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Lack of agreement for defining 'clinical suspicion of rejection' in liver transplantation: a model to select candidates for liver biopsy

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Summary

The gold standard to diagnose acute cellular rejection (ACR) after liver transplantation (LT) is histological evaluation, but there is no consensus to select patients for liver biopsy. We aimed to evaluate the agreement among clinicians to select candidates for liver biopsy early after LT. From a protocol biopsy population (n = 690), we randomly selected 100 LT patients in whom the biopsy was taken 7–10 days after LT. The clinical information between LT and protocol biopsy was given to nine clinicians from three transplant centres who decided whether a liver biopsy was needed. The agreement among clinicians to select candidates for liver biopsy was poor: κ = 0.06–0.62, being $\kappa < 0.40$ in 76% of comparisons. The concordance between indication for liver biopsy and moderate–severe ACR in the protocol biopsy was $\kappa < 0.30$ in all cases. A multivariate model based on the product age-by-MELD (OR = 0.81; P = 0.013), delta eosinophils (OR = 1.5; P = 0.002) and mean tacrolimus trough concentrations <6 ng/ml within the prior 4 days (OR = 11.4; P = 0.047) had an AUROC = 0.84 to diagnose moderate–severe histological ACR. In conclusion, the agreement among clinicians to select patients for liver biopsy is very poor. If further validated the proposed model would provide an objective method to select candidates for liver biopsy after LT.

Introduction

Acute cellular rejection (ACR) usually occurs within the first 4–6 weeks after liver transplantation (LT), and its prevalence may vary depending on the definition used, ranging from 20% to 60% [1–3]. Although the clinical course is favourable for most patients, and successful response to boluses of steroids occurs in up to 80% of patients [4], several episodes of steroid-resistant ACR or the late onset (defined as an episode after 6 months) may increase the risk of chronic rejection and graft loss [5]. In the past, some liver transplant institutions implemented protocol biopsies early after LT to detect and treat ACR [6]. However, the potential harm of liver biopsy and the reduced impact of ACR under the current immunosuppressive regimens have discouraged this strategy.

Alternative approaches have been developed to select candidates for biopsy, being the most extended the so-called clinical suspicion of rejection, by which only patients with abnormalities in liver function tests, not explained by vascular or biliary complications, undergo a liver biopsy. Other centres are more liberal and treat empirically these patients with boluses of steroids, reserving biopsy for unresponsive cases (defined again as an absence of normalization of liver function tests), although this strategy is not supported by any clinical guideline. Nevertheless, liver

function tests are not specific for histological ACR and are only late markers, while there are no defined thresholds for suspecting rejection [7]. Thus, selecting candidates for biopsy relying on liver function test abnormalities could lead to misdiagnosis of moderate–severe histological ACR in patients with normal liver function tests; inhomogeneous selection of candidates to biopsy among centres; and transformation of ACR from an objective outcome (assessed histologically by validated grading systems [8]) to a subjective outcome (as clinicians would think more of rejection in patients on less potent immunosuppressive regimens), which could introduce bias in randomized trials evaluating immunosuppressants, particularly in those open label and/or multicentre.

In this study, we aimed to evaluate the agreement among clinicians to select candidates for liver biopsy early after LT, and to determine whether an objective method based on the combination of routine clinical and laboratory parameters would benefit clinical practice.

Materials and methods

Patients

From a prospectively collected database of 690 LT patients at the Royal Free Hospital (1997–2007) in whom a protocol liver biopsy was taken between days 7 and 10 after LT, 100 patients were randomly selected to participate in the study. The randomization sequence was automatically generated by SPSS 22.0 (IBM, Chicago, IL, USA). All patients received a tacrolimus-based immunosuppression with or without additional immunosuppressants (i.e. antimetabolites and/or steroids), between the LT and the protocol biopsy. Exclusion criteria were as follows: retransplantation, combined organ transplantation, HIV positivity and vascular or biliary complications reported in the Doppler ultrasound performed immediately before the liver biopsy. A dedicated chart was elaborated for each patient including the clinical information between LT and the protocol biopsy (see an example in Fig. 1): demographic features, primary liver disease, MELD at transplant, daily liver function tests and immunosuppression regimen, including dosage and trough concentrations. This information was given to nine experienced clinicians (mean experience 15 years, ranging from 7 to 25 years) from three LT institutions: Royal Free Hospital, London, United Kingdom (BH, JO and DP); Padova University Hospital, Padova, Italy (GG, MS and PB); Reina Sofía University Hospital, Córdoba, Spain (AP, JM and MM). For each patient, physicians had to decide among the following options: (i) to perform a liver biopsy, (ii) to treat empirically with boluses of steroids or (iii) to 'watch and wait'.

The protocol liver biopsy was obtained between days 7 to 10 after LT percutaneously, except for patients with coagulation abnormalities in whom the transjugular approach was preferred. The histological evaluation was performed prospectively by the same pathologist, who was not aware of the clinical information. The presence and severity of histological ACR was established according to the Banff schema [8] which is based in the presence of mixed mainly portal inflammation, endothelitis and bile duct damage, and classifies ACR into none/indeterminate, mild, moderate and severe. For the present study, we considered that only moderate–severe histological ACR was clinically significant as it is treated with boluses of steroids in most LT institutions, whereas mild ACR has little impact on the management [9].

