# Table of contents

## 1. Abstract

L'adalimumab è un anticorpo monoclonale inibitore del TNF-α che presenta una struttura simile alle IgG1 umane. Nonostante adalimumab sia completamente umanizzato, lo sviluppo di anticorpi anti farmaco è stato riportato in diverse patologie infiammatorie.

L'obiettivo del nostro studio è stato quello di verificare la presenza di anticorpi anti-adalimumab (AAA) e la loro rilevanza clinica in una coorte di pazienti con diagnosi di artrite idiopatica giovanile (AIG) trattati con questo anticorpo monoclonale.

Si tratta di uno studio di coorte osservazionale prospettico. Il dosaggio degli AAA è stato effettuato mediate una metodica innovativa: la risonanza plasmonica di superficie (Biacore® T100).

L'attività di malattia è stata valutata utilizzando il JADAS-10 (Juvenile Arthritis Disease Activity Score a 10 articolazioni). L'analisi statistica è stata condotta mediante l'utilizzo del Mann-Whitney U test, il Wilcoxon signed rank test per campioni appaiati; il chi-quadro ed il test di Fisher sono stati utilizzati per confrontare i dati. I test di correlazione Pearson e Spearman sono stati utilizzati per determinare i coefficienti di correlazione delle diverse variabili.

Dei 27 pazienti inclusi nello studio, 10 (37%) soggetti hanno avuto almeno un campione ematico positivo per AAA.

Lo sviluppo degli AA A è avvenuto tra i 3 e i 38 mesi dopo l'inizio della terapia con adalimumab. Tra i pazienti positivi per AAA, 7 soggetti (70%) hanno presentato almeno una ripresa di malattia comparati ai 4 soggetti (23.5%) appartenenti al gruppo degli AAA negativi (rs 0.45, $p < 0.017$).

In conclusione, mediante l'utilizzo di un metodo innovativo e accurato, abbiamo trovato un'alta incidenza di AAA in una coorte di pazienti con AIG trattati con adalimumab per un periodo di 40 settimane; la presenza degli AAA è stata correlata ad un aumentato numero di ricadute di malattia.

## 2. Introduction

### 2.1 Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatic condition in childhood; the prevalence varies between 16 and 150 per 100,000. JIA is not a single pathological entity, but a term including all arthritides starting before 16 years, lasting for more than 6 weeks and with unknown etiology **[1]**. The International League of Associations for Rheumatology (ILAR) recognizes 7 major categories: oligoarthritis, rheumatoid factor (RF)– negative polyarthritis, RF-positive polyarthritis, juvenile psoriatic arthritis (JPsA), systemic JIA (SoJIA), enthesitis-related arthritis (ERA), and undifferentiated arthritis **[2].** This classification is however unsatisfactory, and efforts are currently being made to improve it **[3].**

The improved physiopathological knowledge and the availability of new therapies acting on specific targets have led to an important improvement of outcome in the past two decades **[4].** The pivotal role of Tumor Necrosis Factor α (TNF-α), Interleukin 1 β (IL-1β) and Interleukin 6 as pro-inflammatory cytokine in JIA is well known, and several different drugs against them, called biologics, has been developed **[5]**.

According to the American College of Rheumatology recommendations, non-steroidal anti-inflammatory drugs (NSAIDs) and/or intraarticular steroids (IAS) are the first line therapy for oligoarticular JIA **[6]**. Methotrexate (MTX) is the most commonly used DMARDs for JIA whereas patients that fail to respond or are intolerant to DMARDs are candidates for biologic therapy.

The ideal goal of therapy is to achieve the status of inactive disease. However, it is not easy to reach and maintain that goal, especially for certain JIA categories such as polyarticular and systemic JIA **[7, 8].** Indeed, some patients maintain active disease and others show loss of efficacy after continued treatment. There is still debate regarding the opportunity of using an early aggressive therapy **[9, 10]** instead of a classical step up approach using several agents sequentially. The lack of defined prognostic response markers to treatments makes the decision between these options challenging.

## 2.2 Immunogenicity

### 2.2.1 Biologic agents and immunogenicity

Biologic agents encompass a rapidly growing class of drugs that includes monoclonal antibodies and fusion proteins targeting cytokines playing a main role in disease pathways. These drugs may evoke a host immune system response impacting their efficacy. Immunogenicity is the ability of a genetically engineered protein to provoke an immune response against themselves. Often, it leads to the production of anti-drug antibodies. The production of anti-drug antibodies represents the final stage of a complex immune process involving antigen presentation followed by activation of both adaptive and regulatory cellular immune responses. It may be influenced by many factors such as biologic molecules designing (chimeric or fully humanized), presence of contaminants and impurities, route of administration,

length of treatment, concomitant medication, and the nature of diseases itself **[11]**.

Immunogenicity may have a clinical impact in terms of decreased drugs efficacy by increasing its clearance or competing with the target for the site of activity and/or by inducing infusion reaction and hypersensitivity reactions **[12, 13]**.

Clinical effects depend on the anti-drug antibodies isotype composition determining different immunological consequences such as complement activation, antibody-dependent cellular cytotoxicity (ADCC) and mast cell sensitization **[14].**

Induced anti-drug antibodies represent a mixture of antibodies of different isotypes, specificities and affinities **[15]** and it may change over the time **[11]**.

