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AUTOIMMUNE HEPATITIS: CLINICAL EXPERIENCE AFTER LIVER TRANSPLANTATION AND MOLECULAR STUDY USING SURFACE PLASMON RESONANCE IMAGING-BASED STRATEGY

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SUMMARY

De novo autoimmune hepatitis in patients with HCV recurrence (HCV-R) after liver transplantation (LT) is of challenging diagnosis and the impact of autoimmune therapy (AT) is still a matter of debate. In the first part of this work the aim was to evaluate clinical, serological, histological characteristics of these patients and the impact of AT. Patients have been evaluated in two European transplant centers .Liver biopsies were retrospectively by experts pathologists. Three parameters, plasma cells infiltrate, interface hepatitis and central vein necrosis, were evaluated applying a new semi-quantitative method. Final diagnosis was of prevalent viral lesions: HCV-R, or prevalent immunological lesions: AIH. Forty patients, transplanted between 1983-2009, were included, 16 (40%) patients were HCV-R and 24 (60%) AIH. High grade of interface hepatitis and confluent central vein necrosis were significantly more represented in AIH patients, moreover AST/ALT were significantly higher in AIH group (p=0.05 and p=0.003, respectively). No difference was found regarding baseline immunosuppression, autoantibodies and gammaglobulin levels. No relationship between HCV antiviral therapy and AIH was observed. Ten years survival was lower for AIH compared to HCV-R patients (65%, *versus* 93%, p=0.050). The AT improved the cytolysis but did not modify long-term survival (50% treated versus 87.5% non treated patients, p=ns), which was impaired by severe HCV disease progression.

Anti-dsDNA autoantibodies (Abs) are highly diagnostic for systemic lupus erythematosus (SLE), however, they can be found in autoimmune hepatitis (AIH) but it remains uncertain which antigen triggers the production of these antibodies. Moreover the characteristics of antigen-antibodies interaction are still a matter of concern. In the second part of this work the aim was to differentiate the binding characteristics of dsDNA and anti-dsDNA Abs obtained from AIH and SLE patient's sera using Surface Plasmon Resonance imaging (SPRi) strategy. Sera from AIH (n= 14), SLE patients (n= 7) with anti-dsDNA Abs positive Farr test, as well as from healthy controls (n= 7) were collected. IgGs and IgMs were purified from sera. Ten different types of oligonucleotides (OG) were spotted over the chip surface of SPRi. Kinetic SPRi study was also performed. All sera from both patients and controls

showed a reactivity signal on SPRi, nevertheless when monoclonal mouse anti-IgGs were injected after the sera injection, only for AIH patients the signal was still evident, being lower for SLE patients and controls. When purified IgGs from sera were injected an interaction signal with OG was observed only for AIH patients. Mean IgGs k*off* were comparable among patients, meaning they have the same dissociation kinetic. SPRi method identifies interactions between sera from AIH, SLE patients and controls and dsDNA of OG used. However using purified IgGs a binding signal is observed only for AIH. These results suggest that immunocomplex found in AIH and SLE patients are different, in SLE patients the complex might require a third partner, or probably, recognize a specific dsDNA conformation.

Results from our work suggest that new promising methods can be applied both in clinical and laboratory field for the comprehension and monitoring of autoimmune hepatitis.

Keywords: liver transplantationa, HCV recurrence, autoimmune hepatitis, autoantibodies, anti-dsDNA, Surface Plasmon Resonance imaging.

RIASSUNTO

L'epatite autoimmune *de novo* che insorge in pazienti con recidiva di epatitie C dopo trapianto di fegato e' di difficile diagnosi e l'impatto della terapia autoimmune rimane oggetto di discussione.

Nella prima parte di questo lavoro lo scopo e' stato di valutare le caratteristiche cliniche, sierologiche e istologiche di questi pazienti e il beneficio della terapia autoimmune. I pazienti sono stati inclusi in due centri trapianto europei. Le biopsie epatiche sono state retrospettivamente revisionate da anatomopatologi esperti che hanno valutato tre aspetti, infiltrato plasmacellulare, epatite d'interfaccia e necrosi perivenulare centrale, applicando un nuovo metodo semiquantitativo. La diagnosi finale si e' basata sulla prevalenza di lesioni virali: HCV-R, o di lesioni autoimmuni: AIH. Quaranta pazienti sono stati inclusi, 16 (40%) HCV-R e 24 (60%) AIH. L'epatite d'interfaccia di alto grado e la necrosi centrale confluente sono state gli aspetti significativamente piu' rappresentati nei pazienti AIH, inoltre AST e ALT sono risultate significativamente piu' elevate nei pazienti AIH. Nessuna differenza e' stata riscontrata riguardo all'immunosuppressione di base, aumento delle gammaglobuline o positivita' autoanticorpale. Alcuna relazione e' stata riscontrata tra la terapia antivirale e lo sviluppo di AIH. La spravvivenza e' risultata inferiore per i pazienti AIH rispetto ai pazienti HCV-R (65% *versus* 93%, p=0.050). La terapia autoimmune migliora la citolisi ma non modifica la sopravvivenza (50% pazienti trattati *versus* 87.5% pazienti non trattati, p=ns), che e' compromessa dalla progressione dell'epatite C.

Gli anticorpi anti-dsDNA sono altamente diagnostici di lupus eritematoso sistemico (LES), tuttavia possono essere riscontrati anche in altre patologie come l'epatite autoimmune, rimane incerto quali antigeni inducano la produzione di questi autoanticorpi. Inoltre le caratteristiche dell'interazione antigene-anticorpo sono state solo parzialmente elucidate. Nella seconda parte di questo lavoro lo scopo e' stato di differenziare le caratteristiche dell'interazione tra dsDNA e anti-dsDNA in pazienti con AIH e LES utilizzando la tecnica innovante Surface Plasmon Resonance imaging (SPRi). I sieri di pazienti con AIH (n=14), LES (n=7), con anti-dsDNA ad alto titolo, riscontrati con il test di Farr, e di controlli sani (n=7) sono

stati raccolti. Le IgG e IgM sono state purificate dai sieri. Dieci diversi oligonucleotidi (OG) sono stati immobilizzati sulla superficie del prisma dell'SPRi. La cinetica d'interazione e' stata inoltre valutata. I seri di tutti i pazienti e dei controlli sani hanno mostrato un segnale di attivazione in SPRi, tuttavia una volta iniettate le IgGs monoclonali murine, il segnale e' rimasto evidente solo per i pazienti AIH, divenedo debole e quasi inesistente rispettivamente per i pazienti LES e i controlli sani. Dopo l'iniezione delle IgGs purficate, il segnale e' stato riscontrato solo per i pazienti AIH. La media delle costanti di dissociazione (k*off*) e' risultata comparabile per tutti i pazienti, a significare che la cinetica di dissocazione era comparabile per tutti i sieri di pazienti AIH. La tecnica SPRi permetti di identificare la presenza di interazioni tra dsDNA e anti-dsDNA in pazienti AIH e LES e controlli, utilizzando le IgG purificate il segnale rimane presente solo per i pazienti AIH. Secondo questi risultati e' possibile affermare che esiste una differenza tra i complessi immuni presenti nei pazienti AIH rispetto ai pazienti LES che richiedono la presenza di un terzo componente per la forazione o stabilizzazione dei complesso, o, probabilmente riconosco delle sequenze oligonucleotidiche precise.

Parole chiave: trapianto di fegato, epatite C recidiva, epatite autoimmune, autoanticorpi, anti-dsDNA, Surface Plasmon Resonance imaging.

INDEX

GENERAL INTRODUCTION

The scientific community has progressively accepted the concept of autoimmunity after several works, which aimed to precise its definition.

With the theory of "*horror autotoxicus*", Paul Ehrlich proposed in 1901 that certain obstacles prevent the immune system to produce autoantibodies against the self-tissues [1].

After a period of eclipses, different studies marked the reemergence of the concept of autoimmunity. The rheumatoid factor was described first by Waaler in 1940 and then by Rose in 1948 in patients with rheumatoid polyarthritis. Coombs developed in 1946 a test in order to detect, in maternal blood, the antibodies directed against the fetal antigen Rhesus, that arise in the mother-fetus incompatibility. The application of this test to the acquired hemolytic anemia led Young, in 1951, to define for the first time a disease as "autoimmune" [2]. The same year Harrington suggested that the thrombocytopenic purpura was of autoimmune nature, highlighting the passage of antiplatelet autoantibodies through the blood transfusion from patients to healthy subjects [3].

Hargraves demonstrated in 1948 that the incubation of plasma of patients with systemic lupus erythematosus (SLE) with the bone marrow of healthy subjects induces the production of lupus erythematosus cells with typical inclusion. These cells are nothing but polymorphonuclear neutrophils that phagocyte the nucleus. About ten years later, the factor responsible for this phenomenon was identified: it was an immunoglobuline, in other words an autoantibody [4].

The discovery of the autoimmune hepatitis (AIH) by Waldentrom in 1950 belongs to the autoimmunity recognition process. Under the definition of chronic autoimmune hepatitis, the disease was firstly described by the presence of typical clinical signs and hypergammaglubulinemia.

The reaction between antigens and autoantibodies has been studied *in vivo* since the XX century. The techniques for the study of antigenic target followed the evolution of the methods for proteins study. At the beginning, the techniques for the visualization of the immune-complex, such as the agglutination, have been developed. Later the methods that can precise the intra-cellular localization of the

auto-antigens, such as the indirect immunofluorescence and electronic immune-microscopy have been used.

Biochemical techniques like electrophoresis and immune-enzymatic assays better responded to the need of functional characterization of the autoantibodies, describing their modifications in cells and tissues. Furthermore the combination of physical, biochemical and informatics supports led to the analysis of the interaction between antigen and antibody.

Although the huge amount of discoveries that have been done in this field, several questions remain unanswered. For example the development of an autoimmune process in the context of transplantation and in the presence of immunosuppressive drugs is not fully understood. Moreover the characteristics of the interaction between autoantigens and autoantibodies are only partially elucidated.

CHAPTER I: *de novo* autoimmune hepatitis after liver transplantation in recipients with HCV recurrence, challenging diagnosis and poor prognosis

I. INTRODUCTION

I.1 Liver Transplantation

Liver transplantation (LT) is the definite treatment for end-stage liver disease, liver hepatocellular carcinoma as well as several other rare liver diseases. Since the first liver transplant performed by T. Starzl in 1963 at Denver Unvirsity in Colorado [5], the continuous research in this field brought to an improvement of surgical techniques and medical therapy, transforming this therapeutical option from a experimental to a consolidate practice. The development of transplantation medicine, thanks to the integration of multidisciplinary knowledge, led to excellent results with a 5-year graft survival of 70% and patient survival of 80% [6].

The end-stage liver disease due to hepatitis C (HCV) cirrhosis is the leading LT indications. Hepatocellular-carcinoma (HCC) on cirrhosis is one of the increasing indication. Among the other common indications there are the alcoholic cirrhosis, cirrhosis of viral origin from B and D virus, the cholestatic hepatitis such as primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis. The pre-LT evaluation is a multidisciplinary work-up with as objectives the confirmation of the LT indication, the exclusion of contraindications and the definition of the best timing for the operation [7].

Every country has its own allocation system but most of them agreed on using the Model of End-Stage-Liver Disease (MELD) to assess the severity of the liver disease and to lead the allocation. Although based on objective biological values (creatinine, INR and bilirubine) the MELD score is not always representative of the patient severity [8].

The donor availability is reduced compared to the number of patients requiring a LT. The organ shortage induced the expansion of donor selection criteria with the acceptance of donor with advanced age, anti-HBc positive, non-heart beating donor, living donor and split donor, with increasingly good results [9].

Nevertheless several complications affect the graft and the patients such as viral recurrence, vascular and biliary complications, rejection, diabetes and metabolic syndrome, renal impairment, *de novo* cancer etc.

Moreover there are still several questions regarding the allo- and autoimmune reactions that develop after the LT, leading to graft dysfunction. The onset of features of autoimmune hepatitis (AIH) in patients not transplanted for AIH (*de novo* AIH) has not been clearly explained. Furthermore the development of *de novo* AIH in the context of HCV recurrence after LT results from the complex interplay of viral and immunological factors, in the presence of immunosuppressive drugs and, in certain cases, of antiviral therapy.

I.2 HCV recurrence after liver transplantation

HCV recurrent infection after LT is universal and the natural history of the disease is accelerated compared to the pre-transplant setting [10] [11]. Histological recurrence is reported in 80% of patients, and development of cirrhosis, within 5 years after the transplant, in 30% [12] [13]. Moreover between 2% and 5% of patients develop a severe form of recurrence called fibrosing cholestatic hepatitis (FCH), characterized by cholestatic hepatitis and peri-sinusoidal fibrosis which lead to early graft failure [14].

Therefore preventing and managing HCV recurrence represents two of the transplant hepatologist's main challenges. Risk factors for HCV recurrence are related to several factors such as donor and recipient characteristics, aspects related to the virus, to surgical intervention and to the immunosuppression employed.

I.2.1 Risk factors for severe recurrence

Donor factors

Donor age has been confirmed in several studies as the most relevant risk factor for HCV recurrence. A cut off age to define what "old" donor means has not been established yet, and a wide range, between 33 and >70 years, was used in

previous studies and correlated to worse outcome [15] Furthermore donor age increased in latest years making the selection of younger donor for HCV positive recipient very difficult [16]. The match of an older donor and an advanced age recipient is a predictor of graft failure after LT [17], as well as the match of an older donor and a female recipient [18]. Moreover, the donor-recipient HLA-DRB1 mismatch affects both the occurrence and the progression of HCV infection-related fibrosis [19].

It has been suggested that donor steatosis might be associated with greater severity of HCV recurrence, accelerated fibrosis progression and, ultimately, poor outcome [20] [21]. Nevertheless others studies have found that mild steatosis of the graft can be safely tolerated in HCV + recipient. [22] [23]. Moreover the presence of diabetes mellitus in the donor has been identified as an independent risk factor for graft loss in HCV positive recipients compared to HCV negative ones [24].

The use of HCV+ grafts in HCV+ recipients is not so frequent, but it will expand the donor pool. In an European-based multicenter study, among 694 patients with HCV-related cirrhosis who underwent LT during the study period, 76 (11%) received the graft from anti HCV+ donors. After excluding 13 of 76 cases from the matched analysis due to the inadequate follow-up liver biopsy after LT and unavailability of information on donor HCV viral load and genotype, 63 recipients of anti-HCV-positive donor livers were matched to the recipients of an anti-HCV-negative donor liver, as controls. Recurrence of hepatitis C tended to be more rapid in the group of patients who received anti-HCV-positive grafts, although this difference did not reach statistical significance [25]. An HCV+ donor of an HCV+ recipient does not affect graft and patient survival nor the rate of HCV recurrence, when compared with the use of HCV- grafts [26] [27] [28] On the contrary, the use of grafts from HCV+ donors is not recommended in HCV- recipients [29].

No data are available on the use of partial grafts from HCV+ donor in living donor liver transplantation (LDLT) as the presence of the virus is considered a contraindication for live donation. Split liver or LDLT have been reported in HCV+ recipients with no observed difference in terms of fibrosis progression or graft survival [30].

Previous report identified LDLT as a risk factor for more severe HCV recurrence compared to deceased donor [31] [32] [33], possibly due to the regeneration that takes place in the partial graft which might be associated with higher viral replication [34], or because of a genetic similarity between donor and recipient, particularly HLA matching [35]. In contrast, it has been recently demonstrated that better results are seen in LDLT because of younger donor age and shorter ischemia time [30] [36] [37] [38] [39] [40].

The impact of donation after cardiac death (DCD) on HCV recurrence is still controversial. Some reports associate DCD to a more severe HCV recurrence with more rapid disease progression compared to donation after brain death (DBD), with higher rate of graft failure [41]. On the other hand, a matched study did not find any difference in DBD versus DCD regarding fibrosis progression, patient and graft survival [42].

Donor-Specific Alloantibodies

Among donor related factors associated with HCV recurrence, the study of Donor-Specific Alloantibodies (DSA) is a new field of interest for the transplant community. The immunological insult to the liver is always believed to be cellular. Nevertheless it has been recently reported that DSA, class I and II are independent predictors of fibrosis progression to stage 2-4, and increased risk of death, in patients with HCV recurrence [43].

Recipient factors

Diabetes and metabolic syndrome are frequently reported after LT and can be associated with HCV infection. HCV leads to hyperglycemia and insulin resistance due to a direct alteration of the insulin signaling pathway [44]. Hyperglycemia is associated with HCV disease progression as well as hyperlipidemia and steatosis [45] [46]. Likewise, metabolic syndrome is associated with fibrosis progression after 1 year post-LT [47] [48].

The negative impact of female gender on HCV recurrence has been recently reported, possibly related to the postmenopausal status being associated with increased steatosis [18] or to a more aggressive virological disease [49].

The HIV-HCV coinfection is associated with higher progression to severe fibrosis and lower survival rate compared to monoinfected patients, but the survival benefit after LT is satisfactory for these patients [50] [51]. Moreover in HIV/HCV coinfected patients a higher rate of FCH has been reported, therefore a strict follow-up and an early administration of antiviral therapy is mandatory [52].

CMV and HHV6 reactivation have been correlated with more severe HCV recurrence; however is not clear if CMV and HHV6 can be considered as an independent risk factor or just a marker of over-immunosuppression [53].

The presence in both donor and recipient of the interleukin-28B (IL28B) polymorphism, in the gene region, which encodes for interferon (IFN)-lambda3, is strongly predictive of HCV recurrence. Genotype TT of polymorphism rs12979860 is associated with more rapid fibrosis progression. The relationship between recipient IL28B genotype and time-to-recurrence of hepatitis C has been shown, however, to be independent from the donor IL28B genotype [54]. This finding was confirmed by the finding that recipient IL28B CC genotype is associated with lower alanine aminotransferase levels and viral load at recurrence and a lower frequency of fibrosis ≥2 on liver biopsy at 1 year after LT, when compared with the non-CC genotype. The opposite has been observed in LT recipients of CC genotype donors [55]. The non-CC genotype also seems associated with the more aggressive form of HCV recurrence: FCH [56].

Virological factors

Serum HCV-RNA decreases rapidly after the removal of the infected liver and during the implantation of the new graft. In the first week after the transplant viral kinetics appear highly variable amongst individual [57]. Thereafter, the new liver becomes infected and serum HCV-RNA reaches or even exceed levels before LT [58]. However, the impact of viral load on clinical outcome is still controversial. From some studies it seems associated with fibrosis development at 1 year after

LT [59] and increased mortality [60]. The influence of HCV genotype is debatable, as it has been reported that genotype 1b is associated with more severe recurrence [61] [62] [10] but also that the genotype has no influence on viral recurrence [63].

Factors related to the transplant

Ischemia-reperfusion injury, which depends on several peri-operative factors such as cold and warm ischemia time, preservation solution and technical factors during graft removal, donor status (DCD/DBD), and type of reperfusion, contributes to increase morbidity and mortality after LT. In particular, it has been observed that in HCV + recipients, early preservation injury on biopsy is associated with progression to stage 3-4 of fibrosis and poorer survival rates compared to HCV patients without preservation injury or HCV- patients with preservation injury [64]. Biliary complications after LT are diagnosed more frequently in HCV+ patients but HCV recurrence itself, rather than this complication, is correlated with graft poor outcome [65].

Immunosuppression

Immunosuppression is a key factor for HCV recurrence [66].

Pulsed intravenous methylprednisolone treatment for acute cellular rejection is associated with transient increases in HCV RNA levels [58], and with a more rapid disease progression [67]. On the contrary, the slow tapering of steroids seems to reduce HCV progression [68] [69]. Complete steroids avoidance has not been correlated with a reduction of HCV recurrence or an improvement in patient or graft survival, although this regimen is safe and effective as demonstrated by two multicenter randomized trial [70] [71] [72].

Cyclosporine (CsA) has an antiviral effect on HCV in vitro [73] [74] [75], that has, however, never been confirmed in vivo. Nevertheless a recent study found that Csa in steroid-free regimen is associated with less fibrosis at 1 year after LT [76]. CsA was preferred in the past since it was associated with higher sustained virological response (SVR) when patient were treated with peg-IFN and RBV; this association

was not confirmed for the new generation antivirals. In calcineurine inhibitors (CNI) and steroids regimen no difference between the two CNI has been found [72]. Yet, tacrolimus has been preferred in recent years because of its association with a better survival [77].

The impact of azathioprine (AZA) and mycofenolate mofetil (MMF) is still controversial. Some studies have reported an association between MMF and HCV increased severity [78], while others have observed a stabilization of the disease, and yet in other studies, a disease worsening was noted. Similarly, AZA seems to have a beneficial effect [66]. The above data do not allow for any recommendation to be made regarding the choice of immunosuppression in this scenario.

I.2.2 Natural history

Fibrosis progression

The natural history of HCV liver disease after LT is characterized by the development of fibrosis and its progression to cirrhosis. Fibrosis progression is not linear and may vary according to different time points after LT [79]. Serial liver biopsies have shown that the histological activity at HCV recurrence diagnosis, predicts the risk of cirrhosis development [80]. However, in recent years, recipients have reportedly higher fibrosis rate compared to the past, for similar grading score [81]. This finding suggests that other factors may be responsible for the increase in liver fibrosis.

Fibrosis progression in HCV transplant recipients is associated with early activation of hepatic stellate cells (HSC), a process that appear to be partially independent from necro-inflammatory activity [81] [82]. HSC are the predominant source of type I collagen in the liver and their activation is considered a fundamental step in hepatic fibrogenesis [83, 84].

Tilman et al. compared the traditional fibrogenesis model in which fibrosis development is induced by tissue damage and begins with HSC activation to the telomere model, in which continuous hepatocytes damage and hepatocyte

telomere shortening constitute the main process. Telomere shortening exhausts the hepatocyte's ability to replicate and it fosters fibrogenesis. Similar to the telomere model, a stress-induced model of p21-mediated hepatocyte mito-inhibition emphasizes the importance of a replicative disadvantage for hepatocytes [85]. Kobayashi et al. showed that HCV replication upregulates hepatocyte p21 [86]. Furthermore, it has also been reported that fibrosis progression in HCV-infected liver with steatosis was directly proportional with p21-mediated inhibition of hepatocyte proliferation and enhanced ductular reaction at the interface zone [87]. Nevertheless, an important difference is that hepatocyte inhibition is potentially reversible if stressors are minimized or eliminated. This model explains why any stressors to hepatocytes, such as steatosis, iron, inflammation, HCV + replication, spontaneously increase of p21 expression that occurs with age, can accelerate fibrosis progression [85] [84]. This model suggests also that the process is potentially reversible. Recently, the pivotal role of ductular reaction in the development of progressive fibrosis in HCV recurrence has been recongnized [88].

