

# Ischemic preconditioning induces autophagy and limits necrosis in human recipients of fatty liver grafts, decreasing the incidence of rejection episodes

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Whether ischemic preconditioning (IP) reduces ischemia/reperfusion (I/R) injury in human normal and fatty livers remains controversial. We compared two independent groups of liver donor transplants with *versus* without steatosis to evaluate IP consequences. Liver donors with ( $n = 22$ ) or without ( $n = 28$ ) steatosis either did or did not undergo IP before graft retrieval. Clinical data from the recipients, as well as histological and immunohistological characteristics of post-reperfusion biopsies were analyzed. Incidence of post-reperfusion necrosis was increased (10/10 *versus* 9/14, respectively;  $P < 0.05$ ) and the clinical outcome of recipients was worse for non-IP steatotic liver grafts compared with non-IP non-steatotic grafts. IP significantly lowered the transaminase values only in patients receiving a non-steatotic liver. An increased expression of beclin-1 and LC3, two pro-autophagic proteins, tended to decrease the incidence of necrosis ( $P = 0.067$ ) in IP steatotic livers compared with non-IP steatotic group. IP decreased the incidence of acute and chronic rejection episodes in steatotic livers (2/12 *versus* 6/10;  $P = 0.07$  and 2/12 *versus* 7/10;  $P < 0.05$ , respectively), but not in non-steatotic livers. Thus, IP may induce autophagy in human steatotic liver grafts and reduce rejection in their recipients.

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In steatotic livers, hepatocytes cell death and inflammatory response have a great impact in both surgical and medical context.<sup>1,2</sup> Indeed, steatotic livers are more sensitive to ischemia–reperfusion (I/R) injury.<sup>3,4</sup> Operative mortality rate associated with steatosis after major liver resection has been reported as high as 14% compared with 2% for healthy livers.<sup>4</sup> Steatotic liver grafts are more frequently used in the context of transplantation and they are associated with a primary non-function rate of 60% compared with <5% for non-steatotic grafts.<sup>5,6</sup> Therefore, developing protective strategies to minimize the adverse effects of I/R injury in steatotic livers is of paramount importance.

Ischemic preconditioning (IP), first described in the heart by Murry *et al.*,<sup>7</sup> consists of brief periods of vascular occlusion which confer protection against subsequent I/R via endogenous protective mechanisms. In the liver, IP was shown to lower transaminase levels and reduce endothelial cell injury.<sup>8</sup> This protection has been linked to various mechanisms, such as decreased apoptosis,<sup>9</sup> preservation of the ATP content in liver tissue,<sup>8,10</sup> or overproduction of pro-survival and anti-inflammatory proteins.<sup>11</sup>

The beneficial effects of IP in human liver hepatectomy, associated with warm and short ischemia have been

demonstrated in young patients and patients with liver steatosis.<sup>8</sup> In liver transplantation, some previous studies have demonstrated controversial results of the role of IP on liver grafts. Our group was the first to show a significant decrease in transaminase levels after IP counterbalanced by decreased early function of the graft.<sup>12</sup> Jassem *et al.*<sup>13</sup> found a significant reduction of transaminase levels in IP patients compared with non-IP patients in the post-operative period, whereas Koneru *et al.*,<sup>14</sup> found a paradoxical effect of higher transaminase peak in IP livers. These studies have shown that steatosis was a significant worsening parameter for I/R injuries, although none of them have specifically studied the role of IP on steatotic or non-steatotic livers. Recently, Franchello *et al.*<sup>15</sup> compared normal livers with marginal livers, including mostly livers from donors over the age of 60. They have shown that IP reduced the AST and ALT mean levels 5 min after reperfusion during the first 3 operative days. Interestingly, they also observed that IP induced a lower rate of hepatocyte cell death as assessed by TUNEL assay, the apoptotic index being higher in marginal livers compared with normal livers outside the context of preconditioning. Therefore, our objective was to focus on macro–microvesicular

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steatotic livers that underwent cold ischemia, and preservation during liver transplantation to determine whether and how IP could influence cell death and clinical efficacy or safety in the recipients.

## Results

**Donor, recipient and intraoperative data.** In all, 22 donor livers out of 50 showed steatosis. Steatosis was globally graded as mild (<30% of steatotic hepatocytes) in 16 livers and moderate in 6 (steatotic hepatocytes >30% but <60%). In all, 17 livers showed mixed macro and microvesicular steatosis, with macrovesicular ranging from >1 to ≤30% and microvesicular ranging from >5 to ≤50%. Three steatotic liver grafts showed only microvesicular steatosis ranging from 20 to 40% of hepatocytes. Two others showed only macrovesicular steatosis affecting 10% of hepatocytes. There was no significant difference in distribution of age and hospitalization stay for graft donors before transplantation between groups (Table 1). No significant differences were observed in recipients with regard to age, gender or initial

disease before transplantation (Table 2), nor for surgical parameters (Table 3).

