ORIGINAL ARTICLE

Insight on ALPPS – Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy – mechanisms: activation of mTOR pathway

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Abstract

Background: ALPPS procedure has been introduced to increase the volume of future liver remnant. The mechanisms underlying the accelerated regeneration observed with ALPPS are unknown. It was hypothesized that AMPK/mTOR is activated as an integrating pathway for metabolic signals leading to proliferation and cell growth. Our aim was to analyze increase in liver volume, proliferation parameters and expression of AMPK/mTOR pathway-related molecules in patients undergoing ALPPS.

Methods: A single center prospective study of patients undergoing ALPPS was performed from 2013 to 2015. Liver and serum samples, clinical laboratory results and CT-scan data were obtained. ELISA, Ki-67 immunostaining and qRT-PCR were performed in deportalized and remnant liver tissue in both stages of the procedure.

Results: 11 patients were enrolled. Remnant liver volume increased 112 ± 63% ($p < 0.05$) in 9.1 ± 1.6 days. Proliferation-related cytokines IL-6, TNF-α, HGF and EGF significantly increased, while higher Ki-67 immunostaining and cyclin D expression were observed in remnant livers after ALPPS. mTOR, S6K1, 4E-BP1, TSC1 and TSC2 expression were significantly increased in remnant livers at second stage, while AMPK and Akt increased only in deportalized liver samples.

Conclusion: Rapid liver regeneration with ALPPS might be associated with hepatocyte proliferation induced by mTOR pathway activation.

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Introduction

Liver resection is the treatment of choice for most primary and metastatic liver tumors. Complete resection is often the only treatment strategy with a curative intent, achieving up to 40–67% 5-year survival in patients with colorectal liver metastases.[1–3] Unfortunately, patients are usually deemed unresectable and only 15–20% are surgical candidates.[4,5]

A primary reason for unresectability is an insufficient future liver remnant (FLR), which is associated with a high risk of liver failure.[6] Surgical techniques have been developed to induce liver enlargement and achieve an appropriate FLR, including portal vein embolization (PVE) and portal vein ligation (PVL).[7,8]

Although PVL and PVE obtain similar results in terms of liver growth, time interval between PVE/PVL and hepatectomy is highly variable[9] and up to 35.2% fail to complete a second stage hepatectomy, due to disease progression or insufficient FLR volume.[10–13]

In recent years, a new surgical strategy has been developed: Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS).[14] ALPPS was first performed in Germany, and it consists of a two-stage procedure. During the first surgery, right portal vein ligation and transection of the liver is performed in the site of the future resection. Clearance of metastases of the left lobe is completed as completion hepatectomy

is undertaken during a second procedure.[15] In 2012 Schnitzbauer *et al.* published the first multicentric series of 25 patients undergoing ALPPS with significant liver volume gaining (74%), in a mean period of 9 days. Morbidity rate of 64% and 12% mortality were reported.[15] After this initial experience, several groups have published their experience and an initiative (www.alpps.org) has been developed to analyze the results.[16–18]

ALPPS optimizes liver growth, allowing the remnant liver to proliferate in a shorter time-frame compared with PVL/PVE.[19,20] As a result, this can increase the rate of resectability, operative safety, likelihood of R0 resections with negative margins and therefore decrease the risk of disease progression.[21] Mechanisms involved in the accelerated liver regeneration induced by ALPPS have not been studied in humans.

Nutrients and energy are essential for cellular proliferation. Nutrient entrance to the liver is mainly through portal flow. mTOR (mammalian Target of Rapamycin) is a 289-kDa serine/threonine kinase, that is an essential element in the signaling pathway involved in cell growth and proliferation control.[22] The inhibition of mTOR impairs liver regeneration after hepatectomy.[23] AMPK (5′ adenosine monophosphate-activated protein kinase) is a key energy sensing kinase that can modulate mTOR: increase in AMP/ATP ratio activates AMPK, which through the phosphorylation of TSC (Tuberous sclerosis complex)-2 silences mTOR.[24] On the other hand, AMPK would also have a role in liver regeneration, since in AMPK−/− animals there is a delay in S-phase entry after hepatectomy.[25]

It was hypothesized that the liver volume increase after ALPPS is achieved through a regenerative process related to molecules entering the remnant liver tissue through portal flow, which, in turn, modulate the mTOR/AMPK pathway.

Methods

Study design and eligibility criteria for ALPPS

A prospective study was conducted in patients referred to the Hepatobiliary Surgery program at Hospital del Salvador, Santiago, Chile, between 2013 and 2015. The study was approved by the Institutional Review Board and Ethics Committee. Eligibility criteria included patients considered unsuitable for one-stage hepatectomy with insufficient FLR volume. Exclusion criteria included age <18 years old, known liver cirrhosis (Child B or C), extrahepatic metastases and anesthesia risk ASA > II (Supplementary Table 1). All patients were presented at a Multidisciplinary Tumor Conference for evaluation. An informed consent was obtained before enrollment. All procedures fulfilled ethical standards of Helsinki's Declaration of 1975.