The measurement of fibrosis has been always of great interest in order to predict the speed of disease progression, and to this end, non-invasive fibrosis markers have been developed. These tests can be divided as follow: serum biochemical biomarkers (APRI, fibrotest), imaging techniques such as fibroscan or magnetic resonance, or genetic and molecular markers.

Currently, however, liver biopsy, albeit an invasive method, remains the gold standard to assess fibrosis progression. Several histological score have been used such as Ishak, Knodell and Metavir with non-superiority of one over the other [89].

In order to control fibrosis progression, antiviral therapy (AT) is administered in patients with HCV recurrence. Despite the evaluation of AT response is often based more on virology than on histology, it has been reported that interferon (IFN) may play an anti-fibrotic effect even in those patients in whom an anti-viral effect is not observed [90]. Moreover, AT seems to slow down disease progression when SVR is achieved [91], and increases patient survival in treated LT recipients [92]. Similar results emerged from an Italian multicenter database of patients who underwent standard AT after LT. Furthermore, in this

study, older donor, pre-therapy higher fibrosis stage, diabetes and female gender were identified as risk factors for fibrosis progression, independently from antiviral therapy [93]

Retransplantation

Survival of HCV positive LT recipients is significantly lower compared to patients transplanted for other indications [94], and weather to re-transplant or not LT recipients with decompensated HCV cirrhosis remains a relevant and mulch debated issue, since patient and graft survival is lower after re-LT compared to the first transplant [95] [96]. Moreover most of the death after re-LT are related to post-operative complications, especially sepsis [97] [98].

Some study suggested that HCV infection is a risk factor for mortality [99] [100] [101]; however, on multivariate analysis other variables such as recipient age, model for end-stage liver disease (MELD) >25, re-LT during the first year after LT, donor age >60 and, a warm ischemia time ≥75 min have emerged as independent predictors of mortality. On the other hand, the association between HCV and mortality risk has been clearly demonstrated, in other studies, with HCV-infected recipients having a 30% higher risk of mortality than those without HCV-infection [102].

The International Liver Transplantation Society Expert Panel established that bilirubin >10 mg/dl, creatinine >2.0 mg/ dl (or creatinine clearance <40 ml/min), recipient age >55, donor age >40 and early HCV recurrence (cirrhosis <1 year after LT) are variables associated with a worse outcome after RT [103]. The use of prognostic score is helpful for deciding who would benefit from re-LT avoiding futile transplantation [104].

Interestingly, multiple transplants can be safely performed [105]. Nevertheless, taking into account organ shortage and costs related to the procedure, it becomes mandatory to prevent the need of re-LT by achieving viral clearance and halting disease progression.

I.2.3 Antiviral therapy

Combined peg-IFN and weight-based RBV was the standard of care treatment for patients with established HCV recurrence after LT. The standard weekly doses of peg-IFN-α-2a and peg-IFN-α-2b were 180 μg/week (fixed dose) and 1.5 μg/kg/week, respectively [106]. A weight adjusted RBV daily dose of between 1000 mg (weight <75 kg) and 1200 mg (weight >75 kg), divided in two administrations, was recommended for all genotypes. Increasing the dose has been shown not to improve the outcome.

When to start antiviral therapy has been always a controversial subject.

Pre-emptive AT, defined as therapy before histological disease recurrence is present, is not recommended as the efficacy has been demonstrated by several studies to be rather poor [107] [108] [109]. The pre-emptive strategy, however, might eventually be used with the new generation antivirals, as they are better tolerated compared to Peg-IFN.

Carrión et al. randomized patients with mild recurrent hepatitis C (fibrosis stages F0 to F2) to either no therapy or treatment with peg-IFN- α-2b and RBV for 48 weeks. When the group of patients who received early treatment was compared against those who were treated only after a worsening fibrosis score (F3 to F4) their fibrosis progressed by at least one stage in 26% of cases compared to 54%, respectively [91].

Roche et al. evaluated 113 HCV transplant recipients who underwent AT and found that the rates of liver failure leading to death or retransplantation induced by AT were higher in patients with baseline fibrosis ≥ 3 (Metavir score) when compared to patients with baseline fibrosis <3 (20 vs 1%; p = 0.0001); moreover, no improvement of fibrosis stage was seen during AT [110]. These data, in line with other studies [92], suggest that treatment at the early stages of hepatitis C recurrence is probably the best strategy. Concerning FCH, a review analyzed 16 studies of patients with FCH who underwent AT and specified the rate of viral response: of a total of 42 patients, 13 experienced a virological and biochemical

response, three underwent re-LT, 19 died, and the outcome was not reported for seven patients [111].

Therapy duration is still an open issue; the recommended duration of treatment for all genotype is 48 weeks, except for genotype 1 patients who do not achieve a negative HCV-RNA at week 4 and a decrease of more than two log in HCV-RNA at week 12, and should, therefore, be treated for 72 weeks. Patients with genotypes 2 or 3, low viral load (<400,000 U/ml), mild fibrosis, and in whom HCV-RNA becomes undetectable in 4 weeks (i.e., RVR) may need only 24 weeks of therapy, but this can increase the relapse rate [112]. Several authors have proposed long-term AT for patients who have not responded after 48 weeks of treatment. Walter et al. prolonged antiviral therapy for >12 weeks in 26 patients and for >18 weeks in 21 patients, observing that at 48 weeks, the rate of viral response was 24.3% and at the end of follow-up it was 35.7%. Only 17% of patients had to discontinue antiviral therapy for side effects and poor tolerance. Moreover, the study showed histological benefit of long-term antiviral therapy in slowing fibrosis progression [113].

Kornberg et al. started a prolonged treatment in 14 recipients, which was tailored according to individual long-term tolerability and compliance. Unlimited continuation of full-dose AT for 14–100 months (median: 61 months) was feasible in nine patients. In virological responders, maintaining the therapy led to further regression of inflammation and fibrosis score. Despite persistent viremia, continued AT prevented progression to severe allograft inflammation in virological non-responders, suggesting an antifibrotic efficacy of AT [114].

Regarding the AT related side effects the most common are neutropenia, and anemia, treated with the administration of growth factors such as erythropoietin and granulocyte colony-stimulating factors. Several immunological derangements such as acute cellular rejection, chronic ductopenic rejection, and autoimmune-type graft hepatitis have been reported [115]. With combination therapy, the risk is below 10%, and controlled studies have revealed no differences in acute rejection rates between treated patients and untreated controls.

The association of IFN plus RBV with first generation proteases inhibitors, boceprevir and telaprevir, has been correlated with a higher SVR but with severe side effects such as anemia for boceprevir, and skin rush for telaprevir [116].

In fact, the use of this combination therapy is not recommended anymore after LT.

New antivirals

IFN-free combinations are rapidly taking over, as they are associated to better response, shorter treatment duration and milder side effects.

Introduce in clinical practice as compassioned use, some of the new antiviral agents have now been approved at the European board and some others will be soon approved. In 2014 three drugs were approved: sofosbuvir (SOF), simeprevir (SMV) and daclatasvir (DAC).

SOF is an HCV NS5B nucleotide polymerases inhibitor and it is a pro-drug. It is administered at the dose of 400 mg once daily and recent data suggest its use might be safe in patients with chronic kidney disease [117]. Major side effects are fatigue, headache, nausea, insomnia and anemia.

SMV is an HCV NS3/4A proteases inhibitor. It is administered at the dose of 150 mg, once daily and no data about dose adaptation in patients with Child Pugh B or C cirrhosis are available yet. As it is metabolized by CYP3A, the co-administration of SMV with drugs inducers or inhibitors of CYP3A is not recommended, as they lower or higher SMV exposure; leading to necessary dose adjustment [118].

DAC is administered at the dose of 60mg once daily. It is a substrate of CYP3A and substrate and inhibitor of P-gp. The dose of DAC should be adjusted to 30 mg daily in HIV-infected patients receiving atazanavir/ritonavir and to 90 mg daily in those receiving efavirenz.

The prescription of the combination of new drugs takes into account the viral genotype, the response to a previous viral therapy, the stage of liver disease and drug-drug interaction especially for HIV coinfected patients.

All patients with HCV recurrence after LT should be considered for AT. However, drug-to-drug interaction is particularly important in transplanted patients. No drug interaction between calcineurins inhibitors, Csa and tacrolimus, and SOF, SMV or DAC has been reported. A recently published multicenter study reported the outcome of 40 HCV transplant recipients who underwent combination therapy of SOF and RBV for 24 weeks, most of them were infected with genotype 1, had had previous IFN combination treatment and 40% of them had cirrhosis. SVR 12 weeks after the end of therapy was 70% with major side effects being fatigue, diarrhea and headache [119].

Concerning FCH, the most severe form of HCV recurrence, a case report was recently published in which the patient was effectively treated with the combination of SOF and DAC with no main side effects [120].

The French experience (CUPILT study) reported the safety and efficacy of SOF and DAC for the treatment of 23 patients with histologically confirmed FCH. The diagnosis of FCH was based on biological criteria (Total bilirubine > 34 µmol/l, GGT > 150 UI/ml, AST >70 UI/ml), virological criteria (HCV RNA > 6 log UI/ml à 4 weeks post-transplantation) in the absence of vascular and biliary complications. SVR12 was 100%. The safety was excellent with no interactions with calcineurine inhibitors [121].

The combination of SOF and SIM ± RBV have been administered to genotype 1 patients with histologically proven HCV recurrence. Mean time between LT and antiviral therapy was 29 months. Eleven percent of patients had a FCH and 29% had a METAVIR score of 3 or 4. On intention-to-treat analysis no difference was seen between patients who received RBV (89%) and those who did not (91%) for SVR 12. SVR 12 was significantly higher in genotype 1a patients with F0-F2 fibrosis stage (97%) compared to F3-F4 fibrosis stage (64%) (p=0.01) while in genotype 1b no difference was seen in both groups of patients. The treatment

was well tolerated, only two patients developed serious side effects: acute pancreatitis in one case, the therapy could be reintroduced afterwards, and fatal pulmonary fibrosis in another case [122].

Safety and efficacy of SOF and SMV ± RBV have been also reported in a true life study (TARGET study). Concerning genotype 1 patients (n=131): SVR 4 was 90% (n=60/68), 86% in cirrhotic patients compared to 94% non cirrhotic, slightly better for genotype 1b (95%) compared genotype 1a (83%). On multivariate analysis, the predictive factors of a lower SVR 4 were the presence of cirrhosis, a previous antiviral treatment, a previous decompensation and the Caucasian race [123].

Another combination has been proposed: SOF + ledipasvir (LDV) + RBV for 12 or for 24 weeks with a study reporting a mean time interval between LT and therapy was 4.4 years. Two hundred twenty one patients were genotype 1 and only 2 patients were genotype 4; most of them were non cirrhotic (F0-F3), treatment experienced, non-CC IL28B. No difference was found according to therapy duration 12 weeks versus 24 weeks for F0-F3 patients, CTP A or CTP B. In CTP A patients SVR was slightly higher compared to CTP B patients: 96% versus 85% (for 12 weeks) and 96% versus 83% (for 24 weeks) respectively. However, the groups were not compared amongst each other. Overall, the treatment was well-tolerated with few severe side effects [124].

A phase II study (CORAL-1) presented the safety and efficacy of the combination of ABT-450/r/ombitasvir 150 mg/100 mg/25 mg/j + dasabuvir 250 mg x 2/j + RBV administered in patients with fibrosis stage ≤ 2, naïf of antiviral treatment. A dose reduction was required for the calcineurine inhibitors, in particular tacrolimus: 0.5 mg/week or 0.3 mg every three days; cyclosporine 1/5 of the daily dose in one administration. Most of the patients were genotype 1a (85.3%) and the SVR24 was 97.1% (n=33/34). One patient relapsed at day 3 with the detection of multiple *de novo* mutations: R155K in NS3, M28T + Q30R in NS5A andG554S in NS5B. The main side effect was anemia due to the ribavirin. In conclusion this combination seems promising but with the downside of ribavirin use and the interaction with CNI [125]

I.3 *De novo* autoimmune hepatitis after liver transplantation

The development of an autoimmune hepatitis (AIH) after LT in a recipient in whom the etiology of the end stage liver disease was not AIH, was described for the first time in 1998 from Kerkar and colleagues [126]. This hepatic disease was define *de novo* AIH in order to distinguish it from the AIH recurrence. Some Authors argued that the damage is not "autoimmune" since it affects an allogenic organ and should be called "graft dysfunction mimicking autoimmune hepatitis" [127], but, as reported by Mieli-Vergani, the recurrence of AIH in patients who underwent LT for AIH has never been questioned [128].

The characteristics of the *de novo* AIH are identical of the one of AIH: hypertransaminases, histological features of interface hepatitis, perivenular necrosis, bridge fibrosis, IgG augmentation, autoantibodies positivity. *De novo* AIH is more frequent in children (5%-10%) [128] compared to adults (1%-3%) LT recipients [129], and its appearance is usually observed within two years after the transplant, probably related to the immunosuppression reduction [130].

I.3.1 Definition

At the moment there are no recognized criteria for the diagnosis of de novo AIH. In the immunocompetent patients the diagnosis of AIH is complex and is based on the presence of characteristic features and the exclusion of other diseases. An international group of experts identified clinical, biological and histological characteristics of AIH and realized a score, called International Autoimmune Hepatitis Group (IAIHG) scoring system, which results from the sum of points given to selected variable. The IAIGH score distinguish AIH, pre-treatment, as "probable" (score from 10 to 15) or "definite" (score >15). If applied after the treatment introduction the score changes and the AIH is "probable" if the score is between 12 and 17, and "define" if it is > 17. This scoring system validated in 1993 and revised in 1999 is recommended in particular when the diagnosis is complex [131]. Although it has never been validated for the diagnosis of AIH after LT, as it doesn't take into account the presence of other liver diseases, the impact of

immunosuppression and the rejection, this score has been used to support the *de novo* AIH diagnosis [132].

Furthermore the BANFF group suggested minimal criteria such as high autoantibodies level and hypergammaglobulinemia combined with characteristic histology, after the exclusion of viral infection, drug induced hepatitis, early or late acute rejection or chronic rejection, for the diagnosis of *de novo* AIH. [133]

I.3.2 Histological diagnosis

As said above, in the post-transplant setting the histological diagnosis of *de novo* AIH is complicated by the presence of several concomitant features, therefore a differential diagnosis with early or late acute rejection, chronic rejection, idiopathic hepatitis or viral recurrence is mandatory.

De novo AIH typical features are plasma cells rich infiltrate, interface hepatitis, perivenular necrosis. These observations support the hypothesis that *de novo* AIH are probably the result of an allo-immune response in which immune-mediated lesions are directed towards the hepatocytes rather than the biliary cells or the vascular endothelium. Despite the plasma cells can be part of the inflammatory infiltrate of rejection, especially in the late phase, the severity of the infiltrate is more prominent in *de novo* AIH. Concerning the biliary tract lesions they can be observed at mild level in both *de novo* AIH and HCV recurrence while the extensive cholangitis and the loss of biliary duct are typical of rejection. Perivenular necrosis, extended to centrilobular zone, is absent in HCV recurrence and is characteristic of *de novo* AIH [134].

I.3.3 Autoantibodies and Human Leucocytes Antigen

The present of tissue autoantibodies has been largely identified after solid organ transplantation, particularly after LT [135]. In a study from Salcedo et al. the autoantibodies were tested before and after LT in 179 LT recipients without AIH and were considered positive if ≥ 1:40. The autoantibodies were also tested in 54

cirrhotic patients who were selected as control group and it resulted that 71% of transplanted patients compared to 30% of cirrhotic patients had positive autoantibodies. The actuarial probability to develop autoantibodies was 23%, 42% and 66% at 6, 12 and 36 months after LT respectively. Interestingly, the prevalence of autoantibodies was higher in male rather than in female patients [136].

Amongst 377 children transplanted for hepatic failure, none of them for AIH, 157 (42%) had positive autoantibodies. However, the diagnosis of *de novo* AIH was reported in 5.3% of cases and only 12% of them presented positive autoantibodies [137]. Nevertheless, the same authors reported that the development of autoantibodies against soluble liver antigen (SLA) after LT can predict the later appearance of *de novo* AIH [138].

Autoantibodies are not specific of *de novo* AIH but their detection can help in making the diagnosis of the disease. A sizeable proportion of patients developing *de novo* AIH have atypical LKM antibodies. Salcedo and colleagues found that the serum containing atypical anti-LKM autoantibodies react with the cytosolic fraction rather than the microsomal hepatic fraction, which contains the cytochrome P45002D6, the molecular target of typical anti-LKM [139].

Recently Huguet et al. studied the target of atypical anti-LKM in 8 patients with *de novo* AIH using the proteomic combining bidimensional proteins electrophoresis and the mass spectrometry for proteins identification. They identified as target the isoform III of the carbonic anidrasis, belonging to the glutathione S transferasis (GSTT) family, and the beta1 subunit of the proteosoma. Indeed the presence of these antigens, as potential target of atypical anti-LKM antibodies, needs to be confirmed by the utilization of monoclonal antibodies. Nevertheless this was the first report that identified autoantigens in *de novo* AIH, suggesting new technique for disease investigation [140].

Aguilera and colleagues argued that the *de novo* AIH is the result of a reaction due to the formation of antibodies anti-GSTT1 directed against the transplanted organ, in recipient without GSTT1 [141].

The role of GSTT1 in the development of *de novo* AIH was further confirmed. In another study 29 out of 419 (6.9%) LT recipients had donor/recipients GSTT1 mismatch, and tested positive for GSTT1 antibodies. Twenty out of 27 (74%) patients, for whom data were available, developed *de novo* AIH within a median follow-up of 26 months after LT. The probability of *de novo* AIH development increased over the time, being 11%, 44% and 60% at 12, 24 and 36 months after LT, respectively. No risk factors such as recipient sex, recipient or donor age, rejection episodes, immunosuppression regimen, GSTT1 allelic expression, HLA distribution, CMV infection, were associated with the development of *de novo* AIH. Nevertheless multivariate analysis identified male gender, non- alcoholic etiology and high level of anti GSTT1 as independent predictors of *de novo* AIH [142].

Moreover, other autoantigens seem to be implicated in the *de novo* AIH inset. A study focused on the autoantibodies directed against cytokeratin (CK) 8 and 18. These autoantibodies were tested in 8 patients who underwent LT, without AIH, from a LDLT. A patient developed *de novo* AIH (IAIHG score of 16) about 2 years after the transplant. The autoantibodies against CK 8 and CK 18 tested positive in this patient even after the autoimmune therapy, while they were absent in the other 7 patients and in all the donors. The CK 8 and CK 18 have a protective role towards the damage directed against the hepatocytes. Furthermore, CK 8 and CK 18 mutations can predispose to the development of cryptogenic cirrhosis [143].

Nevertheless the relationship between a precise CK mutation and a specific disease has not been described yet. Murota et al. reported a significantly higher level of antibodies anti-CK8 and anti-CK18 in patients with AIH compared to patients with HCV hepatitis or compared to controls. Probably the damaged hepatocytes release the CK 8 and CK 18 leading to the production of anti-CK8 and anti-CK18 antibodies with, consequently, the development of an autoimmune damage [144].

Despite the relevance of autoantibodies positivity for the diagnosis of *de novo* AIH, their presence cannot be considered as diagnostic and their absence do not allow ruling out the disease.

In 96 pediatric LT recipients, the presence of autoantibodies was found in 74% of cases, but, in only 4 patients, it was associated with hypergammaglobulinemia and organ dysfunction. Furthermore none of them had histological features of AIH nor met the diagnostic criteria of AIH "probable" or "definite" according to IAIHG scoring system. Only one patient, transplanted for Alagille Sdr, had a histological diagnosis of *de novo* AIH, about 2 years after LT, but the autoantibodies were negative. Moreover the presence of autoantibodies had no impact on the outcome in term of graft or patient loss [145]. On the contrary, the presence of autoantibodies after LT has been also associated with rejection and diminished graft and patient survival [146].

The association between AIH and the antigens of the major histocompatibility complex (MHC) DR3/4 is well known for the immunocompetent patients, and has been also studied in the *de novo* AIH. In the first work describing *de novo* AIH, 5 out of 7 LT recipients received a donor with the alleles HLA DR3 and DR4, but no one of the recipient had HLA DR3 or DR4 [126]. On the other hand, Henegan and colleagues found the presence of HLA DR3 and DR4 in all patients with *de novo* AIH [127] as well as Salcedo and colleagues who found the presence of HLA DR3 in all LT recipients studied [139]. Therefore, the role of HLA antigens in the development of the *de novo* AIH is still highly controversial.

I.3.4 Pathogenesis

The pathogenesis of *de novo* AIH after LT has not been clarified yet. One of the possible described mechanisms is the molecular mimicry. The release of autoantigens from a damaged tissue and the exposition to a virus which has the same amino acids sequences of the autoantigens, lead to an immune cross activation. This process is called molecular mimicry. In line with this hypothesis some case of *de novo* AIH was reported after CMV, EBV or Parvovirus infection. The infection due to these viruses is quite common after LT and can stimulate the development of an autoimmune reaction also through other mechanism such as polyclonal stimuli, enhancement and induction of the expression of MHC I and II antigens, interference with immune-regulatory cells.

Interestingly the calcineurine inhibitors, which are the key

immunosuppressive drugs of the transplant, can stimulate the autoimmunity as they interfere with T cells maturation and with T regulatory cells (Treg) function, with, consequently, the possible emergency of autoimmune clones [147] .

The auxiliary liver transplantation is a good model for the study of *de novo* AIH. This model raises the hypothesis that *de novo* AIH is a form of rejection.

