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PREDICTING ACUTE CELLULAR REJECTION AFTER LIVER TRANSPLANTATION: FORM LIVER FUNCTION TEST TO IMMUNE MONITORING

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RIASSUNTO

Lo scopo principale della terapia immunosoppressiva dopo trapianto di fegato è passato dalla prevenzione del rigetto acuto alla preservazione della funzionalità a lungo termine dell'organo trapiantato e alla prevenzione degli effetti collaterali dovuti alla terapia immunosoppressiva. Per perseguire tale scopo è necessaria una gestione ottimale della terapia immunosoppresiva stessa. Tuttavia, la misurazione dei livelli ematici dei farmaci immunosoppressori, generalmente utilizzati come surrogato dei livelli di immunosoppressione, non fornisce informazioni relative alla reale intensità della soppressione del sistema immunitario. Pertanto l'individuazione di marcatori biologici di rigetto acuto e/o di tolleranza risulta fondamentale per poter migliorare la gestione della terapia immunosoppressiva dopo-trapianto di fegato.

Gli scopi degli studi riportati in questa tesi sono: 1) determinare l'incidenza e gli eventuali fattori di rischio di rigetto acuto dopo trapianto di fegato, valutare in che l'influenza del rigetto acuto e della sua severità istologica sulla sopravvivenza dell'organo e del paziente dopo trapianto di fegato; 2) valutare il ruolo degli indici di funzionalità epatica e della conta eosinofilica ematica come potenziali marcatori biologici di rigetto acuto dopo trapianto di fegato, in particolare di grado moderato/severo; 3) valutare, prima e dopo trapianto di fegato l'espressione di specifici marcatori immunologici di rigetto acuto.

I risultati degli studi condotti hanno evidenziato come pazienti con diagnosi di rigetto acuto alla biopsia di protocollo presentino una sopravvivenza di organo e paziente, a 1, 5 e 10 anni dal trapianto di fegato, del tutto sovrapponibile a quella di pazienti senza evidenza istologica di rigetto acuto alla biopsia di protocollo. L'insorgenza di rigetto acuto di grado moderato/severo non sottoposto a trattamento farmacologico è tuttavia associata ad aumentata incidenza di decesso o perdita dell'organo post-trapianto.

Nel valutare potenziali marcatori biologici di rigetto acuto, abbiamo dimostrato che nonostante la conta eosinofilica periferica non sia sufficientemente predittiva per lo sviluppo di rigetto acuto post-trapianto, la differenza nella conta eosinofilica tra la prima e la seconda biopsia epatica può essere considerato un fattore predittivo di miglioramento istologico, indipendentemente dall'utilizzo o meno di terapia con boli steroidei. Non è stata invece evidenziata alcuna associazione tra l'alterazione degli indici di funzionalità epatica e l'insorgenza di rigetto acuto.

Infine, è stato dimostrato che l'insorgenza di rigetto acuto risulta associata ad aumentata espressione di CD28 e CD38 sia sui linfociti T $CD4^+$ che $CD8^+$ e ad un aumento dei livelli di IL-17. Tali alterazioni del sistema immunitario potrebbero essere utilizzate nella pratica clinica per valutare lo stato di soppressione del sistema immunitario in pazienti sottoposti a trapianto di fegato con il fine ultimo di una gestione ottimale e personalizzata della terapia immunooppressiva.

SUMMARY

In recent years, the main end point of immunosuppressive therapy after liver transplantation has moved from the prevention of acute cellular rejection (ACR) toward the preservation of long-term graft function and prevention of immunosuppression-related side effects. This approach requires an optimal management of immunosuppressive therapy according to patient risk factors. However, the concentration of immunosuppressive drugs in the serum of patients, which is generally used as a surrogate for the level of immunosuppression, does not provide information about the magnitude of suppression of the immune system. Therefore a reliable marker for the development of ACR, or to predict patients who could tolerate reduced immunosuppression, would be crucial for improving post-transplant management of liver transplanted patients.

The aims of the studies presented in this thesis were: 1) to assess the incidence of ACR after liver transplantation, to identify potential risk factors for ACR, and to evaluate the impact of ACR and its histological severity on outcomes; 2) to evaluate the role of liver function tests and blood eosinophil count as potential biomarkers for ACR after liver transplantation, with special attention on prediction of histologically proven moderate and severe ACR; 3) to evaluate the expression of specific immunological markers for ACR in patients before and after liver transplantation.

The results of the studies showed that patient and graft survival at 1, 5 and 10 years after liver transplantation were not different with respect to presence or absence of ACR. Only untreated moderate/severe ACR was associated with increased death/graft loss using adjusted Cox regression analysis, whereas mild ACR, whether treated or not, had no effect.

With regards to the evaluation of potential markers of ACR, despite peripheral eosinophilia was not sufficiently predictive of moderate/severe ACR, the delta in eosinophil count between the first and second biopsies was the only independent predictor of histological improvement, irrespective of whether bolus steroids were used.

Lastly, we demonstrated that the increased expression of C28 and C38 on both $CD4^+$ and $CD8^+$ T cells and the increased levels of IL-17. These alterations of immune system could be used routinely in clinical practice to assess the immune status of liver transplanted patients and to properly manage immunosuppressive therapy.

LIST OF ABBREVIATIONS

ACR	Acute cellular rejection
AEC	Absolute eosinophil count
ALD	Alcoholic liver disease
ALF	Acute liver failure
ALP	Alkaline phosphatase
Alpha-GST	Alpha-gluthation S-transferase
ALT	Alanine aminotransferase
APC	Antigen presenting cell
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUROC	Area under receiver operative characteristic
AUTO	Autoimmune liver disease
AZA	Azathioprine
C1q	Complement component 1q
CNI	Calcineurin inhibitors
CsA	Cyclosporine
ELISA	Enzyme-linked immuno sorbent assay
GBP2	Guanylate-binding protein 2
GGT	Gamma glutamyl transpeptidase
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HBV	Hepatitis B virus
HR	Hazard ratio
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
IRF-1	Interferon regulatory factor 1
MELD	Model of End Stage Liver Disease
MHC	Major histocompatibility complex
miR	microRNA
MMF	mycophenolate mofetil
NK	Natural killer cells

NPV	Negative predictive value
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor 1
PBC	Primary biliary cirrhosis
PCR	Polymerase chain reaction
PNF	Primary non function
PPV	Positive predictive value
PSC	Primary sclerosing cholangitis
Pi-GST	Pi-glutathione S-transferase
REC	Relative eosinophil count
SAA	Serum amyloid A protein
sIL-2R	Soluble interleukine-2 receptor
sTNF-RII	Soluble TNF receptor II
TAC	Tacrolimus
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll like receptors
TNFa	Tumor necrosis factor a

1. GENERAL INTRODUCTION

1.1 Acute cellular rejection after liver transplantation

Acute cellular rejection (ACR) was defined in 1995, as “inflammation of the allograft elicited by genetic disparity between the donor and recipient, primarily affecting interlobular bile ducts and vascular endothelia, including portal veins and hepatic venules and occasionally the hepatic artery and its branches” [1].

Viewed from a biological perspective, the recipient’s immune system is activated after transplantation but, because of the baseline immunosuppressive therapy, only some recipients will have clinical manifestations of this. An important distinction has to be made between histological changes of ACR, which may be seen in the absence of any significant clinical or biochemical abnormalities (biological rejection), and those accompanied by clinical signs of graft dysfunction (clinical rejection). Abnormalities of liver function tests are almost universally present, and symptoms absent, so in clinical practice, in the vast majority of the cases, the distinction between clinical and biological rejection can rarely be made.

Most cases occur in the early postoperative period within 30 days, whereas late cases are usually associated with non-adherence to immunosuppressive therapy. However incidence varies according to whether ACR is defined on the basis of clinically significant ACR or simply on the basis of histological abnormalities or a combination of the two. Clinically significant ACR occurs in approximately 50% of patients, whereas histological abnormalities can be seen in up to 80% of protocol biopsies performed at the end of the first week following transplantation [2].

The three main histopathological features are: 1) a predominantly mononuclear, but mixed portal inflammation, containing blast-like or activated lymphocytes,

neutrophils and eosinophils; 2) subendothelial inflammation of portal or terminal hepatic veins (or both); 3) bile duct inflammation and damage (Figure 1.1).

In general, at least two of the above histopathological findings, and biochemical evidence of liver damage, constitute the minimal diagnostic criteria for hepatic ACR. The diagnosis is strengthened if >50% of the ducts are damaged or if unequivocal endothelitis of the portal vein branches or terminal hepatic venules can be identified (Table 1.1).

Early studies of the ACR were focused mainly on inflammatory changes occurring in portal tracts, but also recognized the presence of inflammation involving hepatic venous endothelium and surrounding liver parenchyma [3-5]. During the 8th Banff Conference on Allograft Pathology in 2005, the term central perivenulitis emerged to describe a spectrum of inflammatory regions of the liver that are in most cases thought to be a manifestation of liver ACR. In cases in which perivenular inflammation occurs within the first weeks of liver transplantation and is associated with characteristic portal tract changes, the diagnosis of rejection is straightforward [6]. However in cases where portal inflammation lacks typical features of ACR the term isolated central perivenulitis should be used [7]. Krasinkas et al. [7] demonstrated that isolated central perivenulitis is a common finding in late post-transplant biopsies and that most cases are probably related to rejection and are associated with a worse outcome compared to cases of purely portal-based rejection. In 2004 Lovell et al. [8] found that patients with centrilobular alterations in their first post-transplant biopsy (n=15) developed more frequently ACR (60% vs. 30%; $p<0.04$) and subsequent episodes of chronic rejection (53% vs. 25%; $p<0.04$) when compared to patients, who did not have centrilobular alterations (n=20).

In 1997, an international consensus on a common grading system for ACR was achieved and subsequently it has been prospectively tested and proved to be simple, reliable, and clinically relevant [9-11].

According to this Banff schema, which represents a merger and simplification of many previously published studies, there are two main components: the first is a global assessment of the overall ACR grade (indeterminate, mild, moderate, severe), the second involves scoring the three specific features of ACR semi-quantitatively to produce an overall Rejection Activity Index (RAI) [12].

1.1.1 Immunological basis of acute cellular rejection

1.1.1.1 Mechanisms of acute cellular rejection

After liver transplantation, ACR is initiated by the large number of recipient T cells that recognize donor alloantigens [13, 14]. Donor alloantigens are processed by specialized antigen-presenting cells (APCs), with donor MHC molecules which are internalized by donor and recipient APCs and MHC peptide fragments presented to the recipient's T cells. Antigen presentation involves engagement of these peptide antigenic fragments within a groove on the MHC molecules of the APC surface.

Three non mutually exclusive pathways of allorecognition have been described. In the direct pathway, recipient T cells recognize intact allogeneic MHC molecules on the surface of donor APCs. This pathway is responsible for the large proportion of T cells that have reactivity against alloantigens due to cross-reactivity of the T-cell receptor (TCR) with self and foreign MHC molecules. In the indirect pathway, recipient APCs trafficking through the allograft phagocytose

allogeneic material shed by donor cells and present it to recipient T cells on recipient MHC molecules. Lastly in the semidirect pathway, recipient APCs acquire intact MHC molecules following direct contact with donor APCs and/or through fusion with donor APC-derived exosomes. These chimeric recipient APCs stimulate recipient T cells through direct and indirect pathways [15].

Although the ACR response is mediated primarily by $CD4^+$ T cells C, many activated $CD8^+$ T cells infiltrate the transplant at the time of rejection [16], along with other mononuclear leukocytes. Cells of the innate immune system, such as natural killer (NK) cells, are also present in allografts during rejection. NK cells can recognize alloantigens because they constitutively express inhibitory receptors that are specific for self- MHC class I antigens [17].

1.1.1.2 Role of inflammation in T-Cell commitment

Newly engrafted organs are subject to intense inflammation. The accrued injury to the transplant, caused organ procurement, cold preservation, surgical trauma, and reperfusion injury, leads to the release of proinflammatory cytokines such as IL-6, TNFα, and IL-1β. The characteristics of the inflammatory environment in which donor-reactive $CD4^+$ T cells recognize donor antigens seems to play a crucial role in determining the commitment of these cells. Thus, depending on the cytokines present when antigen activation occurs, naïve $CD4^-$ helper T cells can acquire a variety of cytopathic and/or immunoregulatory phenotypes [18]. $CD4^+$ T cells activated in the presence of IL-12 become interferon γ–producing Th1 cells with tissue-destructive properties, whereas $CD4^+$ T cells activated in the presence of IL-4 differentiate into Th2 cells. In the absence of pro-inflammatory cytokines, TGF-β induces expression of Foxp3 and differentiation of $CD4^+$ T cells into Tregs. In contrast, expression of TGF-β with IL-6 or IL-21 prevents

development of Tregs, leading activated $CD4^+$ T cells to become cytopathic Th17 cells [18-21].

It was believed that antigen-activated helper T cells became terminally differentiated Th1 or Th2 cells with opposite effects: Th1-dependent cytopathic rejection or Th2-dependent cytoprotective effect. However Th1 and Th2 can each mediate graft rejection [22, 23], whereas Treg cells are the key inhibitors of cytopathic, allospecific immune responses [24-26]. Moreover it has been recently shown that Th17 and Tregs have a significant plasticity and are closely interlinked [27]. Thus, Tregs can differentiate into IL-17–producing cells in the presence of IL-2 and IL-1β [28], whereas in the presence of IL-27, Th17-producing cells also produce IL-10, an immunosuppressive cytokine that prevents them from functioning as destructive effector cells [29].

Therefore the current paradigm is that the development of graft rejection or acceptance, is determined by the balance between Th1 and Th17 $CD4^+$ T cells versus Treg cells, with the level of inflammation being crucial in the microenvironment in which T-cell activation takes place.

1.1.1.2 Memory T-Cell and graft acceptance

Upon re-exposure to donor antigen, donor-reactive memory cells respond faster and more powerfully than naive T cells, producing cytolytic effects on the transplanted tissue [30, 31]. Memory T cells can be divided into “central” and “effector” cells [32]. Central memory T cells recirculate through the spleen and lymph nodes, and are responsible for recall antigen responses, whereas effector memory T cells are excluded from lymphoid tissues, migrating to peripheral tissues where they exert rapid and potent effector functions [32]. Due to the

continuous exposure to foreign antigens, memory T cells represent approximately 50% of the total T-cell pool in adults.

Patients who have not received a transplanted organ can still generate donor-reactive T cells, through immunization by direct exposure to alloantigens via pregnancy or blood transfusion [33]. Furthermore, donor-reactive memory T cells can be generated in the absence of alloantigen exposure, through heterologous immunity, wherein an antigen-specific immune response affects the response to an unrelated antigen through cross-reactivity of the T-cell receptor [34]. Some memory T cells are therefore primed by an antigenic pathogen-derived peptide and cross-react with allogeneic (often MHC-derived) peptides presented by self or donor MHC molecules. Following transplantation, alloreactive naïve T cells can acquire a memory phenotype and generate a substantial pool of donor-reactive memory T cells, even when the recipient is under immunosuppressive therapy.

Because of their capacity to rapidly generate effector immune responses, memory T cells appear to be particularly efficient at mediating allograft rejection [35, 36]. Moreover, memory T cells are less sensitive than naïve T cells to many immunosuppressive strategies, such as T cell–depleting antibodies [37] or inhibitors of mammalian target of rapamycin [38]. Given the lower efficacy of conventional immunosuppressive drugs in the neutralization of previously activated or memory lymphocytes, it is not surprising that memory T cells also exert harmful effects in clinical transplantation.

1.1.2 Risk factors of acute cellular rejection

Several studies have aimed to identify patients with a greater risk for developing ACR, but with some exceptions, they have been limited to a small number of patients and focused on a limited number of risk factors, and the results have been frequently contradictory. For this reason there is no consensus about the majority of factors predisposing to the occurrence of acute rejection after liver transplantation.

Data from Birmingham [39, 40] show that there is a lower incidence of ACR when there is no evidence of immune involvement in the pathogenesis of the original liver disease, for example acute liver failure (ALF) from paracetamol. In contrast, in patients transplanted for primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), in which immune-mediated damage of bile ducts is a feature of the original disease, ACR occurs more frequently and there is more frequent progression to ductopenic rejection. In 63 patients reported by Hayashi et al. [41] patients with autoimmune hepatitis had more acute rejection than patients with alcoholic cirrhosis (81% vs. 46.8%, $p<0.001$) regardless of the type of immunosuppression. Steroid-resistant rejection also occurred more frequently in patients with autoimmune (AUTO) liver disease than in patients with alcoholic liver disease (38.1% vs. 12.8%; $p=0.003$) with a trend towards more chronic rejection (11.1% vs. 2.1%). However, there was no difference in allograft or patient survival at 1 and 3 years. Berlakovich et al. [42] evaluated 252 liver transplanted patients: those who had undergone liver transplantation for alcoholic liver disease (ALD), hepatocellular carcinoma (HCC) and posthepatitic cirrhosis had less ACR and less need of rescue therapy than patients who had received liver transplantation for cholestatic disease (n=42). The cumulative rates of ACR episodes per patient per month at 6 months, when 94% of all ACR episodes

occurred, were: 0.45 for alcoholic cirrhosis, 0.55 for post-hepatitic cirrhosis, 0.65 for HCC and 1.0 for cholestatic disease.

The group, which has been consistently shown to have a lower incidence of acute and chronic rejection is chronic hepatitis B. This might reflect the underlying defect in cell-mediated immunity, which allowed the patients to become chronically infected with the virus in the first place [43, 44].

Farges et al. [44] in a retrospective analysis of 330 patients who were liver transplant recipients for chronic liver disease, found that ACR (48% at 1 year) and chronic rejection (10% at 3 years) were comparable in patients who had undergone liver transplantation for PBC, PSC, AUTO and HCV. However, the incidence of ACR (but not chronic rejection) was significantly lower in patients who had undergone liver transplantation for ALD (29% at 1 year), or hepatitis B virus (HBV) cirrhosis (21% at 1 year) and the latter also had lower chronic rejection (0% at 3 years). Thus some groups of patients can receive less immunosuppresion. In particular as HBV replication is potentiated by immunosuppression, it is also beneficial to reduce immunosuppression in these patients. However, Wiesner et al. [11], using multivariate analysis, showed that the 6-week incidence of ACR in a cohort of 762 consecutive adult liver transplant recipients was not dependent on the underlying disease.

Neuberger et al. [40] showed that the percentage of patient with severe ACR at the liver biopsy performed 7 days after the transplantation was higher among patients transplanted for HCV-related liver disease (69%) compared to other aetiologies.

Although it is difficult to draw firm recommendations from these studies, most centres tend to lessen maintenance immunosuppression for HBV, HCV cirrhosis, alcoholic liver disease and HCC and/or use early steroid withdrawal, from the

outset. Conversely, patients with AUTO, PBC, or PSC may need steroid maintenance and heavier initial immunosuppression.

Gomez-Manero et al. [45] reviewed 133 transplanted recipients to identify predisposing factors for early (≤45 days after liver transplantation) ACR. No protocol liver biopsies were performed. Younger recipients, those with better hepatocellular liver function (Child-Pugh A) and those who underwent transplantation for liver disease other than ALD, had a greater risk for early ACR. Combining these three variables, they developed a mathematical model to allow prediction of the individual risk of each patient.