A mutation of the immunoglobulin complementarity determining region (CDR) genes may occur along with anti-drug antibodies switching to other classes due to the T helper cells activation, both these adjustments lead to a change in the antibodies affinity, this process is called “affinity maturation”.

Moreover a persistent immune response to biologics may elicit further expansion and diversification of B cells leading to a change of binding sites, this phenomenon is called “epitope spreading” **[16, 17]**. Therefore, anti-drug antibodies represent a unique mixture of different antibodies with several potential immunological and clinical effects at any given time point during biologic treatment.

### 2.2.2 Adalimumab and immunogenicity

Adalimumab is a TNF-α blocker antibody indistinguishable in structure and function from natural human IgG1, genetically engineered through phage display technology, that binds both soluble and membrane-bound TNF-α **[18, 19].** It has been proven that adalimumab can promote Th17 cells suppression by inducing Foxp3+ T reg cells expansion in rheumatoid arthritis (RA) **[20].** Adalimumab is approved for use in children aged ≥ 4 years with oligo-extended JIA and polyarticular JIA. It is administered at the dose of 24 mg/m2/ every two weeks (max 40 mg) by subcutaneous injections. Adalimumab effectiveness and safety have been shown in a study that suggested also its increased efficacy when used along with MTX **[21].**

Even if adalimumab is fully humanized, the development of Anti-Adalimumab Antibodies (AAA) has been reported in many inflammatory conditions **[22-25].** Several studies pointed out the correlation between the development of AAA and the worsening/flaring up of disease in many inflammatory conditions (RA, Psoriasis, IBD). The evidences of these effects in JIA patients are still scarce and often contradictory as well as the rate and the time point of their formation **[21,26-27]**. This may in part be related to the difficulties of AAA detection due to the lack of proper secondary reagents discriminating between the drug and the immune-response antibodies, which are both human IgG1, and to the presence of drug-antibody complexes and/or other serum factors, which may alter the results. We already demonstrated the validity of surface plasmon resonance (SPR)-based optical biosensors for the detection of AAA in pediatric patients with different rheumatologic conditions **[28]**. Our goals now is to

understand whether AAA formation may influence disease course.

## 2.3 Surface plasmon resonance phenomenon

### 2.3.1 SPR-based biosensor Biacore

Biacore is a label free system exploiting the phenomenon of the Surface Plasmon Resonance (SPR) to monitor the interaction between molecules in real time. The approach involves attaching one interacting partner called *ligand* to the surface of a sensor chip, and then flowing the samples containing the other interaction partner, defined *analyte*, over the surface. Binding of molecules to the sensor surface generates a response, which is directly proportional to the bound mass; this response is measured in Resonance Units (RU) and is graphically expressed by a sensorgram, showing the progress of the interaction during time. SPR is a phenomenon that occurs in thin conducting films at an interface between media of different Refractive Index (RI). In Biacore system, the two media are the glass of the sensor chip and the sample solution, and the conducting film is a thin layer of gold on the sensor chip surface; the wave length of the incident light and the reflective index of the inner surface are constant, so the SPR phenomenon is used to monitor the change of reflective index in the flowing solution close to the sensor chip surface. Under conditions of total internal reflection, light incident on the reflecting interface leaks an electric field intensity called evanescent wave field across the interface into the medium of lower refractive index, without losing net energy. The amplitude of the evanescent field wave decreases exponentially with distance from the surface, and the effective penetration depth in terms of sensitivity to refractive

index is about 150 nm. At a certain combination of angle of incidence and wavelength, the incident light excites plasmons in the gold film. As a result, a characteristic absorption of energy via the evanescent wave field occurs and SPR is seen as a drop in the intensity of the reflected light (Figure 1). Changes in solute concentration at the surface of the sensor chip cause changes in the RI of the solution, which can be measured as an SPR response and expressed in RU, in particular a difference of 0.1° in the angle of reflection generates a response of 1000 RU that correspond to a variation in mass over the chip surface of about 1 ng/mm$^2$.

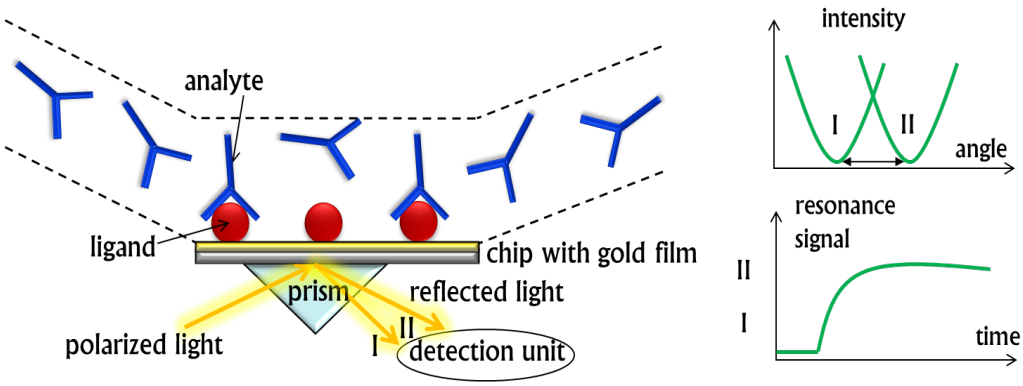


**Figure 1**: Schematic representation of the Surface Plasmon Resonance occurring in the inner core of a Biacore instrument.

