Small-for-size syndrome and hypoxia

A lesson learned from the Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS) rapid liver regeneration model in rats

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Thesis presented for the grade of PhD in medical science

To my husband Kosta and

my children Iris and Alexandre

Always remember

'Οὐδὲν τοῖς θαρροῦσιν ανάλωτον'

Μέγας Αλέξανδρος, 356-323 π.Χ.

PREFACE

When I first step in GAEN Laboratory, I did not know what research was. I am a clinician; basic research was totally unknown to me. All I knew was that I, at least, would have the opportunity to accomplish a teenager's dream! I learned to work with animals, write projects, ask the right questions and define the tools to answer them, analyze the results, write papers, and answer to reviewers .... A thesis is about learning not to take anything for granted, the satisfaction of carrying out a project, the joy and sharing of results, the discovery of personal resources still unsuspected and the enthusiasm of having contributed to a better understanding of a specific field. This great adventure, this unique experience, gave me the opportunity to meet and work with wonderful people, whose support made this project feasible.

I would like to thank Dr Bertrand, probably one of the most charismatic surgeons I have had the chance to work with! I thank him for his support, his confidence and the constant positive pressure which allows to move on. Claude, you transmitted to me your passion for Hepato-Biliary surgery. You generously teached almost all I know in surgery, and you supported my research work! You have always been the "wind beneath my wings", giving me the opportunity to surpass myself and widen my horizons. Crossing your road during my professional life has been the best opportunity I have had.

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pertinent and brilliant ideas to everyone? Your enthusiasm, your scientific rigor and curiosity, your patience are precious! Working with you is a real pleasure!

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Of course, sponsoring is mandatory in research. I would like to thank the “Fondation Godinne” for its financial support. I would also like to thank my PhD Jury, Professeur Jean Luc Balligand, Professeur Maximillien Gourdin, Professeur Peter Stärkel, Professeur Laurence Annet, Professeur Pierre Gianello who followed my steps during this project and helped me to “canalize” my ideas. Special thanks to Thomas Van Gulik and Erik Schadde! What an honor to have had the opportunity to meet you. Both of you really upgraded my “private defense” with a high-level discussion! Your questions, remarks and ideas are an inspiration for my future research work!

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for their love, my friends Valérie, Anne, Christa for believing in me and my operating-theater mates for encouragements!

Last, but not least, I thank my husband, Kosta, my best friend and soul mate! You support every step I make. What a chance and inspiration to have you by my side! Iris and Alexandre, I apologize…mom has not always been very present…. Thank you for giving me your unconditional love, a real energy burst in my everyday life! I love you more than everything in the world and I really hope that this experience will be a lesson for you. You should always remember that “nothing is impossible for the one who dares to just try” …

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LIST OF ABBREVIATIONS

ALPPS: associating liver partition and portal vein ligation for staged hepatectomy
ASAT: aspartate aminotransferase
ALAT: alanine aminotransferase
ATP: adenosine triphosphate
BrdU: bromodeoxyuridine
CD31: cluster of differentiation 31
CD44: cluster of differentiation 44
C/EBPb: enhanced binding protein b
CL: rodent caudate liver lobe
DAMPS: damage associated molecular patterns
DLL-1: delta like canonical ligand 1
DMOG: dimethyloxalylglycine
DNA: deoxyribonucleic acid
EGF: epidermal growth factor
EPC: endothelial progenitor cells
FGF: fibroblast growth factor
FLR: future liver remnant
FLRV: future liver remnant volume
GFP: green fluorescent protein
GRWR: graft to recipient weight ratio
GV/SLV: graft volume/standard liver volume
HABR: hepatic arterial buffer response
HGF: hepatocyte growth factor
HIF: hypoxia-inducible factor
HMGB1: high mobility group box 1
HNF4α: hepatocyte nuclear factor 4α
HPVE: hepatic and portal vein embolization
HRE: hypoxia-responsive elements
H_2S: hydrogen sulphide
Id1: inhibitor of DNA binding protein 1
IL: interleukin
INR: international normalized ratio
iHAF: indexed hepatic artery flow (hepatic artery flow/perfused liver)
iPVF: indexed portal venous flow (portal venous flow/perfused liver)
LLL: rodent left lateral liver lobe
L-NAME: N G-nitro-L-arginine methyl ester
LSEC: liver sinusoidal endothelial cells

LT: liver transplantation
LW: liver weight
Lyve-1: lymphatic vessel endothelial hyaluronan receptor 1
MER/ERK: mitogen-activated protein kinase/extracellular signal regulated kinase
mG: membrane green fluorescent protein
ML: rodent median liver lobe
mT: membrane tomato fluorescent protein
NK-κB: nuclear factor kappa B
NO: nitric oxide
PCNA: proliferating cell nuclear antigen
PDK: pyruvate dehydrogenase kinase
PHD: propyl-hydroxylase domain
PHLF: post-hepatectomy liver failure
PHx: partial hepatectomy
PIGR: polymeric immunoglobin receptor
POD: post-operative day
PVE: portal vein embolization
PVF: portal venous flow
PVL: portal vein ligation
PVO: portal vein occlusion
PVP: portal venous pressure
RAGE: advanced glycation end products receptor
RALPPS: radiofrequency assisted ALPPS
RL: rodent right liver lobe
ROS: reactive oxygen species
SDF-1: stromal derived factor 1
SFSS: small for size syndrome
SIN-1: 3-morpholinosydnonimine-1
STAT2: signal transducer and activator of transcription 2
TGF-α or β: transforming growth factor α or β
TLR-4: Toll-like receptor 4
TLV: total liver volume
TNF-α: tumor necrosis factor α
TSH: two-stage hepatectomy
VCAN-1: versican 1
VE-cadherin: vascular endothelial cadherin
VEGF-A: vascular endothelial growth factor A
VEGF-R2: vascular endothelial receptor 2
VHL: Von Hippel–Lindau tumor suppressor gene
Wnt2: wingless-type MMTV integration site family, member 2

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INTRODUCTION

CHAPTER I: LIVER REGENERATION

1.1. THE LIVER

1.1.1. Liver function

The liver is a multi-functional organ that has a central role in metabolic homeostasis, as it metabolizes, synthesizes, stores and distributes nutrients and vitamins to the organism. It controls physiological processes such as nutrient metabolism following intestinal absorption, waste processing and excretion (such as urea cycle and bile synthesis), detoxification of xenobiotics, energy storage and regulation, production of serum proteins (coagulation factors, albumin) and hormones.[1,2] These functions are so essential that liver mass is controlled within a very narrow range in relation to the overall body mass.[3] Consequently, liver-to-body weight ratio has become a criterion for liver transplantation.[4]

1.1.2. Liver anatomy

In mammalians, the liver is the largest organ of the body and constitutes 2.5% of the body weight. In humans, thorough knowledge of liver anatomy is largely due to the work of a French surgeon and anatomist, Claude Couinaud, who demonstrated that hepatic functional anatomy is based on vascular and biliary relationships. According to Couinaud's classification, the human liver is divided into eight independent functional units termed the 'segments' (Fig. 1A). Each segment has its own individual, dual vascular inflow, biliary and lymphatic drainage. In general, each segment can be considered as wedge-shaped with the apex directed towards the hepatic hilum. At the apex there is a single segmental branch of the portal vein, hepatic artery and bile duct. Along the boundaries of each segment

there is venous outflow through the hepatic veins, so that one hepatic vein drains two adjacent segments (and each segment thus has multiple draining hepatic veins)[5] (Fig. 1B).

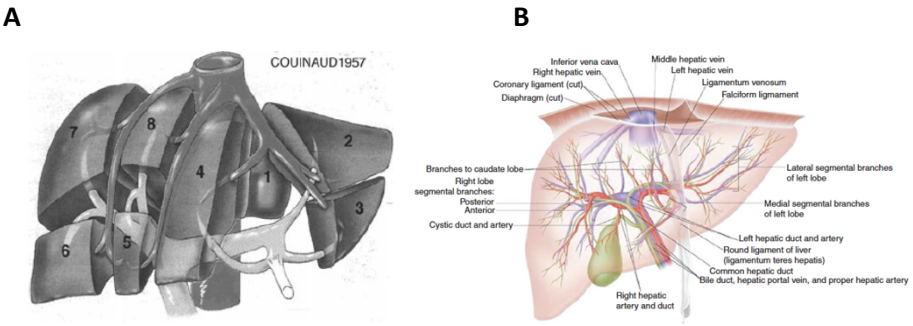

Figure 1. (A) Couinaud's compartmentation of the liver: the original illustration (from Couinaud, *Masson*, 1957).
(B) Vascular distribution in the human liver (from Mulaikal-Emond, *Liver Anesthesiology and Critical Care Medicine*, 2018).

In rodents, anatomical studies based on vascular corrosion casts show similarities to human anatomy considering the hepatic in- and outflow.[6] One major difference to human anatomy is that the rodent liver is partitioned. It is divided into four main lobes: caudate lobe (CL), right liver lobe (RLL), median lobe (ML, right and left segment) and the left lateral lobe (LLL)[7](Fig. 2A). Importantly, in both rat and mouse, the inferior vena cava is intrahepatic. The liver tissue surrounding the inferior vena cava (the 'para-caval' liver) comprises approximately 2.5–3% of the liver mass and is not a distinct anatomical unit (Fig. 2B). Therefore, resection of the para-caval tissue during partial hepatectomy (PHx) is necessarily incomplete, in order to avoid intrahepatic vena cava injury.

A **B**

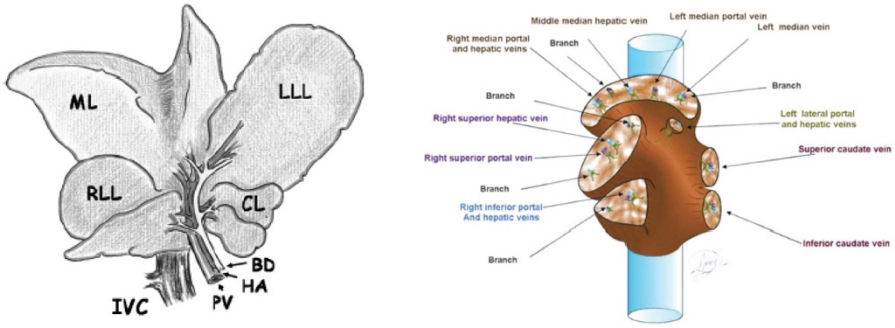

Figure 2. Rodent liver. (A) Schematic representation of the rodent liver showing the partitioned liver lobes. ML: median lobe; LLL: left lateral lobe; RLL: right liver lobe; CL: caudal lobe (from Aller *et al*., *Liver International*, 2009).[8]
(B) The para-caval tissue in rodent livers and the intrahepatic location of the inferior vena cava (from Madrahimov, *Annals of Surgery*, 2006)[6].

1.1.3. Liver architecture

The liver mass is composed mainly of the parenchymal cells (the hepatocytes), performing all the essential functions of the organ and responding to the organism's metabolic demand. They are organized into cords that lie on a specialized capillary bed (the sinusoids) with fenestrated endothelial cells. The fenestrae are clustered together in sieve plates. Their size and number differ according to their location in the lobule (Fig. 3A). The liver sinusoids are dynamic structures that contract and dilate in response to alterations of the sinusoidal blood flow and perfusion pressure, thanks to hepatic stellate cells that act as contractile pericytes. Because the sinusoidal domain of the hepatocytes faces the perisinusoidal space of Disse (the space between the hepatocytes and the fenestrated endothelial sinusoidal lining cells), the hepatocytes are directly exposed to the portal flow, its content and its fluctuations through the fenestrae. The apical pole of hepatocytes delineates the bile canaliculi in which hepatocytes

secrete the bile (Fig. 3B). The portal vein and hepatic artery are organized in a cluster with bile ducts within liver parenchyma forming the 'portal triad' and spaced out at the corners of a hexagonal unit named 'the hepatic lobule' (Figs 3B and 4). The vascular network allows a unidirectional flow from the portal vein and the hepatic artery through the sinusoids to the central vein. Thus, blood flows from the portal triade to central veins, while bile flows in the opposite direction. Liver function is orchestrated by the lobular architecture.[9]

A **B**

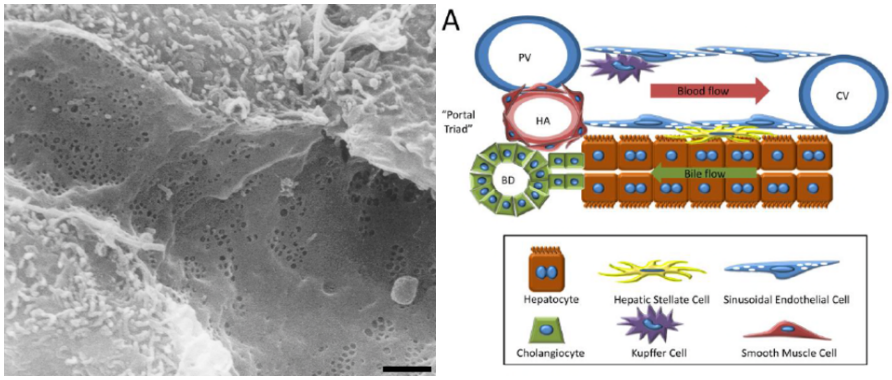

Figure 3. (A) Low magnification scanning electron micrograph of the sinusoidal endothelium from rat liver showing the fenestrated wall. Note the clustering of fenestrae in sieve plates. Scale bar: 1 µm (from Baert, *Comparative Hepatology*, 2002).
(B) Representation of the liver lobule and liver cells (from Kang *et al*., *Cells*, 2012).[1]

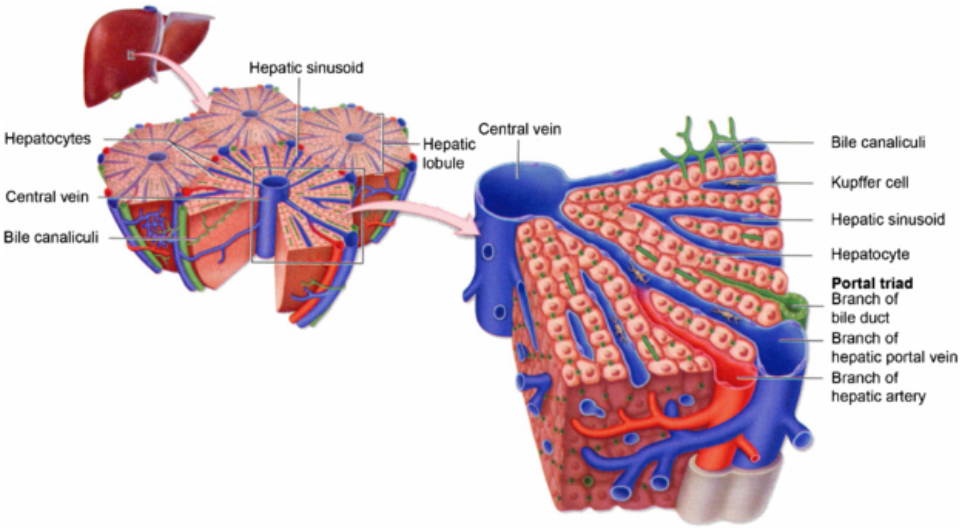

Figure 4. Structure of the liver lobule (from Mescher, *Junqueira's Basic Pathology: Text and Atlas*, 2010; New York: McGraw-Hill).

Besides sinusoidal endothelial cells, resident macrophages (Küpffer cells) and hepatic stellate cells (acting as contractile pericytes and reported to be involved in the regulation of blood flow) are the main cell types of the non-parenchymal cell compartment.[10]

1.1.4. Liver haemodynamics

The liver receives 10–15% of the total blood volume and 25% of cardiac output via a dual blood supply, the portal vein and the hepatic artery.[11] The blood from the hepatic artery and portal vein is incorporated into the sinusoids, draining into hepatic venules that merge into liver veins. Hepatic veins drain the blood into the inferior vena cava and the right atrium. Forty per cent of the total liver blood is in the large vessels, whereas the remaining 60% circulates in the sinusoids.[12] The portal vein is a valveless vessel that passively drains the blood from the capillary system of the intestine, spleen, pancreas, omentum and gallbladder.[10] Thus, the

portal vein provides partially deoxygenated blood to the liver. The portal blood is nutrient-rich during the post-prandial period. In the fasting state, the oxygen saturation in portal blood is approximately 85%, and portal vein blood contributes to 75–80% of the total hepatic inflow (90 ml/min/100 g of liver weight) and 50–70% of the liver oxygen requirement. The hepatic artery contributes 20–25% of the liver inflow (30 ml/min/100 g of liver weight). It supplies well-oxygenated blood to the organ (95% oxygen saturation) and provides 30–50% of the liver oxygen requirement.[13] Oxygen consumption by the liver accounts for 20% of total body oxygen consumption. The hepatic artery is a vessel of resistance, while the portal and hepatic veins are vessels of capacitance, containing most of the liver blood. Thus, the liver is interposed in an arterial high-pressure and a venous low-pressure system.

There is an intimate dynamic adaptive relationship between the hepatic artery and the portal vein: the 'hepatic arterial buffer response' (HABR),[14,15] The HABR represents the ability of the hepatic artery to produce compensatory flow changes in response to fluctuations in the portal venous flow. An increase in portal perfusion causes a reduction of the hepatic artery flow. Conversely, hepatic arterial blood flow is capable of buffering up to a 25–60% decrease of the portal flow.[16]

Physiologically, the purpose of the HABR is to minimize the influence of portal venous flow fluctuations on hepatic perfusion and to maintain adequate oxygen supply to the tissue.[17] This latter function seems to be of minor importance, as the liver normally receives oxygen in excess and is able to increase oxygen extraction in the case of decreased delivery.[18] Accordingly, oxygen concentration does not trigger the HABR. Indeed, low oxygen concentration in the portal blood, such as seen after haemodilution, does not alter the arterial flow as long as the portal perfusion is unchanged. Similarly, a massive increase of oxygen consumption by the hepatocytes does not cause vasodilatation of the hepatic artery.[18]

The current hypothesis underlying the HABR is related to the adenosine 'washout' theory, as proposed by Lautt in 1985.[19] This hypothesis postulates that adenosine is constantly secreted by hepatocytes in the so-called space of Mall, a microscopic fluid compartment surrounding the portal triad (interstitial space). The concentration of adenosine in this space is directly regulated by the portal vein flow. When portal blood flow is reduced, less adenosine is washed away from the space of Mall and the elevation in adenosine levels mediates the dilation of the hepatic artery with a subsequent increase in hepatic arterial flow.[20]

The role of adenosine in the regulation of the hepatic arterial flow is supported by several experimental observations: adenosine causes hepatic arterial dilatation[19] via p1-purinoreceptor-subtype-A2;[21] the portal infusion of adenosine modulates arterial flow;[20] and the arterial flow adaptation to a change in portal flow is abrogated in the presence of adenosine antagonists.[22] Besides adenosine, other vasoactive compounds, such as nitric oxide (NO)[23] and hydrogen sulphide (H_2S),[24] have been identified as regulators of the arterial flow and may contribute to the HABR. Intrahepatic arteries are richly innervated. Biernat *et al*. proposed that capsaicin-sensitive sensory fibers are also involved in the HABR.[25] However, the contribution of vasoactive neuropeptides is far from clear, as the HABR is lost upon brain death[26] while it is unequivocally maintained in liver transplants.[27,28]

The HABR is particularly relevant in the context of liver surgery. In the case of liver resection, irrespective of the extent of the resection, the portal flow is directed through a reduced sinusoidal network leading to elevated portal perfusion of the liver remnant with a reflex constriction of the hepatic artery. This phenomenon becomes of utmost importance when the remnant liver is critically small and insufficient for survival. After extended hepatectomy or transplantation of a small graft, redirection of the whole portal blood into a critically small sinusoidal bed causes portal hyperperfusion, with a critical compensatory constriction of the

common hepatic artery. The ensuing 'de-arterialization' of the remnant liver is proposed as the primary factor for life-threatening postoperative liver failure, a condition fraught with high postoperative mortality rates, called 'post-hepatectomy liver failure' (PHLF) or the 'small-for-size' syndrome (SFSS)[29,30] (see section 2.2) (Fig. 5).

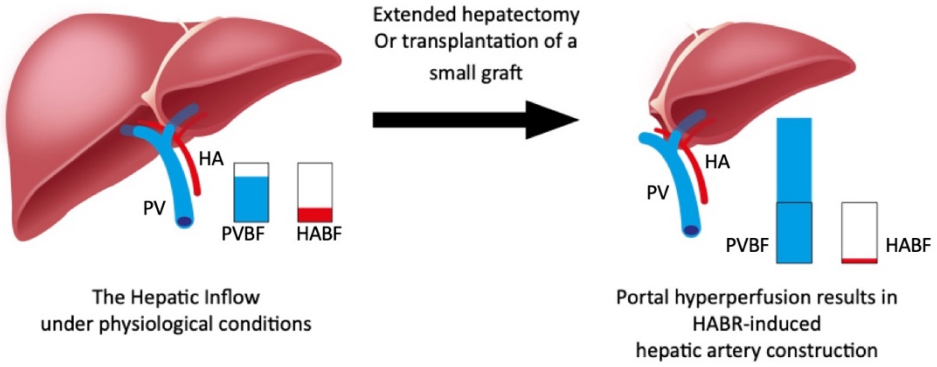

Figure 5. The hepatic arterial buffer response after extended hepatectomy. HA: hepatic artery; PV: portal vein; HABF: hepatic artery blood flow; PVBF: portal vein blood flow (adapted from Eipel, *World Journal of Gastroenterology*, 2010)[15]
Design by Gaêlle De Jesus Silva

The HABR is not universally accepted by researchers; it has been questioned by Dold *et al.*[31] in a model of liver resection in rats. The investigators evaluated the portal and hepatic artery inflows post-hepatectomy and observed, despite a stepwise increase in portal perfusion, constant arterial perfusion of the remnant, whether 30, 70 or 90% of the parenchyma was removed. The authors did, however, observe hypoxia in the remnant liver. They attributed this to an increased oxygen consumption relative to hepatocyte proliferation and increased hepatocyte metabolism, rather than to arterial constriction. With regard to liver transplantation of small grafts, even though several reports link arterial flow reduction to portal hyperperfusion,[28,32] Hickman postulated that increased

noradrenaline serum concentrations after orthotopic liver transplantation with a small graft may be the main cause of arterial constriction.[33]

By activating sensory nerves locally in the liver, adenosine may also stimulate the hepatorenal reflex, an extrahepatic adaptation to the reduced liver inflow that may contribute to liver haemodynamics.

Taken together, current data support that while most of the observations link modulation of the hepatic artery perfusion in response to fluctuations of the portal flow, the conditions in which this process occurs, the mechanisms at play and the contribution of systemic haemodynamics are far from being deciphered. Ideally, in order to accurately analyze the role of the buffer capacity of the hepatic artery, changes in portal flow should be experimentally modulated in the absence of hormonal or compensatory systemic haemodynamic reflex (arterial pressure fluctuation). These conditions are virtually impossible to model *in vivo*.

1.2. LIVER REGENERATION

Liver regeneration is a programmed proliferative response to loss of liver function. After partial hepatectomy (PHx), liver regeneration can be more precisely defined as a compensatory hypertrophy/hyperplasia where, in the remaining liver, tissue expands to restitute the initial liver mass and meet the metabolic demands of the organism.[1] Unlike true regeneration, the expanding liver does not anatomically and spatially replace the lost/resected part of the organ. The liver mass is maintained within a very narrow range in relationship to the body mass. The sensing of a change in liver functional mass and the adapted response to maintain liver function or liver mass within tight limits, by regeneration in case of tissue loss, or by

apoptosis in case of liver enlargement or transplantation of a large graft,[34] implicate numerous and probably redundant pathways collectively called the 'hepatostat'.[35]

1.2.1. Hepatocyte regeneration

Hepatocytes are resting cells, but they have a remarkable capacity to re-enter the cell cycle and to actively divide. Studies report a capacity of up to 69 cell-doublings.[1,36] After PHx, 95% of hepatocytes synchronously enter the cell cycle.[2] Cell fate mapping studies have shown that the vast majority (99%) of newly generated hepatocytes come from division of pre-existing mature hepatocytes,[37,38] and that the process does not rely upon liver progenitors[37,38] or bone marrow progenitors.[39–40] Kanazawa and co-workers[39] explored whether hepatocytes originated from bone marrow cells in three different models of liver injury, and concluded that there was little or no contribution of bone marrow cells to hepatocyte renewal. Interestingly, Fujii *et al*.,[41] in a model of chimeric irradiated green fluorescent protein (GFP)-negative mice reconstituted with GFP-positive bone marrow, identified GFP-positive endothelial cells and Küpffer cells, but no hepatocytes. A similar result was obtained by Dahlke *et al*.,[40] using a model of acute liver failure. Taken together, these experimental data support that, during liver regeneration, bone marrow progenitor cells do not contribute to hepatocyte proliferation, while they contribute to sinusoidal endothelial cell renewal and regeneration of the sinusoidal bed.

Liver resection induces a synchronized proliferation of hepatocytes. Hepatocyte mitosis is submitted to a circadian rhythm,[42] while hepatocyte DNA replication is independent of the nychthemeral cycle. In the early phase after PHx (0–3 days) parenchymal cell mitosis and DNA replication evolve in a biphasic manner,[43,44] the first wave of proliferation being of the highest magnitude (Fig. 6). The proportion

of hepatocytes entering the cell cycle, but not the timing for DNA replication or cell division, depends upon the extent of liver resection.[45,46] The timing of hepatocyte DNA synthesis is species-specific. In the rat liver, the first burst of hepatocyte DNA synthesis peaks at 24 h, whereas in mice it occurs 20 h later, reflecting a longer G1 phase.[47] In rodents, liver mass is completely restored within 5–7 days after PHx,[48,49] even though regeneration continues for 15–16 days.[50] In humans, there are few data on the precise timing of hepatocyte DNA replication[51] after PHx. In culture, however, human hepatocytes respond to the same growth stimuli as rodent cells.[52] The volumetric and functional recovery of the liver has been studied more extensively after hepatectomy in living donors. In a series of 27 donors who underwent a right hepatectomy, the remnant liver volume (approximatively one third of the initial liver mass)[53] increased to 74% at postoperative day 10 (and grew to reach 83% of the liver's original volume at 12 months). The functional recovery, however, evaluated by galactose elimination, was slower and normalized at 6 months.[54]

1.2.2. Non-parenchymal cell regeneration

The proliferation of non-parenchymal cells is delayed compared to that of hepatocytes. Studies estimate that the peak of proliferation of Küpffer and biliary cells occurs at 48 h post-hepatectomy, while it occurs after 96 h for liver sinusoidal endothelial cells[2,55] (Fig. 6).

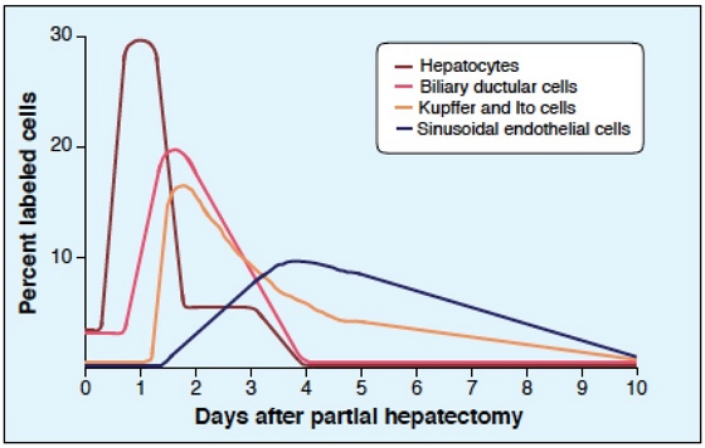

Figure 6. Time kinetics of DNA synthesis in different liver cell types after partial hepatectomy in a rat model of liver regeneration. The four major cell types of the liver undergo DNA synthesis peaks at different time-points. Note that hepatocyte DNA synthesis evolves in a biphasic manner (from Michalopoulos *et al.*, *Science*, 1997).[55]

1.2.3. Temporospatial disorganization of the lobule during liver regeneration

During liver regeneration, there is an asynchronism between proliferation of hepatocytes and liver sinusoidal endothelial cells. This process is at the origin of a transient perturbation of the lobular architecture, with proliferating hepatocytes forming avascular clusters. After 70% PHx this architectural disturbance is not clinically relevant, as the procedure does not associate with loss of liver function or mortality in rodents.[56,57] However, as the hepatocyte proliferation correlates to the extent of hepatectomy,[46] a major resection will trigger a large regenerative response of hepatocytes in the liver remnant, causing potentially intensive lobular derangement. Ninomiya *et al.*'s work[58] demonstrated that the hepatocyte proliferation after 90% PHx is significantly higher compared to 70% PHx, even though 90% hepatectomized animals had higher mortality rates. 90% hepatectomy was associated with avascular hepatocyte clusters formation and lobular

architectural disruption. When the investigators mitigated hepatocyte proliferation in mice after 90% PHx (by injection of an inhibitor of the MEK/ERK pathway), they observed a preserved liver lobular architecture and animal survival. The presence of avascular hepatocyte 'islands' within the regenerative liver could explain that the remnant's mass recovery, reflecting hepatocyte renewal, does not always indicate a functional recovery,[59] as is observed in small-for-size grafts.[60] It is also conceivable that hepatocyte clusters in the regenerating liver are submitted to transient hypoxia according to the extent of resection and the ensuing magnitude of hepatocyte proliferation.[61]

Taken together, these data indicate that islets of regenerating hepatocytes disconnected from the sinusoids may not be functional. Therefore, symmetry in magnitude and timing of hepatocyte proliferation and sinusoidal renewal is essential to maintain lobular architecture and, hence, organ function.

1.2.4. Angiogenesis during regeneration

Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels.[62] The process is characterized by sprouts of endothelial cells which grow towards an angiogenic switch molecule, such as vascular endothelial growth factor A (VEGF-A). It is usually initiated in poorly perfused tissues, when oxygen-sensing mechanisms detect hypoxia that, to resolve, requires the formation of new blood vessels.[63] In the regenerating liver, three types of endothelial cells have been identified: intrahepatic or resident endothelial progenitor cells, mature liver sinusoidal endothelial cells (LSEC) and bone marrow-derived sinusoidal progenitor cells (BM-SPC).[64] It has been estimated that resident sinusoidal endothelial progenitor cells represent 1–7% of the LSEC in the normal rodent liver and probably contribute to LSEC regeneration, although such functionality has not yet been demonstrated.[64] Mature LSEC are quiescent in the

physiological state with a low proliferative rate,[65] and proliferate after liver resection (peak proliferation rate 96 h after PHx) when stimulated by growth factors such as VEGF and fibroblast growth factor (FGF).[64] BM-SPC do not participate in LSEC turnover in the quiescent state. In contrast, after liver injury or partial hepatectomy, they are recruited to the liver and contribute to sinusoidal renewal and liver regeneration.[66]

It is accepted that LSEC are key for optimal liver regeneration, in part because they release hepatocyte growth factor (HGF), wingless-type MMTV integration site family member 2 (Wnt2) and angiopoietin-2[67,68] necessary for hepatocyte proliferation and sinusoidal reconstruction. However, LSEC, whether they originate from BM-SPC differentiation or from mature LSEC division share the same size and surface markers. Current literature does not specify how to determine which of these two cell types is the major driver of initiation of liver regeneration.

After 70% PHx, the expression of hepatocyte mitogens such as HGF and Wnt2 is up-regulated in LSEC through activation of the VEGF/VEGF-receptor 2 (R2) pathway.[68] The phenomenon is dependent upon Id1, a transcription factor, as, after PHx, HGF and Wnt2 are not up-regulated in LSECs from Id1 knockout mice. This is restored when LSECs isolated from wild-type mice after PHx are transplanted into the Id1 knockout mice. However, other experimental work supports that mature LSEC express very little HGF,[66] while BM-SPC that engraft in the liver after resection have a high HGF concentration. Indeed, the production of HGF, the essential mitogen for hepatocyte proliferation, by bone marrow-derived sinusoidal cultured cells was twice as high compared to that of LSEC. Consequently, HGF-rich BM-SPC will, in turn, promote hepatocyte proliferation (Fig. 7).

In their report, Wang *et al.* examined the role of BM-SPC recruitment on normal liver regeneration in a rat model of 70% PHx with or without selective hind limb

irradiation (suppressing the peripheral leucocyte count by nearly 40%). When these irradiated rats underwent PHx liver regeneration was impaired by 40%, with minimal hepatocyte proliferation on day 5. Infusion of BM-SPC in the irradiated rats promoted hepatocyte proliferation and restored the liver weight. Interestingly, restoration of liver regeneration was achieved when these cells were infused on day 1 but not on day 3 after PHx. This observation points out the essential role of BM-SPC and the timing of neoangiogenesis, which should occur during the first wave of hepatocyte proliferation, to rescue functional regeneration. Interestingly, the recruitment of HGF-rich BM-SPC is also VEGF-dependent [VEGF- stromal derived factor 1 (SDF-1) activation].[67,69]

In conclusion, efficient vascularization of the regenerating hepatic plates involves a 'crosstalk' between hepatocytes, liver endothelial cells and BM SPC cells in order to maintain lobular architecture.

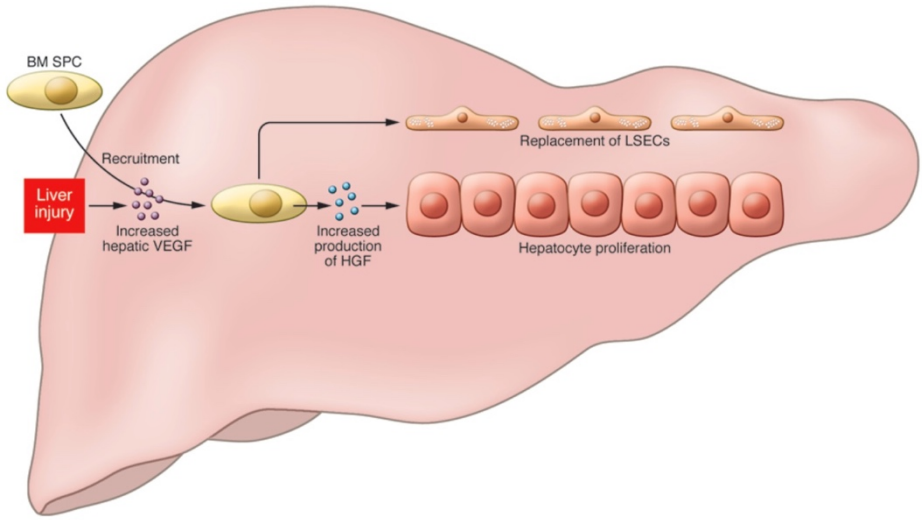

Figure 7. Schematic representation of the intimate 'crosstalk' of liver cells and BM SPCs to liver regeneration. Liver injury or PHx induces increased hepatic VEGF expression, which drives recruitment of HGF-rich BM SPCs. HGF, in turn, stimulates the proliferation of hepatocytes in liver regeneration (from De Leve, *Journal of Clinical Investigation*, 2013)[64]

1.2.5. Triggers of liver regeneration

The literature proposes two main physiological triggers for post-hepatectomy liver regeneration[70] based, respectively, on the sensing of liver metabolic load and on haemodynamic changes.

1.2.5.1. *The metabolic load theory*

According to this theory, liver regeneration is a compensatory response to parenchymal loss, in order to preserve the organ's functional activity. This implies a 'sensing' of metabolic load. Partial hepatectomy imposes an increased metabolic workload to the remaining hepatocytes that generates early stress signals.[71] As early as 2 h after PHx, more than 100 genes are up-regulated as a 'stress' response to the metabolic overload.[72] These genes (c-fos, c-jun, c-myc), cytokines [such as tumor necrosis factor a (TNF-α) and interleukin (IL)-6][73] and transcription factors [signal transducer and activator of transcription (STAT3), nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), enhanced binding protein b (C/EBPb)] 'prime' the hepatocytes, so that they can enter the cell cycle (G1-phase) and respond to hepatic and 'blood-brought' growth factors [HGF, transforming growth factor α (TGF-α), endothelial growth factor (EGF), etc.] and other signaling molecules, i.e. noradrenaline, prostaglandins, oestrogens and insulin.[52] These factors constitute the 'humoral stimuli' for liver regeneration, as proposed by Fausto.[36,74]

The theory of functional liver mass had already been supported in 1987 by the work of Rozga *et al.*[75] and confirmed by others.[76] Rozga and co-workers observed that after segmental portal vein occlusion, the portal-occluded liver segment atrophies, while in the rest of the liver hypertrophy is proportional to the tissue loss. This mechanism assures the metabolic needs of the organism. This observation is also supported by the work of Picard *et al.*[77] These authors reported that, after

segmental portal occlusion, when the hypertrophy of the non-occluded segments is inhibited, cell death in the occluded segments is mitigated, suggesting that there is a homeostatic mechanism that balances cellular events to maintain a stable functional liver mass. However, the metabolic theory fails short to explain an early regenerative response in the non-occluded liver after portal vein occlusion, even before atrophy of the occluded segments occurs.[78] This suggests that the early phase of regeneration is unrelated to the reduction of functional liver mass, and that the initiation of the regenerative process is primed by earlier, unspecific stress signals.

1.2.5.2. *The haemodynamic load theory*

This is another theory put forward to explain how liver regeneration is triggered. After PHx, segmental portal vein ligation or liver transplantation of a small graft, the remaining liver (or portally non-occluded liver segments) receives a generous blood supply, as it has to accommodate portal vein blood previously destined to the whole liver. This increase of portal flow to the liver remnant has been proposed as a trigger of liver regeneration. Two non-mutually exclusive mechanisms that implicate the increased delivery of hepatotrophic factors and the vascular sinusoidal stress or shear stress have been implicated.

1.2.5.2.1. *Hepatotrophic factors of the portal blood*

The immediate rise of the portal flow per unit of liver mass raises the available growth factors and cytokines per hepatocyte. Such factors, carried to the liver by portal blood, include insulin,[79] EGF from salivary glands and duodenal Brunner's glands,[80] endotoxin, serotonin,[81] noradrenaline[75,82] and nutrients derived from the food supply (such as branched amino acids for DNA synthesis[83]), lipids and

carbohydrates.[1,84] These factors could enhance hepatocyte replication by stimulating cell cycle entry and/or supplying the energy needed for this anabolic process.

The assumption that a higher availability of hormonal and nutritional hepatotrophic factors triggers liver regeneration is supported by experiments with canine models of split porto-caval transposition. In this model one portal branch is perfused with blood derived from inferior vena cava, while the contralateral portal branch is perfused with mesenteric blood. The flow rates and the oxygen tension are controlled to be similar in the two portal branches. After 3 months of this vascular rearrangement, researchers observed hypertrophy on the portion of the liver receiving splanchnic blood and atrophy on the segments receiving systemic blood.[85] Moreover, substituting the blood flow from the inferior vena cava with arterial oxygen blood over 3 months did not prevent atrophy or compensate for the qualitative loss of the portal blood stimulus.[86] This suggests that the portal flow brings to the liver factors that are essential for regeneration.

To test the impact of portal transported hepatotrophic factors on liver regeneration without liver resection, Mortensen *et al.*[74] constructed an aorto-portal shunt to the left portal vein branch in pigs increasing the flow into segments II, III and IV, while portal perfusion was intact in the rest of the liver. Acute gene response and late liver regeneration were assessed. Hypertrophy was higher in the portally perfused segments. The authors concluded that this was due to the delivery of hepatotrophic factors.

In line with this, Stärkel *et al*.[87] examined the expression of various cytokines and immediate early genes involved in hepatocyte priming, and demonstrated that the initial activation of these molecules after portal vein occlusion occurs both in the occluded and the non-occluded liver lobes. These results suggest that hepatocyte

priming can be induced by non-specific stimuli (i.e. surgical stress) and the final fate of the cells – atrophy or proliferation – is determined at a later time-point in the late G1 phase. In a subsequent study, the authors[88] suggested that in the occluded lobes the absence of portal hepatotrophic factors and nutrients leads to the induction of inhibitory mediators (TGF-β and IL-1β) and consequently the shutdown of the cell cycle, apoptosis and atrophy.

Taken together, these experiences suggest that the 'quality' or the molecular content of the portal blood is essential for maintaining liver mass and that an increased delivery of portal factors is key for stimulating optimal liver regeneration. This concept has, however, been challenged by others.[89,90] Indeed, Fan and co-workers observed similar liver regeneration regardless of whether the liver blood supply was from a portal or arterial origin.[89]

1.2.5.2.2. *Shear stress*

The 'shear stress' is the frictional force per unit area created when a tangential force (blood flow) acts on a surface (endothelium) so, whenever flow occurs, shear stress exists. Shear stress (τ) is defined by the Haagen–Poisseuille equation shown in Fig. 8. However, it is important to emphasize that this equation is applicable *in vivo* only if we assume that the blood is a Newtonian fluid, the vessel cross-sectional area is cylindrical, the vessel is straight and inelastic and blood flow is steady and laminar.[91]

$$\tau = 32 \cdot \mu \cdot \frac{Q}{\pi \cdot d^3}$$

Figure 8. The Hagen–Poisseuille equation for measurement of the vascular wall shear stress. Shear stress depends upon blood viscosity (haematocrit), velocity (flow) and vessel size; μ is the viscosity, Q the flow and d the vessel diameter. Note that the equation does not take into account either the direction of the blood flow (linear or turbulent) or the vascular elasticity (quality of liver parenchyma).[91]

In most liver studies the shear stress is supposed to be proportional to portal pressure[92–93] or flow.[94] Thus, portal pressure and/or flow are used as a surrogate for shear stress even though, given the limitation explained above, it is impossible to measure the shear stress exactly (intrahepatic vascular diameter inconstant, vascular elasticity depending on the liver stiffness/quality, intrahepatic shunts).[95–96] Other researchers have used indicators to indirectly measure the endothelial stress, such as NO or intravascular release of components of the endothelial glycocalyx.[97] However, the limitations of these approaches are evident, as changes in liver perfusion have a direct impact on systemic haemodynamics.