1.1.3 Prognostic factors of acute cellular rejection

1.1.3.1 Histological severity

McVicar et al. [46] described a group of patients who had focal rejection in the hepatic allograft biopsy defined as lymphocytic infiltration involving less than 20% of portal tracts. In the follow up of patients showing focal or mild rejection, only six (15%) patients subsequently developed abnormal liver function tests and required treatment with additional immunosuppression for ACR [47], suggesting additional immunosuppression is not needed in these patients, and close follow-up would identify the small number requiring therapy.

In Birmingham, during follow up of 151 patients to assess the effect of not treating mild ACR (protocol 7-day biopsies), 97 had histologically mild rejection: 50 had biochemical dysfunction and received prednisone for 3 days, while the remaining 47 cases with stable biochemistry had no additional treatment. Fifty-

four patients with no ACR were included for comparison. The outcome at 3 months in all three groups was similar [2].

Wiesner et al. [11], in a cohort study of 762 consecutive adult liver transplant recipients, examined the association of histological severity of ACR and overall patient outcome. Using univariate analysis, ACR overall, including mostly the milder grades, was significantly associated with an increased patient survival (RR 0.71, p=0.05) and a trend toward improved graft survival. Moreover, adjusting for other risk factors such as age and renal insufficiency revealed no significant decrease in survival among patients who had ACR. These findings were similar to those of Fisher et al. [48], who analysed nine studies (comprising a total of 1473 patients), and found that there was no correlation between mortality and incidence of treated ACR.

These findings in liver transplantation are in contrast to renal transplantation in which acute rejection is significantly associated with decreased patient and graft survival. Why ACR in liver transplant recipients is not associated with decreased patient and graft survival remains unexplained. It is possible that ACR in the setting of controlled alloreactivity exerts a tolerizing effect, making the graft less susceptible to further immunological attack. However, it should be noted that successful treatment for ACR occurs in nearly all cases. Thus the correct interpretation of the finding reported above is that the occurrence and successful treatment of ACR does not influence survival in liver transplant patients, but it does imply that abolishing early ACR need not, and indeed, should not be a goal of initial immunosuppression.

1.1.3.2 Timing

As regards timing of ACR, there is no firm consensus to define what is early or late rejection. In three different studies the timing and the outcome varied according to the definition of each centre.

In a retrospective multicentre analysis of 623 liver transplants, the cumulative incidence of biopsy proved ACR was 59% for early episodes (<6 months) and 21% for late episodes (≥6 months). Patient and graft survival did not differ significantly between those who experienced an early ACR episode and those who did not (p=0.49 and p=0.13, respectively). Furthermore, these parameters did not differ significantly between recipients who experienced a late ACR episode and those who did not (patient survival p=0.18 and graft survival p=0.20) (57).

Wiesner et al. [11] analysed 762 consecutive adult liver transplant recipients and found 367 (48%) who developed at least one ACR episode within the first 6 weeks post-transplantation. Multivariate analysis indicated that ACR was not significantly associated with mortality but there was a trend to better survival (RR 0.78, p=0.25) and re-transplantation free survival (RR 0.86, p=0.44). However, severe ACR doubled the risk of death or re-transplantation compared to mild ACR. Using proportional hazards modelling, in the same study, seven factors were identified as independently associated with an increased incidence of early ACR: younger recipient age, lack of renal impairment, lack of oedema, higher AST levels, fewer human leukocyte antigen DR matches, longer cold ischemic times and older donors.

Mor et al. [49] retrospectively reviewed 375 liver transplants, and defined late onset ACR as that which occurred after 6 months. There were 315 episodes of early ACR in 226 patients, and 31 episodes of late ACR in 26 patients. Low

cyclosporine (CsA) levels appeared to account for 58% of these late episodes. Most episodes of rejection responded to pulse corticosteroids, and chronic ductopenic rejection arose in only two patients. There was no difference in survival between patients experiencing early and late ACR.

Anand et al. [50] reviewed late onset ACR, defining it as rejection recognized after the first 30 days post-transplantation. They evaluated 717 patients who had undergone transplantation in Birmingham between 1982 and 1994: 59 (8%) patients had 71 episodes of late ACR. They too found that the most common precipitating event was low levels of calcineurin antagonists, and that most acute episodes of ACR in this timeframe were responsive to standard therapy. However, in contrast to Mor et al. [49], Anand found that 16 (27%) of 59 patients developing late onset ACR progressed to chronic ductopenic rejection and graft loss. Delayed response to an earlier episode of ACR, and centrilobular necrosis or bile duct loss at the time of diagnosis of late ACR, were associated with high risk of progression to chronic rejection and graft loss.

1.1.3.3 Number of episodes

In an abstract, Wiesner et al. [51] showed that the number of episodes of ACR and the histological severity were significantly associated with chronic rejection ($p<0.001$). Dousset et al. [52] prospectively evaluated 170 liver transplanted patients and showed that there was no difference in graft function between patients with a single episode of ACR (n=56) and those without ACR (n=84). Among patients treated for a single episode of ACR, late hepatic function was not influenced by the severity of ACR, nor by the response to corticosteroids. In contrast, patients with more than one ACR episode (n=30) had significant impairment of liver function tests (aspartate aminotransferase, AST $p<0.05$;

alanine aminotransferase, ALT, $p<0.001$; alkaline phosphatase, ALP $p<0.01$, lower dye clearances ($p<0.01$), and more severe histological damage ($p<0.001$). The authors concluded that a single episode of ACR does not impair long-term hepatic function, whereas recurrent episodes can lead to damage to the liver allograft.

1.2. Immunosuppressive therapy and liver transplantation

1.2.1 Role of immunosuppression in liver transplantation

The introduction of calcineurin inhibitors (CNIs) in the 1980s substantially reduced ACR and improved rates of early engraftment [53], however, in the last 20 years, therapeutic regimens have not substantially evolved. Immunosuppressive drugs are combined to achieve potent and safe effects in the immediate post-transplant period, with a gradual decrease of the dose thereafter. In the early post-transplant period immunosuppression protocols usually include a CNI, azathioprine (AZA) or mycophenolate mofetil (MMF), and corticosteroids. The maintenance therapy is based on the same combination, but with lower dose and with or without discontinuation of corticosteroids. Short-term induction therapy, with monoclonal antibodies, is often added to the protocol, especially in patients with pre-transplant renal dysfunction.

In recent years, the main end point of immunosuppressive therapy has shifted from the prevention of ACR toward the preservation of long-term graft function and prevention of immunosuppression-related side effects.

The complete suppression of early ACR in clinical practice is no longer a goal of initial immunosuppression, as it may eliminate the immunological opportunity to provide some tolerance to the graft [40] although paradoxically clinical trials still

have ACR as an endpoint. At the same time, long-term outcomes of patients is becoming the main concern for clinicians, as the long-term side effects of immunosuppressive therapy cause significant morbidity and mortality. These can be due to direct drug toxicity such as renal dysfunction, hypertension, induction of diabetes and dyslipidaemias or a consequence of the immunosuppressive effect on the immune system such as opportunistic infections, *de novo* malignancy [54-57], and influence the severity of recurrent disease, such as HCV and HCC.

Thus there has been a development of new immunosuppressive protocols using a combination of drugs, with different modes of actions, allowing lower doses of each drug, but not necessarily in practice leading to lower immunopotency. In addition, increasing attention has been given to the observation that some patients tolerate their liver graft without need for long-term immunosuppression, or with greatly reduced immunosuppression.

Lastly, it is often forgotten that, liver transplant recipients are a heterogeneous group of individuals with different predisposing factors for the development of ACR and with different co-morbidities (i.e. HCV infection, pre-transplant renal impairment, presence of hepatocellular carcinoma), which means that a single a optimal and universally applicable immunosuppressive protocol is not applicable, but immunosuppression should be tailored on the patient's clinical condition and risk of ACR.

1.2.3 Immune monitoring as measure of real immunosuppressive status

Current immunological monitoring relies heavily on clinical judgment and on therapeutic drug levels and does not adequately assess the functional immunosuppression status of liver transplanted patients. Trough levels of drugs are arbitrary and are more clinically relevant for preventing excessive low or high

blood concentrations. Therefore, the evaluation of the real immunosuppression state in liver transplanted patients is a major challenge, and there is a high interest in the development of specific immune monitoring assays that could be use to assess liver transplanted patients on long-term immunosuppression.

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

Kawalski et al. [58] performed a meta-analysis using this assay in solid organ transplanted patients and showing a high correlation between infections and lower ranges of ATP responses and between ACR and upper ranges of ATP responses. However it has been shown that this assay could be more useful in order to assess over-immunosuppression rather than under-immunosuppression [59]. Moreover recent studies have supported the use of ATP assays in liver transplantation for distinguishing HCV recurrence from ACR [60]. Mendler et al. [61] demonstrated that patients transplanted for HCV-related cirrhosis have low ATP responses immediately after transplant and at the time of the histological diagnosis of HCV recurrence. Moreover, low ATP production has been correlated with ta more rapid development of fibrosis, possibly due to the over-immunosuppression and a lack of virological control [62].

1.3 Biomarkers of acute cellular rejection

1.3.1 Past and present biomarkers

A perfect diagnostic biomarker for ACR should be highly sensitive and specific, non-invasive, and rapidly available, and although many potential biomarkers have been reported to have diagnostic potential, only few have been validated [63].

Rising of liver enzymes after transplantation is often the first trigger to suspect ACR. However, sensitivity and specificity of liver enzymes are low and these enzymes cannot differentiate ACR from others complications. The area under the ROC curve for AST, ALT, GGT, total bilirubin and conjugated bilirubin is about 0.5. For ALP the area under the ROC curve is slightly better (0.69) but its clinical significance remains doubtful [64].

The first potential biomarkers studied were cytokines. Soluble IL-2 receptor (sIL-2R) levels in serum are increased as early as 10 days before ACR, but CMV infection [65, 66], bacterial infections and cholangitis [67, 68] can also be responsible for this increase. Soluble TNF receptor II (sTNF-RII), and IL-10 increase as well during ACR and during infective complications. The proinflammatory cytokines IFN-γ, IL-1β and IL-4 and IL-6 were not of any use [68]. Kita et al. [69] observed showed that an increase in IL-6 levels during ACR and during infections, but without being able to distinguish between both. Plasma levels of IL-15 are also increased during ACR, particularly during steroid-resistant ACR [70]. Levels of TNFα and of β2 microglobulin were found to be increased in patients with ACR, but these markers could not differentiate ACR from infections [71-74].

Considering other proteins related to the inflammatory response, in two studies [75, 76] a rise of CD28 expression up to 6 days before ACR has been observed.

It is well known that the infiltration of leukocytes into the allograft during ACR is regulated by the expression of adhesion molecules [77]. An increase of intercellular adhesion molecule 1 (ICAM-1) and E-selectin in serum was observed in relation to ACR. However, E-selectin [78, 79] nor ICAM-1 [80] could differentiate ACR from an infectious episode. A differentiation was seen between patients with ACR and CMV infection, where ICAM-1 levels did not increase [81].

The role of toll like receptors (TLR) was also studied with respect to ACR. Patients experiencing ACR had significantly higher levels of TLR4 and a greater capacity to produce the pro-inflammatory cytokines TNFα and IL-6 before transplantation but had a down regulation of the TLR4 pathway if they experienced ACR. In contrast there was no correlation between TLR2 levels and ACR [82].

Apoptosis is an important mechanism of cell death during ACR and this is mediated via Fas ligand. Increased serum levels of soluble Fas antigen has been detected in patients during ACR [83].

Several studies illustrate that blood eosinophilia could be an interesting biomarker for ACR [84, 85]. In one study a positive predictive value of 82% was found, but more interesting a negative predictive value of 86% [86]. However, the response was less clear in patients who received steroids and in HCV positive patients.

Another potential biomarker was alpha-glutathion S-transferase (alpha-GST) and Pi-glutathione S-transferase (Pi-GST). GST's are a family of multifunctional detoxifying enzymes implicated in the conjugation of glutathione with several compounds. Alpha-GST is a low molecular weight protein widely present in the

hepatocyte cytosol with a short half-life. Plasma values increase rapidly in case of ACR, but the lack of sensitivity and specificity make this marker difficult to use in clinical practice [87-90]. Pi-GST is an isoenzyme exclusively found in the biliary epithelium of the liver that was also tested but was not found to be related to ACR [89].

In a small patient series serum amyloid A protein (SAA) was significantly increased during the appearance of ACR [91], but it could not differentiate ACR from infections [67].

1.3.2 Future biomarkers

New biomarkers, especially in the fields of genomics, proteomics and transcritomics have been described in recent years as a result of a constant and progressive research in this area.

Considering genomic studies, loci associated with increased risk of ACR [92] or to poor allograft survival [93] have been identified, but no data on loci associated with the acute event of ACR were found.

Several proteins are involved in the complex immunological mechanisms of ACR, therefore the proteomic analysis seems a promising approach to identify reliable biomarkers of ACR. Massoud et al [94] identified more than 40 different serum proteins in the serum of patients experiencing ACR. Amongst these C4 and C1q were independent predictors of ACR, with the best diagnostic performance being achieved by C4. In an animal model of liver transplantation, Cheng et al. [95] showed that 8 proteins were down-regulated in rats with ACR.

Considering transcriptomics Kobayashi et al [96] found that the mRNA level of interferon regulatory factor 1 (IRF-1) and guanylate-binding protein 2 (GBP2) in leucocytes, not related to T-cell mediated immune response, were upregulated in

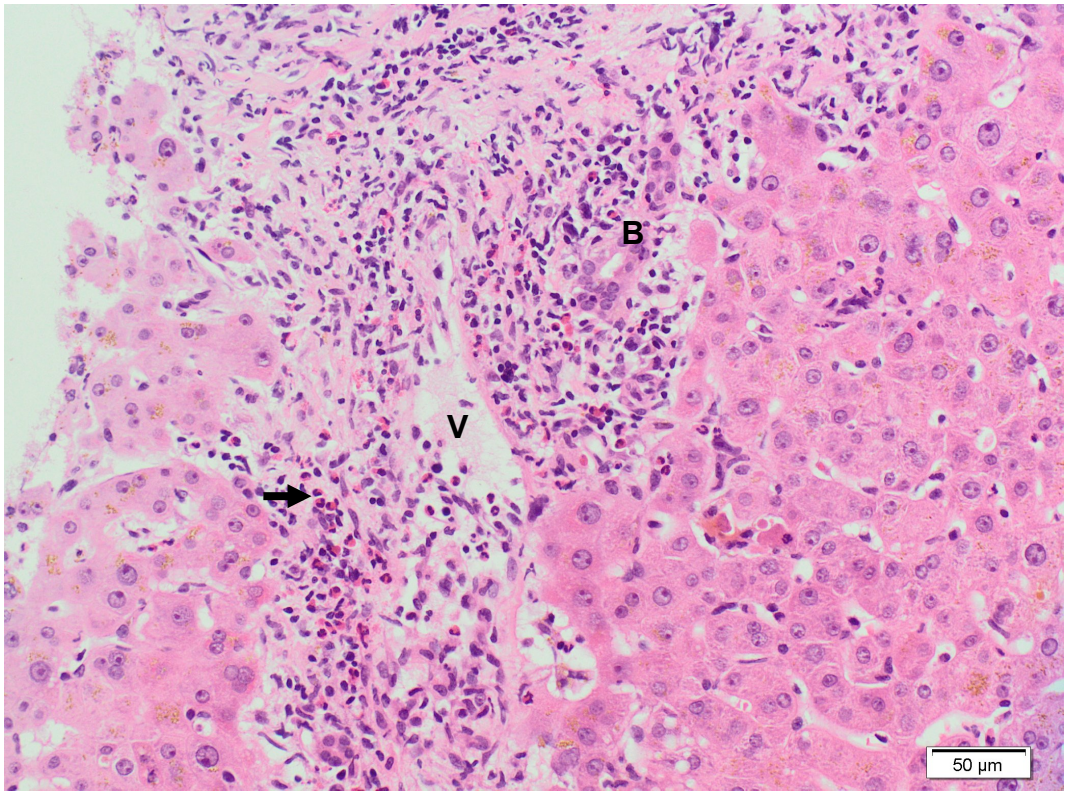
patients with ACR, but only GBP2 reached the statistical significance. ACR after liver transplantation seems also to be associated with a rise of MicroRNA (miR)-122 and miR-148a, starting before the rise of transaminases [97]

1.4 Tables and figures

Table 1.1. Banff schema for grading of acute liver allograft rejection. *Verbal descriptions of mild, moderate and severe acute rejection could also be labeled as grades 1, 2 and 3 respectively.

Overall grade*	Criterion
Indeterminate	Portal inflammatory infiltrate that fails to meet the criteria for the diagnosis of acute rejection
Mild	Rejection infiltrate in a minority of the triads that is generally mild and confined within the portal spaces
Moderate	Rejection infiltrate that expands most or all of the triads
Severe	As for “moderate” but with spillover into periportal areas and moderate to severe perivenular inflammation that extends into the hepatic parenchyma and is associated with perivenular hepatocyte necrosis

Figure 1.1. The picture (HE X20) shows marked expansion of the portal tract by a mixed inflammatory infiltrate containing small lymphocytes with occasional blasts, plasma cells, neutrophils and a moderate number of eosinophils (arrow). The bile ducts (B) are cuffed and infiltrated by lymphocytes and show mild reactive changes. There is subendothelial lymphocytic infiltration involving the portal vein branch (endotheliitis, V). The features are conclusive for acute cellular rejection amounting to moderate degree.



2. PROJECT AIMS

The aim of the first study was to evaluate a large and consecutive cohort of liver transplanted patients, who had protocol biopsies, to assess the incidence of ACR, to identify potential risk factors for ACR, and to evaluate the impact of ACR and its histological severity on outcomes after liver transplantation.

The aim of the second study was to evaluate the role of liver function tests and blood eosinophil count as potential biomarkers for ACR after liver transplantation. We assess whether peripheral blood eosinophil count is predictive of histologically proven moderate and severe ACR, as well as any relationship between changes in blood eosinophil count and clinical course of ACR in both treated and untreated patients.

The aim of the last study was to prospectively evaluate the immunological status in patients with cirrhosis and after liver transplantation, and to identify immunological alterations associated with ACR. The clinical goal of this project is to use the immune monitoring assays to properly manage immunosuppressive therapy, increasing immunosuppression in patients with signs of inappropriate high immune cell function (at risk of infections), and decreasing it in patients with signs of inappropriate low immune cell function (at risk or ACR).

3. ACUTE REJECTION AND OUTCOMES AFTER LIVER TRANSPLANTATION: A PROTOCOL BIOPSY EVALUATION

3.1 Introduction

Diagnosis of ACR is not uniform, being based sometimes on liver biopsy following clinical suspicion, other times simply suspected and treated. Early ACR, which responds to treatment, has no negative long-term effects, and may even be associated with a lower risk for later immunological complications [11, 47]. Thus the more fundamental question is how much rejection is not harmful, such that complete suppression of ACR need not be a primary goal in liver transplantation, and importantly need not be a primary endpoint in clinical studies. However very few studies have evaluated therapy for ACR with respect to clinical outcomes.