Miyagawa-Hayashino and colleagues described the case of a patient affected by biliary atresia who received an auxiliary liver from an HLA identical donor and developed liver dysfunction in the presence of ANA and increased IgG after immunosuppressive therapy discontinuation for sepsis. The biopsy of the auxiliary liver showed features typical of AIH. The patient improved the liver function after high doses of steroids but the disease recurred at steroids discontinuation and during the follow-up the transplanted liver developed massive necrosis. The Authors concluded that *de novo* AIH is a form of rejection, rather than an autoimmune process, nevertheless as biopsies of both organs were not available at the moment of liver dysfunction, taking into account the HLA similarity, an autoimmune damage cannot be excluded and solid conclusion cannot be drawn [148].

At Kyoto University 13 out of 633 (2.1%) pediatric patients had the diagnosis of *de novo* AIH after LT. The indications of LT were biliary atresia in 11 patients, Budd Chiari sdr in 1 patient and Byler disease in 1 patient. Male/female ratio was 2/11. The immunosuppressive protocol consisted in the combination of steroids tapered over a period of 3 to 6 months and tacrolimus. In 9 patients the AIH diagnosis was “definite” according to IAIGH scoring system, while in 4 was “probable”. The patients were treated with metilprednisolone boluses followed by oral prednisone associated with increase dose of tacrolimus. Two patients did not responded to autoimmune therapy and the liver function progressively deteriorated up to the need of retransplantation. The explants showed a massive necrosis with high grade of interface hepatitis. Multivariate analysis identified rejection episodes and the recipient age, between 11 and 15 years, as factors significantly correlated with *de novo* AIH development. No other factors, in particular other recipient ages, recipient sex, donor/recipient sex mismatch, ABO, HLA A, B, DR donor/recipient mismatch, were associated with the disease onset.

Therefore Authors highlighted the role of acute rejection as trigger of *de novo* AIH supporting the hypothesis of an allo- rather than autoimmune disorder [149].

Yoshizawa *et al*. reported the case of a 33 year old recipient who underwent LDLT for primary biliary cirrhosis and who, 4 years after LT, presented a cytolysis, positive ANA and AMA M2, histological features of autoimmunity and a IAIGH score of 16. The patient was treated with AZA and steroids, but at steroids tapering she had an histologically proved new flair of AIH with an IAIGH score post-therapy of 19. The histology showed no signs of primary biliary cirrhosis. The lymphocytes infiltrating the liver were of recipient origin, suggesting that the recipient's T cells attacked the allo-hepatocytes of the donor [150], therefore this report is in favor of the hypothesis that *de novo* AIH is a variant of rejection. `

On the contrary, another study reported the case of a patient transplanted at 25 months of birth with LDLT for a neonatal hemochromatosis. At 13 months after LT, she was diagnosed with *de novo* AIH, without any previous report of acute rejection [151]. This study showed that the *de novo* AIH disease is not necessarily associated with rejection episode.

Another hypothesis explored the possible role of IgG4 in the pathogenesis of *de novo* AIH, but was recently disproved. Four out of 72 (5.6%) patients, 2 male and 2 female, 1 transplanted for biliary atresia, 1 for Budd Chiari Sdr and 2 for primary biliary cirrhosis, had the diagnosis of *de novo* AIH. The IgG4 research on hepatic tissue was mildly positive in 3 patients, while the serological dosage was negative for all of them. Only one patient had an episode of acute rejection, treated with steroid boluses [152].

I.3.5 Autoimmune therapy

No recommendations concerning the treatment of *de novo* AIH have been made yet. The suggested therapy is the one of AIH hepatitis as reported in the first study which described the disease [126]: prednisone 1-2mg/Kg/day, tapered over 6 to 8 weeks until the maintaining dose of 5-10 mg/day, AZA (1.5-2 mg/Kg/day), and increase of baseline immunosuppression, tacrolimus or CsA.

In a retrospective study, 7 out of 1000 adult LT recipients showed features

of *de novo* AIH at a median time interval of 4.3 years after the transplant (range 0.3-7.2). None of them was transplanted for AIH. All patients had increased IgG level, non-organ specific autoantibodies and histological features of AIH, all having a AIH "probable" or "definite" according to the IAIGH scoring system. All of them responded to the treatment with steroids and AZA at standard doses [127].

Tan and Sian Ho reported the contemporary onset of primary biliary cirrhosis and AIH at 5 years after LT with an improvement of the hepatic function after autoimmune therapy with the combination of steroids and AZA [153].

Salcedo and colleagues studied 12 adult LT recipients with *de novo* AIH, and compared 7 patients who received autoimmune therapy to 5 patients who did not, as the diagnosis was made retrospectively. The baseline immunosuppressive therapy consisted of CsA, AZA and prednisone, tapered within one year. The treated patients received high dose of prednisone (1mg/Kg/day) progressively tapered to the maintaining dose, and they showed a significant improvement with a good long term survival compared to the non treated patients who experienced graft loss and retransplantation [139].

Despite the beneficial effect of autoimmune therapy, *de novo* AIH can lead to the development of fibrosis and impair liver function.

In a study a 9-year-old child who was transplanted for a deficit in alfa1 antitripsine, showed, 4 years after LT, clinical-biological and histological features of *de novo* AIH. The treatment consisted in the increase of CsA and the introduction of steroids and MMF. A histological and biological improvement was observed, nevertheless in the long-term follow-up the last biopsy showed a picture of advanced fibrosis [154].

A case-control study on a pediatric population confirmed that steroids dependence is an important characteristic, which can help to define *de novo* AIH. These patients can hardly be maintained on monotherapy with tacrolimus or CsA and require high level of immunosuppression in order to have a normal liver function. Moreover they tend to be on AZA or MMF at the moment of AIH diagnosis,

confirming the over-response of the immune system, despite immunosuppression treatment [155].

In a series of pediatric transplant recipients, patients were on monotherapy with a calcineurine inhibitor at suboptimal dose, at the moment of *de novo* AIH diagnosis. The Authors hypothesized that the non-adequate immunosuppression levels were responsible for the disease development. All were treated with AZA and prednisone similar to treatment for AIH. However, despite aggressive treatment, four patients developed bridging portal fibrosis resulting in graft loss in two patients [156.]

Andreis and colleagues reported that 11 patients with the diagnosis of *de novo* AIH do not respond to increasing CsA or tacrolimus levels and require steroid and AZA. Three had mild to moderate relapse with increase of ALT thereafter [157]

An important issue is the recidivism of the disease at steroids reduction as reported by Spada *et al*. who described an improvement after autoimmune therapy but a disease recurrence at steroids tapering [158].

I.4 HCV recurrence and de novo autoimmune hepatitis after liver transplantation

The presence of autoimmune biological and histological components in patients with HCV pre- and post-LT has been increasingly described and the onset of *de novo* AIH in patients with HCV recurrence has been variably interpreted.

I.4.1 HCV and autoimmunity

In immunocompetent patients HCV is associated with a particular immunopathological pattern that is represented by autoantibody production, crioglobuline mixte, and the B cell lymphoma [159]. Moreover, in patients with chronic HCV infection the presence of autoantibodies and/or a concomitant AIH is not rare.

Non organ specific autoantibody are present in 70% of HCV positive patients, the most frequent are SMA, 60%, followed by ANA, 8%-10% and anti-LKM 1%-5% [160]. Nevertheless, autoantibody positivity is not always associated with histological characteristics of autoimmunity.

A study of 60 HCV positive patients analyzed the prevalence of interface hepatitis, plasma cell infiltrate and lobular hepatitis and found that it was similar in patients with or without autoantibodies, nevertheless the patients with histological autoimmune features had a more severe disease. The presence of HLA DR3 was more frequent in patients with composite viral-autoimmune pattern [161].

The mechanism explaining the extra-hepatic manifestations observed in HCV positive patients seems related to the activation of the immune system induced by the virus. The peculiar ability of C virus to induce autoimmune disease has been partially explained by molecular mimicry, defined by the presence of a linear or structural homology among micro-organism (bacteria, viruses or parasites) and self-antigens. The immune response initially directed against a non-self antigen cross-reacts with homologues sequences of the host inducing the autoimmune reaction. Moreover, this reaction is complicated by the process of inter- and intra-molecular diffusion of epitopes characterized by the ability of the immune response to turn against different epitopes of the same molecule or against an adjacent molecule [162]. Therefore the maintenance of the immune response does not need the antigen by which it was induced [163].

Moreover the C virus can activate natural killer cells through the binding between CD81 receptor and the coat viral protein E2, leading to polyclonal non-antigen specific expansion. This process joint to the ability of the virus to replicate inside the lymphocytes represents another explanation of the autoimmune extra-hepatic manifestation of HCV infection [164].

Moreover the presence of HCV infection and an autoimmune process can impact HCV disease progression.

Interestingly Rigamonti and colleagues found that in HCV+ patients with persistently positive IgG anti-CYP2E1, pre- and post-LT, the prevalence of HCV recurrence was higher, with more severe necro-inflammation and more rapid fibrosis progression at histology, compared to patients with isolated IgG anti-CYP2E1 pre- or post-LT. Multivariate analysis identified that the persistence of IgG anti-CYP2E1 was an independent risk factor for HCV recurrence [165].

I.4.2 HCV antiviral therapy and *de novo* AIH

De novo AIH has been described in the context of antiviral therapy for HCV recurrence after LT, suggesting a possible causal role of this therapy in promoting the disease development.

A study described the case of a patient transplanted for HCV cirrhosis, without autoimmune manifestation and without medical history of viral therapy. The baseline immunosuppression was composed by a triple therapy: daclizulab, prednisolone and tacrolimus, tapered later to only tacrolimus. At 5 years after the transplant the HCV recurrence was diagnosed and a combined therapy of Peg-IFN and RBV was administered with the achievement of early virological response (EVR) and the improvement of liver tests. Nine months after the therapy beginning, increase of liver tests was observed, HCV-RNA was persistently negative, and the tacrolimus trough level was within correct range. A liver biopsy was performed showing features of *de novo* AIH, with no signs of rejection; IgG level was increase but autoantibodies were negative. Considering the negative impact of the steroid therapy on HCV recurrence, only AZA was introduced with the obtainment of transaminases normalization. Thereafter, AZA was discontinued due to severe anemia with a consequent flair of the *de novo* AIH [166].

Merli and colleagues reported three cases of patients who had HCV histological recurrence diagnosed at 6 months, 3 and 4 years after LT. After viral clearance all three patients presented increase of liver tests with positive autoantibodies ANA and ASMA (which were negative before the treatment), increase of gammaglobuline and histological characteristics of *de novo* AIH.

The IAIGH score was 10, 11 and 13. A patient was treated with steroids (1mg/kg/day) and AZA while the other two received only with steroids because of leucopenia. In all patients there was an improvement of the cytolysis and cholestasis indices. HCV infection, however, relapsed, after few months, in all patients [167].

In a study from Bologna, 9 patients underwent antiviral therapy for HCV recurrence after LT, although viral clearance, 8 patients showed increase liver tests. All patients tested positive for autoantibodies in particular ANA and/or ASMA, anti-dsDNA, AMA, had increase IgG and IAIGH score between 10 and 14. The histology was compatible with AIH with severe interface hepatitis and plasma cells infiltrate. Three patients presented a concomitant autoimmune disease such as thyroiditis, overlap sdr and systemic lupus erythematosus. In all patients antiviral therapy was discontinued and the steroid therapy introduced, which was beneficial in 7 patients [132].

Another report described the case of a 52-year-old LT recipient who underwent antiviral therapy (Peg-IFN and RBV) for HCV recurrence, 7 months after LT. Ten months after therapy initiation a rise of liver tests was observed with HCV-RNA persistently negative. The liver biopsy showed histological features of HCV recurrence and AIH. Autoantibodies were mildly positive before the transplant but negative before and during antiviral therapy [168].

A recent study reported the case of a 51-year-old woman with HCV recurrence after LT who underwent antiviral therapy for 17 months and obtained viral clearance 7 months after therapy beginning. The type of interferon was changed (Peg-IFN alfa2a instead of Peg-IFN alfa2b) in order to prolong the therapy, according to Japan's rule. Three months after Peg-IFN switch the patient developed liver dysfunction. The viral load was still negative, ANA were positive and the histology was typical of AIH without signs of acute or chronic rejection. The antiviral therapy was discontinued and the immunosuppressive therapy switched from CsA monotherapy to the association of tacrolimus and steroids. The control biopsy performed one month after therapy change showed the persistence of the

inflammatory infiltrate but the disappearance of plasma cells. However, the virus relapsed thereafter [169].

Recently it has been reported that Peg-IFN can induce immuno-madiated graft dysfunction in patients treated for HCV recurrence after LT with increased graft failure and patients mortality [170].

The interferon can stimulate an autoimmune response as it modulates immunogobuline production, inhibits T suppressor lymphocytes, induces natural killer cells and T cytotoxic lymphocytes, stimulates the expression of the major histocompatibility complex class I and enhance the T helper lymphocytes 1 (Th1) response. This is particularly true for the Peg-IFN, which has a longer midlife compared to the standard IFN. The predominance of Th1 activity has been reported in organ specific autoimmune response and it is well known that the imbalance between Th2 and Th1 response, with the Th1 predominant activity, plays an important role in autoimmune response induced by the interferon [171].

In all cases previously described the onset of AIH appeared at viral clearance, probably due to the immune response stimulated by the interferon. The development of *de novo* AIH, however, is not necessarily due to the IFN.

Interestingly in the series reported by Fiel and colleagues the antiviral therapy was administered in 11 patients who developed *de novo* AIH, 3 of them cleared the virus at the moment of *de novo* AIH diagnosis. Therapy itself was not associated with *de novo* AIH development [172].

Levitsky et al. recently proposed the term interferon-induced graft dysfunction (IGD) for interferon-induced liver graft damage pathologically characterized by PCH, ACR or chronic rejection. One of their most important findings was that overall graft survival was poor in patients who were diagnosed with IGD and achieved SVR, compared to patients without SVR. PCH, a common feature of IGD, is a serious complication that can develop during or after interferon-based treatment for HCV after LT. No prior IFN treatment, the use of

PegIFNa-2a and the presence of PCH in the biopsy performed before antiviral therapy was identified as risk factors for PCH onset [170].

Kugelmas et al. found that IS levels decreased significantly in patients responding favorably to anti-HCV therapy. The viral clearance improves hepatic microsomal function, resulting in lowering of immunosuppression levels. This decrease in IS levels may have a key role in predisposing these patients to ACR [173].

A report described the case of a 23-year-old female transplanted for HCV cirrhosis which complicates a common variable immunodeficiency, she had since the age of 15. The native liver showed no signs of AIH. About 5 months after LT she had histological HCV recurrence motivating the introduction of antiviral treatment, combination of IFN and RBV was administered. She underwent a long-term therapy and at the viral clearance she had a rise in liver tests with the histological diagnosis of *de novo* AIH. Antiviral therapy was discontinued and steroids therapy introduced and the patient normalized liver tests maintaining a viral load persistently negative. It is hard to determine if the emergency of *de novo* AIH was due directly to the IFN or to the restoration of the immune system at the time of viral clearance [174].

Interestingly, Ikegami et al. described the development of PCH during antiviral triple therapy with first generation proteases inhibitors. Nine LT recipients were treated with the combination of Peg-IFN, RBV and teleprevir and 3 (33.3%) of them presented the diagnosis of PCH during the AT. PCH was treated with increase of immunosuppression and introduction of steroids obtaining liver tests improvement and SVR12 [175].

In antiviral triple therapy including telaprevir, which has potent viral clearance activity and strong interference with calcineurine inhibitors metabolism, there might be more chances to have IFN induced PCH. However, the drug-drug interaction between telaprevir and calcineurine inhibitors, which require tacrolimus and cyclosporine dose reduction can expose the patient to sub-optimal IS doses. Calcineurine inhibitors levels should be strictly monitored and promptly augmented at telaprevir discontinuation [176].

Antiviral therapy for HCV recurrence after LT rapidly changed in the past months and nowadays IFN-free regimen is indeed privileged. It will be interesting to see if *de novo* AIH would arise also with the new drug combinations. No data regarding this topic have been published yet.

I.4.3 *De novo* AIH: atypical HCV recurrence or atypical form of rejection?

Hepatic damage characterized by the presence of both viral lesions and autoimmune lesions is of difficult interpretation. To determine which type of lesions are prevalent is complicated as some of the AIH features are part of the spectrum of viral lesions or rejection. The mild damage of the biliary tract is commonly found in both HCV and AIH hepatitis, on the contrary, the lymphocytic severe cholangitis and the biliary tract loss is typical of rejection. The inflammation and necrosis of the central zone is characteristic of AIH and is rarely found in HCV infection. The severe interface hepatitis or the prominent plasma cell infiltrates are also typical of AIH but can be present in patients with HCV recurrence [134, 134].

Khettry and colleagues described the immuno-madiated damage, found in HCV transplant recipients, as a variant of the HCV recurrence and they called it "autoimmune-like (AIH-like)" hepatitis. They studied 92 LT recipients who were grouped according to histological features: 1. No HCV recurrence (n=31), 2. HCV typical recurrence (n=52), 3. Autoimmune-like HCV recurrence (n=9). Six out of 9 patients from the third group had increased gammaglobuline and/or autoantibodies positivity. The patients with AIH-like diagnosis had higher fibrosis progression ≥ 2 (according to Batts classification) and, they presented in the native livers a prominent plasma cells infiltrate, particularly periseptal [177]

Fiel et al. consider the *de novo* AIH as a form of rejection and they called it "plasmacell hepatitis" (PCH). 80% of the patients had recent lowering in immunosuppression or sub-therapeutic calcineurine inhibitors levels and 52% developed PCH within 2 years after LT. Histologic PCH resolution was associated with good outcome, on the contrary, patients not treated or receiving corticosteroids as therapy had poor outcome as 60% of them ended up in

developing cirrhosis, needing of re-LT or death. No histological characteristics in the biopsies performed before the onset of PCH, were predictive of hepatitis resolution. The authors concluded that the PCH is not a truly AIH arguing that AIH rarely develop within the first 2 years after LT and doesn't have a rapid histological resolution, furthermore, autoantibodies present at low level have already been described in patients with HCV recurrence or during rejection episodes. Therefore, based on these observations, authors conclude that the PCH is rather a variant of rejection. Treatment with the optimization of immunosuppression without using steroids appears effective, nevertheless the outcome remains poor [172].

The same group performed a case-control study in which every patient with PCH, developed in the context of HCV recurrence, was compared with two patients with only HCV recurrence, selected according to the date of transplant and biopsies availability. The outcome was evaluated based on the presence of advanced fibrosis or death. 40 patients with PCH diagnosis had a significantly worse outcome compered to the patients without PCH, as they had higher advanced fibrosis (65% versus 40%, p=<0.01), and higher mortality rate (50% versus 30%, p=0.05). The histological analysis of the explant identified plasma cells infiltrate over 30% in 40% of PCH patients versus 18% of control patients (p=<0.01), suggesting that some patient had a predisposition to developing PCH [178].

II. AIM OF THE STUDY

The aim of the present study was to evaluate in patients with HCV recurrence after liver transplantation and the onset of autoimmune histological features:

- prevalence of autoimmune or viral lesions applying a new semi-quantitiavie method, which lead to the identification of two populations
- clinical, biological and histological characteristics of the two population
- impact of autoimmune therapy
- long-term outcome

III. MATERIALS AND METHODS

This study is a bicentric, retrospective study, which analyzed two thousand hundred sixty liver biopsies performed between 2000 and 2012, per protocol or due to transaminases increase, in patients with HCV histological recurrence after LT. The biopsies with features of autoimmune damage were selected and revised by three expert pathologists. The selected biopsies were performed in patients who underwent LT between 1989 and 2009. These patients were included in the study. The last follow-up was July 2013.

The study was performed at two European liver transplant centers: Multivisceral Transplant Unit of Padova, Padova, Italy and Centre Hépato-biliaire, Paul Brousse, Villejuif, France.

III.1 Histological evaluation

In all biopsies three characteristics were evaluated using a semi-quantitative score:

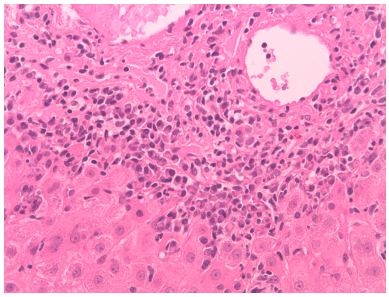
a. Plasma cells infiltrate
   0= absent or mild: if isolated, in less than half of portal tract
   1= moderate: if isolated or in clusters in more than half of portal tract
   2= severe: if in clusters in more than half of portal tract and constitute more than 30% of the inflammatory infiltrate

b. Interface hepatitis
   0= low grade: focal or complete in half of portal tract
   1=high grade: focal or complete in more than half of portal tract

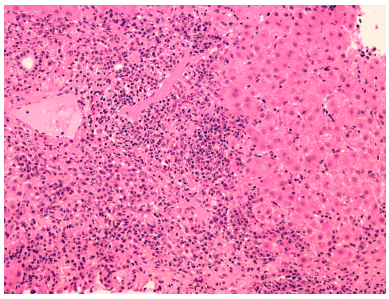
c. Central vein necrosis
   0=absent
   1=present: focal
   2=present: confluent

Images of the three histological characteristics are showed in **Fig. 1**.

a.



b.



c.

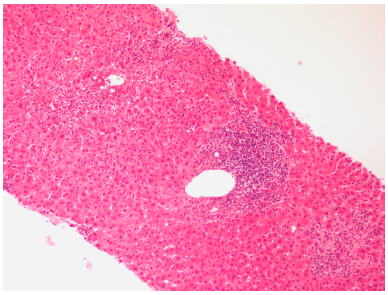


Figure 1. Histological images of: a. plasma cells infiltrate, b. interface hepatitis, c. central vein necrosis

These characteristics have been used to formulate the final histological diagnosis based on the prevalent lesions:

- HCV-R: prevalence of viral lesions
- AIH: prevalence of immunological lesions

The histological activity index (HAI) and the fibrosis stage (S) have been evaluated in the index biopsies, in control biopsies performed after autoimmune therapy as well as in the last available biopsies, according to the Ishak classification, with a score from 0 to 18 for the HAI, and from 0 to 6 for the S.