Surgical technique and clinical outcomes

ALPPS was performed as previously described.[17] All patients were managed in the Intensive Care Unit. On the 9th postoperative (PO) day, computed tomography (CT) was performed to assess growth of the remnant liver. Extended right hepatectomy was performed if sufficient liver enlargement was obtained to secure a future remnant of at least 30% of total liver volume, or remnant liver volume to body weight ratio was ≥0.5.[26] Posthepatectomy liver failure (PHLF) was defined according "50-50 criteria" proposed by Balzan *et al.*[27] and surgical complications were evaluated according to Clavien Dindo classification.[28]

Tissue and serum samples

Upon initial laparotomy (ALPPS Stage I), 1 mL of liver tissue was taken from the right and left lobes, in tumor-free areas. Additional samples of both lobes were taken immediately before hepatectomy at ALPPS Stage II. A sample was kept in formalin 10% for histological analysis and the remaining was stored at −80 celsius degrees for qRT-PCR studies. Blood samples were also taken before ALPPS Stage I and immediately before hepatectomy during Stage II for ELISA studies. Transaminases, bilirubin and prothrombin time/international normalized ratio levels were measured during ICU stay.

Table 1 Clinical characteristics of patients recruited for the study

Variable	Patients (n = 11)
Age (years), mean (±SD)	58.8 (±8)
Male/female, n (%)	6/5 (54.5%/45.5%)
Primary tumor	
Colorectal cancer metastases, n (%)	9 (81.8)
Neuroendocrine tumor metastases, n (%)	1 (9.1)
Hepatocellular carcinoma, n (%)	1 (9.1)
[a]Age-adjusted Charlson comorbidity index, median (IQR)	8 (7–9)
Performance status, median (IQR)	0 (0–0)
Preoperative chemotherapy, n (%)	10 (90.9%)
Chemotherapy, n (N° cycles per patient)	
XELOX	6 (3, 3, 3, 6, 8 and 8 cycles)
FOLFOX	1 (4 cycles)
FOLFOXIRI	1 (6 cycles)
FOLFIRI + Bevacizumab	1 (4 cycles of FOLFIRI + 3 cycles of Bevacizumab)[b]
XELODA	1 (3 cycles)
90YDOTATOC/Lu177-DOTATATE	1 (4 doses of Lu177-DOTATATE)
Preoperative radiotherapy, n (%)	1 (9.1%)

XELOX, oxaliplatin, capecitabine; FOLFOX, folinic acid, 5-fluorouracil, oxaliplatin; FOLFOXIRI, folinic acid, 5-fluorouracil, oxaliplatin, irinotecan; FOLFIRI, 5-fluorouracil, irinotecan, leucovorin; XELODA, capecitabine.
IQR, interquartile range; SD, standard deviation.
[a] Charlson index is a validated method to quantify comorbidities.
[b] The patient who received this type of chemotherapy, also was treated with 8 cycles of XELOX.

