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ORIGINAL ARTICLE

Biological Substrate of the Rapid Volumetric Changes Observed in the Human Liver During the Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy Approach

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Abstract
Background The associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) strategy induces rapid future liver remnant (FLR) hypertrophy. Hepatocyte cellular and molecular changes associated with liver hypertrophy during ALPPS remain ill-defined in humans.
Methods Patients undergoing the ALPPS approach between June 2011 and October 2014 were extracted. Biopsies from the FLR were obtained during the first and second stages. Hematoxylin–eosin staining and immunohistochemical analysis for expression of the proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) were performed. The proliferative index was defined as: PCNA–TUNEL ratio.
Results Eleven of 34 patients treated were studied during both stages. Median FLR hypertrophy was 104 % in 6 days, with a mean difference between preoperative and postoperative volume of 361 ml ($P<0.001$). The mean hepatocyte number increased from 52.7 cells/mm^2 in the first stage to 89.6 cells/mm^2 in the second stage ($P=0.001$). PCNA expression increased by 190 % between stages with a linear correlation ($r=0.58$) with macroscopic hypertrophy. The proliferative index increased from −3.78 cells/mm^2 in first stage to 2.32 cells/mm^2 in the second stage ($P=0.034$).
Conclusions The results of the present study indicate that the rapid FLR volumetric increase observed in ALPPS is accompanied by histological and molecular features of hepatocyte cell proliferation.

Keywords Liver failure · Hypertrophy · Future liver remnant · Two stage hepatectomy · Immunohistochemical analysis

Introduction

Hepatic resection is the only potentially curative treatment for primary and metastatic liver tumors.[1] Extensive liver resections are often required to achieve tumor-free (R0) surgical margins. The modern concept of liver functional reserve is essential during preoperative planning for major liver surgery due to the fact that posthepatectomy liver failure (PHLF) represents the most important cause of mortality after extensive resections.[2,3] This fact is reflected in the trend of current liver surgery to remove the entire tumor load while maintaining a sufficient volume of future liver remnant (FLR). Safe liver surgery requires an FLR of at least 25 % of total liver volume (TLV) in patients with healthy liver parenchyma and 40 % in patients with a diseased parenchyma.[1–3] Portal vein occlusion, either as embolization (PVE) or ligation (PVL), has become the gold standard strategy to increase FLR volume with a low morbidity.[1–5] The recently proposed associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) strategy defies the classical approaches claiming higher tumor resectability rates through a more rapid FLR hypertrophy.[6–9] However, the exact substrate of such accelerated volume increases and the histological modifications accompanying these macroscopic changes are still unknown. The impressive

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short-term hypertrophy obtained with ALPPS has led many authors to question the real substrate of this volumetric modification, suggesting that these changes should be interpreted with caution since acute events as vascular congestion or even inflammation could interfere in this phenomenon.

The aim of the present study was to evaluate the cellular and molecular changes of the hepatocytes associated with the volumetric liver increase observed in humans during the ALPPS approach.

Methods

Data for patients undergoing two-stage hepatectomies with the ALPPS approach at the Hospital Italiano de Buenos Aires between June 2011 and October 2014 was extracted from a prospectively maintained database. All patients were routinely discussed in a multidisciplinary council prior to initiation of treatment. Informed consent was obtained for all patients before surgery, and the Hospital Italiano University Ethics Committee gave ethical approval to perform this study (no. 2110). In addition, every patient of this study has entered the ALPPS International Registry.[10] Both ALPPS stages were performed according to the previously described specifications.[9] Liver volume increase was measured with a multidetector computer tomography (MDCT) and volumetric reconstruction performed by a single experienced radiologist both preoperatively and 6 days after the first surgical stage.[11] In case of insufficient hypertrophy, an additional CT volumetry was performed weekly until a sufficient FLR volume was achieved. The TLV was calculated using the formula: $-794.41+1\,267.28\times$body surface area (BSA, m2; Mosteller formula).[12] The FLR volume as well as the FLR/TLV ratio was estimated before each stage to determine the grade of hypertrophy. Once volumetric CT analysis demonstrated enough FLR hypertrophy and provided the patient was in good condition, the second stage was carried out the next available operative day resecting the disease hemi-liver (DH). PHLF was classified according to the definition proposed by the International Study Group of Liver Surgery.[13]

