TITLE PAGE

AN OBJECTIVE DEFINITION FOR CLINICAL SUSPICION OF T-CELL MEDIATED REJECTION AFTER LIVER TRANSPLANTATION

AUTHORS: Manuel Rodríguez-Perálvarez[1]; Laura De Luca[2]; Gonzalo Crespo [3]; Ángel Rubin[4]; Sandra Marín[1]; Salvador Benlloch[4]; Jordi Colmenero[3]; Marina Berenguer[4]; Miguel Navasa[3]; Emmanuel Tsochatzis[2]; Manuel De la Mata[1].

AFFILIATIONS:

[1]Department of Hepatology and Liver Transplantation. Reina Sofía University Hospital, IMIBIC, CIBERehd. Córdoba (Spain).

[2]UCL Institute for Liver and Digestive Health and Sheila Sherlock Liver Unit. Royal Free Hospital and UCL, London (United Kingdom).

[3]Liver Transplant Unit. Hospital Clinic, IDIBAPS, CIBERehd. Barcelona (Spain).

[4]Hepatology and Liver Transplantation Unit. La Fe University Hospital, CIBERehd. Valencia (Spain).

AUTHORSHIP

- Manuel Rodríguez-Perálvarez: Data analysis and drafted the manuscript.

- Laura De Luca: Data aquisition and contribution with important reagents.

- Gonzalo Crespo: Data aquisition and contribution with important reagents.

- Ángel Rubin: Data aquisition and contribution with important reagents.

- Sandra Marín: Data aquisition and contribution with important reagents.

- Salvador Benlloch: Data analysis and contribution with important reagents.

- Jordi Colmenero: Data analysis and contribution with important reagents.

- Marina Berenguer: Revised the manuscript critically for important intellectual content.

- Miguel Navasa: Revised the manuscript critically for important intellectual content.

- Emmanuel Tsochatzis: Original conception and design. Revised the manuscript critically for important intellectual content.

- Manuel De la Mata: Original conception and design. Revised the manuscript critically for important intellectual content.

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Corresponding author contact information: Prof. Manuel de la Mata, MD, PhD. Mail: mdelamatagarcia@gmail.com; Address: Avda. Menéndez Pidal s/n. Córdoba (Spain). Phone: 0034 957010328 Fax: 0034 957736014

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ABSTRACT

A uniform definition of clinical suspicion of T-cell mediated rejection (TCMR) in liver transplantation (LT) is needed to homogenize clinical decisions, especially within randomized trials. The present multicentre study included a total of 470 primary LT recipients. The derivation cohort consisted of 142 patients who had clinically-driven liver biopsies at any time after LT. The external validation cohort included 328 patients who underwent protocol biopsies at day 7-10 after LT. The rates of moderate-severe histological TCMR were 33.8% in the derivation cohort and 43.6% in the validation cohort. Independent predictors (ie. risk factors) of moderate-severe TCMR in the derivation cohort were: serum bilirubin >4mg/dL (OR=5.83; p<0.001), rising bilirubin within the 4 days prior to liver biopsy (OR=4.57; p=0.003), and blood eosinophils count $>0.1*10^9$/L (OR=3.81; p=0.004). In the validation cohort, the number of risk factors was an independent predictor of moderate-severe TCMR (OR=1.74; p=0.001), after controlling for hepatitis C status. The number of risk factors paralleled the rates of moderate-severe TCMR in the derivation and validation cohorts (p<0.001 in both comparisons). In conclusion, increased serum bilirubin, rising bilirubin and eosinophilia are validated risk factors for moderate-severe histological TCMR, and could be used as objective criteria to select candidates for liver biopsy.

KEYWORDS: Immunosuppression, liver biopsy, liver transplantation, T-cell mediated rejection.

ABBREVIATIONS: IQR, interquartile range; LT, liver transplantation; TCMR, T-cell mediated rejection.

INTRODUCTION

Acute T-cell mediated rejection (TCMR) is a frequent complication in liver transplantation (LT), and forms the primary efficacy endpoint in most randomized trials evaluating immunosuppressive drugs. The gold standard to diagnose and grade TCMR is histological assessment by using the Banff classification[1]. In the past, some institutions implemented protocol liver biopsies early after LT, and reported that up to 80% of patients show histological features of TCMR. However, most of these episodes were mild and without any detrimental impact on graft survival[2-4]. Nowadays, protocol liver biopsies have been abandoned and only patients with clinical suspicion of rejection, which usually means raising transaminases otherwise unexplained, undergo a liver biopsy. However, transaminases are poor predictors of TCMR, and do not mirror its histological severity[2,5].

