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Post-translational modification of relevant proteins in tissue-specific autoimmunity

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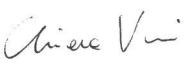
STATEMENT OF ORIGINALITY

The work described in this thesis was carried out at the William Harvey Research Institute, Barts and the London Queen Mary's School of Medicine and Dentistry, department of Biochemical Pharmacology, University of London, in collaboration with the Unit of Endocrinology and Diabetes at University Campus Bio-Medico of Rome.

Unless otherwise stated, the author performed the experiments described.

I hereby state that this thesis entitled "Post-translational modification of relevant proteins in tissue-specific autoimmunity" has not been submitted for a degree or any other qualification at any other university.

Chiara Vinci, 05/06/2018



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ABSTRACT

Introduction: Autoimmune diseases are often associated with increased production of reactive oxygen species (ROS). ROS may post-translationally modify proteins generating neoepitopes which may be a key aspect in the development of autoimmunity. Type 1 diabetes (T1D) and rheumatoid arthritis (RA) are autoimmune diseases that share some factors, such as association with the HLA-DRB1*04 and increased oxidative stress. My group has previously reported the presence of insulin oxidized by ROS (oxPTM-INS) in T1D patients and that antibodies to oxPTM-INS (oxPTM-INS-Ab) are detected in newly diagnosed T1D patients. My group has also reported an autoimmune reactivity against ROS modified type II collagen in rheumatoid arthritis patients (oxPTM-CII).

Hypothesis: This study is based on the hypothesis that ROS released during chronic inflammation are able to post-translationally modify proteins leading to the formation of neoepitopes. The main hypothesis is that these oxPTM play a key role in autoimmune response and thus influence the diseases pathobiology.

Objectives: The main objective of this study was to increase extend the understanding of oxPTM in autoimmunity and in arthritis.

Specific aims:

1 - Testing the reactivity to oxPTM-INS in a longitudinal cohort of patients prior to diagnosis of T1D.

2 - Mapping oxPTM-INS neoepitopes.

3 - Testing reactivity to oxPTM-CII in large cohorts of patients with inflammatory arthritis.

4 - Mapping the oxPTM-CII neoepitopes.

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Methods: Modification of CII and insulin were confirmed by PAGE and, in the case of insulin, mass spectrometry. Serum samples from subjects with inflammatory arthritis (including RA and spondyloarthritis) and T1D were used to evaluate the presence of oxPTM-CII-Ab and oxPTM-INS-Ab. The correlation between these antibodies with clinical, biochemical and HLA genotype was assessed. The epitope mapping for oxPTM-CII and oxPTM-CII was studied.

Results:

1 - Antibodies to at least one oxPTM-INS were present in over the 90% of children progressing to T1D (progr-T1D). •OH-INS-Ab were more common in progr-T1D children than in children non progressing to T1D positive for standard diabetes antibody marker (NP-AAB+) (82.6% vs 19%; $p < 0.001$) and allowed discrimination between progr-T1D and NP-AAB+ children with 74% sensitivity and 91% specificity. None of the children non progressing to T1D negative for standard diabetes antibody marker NP-AAB- children were positive for oxPTM-INS-Ab.

The comparison of plasma and serum from the same patients has confirmed all the previous data. In addition, we have shown that the correlation between plasma and serum analysis is very high.

2 - Mass spectrometry analysis confirmed previous data and has identified more amino acids modifications: cysteine oxidation and amino acid conversion.

Comparing 2D gel-western blot analysis of insulin and oxPTM-INS performed using the most reactive serum samples from T1D patients was leading to the identifications of some interesting protein spots.

3 - In cohort 3 a stronger binding to oxPTM-CII was observed in serum samples from axSpA (74%) compare to PsA (33%), UA (35%) and FM (16%), ($p<0.0001$). Interestingly, binding of axSpA samples was similar to binding of serum samples

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from ERA (91.7%). Binding to ROS-CII was directed to a range of ROS-CII fragments between 25 and 150 kDa.

In cohort 4 binding of AS serum samples to oxPTM-CII was significantly higher than binding to native CII ($p<0.0001$) with a percentage of autoreactivity of 30% to CII and 67.5% to at least one oxPTM-CII.

4 - Comparing 2D gel-western blot analysis of CII and oxPTM-CII performed using the most reactive serum samples from ERA patients was leading to the identifications of some interesting protein spots.

Conclusions: For the first time I have demonstrated reactivity to oxPTM-CII in axSpA patients and these data may support a further understanding on axSpA pathogenesis. Moreover, oxPTM-CII might mark diseased-tissue specific targets.

I have shown that oxPTM-INS auto-reactivity is present before the diagnosis of T1D in over 90% of cases, for this reason oxPTM-INS-Ab may become a biomarker to predict children progressing to T1D and for early diagnosis of T1D.

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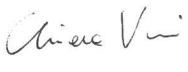

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ABBREVIATIONS

2D two dimension

2D GEL two dimensional gel electrophoresis

ABIS All Babies in Southeast Sweden

AGEs advanced glycation end products

AS ankylosing spondylitis

ASAS Assessment of SpondyloArthritis international Society

axSpA axial spondyloarthritis

BASDAI Bath Ankylosing Spondylitis Disease Activity Index

BIO treatment with biologics

BSA bovine serum albumin

CASPAR classification for psoriatic arthritis

CCP cyclic citrullinated peptide

CD Crohn's disease

CII type II collagen

CRP C-reactive protein

dH_2O distilled water

DMARD disease-modifying anti-rheumatic drugs

e- electron

ELISA enzyme-linked immunosorbent assay

ERA early rheumatoid arthritis

ESR erythrocyte sedimentation rate

Fe3+ iron cation

GABA gamma-aminobutyric acid

GAD glutamic acid decarboxylase

GLYC CII glycated type II collagen

GLYC INS glycated insulin

GWAS genome-wide association study

H+ hydrogen cation

H_2O_2 hydrogen peroxide

HSA human serum albumin

HC healthy control

HEL Hen Egg Lysozyme

HLA human leukocyte antigen

HOCl hypochlorous acid

HOCl CII chlorinated type II collagen

HOCl INS chlorinated insulin

HRP horseradish peroxidase

IA inflammatory arthritis

IA-2A tyrosine phosphatase IA-2 antibodies

IAA anti insulin autoantibodies

IBD inflammatory bowel disease

ICA Islet Cell Autoantibodies

IDF International Diabetes Federation

IL interleukin

IPTG isopropyl β-D-thiogalactoside

LADA latent autoimmune Diabetes in Adult

MHC major histocompatibility complex

MMP matrix metalloproteinases

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MS multiple sclerosis

NAC N-acetyl-cysteine

NIL non treated

NO nitric oxide

NOD non-obese diabetic mouse

NP-AAB$^-$ children antibodies negative non progressing to diabetes.

NP-AAB$^+$ children antibodies positive non progressing to diabetes.

Nr-axSpA non radiographic axial spondyloarthritis

NSAID non-steroidal anti-inflammatory drugs

O_2 oxygen

OD optical density

OH hydroxide

OH INS oxidised insulin

oxPTM oxidative post-translational modification

OxPTM-CII oxidative post-translational modified type II collagen

OxPTM-CII-Ab oxidative post-translational modified type II collagen antibodies

OxPTM-INS oxidative post-translational modified insulin

OxPTM-INS-Ab oxidative post-translational modified insulin antibodies

PAGE polyacrylamide gel electrophoresis

PBS Phosphate-buffered saline

PROGR-T1D children progressing to type 1 diabetes

PS psoriasis

PsA psoriatic arthritis

PTM post-translational modification

RF rheumatoid factor

ROS reactive oxygen species

scFv single chain fragment variable

SDS sodium dodecyl sulfate

-SH thiol group

SLE systemic lupus eritematous

SNPs small nucleic polymorphisms

SOD superoxide dismutase

SpA spondyloarthritis

-S-S- disulphide bridge

T1D type 1 diabetes

T2D type 2 diabetes

TEMED Tetramethylethylenediamine

TEDDY the environmental determinants of diabetes young

TMB 3, 3', 5, 5'-tetramethylbenzidine

TNF tumor necrosis factor

UA undifferentiated arthritis

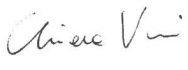
UC ulcerative colitis

VH heavy chain

VL light chain

WHO world health organization

ZnT8A zinc transporter 8 antibodies



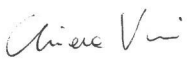
CHAPTER 1

GENERAL INTRODUCTION AND HYPOTHESIS

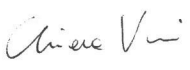
1.1 Background

Autoimmunity is defined as the set of reactions that lead to an immune response against its own organism, which is due to a breakdown of mechanisms responsible for self-tolerance. Self-tolerance is pursued in two main forms: central and peripheral tolerance. Central tolerance is established during the development and maturation of immune cells in the primary lymphoid organs. B cells in the bone marrow and T cells in the thymus are screened for their ability to discriminate between self and non-self through a receptor-antigen recognition system[1]. In certain conditions immune tolerance towards self-components dramatically fails causing aberrant immune responses, collectively referred to as autoimmunity. Discrimination between self and non-self recognition is fundamental. This is partly accomplished by the major histocompatibility complex (MHC), which can be either class I or class II. If an anomaly is detected in presentation by MHC, death of the defective cell is programmed by apoptosis. The epitope-MHC complex is presented to CD4+ T cells that then activate B cells, which will then start to produce specific antibodies. B cells can also recognize antigens by themselves as the B cell receptors expressed are antigen-specific leading to the production of specific antibodies. Thus, self and non-self recognition is strongly dependent on the presentation of epitopes to T lymphocytes by MHC molecules or direct recognition by B lymphocytes. The action of autoantibodies and self-reactive T cells against self-components causes a state of chronic inflammation and permanent damage that are features of several autoimmune diseases[2]. Under normal circumstances, the self and non-self are well differentiated. A balance between immune response and regulatory components of the immune system allows the whole mechanism to be kept under control. An autoimmune disease is characterized by loss of the tolerance to self and an autoreactivity against self-

antigens. These attacks of the immune system can be directed against a particular type of cells, or instead can be more systemic and widespread and involving different elements such as genetics, hormones, diet, chemical and/or exposure to infections that may influence the immune response and potentially lead to autoimmune diseases[3]. Three pathways for immune tolerance breakdown are outlined in the following sections: by loss of the immune system regulation, by cross reactivity of a self-protein with microbial specific antibodies (molecular mimicry), and by chemical modification of self-proteins (aberrant post-translational modifications). T regulatory cells (formerly known as T suppressor cells) play a crucial role in the control of this balance, by being responsible for the elimination of autoreactive T cells. They downregulate or suppress the reactivity of the CD4+ and CD8+ cells towards self- components of the organism. If, for any reason, the balance between effector T cells (helpers and cytotoxic) and regulatory T cells is disrupted, then the immune system downregulation is lost, and an immune process can be initiated[1]. Due to the wide diversity of molecular structures, it is possible to have exogenous pathogens that have similar structure and/or conformation to mammalian surface glycoproteins and/or glycolipids. A possible outcome is the cross-reactivity of the self-molecule and the exogenous antigen, leading to activation of autoreactive lymphocytes[1]. Post-translational modifications (PTM) are chemical modifications of proteins, after their synthesis or during their life cycle. PTM are diverse and abundant, and most proteins undergo proteolytic processing and/or the attachment (or removal) of functional groups to control protein functions, structure and localization. Common examples of PTM include glycosylation, oxidation, phosphorylation, methylation, and amino acid conversions[4]. Most PTM are the result of controlled enzymatic action, but modifications may also be introduced because of aging or exposure of proteins to chemically reactive environments. Such alterations in amino acid structures or sequences can generate neoepitopes from self-proteins, and thus result in the loss of immune recognition[5].



Autoimmune diseases affect around 3-8% of the world's population. The female/male ratio shows that women have a higher risk of autoimmunity[6]. Autoimmune diseases can be defined as either localized or systemic. Multiple sclerosis (MS) and type 1 diabetes (T1D) are, for example, classified as localized autoimmune diseases since they are restricted to a specific tissue or organ[7, 8]. Conversely, diseases like systemic lupus erythematous (SLE) and rheumatoid arthritis (RA) are classified as systemic diseases because the autoantibodies present in these diseases can cause multiple organ impairment[9,10]. Sometimes these diseases can be silent for a long period of time before presenting general and/or specific symptoms. The manifestation of two or more autoimmune diseases in the same patient occurs with a rate greater than expected by chance. The coexistence of several autoimmune diseases in the same patient (polyautoimmunity) is referred to as a kaleidoscope of autoimmunity[11]. Polyautoimmunity suggests the existence of shared pathological mechanisms among autoimmune diseases and the possibility to have a single therapeutic approach for at least those conditions that are strongly associated with each other. The therapeutic treatments of autoimmune diseases are often primarily directed at relieving the symptoms. Other approaches aim to help patients by administering either lacking vital products or nutrients that have been affected by the impairment of target organs. Two examples of these kind of approaches are insulin in the case of T1D[12] or vitamin B12 for SLE[10]. Anti-inflammatory drugs such as aspirin or glucocorticoids are used to reduce inflammation and pain associated with autoimmunity. However, these therapeutic approaches address only the symptoms instead of addressing the underlying cause/causes of the disease. Biologics are a class of drugs that includes a wide range of products, they are isolated from a variety of sources such as human, animal, or microorganism and may be produced by biotechnology methods and other cutting-edge technologies. Among them there are monoclonal antibodies, which have achieved resounding success in the treatment of RA and MS[13,14]. Despite biologics being known for their therapeutic effects in autoimmune diseases, their mechanisms of action remain



largely elusive. Treatments using biologics confer several advantages including high tolerability by the patients and greater target selectivity[14]. However, they are rarely curative and are unfortunately accompanied by serious side effects[14]. Biomolecules present in the body are exposed to post-translational modifications that can affect their function and stability. Reactive oxidants physiologically play an important role in human health, but under inflammatory conditions their levels are abnormally high. Studies have shown that reactive oxygen (ROS) released during chronic inflammation can change structure and shape of proteins[15]. Generation of neo-antigenic proteins by ROS may contribute to epitope spreading in autoimmunity[5]. These neoepitopes could stimulate autoimmunity and lead to the production of auto-antibodies to proteins oxidative post translationally modified (oxPTM) by ROS[16].

1.2 Reactive oxygen species (ROS)

A free radical is an element that has unpaired valence electrons[17]. The presence of the unpaired electrons causes the free radicals to react with other substances, or themselves with high affinity; molecules with free radicals often dimerize or polymerize due to their poor stability. ROS is a term used to collectively describe both reactive molecules and free radicals with unpaired valence electrons that are derived from molecular oxygen (figure 1). They have been well-documented to be a component of the killing response of immune cells to microbial invasion. Recent evidence has shown that ROS play a key role as a messenger in normal cell signal transduction and cell cycling[18]. ROS are physiological molecules generated by the metabolism of oxygen and recent works have described their role in activation of cell signalling cascades, apoptosis and gene expression[18]. It is important to note that ROS can serve as both intra and intercellular messengers. The production of ROS occurs during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalysed oxidation. The most important intermediate for the formation of ROS in biological system is the oxygen, which, although essential for the life of aerobic organisms, has the potential to become

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toxic in high concentrations. It is not toxic by itself, but it is as a result of the reactive intermediates of reduction.

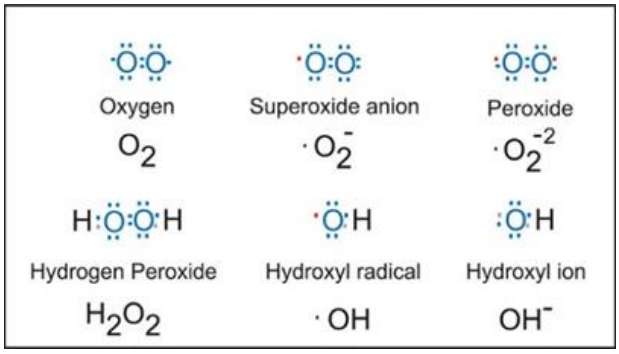

Figure 1. ROS electron structures, names and chemical formulas. Each ROS is represented by the chemical formula, the unpaired electrons are in red [Adapted by Kim et al. Experimental Neurobiology].

Superoxide anion

Starting from molecular oxygen (O_2), and adding an electron (e-) in the outer orbital generates the first product of the reduction: the superoxide anion. This highly toxic species is produced during oxidative phosphorylation, by enzymes and leukocytes. The toxicity is due to its ability to generate the hydroxyl radical (•OH) reacting with ions of transition metals through Fenton reaction, reduction reaction of the iron cations (Fe^{3+}) and Haber-Weiss reaction. For this reason, aerobic organisms have developed isoforms of the enzyme, Superoxide Dismutase (SOD), in humans the isoform SOD1 is soluble and cytoplasmic, the isoform SOD2 is mitochondrial and the isoform SOD3 is extracellular. SOD is a very efficient enzyme, it combines O_2-• with 2 hydrogen cations (H^+) to obtain hydrogen peroxide (H_2O_2) and O_2 and it is an important antioxidant defence in nearly all living cells exposed to oxygen[19, 20].

Hydrogen peroxide

Hydrogen peroxide is often produced by glutathione peroxidase, or by oxidases contained in peroxisomes. It is metabolized by:

-catalase (peroxisomes) in H_2O and O_2;

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-glutathione peroxidase (cytosol and mitochondria).

H_2O_2 could also interact with chloride ions present in plasma leading to the formation of hypochlorite (HOCl), a potent antimicrobial agent generated by the enzyme myeloperoxidase[19, 20].

Hydroxyl radical

Hydroxyl radical is usually produced during water hydrolysis by radiation, or by Fenton reaction. It has a very short half-life (~ 1 nanosecond) and it reacts at the site of generation, in any cellular component. It is considered to be the most toxic and reactive free radical because it can cause damage to the plasma membrane, proteins and nucleic acids. However, it can be inactivated upon conversion to H_2O by glutathione peroxidase[19].

The unpaired electron of ROS likes to find a partner electron by stealing it from carbohydrates, enzymes, proteins, cell membranes and DNA to achieve stability compromising their function. ROS may also react with other molecules, transforming their targets in free radical chain reactions causing extensive damages in the cell, and consequently degenerative diseases and tumours[19, 21].

Table 1. ROS target and caused damages[18].

TARGET	DAMAGES
Lipids	Lipid peroxidation with formation of lipid radical intermediates, and toxic compounds. Damage to cell membranes.
Lipoproteins	Lipid oxidation (cholesterol and unsaturated fatty acids). Oxidation of apoproteic component.
Proteins	Oxidation of SH-groups. Destruction of coenzymes. Formation of covalent bonds with other molecules.
Amino acids	Degradation of easily oxidizable amino acid (histidine, tryptophan, lysine, etc.).
Carbohydrates	Direct oxidation and polymerization. Maillard reaction.
DNA, RNA	Hydroxylation bases with possible mutagenesis.