Histological Studies

Fragments of non-tumoral parenchyma from the FLR were intraoperatively obtained by excisional biopsy at the end of the first (S1) and second (S2) surgical stages, respectively. They were received and fixed in 4 % paraformaldehyde for 24 h and then included in paraffin. Fragments of 4 μm thickness were colored in hematoxylin–eosin (HE) staining for conventional workup. The number of hepatocytes per square millimeter of liver tissue was counted in each sample. To evaluate morphological (volumetric) changes at the cellular level, 100 hepatocytes were taken randomly in 10 fields for each surgery and for each patient. Subsequently, the area (μ^2) of every hepatocyte and its core were measured. ImageJ (software for free use of the NIH) was used for measurements. They were turned over to a spreadsheet where the average of the areas and the major and minor diameters together with their standard deviations (SD) were obtained.

Immunohistochemical Analysis

Immunohistochemical analysis for expression of the proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) were performed to assess hepatocyte proliferation in the tissue fragments.

For PCNA immunostaining, immunoreactive cells were counted according to the following protocol: (1) The obtained samples were washed with phosphate-buffered saline (PBS) at pH 7.2. (2) Then, antigen retrieval was performed by heating in pH 6 citrate buffer in a microwave oven, cuts were delineated with a waterproof pen, and the inhibition of nonspecific sites was performed for 5 min with Power Block® (BioGenex). (3) The sections were incubated with primary antibody mouse anti-proliferating cell nuclear antigen (mouse anti-PCNA—Millipore) overnight at 4 °C in a humid chamber. The next day, the specific biotinylated secondary antibody (Vector Laboratories, USA) was added for 1 h at room temperature followed by Streptavidin Texas Red® (Vector Laboratories, USA) incubation in PBS, pH 8.2, for 1 h at room temperature. Contracoloration was performed with Hoechst (Sigma-Aldrich). Finally, the samples were examined and counted under fluorescence microscope Nikon Eclipse E400 at 400×. The total number of PCNA-positive hepatocytes were counted and expressed as cells per square millimeter of tissue for each surgery and for each patient.

For the TUNEL technique, a detection kit was used (In Situ Cell Death Detection Kit, Fluorescein—Roche/11 684 795 910). Liver tissues of 4 μm thickness were treated with a permeabilization solution of 0.1 % Triton X-100 and 0.1 % sodium citrate for 10 min, incubated with an enzyme solution for 1 h at 37, and rinsed with PBS, and the nuclear contracoloration was performed with Hoechst. Finally, all TUNEL-positive cells were examined and counted under fluorescence microscope Nikon Eclipse E400 at 400×. The total number of TUNEL-positive hepatocytes were counted and expressed as cells per square millimeter of tissue for each surgery and for each patient. We calculated a proliferative index (PI) to measure the balance between the number of cells that are dividing and those that are dying (calculation: PCNA–TUNEL ratio).

Statistical Analysis

Categorical variables are described using percentages. Continuous variables are expressed as means (SD) and median (range) for symmetrically distributed and nonsymmetrically distributed data, respectively. Paired *t* test was used for continuous variables. A *P* value of <0.05 was regarded as statistically significant. Pearson's correlation coefficient was used to measure the strength of the linear relationship between liver volumetric increase in MDCT images and histological or preoperative characteristics. Analyses were performed using the NCSS software (version 2007, NCSS, Kaysville, UT, USA).

Results

General Characteristics

Thirty-four patients with primary or secondary liver tumors were treated with the ALPPS approach during the study period. Biopsies of the FLR during both surgical stages were obtained in 11 patients that represented the study population. Patients with insufficient tissue sampling or lack of biopsy in either S1 or S2 were excluded from the analysis. The baseline demographic and clinical characteristics of these patients are summarized in Table 1. Seven patients were males (64 %), and the mean age was 61.5 years (range=39–80). Primary diagnoses were colorectal liver metastases (CRLM) in eight patients, hepatocellular carcinoma (HCC) in two patients, and cholangiocarcinoma in one patient.