Immediately after PHx or segmental portal vein occlusion, portal hyperperfusion in the future liver remnant (FLR) imposes a high physical force on the endothelial wall. The increase in shear stress is considered to be one of the most important factors in the activation of the regenerative cascade. Lauber and co-workers[98] demonstrated that the degree of portal hyperperfusion correlates with the hepatocyte mitotic index in the remnant liver in a rat model of 70–80 and 90% portal vein ligation. Corroborating these results, multiple experimental and clinical studies indicate that after PHx, the increased portal pressure and flow per gram of remaining liver tissue and, supposedly, sinusoidal shear stress, may be a primary stimulus to regeneration.[23,99,92–100] Similarly, studies on grafts after liver transplantation indicate that hepatocyte proliferation is proportional to the portal hyperperfusion.[101,102] Patients with a small liver graft show an immediate increase of portal pressure and accelerated regeneration through immediate induction of IL-6 and HGF[103] compared to patients with a large liver graft. In a reverse experiment, occlusion of the superior mesenteric artery during 70% PHx (preventing portal hyperflow and shear stress in the remnant liver) caused decreased regeneration, confirming that prevention of shear stress following PHx precludes the activation of the regeneration cascade.[93]

Several studies support the role of shear stress-induced NO in liver regeneration.[93,104,105] Liver regeneration is inhibited by administration of the NO synthase antagonist N G-nitro-L-arginine methyl ester (L-NAME) and restored by the NO donor 3-morpholinosydnonimine-1 (SIN-1).[106,93] Moreover, NO is a key molecule for VEGF-induced liver endothelial cell proliferation[49] and angiogenesis.[107] Thus, stimulation of neoangiogenesis in the regenerating remnant depends upon the extent of liver resection, the portal perfusion and ultimately on shear stress.

Mechanoreceptors are needed in order to translate mechanical forces detected by the endothelium into biological signals.[108,109] A number of intracellular pathways activated by fluid shear stress include stimulation of transmembrane proteins, activation of ion channels, intracellular calcium mobilization,[93,97] Notch1 signaling, expression of VCAN-1 and CD44, as well as c-fos, c-myc and c-jun.[106,110,111] As these genes are crucial for hepatocyte 'priming' during liver regeneration,[52] a causal link between increased shear stress/portal hyperperfusion and the initiation of liver regeneration has been proposed.

Although shear stress triggers a regenerative response in the FLR, experimental data suggest that there is a slight difference in the regulation of liver regeneration between portal vein occlusion (PVO) and hepatectomy even when portal hyperperfusion is equal. Compared to PVO, after PHx FLR mass recovery is higher,[112] with an earlier peak in DNA synthesis[78,113,114] and hepatocyte mitotic activity.[116,115] After PHx, the induction of immediate genes (such as EGR-1/PAI-1) is also earlier and higher compared to PVO, as are cytokine (such as IL-6/IL-1β) concentration.[115] These observations emphasize that shear stress is a crucial but not exclusive regulator of liver regeneration.

Finally, the role of shear stress on liver regeneration has been questioned in the report by Clark *et al.*,[116] in which a massive increase in the portal flow by

arterialization of the portal vein was not found to be associated with a dramatic increase in the rate of cell division. The authors suggested that the shear stress may not be sufficient to induce liver regeneration. It should, however, be noted that Clarke performed a complete aorto-portal shunt, thus abrogating the splanchnic inflow of the portal vein (and all hepatotrophic factors).

1.2.6. Conclusion

The current literature supports that liver regeneration is a highly regulated process which requires an equilibrium between the proliferation of hepatocytes and angiogenesis in order to maintain liver function. Stress factors, metabolic load and haemodynamic changes (portal hyperperfusion of splanchnic, nutrient-rich blood) contribute to trigger the early events of liver regeneration. However, the importance of haemodynamic events and the relative proportion of the portal to the arterial blood are not completely understood.

CHAPTER II: POST-HEPATECTOMY LIVER FAILURE (PHLF) AND 'SMALL-FOR-SIZE' SYNDROME (SFSS)

2.1. BASIC CONCEPTS

2.1.1. Liver resection

Surgical resection represents the first-choice treatment for primary or secondary tumor, giving the patient the best chance for long-term survival.[117,118] Case-series show that liver resection for colorectal liver metastases, even multiple and initially unresectable, can achieve higher survival rates compared to chemotherapy alone.[119] Even though tumor burden and biology have an important impact on survival,[120–121] recent guidelines propose attempting resection with complete tumor clearance.[122,123]

Advances in pre-operative management, extensive knowledge of liver anatomy, more dedicated surgical techniques and postoperative care have made liver resection safer over the years,[117,124,125] expanding the resection criteria and the extent of hepatectomy. Similarly, evolution in surgical techniques, immunosuppressive therapy and the prevention of infectious and non-infectious complications have rendered liver transplantation (LT) safer during the past decades. Consequently, the initial validated indications for LT (decompensated cirrhosis, acute liver failure, metabolic diseases and hepatocellular cancer within the Milan criteria) are currently and continuously expanding.[126] The increasing success of LT has accentuated the imbalance between organ supply and patient demand. As organ shortage is a major problem worldwide, alternative techniques

have been developed to expand sources of grafts, such as spit-LT or living donor-LT, using small grafts.[127,128]

However, despite improvements expanding the frontiers of resection or LT, hepatic surgery still carries some risk of post-operative liver failure, a condition associated with high morbidity and mortality rates.[129] Thus, for a safe outcome, the surgeon must assess and respect the 'liver-related' limits, determined by the size and function of future liver remnant (FLR).[130]

2.1.2. The future liver remnant (FLR)

The future liver remnant can be defined as the liver mass remaining after liver resection. In this manuscript it also designates the liver grafted during transplantation. From the anatomical viewpoint, adequate blood supply (portal and arterial), hepatic outflow (hepatic vein drainage) and biliary drainage are crucial for optimal function. From the physiological viewpoint, a preserved lobular architecture is key to support function.[9]

2.1.3. The optimal future liver remnant

The optimal remnant volume differs between LT and extended hepatectomy, due probably to graft denervation, immunosuppressive therapy and severity of ischaemia–reperfusion injury. In living donor LT, rigorous standardized criteria for optimal graft volume according to the recipient's weight [graft-to-recipient weight ratio (GRWR)>0.8%] or the graft volume to the standard liver volume ratio (GV/SLV>40%) have been established.[131] Similarly, for liver resection, the optimal FLR was mainly defined according to the liver volume. This is based on the

estimation of the FLR volume (FLRV, i.e. the liver volume left after hepatectomy) to the total liver volume (TLV).

When using volume as a reference, it is assumed that liver volume reflects liver function, and that function is homogeneous throughout the liver. However, more recent studies have emphasized the limitations of an approach that only considers volume. Limitations are particularly relevant and of concern when assessing FLR mass recovery after pre-operative portal vein occlusion[132,133,134] or when the parenchyma is diseased.[135,136] Conditions such as steatosis, steatohepatitis, fibrosis, cirrhosis, chemotherapy-induced liver injury and cholestasis have been reported to increase the risk of PHLF. In a diseased liver, liver function may be dramatically decreased and severely overestimated if only size is relied upon.[132] Additional factors, such as the age of the patient,[137] obesity status or the presence of diabetes mellitus,[136] should also be taken into account as they may, independently of concomitant liver disease, impair liver regeneration and worsen the outcome after resection.[138]

Thus, recent literature proposes to assess the volume and the function of FLR before major hepatectomy.[139] Even though the most appropriate test or score for estimating FLR function remains to be validated, hepatobiliary scintigraphy (HBS), evaluating the hepatic uptake of technetium-labelled Mebrofenine (^{99m}Tc-mebrofenine) in the FLR appears to be the best method today.[140] In contrast to other dynamic liver function tests, the main advantage of HBS is to enable the non-invasive assessment of the function in the anatomical regions of interest. The cut-off of the FLR's Mebrofenine uptake for safe hepatectomy has been calculated at 2.7%/min/m^2 and revised at 2.3%/min/m^2, according to recent data.[132,133] When volume is assessed, current data suggest to adapt the minimal FLR to the quality of the parenchyma , with minimal FLRV% cut-off at 20% for normal parenchyma (or at 0.5% according to the ratio to body weight[141]), at 30% after extended

chemotherapy and at up to 40% in the case of cholestatic liver disease, severe fibrosis or cirrhosis[130,142] (Fig. 9).

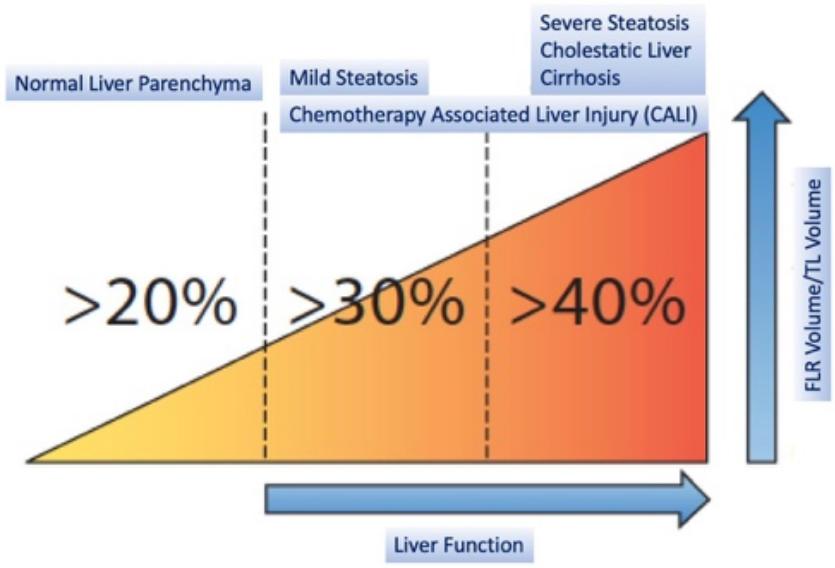

Figure 9. Schematic representation of FLR limits to partial hepatectomy according to liver function. The FLR volume is adapted to the parenchymal quality (adapted from Guglielmi, *Digestive Surgery*, 2012).[130]

It is universally accepted that if these volumetric and functional 'rules' are not respected during extended liver resections or LT, the patient is at risk of developing post-hepatectomy liver failure, or the 'small-for-size syndrome' respectively.

2.2. SFSS AND PHLF

2.2.1. Definition

In liver transplantation the SFSS is a distinct clinical entity in which a small graft [graft weight/recipient weight (GWRW) ratio <0.8] exhibits signs of dysfunction during the first postoperative week in the absence of technical complications (e.g. arterial or portal occlusion, outflow congestion, bile leak), immune rejection or infection. The SFSS is diagnosed when two of the following criteria are recorded on 3 consecutive postoperative days: serum bilirubin >100 µmol/L (6 mg/dl), international normalized ratio (INR) >2, ascites >1l and the presence of encephalopathy grades III or IV during the first week.[143] Other authors adjusted this initial definition according to later outcomes, such as ascites >1l on postoperative day 14 or >500 ml on postoperative day 28, and hyperbilirubinaemia 5 mg/dl on postoperative day 14.[144]

The same concept is applicable to the field of hepatic surgery, where extended resections (e.g. FLRV<20–25% for normal parenchyma) can lead to post-hepatectomy liver failure. In 2011, Rahbari *et al.*[145] suggested a definition of PHLF as: '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'. They differentiated severity into three grades, according to whether changes in clinical management of the patient or invasive treatments are required (Fig. 10). The risk of peri-operative mortality with grades B and C is 12 and 54%, respectively. The incidence of PHLF after extended liver resection ranges between 1, 2 and 35%, according to the series published.[129]

SFSS and PHLF can been viewed as the same manifestation of liver dysfunction and are fraught with high mortality rates of up to 70%.[146,147]

Definition	A postoperatively acquired deterioration in the ability of the liver (in patients with normal and abnormal liver function) to maintain its synthetic, excretory, and detoxifying functions, characterized by an increased INR (or need of clotting factors to maintain normal INR) and hyperbilirubinemia (according to the normal cut-off levels defined by the local laboratory) on or after postoperative day 5. If INR or serum bilirubin concentration is increased preoperatively, PHLF is defined by an increasing INR (decreasing prothrombin time) and increasing serum bilirubin concentration on or after postoperative day 5 (compared with the values of the previous day). Other obvious causes for the observed biochemical and clinical alterations such as biliary obstruction should be ruled out.
Grade	
A	PHLF resulting in abnormal laboratory parameters but requiring no change in the clinical management of the patient.
B	PHLF resulting in a deviation from the regular clinical management but manageable without invasive treatment.
C	PHLF resulting in a deviation from the regular clinical management and requiring invasive treatment.

Figure 10. Consensus definition and severity grading of post-hepatectomy liver failure (PHLF) by the International Study Group of Liver Surgery (ISGLS) (adapted from Rahbari, *Surgery*, 2011).[145]

2.2.2. Factors predicting the occurrence of SFSS and PHLF

Initially, most clinical and experimental studies identified the extent of resection, and consequently the FLR's volume and function, as the essential parameter in establishing the operability of a given patient and the risk of PHLF (see Fig. 9). As mentioned above, other factors besides FLR are now associated with a high risk of PHLF, such as patient-related factors (e.g. age >65 years, male sex and diabetes mellitus and obesity)[138] and the presence of an underlying liver disease.

In the setting of LT, beside the volume and the graft quality (e.g. steatosis < or >30%), outflow obstruction, donor age (>50 years), prolonged intensive care unit stay (>5 days), prolonged cardiac/respiratory arrest, long ischaemia duration, administration of high-dosage vasopressors and severe systemic sepsis have also

been identified as factors contributing to SFSS. Additionally, recipient-related issues such as the extent of end-stage liver disease (Child–Pugh scores B or C) are of major importance.[144,148]

Clinical studies now also support that the haemodynamic parameters are key for the onset of SFSS or PHLF. Studies show that elevation of portal blood pressure and/or flow is a major causal and predictive factor.[96,149] This would explain the development of liver failure in patients with FLR considered to be safe, as well as the absence of liver failure in patients with a threshold of FLR considered 'unsafe'. Indeed, grafts with GRWR<0.8 have been transplanted with success, provided that portal vein pressure (PVP) and portal vein flow (PVF) are maintained below a threshold.[149] Allard *et al*.[150] have recently shown that a rise of PVP >20 mmHg after hepatectomy predicts the risk of PHLF. The Kyoto group proposed a PVP threshold of <15 mmHg to prevent SFSS after LT.[151] In physiological conditions, such as in a healthy donor, the portal flow is around 90 ml/min/100 g of liver. After partial graft, up to a two-times increase in portal flow (i.e. 180 ml/min/100 g of LW) in regarded as safe,[152] while the risk of graft failure significantly rises when PVF is ≥4 times the physiological values or 360 ml/min/100 g of LW.[96]

Taken together, these data suggest that portal vein haemodynamics are even more important than FLR size in the pathogenesis of the small-for-*size*-syndrome,[29] which prompts some authors to rename this clinical entity as small-for-*flow*-syndrome.[30]

2.2.3. Pathophysiology for SFSS and PHLF

During LT with a small graft or after extended hepatic resection, the entire splanchnic blood is distributed to a reduced sinusoidal network in the FLR, with consequent portal hyperperfusion. As mentioned above, portal perfusion over a certain threshold (PVF≥4 times the physiological baseline) is considered to be the

main factor leading to liver insufficiency. This is translated by focal disruption of the sinusoidal endothelial lining, endothelial denudation, connective tissue haemorrhage and oedema in SFSS liver histopathological findings early after surgery.[27] Furthermore, sinusoidal congestion, irregular large gaps of sinusoidal lining cells, collapse of the space of Disse and hepatocyte ballooning are features described.

Additionally, the role of arterial perfusion in SFSS-setting FLRs is emphasized, but less studied. As explained above (see section 1.1.4), by virtue of the HABR, portal hyperperfusion results in a compensatory arterial hypoperfusion. Initially, low hepatic artery flow was thought to be related to diversion of blood through the splenic artery, which was called the 'splenic artery steal syndrome' but is now related to the homeostatic hepatic artery response to portal hyperperfusion, or the HABR.[28] This FLR de-arterialization is predicted to cause ischaemic injury and to be the major pathophysiological mechanism for SFSS.[15,84,94] This hypothesis was supported by histopathological observations of arterial vasospasm and thrombosis, accompanied by perihilar bile duct necrosis, cholangitis abscesses and scattered parenchymal infracts at late stages after SFSS liver transplantation (10–20 days).[27,153] Although SFSS livers show hypoxia, controversy exists as to whether this hypoxic state is due to arterial hypoperfusion or to increased oxygen demand in the regenerative liver.[31]

Although portal hyperperfusion is considered the major causal factor in the onset of SFSS, we have seen above that several animal and human studies have shown that increased portal perfusion is needed for liver regeneration, with an ideal target of PVF of twice the baseline. Additionally, it seems that the magnitude of the regenerative stimulus is proportional to the rise of portal blood flow.[57,60,153–162] On the other hand, Yagi *et al.* showed that after LT a portal pressure >20 mmHg was associated with increased concentrations of hepatocyte growth factor (HGF) and

accelerated hypertrophy of the graft but also ascites, coagulopathy and hyperbilirubinaemia.[154] In a clinical study, Gruttadauria *et al.*[60] observed a significant association of SFSS and hepatocyte proliferation as assessed by Ki67 immunohistochemistry marker, concluding that a greater degree of liver proliferation was associated with a higher risk of liver insufficiency after SFSS-setting hepatectomy or LT. Similarly, Nimomiya *et al.* observed that high regenerative rates in SFSS-setting hepatectomized mice were associated with mortality.[58] In fact, in this study, a high regenerative response was accompanied by the formation of avascular hepatocyte clusters and rupture of the lobular architecture (see section 1.2.3). Loss of lobular architecture rather than regenerative failure is seen as the cause of liver dysfunction and organ failure in SFSS.

Thus, portal overflow, disturbance of hepatic microcirculation and lobular disorganization associated with hepatocyte hyper-proliferation appear as the main pathophysiological mechanisms for hepatocyte dysfunction and SFSS.

2.2.4. Prevention of SFSS and PHLF

2.2.4.1. *Prevention of SFSS by portal venous inflow modulation*

Because portal hyperperfusion is thought to be central to the pathogenesis of the SFSS, modulation of the graft inflow was attempted in order to prevent SFSS in both experimental and clinical settings.[154,155] During liver transplantation, if the measured portal inflow is >20 mmHg, several measures might be considered to reduce the pressure according to algorithms and decision trees, such as in Fig. 11.[96] These techniques include splenectomy,[156] ligation of the splenic artery,[157] splenorenal shunting,[154] hemiporto-caval shunt[158] and mesocaval shunt with ligation of the superior mesenteric artery.[159] For instance, splenic artery ligation (or

embolization) reduces the portal flow by 52% and seems to be a suitable and effective technique to reduce portal inflow whether pre-operatively, per-operatively or even during the immediate postoperative course.[160] If a single technique is insufficient to reduce portal inflow, a combination of these procedures may be applied (Fig. 11). Of course, strategies should be cautiously applied, as they may have side effects. Indeed, porto-systemic shunt may cause a hepatofugal flow, increase the risk of encephalopathy and impair liver regeneration,[160] while splenectomy increases the risk of sepsis and of portal venous thrombosis.[149]

In the setting of extended liver resection, as in liver transplantation, post-hepatectomy portal vein pressure (PVP>20 mmHg) has been described as an independent predictive factor for PHLF. To control PVP after major hepatectomy, porto-caval shunts, splenic artery embolization or ligation and splenectomy have also been proposed.[150]

Additionally, the mastering of surgical techniques can minimize intra-operative complications. When feasible, a combination of surgical techniques aiming to reduce the amount of liver parenchyma resected by performing 'atypical' (non-anatomical or subsegmental) hepatectomy is an effective strategy to prevent PHLF.[161–162] Postoperative complications (such as biliary fistula, infection, sepsis) should be suspected and treated as soon as possible, as these are known to exacerbate the reduced function after liver surgery.

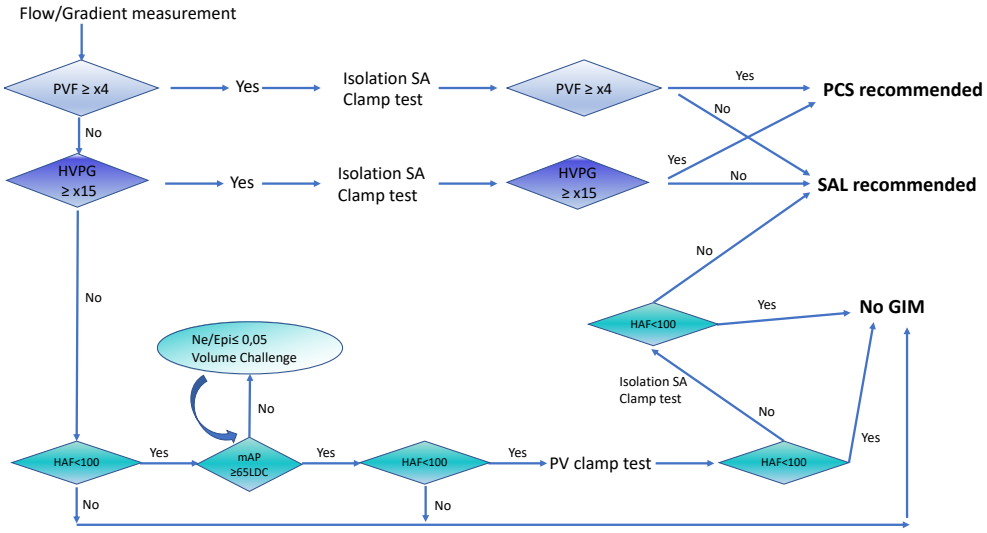

Figure 11. Algorithm for graft inflow modulation (GIM) as proposed by the Gent group based on portal vein flow, hepatic venous pressure gradient and hepatic arterial flow. PVF: portal venous flow, SA: splenic artery; PCS: porto-systemic shunt; HVPG: hepatic venous pressure gradient; SAL: splenic artery ligation; LDC: low-dose catecholamine; MAP: mean arterial pressure; HAF: hepatic artery flow (from Sainz-Barriga, *Liver Transplantation*, 2011).[96]

2.2.4.2. *Pre-operative preparation of the future liver remnant*

As the volume and function of the FLR are essential predictors of PHLF and SFSS, strategies have been proposed to increase, when necessary, the size and function of the FLR prior to resection, in order to prevent post-hepatectomy liver failure.

Portal vein occlusion (PVO) (whether embolization-PVE or ligation -PVL) is the most widely used FLR volume optimization strategy for patients in need of major hepatectomy[119,163] (Fig. 12). In 2000, two-stage hepatectomy (TSH) was introduced to enable complete resection of bilateral multi-nodular hepatic metastasis not amenable to resection in a single procedure, not even after down-sizing chemotherapy.[163] The strategy of this approach was to combine tumor clearance of one hemi-liver with simultaneous contralateral portal vein occlusion to stimulate the growth of the FLR. When the planned operations are completed, comparable

survival rates can be expected after TSH and after for initially resectable colorectal liver metastasis.[119]

The underlying principle of portal vein occlusion is to interrupt the portal venous flow into the liver segments that are planned to be respected, so that portal hyperperfusion of the future liver remnant increases and induces FLR hypertrophy. Portal vein occlusion extends the limits of liver resection. In a cohort study[164] of 265 patients suffering from colorectal liver metastasis in need of extended right hepatectomy, 52.5% had insufficient FLR volume at presentation and underwent PVE, thus raising the curative resection rate up to 79.2%. A meta-analysis of the current literature indicated that PVO causes a mean increase of FLR up to 37.9% in a mean period of 36.9 days, with wide ranges of time intervals between PVO, FLR's volumetric assessment and liver resection reported (from 14 to 42 days).[165] This same review of 1179 patients who underwent PVE demonstrated that the technique was successful in 96.1%;[165] 3.9% of patients did not undergo surgery because of failure of the technique mainly because of insufficient hypertrophy (2.8% of patients). The initial size of the FLR, the low degree of post-PVO hypertrophy (kinetic growth ratio below 2% of FLR mass recovery per week) and a low initial FLR function, as indicated by a mebrofenin uptake <1.72%/min/m^2, are factors strongly associated with insufficient post-PVO mass recovery, postoperative hepatic dysfunction[166,167] and hepatic-related 90-day mortality.[168]

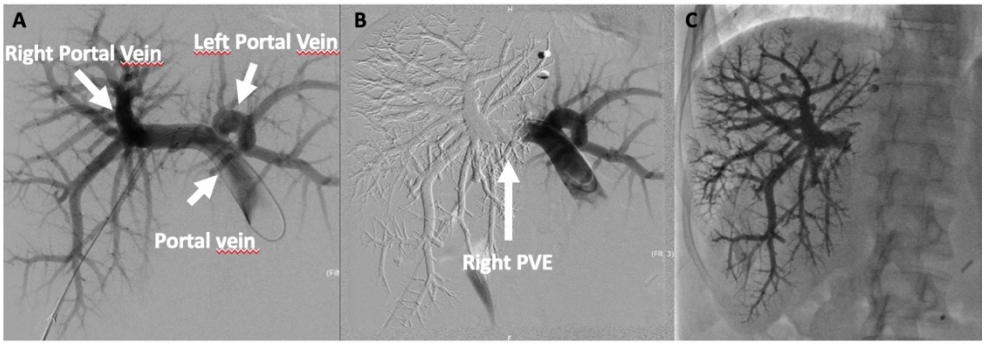

Figure 12. Portal vein embolization by transhepatic ispilateral puncture of the right liver. (A) Dedicated venogram should be performed to delineate individual portal anatomy and patency. **(B)** Portography after full right branch embolization (glue/lipiodol = 1/10)**. (C)** Radiography after right portal vein embolization showing lipiodol infused into the main and distal portal branches (courtesy of F. Deprez, CHU-UCL-Namur, site of Godinne).

After PVO, time is needed for the FLR to hypertrophy (4–6 weeks), and this has been considered as a drawback for the technique, as during this period tumor may progress. Approximately 20% of the originally planned liver resections are cancelled[165] mainly because of disease progression, medical comorbidities and insufficient FLR hypertrophy.[165,169] Of note, recent clinical studies suggest that, after PVO, the targeted rise in FLR's function is obtained prior to the targeted increase in volume.[167,170] These data suggest that the necessary waiting time from PVO to resection could be shorter than indicated by volumetric parameters only. Thus, the disadvantage of the waiting time between PVO and resection is no longer a valid drawback of the technique.

Although disease progression can be subjugated by a smaller interval between PVO and resection and careful patient selection (stable oncological disease), recusing a patient for curative intent treatment because of insufficient FLR still remains a major problem. For patients with very low initial FLR volume/function, in need of enhanced FLR hypertrophy for safe hepatectomy, or who do not respond to PVO, other strategies for FLR modulation prior to resection have been developed. These

techniques include embolization of the ipsilateral hepatic artery (this technique is burdened with a high risk of liver abscess secondary to ischaemic cholangitis),[171] PVE combined with adjuvant stem cell transplantation[172] and sequential transarterial embolization combined with PVE.[173] However, additional research is required to delineate the outcomes of these strategies.

Recently, combined or sequential portal vein and hepatic vein embolization (HPVE)[174–175] and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS)[176–177] have been introduced in order to enhance FLR hypertrophy. As ALPPS was the starting point of our research project, clinical considerations and mechanistic insights will be discussed in a dedicated chapter (Chapter III).

2.2.5. Treatment of SFSS and PHLF

Despite medical and surgical advances during the past decades and growing understanding of the pathophysiology of liver failure, PHLF and SFSS remain the major causes of morbidity and mortality after liver surgery.

Besides prevention of aggravating factors such as infection, vascular complication (arterial or venous thrombosis), biliary fistula and malnutrition, avoidance of hepatotoxic and nephrotoxic drugs and treatment of associated coagulopathy, a reduction of portal hyperperfusion is proposed when portal vein pressure exceeds 20 mmHg. Invasive strategies implicate the creation of portosystemic shunts to achieve portal decompression to below 15 mmHg, splenic artery embolization/ligation and splenectomy, as discussed above. Additionally, pharmacological modulators of the portal flow have been tested with encouraging results, but most of them have been tested in small animal models. These agents

include Olprione, a phosphodiesterase inhibitor with vasodilating properties,[178] prostaglandin E1,[179] nitric oxide[180] and endothelin receptor A antagonist.[181] Xu *et al*.[182] used somatostatin in a rat model of SFFS-liver transplantation and demonstrated decreased portal hyperperfusion, improved liver function and graft survival. Down-regulation of endothelin-1 (a sinusoidal vasoconstrictor) and up-regulation of heme oxygenase-1 (a vasodilatator with antioxidant properties) appeared to participate in these positive effects. In a model of SFSS-setting hepatectomy in pigs, Mokham *et al.*[183] also demonstrated the beneficial effect of somatostatin on portal hyperperfusion, and suggested that somatostatin infusion could become an effective modality for inflow modulation in SFSS-setting hepatectomies.

The use of artificial liver [albumin dialysis, molecular absorbent recirculating system (Mars®)] for the management of PHLF is still controversial and not well established, as few studies have been conducted[184]. Van de Kerkhove *et al.*[185] showed disappointing results, as there was no improvement in survival, while Gilg *et al.*[186] suggested that Mars® could be a salvage strategy provided it is initiated in the early phase of PHLF. More studies are needed to clarify the role of artificial liver in this setting.

Finally, liver transplantation can be an option to treat SFSS in a selected group of patients who otherwise meet the criteria for LT.

CHAPTER III: ASSOCIATING LIVER PARTITION AND PORTAL VEIN LIGATION FOR STAGED HEPATECTOMY

3.1. THE ORIGINAL TECHNIQUE

In its original form, ALPPS is a two-step extended right hepatectomy (right trisectionectomy). Step I includes surgical exploration and ligation of the right portal vein, while biliary and arterial structures and sus-hepatic venous drainage of the right liver are left intact (Fig. 13A, B). The liver parenchyma is then split *in situ* along the right side of the round ligament. Portal, arterial and biliary branches to segment IV are identified and divided. If exploration of the FLR shows metastatic disease, tumors are excised. During step II, the right hepatic artery, bile duct and hepatic vein are ligated, and the extended right lobe is removed (Fig. 13C, D).

ALPPS can be tailored to the patient's needs with modifications of the type and extension of hepatectomy, the technique for portal vein occlusion (PVL or PVE) and the type and degree of parenchymal division. Thus, besides right trisectionectomy, all possible anatomical variants have been proposed to be prepared by ALPPS in a staged setting.

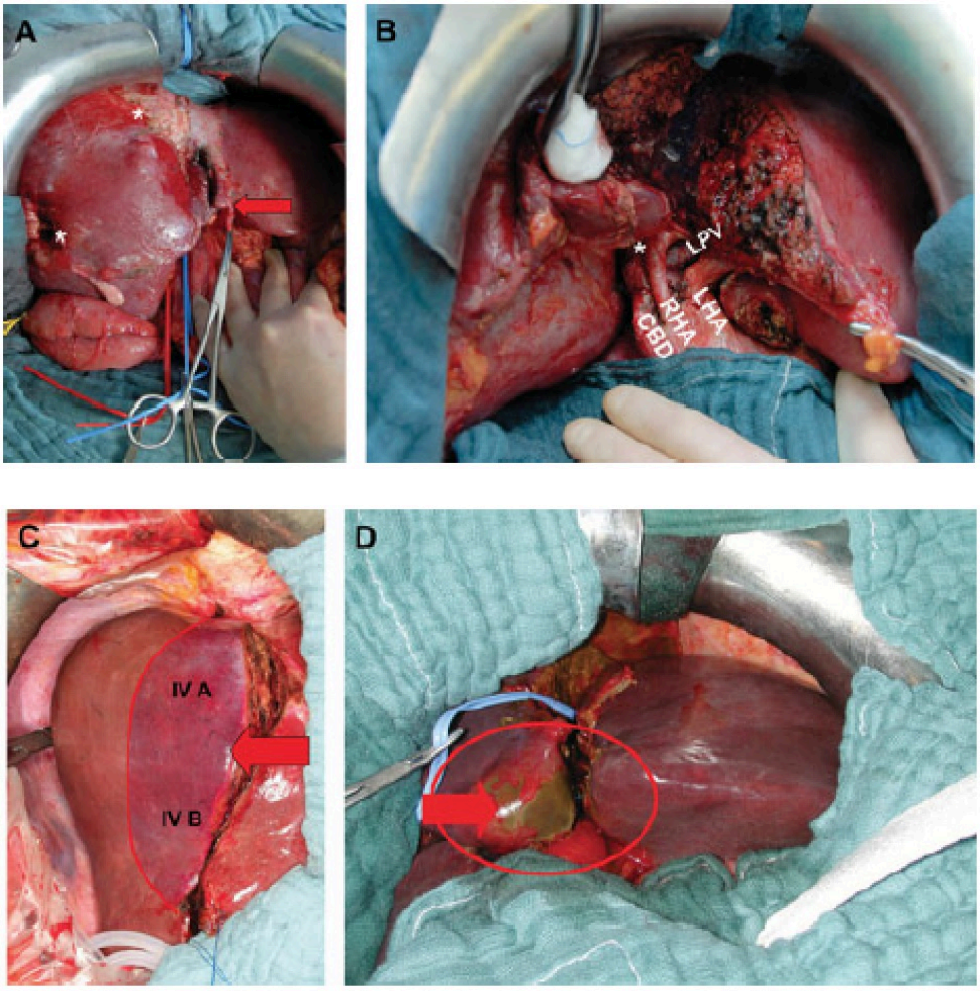

Figure 13. Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS). The original technique of two-staged hepatectomy before right trisectionectomy. **(A) Situs during first surgical procedure before right portal vein ligation and *in-situ* split along the round ligament. (B) Situs after nearly total *in-situ* split and right portal vein division. (C) Segments IV A and B showing reduced perfusion (ischaemia) after dissecting and closing supplying portal venous and arterial structures (red arrow) at the end of *in-situ* split procedure. (D) Partially necrotic liver segment IV B during completion surgery after a hypertrophy interval of 9 days.** Note vital and hypertrophied left lateral liver lobe at the end of completion surgery (red circle and red arrow). CBD: common bile duct; LHA: left hepatic artery; LPV: left portal vein; RHA: right hepatic artery. ∗Marks tumor inside the right liver lobe and in segment IV (adapted from Schnitzbauer *et al*., *Annals of Surgery*, 2012).[176]

3.2. CLINICAL CONSIDERATIONS

Associating liver partition and portal vein ligation for staged hepatectomy is an innovative concept to induce rapid and boosted hypertrophy of the FLR, insofar never observed in either clinical or experimental studies. In 2012 a series of 25 cases was published, including 14 patients with colorectal liver metastases. In this study, a median hypertrophy rate of 74% of the remnant liver after 9 days and a 100% resection rate in patients with otherwise unresectable tumors was achieved.[176] The accelerated liver volume increase was confirmed in subsequent reports. Moreover, these reports showed that ALPPS was an effective technique to induce FLR volume increase even after the failure of FVE.[187,188] In an early metaanaysis it was shown that ALPPS enable hepatectomy (step II) in up to 97% of the cases, compared to 77% after conventional two-stage hepatectomy.[189] This was insofar remarkable, as ALPPS was mainly applied in cases with a higher tumor burden.[190] Other teams reported median FLR hypertrophy rates up to 160% (range 93–250%) in the so-called monosegment ALPPS performed in selected cases to enable resection of all but one liver segment with no postoperative mortality, in these very selected patients.[191,192]

Because of the accelerated and enhanced regeneration it achieved, enabling extensive liver resection, ALPPS attracted tremendous interest and hepatobiliary surgeons from all over the world began to implement this complex surgical technique, even before meticulous evaluation and understanding of the underlying mechanisms.[193–194] However, a systematic review on ALPPS indicated that major complications (Dindo-Clavien >IIIa) occurred in 44% of patients, together with high 90-day mortality rates of 8–16%.[189] In a French series, Truant *et al.*[195] 37.5% of reported deaths were attributed to systemic, non-liver-specific complications

(sepsis, acute renal failure, ARDS, arrythmia, cardiac arrest). This is in sharp contrast to the results of the classical two-stage hepatectomy, in which mortality is exclusively related to post-hepatectomy liver failure. Extrahepatic complications in ALPPS have been attributed to massive release of cytokines and inflammation during the first surgical stress due to the large surgical trauma and ischaemic segment IV liver left in place.[196] Additionally, several investigators reported liver failure with ALPPS, with 14% of patients meeting liver failure criteria after ALPPS step I (i.e. before any liver resection) while 75% of 90-day mortality was due to PHLF.[197] Compared to the 3% mortality rate after conventional two-stage hepatectomy,[198] mortality after ALPPS was considered unacceptable, so the initial wave of enthusiasm was rapidly followed by skepticism and opposition from many experienced hepatobiliary surgeons.[199,200]

Thus, surgeons have attempted to assess factors impacting the outcome of ALPPS and to propose guidelines for cautious implementation of the technique.[201] These factors are briefly summarized below.

- Patient-related factors such as age (>60 or 67, according to the study) and obesity and surgery-related factors such as duration of step I surgery, intra-operative blood loss and postoperative biliary fistula[196,197,202] seem to impact survival.
- Tumor type (primary versus metastasis) impacts the outcome of ALPPS surgery. In the analysis performed by the international ALPPS registry,[202] 90-day mortality after ALPPS for perihilar cholangiocarcinoma, intrahepatic cholangiocarcinoma and gallbladder carcinoma was 23, 13 and 33%, respectively. In addition, other series report 4.5% mortality rates after ALPPS for colorectal liver metastasis, but up to 12.5% for patients with hepatocellular carcinoma and even 30% in those with biliary malignancies.[203,204] Based on these data, the first international expert

meeting recommended ALPPS as an option for the treatment of colorectal liver metastasis. However, ALPPS must be considered with great caution for the treatment of primary liver tumours.[201] These recommendations have been confirmed by the first randomized controlled trial published from the Scandinavian group (LIGRO trial)[205] comparing ALPPS to TSH after PVE (including embolization of segment IV) for patients suffering from colorectal metastasis with insufficient FLR (<30%). The study showed a better resection rate in the ALPPS group, with no differences in severe complications and similar 90-day mortality (8.3 versus 6.9%, respectively), even though the mortality rates were higher than the ones reported in the literature.

- The interstage course also seems determinant for survival, as 35.7% of patients who develop major systemic non-liver-specific complications after step I died after step II, versus 6.3% in the absence of major complications.[196] In particular, signs of liver failure (ISGLS criteria or model of end-stage liver disease score >10) after step I are highly predictive for mortality after ALPPS step II.[197,202–206] To allow full recovery after the step 1 procedure, it was proposed to increase the interval between the two steps of ALPPS (e.g. 24 days for the Hambourg group[207]), especially if signs of liver dysfunction appear after step I operation,[197] if the FLR mass recovery does not reach >30% of the initial total liver volume[205] or in the setting of interstage complications.[201] Increasing the interstage waiting time is even more relevant, as some authors observed a delay in FLR functional gain in ALPPS interstages compared to volumetric gain (relative increase in FLR volume of 42.6% versus in FLR function of 12.5% 7 days after ALPPS step I).[208–209]

- As inflammatory factors and segment IV necrosis produced during *in-situ* split have been implicated in postoperative complications, several modifications of the initial technique have been proposed to limit invasiveness during ALPPS step I and thus reduce the interstage morbidity.[201,210,211] The main advances have been attributed to the preservation of the middle hepatic vein during the first-stage procedure, leading to a partial hepatic transection named 'partial ALPPS'.[210,207] Indeed, transection of only 50–80% of liver parenchyma is associated with a significant reduction of peri-operative morbidity (less ischaemia in segment IV), while it does not compromise the magnitude of the induced FLR hypertrophy (the "ALPPS effect"),[211] even though the degree of hypertrophy corelates to the degree of parenchymal transection.[212] Consequently, a complete *in-situ* split during step I is currently left to those situations where tumor invasion into the FLR between stages is to be avoided. Other adaptations include a combination of partial ALPPS and PVE, either simultaneous or subsequent,[210,213,214,215] the use of a tourniquet or radiofrequency/microwave ablation to create a virtual liver partition in combination with portal vein ligation[216–217] or laparoscopic ALPPS.[218] Although there is no clear evidence that variant ALPPS are superior to conventional ALPPS,[219] it seems that a less invasive step I ALPPS, together with an optimal patient selection, reduces the 90-day mortality rate.[220] Recently a randomized control trial (REBIRTH) compared PVE prior to major hepatectomy versus ALPPS assisted with radiofrequency (RALPPS) and showed significantly higher completion rates in the RALPPS group, with similar morbidity and 30-day mortality.[221]

The current literature suggests that awareness of risk factors and risk adjustment in patient selection, technical modifications towards less invasive ALPPS

procedures, improved interstage assessment of FLR function and interstage management decrease the initially reported high morbidity and mortality rates of ALPPS.[177,207,220–197] While meta-analyses by Moris *et al*.[222] and Liu *et al.*[223] still attributed higher morbidity and mortality in ALPPS compared to conventional TSH, there was no difference in liver-related mortality. However, as most data derive from individual cohorts with small numbers of patients or from the international ALPPS registry (a substantial risk of selection bias in a voluntary registry without independent monitoring exists), results should be interpreted with caution.[224] However, two recent randomized controlled trials comparing ALPPS to TSH showed increased resection rates with comparable surgical tumor margins, complication and short-term mortality rates in selected patients suffering from colorectal liver metastasis, even though the mortality rates reported in LIGRO trial were higher than the ones reported in the litterature.[205,221]

3.3. ALPPS OR CONVENTIONAL HEPATECTOMY POST-PORTAL VEIN OCCLUSION?

Initially, the argument to perform ALPPS has been the shorter interstage period compared to PVE/TSH, in order to decrease the dropout of patients whose neoplastic disease progresses during the waiting period. However, this argument seems not to be of outmost importance. First, recent data suggest that functional recovery after PVE precedes the volumetric recovery of the FLR,[167,170] thus hepatectomy could be completed after a shorter lag time. Conversely, FLR functional recovery in ALPPS could sometimes be slower than volumetric recovery,[208] so many patients should probably wait more than 1 week (as initially proposed) to be resected. Thirdly, if the patients have a rapidly evolving oncological disease that is not stabilized by (chemo-)therapy, they are probably poor

candidates for any kind of resection, especially technically complex surgery as TSH or ALPPS.