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM). The kappa coefficient (κ) was used for the study of concordance. We made two different analyses of concordance: (i) agreement among clinicians to establish the 'clinical suspicion of ACR', (ii) agreement between the indication of liver biopsy and empirical treatment with boluses of steroids, with the presence of moderate–severe ACR in the protocol liver biopsy. The grade of concordance was considered appropriate if $\kappa > 0.60$, suboptimal if $\kappa = 0.40$–0.59, poor if $\kappa = 0.20$–0.39 and very poor if $\kappa < 0.19$.

A multiple logistic regression model based on the patient features, immunosuppression protocol and routine blood tests was designed to predict moderate–severe ACR. Those variables with $P < 0.30$ in the univariate analysis were selected to enter the initial model. The elimination of not significant variables was performed in a backward stepwise process. All possible interactions between the included variables were tested and considered significant if their combination in the model had a $P < 0.05$. The accuracy of the final model was determined by the area under ROC curve. To build the algorithm, a high-sensitivity threshold was chosen to indicate a liver biopsy, and a high-specificity threshold was used to select candidates for empirical treatment of ACR. Every hypothesis was two-tailed and considered significant if $P < 0.05$.

Sample size calculation

The minimum sample size required was calculated using EPIDAT 3.1, aiming to establish the concordance between the indication of liver biopsy and empirical treatment of ACR, with the presence of moderate–severe histological ACR in the protocol liver biopsy. As there are no previous studies addressing this issue, we assumed the worst clinical scenario (i.e. reduced concordance and more patients needed). The following assumptions were made: (i) confidence interval: 95%, (ii) global accuracy: 20%, (iii) propor-

CASE NUMBER ----

Gender: Female Aetiology for liver transplantation: Primary biliary cirrhosis

Age: 54 MELD at transplantation: 23

Immunosuppression protocol: Tacrolimus, mycophenolate and steroids

Ascites: Yes Encephalopathy: No

BIOCHEMICAL PARAMETHERS

DAY AFTER LT	1	2	3	4	5	6	7	8	9
Temperature (°C)	37.5	36.4	36.5	37.3	37	36.8	36.8		
Bilirubin (µmol/L) [NR<19]	139	60	49	61	71	96	79		
AST (IU/L) [NR<31]	868	509	320	115	57	63	45		
ALT (IU/L) [NR<31]	568	629	609	441	309	235	166		
ALP (IU/L) [NR<31]	80	77	71	80	103	203	176		
GGT (IU/L) [NR 5-36]	57	50	43	71	174	612	499		
WBC* (x 10^9/L) [NR 3.5-11]	15.8	8.6	6.8	5.5	7.2	12.9	14.5		
Eosinophils (x 10^9/L) [NR 0.1-0.46]	0.03	0.04	0.11	0.25	0.21	0.52	0.83		
INR	1.6	1.9	1.7	1.7	1.6	1.5	1.4		
PCR CMV (+/-; n° copies)				Neg			Neg		
CNI levels (TAC)			2		3.8		4.8		
TAC dose (mg)	0/1	1/2	1/3	3/3	3/5	4/4	4/4		
MMF dose (mg)	-	1000	1000	1000	1000	1000	1000		
Prednisolone dose (mg)	20	20	20	20	20	20	20		

NR: normal range; Neg: negative; Pos: positive; *White blood cell count (x1000)

Doppler ultrasound did not find any vascular or biliary complications

After considering information provided above, please choose one of the following options:

Perform a liver biopsy ☐

Treat empirically rejection with boluses of steroids ☐

Wait and see ☐

Figure 1 Chart given to the participating clinicians. The clinical information between the liver transplantation and the protocol liver biopsy was summarized for each patient.

tion of patients in whom liver biopsy/empirical treatment would be recommended: 25–35%, (iv) κ coefficient expected: 0.3. Under these premises, a minimum of 95 patients were required. We finally included 100 patients. This cohort was sufficient large to design the multivariate logistic regression model to predict moderate–severe histological ACR. Indeed, according to the formula proposed by Peduzzi *et al.* [10], and considering three covariates, 72 patients would be needed.

Results

In the protocol biopsy obtained at day 7 to 10 after LT, 21 patients had no features of ACR (21%), 37 patients had mild ACR (37%), 36 patients had moderate ACR (36%) and 6 patients showed severe ACR (6%). The features of the patients depending on the grade of histological ACR in the protocol biopsy are displayed in Table 1. There was an increased risk of moderate–severe histological ACR in

women ($P = 0.024$), in patients with mean tacrolimus trough concentrations <6 ng/ml in the 4 days prior to liver biopsy ($P = 0.017$) or with increased serum GGT on the day of the biopsy ($P = 0.013$). Patients with lower MELD at transplant or autoimmune cirrhosis had a trend to an increased risk of moderate–severe ACR ($P = 0.09$ and $P = 0.06$, respectively). The immunosuppression protocol did not influence the risk of moderate–severe histological ACR ($P = 0.53$).