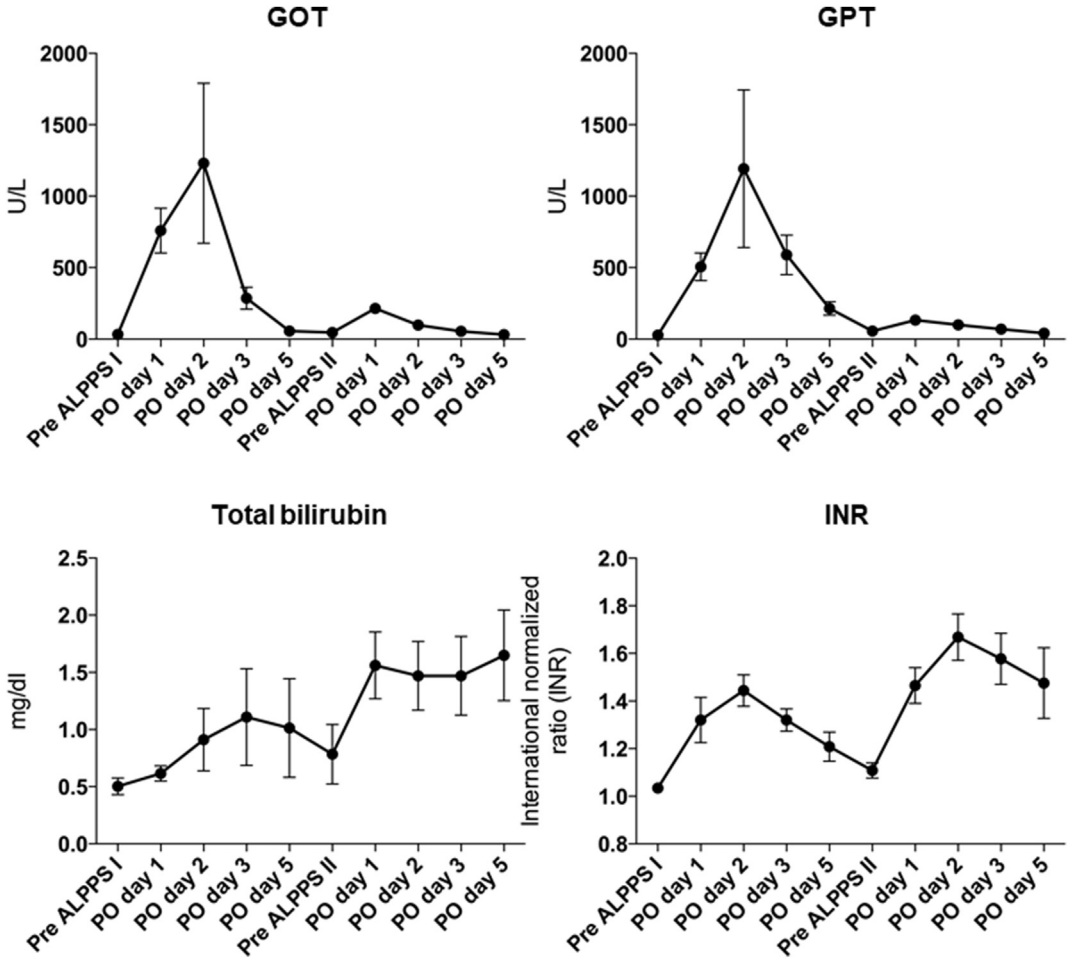

Figure 1 Perioperative function tests and liver injury in ALPPS patients. PO, postoperative day; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; INR, international normalized ratio. Values are shown as mean ± SEM (n = 11)

Liver volume assessment

Patients' weight, height, BMI and volumetric measurements of the liver were recorded before and after ALPPS Stage I. All liver volumetric assessments were performed using CT and 3D reconstructions with Osirix® software. As volumetric sufficiency index of remnant liver, future liver remnant over body weight ratio (FLR/BW) was calculated for each patient.

Histological and immunohistochemical studies

Samples were fixed, embedded in paraffin, sliced and stained with hematoxylin and eosin (HE). Ki-67 immunostaining (Ki-67 antibody, DAKO, Kyoto, Japan) was performed in deparaffinized sections after antigen retrieval. Positive Ki-67 hepatocytes' nuclei were counted in 10 adjacent HPF (High Power Field) per slide and expressed as number of positive nuclei per HPF.

ELISA studies

Liver proliferation markers (TNF-α, IL-6, HGF, EGF and TGF-β) were studied in serum samples, with commercially available ELISA kits (RyD Systems, Inc., USA) according to manufacturer instructions.

Quantitative real-time PCR

Total RNA was extracted using RNeasy® Mini Kit (QUIAGEN Sciences, USA) according to the user's manual. After generation of cDNA (ImProm-II™ System, Promega Corporation, USA), quantitative real-time PCR amplification and analysis was carried out in a Stratagene Mx3005P, using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, USA) following the manufacturer's protocols. Gene specific human primer sequences used are shown in Supplementary Table 2. The expression level was normalized against β-actin and relative expression was calculated through ΔΔCT.

Statistical analysis

Data are expressed as mean ± SD or SEM. Differences between groups were assessed by T-student or Wilcoxon tests. The

statistical significance was $p < 0.05$. Analysis was performed with GraphPad Prism 5.0.

Results

Clinical characteristics, clinical outcome and liver function

Thirteen patients underwent ALPPS, 11 had complete records and were included in the study. All patients had an optimum performance status before surgery, without major comorbidities. Clinical characteristics are presented in Table 1.

All patients completed ALPPS Stage II at a median of 14 days (IQR 12–14 days). Control CT was performed 9.1 ± 1.6 days following the first stage of ALPPS (Supplementary Table 3).

Following ALPPS Stage I, a significant increase in serum levels of transaminases GOT/GPT was observed, reaching peak levels at 48 h after surgery (1230 ± 559.9 U/L and 1192 ± 552.4 U/L), decreasing after day 3 PO. Serum total bilirubin had a slight increase after ALPPS Stage I, followed by a second peak after hepatectomy, reaching maximum levels at day 5 PO (1.6 ± 0.4 mg/dL). INR increased twice, 2 days post first stage and 2 days after hepatectomy, being higher after the second stage (1.7 ± 0.1) (Fig. 1).