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Chronic inflammation can consume increased amounts of oxygen, resulting in the generation of reactive oxidants such as O_2-•, hydrogen peroxide H_2O_2, hydroxyl radical •OH, HOCl and nitric oxide (NO). These ROS are involved in acute and chronic inflammation. Oxidative stress is also associated with sequential oxidative reactions, which generate advanced glycation end products (AGEs) that have damaging effects on proteins[22] (figure 2).

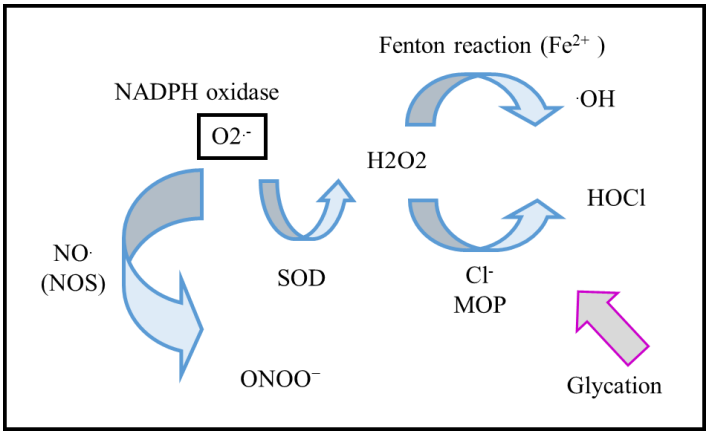

Figure 2. Generation of ROS. Activation of O_2 occurs by two different mechanisms. Reduction of O_2 leads to formation of O_2^-, H_2O_2, and •OH. O_2^- is easily dismutate to H_2O_2 by superoxide dismutase (SOD) or react with nitric oxide (NO^-) generating peroxynitrate ($ONOO^-$). The H_2O_2 can either go through Fenton reaction leading to the formation of •OH and react with a chloride anion (Cl^-) to get HOCl, the reaction is catalysed by myeloperoxidase (MOP) [From Nissim A, Lectures figures].

1.2.1 Oxidative post-translational modifications (oxPTM)

Changes induced by ROS may lead to cellular damage, alteration of cell signalling and affected gene regulation and expression. They also participate in controlling the cellular cycle, apoptosis and immune responses[23]. Structural changes including cleavage, aggregation or abnormal cross-links induced by ROS result in loss of functional activity[16]. Reactive oxygen can modify proteins by generating a range of reactive products. The main target for oxidative modification are cysteine residues since the free thiol group (–SH) can undergo oxidation to disulphide bond

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with another free thiol (–S-S–). In presence of •OH, cysteine may also be oxidized to cysteine sulfenic, sulfinic and sulfonic acid products[24, 25]. Other amino acids are a known target of oxidative modifications. For example, methionine may be oxidised to methionine sulfoxide and further to methionine sulfone[26] while tryptophan can be oxidized to hydroxytryptophan and further to kynurenine[26, 27]. Other oxidative modifications include tyrosine oxidation to 3, 4-tyrosyl radicals[26].

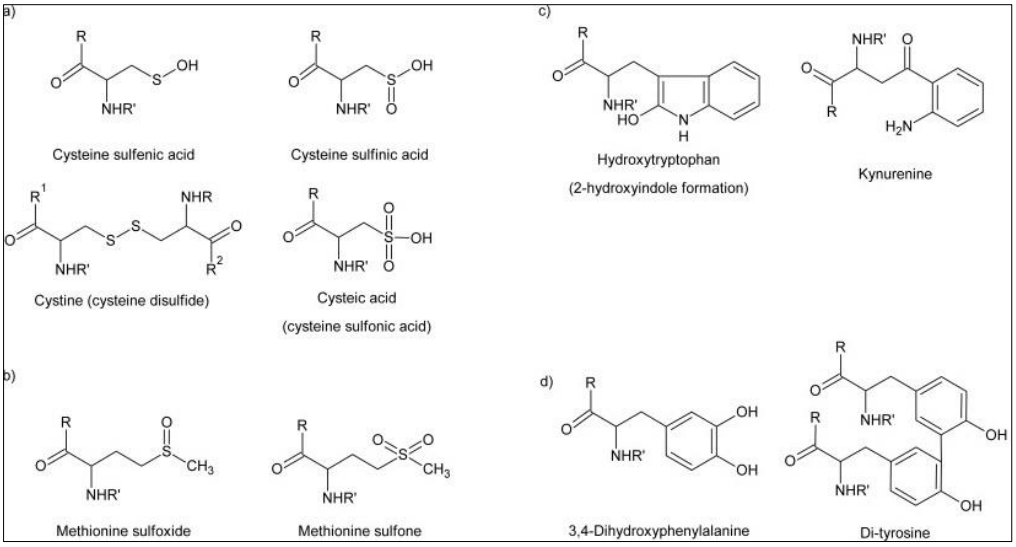

Figure 3. Structures of amino acids after post-translational modifications by reactive oxygen, nitrogen and chlorine species. Structures of some of the post-translationally modified forms of (a) cysteine residues, (b) methionine residues, (c) tryptophan residues and (d) tyrosine residues. [Adapted by Ryan et al. Redox Biology]

1.2.2 Glucose oxidation

All blood sugars are reducing molecules. Glycation is a non-enzymatic reaction that results in the covalent binding between proteins and sugar molecules. Increased oxidative stress and other metabolic stresses such as hyperglycaemia may induce the formation of AGEs through a multi-step process known as Amadori reactions, Schiff base reactions, and Maillard reactions[16]. Some of these molecules are stable, but others AGEs are very reactive, and are therefore implicated in many chronic diseases such as cancer and other diseases such as dissociated sensory loss[28, 29]. These diseases are the result of the interference of glycation with

molecular and cellular function and interference of highly oxidizing side-products such as hydrogen peroxide with physiological molecules. Long-lived cells, long-lasting proteins and DNA may accumulate damage over time[30].

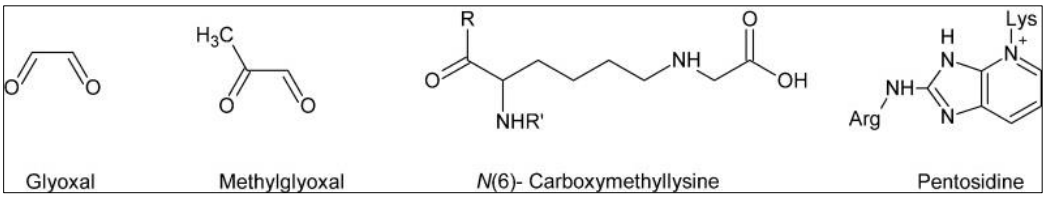

Figure 4. Structures of the products of glucose oxidation. Glyoxal and methylglyoxal, and the AGEs carboxymethyllysine and pentosidine [Adapted by Ryan et al. Redox Biology].

HYPOTHESIS AND AIMS

This study is based on the hypothesis that ROS released during chronic inflammation are able to post-translationally modify proteins. This may result in conformational changes of native proteins, leading to the formation of neoepitopes. These newly formed neoepitopes are not recognised as "self" by the immune system, causing an immune response.

My hypothesis is that these oxPTM play a key role in autoimmune response and influence the diseases pathobiology.

To establish this hypothesis, I have analysed a known inflammatory autoimmune disease, T1D, and I have also studied the involvement of oxPTM in other inflammatory arthritic diseases. These diseases were chosen as they will allow for a broad study on the effect of oxPTM in stimulating immune response.

This PhD project has therefore been divided into two main topics:

1. T1D

My group has previously reported the presence of insulin oxidized by ROS (oxPTM-INS) in T1D patients. Hence, we previously showed that antibodies to oxPTM-INS (oxPTM-INS-Ab) were detected in T1D diagnosed patients.

The aim of my study has been to confirm the presence of oxPTM-INS-Ab and to evaluate their predictive potential in T1D children before the diagnosis.

2. SPONDYLOARTHRITIS (SpA)

It has been previously reported in several studies an autoimmune reactivity against ROS modified type II collagen (oxPTM-CII) in rheumatoid arthritis patients. My group has already established that rheumatoid arthritis patients generate antibodies against oxPTM type II collagen (oxPTM-CII-Ab).

The aim of my study was to investigate the involvement oxPTM-CII-Ab in SpA pathogenesis.

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Part of the project was also dedicate to identify and map the neoepitopes responsible for the autoreactivity within the protein sequences, in the two studies described, oxPTM-INS and oxPTM-CII.

Despite similarities between the two subjects and that many experiments were performed in parallel, the two autoimmune disease studies have been described in two different chapters to allow the details of the methods and the relevance of the results to be described with more clarity.

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CHAPTER 2

TYPE 1 DIABETES: INTRODUCTION

2.1 Background diabetes

Diabetes is a metabolic condition caused by the lack of insulin, resistance to its effects, or both[31]. Patients universally experience hyperglycaemia as a result of the inability to maintain normal blood glucose levels through homeostatic mechanisms. Hyperglycaemia is the medical term describing an abnormally high blood sugar level. Blood sugar levels can be measured in a laboratory either alone or with other blood tests, or it can be measured using a handheld glucometer, a small device that allows frequent monitoring of blood glucose levels without the need for a doctor's office or laboratory. Hyperglycaemia is a hallmark sign of diabetes in type 1 and type 2 diabetes, as well as in prediabetes. Hyperglycaemia has been defined by the World Health Organisation as:

- Blood glucose levels greater than 7.0 mmol/L (126 mg/dl) when fasting;
- Blood glucose levels greater than 11.0 mmol/L (200 mg/dl) 2 hours after meals.

The World Health Organization estimates that high blood glucose is the third highest risk factor for premature mortality, after high blood pressure and tobacco use[32]. Life expectancy is reduced, on average, by more than 20 years in people with diabetes[33]. There are considerable financial costs associated with diabetes. It is currently estimated that about 673 - 1,197 billions of dollars is spent on diabetes and its complications worldwide[34]. In addition, 1 in 10 people admitted to hospital are diabetic. As for other chronic conditions, the impact of diabetes on individuals and society cannot be underestimated. It affects one in eleven adults and a total of 415 millions of people worldwide[34] and this figure is predicted to continue to rise in the coming decades. The World Health Organisation (WHO) and the International Diabetes Federation (IDF) predict that by 2030 between 366 and 438 million individuals will be suffering from diabetes. There are several kinds of

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diabetes and based on the aetiology, they can be classified as shown in the table below. Using the current system of diagnosis and follow up procedures, assigning one type of diabetes to a patient may be difficult as it mainly depends on the symptoms present at the time of diagnosis and therefore many diabetic patients cannot be defined by a single class.

Table 2. Classification of diabetes

TYPE 1 DIABETES: it is caused by an autoimmune response and idiopathic β cell disruption and it is characterized by a full lack of insulin. Patients with T1D are the 10% of the total diabetic patients and are mainly children. The Latent Autoimmune Diabetes in Adults (LADA) is an adult form of T1D with a slow progression[31].
TYPE 2 DIABETES: it is the most common form, 90% of diabetic patients have T2D. T2D patients are mainly over 40 and overweight. It is characterized by insulin deficit and/or insulin resistance. In both case the consequence is hyperglycaemia[35].
GESTATIONAL DIABETES: it is similar to the type 2 diabetes and the diagnosis and progression of the disease is related to the pregnancy. Of pregnant women around 8% are affected[36].
DIABETES INSIPIDUS: it is a rare disease characterized by high diuresis due to an abnormal work of the vasopressin hormone and not related to insulin problems[37].
OTHERS: diabetes induced by drugs, chemicals, infections, diseases related to the exocrine pancreas and endocrinopathies.

T1D= type 1 diabetes, LADA=latent autoimmune diabetes in adult, T2D=type 2 diabetes.

2.2 Type 1 diabetes (T1D)

Type 1 diabetes is a chronic autoimmune inflammatory disease in which environmental factors and genetic predisposition amalgamate to induce

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pancreatic β cells disruption, consequently causing to functional failure of β cells and leading to the aberrant control of blood glucose. This causes long term complications, even under insulin therapy. T1D is less common than type 2 but it is still increasing by around 3% per year, particularly among children. Around 86,000 children develop T1D every year. In 2015 the number of children with type 1 diabetes exceeded 0.5 million. The incidence of T1D varies significantly between countries and racial groups, with countries such as Venezuela and China having an incidence of less than 0.1 children diagnosed per 100,000 per year whilst Northern European countries have a much higher incidence. Europe has the highest number of children with T1D, approximately 140,000[34]. B cell destruction results in an absolute deficiency in endogenous insulin production[31]. Specific loss of the β cells occurs when the islets of Langerhans are infiltrated with dendritic cells, macrophages and $CD4^+$ and $CD8^+$ T lymphocytes[38]. The autoimmune nature of β cell destruction is augmented by the presence of a pool of antibodies against them[12]. Most cases of T1D are characterised by the presence of one or more autoantibodies to islet cells (ICA), insulin (IAA), glutamic acid decarboxylase (GADA) or to the tyrosine phosphatase IA-2A and the zinc-transporter 8 (ZnT8A). About 90% of individuals have one or more of these autoantibodies present at diagnosis and these are present in the blood before acute symptoms appear[31, 38]. The cause of T1D is still unknown, however it is believed to involve a combination of genetic and environmental factors. Despite the underlying mechanism of β cells in the pancreas being caused by autoimmunity other causes may be genetic susceptibility, diabetogenic trigger and exposure to antigens[39, 40].

Genetic predisposition to T1D

T1D is associated with more than 50 genes[41] and about half of these genes are variants in the human leukocyte antigen (HLA) region[42]. The HLA region is situated on chromosome 6p21 containing up to 250 genes[43] (figure 5).

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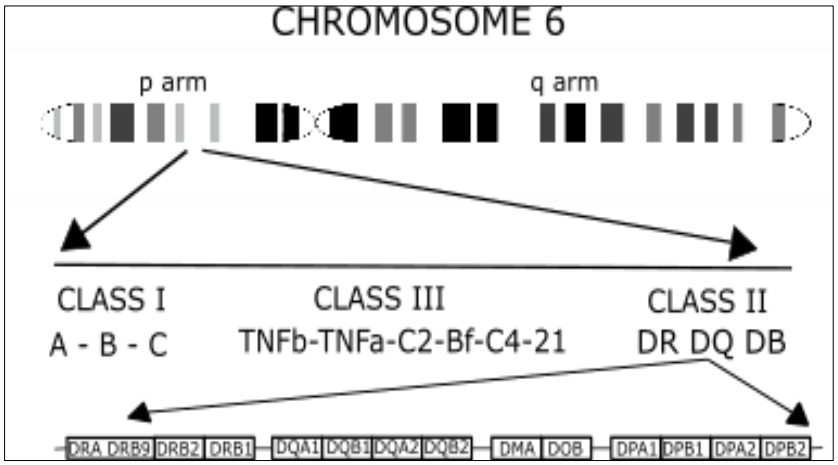

Figure 5. The HLA region on chromosome 6. The HLA associated with T1D are DRB1*03 – DRB1*02 and DRB1*04 – DQB1*0302 [adapted from Mehers and Gillespie 2008].

Variants in HLA class I genes may affect the age of the T1D onset as opposed to the risk of developing the disease. However, it should be noted that some of the HLA class I variants may also correlate with the risk of T1D development[44]. Molecules of HLA class II are heterodimers coded by genes at the HLA-DR, HLA-DQ and HLA-DP loci. Combinations of variants in these loci can influence the risk of the appearance of Islet cell autoantibodies (ICA) increasing/deceasing the risk of developing T1D. It is known that HLA-DR, HLA-DQ and HLA-DP correlate to the four ICA at the onset time[45, 46].

Table 3. HLA genetics association of T1D. [Adapted from F. Pociot and M. F. McDermott]

Association with susceptibility to T1D	
HLA-DQ alleles	**HLA-DR alleles**
A1* 0301 – B1*0201	DRB1*04
A1* 0301 – B1*0302	DRB1*04/ DRB1*801
A1* 0301 – B1*0303	DRB1*301
A1* 0301 – B1*0401	DRB1*301/ DRB1*04
A1* 0301 – B1*0402	DRB1*701
A1* 0501 – B1*0201	DRB1*901
A1* 0501 – B1*0302	

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Association with protection from T1D	
HLA-DQ alleles	**HLA-DR alleles**
A1* 0102 – B1*0602	DRB1*04
A1* 0103 – B1*0603	DRB1*1101
A1* 0301 – B1*0301	DRB1*1301
A1* 0501 – B1*0301	DRB1*1501

Several important proteins to the immune system are coded by the HLA class III[43]. It may be that some small nucleic polymorphisms (SNPs) in the HLA III are associated with the development of the ICA as shown in The Environmental Determinants of Diabetes Young (TEDDY)[47]. There are also several genes, not related with HLA, which appear to strongly influence the development of T1D. The most important of these genes are: PPI that encodes for preproinsulin; PTPN22 that encodes for a tyrosine phosphatase involved in T cell receptor signalling; IL2RA that encodes the α subunit of the interleukin 2 receptor on lymphocytes[48]. Genetic factors undoubtedly play an important role in developing diabetes. The risk of developing T1D is about 5% if the father has it, about 8% if a sibling has it, and about 3% if the mother has it[42].

Environmental factors in T1D

If one identical twin is affected by T1D there is approximately a 50% chance the other will also be affected[49]. This suggests that despite each twin processing exactly the same genes, there are other factors that may influence both the first appearance of autoantibodies and the progression of the disease. One possibility is that T1D is a viral-triggered autoimmune response[50, 51]. Some of the viruses studied in relation with T1D are enterovirus, rubella virus and coxsackie virus during pregnancy[40, 52,53]. Some dietary components, such as cow's milk, have been associated with the onset and progression of T1D[54]. Factors that increase the blood sugar, such as some types of food, alcohol and stress, may accelerate the disease progression[55] whereas vitamin D seems to have a protective effect from T1D and other autoimmune diseases[50]. By limiting the use of certain chemicals and

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drugs that are toxic to β cells can help lower the risk. However, unfortunate events that can also lead to loss of insulin production include trauma, pancreatitis or tumours. Even if the cause of T1D is unknown, the immune-mediated destruction of insulin-secreting cells of the pancreas and the autoimmune component have been shown to have a fundamental role.