**Clinical and histological outcomes in patients receiving non-steatotic or steatotic allografts without IP.** When comparing the two groups of patients who did not undergo IP, higher mean values in both peak and day 5 levels of AST and ALT were observed in recipients of steatotic grafts compared with recipients of non-steatotic grafts, although these differences were not statistically significant because of high inter-patient variability (Table 4). Mean AST and ALT peaks were 862 and 728 IU/l in patients receiving steatotic grafts, whereas they were 458 and 577 IU/l in patients receiving non-steatotic grafts. AST and ALT day 5 levels were 139 and 423 IU/l in patients receiving steatotic grafts and 79 and 247 IU/l in patients receiving non-steatotic grafts (Table 4). Levels of serum bilirubin at day 7 were significantly higher in steatotic livers compared with non-steatotic livers, whereas no difference was observed in PT levels (Table 4). No significant differences in post-transplantation intensive care unit stay (12 days *versus* 11 days) or total hospitalization stay

**Table 1** Allografts characteristics before transplantation in IP and non-IP donors

Variable	Non-steatotic allografts		Steatotic allografts		P-value I versus II <sup>a</sup>	P-value III versus IV <sup>a</sup>	P-value I versus III <sup>a</sup>	P-value II versus IV <sup>a</sup>
	IP (n = 14) group I	Non-IP (n = 14) group II	IP (n = 12) group III	Non-IP (n = 10) group IV				
Age of donor (years)	47.9 ± 13.6	50.1 ± 12.1	50.5 ± 8.4	54.7 ± 10.3	0.65	0.30	0.57	0.34
Donor hospitalization (days in intensive care unit)	1.9 ± 1.1	1.8 ± 1.0	2.1 ± 1.3	1.6 ± 0.9	0.67	0.38	0.73	0.77
Body mass index	21.7 ± 3.3	23.7 ± 2.9	25.1 ± 3.7	24.9 ± 2.3	0.11	0.87	0.02	0.29
<i>Histology before reperfusion</i>								
Necrosis								
Absent	12/14	13/14	12/12	9/10	0.39	0.45	0.28	0.51
Mild	1/14	1/14	0/12	0/10	0.52	—	0.54	0.58
Moderate	1/14	0/14	0/12	1/10	0.50	0.45	0.54	0.42
Steatosis								
Absent	14/14	14/14	0/12	0/10	—	—	10 <sup>-7</sup>	5 × 10 <sup>-7</sup>
Mild (0–30%)	0/14	0/14	8/12	8/10	—	0.30	3 × 10 <sup>-4</sup>	6 × 10 <sup>-5</sup>
Moderate (30–60%)	0/14	0/14	4/12	2/10	—	0.30	0.03	0.16
High glycogen content (grading ≥2)	6/14	9/14	8/12	9/10	0.16	0.19	0.15	0.15

<sup>a</sup>Means were compared using the Student's *t*-test  
Dichotomous variables were compared using the Fisher's exact test

**Table 2** Patients (recipients) characteristics before receiving IP or non-IP livers

Variable	Non-steatotic allografts		Steatotic allografts		P-value I versus II <sup>a</sup>	P-value III versus IV <sup>a</sup>	P-value I versus III <sup>a</sup>	P-value II versus IV <sup>a</sup>
	IP (n = 14) group I	Non-IP (n = 14) group II	IP (n = 12) group III	Non-IP (n = 10) group IV				
Age of recipients (years)	50 ± 14	45 ± 14	47 ± 12	45 ± 13	0.38	0.68	0.55	0.89
Gender (M/F)	8/6	9/5	10/2	9/1	0.28	0.43	0.13	0.15
<i>Etiology</i>								
Alcoholic cirrhosis	2/14	2/14	4/12	3/10	0.40	0.35	0.20	0.26
HBV cirrhosis	3/14	3/14	1/12	1/10	0.35	0.52	0.29	0.34
HCV cirrhosis	4/14	4/14	3/12	3/10	0.32	0.77	0.32	0.64
Others	5/14	5/14	4/12	3/10	0.30	0.73	0.70	0.33

<sup>a</sup>Means were compared using the Student's *t*-test  
Dichotomous variables were compared using the Fisher's exact test