The second argument for performing ALPPS is that it is followed by an enhanced recovery of the liver mass compared to PVO. As such, ALPPS could be a suitable option for patients suffering from colorectal liver metastasis who demonstrate insufficient growth of FLR after PVO techniques,[187] or for patients with an extremely low initial FLR volume/function in whom hypertrophy of the FLR after conventional PVO is unlikely to be sufficient to bring them to second-stage surgery.[166] In such situations, ALPPS may offer a chance of complete tumor removal, prolonged survival and even a chance for cure. In our opinion, TSH and ALPPS should not be evaluated as competing therapeutic strategies, but rather as complementary strategies to treat selected patients who are at the far end of the resectability spectrum.

To conclude, it is interesting to note that even if, in a cohort analysis, 14% of patients developed PHLF after the first step of ALPPS,[197] the vast majority (the remaining 86%) exhibited rapid liver hypertrophy without developing liver dysfunction. ALPPS demonstrates a fascinating rapid hypertrophy of the FLR and, as such, the technique offers an excellent and unique model to study accelerated liver regeneration.

3.4. MECHANISTIC INSIGHTS: FROM CLINICAL OBSERVATIONS TO BASIC RESEARCH

Several animal models, mainly in rodents, have been developed in order to understand the underlying mechanisms of rapid liver hypertrophy achieved with ALPPS (Table 1). Rodent models include a similar transection plane of the median

lobe but differ in the selection and size of the FLR, the extent of portal vein occlusion and the addition or not of a partial hepatectomy during step I. The mouse models of Schlegel *et al.*[225] and Langiewicz *et al.*[226] consisted of a 55% PVL with a simultaneous 30% PHx, thus leaving an FLR of 13% in the first step followed by a step II resection of the ligated lobes. Shi *et al.*[227] performed the same procedure in rats. In contrast, the rat models of Yao *et al.,*[228] Dhar *et al.,*[229] Almau Trenard *et al.,*[230] Andersen *et al.*[231] and Schadde *et al.*[232] comprised a portal vein ligation of all lobes except the right median lobe, keeping an FLR of ±30%. Wei *et al.*[233] combined an approximatively 60% PVL and 10% resection in stage I (FLR of 30%) and examined the outcome of the stage II resection. Garcia *et al.*[234] evaluated first- and two-step procedures with an FLR of 21% in a rat model, while Tong *et al.*[227] performed the same study design with an FLR of 30% (Table 1). In all these models, ALPPS step I achieved increased and accelerated FLR liver regeneration compared to PVL. Amplified and accelerated regeneration was observed even in a setting of classical 70% PHx, considered to be the model of physiologically optimal liver regeneration.

The higher FLR mass recovery seems to correspond to true liver regeneration, not interstitial oedema or intracellular steatosis.[235,236] A common finding in experimental and also clinical ALPPS[235,236] is an increased number of hepatocytes entering the cell cycle, as attested by the increased proportion of ki67, bromodeoxyuridine (BrdU), proliferating cell nuclear antigen (PCNA)-positive hepatocytes. [234,225,227,229,233,237–238] However, observation of small, highly proliferative (Ki67+), hepatocytes with vacant-appearing, glycogen-rich cytoplasm on biopsies of human liver remnants 1 week after ALPPS step I procedure, as in animal models, has been interpreted as a sign of 'hepatocyte immaturity',[237,239] although not associated with liver failure.

All research work consolidates that parenchymal transection, combined to PVO, is at the origin of boosted hypertrophy, with at least 50% of parenchymal split needed

to obtain the 'ALPPS effect'.[211] In a translational study of partial ALPPS in humans and mice, when the median amount of parenchymal transection in partial ALPPS was of 61%, FLR hypertrophy was equivalent to partial and complete ALPPS.

Two possible mechanisms may explain the impact of an *in-situ* split on liver regeneration.

A first explanation for enhanced regeneration after in *in-situ* split is that the liver transection causes tissue damage and excessive release of inflammatory mediators or of putative growth factors, which drive and accelerate liver regeneration. In their study, Schlegel *et al.*[225] showed that parenchymal transection causes an inflammatory response and a release of growth factors significantly stronger than after PVL alone. The authors postulated that such mediators are mainly blood-borne, as a similarly intense regenerative response occurred in animals with PVL that received a transfusion of plasma from liver-transected animals. Intriguingly, a similar effect was observed when PVL animals received plasma from animals that were submitted to extrahepatic organ damage.[225] This suggests that damaged or necrotic tissue, whether or not in the liver, releases factors that amplify liver regeneration. High inflammation is also present in human ALPPS, as IL-6 and TNF-α were found to be increased in liver tissue and plasma 1 h after step I ALPPS compared to PVL alone.[225] In an experimental setting, Dhar *et al.*[229] observed high levels of cytokine-induced neutrophil chemoattractant-1 (CINC-1) and IL-6, as well as increased early infiltration of the liver by inflammatory cells in ALPPS liver biopsies compared with PVL biopsies. The role of inflammatory cytokines was also stressed by Yao *et al.*,[228] who found increased levels of TNF-α, IL-6 and HGF after ALPPS upon comparison with the PVL group. Supportive of these findings is a report by Shi *et al.*,[227] who found elevated NF-κB, p65, STAT3 and YAP (yes-associated protein) expressions after ALPPS; these mediators have a cardinal role in liver regeneration.

A second explanation for enhanced regeneration after the *in-situ* split is that, by definition, it abolishes intraparenchymal porto-portal shunts,[233,212] thus resulting in more portal blood flowing through the FLR. As a consequence, intrasinusoidal pressure increases[232],[229] causing more shear stress, a major trigger of liver regeneration, as we have previously discussed (see section 1.2.5.2). In addition, Schadde *et al.*[232] demonstrated parenchymal hypoxia associated with increased portal inflow in ALPPS step I compared to PVL alone, with an FLR representing 30% of the initial liver volume. Activation of hypoxia signaling pathways by a propyl-hydroxylase inhibitor boosted hypertrophy after PVL, while increasing oxygen delivery to the tissue by administration of myo-inositol tri-pyrophosphate abrogated the accelerated regeneration observed after PVL and transection. The authors suggested that portal hyperperfusion due to abrogation of collateral flow results in a compensatory arterial hypoperfusion (HABR) and hypoxia in the FLR, and this phenomenon could play a key role in modulating regenerative kinetics.

Thus, current data support that the mechanisms underlying increased liver regeneration in ALPPS is due to cytokine release and/or haemodynamic factors in response to parenchymal *in-situ* split associated with portal vein occlusion. Interestingly, hypoxia may be another piece in the complex puzzle of liver regeneration.

Table 1. Animal models used for the study of ALPPS: their study design, proposed mechanistic insights and results PVL: portal vein ligation, PVLT: portal vein ligation and parenchymal transection, RFA : radiofrequency ablation, LLL : left lateral lobe, RML : right median lobe, LML: left median lobe, CL: caudate lobe, FLR: future liver remnant, CT: computer tomography, IHC: immunohistochemistry; in rodents: green: the FLR; red: the resected or portally deprived liver, PVP: portal vein pressure, PVF: portal vein flow.

Author	Species	Study design	ALPPS Step I	ALPPS Step II	Timing Step II (days)	Initial FLR	PHx in Step I	Hemodynamic analysis	FLR Evaluation	Peak of proliferation	Proposed mechanistic insights	SFSS-setting surgery	Mortality
Liao et al JSR 2017	Rabbit	PVL, T, 20, 50, 70% PVLT	(+)	(-)	(-)	80, 50, 30% of total liver	(-)	(-)	CT IHC	NA	(-)	(-)	(-)
Andersen Surgery 2017	Rat	PVL, PVLT, PVL/RFA-split, PVL/RFA-necrosis, Sham	(+)	(-)	(-)	30% (RML) 	(-)	(-)	FLR Weight	4	Necrosis	(-)	(+)
Dhar Ann Surg Innov Res 2015	Rat	PVL, PVLT, Sham,	(+)	(-)	(-)	30% (RML) 	(-)	Portal pressure	FLR Weight	2	Necrosis, cytokines (CINC-1, IL6, IL13, GM-CSF, VEGF, INF-γ)	(-)	(-)
Yao PLOS One 2014	Rat	PVL, PVLT, Sham	(+)	(-)	(-)	30% (RML) 	(-)	Laser Speckle Contrast Imaging	FLR Weight	2	Necrosis, cytokines (TNF-α, IL-6, HGF)	(-)	(-)
Shi Sientific Reports 2015	Rat	LLL Resec-tion, PVL, PVLT, Sham, T	(+)	(+)	3	13% (LML) 	(+)	(-)	FLR Weight	2	Trauma, cytokines (IL-6, NF-kB p65, STAT 3, TMN-α, EGF, HGF, ERK ½, YAP, HGF)	(-)	(+)

Garcia-Perez PLOS One 2015	Rat	PVL, PVLT, Sham	(+)	(+)	8	21% (LML+CL)		(-)	(-)	FLR Weight	2	Necrosis, cytokines (HGF, TNF-α)	(-)	(+)
Wei Surgery 2016	Rat	Control, T, PVL, PVLT	(+)	(+)	2	30% (RML)		(+)	(-)	FLR Weight	1	Intrahepatic collaterals	(-)	(+)
Trenard Cirurgia Espagnola 2014	Rat	PVL, PVLT	(+)	(-)	(-)	30% (RML)		(-)	(-)	FLR Weight	NA	(-)	(-)	(-)
Schlegel Ann Surg 2014	Mice	LLL resection, T, PVL, PVLT	(+)	(+)	2	13% (LML)		(+)	(-)	FLR Weight	4	Inflammation, cytokines (IL6, TNF-α, STAT 3)	(+)	(+)
Langiewicz Journal of Hepatology 2016	Mice	LLL resection, T, PVL, PVLT	(+)	(-)	(-)	13% (LML)		(+)	(-)	FLR Weight	4-8h 2d	Hedgehog pathway	(+)	(-)
Croome HPB 2015	Pig	PVLT	(+)	(+)	7	20%		(-)	(-)	CT	NA	(-)	(+)	(+)
Schadde Surgery 2016	Rat	PVL, PVLT, Sham	(+)	(-)	(-)	30% (RML)		(-)	PVF, PVP	CT	3-24h 3d	Hypoxia: HIF-1α pathway	(-)	(-)
Tong WJG 2018	Rat	PVLT, PHx, Sham	(+)	(+)	5	30% (RML)		(-)	(-)	FLR Weight	2-5d	(-)	(-)	(-)

THE AIM OF THE THESIS

When we initiated our research study in 2015 we were intrigued and fascinated by ALPPS, a unique surgical technique that enhances not only the degree but also the kinetics of future liver remnant mass recovery. Such accelerated regeneration had never been described previously with any other surgical manipulation or drug treatment.

Based on our knowledge on liver regeneration, but also on the pathophysiology of SFSS, our reasoning was based on the following arguments.

1. Post-hepatectomy liver failure or small for size syndrome occurs with a minimal, insufficient-for-survival, future liver remnant after major hepatectomy or liver transplantation.
2. After extended hepatectomy or small graft transplantation, redirection of the whole portal flow into a small FLR causes excessive sinusoidal shear stress[30] and a compensatory constriction of the common hepatic artery (the hepatic arterial buffer response-HABR).[14] The ensuing 'de-arterialization' and hypoxia of the remnant liver are the proposed primary factors for life-threatening postoperative liver failure.[28,29,48,163]
3. Portal vein occlusion triggers a slow and limited FLR hypertrophy. The occurrence of intrahepatic porto-portal collaterals attenuates the portal hyperperfusion of the FLR, thus protecting the liver from SFSS but also limiting the FLR's mass recovery.
4. While leaving in place a minimal, insufficient-for-survival, future liver remnant (SFSS-setting FLR), ALPPS leads to rapid and, most importantly, enhanced liver regeneration, well tolerated by most patients.
5. In the ALPPS step I procedure, portal vein ligation redirects the entire portal blood flow into a small liver sinusoidal network (as with portal vein occlusion), while the parenchymal transection excludes all the intrahepatic

sinusoidal collaterals and, hence, possibilities of intrahepatic shunts. In theory, this should set the FLR in the same portal inflow haemodynamic condition as in an upfront SFSS-setting hepatectomy.

We thus considered that the two surgical settings (ALPPS step I and SFSS-setting hepatectomy) share common features: insufficient-for-survival FLR and presumably equal degrees of portal hyperperfusion (Fig. 14). However, clinical observations suggested that, while a severe portal haemodynamic stress is considered to be at the origin of liver failure in SFSS-setting hepatectomy,[30] a presumably similar haemodynamic condition in a critically small FLR in ALPPS is linked to enhanced liver regeneration.[240,232] This amazing paradox initiated our research project, so we attempted to decipher the differences and similarities of the hepatic inflow in these two surgical conditions. To initiate our study, we needed to establish a model of ALPPS with a SFSS-setting, insufficient-for-survival future liver remnant without prior parenchymal resection in order to faithfully mimic the human procedure, before assessing hepatic inflow.

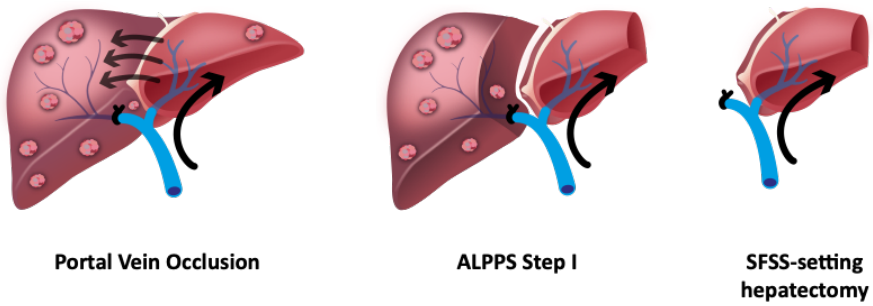

Figure 14: Our hypothesis: the impact of portal vein occlusion, ALPSS step I and SFSS-setting hepatectomy on portal vein haemodynamics.
Design by Gaêlle De Jesus Silva

Our specific aims were the following:

1. To develop an animal model of ALPPS that resembles human procedure implying a small-for-size FLR but without prior reduction of the liver size.
2. To test if ALPPS boosts FLR mass recovery compared to PVL and evaluate survival.
3. To compare FLR mass recovery and outcome in ALPPS and a SFSS-setting hepatectomy with exactly the same small initial FLR.
4. To assess and compare haemodynamic variations in portal vein as well as in hepatic artery caused by PVO, ALPPS and SFSS hepatectomy.
5. To address whether haemodynamic changes in ALPPS are responsible for the prevention of liver failure and for the accelerated liver regeneration in a small-for-size setting.

RESULTS

CHAPTER I: ESTABLISHMENT OF AN ANIMAL MODEL FOR ALPPS WITH INSUFFICIENT-FOR-SURVIVAL FUTURE LIVER REMNANT

In this chapter, we describe a rat model of an ALPPS steps I and II procedure, with minimal FLR, leading to high mortality due to SFSS unless ALPPS is applied. We demonstrated that an *in-situ* split combined with portal vein ligation enhances the degree of liver growth and the kinetic growth ratio compared to PVL alone. Interestingly, our work further suggests that a second benefit might be expected, as hepatectomy induces a higher rate of hepatocyte proliferation that concurs with increased liver mass recovery after ALPPS.

This novel 'clean' rodent model reproduces the objectives intended in human conventional ALPPS and should be of great value for studying the physiological mechanisms leading to accelerated regeneration and rescue from SFSS.

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TECHNICAL REPORT

Associating liver partition and portal vein ligation for staged hepatectomy: establishment of an animal model with insufficient liver remnant

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Abstract
Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) allows extended hepatectomy in patients with an extremely small future liver remnant (FLR). Current rodent models of ALPPS do not include resection resulting in insufficient-for-survival FLR, or they do incorporate liver mass reduction prior to ALPPS. Differences in FLR volume and surgical procedures could bias our understanding of physiological and hemodynamic mechanisms. We aimed to establish a rat ALPPS model with minimal FLR without prior parenchymal resection. In rodents, the left median lobe (LML) represents 10% of total liver. Partial hepatectomy (PHx) sparing LML and pericaval parenchyma represents our reference 87% resection. The first step in the procedure is either portal vein ligation (PVL) corresponding to ligation of all but the LML portal branches, or PVL with transection between the left and right median lobe segments (PVLT), and is defined as ALPPS stage-1. Second, ligated lobes were removed: PVL-PHx represents a conventional 2-stage hepatectomy, while PVLT followed by PHx is a strict reproduction of human ALPPS. In Group A, liver hypertrophy was analyzed after PVL ($n = 38$), PVLT ($n = 47$), T ($n = 10$), and sham ($n = 10$); In group B, mortality and FLR hypertrophy was assessed after PHx ($n = 42$), Sham-PHx ($n = 6$), PVL-PHx ($n = 37$), and PVLT-PHx ($n = 45$). In group A, PVLT induced rapid FLR hypertrophy compared to PVL ($p < 0{,}05$). Hepatocyte proliferation was higher in PVLT remnants ($p < 0{,}05$). In group B, PHx had a 5-day mortality rate of 84%. Sham operation prior to PHx did not improve survival ($p = 0.23$). In both groups, major fatalities occurred within 48 h after resection. PVL or PVLT prior to PHx reduced mortality to 33.3% ($p = 0{,}007$) or 25% ($p = 0.0002$) respectively, with no difference between the 2 two-stage procedures ($p = 0.6$). 7-day FLR hypertrophy was higher after the PVLT-PHx compared to PVL-PHx and PHx ($p = 0.024$). Our model reproduces human ALPPS with FLR that is insufficient for survival without liver resection prior to the stage-1 procedure. It offers an appropriate model for analyzing the mechanisms driving survival rescue and increased hypertrophy.

Introduction

Surgical resection is the only treatment with curative intent for patients suffering from a primary or secondary liver tumor. The extend of liver resection depends on tumor location, and/or burden. Case series show that, when operated upon, patients with colorectal liver metastases, even multiple and initially unresectable, can achieve higher survival rates compared to chemotherapy alone [1]. Tumor burden but also tumor biology may have an important impact on survival [2, 3]. Recent guidelines propose to attempt curative resection [4, 5, 6], and this is also recommended for multiple metastases in spite of the lack of evidence from randomized controlled trials [7]. In order to propose resection, the surgeon must respect oncological limits and "liver-related" limits determined by the size and function of future liver remnant (FLR). Resection must leave in place at least 20–25% of the initial liver volume when the parenchyma is healthy, or 40% in case of diseased liver with reduced functional capacity [8]. If resection is performed without respecting hepatic-related limits, patients risk postoperative liver insufficiency due to

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"small for size syndrome (SFSS)". SFSS is a clinical entity that combines postoperative hyperbilirubinemia, ascites and coagulopathy and is related to high mortality rates [9, 10].

To increase, when needed, the size of the future liver remnant, the gold standard is embolization or ligation of portal branches (PVL/E) nourishing the diseased, to-be-excised, liver in order to stimulate hypertrophy of the FLR by compensatory growth [11]. Portal vein occlusion achieves hypertrophy of the future liver remnant up to 52.8% of its initial size within 4–6 weeks, in some reports. However, up to 30% of the patients who gain insufficient liver mass recovery or experience a progression of their oncogenic disease during this period will be ineligible for liver resection [12].

Associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a technique that combines portal vein ligation and parenchymal in situ split to obtain rapid and enhanced hypertrophy of the future liver remnant [13]. Although the use of the technique is controversial [12], the cautious selection of patients and indications seem paramount to reduce the high morbidity and mortality rates initially reported [14, 15, 16]. ALPPS is proposed for patients with an extremely small FLR [17], or even as a rescue technique for patients whose liver failed to hypertrophy after portal vein occlusion [18]. ALPPS patients obtain accelerated and enhanced FLR hypertrophy [19] without developing liver insufficiency [13, 20], despite the extent of parenchymal resection.

Since the first report published in 2012 [13], experimental attempts have been made to study the mechanism of enhanced FLR hypertrophy in ALPPS. To this end several ALPPS rodent models were developed. However, these models do not include liver resection with insufficient-for-survival FLR [21–26], or they do incorproate liver mass reduction prior to ALPPS technique [27, 28, 29]. Differences in volume of FLR and in surgical events could introduce bias in our understanding of pathophysiological and hemodynamic mechanisms. This study aimed therefore to establish a rat model of ALPPS with extended hepatectomy (±90%) without prior parenchymal resection mimicking the human procedure.

Methods

Animals

All animal experiments were conducted in accordance with European regulations and FELASA guidelines for human care for laboratory animals established by the Université catholique de Louvain (UCLouvain, Belgium), and the study protocol was approved by the university ethics committee. Data were reported according to the ARRIVE guidelines. To avoid gender-related effects only male Wistar rats of ~350 g (UCLouvain Medical school, Brussels, Belgium) were used. Animals were housed in a temperature- and humidity-controlled environment in a 12-h light/12-h dark cycle. They had free access to food and water at all times before and after the surgical procedure. In order to take into account circadian variations in liver regeneration, all procedures were performed between 7 and 12 am. In a preliminary experiment, we measured the volume of the liver and of each individual lobe in 10 control rats.

Surgical procedures and experimental design

During the operative procedures, animals were allowed to breathe spontaneously in a glass cylinder filled with a mixture of oxygen (2 l/min) and isoflurane (2.5%) (IsoFlo, Zoetis BelgiumSA, Louvain-la-Neuve, Belgium). Animals had a median laparotomy after subcutaneous injection of 5cc of warm physiological sterile solution to increase the circulatory blood volume. Liver ligaments were gently freed and left lateral lobe (LLL) and median lobe (ML) were lifted with cotton-tipped sticks out of the abdomen and wrapped in gauzes to keep them humidified.

Conversely to the human liver, the rat liver is already partitioned, and the only lobe that receives two distinct glissonian pedicles is the median one: the right segment of the median lobe (RML) receives a pedicle directly from the hilum, whereas the left segment (LML) shares a common pedicle with the left lateral lobe (LLL). This bifurcation usually takes place into the LLL parenchyma, rendering the dissection of the portal vein branch from the arterial and biliary elements difficult. Indeed, into the parenchyma, glissonien elements are fragile, adherent, and surrounded by connective tissue. Based on microsurgical techniques already described [30, 31, 32], microscope-assisted dissection (×20 magnification) and pedicular hydrodissection permitted to individualize precisely the portal vein branch irrigating the LLL, which is posterior to the arterial and biliary elements. Arterial and biliary elements of the LLL were lifted with a Prolene 7/0 (Ethicon) to avoid damage. Cautious dissection allowed preservation of the whole glissonien pedicle destined to LML. LML represents 10% of the total rat liver mass [33, 31] and can be partitioned from the RML. Therefore, in all procedures (ALPPS, portal vein ligation or extended hepatectomy, see below), LML is the future liver remnant. Another technically difficult part of the operation is the dissection of the right lobe and of the pericaval parenchyma (evaluated at 2–5% of total liver weight [33, 31]) without damaging the inferior vena cava. Even with gentle fine microdissection, liver pericaval parenchyma could not completely be removed.

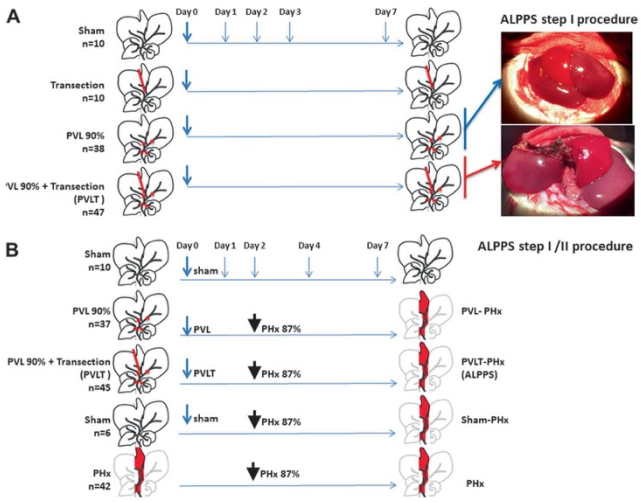

Fig. 1 Schematic representation of the study design for (**a**) step I in the associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) procedure. Sham $n = 10$; Transection: Parenchymal Transection between LMR and left segment of median lobe (LML), $n = 5$ per time points; PVL 90%: Portal vein ligation of all branches but the one irrigating the LML, day 1 ($n = 11$), day 2 ($n = 5$), day 3 ($n = 5$), day 7 ($n = 17$); PVLT: portal vein ligation of all branches but the one irrigating the LML *and* parenchymal transection between LMR and LML, day 1 ($n = 11$), day 2 ($n = 7$), day 3 ($n = 10$), day 7 ($n = 19$) and (**b**) ALPPS step I/step II procedure: PHx: 87% Partial hepatectomy of all liver segments except the LML and pericaval parenchyma at day 0, day 1 ($n = 11$), day 2 ($n = 7$), day 4 ($n = 5$), day 7 ($n = 19$); PVLT-PHx (ALPPS): PVLT at day 0 and resection of all deportalized segments at day 2: day 1 ($n = 11$), day 2 ($n = 7$), day 4 ($n = 11$), day 7 ($n = 16$) after initial operation; Sham: $n = 10$. PVL-PHx: PVL at day 0 and resection of all deportalized segments at day 2: day 1 ($n = 11$), day 2 ($n = 5$), day 4 ($n = 9$), day 7 ($n = 12$)

Description of the ALPPS rat model

ALPPS step I (first step procedure): portal vein ligation and transection (PVLT)

We separately ligated the portal branches for the right, caudate, left lateral lobes and the right segment of the median lobe (90% PVL). This was combined with complete transection between the left and the right segment of the median lobe (PVLT, $n = 47$). Rats undergoing PVLT were compared to animals with PVL alone (PVL, $n = 38$), transection alone (T, $n = 10$) or sham ($n = 10$) (Fig. 1a).

ALPPS step II (complete procedure):

2 days after PVLT described above, we resected all deportalized lobes leaving in place the LML and pericaval tissue (10 and 3% of total liver volume, respectively). This 2-step procedure is thereafter denoted as PVLT-PHx or ALPPS ($n = 45$). The 2-step procedure undergone by the animals in this group is a strict copy of conventional ALPPS procedure in humans. PVLT-PHx (or ALPPS) was compared to the resection of all deportalized segments 2 days after PVL (PVL-PHx, $n = 37$), to a one-step extended 87% hepatectomy (PHx, $n = 42$) and to an extended hepatectomy 2 days after sham operation (Sham-PHx, $n = 6$). In all groups partial hepatectomy corresponded to resection of all liver lobes but the LML segment (10%) and pericaval tissue (3%) (Fig. 1b).

Each animal was clinically observed during the first 6 h post-surgery and twice daily thereafter and mortality recorded. At selected time points (1, 2, 4 and 7 days), systemic (cardiac puncture) blood was obtained and liver harvested. When indicated, rats received 50 mg/Kg 5-bromo-2-deoxyuridine (BrdU) 2 h prior to harvesting. Our experimental protocol and the number of animals are shown in Fig. 1. In the figure legends, we report the number of sample on which each analysis is performed.

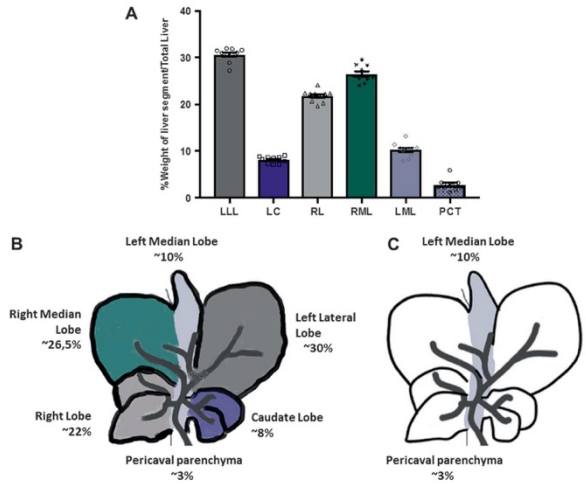

Fig. 2 LML is the ideal future liver remnant (FLR) as it represents approximatively 10% of the total liver parenchyma and can be partitioned from its right counterpart Analysis of relative weight of rat liver lobes compared to total liver weight in 10 control rats (**a** + **b**). Schematic representation of a partial hepatectomy leaving as FLR the left segment of the median and the pericaval tissue (87% of total liver weight) (**c**). LLL left lateral lobe, LC caudate lobe, LR right lobe, RML right medial lobe, LML left median lobe, PC pericaval tissue

Assessment of liver growth and cell proliferation

Five markers of liver regeneration were evaluated: (a) The restitution of liver mass was determined as the weight of LML to body weight ratio (FLR/BW); (b) The Kinetic Growth Ratio (KGR) was assessed as the gain of LML weight/BW/day; (c) Immunohistological staining (IHC) and morphometrical quantification of nuclear expression of the proliferation marker Ki67; (d) DNA synthesis determined by immuno-detection of BrdU incorporated in newly synthesized DNA, and (e) mitotic figures of hepatocytes were counted on 8–10 high power fields (×20 magnification) per sample. To confirm the mitotic cell type, we performed double immuno-fluorescence staining. HNF4α (R&D systems; PP-H1415-00; 1/350) for hepatocyte, pHH3 (Cell Signalling; 9701-S; 1/200) for mitotic cells, Hoechst for nuclear staining and Lyve-1 (R&D Systems; AF2125-SP; 1/100) with TSA (Thermo Fisher; Alexa Fluor™) for liver sinusoidal cell.

Liver injury assessment

Serum aspartate (ASAT) and alanine (ALAT) amino-transferases were measured using an automatic procedure in a serum multiple biochemical analyzer (FUJI DRI-CHEM NX500, Fujifilm Corporation, Tokyo).

Statistical analysis

GraphPad Prism software (San Diego, CA, USA) was used for graphs and statistics. In graphs, we report individual data (dots), the mean ± standard error (SEM). Survival curves were analyzed using the LogRang test (Mantel-Cox). Unpaired two-tailed *t*-test was used for simple comparison or one-way and two-way ANOVA followed by Bonferroni's post-hoc correction for multiple comparison. Statistical significance was assumed for p values < 0.05 ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

Results

The left segment of median lobe, an ideal FLR for our study

Ten rats were harvested as control group to analyze the relative weight of each lobe or segment compared to the total liver weight (Fig. 2a, b). In accordance with other authors [31, 33] the left segment of the median lobe represents 10.24% (range: 7.29–13.1%), while the pericaval parenchyma represents 2.7% (range: 1.1–5.8%) of the total liver (Fig. 2c). Thus, a partial hepatectomy leaving the LML+ pericaval parenchyma as FLR corresponds approximatively to a 87% hepatectomy.

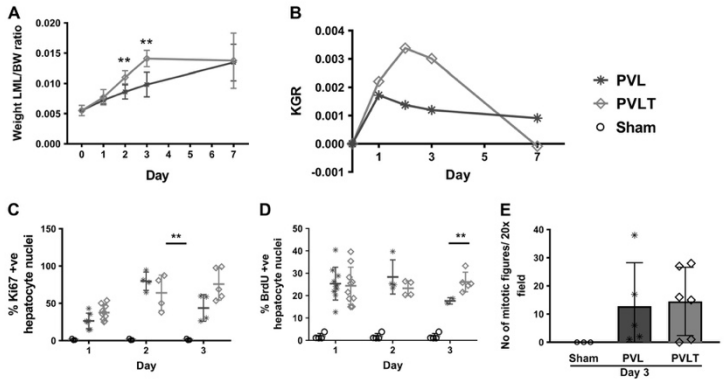

Fig. 3 Accelerated liver regeneration after ALPPS step I procedure. **a** Weight of future liver remnant (LML)/ body weight evolution (two-way ANOVA: surgery: $p < 0.005$, timing: $p < 0.0001$; interaction: ns), **b** Kinetic Growth Ratio (KGR) calculated as the gain of LML weight/ BW/day as per Fig. 1a protocol. **c** Hepatocyte proliferation assessed by quantification of Ki67 positive hepatocyte nuclei after PVLT: day 1 ($n = 10$), day 2 ($n = 4$), day 3 ($n = 5$); PVL: day 1 ($n = 7$), day 2 ($n = 4$), day 3 ($n = 5$); or Sham: ($n = 3$), (two-way ANOVA: surgery: $p < 0.0001$, timing: $p < 0.0001$, interaction: $p < 0.001$). **d** Quantification of BrdU immunostaining (% of positive hepatocyte nuclei) after PVLT: day 1 ($n = 10$), day 2 ($n = 4$), day 3 ($n = 5$); PVL: day 1 ($n = 10$), day 2 ($n = 4$), day 3 ($n = 2$), Sham: ($n = 4$), (two-way ANOVA: timing: ns, surgery: $p < 0.001$, interaction: ns). **e** Mitotic count (per ×20 microscopic field) in day 3 PVLT ($n = 6$); PVL ($n = 5$) and sham ($n = 3$) (One-way Anova: ns). $^{*}P < 0.05$ and $^{**}p < 0.01$ using Student *t*-test. ns not significant

Compared to PVL, PVLT (ALPPS step I) boosts the hypertrophy of the FLR

There was an important release of ASAT and ALAT at day 1 after PVL and PVLT with no significant difference between the groups (data not shown). Both PVL and PVLT induced a hypertrophy of the FLR. The hypertrophy was however greater after PVLT than after PVL at day 2 and 3 ($p < 0.05$) but not at day 7 post-surgery (Fig. 3a). The kinetic growth ratio assessing the daily rate of growth confirmed that the recovery occurred during the first 3 days in PVLT while growth is more constant over the 7 days period after PVL (Fig. 3b). There was no hypertrophy of the LML in animals undergoing transection alone or in sham-operated animals (data not shown). The proportion of cycling hepatocytes, assessed by Ki67 (Figs. 3c, 4c) and the proportion of hepatocytes engaged into the DNA synthesis phase of the cell cycle (BrdU+) (Figs. 3d, 4b) were significantly higher in PVLT than in PVL at day 3. The number of mitotic figures at the 3 day time point was however similar between the 2 groups (Figs. 3f, 4a). Immunohistochemistry and double immunofluorescent staining confirmed that hepatocytes were the main cell type undergoing DNA synthesis and cell division at this time point (Figs. 4 and 5). Thus, PVL associated with parenchymal in situ split accelerates FLR hypertrophy compared to PVL without transection.

ALPPS reduces mortality due to SFSS

Extended 87% partial hepatectomy (PHx) is associated with a high 84.21% mortality (16 out of 19) at day 7 (Fig. 6). Sham operation prior to 87% partial hepatectomy (Sham-PHx) did not improve survival compared to the upfront extended hepatectomy (83.33%, 5 out of 6, $p = 0.23$). In both groups, major fatalities occurred within the first 48 h after extended resection (PHx: 68.4% and Sham-PHx:66.6%). Thus, the extended PHx provokes a SFSS.

When PHx is preceded by PVL (PVL-PHx) or PVLT (PVLT-PHx or ALPPS) the seven-day mortality caused by the hepatectomy significantly dropped, compared with PHx, to 33.3% (4 out of 12, $p = 0.007$) or 25% (4 out of 16, $p = 0.0002$), respectively, with no significant difference between the 2 two-stage procedures ($p = 0.6$) (Fig. 6a). Our surgical strategy that models ALPPS dramatically reduced the mortality due to SFSS that occurs with a minimal FLR.

5 days after hepatectomy, FLR hypertrophy was significantly higher after the PVLT-PHx (or ALPPS) procedure compared to PVL-PHx ($p = 0.024$) and to SFSS (PHx) survivors ($p = 0.024$; Fig. 6b).

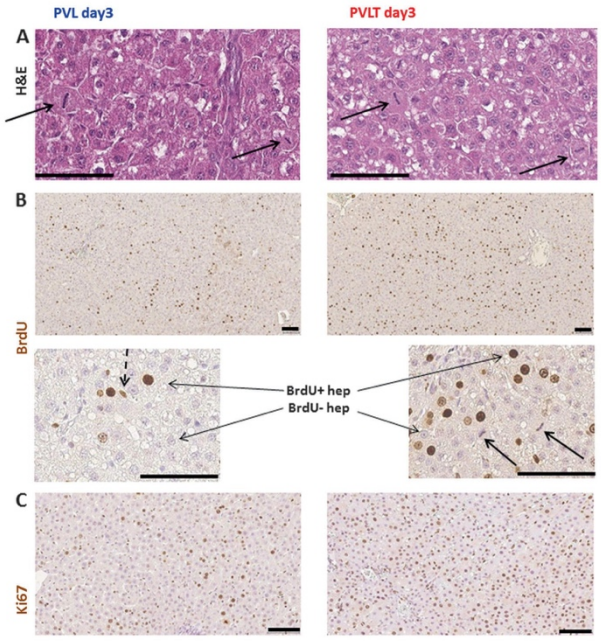

Fig. 4 Histological illustrations of hepatocyte proliferation PVL and PVLT at day 3 after the operation. Representative pictures from **a** H&E-stained liver sections readily showing hepatocyte mitosis (black arrows point to the mitotic figures). **b** Identification of BrdU incorporation into newly synthtised DNA. Lower panel are a higher magnification and show BrdU incorporation preferentially in hepatocytes, although few non parenchymal cells (broken-line arrow) are also in the synthesis phase of the cell cycle. **c** Ki67, a marker of cycling cells, IHC. Bar size: 100 µm

At day 1 after 87% partial hepatectomy, hepatocyte proliferation was already engaged as shown by the increased number of Ki67 positive hepatocyte nuclei (Fig. 6c). PVL triggered hepatocyte proliferation as at day 1 post PVL the proportion of Ki67 positive nuclei rose to the same extend as after PHx ($p = 0.5$). Importantly, PVLT alone, on the first day after the initial step, triggered an even higher regenerative response ($p = 0.0085$ compared to PHx; Fig. 6c). In addition, at day 5 post hepatectomy (step II procedure), the number of hepatocytes still engaged in DNA synthesis (BrdU + hepatocytes in Fig. 7) or in mitosis was significantly higher in PVLT-PHx compared to PVL-PHx ($p = 0.0027$; Fig. 6d). This supports that in addition to initial higher liver hypertrophy obtained after step I procedure, there is a higher rate of hepatocyte proliferation following hepatectomy concurring to increased liver mass recovery after ALPPS (Fig. 6b).

Discussion

ALPPS is a surgical technique implemented for patients needing hepatectomy with very small FLR. It achieves a rapid boost in FLR hypertrophy compared to the classical two-stage hepatectomy with portal vein ligation/occlusion. This accelerated and enhanced hypertrophy could diminish the drop out of planned resections after portal vein ligation or embolization due to progression of the underlying primary or secondary tumor or insufficient liver regeneration [34].

Since 2014, 9 publications describe rodent models of ALPPS. Dahr et al. initially proposed a model in which the right median lobe (corresponding to 26–28 % of the original liver mass (+3% of pericaval liver tissue) is the future liver remnant [21]. This model was subsequently used by several groups [26, 22, 24, 27]. In another model described by Perez et al. [23], the left median lobe, caudate lobe and

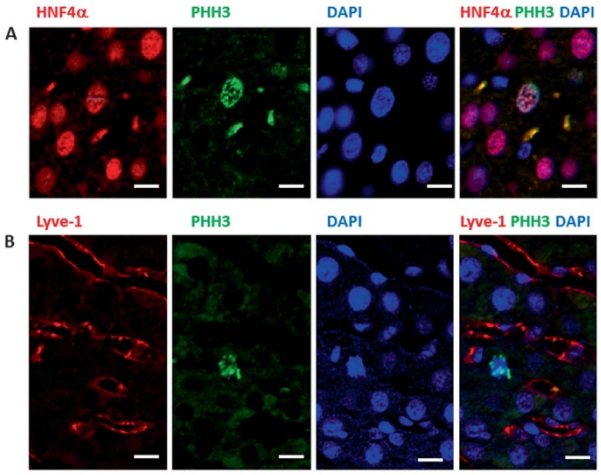

Fig. 5 Immunofluorescent staining of **a** HFN4a, a hepatocyte specific transcription factor, in red, phophohistone H3 (PHH3), a marker of mitotic cells, in green and nucleus in blue (DAPI) and **b** Lyve-1, a endothelial cell marker, in red, PHH3, in green and nucleus in blue (DAPI) in PVLT liver 3 days after first procedure confirming that hepatocytes (DAPI-HNF4α + cells) but not LSEC (Lyve-1 + cells) undergo cell proliferation. Bar size 10 µm

pericaval tissue amounting to 39% of the total liver volume represent the FLR. It is well documented that the rate of liver regeneration is related to the extent of hepatic resection [35] as well as to the extent of occlusion of the portal bed after PVL [36, 35, 37, 38]. In the context of extended hepatectomy Zhang et al [37] even highlighted that a 5% difference in the volume resected significantly impacts on mortality and regeneration rate. In all ALPPS models mentioned above, a large future liver remnant is left in place such as the resection corresponds to a 70% partial hepatectomy, an historical model with excellent survival and in which 7 days suffice to achieve full regeneration [39]. In this regards, the above models do not mimic the minimal, insufficient for survival FLR that would impose an ALPPS procedure in humans.

Other investigators resect part of the liver during the first step of the procedure: Wei and co-workers [27] resected the caudate lobe while Schlegel et al. [28]. and Shi et al. [40]. remove the left lateral lobe in mouse and rat, respectively, at the time of PVLT. This corresponds to an 8–30% upfront partial hepatectomy respectively or a wedge hepatectomy during human ALPPS procedure. Previous literature abundantly supports that such an hepatectomy cannot be seen as trivial as it is sufficient to prime the liver and engage a regenerative response [41, 42], thereby introducing a confounding factor when analyzing the ALPPS-dependent effects.

In the model we describe here a minimal, incompatible with survival FLR is left in place as proved by the tremendous mortality rate after PHx, unless ALPPS is performed. In addition, no liver tissue is resected during the first step of the procedure. This offers thus a clean model to analyze and understand the mechanisms driving the increase in hypertrophy of the FLR and the rescue of survival. We showed that the first step ALPPS procedure, namely PVL and parenchymal transection, rapidly engaged hepatocytes into cell cycle in an accelerated fashion and to a greater extent that conventional PVL. This accelerated entrance into regeneration allows for significantly increased mass and function recovery and animal survival after hepatectomy.