2.2.1 Autoantibodies in T1D

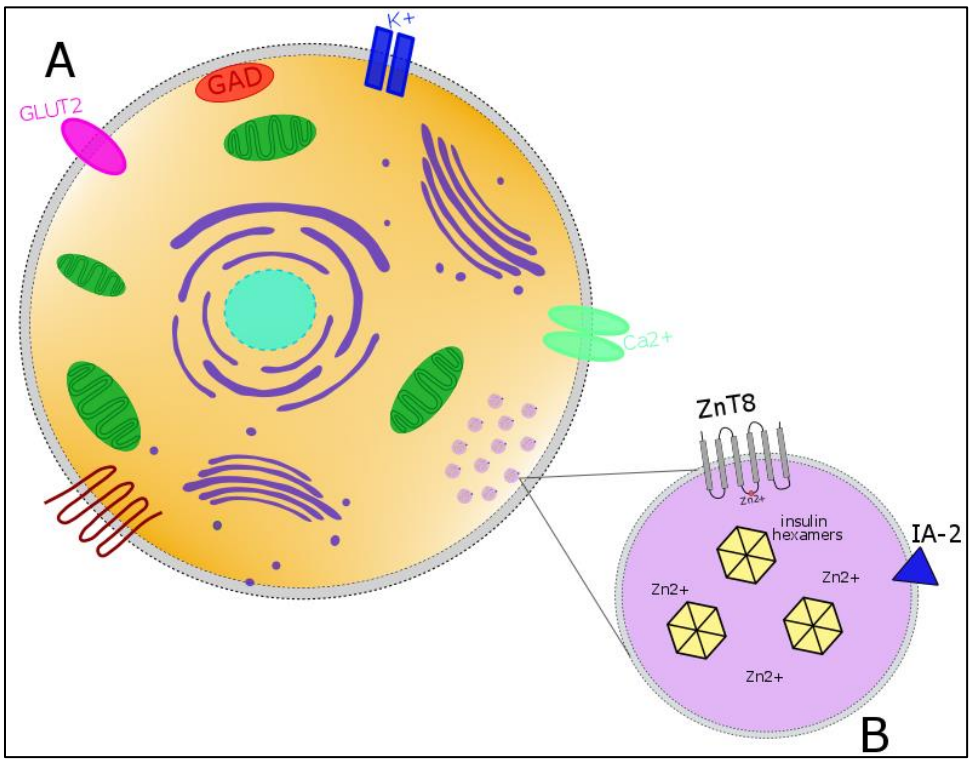

Figure 6. β cell, insulin granule and T1D-related autoantibodies. Figure A shows a β cell with the classic cellular organelles. K+ is a potassium channel; Ca+ is a voltage gated calcium channel; GLUT2 is the glucose transporter 2; GAD is the glutamic acid decarboxylase. Figure B shows an insulin granule containing insulin hexamers and zinc ions (Zn2+); ZnT8 is the zinc transporter 8; IA-2 is the tyrosine phosphatase IA-2.

Islet Cell Autoantibodies (ICA)

The first evidence to suggest the presence of autoantibodies in T1D patients was discovered by the association between the development of T1D and the identification of cytoplasmic ICA, which were identified on the islet cells in the

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human pancreas[56]. ICA are considered markers that appear when β cells are damaged and they can be detected before the complete destruction of β cells. These markers are used to estimate the risk of developing T1D, to discriminate and classify the type of diabetes and ultimately be used decide upon the best treatment for patients. The production of autoantibodies a long time before the development of T1D suggests that there is a big 'window of opportunity' to either delay or prevent T1D, if an effective treatment to slow the underlying autoimmune processes could be found[31]. The test for ICA was very useful for identifying individuals who are likely to develop diabetes and to identify the subgroup of patients that are more likely to quickly progress to insulin treatment. However, this has been largely superseded by tests for autoantibodies against specific proteins[57]. About 96% of T1D patients are positive for at least one of the following four autoantibodies: IAA, GADA, IA-2A & ZnT8. These are explained in more detail below[58].

Insulin AutoAntibodies (IAA)

Insulin autoantibodies are a good diagnostic marker as they have been shown to be correlated inversely with age of onset[59]. The tissue-specific expression of proinsulin in β cells is reflected in a specific targeting of T-cell mediated autoimmune response. IAA are present in approximately 70% of young patients at the diagnosis of T1D and are also found in the non-obese diabetic (NOD) mouse, these together with the early appearance of IAAs in patients support the idea that the disease might be driven by a loss of tolerance toward insulin. The presence of these autoantibodies was first shown in patients treated with exogenous insulin, suggesting that insulin preparations purified from other species were able to trigger insulin autoimmune reactions[60]. In 1983, the presence of IAA was shown in patients with new-onset T1D before administration of exogenous insulin therapies[61]. IAA are the first marker of β cell autoimmunity shown in young individuals affected by T1D and they may appear as early as 6 months of age in children with a high genetic risk for T1D. The highest rate of IAA is between the

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first and second year of age as demonstrated from data about the BABYDIAB and DIPP birth cohorts[62, 63]. It has been shown that both prevalence and levels of IAA are strongly associated with HLA-DRB1*04 in patients and their relatives, an association has also been shown for HLA-DQA1 alleles, with DQA1-*0101, *0102, *0103, *0201, or *0301[46].

Glutamic Acid Decarboxylase Antibodies (GADA)

The glutamic acid decarboxylase (GAD) is an enzyme that catalyses the decarboxylation of glutamate to gamma-aminobutyric acid (GABA) within the nervous system and pancreatic cells. The main isoforms present in humans are GAD1, expressed in the brain and pancreas, and GAD2, expressed only in the pancreatic alpha and β cells[59]. Antibodies to GAD were first shown in subjects with a neurological disorder called the Stiff-person Syndrome and then were subsequently found in patients with new-onset T1D[64]. These autoantibodies are present in almost 75% percent of T1D patients at diagnosis and represent one of the main markers in adult T1D individuals[59] .

Tyrisine phosphatase IA-2 Antibodies (IA-2A)

Another variety of autoantibody detected in patients affected by T1D are IA-2A. These antibodies are less common compared with GADA and IAA but are thought to be associated with the development of the disease and associated with a quicker progression. Tyrosine phosphatase is expressed in neuroendocrine cells such as those in the pancreatic islets and it is bound to the membrane of the insulin granules[59]. The incidence of these antibodies is between 60% and 80% in early T1D but only 45% in individuals developing the disease after the age of 20[65].

Zinc Transporter-8 Antibodies (ZnT8A)

The ZnT8 autoantibodies were discovered more recently in comparison to the other autoantibodies described[66]. ZnT8 is a zinc transporter anchored to the membrane of insulin granules modulating the zinc flux and then the formation of

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insulin complexes in storage crystals. Reactivity to ZnT8 is evident in 70% of T1D patients. Polymorphisms involving the ZnT8-encoding gene SLC30A8 may influence diabetes risk with the presence of a homozygous SNP at position 325 for either arginine or tryptophan leading to the greatest risk of disease progression[67].

Disruption of β cells and their consequent loss of function seems to start after the onset of autoimmunity[68]. The loss of β cells leads to fluctuations in blood glucose levels which explains why, even if there are still β cells present at onset of T1D, these would not be enough to prevent hyperglycaemia. It is estimated that the initial clinical symptoms of T1D can occur with loss of 40% of β cells[69]. It seems insulin therapy can induce a partial and temporary remission in around 80% of young T1D patients[70]. Despite this there are studies demonstrating the failure of anti-hyperglycaemic therapy remission[71].

2.3 Insulin

Insulin is the most specific antigen for pancreatic β-cells and a primary potential auto-antigen in type 1 diabetes[61]. Insulin is a small protein of 5.8 kDa discovered by James Macleod and Frederick Grant Bating in 1921. It is synthesized in the pancreas within the β-cells and consists of an α-chain (21 amino acids) and a β-chain (30 amino acids), linked by three disulfide bonds Cys6-Cys11 of α -chain, Cys7 of α-chain - Cys7 of β-chain, Cys20 of α-chain – Cys19 of β-chain. Insulin is an anabolic hormone responsible for the metabolism of carbohydrates, fats and protein by promoting the absorption of glucose from the blood into fat, liver and skeletal muscle cells[72].

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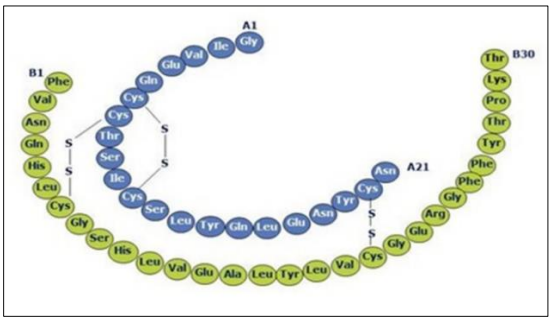

Figure 7. Insulin sequence and primary structure. A1-A21 is the α chain and B1-B30 is the β chain. The structure presents the amino acid sequence with the disulphide bridges cys6-cys11 in the α chain, cys20-cys19 and cys7-cys7 interchain.

In humans, insulin is synthesized as preproinsulin that contains, at its N -terminus, a signal sequence of 24 amino acids that ensure entry into the lumen of the endoplasmic reticulum. Once it reaches this site, the signal sequence is cleaved to obtain proinsulin. In the rough endoplasmic reticulum the proinsulin folds into the correct three-dimensional conformation and 3 disulphide bonds are formed[73,74]. The proinsulin is carried to the Golgi apparatus where it undergoes a new proteolytic degradation by endopeptidase to cut a sequence of 33 amino acid called the C peptide. With an increase of insulin concentration, monomers tend to aggregate into dimers first and then hexamers. Insulin is stored in storage granules until secretion into circulation where the hexamers dissociate into bioactive insulin monomers[73,74].

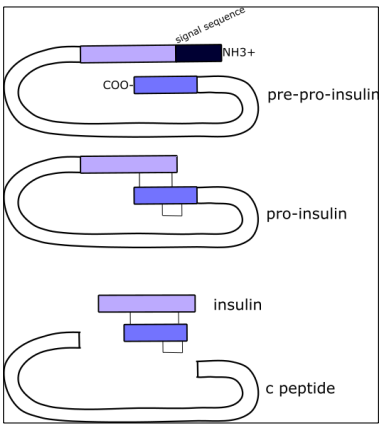

Figure 8. Insulin synthesis. Insulin is synthetized as pre-pro-insulin, the signal sequence is cleaved to obtain pro-insulin and, in the last step of the synthesis, the c peptide is degraded and the insulin is ready as monomer.

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2.3.1 oxPTM-INS and Type 1 diabetes

Currently the most widely accepted mechanism of tissue damage is that of the non-enzymatic glycation of macromolecules such as structural proteins. When high levels of glucose are present in the blood this reducing sugar is able to form irreversible cross links with proteins through non-enzymatic rearrangement with amino acids to form AGEs. Since the cross links are irreversible AGE accumulate in the affected tissue resulting in the loss of functionality. Furthermore, this also acts as a stimulus for pro-inflammatory cytokine release via interaction with the Receptor for Advanced Glycation End products (RAGE) which reside on surfaces of both immune and local tissue cells[75]. Studies have shown that the interaction between AGEs and AGE binding receptors (RAGE) results in activation of pro inflammatory pathways[76]. This chronic, low level inflammatory response to these altered proteins perpetuates the tissue damage through the activation of downstream pro-inflammatory molecules such as cytokines, chemokines and arachidonic acid metabolites. These in turn have the ability to cause tissue breakdown through the up-regulation of molecules such as matrix metalloproteinases (MMPs)[77] and osteolytic activators[78]. Activation of RAGE leads to induction of an oxidative process within the affected tissue, which could account for a continued increase in tissue damage through the direct production of free radicals. Free radicals cause tissue damage as their oxidative reactivity can potentially structurally modify proteins as previously described. Hyperglycaemia and the high production of AGE are two of the main causes of ROS production in T1D patients[79]. The main toxic effect of ROS is abnormal post-translational modifications of β cells self-antigens and thus the generation of neoantigens, which may contribute to the development of autoimmune responses[5]. It is already widely known that ROS play an important role in other autoimmune diseases such as RA[9, 80] and it has previously shown T1D patients present an increased level of autoantibodies to oxPTM-CII[81] and oxPTM-INS[82] in addition to the presence of autoantibodies against oxidized GAD[83]. Insulin is a small molecule with a short half-life (5-10 minutes), but nonetheless it can still undergo post-translational

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modifications. Glycation of insulin or proinsulin can occur at any point during synthesis and storage and glycated insulin is detected in murine models of type 2 diabetes[84]. The process of glycation can be easily induced *in vitro* by the exposure of islets or cells to high glucose concentration in tissue culture, and it is shown that three free amino groups are available in the insulin molecule for glucose binding[85]. The Fenton reaction and chlorination with HOCl can be used to generate hydroxyl radicals and chemical changes within the insulin structure, such as the formation of phenylalanine hydroxyl derivatives, chlorination of tyrosine residues, peroxidation products on amino acids side chains and increased concentration of free carbonyl groups. Oxidative post-translational modification of insulin has been shown using MALDI-TOF analysis, and subsequently a map of amino acid modifications in the structure has been drawn (figure 9)[82]. Mass spectrometry analysis mapped oxPTM to chlorination of Tyr16 and Tyr26; oxidation of His5, Cys7 and Phe24; and glycation of Lys29 and Phe1 in β-chain. Our observations correlate with previous studies, demonstrating that the glycation sites of insulin are at N-terminal Phe1 and Lys29[84, 85] of β-chain. Similarly, oxidation of insulin that is more centrally located in the sequence correlates with a previous report implicating Tyr16 and Tyr26, Phe24 and Cys19 as the preferred sites of modifications[85]. It is interesting to note that one of the observed modifications that involve chlorination of Tyr16 of β-chain (Tyr B16) is within the pre-described region 9–23 of β-chain (B: 9–23). This region is widely recognized as a dominant epitope in autoimmune diabetes[86] whereby Tyr16 plays a crucial role in immunoreactivity. Substitution of Tyr in position 16 to Ala (Tyr B16: Ala) has been shown to abrogate T cell reactivity[87, 88], and administration of a modified B: 9–23 (Tyr B16: Ala) peptide has been reported to suppress expression of IAA and prevent diabetes in the NOD mouse[89]. Conversely, it is possible that Tyr16 modification by HOCl might enhance immunogenicity of this insulin epitope.

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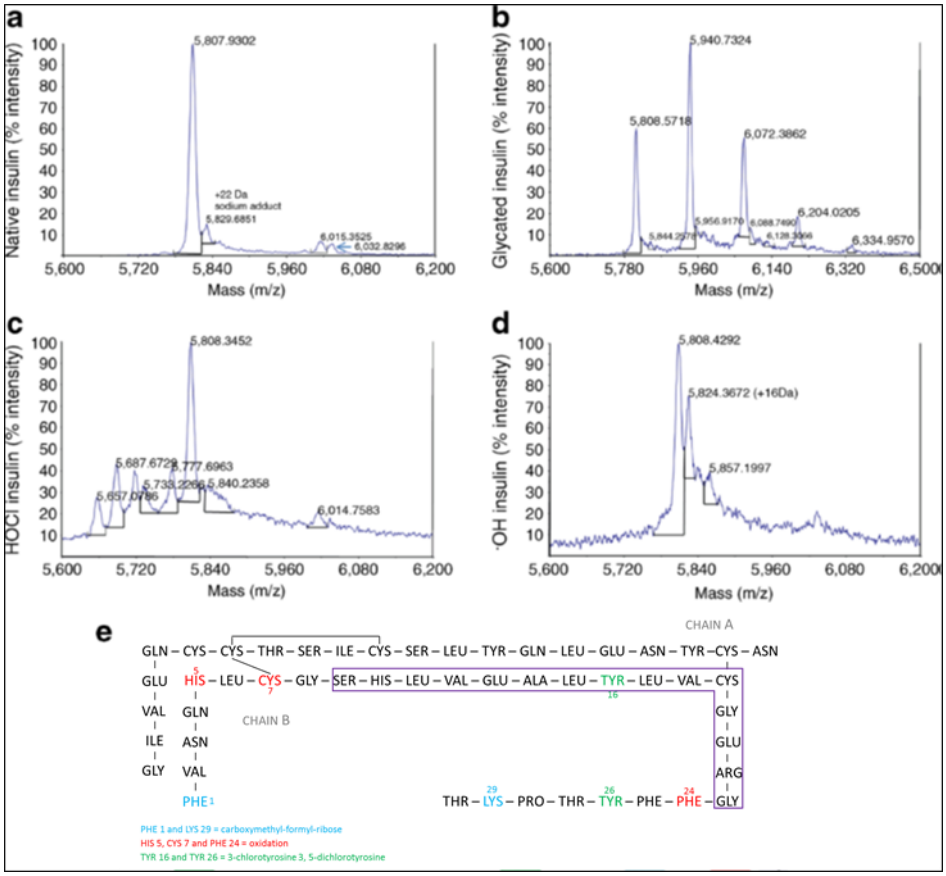

Figure 9. Modification of insulin structure by ROS. **(a–d)** Analysis of native insulin and oxPTM-INS by mass spectrometry. **(a)** Native insulin; **(b)** after glycation, four additional peaks corresponding to the mono-, di-, tri- and tetra-glycated insulin forms; **(c)** Exposure to HOCl, additional peaks at lower m/z indicating degradation products; **(d)** after •OH exposure, one additional pick corresponding to one oxidation. **(e)** Main oxidative changes induced by •OH and HOCl on insulin amino acids[82]. Carboxymethyl-formyl-ribose is indicated in blue, oxidation in red, 3-chlorination and 3, 5-dichlorination are in green.

OxPTM neo-antigenic epitopes are involved in insulin reactivity in the large majority of children diagnosed with type 1 diabetes. It has been previously demonstrated in my group, for the first time, that autoreactivity to oxPTM-INS in individuals with newly diagnosed T1D is significantly more prevalent than IAAs measured by the gold-standard radio binding assay (RBA). Altogether, oxPTM-INS-Ab and IAAs by RBA were detected in 95% of patients with newly diagnosed type 1 diabetes. This data indicate that insulin β-chain is the main site of oxPTM[82]. The

high prevalence of reactivity to oxPTM-INS indicates that the imbalance of the redox state takes place in a large proportion of patients with T1D.

2.4 Aim of T1D project

My group has previously shown that autoimmunity to insulin in T1D may result from neoepitopes induced by oxPTM and it has been reported that oxPTM-INS-Ab are present in about 85% newly diagnosed individuals with T1D and are more common than IAA.

The aim of this part of the study has been the evaluation of oxPTM-INS-Ab as biomarker for the diagnosis of T1D and their predictive potential in children before the diagnosis. Part of the project was also focused on the oxPTM-INS neoepitopes mapping.

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CHAPTER 3

TYPE 1 DIABETES: METHODS

3.1 Chemical modifications of Insulin.

In vitro chemical oxPTM were performed using ROS. For insulin modifications the protocol by Strollo et al[82] was followed. Following modification, protein was tested by native polyacrylamide gel electrophoresis (PAGE) to detect degradation through a concentration range, and enzyme-linked immunosorbent assay (ELISA) to check the binding to oxPTM proteins.

