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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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RUNNING TITLE: Hypoxia prevents mortality in SFSS

Mini-abstract:

We experimentally studied the hemodynamics in ALPPS and SFSS-setting hepatectomies. ALPPS and SFSS hemodynamically differ by a much lower arterial flow per unit liver mass in ALPPS. We show that the ensuing hypoxic response is essential for the function of the regenerating liver by preserving sinusoidal morphology.

LIST of ABBREVIATIONS:

ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy

BW: Body Weight

DAB: 3,3'-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

iHAF: indexed hepatic artery flow

IF: Immunofluorescence

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

qPCR: quantitative Polymerase Chain Reaction

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

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

ABSTRACT

Portal hyperperfusion and "dearterialization" of the liver remnant are the main pathogenic mechanisms for Small For Size syndrome (SFSS). ALPPS induces rapid remnant hypertrophy. We hypothesized similar increase in portal pressure/flow into the future liver remnant in ALPPS and SFSS-setting hepatectomies.

Methods: 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.

Results: 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 up-regulation 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.

Conclusion: 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.

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 [2,3,4] 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 "dearterialisation" and hypoxia of the remnant liver are the proposed primary factors for life-threatening postoperative liver failure[7,8,9,10]. 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[11,12,13,14,15] 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.

Methods:

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 12am. Because it fluctuates with food intake, the portal flow was measured on 12-hours fasten animals.

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 5cc 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, 2mm 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 (i.e. 13% of the initial volume) (PVLT-PHx or ALPPS).

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 harvested. **Fig.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) (200mg/Kg BW) intraperitoneally 12h before extended partial hepatectomy (DMOG-PHx)[26].

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); b) the expression of ki67 in hepatocyte nulcei by immunohistological staining (IHC) and morphometrical quantification (see supplementary materials for technical details).

Blood Flow analysis

After an overnight fasting, animals underwent a laparotomy and microscope-assisted dissection (x20 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 (2mm) and MA0.5PSB (0,5mm) flow probes (Transonic Systems Inc, Ithaca, NY), respectively. PowerLab hard- and softwares 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 x 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 x 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).

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).

Gene and Protein analyses

Gene expression of angiogenic proteins[30] was measured by qPCR on RNA extracted from whole LML tissue (primer sets in **Suppl Fig 1).** We used 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 (LSBio, Seattle, USA, LS-F11633).

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 5 images (20x 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.

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

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 **(Fig. 1B).** When PVLT (PVLT-PHx or ALPPS) preceded PHx the seven-day mortality was significantly lower (25%, p=0.0002) **(Fig 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=0.23) **(Fig 1C).** ALPPS, but not Sham operation, dramatically reduced the mortality that occurs after a SFSS-setting hepatectomy.

Hepatocyte do 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=0.007). FLR mass recovery was also significantly enhanced in the PHx group compared to Sham (p=0.0002) **(Fig 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<0.0001 and p=0.05, 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=0.006) **(Fig 1E).**

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<0.0001) **(Fig. 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 4 to 5 compared to the flow into LML in Sham animals (p=0.0001), with no difference between PVLT and PHx (p=0.2) **(Fig. 2B)**. The total hepatic artery flow (HAF) decreased in both PVLT and PHx compared to Sham (p<0.0001) **(Fig. 2C)** and was significantly lower after PHx compared to PVLT (p=0.0004) **(Fig 2C).** While arterial blood is distributed to the entire liver in PVLT, it

perfused only the remnant in PHx (i.e. the 13% of the original liver mass). Thus, compared to Sham, the indexed arterial flow into the FLR (iHAF) was increased after PHx (p<0.0002) while it tended to decrease after PVLT (p=0.05) **(Fig. 2D).** The iHAF in PVLT was significantly lower compared to PHx (p<0.0001). 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=0.0002 and p=0.03, respectively), supporting hypoxia in the FLR. At 24h, staining was significantly more intense in FLR tissue after PVLT than after PHx (p=0.01) **(Fig. 2E, Suppl Fig 2** for quantification**)**. The difference persisted, but to a lower extend, on day 2 *(data not shown)*.

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 response[30]. The ratio of nuclear to cytosolic HIF-1α was higher in PVLT than in PHx 4 hours after intervention (p=0.01) **(Fig 3A).** Similarly, at the same timepoint, HIF-2α nuclear to cytoplasmic ratio was also higher in PVLT compared to PHx (p=0.01) **(Fig 3B).** On the first postoperative day, VEGF gene expression was significantly increased in PVLT compared to PHx (p=0.02), so was the expression of VEGF Receptor 2 (KDR)(p=0.007) **(Fig 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 6h after PVLT compared to PHx (p=0.0004) **(Fig 4A).** In parallel, the expression of RAGE and TLR4, two receptors on which HMGB1 binds to activate neoangiogenesis[33,34,35], was significantly higher in FLR after PVLT than after PHx (p=0.0007) **(Fig 4B+C).** All angiogenic factors tested showed higher gene expression in the ALPPS step I group compared to PHx **(Suppl Fig 3).** The expression of VE-Cadherin, a glycoprotein indispensable for proper vascular development, and of CD31, which reflects the endothelial cells pool, were significantly up-regulated in PVLT compared to PHx (p=0.0007 and p=0.04, respectively) **(Fig 5A, B).**