Metrics in FLR mass recovery were evaluated by mass measurements during harvesting. In order to integrate inter-individual differences in animals' body weight, we reported FLR mass as liver mass-to-body weight ratio, a valuable and largely used method to assess liver mass and liver mass recovery. Repeated CT or MRI volumetry, in which each animal serves as its own control, would probably be more adequate to appreciate FLR recovery but this would be at the cost of a higher stress to the animal and perhaps interference with the normal regenerative process. While 90% partial hepatectomy in rats usually associates with high mortality, some groups report little lethality, especially when the caudate lobe is left in place [31]. The capacity of regeneration of the individual remnant lobes is significantly

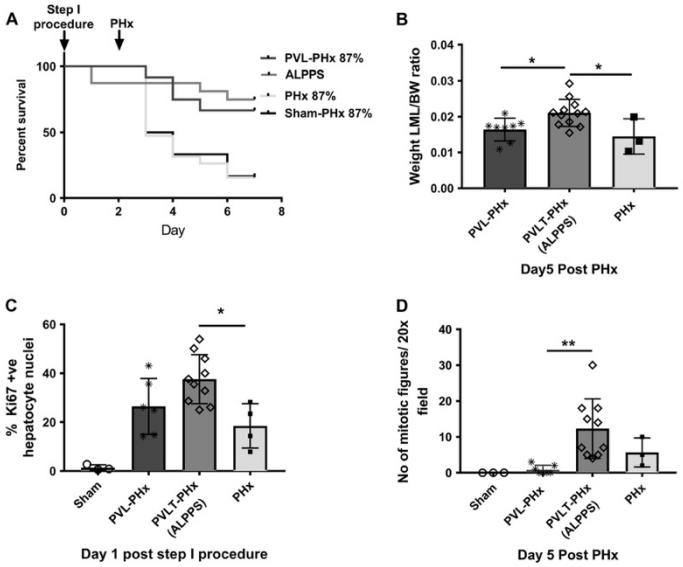

Fig. 6 ALPPS prevents mortality caused by extended hepatectomy. **a** Kaplan–Meier survival curve after PHx ($n = 19$), PVLT-PHx (or ALPPS) ($n = 16$), PVL-PHx ($n = 12$) and Sham-PHx, $n = 6$). PVLT-PHx but also two-stage hepatectomy (PVL-PHx) procedure significantly improved survival ($p = 0.0001$ using Log-rank (Mantel-Cox) test). However, Sham operation prior to 87% partial hepatectomy had no favorable impact on survival compared to an upfront extended resection ($p = 0.23$ using Log-rank (Mantel-Cox) test). The high mortality rate of PHx proved that our animal model represents SFSS. **b** Future Liver remnant (FLR) mass recovery after PVLT-PHx procedure compared to extensive 87% hepatectomy and to PVL-PHx 5 days after surgery (ALPPS $n = 12$, PHx $n = 3$ survivors, PVL-PHx $n = 8$). One-way ANOVA proved a significant improvement in FLR mass recovery in ALPPS compared to PVL-PHx and to PHx ($p = 0.024$, respectively). **c** Ki67 HIC at day 1 after operation (87% hepatectomy, PVLT = ALPPS step I procedure and PVL = PVL-PHx step I procedure) (Sham ($n = 3$), PVL-PHx ($n = 7$), PVLT-PHx ($n = 10$), PHx ($n = 4$)). ANOVA one-way analysis showed a significant difference in proliferation of PVL-PHx and PVLT-PHx compared to Sham ($p = 0.0055$ and $p < 0.0001$ respectively). Hepatocyte proliferation was significantly more important in the PVLT-PHx group compared to PHx ($p = 0.0085$), whereas no significant difference was observed between PVL-PHx and PHx operations ($p = 0.59$). **d** Mitotic count (per ×20 microscopic field) in day 5 post hepatectomy PVLT-PHx ($n = 10$); PVL-PHx ($n = 7$), PHx ($n = 3$), and sham ($n = 3$) (One-way ANOVA and Bonferroni post hoc: $p = 0.0027$ in PVLT-PHx compared to PVL-PHx)

different [43]. Differences in the nature of the FLR and in surgical dissection imposed by various anatomical localizations, genetic strains of the animals or age at the time of procedure may contribute to variable survival. The experiments reported in this study were performed by a senior surgeon with large experience in rodent liver surgery (>500). In addition, mortality curve in the cohort reported in the paper is similar to that of a separate cohort repeated months later.

In conclusion, we here describe the first rat model with minimal FLR, leading to high mortality due to SFSS unless ALPPS is applied. The degree of liver growth and kinetic growth ratio confirms that in situ split combined to PVL boosts liver hypertrophy more than PVL alone. In addition, a second benefice might be expected as hepatectomy induces a higher rate of hepatocyte proliferation that concurs to increased liver mass recovery after ALPPS. This clean model reproduces the objectives intended in human conventional ALPPS and should be of great value for studying the physiological mechanisms leading to accelerated regeneration and rescue from SFSS.

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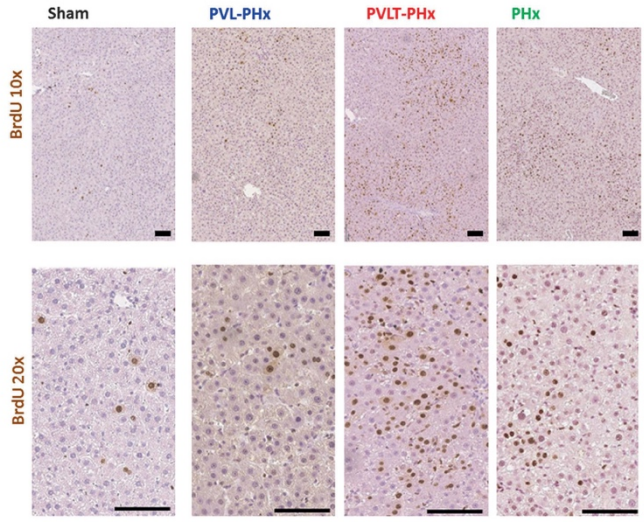

Fig. 7 BrdU immunostaining in Sham, PVL-PHx, PVLT-PHx (ALPPS), PHx 5 days post hepatectomy at low magnification (upper panels) and higher magnification (lower panels). Bar size: 100 µm

UCLouvain, Brussesls, Belgium) and Dr Carola Dahrenmöller (GAEN Lab, IREC, UCLouvain, Brussels, Belgium) for critical review of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Brouquet A, Abdalla EK, Kopetz S, et al. High survival rate after two-stage resection of advanced colorectal liver metastases: response-based selection and complete resection define outcome. J Clin Oncol. 2011;29:1083–90.
2. Blazer DG, Kishi Y, Maru DM, et al. Pathologic response to preoperative chemotherapy: a new outcome end point after resection of hepatic colorectal metastases. J Clin Oncol. 2008;26:5344–51.
3. Sasaki K, Morioka D, Conci S, et al. The tumor burden score: a new "metro-ticket" Prognostic Tool for colorectal liver metastases based on tumor size and number of tumors. Ann Surg. 2018;267:132–41.
4. Garden OJ, Rees M, Poston GJ, et al. Guidelines for resection of colorectal cancer liver metastases. Gut. 2006;55:1–8.
5. Adams RB, Aloia TA, Loyer E, Pawlik TM, et al. Selection for hepatic resection of colorectal liver metastases: expert consensus statement. HPB. 2013;15:91–103.
6. Charnsangavej C, Clary B, Fong Y, Grothey A, et al. Selection of patients for resection of hepatic colorectal metastases: expert consensus statement. Ann Surg Oncol. 2006;13:1261–8.
7. Veereman G, Robays J, Verleye L, et al. Pooled analysis of the surgical treatment for colorectal cancer liver metastases. Crit Rev Oncol Hematol. 2015;94:122–35.
8. Pruvot FR, Truant S. Major hepatic resection: from volumetry to liver scintigraphy. HPB. 2016;18:707–8.
9. Dahm F, Georgiev P, Clavien PA, et al. Small-for-size syndrome after partial liver transplantation: definition, mechanisms of disease and clinical implications. Am J Transplant. 2005;5:2605–10.
10. Asencio JM, García Sabrido JL, Olmedilla L. How to expand the safe limits in hepatic resections? J Hepatobiliary Pancreat Sci. 2014;21:399–404.
11. Makuuchi M, Thai BL, Takayasu K, et al. Preoperative portal embolization to increase safety of major hepatectomy for hilar bile duct carcinoma: a preliminary report. Surgery. 1990;107:521–7.
12. Sun Z, Tang W, Sakamoto Y, et al. A systematic review and meta-analysis of feasibility, safety and efficacy of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) versus two-stage hepatectomy (TSH). Biosci Trends. 2015;9:284–8.
13. Schnitzbauer AA, Lang SA, Goessmann H, et al. Right portal vein ligation combined with in situ splitting induces rapid left lateral liver lobe hypertrophy enabling 2-staged extended right hepatic resection in small-for-size settings. Ann Surg. 2012;255:405–14.

14. Schadde E, Schnitzbauer AA, Tschuor C, et al. Systematic review and meta-analysis of feasibility, safety, and efficacy of a novel procedure: associating liver partition and portal vein ligation for staged hepatectomy. Ann Surg Oncol. 2015;22:3109–20.
15. Schadde E, Ardiles V, Robles-Campos R, et al. Early survival and safety of ALPPS. Ann Surg. 2014;260:829–38.
16. Rosok B, Bjrnsson B, Sparrelid E, et al. Scandinavian multicenter study on the safety and feasibility of the associating liver partition and portal vein ligation for staged hepatectomy procedure. Surgery. 2016;159:1279–86.
17. Schadde E, Malagó M, Hernandez-Alejandro R, et al. Monosegment ALPPS hepatectomy: extending resectability by rapid hypertrophy. Surgery. 2015;157:676–89.
18. Tschuor C, Croome KP, Sergeant G, et al. Salvage parenchymal liver transection for patients with insufficient volume increase after portal vein occlusion - an extension of the ALPPS approach. Eur J Surg Oncol. 2013;39:1230–5.
19. Montalvá Orón EM, Maupoey Ibáñez J, Bañuelos Carrillo R, et al. Monosegment ALPPS: a new variant of the techniques for rapid hepatic regeneration. Critical review of the initial results of our series. Cir Esp. 2016;93:436–43.
20. Linecker M, Björnsson B, Stavrou GA, et al. Risk adjustment in ALPPS is associated with a dramatic decrease in early mortality and morbidity. Ann Surg. 2017;266:779–86.
21. Dhar DK, Mohammad GH, Vyas, S et al. A novel rat model of liver regeneration: possible role of cytokine induced neutrophil chemoattractant - 1 in augmented liver regeneration. Ann Surg Innov Res. 2015;9:1–10.
22. Almau Trenard HM, Moulin LE, Padín JM, Stringa P, Gondolesi GE, Barros Schelotto P. Development of an experimental model of portal vein ligation associated with parenchymaltransection (ALPPS) in rats. Cir Esp. 2014;92:676-81. https://doi.org/10.1016/j.ciresp.2013.11.005
23. García-Pérez R, Revilla-Nuin B, Martínez CM, et al. Associated Liver Partition and Portal Vein Ligation (ALPPS) vs selective Portal Vein Ligation (PVL) for staged hepatectomy in a rat model. Similar regenerative response? PLoS ONE. 2015;10:1–14.
24. Schadde E, Tsatsaris C, Swiderska-Syn M, et al. Hypoxia of the growing liver accelerates regeneration. Surgery. 2017;161:666–79.
25. Budai A, Fulop A, Hahn O, et al. Animal models for associating liver partition and portal vein ligation for staged hepatectomy (ALPPS): achievements and future perspectives. Eur Surg Res. 2017;58:140–57.
26. Yao L, Li C, Ge X, et al. Establishment of a rat model of portal vein ligation combined with in situ splitting. PLoS ONE. 2014;9:1–8.
27. Wei W, Zhang T, Zafarnia S, et al. Establishment of a rat model: Associating liver partition with portal vein ligation for staged hepatectomy. Surgery. 2016;159:1299–307.
28. Schlegel A, Lesurtel M, Melloul E, et al. ALPPS: from humain to mice highlighting accelerated and novel mechanisms of liver regeneration. Ann Surg. 2014;260:839–47.
29. Shi H, Yang G, Zheng T, et al. A preliminary study of ALPPS procedure in a rat model. Sci Rep. 2015;5:17567.
30. Aller MA, Arias N, Prieto I, et al. A half century (1961-2011) of applying microsurgery to experimental liver research. World J Hepatol. 2012;4:199–208.
31. Madrahimov N, Dirsch O, Broelsch C, et al. Marginal hepatectomy in the rat. Ann Surg. 2006;244:89–98.
32. Martins PauloNey, Aguiar PN. Hepatic lobectomy and segmentectomy models using microsurgical techniques. Microsurgery. 2008;28:187–91.
33. Martins PNA, Theruvath TP, Neuhaus P. Rodent models of partial hepatectomies. Liver Int. 2008;28:3–11.
34. Van Lienden KP, Van Den Esschert JW, De Graaf W, et al. Portal vein embolization before liver resection: a systematic review. Cardiovasc Intervent Radiol. 2013;36:25–34.
35. Meier M, Andersen KJ, Knudsen AR, et al. Liver regeneration is dependent on the extent of hepatectomy. J Surg Res. 2016;205:76–84.
36. Lauber DT, Tihanyi DK, Czigany Z, et al. Liver regeneration after different degrees of portal vein ligation. J Surg Res. 2016;203:451–8.
37. Zhang DX, Li CH, Zhang AQ, et al. mTOR-dependent suppression of remnant liver regeneration in liver failure after massive liver resection in rats. Dig Dis Sci. 2015;60:2718–29.
38. Rozka J, Jeppsson B, Bengmark S. Portal branch ligation in the rat reevaluation of a model. Am J Pathol. 1986;125:300–8.
39. Mao SA, Glorioso JM, Nyberg SL. Liver regeneration. Transl Res. 2014;163:352–62.
40. Shi H, Yang G, Zheng T, et al. A preliminary study of ALPPS procedure in a rat model. Sci Rep. 2015;5:1–12.
41. Michalopoulos GK. Liver regeneration after partial hepatectomy. Am J Pathol. 2010;176:2–13.
42. Fausto N. Liver regeneration. J Hepatol. 2000;32:19–31.
43. Inderbitzin D, Studer P, Sidler D, et al. Regenerative capacity of individual liver lobes in the microsurgical mouse model. Microsurgery. 2006;26:465–9.

CHAPTER II: HYPOXIA PROTECTS THE LIVER FROM SFSS

Having established an animal model, we now searched:

1. To compare FLR mass recovery and outcome in ALPPS and a SFSS-setting hepatectomy with the exact same small initial FLR.
2. To assess and compare haemodynamic variations in portal veins as well as in hepatic arteries caused by ALPPS and SFSS-hepatectomy.
3. To address whether haemodynamic changes in ALPPS are responsible for the prevention of liver failure and for the accelerated liver regeneration in a small-for-size setting.

We observed that ALPPS shows significantly higher survival rates compared to SFSS-setting hepatectomy. Analysis of the hepatic inflow showed a severe portal haemodynamic stress in both ALPPS step I and SFSS-setting hepatectomy, suggesting that portal hyperperfusion is not the sole causal factor for liver-related high mortality. Astonishingly, the hepatic artery flow into the FLR was significantly reduced in the ALPPS livers compared to SFSS, and this was associated with a preserved sinusoidal morphology. When we induced activation of hypoxia sensors in an upfront SFSS-setting hepatectomy, we rescued survival and recovered an efficient sinusoidal bed. This leads us to propose the counterintuitive and provocative concept that hypoxia protects survival from SFSS by inducing an early angiogenic switch to preserve the hepatic lobular architecture and maintain the function of the proliferating hepatocytes.

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ORIGINAL ARTICLE

AJT

Hypoxia protects the liver from Small For Size Syndrome: A lesson learned from the associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) procedure in rats

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Portal hyperperfusion and "dearterialization" of the liver remnant are the main pathogenic mechanisms for Small For Size syndrome (SFSS). Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) induces rapid remnant hypertrophy. We hypothesized a similar increase in portal pressure/flow into the future liver remnant in ALPPS and SFSS-setting hepatectomies. In a rodent model, ALPPS was compared to SFSS-setting hepatectomy. We assessed mortality, remnant hypertrophy, hepatocyte proliferation, portal and hepatic artery flow, hypoxia-induced response, and liver sinusoidal morphology. SFSS-hepatectomy rats were subjected to local (hepatic artery ligation) or systemic (Dimethyloxalylglycine) hypoxia. ALLPS prevented mortality in SFSS-setting hepatectomies. Portal hyperperfusion per liver mass was similar in ALLPS and SFSS. Compared to SFSS, efficient arterial perfusion of the remnant was significantly lower in ALPPS causing pronounced hypoxia confirmed by pimonidazole immunostaining, activation of hypoxia sensors and upregulation of neo-angiogenic genes. Liver sinusoids, larger in ALPPS, collapsed in SFSS. Induction of hypoxia in SFSS reduced mortality. Hypoxia had no impact on hepatocyte proliferation but contributed to the integrity of sinusoidal morphology. ALPPS hemodynamically differ from SFSS by a much lower arterial flow in ALPPS's FLR. We show that the ensuing hypoxic response is essential for the function of the regenerating liver by preserving sinusoidal morphology.

KEYWORDS
animal models, basic (laboratory) research/science, disease pathogenesis, liver disease, liver transplantation/hepatology, translational research/science

Abbreviations: ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; BW, body weight; DAB3, 3′-diaminobenzidine; DAMPs, Damage Associated Molecular Patterns; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; ELISA, enzyme-linked immunosorbent assay; FLR, Future Liver Remnant; HABR, hepatic arterial buffer response; HAF, total hepatic artery flow; HGF, Hepatic Growth Factor; HIF, Hypoxia Inducible Factor; HMGB1, High Mobility Group Box 1; IF, Immunofluorescence; iHAF, indexed hepatic artery flow; IHC, immunohistochemistry; IL1beta and 6, Interleukin 1b and 6; IP, intraperitoneal; iPVF, indexed portal vein flow; LLL, left lateral lobe; LML, left segment of median lobe; LSEC, Liver Sinusoidal Endothelial Cells; LT, liver transplantation; Lyve-1, Lymphatic Vessel Endothelial Hyaluronan Receptor 1; ML, median lobe; PC, pericaval tissue; PHLF, Post-Hepatectomy Liver Failure; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; PVF, total portal vein flow; PVL, portal vein ligation; PVLT, portal vein ligation and transection, ALPPS step I procedure; PVLT-PHx, portal vein ligation and transection followed by 87% partial hepatectomy at day 2; qPCR, quantitative Polymerase Chain Reaction; RML, right segment of the median lobe; SFSS, small for size syndrome; Sham-PHx, sham operation followed by 87% partial hepatectomy at day 2; VEGF, vascular endothelial growth factor.

1 | INTRODUCTION

Post-hepatectomy liver failure (PHLF) and small for size syndrome (SFSS) are clinical entities characterized by persistent hyperbilirubinemia, coagulopathy, intractable ascites, and delayed recovery of synthetic function leading to liver insufficiency.[1] They develop after major hepatectomy leaving a very small future liver remnant (FLR) or after liver transplantation (LT) with a small graft. PHLF is responsible for a 90-day mortality rate up to 75% after extended liver resection[2-4] and the size of the graft has been recognized as the only independent predictor factor of SFSS after LT.[2]

Redirection of the whole portal flow into a small FLR causes excessive sinusoidal shear stress[5] and a compensatory constriction of the common hepatic artery (the hepatic arterial buffer response-[HABR]).[6] The ensuing "dearterialization" and hypoxia of the remnant liver are the proposed primary factors for life-threatening postoperative liver failure.[7-10] Hence, reduction of the portal inflow into the remnant liver is a suggested strategy to prevent SFSS. Somatostatin treatment, creation of portosystemic shunts, ligation of splenic artery or splenectomy[11-15] have been employed to this end.

Conversely, other reports claim the beneficial effect of increasing the portal flow, and the inflow of hepatotrophic factors,[16] on liver regeneration.[9,17,18] Based on this observation, portal vein occlusion of the branches nourishing the diseased, to be excised, liver, either by radiological embolization or by surgical ligation, has been proposed for patients in need of major hepatectomy.[19] Portal vein occlusion is now the gold standard procedure before extended liver resections. However, hypertrophy of the FLR requires time (4-6 weeks). Recently, a new technique best known under the acronym ALPPS for "associated liver partition and portal vein ligation for staged hepatectomy" has been proposed to boost liver regeneration.[20] This variant of the standard two-stage hepatectomy combines portal vein ligation (PVL) and parenchymal transection between the diseased and healthy liver in the first step procedure (ALPPS step I) to obtain rapid and enhanced FLR hypertrophy.

Portal ligation in the ALPPS step I procedure redirects the entire portal blood flow through a small liver remnant sinusoidal network, while parenchymal transection excludes all intrahepatic collaterals and possibilities of neo-vascularization. Studies have shown that the extent of the parenchymal split, and thus the number of intrahepatic collaterals abrogated, correlates with the mass recovery of the FLR.[21,22] The contribution of the parenchymal transection to accelerated and enhanced hypertrophy is further underlined by the observation that in-situ split has been used as a rescue technique for patients whose liver fails to hypertrophy after portal vein occlusion.[23,24] Clinical studies document that ALPPS procedure causes an immediate and steep rise of the portal pressure and flow into the liver remnant accompanied by a compensatory arterial constriction (HABR).[25,26] In experimental models, in ALPPS step I, but not in portal vein occlusion alone, the HABR was associated with hypoxia.[26]

While enhanced liver regeneration has been linked to a severe portal hemodynamic stress in a critically small FLR in ALPPS, a similar hemodynamic condition is considered to be at the origin of liver failure in SFSS-setting hepatectomy. We hypothesized that the hypoxic response in ALPPS step I procedure has a protective impact on liver function. We therefore compared liver hemodynamic in a SFSS-setting hepatectomy and in a model of ALPPS leaving a too small for survival FLR recently published by our group.[27] We showed that hypoxia and hypoxia-induced signaling associated with accelerated regeneration and better survival in ALPPS. We further experimentally demonstrated that induction of hypoxia improves survival in SFSS conditions, confirming the protective role of hypoxia.

2 | METHODS

2.1 | Animals

All animal experiments were conducted in accordance with the European regulations and guidelines for human care for laboratory animals. The study protocol was approved by the university ethics committee. Wistar rats (UCLouvain Medical school, Brussels, Belgium) were housed in a temperature- and humidity-controlled environment in a 12-h light/12-h dark cycle. They had free access to food and water at all times. Because hormonal status[28] and circadian cycle influence regeneration, all procedures were performed in male rats and between 7 and 12 AM. Because it fluctuates with food intake, the portal flow was measured on 12-hours fasten animals.

2.2 | Surgical procedures

We recently described an animal model of ALPPS in rats with a minimal, insufficient for survival FLR.[27]

The left segment of the median lobe (LML—10% of the total rat liver mass[27,29]) can be partitioned from the right segment of the median lobe (RML). Therefore, LML is the future liver remnant in all procedures. As the pericaval parenchyma (evaluated at 2%-5% of total liver[27,29]) cannot be resected without damaging the inferior vena cava, the resection amounts to 87% of the initial liver weight.

During operation, animals were allowed to breathe spontaneously in a glass cylinder filled with a mixture of oxygen (2 L/min) and isoflurane (2.5%) (IsoFlo, Zoetis BelgiumSA, Louvain-la-Neuve, Belgium). All animals had a median laparotomy after subcutaneous injection of 5 cc of warm physiological sterile solution to increase the circulatory blood volume.

Sham operation: liver ligaments were gently freed, left lateral and median lobes were lifted with cotton-tipped sticks out of the abdomen and wrapped in gauzes to keep them humidified. The hilum was gently manipulated.

ALPPS model: In step-one procedure we ligated all portal veins but the one perfusing the LML (90% PVL) and transected between the left and right segments of the median lobe (PVLT). As inferior vena cava is intrahepatic in rodents, 2 mm of liver parenchyma was left around the vena cava to avoid vascular trauma. Two days after PVLT, we resected the deportalized lobes leaving in place the LML and pericaval tissue (ie, 13% of the initial volume) (PVLT-PHx or ALPPS).

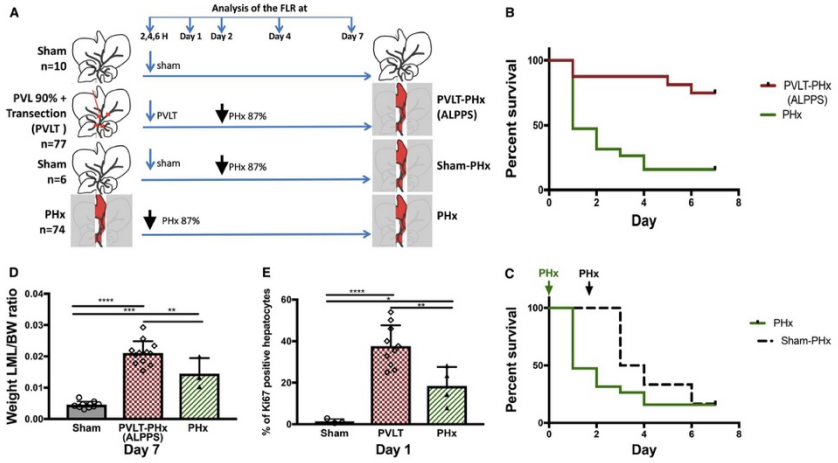

FIGURE 1 Eighty-seven percent partial hepatectomy causes a SFSS and ALPPS procedure prevents extended hepatectomy mortality. Schematic representation of the study design (A). For PHx, 87% of the liver mass was resected by removing all liver segments except the LML and pericaval parenchyma at day 0. A total of 74 animals were included with n = 10 randomized at 2 hours timepoint, n = 10 at 4 hours, n = 12 at 6 hours, n = 11 at day 1, n = 7 at day 2, n = 5 at day 4, and n = 19 at day 7. Animals in the Sham-PHx group underwent laparotomy and mobilization of hepatic ligaments at day 0 and 87% extended hepatectomy at day 2 (n = 6). PVLT-PHx (ALPPS): during the first procedure at day, we ligated all portal vein branches but the one irrigating the LML and transected the parenchyma between RML and LML; we resected all deportalized segments on day 2. A total of 77 animals were investigated, n = 6 at 2 hours, n = 15 at 4 hours, n = 11 at 6 hours, n = 11 at day 1, n = 7 at day 2, n = 11 at day 4, and n = 16 at day 7 after the initial intervention. Sham-operated animals were no hepatectomy controls (n = 10). Kaplan-Meier survival curves (B) after PHx (n = 19), PVLT-PHx (or ALPPS) (n = 16) and (C) PHx (n = 19) and Sham-PHx (n = 6). Arrows in (C) indicate the timing of hepatectomy. Extended PHx causes high mortality, ALPPS significantly improved survival ($P = .0002$ using Log-rank [Mantel-Cox] test) and Sham operation prior to 87% partial hepatectomy had no favorable impact on survival compared to an upfront extended resection ($P = .23$ using Log-rank [Mantel-Cox] test). D, FLR mass recovery expressed as liver remnant weight in relation to body weight after PVLT-PHx compared to extensive 87% hepatectomy and Sham 7 days after surgery (ALPPS [n = 12], PHx [n = 3 survivors], Sham [n = 10]). One-way ANOVA proved a significant improvement in FLR mass recovery in ALPPS and in PHx compared to Sham ($P = .0001$ and $P = .0002$, respectively). Postoperative FLR hypertrophy was more important in PVLT-PHx group compared to PHx ($P = .007$). E, Ki67 HIC at day 1 after operation (87% hepatectomy [n = 4], PVLT = ALPPS step I procedure [n = 10] and Sham [n = 3]). ANOVA one-way analysis showed a significant difference in proliferation of PVLT-PHx and PHx compared to Sham ($P < .0001$ and $P = .05$, respectively). Hepatocyte proliferation was significantly more important in the PVLT-PHx group compared to PHx ($P = .006$). ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; FLR, future liver remnant; HIC, immunohistochemistry; LML, left segment of the median lobe; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; RML, right segment of the median lobe; SFSS, small for size syndrome. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

Extended PH: We resected all liver lobes but the LML segment (10%) and pericaval tissue (3%), achieving an one-step extended 87% partial hepatectomy (PHx). To evaluate the effect of laparotomy on survival, Sham operation 2 days prior to 87% PHx was also performed (Sham-PHx).

We clinically observed each animal during the first 6 hours post-surgery and twice daily thereafter and recorded mortality. At selected time points (2, 4, 6 hours and days 1, 2, 3, and 7, as indicated), portal and systemic (cardiac puncture) blood was obtained and liver recovered. Figure 1A depicts our experimental protocol. In the figure legends, we report the number of animals and samples on which each analysis was performed.

Induction of hypoxia: To evaluate the impact of hypoxia on animal survival and FLR mass recovery, we ligated the hepatic artery (LHA) at the level of the hepatic pedicle (PHx-LHA) at the time of extended partial hepatectomy. In a separate group of animals, we injected Dimethyloxalylglycine (DMOG) (200 mg/kg BW) intraperitoneally 12 hours before extended partial hepatectomy (DMOG-PHx).[26]

2.3 | Assessment of liver proliferation

We evaluated two markers of liver regeneration: (A) The restitution of liver mass was determined as the weight of LML to body weight ratio (FLR/BW); and (B) the expression of ki67 in hepatocyte nulcei by

immunohistological staining (IHC) and morphometrical quantification (see Supplementary Materials for technical details).

2.4 | Blood flow analysis

After an overnight fasting, animals underwent a laparotomy and microscope-assisted dissection (×20 magnification) with hydrodissection of the liver hilum to individualize precisely portal vein and common hepatic artery. A Sham, a PVLT (ALPPS step I procedure) or a PHx was performed. Total portal flow (PVF) and hepatic artery flow (HAF) were measured for 6 minutes once a steady point was reached with MA2PSB (2 mm) and MA0.5PSB (0.5 mm) flow probes (Transonic Systems Inc., Ithaca, NY), respectively. PowerLab hardware and software were used for analysis. As the portal vein perfuses the whole liver in Sham, but only 10% of the liver in PHx and PVLT, we calculated the "indexed" portal vein flow into the FLR (iPVF) as followed (FLR = LML = 10% of the total liver mass): Sham: iPVF = total PVF × 0.1; PVLT: iPVF = total PVF (as all the segmental branches of the PV were ligated except the one vascularizing the LML [FLR]). PHx: iPVF = total PVF. The indexed hepatic artery flow (iHAF) in the FLR was calculated similarly: Sham: iHAF = total HAF × 0.1; PHx: iHAF = total HAF; PVLT: iHAF= total HAF x 0,1 (as in PVLT, we performed 90% PV ligation whereas the arterial perfusion was preserved for the entire liver).

2.5 | Pimonidazole staining for quantification of tissue hypoxia

In hypoxic cells, pimonidazole undergoes chemical reduction, binds to SH-containing molecules, and the resulting complexes accumulate in tissues.[26] Pimonidazole (60 mg/gr BW) was injected in the tail vein 1 hour prior to surgery and detected tissue-bound pimonidazole by IHC (see Supplementary Materials).

2.6 | Gene and protein analyses

Gene expression of angiogenic proteins[30] was measured by qPCR on RNA extracted from whole LML tissue (primer sets in Supporting Information, Figure S1). We used enzyme-linked immunosorbent assay (ELISA) to measure serum concentration of IL1-beta, IL-6, VEGF, HGF (RLB00, R6000B, RRV00 and MHG00, respectively, R&D systems, UK), and High Mobility Group Box 1 (HMGB1) (ST51011, Gentaur, IBL International GmbH). Hypoxia Inducible Factor (HIF) 1α and 2α were quantified in liver nuclear and cytosolic exctracts[31] using ELISA (LS-F11633; LSBio, Seattle).

2.7 | Assessment of the liver endothelial bed

Immunofluorescent (IF) staining of Lyve-1, a molecular marker of lymphatic and sinusoidal endothelial cells[32] was performed (see supplementary materials). We analyzed five images (20× magnification) taken ad random per liver. The smallest diameter of the transversally cut sinusoids was manually measured by two investigators on anonymized images using Fiji software (LOCI, University of Wisconcin, Madison, WI).

2.8 | Statistical analysis

GraphPad Prism software (San Diego, CA) was used for graphs and statistics. In graphs, we report individual data (dots), the mean ± standard error (SEM). Survival curves were analyzed using the LogRang test (Mantel-Cox). Unpaired two-tailed t-test was used for simple comparison or one-way and two-way ANOVA followed by Bonferroni's post-hoc correction for multiple comparison. Statistical significance was assumed for $P < .05$ (*$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$).

3 | RESULTS

3.1 | ALPPS reduces mortality due to SFSS

Extended 87% partial hepatectomy (PHx) is associated with a high 84.2% mortality (16 out of 19 rats) at day 7 with the majority of death (66%) occurring in the first 48 hours reflecting a SFSS (Figure 1B). When PVLT (PVLT-PHx or ALPPS) preceded PHx the 7-day mortality was significantly lower (25%, $P = .0002$) (Figure 1B). Sham operation prior to 87% partial hepatectomy (Sham-PHx) did not modify survival compared to the upfront extended hepatectomy (83.3%, 5 out of 6, $P = .23$) (Figure 1C). ALPPS, but not Sham operation, dramatically reduced the mortality that occurs after a SFSS-setting hepatectomy.

3.2 | Hepatocyte does regenerate in SFFS but ALPPS enhances FLR mass recovery

After 7 days, FLR hypertrophy was significantly higher in PVLT-PHx (or ALPPS) compared to SFSS (PHx) survivors ($P = .007$). FLR mass recovery was also significantly enhanced in the PHx group compared to Sham ($P = .0002$) (Figure 1D). At day 1 after surgery, hepatocytes were already engaged into the cell cycle as shown by the increased number of Ki67 positive hepatocyte nuclei in both PVLT and PHx groups compared to Sham ($P < .0001$ and $P = .05$, respectively). Thus, hepatocytes proliferate after extended PHx. PVLT alone, on the first day after the initial step, triggered a higher regenerative response compared to PHx ($P = .006$) (Figure 1E).

3.3 | FLR are exposed to severe hypoxia after ALPPS step I

We measured the flow in the portal vein and hepatic artery for 6 minutes directly after completion of the surgical procedure. Total portal vein flow (PVF) was equally reduced after PVLT and PHx compared to Sham ($P < .0001$) (Figure 2A). Because 87% of the liver parenchyma was excluded from the portal circulation in PVLT and PHx, the portal flow per mass of perfused liver parenchyma (indexed PVF or iPVF) increased by a factor of 4 to 5 compared to the flow into LML in Sham animals ($P = .0001$), with no difference between PVLT and PHx ($P = .2$) (Figure 2B). The total hepatic artery flow (HAF) decreased in both

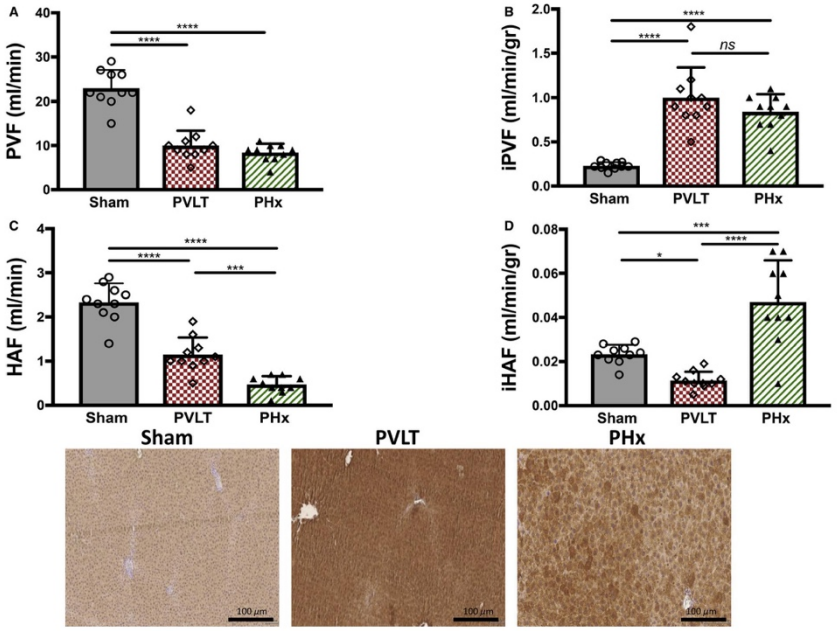

FIGURE 2 Hemodynamic variations after ALPPS step I (PVLT) and PHx. A, Total Portal Flow (PVF) (mL/min) and B, portal flow per liver mass perfused (iPVF) (mL/min/g) in Sham (n = 10), PVLT (n = 10), and PHx (n = 10) procedure. ANOVA one-way analysis showed a significant reduction in the total portal flow in both procedures compared to Sham ($P < .0001$) and a significant increase in the indexed portal flow in both PVLT and PHx compared to Sham ($P < .0001$), with no difference in the inflow between the two procedures ($P = .2$). C, Total HAF and D, hepatic artery flow per liver mass perfused (iHAF) in Sham (n = 10), PVLT (n = 10) and PHx (n = 10). ANOVA one-way analysis showed a significant reduction of the total hepatic artery inflow in both procedures compared to Sham ($P < .0001$). Compared to Sham, there was a tremendous increase in the indexed arterial flow in PHx ($P < .0002$) but a decrease in PVLT ($P = .05$). Thus, FLRs in ALPPS step I receive significantly less arterial blood compared to PHx ($P < .0001$). E, Representative histological pictures of Pimonidazole IHC on FLR on the 1st postoperative day in Sham (n = 2), PVLT (n = 4), and PHx (n = 4). Bar size = 100 µm. ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; FLR, future liver remnant; HAF, hepatic artery flow; iHAF, indexed hepatic artery flow; iPVF, indexed portal vein flow; *ns*, not significant; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection. *$P < .05$, ***$P < .001$, ****$P < .0001$

PVLT and PHx compared to Sham ($P < .0001$) (Figure 2C) and was significantly lower after PHx compared to PVLT ($P = .0004$) (Figure 2C). While arterial blood is distributed to the entire liver in PVLT, it perfused only the remnant in PHx (ie, the 13% of the original liver mass). Thus, compared to Sham, the indexed arterial flow into the FLR (iHAF) was increased after PHx ($P < .0002$) while it tended to decrease after PVLT ($P = .05$) (Figure 2D). The iHAF in PVLT was significantly lower compared to PHx ($P < .0001$). This supports that the FLR receives less arterial, well-oxygenated blood in ALPPS than in SFSS.

Pimonidazole staining, an indicator of hypoxia, was significantly increased in PVLT and PHx compared to Sham on the first postoperative day ($P = .0002$ and $P = .03$, respectively), supporting hypoxia in the FLR. At 24 hours, staining was significantly more intense in FLR tissue after PVLT than after PHx ($P = .01$) (Figure 2E, Supporting Information, Figure S2 for quantification). The difference persisted, but to a lower extent, on day 2 (data not shown).