SPR arises in any thin conducting film under the conditions above described, although the wavelength at which resonance occurs and the shape of the energy absorption profile differ with different conducting materials. Gold is used in Biacore sensor chips because it combines favorable SPR characteristics with stability and a high level of inertness in biomolecular interaction contexts.

This advanced technology can be used to investigate the specificity of an interaction, by testing the extent of binding between different pairs of molecules, or to describe the kinetics and affinity of an interaction, by

analyzing the binding behavior in terms of mathematical interaction models, or to quantify the concentration of specific molecules present in the sample by measuring the response obtained from the sample itself.

Biacore studies can be applied to a wide range of biomolecules including proteins, lipids, nucleic acids, carbohydrates and small molecules such as synthetic drugs. Analytes to be detected should have a molecular weight above 100 Da in order to produce a sufficient change in the reflective index; considering that the evanescent wave penetration depth is 300-400 nm, molecules longer than 400 nm can be detected but not accurately characterized because they produce a non-linear signal.

The heart of Biacore systems is the sensor chip, which provides the physical conditions necessary to generate the SPR signal and to monitor the interactions occurring on its surface.

Different biological compatible matrices are commercially available offering the opportunity to immobilize the ligand through several approaches such as a covalent immobilization, where it is linked to the surface through a covalent chemical link or a high affinity capture, where it is attached by noncovalent but high specific interaction with another molecule and a hydrophobic adsorption, which exploits more or less specific hydrophobic interactions to attach either the molecule of interest or a hydrophobic carrier, such as a lipid monolayer or bilayer, to the sensor chip surface (Figure 2).

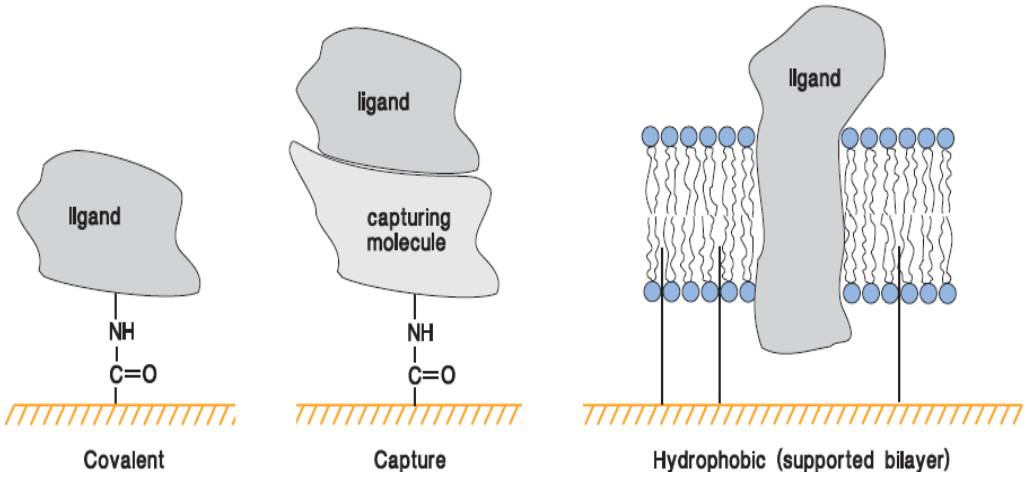


**Figure 2**: Three most commonly used approaches for attaching biomolecules to the sensor chip surface.

The most commonly used sensor chip is the CM5 type, carrying a matrix of carboxymethylated dextran covalently attached to the gold surface (Figure 3), the dextran matrix is flexible, allowing relatively free movement of attached ligands within the surface layer and it provides a high surface capacity for immobilizing a wide range of ligands with every chemical strategies.

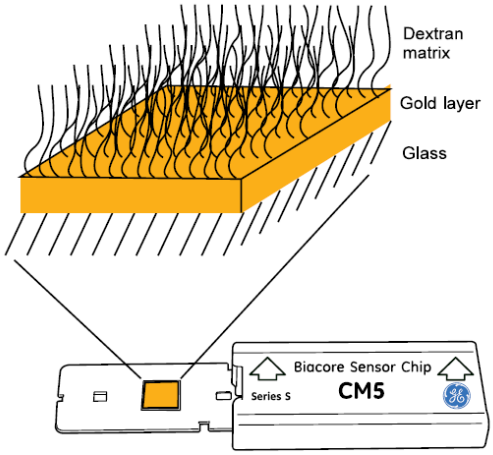


**Figure 3**: Schematic illustration of the structure of a CM5 sensor chip.

Sample containing analyte can be injected over the immobilized ligands thanks to a sophisticated microfluidic system. The sensor surface itself forms one wall

of a flow cell, which is an integral part of the microfluidic system that consists of a series of channels and valves in a plastic block, the Integrated Microfluidic Cartridge (IFC).

Thanks to the valves, the operator can regulate the flow and chose in how many channels the sample has to be injected (Figure 4).