No patients met criteria for PHLF at day 5 PO; however, one presented with progressive elevation of total bilirubin and INR, reaching levels of 3.9 mg/dL and 2.9 respectively on postop day 10. Another patient had clinical signs of liver dysfunction with total bilirubin 5.2 mg/dL and normal INR. Both patients demonstrated clinical improvement and complete normalization of biochemical parameters.

In the assessment of surgical complications, twenty type I or II events were observed in 7 patients. Type IIIB complications were observed in two patients: one had diffuse peritonitis secondary to ileal perforation at day 5 after ALPPS Stage II. This complication was early diagnosed at postoperative period, and the patient was quickly reoperated having a favorable outcome without multi-organ dysfunction. Another patient developed intestinal obstruction after ALPPS Stage I, which was related to the non-resected primary colonic tumor. Type IVa complications were observed also in two patients, who showed clinical signs of PHLF. Both had a favorable outcome with complete clinical regression, without delay in ALPPS Stage II. No mortality was found (Supplementary Table 4). The overall survival was 59% at 12 months and 47% at two years.

Liver regeneration parameters: FLR hypertrophy rates, Ki-67, cyclin D and serum parameters of proliferation

The mean volume of FLR was 299 ± 103 mL before surgery, increasing to 589 ± 141 mL after ALPPS Stage I. This was equivalent to an absolute volume gain of 290 ± 108 mL between stages in a period of 9 days, reaching a rate of 34 ± 17 mL per day. Although, at the group level the absolute remnant liver volume increased by 97% in 9 days, the quantification that better shows the volumetric changes observed at clinical practice is the mean of relative remnant liver volume increment, considering the relative liver enlargement reached by each patient. In this way, the mean was 112 ± 63%, which ranged between 35 and 214% (Fig. 2a). Future liver remnant over body weight ratio (FLR/BW) increased from 0.4 ± 0.1% to 0.8 ± 0.2% between both stages (Fig. 2a–b).

In the right lobe no significant changes were distinguished in Ki-67 positive nuclei/HPF counting between stages (1.1 ± 0.3 at Stage I vs. 0.5 ± 0.1 positive nuclei/HPF at Stage II; $p > 0.05$); while in the left lobe a significant increase in Ki-67 positive nuclei/HPF was observed, 1 ± 0.3 positive nuclei/HPF at Stage I vs. 2.8 ± 0.6 positive nuclei/HPF at Stage II; $p < 0.01$ (Fig. 3a–b). Cyclin D mRNA relative levels varies from 1.3 ± 0.2 in the left lobe in ALPPS Stage I to 3 ± 0.4 in Stage II (t test, $p < 0.01$), while it has no significant difference in the right lobe (Fig. 3c).

A significant rise in serum proinflammatory cytokines involved in liver regenerative response was detected. IL-6 rose from 8.5 ± 4.4 ρg/mL to 77.3 ± 19 ρg/mL ($p < 0.05$); while TNF-α levels increased from 3.5 ± 2.2 ρg/mL to 21.3 ± 7.7 ρg/mL ($p < 0.05$) at ALPPS Stage II. Hepatocyte growth factor (HGF)

a

Variable	Mean (SD)
Remnant liver volume increase, (ml)	290 (±108)
Relative remnant liver volume increase, (%)	112 (±63)
Kinetic of volumetric gain, (ml/day)	34 (±17)
FRL/BW before ALPPS stage I, (%)	0,4 (±0,1)
FRL/BW before ALPPS stage II, (%)	0,8 (±0,2)

b

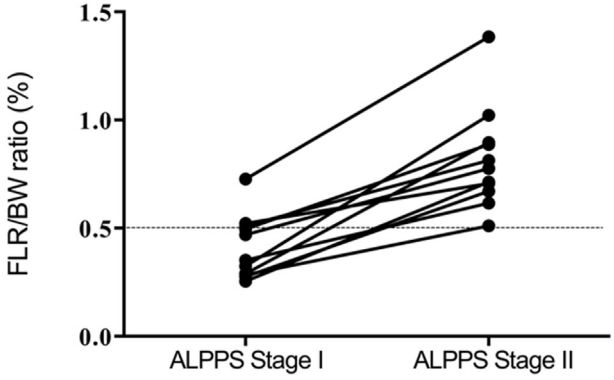

Figure 2 Liver volume increase in ALPPS patients. The mean of relative remnant liver volume increase was quantified considering the relative liver enlargement reached by each patient of this group. FRL/BW is the ratio between the estimated remnant liver weight (g) and total body mass (g), quantified in %. Dashed line shows FRL/BW = 0.5%. SD, standard deviation

serum levels also increased significantly, going from 1255 ± 113 ρg/mL at Stage I to 1586 ± 127 ρg/mL Stage II ($p < 0.01$) (Fig. 4).