Moreover there have only been a few studies evaluating risk factors for ACR; these are insufficient for evidence-based differentiated protocols for preventing and treating ACR [11, 39, 42, 44, 45, 48, 98]. Lastly only three studies used protocol liver biopsies with [11, 44, 98] patients, and no studies evaluated the severity of ACR, rather than just its occurrence. Furthermore, the impact of ACR on subsequent patient and graft outcome remains poorly defined.

3.2 Methods

3.2.1 Study cohort

From October 1988, all patients undergoing liver transplantation at our centre have been prospectively followed with detailed information stored on a computerised database. Pre-transplant data include demographic variables, past

medical history, blood group, lifestyle, aetiology of liver disease, ascites and encephalopathy, pre-transplant renal support or ventilation, and laboratory data. Severity of liver disease at liver transplantation was defined by Child-Pugh and with model for end-stage liver disease (MELD) scores. Donor variables include sex, age at death, blood group, ABO donor/recipient group matching and surgeon's assessment of donor liver appearance. Surgical variables include cold and warm ischemia time, details of anastomoses, and transfusion of blood products and anti-fibrinolytics intraoperatively.

The present analysis includes all patients undergoing a first liver transplant from October 1988 to May 2008. Minimum follow-up was until June 2009, thus all patients had the potential for at least 12 months follow-up after transplantation. Children aged <16 years, multiorgan transplants and deaths or re-transplantation within three days post-transplantation were excluded.

3.2.2 Immunosuppression protocols

The basic immunosuppression policy was to use triple immunosuppression therapy: CsA or Tac in combination with AZA and low dose steroids, which were tapered and stopped between 3-6 months after transplantation. Immunosuppression started immediately after transplant with intravenous methylprednisolone (1 mg/kg/day until July 1997 or 16 mg daily thereafter, followed in both cases by 20 mg oral prednisolone daily once gut function was restored) and AZA (1mg/kg/day) in addition to either Tac (initially 0.1 mg/Kg/day in two divided doses) or CsA (initially 10 mg/kg/day in two divided doses). CNI doses were run on the lower side of the therapeutic range and adjusted according to serum levels, suspicion or proven infection, renal function or toxicity. Between October 1996 and January 1997 patients were randomised to receive

monotherapy with Tac versus CsA [99]. From May 1997 to April 1999 patients were randomised to triple therapy with either Tac or CsA [100]. Thereafter a patient cohort received Tac monotherapy [101]. No antilymphocyte or antibody induction therapy was used and no IL-2 receptor blockers were used in this population.

3.2.3 Protocol biopsies after liver transplantation

The first protocol liver biopsy was planned between 5 and 10 days after liver transplantation. However, due to operational delays some biopsies were performed later, and so we considered the protocol liver biopsy to be performed within 14 days.

All biopsy samples were fixed in formalin and embedded in paraffin. Histological sections (4μm thick; 3 levels through the tissue block) were stained with hematoxylin and eosin, and other histochemical stains were prepared as required. Immunohistochemical stains were used for biliary cytokeratins and viral antigens as necessary.

3.2.4 Diagnosis of ACR

In this study, we only considered histologically diagnosed ACR episodes, whether treated or not treated. The histological criteria for ACR included the presence of: 1) mixed portal inflammation including “activated” lymphocytes and frequently eosinophils; 2) bile duct inflammation/damage; and 3) endothelitis of portal and/or terminal hepatic veins. The degree of ACR was classified as none, mild, moderate or severe using the Royal Free Hospital scoring system [102], which applies the same criteria as the subsequently published Banff acute cellular rejection activity [12], but also includes graft eosinophilia.

3.2.5 Treatment of ACR

ACR episodes were treated with a 1g daily bolus intravenous methylprednisolone for 3 days consecutively, followed by the baseline steroid dose of 20mg/day. If after the first cycle of bolus methylprednisolone, clinical and histological evidence of persistent ACR remained (further liver biopsy was performed 2 days after the last bolus), a second cycle of methylprednisolone was given. If clinical and histological ACR persisted, OKT3 was administered intravenously (5 to 10 days at 5 mg/d) or ATG or ALG.

3.2.6 Statistical analysis

Patients were stratified into those with ACR (mild, moderate or severe) and those with normal biopsy or with other conditions present. As there were few patients with severe ACR, moderate and severe rejection were combined for analysis.

Liver diseases were grouped and analysed as follows: ALF, ALD, HBV, HCV, PBC/PSC/AUTO, and other liver aetiologies. Patients with HCC were classified according to the underlying liver disease.

Factors associated with ACR at first protocol biopsy were identified using univariate and multivariable logistic regression. Recipient-related variables were: gender, age, blood group, aetiology of liver disease, MELD score and Child-Pugh score, ascites, encephalopathy, pre-transplant ventilation, pre-transplant renal support, previous abdominal surgery, serum albumin, bilirubin, creatinine, INR and urea levels at the time of transplant, eosinophil levels the day before liver biopsy. Donor-related variables were: gender, age, blood group, donor/recipient ABO group matching, and organ appearance (evaluated by the transplanting surgeon). Liver transplant-related variables were: year of liver transplantation, immunosuppressive regimen at day 1 post-transplant, hepatic artery

anastomosis, cold ischemia time (min), reperfusion time (min), units of blood, cryoprecipitate, plasma and platelets received intraoperatively. Factors associated with ACR univariately ($p<0.10$) were included in the multivariable logistic regression model to identify independent associations with ACR (backward selection process). If MELD or Child Pugh score were significant univariately, as well as their components, the score was used in the multivariable analysis, rather than the individual components.

Follow-up was to time of first re-transplant or death, whichever occurred first, or up to December 2011.

The causes of death or graft failure after first liver transplant were evaluated as follows: primary-non-function (PNF) or acute vascular occlusion (grouped together), infection, chronic rejection, multi-organ failure, recurrence of primary liver disease, tumour recurrence, “de novo” tumours, and other causes.

Survival after first liver transplant was evaluated according to: presence/absence of ACR, histological severity of ACR and treatment/no treatment of ACR episodes. This was performed using the life-table method with comparisons between groups made by the log-rank test.

Discrete variables are shown as percentages and continuous variables with Normal distribution (Kolmogorov-Smirnov test) as mean values±SD, otherwise as median (range). Chi-square test was used to compare discrete variables, Student’s t -test for continuous variables, and ANOVA analyses for comparisons of three groups or more. Statistically significant differences were defined by a p-value ≤ 0.05.

Factors associated with death or graft failure were identified using Cox proportional hazards regression models. Variables with statistically significant

hazard ratios in univariate analyses ($p<0.1$) were included in the multivariable analysis.

3.3 Results

Liver transplantation was performed in 733 patients between October 1988 and May 2008. Amongst these, 648 patients had a protocol liver biopsy within 14 days after transplantation: 6 patients died within the first 3 days after transplantation and 79 did not undergo protocol liver biopsy.

In the 648 liver biopsies, ACR was diagnosed in 504 (77.8%): 266 (41%) mild, 210 (32.4%) moderate and 28 (4.3%) severe; 76 (11.7%) had a normal biopsy and 68 (10.5%) had other abnormal histopathological features.

Of the 504 patients with ACR diagnosed at protocol biopsy, 266/504 (52.7%) received methylprednisolone: 60/266 (22.6%) with mild ACR, 180/210 (85.7%) with moderate ACR and 26/28 (92.9%) with severe ACR. Of the 144 patients without signs of rejection, 8 (7%) received methylprednisolone before a final diagnosis, and all 8 had other histopathological abnormalities.

The demographic and clinical characteristics of the 648 patients with a protocol biopsy between day 4 to day 14 after transplant are shown in Table 1. Recipient, donor and transplant variables were evaluated according to the protocol biopsy histology. Patients with moderate/severe ACR were significantly younger (median 48 years) compared to patients with mild ACR (51 years) and no ACR (52 years) ($p=0.009$). The diagnosis of histopathological abnormalities other than ACR was more frequent in patients transplanted for ALF compared to other aetiologies ($p=0.029$), whereas the moderate/severe ACR was more frequent in patients

transplanted for PBC/PSC/AUTO compared to other groups (p=0.029) (Table 3.1).

3.3.1 Risk factors for ACR

Using univariate logistic regression, factors associated with a greater likelihood of ACR in the first 14 days were later calendar year (p=0.005), absence of ascites (p=0.05), absence of pre-transplant renal support (p<0.001), absence of encephalopathy (p=0.01), having a PSC/PBC/autoimmune aetiology (p=0.018), higher level of serum albumin (p=0.02), a Child-Pugh score A (p=0.001) and sub-optimal organ appearance (p=0.01). Conversely, a high MELD score (p<0.001), a high blood level of urea (p=0.003), creatinine (p=0.04), bilirubin (p=0.002), and INR (p=0.001) and the transfusion of blood (p=0.009), or plasma (p=0.03) or platelets (p=0.02) during surgery were all associated with a lower risk of ACR (Figure 3.1A). No association was found between ACR and donor or recipient age or gender, cold ischemia or reperfusion time, use of cryoprecipitate intraoperatively, previous abdominal surgery, eosinophil level, and immunosuppressive regimen.

With multivariate logistic regression, the factors associated with ACR at first protocol biopsy were: absence of ascites (OR 1.59, 95%CI 1.05-2.43; p=0.003), no requirement for renal support (OR 3.16, 95%CI 1.54-6.47; p=0.002), and a suboptimal organ appearance (OR 2.67, 95%CI 1.38-5.17; p=0.004), whereas a higher MELD score (OR per 5 MELD points 0.85, 95%CI 0.76-0.95; p=0.01) was associated with a lower risk of ACR (Figure 3.1B).

In a separate multivariable logistic regression analysis the factors associated with moderate/severe ACR at first protocol biopsy (versus no rejection, mild rejection or other conditions) were female recipient sex (OR: 1.52, 95%CI 1.09-2.11;

p=0.014) and no requirement for pre-transplant renal support (2.28, 95%CI 1.31-5; p=0.04), whereas a higher recipient age (OR per 10 years of age: 0.83, 95%CI 0.971-0.97; p=0.017) and number of blood units required (OR per 5 units: 0.84, 95%CI 0.75-0.94; p=0.002) were both associated with a lower risk of moderate/severe ACR (Figure 3.1C).

3.3.2 ACR and survival after liver transplantation

Over the median follow-up period of 87.5 months (range 0.3-253.4), 190 patients (29.3%) died and 45 (6.9%) were re-transplanted. Overall, patient and graft survival at 1, 3, 5 and 10 years after transplantation were 84%, 79%, 75%, 65% and 81%, 75%, 71%, 62% respectively.

Patient and graft survival at 1, 3, 5 and 10 years after liver transplantation, according to the absence/presence of ACR at protocol biopsy, were not significantly different between the two groups (patient survival: 83%, 81%, 76%, 63% vs. 84%, 78%, 75%, 66%; p=0.928; graft survival: 81%, 78%, 73%, 61% versus 82%, 74%, 71%, 62%; p=0.57) (Figure 3.2).

After removing patients with other histological abnormalities from the group with no rejection, there was still no difference in patient (p=0.528) or graft survival (p=0.17).

Stratifying patients according to severity of ACR showed that moderate/severe ACR was associated with greater patient and graft survival at 1, 3 5 and 10 years post-transplant (90%, 84%, 80%, 73% and 87%, 80%, 76%, 70% respectively) compared to mild ACR (80%, 73%, 70%, 61% and 77%, 69%, 66%, 56%; p=0.04 and p=0.016 respectively). Conversely there was no difference in patient or graft survival between patients with no ACR or those with any degree of ACR, or patients with other histopathological abnormalities (Figure 3.3).

When treatment of ACR (yes/no) was evaluated with respect to patient and graft survival treatment was associated with better patient (p=0.001) and graft survival (p=0.006) (Figure 3.4).

This was due solely to patients with moderate/severe ACR who underwent treatment compared to moderate/severe ACR untreated patients (p=0.002 and p=0.018 respectively). Conversely no statistical difference in patient and graft survival was found in patients with mild ACR whether treated or not (p=0.589 and p=0.988 respectively) (Figure 3.5).

An adjusted Cox regression analysis, using variables that were independently associated with outcomes (year of transplantation, donor/recipient ABO blood group matching, and number of blood units used during surgery), showed that ACR overall was not significantly associated with death or graft loss, except for patients whose ACR was not treated (HR 1.54, 95%CI 1.09-2.17; p<0.001). Stratifying this group of patients according to histological severity of ACR showed that this was due to untreated patients with moderate/severe ACR (HR 2.29, 95%CI 1.18-4.44; p<0.001), whereas mild ACR whether treated or not, had no effect on graft survival (Table 3.2).

3.4 Discussion and conclusions

This study evaluated the incidence ACR and risk factors for developing ACR and its impact on patient and graft survival, using a well documented database with patients undergoing protocol liver biopsies at a single Centre.

The impact of ACR on patient and graft survival after liver transplantation has only been properly evaluated in one study [11], which showed that ACR did not influence patient survival. However we now add to these observations, as we

considered not only the presence/absence of ACR, but also stratified patients according to the histological severity of ACR. We confirm that patient and graft survival are not influenced by the presence or absence of ACR. Patients experiencing mild ACR had worse patient and graft survival compared to patients experiencing moderate/severe ACR (p=0.008 and p=0.006 respectively), but by Cox regression analysis this was not confirmed so that this difference was associated with other risk factors in the model.

Importantly our study is the first to evaluate therapy for ACR and not just the occurrence of ACR. Untreated ACR was associated with worse graft survival using adjusted Cox regression analysis (HR 1.54, 95%CI 1.09-2.17; p<0.001), but this was solely due to patients with untreated moderate/severe ACR (HR 2.29; 95%CI 1.18-4.44; p<0.001). Mild ACR treated or not did not influence graft or patient survival. Importantly moderate/severe ACR was associated with more chronic rejection, which was further increased in untreated patients, as a cause of death or graft loss (4/14 vs. 8/56; p<0.001).

The associations of ACR in our cohort, in which we aimed at the lower end of the recommended CNI trough level range, confirm the observation that the liver is a privileged organ in terms of immunological interactions, substantially differing from kidney transplantation, in which even a single episode of ACR can lead to graft loss. Our findings, and previous ones, suggest that ACR in liver transplantation needs to be understood from a different perspective. Firstly, the complete suppression of early cellular rejection should not be not considered as a primary goal of initial immunosuppression, not only because ACR “per se” does not negatively affect graft and patient survival, but also because it may eliminate the immunological opportunity to provide some tolerance to the graft [103, 104]. Secondly, in clinical practice, less potent immunosuppressive regimens can be

safely used after liver transplantation, which will reduce immunosuppression-related side effects such as infection, renal dysfunction, cardiovascular complications and *de novo* malignancies. Thirdly immunosuppressive regimens can be tailored individually according to the risk of developing moderate/severe ACR, and/or using an initial low immunopotent regimen as standard, and then "responding" to moderate/severe ACR if it occurs, by treating it. Thus post-transplant protocol liver biopsies could be planned in the subgroup of patients at risk of moderate/severe ACR to obtain a definitive diagnosis of the severity of ACR, as if not treated, moderate/severe ACR may lead to a worse patient and graft survival. In contrast, mild ACR (which may be more frequent with low immunopotency regimens) does not influence patient and graft survival, whether treated or not.

Therefore it becomes crucial to identify patients at risk of moderate/severe ACR. Although several studies have previously attempted to assess potential risk factors for developing ACR after liver transplantation, none stratified patients according to histological severity, as we have done.

In our study, the risk of ACR, independently from its histological grade, was greater in patients without ascites or encephalopathy pre-transplant, and in recipients receiving suboptimal organs. On the other hand patients with MELD score>25 experienced a significantly lower incidence of ACR, independent from its histological severity. These findings demonstrate that less sick recipients are at greater risk of developing ACR compared to patients who are transplanted in worse clinical condition. The influence of liver function at the time of liver transplantation on ACR has previously been previously evaluated in 133 patients (but without protocol biopsies) [45], showing that patients with who were Child-Pugh score A had a greater risk of ACR, within the first 45 days after liver

transplantation. Similarly, in our study, univariately Child-Pugh score A was a risk factor for ACR (OR 2.63, 95%CI 1.47-4.69; p=0.001); absence of ascites, one of the components of Child-Pugh score, remained significant in the multivariate analysis. Patients with higher MELD scores were at less risk of ACR, and this was confirmed in the multivariate analysis (OR per 5 MELD points 0.85, 95%CI 0.76-0.95; p=0.01). This finding reflects clinical experience; we presume that sicker patients have a more depressed immunity and are less likely to reject.

The association between the use of suboptimal organs and ACR is more difficult to understand. However it has been previously shown that using grafts from older donors is *per se* an independent risk factor for ACR, suggesting that non allogenic differences can trigger immunological reactions [105, 106]. These findings are important because increasingly there is a general deterioration in the quality of donor grafts (i.e. increased donor age, high incidence of steatosis), but also an increased use of donors after cardiac death in some countries. Moreover the number of patients transplanted for hepatocellular carcinoma is constantly increasing, and most of these patients are transplanted with better liver function. All these factors could be responsible for the increased incidence of ACR with more recent transplant era, which occurred in our cohort.

Patients who were most at risk of developing moderate/severe ACR were female recipients, most frequently with autoimmune aetiology, (OR 1.52, 95%CI 1.08-2.11), and patients who did not require pre-transplant renal support (OR 2.28, 95%CI 1.31-5).

It has been demonstrated that patients with good pre-transplant renal function are at greater risk of developing ACR, but no distinction was made according to histological severity of ACR [11]. Our confirmatory findings are important, as more patients are requiring renal support, particularly in centres using MELD or

UKELD allocation systems, and these patients are often those most at risk of sepsis, which in turn is increased with immunosuppression. Therefore the rationale behind the use of low dose immunosuppressive regimens in these patients, would be not only to prevent post-transplant renal dysfunction and to reduce the incidence of sepsis, but also because of lower risk of moderate/severe ACR. In addition older patients (OR per 10 year increase 0.83, 95%CI 0.71-0.97) and those receiving a greater number of blood units during surgery (OR per 4 unit increase 0.84, 95%CI 0.75-0.94) were also at a lower risk of moderate/severe ACR. Thus easily obtained clinical factors can provide a risk stratification for moderate/severe ACR.

The association between reduced incidence of moderate/severe ACR and the increased use of blood units is of particular interest because, to our knowledge, this is the first time this association has been reported in liver transplantation, whereas it was first recognised in kidney transplantation [107]. This phenomenon is known as transfusion-associated immunomodulation [108]. Transfusion of allogenic blood products can induce a down-regulation of immune responses, including decreased helper to suppressor T-lymphocyte ratio, decreased NK cell function, defective antigen presentation and reduction in cell mediated immunity [109-111]. The depression of the immune system might also be the explanation for the significant association between reduced incidence of moderate/severe ACR and older recipient age.