Human recombinant Insulin (I2643, SIGMA) in PBS (1mg/mL) was chemically modified using different concentration of the following systems:

1) Non-enzymatic glycosylation with 2M D-Ribose (R7500, SIGMA) at 37°C for three days (GLYC-INS);

2) 9mM H_2O_2 (10736291, Fisher scientific) and 4.5mM $CuCl_2$ (222011, SIGMA) at 37°C overnight. This reaction was used to produce hydroxyl radical (•OH) by the Fenton reaction (OH-INS);

3) 9mM sodium hypochlorite (230393, VWR) (HOCl-INS) at 37°C overnight.

The Hen Egg Lysozyme (HEL) (L6876, SIGMA) was selected as a control protein based on its small molecular weight similar to insulin and this was modified in parallel in all experiments.

3.1.1 Monitoring insulin modifications

Changes in molecular weight and structure (fragmentation, aggregation, etc.) induced by ROS modifications were evaluated using native PAGE (20% gel), and modifications were monitored by Coomassie blue staining and immunoblotting. To ensure modifications remained reproducible and consistent, ELISA assays were performed by utilising the most reactive samples used in a previous study[82] as primary antibodies, to verify their ability to bind of the modified protein. Insulin

modifications were firstly monitored using a 20% SDS-PAGE since Insulin is a low molecular weight (5-6 kDa) protein. This SDS-PAGE provided information on molecular weight changes mainly for glycated insulin. A 20% native-PAGE was also used to visualise the different ROS-modified insulin forms at a better resolution.

Modifications were also tested monthly to check the stability of the modified proteins longitudinally following the production of large batches. All samples were stored at 4°C. Native PAGE and ELISA were used to monitor the quality control.

3.1.2 Stabilizing insulin modifications

To improve the stability of oxPTM-insulin and inactivate the excess of ROS present in the modified protein mixtures, 5 different scavengers have been tested. Insulin was modified following the protocol previously described (in 3.1) and the oxPTM-insulin was incubated with different concentrations of the scavengers as described in the table 4 and tested at time 0 (after an overnight incubation), and every week for 3 weeks thereafter.

The scavenger used are:

- N-acetyl-cysteine (NAC) (A9165, SIGMA);
- chelex 100 resin (1421253 , BioRad) with a styrene divinylbenzene matrix and the functional group R-CH2N(CH2COO-)2, alone and in combination with L-methionine, taurine and N-acetyl-tyrosine (64319-86329-T4446, SIGMA).

Table 4. Concentration of scavengers used to stabilize oxPTM-insulin.

SCAVENGERS	**QUANTITY (concentration in mM)**			
TYR-TAU	0.5	1	2	5
MET-TYR-TAU	0.5	1	2	5
NAC	0.5	1	2	5
CHELEX	50mg			
CHELEX + AA	50mg			
	0.5	-	2	-

Tyr=tyrisine, tau=taurine, met=methionine, nac=cysteine, chelex=chelating beads.

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3.2 PAGE

In SDS-PAGE the SDS included in the system denatures proteins. Thus, any charge in the protein molecules could be masked by the negative charge by the SDS. During electrophoresis, the proteins migrate and separate in the gel mainly depending on their size. For this reason, SDS-PAGE is commonly used to determine the molecular weight of proteins. However in native PAGE, in the absence of SDS, proteins retain their natural conformations and migration through the gel depends on the charge of the protein, the size, shape and any complex formations.

3.2.1 Native PAGE

A 20% Native gel was used to check insulin modifications. Both the resolving and stacking gels were cast in the absence of SDS. The samples were prepared using a non-reducing sample buffer (62.5mM Tris- HCl pH6.8; 40% Glycerol; 0.01 % Bromophenol Blue) that did not contain beta-mercaptoethanol or dithiothreitol (DTT). The gel plates were placed into the holder (figure 10) and a resolving gel was cast according to the percentage needed (Table 5).

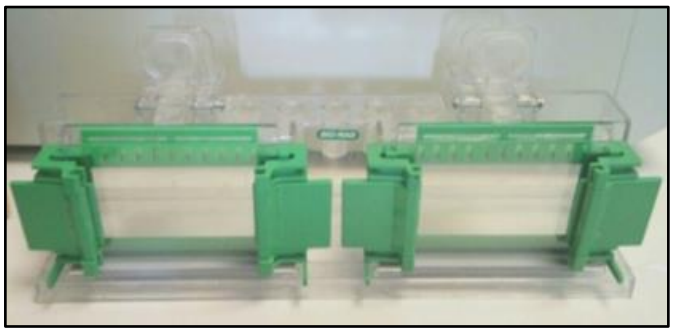

Figure 10. Mini-PROTEAN® 3 Cell (BioRad): gel holder.

Just before pouring the mixed media, 10% ammonium persulfate (A3678, SIGMA) and TEMED (T9281, SIGMA) were added. The resolving gel mixture was overlaid with dH_2O and the gel was allowed to set at room temperature.

Table 5. Recipe for the preparation of 2 native polyacrylamide 20% running gels.

H2O	2ml
1.5 M Tris-HCl, pH 8.8	2.5ml
Acrylamide/Bis-acrylamide	6.6ml
10% ammonium persulfate (APS)	50ul
TEMED	5ul

Once set, the water layer was removed and the stacking gel was added on the top of the resolving gel. Stacking gel was made according to the table below (table 6):

Table 6. Recipe for the preparation of 2 native polyacrylamide stacking gels.

H2O	3.075ml
0.5 M Tris-HCl, pH 6.8	500ul
Acrylamide/Bis-acrylamide	670ul
10% ammonium persulfate (APS)	30ul
TEMED	5ul

A comb was inserted into the top of the gel before it was cast to create wells. The gel was left to polymerise for 15-30 min. The gel was clamped into the electrode chamber with the smaller plate facing inwards. The inner chamber of the apparatus was filled with 1x Running buffer (25mM Tris, 192mM glycine, pH8.3). The other chamber of the apparatus was also filled to the top with 1x running buffer and subjected to electrophoresis under constant voltage of 100 - 120 V. The whole process is shown in the figure 11.

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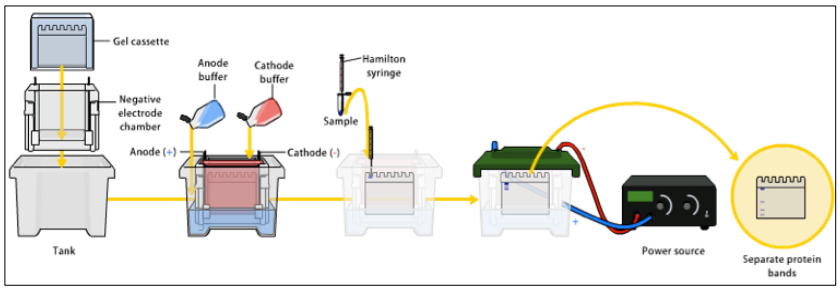

Figure 11. Representation of the PAGE preparation and run[90]. The gel was clamped into the electrode chamber that was filled with 1x Running buffer. The apparatus was subjected to electrophoresis under constant voltage.

After completion of electrophoresis, the gel was incubated in a fixing solution (40% methanol and 10% acetic acid) for 20 minutes prior to staining with Coomassie Blue (0.1%w/v Coomassie Blue, 45% methanol and 10% acetic acid) for 30 minutes at room temperature. The gel was transferred into destaining solution (40% methanol and 10% acetic acid) for a 4 hours.

3.2.2 Tricine SDS 10-20% PAGE

Gradient tricine gels (Invitrogen) is an SDS-PAGE that provide separation of low molecular weight proteins and peptides. This system is specifically developed for studying small proteins, peptides, and particularly useful for degraded and modified proteins. In the tricine system the glycine is replaced by tricine resulting in more efficient a greater separation of smaller proteins. The precast gradient tricine gel system includes specific buffers for electrophoresis, protein transfer and sample loading (EC6625BOX, LC1676, LC1675, LC3675 Thermofisher). The cast gel was clamped into the electrode chamber and the inner chamber was filled with 1x running buffer. The electrophoresis was performed by applying a constant voltage of 100 - 120 V. Coomassie blue staining was conducted as described previously.

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3.3 Western blotting

OxPTM-INS, native INS, oxPTM -HEL and native HEL (10µg) were electrophoresed either using a 20% native gel or a 10-20% gradient tricine gel and electroblotted under native conditions for 40 minutes at 80V (the process is shown in the figure 12). Due to the dimension of insulin a nitrocellulose membrane with 0.2µm Pore Size was used. Nonspecific binding was blocked by incubating with 5% BSA/PBS for 1 hour at room temperature. Membranes were then incubated with a 1:100 dilution of serum samples in 5% Milk/PBS (pre-incubated during blocking) overnight at 4°C. The following day, membranes were incubated for 1 hour with 1:1000 dilution of anti-human IgG-HRP (company) in 5% Milk/PBS. Membranes were washed and developed with Luminata Forte Western HRP substrate (WBLUF0100, Fisher Scientific), a film was exposed for the required time in a hypercassette in a dark room, and then developed in the film processor (SRX 101A Konica Minolta).

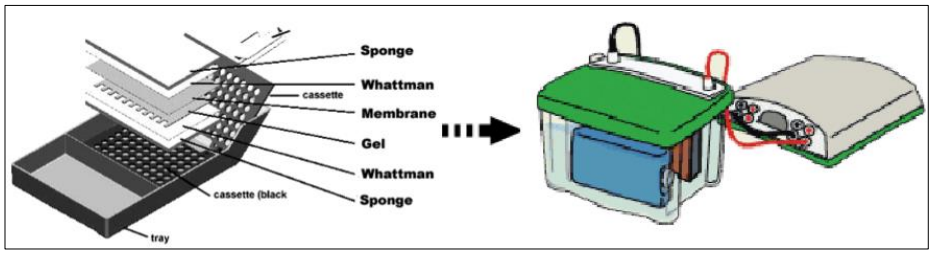

Figure 12. Representation of the western blot preparation and run[90]. The transfer sandwich was assembled with: on bottom half of plastic transfer cassette, sponge, filter paper then place gel on top of filter paper. Transfer membrane has to be placed directly on top side of gel and then filter paper and sponge. The plastic transfer cassette was moved in the transfer tank filled with transfer buffer and subjected to constant voltage.

3.4 Patients in cohort 1: ABIS study

Samples analysed were from ABIS biobank, located in Sweden. ABIS is a large prospective study involving 17,055 unselected children born between 1st October 1997 and 1st October 1999. The samples consist of cord blood and capillary blood collected at 1, 2.5, 5 7 and 11 years. All samples are characterised for HLA and autoantibodies involved in T1D pathogenesis. The aim of the ABIS is to study the

effects of several factors, such as the environment on the development of autoimmune diseases, including T1D. Among the children screened, 116 developed type 1 diabetes during the clinical follow-up[91]. In this study, we have analysed 51 samples from the 23 children who progressed to type 1 diabetes (progr-T1D). Samples were collected longitudinally before diagnosis at three different time points: 5, 7 and 11 years. Not all time points were available for every child. For example, only one time point was available from the seven progr-T1D children before collection of the second time point. As controls, we used samples from 63 children who did not progress to type 1 diabetes over the course of time. The control group includes 32 children positive to at least one islet-antibody marker (NP-AAB+) and 31 samples from autoantibody-negative children (NP-AAB−) and yet both groups did not progress to T1D. Informed consent was obtained from parents prior to collection of blood. The study was approved by the Research Ethics Committees of the Medical Faculties of Linköping University, Linköping and Lund University, Lund, Sweden and the Ethics Committee of the University Campus Bio-Medico di Roma.

3.5 Patients in cohort 2: plasma vs serum

Samples analysed were from a group of young patients, kindly provided by Professor Johnny Ludvigsson. Serum samples were collected from 50 patients taken fasting at 10 days after onset and under insulin therapy for the 10 days. Clinical features such as HLA genotyping and level of IA-2A and GADA were recorded for most of the patients.

3.6 Islet autoantibodies

Islet antibodies are considered markers of b-cell disruption and are used extensively in the clinic. In collaboration with the Linköping University (Division of Paediatrics, Department of Health and Environment, Faculty of Health Sciences) islet antibodies were evaluated according to standardised protocols[91]. Different protocols were followed depending on the type of islet antibody. GADA and IA-2A

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were determined following the protocol as described by Wahlberg et al[92]. ZnT8A, in the three most frequent variant (ZnT8RA, ZnT8QA, ZnT8WA), were measured as previously described[93]. For all three markers the threshold cut-off was considered at the 98th percentile. The IAA were measured according to Williams et al and Holmberg et al[94, 95] and the cut off was defined as equivalent to 95th percentile.

3.7 HLA genotyping

HLA genotyping was performed in collaboration with Linköping University[91] following a protocol previously described[96]. The different genotypes were classified as susceptibility-associated, neutral or protective based on the categorisation done by Hermann et al[96].

3.8 ELISA

The ELISA was performed to establish the reactivity of T1D patient sera to native and modified insulin.

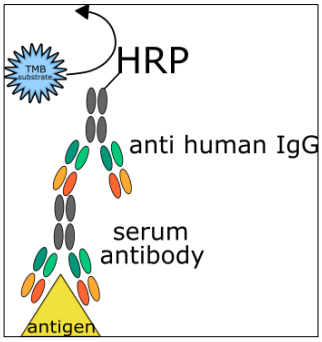

Figure 13. Representation of the antibody binding in ELISA assay. Plate was coated with the antigen and, after blocking, diluted serum samples were added. The secondary antibody used was an anti-human IgG-HRP and after incubation the TMB substrate was used.

3.8.1 ELISA settings

The ELISA was optimized[82] following this protocol: a 96well NUNC assay plate (442404, Thermofisher) was coated and incubated overnight at 4°C with 100 µl per well of modified and native insulin in 0.05 M Carbonate/Bicarbonate Buffer pH

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9.6 concentrated 10µg/mL. The following day, coated plates were washed 3 times with 0.1% Tween PBS and blocked using 5% BSA/0.1% Tween/PBS for 2 hours. After blocking, 100 µl per well of 1:200 diluted serum samples in 5% BSA/0.1% Tween/PBS were added to each well and incubate for 2 hours at room temperature. The secondary antibody used after incubation with human serum samples is a 1:1000 dilution of anti-human IgG-HRP (A8792, SIGMA) in 5% BSA/0.1% Tween/PBS for 2 hours at room temperature. The ELISA plates were washed, and 100µl/well of a 1:100 dilution of 10mg/mL TMB (3, 3', 5, 5'-tetramethylbenzidine) (860336, SIGMA) in 0.1M sodium acetate pH 6 plus 2µl/10mL of H2O2 was added. The reaction was stopped using 20% sulphuric acid. The optical density (OD) was measured at 450 nm using the Thermofisher multiscan fc plate reader.

3.8.2 Competitive ELISA

The competitive ELISA was performed to assess the serum binding specificity. The difference between a standard ELISA and a competitive ELISA is a competitive binding process between the primary antibody and another antibody and/or compound as shown in figure 14. Herein, the competitive ELISA was set up using a 2 hour incubation between T1D serum samples and native or OH-INS. The primary antibody was incubated with native or OH-INS, antibody-antigen complexes were then added to the ELISA plate pre-coated with native insulin and ROS-modified insulin. Unbound antibodies were removed by washing the plate, as in the standard protocol, just before adding the secondary antibody (anti-human IgG HRP conjugated) that is specific for the human serum samples. Plates were developed as previously described. The major advantage of a competitive ELISA is the high specificity and, in our case, the opportunity to confirm the data from the standard ELISA.

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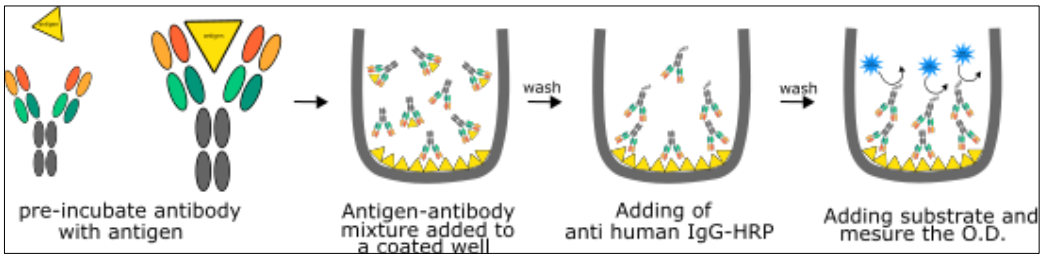

Figure 14. Competitive ELISA. Plate was coated with the antigen and, after blocking, diluted serum samples previously incubated with a competitor antigen were added. The secondary antibody used was an anti-human IgG-HRP and after incubation the TMB substrate was used.

3.9 Statistical analysis

Data analysis was performed using Prism Software version 6.01 (GraphPad, San Diego, CA, USA). HEL and oxPTM-HEL were used as background controls. The Mann-Whitney test was used to test and compare the antibody binding. Longitudinal changes in antibody binding were evaluated by the Wilcoxon paired test. To determine predictive discrimination between T1D and healthy control groups, we used the 99th percentile of the healthy individuals as cut-off point absorbance units to construct a contingency table of positive binders to oxPTM-CII and tested it by Fishers Exact Test.

3.10 Epitope mapping

In patients with T1D, autoantibodies against insulin are often the first autoantibodies detected, and in some cases can be detected before the onset. For this reason, molecular interactions between autoantibodies and insulin have a great interest from both diagnostic and therapeutic points of view[61]. These autoantibodies and the epitope/epitopes responsible for the binding are not well characterised. The process to identify the binding site responsible of molecular interactions between autoantibodies and their target is called epitope mapping[97].

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Classification of epitopes is generally made in 3 classes: linear epitopes, conformational epitopes and discontinuous epitopes. The linear epitopes are the primary amino acid sequence of a particular region of the protein; conformational epitopes are defined by a primary amino acid sequence and by its spatial conformation; discontinuous epitopes consist of non-adjacent parts of the protein sequence, which are together in 3D structure. The majority of interactions between antigen and antibody are due to conformational epitopes[98]. The importance of epitope mapping is the opportunity of use the epitope sequence to design specific antibodies and vaccines for therapy and/or diagnostic tests blocking, trapping and detecting proteins[99]. Mapping proteins of medical interest, such as insulin, is very important even if it is not easy, since many of them are only formed in the native structure of protein presenting a conformational or discontinuous epitope[98, 100]. The fast technological development of proteomic methods including two-dimensional (2D) gel electrophoresis, mass spectrometry, BIAcore, antibody and tissue arrays, have been used to identify proteins and/or protein fragments. Epitopes involved in the autoimmune binding of autoantibodies in T1D have been studied using monoclonal antibodies, and their Fab[101, 102, 103] but there is now much knowledge about the insulin and the oxPTM-INS epitopes.