EGF (Epidermal growth factor) and TGF-β (transforming growth factor beta) have also been described in progression and termination of liver regeneration, respectively. In our samples, EGF increased from 346.5 ± 45.9 to 648.9 ± 116.9 ρg/mL between stages ($p < 0.05$) (Fig. 4), while TGF-β levels did not change significantly (Data not shown).

Activation of mTOR/AMPK pathway

A significant increase in mRNA levels of mTOR was observed in remnant liver samples taken at ALPPS Stage II: 2.079 ± 0.4 vs 1.073 ± 0.2; ($p < 0.05$) (Fig. 5, upper panel), which is consistent

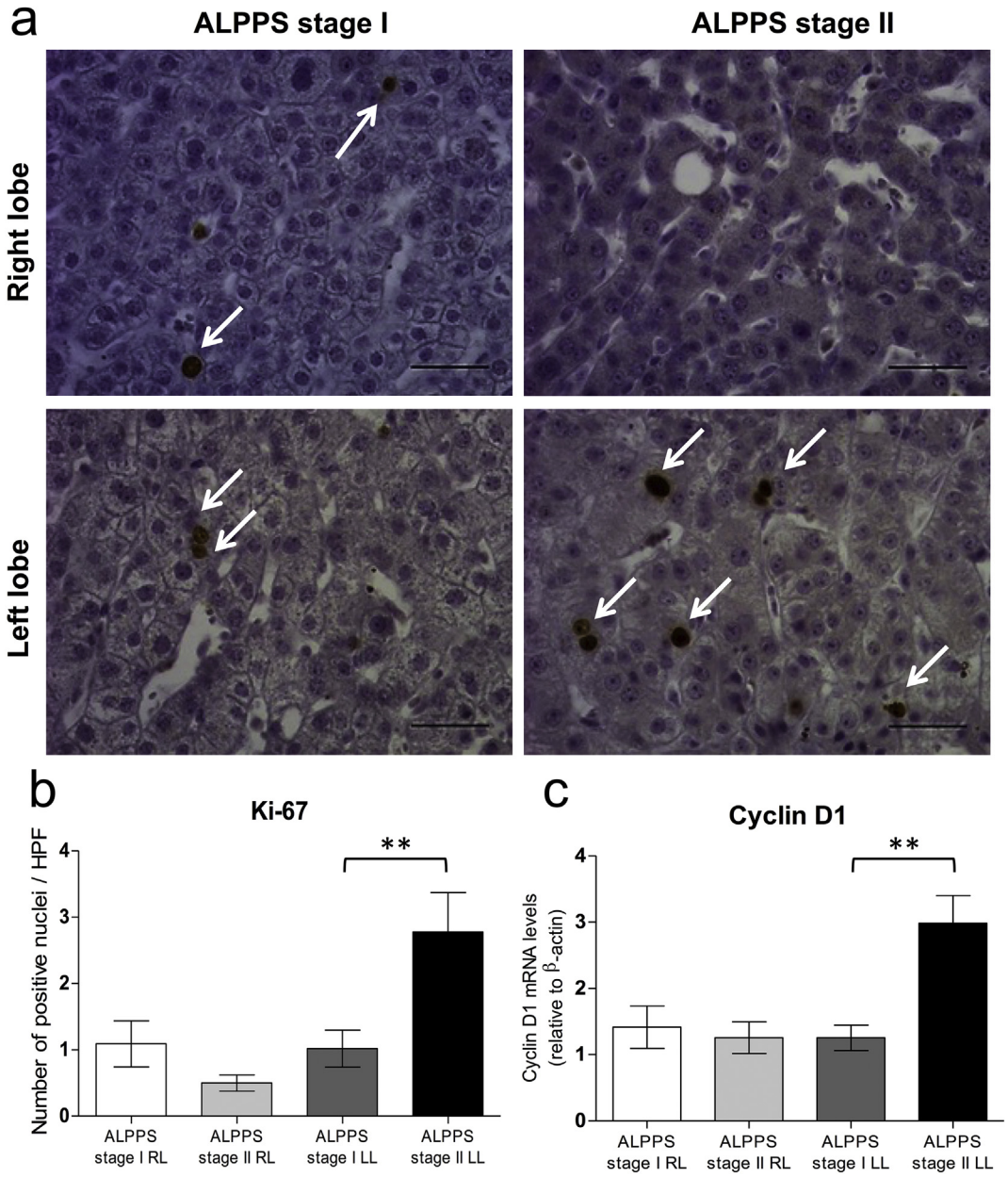

Figure 3 Proliferative parameters in ALPPS patients. Ki-67 positives hepatocytes in deportalized and remnant liver samples before ALPPS stage I and stage II. (a). Histological slides in high power field (HPF = 400×) of remnant liver (Left lobe) and deportalized lobe (Right lobe) of one patient. Arrow indicates hepatocyte nuclei Ki-67 (+) and scale bar is 50 μm. (b). Quantification of hepatocyte nuclei Ki-67 (+) in both remnant liver and deportalized lobes in all patients. (c). mRNA levels of Cyclin D1. For (b) and (c) values are shown as mean ± SEM (n = 11). Statistical significance was performed by Student's t-test for unpaired data (*$p < 0.05$, **$p < 0.01$)

with a significant increment in the expression of genes downstream of mTOR: S6K1 (S6 Kinase), and eIF4EBP1 (Eukaryotic Translation Initiation Factor-4E-Binding Protein-1, or 4EBP1) (Fig. 5).

