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REVIEW

Markers of acute rejection and graft acceptance in liver transplantation

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Abstract

The evaluation of the immunosuppression state in liver transplanted patients is crucial for a correct post-transplant management and a major step towards the personalisation of the immunosuppressive therapy. However, current immunological monitoring after liver transplantation relies mainly on clinical judgment and on immunosuppressive drug levels, without a proper assessment of the real suppression of the immunological system. Various markers have been studied in an attempt to identify a specific indicator of graft rejection and graft acceptance after liver transplantation. Considering acute rejection, the most studied markers are pro-inflammatory and immunoregulatory cytokines and other proteins related to inflammation. However there is considerable overlap with other conditions, and only few of them have been validated. Standard liver tests cannot be used as markers of graft rejection due to their low sensitivity and specificity and the weak correlation with the severity of histopathological findings. Several studies have been performed to identify biomarkers of tolerance in liver transplanted patients. Most of them are based on the analysis of peripheral blood samples and on the use of transcriptional profiling techniques. Amongst these, NK cell-related molecules seem to be the most valid marker of graft acceptance, whereas the role $CD4^+CD25^+Foxp3^+$ T cells has still to be properly defined.

Key words: Liver transplantation; Acute cellular rejection; Tolerance; Biomarkers

Core tip: This review explores the available data in the literature concerning potential markers of acute cellular rejection and graft acceptance after liver transplantation, as well as their impact on decision-making for clinicians.

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INTRODUCTION

During the past 25 years liver transplantation has become the standard therapy for acute and chronic liver failure. Nowadays, with a 5-year patient survival rate of 73%[1], long-term outcome of patients is becoming the main concern for clinicians, who have to deal with the side effects of immunosuppressant drugs in the long-term.

Current immunological monitoring after liver transplantation relies mainly on clinical judgment and on measurement of immunosuppressive drug levels, without a proper assessment of the real suppression of the immunological system. Therefore, the evaluation of the immunosuppression state in liver transplanted patients is crucial for a correct post-transplant management and constitutes a major step towards the personalisation of immunosuppressive therapy.

The ideal diagnostic biomarker should be highly sensitive and specific, non-invasive, and rapidly available[2]. Despite the elevated interest in the evaluation of potential biomarkers of acute cellular rejection (ACR) and graft acceptance, and in the development of specific immune monitoring assays, only few of them are used routinely in clinical practice.

The aim of this review was to explore the available data in the literature concerning potential markers of ACR or graft acceptance after liver transplantation, as well as their impact on decision-making for clinicians.

MARKERS OF ACUTE CELLULAR REJECTION

Various markers have been studied in an attempt to identify a specific indicator of graft rejection after liver transplantation. However, the use of these markers has been hampered by the fact that there is considerable overlap with other conditions, and currently only a few of them have been validated[3].

Liver enzymes

The suspicion of ACR is usually driven by the rise of liver enzymes after transplantation. However, several reports have clearly shown that elevated standard liver tests have a low sensitivity and specificity for ACR and show a weak correlation with the severity of histopathological findings[4,5]. Moreover, liver enzymes do not allow for ACR to be differentiated from others complications. A study based on 70 post-transplant liver biopsies demonstrated that there is no single chemical parameter nor a combination of parameters that can statistically or clinically distinguish patients with ACR from those with other causes of graft dysfunction[6]. More recently, Rodriguez-Peralvarez *et al*[7] showed that patients with moderate/severe ACR are characterized by higher bilirubin levels and cholestasis parameters, with lower aspartate aminotransferase (AST), AST/alanine aminotransferase (ALT) ratio than those with mild or no ACR. However, the combination of these serum parameters in the logistic regression analysis had only a sensitivity of 73% and a specificity of 52.9%. ALT value was not related to the presence or grading of ACR, and although ALP values were related to ACR, this enzyme cannot be used as a marker of ACR nor its severity, due to the myriad of disorders in which it is elevated.

Cytokines

After liver transplantation, the characteristics of the inflammatory environment in which T cell recognition of the alloantigen takes place determines the lineage commitment of these cells. Thus, depending on the cytokines that are present when antigen activation occurs, naïve $CD4^+$ helper T cells may acquire cytopathic and/or immunoregulatory phenotypes[8,9].

Based on this immunological background, the first potential biomarkers studied to predict ACR were cytokines. Products of activated T lymphocytes, such as IL-2 or soluble components of its receptor (sIL-2R), have been particularly well studied.