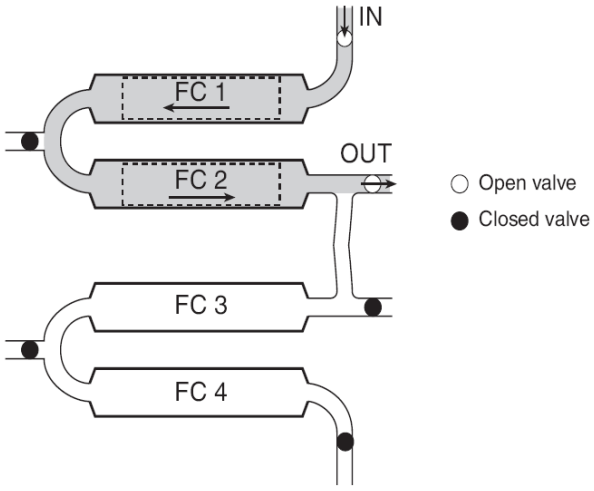


**Figure 4**: Schematic representation of the 4 flow cells and of the valves involved in the control of the flow; in this case sample flows in channel 1 and channel 2.

The signal registered in the reference channel will be subtracted from the one obtained in the active channels. This subtraction has the advantage of removing the aspecific interactions that can be registered on a gold surface when injecting a complex matrix. The other advantage is the removal of the so call bulk effect that can mask interactions involving low molecular weight analytes and that is due to a high difference in the reflective indices of the sample solution and of the running buffer.

Covalent immobilization to the dextran matrix is the most commonly used approach for attaching the ligand to the surface, and it is also the method of choice for immobilizing capturing molecules. Covalent immobilization

generally results in stable attachment of the ligand to the surface under the buffer conditions normally used for surface regeneration that removes bound analyte at the end of each analysis cycle but leaves the ligand attached to the surface.

To obtain a good immobilization level, the electrostatic pre-concentration of ligands in the dextran matrix must be achieved. It's important to consider that at pH values above 3.5 the carboxymethylated dextran on the sensor chip surface is negatively charged, so the primary requirement for the electrostatic pre-concentration on the surface is that the pH of the ligand solution should lie between 3.5 and the isoelectric point of the ligand, so that the surface and the ligand carry opposite net charges (Figure 5).

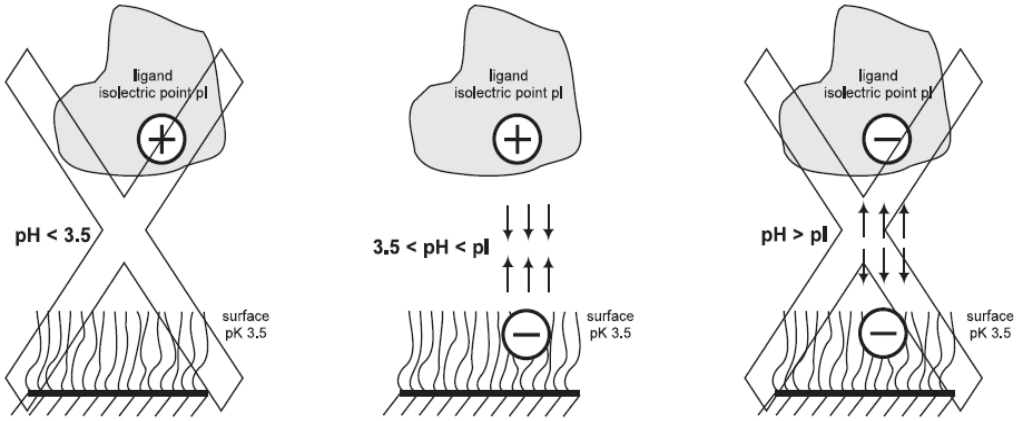


**Figure 5**: Schematic representation of ligand pre-concentration on chip surface; ligand is concentrated electrostatic attraction between ligand and chip is possible only when the pH lies between the isoelectric point of the ligand and the $pK_a$ of the surface.

The general pattern for essentially all covalent immobilization methods consists in the following steps:

- chip surface activation by the injection of appropriate reagents
- ligand injections in the previously identified best immobilization buffer up to reaching a satisfactory immobilization level

- injection of a reagent to deactivate remaining active groups on the surface and to remove non-covalently bound ligand

The most common immobilization chemistries (Figure 6) are:

- amine coupling, exploiting primary amine groups of the ligand after activation of the surface
- thiol coupling, exploiting thiol-disulfide exchange between thiol groups and active disulfides introduced on either the ligand (surface thiol coupling) or the surface matrix (ligand thiol coupling)
- aldehyde coupling, using the reaction between hydrazine or carbohydrazide groups introduced on the surface and aldehyde groups obtained by oxidation of carbohydrates in the ligand