The agreement among transplant physicians to define clinical suspicion of rejection and to advise biopsy after LT was evaluated in a study including 100 LT patients with protocol liver biopsies at day 7[6]. The concordance among clinicians was poor or very poor in 76% of comparisons (κ coefficient<0.40), and most importantly, the concordance between clinical suspicion of rejection and the histological assessment was very poor in all cases (κ coefficient <0.30). In randomized trials using biopsy-proven TCMR as primary efficacy endpoint, this heterogeneity in selecting candidates for liver biopsy may be translated into increased risk of bias, particularly in those with open label design and with multicenter involvement[7]. Therefore, if protocol liver biopsies are no longer to be used, a systematic approach to select candidates for liver biopsy according to their individual risk of moderate-severe TCMR is mandatory. This method should be accurate, reproducible, and based on routine parameters whenever possible. In addition, it should not include information regarding immunosuppression, so it can be

implemented in randomized trials without any confounding effect. Although previous studies have identified some clinical predictors of TCMR, such as younger age, low MELD score, underlying autoimmune liver disease, elevated blood eosinophils count and vitamin D deficiency [2,5,8,9], they are not sufficiently validated to guide clinical decisions. Biomarkers and immune function assays have been proposed, but again results are contradictory and they are not widely available[10-17].

Within this clinical scenario, the aims of the present study were: 1) To identify independent predictors of histological TCMR among routine clinical and laboratory parameters, and 2) to design and validate an objective method to select patients at increased risk of TCMR after LT, and thus able to homogenize the selection of candidates for liver biopsy both in randomized trials and clinical practice.

PATIENTS AND METHODS

Study design, patients and variables

This is a retrospective multicenter study including 470 adult primary LT patients who subsequently underwent a liver biopsy. Patients were considered for inclusion if they had at least two determinations of complete blood count and liver tests (ie. transaminases, bilirubin and cholestatic parameters) within the 4 days prior to liver biopsy. Exclusion criteria were: multiorgan transplantation, HIV infection, ongoing vascular/biliary complications, treatment with boluses of steroids one month prior to liver biopsy and primary immunosuppression not based on tacrolimus. Although concomitant immunosuppressive drugs were allowed, patients with cyclosporine-based immunosuppression were excluded in order to increase homogeneity. Cyclosporine-based protocols are seldom used nowadays given the increased risk of TCMR and graft

loss as compared with tacrolimus[18]. Included patients were divided in two cohorts: 1) A derivation cohort, which consisted in 142 patients transplanted from 2008 to 2014 in 3 transplant institutions, and who had clinically-driven liver biopsies; 2) An external validation cohort including 328 consecutive patients transplanted in a single institution from 2000 to 2007 with protocol liver biopsies at day 7-10 after LT.

All liver biopsies were systematically evaluated to diagnose and grade acute TCMR according to the Banff schema, which is based in the presence of mixed mainly portal inflammation, endothelitis and bile duct damage, and classifies TCMR as none/indeterminate, mild, moderate and severe[1]. Only moderate-severe histological TCMR was considered clinically significant for the present study, since mild TCMR does not require treatment in most cases and its prognostic impact is negligible[7]. In the derivation cohort, liver biopsies were indicated at any time after LT by the responsible transplant clinician in each center, either because of clinical suspicion of rejection, or under any other indication (mainly graft dysfunction or suspicion of hepatitis C recurrence). In the validation cohort, liver biopsies were scheduled between days 7-10 post-LT irrespective of graft function. A multicenter population with clinically-driven liver biopsies is “a priori” more heterogenous, whereas a cohort with protocol liver biopsies is expected to be more uniform and closer to the gold standard to diagnose TCMR. Therefore, the former population was used as a derivation cohort while the later served as validation. In both cohorts, liver biopsies were performed percutaneously, except for patients with abnormal coagulation parameters, in whom the transjugular approach was preferred.

Risk factors of histological moderate-severe TCMR were screened among routine clinical and biochemical parameters including recipient age, pre-LT MELD score, gender, aetiology of liver disease, interval from LT to liver biopsy, mean tacrolimus

trough concentrations within the four days prior to liver biopsy, concomitant immunosuppressive drugs, transaminases (AST/ALT), cholestatic parameters (ALP/GGT), bilirubin, INR, serum creatinine and blood eosinophils count. The kinetics of liver tests within the 4 days prior to liver biopsy, calculated as the relative delta change within this period, were also analyzed as possible predictors of significant TCMR.