**Table 3** Surgical data during liver transplantation in patients receiving a IP and non-IP allograft

Variable	Non-steatotic allografts		Steatotic allografts		P-value I versus II <sup>a</sup>	P-value III versus IV <sup>a</sup>	P-value I versus III <sup>a</sup>	P-value II versus IV <sup>a</sup>
	IP (n = 14) group I	Non-IP (n = 14) group II	IP (n = 12) group III	Non-IP (n = 10) group IV				
Allograft weight (g)	1333 ± 192	1533 ± 403	1622 ± 494	1540 ± 314	0.11	0.67	0.06	0.96
Portal ischemic time (min)	435 ± 127	476 ± 151	424 ± 134	429 ± 76	0.44	0.91	0.83	0.38
Arterial ischemic time (min)	36 ± 25	35 ± 24	50 ± 22	40 ± 15	0.88	0.26	0.16	0.56
Blood units	8.1 ± 10.2	5.9 ± 5.2	7.8 ± 11.1	7.5 ± 9.2	0.46	0.94	0.94	0.58
Surgical complications (vascular or biliary)	3/14	2/14	3/12	3/10	0.34	0.35	0.35	0.27

<sup>a</sup>Means were compared using the Student's *t*-test  
Dichotomous variables were compared using the Fisher's exact test

**Table 4** Clinical data during liver transplantation in patients receiving a IP and non-IP allograft

Variable	Non-steatotic allografts		Steatotic allografts		P-value I versus II <sup>a</sup>	P-value III versus IV <sup>a</sup>	P-value I versus III <sup>a</sup>	P-value II versus IV <sup>a</sup>
	IP (n = 14) group I	Non-IP (n = 14) group II	IP (n = 12) group III	Non-IP (n = 10) group IV				
<b>Hepatic function</b>								
AST at day 5 (IU/l)	42 ± 33	79 ± 57	55 ± 40	139 ± 164	0.047	0.10	0.37	0.22
ALT at day 5 (IU/l)	82 ± 39	247 ± 194	222 ± 246	423 ± 409	0.005	0.17	0.046	0.17
AST peak (IU/l)	235 ± 189	458 ± 308	881 ± 1323	862 ± 762	0.029	0.96	0.08	0.09
ALT peak (IU/l)	187 ± 94	577 ± 361	795 ± 796	728 ± 724	0.0006	0.84	0.009	0.51
PT (%) at day 5	61 ± 13	67 ± 10	63 ± 20	68 ± 9	0.18	0.48	0.73	0.74
Bilirubin at day 7 (μmol/l)	46 ± 44	38 ± 21	106 ± 148	78 ± 45	0.55	0.57	0.16	0.007
<b>Hospitalization</b>								
Intensive care unit stay (days)	12 ± 7	11 ± 6	15 ± 10	12 ± 5	0.67	0.42	0.47	0.59
Total stay in hospital (days)	28 ± 8	29 ± 13	52 ± 43	33 ± 9	0.70	0.18	0.049	0.53
Retransplantation	1/14	1/14	0/12	0/10	—	—	0.52	0.52
<b>Rejection</b>								
Acute rejection	2/14	5/14	2/12	6/10	0.15	0.048	0.40	0.17
Rejection activity index (mean ± S.D.)	5 ± 1.4	5.2 ± 1.3	5 ± 1.7	4.7 ± 0.9	0.86	0.73	1.00	0.66
Acute rejection delay (median in month)	0.5	1.25	0.5	2	0.84	0.92	0.99	0.99
Chronic rejection	0/14	2/14	2/12	7/10	0.24	0.017	0.20	0.009
Ductopenia (mean ± S.D. in %)	—	44.5 ± 15.6	49.7 ± 23.1	36.5 ± 10.2	—	0.15	—	0.22
Chronic rejection delay (median in month)	—	42	9	36	—	0.09	—	1.00
Follow-up (mean ± S.D. in months)	53 ± 18	60 ± 30	47 ± 27	64 ± 26	0.44	0.16	0.51	0.76

<sup>a</sup>Means were compared using the Student's *t*-test  
Dichotomous variables were compared using the Fisher exact test  
Medians were compared with Kolmogorov–Smirnov test

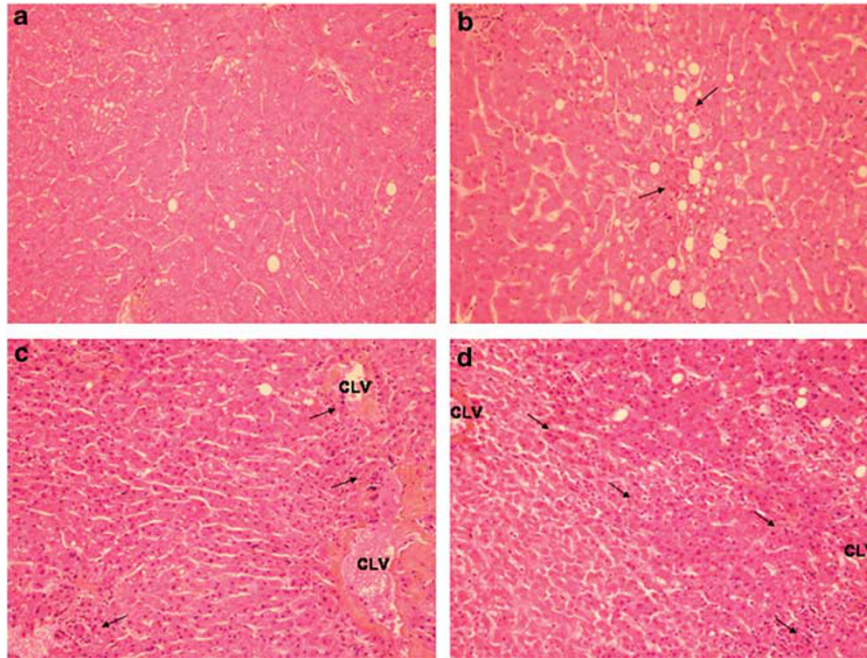
(33 days *versus* 29 days) were observed between the 10 steatotic and the 14 non-steatotic grafts recipients (Table 4).