III.2 Clinical and biological characteristics

For each patient the following characteristic have been evaluated: sex, transplant age, transplant indication, early complications after LT, HCV genotype, baseline immunosuppression (cyclosporine or tacrolimus +/- mycophenolate mofetil, prolonged steroid therapy more than three months after the transplant, rejection episodes, presence of extra-hepatic autoimmune disease, diabetes.

Interval time between LT and liver biopsies was registered as well as time and cause of death. For each donor sex and age were evaluated.

At the time of liver biopsy the following data were collected:

- non organ specific autoantibodies tissue anti-nuclear (ANA), distinguished based on their titer on: mild (between 1:40 and 1:160), moderate (1:320), elevated (≥1:640); anti-smooth muscle (ASMA), anti-liver-kidney microsome (LKM) and anti-mitochondria (AMA).
- Trough level of calcineurine inhibitors considered adequate, over one year after LT, if >4 ng/ml for the tacrolimus and >300 ng/ml for the cyclosporine dosed at 2 hours after the administration
- Aspartate aminotransferasis (AST, N 10-45 U/l), alanine aminotransferasis (ALT, N 10-50 U/l), total bilirubin (N 1.7-17 umol/l), alkalin phosphatases (PAL, N 56-128 U/l), gamma-glutamil transpeptidases (GGT, N 3-65 U/l), gammaglobuline (N 11-18.6%)
- Viral load quantified with the following test: COBAS AMPLICOR HCV MOMITOR test, COBAS TaqMan – Roche, and reported in IU/ml

III.3 Antiviral therapy

The antiviral therapy administration was reported. The therapy consisted in the combination of Peg-IFN: Peg-IFNα2a or Peg-IFNα2b and ribavirin. The starting doses were the following: 135 o 180 μg/kg/week for the Peg-IFNα2a and 1.5 μg/kg/week for the Peg-IFNα2b. Ribavirin was administered at 800-1200 based on body weight, taking into account renal function and anemia. Viral clearance 6 months after the end of therapy (sustained virological response, SVR) was reported.

III.4 Autoimmune therapy

Therapy of *de novo* AIH consisted in the introduction of prednisolone at 1 mg/kg/day, and/or micophenolate mofetil at 1.5-2 gr/day or azathioprine 50-100 mg/day. The steroids were tapered to a maintenance dose of 5-10 mg/day.

III.5 Statistical analysis

The comparison between patients with prevalent viral lesions (HCV-R) and those with prevalent autoimmune lesions (AIH) have been made for each variable. The variables have been evaluated with Mann-Whitney, Chi-square and Fisher test, with significance at $p<0.05$. The graft and patients survivals have been evaluated according the non-parametric Kaplan-Meier model for the general population and have been compared between HCV-R and AIH patients.

IV. RESULTS

IV.1 Population

IV.1.1 Study design

Forty out of 1083 (3.7%) patients, who underwent LT for HCV cirrhosis and had histological HCV recurrence, presented features of autoimmune damage in at least one liver biopsy.

According to the pathological revision the 40 patients were divided in two groups, based on the prevalence of viral or autoimmune lesions:

- HCV-R 16 (40%)
- AIH 24 (60%)

Figure 2 illustrates patient's diagnosis, treatment and outcome. The autoimmune therapy (AT) was administered to 4 out of 16 (25%) HCV-R patients versus 16 out of 24 (67%) AIH patients (p=0.009). Eleven patients (among them one was retransplanted) died and two were retransplanted. Among HCV-R patients: 2 patients died and 1 was retransplanted. Among AIH patients: 1 patient was retransplanted and then died, 8 died and 1 was retransplanted and is still alive.

The cause of death (D) / retransplantation (R)

- HCV end stage disease (1R and D/4D) 5
- HCV cirrhosis + autoimmune (2R/1D) 3
- Fibrosant Cholestatic Hepatitis 1
- Sepsis 3
- HCC recurrence 1

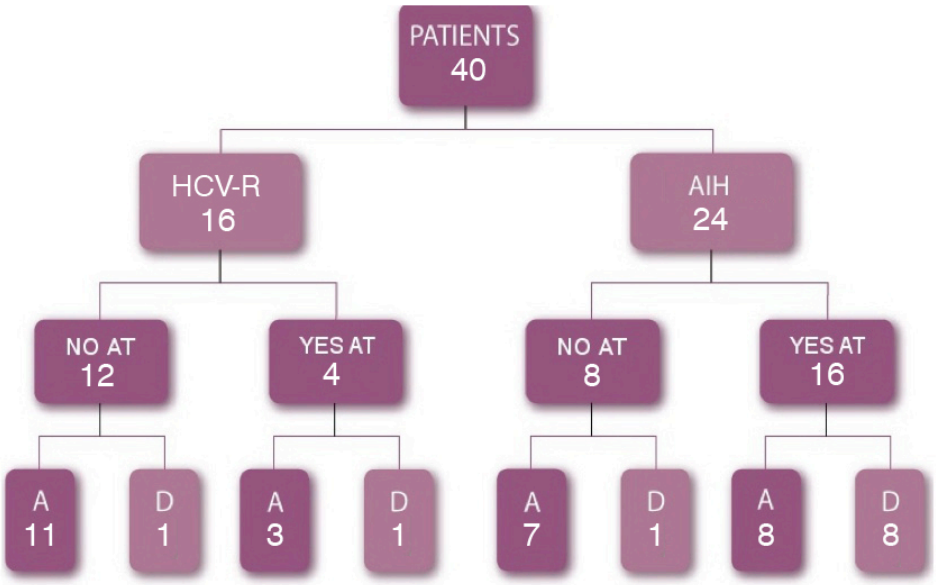


Figure 2. Flow chart resuming the study design. HCV-R: prevalence of viral lesions; AIH: prevalence of autoimmune lesions; AT: autoimmune therapy; A: alive; D: dead.

IV.1.2 Clinical and biochemical characteristics

The study population was represented by 33 male and 7 female, median age 54 years (27-69). The median follow-up time was from LT 71 months (26-149). The median time between index biopsy and LT was 24 months (4-221). Donor prevalent gender was male (n=26) compared to female (n=14), and donor median age was 51.5 (17-79). The main immunosuppressive drugs were tacrolimus in 50% of patients and cyclosporine in the other 50%. The calcineurine trough level was considered in a satisfactory range in 33 (82.5%) of patients at the time of index biopsy. Acute rejection episodes were found in 9/40 (22.5%) patients and in 7 patients rejection was classified as moderate/severe and required steroid boluses. The early complications after the LT were found in 13 out of 40 (32.5) patients, most of them were vascular (n=7), followed by infections (n=4), and biliary complications (n=3). Sixteen (40%) of patients had diabetes and only 8 (20%) presented an extra-hepatic autoimmune disease. The clinical and biochemical features of the study population are resumed in **Tab. 1**. Overall 19 out of 32 (59%) patients with available data had high titer ANA. The immunological characteristics of the study population are showed in **Tab. 2**.

	Patients N=40
Gender M/F	33/7
Age at LT median (range) ys	54 (27-69)
Genotype	
1a/1b/4 N(%)	28 (70)
2a2c N(%)	10 (25)
Diabetes N(%)	16 (40)
Extrahepatic autoimmune disease N(%)	8 (20)
Donor gender M/F	26/14
Donor age median (range)	51.5 (17-79)
Cyclosporine N(%)	20 (50)
Tacrolimus N(%)	20 (50)
Correct immunosuppressor trough level N(%)	33 (82.5)
Acute rejection N(%)	9 (22.5)
Interval LT-index LB median (range) months	24 (4-221)
AST median (range) U/l	153 (24-562)
ALT mdian (range) U/l	137 (28-1282)
Total Bilirubin median (range) umol/l	23 (1.7-673)
ALP median (range) U/l	125 (41-451)
GGT median (range) U/l	143.5 (14-1303)
HCV-RNA median (range) IU/ml	4.420.000 (0-21.046.080)

Table 1. Clinical and biochemical characteristics of the overall population

	Patients N=40
Gammaglobulin median (range) g/L	22.8 (15-46)
ANA	N=32
negative	13 (41)
> 1:160 (%)	11 (34)
> 1:320 (%)	4 (12.5)
> 1:640 (%)	4 (12.5)
AMA	
negative	32
ASMA	
Positive	9 (28)
Anti-LKM neg	32

Table 2. Autoimmune biochemical characteristics of the overall population

IV. 2 Comparative study: AIH versus HCV-R

The study population was divided in two groups, based on the prevalence of histological lesions, viral versus autoimmune, as previously mentioned: 16 (40%) HCV-R patients and 24 (60%) AIH patients.

IV.2.1 Clinical and biochemical characteristics

The male gender was prevalent in both groups, M/F: 15/1 in HCV-R compared to 18/6 in AIH patients. The median age was 55 years (30-63) for HCV-R patients compared to 52 (31-69) for AIH patients. Donor sex and age were also not significantly different between the two groups. No difference was observed regarding the calcineurine inhibitor used as major immunosuppressive drug (cyclosporine versus tacrolimus). Nine out of 40 (22.5%) patients had moderate/severe acute rejection, which was treated in 8 patients with steroids boluses. The number of patients who had at least one episode of acute rejection was higher in AIH compared to HCV-R patients, 2 (13%) versus 7 (39%); although the difference was not significant.

The median interval time between LT and the index biopsy was 23 (6-209) months in HCV-R compared to 26.5 (5-224) months in AIH patients. The mean interval time between LT and index biopsy for patients who had acute rejection was 22 months (range 8 - 223) as the overall population.

Concerning the C virus, the genotypes were equally distributed; with the prevalence of genotype 1 and 4 compared to genotype 2 in both HCV-R and AIH patients. Interestingly viral load was higher in HCV-R patients even if the difference was not statistically significant.

Among biochemical variables AST and ALT were significantly higher in AIH patients compared to HCV-R patients: AST 86 U/l (27-448) versus 175 U/l (24-562) respectively (p=0.053), ALT 83 U/l (28-319) versus 227 U/l (29-1282), respectively (p=0.003). All the clinical and biological characteristics are summarized in **Tab. 3**.

	HCV-R N=16	AIH N=24	p
Gender M/F	15/1	18/6	ns
Age at LT median (range) ys	55 (30-63)	52 (31-69)	ns
Genotype			
1a/1b/4 (%)	13 (81)	15 (62)	ns
2a2c (%)	3 (19)	7 (29)	ns
Diabetes (%)	9 (56)	7 (32)	ns
Extrahepatic autoimmune disease (%)	2 (12.5)	6 (25)	ns
Donor Gender M/F	8/8	18/6	ns
Donor age median (range)	56 (24-75)	55 (17-79)	ns
Cyclosporine (%)	7 (44)	13 (54)	ns
Tacrolimus (%)	9 (56)	11 (46)	ns
Acute rejection (%)	2 (13)	7 (39)	ns
Interval LT-index LB median (range) months	23 (6-209)	26.5 (5-224)	ns
AST median (range) U/l	86 (27-448)	175 (24-562)	**0.053**
ALT mdian (range) U/l	83 (28-319)	227 (29-1282)	**0.003**
Total Bilirubin median (range) umol/l	18 (1-673)	27 (1.3-223)	ns
ALP median (range) U/l	122.5 (42-286)	156.5 (41-451)	ns
GGT median (range) U/l	82.5 (19-799)	168 (14-1303)	ns
HCV-RNA median (range) IU/ml	3.815.009 (5.785-11.777.430	1.609.032 (0-21.046.080)	ns

Table 3. Patient's clinical and biological characteristics at time of index biopsy. Comparison between AIH and HCV-R patients.

The evaluation of autoimmune biochemical characteristics showed no significantly differences between HCV-R and AIH patients concerning gammaglobuline level or non-organ specific autoantibodies titer (**Tab. 4**).

	HCV-R N=16	AIH N=24	p
Gammaglobumine median (range) g/L	23 (8-38)	21 (12.5-46)	ns
ANA			
negative	5 (42)	8 (40)	ns
> 1:160 (%)	4 (33)	7 (35)	ns
> 1:320 (%)	2 (17)	2 (10)	ns
> 1:640 (%)	1 (8)	3 (15)	ns
AMA			
negative	12	20	ns
ASMA			
Positive	4 (33)	5 (25)	
Anti-LKM negative	12	20	ns

Table 4. Autoimmune biochemical characteristics. Comparison between AIH and SLE patients.

IV.2.2 Histological characteristics

The three histological characteristics were evaluated in order to distinguish AIH from HCV-R patients. In AIH patients the high-grade interface hepatitis were present in a significantly higher number of patients compared to HCV-R patients: 24/24 (100%) versus 9/16 (56%) (p=0.010) respectively. The confluent central vein necrosis was also more frequent in AIH compared to HCV-R patients: 20/24 (87%) versus 0 (p=<.0001) respectively. Concerning the plasma cells infiltrate it was moderate/severe in a higher number of AIH patients compared to HCV-R patients, although the difference was not statistically significant, probably to the small sample size.

The histology of liver biopsies was compared according to Ishak score: the activity index (HAI) was significantly higher in patients with AIH compared to HCV-R patients: 16.23±17.13 (mean±SD) versus 8.09±17.13 (mean±SD) respectively (p=0.005), nevertheless no difference was observed in term of fibrosis stage (S): 3.23±1.36 (mean±SD) versus 3.00±1.26 (mean±SD) respectively (p=ns) (**Tab. 5**).

	HCV-R N=16	AIH N=24	p
HAI (mean±SD)	8.09±17.13	16.23±17.13	0.005
S (mean±SD)	3.23±1.36	3.00±1.26	ns

Table 5. Histological evaluation according Ishak score in HCV-R and AIH patients.

HAI=histological activity index, S=fibrosis stage.

IV.2.3 Antiviral therapy

The antiviral therapy was administered in 16 out of 40 (40%) patients and consisted in the combination of Peg-IFN and ribavirin.

In 6 patients antiviral therapy was administered 12 to 60 months before the index biopsy. In 8 patients antiviral therapy was administered after immune damage diagnosis. In 2 patients the index biopsy was made during the antiviral therapy. Six out of 16 (37.5%) patients obtained sustained virological response.

IV.2.4 Long term outcome

Overall graft survival was 67.5 % (27/40) and overall patient survival was 72.5 % (29/40) 10 years after LT.

Patient survival 10 years after LT was significantly lower for AIH patients compared to HCV-R patients: 65% versus 93% respectively (p=0.050) (**Fig. 3**).

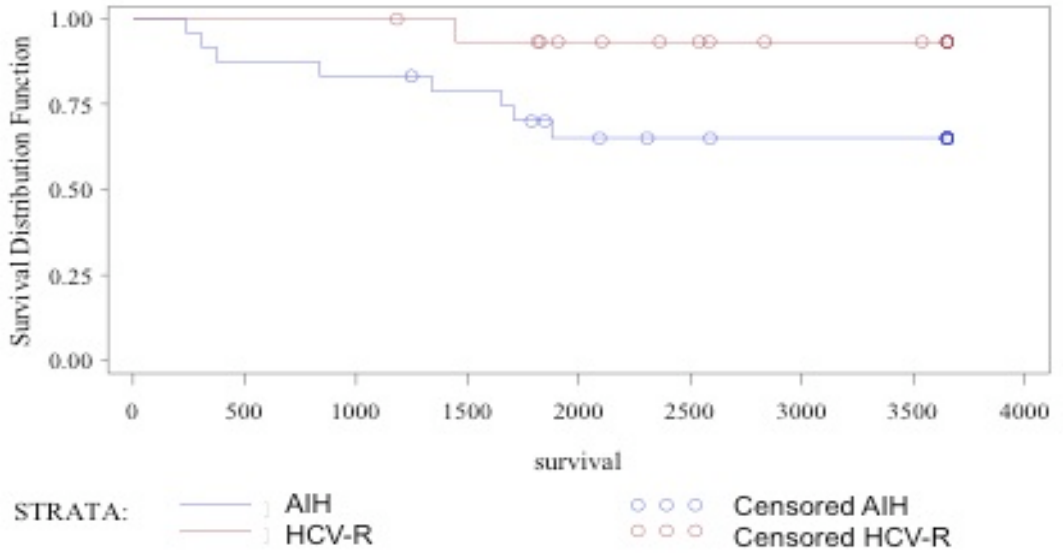


Figure 3. Patient survival according to AIH or HCV-R (p=0.050)

IV.3 Autoimmune therapy (AT) in AIH patients

IV.3.1 Autoimmune therapy

Retrospectively AIH patients were more frequently treated with autoimmune therapy (AT) comparing to HCV-R patients: 16 (67%) versus 4 (25%), respectively (p=0.009) (**Tab. 6**).

The 4 HCV-R patients treated with AT presented either a positive autoantibody or hypergammaglobulinemia which explains why the clinicians chose to introduce AT.

The study focused on the 16 AIH patients treated with AT. Almost every patient (15 out of 16) received high doses of steroids, in 6 patients AZA was added while in 10 patients MMF was added. No significant difference was found in the number of patients who was treated with AZA or MMF. Steroids were administered at the dose of 1 mg/Kg/day for a month and then tapered, with a maintenance dose of 3 to 5 mg/day.

	HCV-R N=16	AIH N=24	p
AT YES N(%)	4 (25)	16 (67)	0.009
AT No N(%)	12 (75)	8 (33)	

Table 6. Autoimmune therapy in HCV-R and AIH patients. AT= Autoimmune Therapy

IV.3.2 Biochemical characteristics before and after AT

At a median interval time of 11 months (range 1 – 59 months) a control biopsy was performed in all AIH patients. At that time biochemical characteristics were recorded (**Tab. 7**). A statistically significant decrease of transaminases level was observed: AST pre-AT 204 (36-562) versus AST post-AT 74.5 (29-241) (p=0.0050), ALT pre-AT 295 (29-1282) versus ALT post-AT 104.5 (35-173) (p=0.0012).

	AIH N=16		
	Before AT	After AT	p
AST median (range) U/l	204 (36-562)	74.5 (29-241)	**0.0050**
ALT median (range) U/l	295 (29-1282)	104.5 (35-173)	**0.0012**
Total Bilirubin median (range) umol/l	30 (1.3-187)	25 (7.5-789)	ns
ALP median (range) U/l	168 (88-451)	112 (62-275)	ns
GGT median (range) U/l	189 (14-1303)	88 (29-1544)	ns
HCV-RNA median (range) U/l	1.820.000(0-21.046.080)	1.320.000 (0-11.786.323)	ns

Table 7. Biochemical characteristics of AIH patient before and after AT.

AT = autoimmune therapy.

The autoimmune biochemical characteristics were evaluated before and after AT. Even though there was a decrease in gammaglobuline concentration and in high level of autoantibodies, the differences were not statistically significant (**Tab. 8**).

	AIH N=16		
	Before AT	After AT	p
Gammaglobuline median (range) g/L	26 (16.5-46)	21 (15-46)	ns
ANA	N=14	N=12	
> 1:160 (%)	10 (71)	8 (67)	ns
ASMA	N=14	N=10	
Positive (%)	5 (36)	2 (20)	

Table 8. Autoimmune characteristics of AIH patients before and after AT.

AT = autoimmune therapy.

IV.3.3 Histological changes before and after AT

The changes of histological characteristics were evaluated in AIH patients in liver biopsies performed before and after AT. A reduction from moderate/severe to mild plasma cells infiltrate and a reduction from high grade to low grade interface hepatitis was observed. However these changes were not statistically significant. The regression of central necrosis was observed in 14/16 (87.5%) versus 2/16 (12.5%) patients (p=0.045).

The histology of biopsies performed before and after AT was also compared according to Ishak score. There was a significant improvement of the activity index (HAI): 11±2.78 (media±DS) versus 4.89±2.80 (media±DS) (p=0.007), before and after AT respectively.

On the other hand no significant difference was observed concerning the fibrosis stage (S): 3.33±1.22 (media±DS) versus 3.22±2.28 (media±DS) (p=ns), before and after AT respectively (**Tab. 9**).

AIH N=16			
	Before AT	After AT	p
HAI (mean±SD)	11±2.78	4.89±2.80	0.007
S (mean±SD)	3.33±1.22	3.22±2.28	ns

Table 9. Histological evaluation according Ishak score in AIH patients before and after autoimmune therapy (AT). HAI=histological activity index, S=fibrosis stage.

IV.3.4 Long term survival according to AT

Patients survival 10 years after LT in AIH group was higher for non-treated compared to treated patients, although the difference was not statistically significant: 87.5% versus 50%, respectively (p=0.1980). (**Fig. 4**).

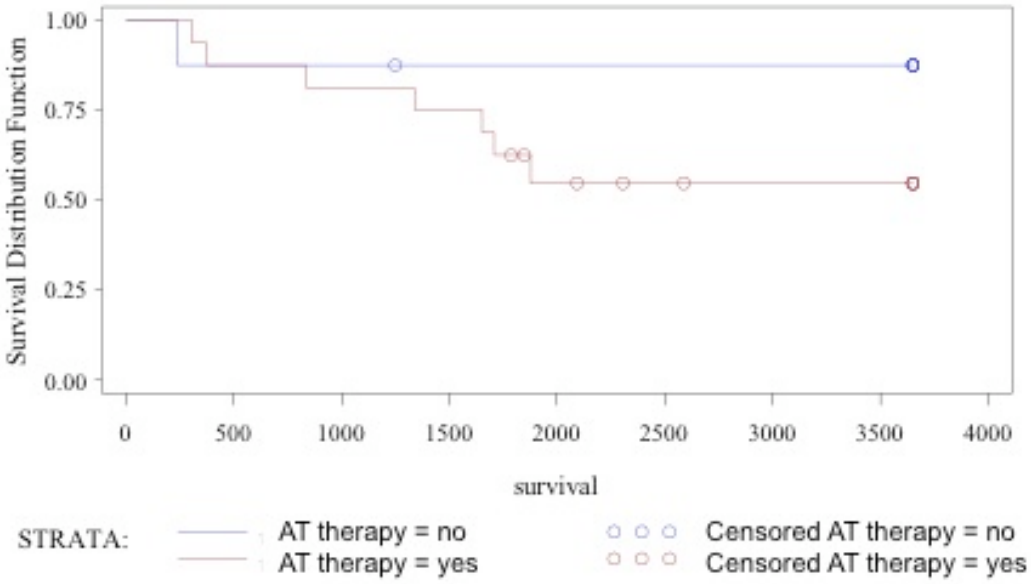


Figure 4. Patient survival in AIH patients who underwent autoimmune therapy versus patients who did not (p=ns).