3.10.1 Native 2 dimensions electrophoresis (2D Gel)

Conventional protein gels only run in one dimension, but as the name implies, 2D gels run in 2 dimensions. Common separation characteristics are isoelectric point in the first dimension, followed by mass/size separation in the 2nd dimension. Proteins have their own isoelectric point that comes about as an average of their amino acid isoelectric point and for this reason they are charged at different pHs. Proteins loaded in 2D Gel strips, and stimulated by voltage, move across the pH gradient, when they reach the region of the gel that matches their isoelectric point, they stop moving. After that the proteins have been separated by isoelectric point, they can be analysed based on their mass (figure 15).

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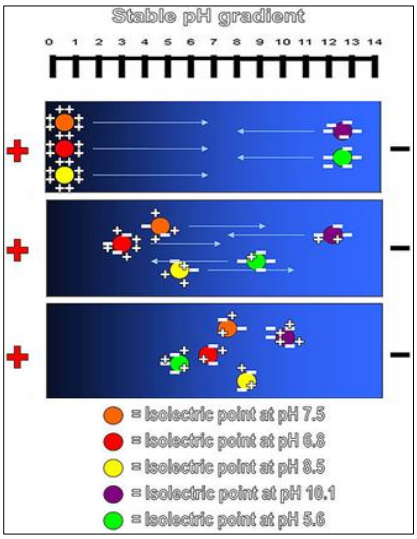

Figure 15. Scheme of the first dimension of 2D gel: isoelectrofocusing. Proteins loaded onto a stable pH gradient strip, stimulated by voltage, move across the pH gradient, stopping when they are at the region of the strip that matches the isoelectric point.

First dimension: IsoElectroFocusing (IEF)

System used: Ettan IGPhor Isoelectrofocusing system (GE Healthcare) (figure 16).

Figure 16. IPGphor isoelectrofocusing system. [www.labmerchant.com]

Firstly, Immobiline Dry Strip pH 3-10NL (17600112, GE Healthcare) was rehydrated using 25-30µg of protein and 100µl of IPG buffer (17-6000-87, GE Healthcare) 0.5%

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at room temperature at least for 10 hours in the IGP strip holder. No voltage was applied at this stage. The strip was positioned with the gel side down and the pointed (anodic) end of the strip directed toward the pointed end of the strip holder. DryStrip cover fluid was applied to cover the IPG strip to assure a complete wetting. After the rehydration strips were processed isoelectrofocusing using the Pharmacia Biotech IPGphor Isoelectric Focusing System following this protocol:

1. Step-on-hold 100V 30minutes
2. Gradient 300V 30minutes
3. Step-on-hold 300V 10minutes
4. Gradient 1000V 3hours
5. Gradient 3000V 4hours
6. Gradient 4000V 4hours
7. Gradient 5000V 4hours
8. Step-on-hold 5000V 3hours

Second dimension: Native-PAGE

After the IEF, each strip was equilibrated 30minutes in equilibration buffer (75mM TRIS-HCl PH 8.8 30% Glycerol 0.002% Bromophenol Blue). Strips were then loaded on a 20% native gel, the running gel was made as previously described, the stacking gel was made based on the strip. The strip was placed with the "plus-end" on the left lowered so the entire bottom edge of the strip was in contact with the top surface of the gel. The IGP strip was sealed in place using a 0.5% agarose gel. The gel was then subjected to electrophoresis under constant voltage of 100V. The gel was then removed and stained with Coomassie Blue or used for a western blot.

3.10.2 Mass spectrometry

Mass spectrometry is a technique based on the ionizations of proteins and the sorting of the neo formed ions depending on their mass-to-charge ratio. This kind of methods are currently used in many different fields, in proteomics they are important to identify proteins, elucidate protein modifications, reading functional

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interactions, such as the antibody-antigen binding. During a mass spectrometry analysis, the samples is firstly ionised, this can be done following several methods such as bombarding the samples with electrons. The ions resulting are then separated according to their mass-to-charge ratio, can be detected by a mechanism capable of detecting charged particles. The results of this analysis are displayed as a spectrum representing a plot of the ion signal as a function of the mass-to-charge ratio[104].

The mass spectrometry analysis was conducted in collaboration with the Cambridge Centre for Proteomics based at the University of Cambridge. In order to obtain the amino acid sequence of the modified insulin the proteins were digested using chymotrypsin and the MS/MS ion search was set up using the ESI-ORBITRAP-HCD (High energy Collision Dissociation).

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CHAPTER 4

TYPE 1 DIABETES: RESULTS

4.1 *In vitro* insulin modifications

4.1.1 PAGE analysis of insulin and oxPTM-INSULIN

Modification of insulin was conducted as previously described[82] and checked by SDS and native page. The SDS-PAGE, shown in figure 17A, presented a similar profile of bands for native and modified insulin, all of which are around 5 kDa. The resolution of the SDS-PAGE does not consent a good discrimination between insulin and oxPTM-insulin, for this reason the native PAGE was used to check the modifications. The native PAGE, shown in figure 17B, showed a difference in the run between native and oxPTM-insulin; glycation by ribose induce a small shift to a higher molecular weight, the oxidised and chlorinated insulin present the appearance of several additional bands with slower mobility and a smear of protein along the whole lane that suggest fragmentation of insulin.

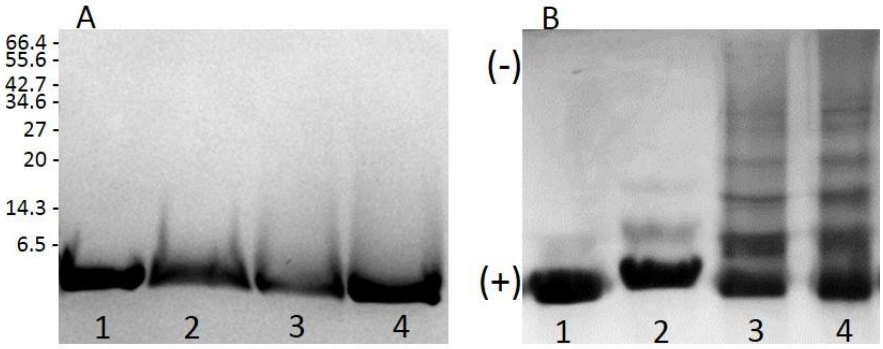

Figure 17. Native and SDS-PAGE analysis of insulin and oxPTM-INS. Figure A shows results using a 20% SDS-PAGE. Native insulin (lane 1) showed an electrophoretic band that migrated to the region of 5-6 kDa protein marker; glycation by ribose (lane 2) presented a small shift to an higher molecular weight; oxidised (lane 3) and chlorinated (lane 4) insulin resulted in a smear of protein, indicating fragmentation. Figure B shows results using a 20% native PAGE. Native insulin (lane 1) showed a profile similar to the SDS-PAGE; glycation by ribose (lane 2) presented a reduction in mobility and the appearance of two bands with slower mobility; oxidised (lane 3) and chlorinated (lane 4) insulin resulted in a smear of protein and also the appearance of additional and slower mobility bands.

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Insulin modifications were also checked using a precast gradient 10-20% tricine gels. The gel was stained with Coomassie blue, the result of which is shown in figure 18. The gradient tricine gel shows a difference in the run between native and oxPTM-insulin; the oxidised insulin presents the appearance of several additional bands with higher molecular weight, and chlorinated insulin presents the appearance of similar additional bands with higher molecular weight and a smear of protein along the whole lane, which suggests fragmentation of insulin.

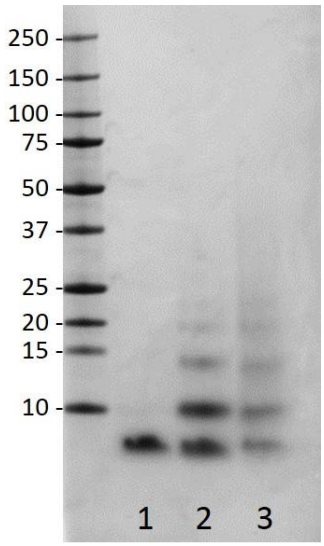

Figure 18. Analysis of insulin and oxPTM-INS using a 10-20% gradient tricine gel. Native insulin (lane 1) showed an electrophoretic band that migrated to the region of 5-6 kDa; oxidised (lane 2) insulin showed a band that migrated to about 5-6 kDa plus three extra bands with a higher molecular weight; chlorinated (lane 3) insulin resulted in a smear of protein, indicating fragmentation, and few additional bands.

4.1.2 Western blot

Binding to native and modified insulin was also detected by western blot to confirm the specificity of the binding. Figure 19 shows the binding of the most reactive samples used previously[82]. Binding to oxidised and chlorinated insulin was stronger and directed to a diffuse fragment with a slower mobility compared to the native protein. There is no binding to glycated insulin.

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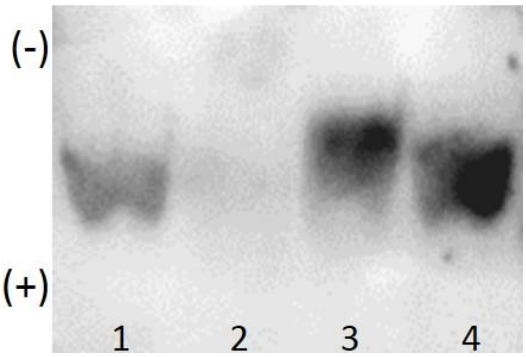

Figure 19. Binding to native insulin and oxPTM-INS detected by western blot. Binding to NT-INS (lane 1) and a stronger-intensity binding to a smear of a smaller mobility fragment of oxidised insulin (lane 3) and chlorinated insulin (lane 4) was observed. No binding to glycated insulin (lane 2) was detected.

4.1.3 ELISA

Modified insulin reactivity was also checked by ELISA using positive samples previously used[82] following the protocol described below. As shown in figure 20, binding to oxidised and chlorinated insulin was stronger compared to the binding to native insulin, and reactivity of T1D serum samples was significantly higher than HC ($p<0.0001$) confirming the data published[82, 105].

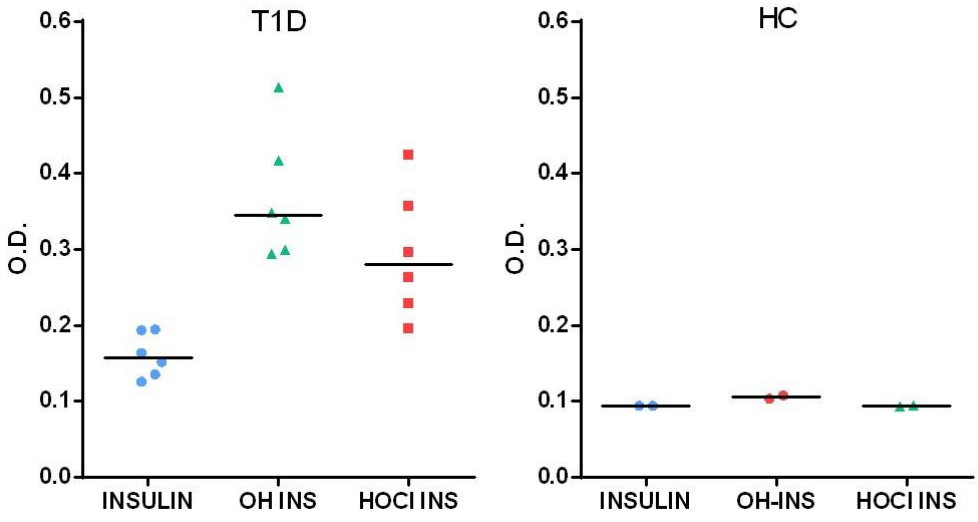

Figure 20. Binding to native insulin and oxPTM-INS detected by ELISA Binding to oxPTM-INS was stronger compare to native insulin and reactivity of T1D serum samples was higher than HC.

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4.1.4 Mass spectrometry

The results from mass spectrometry conducted at the Cambridge Proteomics Core facility are shown in the figure 21, the oxPTM-INS amino acid sequence obtained confirms the amino acids modifications previously published[82] and shown in figure 9. In addition to these modifications the mass spectrometry results show two amino acid conversions (figure 22 yellow squares): His 10 was converted to Trp and Leu 17 to Tyr. Additional mono-oxidation of Cys 7 and 19 on β-chain and Cys 6, 7, 11 and 20 on α-chain were also detected, in addition also oxidation of the Tyr 14 was detected (figure 22 red squares).

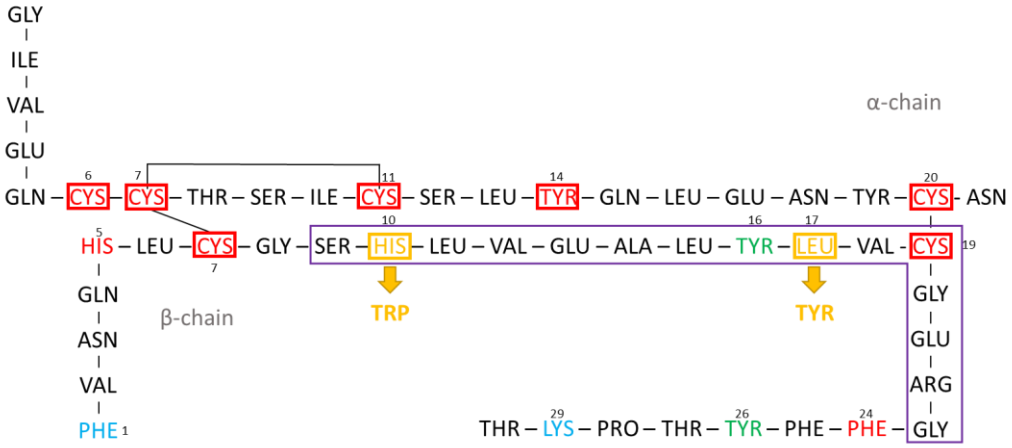

Figure 21. Mass spectrometry analysis of oxPTM-INS. In addition to the modifications shown in figure 9 the new modifications mapped are shown in squares, amino acid conversions in yellow and oxidation in red [Cambridge Proteomics Core facility].

4.2 Longitudinal stability study

4.2.1 Native PAGE analysis

To be sure that the assay and modifications used in experiments are stable, the modifications were checked over different time points. Figure 22 shows the comparison between fresh modifications and 1 month old modifications. The figure shows the native PAGE stained with Coomassie blue. Native and oxidised

insulin look similar while the new chlorinated insulin is not clearly represented in the gel and therefore not comparable with the old chlorinated insulin.

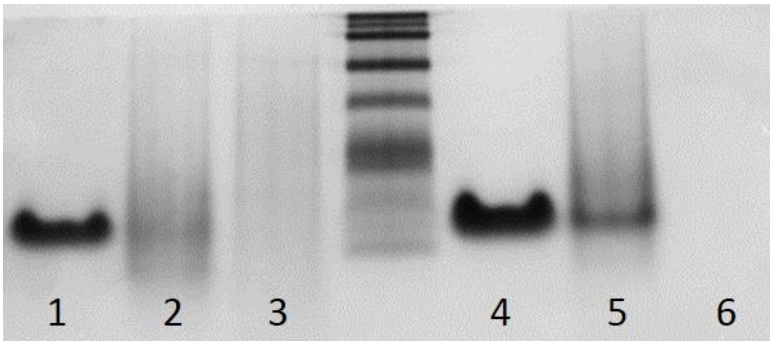

Figure 22. Native PAGE analysis of insulin and oxPTM-INS over time. Lanes 1, 2 and 3 were loaded with new modifications; lanes 4, 5, and 6 were loaded with old modifications. Native insulin (lanes 1 and 4) had almost the same running profile; oxidised insulin (lanes 2 and 5) are very similar; chlorinated insulin (lanes 3 and 6) are not comparable because the chlorinated insulin in the lane 6 is almost completely degraded.

4.2.2 ELISA

Fresh and 1-month old modifications were also checked in ELISA to assess the changes in binding over time. Some of the most reactive samples from different cohorts of T1D patients were used as positive controls to analyse the modifications. The results of these ELISA analysis are shown in figure 23. The reactivity to old and fresh modifications are similar and not significant, although one sample has shown a decrease of reactivity to old insulin modification.

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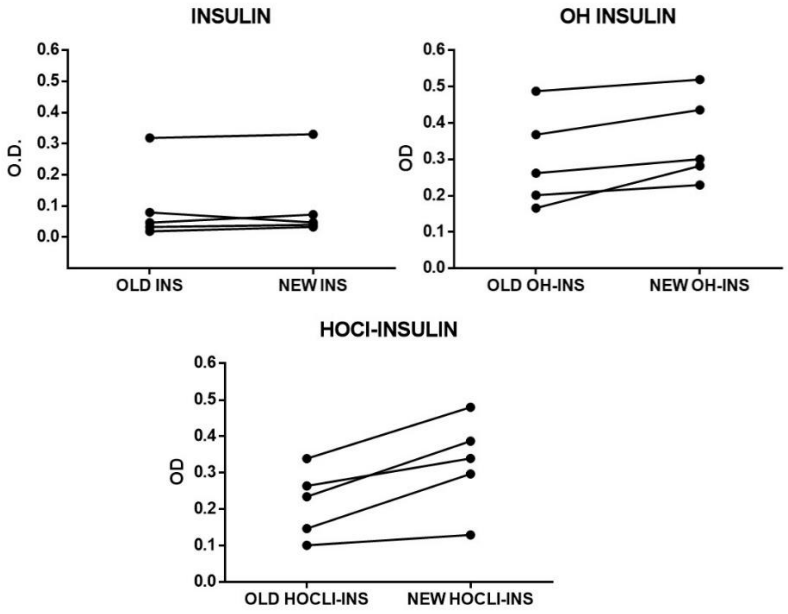

Figure 23. Comparison of ELISA using new and old modifications. Some of the most reactive samples from different cohort of patients with T1D were used to check if there were any differences in the binding to old and new modified insulin. The difference is not significant.

4.3 Stabilizing insulin modification: scavengers

The scavengers used to improve the stability of the modified insulin were N-acetyl-cysteine and chelex 100 resin, alone and in combination with L-methionine, taurine and N-acetyl-tyrosine.

Some of the most reactive serum samples previously used[82] were used as primary antibodies in ELISA to check the stability of oxPTM-insulin plus scavengers through 3 weeks and 4 time points in comparison with insulin modifications newly prepared.

4.3.1 Scavengers: chelex 100 resin

The chelating resin was tested to stabilise oxidised insulin, the resin was used alone and in combination with 2 different concentration of a mix of amino acids (methionine, tyrosine and taurine).

The graph in the figure 24 shows the results of the 3 conditions used for the chelating resin. The pink and purple colour indicate insulin and oxidised insulin. These controls were prepared the day before the checking of each time point to compare the reactivity to fresh oxPTM-INS with the reactivity to old modifications plus scavengers. The results show that reactivity towards oxidised insulin is stable through time when the chelating resin is used, regardless of the presence of the amino acid mix.

