fatty infiltration of the liver after partial resection has been known for 70 years [70]. After hepatectomy, circulating fatty acids are inefficiently metabolized by the remnant liver, and infiltrate parenchyma, resulting in diffuse hepatocellular steatosis and, in some instances, to cell death [70–72]. This peculiar post-resection injury has been shown to peak between days 1 and 2 after 70%Hx, while it resolves and disppears thereafter in surviving rats. The RNAseq performed on day-1 liver samples showed that roxadustat tilts the balance of cellular functions, favoring lipid metabolism rather than cell replication, exactly by the moment of the worst lipid overload. Whether such a shift streamlines the postoperative recovery has to be ascertained. Still, Vic et al. previously proved that relieving steatosis via testosterone administration raised rat survival rate after 90% hepatectomy [71]. In set of additional experiences, we evaluated whether the level of intrahepatic or circulating portal and peripheral lipids might contribute to the explanation of our findings but the results of these extra measurements did not show differences between 70%Hx-RXD25 and 70%-placebo groups (Supplementary Information S6).

The improvement in cellular stress obtained after roxadustat treatment was an unforeseen finding and it may pave the way for the exploration of the role of selective HIF1α stabilization after liver resection. In other experimental settings, Roxadustat has shown to alleviate liver fatty infiltration in two zebrafish atp7b deficiency models of human Wilson's disease [73]. Since, in our surgical model, transient steatosis tends to physiologically vanish in surviving rats after d2, we could not expect to find a statistically significant difference after this time point, and we decided to discontinue the experiment on the grounds of ethical considerations.

It remains to be established whether isoquinolines are a treatment for fatty liver infiltration at large thanks to their action on steatosis and ballooning. Nonetheless the effect of roxadustat on steatosis and ballooning looks promising for the constellation of non-alcoholic fatty liver disease and steatohepatitis, whose incidence is alarmingly soaring in the general population. These conditions are known to be associated with defective liver regeneration after resection and with increased rate of primary non-function after transplantation if steatotic livers are used as grafts. The mechanism by which roxadustat might prompt the liver to process lipids is elucidated by the RNAseq analysis: a selective HIF1α stabilization largely elicited the transcription of genes coding for lipid metabolism and oxidative phosphorylation. What is more elusive is the possible significance of this effect in the context of hypoxia. Anaerobic metabolism is usually preferred in conditions of hypoxia [26,74], and, in fact, PHDs deletion [75–77] and vHL loss [78–80] originate the classical cellular response to hypoxia: decreased mitochondrial activity, increased glycolysis, and glycogen and lipid accumulation. However, increased cellular respiration results in a positive energy balance, a prerequisite for an improved liver function during regeneration. While not manifested by indicator other than decreased cell stress and ballooning in a paraphysiological standard 70%Hx, the exploration of roxadustat in hepatic surgery on steatotic livers is an intriguing perspective.

In conclusion, it remains unproven whether a roxadustat-induced selective HIF stabilization enhances function or proliferation during the early postoperative period after standard hepatectomy, when hypoxia responses are at their acme. However, roxadustat improved hepatocellular ballooning and fatty infiltration, signs of hepatocellular suffering and metabolic dysfunction, and this finding claims for further investigation of roxadustat in fatty liver diseases and of the role of hypoxia-simulating treatments after liver resection.

Clinical perspectives

- Hypoxia triggers liver regeneration after resection but it is controversial to actively induce hypoxia in patients undergoing hepatectomy. However, a hypoxic response can be simulated by means of roxadustat, a selective prolyl hydroxylase inhibitor that artificially stabilizes hypoxia inducible factor-1α (HIF1α) levels.
- The present study showed the administration of roxadustat concomitant with liver resection alleviated postoperative steatosis and ballooning, two hallmarks of liver suffering after resection, by raising HIF1α and increasing oxidative phosphorylation.
- Selective HIF stabilization does not accelerate liver regeneration but promotes cellular respiration and mitigate steatosis, suggesting a possible role for roxadustat in the surgery of steatotic livers and in the treatment of fatty liver disease at large.

Data Availability

The data that support the findings of the present study are available from the corresponding author, upon reasonable request. The RNAseq data will be freely available from https://www.ncbi.nlm.nih.gov/sra, under the BioProject ID: PRJNA705692.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Samuele Iesari: Conceptualization, Data curation, Formal analysis, Validation, Investigation, Methodology, Writing—original draft, Writing—review & editing. **Isabelle Leclercq:** Conceptualization, Supervision, Methodology, Writing—original draft, Writing—review & editing. **Nicolas Joudiou:** Data curation, Investigation, Writing—review & editing. **Mina Komuta:** Data curation, Formal analysis, Writing—original draft, Writing—review & editing. **Aurélie Daumerie:** Data curation, Investigation, Writing—review & editing. **Jérôme Ambroise:** Software, Formal analysis, Writing—review & editing. **Alexandra Dili:** Conceptualization, Methodology, Writing—review & editing. **Natacha Feza-Bingi:** Investigation, Methodology, Writing—review &

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editing. **Daela Xhema:** Investigation, Methodology, Writing—review & editing. **Caroline Bouzin:** Data curation, Investigation, Writing—review & editing. **Bernard Gallez:** Conceptualization, Methodology, Writing—review & editing. **Francesco Pisani:** Supervision, Validation, Project administration, Writing—review & editing. **Eliano Bonaccorsi-Riani:** Supervision, Validation, Methodology, Writing—review & editing. **Pierre Gianello:** Conceptualization, Resources, Supervision, Funding acquisition, Validation, Methodology, Project administration, Writing—review & editing.