Kinetics of liver function tests and eosinophil count are shown in Fig. 2. The serum concentrations of transaminases decreased within the 4 days prior to liver biopsy similarly in patients with or without moderate–severe ACR: AST decreased 69% in patients with none–mild ACR and 61% in patients with moderate–severe ACR ($P = 0.10$); ALT decreased 53% in patients with none–mild ACR and 59% in patients with moderate–severe ACR ($P = 0.37$). Conversely, the cholestatic enzymes rose within the 4 days prior to liver biopsy, and the increase was more pronounced in patients with moderate–severe ACR: 18% vs. 61% for ALP ($P = 0.021$) and 16% vs. 78% for GGT ($P = 0.07$). Peripheral eosinophil counts within the 4 days prior to liver biopsy were the most discriminative parameter of moderate–severe ACR. In patients with none–mild ACR eosinophil count remained almost unchanged (15% increase), whereas patients with moderate–severe ACR nearly tripled eosinophil count within this period (180% increase) ($P < 0.001$).

Study of concordance

The most frequently recommended approach by the participating clinicians was 'watch and wait' which ranged from 39% to 79% (median 61%). A liver biopsy was advised for 17–43% of patients (median 28%), whereas empirical treatment of rejection was recommended for 0–25% of patients (median 11%). The proportion of each recommendation given was neither influenced by the institution ($P = 0.36$ for 'watch and wait'; $P = 0.58$ for liver biopsy; $P = 0.11$ for empirical treatment) nor modified by the experience (number of years dealing with LT patients) of the clinician involved ($P = 0.64$ for 'watch and wait', $P = 0.20$ for liver biopsy and 0.46 for empirical treatment). The agreement among clinicians to select patients for liver biopsy or empirical treatment ranged between $\kappa = 0.06$ and $\kappa = 0.62$ (Fig. 3). From the 72 comparisons made resulting from the different combinations of the 9 clinicians involved, the agreement was very poor ($\kappa < 0.19$) in 25% of comparisons, poor ($\kappa = 0.20$–0.39) in 50% of comparisons, suboptimal ($\kappa = 0.40$–0.59) in 22.3% of comparisons and finally appropriate ($\kappa > 0.60$) in only 2.7% of comparisons. The agreement between the indication of liver biopsy and

Table 1. Univariate analysis of clinical and laboratory data depending on the grade of acute cellular rejection found in the protocol liver biopsy at day 7–10 after liver transplantation. Liver function tests and blood eosinophils count are shown on the day of the protocol biopsy.

Variable	Whole cohort ($n = 100$)	None–mild ACR ($n = 58$)	Moderate–severe ACR ($n = 42$)	*P*
Age	48.9 ± 10.9	49.7 ± 10	47.6 ± 11	0.35
Gender (%)				
Male	35%	74.1%	52.4%	0.024
Female	65%	25.9%	47.8%	
Pre-LT MELD	18.6 ± 8.4	19.9 ± 8	16.9 ± 8	0.09
Pre-LT liver disease				
Autoimmune cirrhosis*	26%	19%	35.7%	0.06
Other aetiologies	74%	81%	64.3%	
Immunosuppression protocol				
Tacrolimus monotherapy	44%	41.4%	47.6%	0.53
Tacrolimus + antimetabolites or steroids	56%	58.6%	52.4%	
Mean tacrolimus trough levels				
<6 ng/ml	41%	31%	54.8%	0.017
>6 ng/ml	59%	69%	45.2%	
AST (IU/l)	68.3 ± 59	63.8 ± 57	74.3 ± 62	0.39
ALT (IU/l)	223 ± 184	214 ± 154	235 ± 220	0.59
ALP (IU/l)	192 ± 151	172 ± 163	220 ± 131	0.12
GGT (IU/l)	285 ± 216	230 ± 166	353 ± 253	0.013
Bilirubin (μmol/l)	103 ± 82	102 ± 75	104 ± 92	0.89
Eosinophils ($\times 10^9$)	0.53 ± 0.4	0.48 ± 0.3	0.6 ± 0.5	0.20

ACR, acute cellular rejection; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyltransferase; LT, liver transplantation; MELD, model for end-stage liver disease.
*Autoimmune cirrhosis comprises primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis.