Boleslawski *et al*[10] evaluated the intracellular IL-2 quantification in $CD3^+CD8^+$ cells in 21 liver transplant recipients for 6 mo after liver transplantation, showing that intracellular IL-2 expression in $CD8^+$ T cells before transplantation was closely related to the development of ACR. These results were later confirmed by Akoglu *et al*[11], who demonstrated that patients experiencing ACR showed a significantly higher intracellular percentage of $IL\text{-}2^+$ in $CD8^+$ T cells compared to stable liver transplant recipients. They also showed a good correlation between the percentage of $CD8^+IL\text{-}2^+$ cells and Banff score (Spearman's rho = 0.81; $P = 0.027$) (Table 1).

When the expression of IL-2 and IL-2 receptor was evaluated in liver grafts of patients with and without ACR, IL-2 and IL-2 mRNA were absent, with minimal expression of IL-2 receptor in patients experiencing ACR, whereas IL-4 and IL-4 mRNA were highly expressed during ACR, being absent in stable liver transplant recipients[12] (Table 1).

In a recent study, Millán *et al*[13] evaluated the intracellular expression and soluble production of IFN-γ and IL-2 in 47 liver transplanted patients. A pre-transplant cut-off value of 55.8% for the percentage of $CD8^+IFN\text{-}\gamma^+$ identified patients at high risk of ACR (sensitivity = 75% and specificity = 82%). In the first week after transplantation, patients with a percentage of inhibition for soluble IFN-γ, a percentage of $CD8^+IFN\text{-}\gamma^+$ and a percentage of $CD8^+IL2^+$ lower than 40%, developed ACR.

Regarding TNF-α, it has been shown that pre-

Table 1 Marker of acute cellular rejection after liver transplantation

Biomarker	Sample size	Ref.
Citokines		
IL-2	66	Akoglu *et al*[11]
	21	Boleslawski *et al*[10]
IL-4	20	Conti *et al*[12]
IL-6	169	Kita *et al*[17]
IL-15	35	Conti *et al*[18]
IL-18	Rat model	Fábrega *et al*[19]
IL-23	50	Fábrega *et al*[20]
IFN-γ	47	Millán *et al*[13]
TNF-α	50	Imagawa *et al*[15]
Other markers related to inflammation		
CD28	55	Minguela *et al*[22]
	237	Minguela *et al*[23]
	52	Boleslawski *et al*[24]
CD38	52	Boleslawski *et al*[24]
CD25	55	He *et al*[25]
ICAM-1	NA	Adams *et al*[26]
	12	Romero *et al*[27]
Bile markers		
Bikle acid concentrations	41	Janssen *et al*[30]
IL-6	51	Umeshita *et al*[31]
IL-8	45	Warlé *et al*[32]
Alanine Aminopeptidase N	9	Kim *et al*[33]
Ascites markers		
IL-2 receptor	30	Ganschow *et al*[34]
IL-6	30	Ganschow *et al*[34]
IL-1 receptor antagonist	30	Ganschow *et al*[34]

IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor; ICAM-1: Intercellular adhesion molecule 1.

transplant "*in vitro*" production of this molecule was significantly increased in patients with post-transplant ACR (n = 9) compared with those who did not develop ACR (n = 12)[14]. When plasma levels of TNF-α were measured in 50 adult patients following liver transplantation, its concentration was significantly higher in patients experiencing ACR than in those with a stable clinical course (941 ± 83 pg/mL *vs* 240 ± 6 pg/mL, P = 0.0001)[15] (Table 1).

An important role of IL-18 in liver allograft rejection has been postulated in a recent study using a rat model of liver transplantation, which showed that specific suppression of IL-18 was associated with significantly decreased serum alanine amino-transferase levels and less histologic hepatic injury early after transplantation[16] (Table 1).

In another study, serum levels of IL-6 were evaluated in 20 liver transplanted patients with no infections, and it was demonstrated that levels of this cytokine were significantly higher 0-4 d before histological diagnosis of ACR compared to those of patients without ACR (131 ± 78 pg/mL *vs* 40 ± 21 pg/mL, P < 0.01). IL-6 elevation due to ACR appeared to be distinguishable from increases caused by infection, being serum IL-6 levels unrelentingly elevated during bacterial infection (> 1000 pg/mL). However, there was no correlation between IL-6 elevation of and histological grade of ACR[17] (Table 1).