3.4 | Hypoxia in ALPPS step I induces an angiogenic response

Hypoxia is known to activate and induce nuclear translocation of HIF-1α and 2α transcription factors, driving thereby an angiogenic

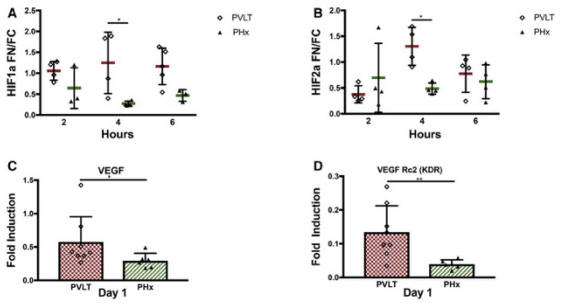

FIGURE 3 Angiogenic response to hypoxia. Ratio of nuclear to cytoplasmic HIF1α (A) and HIF2α (B) as measured by ELISA 2, 4, and 6 hours post-procedure in PVLT (n = 4, n = 4 and n = 5, respectively) and PHx (n = 4, n = 4, and n = 4, respectively). Data were analyzed by two-way ANOVA. VEGF (C) and VEGF Rc2 (D) gene expression measured by qPCR one day after the operation in PVLT (n = 8) and PHx (n = 6). Data are expressed as fold induction compared to Sham (controls) and analyzed using the Mann-Whitney test. Individual data mean and SEM per group are presented. ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; HIF, hypoxia inducible factor; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; qPCR, quantitative polymerase chain reaction; Rc2, Receptor 2; SEM, standard error of the mean; VEGF, vascular endothelial growth factor. *$P < .05$, **$P < .01$

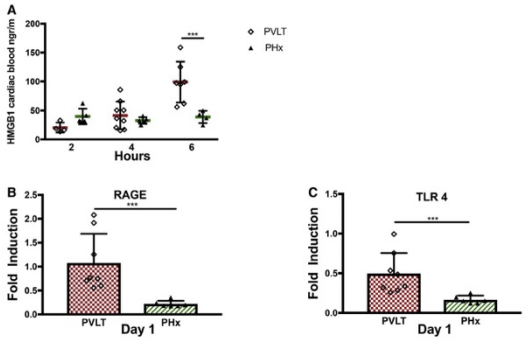

FIGURE 4 DAMPS response to hypoxia. HMGB1 concentrations measured by ELISA in serum prepared from cardiac blood (A) in PVLT (2 hours, n = 3; 4 hours, n = 9;6 hours, n = 7) and PHx (2 hours, n = 5; 4 hours, n = 5; 6 hours, n = 4). Data are analyzed by two-way ANOVA. RAGE (B) and TLR4 (C) gene expression measured by qPCR one day after the operation in PVLT (n = 8) and PHx (n = 6). Data are expressed as fold induction compared to Sham (controls) and analyzed using Mann-Whitney test. Individual data mean and SEM per group are presented. ANOVA, analysis of variance; DAMPS, damage-associated molecular patterns; ELISA, enzyme-linked immunosorbent assay; HMGB1, High Mobility Group Box 1; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; qPCR, quantitative polymerase chain reaction; RAGE, receptor for advanced glycation end products; SEM, standard error of the mean; TLR, toll like receptor. ***$P < .001$

response.[30] The ratio of nuclear to cytosolic HIF-1α was higher in PVLT than in PHx 4 hours after intervention ($P = .01$) (Figure 3A). Similarly, at the same timepoint, HIF-2α nuclear to cytoplasmic ratio was also higher in PVLT compared to PHx ($P = .01$) (Figure 3B). On the first postoperative day, VEGF gene expression was significantly increased in PVLT compared to PHx ($P = .02$), so was the expression of VEGF Receptor 2 (KDR) ($P = .007$) (Figure 3C,D). Serum levels of VEGF, IL1-β, IL-6, and HGF measured by ELISA were similar in PVLT and PHx (data not shown). Damage-associated molecular patterns (DAMPs) are released by damaged or hypoxic cells. HMGB1, one of such DAMPs, was significantly increased in systemic blood at 6 hours after PVLT compared to PHx ($P = .0004$) (Figure 4A). In parallel, the expression of the receptor for advanced glycation end products (RAGE) and Toll like receptor 4 (TLR4), two receptors

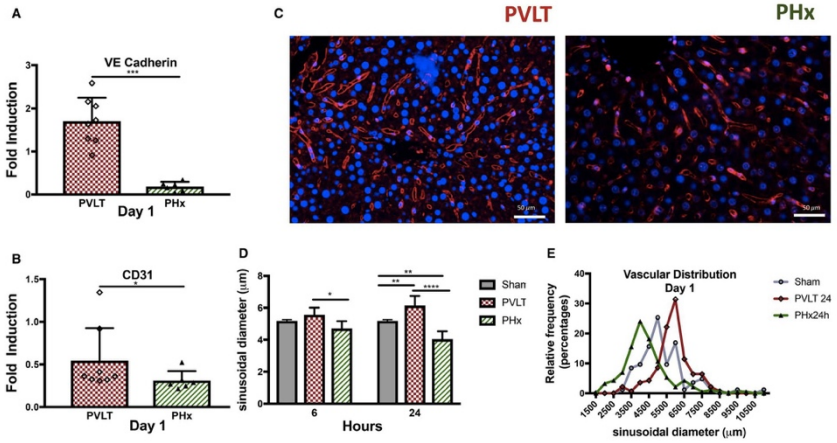

FIGURE 5 In ALPPS FLRs the sinusoidal bed is preserved. VE-Cadherin (A) and CD31 gene expression (B) one day after the operation in PVLT (n = 8) and PHx (n = 6) expressed as fold induction compared to Sham (controls) and analyzed using the Mann-Witney test. Lyve-1 fluorescent staining of sections at 6 hours, and 1 day after PHx and PVLT: (C) Representative pictures at day 1 showing collapsed sinusoids in PHx while they remain patent in PVLT. Lyve-1 in red and nuclei in blue. Bar size = 50 μm. (D) Sinusoidal diameter in Sham (n = 4), PVLT (6 hours, n = 7; day 1, n = 9) and PHx (6 hours, n = 5; day 1, n = 5) (mean + SEM, data analyzed by ANOVA) and (E) distribution of sinusoid diameter at day 1 post-surgery in Sham (n = 4), PVLT (n = 9), and PHx (n = 5). At 6 hours after surgery, sinusoidal diameter was already significantly larger in PVLT compared to PHx ($P = .01$) and this difference widened at day 1 ($P < .0001$). Of note, 1 day after surgery, sinusoids in PHx are collapsed compared to Sham ($P = .002$). ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; FLR, future liver remnant; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; SEM, standard error of the mean . *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$

on which HMGB1 binds to activate neoangiogenesis,[33-35] was significantly higher in FLR after PVLT than after PHx ($P = .0007$) (Figure 4B,C). All angiogenic factors tested showed higher gene expression in the ALPPS step I group compared to PHx (Supporting Information, Figure S3). The expression of VE-Cadherin, a glycoprotein indispensable for proper vascular development, and of CD31, which reflects the endothelial cells pool, were significantly upregulated in PVLT compared to PHx ($P = .0007$ and $P = .04$, respectively) (Figure 5A,B).

3.5 | ALPPS step I rescues liver sinusoidal morphology

As visualized on Lyve-1 immunostaining (Figure 5C), the hepatic sinusoids appeared large in PVLT and small and sometimes collapsed in PHx FLRs. Morphometric quantification confirmed the difference. Compared to Sham, the mean diameter of the sinusoids was significantly larger in PVLT after 6 hours ($P = .01$) and even more so at day 1 ($P < .0001$) while sinusoids were significantly smaller after extended hepatectomy (Figure 5D). The curve of distribution of sinusoid size confirmed this finding (Figure 5E).

3.6 | Hypoxia improves survival in SFSS without affecting FLR mass recovery

Thus, our data support the hypothesis that ischemic signals in the FLR in ALPPS protect from SFSS. To test this, we induced hypoxia upon extended partial hepatectomy either by ligation of hepatic artery during PHx (PHx-LHA) or by administration of DMOG, an inducer of hypoxic signaling,[26] prior to PHx (DMOG-PHx) (Figure 6A). We confirmed increased pimonidazole staining with the two procedures compared to extended PHx alone (Figure 6B, Supporting Information, Figure S4A for representative histological pictures). As shown in Figure 6C, hepatic artery ligation and DMOG treatment significantly improved survival compared to extended PHx alone (66.7% and 83.3%, respectively compared to 15.8%, $P < .0001$), such as survival is comparable to that obtained with ALPPS. Neither local nor systemic hypoxia affected the final FLR mass recovery (Figure 6D). Hepatocyte proliferation attested by Ki67 IHC was induced in all three groups compared to Sham ($P < .05$) but was higher in hepatic artery ligation model (PHx-LHA) compared to PHx alone ($P = .01$) (Figure 6E).

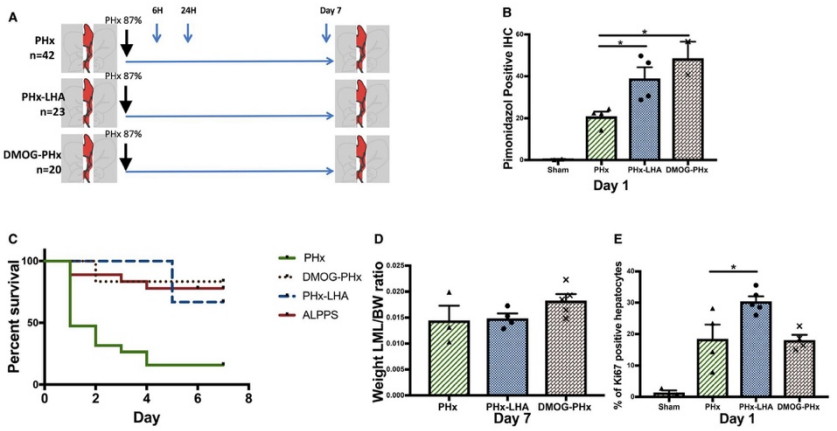

FIGURE 6 Hypoxia in SFSS rescues survival. (A) Schematic representation of the study designed: 42 animals underwent 87% extended hepatectomy (PHx; n = 12 at 6 h, n = 11 at 1 day, and n = 19 at 7 days); 23 animal had a ligature of the hepatic artery at the time of extended PHx (PHx-LHA; n = 12 at 6 hours; n = 5 at day 1 and n = 6 at day 7); 20 animals received a DMOG injection (I.P.) 12 hours prior to extended PHx (DMOG-PHx; n = 9 at 6 hours; n = 5 at day 1, and n = 6 at day 7). (B) Morphometrical quantification of Pimonidazole IHC on FLR on the first postoperative day in Sham (n = 2), PHx (n = 4), PHx-LHA (n = 4), and DMOG-PHx (n = 2). ANOVA one-way analysis showed hypoxia in FLR tissues in PHx-LHA and DMOG-PHx ($P = .04$ and $P = .01$, respectively). (C) 7-day Kaplan Meier survival curve after ALPPS (n = 16), PHx (n = 19), PHx-LHA (n = 6), and DMOG-PHx (n = 6). Hepatic artery ligation or DMOG injection prior pour PHx had a favorable impact on survival compared to PHx ($P < .0001$ using Log-rank [Mantel-Cox] test). Hypoxic model PHx showed similar survival to ALPPS. (D) Mass of the liver remnant relative to body weight at day 7 after initial hepatectomy, and (E) morphometrical quantification of the percentage of Ki67+ hepatocyte nuclei in Sham (n = 3) and at day 1 after hepatectomy in PHx (n = 4), PHx-LHA (n = 5), and DMOG-PHx (n = 4). ANOVA one-way analysis showed a significant difference in proliferation index in PHx-LHA compared to PHx ($P = .01$). All models of partial extended hepatectomy showed significant proliferation index compared to Sham ($P = .0036$ for PHx, $P < .0001$ for PHx-LHA and $P = .004$ for DMOG-PHx). Individual data, mean and SEM are presented. Data are analyzed by ANOVA. ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; FLR, future liver remnant; IHC, immunohistochemistry; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; SEM, standard error of the mean. *$P < .05$

3.7 | Hypoxia improves sinusoidal morphology

There was a significant activation in HIF-1α (Figure 7A), but not in HIF-2α (Figure 7B), 6 hours after surgery in the PHx-LHA group compared to PHx ($P = .04$). Expression of angiogenesis markers Angiopoietin 1 (Figure 7C), Angiopoietin 2 (Figure 7D), and Platelet-derived growth factor b (Figure 7E) as well as VE-Cadherin gene expression (Figure 8A) was also enhanced in the PHx-LHA group compared to PHx. Compared to PHx, hepatic artery ligation did not enhance HMGB1 systemic release. Systemic DMOG administration associated with high circulating HMGB1 compared to PHx ($P = .01$) at early time points (Supporting Information, Figure S4B). Induction of hypoxia in SFSS, whether through hepatic artery ligation or DMOG treatment, did not alter expression of HMGB1 receptors RAGE and TLR4 (Supporting Information, Figure 4C,D).

Lyve-1 IF evidenced that local hypoxia as well as DMOG treatment preserved sinusoidal diameter compared to collapsed sinusoids in PHx (PHx-LHA vs. PHx, $P < .0001$, DMOG-PHx vs. PHx, $P = .0001$) (Figure 8B). Thus, with the imposition of hypoxia, the diameter of sinusoids and the distribution of the sinusoids' size upon extended PHx (PHx-LHA or DMOG-PHx) are similar than after ALPPS (Figure 8C,D).

4 | DISCUSSION

The present study provides evidence that hypoxia, experimentally induced by the ligation of the hepatic artery or systemic administration of DMOG, rescues survival after extended hepatectomy. This sharply contrasts with the current theory on the origin of post-hepatectomy liver failure.[5,36] Our understanding of SFSS is incomplete but has been related to a small for flow syndrome, ie, a damage caused by an excessive portal blood flow into a small remnant.[5] Works over the years indeed support a major contribution of excessive shear stress and endothelial damage to liver failure.[5,37] When the full portal flow enters a much-reduced liver parenchyma, the pressure building up in the portal vein not only damages the sinusoidal bed but effectively vasoconstricts hepatic

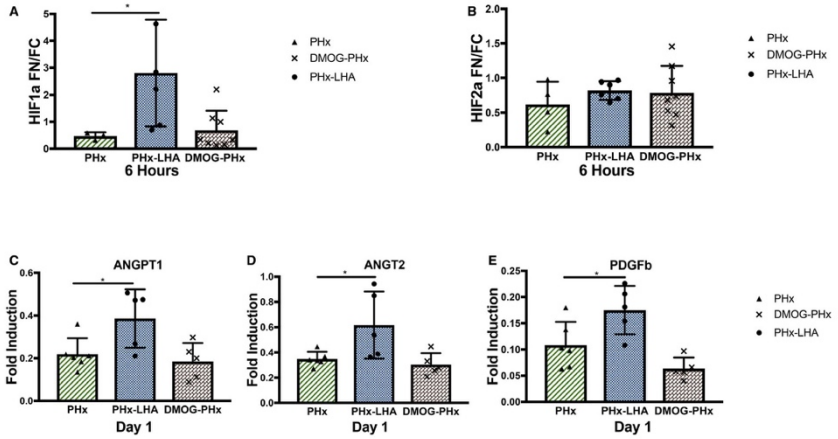

FIGURE 7 Hepatic artery ligature induced a hypoxic response the liver remnant. Ratio of nuclear to cytoplasmic HIF1α (A) and HIF2α (B), as measured by ELISA 6 hours post procedure in PHx (n = 3), PHx-LHA (n = 6), DMOG-PHx (n = 8). Angiopoietin 1 (C) and 2 (D) and PDGFb (E) gene expression measured by qPCR 1 day after the operation in PHx (n = 6), PHx-LHA (n = 5), DMOG-PHx (n = 5). Individual data, mean, and SEM are presented. Data were analyzed using ANOVA. ANOVA, analysis of variance; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; ELISA, enzyme-linked immunosorbent assay; HIF, hypoxia inducible factor; PDGFb, platelet-derived growth factor b; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean. *$P < .05$

arterioles. Such "de-arterialization" of the FLR is considered to be the cause of SFSS.[8] On the other hand, clinical and experimental studies support that increased portal flow/pressure triggers liver regeneration.[38] Shear stress is needed for regeneration[39] but increased flow itself is insufficient to stimulate liver growth as demonstrated by the inefficacy of arterio-portal shunt to enhance liver regeneration.[40]

In our experimental model, preconditioning of the FLR by portal vein ligation and in-situ split (ALPPS step I) prevented mortality and boosted liver hypertrophy[27] although the procedure caused the same sudden and steep rise of efficient portal inflow into the FLR as in SFSS.

The total portal flow significantly dropped after PVLT compared to Sham. This was however not associated with a compensatory rise of the total hepatic artery flow, as it would be expected according to the HABR concept. Data pertaining to liver hemodynamics in ALPPS in humans are scarce and conflicting. In their study, Schadde et al[26] found no difference in total portal venous flow during PVLT compared to baseline while Tomassini et al[25] recorded a significant reduction. In the sectorial right and left (to the FLR) arterial branches, the former but not the latter observed an HABR. The divergence between these two studies in similar surgical context highlights that the adaptation of liver hemodynamics during ALPPS step I is complex. The HABR is likely triggered at the level of hepatic microvasculature according to the "adenosine washout" theory, and involves multiple interrelated mechanisms for a dynamic regulation of hepatic inflow as to maintain, acutely and chronically, a constant hepatic blood flow-to-liver mass ratio.[6]

In our study, we measured the portal and arterial flow at the main vascular trunks, while the efficient portal or arterial inflow into the FLR was calculated (indexed portal/arterial flow). The indexed portal venous flow was significantly and similarly increased up to four times over controls both in ALPPS step I and in SFSS. Bucur et al recently showed that a portal venous flow per unit of liver mass exceeding four times the normal amount is incompatible with survival.[41] In our hands, the overflow was associated with mortality in the SFSS group but with survival and liver mass recovery in the ALPPS group. The hemodynamic differences came from the hepatic artery. The indexed arterial flow is much lower after ALPPS than SFSS. Thus, perfusion of the FLR with oxygenated blood is lower in ALPPS. We further demonstrate that FLRs in ALPPS are more hypoxic than after SFSS-setting hepatectomy. This suggests that although portal hyperperfusion plays a pivotal role in triggering regeneration, hypoxia is key to preventing liver insufficiency.

In accordance with clinical[42] and experimental studies,[43,44] we show here that liver insufficiency after hepatic resection in SFSS-setting is not due to the failure of hepatocytes to regenerate. Ninomya et al, however, suggested that liver failure is related to an asynchronous proliferation between hepatocytes and liver sinusoidal endothelial cells (LSEC), keeping clusters of newly generated hepatocytes away

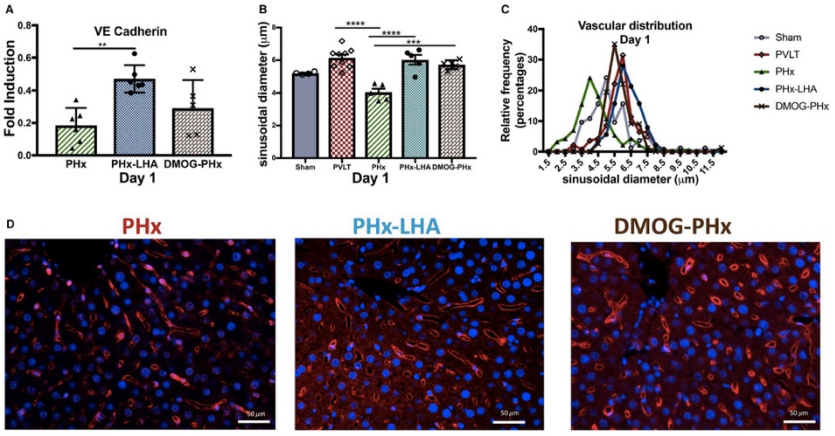

FIGURE 8 Induction of hypoxia maintains sinusoid patency in post-extended PHx remnants. (A) VE-Cadherin gene expression, (B) sinusoidal diameter, (C) distribution of sinusoid diameter as measured on Lyve-1 IF pictures in Sham (n = 4), and at day 1 in PVLT (n = 9), PHx (n = 6), PHx-LHA (n = 6), and DMOG-PHx (n = 5). (D) Representative picture of Lyve-1 IF in PHx, PHx-LHA, and DEMOG-PHx. Lyve-1 in red and nuclei in blue. Bar size = 50 μm. ANOVA one way showed significant increase in sinusoidal diameter in PHx-LHA and DMOG-PHx compared to PHx ($P < .0001$ and $P = .0001$, respectively), so as there is no difference in vascular morphology of these hypoxic models compared to ALPPS step I. ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; PVLT, portal vein ligation and transection. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

from sinusoidal structures. The authors showed that deceleration of hepatocyte division (using MEK/ERK pathway blockers) rescued animals' survival after 90% partial hepatectomy.[44] This could explain recent publications that claim that volume and postoperative FLR hypertrophy are not always predictive of function.[45] In our model, SFSS survivors had a significantly increased hepatocyte proliferation resulting in an enlargement of the liver remnant. Hypoxia did not affect liver mass recovery. Thus, the beneficial effect of hypoxia on survival in SFSS-setting hepatectomy is not due to enhanced parenchymal cell proliferation.

LSEC proliferation is needed to support the function in the regenerating liver.[46] Hypoxia might account for preservation of the sinusoidal bed, hepatic architecture, and thus function during regeneration. We indeed demonstrate that hypoxia in ALPPS step I was associated with early activation of hypoxia sensors HIF-1α and HIF-2α, known to play an important role in neovascularization, and with upregulation of VEGF and VEGF Rc2, the HIF-driven mediators of neo-angiogenesis. Kron et al[47] recently reported that hypoxia induces LSEC proliferation after 68% partial hepatectomy in mice. The authors suggested that HIF-2α was the hypoxic signal inducing LSEC proliferation. In our model, even if we observed both HIF-1α and HIF-2α upregulation in ALPPS compared to SFSS-setting hepatectomy, HIF-2α was not upregulated when hypoxic signals were induced by DMOG or hepatic artery ligation. Thus, the favorable impact of hypoxia on survival and sinusoidal preservation cannot be solely ascribed to HIF-2α activation. A surge of hypoxia in extended hepatectomy (such as obtained by hepatic artery ligation) triggers an early neo-angiogenic response as supported by upregulation of pro-angiogenic genes and VE cadherin. Activation of angiogenic signals, both in ALPPS and upon hepatic artery ligation, associates with enlarged sinusoidal diameter patency. DMOG administration had exactly the same effect. It is thus tempting to speculate that increased hypoxic signals in FLR stimulate early neo-angiogenesis (thus overcoming the asynchronism in hepatocyte-LSEC proliferation), rebuild an efficient sinusoidal bed and preserve the hepatic lobular architecture, thus maintaining the function of proliferating hepatocytes.

The release of danger signals by hypoxic hepatocytes might be an additional mechanism favoring the regeneration of the hepatic sinusoids. Damage associated molecular patterns such as HMGB1 are intranuclear proteins that translocates in the cytoplasm before being released in the extracellular space by necrotic cells or cells exposed to life-threatening stress, including hypoxia.[35,48] Besides activation of immune response, HMGB1 has been recognized as a putative proangiogenic factor capable to stimulate endothelial cell proliferation, attract circulating endothelial progenitors and improve neo-angiogenesis.[33,34,49,50] Here, we found enhanced circulating HMGB1

at early time points in ALPPS and DMOG-treated extended PHx, but not after hepatic artery ligation. Several groups studied ALPPS step I procedure and suggested that rapid liver regeneration was the response to the trauma caused by parenchymal transection.[51-53] In our experience, the rise in HMGB1 was mostly attributed to hypoxia due to portal vein ligation and abrogation of intrahepatic collaterals. Indeed, in our study, HMGB1 release was not ascribed to surgical trauma alone, as liver transection alone or kidney necrosis did not increase circulating HMGB1 (data not shown). This observation is shared by others.[48] Whether in a too small for survival liver remnant, HMGB1 release helps hepatic cells to turn hypoxic signals to their advantage and triggers tissue repair via neo-angiogenesis remains to be explored.

To our knowledge, this is the first study that compares FLR inflow hemodynamics in ALPPS and in SFSS-setting hepatectomy. We bring experimental evidence that sensing of hypoxia may be the catalyst of early neo-angiogenesis favoring the functional organization of the proliferating hepatocytes. Further studies are required to dissect the mechanisms that have a protective impact on SFSS-setting hepatectomy and test whether they apply in conditions of transplantation of a small graft. If verified, induction of hypoxia or pharmacological mimicry of hypoxia-driven response could be proposed during transplantation of a small graft at risk of SFSS.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AD, CB, IL. Performed the experiments: AD, VL, BP. Analyzed the data: AD, VL, BP, CB, IL. Contributed to writing: AD, CB, IL. Revised the manuscript: AD, CB, IL.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

All data are enclosed in the manuscript, there are no data available elsewhere.

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REFERENCES

1. Asencio JM, García Sabrido JL, Olmedilla L. How to expand the safe limits in hepatic resections? *J Hepatobiliary Pancreat Sci*. 2014;21:399-404.
2. Gad E, Shoreem H, Soliman H, et al. Small for size syndrome difficult dilemma: lessons from 10 years single center experience in living donor liver transplantation. *Transplantation*. 2014;98:783-784.
3. Virani S, Michaelson JS, Hutter MM, et al. Morbidity and mortality after liver resection: results of the patient safety in surgery study. *J Am Coll Surg*. 2007;204:1284-1292.
4. Balzan S, Belghiti J, Farges O, et al. The '50-50 criteria' on postoperative day 5. *Ann Surg*. 2005;242:824-829.
5. Asencio JM, Vaquero J, Olmedilla L, García Sabrido JL. "Small-for-flow" syndrome: shifting the "size" paradigm. *Med Hypotheses*. 2013;80:573-577.
6. Lautt WW. Regulatory processes interacting to maintain hepatic blood flow constancy: vascular compliance, hepatic arterial buffer response, hepatorenal reflex, liver regeneration, escape from vasoconstriction. *Hepatol Res*. 2007;37:891-903.
7. Asakura T, Ohkohchi N, Orii T, et al. Portal vein pressure is the key for successful liver transplantation of an extremely small graft in the pig model. *Transpl Int*. 2003;16:376-382.
8. Smyrniotis V, Kostopanagiotou G, Kondi A, et al. Hemodynamic interaction between portal vein and hepatic artery flow in small-for-size split liver transplantation. *Transpl Int*. 2002;15:355-360.
9. Michalopoulos GK. Liver regeneration after partial hepatectomy. *Am J Pathol*. 2010;176:2-13.
10. Vasavada BB, Chen CL, Zakaria M. Portal flow is the main predictor of early graft dysfunction regardless of the GRWR status in living donor liver transplantation—a retrospective analysis of 134 patients. *Int J Surg*. 2014;12:177-180.
11. Kinaci E, Kayaalp C. Portosystemic shunts for "too small-for-size syndrome" after liver transplantation: a systematic review. *World J Surg*. 2016;40:1932-1940.
12. Troisi R, Ricciardi S, Smeets P, et al. Effects of hemi-portocaval shunts for inflow modulation on the outcome of small-for-size grafts in living donor liver transplantation. *Am J Transplant*. 2005;5:1397-1404.
13. Umeda Y, Yagi T, Sadamori H, et al. Effects of prophylactic splenic artery modulation on portal overperfusion and liver regeneration in small-for-size graft. *Transplantation*. 2008;86:673-680.
14. Eipel C, Abshagen K, Ritter J, Al E. Splenectomy improves survival by increasing arterial blood supply in a rat model of reduced-size liver. *Transpl Int*. 2010;23:998-1007.
15. Mohkam K, Darnis B, Schmitt Z, Duperret S, Ducerf C, Mabrut JY. Successful modulation of portal inflow by somatostatin in a porcine model of small-for-size syndrome. *Am J Surg*. 2016;212:321-326.
16. Lauber DT, Tihanyi DK, Czigany Z, et al. Liver regeneration after different degrees of portal vein ligation. *J Surg Res*. 2016;203:451-458.
17. Eguchi S, Yanaga K, Sugiyama N, et al. Relationship between portal venous flow and liver regeneration in patients after living donor right-lobe liver transplantation. *Liver Transpl*. 2003;9:547-551.
18. Szijártó A, Fülöp A. Triggered liver regeneration: from experimental model to clinical implications. *Eur Surg Res*. 2015;54:148-161.
19. Makuuchi M, Thai BL, Takayasu K, et al. Preoperative portal embolization to increase safety of major hepatectomy for hilar bile duct carcinoma: a preliminary report. *Surgery*. 1990;107:521-527.
20. Schnitzbauer AA, Lang SA, Goessmann H, et al. Right portal vein ligation combined with in situ splitting induces rapid left lateral liver lobe hypertrophy enabling 2-staged extended right hepatic resection in small-for-size settings. *Ann Surg*. 2012;255:405-414.
21. Chan ACY, Chok K, Dai JWC, Lo CM. Impact of split completeness on future liver remnant hypertrophy in associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) in

hepatocellular carcinoma: complete-ALPPS versus partial-ALPPS. *Surgery*. 2017;161:357-364.
22. Linecker M, Kambakamba P, Reiner CS, et al. How much liver needs to be transected in ALPPS? A translational study investigating the concept of less invasiveness. *Surgery*. 2017;161:453-464.
23. Tschuor C, Croome KP, Sergeant G, et al. Salvage parenchymal liver transection for patients with insufficient volume increase after portal vein occlusion—an extension of the ALPPS approach. *Eur J Surg Oncol*. 2013;39:1230-1235.
24. Oldhafer KJ, Stavrou GA, Van Gulik TM, et al. ALPPS—Where do we stand, where do we go? Eight recommendations from the first international expert meeting. *Ann Surg*. 2016;263:839-841.
25. Tomassini F, D'Asseler Y, Giglio MC, et al. Hemodynamic changes in ALPPS influence liver regeneration and function: results from a prospective study. *HPB (Oxford)*. 2019;21:557-565.
26. Schadde E, Tsatsaris C, Swiderska-Syn M, et al. Hypoxia of the growing liver accelerates regeneration. *Surgery*. 2017;161:666-679.
27. Dili A, Lebrun V, Bertrand C, Leclercq IA. Associating liver partition and portal vein ligation for staged hepatectomy: establishment of an animal model with insufficient liver remnant. *Lab Invest*. 2019;119:698-707.
28. Kao TL, Kuan YP, Cheng WC, et al. Estrogen receptors orchestrate cell growth and differentiation to facilitate liver regeneration. *Theranostics*. 2018;8:2672-2682.
29. Madrahimov N, Dirsch O, Broelsch C, Dahmen U. Marginal hepatectomy in the rat. *Ann Surg*. 2006;244:89-98.
30. Krock BL, Skuli N, Simon MC. Hypoxia-induced angiogenesis: good and evil. *Genes Cancer*. 2011;2:1117-1133.
31. Da Silva Morais A, Lebrun V, Abarca-Quinones J, et al. Prevention of steatohepatitis by pioglitazone: implication of adiponectin-dependent inhibition of SREBP-1c and inflammation. *J Hepatol*. 2009;50:489-500.
32. Carreira CM, Nasser SM, Tomaso E, et al. LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res*. 2001;61:8079-8084.
33. Schlueter C, Weber H, Meyer B, et al. Angiogenetic signaling through hypoxia: HMGB1: an angiogenetic switch molecule. *Am J Pathol*. 2005;166:1259-1263.
34. Yang S, Xu L, Yang T, Wang F. High-mobility group box-1 and its role in angiogenesis. *J Leukoc Biol*. 2014;95:563-574.
35. Tsung A, Klune JR, Zhang X, et al. HMGB1 release induced by liver ischemia involves Toll-like receptor 4-dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med*. 2007;204:2913-2923.
36. Abshagen K, Eipel C, Vollmar B. A critical appraisal of the hemodynamic signal driving liver regeneration. *Langenbeck's Arch Surg*. 2012;397:579-590.
37. Fondevila C, Hessheimer AJ, Taurá P, et al. Portal hyperperfusion: mechanism of injury and stimulus for regeneration in porcine small-for-size transplantation. *Liver Transplant*. 2010;16:364-374.
38. Sato Y, Koyama S, Tsukada K, Hatakeyama K. Acute portal hypertension reflecting shear stress as a trigger of liver regeneration following partial hepatectomy. *Surg Today*. 1997;27:518-526.
39. Mortensen KE, Conley LN, Hedegaard J, et al. Regenerative response in the pig liver remnant varies with the degree of resection and rise in portal pressure. *AJP Gastrointest Liver Physiol*. 2008;294:G819-G830.
40. Clarke AM, Thomson RYFG. Vascular factors in liver regeneration. *Surg Gynecol Obs*. 1968;126:45-52.
41. Bucur PO, Bekheit M, Audebert C, et al. Modulating portal hemodynamics with vascular ring allows efficient regeneration after partial hepatectomy in a porcine model. *Ann Surg*. 2018;268:134-142.
42. Gridelli BG. Liver volume restoration and hepatic microarchitecture in small-for-size syndrome. *Ann Transplant*. 2015;20:381-389.
43. Ninomiya M, Harada N, Shiotani S, et al. Hepatocyte growth factor and transforming growth factor beta1 contribute to regeneration of small-for-size liver graft immediately after transplantation. *Transpl Int*. 2003;16:814-819.
44. Ninomiya M, Shirabe K, Terashi T, et al. Deceleration of regenerative response improves the outcome of rat with massive hepatectomy. *Am J Transplant*. 2010;10:1580-1587.
45. Olthof PB, Schadde E, van Lienden KP, et al. Hepatic parenchymal transection increases liver volume but not function after portal vein embolization in rabbits. *Surgery*. 2017;162:732-741.
46. Ding B-S, Nolan DJ, Butler JM, et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature*. 2010;468:310-315.
47. Kron P, Linecker M, Limani P, et al. Hypoxia-driven Hif2a coordinates mouse liver regeneration by coupling parenchymal growth to vascular expansion. *Hepatology*. 2016;64:2198-2209.
48. Liu A, Dirsch O, Fang H, et al. HMGB1 translocation and expression is caused by warm ischemia reperfusion injury, but not by partial hepatectomy in rats. *Exp Mol Pathol*. 2011;91:502-508.
49. Vénéreau E, Ceriotti C, Bianchi ME. DAMPs from cell death to new life. *Front Immunol*. 2015;6:1-11.
50. Chavakis E, Hain A, Vinci M, et al. High-mobility group box 1 activates integrin-dependent homing of endothelial progenitor cells. *Circ Res*. 2007;100:204-212.
51. Schlegel A, Lesurtel M, Melloul E, et al. ALPPS: from human to mice highlighting accelerated and novel mechanisms of liver regeneration. *Ann Surg*. 2014;260:839-847.
52. Yao L, Li C, Ge X, et al. Establishment of a rat model of portal vein ligation combined with in situ splitting. *PLoS ONE*. 2014;9(8):1-8.
53. Dhar DK, Mohammad GH, Vyas S, Broering DC, Malago M. A novel rat model of liver regeneration: possible role of cytokine induced neutrophil chemoattractant-1 in augmented liver regeneration. *Ann Surg Innov Res*. 2015;9:11.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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DISCUSSION

When I started this research work in 2015, ALPPS was a newborn technique with scarce clinical data reporting accelerated and enhanced liver hypertrophy. As a liver surgeon, I was fascinated by this new technique able to enhance the kinetic growth ratio in future liver remnants. ALPPS allowed curative treatment in patients who did not have enough FLR hypertrophy after portal vein occlusion to undergo liver resection. Laboratory data on ALPPS's mechanistic insights were scarce. A few studies suggested that liver partition in ALPPS step I was the trigger for liver hypertrophy, and the mechanism proposed was the massive release of inflammatory mediators. I was very eager to unravel the 'ALPPS-effect' as it may provide clues to trigger and accelerate liver regeneration in patients in need of extended liver resection. This was the starting point of this thesis.

1. ESTABLISHMENT OF A NOVEL ALPPS MODEL

First, we established a rat model to reproduce ALPPS. In 2015, several rodent models of ALPPS were published (Table 1) and attributed the enhanced liver regeneration to the *in-situ* split accompanying portal vein occlusion. However, for anatomical reasons linked to rodent liver vascular anatomy, researchers proposed a model of ALPPS keeping the right median lobe as a future liver remnant. This represents approximately 30% of the total liver volume. Thus, a large FLR is left in place, as the resection corresponds to a 70% partial hepatectomy, a historical model with excellent survival in which 7 days suffice to achieve full regeneration.[48] This contrasts with FLR in the ALPPS setting which is, by definition, small and insufficient for survival. It is well documented that the rate of liver regeneration is related to the extent of hepatic resection and occlusion of the portal bed.[46,75,98] In the context of extended hepatectomy, Zhang *et al.*[241] even highlighted that a 5% difference in the volume resected significantly impacts upon mortality and

regeneration rates. In this regard, in our opinion the above model does not mimic the minimal, insufficient-for-survival FLR that would impose an ALPPS procedure in humans.

Other investigators combined partial liver resection (8–30%) during the first step of ALPPS. Previous literature abundantly supports that such a resection cannot be seen as trivial, as it is sufficient to 'prime' the liver and engage a regenerative response.[36] Even a minimal resection can cause systemic or hepatic haemodynamic alterations, thereby introducing a confounding factor when analyzing the ALPPS-dependent effects.

Based on these considerations, we chose to develop a rat animal model with insufficient-for-survival FLR, without previous liver resection. Thus, we first evaluated the relative volume of each segment in rat controls and decided to use the left part of the median lobe (10% of the total liver) as FLR. This, together with the peri-caval tissue, would correspond to an 87% partial hepatectomy, which proved to be an SFSS-setting hepatectomy given the high 84% mortality rate. Importantly, most of the deaths occurred during the first 2 postoperative days, a timing that corresponds to the first round of hepatocyte proliferation in rats. When the ALPPS step I procedure preceded the SFSS-setting hepatectomy, the 7-day survival rate increased to 75% ($P = 0.0002$). Survival was associated with accelerated liver growth in the ALPPS group compared to the PVL-PHx group, while there was no significant difference in FLR hypertrophy between ALPPS and SFSS-setting hepatectomy during the first 3 postoperative days. The degree of liver hypertrophy (Weight LML/BW at the time of sacrifice) and kinetic growth ratio confirmed that parenchymal transection combined with PVL triggers FLR mass recovery more than PVL alone, which corroborates the clinical observation in humans. After the 1st step procedure (PVL or PVLT), the body weight of all animals dropped, suggesting a catabolic general state relative to the operation. However,

there was no significant difference in body weight evolution between the 2 surgical procedures, as the median in decrease body weight was 6,66% (range 2,4-8,38) in the PVL group and 6,64% (range 5,78-6,95) in the PVLT group, even though animals in PVL group tended to fully recover their initial body weight on POD7 compared to PVLT group animals. When we analyzed the FLR (left median lobe) mass recovery reported to the initial body weight, there was still a significant increase in the liver mass in the PVLT group on post-operative day 3 ($p<0,05$) *(data not shown).* Triggered hypertrophy in ALPPS was associated with signs of liver parenchymal stress as assessed by transaminase measures (aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), significantly higher in ALPPS at post-operative day 1 ($p<0,0001$) and 2 ($p=0,004$) compared to SFSS-hepatectomy survivors. Cytolysis in the ALPPS group significantly dropped by post-operative day 3 and normalized by POD 7 *(data not shown)*. Of note, in SFSS-hepatectomy, serum bilirubin was significantly higher compared to ALPPS step I at POD1 ($p=0,0002$) and POD2 (0,03).

Moreover, the boost of FLR hypertrophy in our model was associated with hepatocyte proliferation, as assessed by BrdU and ki67-positive hepatocyte nuclei in ALPPS step I. The same features were observed by other investigators[242,238], supporting that FLR mass recovery in ALPPS is not related to interstitial oedema or intracellular fat accumulation.[236,235] Some authors suggested that highly proliferative small hepatocytes might be non-functional.[237,239] Nevertheless, highly proliferative hepatocytes were associated with survival in our study, suggesting that ALPPS conferred functional liver regeneration.

Thus, we established of a novel 'clean' rat model of ALPPS, with minimal FLR, leading to high mortality rates unless ALPPS is applied.

2. ALPPS MECHANISTIC INSIGHTS

In the early reports of ALPPS, most authors had already attributed the enhanced FLR mass recovery in ALPPS step I compared to portal vein occlusion to the parenchymal transection associated to PVO. An *in-situ* split causes parenchymal necrosis (cellular and tissular), stresses and trauma, all processes resulting in a massive proinflammatory surge immediately after the operation. According to Fausto's humoral theory, such a release of inflammatory factors triggers liver regeneration (see section 1.2.5.1). The same effect may also explain and be responsible for the systemic inflammatory response syndrome, also known as SIRS or SIRS-like syndrome,[243,244,] observed in some ALPPS patients in clinics. Of note, surgical procedures of similar invasiveness, i.e. split liver in liver transplantation or living liver donation, which also include a complete liver transection, were shown to induce comparable systemic inflammation and cytokine release in patients.[245] If we combine the inflammatory cytokine systemic rush to the organism's catabolic state[230] in the context of a major operation, we can probably explain the high morbidity rates initially reported in ALPPS. If this inflammatory storm is effectively happening, then the proposition of several authors to prolong the interstage period to allow the organism to recover prior to ALPPS step II makes a great deal of sense (see section 3.2). However, as we (data not shown) and others[225] observed, isolated parenchymal transection prior to extended hepatectomy, without PVL-redirection of the portal flow, has absolutely no effect on FLR hypertrophy. This observation supports that portal hyperperfusion, next to the inflammatory storm, is key to obtain the 'ALPPS effect'.

After portal vein occlusion, the reperfusion of the intraparenchymal portal branches of the occluded part of the liver is possible via already-existing as well as newly formed porto-portal shunts.[170,177,233] Such intrahepatic collaterals mitigate

the portal hyperperfusion in the FLR.[246,247] In the case of ALPPS, *in-situ* split, by definition, abolishes intraparenchymal porto-portal shunts.[233] As a result the portal blood flowing through the FLR is higher that PVO alone. Indeed, in our study we observed a significantly higher increase in portal flow in the FLR immediately after the ALPPS step I procedure compared to PVL. This increased intrasinusoidal pressure[229] and flow may cause more shear stress, a contributing trigger for liver regeneration. Thus, ALPPS-boosted hypertrophy could be explained by the 'haemodynamic theory' of liver regeneration, developed in Chapter I (see section 1.2.5.2).

More recent experimental work has suggested that portal hyperperfusion due to abrogation of collateral flow results in compensatory arterial hypoperfusion (HABR) and hypoxia in the FLR, and this phenomenon could also play a key role in modulating regenerative kinetics.[232]

3. PORTAL HYPERPERFUSION: A DOUBLE-EDGED SWORD

Our experimental study of hepatic inflow showed portal hyperperfusion of the FLR in both PVL and ALPPS step I models compared to baseline. While after PVL there was a two-fold increase of the indexed portal inflow (iPVF = portal flow per gram of perfused tissue) (data not shown), in ALPPS it increased as high as four times the baseline. Moreover, immediately after the operation, the iPVF significantly increased in the ALPPS step I procedure compared to the PVL (P = 0.004), condition associated with enhanced FLR mass recovery.

As mentioned in the Introduction, portal hyperperfusion, the ensuing increased shear stress and the delivery of hepatotrophic factors, into the liver remnant have been proved to be a stimulus of liver regeneration (see section 1.2.5.2).[1] In the

setting of critically small grafts in liver transplantation, portal hyperperfusion is linked to high hepatocyte proliferation rates, even if SFSS and mortality occurs.[60,248] Conversely, a four-fold increase in portal flow has been shown to induce liver damage that compromises survival. The negative impact of portal hyperperfusion on FLR survival is so well established that authors propose to rename the SFSS as ***'small-for-flow syndrome'***. Moreover, reduction of the portal inflow is now part of the prevention and therapeutic arsenal against SFSS (see sections 2.2.4.1 and 2.2.5). Despite this, our work supports that a dramatic increase in portal inflow prevents mortality and induces functional liver regeneration in ALPPS.

In ALPPS, portal vein ligation redirects the entire portal blood flow into a small liver sinusoidal network (as with portal vein occlusion), while the parenchymal transection excludes all the intrahepatic sinusoidal collaterals and, hence, possibilities of intrahepatic shunts. In line with our study, other authors report an immediate and steep rise of the portal pressure and flow into the liver remnant in ALPPS step I.[232,240] However, while severe portal haemodynamic stress in critically small FLR in ALPPS is considered to be the origin of enhanced hypertrophy, a similar haemodynamic condition is linked to liver failure in an SFSS-setting hepatectomy.