No differences in the incidence of acute rejection (AR 6/10 *versus* 5/14) nor in rejection activity index were observed (Table 4). However, a statistically significant increase in the incidence of chronic rejection (CR) was observed in recipients of steatotic grafts compared with recipients of non-steatotic grafts (7/10 *versus* 2/14;  $P < 0.01$ ), whereas no differences in severity of ductopenia was observed (Table 4).

Histological changes in livers following transplantation were analysed. Necrotic cells associated with polymorphonuclear infiltrate were observed in centrilobular areas, throughout the lobule and periportal spaces. Surgical necrosis was more frequently observed in patients receiving steatotic grafts compared with patients receiving non-steatotic grafts (10/10 *versus* 9/14  $P < 0.05$ ; Figure 1, Table 5). However, no differences in necrotic index was observed between the two

groups (Table 5). Apoptotic cell death was evaluated by activated caspase 3 immunostaining. Apoptotic cells were mostly detected in centrilobular areas in both steatotic and non-steatotic hepatocytes (Figure 2a). In particular, activated caspase 3 was more frequently detected in patients receiving steatotic grafts compared with non-steatotic grafts (3/10 *versus* 0/14;  $P = 0.059$ ).

**Effects of IP on clinical and histological outcomes of patients receiving steatotic allografts.** IP did not significantly change transaminase peaks and day 5 levels in recipients of steatotic allografts. In particular, mean AST and ALT peaks were 881 and 795 IU/l in IP graft recipients compared with 862 and 728 IU/l in non-IP graft recipients. Mean AST and ALT day 5 levels were lower, but not significantly, in IP graft recipients compared with non-IP graft recipients (55 and 222 IU/l *versus* 139 and 423 IU/l,



**Figure 1** Representative images of different grades of necrosis. (a) Absence of necrosis ( $\times 200$ ). (b) Mild necrosis: few foci of polymorphonuclear cells (arrows) can be observed through the lobule surrounding few necrotic hepatocytes ( $\times 200$ ). (c) Moderate necrosis: some foci of polymorphonuclear cells can be observed around the section of the centrolobular vein (CLV) associated to some necrotic hepatocytes ( $\times 200$ ). (d) Severe necrosis: large necro-inflammatory area with bridging between two centrolobular veins (CLV;  $\times 200$ )

**Table 5** Histological and immunohistochemical and analysis on post-reperfusion livers

Variable	Non-steatotic allografts		Steatotic allografts		<i>P</i> -value I versus II <sup>b</sup>	<i>P</i> -value III versus IV <sup>b</sup>	<i>P</i> -value I versus III <sup>b</sup>	<i>P</i> -value II versus IV <sup>b</sup>
	IP ( <i>n</i> = 14) group I <sup>a</sup>	Non-IP ( <i>n</i> = 14) group II	IP ( <i>n</i> = 12) group III	Non-IP ( <i>n</i> = 10) group IV				
<i>Surgical necrosis (presence)</i>	9/14	9/14	8/12	10/10	0.30	0.067	0.32	0.047
Absent	5/14	5/14	4/12	0/10	0.30	0.067	0.32	0.047
Mild	7/14	7/14	5/12	7/10	0.29	0.15	0.28	0.21
Moderate	1/14	1/14	2/12	3/10	0.52	0.30	0.36	0.16
Severe	1/14	1/14	1/12	0/10	0.52	0.54	0.52	0.58
Necrotic index	0.71 $\pm$ 0.67	0.67 $\pm$ 0.61	0.80 $\pm$ 0.71	0.97 $\pm$ 0.32	0.85	0.50	0.75	0.17
Activated caspase 3	0/12	0/14	1/12	3/10	—	0.20	0.50	0.059
Beclin 1	2/12	0/14	9/12	2/10	0.20	0.015	0.006	0.16
LC3	2/12	2/14	9/12	3/10	0.40	0.046	0.006	0.26
Beclin 1+ LC3	1/12	0/14	7/12	2/10	0.46	0.071	0.014	0.16