Plasma levels and "*in situ*" expression of IL-15 are enhanced during ACR compared with patients without ACR (5.2 ± 1.3 pg/mL *vs* 0.6 ± 0.4 pg/mL, P = 0.02), with this expression being particularly evident when patients with steroid-resistant ACR were considered (6.9 ± 1.1 pg/mL)[18] (Table 1).

The role of IL-9, IL-23 and IL-17 in liver transplantation remains to be clarified. As far as IL-9 is concerned, when serum levels were determined in 50 liver transplanted patients (15 patients with ACR episodes, and 35 patients without ACR) on day 1 and 7 after liver transplantation and on the day of liver biopsy, no difference was found between patients with and without ACR[19]. Similarly, the serum concentrations of IL-23 and IL-17 were not different early in the post-transplantation period. However, a significant increase in serum IL-23 levels in the ACR group was seen at the time of liver biopsy[19,20]. These data were confirmed by a latter prospective study[21] showing that the levels of circulating $CD4^+IL\text{-}17^+$ T cells were higher in patients with ACR than those with no ACR (2.56% ± 0.43% *vs* 1.79% ± 0.44%, P < 0.001). Moreover, the frequency of $CD4^+IL\text{-}17^+$ cells in peripheral blood was correlated with the histological severity of ACR (r = 0.79, P = 0.0002) (Table 1).

In conclusion, pro-inflammatory and immuno-regulatory cytokines have been the most studied markers to predict ACR. Despite most of them showed an increased expression during ACR, many of these cytokines cannot differentiate between ACR and infections, making their utility limited in clinical practice.

Other markers related to inflammation

One of the first studies evaluating the expression of CD28 after liver transplantation demonstrated that patients experiencing ACR showed a clear increase with respect to patients without ACR, and to healthy controls. Significant differences in the total-$CD28^+$ lymphocytes between the ACR and non-ACR groups were reached on days 7 to 9 (P < 0.01) and 10 to 13 (P < 0.05) after transplantation[22]. The same group, in a subsequent study, showed that ACR and virus re-infection could be distinguished from each other because CD28 was up-regulated on $CD4^+$ lymphocytes only in recipients with ACR, irrespective of their HBV/HCV infection status[23] (Table 1).

The expression of CD28 and CD38 was also analysed on $CD3^+$, $CD4^+$ and $CD8^+$ cells in 52 liver transplanted patients in another study. The mean frequencies of CD28 and CD38-expressing T cells were significantly higher in patients with ACR (P = 0.01 and P = 0.001, respectively). Moreover, at multivariate analysis, only CD28 and CD38

frequencies at day 14 were independently associated with ACR (HR = 1.27, P = 0.04 and HR = 1.11, P = 0.01 respectively)[24] (Table 1).

CD25 expression may also constitute a biological marker of immune activation in transplant recipients. Circulating $CD4^+CD25^{high}FoxP3^+$ T cells were significantly lower in patients with ACR compared with patients not experiencing ACR (2.23% ± 0.54% *vs* 2.99% ± 0.86%, P = 0.01) in a prospective analysis of 55 patients who underwent liver transplantation. Longitudinal analysis revealed circulating $CD4^+CD25^{high}FoxP3^+$ T cells of patients in the rejection group to be significantly lower during ACR than during quiescence (2.23% ± 0.54% *vs* 3.68% ± 0.70%, P = 0.0001). Furthermore, the frequency of circulating $CD4^+CD25^{high}FoxP3^+$ T cells negatively correlated with Rejection Activity Index (r = 0.80, P = 0.01)[25] (Table 1).

During graft rejection, adhesion molecules play a crucial role in infiltration, activation, and binding of effector cells to target tissues. The expression of intercellular adhesion molecule 1 (ICAM-1), for instance, has been studied on liver tissue after transplantation. It has been shown that ICAM-1 expression on bile ducts, endothelium, and perivenular hepatocytes (structures affected by the rejection process) is greater in patients with ACR than in patients with no ACR. Moreover, it was demonstrated that in patients with a resolving episode of rejection ICAM-1 expression was greatly reduced after high-dose corticosteroid treatment[26]. The effect of steroid therapy on ICAM-1 expression in liver biopsies of patients with post-transplant ACR was confirmed in the study by Romero *et al*[27]. After steroid treatment, the intensity of ICAM-1 expression decreased significantly in sinusoids (1.5 ± 0.67 *vs* 2.41 ± 0.66, $P < 0.05$) and in perivenular hepatocytes (0.25 ± 0.86 *vs* 0.83 ± 0.57, $P < 0.05$) compared to the pre-treatment liver biopsy samples (Table 1).