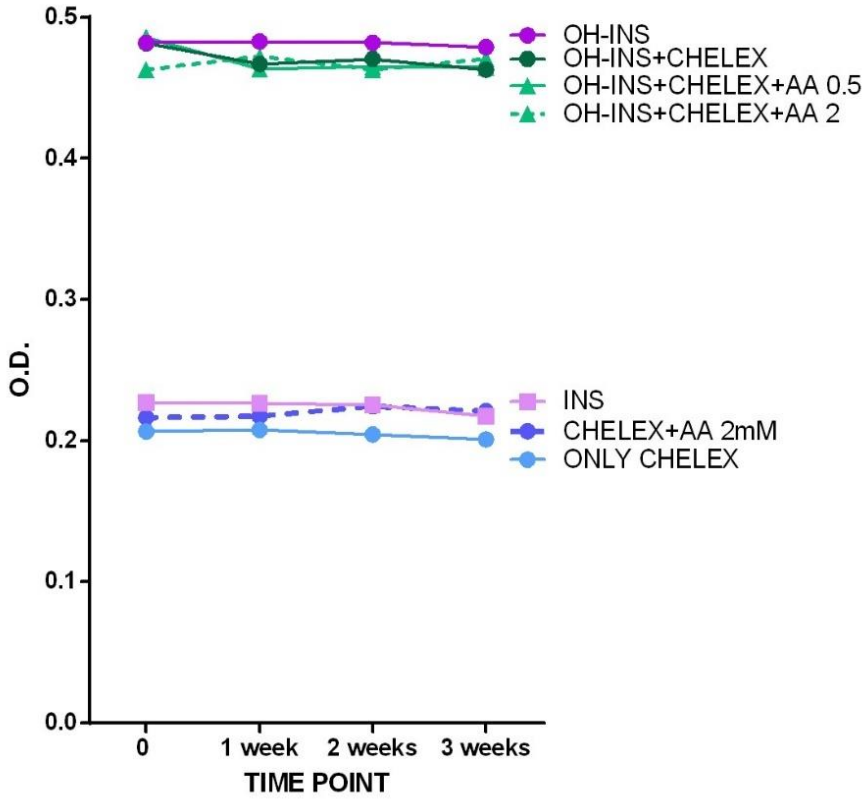

Figure 24. Stabilization using chelex 100 resin as scavenger. Reactivity of serum samples to insulin and oxidised insulin fresh modified in comparison with oxidised insulin 1, 2 and 3 weeks old incubated with a chelating resin in combination with a mix of amino acids.

4.3.2 Scavengers: N-acetyl-cysteine (NAC)

N-acetyl-cysteine was tested at 4 different concentrations. Figure 25 shows the results of two of the 4 concentrations tested as example. The pink and purple

colour indicate insulin and oxPTM-INS. As explained about the chelex scavenger, oxPTM-INS were prepared the day before the checking of each time point to compare the reactivity to fresh oxPTM-INS with the reactivity to old modifications plus scavengers. The results show that reactivity is stable through time and the values are very similar to fresh insulin modifications.

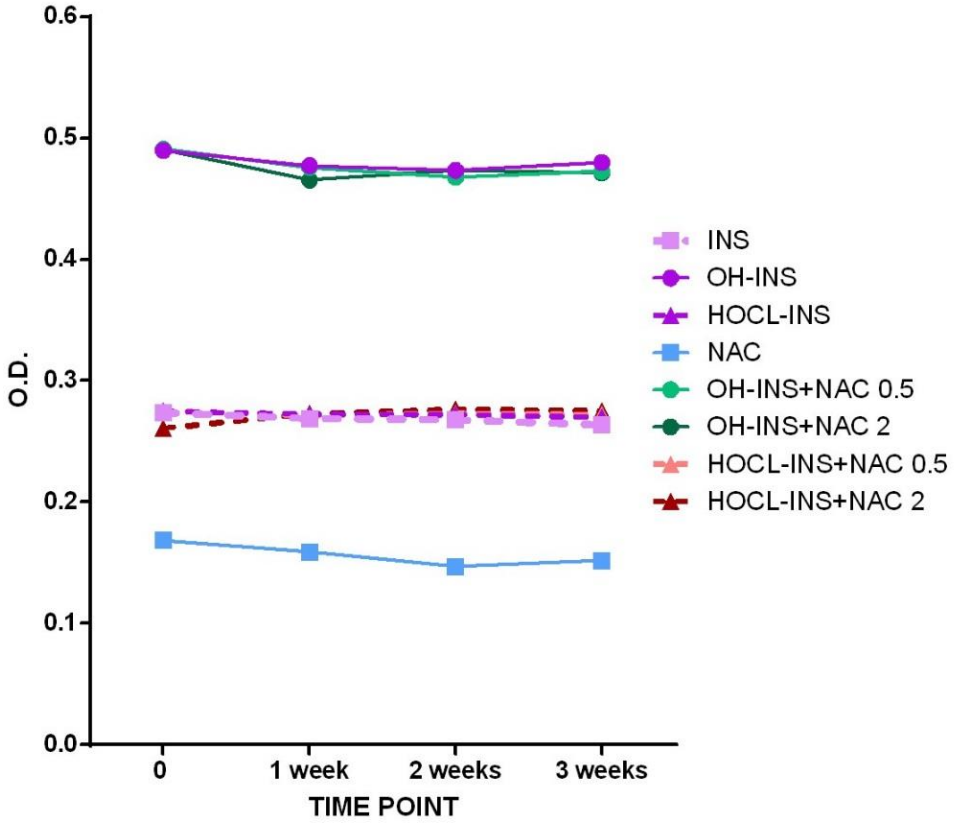

Figure 25. Stabilization using NAC as scavenger. Reactivity of serum samples to insulin and oxPTM-INS fresh modified in comparison with oxPTM-INS 1, 2 and 3 weeks old incubated with NAC.

4.4 Patients analysis cohort 1: ABIS study

The characteristic of the patients included in the ABIS cohort are shown in table 7. The ABIS study includes all the babies born in south east Sweden between 1st October 1997 and 1st October 1999. The cohort was divided in 3 groups: children negative for ICA who did not progress to T1D (NP-AAB$^-$), children positive for ICA who did not progress to T1D (NP-AAB$^+$) and children positive for at least one of the

ICA who did progress to T1D (PROGR-T1D). The characteristics reported in the tables below refer to the first time point available for each subject. The age of the children was comparable even if the age in the NP-AAB$^-$ and NP-AAB$^+$ groups were slightly higher than PROGR-T1D group; the children were mainly males but among the groups this characteristic was comparable (between 67% and 72%). The 79% of the PROGR-T1D children were positive for more than one ICA compare to the 27% in the NP-AAB$^+$ group.

Table 7. Characteristics of the patients included in the cohort 1. (Data are presented as means ± SD and n (%)).

CHARACTERISTICS	PROGR-T1D (N=24)	NOT PROGRESSING T1D	
		NP-AAB$^+$ (N=33)	NP-AAB$^-$ (N=25)
Age at baseline (y)	6.02 ± 1.65	7.36 ± 2.44	7.28 ± 2.49
Sex, male	16 (67%)	22 (67%)	18 (72%)
Multiple autoantibodies (≥2)	19 (79%)	9 (27%)	NA

PROGR-T1D=children progressing to T1D, NP-AAB$^+$=children not progressing to T1D but ICA positive, NP-AAB$^-$= children not progressing to T1D but ICA negative.

The information about the islet antibodies in subjects who did and did not progress to T1D are reported in table 8. Data for GADA and IA-2A were available for most of the samples (51 out of 57), IAA were available only in 45 children and data for ZnT8A were available in 22 children and it is considered positive when one or more variants are positive.

In the PROGR-T1D group the percentage of positivity to GADA was 78%, to IA-2A 70%, to IAA was 56% and positivity to ZnT8A was 50%. Both titres and prevalence of GADA and ZnT8A were similar in PROGR-T1D and NP-AAB$^+$, also for the IAA the difference between the two groups was not very high. IA-2A was the most specific antibody among the ICA markers being higher in the group of progr-T1D than NP-AAB+ ($p<0.001$) with a difference of 56%.

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Table 8. Prevalence of ICA in cohort 1.

	PROGR-T1D	NP-AAB$^+$
GADA	18 (78%)	25 (89%)
IA-2A	16 (70%)	4 (14%)
IAA	10 (56%)	8 (35%)
ZnT8A	7 (30%)	3 (30%)

PROGR-T1D=children progressing to T1D, NP-AAB+=children not progressing to T1D but ICA positive. GADA=anti glutamic acid decarboxylase antibody, IA-2A=anti tyrosine phosphatase IA-2 antibody, IAA=anti insulin autoantibodies, ZnT8A=anti zinc transporter 8 antibody.

The data from the HLA genotyping are reported in table 9. The information, available only for 43 subjects, are divided into several groups based on the association with susceptibility or protection to T1D following the classification described[44], in which S = susceptible, N = neutral and P = protective. The haplotypes DR4-DQ8(DRB1*0401/2/4/5-DQB1*0302) and DR3-DQ2 (DQA1*05-DQB1*02) were classified as S; the haplotypes DR2-DQ6 (DQB1*0602), DR11/12/1303-DQ7 (DQA1*05-DQB1*0301), DR7-DQ3 (DQA1*0201-DQB1*0303), DR14-DQ5 (DQB1*0503), DR403-DQ8 (DRB1*0403-DQB1*0302) and DR1301-DQ6 (DQB1*0603) were classified as P. The higher percentage of PROGR-T1D is shown by the haplotype group called SS (47%) whereas in the NP-AAB$^+$ group there is not a predominant haplotype group.

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Table 9. Prevalence of the HLA genotypes in cohort 1. (Data are presented as number of subjects).

HLA SUSCEPTIBILITY CATEGORY	PROGR-T1D	NOT PROGRESSING T1D	
		NP-AAB$^+$	NP-AAB$^-$
SS	8	3	0
SN	6	3	1
SP	3	3	4
NN	0	2	2
NP	0	2	2
PP	0	1	1

PROGR-T1D=children progressing to T1D, NP-AAB+=children not progressing to T1D but ICA positive, NP-AAB-= children not progressing to T1D but ICA negative. HLA haplotypes: S=susceptible, N=neutral and P=protective.

4.4.1 OxPTM-INS autoreactivity in cohort 1

Samples were blindly analysed for the reactivity to oxPTM-insulin, glycated, oxidised and chlorinated. Samples analysed in a previous study[82] were used as positive controls and healthy subject were used as negative controls. Binding to oxPTM-insulin has been evaluated in the 3 groups of samples (NP-AAB$^-$, NP-AAB$^+$ and PROGR-T1D) at the first time point available per subject. Comparing the 3 groups, binding to oxPTM-insulin (both oxidised and chlorinated) was significantly higher in PROGR-T1D group ($p<0.0001$). In figure 26, values above the dashed lines were defined as positive for antibodies to NT-INS and oxPTM-INS (95th percentile of NP-AAB-). In PROGR-T1D group, 23 out of 24 samples were positive for at least one oxPTM-insulin (96%) and 19 samples were positive for both, oxidised and chlorinated insulin (79%). In the PROGR-T1D group binding to oxidised insulin was significantly higher than binding to native insulin ($p<0.0001$).

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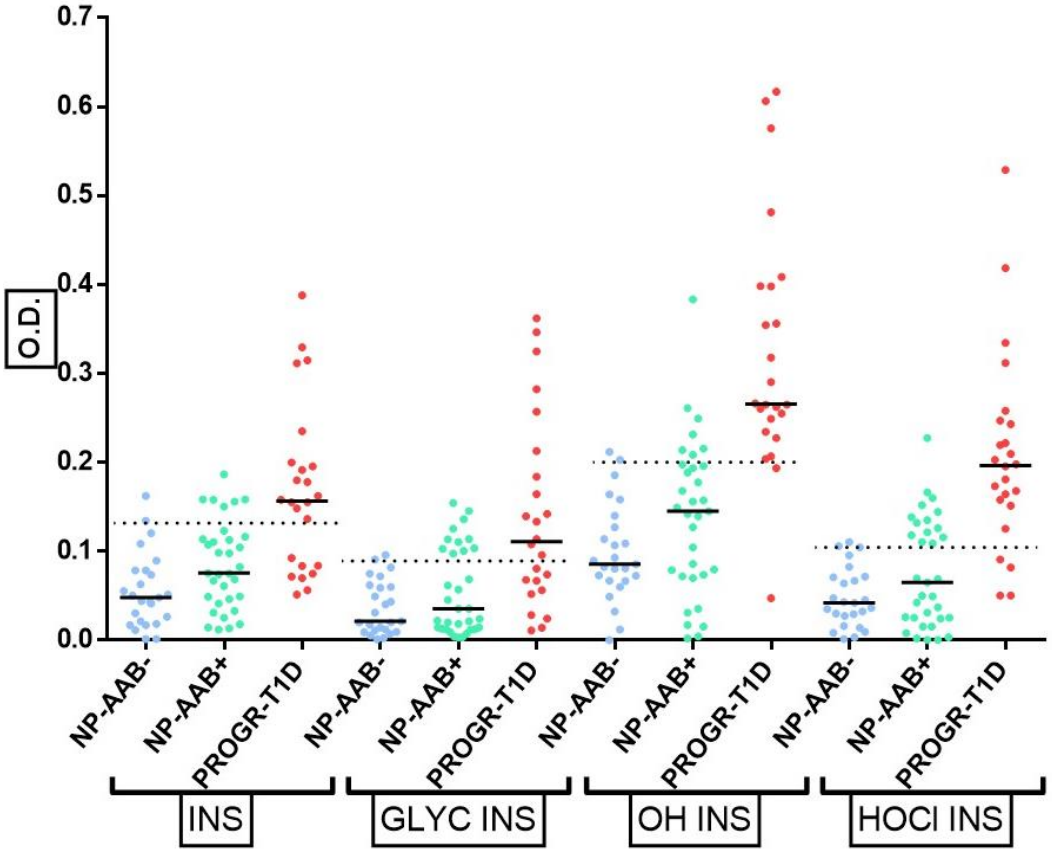

Figure 26. Evaluation of antibody binding to insulin and oxPTM-INS in cohort 1 (data are on the earliest time point available). Reactivity to NT-INS and oxPTM-INS was significantly higher in samples from progr-T1D children compared with non-progressing children, regardless of whether they were NP-AAB^{+} or NP-AAB^{-} to the standard islet autoantibody markers ($p < 0.006$). Binding to oxPTM-INS modified by •OH was significantly higher than to NT-INS in progr-T1D children ($p < 0.0001$).

This data indicated that oxidised insulin allowed discrimination between children progressing to T1D and children who did not progress even if they are positive for at least one ICA ($p < 0.006$ that was the higher p value in the statistical analysis), with 79% sensitivity and 92% specificity.

4.4.2 Longitudinal analysis of oxPTM-INS autoreactivity

Binding to insulin and oxPTM-insulin was also evaluated longitudinally in the 3 groups. Figure 27 shows the longitudinal changes of binding to insulin and oxPTM-

insulin in the NP-AAB$^-$ group. There were no substantial changes over the 3 time points (5, 8 and 11 years), most of the samples were binding insulin and oxPTM-insulin weakly with the exception of 2 samples that demonstrated high reactivity to oxidised and chlorinated insulin- but only at the first time point.

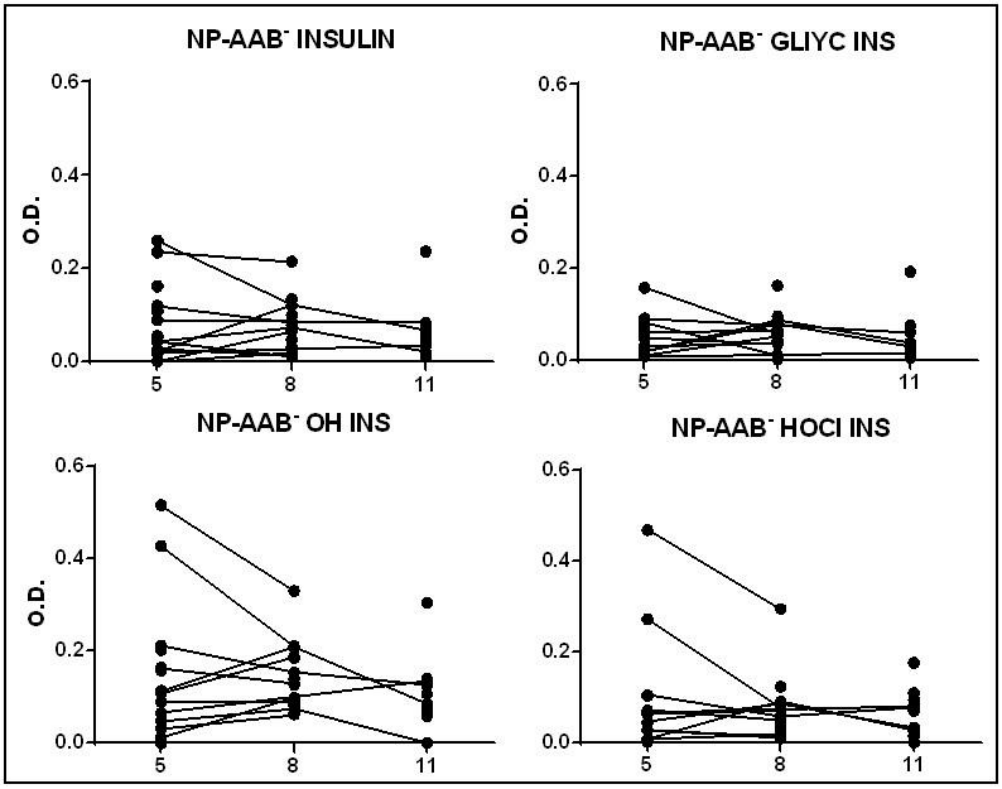

Figure 27. Longitudinal changes of binding to insulin and oxPTM-INS in NP-AAB$^-$ children according to age.

In figure 28, longitudinal changes of binding to insulin and oxPTM-insulin are shown. In the NP-AAB$^+$ group, binding to insulin and glycated insulin can be considered minimal background levels for most of the samples. Binding to oxidised and chlorinated insulin was weak, except for few samples that have an increase in the reactivity between the first and the second time point. Unfortunately, the latest time point for these subjects were not available.