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Abbreviations

AGPT2, angiopoietin-2; ALPPS, associating liver partition and portal vein ligation technique for two-stage hepatectomy; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the curve; DMOG, dimethyloxalylglycine; DMSO, dimethyl sulfoxide; EDHB, ethyl-3,4-dihydroxybenzoate; EdU, 5-ethynyl-2′-deoxyuridine; Gd-EOB-DTPA, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid; GO, gene ontology; GSEA, gene set enrichment analysis; HDL, high-density lipoprotein; HIF, hypoxia-inducible factor; MRI, magnetic resonance imaging; OATP, organic anion-transporting polypeptide; ORA, over-representation analysis; PBS, phosphate-buffered saline; PHD, prolyl hydroxylase domain protein; RLBWR, remnant-liver-to-body-weight ratio; RNAseq, RNA sequencing; ROI, region of interest; RT-qPCR, real-time quantitative polymerase chain reaction; RXD, roxadustat; SFSS, small-for-size syndrome; 70%Hx, 70% hepatectomy.

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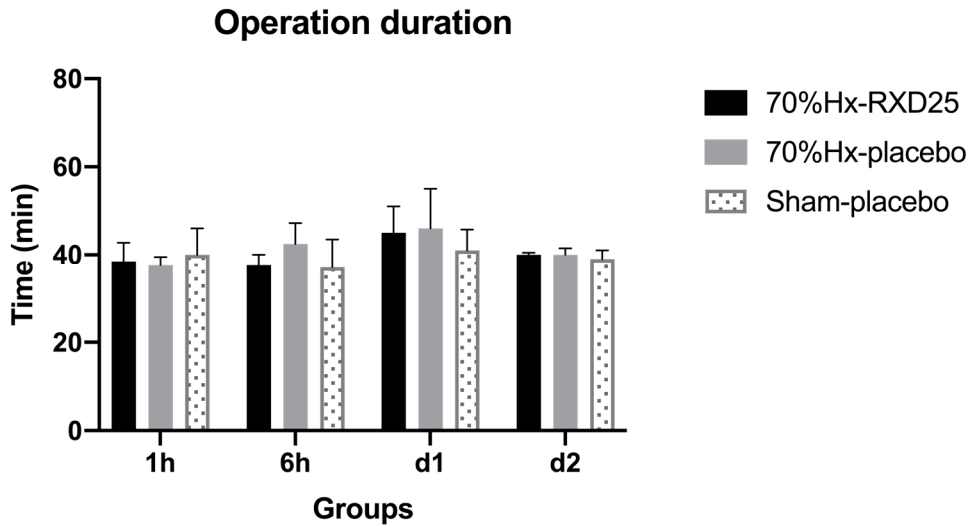
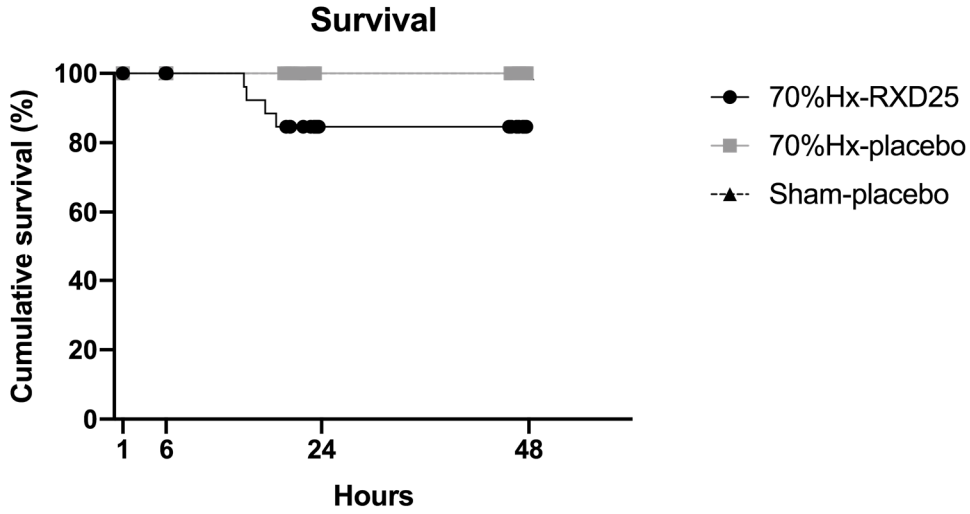
Supplemental Information 1: Operation duration. There were no differences within every batch of experiments (1h: P=0.604; 6h: P=0.368; d1: P=0.220; d2: P=0.653).



Supplemental Information 2: Survival curve (Log rank P=0.073).



Supplemental Information 4: Analysis of the RNAseq results, showing the differences between the 70%Hx-RXD and the 70%Hx-placebo groups.