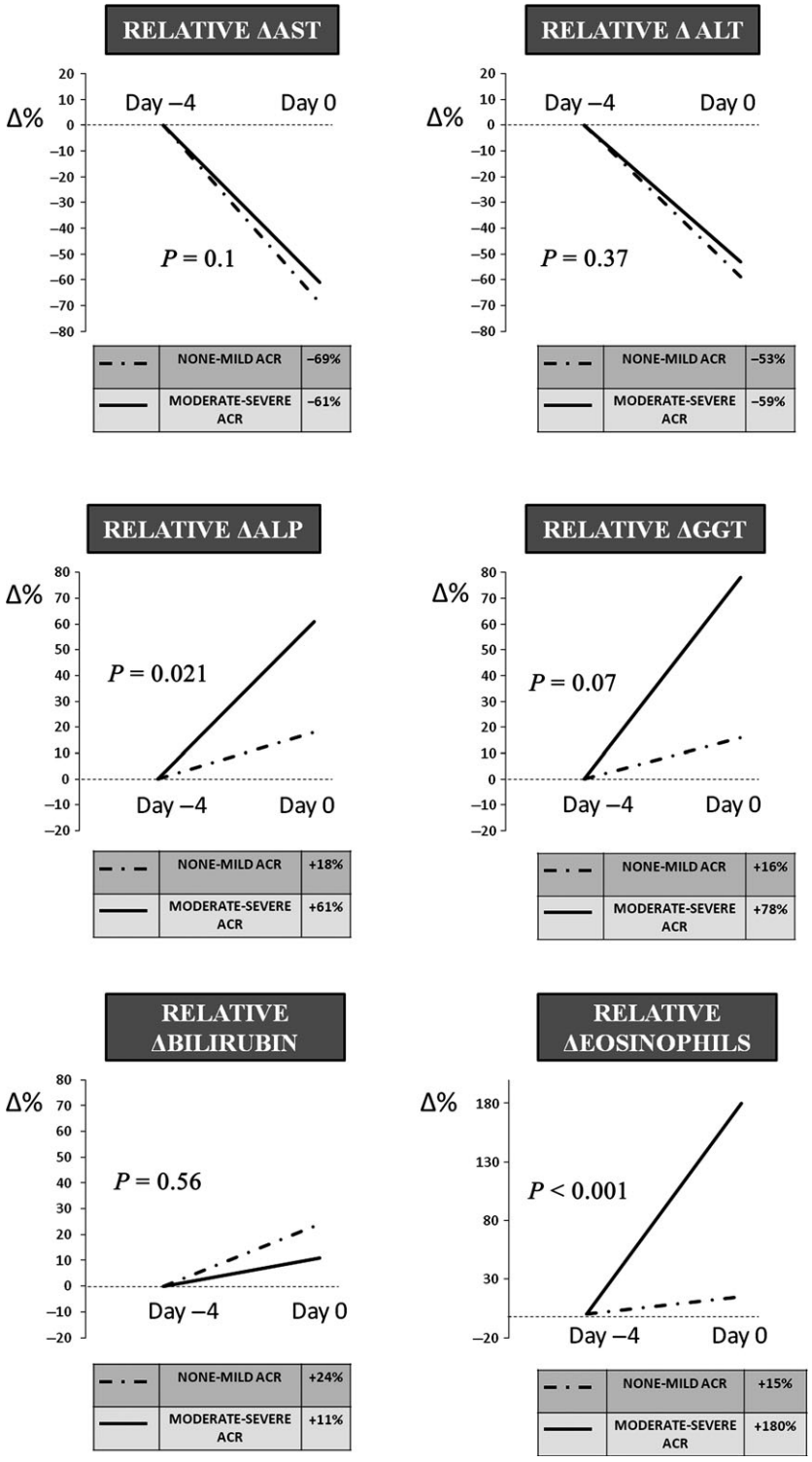


Figure 2 Kinetics of liver function tests and eosinophil count within the 4 days prior to liver biopsy in patients with none–mild acute cellular rejection compared with patients with moderate–severe acute cellular rejection. Results are presented as the delta relative change (Δ%).

empirical treatment made by the clinician with the presence of moderate–severe ACR in the protocol biopsy (Fig. 3, last row) was poor or very poor in all cases (11.1% and 88.9%, respectively). The experience of the clinician did not influence the grade of concordance between the clinical suspicion of ACR and the histological findings (correlation coefficient $r = -0.07$; $P = 0.85$).

were as follows: product of age by pre-LT MELD (OR = 0.81; $P = 0.013$); rising blood eosinophil count within the 4 days prior to liver biopsy (OR = 1.50; $P = 0.002$); and reduced immunosuppression (OR = 11.4; $P = 0.047$) defined as tacrolimus monotherapy with mean trough concentrations <6 ng/ml. The model score can be calculated through the following formula:

$$\text{Score} = \frac{e^{(-0.07093)+0.00206\times(\text{Age}*\text{MELD})-2.43720\times(\text{Red\_Immunos})-0.00407\times(\Delta\text{EOS})}}{1+e^{(-0.07093)+0.00206\times(\text{Age}*\text{MELD})-2.43720\times(\text{Red\_Immunos})-0.00407\times(\Delta\text{EOS})}}$$

Where:

Age * MELD: multiplication of the recipient age and pre-LT MELD score.

Multivariate analysis

The initial multivariate model was composed by the following variables: gender, pre-LT MELD, underlying liver disease, mean tacrolimus trough concentrations, ΔALP, ΔGGT and Δeosinophils within the 4 days prior to liver biopsy. Gender, aetiology of liver disease, ΔALP and ΔGGT were excluded from the model because of lack of statistical significance ($P = 0.21$, $P = 0.22$, $P = 0.49$ and $P = 0.62$, respectively). All possible interactions between variables were tested, and none of them was significant excepting the one involving age and pre-LT MELD, which was kept in the model. The final model is shown in Table 2. The independent predictors of moderate–severe histological ACR

Table 2. Multivariate logistic regression model to predict moderate–severe histological acute cellular rejection in the protocol biopsy at day 7–10 after liver transplantation.