In conclusion, the results of our study clearly show that ACR after liver transplantation, with the exception of untreated moderate/severe ACR, does not influence patient and graft survival. Moreover it confirms what is practised in many centres based on experience, that it is not mandatory to treat patients with

mild ACR, as it does not influence graft or patient survival. However attention needs to be focussed on patients at risk of moderate/severe ACR, as if not treated, this increases the rates of graft loss and death. Our findings should lead clinicians to consider returning to perform protocol biopsies for those at risk of moderate/severe ACR, and reducing immunopotency of treatment protocols in those at least risk (older age, worse liver and/or renal function, blood transfusion). A general policy of a universal and initial immunosuppressive regimen with low immunopetency could be used, which would be escalated only if moderate/severe ACR occurs (and then treated), as this would reduce complications of immunosuppression and maintain, if not increase, graft and patient survival following liver transplantation [112]. Future randomized clinical trials should only focus on moderate/severe ACR, and not on the overall rejection rate. Therefore protocol biopsies will be necessary or alternatively appropriate and validated non invasive methods such as biomarkers [87], should be used.

3.5 Tables and figures

Table 3.1 Demographic and clinical characteristics of all 648 transplanted patients who underwent first protocol liver biopsies between 4-14 days after liver transplantation and according to histological severity of ACR. P-values refer to the comparison between different histological severity of ACR.

	Total cohort n=648 (%)	**Mild ACR** N=266 (%)	**Mod/Sev ACR** n=238 (%)	**No ACR** n=76 (%)	**p**
Male recipient	60.3	65.8	52.9	61.8	0.029
Recipient age, years median (range)	49 (17-70)	52 (17-70)	48 (17-66)	53 (19-67)	0.009
Liver disease					
ALF	7.7	4.9	8.4	9.2	
ALD	20.5	22.9	18.9	19.7	
HBV	10	10.9	8.8	10.5	0.029
HCV	24.2	27.8	19.3	32.9	
PBC/PSC/AUTO	24.2	22.2	29.4	19.7	
Other	13.3	11.3	15.1	7.9	
Ascites, no (%)	36.1	37.2	39.1	28.9	0.26
Encephalopathy (no)	86.2	88.6	87.3	85.5	0.011
Ventilation (no)	94.8	96.2	95	96.1	0.017
Renal support (no)	93.8	96.2	96.2	88.2	<0.001
MELD, median (range)	16 (6-40)	15.2 (6-40)	15.5 (6-40)	17.2 (6-40)	<0.001
Child-Pugh					
A	18.8	20.7	20.6	11.8	0.001
B	48.6	49.2	52.1	51.3	
C	32.6	30.1	27.3	36.8	
Abdominal surgery (no)	87.5	89.5	84.9	84.2	0.18
Donor gender (male)	48.5	47.4	48.3	50	0.93
Donor age, years median (range)	49 (9-75)	43 (11-73)	41 (9-76)	37 (11-69)	0.38
ABO-group matching					
Identical	88.4	91	88.2	88.2	0.15
Compatible	9	6	10.1	9.2	
Incompatible	2.6	3	1.7	2.6	
Suboptimal organ	15.7	19.9	15.1	3.9	0.009
Years of LT					
2003-2008	28.4	27.8	34.9	6.6	<0.001
1999-2002	31	29.3	31.1	32.9	
1988-1998	40.6	42.9	34	60.5	
IS regimen at day 1					
Mono	43	42.8	46.8	32.9	
Double	23	23.9	19	31.6	0.50
Triple	26.7	26.1	27.4	28.9	
None	7.3	7.2	16.8	6.6	
Cold ischemia time, min median (range)	632 (137-1194)	638 (137-1194)	619 (290-1043)	637.5 (290-1194)	0.26
Reperfusion time, min median (range)	43 (22-106)	44 (23-85)	43 (19-85)	42.5 (24-106)	0.49
Blood products, unit (median)					
Blood	6 (0-68)	6 (0-65)	5 (0-58)	6.5 (0-50)	0.001
Plasma	6 (0-35)	6 (0-30)	4 (0-56)	6.5 (0-19)	0.012
Platelets	2 (0-30)	2 (0-30)	1 (0-30)	2 (0-25)	0.002
Cryoprecipitate	0 (0-40)	0 (0-40)	0 (0-30)	0 (0-20)	0.19

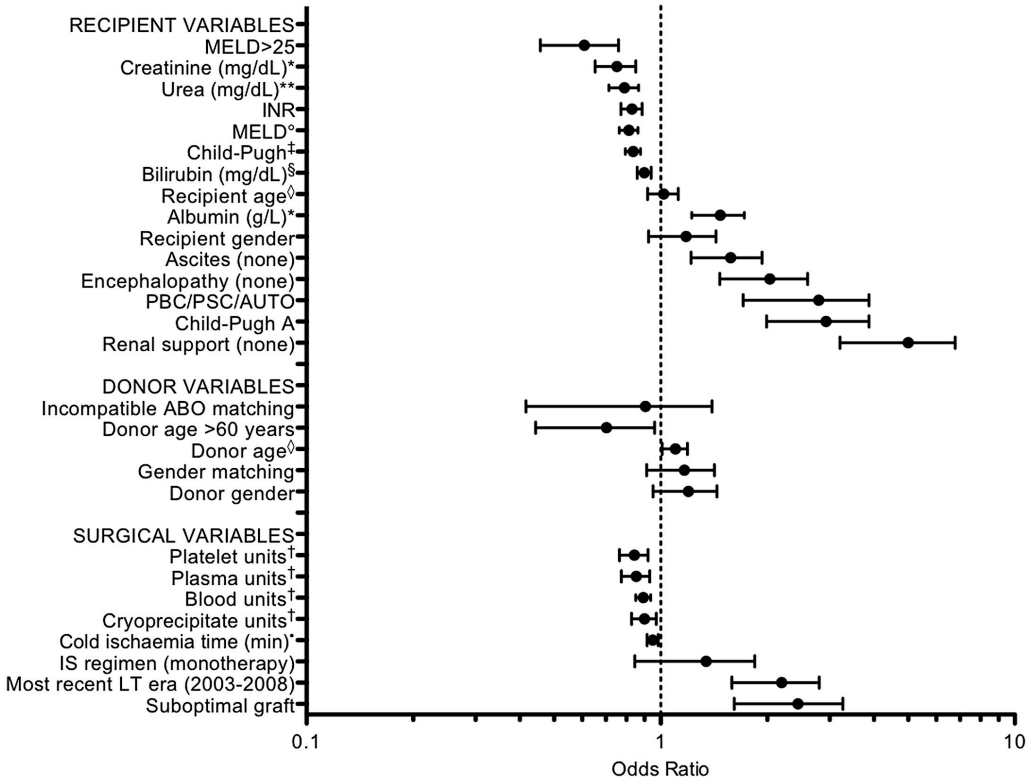
Table 3.2. Univariate and multivariate Cox regression analysis for risk of death or graft survival after liver transplantation in 648 transplanted patients who underwent first protocol liver biopsies between 4-14 days after liver transplantation.

	Unadjusted			Adjusted*		
	RH	95%CI	p	RH	95%CI	p
No ACR (reference)						
Any ACR	1.09	0.80-1.49	0.57	1.27	0.85-2.09	0.38
No ACR (reference)						
Mild ACR	1.59	1.03-2.46	0.016	1.48	0.90-2.53	0.082
Mod./Sev. ACR	1.05	0.67-1.66		1.21	0.76-1.92	
Other abnormalities	1.55	0.89-2.69		1.56	0.89-2.75	
No ACR (reference)						
Untreated ACR	1.34	0.96-1.88	0.017	1.54	1.09-2.17	<0.001
Treated ACR	0.89	0.63-1.26		0.98	0.69-1.39	
No ACR (reference)						
Mild untreated ACR	1.43	0.91-2.27	0.13	1.50	0.95-2.38	<0.001
Mild treated ACR	1.58	0.91-2.73		1.53	0.88-2.65	
Mod./Sev. untreated ACR	1.88	1.01-3.62		2.29	1.18-4.44	
Mod./Sev. treated ACR	0.95	0.59-1.52		1.08	0.67-1.74	
Other abnormalities	1.55	0.89-2.69		1.56	0.89-2.74	

**Adjusted for year of transplantation, ABO-group matching, units of blood received*

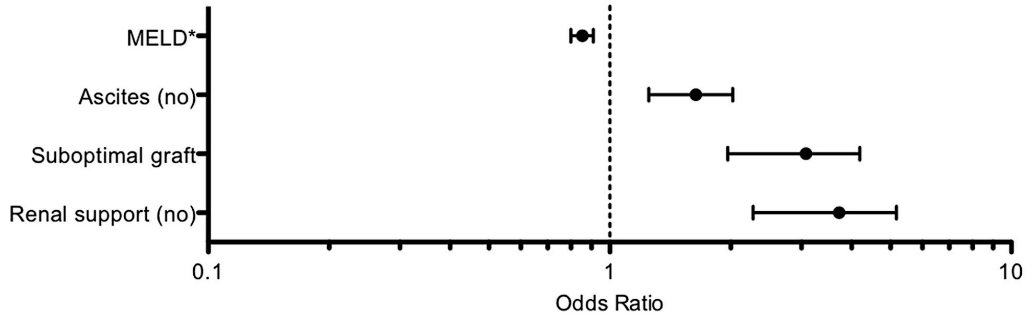
Figure 3.1. Risk factors for the development of ACR at first protocol biopsy in 648 patients between 4-14 days after liver transplantation. Figure 1A, 1B: univariate and multivariable logistic regression analysis of risk factors for ACR (mild/moderate/severe vs. no ACR/other abnormalities). Figure 1C: multivariable logistic regression analysis of risk for moderate/severe ACR (vs. no/mild ACR/other abnormalities).

A.



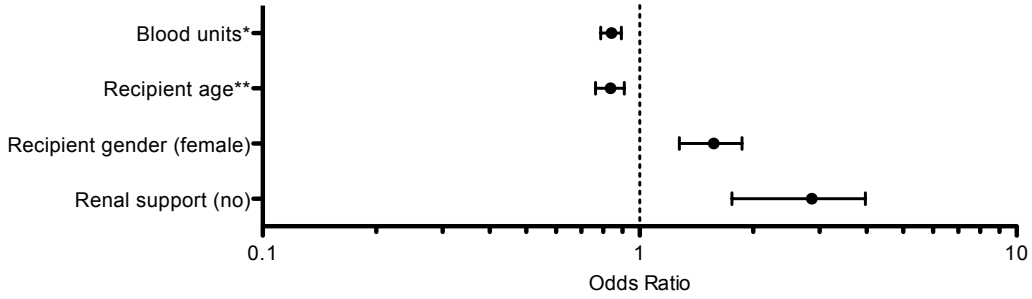
**per 1 unit increase; ** per 3 unit increase; °per 5 point increase; ‡per 1 point increase; §per 5 unit increase; ◊per 10 year increase; †per 4 unit increase; •per 60min increase*

B.



**per 5 unit increase*

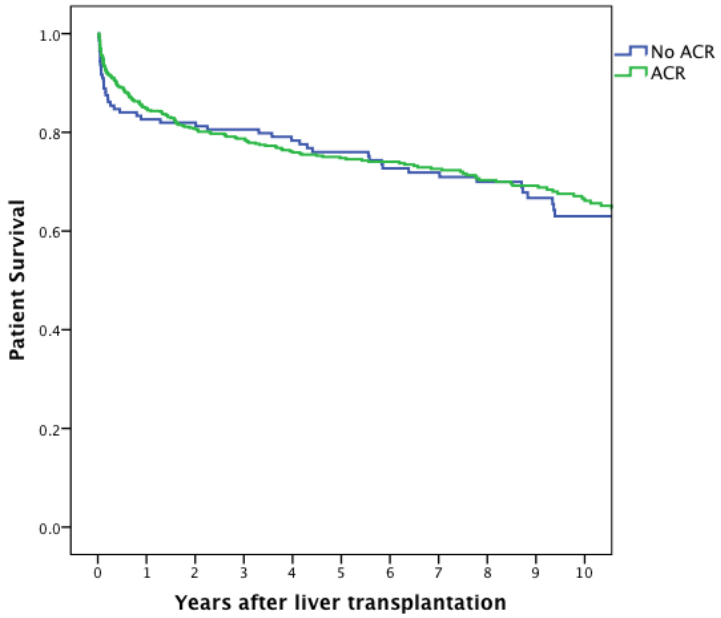
C.



** per 4 unit increase; ** per 10 year increase*

Figure 3.2. Patient (A) and graft (B) survival after liver transplantation according to presence/absence of acute cellular rejection at the first protocol biopsy.

A. 648 patients; log-rank p=0.92



B. 648 patients; log-rank p=0.57

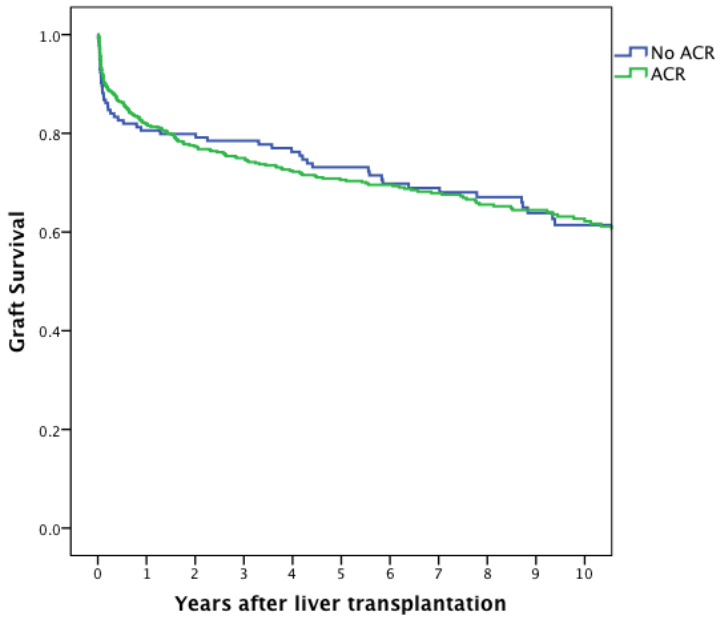
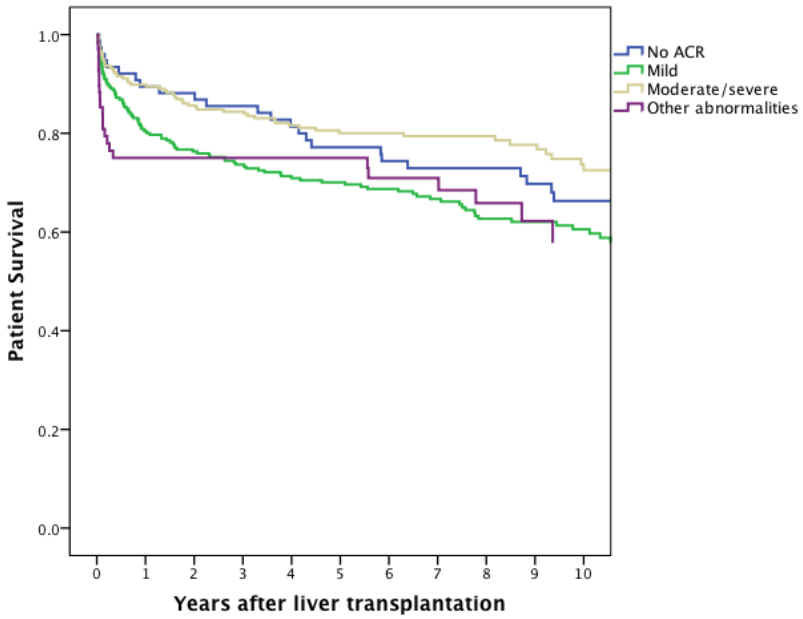


Figure 3.3. Patient (A) and graft (B) survival after liver transplantation according to histological severity of acute cellular rejection at the first protocol biopsy.

A. 648 patients; global log-rank p=0.04



B. 648 patients; global log-rank p=0.016

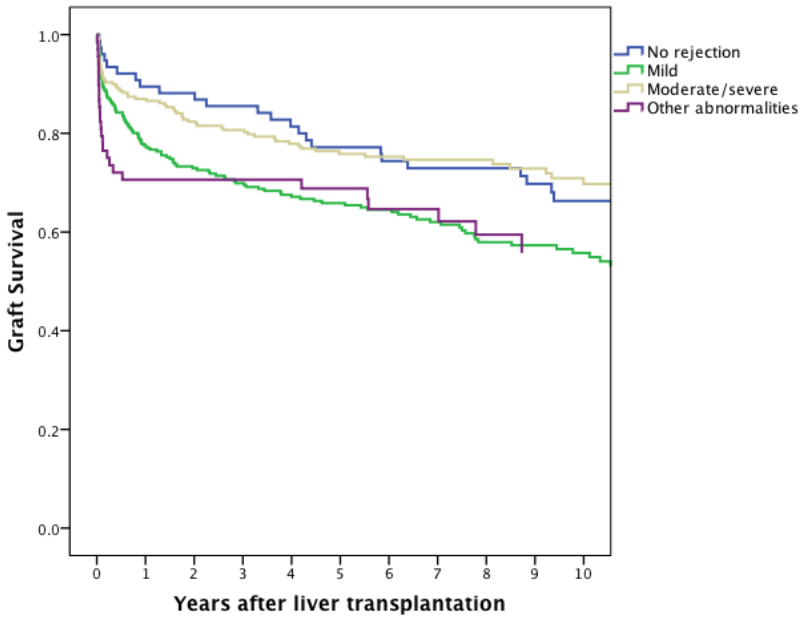
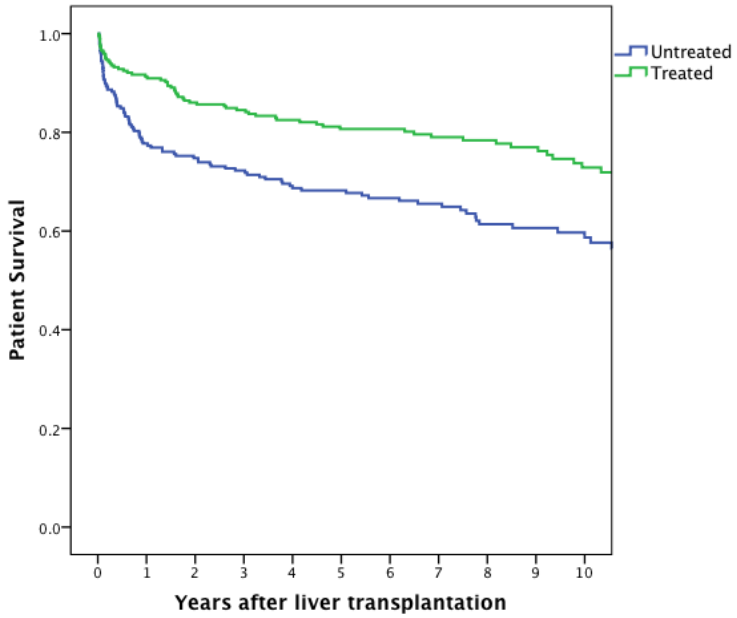


Figure 3.4. Patient (A) and graft (B) survival after liver transplantation according treatment or no treatment of the acute cellular rejection diagnosed at the first protocol biopsy.