Clustering of over-represented pathways

The analysis was based on six reference sets including *Biological Process* (BP), *Cellular Component* (CC), and *Molecular Function* (MF), from *Gene Ontology* (GO),[1,2] as well as the *Kyoto Encyclopaedia of Genes and Genomes* (KEGG),[3] the *Reactome*,[4] and the *Panther* databases.[5]

Within the KEGG database, there was an overrepresentation of pathways related to: 1) cell growth and death (rno04110, rno03030, rno03440, rno03430, rno03460); 2) lipid metabolism (rno00071, rno04146, rno03320, rno01212, rno04979, rno04714); 3) energy production (rno00190, rno01200, rno01210, rno00010); 4) xenobiotic metabolism (rno00982, rno00980); 5) oxidative stress (rno00480); and 6) endocrine signalling (rno00140) (Supplemental Information 6). Among the most upregulated gene sets, there were 1) the HIF1α signalling pathway (rno04066), 2) the gene sets pertaining to energy production (rno00190), and 3) the lipid metabolism (rno00062). A negative regulation involved genes related to: 1) cell growth and death (rno04218, rno00240, rno04914, rno04114, rno03420, rno03410, rno03460, rno04110, rno03440, rno03430, rno03030); 2) oxidative stress (rno00480); 3) xenobiotic metabolism (rno00982, rno00983, rno00980); and 4) endocrine signalling (rno00140). (Supplemental Information 7).

Within Gene Ontology, the bioinformatics analysis of Biological Process distinguished an overrepresentation of gene sets related to: 1) cell growth and death (GO:0034501, GO:0006336, GO:2000816, GO:0010824, GO:0006261, GO:0032508, GO:0044786, GO:0006260, GO:0000731, GO:0010965, GO:0051310, GO:0000070, GO:0000819, GO:0140014, GO:0061640, GO:0098813, GO:0007098, GO:0007059, GO:0006323, GO:0044843, GO:0000724, GO:0000280, GO:1903201, GO:0007052, GO:1903046, GO:0072527, GO:0048285, GO:0000075, GO:0045931, GO:1903047,

GO:0009145, GO:1901988, GO:0001889, GO:0006281, GO:1901987, GO:1901292, GO:0000278, GO:0010564, GO:0009266, GO:2001252, GO:0009260, GO:2001234, GO:2001020, GO:0009259, GO:0006974, GO:0051052, GO:0000226); 2) lipid metabolism (GO:0034368, GO:0046461, GO:0090181, GO:0006639, GO:0042632, GO:0016042, GO:0016125, GO:0019395, GO:0019216); 3) energy production (GO:0071377, GO:0042773, GO:0006096, GO:0046395, GO:0006091, GO:0046034, GO:0032787, GO:0015980, GO:0019362); 4) xenobiotic metabolism (GO:0042738, GO:0032354, GO:0046164, GO:0017144, GO:0006066, GO:0009636, GO:1901361); 5) oxidative stress (GO:0019430, GO:0006749, GO:0098869, GO:0034614); 6) endocrine signalling (GO:0034433, GO:0006953, GO:0006695, GO:0006690, GO:0071385, GO:0031960, GO:0007565, GO:0010817); 7) HIF1α signalling pathway (GO:0042311, GO:0006879, GO:0061045, GO:0017015, GO:0036293, GO:0009611); 8) inflammation (GO:0006958, GO:0016064); and 9) transmembrane transporters (GO:0015711) (Supplemental Information 8). In parallel to energy storage, the feeding behaviour (GO:0007631) appeared upregulated, while gene sets related to: 1) cell growth and death (GO:0032886, GO:0051052, GO:0006333, GO:0007064, GO:0051294, GO:0044843, GO:0072698, GO:0051382, GO:0098534, GO:0019985, GO:0007098, GO:1902749, GO:0031570, GO:0045005, GO:0007131, GO:0032392, GO:0071459, GO:0006302, GO:0006281, GO:0000724, GO:0007127, GO:1901987, GO:1901988, GO:1901989, GO:0000226, GO:1901990, GO:0050000, GO:0051303, GO:0051301, GO:0000281, GO:0034502, GO:2001251, GO:0007346, GO:0045132, GO:0010564, GO:0000910, GO:0051321, GO:0044786, GO:0071824, GO:0006323, GO:0051310, GO:1903046, GO:0065004, GO:1905819, GO:0007052, GO:0000278, GO:0051306, GO:0000280, GO:0098813, GO:0006260, GO:0006261), 2) oxidative stress (GO:0006749), 3) xenobiotic metabolism (GO:0006805), and 4) inflammation (GO:1901623) were downregulated (Supplemental Information 9).

In Cellular Component, we identified an overrepresentation of pathways linked to: 1) cell growth and death (GO:0043601, GO:0005657, GO:0000777, GO:0000779, GO:0000780, GO:0090734, GO:0000776, GO:0000775, GO:0098687, GO:0000793, GO:0005875, GO:0000228, GO:0044454, GO:0005819, GO:0000792, GO:0000785, GO:0005813, GO:0005635, GO:0005815, GO:0044450); 2) energy production (GO:0045277, GO:0070469, GO:0005746, GO:0030964, GO:0019867, GO:0031970, GO:0016469, GO:1904949, GO:0005743, GO:0019866, GO:0031966, GO:0005740, GO:0031301); and 3) lipid metabolism (GO:0034358, GO:0005782, GO:0031907, GO:0044438, GO:0005777, GO:0042579) (Supplemental Information 10). At GSEA, pathways associated with cell growth and death were downregulated (GO:0000922, GO:0044815, GO:0051233, GO:0000793, GO:0005876, GO:0098687, GO:0000777, GO:0005819, GO:0072686, GO:0000775, GO:0043596, GO:0000780, GO:0005657, GO:0032993, GO:0005875, GO:0005813, GO:0044454, GO:0000228, GO:0005874, GO:0000307, GO:0005815, GO:0043601, GO:0044450) (Supplemental Information 11).