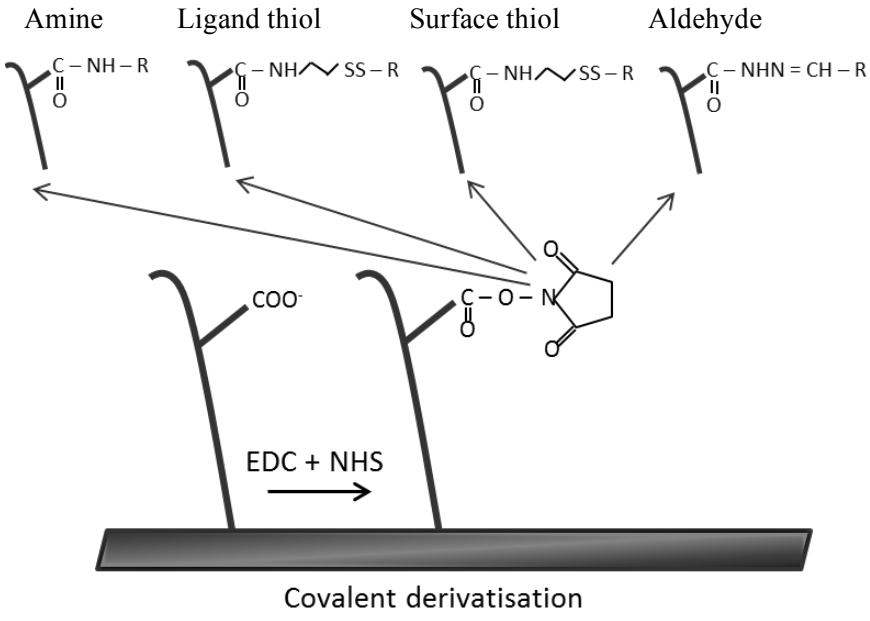


**Figure 6**: Covalent immobilization approaches for attaching the ligand to the surface.

### 2.3.2 Binding studies

Once the ligand has been immobilized on the surface of the chip, binding measurement can be done to quantify the interaction between the ligand and the samples containing the analyte we want to reveal.

A binding experiment is performed for each sample to be tested, by repeating the same cycle of analyses consisting in the following steps: sample injection under a continuous flow of running buffer to allow the formation of the complex, running buffer flow to obtain a partial dissociation, chip surface regeneration to completely remove the analyte that interacted with the ligand without altering the ligand itself. During the injection time the interaction between ligand and analyte generates a change in the reflective index and is converted into an instrumental signal that is directly related to the change in mass concentration on the surface, so that molar responses are proportional to the size of the molecule involved. A given response will represent a higher molar concentration of a small molecule than a large one: conversely, a given number of molecules binding to the surface will give a lower response if the molecule is small. This response is graphically expressed by a sensorgam (Figure 7) showing the progress of the interaction during time.

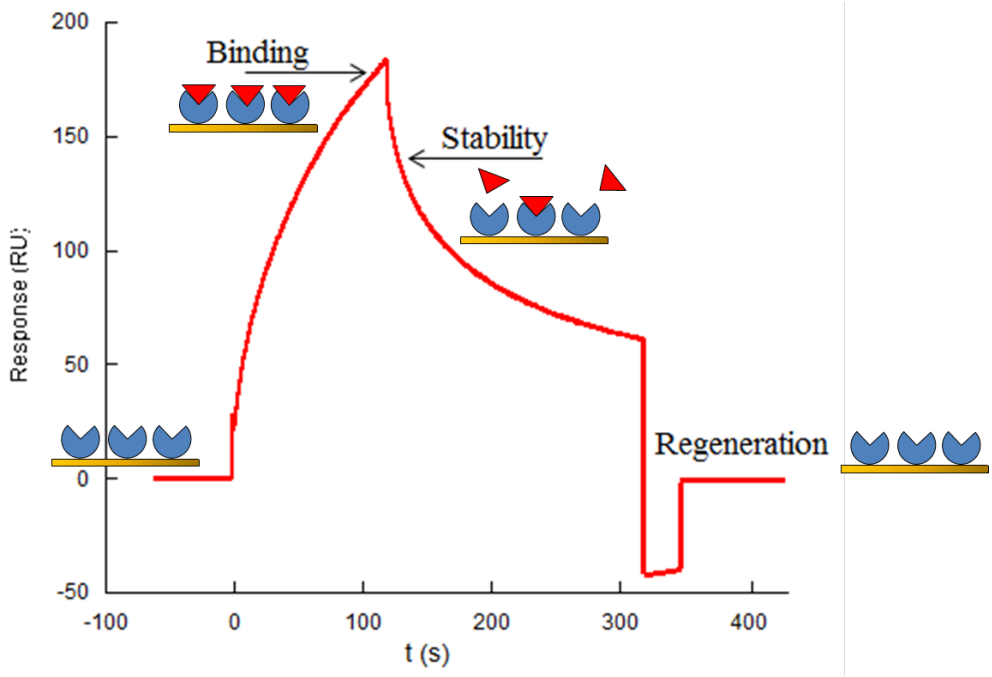


**Figure 7**: Typical sensorgram resulting from a binding experiment; the signal in Resonance Units is direclty related to the quantity of the analyte that binds to the ligand.

To compare the results obtained injecting different samples, two kinds of reference points illustrated in Figure 7 can be considered: the *binding* values that indicate the maximum interaction signal that is revealed at the end of the injection or the *stability* values that indicate the signal registered 20 seconds after the end of the injection and that only depends on very high specific interactions.