Liver Volume Assessment and Operative Outcomes

All patients showed an FLR volume increase between both surgical stages (Fig. 1). The median hypertrophy of the FLR was 104 % (range=31–199) in a median of 6 days (range=4–12), which represented a mean difference between the preoperative and postoperative FLR volume of 361 ml (SD=138) ($P<0.001$). The mean FLR/TLV ratio increased from a baseline value of 23.8 % (SD=5.1) to 49.1 % (SD=8.5) before reoperation. All patients successfully underwent both stages of ALPPS. Six patients suffered postoperative complications (54 % overall morbidity), and one patient died during hospital stay. Two patients developed PHLF (one grade A and one grade B). The median hospital stay was 16 days (range=12–46).

Hepatocyte Morphology and Proliferation (PCNA and TUNEL)

The mean number of hepatocytes in the FLR increased from 52.7 cells/mm^2 (SD=13.6) in S1 to 89.6 cells/mm^2 (SD=48.5) in S2 ($P=0.001$) (Fig. 2). Hepatocyte hypertrophy (HH) was found in 10 of the 11 patients (91 %) of the study (Fig. 3). The mean hepatocyte volume was 1149 μ^2 (SD=9.75) in S1 and 1296.2 μ^2 (SD=3.67) in S2 ($P=0.43$), representing a mean increase in hepatocyte volume by 12.8 %. Nuclear hypertrophy (NH) was found in 9 of 11 patients (82 %). The mean nuclear volume was 399.2 μ^2 (SD=19.2) in S1 and 795.3 μ^2 (SD=24.9) in S2 ($P=0.003$), representing a mean nucleus volume increase of 99.2 %. The mean PCNA expression was 1.38 cells/mm^2 (SD=2.55) in S1 and 4 cells/mm^2 (SD=7) in S2 ($P=0.29$), representing a mean overall increase in PCNA

Table 1 Patient characteristics

Patient	Diagnose	Age	Preoperative FLR (ml)	Postoperative FLR (ml)	Preoperative chemotherapy	Regimen type	Interval between surgeries (days)
1	CRLM	48	352	828	Yes	FOLFOX+Cetuximab FOLFIRI+Bevacizumab	7
2	HCC	77	412	541	No	–	7
3	CRLM	53	572	853	Yes	FOLFOX	5
4	CRLM	57	380	538	Yes	CAPOX+Bevacizumab	7
5	CRLM	39	430	1030	No	–	7
6	CRLM	50	400	832	Yes	FOLFOX+Bevacizumab FOLFIRI+Cetuximab	12
7	CRLM	76	500	879	Yes	Capecitabine	10
8	CRLM	63	445	830	Yes	FOLFIRI+Cetuximab	14
9	HCC	80	434	781	No	–	10
10	CCA	57	317	640	No	–	6
11	CRLM	77	305	771	Yes	FOLFIRI	12

CRLM colorectal liver metastases, *HCC* hepatocarcinoma, *CCA* cholangiocarcinoma, *FLR* future liver remnant, *FOLFOX* folinic acid, 5-fluorouracil, and Oxaliplatin, *CAPOX* capecitabine and oxaliplatin, *FOLFIRI* folinic acid, 5-fluorouracil, and irinotecan