Lastly, graft eosinophilia has been identified as an independently associated feature of ACR in liver transplantation[28]. In one study, the absence of peripheral eosinophilia predicted the absence of moderate/severe ACR, however it could not be used to predict or to assess the response to corticosteroids for the treatment of acute rejection[29]. In a more recent study, based on 690 consecutive first liver transplant patients and using protocol liver biopsies, peripheral eosinophil count was strongly associated with moderate-severe ACR (OR = 2.15; P = 0.007), although the area under ROC curve (AUROC) was 0.58. These investigators also found that the delta in eosinophil count between the biopsies performed before and after ACR treatment was the only independent predictor of histological improvement (OR = 3.12; P = 0.001)[7] (Table 1).

In conclusion, the expression of CD28 and CD38 on T cells at specific interval time form liver transplantation, seems to be a reliable marker of ACR, being able to differentiate between ACR and infection. Promising results have been found when eosinophil count was evaluated, especially because it is strongly associated with moderate-severe ACR, which often require steroid treatment.

Bile and ascites markers

Contrasting data are available on the role of bile and ascites markers as potential tools for predicting which patients will develop ACR after liver transplantation.

In a study on 41 patients who underwent liver transplantation, the investigators performed serum bile acid concentration measurements and correlated these with findings at liver biopsy. In patients with ACR, bile acid concentrations were statistically significantly increased 3 d prior to liver biopsy (from a mean of 37 ± 31 μmol/L to 118 ± 46 μmol/L; P = 0.001). Moreover, successful antirejection treatment correlated with a significant decrease of serum bile acid as early as 1 d after initiation of therapy (P = 0.008)[30] (Table 1).

Patients with ACR showed a significant increase of bile IL-6 compared with patients who had uneventful postoperative courses (1090 ± 990 pg/mL *vs* 18 ± 3 pg/mL, $P < 0.05$) in a study performed on 51 liver transplant recipients[31]. In a prospective study on 45 patients who underwent liver transplantation, biliary IL-8 levels were also demonstrated to be significantly increased at the onset of ACR (11.62 ± 4.25 pg/mL, $P < 0.001$) compared with patients with an uneventful course and those with infectious complications[32] (Table 1).

Lastly, in a more recent study, alanine aminopeptidase N (APN/CD13) enzyme activity in bile samples collected within 3 d before post-transplant liver biopsy was significantly higher in patients with ACR (584 ± 434 U/g protein) than in those free of ACR (301 ± 271 U/g protein) (P = 0.004)[33] (Table 1).

In another study, the value of cytokine quantification in drained ascites was evaluated in 30 children in the first 2 wk after liver transplantation. There were no significant elevations of IL-2 receptor and IL-6 in serum and ascites between patients with and without ACR. However, the concentration in ascites of the IL-1 receptor antagonist increased 48 h before ACR (P = 0.01 *vs* no ACR). The IL-1 receptor antagonist concentration in ascites was up to 11-fold higher than in serum during ACR (15.43 *vs* 1.38 ng/mL)[34] (Table 1).

In conclusion, despite encouraging results, bile and ascites markers have a controversial use in diagnosing ACR. The main limit of these diagnostic approaches is that they often requires invasive procedures such as the position of a T tube (which is no longer used in most of the liver transplant centres) or performing a paracentisis. Therefore

this aspect is of great relevance in clinical practice because, liver biopsy, which is the gold standard for ACR diagnosis, is an invasive procedures and the clinical attention is posed mainly to non-invasive markers.

Future markers

To date, the Cylex ImmuKnow assay, which quantifies the amount of adenosine triphosphate produced by $CD4^+$ T cells after *in vitro* stimulation by a non-donor-specific mitogen (phytohemagglutinin-L), is the only commercially available test to evaluate the immune status in transplanted patients.