To the best of our knowledge, no study has compared the portal haemodynamics in these two surgical situations. This paradox, but also the assumption that SFSS-FLRs in ALPPS step I would be submitted to the same portal haemodynamic stress as in SFSS-setting hepatectomies, prompted us to explore the haemodynamics of FLR inflow. We therefore compared portal flow in ALPPS step I and SFSS-setting upfront hepatectomy, with exactly the same initial future liver remnant, and observed that the portal flow through the FLR is significantly increased up to four-fold of the baseline, with no difference between ALPPS step I and SFSS-setting hepatectomy. Bucur *et al.*[249] showed that a portal venous flow per unit of liver mass

equal to or greater than four times the control is incompatible with survival. Similarly, in clinics, when the portal inflow increases to four times the baseline, graft inflow modulation is recommended to prevent SFSS.[96] In our hands, the portal hyperperfusion was indeed associated with high mortality rates in the upfront SFSS-setting hepatectomy (84% at 7 days), but with significantly improved survival when the liver was 'preconditioned' by ALPPS step I. Moreover, survival in ALPPS was associated with rapid and enhanced hypertrophy.

Thus, our study not only supports that portal hyperperfusion is a trigger for liver regeneration, but also suggests that portal haemodynamic stress is not the sole pathogenic factor of SFSS.

4. HEPATIC ARTERY INFLOW

4.1. Arterial inflow in ALPPS

When the whole portal blood is redirected through a small sinusoidal network, there is a compensatory constriction of the hepatic artery (HABR). The ensuing de-arterialization of the remnant is considered to be the main cause of liver dysfunction in an SFSS-setting hepatectomy.[15] Surprisingly, in our model the hepatic artery flow entering the FLR in the extended hepatectomy group (animals that died) was significantly higher compared to ALPPS step I (animals that survived). ALPPS arterial inflow was also significantly lower compared to controls. We further confirmed that, immediately after the operation, FLRs in ALPPS were hypoxic compared to controls and significantly more hypoxic than SFSS livers.

4.2. The hepatic arterial buffer response (HABR) and the SFSS

When analyzing the arterial inflow in the FLRs in the upfront SFSS-setting hepatectomy of our model, we observed that the hepatic artery flow per tissue perfused (iHAF=indexed hepatic artery flow) was dramatically increased compared to controls, suggesting the absence of HABR in this context. This surprising finding has also been reported by Dold *et al.*[31] The authors observed that a patent portal hyperperfusion after 90% PHx in rats did not induce a HABR, even though FLRs showed decreased hepatocellular oxygenation together with a reduced mitochondrial redox state. In line with Dold *et al*.'s work we also observed that, while the hepatic artery flow into in the FLR of upfront extended hepatectomy was significantly increased compared to controls, SFSS livers were hypoxic while controls not. Another confusing consideration is that, even if the HABR exists in SFSS-setting hepatectomies (as suggested by several authors), we have seen in the Introduction that after PHx there is an increase of portal inflow into the remnant. Indeed, after 70% PHx there is a two-fold increase of portal perfusion which increases to four-fold after 90% PHx.[31] Even if the portal blood is less oxygenated than arterial blood, it does contribute to 50–70% of the liver oxygen supply/requirements under resting conditions (see section 1.1.4). These considerations raise the question of the mechanisms for hypoxia in the remnants in SFSS-setting hepatectomy.

4.2.1 *Why are SFSS remnants hypoxic?*

To the best of our knowledge, the role of portal oxygen supply in a regenerating liver is not well elucidated. In trying to understand this observation, it must be considered that portal hyperperfusion is associated with an increased shear stress

which is capable of causing endothelial damage, including cell swelling, sinusoidal narrowing and denudation.[27,60,250] This could be the cause of impaired oxygen diffusion and uptake by the liver. This hypothesis is, however, contested by authors who found that the functional sinusoidal density after extended PHx is only slightly affected.[31] Another possible explanation could be that, after extended PHx or LT of a small graft, the metabolic demands on the liver remnant increase (increased metabolic load to the reduced liver together with the energy requirement for DNA replication and cell division). Hence, high oxygen consumption renders the liver hypoxic. Finally, we have seen in the Introduction that during the early phase of liver regeneration after PHx, the proliferation of LSEC is initiated after the first round of hepatocyte proliferation. The asynchrony between these events may cause a transient disorganization of the lobule and explain the observed hypoxia in SFSS-setting FLRs. This hypothesis may also explain the correlation between the degrees of liver resection (and thus of hepatocyte proliferative stimulus) and tissue hypoxia.

4.3. Portal and arterial inflow in ALPPS

Analysis of the hepatic inflow in this experimental work supports that portal hyperperfusion is equal in SFSS-setting hepatectomy and ALPPS step I, while ALPPS FLRs receive less arterial flow and are significantly more hypoxic. This compels us to conclude that, in striking contrast of what is reported in the literature, although portal hyperpefusion plays a pivotal role in triggering regeneration, liver de-arterialization and hypoxia could be key to the prevention of liver insufficiency during regeneration of a small liver remnant.

5. SFSS AND HYPOXIA: A LESSON LEARNED FROM THE ALPPS PROCEDURE IN RATS

We know that the regenerative liver requires enormous amounts of oxygen to satisfy the increased metabolic demand imposed by cell proliferation.[90] Mitochondrial oxidative phosphorylation consumes approximately 90% of the cellular O_2 to produce ATP. In the face of a large proliferative stimulus such as a small FLR, sustained hepatocyte proliferation occurs prior to efficient angiogenesis causing (transient) microcirculatory disturbances and temporospatial disorganization of the lobule. This may lead to insufficient oxygen and energy delivery, alteration of the mitochondrial redox state[251] and impaired ATP production.[252] It is thus temping to conclude that hypoxia in the regenerative liver has a deleterious impact.

However, the impact of hypoxia on liver regeneration is far from elucidated. Adding to the complexity, there is a physiological gradient of oxygen tension across the hepatic lobule from the periportal to perivenous hepatocytes, and, thus, the way the hepatocytes tolerate, adapt and respond to hypoxic stress may differ according to their location. Hypoxia induces an impaired intracellular redox state that produces reactive oxygen species (ROS). ROS can act as signaling molecules and activate mitogen pathways such as MAPK cascade (ERK1/2, c-jun),[253] cascades known to actively initiate and participate in liver regeneration.

Moreover, hypoxia elicits 'anapyrexia', a regulated decrease of body temperature. Anapyrexia reduces oxygen consumption, increases the affinity of hemoglobin for oxygen, and blunts the energetically costly responses to hypoxia (ventilatory and cardiovascular responses).[254] Most importantly, as stipulated by the Arrhenius

equation, the cellular metabolic rate is reduced by 50% for every 10°C drop in temperature.[255] Thus, anapyrexia could contribute to the preservation of ATP by reduction in hepatocyte metabolic rate and reduction in ATP consumption. As a result, acidosis is avoided, which would otherwise be associated with metabolic dysregulation, while ATP depletion is deterred, which could otherwise lead to necrosis. As such, in liver transplantation, hypothermia decreases the risk of ischemia/reperfusion injury.[255]

In addition, oxygen-sensing mechanisms respond rapidly to low oxygen by activating hypoxia-inducible factors (HIFs). HIF-1α and -2α are cytosolic heterodimeric transcription factors consisting of two subunits in the active state (α and β). While the β-subunit is constitutively expressed, the oxygen-sensitive α-subunit is constantly hydroxylated by propyl-hydroxylase domain (PHD) proteins under normoxia. Of note, PDH hydroxylase activity is not only regulated by oxygen. Other co-factors may regulate its activity, such as 2-oxo-glutarate, iron and ascorbate.[256] Hydroxylated α-subunits are scaffolded on a multimeric protein complex of the Von Hippel–Lindau tumor suppressor gene (VHL), which leads to rapid ubiquitination and proteasomal degradation. Under hypoxia or alterations of the redox cellular state, HIF α subunits escape hydroxylation, form dimers with β-subunits, translocate into the nucleus where they bind with hypoxia-responsive elements (HRE) and regulate the expression of target genes. Although hypoxia is the principal regulator for HIF activity, other conditions such as ionizing radiation, environmental stress and reactive oxygen species may result in HIF nuclear accumulation[63] (Fig. 15).

Hypoxia inducible factors may favorably impact liver regeneration by the following pathways:

- First, HIF-induced target genes adapt the cell metabolism to the hypoxic condition by reducing cell oxygen consumption: HIF induces glycolysis,[63] increases glucose uptake and gluconeogenesis[257] and reduces mitochondrial respiration.[258] As mentioned above, Matsuo *et al.*[237] performed liver biopsies in patients undergoing rapid liver regeneration after the ALPPS step I procedure and observed high glycogen concentrations in regenerating hepatocytes. Increased cell glycogen may sign cellular adaptation to hypoxia (HIF-1α-dependent), so that cells can confront low oxygen concentrations, survive glucose deprivation[259] and divide during liver regeneration.[257] In addition, experimental data support that loss of PHD1, as in PHD knockout mice, protects hepatocytes against ischaemic stress compared to wild-type animals in a HIF-2α-dependent manner. Upon hepatectomy, loss of PDH-1 reduced the oxidative stress [as attested by 8-oxo-2'-deoxyguanosine immunohistochemistry (IHC)], lowered oxygen cellular consumption [as attested by up-regulation of puryvate dehydrogenase kinase (PDK-1)], attenuated hepatocyte swelling and the resultant compression of sinusoids, thus improving microcirculation in the remnants.[260,261] Treatment of hepatectomized mice with a PHD-inhibitor increased the expression of cell cycle-promoting cyclins in FLRs, thus triggering liver regeneration. In our experimental work, we also found an up-regulation of HIF-2α in ALPPS step I remnants compared to SFSS. However, when we induced hypoxia in SFSS-setting hepatectomy by hepatic artery ligation or activated hypoxia sensors by dimethyloxalylglycine (DMOG), we did not find any difference in HIF-2α nuclear concentrations between the animals that survived and those that

died. This observation suggests that in our experimental setting, at least, HIF-2α is not key for hepatoprotection and prevention of SFSS.

- Secondly, HIF-1α (and HIF-2α) activates the transcription of proangiogenic genes that increase vascular permeability and promote endothelial cell proliferation, sprouting, migration, adhesion and neo-vessel formation[63] (Fig. 15). As such, HIFs factors are the 'master regulators' of angiogenesis. In addition, activation of hypoxia sensors in the liver promotes the proliferation, mobilization and engraftment into the regenerating liver of BM-SPC via a VEGF and SDF-1 signaling pathway.[67,69,262] As developed in Chapter I (see section 1.2.4), the crucial role of angiogenesis and BM-SPC in liver regeneration has been repeatedly pointed out by several research groups.[62,67,69,262] Several studies have also reported the bone marrow-derived sinusoidal progenitor cells as a valuable source of hepatocyte growth factors once homed in the regenerating liver.[64,69,262] Whether this occurs during physiological regeneration or in conditions that cause endothelium damage is still debated.[263] Data from our work support that, during the early time-points after surgery, hypoxia sensors (HIF-1α and -2α) were highly activated in ALPPS step I compared to SFSS-setting FLRs, and this was linked to up-regulation of several neoangiogenic genes, sinusoidal endothelial cell proliferation (as assessed by up-regulation of CD31 and VE-cadherin) and a preserved morphology of the liver sinusoids.

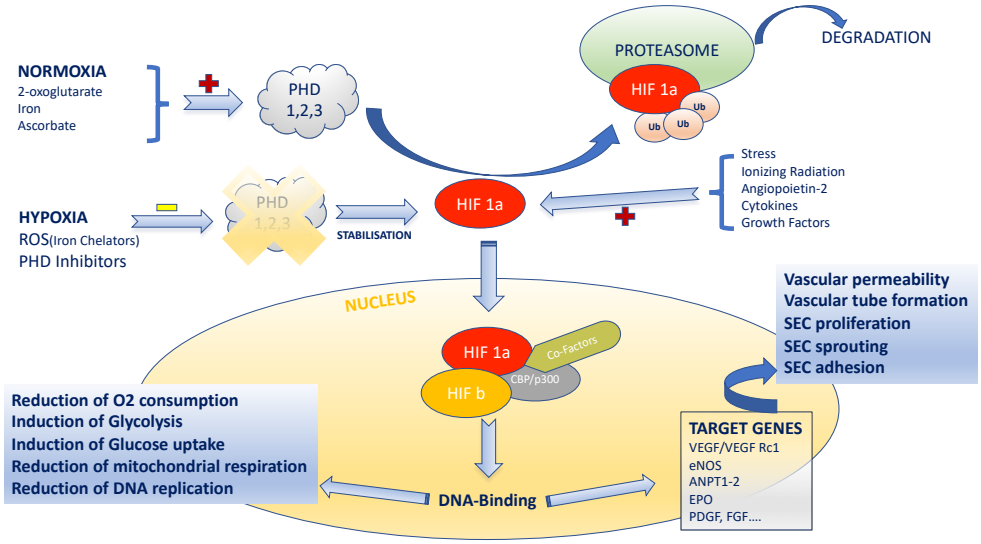


Figure 15. HIF-1α activation mechanism and effects on cellular metabolism and neoangiogenesis (inspired and adapted from Fong, *Angiogenesis*, 2008 and Krock, *Genes and Cancer*, 2011).[63]

Other hypoxia-induced mechanisms may be at play to induce angiogenesis into the avascular regenerating hepatocyte clusters. Damage-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1), are nuclear molecules that have a physiological 'daytime job' inside the cell (regulation of genome replication, recombination, DNA repair). They are passively released in the extracellular space when cells undergo a life-threatening insult such as hypoxia. There they signal the cell damage to the environment. HMGB1, through advanced glycation end products receptor (RAGE) and Toll-like receptor 4 (TLR-4), mediates various actions such as the release of angiogenic cytokines and the proliferation of bone marrow sinusoidal progenitor cells, as well as their migration, sprouting and homing in the damaged tissue. Consequently, HMGB1 drives angiogenesis[264,265] (Fig. 16). In ALPPS, Schlegel *et al.*[225] correctly interpreted HMGB1 release as a marker of tissue damage. For us, increased systemic HMGB1 release observed in our ALPPS model is also a mechanism by which hepatic cells can turn ischaemic signals to their advantage and

trigger ‘tissue repair’ by neoangiogenesis. In our animal model, the magnitude of HMGB1 systemic release early after the operation correlated with the degree of tissue hypoxia in the FLR. As such, there was significantly more HMGB1 ELISA release in the circulation after ALPPS step I and SFSS-setting hepatectomy combined with hepatic artery ligation compared to SFSS-setting hepatectomy alone.[266] By contrast, complete organ necrosis, by kidney or splenic trauma, did not induce a significantly higher systemic HMGB1 translocation, suggesting that complete cell necrosis does not result in DMAPS translocation (*data not shown).*

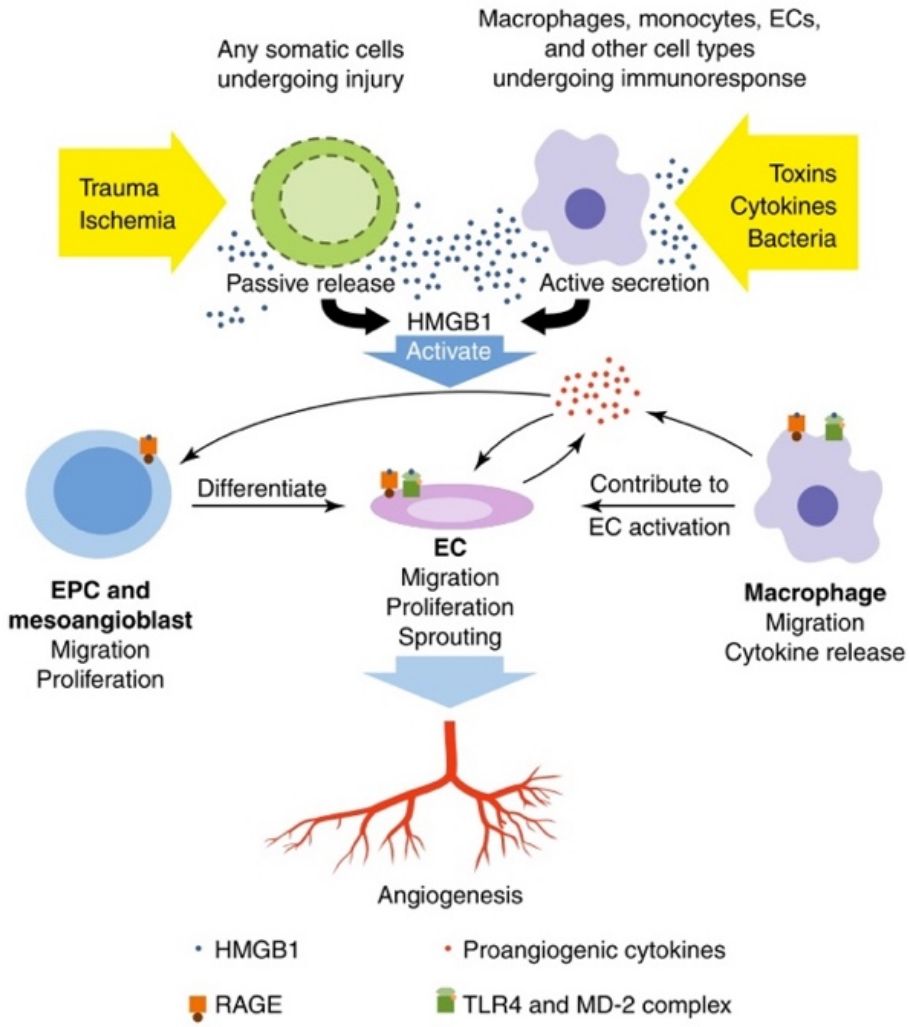


Figure 16. Schematic representation of the HMGB1 contribution to angiogenesis in response to hypoxia. HMGB1 directly contributes to vessel formation by promoting endothelial cell proliferation, migration and sprouting (from Yang, *Journal of Leucocyte Biology*, 2014).[265]

We have seen that after partial hepatectomy there is a transient perturbation of the lobular architecture, with proliferating hepatocytes forming avascular clusters. As the magnitude of hepatocyte proliferation correlates with the extent of hepatectomy,[46,267,268] a major, SFSS-setting resection triggers a large regenerative response of hepatocytes and consecutively large avascular hepatocyte islands.[58] We hypothesize that such lobular disorganization resulting from a high regenerative response causes liver dysfunction and that hypoxia in ALPPS activates an early angiogenic response to prevent mortality and liver failure. Deceleration of hepatocyte division allowed survival improvement in an SFSS-setting hepatectomy, due probably to a better synchronization between hepatocytes and LSEC proliferation.[58,267] As highlighted by Belghiti *et al.*[268] and Gruttadauria *et al.*,[60] among others, the excess of regeneration after major PHx or LT of a small graft in humans independently predicts the SFSS.

Accordingly, **we propose that the primary goal of therapy or prevention of SFSS or PHLF should be to trigger an angiogenic switch to balance angiogenesis with hepatocyte proliferation during the early phase of liver regeneration**.

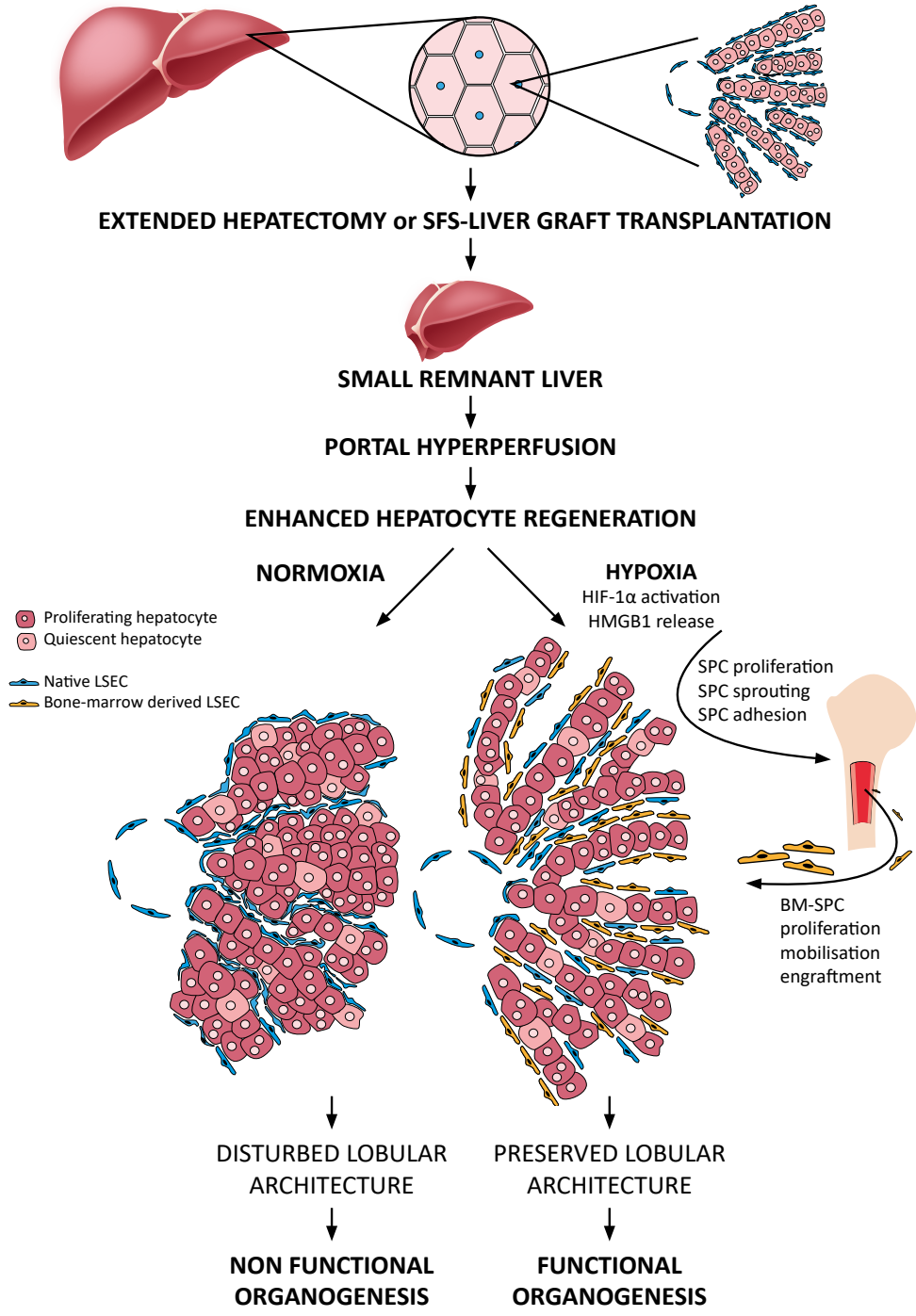
In our animal model, we observed similar FLR hypertrophy in both ALPPS step I and SFSS-setting hepatectomy linked to portal hyperperfusion. We also observed hypoxia in FLR after both surgical technics. Hypoxia has been reported to be at the origin of vascular leakage and edema in several brain or pulmonary diseases[269,270] while within seconds of cessation of blood flow, energy metabolism shifts from mitochondrial respiration to anaerobic glycolysis, lactate and protons accumulate in cells, inducing acidosis, osmotic load and then cell edema.[271] In our animal model, tissue edema, that could be relative to hypoxia, has not been assessed. However, FLR hypertrophy did correlate with hepatocyte proliferation (as assessed by Ki67 positive hepatocytes) and a higher mitotic index in the ALPPS

group compared to the PVL group and to the SFSS-setting hepatectomy.[272] Our observation is in accordance to the human study conducted by Eshmuminov et al in 2017.[236] The authors investigated the hepatocyte proliferation and water fraction (interstitial edema or cell swelling) by magnetic resonance imaging in the FLR of patients that underwent ALPPS. They observed that ALLPS's remarkable increase of the FLR represents efficient hepatocyte proliferation rather than increased steatosis or cellular edema.

Here, we provide experimental evidence to the concept that a surge of hypoxia drives a very early neoangiogenic switch (at 6 and 24 h after PHx), resulting in preserved sinusoidal diameter, patency and morphology during the first phase of liver regeneration. This remodeling of the sinusoidal architecture is of the utmost importance in the critical situation of SFSS-setting hepatectomy, as it enables functional organization of the regenerating hepatocytes with a favorable impact on survival. When we experimentally induced hypoxia in SFSS-setting hepatectomies, survival rates significantly improved. This fascinating event could not be attributed to improved FLR mass recovery, as there was no difference between the groups. Upon hypoxia that prevents mortality, the density of patent sinusoids was higher early after extended SFSS-setting hepatectomy compared to non-hypoxic lethal SFSS-FLRs, supporting that there is an extension of the liver sinusoidal vascular network (angiogenesis).[266] Based on the data we have shown, we would like to propose the hypothesis that hypoxia induces an early angiogenic switch, improves cellular 'crosstalk' mainly by increasing the recruitment of endothelial progenitor cells, preserves sinusoidal bed morphology and restores the lobular liver architecture allowing efficient liver regeneration after major hepatectomy[68,69,273] (Fig. 17). Of course, this calls for future studies focusing on the mechanism and the necessary degree of activation of hypoxia sensors to obtain early neoangiogenesis without harming the patient.

Figure 17: Hypoxia in the rapid regenerating remnant promotes neoangiogenesis as to preserve the functional architecture

design by Gaêlle De Jesus Silva


EXTENDED HEPATECTOMY or SFS-LIVER GRAFT TRANSPLANTATION
SMALL REMNANT LIVER
PORTAL HYPERPERFUSION
ENHANCED HEPATOCYTE REGENERATION
NORMOXIA
HYPOXIA
HIF-1α activation
HMGB1 release
Proliferating hepatocyte
Quiescent hepatocyte
Native LSEC
Bone-marrow derived LSEC
SPC proliferation
SPC sprouting
SPC adhesion
BM-SPC
proliferation
mobilisation
engraftment
DISTURBED LOBULAR ARCHITECTURE
PRESERVED LOBULAR ARCHITECTURE
NON FUNCTIONAL ORGANOGENESIS
FUNCTIONAL ORGANOGENESIS

FUTURE PERSPECTIVES & TRANSLATIONAL POTENTIAL

Unsolved questions remain. First, confirming our findings in other models of hypoxic SFSS-setting hepatectomy appears unavoidable. Even if ligation of the common hepatic artery allowed us to take the first step in the understanding of hypoxia-induced hepatoprotection, other, less invasive, approaches to induce hypoxia and early angiogenesis should be investigated, especially if translation of the data is envisioned. Secondly, we need to better characterize the degree and timing of sinusoidal endothelial cell proliferation and the dynamics for effective angiogenesis. Thirdly, it would also be interesting to assess if hypoxia-induced angiogenesis relies upon liver-native sinusoidal cells and/or endothelial progenitor cells and to identify the mechanisms driving the angiogenic program. Fourthly, given that most of the extended hepatectomies are performed for the treatment of liver tumors, we could not omit investigating the effect of hypoxia-induced angiogenesis on tumor growth and biology. Lastly, we should assess the minimal degree of hypoxia necessary to trigger effective liver regeneration.

We propose to investigate these questions in a step-by-step approach in the perspectives of this thesis work.

1. MOUSE MODEL OF SFSS-SETTING HEPATECTOMY AND HYPOXIA CHAMBERS

We currently use a mouse model of SFSS-setting hepatectomy. In contrast to our previous work on rats, but in line with others,[58] resection of all liver lobes except the caudate (90% PHx) show 100% mortality during the first 24 h after the operation. This very early mortality probably reflects major systemic haemodynamic perturbation associated with resection rather than the induction of SFSS, so we abandoned this technique. We thus established an SFSS-setting partial hepatectomy of 80% (PHx 80%), keeping the posterior part of the right lobe and the caudate lobe as FLR. This model shows high mortality rates of up to 68% at 7 days

while, as in our rat model, most deaths occur during the first 72 h after the operation (56%). We use a 70% partial hepatectomy model (resection of the left lateral and median lobe) as control animals, as this well-established model of liver regeneration is perfectly tolerated.[274]

To test the impact of hypoxia, we first ligate the hepatic artery at the hilum, while proceeding with PHx 80% (PHx 80%-HAL). The procedure tends to have a favorable, although not significant, impact on survival (75% on day 3, 56% on day 7 after the operation) upon PHx 80% (Fig. 18).

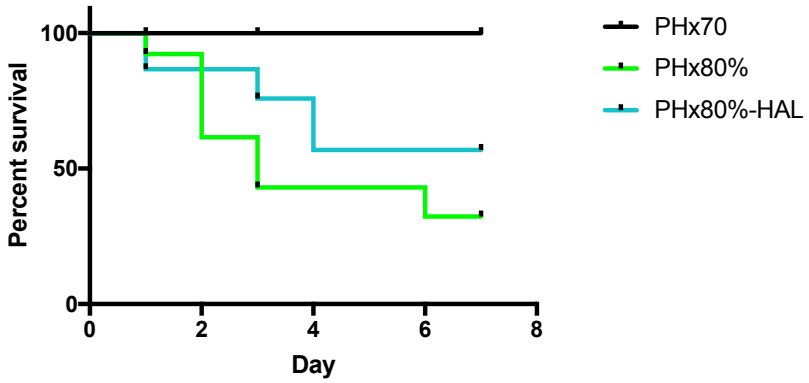


Figure 18. Survival rates on different groups of SFSS-setting hepatectomy. Kaplan–Meier survival curve after PHx 70% (*n* = 15), PHx 80% (*n* = 26) and PHx 80%-HAL (*n* = 15). When we ligated the hepatic artery while performing SFSS-setting survival tended to improve, mainly on the third postoperative day, without reaching significance.

In our hands, hepatic artery ligation in mice is a technically challenging and lengthy operation. This can impact upon the animal's survival. Moreover, the hepatic artery in mice is already ramificated in the hilum, so we are unsure that hepatic artery ligation of the FLR is complete and effective. DMOG is a drug that mimics hypoxia-induced signals as it stabilizes HIF-1α, so it could be used to mimic the effects of hypoxia. However, it also has other effects and anti-oxidative properties that may

confound interpretation.[260] We therefore chose to expose mice to a hypoxic environment. We place them into hypoxia chambers to induce, with no extra surgical burden, titratable hypoxia in the liver remnant. In these large plexiglass chambers, ambient oxygen concentration is strictly controlled. Reduction of the inspired oxygen fraction to 11% (FiO_2) has been proven to be well tolerated and to induce HIF activation and neoangiogenesis, mainly in lung and heart disease animal models.[275–276] Therefore, we expose mice to continuous hypoxia ($Fi0_2$ of 11) immediately after 80% PHx for 3 consecutive days (PHx 80%-HC). Survival and FLR mass recovery are assessed. The design of the current preliminary study is shown in Fig. 19.

We would also like to titrate hypoxia in order to determine the threshold in which survival is improved. Therefore, in consecutive experiments, we will assess the degree, timing, duration and rhythmicity (continuous vs intermittent) of hypoxic events necessary to efficiently activate neoangiogenesis. Simultaneously, it would be of interest to study the body temperature in hepatectomized animals in hypoxic and normoxic conditions, in order to investigate if systemic hypoxia in our experimental model results in anapyrexia, known to have an hepatoprotective effect.[254,255]

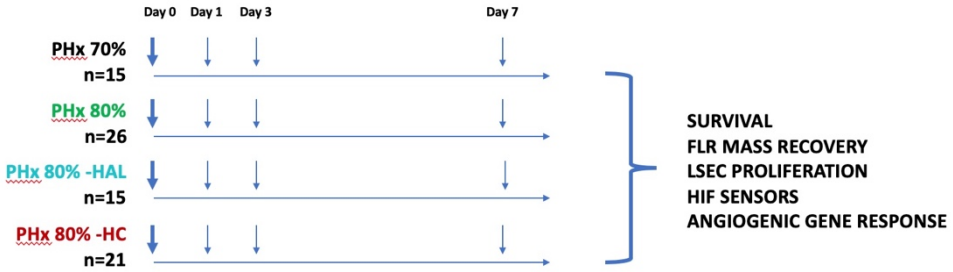


Figure 19. Schematic representation of the current study design. PHx 70%: resection of the left lateral and median lobe (*n* = 5 per time-points), PH× 80%: resection of the left lateral, median and anterior part of the right lobe, day 1 (*n* = 9), day 3 (*n* = 10), day 7 (*n* = 6), PHx 80%-HAL: PHx 80% combined to common hepatic artery ligation (*n* = 5 per time-points), PHx 80%-HC: PHx 80% in mice placed into the hypoxic chamber with continuous hypoxia for the first 3 postoperative days (FiO2: 11%), day 1 (*n* = 5), day 3 (*n* = 5), day 7 (*n* = 11).

Preliminary results of our ongoing work show that survival is significantly higher (95%) when animals subjected to SFSS-setting hepatectomy are placed into hypoxia chambers (FiO_2 11%) compared to animals with SFSS-FLRs in ambient air (FiO_2 21%) (*P* = 0.0007) (Fig. 20A). As observed in our rat model, the improved survival in hypoxic animals cannot be attributed to a better FLR mass recovery (reflection of hepatocyte proliferation), as no difference is observed between the groups (Fig. 20B).

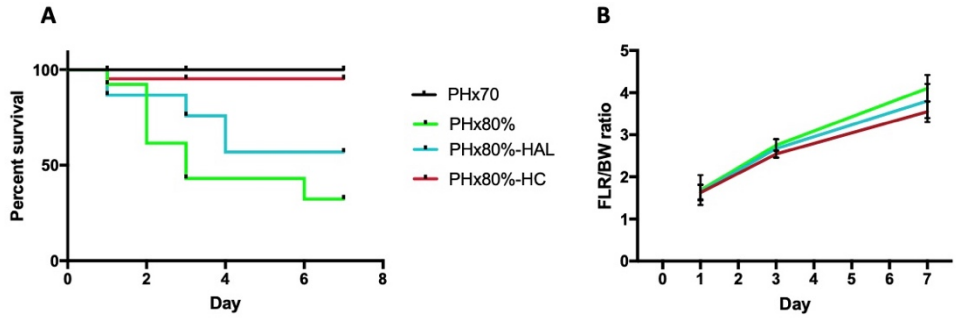


Figure 20. Hypoxia prevents mortality due to extended hepatectomy and has no impact on FLR mass recovery. (A) Kaplan–Meier survival curve after PHx 70% (*n* = 15), PHx 80% (*n* = 26), PHx 80%-HAL (*n* = 15) and PHx 80%-HC (*n* = 21). When animals submitted to the SFSS-setting hepatectomy are placed into hypoxic chambers, survival is significantly improved compared to animals having the same operation under normoxia (*P* = 0.0007, using the log-rank (Mantel–Cox) test). **(B) Future liver remnant mass recovery in the SFSS-setting hepatectomy.** After PHx 80%, day 1 (*n* = 9), day 3 (*n* = 10), day 7 (*n* = 6), PHx 80%-HAL (*n* = 5 per time-points), PHx 80%-HC, day 1 (*n* = 5), day 3 (*n* = 5) and day 7 (*n* = 11) showing no difference between the groups.

At this point, immunofluorescence staining of hepatocyte nuclear factor 4α (HNF4α; marker of hepatocytes), lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1; marker of lymphatic and sinusoidal cells) and Ki67 (marker of cell proliferation) will allow us to assess the degree and timing of hepatocyte and sinusoidal cell proliferation. To assess the effect of hypoxia on the vascular bed, we will assess liver vascular density and the diameter of sinusoid vessels (Feret's diameter) by morphometrical analysis after Lyve-1 staining, as we have previously described.[24,277] We would also like to confirm effective angiogenesis in a dynamic manner by using intravital multi-photon microscopy in the SFSS mouse model under normoxia and hypoxia.[278,279]

Preliminary results demonstrate that hepatocyte proliferation (HNF4α+/Lyve-1-/Ki67% cells) 3 days after the operation is significantly enhanced in PHx80%

compared to PHx70% (p=0,03), while sinusoidal cell proliferation (HNF4α-/Lyve-1+/Ki67% cells) is comparable (p= ns) (data not shown). Considering the impact of hypoxia on sinusoidal endothelial cell proliferation, preliminary data support that local hypoxia, by hepatic artery ligation, and systemic hypoxia, by placing the mice in hypoxic chambers with $Fi0_2$ of 11%, significantly triggers sinusoidal cell proliferation, as supported by a significantly higher proportion of Ki67 positive Lyve-1 positive endothelial cells (ratio of Ki67+/Lyve-1+/HNF4α- cells on the total amount of Lyve-1+/HNF4α- endothelial cells in %) (Fig 21). This occurs even though there is no difference in FLR mass recovery (Fig 20B).

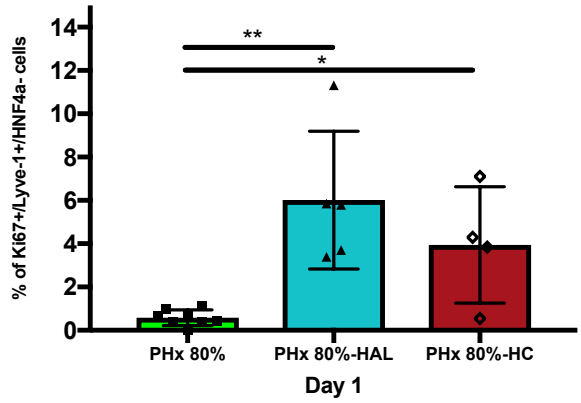


Figure 21: Hypoxia triggers early sinusoidal endothelial cell proliferation in SFSS-setting hepatectomy Preliminary results: After PHx80% n=8, PHx80%-HAL n=5, PHx80%-HC n=4. Local hypoxia induced by hepatic artery ligation (PHx80%-HAL) and systemic hypoxia (PHx80%-HC) triggers endothelial cell proliferation one day after a SFSS-setting hepatectomy (PHx80% vs PHx80%-HAL p=0,001; PHx80% vs PHx80%-HC p=0,04)

We will also evaluate by hypoxia by pimonidazole staining, as previously reported,[266] while endogenous hypoxia markers, such as carbonic anhydrase IX and HIF-1a IHC will help as to evaluate the dynamic evolution of tissue hypoxia and reoxygenation.[280] In vivo hypoxia could be assessed by magnetic resonance imaging,[281] while electron paramagnetic resonance will be avoided. Indeed, in a

preliminary experiment, we inserted lithium phtalocyanine microcrystals (LiPc) in the FLR to assess tissue oxygenation after PVL, PVLT and SFSS-setting hepatectomy *(data not shown)*. However, we stopped the experiment as the spatial resolution of this technic is not suitable for deep located organs, and proved to be too invasive.[282] Liver parenchymal stress and synthetic function will be assessed by serum concentrations of transaminases, bilirubin and INR. We will also assess the activation of hypoxia sensors, downstream targets, the expression profile of proangiogenic genes [angiopoietin 1 and 2, SDF-1, delta like canonical ligand (DLL)-1 and -4, endothelial cell nitric oxide synthase (eNOS), polymeric immunoglobulin receptor (PIGR)] and the cytoplasmic translocation and cell release of HMGB1 and its receptors RAGE and TLR-4. This will help us to unravel the most essential mechanisms at play. Possible pathways may be VEGF-dependent, as this is largely explored in oncology, or dependent upon the release of HMGB1, known to mobilize endothelial progenitors in the marrow, chemoattract them in the liver and stimulate angiogenesis and sprouting.[265] Experimental manipulations will confirm the engagement of these mechanisms.

2. RELATIVE CONTRIBUTION OF NATIVE LSEC AND ENDOTHELIAL PROGENITOR CELLS IN THE RAPIDLY REGENERATING LIVER

As stated above, based on our rat model, our hypothesis is that acute postoperative hypoxia triggers an earlier peak in LSEC proliferation and probably the recruitment of endothelial progenitor cells to the liver remnant, reported as a major source of growth factors for hepatocytes.[64,69,262] It is thus essential to assess the relative contribution of liver-native sinusoidal cells and/or to endothelial progenitor cells in hypoxia-induced angiogenesis. Because liver sinusoidal endothelial cells, whether originating from endothelial progenitor cell (EPC) differentiation or mature LSEC division, share the same markers,[263,23] we use cell-tracking tools to address our

question. We use a lineage-tracing murine Cre-loxP-dependent model[283] (Cdh5-PAC-CreERT2) to permanently label endogenous LSEC (and their progeny). This tool will enable us to determine the contribution of LSEC and recruited progenitors to the remodeling of the sinusoids upon hypoxic small-for-size hepatectomy *in vivo*. Here, animals expressing the oestrogen-sensitive Cre recombinase under the control of endothelial-specific E-cadherin promotor are crossed with Rosa26R mT/mG mice. In the off springs, all cells express membrane Tomato (mT+) fluorescent protein. Injection of tamoxifen will activate the Cre recombinase (only expressed in endothelial cells) to excise the floxed sequence in the Rosa locus to permanently silence membrane Tomato (mT–) and express membrane green (mG+) fluorescent protein (Fig. 22). Thus, native liver sinusoidal endothelial cells and their progeny are fluorescent green (mG+), while other cells, and in particular endothelial progenitor cells and their progeny, remain fluorescent red (mT+) (Fig. 22).

With this system, we will be able to appreciate the relative contribution of liver-native endothelial cells (mG+) and endothelial cells recruited from progenitors (mT+). We quantify amongst Lyve-1 positive endothelial cells the proportion of mG+ (native) and mG– / mT+ progenitors recruited to and differentiated in endothelial cells in the liver sinusoids in SFSS remnants of transgenic mice in normoxic and hypoxic conditions after SFSS-setting hepatectomy. Transgenic animals are submitted to the study design described in Fig. 23.