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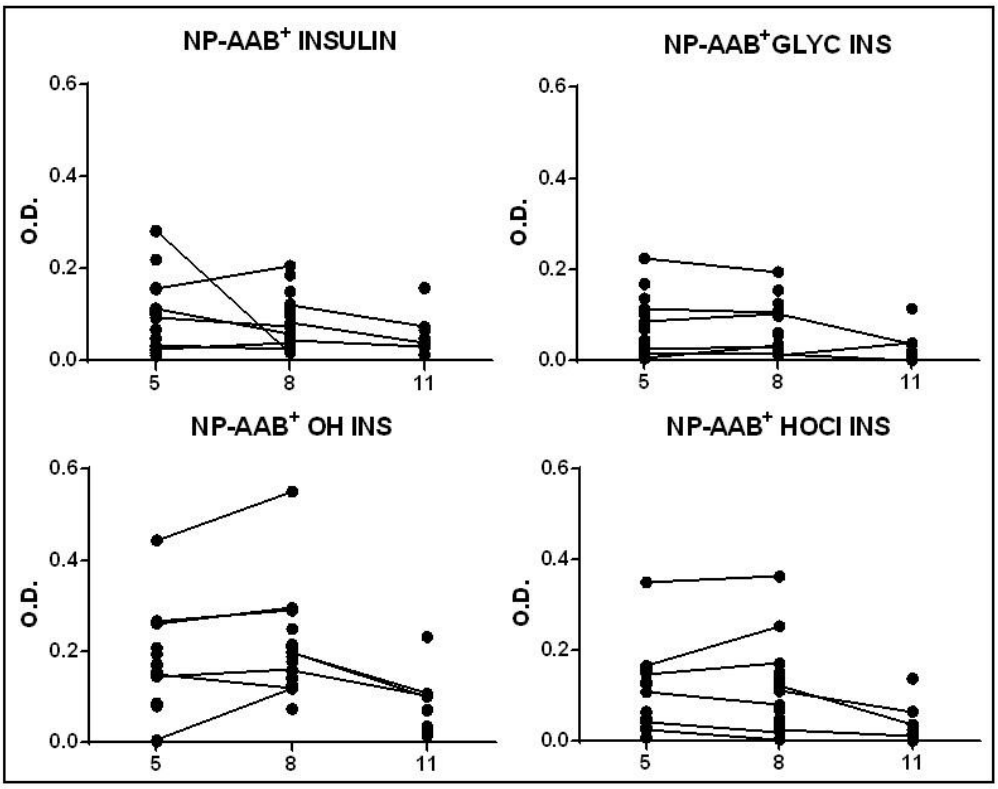

Figure 28. Longitudinal changes of binding to insulin and oxPTM-INS in NP-AAB$^+$ children according to age.

Longitudinal changes of binding to insulin and oxPTM-insulin in the PROGR-T1D group are shown in Figure 29. Binding to insulin and glycated insulin was comparable over the 3 time-points. Binding to chlorinated insulin was stable throughout the time course with the exception of 2 samples between the first and the second time-point. As example of oxPTM-insulin the insulin oxidised by H_2O_2 was considered for a deeper analysis. In general, the binding to oxidised insulin did not change significantly across the time points. Seroconversion occurred once between the first and the second time-point, with one of the negative samples at the beginning becoming positive at the second time-point. On the contrary, one patient negative at the first time-point was positive at the last one, and two samples negative at the last time point were both positive before.

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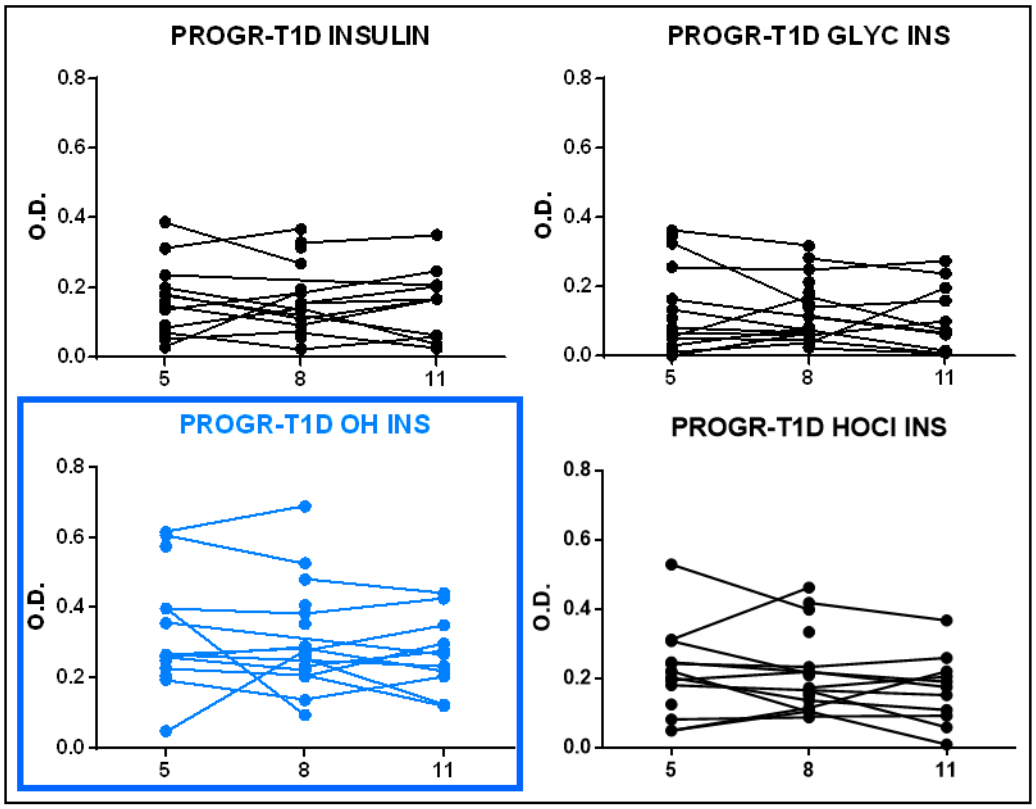

Figure 29. Longitudinal changes of binding to insulin and oxPTM-insulin in PROGR-T1D children according to age. •OH-INS is shown in evidence.

4.4.3 ICA and oxPTM-INS-Ab overlapping

IA-2A was the most specific autoantibody among the ICA markers, the IAA was the only other autoantibody that was showing an higher prevalence in PROGR-T1D compared to NP-AAB^{+}. Figure 30A shows the overlapping binding between oxPTM-insulin (oxidation was picked as an example of oxPTM) and IA-2A. In the PROGR-T1D, 15 subjects were reactive to both oxPTM-insulin and IA-2A, 6 of them were positive for oxPTM-insulin only and only 1 was positive for IA-2A. One of the 23 samples was negative for both autoantibodies. In figure 30B, the overlapping binding between oxPTM-insulin, IAA and IA-2A in the PROGR-T1D group is shown.

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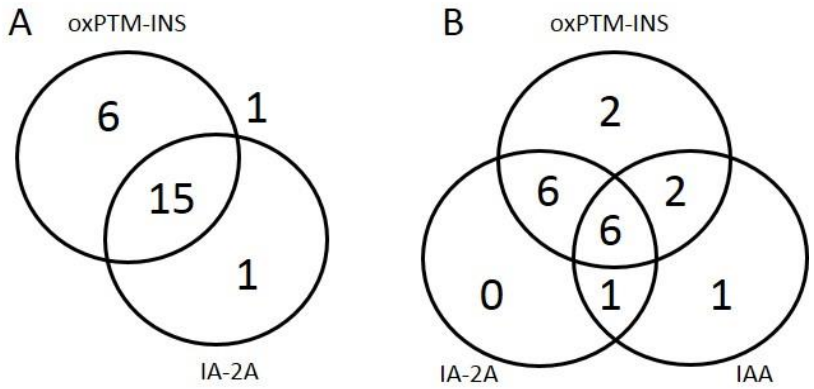

Figure 30. Overlapping reactivity to oxPTM-INS and T1D-associated antigens in children PROGR-T1D. Figure A shows the overlapping reactivity between oxidised insulin and IA-2A. Figure B shows the overlapping reactivity between oxidised insulin, IA-2A and IAA.

Most of the samples are characterised by the presence of one of the ICA (IAA and/or IA-2A) with oxPTM-insulin autoantibodies (78%) and this combination of autoantibody analysis led to the identification of 100% of PROGR-T1D subjects with a low percentage of false-positive (6 out 23 i.e. 26%), as shown in figure 31.

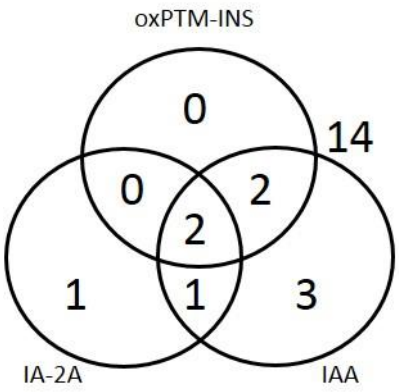

Figure 31. Overlapping reactivity to oxPTM-INS and T1D-associated antigens in children NP-ABB^{+}. Overlapping reactivity between oxidised insulin, IA-2A and IAA is demonstrated.

4.4.4 Risk of progression to T1D

In order to understand which biomarkers are the best to predict and diagnose T1D, probability of progression to T1D has been analysed. The positivity to one, or more than one, of the standard autoantibodies, ICA, and to oxPTM-INS autoantibodies

were evaluated. In figure 32A, the positivity to one ICA and more than one ICA were compare. The probability to develop T1D increases dramatically when there is positivity to at least two standard autoantibodies. The 10-year risk (95% confidence limits) in children positive for one autoantibody was 6% (1-67%), while for more than one was 70% (47-84). Figure 32B compares, in the risk group of subjects positive to more than one ICA, the difference between the sub-groups of positive and negative to oxPTM-INS-Ab. The risk to develop T1D was significantly higher (p=0.03) in individuals who were also positive to oxPTM-INS-Ab, compared with those who were negative for oxPTM-INS-Ab. Figure 32C represents the measurement of the probability to develop T1D in children positive to GADA, IA-2A and IAA. This graph shows a comparison between the standard antibodies and GADA and IA-2A associated with oxPTM-INS autoantibodies. Interestingly, children that were in this second group, had higher risk of progression to T1D within 5 years and, at 10 years of follow-up, T1D risk increased to 100%.

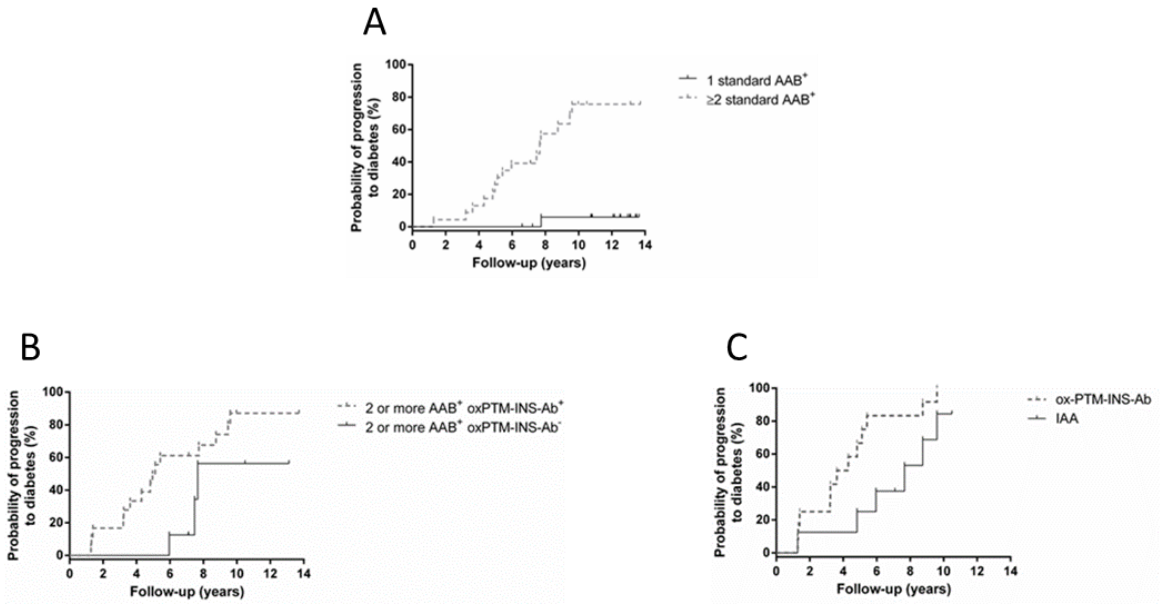

Figure 32. Probability of progression to T1D in cohort 1 from the ABIS study. Figure A shows the probability of progression to T1D based on the number of standard autoantibodies positivity; figure B shows the probability of progression according to the positivity to oxPTM-INS autoantibodies; figure 3 shows the difference in the risk to develop T1D when oxPTM-INS autoantibodies were used in place of IAA.

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4.5 Patients analysis cohort 2: plasma vs serum

The characteristics of the patients included in cohort 2 are shown in table 10, with the HLA genotyping is shown in table 11. Cohort 3 includes 50 patients and 100 samples, 1 serum samples and 1 plasma sample per patients. The patient's samples were taken fasting 10 days after diagnosis and patients were under insulin therapy since diagnosis. Information about the HLA genotype and ICA positivity were available for more than 84% of the patients. C-peptide has been analysed for all the patients at the time of diagnosis (T0) and for 47 out of 50 patients after 10 days (T10). Insulin and c-peptide are secreted in equimolar amount from the pancreatic beta-cells, for this reason the analysis of c-peptide levels is an indication of the beta-cells secretory activity. The IA-2A and the GADA were evaluated in 43 and 42 patients respectively, showing 67% and 76% of positivity.

Table 10. Characteristics of the patients included in cohort 2. (Data are presented as mean (the range value are in brackets) and percentage (n)).

Gender ratio (F/M)	0.67
Age average (y)	11.66 (3-20)
Age of diagnosis average (y)	10.6 (3-18)
C peptide day 0	0.22 (0.06-0.85)
C peptide day 10	0.11 (0.03-0.61)
IA-2A positivity	67% (29/43)
GADA positivity	76% (32/42)

The overlapping reactivity of the ICA is shown in figure 33. IA-2A alone is positive in 18.4% of patients, GADA in 26.3% and the positivity to both autoantibodies is 55.3%.

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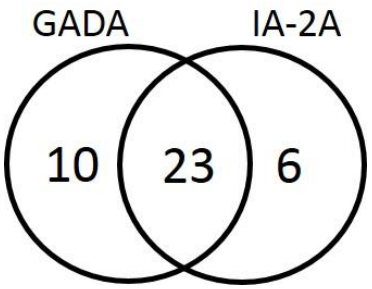

Figure 33. Overlapping reactivity of ICA. GADA and IA-2A overlaps in more than half of the samples tested.

Table 11 shows the prevalence of the HLA genotypes in cohort 2. The HLA haplotypes most represented in group 1 are DQA1*03-DQB1*0302 (15 patients), DQA1*05-DQB1*02 (17 patients); the haplotypes most represented in group 2 is and DQA1*03-DQB1*0302 (19 patients). It is known that the haplotype DQA1*03-DQB1*0302 confers susceptibility to T1D[106] meanwhile the haplotype DQA1*05-DQB1*02 is associated to T1D but more often associated to coeliac diseases[45, 107]

Table 11. Prevalence of the HLA genotypes in the patients included in the cohort 2.

HLA HAPLOTYPE 1	NUMBER PATIENTS	HLA HAPLOTYPE 2	NUMBER PATIENTS
DQA1*01-DQB1*05	2	**DQA1*01-DQB1*05**	5
DQA1*01-DQB1*06	3	**DQA1*01-DQB1*06**	3
DQA1*02-DQB1*02	1	**DQA1*01-DQB1*0602**	3
DQA1*03-DQB1*0302	**15**	**DQA1*02-DQB1*02**	1
DQA1*03-DQB1*0303	1	**DQA1*03-DQB1*0301**	2
DQA1*03-DQB1*04	1	**DQA1*03-DQB1*0302**	**19**
DQA1*04-DQB1*04	2	**DQA1*04-DQB1*04**	1
DQA1*05-DQB1*02	**17**	**DQA1*05-DQB1*02**	9
DQA1*05-DQB1*0301	1	**DQA1*05-DQB1*0301**	1

4.5.1 Autoreactivity in cohort 2

The graph in figure 34A shows the reactivity of the 50 samples, plasma and sera. This data confirmed the high reactivity of T1D patients to oxPTM-INS, in this cohort

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there is no statistical difference between reactivity to oxidised and chlorinated insulin. There is also no difference between native and oxPTM-INS.

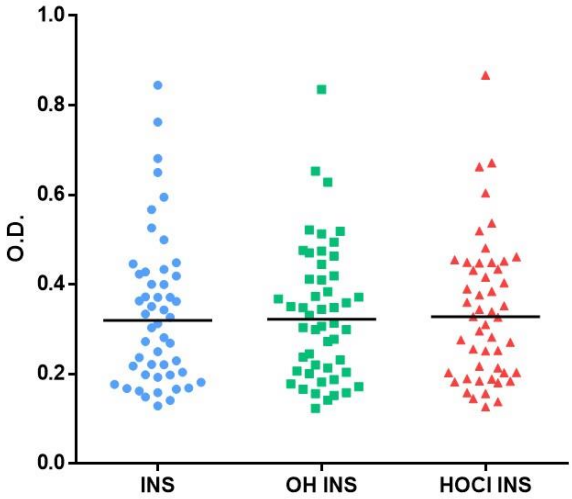

Figure 34A. Autoreactivity to insulin and oxPTM-insulin in cohort 2. Reactivity to insulin and oxPTM-INS is high but there are no differences among the three antigens tested.

The overlapping reactivity of the ICA (IA-2A and GADA) and oxPTM-INS is shown in figure 34B. The analysis of the three antibodies increased the detection of T1D patients identifying also 7 subject (15%) who were negative for GADA and IA-2A.

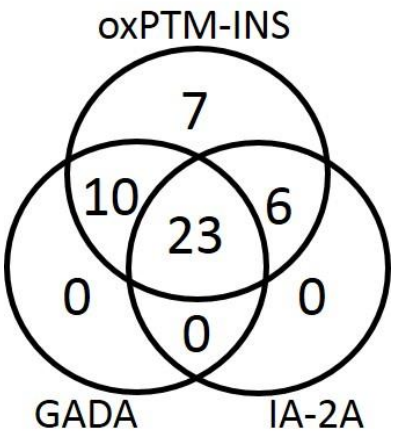

Figure 34B. Autoreactivity to insulin and oxPTM-insulin in cohort 2. Overlapping of oxPTM-INS reactivity and ICA in cohort 2. OxPTM-INS reactivity and GADA were overlapping in 33 patients; IA-2A were overlapping in 29; oxPTM-INS-Ab, GADA and IA-2A

4.5.2 Correlation serum-plasma reactivity

The 100 samples included in cohort 3 have been tested in ELISA to demonstrate the correlation between sera and plasma reactivity. The results are shown in figure 35A and 35B. Serum samples and plasma reactivity to oxPTM-insulin correlated with an R squared value of 0.94 for oxidised insulin and 0.90 for chlorinated insulin, both with a significant p value ($p<0.0001$).