Variable	OR	95% CI	*P*
Product age-by-pre-LT MELD	0.81	0.69–0.95	0.013
Minimized immunosuppression*	11.4	1.1–125	0.047
ΔEosinophils in the previous 4 days	1.5	1.15–1.95	0.002

*Defined as mean trough concentrations of tacrolimus <6 ng/ml without other immunosuppressants (excepting steroids).

		RFH			PUH			HURS		
		R1	R2	R3	P1	P2	P3	H1	H2	H3
RFH	R1		0.43	0.35	0.21	0.28	0.13	0.20	0.37	0.18
	R2	0.43		0.56	0.17	0.38	0.22	0.34	0.51	0.31
	R3	0.35	0.56		0.17	0.42	0.14	0.44	0.62	0.36
PUH	P1	0.21	0.17	0.17		0.32	0.06	0.32	0.19	0.21
	P2	0.28	0.38	0.22	0.32		0.27	0.40	0.41	0.44
	P3	0.13	0.22	0.14	0.06	0.27		0.35	0.08	0.39
HURS	H1	0.20	0.34	0.44	0.32	0.40	0.35		0.44	0.40
	H2	0.37	0.51	0.62	0.19	0.41	0.08	0.44		0.33
	H3	0.18	0.31	0.36	0.21	0.44	0.39	0.40	0.33	
HISTOLOGY Moderate-severe rejection		0.06	0.17	0.04	0.18	0.11	0.17	0.18	0.11	0.30

NOTICE: each hepatologist is named with his/her hospital's initial and a number ranging from 1 to 3.

RFH: Royal Free Hospital. London (UK).
PUH: Padova University Hospital. Padova (Italy).
HURS: Reina Sofía University Hospital. Córdoba (Spain).

Figure 3 Summary of the study of concordance. Each clinician is represented in a column/row as the initial of his/her institution plus a number ranging from 1 to 3. Kappa coefficients are displayed. The highlighted row indicates the concordance between clinical suspicion of rejection and histological evaluation (gold standard).

Red Immunos: reduced immunosuppression defined as tacrolimus <6 ng/ml within the 4 days prior to liver biopsy without concomitant immunosuppressants (no = 0; yes = 1).

ΔEOS: relative delta eosinophil count within the prior 4 days calculated as:

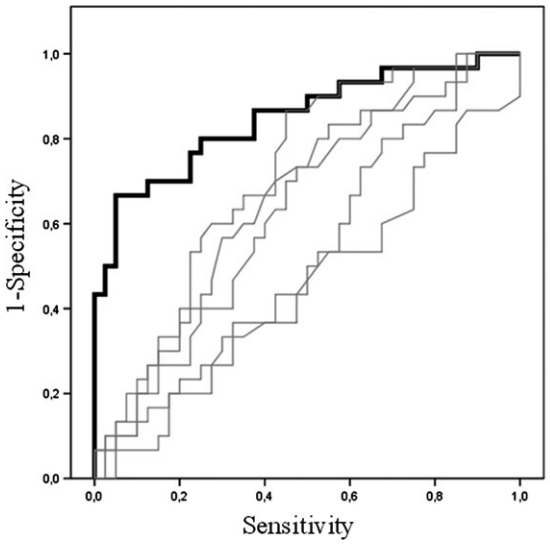
$$\left(\frac{\mathrm{EOS}_{\mathrm{day0}} - \mathrm{EOS}_{\mathrm{day-4}}}{\mathrm{EOS}_{\mathrm{day0}}} \times 100\right)$$

The area under ROC curve of the model was 0.84, which was significantly higher than those obtained from AST (0.61), ALT (0.50), ALP (0.65), GGT (0.64) and bilirubin (0.44) (Fig. 4). The best threshold for the model was 0.28, with 79% sensitivity and 70% specificity. An algorithm based on the model was designed to aid the decision-making process (Fig. 5). If the score derived from the model was ≥0.81, the positive predictive value for moderate–severe ACR was 100% (positive likelihood ratio = 22), and thus, the immunosuppression regimen should be strengthened, and treatment with boluses of steroids should be implemented whenever needed. On the other hand, if the score was ≤0.24, the negative predictive value was 90% (negative likelihood ratio = 0.14), and no action would be needed except from routine surveillance. If the score was between 0.25 and 0.80, immunosuppression should be optimized and the score calculation repeated after 48 h. If the score remained unchanged (0.25–0.80), the risk of moderate–severe ACR was 55%, and a liver biopsy should be performed.

In our cohort, the distribution of patients following the algorithm is shown in Fig. 5. The first calculation of the model would have recommended empirical treatment for 20 patients (20%) and routine surveillance for 34 patients (34%). In the remaining patients (n = 46; 46%), a second calculation of the model would be required after 48 h. If a similar distribution of patients as in the first calculation was assumed, eight patients of 46 (20%) would change their status to empirical treatment and 16 patients of 46 (34%) would change to routine surveillance. After the second assessment, 22 patients (22%) would remain in the 'grey area' of the model in which a liver biopsy is recommended. In total, according to this algorithm, 28 patients (28%) would have been treated empirically with boluses of steroids, 22 patients (22%) would have received a liver biopsy and 50 patients (50%) would have required routine surveillance. The rates of misdiagnosis of moderate–severe ACR were 10%, and among patients having none–mild ACR, 16 of 42 (38.1%) would have received a liver biopsy. No patient with none–mild ACR would have received empirical treatment with boluses of steroids.