The enquiry of Molecular Function pinpointed an overrepresentation of gene sets connected with: 1) energy production (GO:0016675, GO:0004129, GO:0015002, GO:0016676, GO:0016628, GO:0016712, GO:0009055, GO:0050136, GO:0004497, GO:0020037, GO:0046906, GO:0005506, GO:0016705, GO:0016829, GO:0031406); 2) lipid metabolism (GO:0047617, GO:0070325, GO:0016788); 3) cell growth and death (GO:0003887, GO:0003678, GO:0004536, GO:0042393, GO:0052689, GO:0008017); 4) transmembrane transporters (GO:1901618, GO:0046943, GO:0005342, GO:0015318, GO:0015075); 5) oxidative stress (GO:0004364, GO:0004601); 6) xenobiotic metabolism (GO:0008106, GO:0016616); and 7) endocrine signalling (GO:0008395) (Supplemental Information 12). At GSEA, there was a downregulation of gene sets associated with: 1) cell growth and death (GO:0008094, GO:0003777, GO:0140097, GO:0003688, GO:0035173, GO:0003887, GO:0003684,

GO:0015631, GO:0016779, GO:0043142, GO:0042393); 2) xenobiotic metabolism (GO:0004032, GO:0008106, GO:0016616, GO:0004033, GO:0016614); 3) endocrine signalling (GO:0016229, GO:0008395); and 4) inflammation (GO:0008392) (Supplemental Information 13).

The exploration of Reactome revealed as overrepresented pathways the following clusters: 1) cell growth and death (R-RNO-69190, R-RNO-174417, R-RNO-68962, R-RNO-5358565, R-RNO-5655862, R-RNO-176187, R-RNO-2299718, R-RNO-5693537, R-RNO-73886, R-RNO-606279, R-RNO-69002, R-RNO-69306, R-RNO-5693538, R-RNO-141424, R-RNO-141444, R-RNO-69239, R-RNO-5685938, R-RNO-69481, R-RNO-69206, R-RNO-453279, R-RNO-69242, R-RNO-69620, R-RNO-2500257, R-RNO-69278, R-RNO-1640170, R-RNO-68877, R-RNO-187577, R-RNO-68882, R-RNO-68886, R-RNO-453276, R-RNO-453274, R-RNO-73894); 2) energy production (R-RNO-70171, R-RNO-70326); 3) xenobiotic metabolism (R-RNO-211897, R-RNO-211859); and lipid metabolism (R-RNO-2173782) (Supplemental Information 14). At GSEA, there was an upregulation of genes associated with energy production (R-RNO-70263) and inflammation (R-RNO-2142691), and a downregulation of gene sets associated with: 1) cell growth and death (R-RNO-380270, R-RNO-3371453, R-RNO-69563, R-RNO-6781827, R-RNO-5358508, R-RNO-2559582, R-RNO-6804757, R-RNO-69231, R-RNO-3700989, R-RNO-110312, R-RNO-176412, R-RNO-5693537, R-RNO-69202, R-RNO-5693616, R-RNO-5633007, R-RNO-73894, R-RNO-73884, R-RNO-2299718, R-RNO-5651801, R-RNO-110313, R-RNO-174143, R-RNO-69190, R-RNO-73893, R-RNO-174417, R-RNO-69473, R-RNO-5693532, R-RNO-180786, R-RNO-5656169, R-RNO-157579, R-RNO-69273, R-RNO-5693538, R-RNO-606279, R-RNO-69002, R-RNO-69239, R-RNO-141444, R-RNO-1640170, R-RNO-69278); 2) oxidative stress (R-RNO-156590); and 3) endocrine signalling (R-RNO-8957322) (Supplemental Information 15).

In Panther, we observed that a gene set connected cell growth (P00017) was overrepresented and upregulated (Supplemental Information 16-17).

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Supplemental information 6: Additional measurements