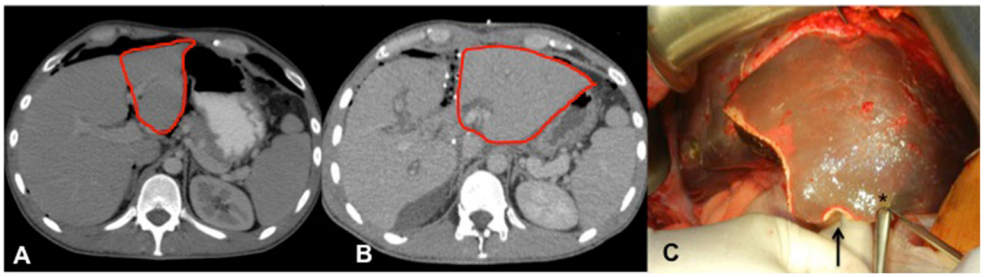

Fig. 1 Patient (aged 57) with colorectal liver metastases who received preoperative chemotherapy. **a** Preoperative CT scan demonstrating a future liver remnant (FLR) volume of 380 ml comprising the left lateral segment. **b** CT scan after 7 days of the first stage showed an FLR volume of 538 ml. **c** Intraoperative image of the second stage, in which a right trisectionectomy was performed. A biopsy of the FLR is taken at the beginning of the second stage (*asterisk*). The small parenchymal scar in the FLR corresponds to the previous biopsy taken during first stage (*arrow*)

expression by 190 % (Fig. 4). In those patients with PCNA increase between surgeries, there was a linear correlation (r=0.58) with the FLR hypertrophy observed in images and inverse correlation (r=−0.6) with patients' age.

The mean TUNEL expression was 5.16 cells/mm^2 (SD=7.8) in S1 and 1.68 cells/mm^2 (SD=1.9) in S2 (P=0.12), representing a mean decrease in TUNEL expression by 67.5 %. The PI increased from −3.78 cells/mm^2 (SD=6.8) in S1 to 2.32 cells/mm^2 (SD=7.5) in S2 (P=0.034). However, three patients experienced a reduction in the PI between both surgeries. One of these patients was a 77-year-old woman with a giant HCC in the noncirrhotic liver. The other two patients had CRLM, and one of them was a 76-year-old man. Both received long-course chemotherapy and had liver steatosis.

Hepatocyte morphology and proliferative activity are summarized in Table 2.

Discussion

The recently described short-term two-stage hepatectomy known as the ALPPS approach has had an impressive worldwide impact in the surgical community.[14] Even though there are many reports in the literature that have confirmed the impressive feature of rapid FLR volumetric increase with this technique, there are still many uncertainties regarding the biological substrate of these rapid volumetric changes. It is known that edema, liver congestion, and inflammation can affect macroscopic liver volume after major liver resection.[15] In an attempt to differentiate between a true increase in liver tissue and postoperative parenchymal edema during ALPPS, Knoefel et al.[16] performed densitometric analysis of CT scans and found only minor differences in Hounsfield units before and after ALPPS, therefore assuming that there was true parenchymal hypertrophy of the FLR. However, only scarce data is provided in most published series regarding microscopic analysis of resected specimens as gold-standard evaluation of histoarchitectural changes in the FLR. Histological analysis was performed in one patient only from a total of 25 patients included in the inaugural cooperative German series.[7] In that particular patient, increased proliferation and features consistent with hyperplasia were observed. More recently, Hernandez-Alejandro et al.[17] analyzed the Ki-67 index in FLR biopsies and found an increase from 0 to 14 % after ALPPS. Using a rodent model, the group from the University of Zurich found that Ki-67 expression was significantly higher in ALPPS mice compared to other groups and that accelerated liver hypertrophy was supported by volume increase and hepatocyte proliferation.[18] While data from animal models are very helpful, it has to be taken into account that the kinetics of regeneration differ between species and that hepatocyte replication in humans often takes place in diseased livers, many times with impaired function or tissue architecture grossly altered.

The results found in the present study provide solid evidence demonstrating that the rapid volumetric increase of the FLR during the ALPPS approach in humans has direct correlation with histological and molecular features of hepatocyte cell proliferation. It is known that liver cell replication initiates as soon as 12 h after surgery by massive changes in gene expression.[19] The PCNA is a nuclear protein synthetized in the early phase of the cellular cycle (G1 and S phase of cellular cycle) that marks proliferative activity by detecting cycling cells. In the present study, there was a mean overall increase in PCNA expression of 190 % between both surgical procedures with a linear correlation with the FLR hypertrophy observed in the MDCT. On the other hand, the paramount role of liver cell apoptosis has been already emphasized after liver resection in mice.[20,21] Apoptosis is evaluated with TUNEL, which identifies DNA fragmentation. In the present study, we found a mean reduction in TUNEL expression of 67.5 % between both surgical stages. However, neither PCNA nor TUNEL alone explains the remarkable regenerative phenomenon seen during the ALPPS approach. In this regard, the PI, which relates both molecular markers, increased from −3.78 cells/mm^2 in S1 to 2.32 cells/mm^2 in S2 (P=0.034). Similar results have been recently reported in an animal model after assessing Ki-67 as PI, with significantly higher values in mice