<sup>a</sup>In all, 12 paraffin blocks were available for group I to allow further immunohistochemical analysis

<sup>b</sup>Means were compared using the Student's *t*-test

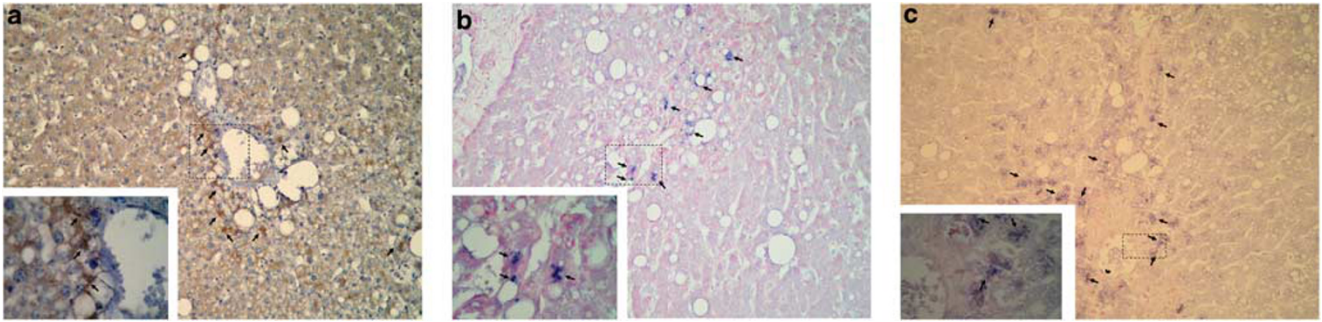
Dichotomous variables were compared using the Fisher's exact test

respectively). Lower PT and higher serum bilirubin, although not significantly, were observed in IP grafts compared with non-IP grafts (Table 4). No significant differences in post-transplantation intensive care unit stays were observed between the two groups (15 days *versus* 12 days). Total hospitalization stay was longer, but not significantly different, for IP graft recipients compared with non-IP recipients (52 days *versus* 33 days; Table 4).

Interestingly, IP significantly decreased the incidence of both AR and CR in recipients of steatotic grafts compared with recipients of non-IP steatotic grafts (2/12 *versus* 6/10 for AR and 2/12 and 7/10 for CR;  $P < 0.05$ ; Table 4). However, IP did not change the rejection activity index, nor severity of ductopenia in steatotic allografts (Table 4).

Histological examination showed that IP tended to decrease the incidence of surgical necrosis in steatotic grafts (8/12 *versus* 10/10;  $P = 0.067$ ). However, no differences in necrotic index was observed between the two groups (Table 5). No difference in the detection of activated caspase 3 was observed in IP steatotic livers compared with non-IP steatotic livers (Table 5).

**Effects of IP on clinical and histological outcomes of patients receiving non-steatotic allografts.** IP significantly decreased both AST and ALT peak and day 5 levels in non-steatotic allografts recipients. In particular, mean AST and ALT peak were 235 and 187 IU/l in recipients of IP livers compared with 458 and 577 in recipients of non-IP livers



**Figure 2** Assessment of activated caspase 3, beclin-1 and LC3 in steatotic preconditioned livers. (a) Activated caspase 3 expression in the hepatocytes of a centrolobular region (arrows;  $\times 400$ ). Hepatocytes expressing activated caspase 3 circumscribed within the dashed lines are shown enlarged in the inset ( $\times 1000$ ). (b) Beclin-1 expression in the hepatocytes located in a centrolobular region and also in the lobule ( $\times 400$ ). Centrolobular hepatocytes expressing beclin-1 circumscribed within dashed lines are shown enlarged in the inset ( $\times 1000$ ). (c) LC3 expression in the hepatocytes located in a centrolobular region and also in the lobule ( $\times 400$ ). Centrolobular hepatocytes expressing LC3 circumscribed within dashed lines are shown enlarged in the inset ( $\times 1000$ )

( $P < 0.05$  and  $P < 0.01$  for AST and ALT peaks, respectively). Mean AST and ALT day 5 levels were 42 and 82 IU/l in recipients of IP livers compared with 79 and 247 IU/l in recipients of non-IP livers ( $P < 0.05$  and  $P < 0.01$  for AST and ALT day 5 levels, respectively). Lower PT and higher serum bilirubin were observed, although not significantly, in IP grafts compared with non-IP grafts (Table 4). No significant difference in post-transplantation intensive care unit stays (12 days *versus* 11 days) or total hospitalization stays (28 days *versus* 29 days) was observed between the IP or non-IP non-steatotic graft recipients (Table 4).