Discussion

This is the first study addressing the physician agreement to evaluate ACR in a population with protocol biopsies early after LT. A poor concordance among hepatologists to define clinical suspicion of ACR was found, leading to a wide heterogeneity in candidate selection for liver biopsy. The proposed model and the derived algorithm would have significantly reduced the rates of misdiagnosis of ACR, while minimizing the number of unnecessary liver biopsies.



	AUROC
MODEL	0.84*
AST	0.61
ALT	0.50
ALP	0.65
GGT	0.64
Bilirubin	0.44

*$P < 0.05$ in all comparisons

Figure 4 ROC curves comparing the accuracy of the multivariate model (composed by the product age-by-MELD, reduced immunosuppression and delta blood eosinophil count) with conventional liver function tests to predict moderate–severe histological acute cellular rejection.

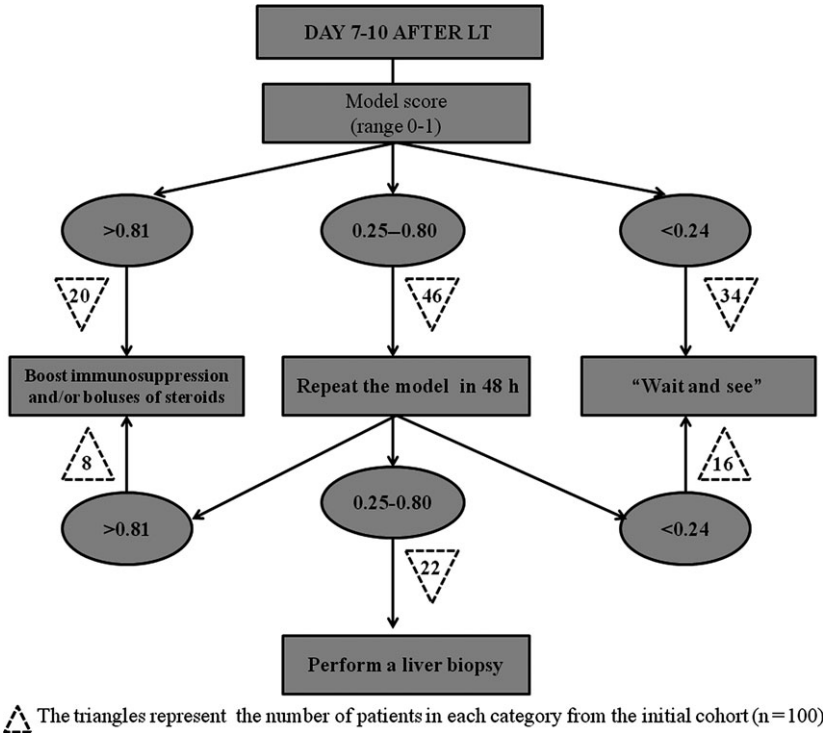


Figure 5 Algorithm to diagnose acute cellular rejection early after liver transplantation based in the multivariate logistic regression model.

In the current scenario, the gold standard to diagnose and grade ACR is the histological evaluation through the Banff criteria [8]. As the influence of early ACR proved to be a minor problem in terms of graft loss and mortality [11,12], and mild ACR has even been proposed to be beneficial for some patients [9,13], protocol biopsies were abandoned in most LT institutions. Nowadays, only patients with rising liver function tests without alternative explanation undergo a liver biopsy in what has been termed 'clinical suspicion of ACR'. In previous studies including patients with protocol biopsies, liver function tests were neither sensitive nor specific to diagnose ACR, and indeed, the thresholds for these parameters are not uniform in the literature [7,14,15]. Therefore, this strategy transforms the histological evaluation, which is an objective and validated method to diagnose and grade ACR [8], in a subjective approach which may vary widely, not only among LT centres, but also between clinicians within the same institution. The consequences of using the 'clinical suspicion of ACR' approach were not surprising: 28% to 71% of patients with moderate–severe ACR would have been timely diagnosed or misdiagnosed. Conversely, 15% to 43% of patients with none or mild ACR would have undergone a liver biopsy and up to 24% would have received empirical treatment with boluses of steroids with no underlying ACR. The poor agreement to select candidates for liver biopsy may be particularly relevant for randomized controlled trials evaluating different immunosuppression protocols after LT, particularly for those that are open label and/or multicentre, in which protocol biopsies are seldom used. This may explain in part the different rates of ACR found in randomized controlled trials with similar immunosuppression schedules [16]. For instance, among randomized trials using reduced tacrolimus, mycophenolate mofetil and steroids, the incidence of biopsy-proven ACR after clinical suspicion ranges from 15% to 43% [17,18]. In addition, the clinician would be more worried of ACR in patients on less potent immunosuppression, and the rates of liver biopsies and thus the rates of moderate–severe ACR may be increased in this group, leading to a classification bias. Future randomized trials evaluating immunosuppressants after LT should use either protocol liver biopsies, or an objective and uniform method to select candidates for liver biopsy.