Statistical analysis

Statistical analysis was performed by using SPSS 22.0 (IBM, Chicago, USA) and SAS/STAT® software. Variables are displayed as frequency tables or mean ± standard deviations, except for asymmetric distributions which are presented as median and interquartile range (IQR). For continuous variables, optimal thresholds were obtained by using ROC curves. In the derivation cohort, the risk factors of moderate-severe histological TCMR were screened by using univariate analysis: Chi-square test was used for frequencies, Student's t test or ANOVA for continuous variables, and Mann–Whitney's U or Kruskal–Wallis for asymmetric distributions. Multiple logistic regression was used to identify independent predictors of moderate-severe histological TCMR in the derivation cohort, and to explore their utility in the validation cohort. Those variables with $p<0.20$ in the univariate analysis were selected to enter the initial multivariate model. Not significant covariates were eliminated from the model in a backward stepwise process. The final model included independent predictors, significant interactions (if any) and confounding factors (both hepatitis C status and interval from LT to liver biopsy were potential confounding factors from the clinical point of view, and were controlled irrespective of their statistical behavior). A nomogram based on the multivariate logistic regression model was created to calculate

the individual risk of moderate-severe TCMR. Every hypothesis was two-tailed and considered significant if $p<0.05$.

Sample size calculation

The minimum sample size required was calculated by using EPIDAT® 3.1 (Xunta de Galicia). The following assumptions were made:

- Expected negative predictive value (ability of the model to exclude moderate-severe histological TCMR): 80%-95%

- Expected positive predictive value (ability of the model to predict moderate-severe histological TCMR): 55%-75%

- Prevalence of moderate-severe TCMR in the included population: 35%

- Statistical power: 80%; Alpha error: 5%

Under these premises, the minimum sample size required was n=127 (n=142 after applying Yates' correction). In the present study, the derivation cohort included 142 patients and the validation cohort comprised 328 patients.

RESULTS

Descriptive study

A descriptive evaluation of both derivation and validation cohorts is shown in table 1. Both cohorts were comparable in terms of age, pre-LT MELD score and gender. Since the derivation cohort consisted of clinically-driven liver biopsies at any time post-LT, the interval from LT to liver biopsy was longer and more heterogeneous among

patients, as compared with the validation cohort (33 days [IQR 13-161] vs 6 days [IQR 5-8] respectively; p<0.001). Moderate-severe histological TCMR was present in 33.8% of patients in the derivation cohort and 43.6% of patients in the validation cohort. At the time of liver biopsy, mean tacrolimus trough concentrations were lower in the derivation cohort (8±3.9 ng/mL vs 9.2±5.5 ng/mL; p=0.019), but concomitant immunosuppressants were more frequent (62.7% vs 45.5%; p<0.001). Regarding biochemical parameters on the day of the liver biopsy, the derivation cohort was characterized by increased levels of AST, ALP and GGT, but lower eosinophils count, INR and creatinine, as compared with the validation cohort. In the derivation cohort, transaminases and cholestatic parameters were stable within the 4 days prior to liver biopsy (ie. relative delta change close to 100%). In the validation cohort, transaminases were decreasing (ie. relative delta change <100%) while cholestatic parameters were rising (ie. relative delta change >100%) within the same timeframe, as it would be expected at day 7-10 after LT.

Risk factors of histological moderate-severe TCMR in the derivation cohort

The histological evaluation of 142 included patients showed: indeterminate/none TCMR in 71 patients (50%), mild TCMR in 23 patients (16.2%), moderate TCMR in 29 patients (20.4%) and severe histological TCMR in 19 patients (13.4%). Other significant histological findings among patients without TCMR were: hepatitis C recurrence (n=20; 28.1%), unspecific/minimal inflammatory changes (n=20; 28.1%), cholestasis (n=6; 8.4%), ischemia-reperfusion injury (n=4; 5.6%), steatosis/steatohepatitis (n=3; 4.2%), and venooclusive disease (n=3; 4.2%). Univariate analysis comparing patients with none/mild TCMR vs moderate-severe TCMR is shown in table 2. Patients with moderate-severe TCMR had a shorter interval between LT and liver biopsy (13 days [IQR 9-218] vs 50.5 days [IQR 17-160]; p=0.004), and less