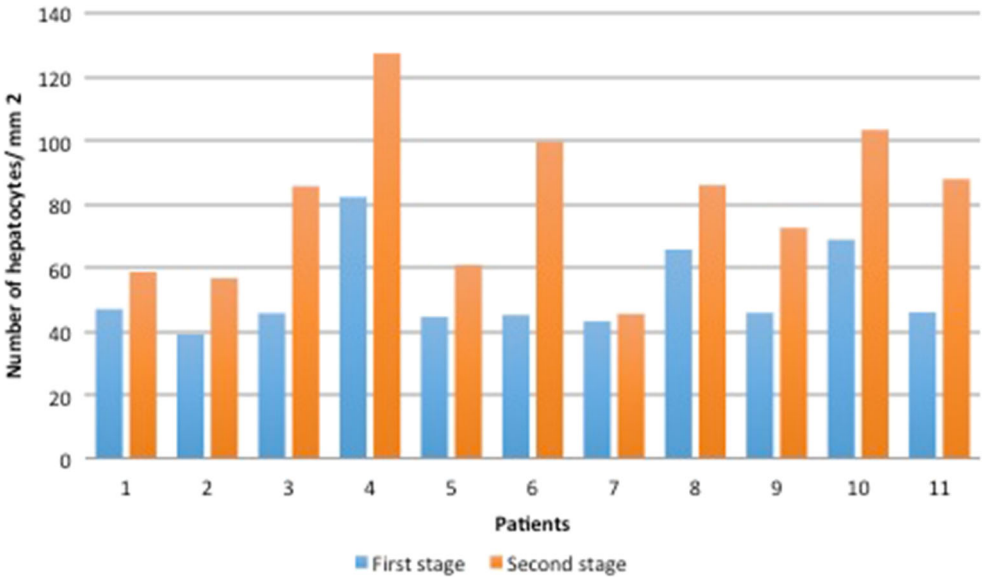

Fig. 2 Number of hepatocytes in the future liver remnant per square millimeter of tissue in each patient

subjected to ALPPS when compared to other groups.[18] Despite these modifications in gene expression of proliferation-associated molecules, the rapid volume growth of the FLR seen in ALPPS might be explained by a global proliferation of many hepatocytes as well as the recruitment of liver progenitor cells. In the present study, the mean number of hepatocytes in the FLR increased from 52.7 cells/mm^2 to 89.6 cells/mm^2 (P=0.001). Furthermore, experiments in rats showed that not only is hepatocyte replication involved in liver regeneration after hepatectomy, but also that cellular hypertrophy makes a great contribution to this phenomenon.[22–24] Even though most patients in our series presented HH and NH, only NH had a significant increase between both surgical procedures (99.2 %, P=0.003).

Age represents a main issue in liver surgery, because more than 50 % of liver tumors occur in patients aged 65 years or more.[25] Liver resection in old patients is no longer a formal contraindication, and frequently surgeons perform hepatectomy in the elderly with similar outcomes than in younger patients.[26] Nevertheless, there is evidence suggesting that functional hepatocellular regeneration is similar between old and young patients; experimental data from rats consistently suggests that liver regeneration is more favorable in younger rats.[27,28] When analyzing patient age as a preoperative factor that could modify molecular responses after liver resection in our study, we found that patient age had an inverse correlation with PCNA expression, suggesting that older patients might have impaired regenerative capacity. However, the small sample size and the lack of multivariate analysis in this study prevents to draw any strong conclusion in terms of associating age and proliferation.