In the present study, the rates of moderate–severe histological ACR were slightly above the recent randomized trials using biopsy-proven ACR [19,20], but similar to those consistently reported in randomized trials using protocol biopsies [2]. We identified a multivariate model composed by clinical and routine laboratory parameters able to predict moderate–severe ACR in the liver biopsy. Younger patients with reduced MELD at transplantation were more likely to have moderate–severe ACR, whereas the sickest patients at transplantation (older and with

increased MELD) were protected against ACR, as previously reported [5,9,21,22]. Regarding immunosuppression, the use of much reduced tacrolimus trough concentrations (<6 ng/ml) without antimetabolites was an independent predictor of ACR. In previous randomized trials, reduced tacrolimus early after LT has been successfully implemented with no increase in ACR rates, by adding other immunosuppressants [23,24] or implementing induction immunosuppression [25]. The use of tacrolimus trough concentrations <6 ng/ml early after LT without additional immunosuppressants is very uncommon in clinical practice, as it was in our cohort, and it increases the risk of ACR and graft loss [13]. Finally, the change in blood eosinophil counts provided useful information about the presence and grade of ACR according to previous reports [7,26], and it was confirmed in the present study.

The model was formed by a static variable (product age-by-MELD at transplantation), and two variables able to change over time (immunosuppression and blood eosinophil count). Thus, the product of the model can be modified either by rising immunosuppression and/or by repeating the analysis of blood eosinophil count. In the proposed algorithm, this possibility was taken into account and, if the first calculated score was between 0.21 and 0.80, a second assessment after 48 h was recommended. This strategy may increase the AUROC of the model (>0.84) although this could not be measured in the study because paired biopsies were lacking. The model would be also useful for monitoring the response of ACR after receiving boluses of steroids. In previous studies, the delta eosinophil count alone was predictive for histological improvement of ACR irrespective of whether boluses of steroids were used [7].

The pathogenesis of ACR after LT is mediated by an immune response in which donor antigens coming from the major histocompatibility complex are presented to T cells, causing graft damage and the typical histopathological findings [27]. Despite the advances in flow-cytometry, genomewide analyses, proteomics and metabolomics, not invasive biomarkers of ACR after LT are still lacking. Further studies are needed to identify the critical nodes of ACR after LT in order to improve diagnosis and also to design more specific immunosuppressive drugs against rejection. The addition of not invasive biomarkers to the proposed model would increase the accuracy and would reduce the need of liver biopsies early after LT.

Patients transplanted with hepatitis C deserve a mention apart. As the use of boluses of corticosteroids was related to a more aggressive viral recurrence, fibrosis progression and increased risk of graft loss [28,29], the presence of moderate–severe ACR should be confirmed histologically in all cases. If the score of the model is above 0.80, a liver biopsy should be performed and the levels of calcineurin inhibitors rose whenever needed. Boluses of steroids should be reserved for patients with persistent moderate–severe ACR.

The limitation of the present study is the limited sample size, which did not allow for a validation of the predictive model of ACR. Further studies in independent populations are needed to validate both the accuracy of the model and the chosen thresholds.

In conclusion, the lack of criteria to define 'clinical suspicion of ACR' introduces a subjective component in the evaluation of ACR and therefore increases the risk of misdiagnosis. In randomized controlled trials including ACR as an endpoint, particularly in those that are multicentric and/or open label, the selection of patients undergoing liver biopsy should be based on objective parameters. If further validated, the proposed model based on clinical and conventional laboratory features may be a reliable tool to guide clinical decisions early after LT and to minimize the risk of bias in randomized controlled trials assessing ACR. The addition of novel biomarkers of ACR may improve the accuracy of the model.

Authorship

MR-P: participated in the research design, in the data analysis and wrote the paper. CG-C: collected and analysed data. ET: collected data and contributed important reagents. GG, BH, AP-G, JO'B, MS, JLM-Á, DP, PB, JB and APD: participated in the performance of the research and contributed important reagents. MG-M: participated in the performance of the research. PB and AKB: participated in research design and contributed important reagents. MDM: participated in research design, in the data analysis and contributed important reagents.

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References

1. Shaked A, Ghobrial RM, Merion RM, *et al.* Incidence and severity of acute cellular rejection in recipients undergoing adult living donor or deceased donor liver transplantation. *Am J Transplant* 2009; **9**: 301.