The activation of mTOR is regulated by a heterodimer consisting of two gene products, TSC1 and TSC2. A significant increase in mRNA levels of both genes was observed after ALPPS in the left lobe, while a significant decrease of TSC1 was observed in the right lobe (Fig. 5).

Upstream mTOR, the expression of two genes were evaluated: AMPK catalytic subunit α increase significantly in the right lobe, but did not have changes in the left lobe. Akt exhibits the same behavior than AMPK in both lobes (Fig. 5).

Discussion

ALPPS was first described 5 years ago as a technique that optimizes liver regeneration in patients who require an extended hepatectomy, but have an insufficient future liver remnant. In this series of patients the volumetric increase observed post ALPPS, reached 112 ± 63%, with a final FLR/BW ratio of 0.8 ± 0.2% within 9 days. These results are consistent with initial reports of ALPPS,[29–31] as well as larger series of patients reported by the ALPPS registry.[32]

Despite the advantages of ALPPS, there is still concern regarding the clinical safety of the procedure. Morbidity, from initial series, has improved in most recent communications, reflecting more experience with this technique.[33] In our experience a critical point to decrease complications and mortality is a careful selection of candidates using a multidisciplinary evaluation.

Initial inclusion criteria considered only patients with CRC metastases, because their best clinical outcomes.[34] Liver involvement by primary or non CRC metastatic liver tumors was defined as a relative exclusion criteria. Lately, two patients with non CRC liver metastases were included, because they fulfilled all others inclusion criteria and presented excellent clinical status. The first patient, a 70 years old male with HCC had a healthy liver with optimal clinical status. He developed no perioperative complications and no relapse at 36 months post ALPPS.

The second was a patient who had a history of a neuroendocrine tumor located on the tail of the pancreas in 2003. A distal pancreatectomy with splenectomy was performed at that time, followed by medical therapy based on 90Y/DOTATOC and 177Lu/DOTATATE. The patient had no recurrence until 2013, when presented with multiple bilobar liver lesions. Initially, medical treatment with 90Y/DOTATOC-177Lu/DOTATATE was recommended, but PET/SCAN showed progression of liver