V. DISCUSSION

HCV disease recurrence after LT is almost universal leading to reduce graft and patient survival compared to other liver disease [94].

The development of *de novo* AIH (otherwise called plasma cell hepatitis) in the context of HCV recurrence is of challenging diagnosis and lead to a worse outcome.

Diagnosis of *de novo* AIH in HCV recipients needs a differential diagnosis with the immunological damage due to the virus *per se*. The peculiar ability of C virus to induce autoimmune damage is in part explained by the molecular mimicry and the cross reaction between viral antigens and self-antigens . Moreover the maintenance of the immune response doesn't need the presence of the antigen by which it was originated [163].

The aim of this study was to distinguish patients with prevalent immunological lesions from patients with prevalent viral lesions, with a new, strictly defined, semi-quantitative method, which takes into account three parameters: plasma-cell infiltrate, interface hepatitis and central necrosis. Moreover this study wants to identify the clinical, biochemical and autoimmune characteristics of the two different populations and their outcome.

Forty out of 1083 patients (3.7%), transplanted in 2 liver transplant centers, having HCV recurrence after LT and at least a liver biopsy, performed between 2000 and 2012, showing immunological damage, were identified. The index biopsies were reviewed according to the new method.

After the pathological revision 24 out of 40 LT recipients with HCV recurrence and immunological lesions, had the final diagnosis of *de novo* AIH, for the prevalence of the autoimmune lesions (AIH patients). This group of patients was compared with 16 patients with prevalent viral damage and a definitive diagnosis of HCV recurrence (HCV-R). AIH patients presented more frequently high-grade interface hepatitis ($p=0.010$) and central necrosis ($p<0.0001$), compared to HCV-R patients.

The plasma cells infiltrate moderate/severe was more represented in AIH patients but the difference was not statistically significant.

As expected the histological activity evaluated according the Ishak score was significantly higher in AIH patients (p=0.005), while no difference was found for the fibrosis stage.

The histological features of the *de novo* AIH are the same of the AIH pre-transplant; only the inflammation infiltrate and the necrosis tend to be more prominent compared to the pre-transplant setting [130].

Interestingly it has been previously reported that transplant recipients with HCV recurrence and PCH presented a plasma cell rich infiltrates in explants liver, meaning that there was immune system impairment before the transplant [178].

Histological diagnosis of HCV recurrence is characterized by a predominantly portal-based mononuclear inflammatory infiltrate including lymphoid aggregates, while interface hepatitis and lobular inflammation are usually mild in severity. Mild steatosis may also be present. Histological features of *de novo* AIH are characterized by plasma-cell rich mononuclear central and portal inflammatory infiltrate, associated with varying degrees of interface hepatitis. Lobular inflammation and necrosis can include central perivenulitis [133].

In accordance with our results, it has been recently demonstrated that a histological pattern of centrilobular injury including increased necroinflammatory activity and increased centrilobular plasma cell infiltration correlates with measurements of autoimmunity in liver recipients [179].

It is still controversial whether this entity represents an autoimmune process, an atypical manifestation of HCV recurrent hepatic disease or allograft rejection .

The study from Khettry and colleagues support the hypothesis that the *de novo* AIH is a variant of HCV recurrence, which they called “autoimmune-like” (AIH-like) hepatitis. The common finding was central venulitis with prominent

plasma cells infiltrate, which is typical of AIH. They argued that in the post-LT setting, the interplay of host's immune system and hepatitis C virus is further complicated by the introduction of donor antigens in the presence of immunosuppressive and anti viral therapy and, in some cases, may result in an AIH-like reaction [177].

On the contrary, Fiel et al. consider *de novo* AIH as form of rejection which they called it "plasma cell hepatitis" (PCH). This hypothesis was supported by the observation that 80% of the patients have suboptimal calcineurines inhibitors levels at the time of the PCH diagnosis. The Authors propose a complex scoring system based on the evaluation of 5 parameters (initial assessment, portal inflammation, interface hepatitis, lobular activity, plasma cells) for histological evaluation. Nevertheless this score was not applied to compare PCH and HCV patients [172].

Although the different etiological hypothesis the reported studies seem to agree concerning the type of the lesions characterizing the autoimmune damage.

Concerning biochemical variables AST and ALT level were significantly higher in AIH patients compared to HCV-R patients. No other predictive factors were found such as recipient age or gender, year of transplantation, donor age or gender.

Autoantibodies positivity and their titer were not significantly associated with AIH diagnosis. Since autoantibodies are not specific, as they could be also present in simply viral recurrence, they can help in making the diagnosis of *de novo* AIH when associated with compatible histological findings but their absence do not rule out the disease.

The IAIGH scoring system, although has not been validated in the post-transplantation setting, can be used in order to help in making the *de novo* AIH diagnosis.

Despite clinical and biochemical factors can be complementary to histological findings and can support disease diagnosis the histological evaluation is still the gold standard for *de novo* AIH identification.

Therefore we support the importance of protocol biopsies in HCV positive patients who are clinically well, with normal graft function since they can detect histological abnormalities, which may have implications for prognosis and treatment.

De novo AIH have been associated with antiviral therapy [115, 132, 168], interestingly in only 3 out of 40 patients from this study, antiviral therapy might be directly responsible for the development of AIH. However, it is always difficult to determine if the *de novo* AIH is induced by INF or by the immune restoration responsible for the viral clearance [174].

In patients with HCV recurrence, the presence of immune features in biopsy performed before antiviral therapy was the most important predictive factor of immune-mediated graft dysfunction development during the therapy [170]. This confirms that immunological damage can develop independently from interferon immunological pressure.

The onset of *de novo* AIH has been also reported during antiviral therapy with first generation proteases inhibitor. In this particular scenario is not clear if the IFN or rather the lowering of immunosuppression, due to the interaction between telaprevir and calcineurine inhibitors was responsible of the disease development [175, 176].

The introduction of IFN-free based antiviral regimen seems to be safer in this group of patients. Nevertheless the experience with the IFN-free regimen is still limited and although no case of *de novo* AIH have been described yet, this complication might be possible.

Indeed the complex interplay between the immune system, HCV, and immunosuppression drug complicate the diagnosis and the management .

Interestingly in our study HCV viral load was higher in the HCV-R group although the difference was not statistically significant. Moreover AIH patients had more episodes of acute rejection compared to HCV-R patients, even if the difference was also not significant.

Moderate/severe acute or chronic rejection is rarely present in patients with very high levels of HCV RNA whereas their occurrence is frequently associated with very low levels or clearance of HCV RNA . Partial or complete weaning of immunosuppression in HCV recipients demonstrated the significant but precarious balance between immunosuppression and disease progression [66, 69].

De novo AIH was attributed to inadequate immunosuppression level, usually during the decrease IS dose, two years after LT [130, 156]. We confirmed the appearance of the disease two years after the transplant but for most of the patients the immunosuppression trough level was within a desirable range. No difference was found regarding cyclosporine or tacrolimus as risk factors for immunological damage.

It has been reported that tacrolimus-based immunosuppression might have a protective role in *de novo* AIH development but the population was HCV- .

In our study AIH diagnosis was associated with a significantly inferior long-term survival compared with HCV-R: 65% compared to 93% of patients respectively (p=0.050).

A case control study in which 40 patients with plasma cell hepatitis complicating HCV recurrence were match with patients with only HCV recurrence (1:2 ratio), confirmed our finding. PCH patients were more likely to have a worse outcome than control patients including increased mortality [178].

There is no consensus concerning *de novo* AIH treatment. It seems that treatment with steroids results in a dramatically improvement of the outcome but in HCV- patients [139].

In the present study, retrospectively, most of AIH patients, compared to HCV-R patients, underwent autoimmune therapy (67% versus 25%, p=0.009) reflecting how clinician based the judgment on histological diagnosis. Treatment was based mostly on increase or introduction of steroids, increase of baseline immunosuppression (tacrolimus or CsA) and the administration of AZA or MMF.

In the 16 AIH patients who received the autoimmune therapy there was an improvement of cytolysis and in the control biopsy performed after therapy, 14 out of 16 (87.5%) patients showed an improvement of autoimmune histological features.

Nevertheless AIH patient survival was lower for treated patients (50%) compared to not treated ones (87.5%) (p=ns), although the difference was not significant probably due to the small simple size. The most frequent cause of death was the exacerbation of HCV recurrence.

In previous report the autoimmune therapy improve liver function tests, nevertheless patients who received steroids had a worse outcome likewise not treated patients . It is well known that high dose of steroids have a negative impact on HCV recurrence, as demonstrated by the treatment of acute rejection.

Probably the best option for the treatment of *de novo* AIH is the combination of low dose of steroids and AZA or MMF, accompanied to the increase of calcineurine inhibitors.

Nevertheless, as the autoimmune therapy promotes HCV disease progression, antiviral therapy should not be delayed, preferring IFN-free regimens. Immunosuppression level needs to be strictly monitored, especially at the time of viral clearance since the restoration of the immune system might direct towards the graft. Thus the increase of immunosuppression is recommended.

VI. CONCLUSION

In conclusion about 3.7% of liver transplant recipients with HCV recurrence can develop immunological damage, particularly around 2 years after the transplant.

Histological diagnosis, with a precise semi-quantitative method let clinicians to make the difference between a true *de novo* AIH and a viral disease with mild immunological damage.

Patients with *de novo* AIH have higher cytolysis compared to patients with prevalent HCV recurrence; they might have increased gammaimmunoglobuline and high titer of non-organ specific autoantibodies, but this finding are not determinant in order to make disease diagnosis.

The main tool remains the evaluation of histological features of central necrosis, interface hepatitis, plasma cell infiltrate.

De novo AIH complicating the HCV recurrence has a worse outcome compared the simple viral recurrence. The antiviral therapy including IFN regimen might have a direct impact on the immune system but the real responsible of autoimmunity onset it is probably the immune restoration after viral clearance. However, administration of IFN-free antiviral therapy might reduce the development of *de novo* AIH but data are still lacking.

Autoimmune therapy based on high dose of steroids improves biochemical and histological findings but does not improve survival, mostly because of HCV disease exacerbation.

The management of this particular population is highly complex. Autoimmune therapy associating low doses of steroids and MMF or AZA should be preferred. Moreover, antiviral therapy needs to be administered paying attention to immunosuppression, particularly at the moment of viral clearance.

CHAPTER II: study of interactions between dsDNA and anti-dsDNA autoantibodies in autoimmune hepatitis and systemic lupus erythematosus using Surface Plasmon Resonance imaging-based strategy

I. INTRODUCTION

I.1 Antinuclear antibodies

Serum antinuclear antibodies (ANA) are the most common marker of autoimmune disease, especially of systemic lupus erythematosus (SLE), however, they can also be found in up to 20% of healthy subjects [180].

The nucleus, described by Bauer in 1802, was the first intracellular structure to be identified. It is highly organized into distinct compartments and plays major functions of cell's life, such as DNA replication and transcription, moreover the processing of heterogeneous nuclear ribonucleic acid (hnRNA) and the biogenesis of ribosome are integrated in its structural framework. The nucleus represents the most important structure amongst the intracellular targets of autoimmune diseases and distinct autoantibodies recognize different array of nuclear antigens.

Indirect immunofluorescence (IIF)-based techniques using frozen tissue sections or cultured cell lines is widely adopted to detect ANA and tissue autoantigens reactive with autoimmune sera. The examination of stained specimens under fluorescence microscopy not only allows clinicians to evaluate the presence of ANA but provides information on serum patterns. Positive staining suggests the localization of reactive nuclear antigens and different patterns may help in the differential diagnosis of diseases.

Different IIF patterns can be observed:

- Rimlike: the peripheral nuclear patterns suggest the presence of autoantibodies directed against proteins of different components of the nuclear envelope: lamina, pore complex, inner membrane (like lamin, gp210, NP62).
- Homogeneous: this pattern includes anti-double stranded DNA (dsDNA), chromatine, and anti-histones antibodies.
- Centromere: this fluorescence pattern consists of discrete nuclear dots in interphase cells that remain associated with condensate chromosome of mitotic cells. Anticentromere antibodies recognize a family of proteins localized in the centromeric heterochromatin

- Speckled: this pattern is indicative of autoantibodies targeting a large family of non-histone antigens, including soluble nuclear antigens (like SS-A, SS-B, Sm, RNP, PCNA),
- Nucleolar: different nucleolar structures (such as fibrillarin, RNAP I-II, Th/To, PmScl) are recognized by nucleolar antibodies.
- Nuclear dot: this pattern is characterized by staining of 3-20 dots of variable size distributed all over the nucleus. Anti-nuclear dot antibodies are directed against proteins as Sp100, PML.

Although the fluorescent ANA assay is still adopted as screening method and provides information of subnuclear localizations of antigens, this technique is often not specific enough for diagnostic purpose as one pattern correspond to several antibodies.

I.2. Antibodies against DNA

I.2.1 Anti-DNA antibodies production

The DNA was the first nuclear antigen identified as targeted of anti-nuclear antibodies in 1957 [181]. Anti-DNA antibodies belong to the normal immune repertoire and display the characteristic features of natural autoantibodies. They are mostly IgM encoded by germline DNA with few or no somatic mutations, moreover, they are poly-reactive and bind DNA with low avidity. These antibodies are non-pathogenic, mainly react with single-strand DNA (ssDNA) and can be detected in up to 20% of healthy individuals [182].

The detection of anti-dsDNA in a patient's serum is known to be highly specific of systemic lupus erythematosus (SLE), is considered as an important marker for the disease diagnosis and it is one of the few examples of autoantibodies that correlate with disease severity. Nevertheless anti-dsDNA can be found in other autoimmune disease such as autoimmune hepatitis or rheumatic disease [183,184].

The anti-dsDNA antibodies found in SLE patients are believed to be high-avidity IgG which are somatically mutated, as the expression of an antigen driven selection process, and react with double stranded molecules [185]. Low avidity DNA antibodies occur in other rheumatic diseases.

The mechanism by which pathogenic autoantibodies develop is uncertain. Production of anti-DNA seems based on an immune reaction towards nucleosomes derived from apoptotic cells that are not efficiently cleared [186]. Anti-dsDNA autoantibodies may arise as a consequence of polyclonal antigen-nonspecific disturbances of B and T cells, which leads to an extensive diversity of anti-DNA autoantibodies, or as a consequence of antigen-driven responses being promoted by polyclonal activation [187].

Studies on genes coding variable heavy (VH) and variable light (VL) chains in Ig synthesis, have pointed out that both V chains are necessary for DNA reactivity of an anti-DNA antibody and that no unique V (variable), D (diversity) or J (joining) gene segments are used for the construction of the antibody. Furthermore, genetic studies have suggested that a process of somatic mutation and clonal expansion, which favors sequences with the accumulation of positively charged aminoacids in the complementary determining regions (CDR) is responsible for anti-DNA production. The antigens that trigger this process are not known but nucleosomes are probably implicated [188].

The cellular origin of natural and autoimmune autoantibodies appears different. The natural anti-DNA antibodies are in fact produced by B1 (CD5 fl) lymphocytes, while the pathogenic ones are secreted by B2 (CD5 neg) lymphocytes, two B cells subpopulations. Naive B cells specific for ssDNA might clonally expand if stimulated by immunogenic DNA and gain specificity for dsDNA as a consequence of somatic mutations under antigenic stimulation pressure.

Antibodies directed against DNA react with DNA either pure or linked with proteins such as the histones into the nucleosome. Sequential as well as backbone determinants of DNA can be the targets of anti-DNA antibodies. Backbone determinants are short regions of DNA helix or short nucleotide sequences.

The B cell paratope and DNA epitope is predominantly based on electrostatic interaction and it is highly dependent of pH and salt concentration. Apart from backbone recognition there is also a selective recognition of DNA epitopes variably expressed on DNA, this type of binding seems more pronounced for ssDNA and is based on the recognition of specific sequences.

The binding site of anti-DNA antibodies requires DNA fragments from 40 to several hundreds of base-pairs length for stable interaction. However, the size dependency differs among antibodies. Both Fabs of an anti-DNA antibody need to be bound via bivalent interactions with antigenic sites distributed along the DNA molecule in order to form a stable complex.

A molecular model of the anti-DNA antibodies and DNA binding was introduced by Radic et al., stressing the importance of the antibody heavy chain (HC) in binding DNA [189]. The HC-CDR1 and HC-CDR2 extended into the major groove of DNA and the HC-CDR3 straddles one of the phosphate backbones. The stretches between the CDRs are termed the framework regions (FWRs) and the loop of the HC-FWR3 is positioned to contribute to contacts within the minor groove whilst the light chain (LC) binds mostly wt LC-CDR1, which reaches into the minor groove (**Fig. 1**).

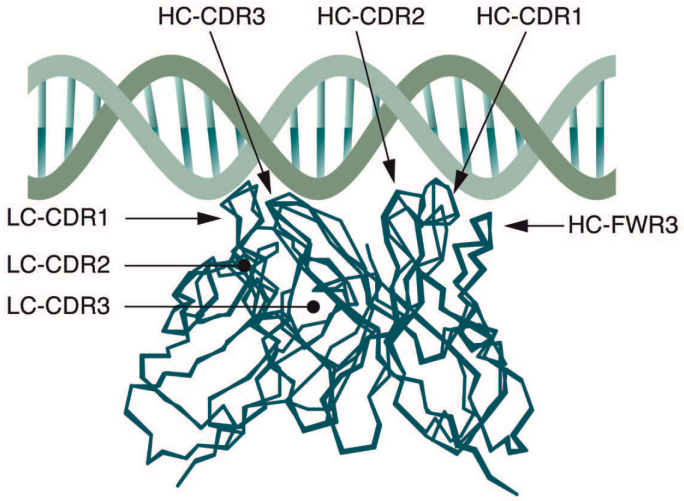


Figure 1. Model of anti-dsDNA antibodies bind to DNA.
Contribution of different CDR and FWR.

The role of DNA in eliciting the production of anti-DNA antibodies has not been yet clarified. It remains uncertain which antigen triggers the production of these antibodies. Moreover the characteristics of antigen-antibodies interaction, in terms of affinity/avidity are still a matter of concern.

I.2.2 Anti-DNA antibodies and diseases

Antibodies directed against DNA molecule have always been claimed to play an important role in the pathogenesis of SLE [190]. The antigen-antibody binding may occur in circulation resulting in an immune complex and depositing in the tissue or it may happen in situ. At the site of deposition, subsequent complement activation leads to inflammation and features of the disease.

Anti-dsDNA detection can help in the diagnosis of SLE and can also be used to follow the clinical course of the disease. For this purpose a test for the identification of the autoantibodies with high specificity to SLE should be used. If screening for the presence of anti-dsDNA is done by an assay that is not selective for high avidity anti-dsDNA then a positive result is not diagnostic of the disease. It has been reported that anti-dsDNA can be detected in up to 55% of patients ranging from 9.3 years to 1 month prior to SLE development [191], moreover in a group of 441 non-SLE patients who presented a positive Farr assay for anti-dsDNA, 85% developed SLE within 5 years after the first positive test [192]

Anti-DNA antibodies have been described in liver disease. Furthermore anti-dsDNA are reported in liver autoimmune-type chronic liver disease, such as auto-immune hepatitis (AIH). On the other hand anti-ssDNA are detected in non autoimmune chronic liver disease and might reflect inflammatory activity [183,193].

I.2.3 Methods of antibodies detection

The mostly used assays developed for the detection of anti-DNA antibodies are:

- radioimmunoassay (RIAs) (Farr assay)
- indirect-immunofluoroscence test (IIFT) on Crithidiae luciliae (CLIFT),
- enzyme-linked immunoadsorbent assays (ELISAs)

The Farr assay is a radioassay in which radiolabelled DNA binds to antibody to native deoxyribonucleic acid contained in the patient sample. After incubation, separation of bound from free DNA is achieved by precipitation with ammonium sulfate. The bound fraction is them counted and patient sample concentrations are read from a calibration curve. The antigen should be bigger than 10^5 but smaller than 10^7 kDa, DNA should be double stranded and monodisperse in size. Circular dsDNA bacteriophage (such as from PM2) or plasmids (such as pUC18) are to be preferred. Results are expressed in international unit. It detects antibodies of M and G isotype (**Fig. 2**). Its specificity for the detection of anti-dsDNA antibodies in LED is high (95-99%) with a sensibility between 32 to 85%.

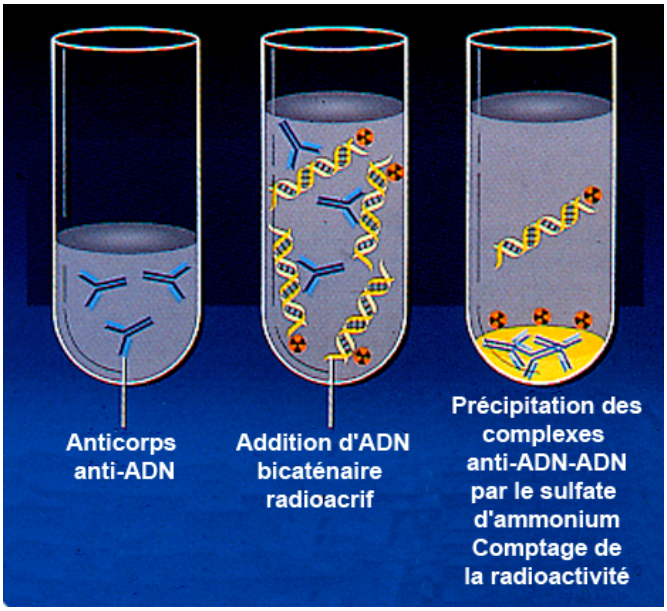


Figure 2. Farr assay is a radioassay for the detection of anti-dsDNA antibodies contained in patient sera.