### 2.3.3 Kinetic and affinity studies

The biosensor Biacore T100 allows the characterization of the kinetic and the affinity of the interaction between the biomolecules of interest. Kinetic and affinity are normally determined from the binding characteristics of a series of analyte concentrations.

The minimal requirements to perform a kinetic and affinity evaluation are the knowledge of the molecular weight of the analyte, the application of a concentration series of analyte with at least four non-zero concentrations and one blank cycle consisting of zero concentration sample.

Experimental data obtained with every sample are fitted to a series of mathematical models describing the interaction. The association phase during sample injection contains information on both association and dissociation processes and allows the calculation of the association rate $k_a$ ($M^{-1}s^{-1}$), from the dissociation phase when buffer flow removes dissociated analyte molecules, the dissociation rate $k_d$ ($s^{-1}$) can be obtained. For the simple 1:1 binding model, the affinity constant $K_D$ (M) is equal to the ratio of the rate constants ($k_d/k_a$) and can therefore be derived from kinetic measurements.

The standard kinetic interaction models provided with Biacore systems are: a) the 1:1 binding that describes one molecule of analyte binding to one molecule of ligand; b) the bivalent analyte that indicates the interaction of a monovalent ligand with analyte molecules that carry two identical and independent binding sites; c) the heterogeneous ligand describing the interaction of one analyte with two independent ligands on the surface; in this case, the observed binding is the sum of the interaction with the two ligands.

In order to obtain a high quality of kinetic and affinity data, it is necessary that the sensorgrams have a sufficient curvature during the association and the dissociation phase; consequently interactions characterized by a very slow kinetic have to be monitored for a longer time and for interactions characterized by a fast kinetic, the flow should be decreased. The optimal

concentration range of the analyte has to be chosen in order to achieve the saturation of the signal at the highest concentration used **[29]**.

### 2.4 Study aim

Aim of this study is to assess, through SPR biosensor assay, the presence of AAA and their clinical relevance in a cohort of patient with JIA receiving Adalimumab.

## 3. Methods

### 3.1 Study design

This is a prospective observational inception cohort study, recruiting JIA children between January 2016 and May 2017 from the Pediatric Rheumatology Unit of Anna Meyer Children's University Hospital, Florence, Italy. During routine follow-up visit, at the study entry and thereafter every 3 months, enrolled children received general physical examination, rheumatologic and laboratory evaluation in order to assess disease activity and drugs toxicity. The present study protocol was approved by our Hospital Ethics Committees and informed consent was obtained from all patients and/or parents, as appropriate.

### 3.2 Patients

All the patients enrolled fulfilled the International League of Associations for Rheumatology (ILAR) criteria for JIA **[2]**. All of them received adalimumab for at least 3 months before study inclusion. Patients were treated with either adalimumab and concomitant methotrexate (MTX) or adalimumab alone. All patients received adalimumab at the dose of 24 mg/m2 subcutaneously every other week. MTX was administrated weekly at average dose of 12.5-15 mg/$m^2$. All included patients have been previously received non-steroidal ant-inflammatory drugs (NSAIDs) and MTX. Exclusion criteria were age at the enrollment older than 18 years, a chronic disease different from JIA, and a previous treatment with biologic treatments different from Adalimumab.

### 3.3 Disease activity measurement

The disease activity was evaluated using the Juvenile Arthritis Disease Activity Score with 10 joints count (JADAS-10) according to the formula published by Consolaro et al. **[30]**. The score was calculated as the sum of the following 4 components: physician global rating of overall disease activity, measured on a 10-cm horizontal visual analogue scale (VAS) (0 = no activity; 10 = maximum activity); parent/child ratings of well-being and pain, assessed on a 10-cm horizontal VAS (0 = best; 10 = worst); the number of active joints assessed in 10 joints (any involved joint to a maximum of 10), and the erythrocyte sedimentation rate (ESR) normalized to a scale of 0–10.

The presence of active uveitis was monitored over the time as possible cause of treatment failure. JADAS-10 was determined at each time point of serum

samples as well as the presence of active uveitis. The achievement of the status of either inactive disease (JADAS-10 below 1 for polyarticular and oligoarticular JIA) or minimal active disease (JADAS-10 below 2 for oligoarticular and below 3.8 for polyarticular JIA) was considered as clinical remission as suggested by Consolaro et al **[31]**. Active disease was defined as a JADAS-10 > 2 for oligoarticular and JADAS-10 > 3.8 for polyarticular and/or presence of active uveitis.

### 3.4 Data collecting

A customized database was developed to collect demographic information (age at inclusion and gender), clinical (JIA subtype, JADAS, uveitis, concomitant therapy, adverse reactions) and laboratory data (AAA serum levels) at each time points.