A recent systematic literature review evaluated the use of ImmuKnow in liver transplant recipients. The study identified five studies analysing ImmuKnow performance for infection and 5 studies analysing ImmuKnow performance for ACR. Considering the ability to predict ACR, the pooled sensitivity, specificity, positive likelihood ratio, diagnostic odds ratio, and AUROC curve for this analysis were 65.6% (95%CI: 55.0%-75.1%), 80.4% (95%CI: 76.4%-83.9%), 3.4 (95%CI: 2.4-4.7), 8.8 (95%CI: 3.1-24.8), and 0.835 ± 0.060 respectively, while the respective values in the setting of infection were 83.8% (95%CI: 78.5%-88.3%), 75.3% (95%CI: 70.9%-79.4%), 3.3 (95%CI: 2.8-4.0), 14.6 (95%CI: 9.6-22.3), and 0.824 ± 0.034, respectively. Notably, heterogeneity was low for infection studies and high for ACR studies[35]. Based on these data, it appears that this assay could be more useful in order to assess over-immunosuppression rather than under-immunosuppression[36].

Due to the high number of proteins involved in the ACR process, proteomic analysis could have a crucial role in identifying a potential biomarker of ACR. However, despite several studies have been performed, the results are not conclusive and these techniques have to progress from the research bench to the clinical routine[37].

In conclusion, future markers of ACR such as ImmuKnow and proteomic analysis, have been evaluated, but well designed, prospective studied are needed in order to better understand their clinical applicability.

MARKERS OF GRAFT ACCEPTANCE

To date, liver biopsy is the gold standard to assess the graft status after liver transplantation, but it is an invasive procedure and is not suitable for monitoring the graft on a daily basis. Moreover, it does not provide any useful information for predicting future development of tolerance[38]. Therefore, biomarkers of graft acceptance could be crucial in order to select patients eligible for enrolment in immunosuppressive drug weaning or withdrawal protocols. Thus, several studies have been performed to identify biomarkers of tolerance in liver transplanted patients; most of them are based on the analysis of peripheral blood samples and on the use of transcriptional profiling techniques[39].

Non-specific genomic analysis

In the first study using microarray gene expression profiling, Martínez-Llordella *et al*[40] found that genes encoding for γδT-cell, for NK receptors, and for proteins involved in cell proliferation arrest were up-regulated in tolerant liver transplanted patients (n = 16) compared to immunosuppression-dependent patients (n = 16) or healthy individuals (n = 10). A second study by the same group, using a larger cohort of patients, confirmed these results. Again, NK cell and $\gamma\delta TCR^+$ T cell transcripts were predominantly expressed in tolerant liver transplanted patients[41].

In a more recent study, transcriptional profiles from 300 samples were examined by microarrays and RT-PCR measurements of blood specimens from paediatric and adult liver transplant recipients and of normal tissues. Tolerance-specific genes were validated in independent samples across two different transplant programs and validated by RT-PCR. A minimal set of 13 unique genes, highly expressed in NK cells (P = 0.03), were significantly expressed in both paediatric and adult liver transplanted tolerant patients, and the performance of this gene set analysis, tested in independent samples, yielded a 100% sensitivity and 83% specificity[42].

Lastly, Bohne *et al*[43] recently reported the results of a multicentre prospective study evaluating 75 liver transplant recipients from whom cryopreserved liver tissue samples had been obtained before the initiation of drug minimization and were available for transcriptional analyses. Amongst these, 33 recipients successfully discontinued all immunosuppressive drugs, while 42 rejected their allografts. Before initiation of drug withdrawal, operationally tolerant and non-tolerant recipients differed in the intragraft expression of genes involved in the regulation of iron homeostasis. Moreover, operationally tolerant patients exhibited higher serum levels of hepcidin and ferritin and increased hepatocyte iron deposition compared to non-tolerant ones.

Peripheral blood immunophenotyping

An increase Vδ1/Vδ2 γδT-cells ratio has been found in operationally tolerant liver transplanted patients (n = 12, ratio = 1.5) when compared with liver transplanted patients on immunosuppression (n = 19, ratio = 0.8; $P < 0.01$) and with age-matched healthy controls (n = 24, ratio = 0.3; $P < 0.05$)[44]. The increase in the number of circulating $V\delta1^+$ T cells in tolerant patients has also been confirmed

in a later study by Martínez-Llordella *et al*[40] who demonstrated that Vδ1$^+$ subtype is the predominant γδT-cell subpopulation in tolerant recipients.

Based on this, altered distribution of the Vδ1 and Vδ2 γδT cells in operationally tolerant liver transplant recipients, γδT cells subset quantification was proposed as a biomarker of immunologic risk in liver transplantation. However, a recent study showed that alterations in the γδT cell compartment are not restricted to tolerant liver recipients, and that most immunosuppressed liver recipients display an enlarged peripheral blood γδT cell pool mainly resulting from an expansion of Vδ1 T cells exhibiting an oligoclonal repertoire and different phenotypic and cytokine production traits than Vδ2 T cells. The authors proposed that persistent viral infection might be the cause of these alterations[45].