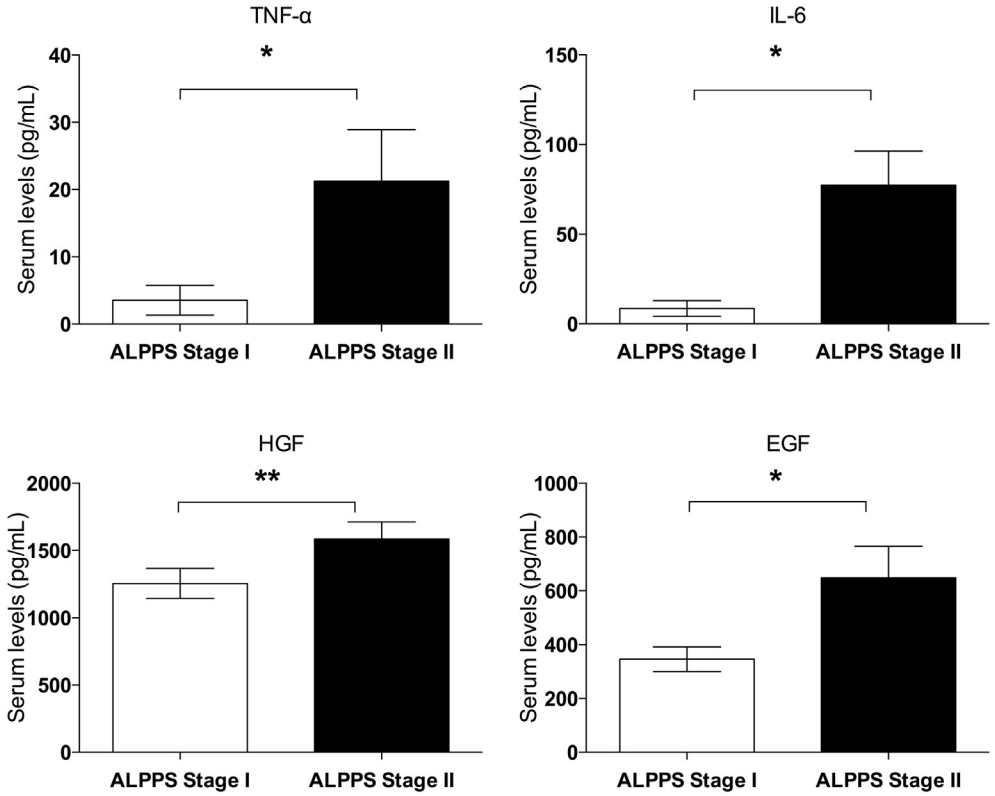

Figure 4 Serum levels of proliferation markers. Serum levels of TNF-α, IL-6, HGF and EGF. Values are shown as mean ± SEM (n = 11). Statistical significance was performed by Student's t-test for unpaired data (*p < 0.05, **p < 0.01)

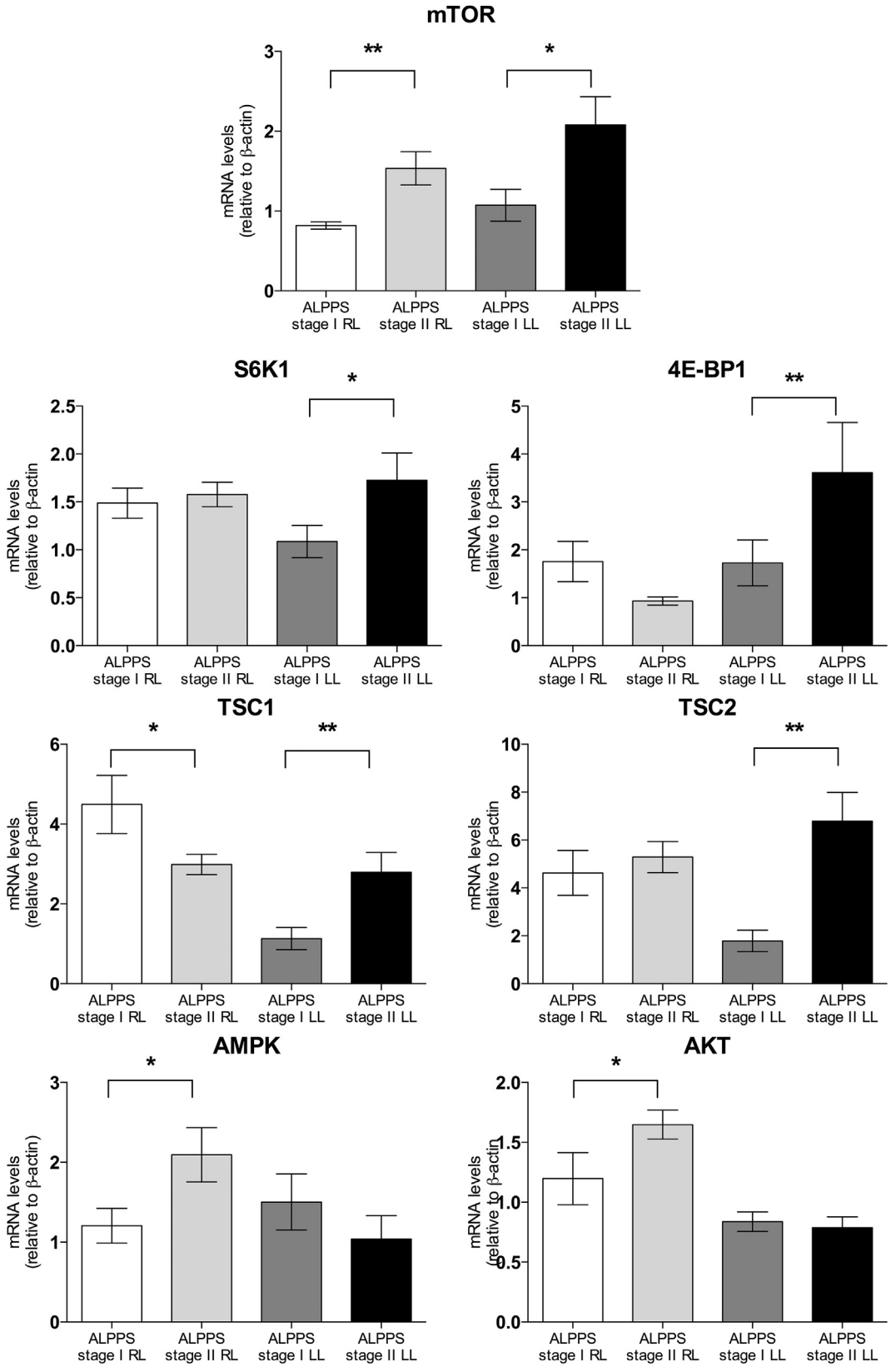

Figure 5 Expression of genes related to mTOR pathway. mRNA levels of mTOR, S6K, 4E-BP1, TSC1, TSC2, AMPK and AKT values are shown as mean ± SEM (n = 11). Statistical significance was performed by Student's t-test for unpaired data (*p < 0.05, **p < 0.01)

metastases without extrahepatic lesions. Surgical treatment of liver metastases with ALPPS was decided, allowing R0 resection. The postoperative course after ALPPS was uneventful. A 25-months disease-free survival post ALPPS to date has justified this technical approach.