frequent chronic hepatitis C as underlying liver disease (p=0.001). Mean tacrolimus trough concentrations within the 4 days prior to liver biopsy were marginally reduced in patients with moderate-severe TCMR as compared with none/mild TCMR (6.8 ng/mL vs 8.2 ng/mL; p=0.042). Patients with low tacrolimus exposure within the 4 days prior to liver biopsy, defined as trough concentrations <6 ng/mL within the first month or <4 ng/mL thereafter, had increased rates of moderate-severe TCMR (51.4% vs 28%; p=0.011). Concomitant immunosuppressive drugs had no influence on the presence and grading of TCMR (p=0.46). Patients with moderate-severe TCMR were biochemically characterized by increased eosinophils count (p=0.001) and increased serum bilirubin (p<0.001) on the day of the liver biopsy, together with raising bilirubin (<0.001) and cholestatic parameters (p=0.001 for ALP and p=0.047 for GGT) within the 4 days prior to liver biopsy. Noteworthy, neither absolute transaminases on the day of the liver biopsy, nor delta relative change of transaminases within the 4 days prior to liver biopsy, were associated with increased risk of TCMR. The initial multivariate model included the following variables: pre-LT MELD, aetiology of liver disease, tacrolimus trough concentrations, absolute eosinophils count, ALT, bilirubin, and relative delta change of ALT, ALP, GGT, bilirubin and INR within the 4 days prior to liver biopsy. Hepatitis C status and interval from LT to liver biopsy were controlled as potential confounding factors. After excluding not significant covariates and exploring potential interactions, the final multivariate model is shown in table 3. Independent risk factors of moderate-severe TCMR were increased serum bilirubin on the day of the liver biopsy (OR=5.83; p<0.001), raising bilirubin within the 4 days prior to liver biopsy (OR=4.57; p=0.003), and increased absolute eosinophils count (OR=3.81; p=0.004). A nomogram based in such model was constructed to allow a simple calculation of the individual risk of moderate-severe TCMR (figure 1). The optimal threshold for each risk factor was as

follows: serum bilirubin on the day of the liver biopsy > 4 mg/dL; relative delta bilirubin change within the 4 days prior to liver biopsy >100% (ie. any increase); Absolute eosinophils count on the day of the liver biopsy >0.1 $x10^9$. The combination of these risk factors had an area under ROC curve to predict moderate-severe histological TCMR of 0.81, which was superior to the area under ROC curve obtained from each component alone (ie. 0.74 for serum bilirubin, 0.70 for relative delta change of bilirubin and 0.66 for absolute eosinophils count). From the whole cohort (n=142), 30 patients did not have any risk factor (21.1%), 42 patients had one risk factor (29.6%), 47 patients had 2 risk factors (33.1%), and 23 patients fulfilled the 3 risk factors (16.2%). The number of risk factors paralleled the risk of moderate-severe TCMR: 0 risk factors=0%; 1 risk factor=21.4%; 2 risk factors=42.6%; 3 risk factors=78.3%; p<0.001 (see figure 2).

Validation of risk factors of moderate-severe TCMR in a protocol biopsy population

Among 328 patients with protocol liver biopsies at day 7-10 after LT, 55 patients (16.8%) had none/indeterminate TCMR, 130 patients (39.6%) had mild TCMR, 127 patients (38.7%) had moderate TCMR, and 16 patients (4.9%) showed features of severe TCMR. The univariate analysis revealed that patients with moderate-severe histological TCMR had increased bilirubin (6.3 mg/dL [IQR 3.7-9.9] vs 4.5 mg/dL [IQR 2.3-8.7]; p=0.003) and increased absolute eosinophils count (0.38 $x10^9$ [IQR 0.23-0.57] vs 0.51 $x10^9$ [IQR 0.30-0.77]; p=0.002) on the day of the protocol liver biopsy as compared with patients with no/mild TCMR. In addition, the delta relative increase of bilirubin within the 4 days prior to liver biopsy was greater among patients with moderate-severe TCMR (152% [IQR 95-253] vs 130% [IQR 78-221]; p=0.044). Some of the optimal thresholds for risk factors had to be adapted to the early phase after LT, which is characterized by a particular biochemical profile consisting in a progressive