Several studies have shown that the numbers of circulating CD4$^+$CD25$^+$ T-cells is increased in operationally tolerant patients after liver transplantation[40,44,46,47]. When peripheral blood mononuclear cell populations were analysed in 12 liver transplant recipients with stable graft function for more than 2 years, the percentage of CD4$^+$CD25^{high+} cells was significantly higher in tolerant patients (n = 12, 2.3% ± 0.6%), compared with patients who were still on immunosuppression (n = 19, 0.9% ± 0.7%; P < 0.01), and with age-matched volunteers (n = 24, 1.8% ± 0.6%; P < 0.05)[44]. This data were confirmed by Pons *et al*[46] who found an increased frequency of CD4$^+$CD25^{high+} cells when immunosuppressive therapy was withdrawn in tolerant patients (n = 5). The most interesting data of this study was that relative mRNA FoxP3 expression increased 3.5-fold before the complete withdrawal of immunosuppression in tolerant patients, and this increase continued when the immunosuppressive therapy was stopped. Conversely, patients who suffered ACR (n = 7) did not exhibit an increase in CD4$^+$CD25^{high+} cells or FoxP3 expression.

When the expression of Foxp3 mRNA and the presence of CD4, CD8, and Foxp3 cells were quantified in liver biopsies from tolerant living-donor liver transplanted patients, it was found that Foxp3 mRNA expression was higher in tolerant patients (n = 28), compared with patients on immunosuppression (n = 29; P = 0.07), but was equivalent to patients who experienced chronic rejection (n = 7; P < 0.01). The number of Foxp3 cells was significantly increased in tolerant patients, compared with patients on immunosuppression (P < 0.05), although the number of CD4 or CD8 cells did not differ between the two groups[48].

CONCLUSION

The evaluation of the real suppression of the immune system after liver transplantation would allow transplant clinicians to modulate the immunosuppressive therapy according to patient needs, identifying, not only patients at risk of acute rejection, and infection, but also understanding if the immunological background would allow a progressive reduction of the immunosuppressive therapy, favouring graft acceptance.

Despite these considerations, the current immunological monitoring after liver transplantation relies mainly on clinical judgment and on immunosuppressive drug levels, without a proper assessment of the real suppression of the immunological system.

Therefore, it becomes crucial to identify potential biomarkers of immune activity, which can be used to tailor immunosuppression after liver transplantation.

In this manuscript, we reviewed available data on studies assessing the role of different biomarkers of ACR and graft acceptance after liver transplantation.

Considering biomarkers for ACR, pro-inflammatory and immunoregulatory cytokines are the most studied ones, showing an increased expression during ACR. However many of these cytokines cannot differentiate between ACR and infections, making their utility limited in clinical practice. The expression of other proteins related to inflammation, not only in the blood, but also in the bile and in the ascites has been evaluated, but the results are controversial. Moreover, the use of ascites markers is an invasive method and it needs the presence of ascites after liver transplantation, therefore it is not applicable on a daily basis.

When we evaluated available markers of graft acceptance after liver transplantation, we found that data are more encouraging compared to biomarkers of ACR. Patients undergoing immunosuppression withdrawal seem to present specific characteristics compared to non-tolerant patients. One of the most reliable blood marker, which could help clinicians to differentiate between tolerant and non-tolerant patients, are NK cells and their related transcripts. It has been clearly demonstrated that they are already present in the blood of tolerant liver transplanted patients before the withdrawal of immunosuppressive therapy. The role CD4+CD25+Foxp3+ T cells, which seem to have a immunoregulatory effect, is less clear due to the use of immunosuppressive drugs, which could alter their expression. Independently from the markers identified, there is a substantial difference between the expression of specific markers in the blood and their expression in the transplanted liver. This difference makes blood-related biomarkers less accurate in order to predict graft acceptance and forces clinician still to use liver biopsy to monitor patients undergoing immunosuppression withdrawal.

Lastly, it is becoming evident that a single biomarker cannot be able to reflect all the alterations of the immune system associated with organ

transplantation. Therefore a panel of different biomarkers will be needed to properly evaluate the immunological suppression and to modify immunosuppressive treatment according to patient needs. Once a panel of markers is identified, it should undergo validation in large multicentre studies in order to prove its real clinical utility.

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