No difference in the incidence of AR or CR was observed in these two groups (2/14 *versus* 5/14 for AR and 0/14 *versus* 2/14 for CR), as well as in rejection activity index or ductopenia severity (Table 4).

Histological examination did not show any difference in the incidence or severity of surgical necrosis neither in necrotic index (Table 5). No apoptotic cells were detected either in IP nor in non-IP biopsies (Table 5).

**IP activates autophagy in steatotic allografts.** As we previously showed that autophagy is activated by IP in steatotic and peliotic livers treated by chemotherapy,<sup>16</sup> we investigated the presence of autophagy biomarkers in post-reperfusion biopsies of this series to evaluate the potential role of autophagy in steatotic livers.

The presence of autophagy markers was assessed by immunohistochemical detection of beclin-1 and LC3, these two proteins being essential for the macroautophagic process and, respectively, representing an early and a late marker of autophagy.<sup>17</sup> Hepatocytes showing positive staining for both proteins were found mainly in the centrolobular areas, although some positive hepatocytes were observed throughout the lobule (Figures 2b and c). Beclin-1 and LC3 staining were rarely observed in post-reperfusion non-steatotic livers. In particular, only one IP non-steatotic liver was positive for both protein stains, whereas 7/12 IP steatotic livers showed positive staining for both beclin-1 and LC3 ( $P < 0.05$ ; Table 5). Double-positive staining for beclin-1 and LC3 was more frequently observed in IP steatotic livers compared with non-IP steatotic livers, although the results did not reach statistical significance (7/12 *versus* 2/10;  $P = 0.07$ ; Table 5). When we considered only immunohistochemical-positive biopsies,

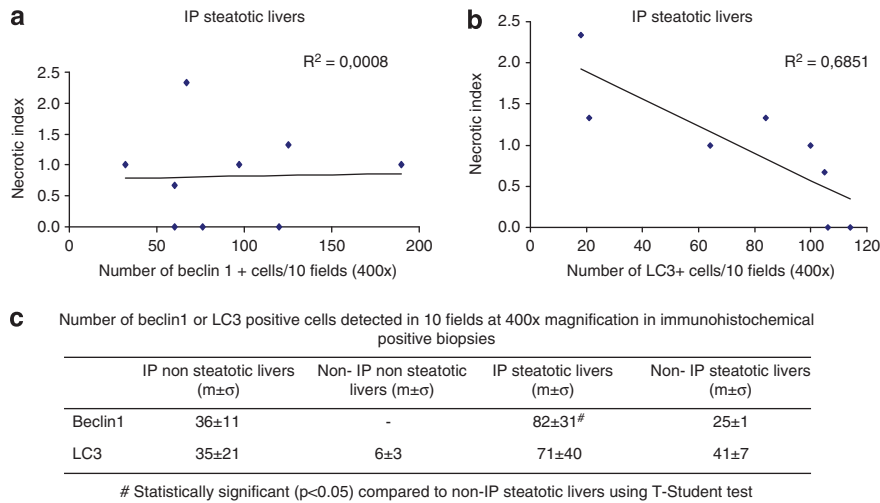
we observed a statistically significant increased number of beclin-1-positive cells in IP steatotic livers compared with non-IP steatotic livers (Figure 3). Interestingly, we observed an inverse correlation between the number of LC3-positive cells and the necrotic index in IP steatotic livers, whereas no correlation was observed in the other groups. No correlation between the number of beclin-1-positive cells and the necrotic index was observed (Figure 3).

## Discussion

In this study, we showed that IP may be beneficial for both patients receiving either steatotic or non-steatotic allografts, although biological mechanisms mediating IP effects did differ. IP tended to reduce parenchyma necrosis and subsequent graft rejection incidence in recipients of steatotic grafts. The activation of autophagy could have an important role to ensure cell homeostasis, and limit necrosis and graft rejection in steatotic livers.

Ischemia was more deleterious in steatotic grafts compared with non-steatotic livers as shown by transaminase levels after transplantation and the presence of surgical necrosis in post-reperfusion biopsies of non-IP livers. Our results are in accordance with previous observations in human or experimental liver surgery.<sup>4,18</sup> Chronic necrosis, generally associated with lower ATP levels<sup>19</sup> and increased inflammatory response in the parenchyma,<sup>20</sup> could explain that steatotic livers are less resistant to I/R injuries.<sup>4,18</sup>

The main parameter considered to assess I/R injury was serum transaminases level. However, contrasting results were published. The discrepancies in the significance of transaminases values can be attributed to different sample timing after transplantation and to high interpatient variability, particularly in marginal grafts such as steatotic livers, as we can observe here, or grafts from donors  $> 60$  years of age.<sup>15</sup> Indeed, we showed that IP significantly decreased transaminases levels in non-steatotic livers, whereas a nonsignificant decrease was observed in IP steatotic liver. Moreover, the groups of patients with improved transaminase levels did not benefit from shorter hospitalization stays, as also observed in other studies.<sup>21,22</sup> Therefore, more accurate biological markers are needed to better assess I/R injuries and the subsequent effects of IP.