Crithidia lucilia is a haemoflagellate particularly suitable as a substrate for anti-dsDNA detection by IIF test, as it contains a highly dense mass of circular dsDNA in its large mitochondrion. In case of positive reaction, antibodies in patients sera bind to dsDNA and the attached antibodies are stained in a second step with fluorecein-labelled anti human antibodies (**Fig. 3**). This test couples high disease specificity (99%) to good sensitivity (13-47%) for SLE (HAMAN). It is possible in the second step to use antibodies to IgM or IgG type, for SLE (HAMAN). It is possible in the second step to use antibodies to IgM or IgG type, giving the isotype of the anti-DNA antibodies contained in the patient sera. Results are semiquantitative

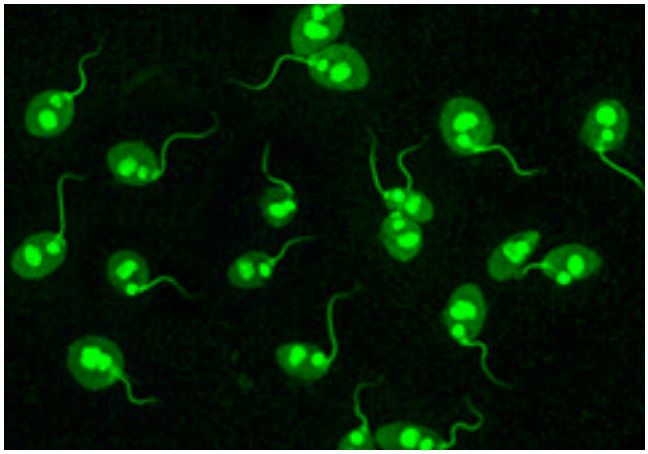


Figure 3. AntidsDNA detected by indirect immunofluorecence on *Crithidia luciliae*. Fluorescence of both nucleus and kinetoplast.

In ELISAs systems, DNA has to be coated to plastic; dsDNA is mostly coated via intermediates such as poly-L-lysine, protamine or methylated bovine serum albumine. These precoats interfere with binding of immune complexes and/or aspecific immunoglobulins to the plates. A better alternative is to use biotinylated DNA and coat this via streptavidin to the plate. Problem with this technique is the denaturation of dsDNA into ssDNA, giving falsepositive results. Its specificity for tee detection of dsDNA is lower (71-97%) with a sensibility of 44-79%.

The different results obtained from the utilization of these tests suggest that more than one test should be helpful in the diagnosis and follow-up of SLE [194].

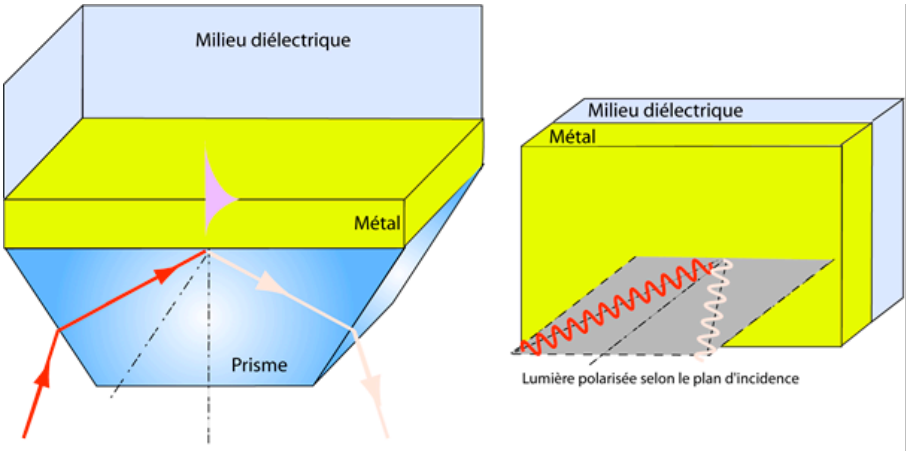
I.3 Surface Plasmon Resonance

I.3.1 Physical principle

A new approach to measure the binding of proteins and other biomolecules is the Surface Plasmon Resonance (SPR) method, first described in 1988, and nowadays one of the most established label-free biosensor techniques.

SPR system is constituted by a prism covered by a gold film, 50 nm thick, over which the ligands are fixed. The gold surface is in contact with a buffer solution, which runs into a flowing chamber. In the flowing chamber is possible to inject the binding partner (analyte) of the ligand that will contact the chip surface. A polarized light from a laser source pass through the prism and reach the back site of the gold surface, at the angle of total internal reflection. This light induces a non-propagative evanescent wave that penetrates into the flow cell. At a given angle, dependent upon the refractive index of the solution in the flow chamber, the interaction between the evanescent wave and the free electrons of the gold layer induces a wavelike oscillation of the free electrons (resonates) of the metal and a reduction in the intensity of reflected light (**Fig. 4**).

The changes of the angle of reflected light correspond to the changes of the refractive index of the solution in the flow cell immediately adjacent to the gold layer. The refractive index is in relation with the mass over the prism represented by either the ligand alone either the ligand bound to the analyte. The output from the photo-detector array, that is used to determine the SPR angle, can be viewed as a 'dip' in the intensity curve of the reflected light (**Fig. 5**).



La résonnance entre l'onde électromagnétique incidente et les électrons libres du métal est obtenue uniquement par une radiation lumineuse de longueur d'onde précise (permettant le couplage avec l'oscillation des électrons) polarisée TM (polarisation parralèle au plan d'incidence) ainsi qu'un angle incidence précis permettant une réflexion totale et la crétion d'une onde évanescente.

Un prisme à indice de réfraction plus élevé est accolé directement au métal et permet d'exiter et d'observer les plasmons de surface. Pour des angles supérieurs à celui de la réflexion totale, le champ évanescent créé à travers le métal peut coupler un plasmon de surface, ce qui impose qu'une portion de la lumière incidente se complexe au plamon. Elle n'est alors plus réfléchie.

Figure 4. Surface Plasmon Resonance physical principle.

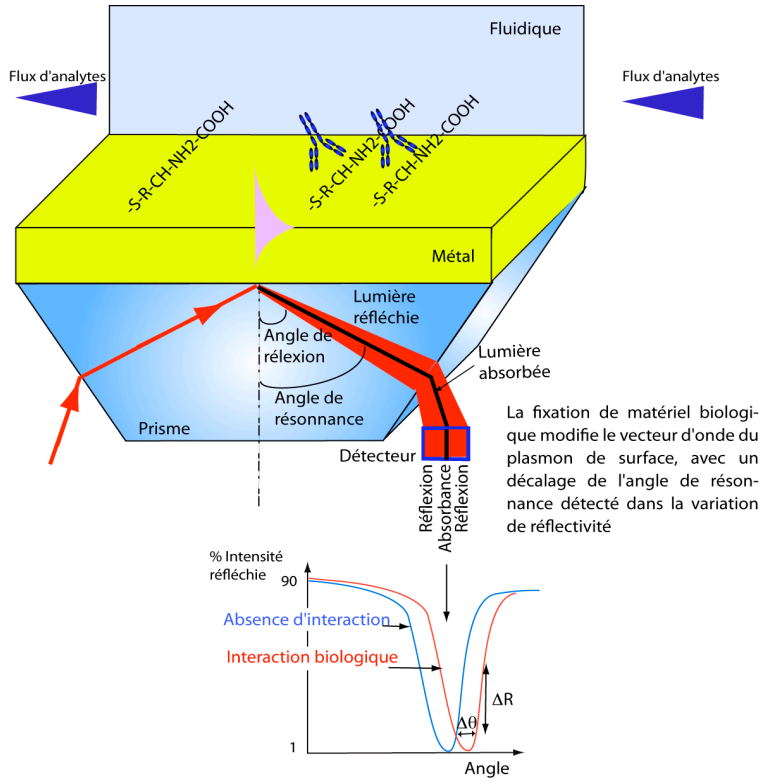


Figure 5. Surface Plasmon Pesonance. Principle of the detection of molecular interaction at the surface of the gold layer

In the surface plasmon resonance imaging (SPRi), the incidence angle is maintained fix and the reflected light is simultaneously detected by a charged coupled device (CCD), the value of a single pixel is proportional to the intensity of the reflected light. An informatics system calculate the pixel value of the obtained images [195] (**Fig. 6**).

After the injection of the analyte in the flow chamber, the chip surface can be regenerated by injecting in the flow chamber a regeneration buffer, which allows the utilization of the prism for the study of several analytes.

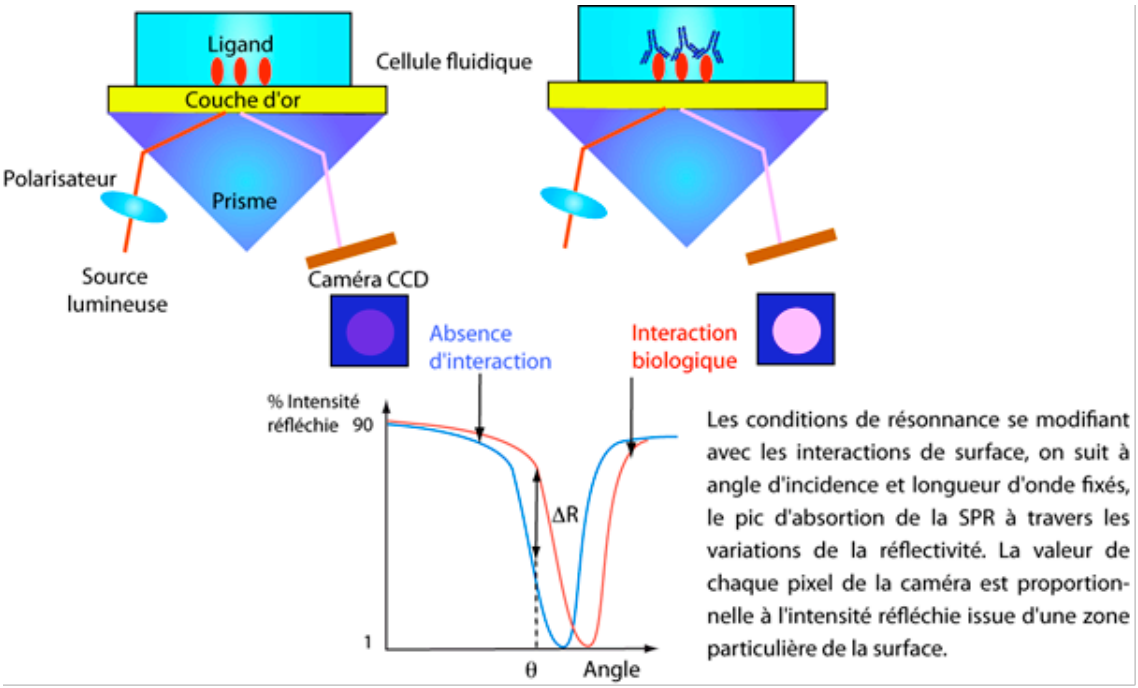


Figure 6. Surface Plasmon Resonance imaging.

Theoretically both the DNA and the antibody can be immobilized on the surface. There are several methods to immobilize the molecules over the gold surface. One of the most common technique use the creation of a bridge gold-thiol as described in the following reaction: R-SH + Au → R-SH-Au +H+ + e-.

Before the link of the molecules on the gold, the surface is treated in order to get an interaction between the gold and thiol. This interaction is not well known but allows the formation of a self-assembling monolayers (SAMs) (approximately 100 nm thick) which favorites the uniform binding of the ligands having a thiol on their extremities.

It has been reported that the SAMs, which is composed by alkane-thiols with ethylene glycol oligomers (OEG-SH), confer to the gold a certain resistance to the proteins binding reducing the non-specific interactions. Moreover, pretreating the SPRi biochip surface, prior to dsDNA adsorption, with a SAM composed of n ethylen glycol (n=4) increases dsDNA accessibility for the analyte avoiding the denaturation of dsDNA oligomer.

The presence of ssDNA 5' end thiol optimizes the DNA density on the surface [196](**Fig. 7**) .

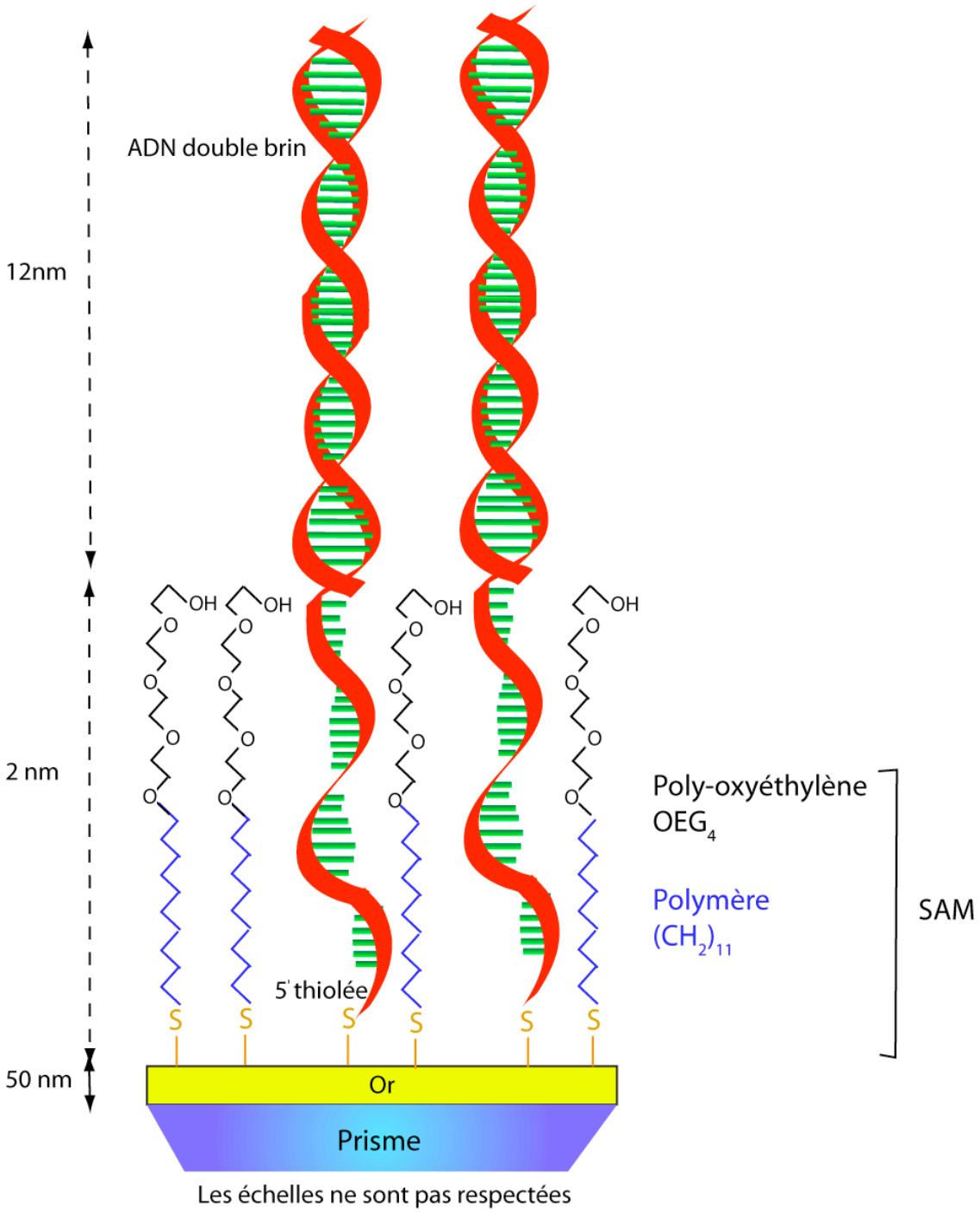


Figure 7. Chip surface design. SPRi biochip is surmounted with a sparse self-assembled monolayer of ethylen glycol (SAM). The dsDNA with a thiol modification in 5' end is adsorbed between the SAM.

I.3.2 Monitor of the kinetic interaction

The different steps of molecular interaction can be synthesized into a sensogram that represent the evolution of the intensity signal expressed by resonance % (R%) over the time (**Fig. 8**).

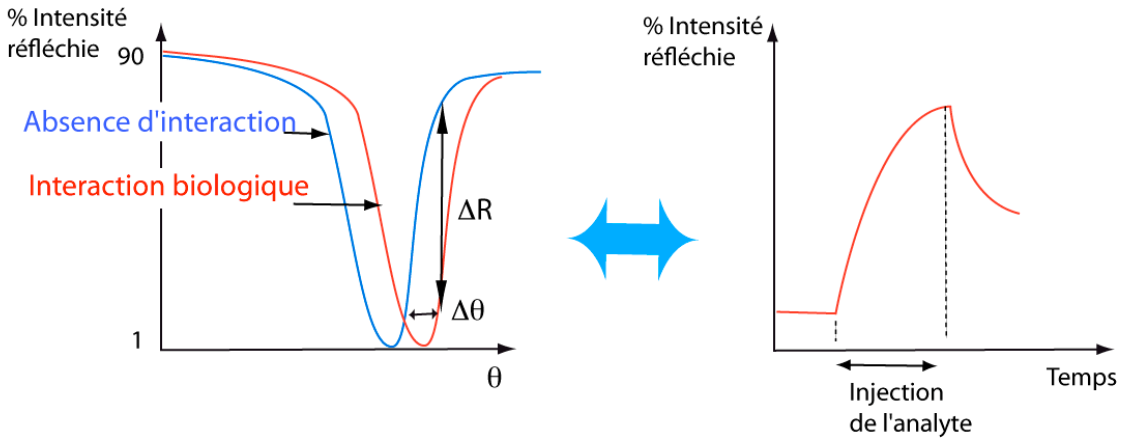


Figure 8. In surface plasmon resonance imaging, the evolution of the reflectivity during the time (left) can be visualized on a sensogram (right).

Since this particular system generates real time binding data makes it well suited to the analysis of binding kinetics. The period during which the analyte is being injected is called the "association phase" whereas the period following the end of the injection is called the "dissociation phase". During the association phase, there is a simultaneous association and dissociation between ligands and analytes. The equilibrium is reached when the association rate equals the dissociation rate.

It is important to note that during the dissociation phase not only dissociation process take place but some re-binding can occur. Nevertheless is possible to calculate the association (k_{on}) and dissociation (k_{off}) constants, which represent the rate of the complex formation for the former and the complex stability for the latter. The k_{off} can be calculated easily during the dissociation phase. Equations of the kinetic of the different phases are given in (**Fig. 9**). The equilibrium constant KD is the ratio of k_{off}/k_{on}.

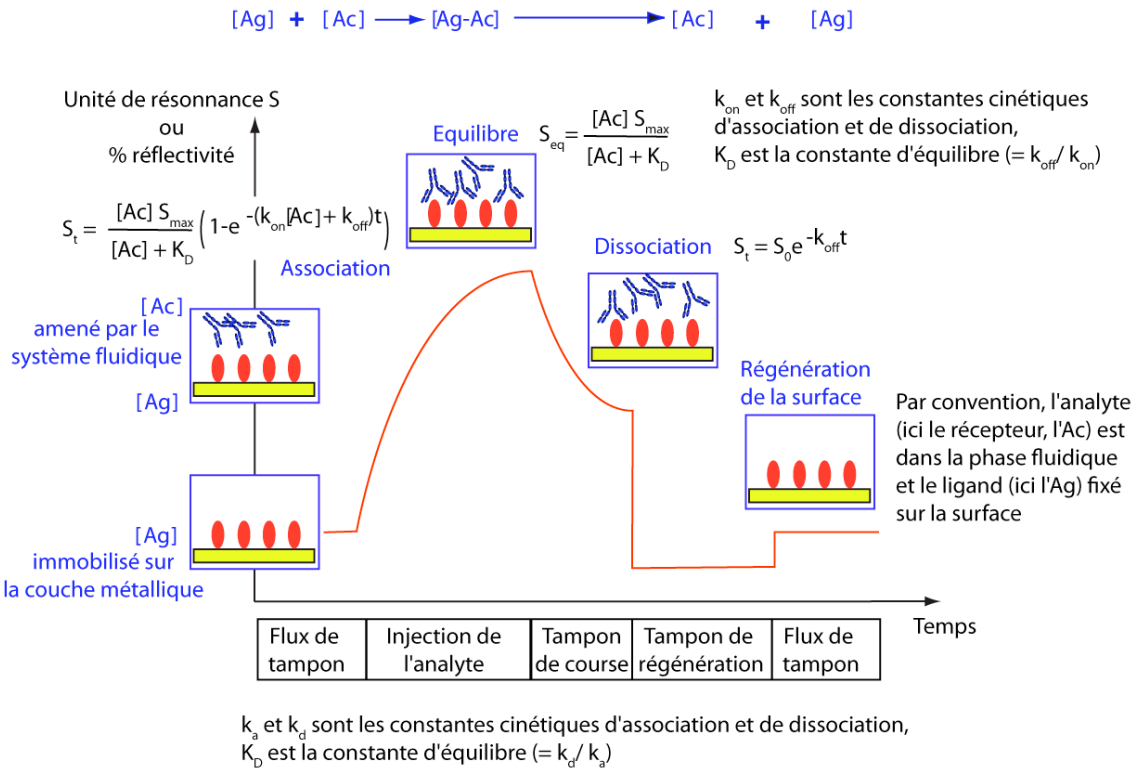


Figure 9. Sensogram and equations at the different phases of the kinetic.

I.3.3 Anti-DNA antibodies by SPR studies

SPR can be used in order to study the kinetics of the binding process between antibody and oligonucleotides immobilized over the chip surface. This system has the technical advantage of using unlabeled reactants and allowing real-time monitoring of their interactions.

Moreover SPR lead to the determination of the k_{on} and k_{off} constants and the intrinsic affinity of molecules interactions. Previous SPR analysis showed that the bind of an antibody to biotinylated pUC18 plasmid was due to a specific affinity between the antibody and that plasmid.

It has already been demonstrated that SPR biosensor is suitable for studying binding kinetics of dsDNA anti-dsDNA antibodies in patient's sera adding a new analytic quality to ancient methods. However, only the interaction between sera and DNA antigen have been analyzed without making distinctions among antibodies isotypes [197].

Using he SPR method, Buhl et al. characterized three monoclonal anti-dsDNA antibodies, two humans and one murine, by measuring the association and dissociation kinetic rates and calculating the functional affinity (avidity) as the ratio of these two rates. While all mAbs exhibited comparable avidities, which could be confirmed by gel shift experiments, one of them proved to have slower association and dissociation kinetics. This was the only mAb providing positive results in the Farr RIA. In inhibition experiments with ss- and ds-oligonucleotides 10, 24 and 42 bp in length, the mAbs acted substantially different. The results suggest that kinetic rate constants seem to be decisive in explaining the behaviour of mAbs. Different reactivity to various DNA species should be taken into account with respect to varying DNA sources in commonly used laboratory assays [198].