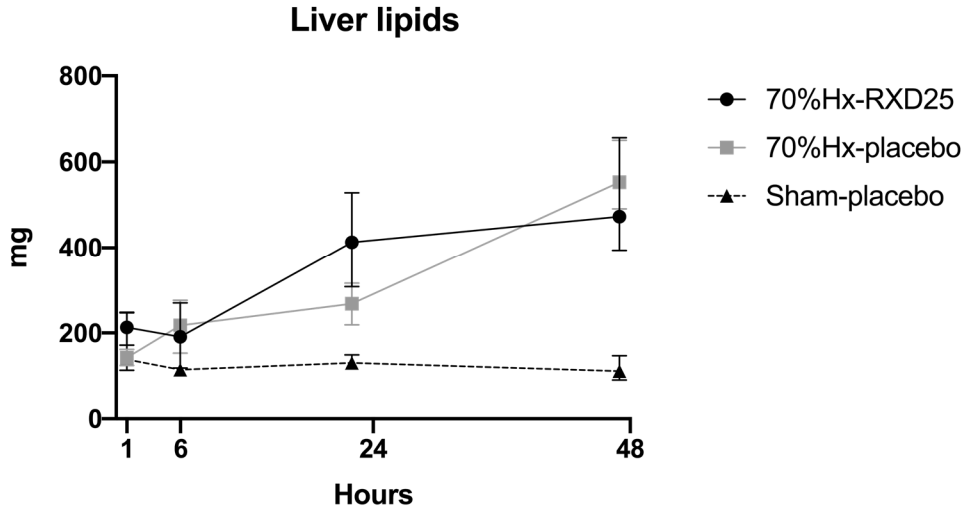

Figure 1. Evolution of lipid content in the remnant liver over time. On d1, 70%Hx-RXD25 vs. sham-placebo (P<0.001), and 70%Hx-placebo vs. sham-placebo (P=0.043); on d2, 70%Hx-RXD25 vs. sham-placebo (P=0.009), and 70%Hx-placebo vs. sham-placebo (P<0.001). Data shown as medians and IQR.

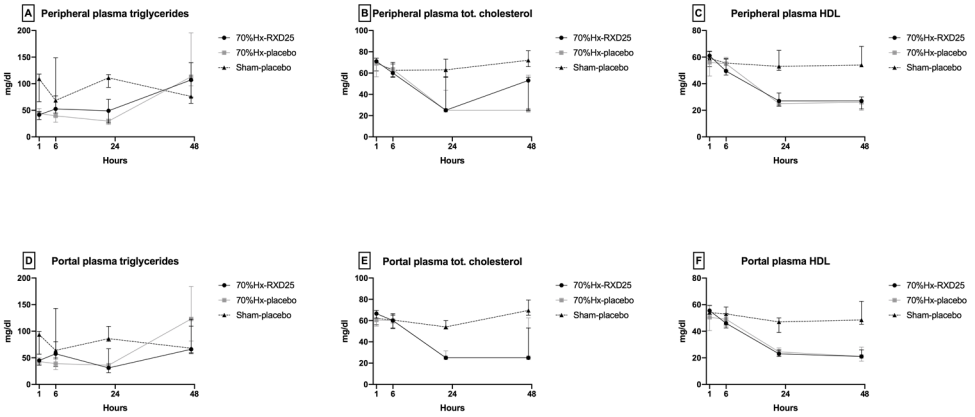

Figure 2. Evolution of peripheral and portal plasma lipids over time. A: Peripheral plasma triglycerides. At 1h, 70%Hx-RXD25 vs. sham-placebo (P=0.042); on d1, 70%Hx-RXD25 vs. sham-placebo (P=0.019), and 70%Hx-placebo vs. sham-placebo (P<0.001). B: Peripheral plasma total cholesterol. On d1, 70%Hx-RXD25 vs. sham-placebo (P=0.014), and 70%Hx-placebo vs. sham-placebo (P=0.001); on d2, 70%Hx-RXD25 vs. sham-placebo (P=0.007), and 70%Hx-placebo vs. sham-placebo (P=0.003). C: Peripheral plasma high-density lipoproteins. On d1, 70%Hx-RXD25 vs. sham-placebo (P=0.019), and 70%Hx-placebo vs. sham-placebo (P<0.001); on d2, 70%Hx-RXD25 vs. sham-placebo (P=0.003), and 70%Hx-placebo vs. sham-placebo (P=0.002). D: Portal plasma triglycerides. On d1, 70%Hx-RXD25 vs. sham-placebo (P<0.001), and 70%Hx-placebo vs. sham-placebo (P=0.019). E: Portal plasma total cholesterol. On d1, 70%Hx-RXD25 vs. sham-placebo (P<0.001), and 70%Hx-placebo vs. sham-placebo (P=0.002); on d2, 70%Hx-RXD25 vs. sham-placebo (P=0.007), and 70%Hx-placebo vs. sham-placebo (P=0.021). F: Portal plasma high-density lipoproteins. On d1, 70%Hx-RXD25 vs. sham-placebo (P=0.001), and 70%Hx-placebo vs. sham-placebo (P=0.019); on d2, 70%Hx-RXD25 vs. sham-placebo (P=0.004), and 70%Hx-placebo vs. sham-placebo (P=0.003). Data shown as medians and IQR.

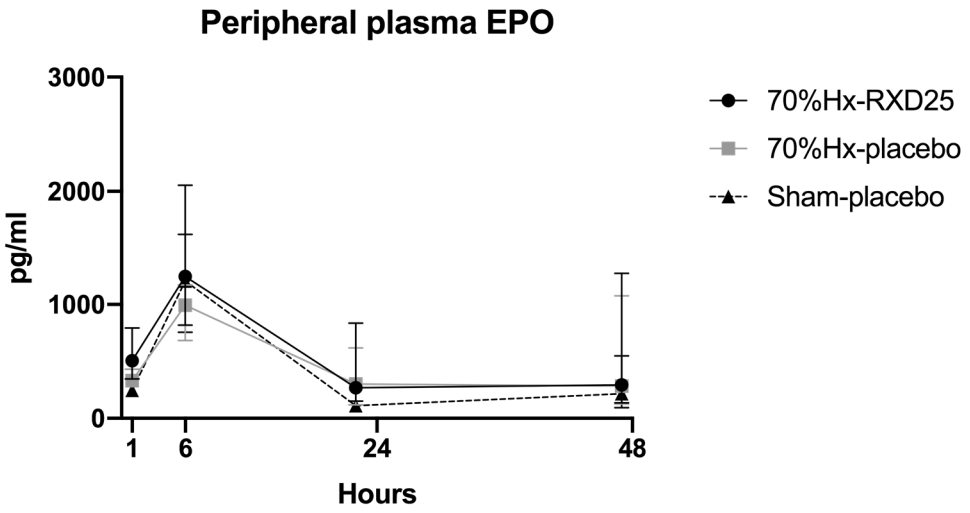

Figure 3. Evolution of peripheral plasma levels of erythropoietin over time. At 1h, 70%Hx-RXD25 vs. sham-placebo (P=0.018); on d1, 70%Hx-RXD25 vs. sham-placebo (P=0.011). Data shown as medians and IQR.