ALPPS step I rescues liver sinusoidal morphology

As visualized on Lyve-1 immunostaining **(Fig 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 6h ($p=0.01$) and even more so at day 1 ($p<0.0001$) while sinusoids were significantly smaller after extended hepatectomy **(Fig 5D).** The curve of distribution of sinusoid size confirmed this finding **(Fig 5E).**

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). **(Fig. 6A)**. We confirmed increased pimonidazole staining with the two procedures compared to extended PHx alone **(Fig. 6B, Supp. Fig. 4A** 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%, $p<0.0001$), such as survival is comparable to that obtained with ALPPS. Neither local nor systemic hypoxia affected the final FLR mass recovery **(Fig. 6D).** Hepatocyte proliferation attested by Ki67 IHC was induced in all three groups compared to Sham ($p<0.05$) but was higher in hepatic artery ligation model (PHx-LHA) compared to PHx alone ($p=0.01$) **(Fig. 6E).**

Hypoxia improves sinusoidal morphology

There was a significant activation in HIF-1α **(Fig 7A)**, but not in HIF-2α **(Fig 7B),** 6hours after surgery in the PHx-LHA group compared to PHx ($p=0.04$). Expression of angiogenesis markers Angiopoietin 1 **(Fig 7C)**, Angiopoietin 2 **(Fig 7D)** and PDGFb **(Fig 7E)** as well as VE-Cadherin gene expression **(Fig 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 ($p=0.01$) at early time points **(Suppl Fig 4B).** Induction of hypoxia in SFSS,

whether through hepatic artery ligation or DMOG treatment, did not alter expression of HMGB1 receptors RAGE and TLR4 **(Suppl Fig 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, $p<0.0001$, DMOG-PHx vs PHx, $p=0.0001$) **(FIG 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 **(Fig 8C, D).**

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, i.e. 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 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 a 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 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 with oxygenated blood of the FLR 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 prevent 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 failure of hepatocyte regeneration. Ninomya et al. rather 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 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 on neovascularisation, and with up regulation 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 observe both HIF-1α and HIF-2α up-regulation in ALPPS compared to SFSS-setting hepatectomy, HIF-2α was not up-regulated 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 obtain by hepatic artery ligation) triggers an early neo-angiogenic response as supported by up-regulation 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 neaoangiogenesis (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 favouring 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[48,35]. 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 neoangiogenesis[49,50,33,34]. 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[51,52,53]. 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 neoangiogenesis 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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AUTHORS CONTRIBUTIONS:

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

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LEGENDS:

Figure 1: 87% 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 2hours 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 LMR and LML; we resected all deportalized segments on day 2. A total of 77 animals were investigated, n= 6 at 2h, n=15 at 4h, n= 11 at 6h, 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=0.0002 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=0.23 using Log-rank (Mantel-Cox) test). **(D)** Future Liver remnant (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=0.0001 and p=0.0002, respectively). Postoperative FLR hypertrophy was more important in PVLT-PHx group compared to PHx (p=0.007). **(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 ($p<0.0001$ and p=0.05, respectively). Hepatocyte proliferation was significantly more important in the PVLT-PHx group compared to PHx (p=0.006).

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 ($p<0.0001$) and a significant increase in the indexed portal flow in both PVLT and PHx compared to Sham ($p<0.0001$), with no difference in the inflow between the two procedures (p=0,2). **(C)** Total Hepatic Artery Flow (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 ($p<0.0001$). Compared to Sham, there was a tremendous increase in the indexed arterial flow in PHx ($p<0,0002$) but a decrease in PVLT (p=0.05). Thus, FLRs in ALPPS step I receive significantly less arterial blood compared to PHx ($p<0.0001$). **(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 = 100μm.

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 Mann-Witney test. Individual data mean and SEM per group are presented.

Figure 4: DAMPS response to hypoxia

HMGB1 concentrations measured by ELISA in serum prepared from cardiac blood **(A)** in PVLT (2h, n=3; 4h, n=9;6h, n=7) and PHx (2h, n=5; 4h, n=5; 6h, 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-Witney test. Individual data mean and SEM per group are presented.

Figure 5: In ALPPS FLRs the sinusoidal bed is preserved

(A) VE-Cadherin and **(B)** CD31 gene expression one day after the operation in PVLT (n=8) and PHx (n=6) expressed as fold induction compared to Sham (controls) and analyzed using 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 = 50µm **(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 ($p=0.01$) and this difference widened at day 1 ($p<0.0001$). Of note, 1 day after surgery, sinusoids in PHx are collapsed compared to Sham ($p=0.002$).

Figure 6: Hypoxia in SFSS rescues survival

(A) Schematic representation of the study designed: 42 animals underwent 87% extended hepatectomy (PHx; n=12 at 6h, n=11 at 1day 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 6h; n=5 at day 1 and n=6 at day 7) ; 20 animals received a DMOG injection (I.P.) 12h prior to extended PHx(DMOG-PHx; n=9 at 6h; n=5 at day 1 and n=6 at day 7). **(B)** Morphometrical quantification of Pimonidazole IHC on FLR on the 1st 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 ($p=0,04$ and $p=0,01$ 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<0.0001 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=0.01). All models of partial extended hepatectomy showed significant proliferation index compared to Sham (p=0.0036 for PHx, p<0;0001 for PHx-LHA and p=0;004 for DMOG-PHx). Individual data, mean and SEM are presented. Data are analyzed by ANOVA.

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 one 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.

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<0.0001 and p=0.0001, respectively), so as there is no difference in vascular morphology of these hypoxic models compared to ALPPS step I.

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