**Figure 3** Expression of autophagy markers in human liver biopsies and correlation with necrotic index. **(a)** Correlation between beclin-1 expression and necrotic index in IP steatotic livers. **(b)** Correlation between LC3 expression and necrotic index in IP steatotic livers. **(c)** Beclin-1 and LC3 expression in immunohistochemical-positive biopsies in all studied groups

Similarly, the decrease of apoptosis has been reported as a biological protective mechanism triggered by IP.<sup>23</sup> It has been mostly assessed by TUNEL assay or caspase 3 or Bcl-2 expression levels evaluation. However, results were not always conclusive.<sup>15,16,23</sup> This can be because of the use of the TUNEL assay that can not discriminate between apoptosis or necrosis cell death,<sup>9</sup> or to the comparison of groups of biopsies that included steatotic livers or livers from donors over the age of 60. Indeed, steatotic livers have been reported to have higher levels of oxidative or nitrosative radicals with subsequent oxidized lipids and proteins,<sup>24</sup> and consequently lower intracellular ATP levels, thus favoring necrotic cell death following I/R injury.<sup>24,25</sup> In our study, we observed that steatotic livers frequently exhibited necrosis although non-steatotic livers did not. Interestingly, IP tended to decrease the incidence of necrosis without changing apoptosis in IP steatotic livers. We can note, as in other studies,<sup>15,26</sup> that apoptosis assessed by caspase 3 activation was not a major mechanism triggered by I/R. Few hepatocytes exhibited caspase 3 activation. We can hypothesize that other apoptotic pathways could be activated or that apoptosis was controlled. Recently, autophagy has been described to be activated in stress conditions to ensure cell survival by limiting necrosis or apoptosis *in vivo*.<sup>27</sup> Autophagy is a catabolic pathway triggered following various stress conditions, such as starvation or transient hypoxia, and aimed to restore adequate intracellular ATP and aminoacids levels and to eliminate damaged organelles.<sup>28,29</sup> It is reported that autophagy cross-talks with apoptotic and necrotic cell death pathways,<sup>17</sup> and that activation of autophagy may favour cellular survival by decreasing reactive oxygen species production.<sup>30</sup> In other cellular contexts, autophagy has been shown to retard cell death by suppressing ER stress.<sup>31</sup> Actually, ER stress has been recently described to have a role in the pathogenesis of liver steatosis<sup>32,33</sup> and ER stress inhibition has been shown to be protective under I/R in an experimental model of steatotic/non-steatotic partial hepatectomy.<sup>34</sup> Thus, we can speculate that in our context, activation of autophagy may be involved in

ER stress attenuation in steatotic livers, and that the modulation of autophagy and ER stress can have beneficial effects in liver pathologies. Accordingly, our group has described that IP can trigger autophagy to switch on/off necrosis and/or apoptosis in steatotic or peliotic livers from patients formerly treated by several courses of chemotherapy.<sup>16</sup> In this study, the decreased trend of incidence of necrosis that we observed was associated with a significant increased expression of autophagy markers in steatotic livers. Thus, the induction of autophagy, especially in IP steatotic livers, was in favor of the restoration of sufficient energetic levels and aminoacids availability in injured hepatocytes to prolong their survival under I/R stress. It is noteworthy that high ATP levels have been correlated with better post-transplantation outcomes.<sup>35,36</sup>

Tissue necrosis and vascular injury caused by I/R has been associated with an increased risk of acute and chronic graft rejection.<sup>37</sup> Interestingly, in our study IP significantly decreased mild AR and mild CR incidences only in recipients of steatotic livers. A decrease in AR was previously observed in recipients of IP liver in other studies<sup>13–15</sup> and in particular Franchello *et al.* showed that IP increased survival rates at 6 months in marginal grafts but not in non-marginal grafts (which had, however, a higher survival rate compared with marginal livers). The biological mechanism involved in the decrease graft rejection after IP remains to be elucidated. However, a decreased sensitivity in necrotic cell death that we and other authors observed<sup>15</sup> and probably a decreased inflammatory response observed by another group<sup>13</sup> may be involved. It has been hypothesized that perioperative allograft injury induced by I/R releases mediators, such as TNF $\alpha$ , IL1 $\alpha$ , HMGB1, and cellular nucleotides, that enhance destructive adaptive immune responses driving vascular inflammation and production of IL1 $\beta$  within grafted organs.<sup>38</sup> Interestingly, TNF $\alpha$  signaling, known as a mediator of inflammation in liver and adipose tissue, has been recently shown to be involved also in NAFLD pathogenesis.<sup>39,40</sup> Thus, the induction of autophagy may promote cell survival by restoring adequate

intracellular ATP levels and consequently decreasing the release of inflammation mediators by injured cells. In our clinical context, we cannot conclude whether autophagy has a pro-survival role or an ATP restoring function, but a better understanding of autophagy activation during IP may lead clinical improvements in post-transplantation outcomes of patients receiving steatotic grafts.