Summary of the main findings of the third work

This experimental work reports for the first time an experience with roxadustat in partial liver resection. Our main findings are wrapped up below:

1. A single intraperitoneal administration of roxadustat effectively increases liver HIF1α levels and this stabilisation vanishes in six hours. Blood cell response and RNA sequencing show the roxadustat-induced upregulation of HIF1α-associated pathways lasts for at least until 22 hours. However, roxadustat did not seem to stabilise HIF2α in the liver.
2. Roxadustat specifically inhibits the expression of OATP1. Accordingly, at contrast-enhanced MRI, Gd-EOB-DTPA uptake appeared generally hindered in roxadustat-treated resected rats, compared to 70%Hx-placebo and sham-placebo. Conversely, roxadustat did not affect Gd-EOB-DTPA biliary excretion, which relies on the ATP-dependent canalicular membrane multidrug resistance proteins. In summary, roxadustat did not appear to enhance post-resection hepatocellular function.
3. Collaterally, contrast-enhanced MRI demonstrated, for the first time in the literature, an overall similarity in MRI parameters between sham-operated and 70%-liver resected rats. This evidence gives a functional basis to the well-known notion this rodent model of 70% liver resection is hardly fatal. Functional MRI documents changes in hepatocellular energetic status, which are essential for an approach to early postoperative liver failure.
4. Roxadustat did not improve postoperative liver growth, as assessed by means of size measurement, ki67 staining, EdU labelling and mitoses count, while it is known that non-selective PHD inhibitors improve hepatocellular proliferation, an effect that, thus, might involve other dioxygenases.
5. The most relevant effect observed upon the administration of roxadustat in this model of hepatectomy is a marked improvement in hepatocellular

ballooning and steatosis, known hallmarks of hepatocellular stress, in the first postoperative day.

6. Day-one RNA sequencing suggested that HIF1α-stabilizing treatment unbalances hepatocellular functions, by prompting lipid metabolism and oxidative phosphorylation, and by dampening cell cycle progression. The effect of this shift on postoperative recovery remains to be investigated.

Conclusions: future perspectives and translational potential

The role of depletive antibodies as induction agents in liver transplantation

Potent immunosuppressive compounds have virtually abolished early graft loss because of acute T-cell-mediated rejection. Still, the main issues in the handling of immune response after liver transplantation have not been adequately tackled despite decades of intense research. Chronic rejection still induces a certain graft attrition, while late patients' morbidity and mortality because of chronic immunosuppression are still a concern. The ideal solution would be graft tolerance without chronic immunosuppression, a much-coveted target in solid organ transplantation. As early as 1998, Starzl first hinted that extended organ engraftment under conventional immunosuppression is a manifestation of only partial tolerance.[405-407] He postulated that complete graft tolerance is attainable through clonal exhaustion-deletion process, by means of recipient pre-treatment and minimised post-transplant immunosuppression and thanks to the high antigen load offered by the liver.[408,409] Nonetheless, none of the cited needs have been satisfactorily met to date.

The phenomenon of T cell exhaustion ensues long-lasting exposure to self and non-self antigens. Exhausted T cells (Texh) show limited capacity to proliferate and produce cytokines in response to specific antigen stimulation and are identifiable because of specific transcriptional, epigenetic, and metabolic signatures.[410] Although proof of the association between T cell exhaustion and a better function of transplanted organs exists,[411] the study of pathways and molecules that directly foster Texh is underway.[412] It appears that FcγRIIb is the only inhibitory Fc receptor.[413] It regulates immune and inflammatory responses by playing a role in innate immunity and in B cells, CD8+ T cells, suggesting its

involvement in successful engraftment. Consequently, the development of safe and effective FcγRIIb agonists may represent a promising way forward in organ transplantation.[410] However, the array of approaches to harness T cell exhaustion is still limited to indirect strategies anticipated almost 25 years ago, namely huge antigen load,[414] induction with depleting agents as opposed to non-depleting agents, and CNI withdrawal.[415,416]

Antigen abundance activates the TCR-NFAT-TOX/NR4A axis that leads to exhaustion in T cells,[417] reduced rejection rates and better graft survival. This is evident in models of larger skin grafts compared to tinier ones,[417] in recipients of dual-kidney transplant as opposed to single-kidney transplant recipients,[418] and, the most suggestive case, in recipients of liver compared to recipients of smaller organs.[414] Although this evidence advocate for tailored immunosuppressive drug management according to the size of the graft, there is not much room to artificially increase the antigen load of an organ in current clinical practice.