To the present, the mechanisms that trigger the rapid and tremendous hypertrophy observed during ALPPS remain unclear. Liver regeneration after partial hepatectomy is mainly explained by two pathways that induce hepatocyte replication. The "humoral theory" involves an increased metabolic demand on the liver remnant and the "blood-flow theory" implies an increased blood supply to the liver remnant with a

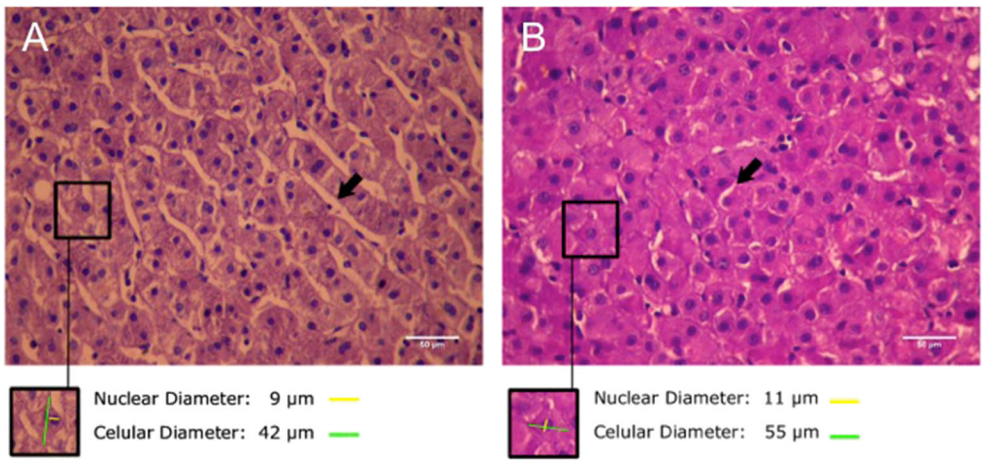

Fig. 3 Hematoxylin–eosin staining. **a** Microscopic image of the future liver remnant (FLR) parenchyma at first stage. The *black arrow* shows the hepatic sinusoid space. **b** Microcopy of the FLR parenchyma at second stage. The *black arrow* shows a reduction in the hepatic sinusoid spaces. Hepatocyte magnification is performed to measure nuclear and cellular diameters at both surgical stages. *Bars*=50 µm

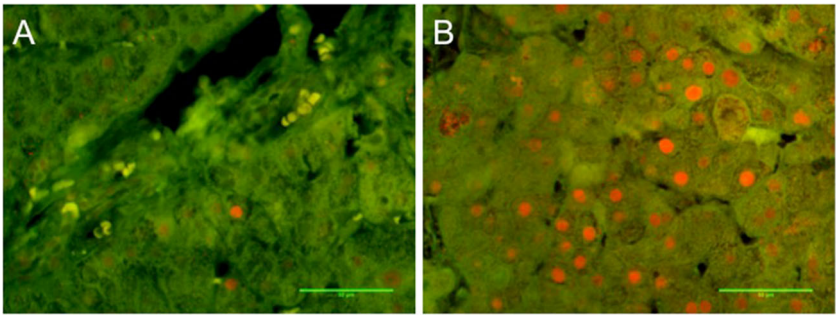

Fig. 4 Immunohistochemical staining of liver parenchyma for proliferating cell nuclear antigen (PCNA) at both surgical stages. Tissue sections show an increase of hepatocytes with PCNA-positive nuclei (*red-fluorescent*) between the first stage (**a**) and the second stage (**b**). *Bars*=50 μm