### 3.5 Surface Plasmon Resonance (SPR) assay

Blood samples were collected sequentially during routine laboratory tests by antecubital venepuncture and the Laboratory of Peptide and Protein Chemistry and Biology of University of Florence performed AAA assays. Each sample was centrifuged, and supernatant aliquoted and stored at −20 °C until assayed. SPR experiments were achieved using a Biacore® T100 instrument (GE Healthcare, Uppsala, Sweden). All binding analyses were performed at +25 ºC using HBS-EP+ (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20) as running buffer. Sensor chips CM5, amine coupling kit, Glycine-HCl, and Running Buffer were purchased by GE Healthcare. Sodium acetate and Sodium hydroxide were purchased by Carlo Erba (Milano, Italy). Commercial

adalimumab was obtained in a 40 mg/0.8 mL prepared for clinical use (Humira® lot 30404XD11; AbbVie Ldt., Maidenhead, United Kingdom). Experiments were executed following the previously described SPR protocol **[28]** properly optimized for the current study. Adalimumab was immobilized on the sensor chip CM5-type surface raising a final immobilization level of 16800 ± 300 Resonance Units (RU). Human serum samples were thawed to ambient temperature and then diluted 1:100 in running buffer. The diluted serum samples were injected at flow rate of 30 μL/min over the immobilized adalimumab during 120 sec and experiments were replicated four times. Responses were measured in Resonance Units (RU) as the difference between active and reference channel. The optimal cut-off point was properly optimized from previously described protocol **[28]** maintaining the same sensitivity and specificity (79% and 99% respectively). A cut-off value greater than 0.85 RU was considered positive. After each sample injection, surface was regenerated with a 10 sec pulse of 50 mM Glycine-HCl pH 2.5 followed by a 10 sec pulse of a solution 1 mM NaOH allowing the complete removal of specifically and non-specifically bounded biological material from the surface. Following this protocol all further experiments were performed not over and above 100 measurements per channel **[28]**.

### 3.6 Statistical analysis

In order to test the presence of AAA in a cohort of JIA children receiving Adalimumab, considering previously reported frequency of AAA in literature

**[22-25, 27]**, a large expected difference was estimated for the sample. A priori power analysis, setting effect size (f) at 0.40, as per Cohen. **[32]** and power at 0.95, showed that 34 participants in all groups combined would be required. The Mann-Whitney U test, Kruskal-Wallis test, Wilcoxon signed-rank test for paired samples, chi-square test, and Fisher's exact test, when appropriate, were used to compare data. Pearson and Spearman correlation tests were used to determine correlation coefficients for different entered variables: demographic data, clinical features, and serologic profile. Analyses were performed on SPSS package for MAC, version 24.0 (SPSS).

## 4. Results

Sixty serum samples from 27 patients were collected: 8 patients had one sample collected, 9 patients two samples collected, 6 patients three, and in 4 patients four samples were collected prospectively. The mean time interval between each sample collection was 6 months (±3.47 SD). The whole population was followed over a mean time of 40 weeks, range 12-64. No patient experienced a uveitis flare during the study. No infusion reactions or other side effects were observed. The main patient characteristics are shown in Table 1.

| | Total | AAA positive | AAA negative |
|---|---|---|---|
| Number of patients (M/F) | 27 (6/21) | 10 (2/8) | 17 (4/13) |
| Age (years) at inclusions (± SD) | 9.5 (± 3.32) | 11.15 (± 3.11) | 8.52 (± 3.12) |
| JIA category | | | |
| Oligoarthritis | 13 | 4 | 9 |
| Polyarthritis | 12 | 4 | 8 |
| Enthesitis-related arthritis | 2 | 2 | 0 |
| ANA positivity | 24 | 9 | 15 |
| Associated uveitis in history | 15 | 6 | 9 |
| Months on concomitant MTX (± SD) | 19.67 (± 22.79) | 22.9 (± 32.27) | 17.76 (± 15.74) |
| Months on adalimumab (± SD) | 35.55 (± 27.96) | 41.6 (± 33.55) | 32 (± 24.51) |
| JADAS-10 (SD) at onset | 5.4 (± 6.6) | 3.7 (± 3.4) | 6.7 (± 8.1) |
| JADAS-10 at last follow-up | 5.1 5.1 | 4.6 5.2 | 5.4 5.3 |
| Months of study follow-up (SDS) | 10 (± 3.57) | 10 (± 4.14) | 10 (± 3.31) |
| Disease duration at study entry (years, ± SD) | 4.79 (± 3.04) | 5.92 (± 4.15) | 4.13 (± 2.03) |

*SD* standard deviation, *JIA* juvenile idiopathic arthritis, *ANA* anti-nuclear antibodies, *JADAS-10* Juvenile Arthritis Disease Activity Score with 10 joint count

**Table 1.** Main patient characteristics

Ten patients (37%) had at least one AAA-positive sample. Among this group, 15 out of 26 samples collected were positive with a mean value of 1.131 RU (range 0.856-3.163 RU). Patients developed AAA between 3 and 38 months after starting adalimumab. Three patients had more than one sample positive and one of them had 4 positive samples, with the highest antibodies titer of the whole cohort.

Before starting adalimumab, all eligible patients received MTX; 16 children continued MTX in association with anti-TNF-α treatment. Five (31%) out of 16 children receiving MTX in addition to adalimumab and 5 (45%) out of 11 receiving adalimumab alone had AAA positive ($\chi^2$ 0.56, p= 0.45). The duration of concomitant MTX administration was not significantly different in AAA positive children than in AAA negative ones: 22.9 ±32.3 *vs* 17.8 ±15.7 months, respectively.

AAA positive group had been on adalimumab longer than AAA negative group, although this did not reach statistical significance: 41.9 ±33.5 *vs* 32 ±24.5 months (p=0.51).