Depleting antibodies have pleiotropic immunoregulatory effects. Alongside broad and direct clearance of leukocytes, the administration of these antibodies has been associated with the upregulation of immune checkpoint, namely the programmed cell death protein 1 (PD1), cell surface receptors that promote apoptosis of antigen-specific T cells and reduce apoptosis in Tregs.[419] There is indeed a certain amount of evidence that antilymphocyte antibodies support the expansion of Tregs.[420] Yet it is entirely to be demonstrated whether these changes are the harbinger of clinical operational tolerance, or, at least, of *prope* tolerance, where graft acceptance is kept at the price of a low, nontoxic maintenance immunosuppression.

CNIs are the mainstay of antirejection prophylaxis after solid organ transplantation but bear significant toxicity, including nephrotoxicity and insulin resistance. Remarkably, calcineurin and NFAT are also necessary to induce TOX, a transcription factor that mediates exhaustion. Therefore, CNIs behave as double players that protect against TCMR-A but impede T cell exhaustion and tolerance.[415] Besides, though post-LT accelerated decline in glomerular filtration

rate is multifactorial,[421] CNI-induced nephropathy is a modifiable event that is influenced by dose and duration of CNI administration.[112,422] The safety and efficacy of induction polyclonal anti-lymphocyte globulins associated with CNI minimisation is implied in retrospective series, with good short-term results,[161,423,424] but never confirmed in randomised controlled prospective trials.[425] Altogether, it is safe to assume that CNI minimisation or withdrawal strategies are conceptually justified but hard to put into clinical practice.

With our experience, while we clearly ascertained that a massive induction with ATLG is beneficial in reducing early histological signs of TCMR-A, we could not prove that this is beneficial in terms of reduced TCMR-C. ATLG administration was not associated either with a reduced need of CNI, a better renal function or an improved patient survival. On the contrary, there was some concern for an excess, though not statistically significant, of early graft loss in the experimental group. Our interpretation for subsequent research is that it would be better to split the massive ATLG dose of 9 mg/kg in the first days after liver transplantation in order to avoid the excess of infusion-related adverse events. Whether ATLG were successful in inducing a tolerogenic phenotype in our liver recipients is entirely to be proved because this requires long-term data that are being collected during the time I am writing this doctoral thesis.

The pharmacological research about tolerance has evolved over time towards new drugs. mTORis clearly influence T cell exhaustion, but the direction of this influence, whether for or against, is less clear. As a consequence, the role of mTOR signalling in dysfunctional T cell states awaits further investigation.[426]

The inhibition of co-stimulation, the secondary crucial signal for the activation of an antigen-specific immune response, might constitute the way forward to get past CNIs. Co-stimulation blockade promises better transplant outcomes with improved safety and tolerability profile. Belatacept, a modified CTLA4-Ig fusion protein, was first approved as a maintenance agent in kidney transplantation to replace CNIs.[427] The research about iscalimab, a fully human anti-CD40 non-depleting

monoclonal antibody is currently underway.[428] However, these compounds are under scrutiny because of an unacceptably higher rate of early acute rejections.[429]

The future of living-donor liver transplantation

Deceased-donor organ scarcity is becoming conceptually unbearable in view of the success of a radical treatment, such as LT, and in light of the expansion of indications. Adult-to-adult LDLT raised great expectations to the same degree of live-donor kidney transplantation. Unfortunately, after initial interest, Western centres have substantially disregarded this tricky procedure (Figure 3). The risks for the donor, the ever-lurking small-for-size syndrome, the problem of a long learning curve, the precise technical requirements that we have extensively described still discourage from this procedure.[430]

A number of possible solutions have been proposed over the years. Among them, dual LDLT is a strategy that successfully increase parenchymal mass in the recipient,[431] but eventually does not appear attractive because it even requests two live donors, seeming ethically questionable. Contrariwise, paediatric LDLT programmes have thriven through a codified procurement of the left lateral sector.[432] In this sense, the solution for donor's safety appears to dwell in standardisation.

In 2015, Line et al. proposed to transfer the concepts of ALPPS to LT.[433] Brilliantly, this group used discarded left lateral sectors, coming from split liver procurements, for a transplant-based two-stage hepatectomy. They named RAPID this procedure that combines left-liver resection, the ligation of the contralateral portal branch and partial LT, and that entails a delayed completion hepatectomy. Not long after, the convincing results of the initial experience opened the door to a natural evolution: Nadalin et al. reported the use of live-donor left lateral sectors as grafts.[50] The risk of liver failure in donors is reduced after left hepatectomy compared to right hepatectomy. Likewise, in the recipient, the presence of residual native liver before completion hepatectomy offers protection against a possible

temporary graft underperformance. This ground-breaking trick gives tiny grafts time to regenerate, while the residual native liver act as a stake that supports recipient's metabolism. Generally, the presence of portal hypertension comes to mind as a contraindication that confines this technique to non-parenchymal liver diseases. Still Balci et al. have recently demonstrated that RAPID is feasible for hepatocarcinoma in compensated cirrhosis, provided that good portal flow modulation is ensured.[434]

From the beginning, this novel two-stage strategy showed the potential to contain intra- and postoperative adverse events and to calm ethic debate about LDLT. It was not long before we embarked in this adventure.[49,366] These contributions are the matter of the thesis of a brilliant colleague of mine (and dear friend). Consequently, we will not dwell on this point herein. We can anticipate that our research group is focussing on three main themes: immunosuppression and pharmacotherapeutics of cancer patients, technical refinements, and extension of oncological indications, three crucial issues for LDLT to become the widespread practice that is needed to be.