II. AIM OF THE STUDY

The aim of the present study was to differentiate the characteristics of the binding between dsDNA and anti-DNA antibodies in patients with autoimmune hepatitis (AIH) presenting or not antinuclear antibodies defined by IIF, and patients with systemic lupus erythematosus (LES) using the SPRi based strategy.

This original method allows label-free, real time monitor of molecules interaction improving the discrimination between anti-dsDNA antibodies and dsDNA antigens interaction in the both diseases.

III. MATERIALS AND METHODS

III.1 Patients sera

Twenty eight sera have been analyzed: 14 have been taken from patients with AIH as defined by the International Autoimmune Hepatitis Group, with high level (>30U) positive anti-dsDNA antibodies detected by Farr test. Among them 9 were positive for antinuclear antibodies by IIF on HEP2-cell monolayers and unfixed cryostat sections of rat liver (cut-off positivity ≥1:80), while 5 were not. Seven sera have been taken from patients with SLE with hight level of anti-ds DNA by Farr test and high anti-nuclear antibodies by IIF and seven sera have been taken from healthy individuals negative for antinuclear and anti-dsDNA antibodies, included into the study as negative controls (**Tab. 1**). All sera were stored at -80°C until use (Laboratoire d'Immunologie, H.pital, Saint-Antoine, Paris, France).

Patients sera, anti-dsDNA by Farr test		
AIH	**SLE**	**Controls**
dsDNA+	**dsDNA+**	**dsDNA-**
n=14	**n=7**	**n=7**

Table 1. The distribution of patients selected according to their disease and Farr test (AIH, autoimmune hepatitis, SLE, systemic lupus erythematosus, ds double strand)

III.2 IgG and IgM purification

IgG were purified from patients sera with 'Protein G HP SpinTrap' kit (GE Healthcare) using the affinity of protein G of streptococcal bacteria coupled to Sepharose© according to kit protocol. Elution buffer was 0.1 M glycine-HCl, pH 2.7, binding buffer was 10 mM sodium phosphate, pH 7.0, neutralizing buffer was 1M Tris-HCl, pH 9.0.

IgM were purified from patients sera, after IgG purification, with a 'HiTrap IgM Purification HP, 1ml' kit (GE Healthcare) according to kit protocol. This purification used a thiophilic affinity medium with 2-mercaptopyridine coupled to Sepharose©. Thiophilic adsorption is incompletely understood and the interaction can result from a combined electron donating and accepting action of the ligand, or as a mixed mode hydrophilic-hydrophobic interaction. Elution buffer was 20 mM sodium phosphate, pH 7.5, binding buffer 20mM sodium phosphate, 0.8 M (NH4)2SO4, pH 7.5, regeneration 20 mM sodium phosphate, pH 7.5 with 30% isopropanol.

Proteins levels were defined using the Bradford protocol with BSA as standart. The IgG and IgM elutes were run on a sodium dodecyl sulfate 10% polyacrylamide gel (SDS-PAGE) electrophoresis. After the electrophoresis the gel was silver-stained allowing the staining of two bands at 50 kDa and 25 kDa for IgG and at 60kDa and 25 kDa for IgM corresponding to the molecular mass of heavy and light chains respectively (data not shown).

III.3 Oligonucleotides

All the oligonucleotides (OG) were from MWG eurofins. Long DNAs were obtained by PCR amplification of a DNA sequence (no EcoRI site) of either 250 or 500 bp cloned into pGEMt vector using the BamHI (Thi-TTTTT-(CH2)3-CTCCCGGCCGCCATGGCCG) and BamH2 15CCTGCAGGCGGCCGCACTA as primers. These two primers generate a 5' thiolated single stranded extremity of five thymines. A quantity of 1.2 ml of standard PCR product was concentrated by ethanol precipitation, resuspended in 7% glycerol, 100 mM NaCl to to 1 **µ**mol, treated with tris(2-carboxyethyl)phosphine (TCEP) during 2 hours at room temperature and then desalted throw a BioSpin 6 column (Biorad) to remove the TCEP.

Double stranded oligonucleotides were obtained by hybridization of two complementary strands in 7% glycerol, 100 mM NaCl solution by slow cooling down from 95°C to room temperature, TCEP treated for 2 hours and desalted through a column (BioSpin 6, BioRad).

Oligonucleotide 02/10, 32 bases long, single stranded sequence: Thi-TTTTTTAGTATGAATTCGCGAATTCTCGTACG was coupled with a 27 bases long sequence CGTACGAGAATTCGCGAATTCATACTG; DL10 dsDNA results from the hybridization of AATTGCTATeATAGCTCCGCACGCTGGTACCCATCTCATGA and Thi-TTTTTTCATGAGATGGGTACCAGCGTGCGGAGCTATATAGCAATT THF dsDNA vas obtained by hybridizing CACTTCGGAPTGTGACTGATCC and Thi-TTTTTGGATCAGTCACATTCCGAAGTG and HP dsDNA is obtained by auto hybridization of

Thi TTTTTGGATCAGTCACATTCCGAAGTGTTTCACTTCGGAPTGTGACTGATCC

(**Tab. 2**).

DNA	bp	Modification	Methylation
o500	500	5'-thiol C3 spacer	No
o500c	500	5'- thiol C3 spacer	CpG (5Me-C)
o500g	500	5'- thiol C3 spacer	GpC (5Me-C)
o250	250	5'- thiol C3 spacer	No
o2/10	27	5'- thiol	No
o2/10c	27	5'- thiol	CpG (5Me-C)
o2/10e	27	5'- thiol	EcoRI (6Me-A)
hp	22	5'- thiol and Hair pin with a 3 bases loop	No
THF	22	5'- thiol and Tetrahydrofurane (abasic site) in position 10	No
DL10	40	5'- thiol and ethenoA in position 10	No

Table 2. Characteristics of oligonucleotides used as antigen (bp, base-pairs)

In vitro methylation: DNA methylases CpG (M. SssI), GpC (M. CviPI) and EcoRI methyltransferases were from NEB. The DNA modifications were performed as recommended by NEB and incubated 2 hours at 37°C. The methylation was verified for oligonucleotides by NruI or EcoRi restriction; after 2 hours incubation at 37°C with the restriction enzymes (NEB), products were separated by PAGE in a 18% acrylamide gel. Oligonucleotides were visualized under UV illumination after Ethydium bromide coloration.

III.4 Prism preparation

The prism with the gold chip surface was treated as follows:
- drowned for 25" in 11-mercaptoundecyl-tetra-ethylene glycol (1mM),
- rinsed in ETOH,
- dried by pure Argon gas.

Different dsDNAs were spotted on the surface at 1µM in glycerol (7%) and incubated for 2 hours in a humid chamber. The prism was then directly inserted into the SPRi apparatus. The buffer was a solution made by 10 mM HEPES and 50 mM KCl, ph 6.9 which flowed across the surface at 25 µl/min.

III.5 Surface Plasmon Resonance imagery (SPRi)

SPRi data were collected using SPRi apparatus purchased from GenOptics (Orsay, France) with incoherent light source (**λ**=830 nm) as described elsewhere [17]. SPR images captured by a time resolved CCD camera and LabView software (GenOptics, France) permitted real-time averaging of the intensity on each spot in order to obtain the reflectivity signals. The reflectivity signal is expressed as % of reflectivity (%R). For SPRi experiments, the sera were diluted 800-fold and purified IgG and IgM 200-fold in 10 mM HEPES and 50 mM KCl, (ph 6.9) buffer. The samples were injected in the flow chamber over 7 minutes at 25 µl/min. This was followed by 20-minute dissociation period. Regeneration was obtained injecting 4 mM NaCl over the chip at 100 µl/min.

Moreover mouse monoclonal anti-human IgGs (AdGene), or IgMs, diluted to 1.25 µg/ml, were injected during the dissociation phase (**Fig. 9**). A signal modification corresponding to an increase of the reflectivity indicates the binding of anti-human immunoglobulin with the anti-DNA antibodies.

In the sensogram, we eliminated the first part of the association and the dissociation phases, because these parts of the curves may be affected by the buffer changes and mass transport phenomenon.

IV. RESULTS

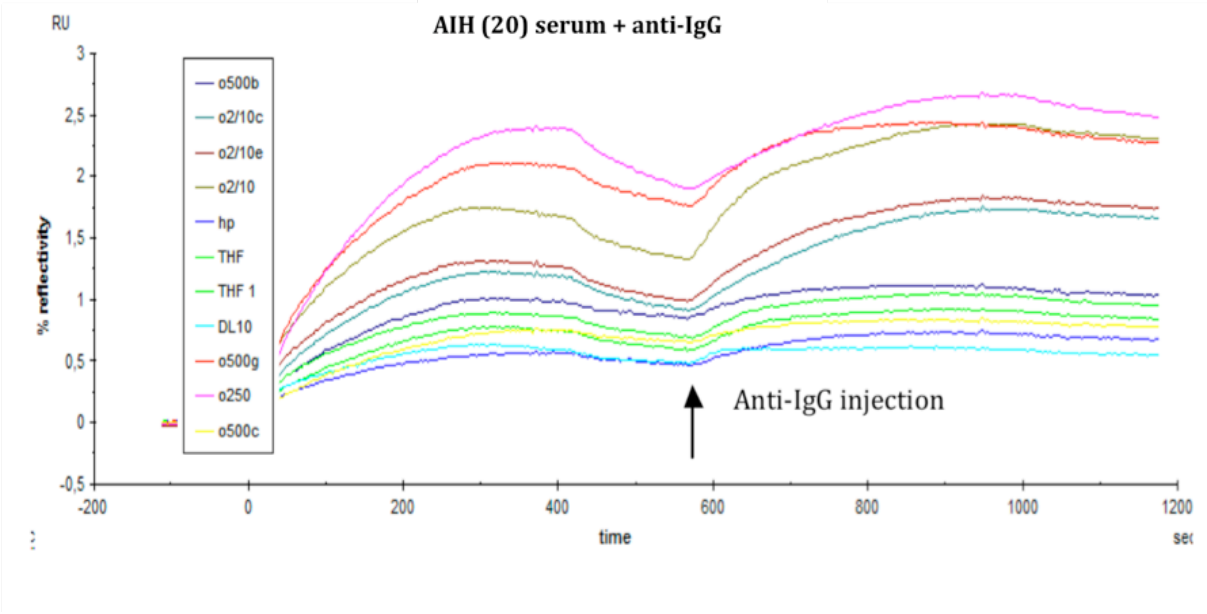
IV.1 Patients sera SPRi studies

When patients' sera were injected in the flow chamber a reflectivity signal was observed for the following OG: o500g, o500, o250, o2/10e, o2/10c, o2/10. No signal was observed corresponding to the OG o500c probably due to the fact that the density of DNA was too low in this spot. Because of this negative result the OG o500c will not be further analyzed. The signals obtained represent the interaction between the DNA spotted over the chip surface and DNA binding proteins present in the sera.

The signal intensity was different among the sera, being higher for sera obtained from AIH patients compared to SLE patients and controls. The noted differences, however, may be due to the variation of DNA binding proteins concentration.

When mouse monoclonal anti-human IgGs were injected during the dissociation phase, the reflectivity signal increased for all the samples but with higher intensity for AIH patients compared to SLE patients and controls.

In the following images are shown three examples of sensograms originated by the injection of the serum obtained from a AIH patient (**Fig. 10**), a SLE patients (**Fig. 11**), and an healthy individual (**Fig. 12**). Each injection of the serum have been followed by the injection of an mouse anti-IgG. Differential images of the chip surface generated from the coupled device are also reported. These examples are representative of the behavior of the patients' sera obtained from the same group.



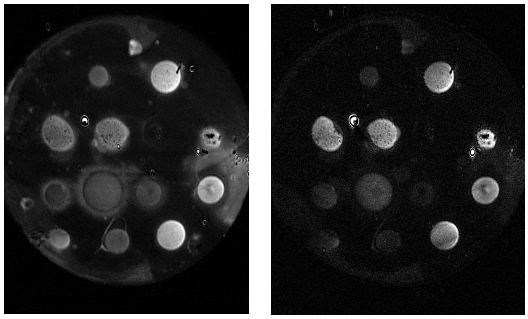


Figure 10. (A) Surface plasmon resonance sensograms obtained from the injection first of an AIH patient's serum (patient number 20) (800fold dilution) and thereafter of anti-IgG over different oligonucleotides (OG) used as antigens. The re-increase of the signal indicates the binding between anti-IgG and the primary antibody. RU (resonance unit). **(B) Differential images of the chip surface at AIH serum injection (left) and anti-IgG injection (right).**

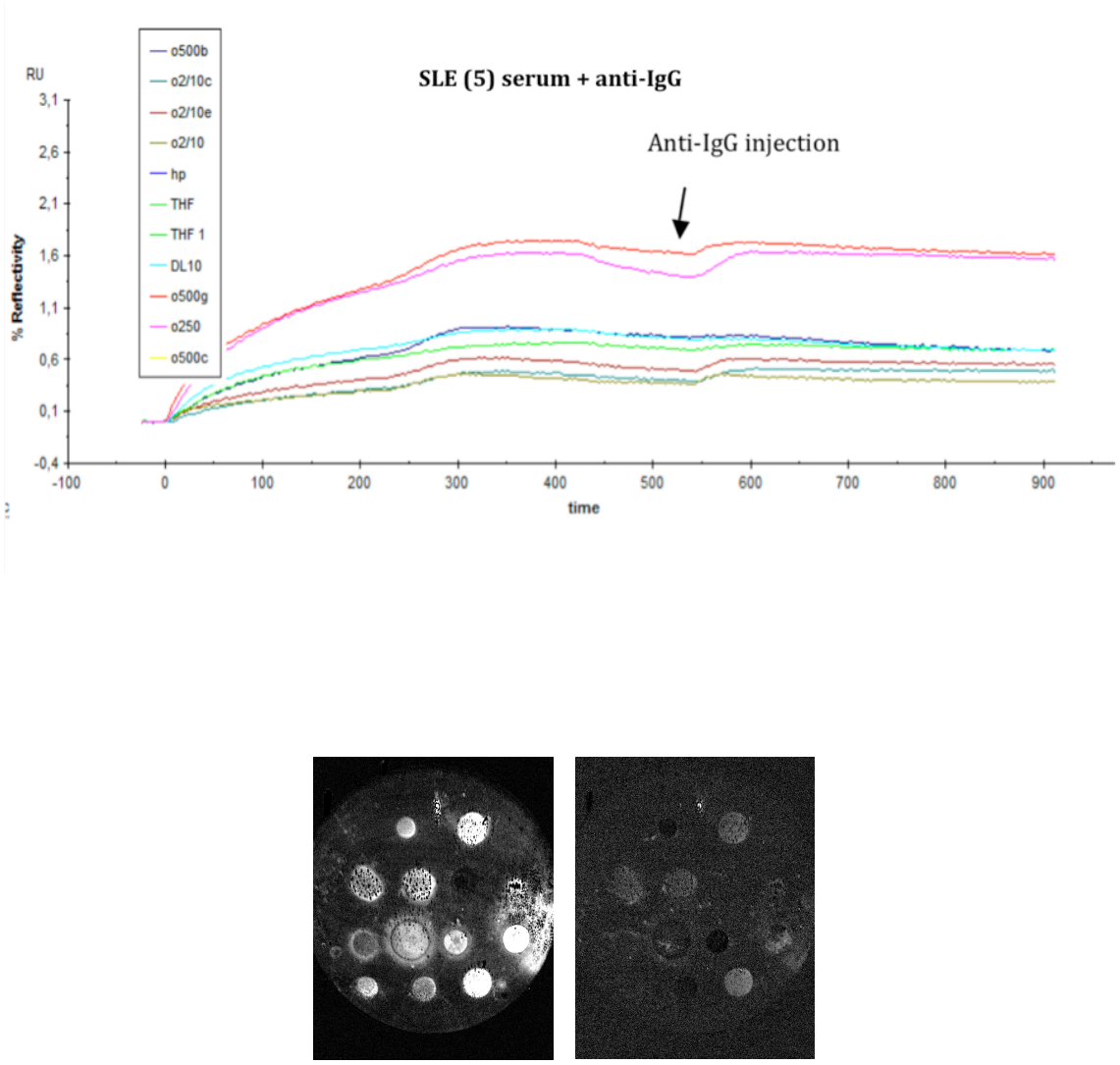


Figure 11. (A) Surface plasmon resonance sensograms obtained from the injection first of a SLE patient serum (patient number 5) (800fold dilution) and thereafter of anti-IgG over different oligonucleotides (OG) used as antigens. The re-increase of the signal indicates the binding between anti-IgG and the primary antibody. The signal modification is moderate. RU (resonance unit). **(B) Differential images of the chip surface at SLE serum injection (left) and anti-IgG injection (right).**

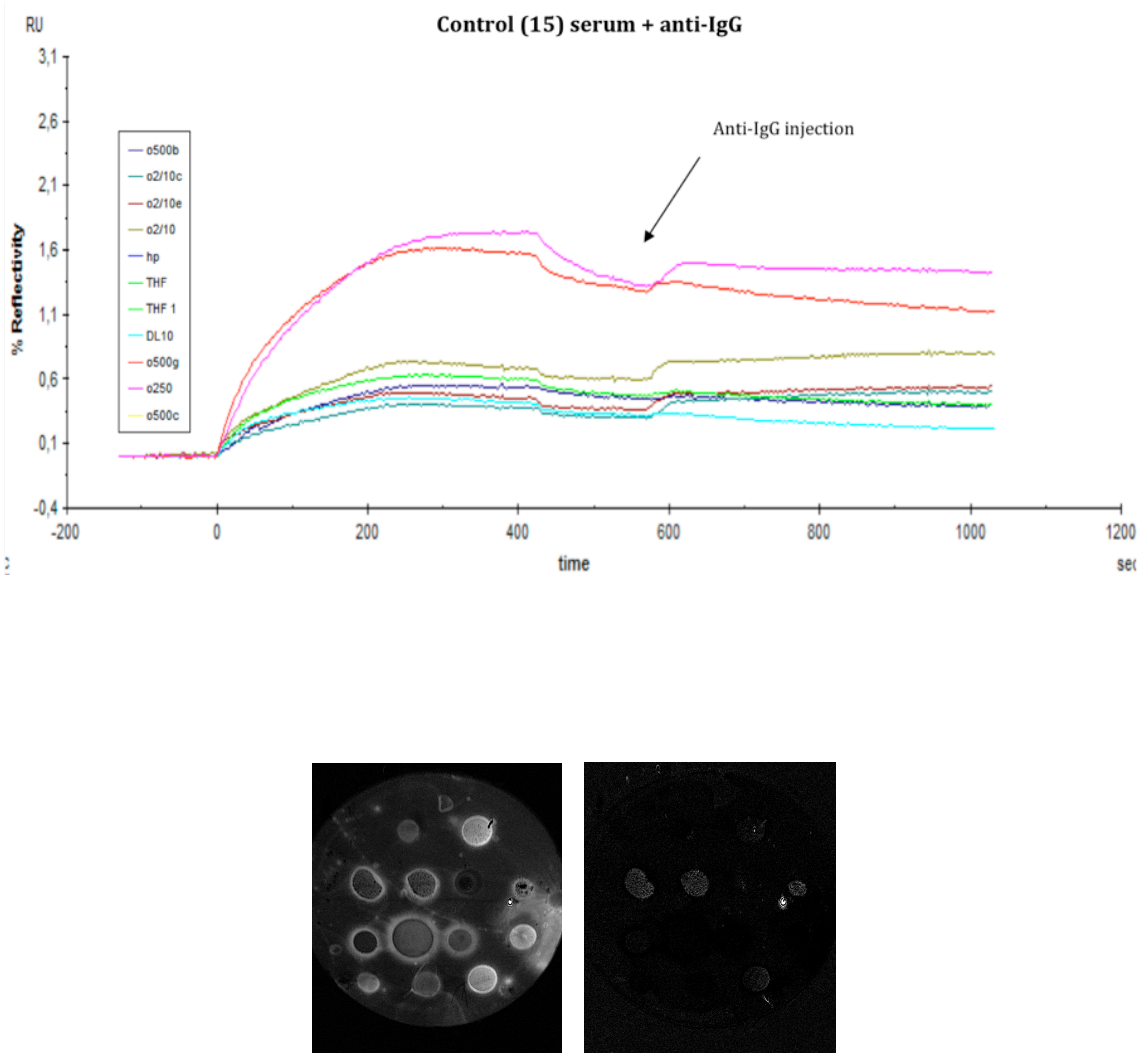


Figure 12. (A) Surface plasmon resonance sensograms obtained from the injection first of a control patient's serum (patient number 15) (800fold dilution) and thereafter of anti-IgG over different oligonucleotides (OG) used as antigens. The re-increase of the signal indicates the binding between anti-IgG and the primary antibody. The signal modification is very moderate. RU (resonance unit). **(B) Differential images of the chip surface at control serum injection (left) and anti-IgG injection (right).**

When mouse monoclonal anti-human IgMs antibodies were injected during the dissociation phase no reflectivity signal was observed neither for patients' sera (AIH or SLE) nor for controls' sera, as showed by (**Fig. 13**) with a representative example of an AIH patient (number 20).