A. 648 patients; log-rank p=0.001



B. 648 patients; log-rank p=0.006

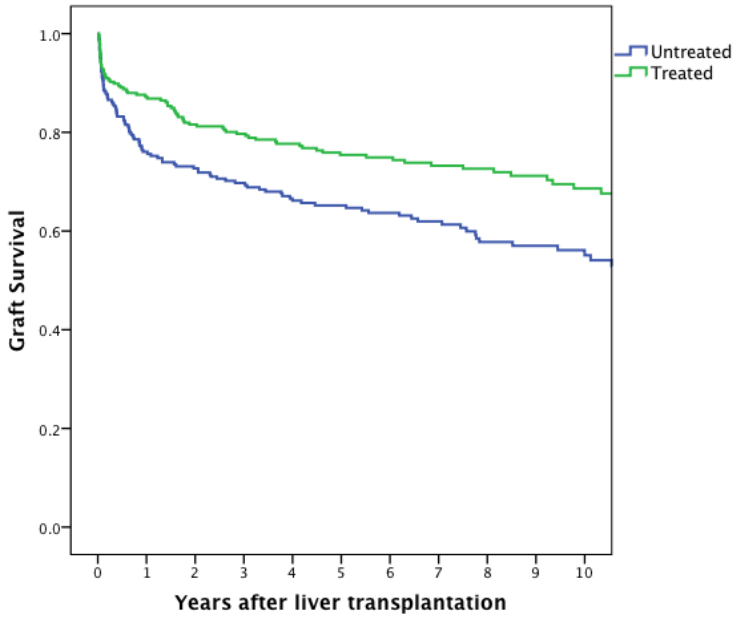
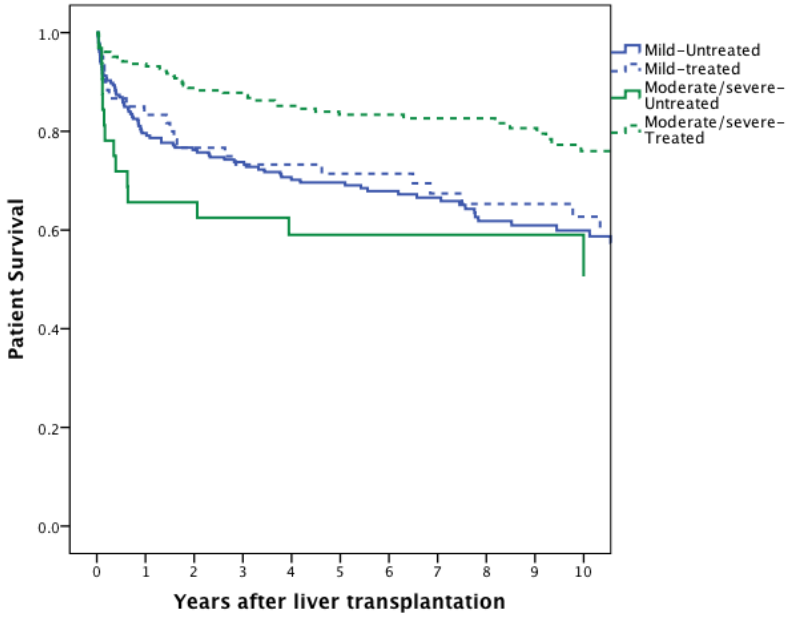
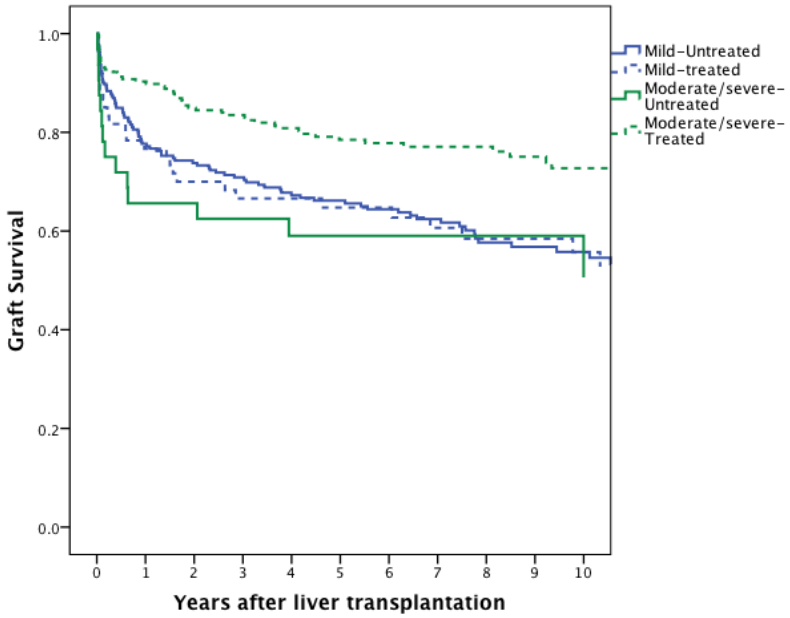


Figure 3.5. Survival after liver transplantation according treatment or no treatment of the acute cellular rejection diagnosed at the first protocol biopsy. Patients were stratified on the basis of histological severity of acute cellular rejection.

A. 648 patients, global log-rank p=0.002



B. 648 patients, global log-rank p=0.008



4. ROLE OF BLOOD EOSINOPHIL COUNT IN PREDICTING SEVERITY AND CLINICAL COURSE OF ACUTE REJECTION AFTER LIVER TRANSPLANTATION

4.1 Introduction

Eosinophils are typically involved in ACR, first reported as an association with ACR in kidney transplantation [113], and subsequently in lung and heart transplants [114]. In the liver graft, a portal tract eosinophilic infiltrate is a typical finding of ACR, which contributes diagnostically, adding to the Banff criteria [102]. As graft eosinophils come from blood, a high peripheral eosinophil count might predict histological ACR after liver transplantation. Absolute eosinophil count (AEC) increases in blood 2–3 days earlier than liver function tests and 3–4 days before ACR is proven histologically [115], and there is a positive correlation with eosinophilia in the liver graft [116].

However the diagnostic utility of blood eosinophilia for ACR has varied. The first report [84] found AEC (threshold >0.5 x 10^9/l) to have a negative predictive value (NPV) of 99% and a positive predictive value (PPV) of 44% for ACR. The only prospective study included only 20 patients [117]. Other studies [86, 115, 118] showed that AEC was a specific predictor of ACR with a high negative predictive power, but with inadequate sensitivity and low positive predictive power. In addition the predictive ability of a reduction in AEC following treatment of ACR is less studied [84, 86, 117, 118].

Previous studies have important limitations. Firstly, the major endpoint was prediction of any degree of ACR. However mild rejection is usually not treated and maintenance immunosuppression is not modified [119]. Secondly the sample size was insufficient to perform multivariate analyses, and inadequate to address

whether combining liver function tests and eosinophil count could predict ACR more accurately. Finally several biopsies were evaluated per patient without differentiating the interval from transplantation, thus introducing systematic errors, and making results less clinically relevant.

4.2 Materials and Methods

We identified 690 patients in our prospectively collected liver transplant database between October 1988 and February 2008 during which interval protocol biopsies were obtained 5-7 days after first liver transplantation to establish the presence and severity of ACR. There were another 75 patients in whom graft biopsy was not available in the first 2 weeks after transplant because of early death (n=20), re-transplant (n=13) or other complications and were not analysed. Routine laboratory tests including liver function profile were evaluated on the day of the biopsy. AEC (normal range 0-0.46 x 10^9/l) was recorded the day before and on the day of the biopsy. Relative eosinophil count (REC) was calculated with the following formula: AEC x 100/total white cell count (threshold 3.5%).

AEC on the day of the second biopsy and ΔAEC between the first and second biopsy were evaluated as potential predictors of clinical course of ACR and response to treatment. The threshold chosen for ΔAEC was the null value (0 x 10^9/l), which meant no change in eosinophil count between the first and second biopsy.

Liver biopsies were examined to assess and grade ACR according to the Royal Free system [102], which predates the Banff schema [12]. The Royal Free ACR system applies the same histopathological diagnostic criteria (mixed mainly portal inflammation, endothelitis and bile duct damage) as the Banff schema, except

that the Royal Free system evaluation of eosinophils in the inflammatory infiltrate is included as a separate additional axis of assessment. The immunosuppression protocol started immediately after transplant with intravenous methylprednisolone (1 mg/kg/day until July 1997 or 16 mg daily thereafter, followed in both cases by 20 mg oral prednisolone daily once gut function was restored) and AZA (1 mg/kg/day) in addition to either Tac (initially 0.1 mg/kg/day in two divided doses) or CsA (initially 10 mg/kg/day in two divided doses). CNI doses were run on the lower side of the therapeutic range and adjusted according to serum levels, the presence of infection or toxicity. Between October 1996 and January 1997 a clinical trial was conducted [99] during which patients were randomized to receive monotherapy with Tac versus CsA. From May 1997 to April 1999 patients were randomized to triple therapy based on either Tac or CsA [100]. Thereafter a cohort of patients received Tac monotherapy [101]. At all times standard treatment for ACR consisted of 1 g of intravenous methylprednisolone given on three consecutive days. However 28 patients (9.5%) received two boluses and 20 patients (6.8%) received just one bolus because of individual clinical circumstances.

In 487 patients, a second biopsy was obtained after 6.1±2 days from the first one to assess the course and response to treatment. We evaluated the whole group for the presence of ACR, and then patients who had or who had not received boluses of steroids in relation to the change in eosinophil count. According to the grade of ACR in the second biopsy, patients were classified in three groups: (i) improvement: when ACR grade improved from moderate or severe to mild or no ACR; (ii) deterioration: opposite of the previous; (iii) no change: when no significant histological change was found.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, USA). Variables are displayed in frequency tables or expressed as means and standard deviations, except those with an asymmetric distribution, which are described with medians and interquartile ranges (IQR). Testing for differences between groups were performed using Chi square test for frequencies, student's t test or anova tests for quantitative variables and Mann–Whitney's U test or Kruskal-Wallis for variables with an asymmetric distribution. The optimal threshold value for peripheral eosinophil count with respect to moderate/severe ACR was established by receiver operating characteristic (ROC) curves. We used multiple logistic regression to control for possible confounding factors and to evaluate the combination of eosinophil count and other routine laboratory tests, which have also been used in previous papers, (AST, ALT, AST/ALT ratio, ALP, GGT, bilirubin, albumin, urea and creatinine) in predicting moderate/severe ACR. The same method was used to identify those variables independently related with histological improvement of ACR. Every hypothesis tested was two tailed and considered statistically significant if $p<0.05$.

4.3 Results

4.3.1 Descriptive evaluation

There were 690 patients of whom 425 (61.6%) were men. Major aetiologies were alcoholic liver disease (17.1%), HCV (13.3%), or their combination (5.1%), HCC (11.4%), PBC (13%), ALF (8%), PSC (7%), HBV (5.8%) and cryptogenetic cirrhosis (5.1%). A protocol liver biopsy was obtained 6±2.5 days after liver transplantation. ACR was found in 532 patients (77.1%) which was mild in 294

(42.6%), moderate in 211 (30.6%) and severe in 27 (3.9%) biopsies respectively and 158 patients (22.9%) had no histological ACR. In 294 cases (42.6%) boluses of corticosteroids were given after the first biopsy, with 90 (30.6% treated) patients having mild ACR, 178 (84.4% treated) moderate ACR and 26 (96.3% treated) severe ACR.

A second biopsy was taken 6.5±2 days after the first one in 487 patients (70.6%). The group who had a second biopsy had more severe ACR on the first biopsy (moderate-severe rate 42.3% vs. 15.8%; $p<0.001$) and subsequently received more corticosteroid boluses and AZA (Table 4.1).

In the group that had received bolus steroids, an improvement was seen in 102 (40.6%) patients, 23 (9.2%) showed deterioration and 126 (50.2%) remained unchanged. With regard to the patients who did not receive bolus of steroids initially, only 17 (7.2%) improved while 56 (23.7%) showed deterioration and 163 (69.1%) remained unchanged. Considering subgroups according to the grade of ACR on the first biopsy, of 99 patients initially classified as 'no ACR' 37 (37.4%) remained unchanged while 62 patients worsened (45.5% to mild ACR and 13.1% to moderate-severe ACR). From 182 patients with mild ACR on the first biopsy, improvement to no ACR was seen in 30 patients (16.5%) and deterioration to moderate-severe ACR occurred in 66 patients (34%). Finally from 206 patients with moderate-severe ACR at baseline, improvement was detected in 119 cases (97 (47.1%) passed to mild ACR, and 22 (10.7%) to no ACR).

4.3.2 Laboratory variables as predictors of moderate or severe ACR

The univariate analysis showed that both AEC and REC were higher in ACR patients especially when moderate-severe ACR occurred (Figure 4.1).

In the ROC analysis, the area under curve was 0.58, 0.59 and 0.57 for AEC, AEC (day-1) and REC respectively. Values of sensitivity, specificity, PPV and NPV tested for several cut-off points related to moderate or severe ACR are shown in Table 4.2.

It is noteworthy that, although sensitivity and specificity vary (occasionally exceeding 90%) depending on the cut-off point chosen, predictive values for moderate/severe ACR are relatively constant and lower than 70% for most scenarios.

The initial immunosuppression regimen used immediately after transplantation, and the indication for liver transplant did not influence either AEC or grade of ACR. Nevertheless those patients with PSC showed higher levels of AEC (0.6 x 10^9/l; IQR 0.14-1.3) at first biopsy than other indications (0.3 x 10^9/l; IQR 0.16-0.52) (p=0.01).

Patients with moderate to severe ACR were also characterized by higher bilirubin and cholestasis parameters with lower AST, AST/ALT ratio, albumin, urea and creatinine (Table 4.3) than those with mild or no ACR. Serum bilirubin, GGT, albumin, urea and AEC on the day of biopsy were independently related with the degree of ACR in the multivariate analysis (Table 4.3).

The combination of these serum parameters in the logistic regression analysis had 73% sensitivity and 52.9% specificity which was only a marginal improvement compared with AEC alone (global precision improved from 0.62 to 0.65). ALP and creatinine were tested within the model instead of GGT and urea respectively, but they did not reach statistical significance. It is noteworthy that the ALT value, which is widely used as a marker of ACR in clinical practice, was not related to the presence or grading of ACR (Figure 4.2). The ALP was related to ACR but because of the wide overlap it cannot be used as a marker of ACR

nor its severity (Figure 4.2). The immunosuppression regimen and the indication for liver transplant (primary sclerosing cholangitis) were both included and then excluded as possible confounding factors for the association between AEC and grade of ACR (Table 4.3).

4.3.3 Peripheral eosinophil count as biological marker for ACR

In the second biopsy, 89 cases (18.3%) showed no ACR, 232 (47.6%) had mild ACR, 135 (27.7%) moderate ACR and 31 (6.4%) severe ACR. Compared with the first biopsy, there was an improvement for 119 patients (24.4%) and deterioration for 79 (16.2%) while 289 (59.3%) remained unchanged. The AEC on the day of the second biopsy and the change in AEC between the first and the second biopsy were closely related with the histological course of ACR (Figure 4.3).

A decrease in AEC was associated with an improvement of the histological grade of ACR. In the ROC curve the AUC for ΔAEC was 0.72 (95% CI 0.66-0.78) while for AEC on the day of the second biopsy it was 0.34 (95% CI 0.27-0.40). The best threshold for ΔAEC was no increase i.e. 0 x 10^9/ l (Sensitivity = 75%; Specificity = 64%). In the subgroup of patients with moderate-severe ACR on the first biopsy, AEC decreased in those patients who achieved histological improvement (ΔAEC = 0.19x10^9/l; IQR 0.007-0.46) while a trend to rise in AEC was seen in patients who remained unchanged (ΔAEC = -0.06x10^9/l; IQR -0.23 – 0.27), with statistically significant differences between them ($p<0.001$). With regard to liver function tests, there was a trend to a rise in parameters of cholestasis (GGT and ALP) between the first and the second biopsy, which was significantly greater for ALP in those cases without improvement in the second biopsy (Table 4.4). Nevertheless in the multiple logistic regression, the only

independent predictors of good histological course were ΔAEC and treatment with boluses of steroids (Table 4.4).

In the present cohort, 108 patients at the first biopsy had ALT levels lower than 100 IU, and among these 39 patients (36.1%) showed moderate or severe ACR. In this subgroup of patients, the ΔAEC was more accurate in predicting clinical course of ACR. In the ROC curve, the area under the curve was 0.81 and, with a threshold of no increase i.e. 0 x 10^9/l, the sensitivity was 82% and specificity was 69%.

4.3.4 Blood eosinophils and histological improvement of ACR

AEC on the day of the first biopsy was comparable between patients who had a second biopsy and patients without a second biopsy (0.33 x 10^9 IQR 0.17–0.58 vs. 0.30 x 10^9 IQR 0.14–0.5; p=0.44). Among the patients with a second biopsy, the subgroup, who received boluses with steroids achieved improvement in the biopsy grade of ACR (102/251; 40.6%), more frequently than the subgroup who did not receive bolus steroids (17/236; 7.2%) (p<0.001). This may be explained in part because of differences in the grade of ACR in the first biopsy (Figure 4.4).

In the subgroup of 251 patients who were given corticosteroids boluses, AEC on the day of the second biopsy and ΔAEC between the first and the second biopsy were related to the likelihood of treatment response (p=0.001 and p<0.001 respectively) (Figure 4.5). In the ROC curve, the area under curve for ΔAEC was higher (0.66) than for AEC on the day of the second biopsy (0.35). An AEC rising higher than 0.3 x 10^9/l between the first and second biopsy was associated with a high likelihood of no response to bolus steroids (78.3%), with a sensitivity for this threshold of 94.8%. Among patients with moderate-severe ACR on the first biopsy who received bolus steroids, the AEC decrease was greater in those

cases with improvement (ΔAEC = 0.19 x 10^9/l; IQR 0–0.48) compared with those who did not (ΔAEC = -0.04 x 10^9/l; IQR -0.23–0.38; p=0.001). Improvement in grade of ACR was also more frequent in patients who received 3 boluses of steroids (97/212; 45.8%) than in those who received a lower dose (5/39; 12.8%) (p<0.001); nevertheless the number of boluses did not influence AEC on he day of the second biopsy (p=0.63) and neither ΔAEC between the first and second biopsies (p=0.21). The delta value of liver function tests (bilirubin, AST, ALT, AST/ALT ratio, ALP and GGT) did not correlate with the likelihood of treatment response (p=0.22, p=0.89, p=0.31, p=0.34 and p=0.11 respectively). The multivariate analysis (which included ΔAEC, steroid dose, immunosuppression protocol and delta of liver function tests) identified ΔAEC as the only independent variable able to predict the histological response of ACR after treatment with boluses of steroids (OR=2.77; 95%CI 1.4-5.5; p=0.004) although the association was marginally less than in the group overall (OR decreased from 3.12 to 2.77).

With regard to the subgroup of 236 patients who had not received steroids, the ΔAEC, but not the AEC, on the day of the second biopsy was related to the likelihood of ACR improvement (p=0.009 and p=0.11 respectively) (Figure 4.5). Delta values of bilirubin, AST, ALT, and GGT between the first and the second biopsies were not related to the likelihood of ACR improvement in the second biopsy (p=0.19, p=0.91, p=0.23 and p=0.08 respectively). As described previously in the whole cohort, a larger difference in ΔALP was seen in the group who did not improve (106 vs. 23 IU/l; p=0.015). Among patients in this group with moderate-severe ACR on the first biopsy, the AEC decreased in those patients who improved (ΔAEC = 0.21 x 10^9/l; IQR 0.02–0.24) while it increased in those who did not (ΔAEC = -0.11 x 10^9/l; IQR -0.37–0; p=0.008). Multivariate analysis

could not be performed in this subgroup because of the small number of patients that improved the grade of ACR in the second biopsy (n=17).