improvement of the liver graft function and variable cholestasis related to ischemia-reperfusion injury. The threshold remained unchanged for serum bilirubin on the day of the liver biopsy (ie. >4 mg/dL), but had to be increased for relative delta change of serum bilirubin within the 4 days prior to liver biopsy, which was placed at >130%, and for absolute eosinophils count on the day of the liver biopsy, which was >0.40 $x10^9$. According these thresholds, 40 patients (12.2%) had 0 risk factors, 97 patients (29.6%) had 1 risk factor, 114 patients (34.8%) had 2 risk factors and 77 patients (23.5%) had 3 risk factors. The risk of moderate-severe histological TCMR increased with the number of risk factors: 27.5% with 0 risk factors, 35.1% with 1 risk factor, 41.2% with 2 risk factors, and 66.2% with 3 risk factors ($p<0.001$). The number of risk factors was an independent predictor of moderate-severe TCMR (OR 1.74 [95%CI 1.3-2.2]; $p<0.001$) after controlling for hepatitis C status (OR 0.53 [95%CI 0.30-0.92]; $p<0.025$).

DISCUSSION

A uniform definition for clinical suspicion of TCMR is paramount to guide clinical decisions and to tailor immunosuppression. In renal transplantation, serum creatinine and glomerular filtration rate mirror graft function, and are established markers of TCMR[19]. A similar utility was expected from transaminases in LT, but these do not provide information about liver function, and they are poor markers of TCMR[2]. The ambiguous meaning of rising transaminases after LT is responsible for the lack of agreement to diagnose TCMR among clinicians[6], and may introduce bias in randomized trials[7], thus hampering the path towards minimal immunosuppression and tolerance. In the present study, a simple and objective model based on routine

biochemical parameters was able to identify patients at increased risk of moderate-severe histological TCMR to undergo a liver biopsy.

Many studies have evaluated the role of non invasive biomarkers of TCMR among serum parameters of inflammation[11,17,20,21] or mediators of T-cell activation[10,22]. Unfortunately, none of these biomarkers has been implemented hitherto because of their complexity, lack of reproducibility/validation, inaccuracy or costs[15,23]. Regarding conventional liver tests, a standardized methodology to select candidates for liver biopsy after LT has been seldom attempted, and never fully accomplished. A recent systematic review of randomized controlled trials in LT published from 2007 to 2015, revealed that only 2 studies out of 30 (6.7%) used predefined criteria to select candidates for liver biopsy[7]. In one study, the indication of liver biopsy relied on subjective symptoms including fever, malaise, back or abdominal pain, tenderness or enlargement of the liver, or change in bile color, with or without rapid increase in transaminases (no thresholds defined)[24]. The second randomized trial considered patients at clinical suspicion of TCMR if they had rising transaminases among 3 consecutive test results (elevated >1.5 times above the baseline) or serum bilirubin elevated by >0.3 mg/dL from baseline[25]. Although less ambiguous, these latter criteria were based on opinion of experts and cut-offs were established arbitrarily. In large observational series, autoimmune liver disease, younger recipient age, eosinophilia and vitamin D deficiency, among others, were identified as risk factors for TCMR after LT, but it is unclear how to combine this information to obtain an individualized risk assessment in each patient [2,5,8,9]. A multivariate logistic regression model based on 100 LT patients with early protocol liver biopsies combined age, pre-LT MELD score, blood eosinophils count and tacrolimus trough concentrations prior to liver biopsy[6]. However, the model lacked of external validation, and was

hampered by its complex calculation. The methodology proposed in the present study to select candidates for liver biopsy is simple, rationale and objective. Although restricted to patients under tacrolimus-based immunosuppression, which currently form the vast majority among the LT population, the model is independent from trough concentrations and co-medications. Its components or risk factors are routine laboratory tests, dynamic and widely available. Their ability to mirror progressive graft dysfunction or spontaneous improvement is well established in clinical practice, but optimal thresholds were unknown, leading to a significant heterogeneity in selecting patients to liver biopsy. The present study provides cut-offs for bilirubin and blood eosinophils at different time-points after LT, thus allowing for a more objective and homogenous assessment. The potential utility of the model to monitor response to boluses of steroids was not analyzed in the present study and requires further investigations.