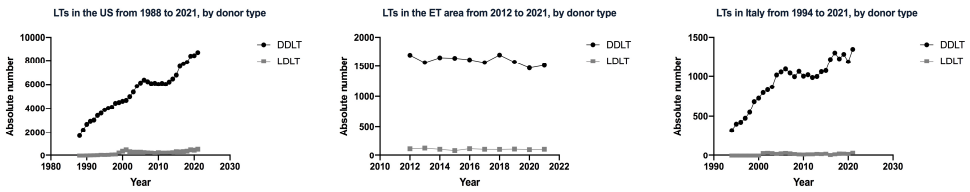

Figure 3: Trends in LDLT compared to DDLT in the US (to the left), in the Eurotransplant area (in the middle), and in Italy (to the right). LDLT volume is strikingly stagnating.

The use of selective prolyl hydroxylase inhibitors in liver surgery

How to translate the benefits that hypoxia might bring to liver surgery into real-world clinical practice? At the beginning of my experimental project, we postulated that PHD inhibitors are a potential chance for the management of PHLF. Our experience with selective pharmacological hypoxia simulators has not brought evidence in favour of PHD inhibition as a mechanism to improve liver regeneration or function after liver resection. Unfortunately, we came to this conclusion in spite of previous works that had been carried out with non-selective PHD inhibitors, as extensively discussed above. We have surmised that hypoxia mediates adaptive changes in the liver that are not, at least, entirely, driven by the HIF pathways. It thus remains unclear the precise mechanism of action of hypoxia and the degree of activation of putative and described hypoxia sensors required to favour regeneration, to induce early neoangiogenesis, and to preserve hepatocellular function. On this score, a promising pathway to explore might be the "normobaric oxygen paradox",[435] where relative fluctuations of physiological and supra-physiological oxygen pressure, rather than steady hypoxic conditions, induce HIFs transcriptional effects.[436]

With our experience, we found out that a selective HIF1α stabilisation surprisingly brings about mitigation in fatty liver infiltration after resection and that this effect is mediated by a promotion of oxidative metabolism.

In baseline conditions, the liver does not stock fats, which happens under stress, like trauma, high lipid and carbohydrate intake, abnormal lipid metabolism, intoxication, and resection.[437,437] Post-resection steatosis can be a direct consequence of increased peripheral lipid mobilization, increased lipid uptake or decreased export, and impaired mitochondrial beta-oxidation, but the easiest explanation is the increased lipid inflow per gram of remnant liver tissue.[437,439,440] While a reduction in ectopic hepatic lipid accumulation does not translate into an

enhancement in liver regeneration after hepatectomy,[437] intrahepatic fat is associated with metabolic dysfunction,[441] lipotoxicity, due to increased endoplasmic reticulum stress, mitochondrial stress, and impaired mitophagy,[442] and an increased susceptibility to IRI, as a result of microcirculatory blood flow and cellular changes.[443] All of these events inevitably impair liver regeneration.[444,445] Understandably, the degree of pre-existent steatosis has an impact on postoperative morbidity and mortality.[446] In liver transplantation, graft steatosis is associated with increased risk of primary nonfunction or dysfunction, and reduced patient survival.[447-450] In liver resection, steatosis has been shown to increase complications, mortality, need for blood transfusions, liver dysfunction, and acute liver failure.[451-453]

In the Western world, alcohol-related liver disease is the reason for LT of about one third of patients, a stable proportion, while non-alcoholic fatty liver disease accounts for 20 to 46% of primary liver diseases, a growing percentage.[454] Besides, secondary liver cancers routinely undergo neo- or adjuvant treatments with 5-fluoruracil, irinotecan, or oxaliplatin, all known to induce chemotherapy-associated steatosis.[455,456] Subsequently, although pre-existent steatosis and resection-induced fatty liver infiltration are two different settings, the reversibility of steatosis springs to mind as a potential target to extend indications and to reduce postoperative adverse events in liver surgery. Indeed, hypo-caloric, hyper-protein diet and exercise already represent usual preconditioning to clear steatosis and promote liver shrinkage before bariatric surgery and donor hepatectomy.[457,458] Unfortunately, this approach requires time. On the contrary, HIF1α has been proved to be a master regulator of lipid metabolism in the liver. Loss of hepatic HIF1α worsens choline-deficient diet-induced fatty liver disease in mice.[459] HIF1α-null mice stock more lipids when exposed to ethanol compared to wild-type counterparts, while the administration of DMOG markedly improve ethanol-induced steatosis.[460] Although the relationship between HIF1α signalling and Cu-induced steatosis has not been clarified, DMOG and roxadustat reduce copper

accumulation and, interestingly, mitigate steatosis in two zebrafish atp7b deficiency models of human Wilson's disease.[461] Altogether, these experiences imply that HIF1α stabilisation can become a key strategy for the clearance of steatosis to be obtained after a short course of PHD inhibitor. The domains where all these enthralling hypotheses need to be explored are plentiful. The spectrum ranges from pre-transplant graft defatting, during machine perfusion, to the treatment of fatty liver disease, to name a few conditions. The way is just open.

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