4.4 Discussion and conclusions

This study evaluated the clinical usefulness and accuracy of peripheral blood eosinophil count for predicting moderate and severe ACR, as well as its clinical course and response to treatment with steroids in a large cohort of liver transplant patients. Since there is no consensus on the definition of ACR based on liver function tests, which are also poorly correlated with its grade, histological ACR was used as the gold standard to evaluate the accuracy of eosinophils.

Absolute eosinophil count measured on the day before or on the day of the biopsy was higher in patients with ACR, which is in agreement with previous reports [84, 86, 116-118], and was related to the histological grade of rejection confirming our previous observations in 275 liver biopsies from 101 patients [86]; this correlation is more likely as eosinophils in the histological infiltrate are an independent marker of ACR [102]. However using the upper limit of normal range (0.46 x 10^9/l) the PPV and NPV were only 66.5% and 51.9% respectively. Even when thresholds with a higher sensitivity and specificity were tested, the predictive values did not exceed 70% in any situation, so this parameter is not itself sufficiently predictive to guide therapeutic decisions. Our results do not confirm the high NPV in 51 patients [115], and in 60 patients [84], nor high specificity in 167 patients [118] of a raised AEC, found in previous studies.

Other studies have shown that several routine biochemical laboratory tests are related to the presence of ACR [87, 90, 120]. When we assessed these parameters in association with the AEC in predicting moderate-severe ACR, the

AEC was the strongest related parameter (OR = 2.15) and a higher bilirubin and GGT with a lower albumin and urea, independently predicted moderate-severe ACR. However, the combination of these tests only marginally improved the global precision of AEC (0.62 to 0.65). Thus the benefit of combining AEC with routine biochemical laboratory tests was limited.

Previous studies [84, 86] did not evaluate relationships between peripheral eosinophil counts and histological changes. A higher AEC or REC before treatment predicted biochemical response to bolus corticosteroids with a sensitivity and specificity ranging from 45% to 50% in one study of 140 paired biopsies [118]. In our study, only the ΔAEC between the first and second biopsy and treatment with bolus steroids were independent predictive factors for histological improvement in the multivariate analysis. In the group overall the sensitivity and specificity of ΔAEC for predicting improvement was 75% and 64% respectively (threshold 0 x 10^9 which meant no difference in ΔAEC between the first and second biopsies). These results were consistent among the group of patients with moderate-severe rejection on the first biopsy. Importantly when transaminases were low (ALT <100 IU/l), the accuracy of DAEC was improved (sensitivity 82% and specificity 69%). Thus ΔAEC is a simple non-invasive parameter that helps to assess the course of ACR, whereas differences in standard liver function tests were not helpful.

In patients treated with boluses of corticosteroids, it is reasonable to expect a lower predictive power for ΔAEC because steroids lower blood eosinophil counts. However this was not a major issue: OR for ΔAEC in the multivariate analysis decreased from 3.12 in the group overall to 2.77 in the bolus corticosteroid group. The ΔAEC was not affected by type of maintenance immunosuppression

including steroids, which were used at much lower doses compared with bolus doses.

In conclusion, we found that, although the AEC is independently related to moderate and severe ACR, its predictive power is not accurate enough to make therapeutic decisions using this parameter alone. The combination of AEC with other ACR independently associated variables (i.e. bilirubin, GGT, albumin and urea) only had a marginal benefit in terms of diagnostic precision. However, the changes in AEC used as a monitoring test can provide valuable information about the histological course and the likelihood of response to boluses of steroids for treatment of moderate-severe rejection. This finding is particularly useful if biopsies are not routinely performed to diagnose acute cellular rejection and assess response to therapy. Nevertheless it would be useful to have a consensus definition for clinical rejection, which might include peripheral eosinophil count and would need a correlation with protocol biopsies.

4.5 Tables and figures

Table 4.1. Baseline characteristics in patients who had a protocol first liver biopsy after transplantation (days 6±2.5) who then had or did not have a second biopsy.

	Group with 2nd biopsy (n=487)	**Group without 2nd biopsy** (n=203)	**p**
Moderate/severe ACR at first biopsy	206 (42.3%)	32 (15.8%)	<0.001
Tacrolimus	343 (70.4%)	155 (76.4%)	0.11
Ciclosporine	113 (23.2%)	38 (18.7%)	0.19
Azathioprine	214 (43.9%)	69 (34%)	0.015
Mycophenolate	50 (10.3%)	19 (9.4%)	0.71
Prednisone	226 (46.4%)	93 (45.8%)	0.88
Steroid boluses	251 (51.5%)	43 (21.2%)	<0.001

Table 4.2. Accuracy of AEC and REC to predict histological moderate or severe rejection in the first (protocol) biopsy after liver transplantation (days 6±2.5). Normal range of AEC: 0-0.46 $x10^9$/L where $0.46x10^9$/L is the upper limit of the normal range.

	Cut-off point	Sensitivity	Specificity	PPV	NPV
AEC ($x10^9$)	1	8.2%	93.6%	44.4%	62.7%
	0.46	43.8%	73%	49.7%	68.1%
	0.2	70.6%	39.5%	41.5%	68.9%
	0.1	86.6%	21%	40%	72%
AEC (day-1) ($x10^9$)	1	2.7%	96.7%	33.3%	61.9%
	0.46	27.3%	79.4%	44.7%	64.1%
	0.2	62.6%	52.6%	44.7%	69.7%
	0.1	78.1%	37.9%	43.5%	70.9%
REC (%)	6	25.7%	76.2%	40%	62.4%
	3.5	57.2%	55.4%	44.2%	66.7%
	2.5	70.1%	45.5%	44.3%	71.1%
	1.2	81.8%	29.4%	41.7%	72.4%

Table 4.3. Relationship between laboratory variables and histological grade of rejection in the first protocol liver biopsy after liver transplantation (days 6±2.5). Univariate analysis and multiple logistic regression (n=690 patients).

	Unadjusted Analysis Histological ACR			Adjusted Analysis Moderate-severe ACR	
	None-mild	**Moderate-severe**	**p**	**OR (95%CI)**	**p**
AEC (x10^9/L)	0.28 (IQR 0.13, 0.5)	0.40 (IQR 0.18-0.64)	<0.001	2.15 (1.2-3.8)	0.007
Bilirubin (µmol/L)	92±82	97.5±76.2	0.39	1.0 (1.0-1.01)	0.04
AST (IU/L)	88 (IQR 50, 206)	74 (IQR 48, 129)	0.01		
ALT (IU/L)	279 (IQR 136, 565)	250 (IQR 132, 531)	0.65		
AST/ALT	0.53 (IQR 0.32, 0.8)	0.43 (IQR 0.3, 0.7)	<0.001		
ALP (IU/L)	106 (IQR 70, 161)	133 (IQR 87, 213)	<0.001		
GGT(IU/L)	189 (IQR 105, 328)	284 (IQR 167, 412)	<0.001	1.0 (1.01-1.03)	0.003
Albumin (g/L)	30±8.2	27.4±7.7	<0.001	0.96 (0.93-0.99)	0.02
Urea (mg/dL)	13.5±8.8	10.6±7.4	<0.001	0.96 (0.93-0.99)	0.04
Creatinine (µmol/L)	133±73	120.3±72.2	0.034		

Confounding factors controlled for: aetiology (primary sclerosing cholangitis, immunosuppression (cyclosporine vs. tacrolimus; maintenance prednisone; maintenance azathioprine)

Table 4.4. Variables related to histological improvement of acute cellular rejection in the second biopsy performed to assess course of rejection (6.1±2 days after first biopsy). Univariate analysis and multiple logistic regression (n=487 patients).

	Univariate analysis			Multivariate analysis (improvement)		
	Improvement	**No change/ deterioration**	**p**	**OR**	**CI95%**	**p**
Bolus steroids (%)	102/119 (85.7%)	149/368 (40.5%)	<0.001	10.09	4.7-21.4	<0.001
ΔAEC ($x10^9$/L)	0.25 (IQR 0.05-0.5)	-0.04 (IQR -0.3, -0.2)	<0.001	3.12	1.5-6.2	0.001
ΔBilirubin (µmol/L)	10.5 (IQR (-25, -38)	5 (IQR -40, -28)	0.16			
ΔAST (IU/L)	15.5 (IQR (-26, -54)	18 (IQR -28, -73)	0.41			
ΔALT (IU/L)	98.5 (IQR 11, 382)	143 (IQR 18, 362)	0.97			
ΔALP (IU/L)	-80 (IQR (-241, -7.5)	-130 (IQR -269, -17)	0.036			
ΔGGT (IU/L)	-38 (IQR (-318, -74)	-138 (IQR -342, 0)	0.095			

Confounding factors controlled for: aetiology (primary sclerosing cholangitis, immunosuppression (cyclosporine vs. tacrolimus; maintenance prednisone; maintenance azathioprine)

Figure 4.1. Absolute eosinophil counts on the day of the biopsy (AEC) and on the day before (AEC day-1) and relative eosinophil count (REC) according to histological grade of rejection in the first protocol biopsy performed 6±2.5 days after liver transplantation. Medians and IQR are shown.

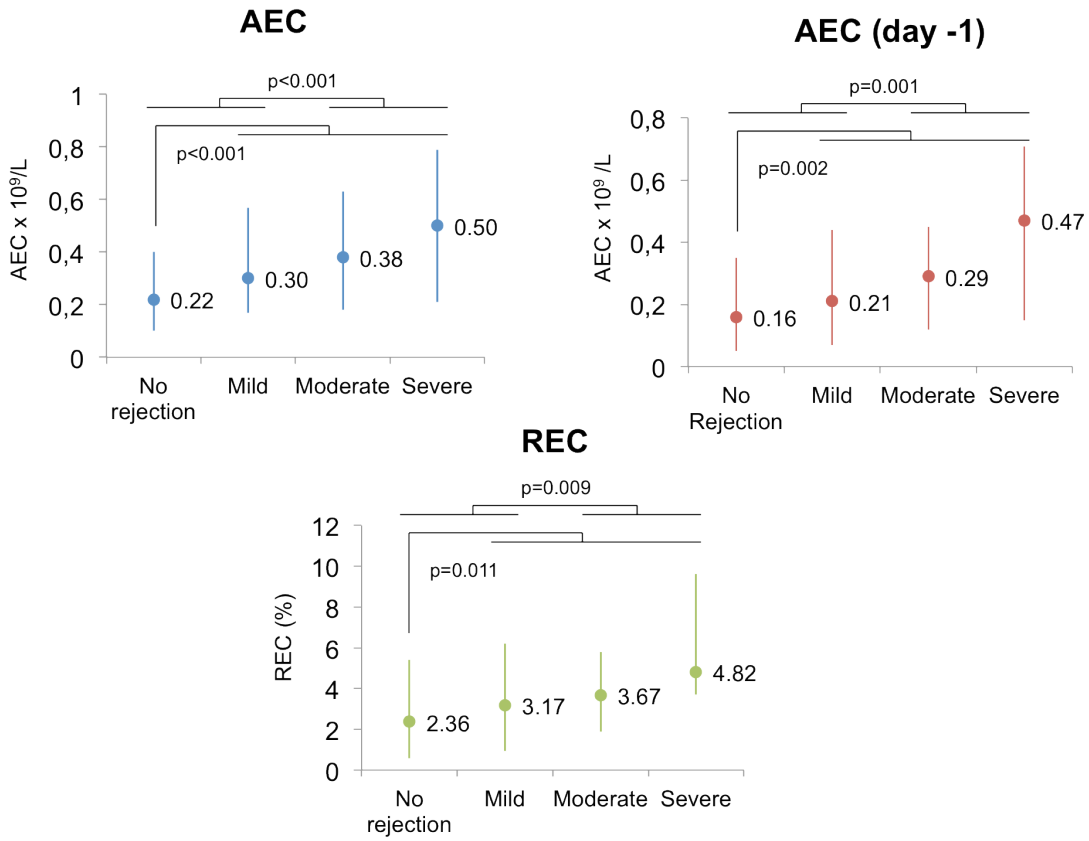
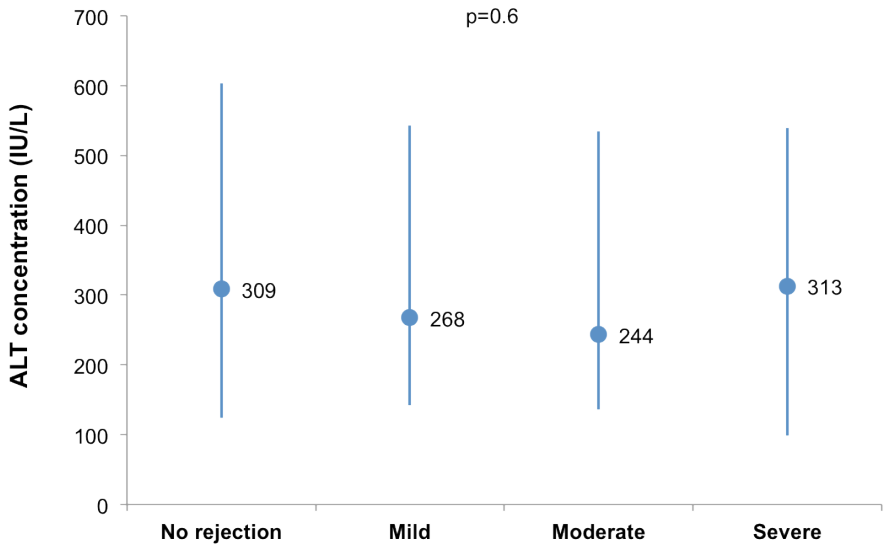


Figure 4.2. ALT and ALP concentration and histological level of rejection in the first protocol liver biopsy performed 6±2.5 days after liver transplantation.

A.



B.

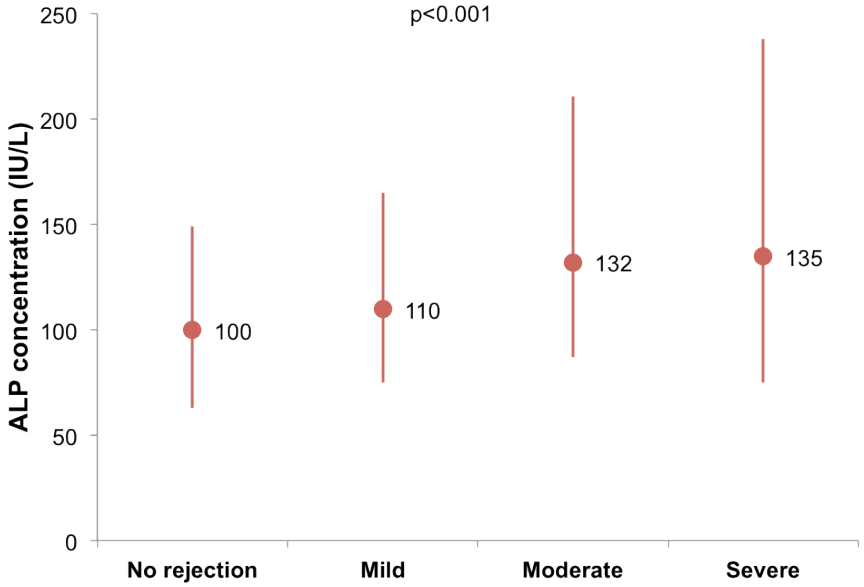
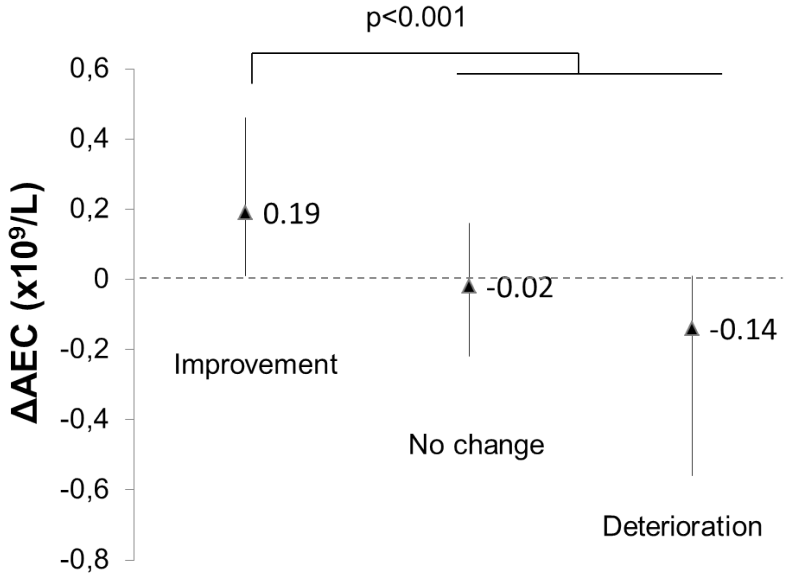


Figure 4.3. Delta of absolute eosinophil count (ΔAEC) between the first protocol biopsy and the second biopsy and absolute eosinophil count on the day of the second biopsy (AEC 2ndbx) after liver transplantation and their relationship with histological change of rejection in the whole cohort (n = 690). Medians and IQR are shown.

A.



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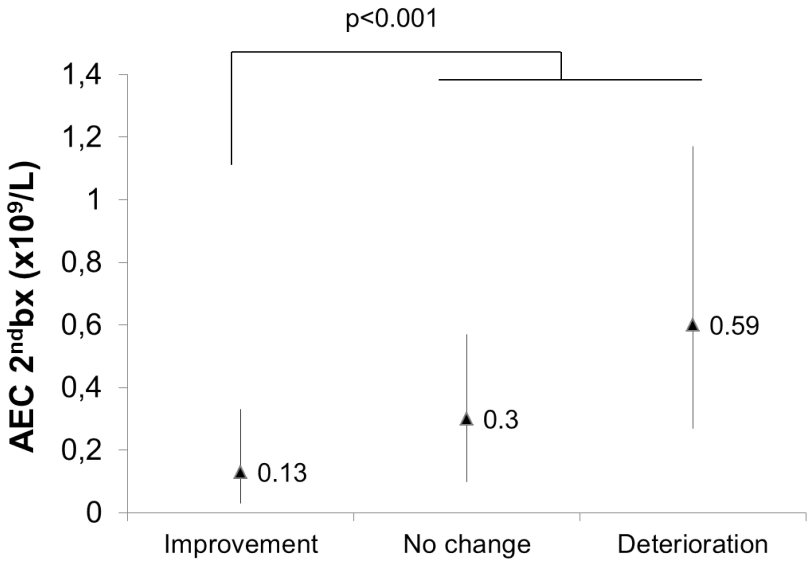


Figure 4.4 Grade of ACR in the first protocol biopsy after liver transplantation depending on whether boluses of steroids were subsequently given. The proportion of moderate/severe rejection was higher at baseline in the steroid group (p<0.001).