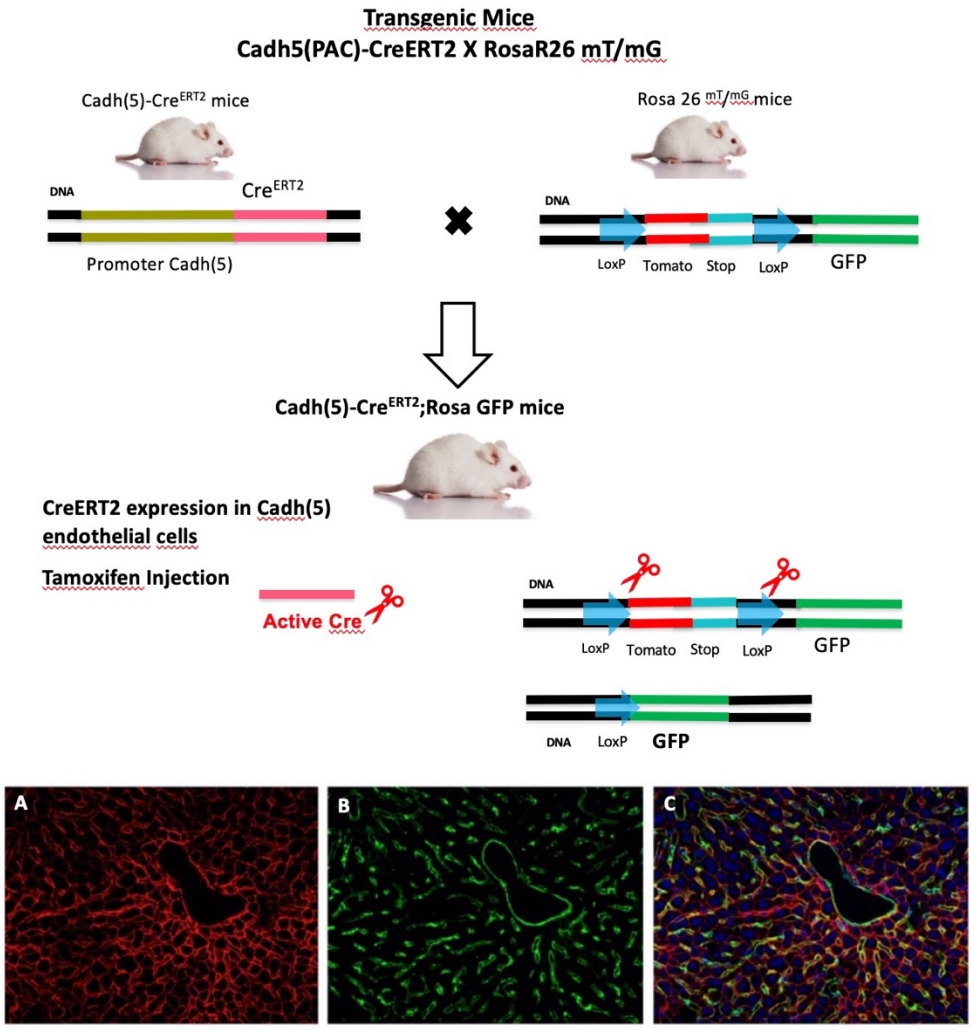


Figure 22. Cdh5(PAC)-CreERT2 crossed with Rosa26 mT/mG mice. All liver cells are red, but liver native sinusoidal endothelial cells are green. **(A) mTomato.** The cell membrane of all cells (hepatocytes, stelate, immune system) is red. **(B) mGreen.** Liver endothelial cell membrane is green. **(C) Merge.**

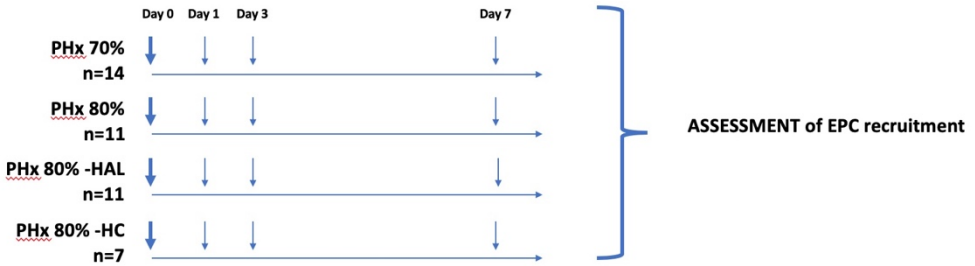
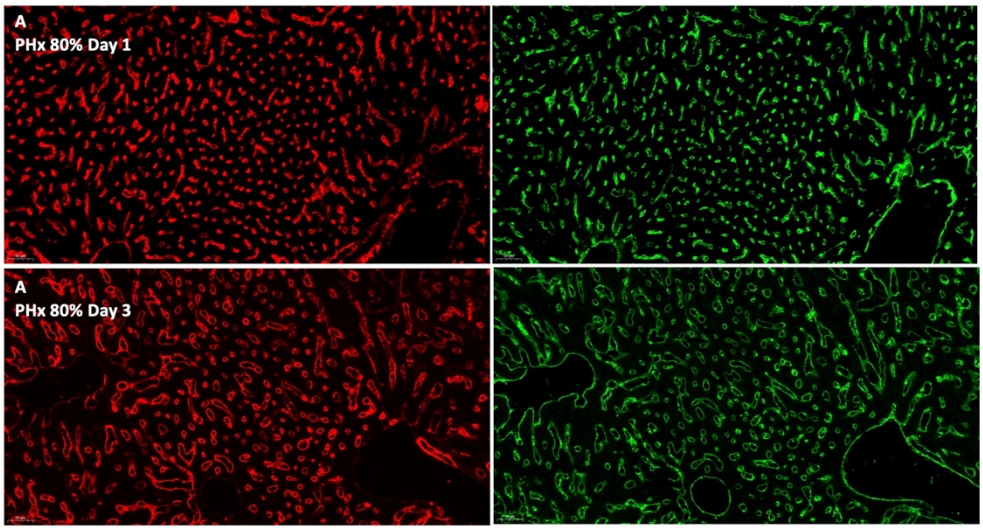
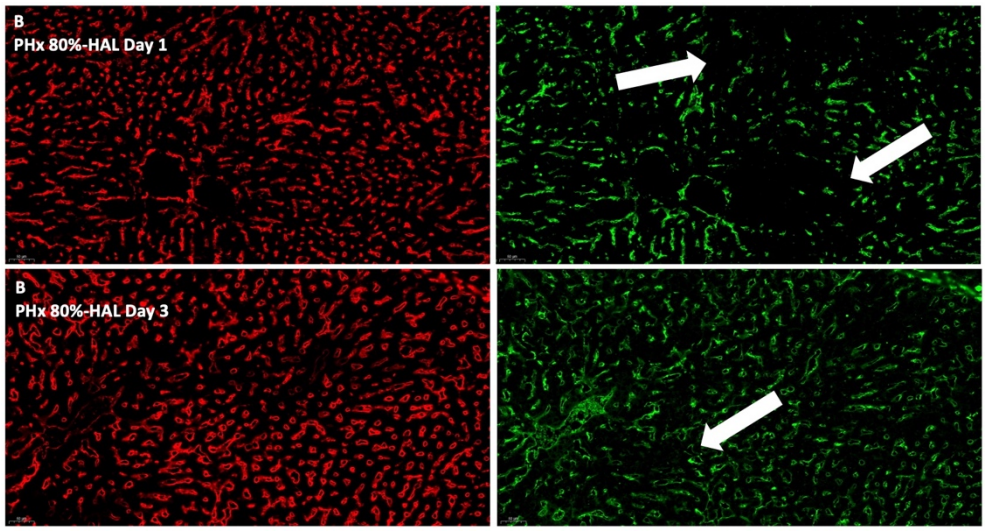


Figure 23. Schematic representation of the current study design on Cdh5(PAC)-CreERT2 crossed with Rosa26 mT/mG mice. PHx 70%: resection of the left lateral and median lobe (*n* = 5 per time-points); PHx 80%: resection of the left lateral, median and anterior part of the right lobe, day 1 (*n* = 6), day 3 (*n* = 5); PHx 80%-HAL: PHx 80% combined to common hepatic artery ligation day 1 (*n* = 8), day 3 (*n* = 3); PHx 80%-HC: PHx 80% in mice placed into a hypoxic chamber with continuous hypoxia for the first 3 postoperative days (FiO2: 11%), day 1 (*n* = 5), day 3 (*n* = 5).

Our preliminary results show that the system works, as almost 100% of endothelial cells in the liver (Tam-Cdh5-Cre mice) are mG+ (Fig 22). Also, 24 h after PHx 80% (a SFSS hepatectomy), the majority of Lyve-1 endothelial cells are mG+ (native) and only a few Lyve-1 endothelial cells are mT+/mG– (i.e. from endothelial progenitors) (Fig 24A). Impressively, upon ligation of the hepatic artery to render the SFSS-FLR hypoxic, large portions of the sinusoidal bed are composed of Lyve-1+/mT+/mG– endothelial cells (Fig. 24B). Similarly, a large part of the sinusoidal bed is composed by Lyve-1+/mT+/mG– cells when SFSS-hepatectomized mice are placed into hypoxic chambers (Fig.24C).

This supports the efficiency of the transgenic system in identifying the origin of cells during remodeling of the sinusoidal network post-hepatectomy. Most importantly, the current data provide a preliminary, but strong, indication that endothelial progenitor cells are recruited and activated in the hypoxic liver remnant.


A
PHx 80% Day 1
A
PHx 80% Day 3


B
PHx 80%-HAL Day 1
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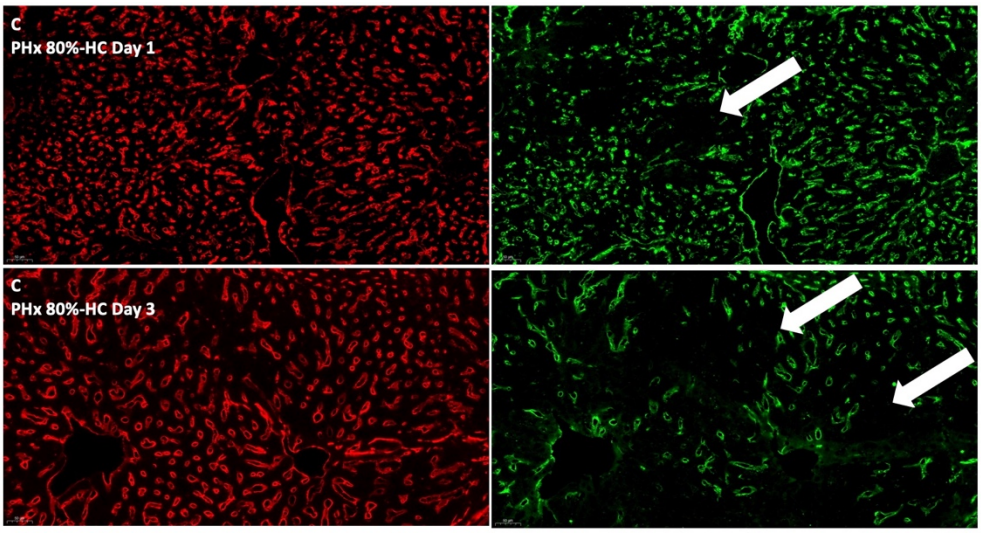

Figure 24. Cdh5(PAC)-CreERT2 crossed with Rosa26 mT/mG mice illustrating in red, Lyve-1+ cells (sinusoidal endothelial cells) and in green mG+ cells (liver native sinusoidal endothelial cells. (A) In a SFSS-setting hepatectomy (PHx80%) most sinusoidal endothelial cells are liver native (Lyve-1/mG+ cells) at 1 and 3 days after the operation. **(B+C)** When animals are submitted to hypoxia during a SFSS-setting hepatectomy (**(B)** local hypoxia by hepatic artery ligation - PHx 80%-HAL, or **(C)** systemic hypoxia by hypoxia chambers- PHx 80%-HC) sinusoidal endothelial bed is not anymore exclusively composed by native liver endothelial cells. Indeed, in discrete (B-hepatic artery ligation) or large (C – hypoxic chamber) part of the Lyve-1 sinusoidal bed (red IF) are of non-endothelial origin (Lyve-1/mG-) (white arrows). Thus, hypoxia induced angiogenesis is mediated by precursors cells, that we suspect to be of bone marrow origin.

3. ORIGIN OF THE CELLS OF THE SINUSOIDAL BED IN THE REGENERATING, HYPOXIC SMALL FOR SIZE REMNANT.

If endothelial progenitor cells (EPC) contribute to hypoxia-driven angiogenesis in the SFSS liver, we need to verify their source. A dormant population of EPC has been described in the liver and represents 1–7% of endothelial cells according to species and age.[64] Nevertheless, a growing body of evidence also supports that a small population of bone marrow-derived mononuclear cells, which express a variety of endothelial cell surface markers (hence designated as EPC), could promote

neovascularization, including in the regenerating liver.[68,68,69] Thus, identifying the EPC origin is of outmost importance. The question will be addressed by using chimeric mice reconstituted with GFP+ bone marrow after irradiation, subjected to extended hepatectomy and maintained in hypoxic conditions.

4. EFFECTS ON TUMOR

Given that most extended hepatectomies are performed for the treatment of liver tumors, we could not neglect investigating whether or not hypoxia-induced angiogenesis triggers tumor growth in the context of liver regeneration. The Amsterdam group demonstrated that PVE only could stimulate tumor progression,[284] an observation also supported by others.[285,286] It is also well documented that hypoxia-induced angiogenesis promotes tumor growth.[287] It could be speculated that if ALPPS allows rapid liver regeneration (compared to PVE) and hypoxia-induced angiogenesis, these same mechanisms would enhance tumor progression. However, a recent translational study suggested that ALPPS does not enhance the growth of colorectal liver metastasis.[288] Clinical data for the long-term oncological outcome after ALPPS are sparse, as most series only comprise a limited number of patients. Hepatic recurrence of colorectal liver metastasis after ALPPS occurs frequently (in up to 65% of patients),[289] however, comparable high rates of recurrence are reported after conventional TSH.[119] Recently reported data on one-site enrolment of the LIGRO trial[205] showed that both ALPPS and TSH were associated with a 1-year recurrence rate of 25%, while 1- and 2-year overall survival rates of the ALPPS registry were reported to be 76 and 63%, respectively. Therefore, we will evaluate neoangiogenesis and tumor growth in a hypoxic model of SFSS-setting hepatectomy (with a tumor-bearing FLR), in order to address this question of outmost importance.

5. TRANSLATION OF OUR FINDINGS TO THE CLINICS: IS IT REALISTIC, CONCEIVABLE OR UNTHINKABLE?

The current study yields some limitations inherent of every experimental work on small animal models, mostly related to the variations in the biliary tree and its arterial blood vascularization between rodents and humans. In order to assess the translational potential of our results, it seems important to define the profile of the hypoxic response and sensors in humans. We could assess HIFs on liver biopsies and HMGB1 release in serum after different degrees of hepatectomy (minor vs major), or after portal vein occlusion and ALPPS. As pimonidazole immunohistostaining is an exogenous marker of hypoxia, that needs to be administered before biopsy, tissue hypoxia could be evaluated by endogenous hypoxia markers in IHC such as carbonic anhydrase IX and HIF-1a, while reoxygenation could be assessed by the dynamic evolution of the co-localization of the above markers.[280,290] In vivo FLR's tissue oxygenation could be evaluated by non-invasive imaging, such as Blood Oxygen Level Dependent MRI, as it is actually proposed for tumor oxygen mapping.[281,291] Thus, a profile of hypoxic sensors and the kinetics of tissue hypoxia in the FLR can be obtained in humans. We could also assess whether there is an enrichment in circulating endothelial precursors upon ALPPS or a hypoxic SFSS-setting remnant compatible with mobilization of bone-marrow derived progenitors. This could be of major importance in patients with bone marrow functional impairment (e.g. aged patient, chemotherapy) as the process of recruitment of bone marrow progenitors might be inefficient and impair liver regeneration. If indeed, it appears that, as in animal models, hypoxia triggers an angiogenic response that associates with functional regeneration and survival in SFSS-hepatectomy, then it will be of interest to define, in an experimental setting, the degree, timing, duration and rhythmicity (continuous vs intermittent) of

hypoxic events necessary to efficiently activate neoangiogenesis. If our hypothesis on the essential role of hypoxia-induced angiogenesis for functional liver regeneration after SFSS-setting hepatectomy is confirmed, the hypoxic urge should be early (during the first wave hepatocyte proliferation), and brief to prevent a deleterious impact on the organism. The importance of the timing of neoangiogenesis through BM-SPC has been pointed out in previous reports showing that restoration of liver regeneration in immunosuppressed rats is achieved when BM-SPCs are infused on day 1 but not on day 3 after PHx.[66] In addition, it also seems essential to know if hypoxia can rescue the liver function even when the SFSS is installed. In that case, hypoxia sensors could be a valid option for treatment.

My aim and wish are to actively contribute to answering these questions in the follow-up to this PhD Thesis. Naturally, as a liver surgeon, the ultimate purpose of this research work would be to define the therapeutic applications in humans as to prevent and/or treat post-hepatectomy liver failure in an extended hepatectomy or SFSS in a liver transplantation with a small graft.

Currently, the main strategy is the modulation of the portal flow either using drugs or physical manipulations (see section 2.2.4.1 and 2.2.5). Other options are being explored such as pharmacological slowing-down of hepatocyte proliferation,[58] stem cells transplantation or injections of immortalized hepatocytes injections, but only for acute liver failure and not for SFSS.[292,293] Autologous bone marrow stem cell injection via the portal vein or the hepatic artery has already been proposed with encouraging results on FLR mass recovery after PVE in a small group of patients.[172] However, human application of these procedures raises many questions, including potentially oncological risks, difficulties in cell sorting of

endothelial stem cells (as cell surface markers include heterogenous cell populations of immature hematopoietic and endothelial stem cells), the lack of effective tracking of cell homing in humans, the need of identification of simultaneous infusion of factors promoting cell homing in the liver and the invasiveness of isolation and administration of bone marrow stem cells.[294] For all these reasons, even if autologous administration of bone marrow stem cells could be an attractive field to explore, we would propose a less invasive, and more 'natural', way to prevent or treat SFSS.

Based on the paradigm of the 'ALPPS effect' unraveled by our experimental work, we think that rapid liver regeneration combines a severe hemodynamic stress, high hepatocyte proliferative response and hypoxia, the latter triggering an angiogenic switch. Thereby, hypoxia enables to resolve the fundamental temporal mismatch between hepatocyte proliferation and sinusoidal regeneration, implicated in the pathophysiology of SFSS. My future aim and research work would focus on actively translate the 'ALPPS effect' in an upfront extended SFSS-setting hepatectomy in humans. This would implicate that, when a SFSS is feared or installed, instead of modulating the hepatic inflow (and thus mitigate hepatocyte proliferation), we could find noninvasive ways to activate hypoxia sensors, mimic an 'hypoxia-like' effect, to promote angiogenesis. A possible route to explore in animal models would be intravenous injection of "low-dose" HMGB1 to activate the migration, sprouting and homing of bone marrow sinusoidal progenitor cells in the FLR. Activation of hypoxia inducible factors by intravenous administration of propyl-hydroxylase inhibitors is another. In our study, we administered DMOG with favorable impact on survival. Research work has already stressed the favorable impact of PHD inhibitors in liver regeneration without increasing liver tumor,[232,295,295] while clinical trials assessing their safety and efficiency are actually

ongoing in patients with kidney diseases.[296] Of course, further pharmacokinetic studies in humans, based on the PHD inhibitors biodistribution are prerequisite for translation into clinical studies, but PHD inhibitors could be an interesting pathway to cope with PHLF. Finally, another attractive pathway is based on the 'normobaric oxygen paradox'.[297] Our experimental study design included induction of hypoxia during SFSS-setting hepatectomy by hepatic artery ligation or placing the animals in hypoxic chambers, conditions that we can difficultly translate in humans. The 'normobaric oxygen paradox' is based on the fact that relative changes of oxygen availability, rather than steady-state hypoxic conditions, play an important role in hypoxia-inducible factor (HIF) transcriptional effects.[298] Thus, we can propose to mimic the activation of hypoxia sensors by pulsed high oxygen therapy.[298] According to this treatment already implemented in clinics,[299] normoxia following an hyperoxic event is sensed by tissues as an oxygen shortage, upregulating HIF-1 activity. Thus, when a SFSS is feared or installed, instead of modulating the hepatic inflow to mitigated hepatocyte proliferation, we could propose the pulsed high oxygen therapy to induce angiogenesis and maintain liver architecture and function. Of course, caution should be paid in patients with bone marrow functional impairment (such as after several lines of chemotherapy or aged patients), as BM-SPC may be implicated in hypoxia-induced angiogenesis. Finally, the question of whether HIF activators can potentially cause cancer or tumor progression in humans is important.[300] Although diligent attention has to be paid to this issue, it is reassuring that, in several clinical trials with gene-therapy-mediated HIF overexpression or with pharmacological HIF activators, no concerns regarding neoplastic diseases were reported.

Our future line of research in clinics will be directed towards the improvement of liver regeneration by preserving the residual hepatocyte function and

microvascular organization, ideally by inducing hypoxia-like effects that can improve early angiogenesis of the rapidly growing liver remnant.

REFERENCES

1. Kang L-I, Mars WM, Michalopoulos GK. Signals and Cells Involved in Regulating Liver Regeneration. *Cells*. 2012;1(4):1261-1292.
2. Taub R. Liver regeneration: From myth to mechanism. *Nat Rev Mol Cell Biol*. 2004;5(10):836-847.
3. Mubbunu L, Bowa K, Petrenko V, Silitongo M. Correlation of Internal Organ Weights with Body Weight and Body Height in Normal Adult Zambians: A Case Study of Ndola Teaching Hospital. *Anat Res Int*. 2018;2018:1-5.
4. Kiuchi T, Kasahara M, Uryuhara K, et al. Impact of graft size mismatching on graft prognosis in liver transplantation from living donors. *Transplantation*. 1999;67(2):321-327.
5. Tani K, Shindoh J, Akamatsu N, et al. Venous drainage map of the liver for complex hepatobiliary surgery and liver transplantation. *Hpb*. 2016;18(12):1031-1038.
6. Madrahimov N, Dirsch O, Broelsch C, Dahmen U. Marginal Hepatectomy in the Rat. *Ann Surg*. 2006;244(1):89-98.
7. Martins PNA, Theruvath TP, Neuhaus P. Rodent models of partial hepatectomies. *Liver Int*. 2008;28(1):3-11.
8. Aller MA, Mendez M, Nava MP, Lopez L, Arias JLA, Arias J. The value of microsurgery in liver research. *Liver Int*. 2009;29(8):1132-1140.
9. Zheng ZY, Weng SY, Yu Y. Signal molecule-mediated hepatic cell communication during liver regeneration. *World J Gastroenterol*. 2009;15(46):5776-5783.
10. Vollmar B, Menger MD. The Hepatic Microcirculation: Mechanistic Contributions and Therapeutic Targets in Liver Injury and Repair. *Physiol Rev*. 2009;89(4):1269-1339.
11. Wakim KG. Basic and clinical physiology of the liver: normal and abnormal. *Anesth Analg*. 1965;44(5):Suppl:632-710.
12. Greenway C V, Stark RD. Hepatic vascular bed. *Physiol Rev*. 1971;51(1):23-65.
13. Kim JH. Effects of portal hyperperfusion on partial liver grafts in the presence of hyperdynamic splanchnic circulation: hepatic regeneration versus portal hyperperfusion injury. *Anesth Pain Med*. 2016;11(2):117-129.
14. Lautt WW. Regulatory processes interacting to maintain hepatic blood flow constancy: Vascular compliance, hepatic arterial buffer response, hepatorenal reflex, liver regeneration, escape from vasoconstriction. *Hepatol Res*. 2007;37(11):891-903.
15. Eipel C, Abshagen K, Vollmar B. Regulation of hepatic blood flow: The hepatic arterial buffer response revisited. *World J Gastroenterol*. 2010;16(48):6046-6057.
16. Lautt WW. Relationship between hepatic blood flow and overall metabolism: the hepatic arterial buffer response. *Fed Proc*. 1983;42(6):1662-1666.
17. Lautt WW. The hepatic artery: Subservient to hepatic metabolism or guardian of normal hepatic clearance rates of humoral substances. *Gen Pharmacol Vasc Syst*.

1977;8(2):73-78.

18. Bredfeldt JE, Riley EM, Groszmann RJ. Compensatory mechanisms in response to an elevated hepatic oxygen consumption in chronically ethanol-fed rats. *Am J Physiol Liver Physiol*. 1985;248(5):G507-G511.
19. Lautt WW, Legare DJ, D'Almeida MS. Adenosine as putative regulator of hepatic arterial flow (the buffer response). *Am J Physiol Circ Physiol*. 1985;248(3):H331-H338.
20. Lautt WW, Greenway C V. Conceptual review of the hepatic vascular bed. *Hepatology*. 1987;7(5):952-963.
21. Ezzat WR, Lautt WW. Hepatic arterial pressure-flow autoregulation is adenosine mediated. *Am J Physiol Circ Physiol*. 1987;252(4):H836-H845.
22. Browse DJ, Mathie RT, Benjamin IS, Alexander B. The role of ATP and adenosine in the control of hepatic blood flow in the rabbit liver in vivo. *Comp Hepatol*. 2003;2:1-10.
23. Cantré D, Schuett H, Hildebrandt A, et al. Nitric oxide reduces organ injury and enhances regeneration of reduced-size livers by increasing hepatic arterial flow. *Br J Surg*. 2008;95(6):785-792.
24. Siebert N, Cantré D, Eipel C, Vollmar B. H2S contributes to the hepatic arterial buffer response and mediates vasorelaxation of the hepatic artery via activation of KATP channels. *Am J Physiol - Gastrointest Liver Physiol*. 2008;295(6):1266-1273.
25. Biernat J, Pawlik WW, Sendur R, Dembiński A, Brzozowski T, Konturek SJ. Role of afferent nerves and sensory peptides in the mediation of hepatic artery buffer response. *J Physiol Pharmacol*. 2005;56(1):133-145.
26. Golling M, Jahnke C, Fonouni H, et al. Distinct effects of surgical denervation on hepatic perfusion, bowel ischemia, and oxidative stress in brain dead and living donor porcine models. *Liver Transplant*. 2007;13(4):607-617.
27. Demetris AJ, Kelly DM, Eghtesad B, et al. Pathophysiologic observations and histopathologic recognition of the portal hyperperfusion or small-for-size syndrome. *Am J Surg Pathol*. 2006;30(8):986-993.
28. Smyrniotis V, Kostopanagiotou G, Kondi A, et al. Hemodynamic interaction between portal vien and hepatic artery flow in small-for-size split liver transplantation. *Transpl Int*. 2002;15(7):355-360.
29. Vasavada BB, Chen CL, Zakaria M. Portal flow is the main predictor of early graft dysfunction regardless of the GRWR status in living donor liver transplantation - A retrospective analysis of 134 patients. *Int J Surg*. 2014;12(2):177-180.
30. Asencio JM, Vaquero J, Olmedilla L, García Sabrido JL. " Small-for-flow" syndrome: Shifting the " size" paradigm. *Med Hypotheses*. 2013;80(5):573-577.
31. Dold S, Richter S, Kollmar O, et al. Portal hyperperfusion after extended hepatectomy does not induce a hepatic arterial buffer response (HABR) but impairs mitochondrial redox state and hepatocellular oxygenation. *PLoS One*. 2015;10(11):1-15.

32. Marcos A, Olzinski AT, Ham JM, Fisher RA, Posner MP. The interrelationship between portal and arterial blood flow after adult to adult living donor liver transplantation. *Transplantation*. 2000;70(12):1697-1703.

33. Hickman R, Stapleton GN, Mets B, Hlatshwayo S, Janicki P. Hepatic blood flow during reduced liver grafting in pigs. A comparison of controls and recipients of intact allografts. *Dig Dis Sci*. 1995;40(6):1246-1251.

34. Bekheit M, Rajakannu M, Bucur P, et al. Serial volumetric assessment of large for size liver grafts after whole cadaveric liver transplant in adults: do large liver grafts shrink in size? *Hpb*. 2016;18(2):200-206.

35. Michalopoulos GK. HEPATOSTAT: Liver regeneration and normal tissue maintenance. *Hepatology*. 2017;Apr(65(4)):1384-1392. doi:10.1002/hep.28988

36. Fausto N. Liver regeneration. *J Hepatol*. 2000;32:19-31.

37. Malato Y, Grimm D, Kay MA, et al. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. *J Clin Invest*. 2011;121(12):4850-4860.

38. Español-Suñer R, Carpentier R, Van Hul N, et al. Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. *Gastroenterology*. 2012;143(6):1564-1575.e7.

39. Kanazawa Y, Verma IM. Little evidence of bone marrow-derived hepatocytes in the replacement of injured liver. *Proc Natl Acad Sci*. 2003;100:11850-11853.

40. Dahlke MH, Popp FC, Bahlmann FH, et al. Liver regeneration in a retrorsine/CCl4 - Induced acute liver failure model: Do bone marrow-derived cells contribute? *J Hepatol*. 2003;39(3):365-373.

41. Fujii H, Hirose T, Oe S, et al. Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice. *J Hepatol*. 2002;36(5):653-659.

42. Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H. Control mechanism of the circadian clock for timing of cell division in vivo. *Science (80-)*. 2003;302(5643):255-259.

43. Fabrikant JI. The kinetics of cellular proliferation in regenerating liver. *J Cell Biol*. 1968;36(3):551-565.

44. Zou Y, Bao Q, Kumar S, Hu M, Wang GY, Dai G. Four waves of hepatocyte proliferation linked with three waves of hepatic fat accumulation during partial hepatectomy-induced liver regeneration. *PLoS One*. 2012;7(2).

45. Kobayashi T, Imamura H, Aoki T, Sugawara Y, Kokudo N, Makuuchi M. Morphological Regeneration and Hepatic Functional Mass after Right Hemihepatectomy. *Dig Surg*. 2006;23(1-2):44-50.

46. Meier M, Andersen KJ, Knudsen AR, Nyengaard JR, Hamilton-Dutoit S, Mortensen FV. Liver regeneration is dependent on the extent of hepatectomy. *J Surg Res*. 2016;205(1):76-84.

47. Weglarz TC, Sandgren EP. Timing of hepatocyte entry into DNA synthesis after partial hepatectomy is cell autonomous. *Proc Natl Acad Sci*. 2000;97(23):12595-12600.

48. Michalopoulos GK. Liver Regeneration after Partial Hepatectomy. *Am J Pathol*. 2010;176(1):2-13.
49. Yoshida D, Akahoshi T, Kawanaka H, et al. Roles of vascular endothelial growth factor and endothelial nitric oxide synthase during revascularization and regeneration after partial hepatectomy in a rat model. *Surg Today*. 2011;41(12):1622-1629.
50. Taki-Eldin A, Zhou L, Xie HY, Zheng SS. Liver regeneration after liver transplantation. *Eur Surg Res*. 2012;48(3):139-153.
51. Mangnall D, Bird NC, Majeed AW. The molecular physiology of liver regeneration following partial hepatectomy. *Liver Int*. 2003;23(2):124-138.
52. Fausto N. Liver regeneration T ". *J Hepatol*. 2000;32:19-31.
53. Abdalla EK, Denys A, Chevalier P, Nemr RA, Vauthey J-N. Total and segmental liver volume variations: Implications for liver surgery. *Surgery*. 2004;135(4):404-410.
54. Nadalin S, Testa G, Malagó M, et al. Volumetric and functional recovery of the liver after right hepatectomy for living donation. *Liver Transplant*. 2004;10(8):1024-1029.
55. Michalopoulos GK. Liver Regeneration. *Science (80-)*. 1997;276(5309):60-66.
56. Mitchell C, Willenbring H. A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. *Nat Protoc*. 2008;3(7):1167-1170.
57. Yang L, Luo Y, Ma L, et al. Establishment of a novel rat model of different degrees of portal vein stenosis following 70% partial hepatectomy. *Exp Anim*. 2016;65(2):165-173.
58. Ninomiya M, Shirabe K, Terashi T, et al. Deceleration of Regenerative Response Improves the Outcome of Rat with Massive Hepatectomy. *Am J Transplant*. 2010;10(7):1580-1587.
59. Pruvot FR, Truant S. Major hepatic resection: from volumetry to liver scintigraphy. *Hpb*. 2016;18(9):707-708.
60. Gruttadauria S, Pagano D, Liotta R, et al. Liver Volume Restoration and Hepatic Microarchitecture in Small-for-Size Syndrome. *Ann Transplant*. 2015;20:381-389.
61. Maeno H, Ono T, Dhar DK, Sato T, Yamanoi A, Nagasue N. Expression of hypoxia inducible factor-1alpha during liver regeneration induced by partial hepatectomy in rats. *Liver Int*. 2005;25(5):1002-1009.
62. Uda Y, Hirano T, Son G, et al. Angiogenesis is crucial for liver regeneration after partial hepatectomy. *Surg (United States)*. 2013;153(1):70-77.
63. Krock BL, Skuli N, Simon MC. Hypoxia-Induced Angiogenesis: Good and Evil. *Genes and Cancer*. 2011;2(12):1117-1133.
64. DeLeve LD. Liver sinusoidal endothelial cells and liver regeneration. *J Cinical Investig*. 2013;123(5):1861-1866.
65. Poisson J, Lemoinne S, Boulanger C, et al. Accepted Manuscript Review Liver sinusoidal endothelial cells: physiology and role in liver diseases Liver sinusoidal

endothelial cells: physiology and role in liver diseases Liver sinusoidal endothelial cells: physiology and role in liver diseases. *J Hepatol*. 2016:1-16.

66. Wang L, Wang X, Xie G, Wang L, Hill CK, Deleve LD. Liver sinusoidal endothelial cell progenitor cells promote liver regeneration in rats. *J Clin Invest*. 2012;122(4):1567-1573.
67. Deleve LD, Wang X, Wang L. VEGF-sdf1 recruitment of CXCR7+ bone marrow progenitors of liver sinusoidal endothelial cells promotes rat liver regeneration. *Am J Physiol - Gastrointest Liver Physiol*. 2016;310(9):G739-G746.
68. Ding B-S, Nolan DJ, Butler JM, et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature*. 2010;468(7321):310-315.
69. Wang L, Wang X, Wang L, et al. Hepatic Vascular Endothelial Growth Factor Regulates Recruitment of Rat Liver Sinusoidal Endothelial Cell Progenitor Cells. *Gastroenterology*. 2012;143(6):1555-1563.
70. Abshagen K, Eipel C, Vollmar B. A critical appraisal of the hemodynamic signal driving liver regeneration. *Langenbeck's Arch Surg*. 2012;397(4):579-590.
71. Crumm S, Cofan M, Juskeviciute E, Hoek JB. Adenine nucleotide changes in the remnant liver: An early signal for regeneration after partial hepatectomy. *Hepatology*. 2008;48(3):898-908.
72. Fausto N. Liver regeneration and repair: Hepatocytes, progenitor cells, and stem cells. *Hepatology*. 2004;39(6):1477-1487.
73. Gilgenkrantz H, Collin de l'Hortet A. New insights into liver regeneration. *Clin Res Hepatol Gastroenterol*. 2011;35(10):623-629.
74. Mortensen KE, Conley LN, Nygaard I, et al. Increased sinusoidal flow is not the primary stimulus to liver regeneration. *Comp Hepatol*. 2010;9(1):2.
75. Cruise J, Houck K, Michalopoulos G. Induction of DNA synthesis in cultured rat hepatocytes through stimulation of alpha 1 adrenoreceptor by norepinephrine. *Science (80-)*. 1985;227(4688):749-751.
76. Gock M, Eipel C, Linnebacher M, Klar E, Vollmar B. Impact of portal branch ligation on tissue regeneration, microcirculatory response and microarchitecture in portal blood-deprived and undeprived liver tissue. *Microvasc Res*. 2011;81(3):274-280.
77. Picard C, Starkel P, Sempoux C, Saliez A, Lebrun V, Horsmans Y. Molecular mechanisms of apoptosis in the liver of rats after portal branch ligation with and without retrorsine. *Lab Investig*. 2004;84(5):618-628.
78. Lambotte L, Li B, Leclercq I, Sempoux C, Saliez A, Horsmans Y. The compensatory hyperplasia (liver regeneration) following ligation of a portal branch is initiated before the atrophy of the deprived lobes. *J Hepatol*. 2000;32(6):940-945.
79. Tseng J-H, Ouyang C-H, Lin K-J, Yeh T-S. Significance of Insulin Signaling in Liver Regeneration Triggered by Portal Vein Ligation. *J Surg Res*. 2011;166(1):77-86.
80. Mitchell C, Nivison M, Jackson LF, et al. Heparin-binding Epidermal Growth Factor-like Growth Factor Links Hepatocyte Priming with Cell Cycle Progression during Liver Regeneration. *J Biol Chem*. 2005;280(4):2562-2568.

81. Furrer K, Rickenbacher A, Tian Y, et al. Serotonin reverts age-related capillarization and failure of regeneration in the liver through a VEGF-dependent pathway. *Proc Natl Acad Sci*. 2011;108(7):2945-2950.

82. Olsen PS, Poulsen SS, Kirkegaard P. Adrenergic effects on secretion of epidermal growth factor from Brunner's glands. *Gut*. 1985;26(9):920-927.

83. Kim S-J, Kim D-G, Lee MD. Effects of Branched-Chain Amino Acid Infusions on Liver Regeneration and Plasma Amino Acid Patterns in Partially Hepatectomized Rats. *Hepatogastroenterology*. 2011;58(109):1280-1285.

84. Michalopoulos GK. Liver regeneration. *J Cell Physiol*. 2007;213(2):286-300.

85. Marchioro TL, Porter KA, Brown BI, et al. The specific influence of nonhepatic splanchnic venous blood flow on the liver. *Surg Forum*. 1965;16:280-282.

86. Marchioro TL, Porter KA, Brown BI, Otte JB, Starzl TE. The effect of partial portacaval transposition on the canine liver. *Surgery*. 1967;61(5):723-732.

87. Stärkel P, Horsmans Y, Sempoux C, et al. After portal branch ligation in rat, nuclear factor κB, interleukin-6, signal transducers and activators of transcription 3, c- fos , c- myc , and c- jun are similarly induced in the ligated and nonligated lobes. *Hepatology*. 1999;29(5):1463-1470.

88. Stärkel P, Lambotte L, Sempoux C, et al. After portal branch ligation in the rat, cellular proliferation in associated with selective induction of c-Ha-ras, p53, cyclin E, and Cdk2. *Gut*. 2001;49(1):119-130.

89. Fan YD, Praet M, Van Huysse J, Lelie B, De Hemptinne B. Effects of portal vein arterialization on liver regeneration after partial hepatectomy in the rat. *Liver Transplant*. 2002;8(2):146-152.

90. Li J, Cai C, Guo H, et al. Portal vein arterialization promotes liver regeneration after extended partial hepatectomy in a rat model. *J Biomed Res*. 2015;29:69-75.

91. Papaioannou TG, Stefanadis C. Vascular wall shear stress: basic principles and methods. *Hellenic J Cardiol*. 2014;46(1):9-15.

92. Sato Y, Koyama S, Tsukada K, Hatakeyama K. Acute portal hypertension reflecting shear stress as a trigger of liver regeneration following partial hepatectomy. *Surg Today*. 1997;27(6):518-526.

93. Schoen JM, Wang HH, Minuk GY, Lautt WW. Shear Stress-Induced Nitric Oxide Release Triggers the Liver Regeneration Cascade. *Nitric Oxide*. 2001;5(5):453-464.

94. Wang PF, Li CH, Chen YW, Zhang AQ, Cai SW, Dong JH. Preserving hepatic artery flow during portal triad blood inflow occlusion improves remnant liver regeneration in rats after partial hepatectomy. *J Surg Res*. 2013;181(2):329-336.

95. Mohkam K, Rode A, Darnis B, et al. Hepatic venous pressure gradient after portal vein embolization: An accurate predictor of future liver remnant hypertrophy. *Surg (United States)*. 2018;164(2):227-232.

96. Sainz-Barriga M, Scudeller L, Costa MG, de Hemptinne B, Troisi RI. Lack of a correlation between portal vein flow and pressure: Toward a shared interpretation of hemodynamic stress governing inflow modulation in liver transplantation. *Liver*

Transplant. 2011;17(7):836-848.

97. Díaz-Juárez JA, Hernández-Muñoz R. Rat Liver Enzyme Release Depends on Blood Flow-Bearing Physical Forces Acting in Endothelium Glycocalyx rather than on Liver Damage. *Oxid Med Cell Longev*. 2017;2017:1-15.

98. Lauber DT, Tihanyi DK, Czigany Z, et al. Liver regeneration after different degrees of portal vein ligation. *J Surg Res*. 2016;203(2):451-458.

99. Niiya T, Murakami M, Aoki T, Murai N, Shimizu Y, Kusano M. Immediate increase of portal pressure, reflecting sinusoidal shear stress, induced liver regeneration after partial hepatectomy. *J Hepatobiliary Pancreat Surg*. 1999;6(3):275-280.

100. Nobuoka T, Mizuguchi T, Oshima H, et al. Portal Blood Flow Regulates Volume Recovery of the Rat Liver after Partial Hepatectomy: Molecular Evaluation. *Eur Surg Res*. 2006;38(6):522-532.

101. Shimazu M, Kato Y, Kawachi S, et al. Impact of Portal Hemodynamic Changes in Partial Liver Grafts on Short-Term Graft Regeneration in Living Donor Liver Transplantation. *Transplant Proc*. 2016;48(8):2747-2755.

102. Eguchi S, Yanaga K, Sugiyama N, et al. Relationship between portal venous flow and liver regeneration in patients after living donor right-lobe liver transplantation. *Liver Transpl*. 2003;9(6):547-551.

103. Oyama T, Sadamori H, Matsukawa H, et al. Small liver graft regenerates through immediate increase of HGF and IL-6--possible involvement of sinusoidal tensile/shear stress in small liver graft. *Hepatogastroenterology*. 2007;54(79):2078-2083.

104. Bauer I, Wanner GA, Rensing H, et al. Expression pattern of heme oxygenase isoenzymes 1 and 2 in normal and stress-exposed rat liver. *Hepatology*. 1998;27(3):829-838.

105. Suematsu M, Kashiwagi S, Sano T, Goda N, Shinoda Y, Ishimura Y. Carbon Monoxide as an Endogenous Modulator of Hepatic Vascular Perfusion. *Biochem Biophys Res Commun*. 1994;205(2):1333-1337.

106. Schoen JM, Lautt WW. Nitric oxide potentiates C-Fos mRNA expression after 2/3 partial hepatectomy. *Proc West Pharmacol Soc*. 2002;45(May):47-48.

107. Pipili-synetos E, Sakkoula E, Maragoudakis ME. Nitric oxide is involved in the regulation of angiogenesis. *Br J rnarmacol kt17Ji,*. 1993;108:855-857.