All patients in this series completed an R0 liver resection; however, absolute volume increase varied from 185 to 697 mL, suggesting that other factors may impact the proliferative response in a case-by-case basis.

The influence of chemotherapy agents on the tumor-free liver parenchyma and its functionality is still unclear. Chemotherapy is a risk factor for steatosis and steatohepatitis, increasing post-operative morbi-mortality.[35] 5-Fluorouracil and leucovorin are related with fatty liver, yielding higher postoperative infection rates.[36] Chemotherapy associated steatohepatitis has been described with irinotecan use, and is associated with poorer outcomes after liver resection, mainly due to insufficient regenerative response.[36] Chemotherapy-induced liver injury has not been systematically studied as a potential factor-modifying outcome after ALPPS and larger series of patients should be analyzed to achieve this aim.

Liver regeneration is a complex sequence of biological events that have been studied in different models of hepatectomy and portal vein occlusion. It is unknown how this process is triggered by ALPPS. It has been suggested that the faster liver volume increase post ALPPS is explained by tissue edema or sinusoidal congestion. This study provides evidence that the increase in liver volume is due hepatocellular hyperplasia, as it is confirmed by Ki-67 and cyclin D results.

IL-6, TNF-α, EGF and HGF are critical promoters of liver regeneration[37] and have also been described in animal models of ALPPS.[38] These cytokines and growth factors were increased in serum from patients before ALPPS Stage II.

These molecular changes are not specific events in ALPPS, and the enhancement of these parameters may occur in other clinical situations such as regular hepatectomies, PVE and PVL. The difference should be in the kinetic changes of all these molecules and gene expressions that finally determines a greater and faster remnant liver volume increase in ALPPS compared to PVE/PVL.

Silencing of mTOR has been related to suppression of remnant liver regeneration after 85% partial hepatectomy.[39] mTOR and two effectors downstream; the ribosomal protein S6K1 and 4E-BP1, which are important for protein synthesis, were increased in the left lobe from ALPPS patients. This is the first communication that associates this pathway with the regenerative response triggered by ALPPS.

Regarding the regulation of mTOR during this process, no significant changes in Akt and AMPK expression in the proliferating lobe were found. AMPK through the phosphorylation of TSC2 inhibits mTOR action,[40] which is congruous with the result found in the left lobe and the increase in mRNA expression found in the right lobe that could be related with decrease in AMP/ATP ratio, due to redistribution of nutrients-rich portal flow.

Insulin and insulin-like growth factor 1 (IGF-1) activate mTOR through PI3K/Akt pathway.[41] The absence of activation of this pathway in the proliferating lobe, in the time frame we studied, suggests an earlier activation or other pathways implied in the regulation of cell cycle progression.

mTOR controls cell growth and metabolism in response to nutrients, growth factors, cellular energy and stress.[40] We found increase expression of mTOR and downstream effectors in proliferating liver tissue from ALPPS' patients. These findings need correlation with protein levels as well as markers of activation of the pathways in animal models.

Inhibition studies with rapamycin in animal models, or model of ALPPS in genetically modified animals would also increase the comprehension of the process and the role of mTOR in it.

70% hepatectomy results in significantly decreased glycemia, loss of systemic body mass and adipose stores as well as accumulation of liver triglycerides. When 85%–90% of the liver is resected, impaired liver regeneration and increased mortality is seen. This suggests liver regeneration can not rescue the function below a certain FLR/BW without additional support,[42] which would be the case of ALPPS. The auxiliary liver contribution and the progression curve of function recovery of the remnant liver, are variables that also need to be assessed, in order to optimize follow up and surgical planning.

No control group of PVL/PVE was included in this study, since liver tissue samples are taken only during hepatectomy, making these techniques not comparable. No baseline measurements could be done prior to liver regeneration, and it would be taken at different time-points compared to ALPPS patients, making these measurements not comparable. For all these reasons, only ALPPS patients were included in this study. To overcome these technical difficulties and limitations, it would be useful to develop an *in vivo* experimental models of ALPPS, as it has been previously described,[38,43] to complement our results.

Finally, this study provides an insight on the molecular mechanisms of liver regeneration triggered by ALPPS in a human model, where we have described a possible association between liver remnant volume increase and a molecular upregulation of mTOR pathway. Additional research is needed in order to confirm these findings.

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Conflicts of interest

All authors have no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.hpb.2018.02.636.