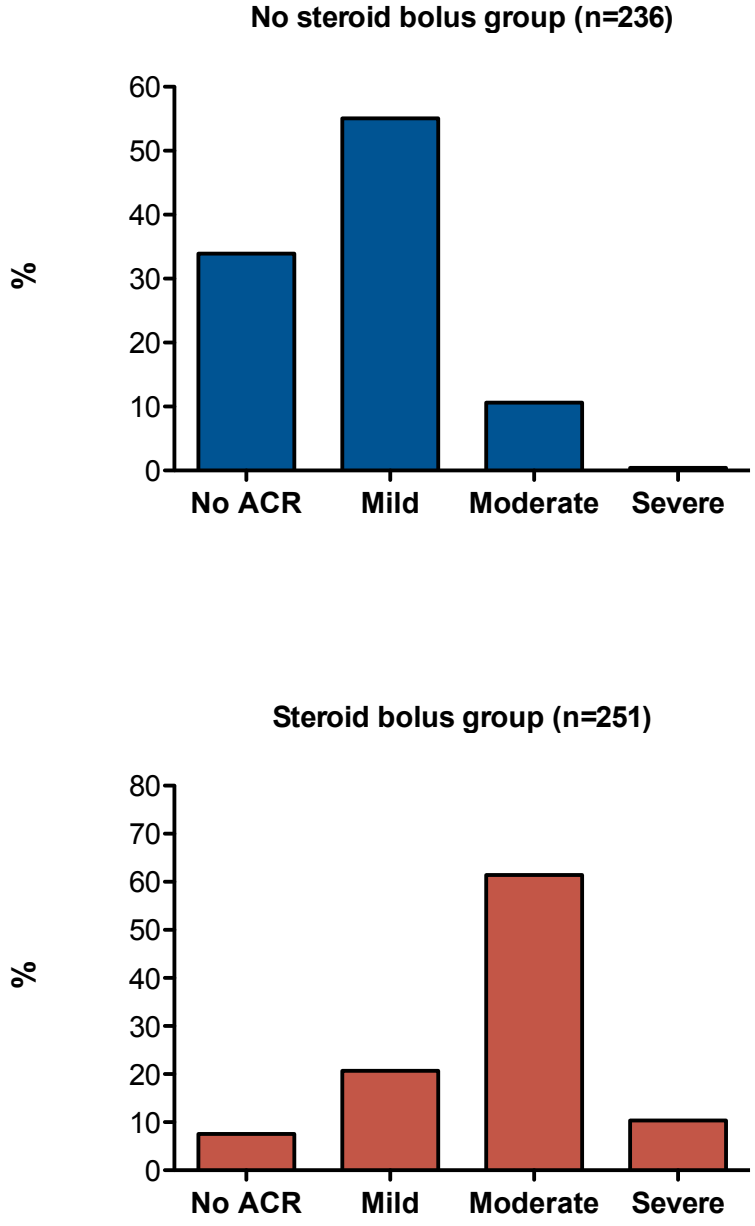
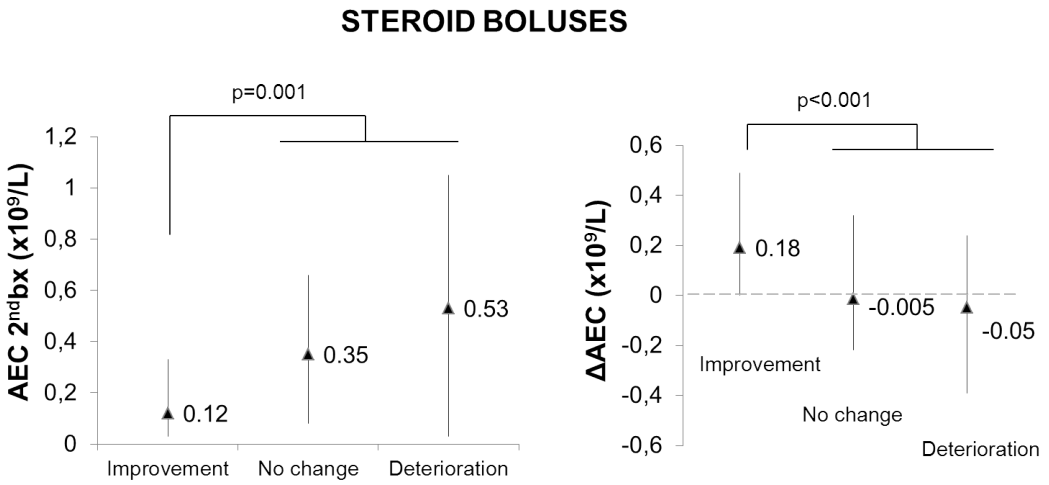
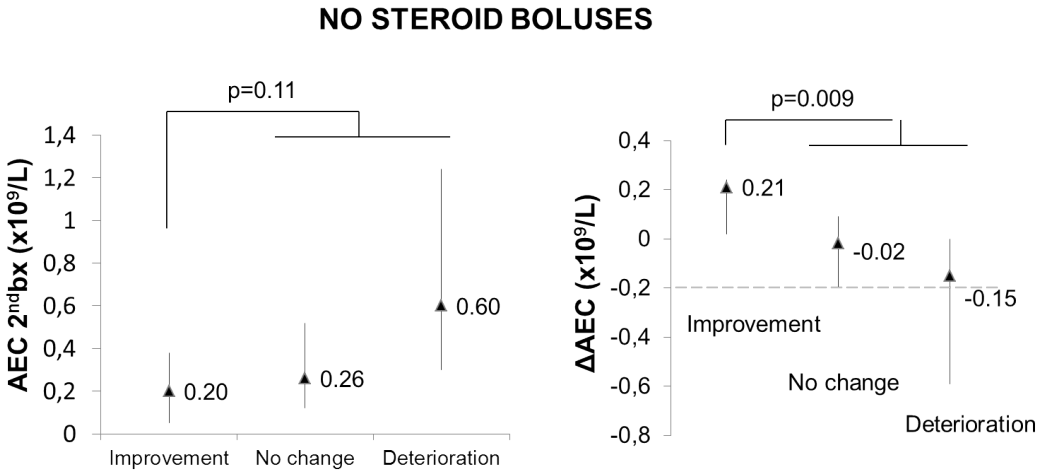


Figure 4.5. Delta of absolute eosinophil count (ΔAEC) between the first protocol biopsy and second biopsy after liver transplantation and absolute eosinophil count on the day of the second biopsy (AEC 2ndbx) related to histological course of rejection whether boluses of steroids were used (n=251) or not (n=236). Medians and IQR are shown.

A.



B.



5. IMMUNE MONITORING BEFORE AND AFTER LIVER TRANSPLANTATION

5.1 Introduction

Measuring the concentration of immunosuppressive drugs in the serum of patients is generally used as a surrogate for the level of immunosuppression, but it does not provide information about the magnitude of suppression of the immune system. In addition, there is generally a poor correlation between ACR and immunosuppression levels or the degree of liver test abnormalities. Therefore a marker for the appearance of ACR, or able to predict patients who could tolerate reduced immunosuppression, would be important for improving post-transplant management of liver transplanted patients.

In the cascade of events inducing ACR, the second signal is represented by the interaction between the CD28 molecule and the B7 ligand [121]. On the other hand the expression of CD38, a marker of activation of T-lymphocytes [122], has been shown to be a marker of cytomegalovirus infection in transplant recipients [123]. Therefore the expression of these two proteins should be evaluated as potential marker of immunological status.

Recent studies have shown that a distinct Treg subset expressing CD4, CD25, the α-chain of the IL-2 receptor, and the transcription factor Foxp3, was able to suppress activation of effector T cells [124, 125]. Tregs can also play a central role in modulating allograft rejection in animal models of transplantation [126-128]. These data have suggested that the presence of Tregs in the periphery may be crucial to allow graft acceptance and possibly to develop tolerance.

However, there is little information about the clinical significance of circulating Tregs in liver transplanted patients.

Lastly, Th17 cells have been cast as major players in autoimmunity, but their specific role in allograft rejection is under investigation. In the peri-transplant period, the production of pro-inflammatory cytokines such as IL-6, TNF-α, TGF-β, IL-12, and IFN-γ promote the acquisition of cytodestructive Th1 and Th17, characterized by the production of IL-17. This induces the generation of graft-destructive lymphocyte populations and simultaneously blocks the development and suppressive function of Tregs [129, 130]. It has been shown that the presence of Th17 cells in the allograft might be a biomarker of detrimental tissue inflammation rather than part of a mechanism that mediates graft destruction [129-134]. Despite in renal transplanted patients a link has been found between IL-17 and ACR [135, 136], data in liver transplant setting are still controverse.

5.2 Materials and Methods

5.2.1 Patient cohort

Patients listed for liver transplantation and transplanted at the Royal Free Hospital (London, UK) were included in this prospective study.

Inclusion criteria were: age ≥17 years, written informed consent, and follow-up after liver transplantation performed at Royal Free Hospital.

Patients transplanted for acute liver failure were excluded from the study.

Patients considered vulnerable, such as patients with learning difficulties or with English as a second language, were included in this study, only if it was felt that the research might have benefit them. Vulnerable patients were enrolled into the

study providing that they had a good general understanding of what is being investigated. Translators were available to explain the study if required.

Peripheral blood samples were obtained from all patients before (at the time of listing and/or at the day/night of transplantation) and after liver transplantation at specific time points (day 3, day 5, day 15, day 30, day 60), as well as at time of protocol liver biopsy or if patients were readmitted after discharge.

The first protocol liver biopsy was planned between 7 and 13 days after liver transplantation. All biopsy samples were fixed in formalin and embedded in paraffin. Histological sections (4 µ thick; 3 levels through the tissue block) were stained with hematoxylin and eosin, and other histochemical stains were prepared as required.

The diagnosis of ACR was based on pathologic findings, using the Banff classification [12]. Patients who developed ACR were treated with 1g of intravenous methylprednisolone given on three consecutive days.

5.2.2 CD25, CD28 and CD38 assessment

Heparinized blood was diluted 1/1 with RPMI 1640, then incubated for 30 min at room temperature in the dark with monoclonal antibodies anti cell surface antigens CD25, CD28 and CD38. Erythrocytes and were lysed by incubation with FACS lysing solution (Becton Dickinson, USA) for 10 min at room temperature in the dark, and then washed in a buffer consisting of phosphate buffered saline containing 2% bovine serum albumin. Mononuclear cells were obtained from heparinized blood by density gradient centrifugation over Ficoll-Paque plus (Amersham Biosciences, United Kingdom). Cells were then fixed by incubation with 0.3% paraformaldehyde-phosphate-buffered saline and stored at 4°C until analysis.

Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, USA). Expression levels of molecules on lymphocytes were measured using 4-color surface staining using Phycoerythrin (PE), fluorescein isothiocyanate (FITC), peridin chlorophyll protein (PerCP), and allophycocyanin (APC)-labelled antibodies: anti-CD4 (mouse IgG1, PerCP), anti-CD8 (mouse IgG1, FITC), anti-CD28 (mouse IgG1, PE) anti CD38 (mouse IgG1, PE), anti-CD25 (mouse IgG1, FITC). All monoclonal antibodies, were obtained from Becton Dickinson. We used the Flow Jo 7.3.5 (Treestar Software, Inc., Ashland, Ore) for Windows system for all data analysis.

5.2.3 Assays of IL-17

Serum samples were obtained from clotted blood after centrifugation at 2000*g* for 15 minutes within 20 minutes of collection, and the serum was stored at -20°C until analysis was performed. IL-17 circulating levels were determined by quantitative enzyme linked immunosorbent assay (ELISA) with the commercial human IL-17A ELISA Ready-SET-Go kit (eBioscience, San Diego, CA).

5.2.4 Statistical analysis

Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, USA). Variables are displayed in frequency tables or expressed as means and standard deviations, except those with an asymmetric distribution, which are described with medians. Testing for differences between groups were performed using Chi square test for frequencies, student's t test or anova tests for quantitative variables and Mann–Whitney's U test or Kruskal-Wallis for variables with an asymmetric distribution.

5.3 Results

Between June 2011 and October 2012, 79 patients were transplanted at the Sheila Sherlock Liver Centre, Royal Free Hospital (London, UK). Amongst these 8 patients were excluded because transplanted for ALF. Amongst the remaining 71 patients, 66 agreed to participate in the study. 5 patients drop out because of non-adherence to indications (n=4) and because of personal reasons (n=1), leaving 56 patients for the analysis.

Nearly half of the patients were male (55.5%), with a mean age±SD of 49.7±10.5. The most frequent indication to liver transplantation was alcohol related liver cirrhosis (28.5%), followed by cholestatic liver disease (21.5%). Mean MELD score at the time of liver transplantation was 15.5±30.5, and the mean time on the waiting list was 188.6±30.5 days (Table 5.1).

Mean donor age was 44.8±15.3, all the transplants were performed with an identical ABO group matching and the mean cold ischemia time was 6.58±2.32 hours (Table 5.1)

ACR was diagnosed in 9 patients: 6 (66.7%) mild, 2 (22.2%) moderate and 1 (11.1%) severe. Demographic and clinical characteristics of patients with ACR are reported in Table 5.1.

5.3.1 Levels of Tregs before and after liver transplantation

Patients with cholestatic liver disease presented the lowest expression of $CD4^{+}CD25^{+}$ (2.9±1.8%) when compared to patients with alcoholic liver disease (4.5±1.5%; p=0.02), virus-related liver disease (6.9±2.4%; p<0.001) and other aetiologies (4.8±1.7%; p=0.02) (Figure 5.1). When levels of Tregs were evaluated according to the interval time from liver transplantation, we found that the lowest levels were within the first week after liver transplantation (1.2±0.4%) followed by

a progressive increase at day 15 (2.6±0.3%; p=0.01), day 30 (3.0±0.2%; p=0.001) and day 60 (3.2±0.2%; p=0.001). No statistical difference was seen in terms of Tregs levels between day 30 and day 60 (Figure 5.2).

Levels of Tregs were then assessed after stratification patients according to the presence/absence of ACR. Patients with ACR showed a similar expression of $CD25^{+}CD4^{+}$ T cells compared with patients without ACR when the assessment was performed before liver transplantation (3.2±0.5% vs. 3.4±0.8%; p=0.57) (Figure 5.3A), whereas after liver transplantation patients who experienced ACR presented a significantly lower percentage of CD25+CD4+ T cells compared to patients without ACR (1.99±0.6% vs. 4.0±0.8%; p=0.001; Figure 5.3B).

5.3.2 CD28 and CD38 as potential markers of ACR

The mean frequencies of CD28+CD4+ T cells and of CD38+CD4+ T cells were significantly higher in patients with ACR compared with patients without ACR at day 5 (52.1±3.2% vs. 45.7±8.4%; p=0.01 and 45.6±9.3% vs. 32.3±8.1%; p=0.002 respectively) and at the day of ACR diagnosis (54.6±4.6% vs. 43.3±5.2%; p=0.001 and 39.4±12.1% vs. 27.5±7%; p=0.001 respectively). No difference between the two groups was found on samples taken on day 3 (Figure 5.4A and 5.4B).

When the expression of CD28 and CD38 was evaluated on CD8+T cells similar results were found. Patients with ACR presented a significantly higher expression of both CD28 and CD38 compared with patients without ACR at day 5 (57.4±9.6% vs. 43.3±8.1%; p=0.03 and 54±8.4% vs. 39.2±7.6%; p=0.001, respectively), and at day of liver biopsy (59.5±8.1 vs. 44.2±6.3%; p=0.02 and 49.1±11.3% vs. 30.4±8.7%; p=0.001 respectively) (Figure 5.5A and 5.5B).

In order to evaluate the potential influence of infections on the expression of CD28 and CD38, the same analysis was repeated after dividing patients into three subgroups: a) patients with ACR; b) patients who experienced an infective complications; c) patients without ACR nor infection. Again patients with ACR presented a significantly higher expression of CD28 and CD38 compared to patients without ACR and without infection. Conversely no difference was found in terms of CD28 and CD38 expression between patients with infection and those without events (data not shown).

5.3.3 Levels of IL-17 after liver transplantation

The concentration of IL-17 at day 3, and day 5 after liver transplantation did not differ between patients who experienced ARE and patients without ACR. Conversely, when the concentration of IL-17 was measured the day of ACR diagnosis, we found that the patients with ACR presented a significantly higher levels of IL-17 compared with patients without ACR (14.2±3.5 pg/mL vs. 7.9±2.4 pg/mL; $p<0.001$) (Figure 5.6). We also observed that, in patients experiencing ACR, the levels of IL-17 tended to increase from day 3 until the day of ACR diagnosis, slightly decreasing afterward (Figure 5.6). In patients without ACR these changes in IL-17 were not seen with IL-17 remaining substantially stable overtime.

5.4 Discussion and conclusions

Today, the administration of immunosuppression is considered more of an art than a science. Indeed, there are not reliable markers of the immunological status of organ transplanted patients. Moreover, there is generally a poor

correlation between ACR and immunosuppression levels, with liver biopsy still remaining the gold standard for the diagnosis of ACR. In this contest it is becoming crucial to identify potential markers of immune activity to adjust immunosuppressive therapy according to the real suppression of the immune system.

In this study, we prospectively assessed patients before and after liver transplantation and we evaluated the potential role of expression of different proteins and interleukin as potential markers of immune status.

Considering Tregs, their role in transplantation has been evaluated mainly in animal studies, which showed that the transfer of Tregs from long-term tolerant mice to allografted posts prevents rejection of transplanted allogeneic pancreatic islets or skin (8,17). These studied concluded that an increased number of circulating Tregs may be beneficial for allograft survival. However their role in mediating ACR in human liver transplant recipients is still controversial.

In our study pre-transplant frequencies of circulating Tregs were not different between patients with and without ACR, therefore their evaluation in the pre-transplant phase might not be useful in order to discriminate between patients who will develop ACR after liver transplantation and those who will not develop it. Conversely, in the post-transplant period we found that patients with ACR presented significantly levels of Tregs compared with patients without ACR. The cause of this low expression in patients with ACR is still under debate, but we could speculate that circulating Tregs cells may be recruited in other compartments, such as the graft or secondary lymphoid organs. Further prospective studies, possibly including analysis of the expression of Tregs in the graft, are needed to properly understand the role of this subset of T cells in modulating ACR and graft acceptance.

With regards to other proteins expressed on activated T cells, CD28 and C38 represents two of the most interesting ones. The interaction between CD28 is the B7 ligand is necessary as a second signal in the ACR pathway [121], and CD38 has been shown to be an early marker of cytomegalovirus infection in transplant recipients [123], but its role on T cell activation is still unclear. In our study patients experiencing ACR showed increased expression of CD28 and CD38 on both $CD4^+$ and $CD8^+$ T cells compared with patients without ACR. Interestingly this difference was evident on day 5 post-transplant and on the day of liver biopsy, whereas no difference was found at baseline between the two groups.

In line with our results, a French group showed that patients with ACR presented a significantly higher expression of CD28 and CD38 on CD3, CD4 and CD8 T cells populations compared to patients without ACR [137]. This expression decreased after anti-rejection therapy. Although in this study the CD28 and CD38 expression levels did not change in patients suffering from an acute CMV infection, previous papers have shown alterations in the CD28 and CD38 pools during CMV infection [138, 139], limiting its clinical use.

Lastly, we observed that IL-17 levels were higher in patients with ACR compared with patients with no ACR, but this difference was evident only the day of liver biopsy. These results are in line with the study Caldwell et al. [140], who found that hepatic ischemia reperfusion injury led to the induction of Th17 cells. In a experimental model of lung ischemia reperfusion injury an early activation of Th17 cells was observed [141]. A potential role of IL-17 in mediating ACR has been also demonstrated in renal [135, 142] and heart [143, 144] transplant recipients.