consequent increase in intrahepatic shear stress that stimulates liver regeneration.[29] Additionally, there is a threefold increase in signaling molecules and nutrients transported by portal flow and a reduced oxygen supply to the liver due to arterial hypoperfusion mediated by the hepatic arterial buffer response.[30] Michalopoulos et al.[31] summarized those molecular factors associated with liver regeneration after hepatectomy, describing those endogenous mediators which induce liver cell replication, like hepatocyte growth factor, tumor necrosis factor (TNF), and the IL6, epidermal, and transforming growth factors. More recently, Yokoyama et al.[32] analyzed those hemodynamic and humoral factors involved in hepatic regeneration after partial hepatectomy and PVE. Despite some differences regarding hepatic hemodynamics following PVE from those following partial hepatectomy, mechanisms and mediators are the same in both procedures. Regarding specifically the ALPPS approach, the distinctive feature of this technique is the parenchymal partition, which disrupts intrahepatic portal collaterals leading to complete portal flow deprivation of the excluded segments and causes surgical trauma that might itself be a regeneration stimulus. Recent experimental evidence suggests that the accelerated regenerative ability of the FLR during ALPPS might be more related to circulatory growth factors rather than a discontinuity of the microcirculation after liver transection and that, significantly, more hepatocytes enter the cell cycle earlier after ALPPS than after PVL.[18] In addition, our group has recently postulated that the hemodynamic changes observed during both stages of ALPPS might also play an important role in rapid regeneration by alleviating FLR hyperflow and portal hypertension in small-for-size settings.[33]

The main limitation of our study is the relatively small number of patients included. However, to the best of our knowledge, there are no other reports in the literature with such detailed histological and immunohistochemical analysis of FLR tissue during the ALPPS approach in humans. Another drawback is that nonparenchymal cells (stem cells, stellate cells, oval cells, and vascular and biliary endothelial cells) which are required to regenerate a mature liver parenchyma as well as other phenomena occurring in the interstitial space such as congestion or inflammation that might have had volumetric impact were not evaluated in the present study. Regarding the relatively low percentage of patients from the overall population that were included in the present study (32 %), it is worth clarifying that this was not due to a selection bias since we began performing routine intraoperative biopsies late in our series, and on the other hand, we excluded patients due to inadequate tissue sampling. Even though the results obtained in this study clarify the histological correlation of the macroscopic hypertrophy seen during ALPPS in a static moment of time, these are dynamic processes that take place over time and have regulatory and triggering signals that remain not fully understood. The fact that having performed histological analyses in various time intervals between patients (r=5–14 days) challenges the significance of histological findings at the time of S2. Moreover, patients who underwent the ALPPS procedure are complex patients who present advanced oncological diseases, chemotherapy

Table 2 Hepatocyte morphology and proliferative activity

Variable	First stage	Second Stage	*P* value
FLR volume, mean (SD), ml	413 (77.7)	774 (148.4)	0.001
Hepatocyte number, mean (SD), cells/mm^2	52.7 (13.6)	89.6 (48.5)	0.001
Hepatocyte volume, mean (SD), μ^2	1149 (9.75)	1296 (3.67)	0.43
Nuclear volume, mean (SD), μ^2	399.2 (19.2)	795.3 (24.9)	0.003
PCNA, mean (SD), cells/mm^2	1.38 (2.55)	4 (7)	0.29
TUNEL, mean (SD), cells/mm^2	5.16 (7.8)	1.68 (1.9)	0.12
Proliferative index, mean (SD), cells/mm^2	−3.78 (6.8)	2.32 (7.5)	0.034

FLR future liver remnant, *PCNA* proliferating cell nuclear antigen, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling, *SD* standard deviation

treatments, and even history of previous surgeries. Clearly, these factors could play an important role in liver regeneration and need further analysis in future investigations.

Conclusion

The results of this study demonstrate that there is true hepatocellular hyperplasia and hypertrophy behind the rapid macroscopic volumetric increase of the FLR observed during ALPPS in humans. We found not only a significantly increased number of hepatocytes in the FLR but also significant morphological changes at the cellular level as well as up-regulation of molecular markers of proliferative activity, all of which had direct correlation with volume gain in MDCT. This indicates that quiescent hepatocytes indeed enter the cell cycle and replicate during ALPPS. However, patient age inversely correlated with PCNA expression, suggesting that elderly patients have less regenerative capacity. The results of this study give new insights that could serve as fertile soil in which future experimental work will be developed to understand the possible mechanisms involved in human liver regeneration during the ALPPS strategy.

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Compliance with Ethical Standards

Conflicts of Interest and Source of Funding None of the authors of this manuscript has any direct or indirect commercial financial incentive associated with the publication of this paper. The funding involved in this work has been provided by our institution.

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