108. Albinsson S, Hellstrand P. Integration of signal pathways for stretch-dependent growth and differentiation in vascular smooth muscle. *Am J Physiol Physiol*. 2007;293(2):C772-C782.

109. Weinbaum S, Zhang X, Han Y, Vink H, Cowin SC. Mechanotransduction and flow across the endothelial glycocalyx. *Proc Natl Acad Sci*. 2003;100(13):7988-7995.

110. Isomura H, Sawada N, Nakajima Y, et al. Increase in portal flow induces c-myc expression in isolated perfused rat liver. *J Cell Physiol*. 1993;154(2):329-332.

111. Gan L, Doroudi R, Hägg U, Johansson A-M, Selin-Sjögren L, Jern S. Differential immediate-early gene responses to shear stress and intraluminal pressure in intact

human conduit vessels. *FEBS Lett*. 2000;477(1-2):89-94.

112. Mueller L, Grotelueschen R, Meyer J, et al. Sustained function in atrophying liver tissue after portal branch ligation in the rat. *J Surg Res*. 2003;114(2):146-155.

113. Heinrich S, Jochum W, Graf R, Clavien PA. Portal vein ligation and partial hepatectomy differentially influence growth of intrahepatic metastasis and liver regeneration in mice. *J Hepatol*. 2006;45(1):35-42.

114. Tashiro S. Mechanism of liver regeneration after liver resection and portal vein embolization (ligation) is different? *J Hepatobiliary Pancreat Surg*. 2009;16(3):292-299.

115. Furrer K, Tian Y, Pfammatter T, et al. Selective portal vein embolization and ligation trigger different regenerative responses in the rat liver. *Hepatology*. 2008;47(5):1615-1623.

116. Clarke AM, Thomson RY FGFG. Vascular factors in liver regeneration. *Surg Gynecol Obs*. 1968;126(1):45-52.

117. Poon RT, Fan ST, Lo CM, et al. Improving perioperative outcome expands the role of hepatectomy in management of benign and malignant hepatobiliary diseases: Analysis of 1222 consecutive patients from a prospective database. *Ann Surg*. 2004;240(4):698-710.

118. Imamura H, Seyama Y, Kokudo N, et al. Single and multiple resections of multiple hepatic metastases of colorectal origin. *Surgery*. 2004;135(5):508-517.

119. Brouquet A, Abdalla EK, Kopetz S, et al. High survival rate after two-stage resection of advanced colorectal liver metastases: Response-based selection and complete resection define outcome. *J Clin Oncol*. 2011;29(8):1083-1090.

120. Blazer DG, Kishi Y, Maru DM, et al. Pathologic response to preoperative chemotherapy: A new outcome end point after resection of hepatic colorectal metastases. *J Clin Oncol*. 2008;26(33):5344-5351.

121. Charnsangavej C, Clary B, Fong Y, Grothey A, Pawlik TM, Choti MA. Selection of patients for resection of hepatic colorectal metastases: Expert consensus statement. *Ann Surg Oncol*. 2006;13(10):1261-1268.

122. Garden OJ. Guidelines for resection of colorectal cancer liver metastases. *Gut*. 2006;55(suppl_3):iii1-iii8.

123. Adams RB, Aloia TA, Loyer E, Pawlik TM, Taouli B, Vauthey JN. Selection for hepatic resection of colorectal liver metastases: Expert consensus statement. *Hpb*. 2013;15(2):91-103.

124. Jarnagin WR, Gonen M, Fong Y, et al. Improvement in perioperative outcome after hepatic resection: analysis of 1,803 consecutive cases over the past decade. *Ann Surg*. 2002;236(4):397-406; discussion 406-7.

125. Clavien P-A, Petrowsky H, DeOliveira ML, Graf R. Strategies for Safer Liver Surgery and Partial Liver Transplantation. *N Engl J Med*. 2007;356(15):1545-1559.

126. Amer A, Wilson CH, Manas DM. Liver transplantation for unresectable malignancies: Beyond hepatocellular carcinoma. *Eur J Surg Oncol*. July 2019;45(12):2268-2278.

127. Song ATW, Avelino-Silva VI, Pecora RAA, Pugliese V, D'Albuquerque LAC, Abdala E. Liver transplantation: Fifty years of experience. *World J Gastroenterol*. 2014;20(18):5363-5374.

128. Fukazawa K, Yamada Y, Nishida S, Hibi T, Arheart KL, Pretto EA. Determination of the safe range of graft size mismatch using body surface area index in deceased liver transplantation. *Transpl Int*. 2013;26(7):724-733.

129. Rahbari NN, Garden OJ, Padbury R, Brooke-smith M. Posthepatectomy liver failure : A definition and grading by the International Study Group of Liver Surgery (ISGLS). 2011;149(5):713-724.

130. Guglielmi A, Ruzzenente A, Conci S, Valdegamberi A, Iacono C. How much remnant is enough in liver resection? *Dig Surg*. 2012;29(1):6-17. doi:10.1159/000335713

131. Brown RS. Live Donors in Liver Transplantation. *Gastroenterology*. 2008;134(6):1802-1813.

132. de Graaf W, van Lienden KP, Dinant S, et al. Assessment of future remnant liver function using hepatobiliary scintigraphy in patients undergoing major liver resection. *J Gastrointest Surg*. 2010;14(2):369-378.

133. Chapelle T, Op De Beeck B, Huyghe I, et al. Future remnant liver function estimated by combining liver volumetry on magnetic resonance imaging with total liver function on 99mTc-mebrofenin hepatobiliary scintigraphy: Can this tool predict post-hepatectomy liver failure? *Hpb*. 2016;18(6):494-503.

134. Rassam F, Olthof PB, van Lienden KP, et al. Functional and volumetric assessment of liver segments after portal vein embolization: Differences in hypertrophy response. *Surg (United States)*. 2019;165(4):686-695.

135. De Meijer VE, Kalish BT, Puder M, IJzermans JNM. Systematic review and meta-analysis of steatosis as a risk factor in major hepatic resection. *Br J Surg*. 2010;97(9):1331-1339.

136. Zarzavadjian Le Bian A, Costi R, Constantinides V, Smadja C. Metabolic Disorders, Non-Alcoholic Fatty Liver Disease and Major Liver Resection: An Underestimated Perioperative Risk. *J Gastrointest Surg*. 2012;16(12):2247-2255.

137. Cieslak KP, Baur O, Verheij J, Bennink RJ, van Gulik TM. Liver function declines with increased age. *Hpb*. 2016;18(8):691-696.

138. Zarzavadjian Le Bian A, Costi R, Sbai-Idrissi MS, Smadja C. Liver resection and metabolic disorders: An undescribed mechanism leading to postoperative mortality. *World J Gastroenterol*. 2014;20(39):14455-14462.

139. Rassam F, Olthof PB, Bennink RJ, Van Gulik TM. Current Modalities for the Assessment of Future Remnant Liver Function. *Visc Med*. 2017;33(6):442-448.

140. de Graaf W, Bennink RJ, Heger M, Maas A, de Bruin K, van Gulik TM. Quantitative Assessment of Hepatic Function During Liver Regeneration in a Standardized Rat Model. *J Nucl Med*. 2011;52(2):294-302.

141. Truant S, Boleslawski E, Sergent G, et al. Liver Function Following Extended Hepatectomy Can Be Accurately Predicted Using Remnant Liver Volume to Body

Weight Ratio. *World J Surg*. 2015;39(5):1193-1201.

142. Zorzi D, Laurent A, Pawlik TM, Lauwers GY, Vauthey JN, Abdalla EK. Chemotherapy-associated hepatotoxicity and surgery for colorectal liver metastases. *Br J Surg*. 2007;94(3):274-286.

143. Dahm F, Georgiev P, Clavien PA et al. Small-for-size syndrome after partial liver transplantation: Definition, mechanisms of disease and clinical implications. *Am J Transplant*. 2005;5(11):2605-2610.

144. Golriz M, Majlesara A, El Sakka S, et al. Small for Size and Flow (SFSF) syndrome: An alternative description for posthepatectomy liver failure. *Clin Res Hepatol Gastroenterol*. 2016;40(3):267-275.

145. Rahbari NN, Garden OJ, Padbury R, et al. Post-hepatectomy haemorrhage: A definition and grading by the International Study Group of Liver Surgery (ISGLS). *HPB*. 2011;13(8):528-535.

146. Gad E, Shoreem H, Soliman H, et al. Small For Size Syndrome Difficult Dilemma: Lessons From 10 Years Single Center Experience in Living Donor Liver Transplantation. *Transplantation*. 2014;98(17):783-784.

147. Balzan S, Belghiti J, Farges O, et al. The “50-50 Criteria” on Postoperative Day 5. *Ann Surg*. 2005;242(6):824-829.

148. Serenari M, Cescon M, Cucchetti A, Pinna AD. Liver function impairment in liver transplantation and after extended hepatectomy. *World J Gastroenterol*. 2013;19(44):7922-7929.

149. Uemura T, Wada S, Kaido T, et al. How far can we lower graft-to-recipient weight ratio for living donor liver transplantation under modulation of portal venous pressure? *Surg (United States)*. 2016;159(6):1623-1630.

150. Allard M-A, Adam R, Bucur P-O, et al. Posthepatectomy Portal Vein Pressure Predicts Liver Failure and Mortality after Major Liver Resection on Noncirrhotic Liver. *Ann Surg*. 2013;258(5):822-830.

151. Ogura Y, Hori T, El Moghazy WM, et al. Portal pressure <15mmHg is a key for the successful adult living donor liver transplantation utilizing smaller grafts than before. *Liver Transplant*. 2010;16(6):718-28.

152. Sainz-Barriga M, Reyntjens K, Costa MG, et al. Prospective Evaluation of Intraoperative Hemodynamics in Liver Transplantation with Whole, Partial and DCD Grafts. *Am J Transplant*. 2010;10(8):1850-1860.

153. Kelly DM, Demetris AJ, Fung JJ, et al. Porcine partial liver transplantation: A novel model of the ?small-for-size? liver graft. *Liver Transplant*. 2004;10(2):253-263.

154. Yagi S, Iida T, Hori T, et al. Optimal Portal Venous Circulation for Liver Graft Function after Living-Donor Liver Transplantation. *Transplantation*. 2006;81(3):373-378.

155. Asakura T, Ohkohchi N, Orii T, et al. Portal vein pressure is the key for successful liver transplantation of an extremely small graft in the pig model. *Transpl Int*. 2003;16(6):376-382.

156. Sato Y, Yamamoto S, Oya H, et al. Splenectomy for reduction of excessive portal

hypertension after adult living-related donor liver transplantation. *Hepatogastroenterology*. 2002;49(48):1652-1655.

157. Troisi R, Ricciardi S, Smeets P, et al. Effects of hemi-portocaval shunts for inflow modulation on the outcome of small-for-size grafts in living donor liver transplantation. *Am J Transplant*. 2005;5(6):1397-1404.

158. Yamada T, Tanaka K, Uryuhara K, Ito K, Takada Y, Uemoto S. Selective Hemi-Portocaval Shunt Based on Portal Vein Pressure for Small-for-Size Graft in Adult Living Donor Liver Transplantation. *Am J Transplant*. 2008;8(4):847-853.

159. Boillot O. Portomesenteric disconnection for small-for-size grafts in liver transplantation: Preclinical studies in pigs. *Liver Transplant*. 2003;9(9):S42-S46.

160. Umeda Y, Yagi T, Sadamori H, Fujiwara T. Small-for-Size Syndrome After Living Donor Liver Transplantation. *Liver Transplant - Tech Issues Complicat*. 2012;86:673-680.

161. Lordan JT, Roberts JK, Hodson J, et al. Case-controlled study comparing peri-operative and cancer-related outcomes after major hepatectomy and parenchymal sparing hepatectomy for metastatic colorectal cancer. *HPB*. 2017;19(8):688-694.

162. Moris D, Ronnekleiv-Kelly S, Rahnemai-Azar AA, et al. Parenchymal-Sparing Versus Anatomic Liver Resection for Colorectal Liver Metastases: a Systematic Review. *J Gastrointest Surg*. 2017;21(6):1076-1085.

163. Adam R, Laurent A, Azoulay D, et al. Two-stage hepatectomy: A planned strategy to treat irresectable liver tumors. *Ann Surg*. 2000;232(6):777-785.

164. Shindoh J, Tzeng C-WD, Aloia TA, et al. Portal vein embolization improves rate of resection of extensive colorectal liver metastases without worsening survival. *Br J Surg*. 2013;100(13):1777-1783.

165. Van Lienden KP, Van Den Esschert JW, De Graaf W, et al. Portal vein embolization before liver resection: A systematic review. *Cardiovasc Intervent Radiol*. 2013;36(1):25-34.

166. Cieslak KP, Huisman F, Bais T, et al. Future remnant liver function as predictive factor for the hypertrophy response after portal vein embolization. *Surg (United States)*. 2017;162(1):37-47.

167. Ribero D, Abdalla EK, Madoff DC, Donadon M, Loyer EM, Vauthey JN. Portal vein embolization before major hepatectomy and its effects on regeneration, resectability and outcome. *Br J Surg*. 2007;94(11):1386-1394.

168. Shindoh J, Truty MJ, Aloia TA, et al. Kinetic growth rate after portal vein embolization predicts posthepatectomy outcomes: Toward zero liver-related mortality in patients with colorectal liver metastases and small future liver remnant. *J Am Coll Surg*. 2013;216(2):201-209.

169. Alvarez FA, Castaing D, Figueroa R, et al. Natural history of portal vein embolization before liver resection: a 23-year analysis of intention-to-treat results. *Surgery*. 2018;163(6):1257-1263.

170. van den Esschert JW, van Lienden KP, de Graaf W, et al. Portal vein embolization

induces more liver regeneration than portal vein ligation in a standardized rabbit model. *Surgery*. 2011;149(3):378-385.

171. Gruttadauria S, Gridelli B. Sequential Preoperative Ipsilateral Portal and Arterial Embolization in Patients with Liver Tumors: Is It Really the Best Approach? *World J Surg*. 2007;31(12):2427-2428.

172. Esch JS am, Schmelzle M, Fürst G, et al. Infusion of CD133+ Bone Marrow–Derived Stem Cells After Selective Portal Vein Embolization Enhances Functional Hepatic Reserves After Extended Right Hepatectomy. *Ann Surg*. 2012;255(1):79-85.

173. Aoki T. Sequential Preoperative Arterial and Portal Venous Embolizations in Patients With Hepatocellular Carcinoma. *Arch Surg*. 2004;139(7):766-774.

174. Hwang S, Lee S-G, Ko G-Y, et al. Sequential Preoperative Ipsilateral Hepatic Vein Embolization After Portal Vein Embolization to Induce Further Liver Regeneration in Patients With Hepatobiliary Malignancy. *Ann Surg*. 2009;249(4):608-616.

175. Guiu B, Quenet F, Escal L, et al. Extended liver venous deprivation before major hepatectomy induces marked and very rapid increase in future liver remnant function. *Eur Radiol*. 2017;27(8):3343-3352.

176. Schnitzbauer AA, Lang SA, Goessmann H, et al. Right portal vein ligation combined with in situ splitting induces rapid left lateral liver lobe hypertrophy enabling 2-staged extended right hepatic resection in small-for-size settings. *Ann Surg*. 2012;255(3):405-414.

177. Eshmuminov D, Raptis DA, Linecker M, Wirsching A, Lesurtel M, Clavien P-A. Meta-analysis of associating liver partition with portal vein ligation and portal vein occlusion for two-stage hepatectomy. *Br J Surg*. 2016;103(13):1768-1782.

178. Kenya Yamanaka, Etsuro Hatano, Masato Narita, Koji Kitamura, Atsuko Yanagida, Hiroyuki Asechi, Hiromitsu Nagata, Kojiro Taura, Takashi Nitta and SU. Olprinone Attenuates Excessive Shear Stress Through Up-Regulation of Endothelial Nitric Oxide Synthase in a Rat Excessive Hepatectomy Model Kenya. *Liver Transplant*. 2007;13(3):465-466.

179. Morioku D, Sekido H, Suito S, Ichikuwa Y, Sbimudu H. Prostaglandin E l Improved the Function of Transplanted Fatty Liver in a Rat Reduced-Size-Liver. 2003;9:79-86.

180. Man K, Lee TK, Liang TB, et al. FK 409 Ameliorates Small-for-Size Liver Graft Injury by Attenuation of Portal Hypertension and Down-Regulation of Egr-1 Pathway. *Ann Surg*. 2004;240(1):159-168.

181. Palmes D, Budny TB, Stratmann U, Herbst H, Spiegel HU. Endothelin-A receptor antagonist reduces microcirculatory disturbances and transplant dysfunction after partial liver transplantation. *Liver Transplant*. 2003;9(9):929-939.

182. Xu X, Man K, Zheng S Sen, et al. Attenuation of acute phase shear stress by somatostatin improves small-for-size liver graft survival. *Liver Transplant*. 2006;12(4):621-627.

183. Mohkam K, Darnis B, Schmitt Z, Duperret S, Ducerf C, Mabrut JY. Successful modulation of portal inflow by somatostatin in a porcine model of small-for-size

syndrome. *Am J Surg*. 2016;212(2):321-326.

184. Chiu A, Fan ST. MARS in the treatment of liver failure: Controversies and evidence. *Int J Artif Organs*. 2006;29(7):660-667.

185. van de Kerkhove MP, de Jong KP, Rijken AM, de Pont ACJM, van Gulik TM. MARS treatment in posthepatectomy liver failure. *Liver Int*. 2003;23(SUPPL. 3):44-51.

186. Gilg S, Escorsell A, Fernandez J, et al. Albumin dialysis with mars in post-hepatectomy liver failure (PHLF): experiences from two HPB centers. *Hpb*. 2016;18(1):e691.

187. Tschuor C, Croome KP, Sergeant G, et al. Salvage parenchymal liver transection for patients with insufficient volume increase after portal vein occlusion - An extension of the ALPPS approach. *Eur J Surg Oncol*. 2013;39(11):1230-1235.

188. Enne M, Schadde E, Björnsson B, et al. ALPPS as a salvage procedure after insufficient future liver remnant hypertrophy following portal vein occlusion. *HPB*. 2017;19(12):1126-1129.

189. Schadde E, Schnitzbauer AA, Tschuor C, Raptis DA, Bechstein WO, Clavien P-A. Systematic Review and Meta-Analysis of Feasibility, Safety, and Efficacy of a Novel Procedure: Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy. *Ann Surg Oncol*. 2015;22(9):3109-3120.

190. Schadde E, Ardiles V, Slankamenac K, et al. ALPPS offers a better chance of complete resection in patients with primarily unresectable liver tumors compared with conventional-staged hepatectomies: Results of a multicenter analysis. *World J Surg*. 2014;38(6):1510-1519.

191. Pineda-Solís K, Paskar D, Tun-Abraham M, Hernandez-Alejandro R. Expanding the limits of resectability: Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) using monosegment 6, facilitated by an inferior right hepatic vein. *J Surg Oncol*. 2017;115(8):959-962.

192. Schadde E, Malagó M, Hernandez-Alejandro R, et al. Monosegment ALPPS hepatectomy: Extending resectability by rapid hypertrophy. *Surg (United States)*. 2015;157(4):676-689.

193. Barkun JS, Aronson JK, Feldman LS, Maddern GJ, Strasberg SM. Evaluation and stages of surgical innovations. *Lancet*. 2009;374(9695):1089-1096.

194. Hirst A, Philippou Y, Blazeby J, et al. No Surgical Innovation Without Evaluation: Evolution and Further Development of the IDEAL Framework and Recommendations. *Ann Surg*. 2019;269(2):211-220.

195. Lam VWT, Laurence JM, Johnston E, Hollands MJ, Pleass HCC, Richardson AJ. A systematic review of two-stage hepatectomy in patients with initially unresectable colorectal liver metastases. *HPB*. 2013;15(7):483-491.

196. Truant S, Scatton O, Dokmak S, et al. Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS): Impact of the inter-stages course on morbi-mortality and implications for management. *Eur J Surg Oncol*. 2015;41(5):674-682.

197. Schadde E, Raptis DA, Schnitzbauer AA, et al. Prediction of mortality after ALPPS Stage-1. *Ann Surg*. 2015;262(5):780-786.

198. Viganò L, Cimino MM, Adam R, Torzilli G. Improving the Safety of ALPPS Procedure. *Ann Surg*. 2017;266(6):e101-e102.

199. Belghiti J, Dokmak S, Schadde E. ALPPS: Innovation for innovation's sake. *Surgery*. 2016;159(5):1287-1288.

200. Wigmore SJ. ALPPS: The argument against. *Eur J Surg Oncol*. 2017;43(2):249-251.

201. Oldhafer KJ, Stavrou GA, Van Gulik TM, et al. ALPPS - Where do we stand, where do we go? Eight recommendations from the first international expert meeting. *Ann Surg*. 2016;263(5):839-841.

202. Schadde E, Ardiles V, Robles-Campos R, et al. Early Survival and Safety of ALPPS. *Ann Surg*. 2014;260(5):829-838.

203. Serenari M, Zanello M, Schadde E, et al. Importance of primary indication and liver function between stages: Results of a multicenter Italian audit of ALPPS 2012-2014. *Hpb*. 2016;18(5):419-427.

204. Nadalin S, Capobianco I, Li J, Girotti P, Königsrainer I, Königsrainer A. Indications and Limits for Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS). Lessons Learned from 15 Cases at a Single Centre. *Z Gastroenterol*. 2014;52(01):35-42.

205. Sandström P, Røsok BI, Sparrelid E, et al. ALPPS Improves Resectability Compared with Conventional Two-stage Hepatectomy in Patients with Advanced Colorectal Liver Metastasis: Results from a Scandinavian Multicenter Randomized Controlled Trial (LIGRO Trial). *Ann Surg*. 2018;267(5):833-840.

206. Linecker M, Stavrou GA, Oldhafer KJ, et al. The ALPPS risk score avoiding futile use of ALPPS. *Ann Surg*. 2016;264(5):763-771.

207. Stavrou GA, Donati M, Fard-Aghaie MH, et al. Did the International ALPPS Meeting 2015 Have an Impact on Daily Practice the Hamburg Barmbek Experience of 58 Cases. *Visc Med*. 2017;33(6):456-461.

208. Truant S, Baillet C, Deshorgue AC, El Amrani M, Huglo D, Pruvot FR. Contribution of hepatobiliary scintigraphy in assessing ALPPS most suited timing. *Updates Surg*. 2017;69(3):411-419.

209. Truant S, El Amrani M, Skrzypczyk C, et al. Factors associated with fatal liver failure after extended hepatectomy. *Hpb*. 2017;19(8):682-687.

210. de Santibañes E, Alvarez FA, Ardiles V, Pekolj J, de Santibañes M. Inverting the ALPPS paradigm by minimizing first stage impact: the Mini-ALPPS technique. *Langenbeck's Arch Surg*. 2016;401(4):557-563.

211. Linecker M, Kambakamba P, Reiner CS, et al. How much liver needs to be transected in ALPPS? A translational study investigating the concept of less invasiveness. *Surg (United States)*. 2017;161(2):453-464.

212. Deal R, Frederiks C, Williams L, et al. Rapid Liver Hypertrophy After Portal Vein Occlusion Correlates with the Degree of Collateralization Between Lobes—a Study

in Pigs. *J Gastrointest Surg*. 2018;22(2):203-213.

213. Tanaka K. Modified ALPPS procedures: more safety through less invasive surgery. *Langenbeck's Arch Surg*. 2017;402(4):563-574.

214. Gall TMH, Sodergren MH, Frampton AE, et al. Radio-frequency-assisted Liver Partition With Portal Vein Ligation (RALPP) for Liver Regeneration. *Ann Surg*. 2015;261(2):e45-e46.

215. Li J, Kantas A, Ittrich H, et al. Avoid "All-Touch" by Hybrid ALPPS to Achieve Oncological Efficacy. *Ann Surg*. 2016;263(1):e6-e7.

216. Robles Campos R, Parrilla Paricio P, López Conesa A, et al. A new surgical technique for extended right hepatectomy: tourniquet in the umbilical fissure and right portal vein occlusion (ALTPS). Clinical case. *Cirugía española*. 2013;91(10):633-637.

217. Cillo U, Gringeri E, Feltracco P, et al. Totally Laparoscopic Microwave Ablation and Portal Vein Ligation for Staged Hepatectomy. *Ann Surg Oncol*. 2015;22(8):2787-2788.

218. Machado MA, Surjan R, Basseres T, Makdissi F. Total Laparoscopic Reversal ALPPS. *Ann Surg Oncol*. 2017;24(4):1048-1049.

219. Donati M, Basile F, Oldhafer KJ. Present status and future perspectives of ALPPS (associating liver partition and portal vein ligation for staged hepatectomy). *Futur Oncol*. 2015;11(16):2255-2258.

220. Linecker M, Björnsson B, Stavrou GA, et al. Risk Adjustment in ALPPS Is Associated with a Dramatic Decrease in Early Mortality and Morbidity. *Ann Surg*. 2017;266(5):779-786.

221. Jiao LR, Puerta ABF, Gall TMH, et al. Rapid induction of liver regeneration for major hepatectomy (REBIRTH): A randomized controlled trial of portal vein embolisation versus ALPPS assisted with radiofrequency. *Cancers (Basel)*. 2019;11(3):1-14.

222. Moris D, Ronnekleiv-Kelly S, Kostakis ID, et al. Operative Results and Oncologic Outcomes of Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS) Versus Two-Stage Hepatectomy (TSH) in Patients with Unresectable Colorectal Liver Metastases: A Systematic Review and Meta-Anal. *World J Surg*. 2018;42(3):806-815.

223. Liu Y, Yang Y, Gu S, Tang K. A systematic review and meta-analysis of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) versus traditional staged hepatectomy. *Medicine (Baltimore)*. 2019;98(15):e15229.

224. Schnitzbauer AA, Schadde E, Linecker M, et al. Indicating ALPPS for Colorectal Liver Metastases: A Critical Analysis of Patients in the International ALPPS Registry. *Surg (United States)*. 2018;164(3):387-394. doi:10.1016/j.surg.2018.02.026

225. Schlegel A, Lesurtel M, Melloul E, et al. ALPPS: From humain to mice highlighting accelerated and novel mechanisms of liver regeneration. *Ann Surg*. 2014;260(5):839-847.

226. Langiewicz M, Schlegel A, Saponara E, et al. Hedgehog pathway mediates early acceleration of liver regeneration induced by a novel two-staged hepatectomy in

mice. *J Hepatol*. 2017;66(3):560-570.

227. Shi H, Yang G, Zheng T, et al. A preliminary study of ALPPS procedure in a rat model. *Sci Rep*. 2015;5:1-12.

228. Yao L, Li C, Ge X, et al. Establishment of a rat model of portal vein ligation combined with in situ splitting. *PLoS One*. 2014;9(8):1-8.

229. Dhar DK, Mohammad GH, Vyas S, Broering DC, Malago M. A novel rat model of liver regeneration: possible role of cytokine induced neutrophil chemoattractant-1 in augmented liver regeneration. *Ann Surg Innov Res*. 2015;9(1):11.

230. Almau Trenard HM, Moulin LE, Padín JM, Stringa P, Gondolesi GE, Barros Schelotto P. Development of an Experimental Model of Portal Vein Ligation Associated With Parenchymal Transection (ALPPS) in Rats. *Cirugía Española (English Ed*. 2014;92(10):676-681.

231. Andersen KJ, Knudsen AR, Jepsen BN, et al. A new technique for accelerated liver regeneration: An experimental study in rats. *Surg (United States)*. 2017;162(2):233-247.

232. Schadde E, Tsatsaris C, Swiderska-Syn M, et al. Hypoxia of the growing liver accelerates regeneration. *Surg (United States)*. 2017;161(3):666-679.

233. Wei W, Zhang T, Zafarnia S, et al. Establishment of a rat model: Associating liver partition with portal vein ligation for staged hepatectomy. *Surg (United States)*. 2016;159(5):1299-1307.

234. García-Pérez R, Revilla-Nuin B, Martínez CM, Bernabé-García A, Baroja Mazo A, Parrilla Paricio P. Associated Liver Partition and Portal Vein Ligation (ALPPS) vs selective Portal Vein Ligation (PVL) for staged hepatectomy in a rat model. Similar regenerative response? *PLoS One*. 2015;10(12):1-14.

235. Tanaka K, Matsuo K, Murakami T, et al. Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS): Short-term outcome, functional changes in the future liver remnant, and tumor growth activity. *Eur J Surg Oncol*. 2015;41(4):506-512.

236. Eshmuminov D, Tschuor C, Raptis DA, et al. Rapid liver volume increase induced by associating liver partition with portal vein ligation for staged hepatectomy (ALPPS): Is it edema, steatosis, or true proliferation? *Surgery*. 2017;161(6):1549-1552.

237. Matsuo K, Murakami T, Kawaguchi D, et al. Histologic features after surgery associating liver partition and portal vein ligation for staged hepatectomy versus those after hepatectomy with portal vein embolization. *Surg (United States)*. 2016;159(5):1289-1298.

238. Uribe M, Uribe-Echevarría S, Mandiola C, Zapata MI, Riquelme F, Romanque P. Insight on ALPPS – Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy – mechanisms: activation of mTOR pathway. *Hpb*. 2018;20(8):729-738.

239. Tong YF, Meng N, Chen MQ, et al. Maturity of associating liver partition and portal vein ligation for staged hepatectomy-derived liver regeneration in a rat model.

World J Gastroenterol. 2018;24(10):1107-1119.

240. Tomassini F, D'Asseler Y, Giglio MC, et al. Hemodynamic changes in ALPPS influence liver regeneration and function: results from a prospective study. *HPB*. October 2018:1-9.

241. Zhang DX, Li CH, Zhang AQ, et al. mTOR-Dependent Suppression of Remnant Liver Regeneration in Liver Failure After Massive Liver Resection in Rats. *Dig Dis Sci*. 2015;60(9):2718-2729.

242. Sheng R fan, Yang L, Jin K pu, et al. Assessment of liver regeneration after associating liver partition and portal vein ligation for staged hepatectomy: a comparative study with portal vein ligation. *Hpb*. 2018;20(4):305-312.

243. Balk RA. Systemic inflammatory response syndrome (SIRS): Where did it come from and is it still relevant today? *Virulence*. 2014;5(1):20-26.

244. Vincent JL, Opal SM, Marshall JC, Tracey KJ. Sepsis definitions: Time for change. *Lancet*. 2013;381(9868):774-775.

245. Von Heesen M, Hülser M, Seibert K, et al. Split-liver procedure and inflammatory response: Improvement by pharmacological preconditioning. *J Surg Res*. 2011;168(1):e125-e135.

246. Szijártó A, Fülöp A. Triggered Liver Regeneration: From Experimental Model to Clinical Implications. *Eur Surg Res*. 2015;54(3-4):148-161.

247. Wilms C, Mueller L, Lenk C, et al. Comparative Study of Portal Vein Embolization Versus Portal Vein Ligation for Induction of Hypertrophy of the Future Liver Remnant Using a Mini-Pig Model. *Ann Surg*. 2008;247(5):825-834.

248. Byun SH, Yang HS, Kim JH. Liver graft hyperperfusion in the early postoperative period promotes hepatic regeneration 2 weeks after living donor liver transplantation. *Medicine (Baltimore)*. 2016;95(46):e5404.

249. Bucur PO, Bekheit M, Audebert C, et al. Modulating Portal Hemodynamics With Vascular Ring Allows Efficient Regeneration After Partial Hepatectomy in a Porcine Model. *Ann Surg*. 2018;268(1):134-142.

250. Palmes D, Budny TB, Dietl K-H, Herbst H, Stratmann U, Spiegel H-U. Detrimental effect of sinusoidal overperfusion after liver resection and partial liver transplantation. *Transpl Int*. 2004;17(12):862-871.

251. Glanemann M, Vollmar B, Nussler AK, Schaefer T, Neuhaus P, Menger MD. Ischemic preconditioning protects from hepatic ischemia/reperfusion-injury by preservation of microcirculation and mitochondrial redox-state. *J Hepatol*. 2003;38(1):59-66.

252. Marubayashi S, Takenaka M, Dohi K, Ezaki H, Kawasaki T. Adenine nucleotide metabolism during hepatic ischemia and subsequent blood reflow periods and its relation to organ viability. *Transplantation*. 1980;30(4):294-296.

253. Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cell Signal*. 2007;19(9):1807-1819.

254. Steiner AA, Branco LGS. Hypoxia-Induced Anapyrexia: Implications and Putative Mediators. *Annu Rev Physiol*. 2002;64(1):263-288.

255. Olthof PB, Reiniers MJ, Dirkes MC, Van Gulik TM, Heger M, Van Golen RF. Protective mechanisms of hypothermia in liver surgery and transplantation. *Mol Med*. 2015;21:833-846.

256. Fong GH. Mechanisms of adaptive angiogenesis to tissue hypoxia. *Angiogenesis*. 2008;11(2):121-140.

257. Tajima T, Goda N, Fujiki N, et al. HIF-1α is necessary to support gluconeogenesis during liver regeneration. *Biochem Biophys Res Commun*. 2009;387(4):789-794.

258. Kim JW, Tchernyshyov I, Semenza GL, Dang C V. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. 2006;3(3):177-185.

259. Pelletier J, Bellot G, Gounon P, Lacas-Gervais S, Pouysségur J, Mazure NM. Glycogen synthesis is induced in hypoxia by the hypoxia-inducible factor and promotes cancer cell survival. *Front Oncol*. 2012;2:1-9.

260. Schneider M, Van Geyte K, Fraisl P, et al. Loss or Silencing of the PHD1 Prolyl Hydroxylase Protects Livers of Mice Against Ischemia/Reperfusion Injury. *Gastroenterology*. 2010;138(3):1143-1154.

261. Kron P, Linecker M, Limani P, et al. Hypoxia-driven Hif2a coordinates mouse liver regeneration by coupling parenchymal growth to vascular expansion. *Hepatology*. 2016;64(6):2198-2209.

262. Ding B, Cao Z, Lis R, et al. Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. *Nature*. 2014;505(7481):97-102.

263. Singhal M, Liu X, Inverso D, et al. Endothelial cell fitness dictates the source of regenerating liver vasculature. *J Exp Med*. 2018;215(10):2497-2508.

264. Vénéreau E, Ceriotti C, Bianchi ME. DAMPs from Cell Death to New Life. *Front Immunol*. 2015;6(AUG):1-11.

265. Yang S, Xu L, Yang T, Wang F. High-mobility group box-1 and its role in angiogenesis. *J Leukoc Biol*. 2014;95(4):563-574.

266. Dili A, Bertrand C, Lebrun V, Pirlot B, Leclercq IA. Hypoxia protects the liver from Small For Size Syndrome: A lesson learned from the associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) procedure in rats. *Am J Transplant*. 2019;19(11):2979-2990.

267. Cai S-R, Motoyama K, Shen KJ, Kennedy SC, Flye MW, Ponder KP. Lovastatin decreases mortality and improves liver functions in fulminant hepatic failure from 90% partial hepatectomy in rats. *J Hepatol*. 2000;32(1):67-77.

268. Belghiti J, Liddo G, Raut V, et al. "Inherent limitations" in donors: Control matched study of consequences following a right hepatectomy for living donation and benign liver lesions. *Ann Surg*. 2012;255(3):528-533.

269. Dehler M. Hypoxia causes permeability oedema in the constant-pressure perfused rat lung. *Eur Respir J*. 2006;27(3):600-606.

270. Juzekaeva E, Gainutdinov A, Mukhtarov M, Khazipov R. Dynamics of the hypoxia—induced tissue edema in the rat barrel cortex in vitro. *Front Cell Neurosci*.

2018;12(December):1-11.

271. Michiels C. Physiological and pathological responses to hypoxia. *Am J Pathol*. 2004;164(6):1875-1882.

272. Dili A, Lebrun V, Bertrand C, Leclercq IA. Associating liver partition and portal vein ligation for staged hepatectomy: establishment of an animal model with insufficient liver remnant. *Lab Invest*.2019;99(5):698-707.

273. Hu J, Srivastava K, Wieland M, et al. Endothelial Cell-Derived Angiopoietin-2 Controls Liver Regeneration as a Spatiotemporal Rheostat. *Science*. 2014;343(6169):416-419.

274. Michalopoulos GK. Liver regeneration after partial hepatectomy: Critical analysis of mechanistic dilemmas. *Am J Pathol*. 2010;176(1):2-13.

275. Ball MK, Waypa GB, Mungai PT, et al. Regulation of hypoxia-induced pulmonary hypertension by vascular smooth muscle hypoxia-inducible factor-1α. *Am J Respir Crit Care Med*. 2014;189(3):314-324.

276. Nakada Y, Lu Z, Canseco DC, et al. Hypoxia induces heart regeneration in adult mice. *Nature*. 2016;541(7636):222-227.

277. Ren P, Kang Z, Gu G, et al. Hyperbaric oxygen preconditioning promotes angiogenesis in rat liver after partial hepatectomy. *Life Sci*. 2008;83(7-8):236-241.

278. Matsumoto S, Kikuta J, Ishii M. Intravital imaging of liver cell dynamics. *Methods Mol Biol*. 2018;1763:137-143.

279. Anton N, Parlog A, Bou About G, et al. Non-invasive quantitative imaging of hepatocellular carcinoma growth in mice by micro-CT using liver-targeted iodinated nano-emulsions. *Sci Rep*. 2017;7(1):1-13.

280. Jankovic B, Aquino-Parsons C, Raleigh JA, et al. Comparison between pimonidazole binding, oxygen electrode measurements, and expression of endogenous hypoxia markers in cancer of the uterine cervix. *Cytom Part B - Clin Cytom*. 2006;70(2):45-55.

281. O'Connor JPB, Boult JKR, Jamin Y, et al. Oxygen-enhanced MRI accurately identifies, quantifies, and maps tumor hypoxia in preclinical cancer models. *Cancer Res*. 2016;76(4):787-795.

282. Presley T, Kuppusamy P, Zweier JL, Ilangovan G. Electron paramagnetic resonance oximetry as a quantitative method to measure cellular respiration: A consideration of oxygen diffusion interference. *Biophys J*. 2006;91(12):4623-4631.

283. Greenhalgh SN, Conroy KP, Henderson NC. Cre-ativity in the liver: Transgenic approaches to targeting hepatic nonparenchymal cells. *Hepatology*. 2015;61(6):2091-2099.

284. Hoekstra LT, van Lienden KP, Doets A, Busch ORC, Gouma DJ, van Gulik TM. Tumor Progression After Preoperative Portal Vein Embolization. *Ann Surg*. 2012;256(5):812-818.

285. Pamecha V, Levene A, Grillo F, Woodward N, Dhillon A, Davidson BR. Effect of portal vein embolisation on the growth rate of colorectal liver metastases. *Br J Cancer*.

2009;100(4):617-622.

286. Maggiori L, Bretagnol F, Sibert A, Paradis V, Vilgrain V, Panis Y. Selective portal vein ligation and embolization induce different tumoral responses in the rat liver. *Surgery*. 2011;149(4):496-503.

287. Chouaib S, Messai Y, Couve S, Escudier B, Hasmim M, Noman MZ. Hypoxia promotes tumor growth in linking angiogenesis to immune escape. *Front Immunol*. 2012;3(FEB):1-10.

288. Kambakamba P, Linecker M, Schneider M, et al. Impact of associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) on growth of colorectal liver metastases. *Surg (United States)*. 2018;163(2):311-317.

289. Wanis KN, Ardiles V, Alvarez FA, et al. Intermediate-term survival and quality of life outcomes in patients with advanced colorectal liver metastases undergoing associating liver partition and portal vein ligation for staged hepatectomy. *Surgery*. 2018;163(4):691-697.

290. Shin KH, Diaz-Gonzalez JA, Russell J, et al. Detecting changes in tumor hypoxia with carbonic anhydrase IX and pimonidazole. *Cancer Biol Ther*. 2007;6(1):70-75.

291. Krohn KA, Link JM, Mason RP. Molecular imaging of hypoxia. *J Nucl Med*. 2008;49(SUPPL.6):129-149.

292. Kobayashi N. Prevention of Acute Liver Failure in Rats with Reversibly Immortalized Human Hepatocytes. *Science (80-)*. 2000;287(5456):1258-1262.

293. Chen Y, Li J, Liu X, Zhao W, Wang Y, Wang X. Transplantation of Immortalized Human Fetal Hepatocytes Prevents Acute Liver Failure in 90% Hepatectomized Mice. *Transplant Proc*. 2010;42(5):1907-1914.

294. Stutchfield BM, Rashid S, Forbes SJ, Wigmore SJ. Practical barriers to delivering autologous bone marrow stem cell therapy as an adjunct to liver resection. *Stem Cells Dev*. 2010;19(2):155-161.

295. Harnoss JM, Platzer LK, Burhenne J, et al. Prolyl hydroxylase inhibition enhances liver regeneration without induction of tumor growth. *Ann Surg*. 2017;265(4):782-791.

296. Bernhardt WM, Wiesener MS, Scigalla P, et al. Inhibition of prolyl hydroxylases increases erythropoietin production in ESRD. *J Am Soc Nephrol*. 2010;21(12):2151-2156.

297. Rocco M, D'Itri L, De Bels D, Corazza F, Balestra C. The "normobaric oxygen paradox": a new tool for the anesthetist? *Minerva Anestesiol*. 2014;80(3):366-372.

298. Cimino F, Balestra C, Germonpré P, et al. Pulsed high oxygen induces a hypoxic-like response in human umbilical endothelial cells and in humans. *J Appl Physiol*. 2012;113(11):1684-1689.

299. Khalife M, Wiams K, Ben Aziz M, Paesmans M, Balestra C, Sosnowski M. Effects of induced relative hypoxia during the postoperative period of abdominal oncologic surgery, on hemoglobin and reticulocyte levels: a prospective, randomized controlled clinical trial. *Crit Care*. 2012;16(Suppl 1):P262.

300. Eltzschig HK, Bratton DL, Colgan SP. Targeting hypoxia signalling for the treatment of ischaemic and inflammatory diseases. *Nat Rev Drug Discov*. 2014;13(11):852-869.