In conclusion, our study demonstrated that there are specific alterations of immune system, which could be used routinely in clinical practice to assess the immune status of liver transplanted patients and to monitor those ones who are at risk of ACR. Due to the small number of patients included in the present study, it's difficult to estimate the reliability of these markers, however we believe that future studies should address the question by a simultaneous evaluation of different parameters and biomarkers. This approach could lead to a more appropriate use of immunosuppression and to a reduced incidence of immunosuppression-related side effects.

5.5 Tables and figures

Table 5.1. Demographic and clinical characteristics of all 56 transplanted patients, and of patients experiencing acute cellular rejection (n=9).

	Study cohort n=56 (%)	**Patients with ACR** n=9 (%)
Recipient gender		
Male	31 (55.3)	3 (33.3)
Female	25 (44.7)	6 (66.7)
Recipient age, years (mean±SD)	49.7±10.5	50.4±11.2
Liver disease		
ALD	16 (28.5)	1 (11.1)
HBV	8 (14.3)	1 (11.1)
HCV	9 (16)	2 (11.1)
Cholestatic	12 (21.5)	3 (33.4)
Other	11 (19.7)	2 (22.3)
MELD (mean±SD)	15.5±30.5	12.5±3.35
UKELD (mean±SD)	52.1±8.3	49.6±12.1
Donor gender		
Male	18 (32.1)	5 (55.6)
Female	38 (67.9)	4 (44.4)
Donor age, years (mean±SD)	44.8±15.3	45.8±16.2
ABO-group matching		
Identical	56 (100)	9 (100)
Compatible	(0)	(0)
Incompatible	(0)	(0)
IS regimen at day 1		
Mono	25 (44.6)	3 (33.3)
Double	13 (23.2)	1 (11.1)
Triple	18 (32.2)	5 (55.6)
Cold ischemia time, min (mean±SD)	6.58±2.32	7.64±1.95

Figure 5.1 Levels of Tregs in patients patients before liver transplantation according to the aetiology of liver disease. Patients with cholestatic liver disease presented significantly lower levels of Tregs compared to other groups ($p<0.001$ vs. viral; $p=0.02$ vs. alcohol and other).

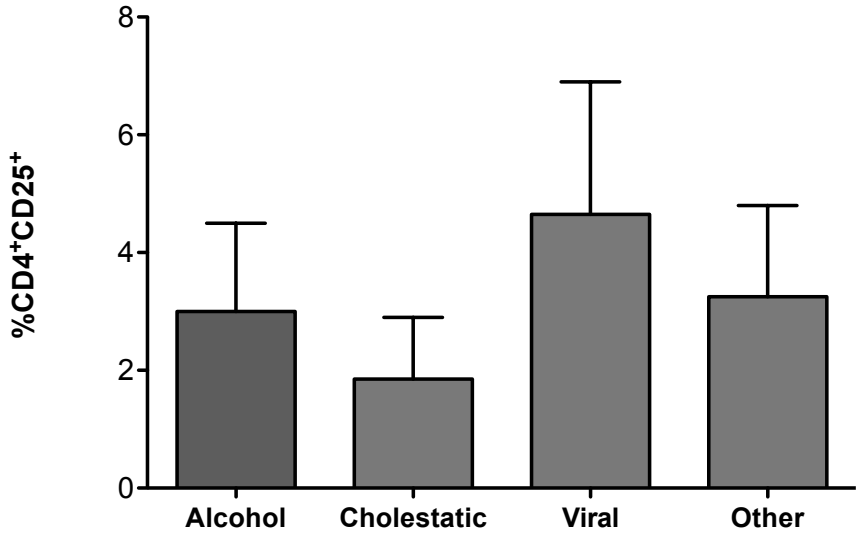


Figure 5.2. Change over time of Tregs levels in patients before and after liver transplantation, evaluated at fixed intervals. Levels at day 5 were significantly lower compared to levels at day 15 (p=0.01), day 30 (p=0.001) and day 60 (p=0.001). No statistical difference was seen in terms of Tregs levels between day 15, day 30 and day 60.

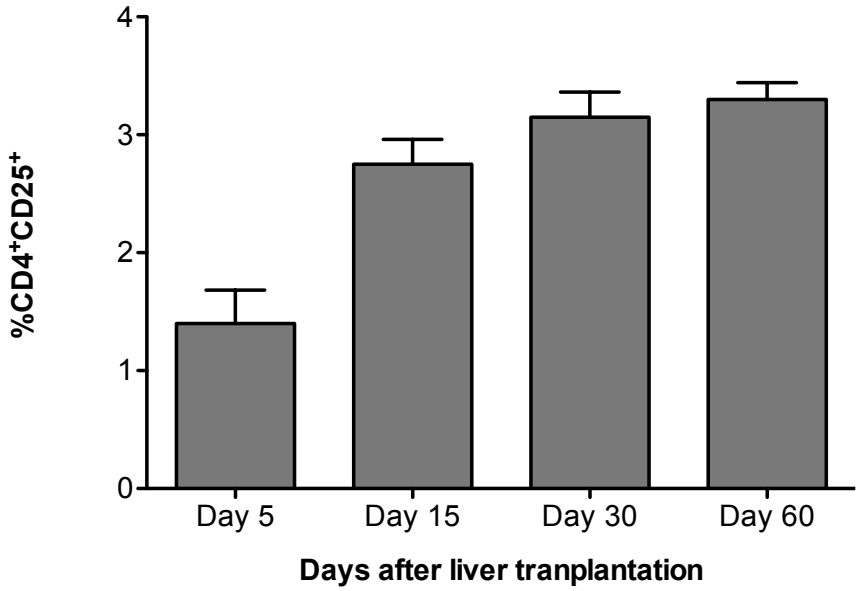
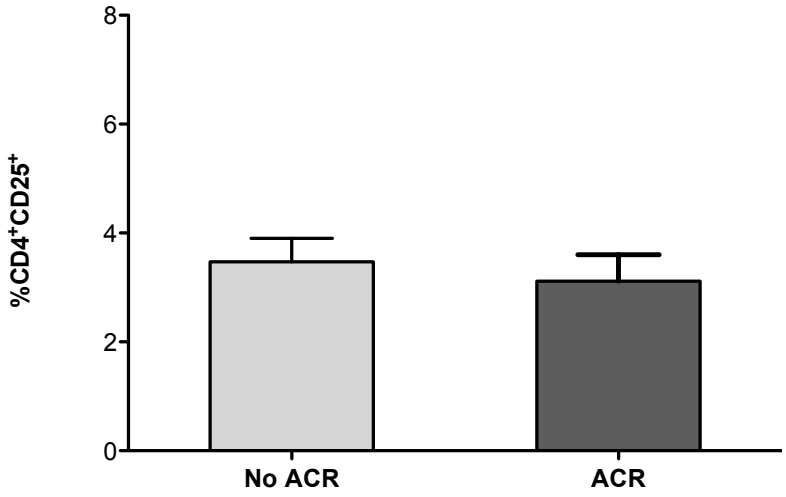


Figure 5.3. Levels of Tregs in patients before (A) and after liver transplantation (B) according to the presence or absence of ACR.

A. Before liver transplantation (p=0.57)



B. After liver transplantation (p=0.001)

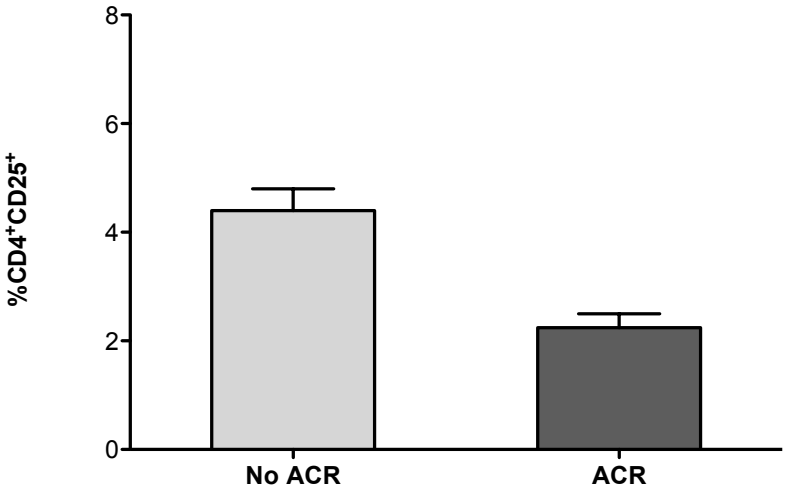
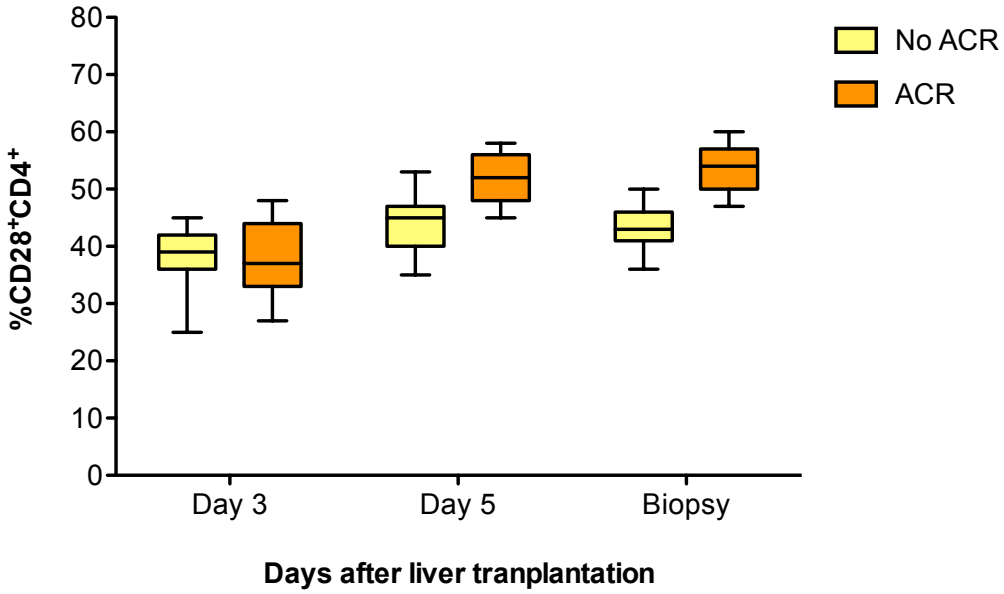


Figure 5.4 Expression of CD28 (A) and CD38 (B) on $CD4^+$ T cells (B) from day 3 until the day of ACR diagnosis. Patients were stratified according to the presence/absence of ACR. Expression of CD28 and CD38 were significantly higher in ACR vs. no ACR patients both at day 5 (p=0.01 and p=0.001 respectively) and at the day of liver biopsy (p=0.002 and p=0.001 respectively).

A.



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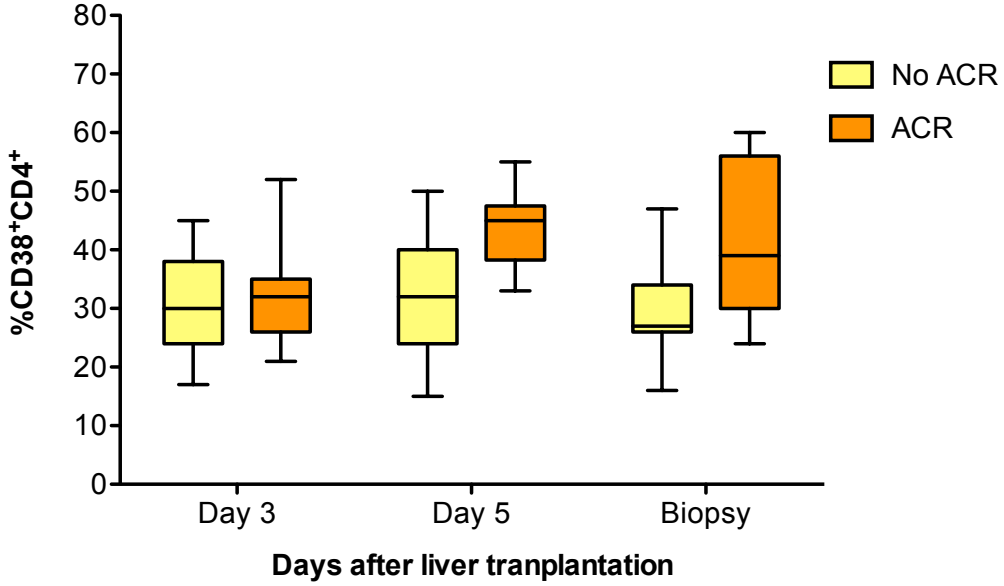
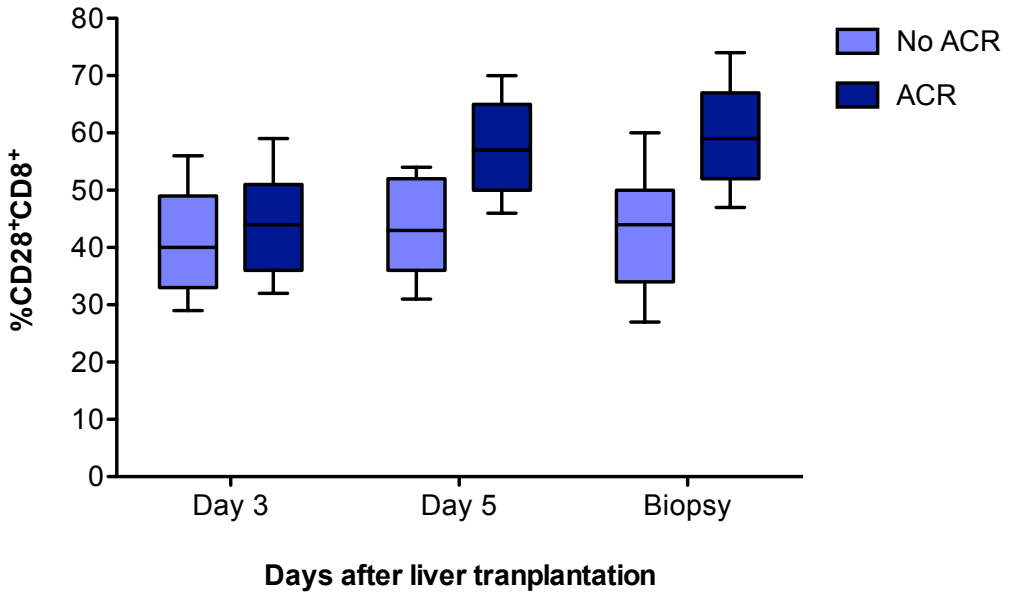


Figure 5.4 Expression of CD28 (A) and CD38 (B) on CD8+ T cells (B) from day 3 until the day of ACR diagnosis. Patients were stratified according to the presence/absence of ACR. Expression of CD28 and CD38 were significantly higher in ACR vs. no ACR patients both at day 5 (p=0.03 and p=0.001 respectively) and at the day of liver biopsy (p=0.02 and p=0.001 respectively).

A.



B.

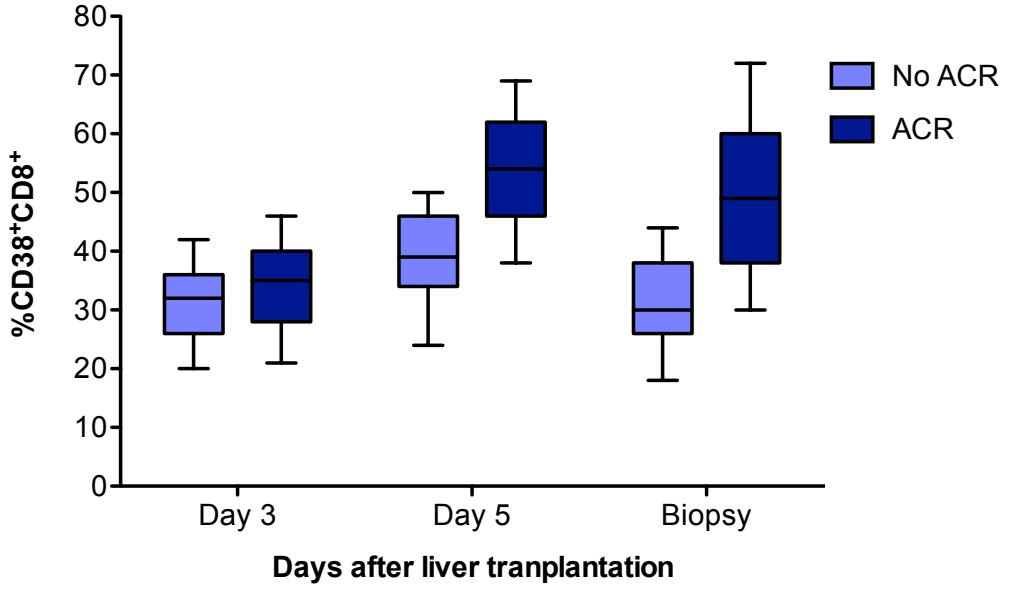
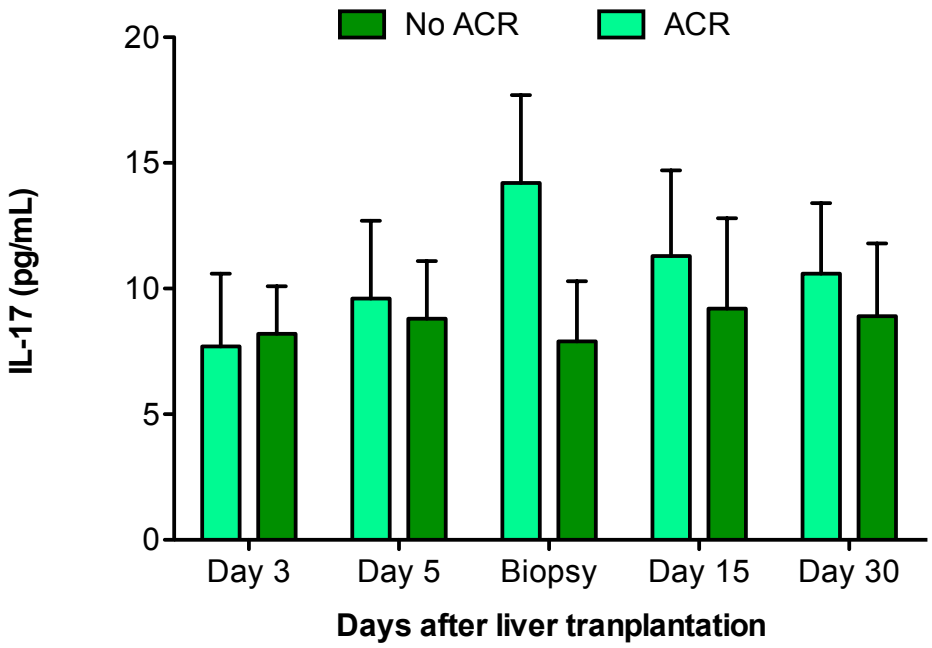


Figure 5.6. Change over time of IL-17 levels at fixed intervals after liver transplantation. Patients with ACR presented significantly higher levels of IL-17 on the day of protocol liver biopsy ($p<0.001$).



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