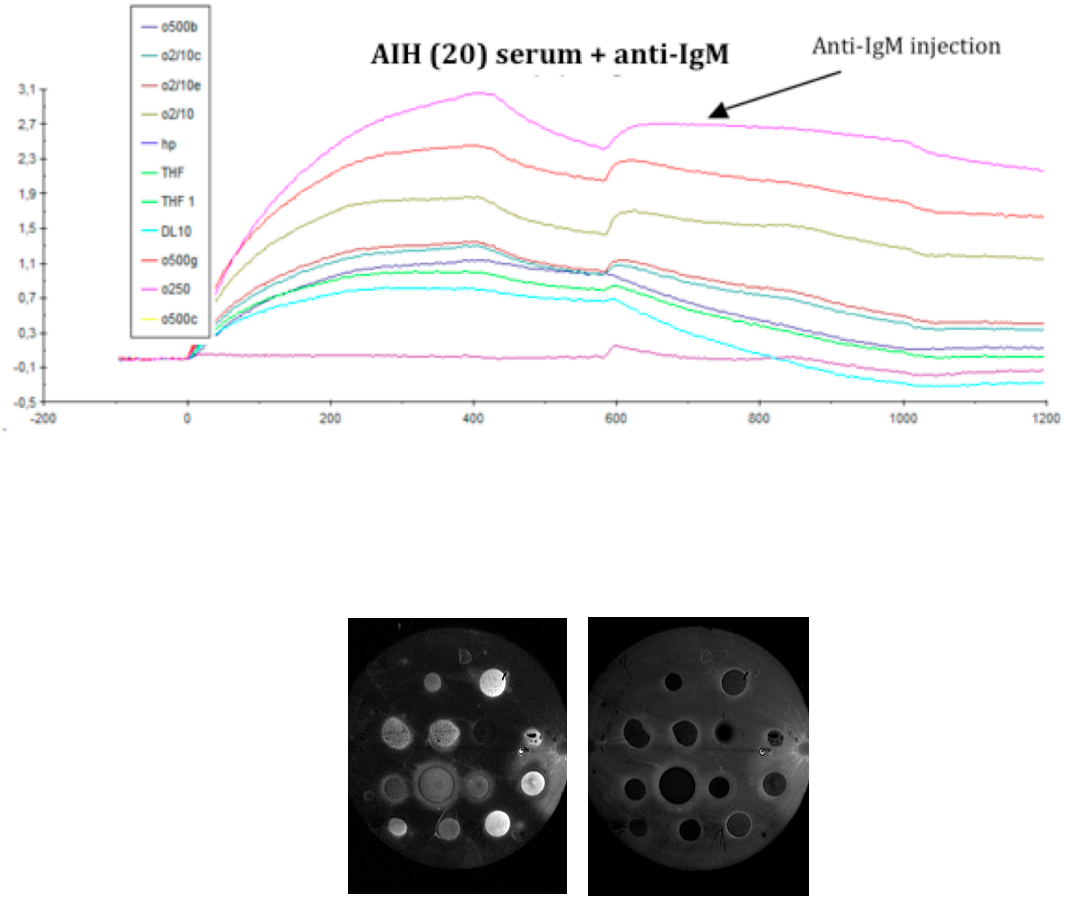


Figure 13. (A) Surface plasmon resonance sensograms obtained from the injection first of an AIH patient's serum (patient number 20) (800fold dilution) and thereafter of anti-IgM over different oligonucleotides (OG). RU (resonance unit). **(B) Differential images of the chip surface at AIH serum injection (left) and anti-IgM injection (right).**

IV.2 Purified IgG SPR studies

Purified IgGs, from patients and controls sera, were injected in the flow chamber. The IgGs from AIH patients showed an reflectivity signal (**Fig. 14 A**), whereas IgGs from SLE patients and controls did not (**Fig. 14 B** and **Fig. 14 C**), except for one SLE patient for whom IgG acted exactly the same of AIH ones.

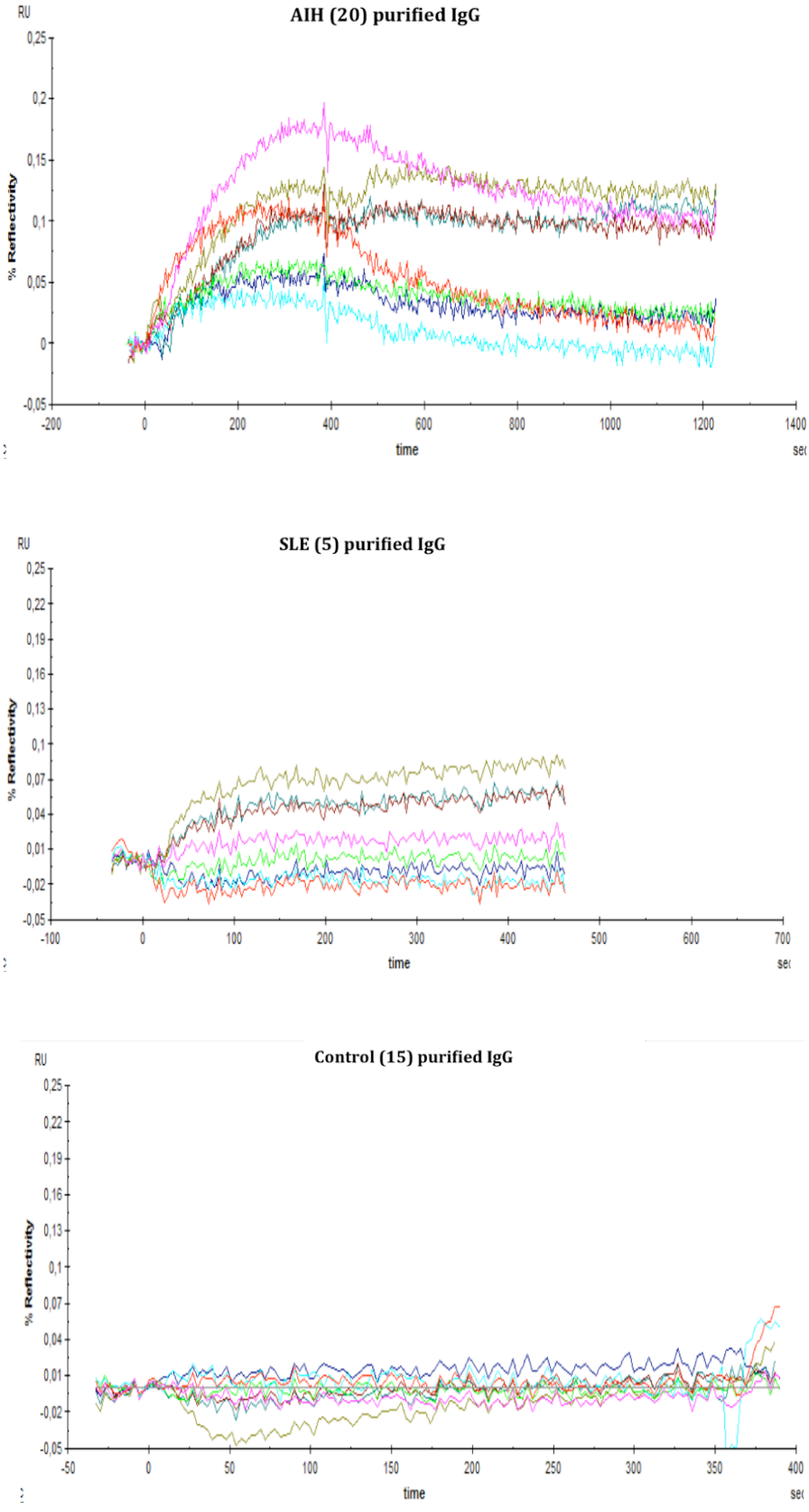


Figure 14. Representative surface plasmon resonance sensograms obtained by the injection of (A) purified IgG of AIH patient number 20, (B) purified IgG of SLE patient number 5, (C) purified IgG of control patient number 15 over immobilized dsDNA.

The reflectivity signal was generated by the interaction between purified IgGs from AIH patients and the following dsDNA, in order of intensity: o250, o500g, o500, o2/10e, o2/10c, o2/10 (**Fig.15**). Amongst the long OG: o250 showed the major intensity signal over the other OG in particular to o500 and o500g. This is possibly explained by the fact that very long OG, as 500 pb, have a lower density compared to shorter OG, when spotted over the chip surface. Concerning the small OG the methylated ones (o2/10c and o2/10e) showed an higher intensity signal compared to non methylated one (o2/10). The hairpin OG (hp) had an intensity signal similar to methylated OG. The hair-pin OG contains ssDNA loop that can also be recognized by antibodies. The intensity signal of the OG with modified bases (THF, DL10) varies according to different sera as methylated and non-methylated OG that could correspond to particular immunological response to each patient's.

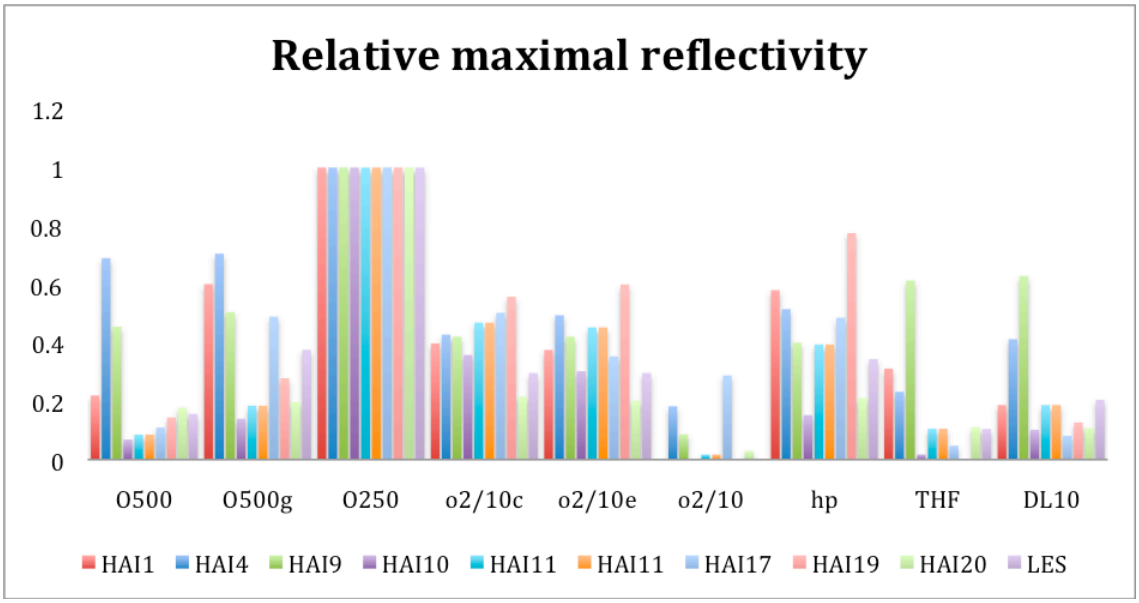


Fig.15 The maximal reflectivity for each DNA is divided by the signal of o250 DNA

IV.3 Purified IgM SPR studies

Purified IgMs were injected in the flow chamber. No reflectivity signal was observed neither for patients (AIH and SLE) nor for controls (**Fig. 16**)

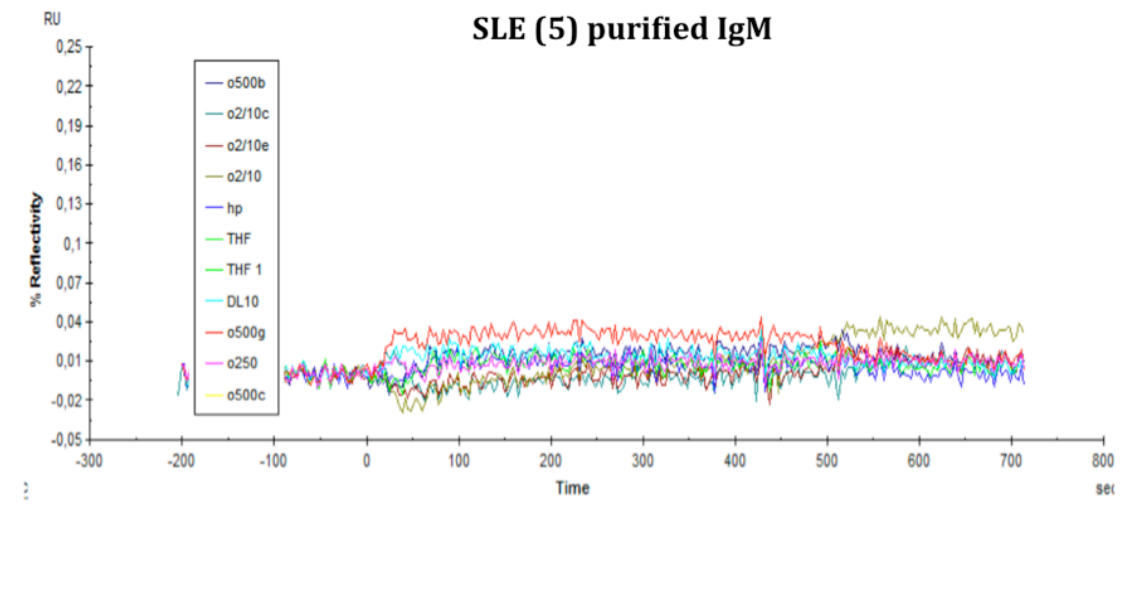


Figure 16. Representative surface plasmon resonance sensogram obtained from the injection of purified IgM of SLE patient number 5, over different immobilized dsDNA.

IV.4 Kinetic SPR studies

The injection of purified IgGs from AIH patients showed an interaction signal, as already mentioned. The kon could not be calculated, as we do not know the concentration of antidsDNA IgGs present in the serum. However koff of the dissociation phase of the immunocomplex were calculated (**Tab. 3**).

No difference was found among AIH patients, particularly AIH/IIF + and AIH/IIF-, regarding dissociation binding kinetic. Regarding the only SLE patient koff were comparable, so immunocomplex stability seems to be not discriminant.

	HAI1	HAI4	HAI9	HAI10	HAI11	HAI17	HAI18	HAI19	HAI20	LES
250b	1.99E-03	1.18E-03	1.98E-03	1.99E-03	2.19E-03	1.01E-03	1.32E-03	2.00E-03	1.22E-03	1.73E-03
o500	ND	7.58E-03	9.48E-04	ND	2.51E-03	4.92E-03	4.92E-03	ND	1.88E-03	2.38E-03
o2/10c	1.19E-03	8.99E-04	3.10E-03	3.02E-03	1.64E-03	8.62E-04	2.11E-03	2.26E-03	1.79E-03	1.99E-03
o2/10e	6.66E-04	1.04E-03	6.67E-03	2.65E-03	1.76E-03	1.09E-03	1.79E-03	2.77E-03	7.29E-04	1.96E-03
o2/10	ND	5.45E-04	1.52E-03	3.31E-02	1.58E-03	8.89E-04	1.08E-03	1.88E-03	1.73E-03	1.52E-03

Table 3. Mean k_{off} of the interactions between purified IgGs from AIH patients and dsDNA on the chip surface. Mean k_{off} are expressed in sec1. ND: not determined, because of the too low intensity.

V. DISCUSSION

Antibodies directed against DNA have been always claimed to play an important role in the pathogenesis of SLE, but they have also been described in chronic liver disease, particularly AIH, and also in healthy individuals. However the anti-DNA detected in patients affected by a disease are directed against dsDNA while the antibodies detected in healthy subjects are directed against ssDNA. The characteristics of antigen-antibodies interaction are still a matter of concern. It has already been demonstrated that SPR biosensor is suitable for the study of the binding kinetics between DNA and anti-DNA antibodies in patients' sera adding a new analytic quality to ancient methods, as it is a label-free and real time detecting device. The aim of the study was to evaluate the characteristics of interaction between dsDNA and anti-dsDNA comparing AIH patients and SLE patients. Twenty eight sera obtained from AIH, SLE patients and healthy individuals were analyzed using the SPRi strategy. The patients' sera contained anti-dsDNA antibodies identified by Farr assay. Ten different DNAs were spotted over the chip surface.

After the injection of the sera in the flow chamber, a reactivity signal was observed for both patient populations (AIH and SLE) and controls. This finding suggests the presence of an interaction between serum DNA binding proteins and dsDNAs immobilized over the chip sensor surface.

The second observation was that the intensity of the signal was a little bit higher for AIH patients compared to SLE patients or controls. Regarding the type of interactions that took place between the molecules, we can exclude that sera components recognize DNA extremities otherwise all the reactivity signals would be the same for AIH, SLE patients and controls that was not the case. On the contrary, we can hypothesized either the occurrence of DNA denaturation due to the adsorption of the DNA molecules over the surface, and the recognition of ssDNA, as reported for healthy individuals either the presence of non-specific binding.

However, as previously mentioned,, pretreatment of gold surface with a sparse self-assembled monolayer composed of 4 ethylen glycol, allows both the

optimization of dsDNA monolayer formation and dsDNA accessibility to proteins while simultaneously preventing non specific interactions of proteins on the entire surface 17. This method enables DNA immobilization over the chip sensor, avoiding modifications or denaturations and non-specific binding.

We can conclude that the reactivity signal was generated from the interactions between DNA binding proteins present in the sera and dsDNA.

It is important to notice that the interaction was not observed for all dsDNA tested, and that it was particularly intense for OG of 250 bp. The difference of the reactivity signal between sera and dsDNA could depend on dsDNA density on the gold surface, dsDNA type and antigens-antibodies affinity.

In order to know if the binding proteins present in the serum were an immunoglobulin, we injected mouse monoclonal anti-IgG during the dissociation phase of the formed complex and studied the evolution of the signal. Surprisingly, when monoclonal mouse anti-IgGs were injected, 7 minutes after serum injection, the reactivity signal observed was of high intensity for AIH patients, of lower intensity for SLE patients and even lower for healthy individuals compared to AIH patients. These results highlight that the immune complex is formed by an IgG anti-dsDNA for AIH patients, on the contrary, it can be possibly due to other molecules for SLE patients and controls or other processes further hypothesized. Nevertheless, whatever was bound to the surface was bound to dsDNA because the signals were from the dsDNA spots and not from the other part of the surface.

The possible presence of IgM bound to dsDNA on the chip surface was excluded. After the injection of the serum monoclonal mouse anti-IgM were injected during the dissociation phase and no reactivity signal was observed for any individuals of the study group. This finding confirms that the isotype of immunoglobuline anti-dsDNA in AIH is IgG. The increase of IgG level is part of diagnostic criteria of AIH [131]; moreover, AIH shows a predominant IgG plasma cell infiltrate in liver biopsies [199]. It is also well known that SLE disease is characterized by IgG positive plasma cell [200].

We studied the interaction between anti-dsDNA antibodies and dsDNA antigens using purified antibodies from patients and controls' sera.
Purified IgG from all AIH patients produced a good reflectivity signal compared to SLE patients and controls, for which, the signal was completely absent or uninterpretable, except for one SLE patient which presented an interaction between purified IgG and dsDNA.

One hypothesis to explain the different behavior of the sera is that a tertiary partner, probably lost during IgG purification, intervenes in the formation of the antigen-antibody complex in SLE patients. The loss of this partner, necessary for the immune-complex formation or involved in his stability, could also explain the difference of signal obtained after the injection of anti-IgG and purified IgG in the flow chamber.

This hypothesis may also explain why DNA itself is not very immunogenic. Indeed an immunogenic non-self peptide tightly bound to a DNA molecule induces the formation of anti-dsDNA autoantibodies with similar characteristics to anti-dsDNA antibodies from lupus prone mice [201].

Similarly, the transgenic expression of polyoma virus T antigen results in the generation of anti-DNA autoantibodies [202]. Therefore the tight association of DNA with an immunogenic DNA-binding protein leads to generation of anti-dsDNA autoantibodies [203].

Why the presence of a third component (immunogenic-DNA binding protein?) is necessary for the binding of anti-dsDNA in SLE patient whereas in AIH is not, remains a concern.

In order to explore this hypothesis, the molecule of the immune-complex can be studied by MALDI-TOF spectrometry. After the SPRi studies, the chip surface can be transferred into the spectrometer, and mass data analysis can help in structure elucidation of the structure of the compounds.

Another way to explore this hypothesis is to use affinity chromatography of oligonucleotides bound on gold surface with whole sera. The components bound to the OG have first to be washed with stringent elution, then studied by 2D electrophoresis and finally identified by mass spectrometry. However, there is a limitation of this approach, as we do not know the nature of the hypothezed third partner, and if its molecular masse is in the game of the resolved proteins by electrophoresis. Furthermore, proteins with high affinity for DNA may be basis, and these proteins are not well resolved by iso electrofocalisation.

The second hypothesis formulated to explain our results is that the anti-dsDNA present in SLE patients recognize a specific dsDNA conformation that was not represented by oligonucleotides spotted over our chip surface. It has been reported that, besides their actuarial sequences and length, the binding affinity of anti-dsDNA autoantibodies and dsDNA antigens depends largely on the structure of oligonucleotides [204].

Pisetsky and colleagues showed that in healthy subjects antibodies can bind to HinfI restriction fragments of bacterial ssDNA under conditions in which SLE anti-DNA binding is dramatically reduced [205]. These finding identify an additional difference between SLE patients and healthy subjects concerning anti-dsDNA antibodies properties and suggest that in healthy subjects anti-DNA antibodies bind linear epitopes on bacterial DNA, whereas in SLE patients anti-DNA antibodies bind antigens with particular conformations present on both bacterial and mammalian DNA.

One of the limits of the SPRi method is that the DNA spotted on the surface is linear, unable to undergo conformational changes such as bending or looping.

Some of the AIH patients were tested with IIF: patients with ANA IIF positive test compared to IIF negative ones showed no significant difference in reactivity signal. In a recent large study of 292 ANA negative patients referred for rheumatic symptom, 5.5% were positive by IIF on *C. luciliae* for anti-dsDNA autoantibodies [206]. This test discrepancy remains to be elucidated.

The k_{off} analysis showed no differences among purified IgGs obtained from AIH patients' sera.

Even though some differences in the intensity of the signal were identified amongst OGs, the dissociation phase was comparable for all OGs, meaning that the signal variations might be due to the association phase.

VI. CONCLUSION

The interaction between autoantigens and autoantibodies has not been well elucidates yet. In particular the immune-complex formation between dsDNA and anti-dsDNA in different autoimmune disease is still an issue.

SPRi is an optical, real time, label-free technique that studies the interaction between a ligand immobilized over a prism surface and an analyte in a solution.

In this study we demonstrated that this method can be applied to the analysis of dsDNA and anti-dsDNA interaction and should be considered as a new strategy in the autoimmune research.

The immune-complex dsDNS/anti-dsDNA is more reactive and stable in AIH then SLE patients as demonstrated using purified IgG from patients' sera.

The first hypothesis that can explain the different behavior between the two disease is the presence of a third partner required for the immune-complex formation in SLE patients, which is possibly lost during IgG purification.

The second, and more probable, hypothesis is that in SLE patients the anti-dsDNA recognizes a particular DNA structure not represented by the oligonucleotides used in this work.

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