## Patients and Methods

**Study population and experimental design.** Among the patients who underwent a liver transplantation in our institution from 2000: (1) in an elective situation, (2) with a whole deceased donor liver, (3) from a donor without cardiac arrest or severe hemodynamical instability before retrieval, we analyzed patients who received an IP steatotic graft ( $n = 12$ ) and we compared them with age-matched patients receiving non-IP steatotic liver grafts ( $n = 10$ ), IP non-steatotic liver grafts ( $n = 14$ ) or non-IP non-steatotic liver graft ( $n = 14$ ). Only patients receiving a graft from a donor staying < 4 days in an intensive care unit and not having an alcohol-induced accident were included in the study. The mean duration stay of donors in an intensive care unit was  $1.9 \pm 1.1$  days. The protocol of preconditioning (10 min of portal triad clamping followed by 10 min of reperfusion followed by multiorgan recovery) is the same as used in the previous clinical studies performed at our institution.<sup>12,21</sup> All post-reperfusion biopsies were taken at the same time before closure of the abdomen. The protocol was approved by our center's investigation and review board, and was always accepted by the teams recovering other organs. The pre-transplant characteristics of graft donors are shown in Table 1. I/R injury was evaluated as transaminase peak levels and transaminase levels 5 days after transplantation (day 5). A routine histological examination on post-reperfusion livers was performed to assess the degree of steatosis and surgical necrosis.

Each group was followed until July 2010 in order to evaluate the incidence and the grading of AR and CR of liver grafts after liver transplantation.

**Histological evaluation.** Samples were fixed in alcohol-formalin-acetic acid, embedded in paraffin and stained with standard haematoxylin eosin safran and picosirius stain. Histological review was made by an experienced pathologist (MS) without knowledge of the state of IP of the graft or other clinical data. The review concerned post-reperfusion surgical biopsies. Histological features, including steatosis and 'surgical' necrosis, were assessed semi-quantitatively. Steatosis of macro and microvacuolar type was graded as mild (0–30%), moderate (30–60%) or severe (60–100%). Severely steatotic livers are discarded and not used for transplantation. When mixed steatosis was observed, the overall degree was calculated adding the macro and microvacuolar degrees. 'Surgical' necrosis was defined as patchy necrosis associated with polymorphonuclear infiltrate and graded as mild (score = 1), moderate (score = 2) and severe (score = 3). To assess a necrotic index, the mean score between lobular, periportal and perivenous necrosis was calculated for each biopsy. The review also concerned liver biopsies performed in the recipient after liver transplantation, especially in order to assess the presence of AR and CR, both graded according to the relevant Banff classifications.<sup>22</sup> A rejection activity index was used to score liver allograft biopsies with AR, whereas severity of ductopenia was used to score liver allograft biopsies with CR.

**Immunohistological procedures.** All but two liver samples were available for immunostaining. Immunohistology was performed on 4  $\mu$ m paraffin-embedded formalin-fixed tissue sections using activated anti-human rabbit caspase 3 (Cell Signaling, Beverly, MA, USA), anti-human mouse beclin-1 (an early and essential autophagic protein; BD Biosciences, Franklin Lake, NJ, USA) and anti-human rabbit anti LC3 (a late autophagic protein; MBL, Nagoya, Japan) antibodies. Antigen retrieval was obtained by heat at 97°C in a citrate buffer at pH 6 and in Tris-citrate buffer for beclin-1 and LC3 and at pH 9 with 0.5% saponine for activated caspase 3. The revelation system was based on a three-step biotin-free immunophosphatase stain using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chromogene substrate (Dako, Glostrup, Denmark) followed by nuclear red counterstaining for beclin-1 and LC3 detection, whereas it was a one-step biotin free immunoperoxidase stain (ImmPress, Vector Laboratories, Burlingame, CA, USA) using 3,3-diamino-benzidine chromogene (Dako) substrate followed by Hemalun counterstaining for activated caspase 3.

**Statistical analysis.** Results are expressed as mean and standard deviation. Mean results were compared in the different groups with the help of variance analysis. Student *t*-tests after normalization of variables were used to compare two means. For dichotomous variables, a Fisher exact test was used. Median was used to compare AR or CR delay. Median results were compared in the different groups with Kolmogorov–Smirnov tests.

## Conflict of Interest

The authors declare no conflict of interest.

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