2. Lerut J, Mathys J, Verbaandert C, *et al.* Tacrolimus monotherapy in liver transplantation: one-year results of a prospective, randomized, double-blind, placebo-controlled study. *Ann Surg* 2008; **248**: 956.
3. Boillot O, Seket B, Dumortier J, *et al.* Thymoglobulin induction in liver transplant recipients with a tacrolimus, mycophenolate mofetil, and steroid immunosuppressive regimen: a five-year randomized prospective study. *Liver Transpl* 2009; **15**: 1426.
4. Wiesner RH, Ludwig J, Krom RA, *et al.* Treatment of early cellular rejection following liver transplantation with intravenous methylprednisolone. The effect of dose on response. *Transplantation* 1994; **58**: 1053.
5. Thurairajah PH, Carbone M, Bridgestock H, *et al.* Late acute liver allograft rejection; a study of its natural history and graft survival in the current era. *Transplantation* 2013; **95**: 955.
6. Burroughs AK, Patch DW, Stigliano R, Cecilioni L. Protocol biopsies in liver transplantation. *Liver Transpl* 2003; **9**: 780.
7. Rodriguez-Peralvarez M, Germani G, Tsochatzis E, *et al.* Predicting severity and clinical course of acute rejection after liver transplantation using blood eosinophil count. *Transpl Int* 2012; **25**: 555.
8. Demetris A, Batts K, Dhillon A, *et al.* Banff schema for grading liver allograft rejection: an international consensus document. *Hepatology* 1997; **25**: 658.
9. Wiesner RH, Demetris AJ, Belle SH, *et al.* Acute hepatic allograft rejection: incidence, risk factors, and impact on outcome. *Hepatology* 1998; **28**: 638.
10. Peduzzi P, Concato J, Kemper E, Holford TR, Feinstein AR. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol* 1996; **49**: 1373.
11. Adam R, Karam V, Delvart V, *et al.* Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). *J Hepatol* 2012; **57**: 675.
12. Bartlett AS, Ramadas R, Furness S, Gane E, McCall JL. The natural history of acute histologic rejection without biochemical graft dysfunction in orthotopic liver transplantation: a systematic review. *Liver Transpl* 2002; **8**: 1147.
13. Rodriguez-Peralvarez M, Germani G, Papastergiou V, *et al.* Early tacrolimus exposure after liver transplantation: relationship with moderate/severe acute rejection and long-term outcome. *J Hepatol* 2013; **58**: 262.
14. Schlitt HJ, Nashan B, Krick P, *et al.* Intragraft immune events after human liver transplantation. Correlation with clinical signs of acute rejection and influence of immunosuppression. *Transplantation* 1992; **54**: 273.
15. Nagral A, Butler P, Sabin CA, Rolles K, Burroughs AK. Alpha-glutathione-S-transferase in acute rejection of liver transplant recipients. *Transplantation* 1998; **65**: 401.
16. Rodriguez-Peralvarez M, Germani G, Darius T, Lerut J, Tsochatzis E, Burroughs AK. Tacrolimus trough levels, rejection and renal impairment in liver transplantation: a systematic review and meta-analysis. *Am J Transplant* 2012; **12**: 2797.
17. Klintmalm GB, Washburn WK, Rudich SM, *et al.* Corticosteroid-free immunosuppression with daclizumab in HCV (+) liver transplant recipients: 1-year interim results of the HCV-3 study. *Liver Transpl* 2007; **13**: 1521.
18. Kato T, Gaynor JJ, Yoshida H, *et al.* Randomized trial of steroid-free induction versus corticosteroid maintenance among orthotopic liver transplant recipients with hepatitis C virus: impact on hepatic fibrosis progression at one year. *Transplantation* 2007; **84**: 829.
19. Neuberger JM, Mamelok RD, Neuhaus P, *et al.* Delayed introduction of reduced-dose tacrolimus, and renal function in liver transplantation: the 'ReSpECT' study. *Am J Transplant* 2009; **9**: 327.
20. Klintmalm GB, Feng S, Lake JR, *et al.* Belatacept-based immunosuppression in de novo liver transplant recipients: 1-year experience from a phase II randomized study. *Am J Transplant* 2014; **14**: 1817.
21. Ikegami T, Bekki Y, Imai D, *et al.* Clinical outcomes of living donor liver transplantation for patients 65 years old or older with preserved performance status. *Liver Transpl* 2014; **20**: 408.
22. Heinbokel T, Hock K, Liu G, Edtinger K, Elkhal A, Tullius SG. Impact of immunosenescence on transplant outcome. *Transpl Int* 2013; **26**: 242.
23. Boudjema K, Camus C, Saliba F, *et al.* Reduced-dose tacrolimus with mycophenolate mofetil vs. standard-dose tacrolimus in liver transplantation: a randomized study. *Am J Transplant* 2011; **11**: 965.
24. Saliba F, De Simone P, Nevens F, *et al.* Renal function at two years in liver transplant patients receiving everolimus: results of a randomized, multicenter study. *Am J Transplant* 2013; **13**: 1734.
25. Turner AP, Knechtle SJ. Induction immunosuppression in liver transplantation: a review. *Transpl Int* 2013; **26**: 673.
26. Barnes EJ, Abdel-Rehim MM, Goulis Y, *et al.* Applications and limitations of blood eosinophilia for the diagnosis of acute cellular rejection in liver transplantation. *Am J Transplant* 2003; **3**: 432.
27. Sanchez-Fueyo A, Strom TB. Immunologic basis of graft rejection and tolerance following transplantation of liver or other solid organs. *Gastroenterology* 2011; **140**: 51.
28. McCaughan GW, Zekry A. Mechanisms of HCV reinfection and allograft damage after liver transplantation. *J Hepatol* 2004; **40**: 368.
29. Berenguer M. What determines the natural history of recurrent hepatitis C after liver transplantation? *J Hepatol* 2005; **42**: 448.