Patients AAA positive experienced more relapses then those without anti-drug antibodies. Indeed the number of total relapses in the AAA positive group was 10, with four children experiencing one relapse and three children two relapses, while in the AAA negative group 4 relapses occurred, with two children showing two relapses (rs 0.45, p< 0.017).

JADAS-10 at last available follow-up did not show a statistical significant difference between children who were AAA positive and those who were AAA negative (mean ±SD: 4.6 ±5.2 *vs* 5.4 ±5.3, p=0.78). JADAS-10 at last available follow-up was not different from JADAS-10 at study inclusion in AAA positive as well as in the AAA negative group (p=0.39, p=0.76, respectively), although an increasing trend of JADAS-10 score for AAA positivity along with a decreasing one for AAA negativity was observed.

During follow-up, three patients discontinued adalimumab: one who was AAA negative, after the achievement of sustained remission, and two for inefficacy, one of them with detectable AAA.

At last available follow-up, 3 out of 10 (30%) children who were AAA positive were in clinical remission compared to 7 out of 17 (41.2%) children with AAA negative ($\chi^2$ 0.33, p= 0.56).

No significant correlation was detected between the presence of AAA and gender, JIA onset type, antinuclear antibodies (ANA) positivity, history of uveitis and disease duration.

## 5. Discussion

Understanding why biologic therapies fail or loss their efficacy over time is crucial for the treatment of children with chronic disease such as JIA. Furthermore, it is important to improve the effectiveness of available drugs and to spare our weapons in lifespan optic.

The risk of immunogenicity and its clinical impact are well recognized in infliximab treated patients **[15, 33],** conversely to etanercerpt **[34]**. Adult literature analyzed the risks and potential consequences of anti-drug antibodies against adalimumab in RA **[25, 35]** while the same topic for JIA is still a matter of debate **[21, 26-27]**.

Conversely to previous data, the present study reports high percentage (37% of patients) of AAA, suggesting that children may produce AAA like adults do, or even easier **[21, 25-27]**. However the different assay used to detect AAA has to be taken into account.

Since AAA positive patients showed higher chance to relapse, these results may to support the hypothesis that AAA presence could influence the disease activity. Our analysis was not able to find any correlation with concomitant MTX treatment and the duration of anti-TNF-α treatment, even if children with positive anti-drug antibodies were on adalimumab treatment for a longer period. It is possible that our study is underpowered to test these potential differences.

Indeed, we found that AAA positive patients tend to increase their JADAS-10 over the time, whereas patients AAA negative showed the opposite.

AAA may appear very early since treatment onset: one patient resulted positive just after 3 months of adalimumab, and withdrew it very soon due to inefficacy. Of note, we did not observe any infusion reactions in both AAA positive and negative groups.

So far few studies on adalimumab immunogenicity in JIA patients have been published and results were controversial. In a big multicenter randomized study, Lovell et al. found detectable AAA in 27 out of 171 (16%) JIA patients treated with adalimumab over an observation time period of 32 weeks [**21**]. In another study, Imagawa et al. [**26**] found detectable AAA over 60 weeks in 6 out of 25 JIA patients (24%) and half of these were receiving concomitant MTX. No evidence of a correlation between disease activity and AAA formation was found in both these studies and anti-drug antibodies were transient. Skrabl-Baumgartner et al. [**27**] had different results in a cohort of 23 JIA patients treated with adalimumab over a longer period of time (208 weeks). Five out of 6 patients with detectable AAA (26%) showed loss of response to adalimumab and the number of patients with concomitant MTX was significantly higher in AAA negative group.

The above mentioned conflicting publications are difficult to compare with our data due to different AAA assays, different observation time, and different type of study.

All previous studies detected anti-drug antibodies using an enzyme-linked immunoassay (ELISA). These tests, albeit specific, may be influenced by the presence of adalimumab in the sample, eventually underestimating the amount of antibody **[33]**. Conversely**,** SPR assay delivers direct information quickly,

with good sensitivity and high specificity without using a secondary antibody; thus the result of antibody assay is not hampered by technical confounders [**28**]. This may, at least in part, explains the higher incidence of AAA in our cohort compared to other studies.

However, both SPR and ELISA are not able to determine if the detected antibodies, independently of their affinity, are neutralizing, i.e. able to block TNF activity. For this reason, the additional evaluation of the adalimumab trough concentration may be required to determine a concentration-effect curve of adalimumab **[34]**. Indeed, the additional evaluation of the adalimumab trough concentration is required to determine a concentration-effect curve of adalimumab treatment.

In conclusion, using an innovative and accurate assay method, we found a high incidence of anti-drug antibodies in a cohort of adalimumab treated JIA patients observed over a mean period of 40 weeks; the presence of AAA seemed to be related to the number of relapses.

The ongoing project ABIRISK (Anti-biopharmaceutical Immunization: Prediction and analysis of clinical relevance to minimize the risk) involving several centers belonging to Paediatric Rheumatology International Trials Organisation (PRINTO) is currently underway and will shed light on the risk of immunogenicity in JIA patients treated with biologic therapies such as adalimumab and the potential clinical impact in a much larger cohort.

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