The model based in risk factors was tested in two different clinical scenarios. The derivation cohort consisted in "a priori" more heterogeneous and multicenter population of patients with clinically-driven liver biopsies obtained at any time after LT. In this setting, lingered to current routine clinical practice, the presence of ≥2 risk factors had 4-fold increased rates of moderate-severe TCMR. There were only 9 false negative patients, and all of them had one risk factor. Noteworthy, in absence of risk factors, no patient had moderate-severe TCMR. The model was then investigated within a larger population with protocol liver biopsies at day 7-10 after LT, which may be considered as more homogeneous and closer to the gold standard. External validation in larger cohorts is often desirable, as it allows to recalibrate the model to different circumstances, and to adapt thresholds[26]. The model based on risk factors was an independent predictor of moderate-severe TCMR in the protocol biopsy evaluation, but

negative predictive values were lower, and up to 1/3 of patients with 0 or 1 risk factor had moderate-severe TCMR. These unnoticed episodes of histological TCMR early after LT without graft dysfunction are thought to have limited prognostic relevance[27], and should not motivate the use of more intense immunosuppression[2,28]. Therefore, although the model would not select these patients for liver biopsy, as they are not within the current clinical scenario, this may not form a significant limitation.

The present model is not a diagnostic test *per se* and it is not intended to waive liver biopsies. Empirical therapy of TCMR without histological confirmation should be strongly discouraged, even among patients with three risk factors. To the contrary, the applicability of the model relies on its capacity to homogenously select patients at "a priori" high risk of TCMR to undergo liver biopsy. This strategy would solve the current lack of agreement to define clinical suspicion of TCMR[6] and it may contribute to reduce variability both in randomized trials and in clinical practice. The derived nomogram would ease the clinical decision-making process at the bedside, and would allow for an informed decision to individually advise liver biopsy.

The independent analysis of this model with much alike results in two contrasting clinical scenarios, as they are clinically-driven liver biopsies and early protocol biopsies, reinforced its external validity. Although serum bilirubin had identical thresholds for both, derivation and validation cohorts (ie.>4mg/dL), cutoffs of relative delta bilirubin and eosinophils count had to be adapted to the early phase after LT in the validation cohort. While any worsening of serum bilirubin was considered a risk factor in long-term stable patients, a more pronounced increase by >30% of serum bilirubin was required early after LT. In other words, a mild increase of serum bilirubin (<30%) does not translate into a significant risk of TCMR early after LT, but should be considered a risk factor if it occurs thereafter. On the other hand, blood eosinophils

count may be physiologically increased in the early post-operative phase. Although the underlying reasons are eluding and more likely multifactorial, this finding is not surprising given the well recognized role of eosinophils in tissue remodeling, inflammation and foreign-body reaction[29]. In the present study, blood eosinophilia was defined >0.4x10^9 early after LT, and >0.1 x10^9 thereafter.

The potential influence of confounding factors should be taken into account. The risk of TCMR is highest within the first weeks after LT, and declines abruptly thereafter being extremely rare after the first year. On the other hand, recurrent hepatitis C has a later onset, but shares some histological features with TCMR, thus forming a challenging differential diagnosis even for experienced pathologists. In the present study, both factors -interval from LT to liver biopsy and hepatitis C status- were controlled in the multivariate analysis to avoid any confounding effect. Other potential confounders such as variable quality of liver biopsy specimens, lack of central pathology reading, or agreement among pathologists from different institutions could not be controlled and may have influenced negatively the accuracy of the model. Despite this, such a large and multicenter population with detailed biochemical and histological evaluation has no precedent in the literature and derived results should be used to improve the quality of randomized trials and to benefit clinical care.

In summary, rising bilirubin over 4 mg/dL and increased blood eosinophils count defined as >0.4x10^9 within the first 10 days after LT, and >0.1x10^9 thereafter, are established risk factors of moderate-severe TCMR, whereas transaminases are not reliable. While awaiting novel biomarkers[23], the presence of more than one of these factors should motivate a liver biopsy to confirm the presence of TCMR and to establish its severity before starting targeted therapy. The implementation of this simple method would homogenize clinical practice, while decreasing the risk of bias in randomized

controlled trials evaluating immunosuppression and using TCMR as the primary efficacy endpoint.

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FIGURE LEGENDS

Figure 1. Nomogram to predict the individual risk of moderate-severe histological T-cell mediated rejection (TCMR). For calculations, trace a vertical line from each predictor (1 to 4) to the first line labeled as “Points”. Then, sum points from each predictor and trace a vertical line from the “Total Points” axis to the “Risk of moderate-severe TCMR” axis.

Figure 2. Rates of moderate-severe histological T cell mediated rejection according to the number of risk factors identified (ie. increased serum bilirubin, rising bilirubin and increased absolute eosinophils count).