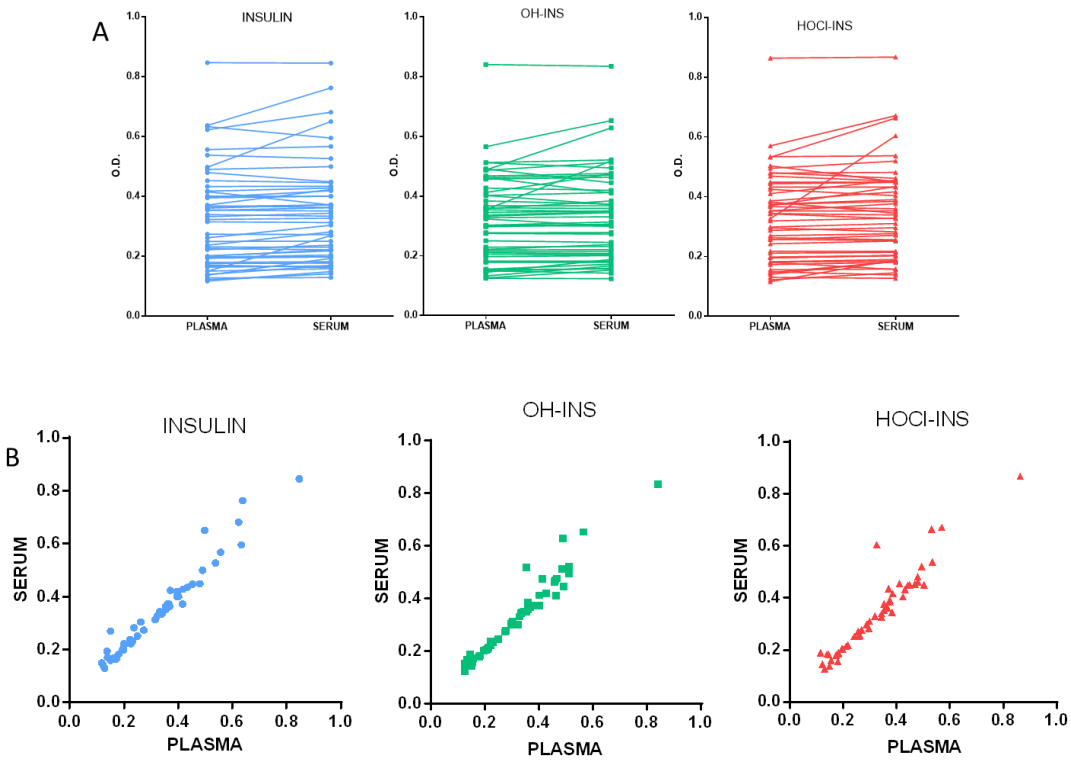

Figure 35. Comparison and correlation between serum samples and plasma reactivity to insulin and oxPTM-INS. Figure A shows a paired comparison between plasma and serum from the same patients. Figure B shows the correlation between reactivity detected in serum samples and plasma samples.

4.6 Insulin and oxPTM-INS epitope mapping

The 2D gel is a higher resolution method and it has the ability to separate whole proteins and their modifications. This ability to separate low molecular weight proteins and peptides may allow investigation of protein degradation and

cleavage fragments. Oxidised and chlorinated insulin were run in a native 2D gel to obtain a high resolution separation of the whole protein and its modifications. The first dimension was an IEF using the IPG4 system, the second dimension was a standard native PAGE in which the stacking gel was replaced by the IEF strip. The gels resulting from the 2D gel were stained with Coomassie blue and Silver Stain kit. Due to the detection limit of these two staining techniques the stained gels are not available.

To map the epitope/epitopes responsible for the autoreactivity in T1D patients, a western blot (after 2D gel) using serum samples from patients as primary antibody was necessary. The results are shown in figure 36. Figure 36A shows the western blot of the native insulin, figure 36B shows the western blot of the oxidised insulin and figure 36C shows the western blot of the chlorinated insulin. When comparing these three figures it was possible to highlight few protein spots presents in oxPTM-INS western blot and not in the native insulin, the protein spots differently expressed are displayed in red circles.

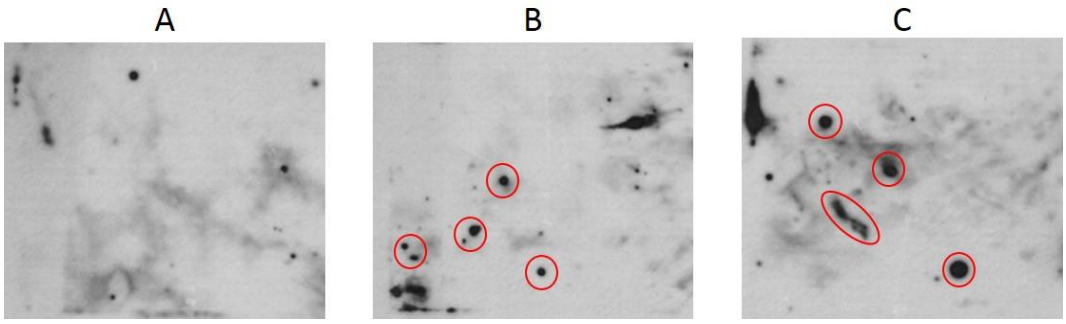

Figure 36. 2D gel-western blot of native and oxPTM-INS. The figure A shows the analysis of native insulin; the figure B shows the analysis of oxidised insulin and the figure C shws the analysis of chlorinated insulin. The red circles highlight the protein spots differently present in oxPTM-INS in comparison with native insulin.

4.7 Summary of the results

In this first half of the project I have shown that insulin is modified by ROS *in vitro*, and that these modifications are clinically relevant. The amino acid modifications in the insulin sequence have been successfully mapped by PAGE and MS/MS analysis. In addition to the changes in amino acid sequence of insulin after modification that was previously published[82], 4 additional modifications on the β-chain (His10 switched to Trp, Leu17 switched to Tyr and Cys7 and 19 were oxidised) and 5 additional oxidations on the α-chain (Tyr14, Cys6, 7, 11 and 20) were identified. I have also shown that using scavengers, in particular NAC and Chelex resin, it is possible to stabilize the post translational modifications and inactivate the residual oxidation for at least one month. The analysis of the ABIS cohort includes the study of samples collected longitudinally from children who did or did not progress to T1D after a median follow up of 10.8 years. These samples were tested for the gold standard islet autoantibodies to insulin (IAA), GAD (GADA), tyrosine phosphatase 2 (IA-2A) and zinc transporter 8 (ZnT8A). The reactivity to oxPTM-INS (oxidised or chlorinated) was evaluated by ELISA Binding and prevalence of oxPTM-INS-Ab were compared between progr-T1D, NP-AAB+ and NP-AAB− children at the earliest time point available. In the progr-T1D group, positivity to at least one oxPTM-INS was over 90%, indicating the possibility to discriminate between children progressing to T1D and children who did not progress, in particular for oxidised modified insulin. Most of the patients are characterised by the presence of one or two of the ICA (IAA, IA-2A GADA, and/or ZnT8A). IA-2A was the most specific marker among ICA and the assessment of autoantibodies to oxPTM-INS in combination with IA-2A and IAA led to the identification of 100% of PROGR-T1D subjects with a low percentage of false-positive. Children positive to at least one ICA were stratified in two sub-groups with different risk based on autoantibodies to oxPTM-INS positivity. The risk to develop diabetes was higher children positive for more than one ICA when they were also positive to autoantibodies to oxPTM-INS. Importantly, replacing IAA with autoantibodies to oxPTM-INS, children had two-fold higher risk to progress

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to T1D within 5 years and 100% of the risk to develop T1D at 10 years of follow-up. The analysis of cohort 2 led to the identification of the best ELISA conditions indicating that, with the right conditions, the assay was stable and confirming the results previously obtained and published[82, 105]. Cohort 3 allowed us to confirm the high reactivity to oxPTM-ins in serum samples and plasma with an almost perfect correlation between serum and plasma from the same patients. In this study I have also started mapping the epitopes responsible for the autoreactivity in T1D patients using the 2D gel technique to separate all the fragments after oxPTM. The preliminary results were promising, highlighting protein spots for the next step, sequencing by mass spectrometry.

This data indicated that oxidised insulin allowed discrimination between children progressing to T1D and children who did not progress, even if they are positive for at least one ICA ($p < 0.006$), with 79% sensitivity and 92% specificity.

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CHAPTER 5

TYPE 1 DIABETES: DISCUSSION

T1D is autoimmune disease characterised by hyperglycaemia and β cells disruption that cause long term complications even under insulin therapy. T1D is becoming a significant epidemic issue with over half a million children effected[34]. Although the importance of the autoimmunity component in the development of the disease is well known the exact pathobiology and cause are not fully understood. Several studies show that insulin is the most specific autoantigen in T1D[86,108,109]. Antibodies against a range of self-antigens IAA, GADA and IA-2A have also been detected in T1D patients and together with antibodies to insulin are used for the diagnosis (combined with blood analysis of sugar level)[31,38]. Chronic inflammation and hyperglycaemia in T1D results in oxidative stress and increased in the generation of reactive oxidants. The abnormal presence of ROS may lead to cellular damage, alteration of the pathways and structural changes of self-proteins as a results of oxidative post translational modification. OxPTM play a predominant role in formation of oxidative insulin neoepitopes. Indeed, autoreactivity to oxPTM-INS is significantly more prevalent than IAA allowing the identification of 34% of patients who were IAA negative [82]. The aim of this first part if the study was to confirm the presence of oxPTM-INS-Ab and to evaluate their predictive value. Hence, part of the study was dedicated to characterisation of the neoepitopes responsible for the development of autoimmunity in T1D.

5.1 OxPTM-INS characterization

The hypothesis of this study was that ROS released during chronic inflammation post-translationally modifies insulin, in the process of balancing their unpaired electronic state[15]. Our group has already demonstrated the presence of antibodies to oxPTM-INS in subjects with T1D [82]. Initially, I have confirmed the data previously

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published by reproducing and validating the protocols for the *in vitro* oxPTM, both by oxidation and chlorination. I next focused my research in establishing long term storage stability of the oxPTM-INS.

In order to be sure that oxPTM-INS was always modified in a comparable way, every batch was tested using several techniques. Three different protocols for PAGE: native, SDS and tricine were used to confirm the changes in insulin after exposure to ROS. Standard SDS-PAGE (figure 18A) was not showing any difference between native and modified proteins whereas using either native (figure 18B) or tricine gels (figure 19) demonstrates the changes in the insulin after exposure to ROS. Each batch was also tested by ELISA and western blot to confirmed the binding of T1D samples to oxPTM-INS as previously described[82].

The mass spectrometry analysis was repeated in collaboration with the Centre for Proteomics based at Cambridge University to precisely identify the exact amino acids that are oxidised following oxPTM of INS. The previously reported oxidised amino acids were confirmed[82] but new oxidised regions were detected (figure 22). This includes oxidation of Cys7 and Cys9 on the β-chain and oxidation of Cys6, 7, 11 and 20 on the α-chain. It is well known that an important class of redox modification occurs to the thiol (-SH) group in the side chain of cysteine residues, which can produce multiple chemically distinct alterations to the protein: sulfenic, sulfinic and sulfonic acid, and disulfides[110]. These oxPTM are shown to affect the protein structure and function[111]. The sulphur atom in the thiol group is electron rich and the availability of different oxidation states permits the formation of a diverse range of ox-PTM[112,113]. Depending on the oxidation states the reaction with cysteine thiol group can lead to the disruption of the disulphide bridges in the insulin structure forcing the separation of the two chains.

Another additional amino acid oxidation was detected on Tyr 14 on the α-chain. The main mechanism of the oxidation of tyrosine residue is well characterised. The first step involves the addition of an OH radical on the cycle followed by a slow OH^- . Using different kind of oxidants, tyrosine can undergo oxidation giving its radical

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cation that deprotonates[114]. These new modifications on the α-chain emphasize the importance of the structure and conformation of the modified protein in the generation of neoepitopes. On the β-chain, in the area of the immunodominant epitope, two amino acid conversions were newly identified. The first amino acid conversion was the His 10 to Trp due to the formation of the benzene ring on the side chain of the tryptophan; the second conversion was Leu 17 to Tyr. The mass spectrometry analysis used helped to identify all the amino acids modified in the insulin sequence after exposure to ROS. The limit of this analysis is the identification of all the amino acids modification present in the mixture of modified insulin molecules that are not homogenously modified, this means that these modifications may all coexist on the same molecule or there may be combination of them on different insulin molecules. The results of these changes in the protein structure are likely to be the biggest process involved in the generation of neoepitopes. Even if there are several amino acids that are target of ROS modifications, due to the insulin sequence and amino acids position and interaction, cysteines seems the target that may lead to the biggest structural changes. To understand how exactly the insulin is modified and which modifications have a clinical relevance, more studies are required.

My observation correlates with studies previously published, although the exact amino acid sequences of the neoepitopes responsible for the autoreactivity in T1D patients is not known yet. Previous data suggested that the immune response was mainly directed to the β-chain of the insulin, and in particular to the immunodominant epitope (B:9-23)[82]. The mass spectrometry mapped oxPTM-INS on the β chain[82] correlating with previous study that described modifications at the N-terminal of the β chain[84,85]. All these studies have confirmed the chlorination of Tyr 16 in the β chain as main modification within the T cell immune dominant epitope[86]. I have shown that the area B: 9-23 is the area containing the largest number of modifications, as expected. I have confirmed the chlorination on the Tyr 16 but additionally I have shown 3 big modifications that may change completely the protein conformation. The oxidation of cysteines involved in the

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disulphide interchain bonds and the conversion of amino acids are massive modifications and draw attention to the possible change of insulin conformation with the consequent generation of neoepitopes.

5.2 OxPTM-INS stability

In order to respect criteria of consistency and reproducibility, especially on the analysis of larger cohorts of patients, it become necessary to analyse the stability of the modified proteins. I have compared fresh modifications and 1 month-old modifications showing that modified insulin was not stable and almost completely degraded after one month (figures 23 and 24). To improve the stability of oxPTM-INS and inactivate the excess of ROS present in the modified protein mixture, several scavengers were tested. The scavenger tested were chelating resin and NAC. Both are well known scavengers in oxidation. The chelating resin, normally used for purification via ion exchange, contain paired iminodiacetate ions which act as chelating group and also as weak cation[115]. The NAC is often used as scavenger because, with its thiol group, it represent an usual target for oxPTM[110]. Experiments performed using a chelating resin to stabilize oxidised insulin were efficacious. Different concentrations and combinations with different amino acids were tested but, regardless the concentration of amino acids or the combinations added, the chelating resin was able to stabilize oxidised insulin. Fresh modifications alone and 1, 2, and 3 weeks-old modifications plus chelex were showing a comparable reactivity with non-significant differences (figure 25). NAC was also tested as scavengers to stabilize both, oxidised and chlorinated insulin. In both cases the reactivity of T1D patients was comparable to the reactivity to fresh modifications, this was happening for all the conditions tested (figure 26). Chelex and NAC were chosen knowing their potential as scavengers and the results obtained confirm that they are both good candidates as scavengers to deactivate ROS and to stop the reaction on the insulin structure that could slowly lead to the degradation of the whole protein.

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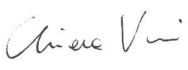
5.3 OxPTM-INS reactivity: ABIS study

The higher the level of ROS production during the hyperglycaemic state in T1D, the lower level of ROS scavengers in the β cell microenvironment. The presence of chronic inflammation, and thus high ROS levels, leads to the increase of oxPTM[82]. This is likely the origin and the cause of the high prevalence of reactivity to oxPTM-INS compared to the native protein, suggesting a relevant role of these ROS-generated neoepitopes in the pathogenesis of T1D. This part of the study supports the idea of oxPTM-INS as prominent antigen in T1D. Indeed, it is known that autoreactivity to oxPTM-INS is significantly more present than the IAA (gold standard method used)[82]. To verify the hypothesis of a predictive value of oxPTM-INS-Ab in T1D patients I have analysed the reactivity in a specific cohort of children from the ABIS study. This study includes samples from collected from all the babies born in south east Sweden between 1-10-1997 and 1-10-1999, the babies were followed up and samples were collected at different time point without selecting the participant according to diabetes risk or any other autoimmunity or autoinflammatory predisposition. The main strength of this study is the prospective evaluation of oxPTM-INS-Ab in a well characterised cohort of children from the general population. Additionally, children progressing to T1D were longitudinally followed up allowing us to investigate whether oxPTM-INS-Ab develop very early in the natural history of the disease. The limitations of this study are represented by the lack of earlier time point in the tested group of children progressing to T1D. Indeed, the earliest time point available for our cohort was 5 years of age. Data from birth cohorts suggest the high possibility of seroconversion in high risk individuals around 2 years of age [116]. Another limitation is represented by the determination of the ZnT8A, the analysis of this autoantibodies was performed only on a limited number of children, due to the insufficient availability of this modern technology when samples were collected. The cohort analysed was divided in 3 groups giving the opportunity to compare seropositive children that progress to T1D to the one that did not progress to T1D positive for the ICA. The



analysis of this cohort confirmed the data previously published, about 80% of the children were double positive for oxPTM-INS and about 96% were positive for at least one of the oxPTM-INS tested detecting more than one third of the children progressing to T1D negative for IAA. Consistently with previous findings, we have shown a low predictive accuracy of ICA when analysed as single test even if the presence of at least 2 ICA can be considered a very early stage of asymptomatic T1D[117] and most of the factor associated with the developing of T1D are also associated with the ICA positivity[118]. OxPTM-INS had a higher predictive accuracy than the other ICA and, used in combination with IAA and IA-2A, were able to detect all the children progressing to T1D. This data shows for the first time the presence of oxPTM-INS-Ab before the T1D clinical onset, this supports the hypothesis that the unbalanced production of ROS take place before the onset, having an important role in the T1D pathogenesis[119]. Thinking about previous publications[82,105] and the results obtained from the analysis of this cohort, it is very important that these findings can be applied to the general population to predict the risk of T1D in new born children. These findings suggest that oxPTM of β cell autoantigen precede T1D onset and that oxPTM-INS-Ab may be used as biomarker for the prediction and diagnosis once this result will be confirmed in larger cohorts. To our knowledge, this is the first study investigating antibody reactivity to oxPTM of a β cell antigen as a predictive biomarker of T1D. Additional neoepitopes derived from PTM of β cell autoantigen have been described in humans and animal modes of T1D, including antibody response to oxidised GAD and reactivity to citrullinated GAD[83,120], C-peptide deamination[121], and hybrid fused peptides[119,122]. The response to modified antigens may also involve proteins that are not proper β cell antigens, such as type II collagen that has been tested previously by members of my research group[81]. The accuracy of oxPTM-INS was better than accuracy of the standard IAA and GADA. This study shows the superiority of oxPTM-INS-Ab over IAA and this result is consistent and confirmed in two different studies carried out by our group[82,105]. In newly diagnosed T1D, oxPTM-INS-Ab were more sensitive than IAA, detecting over one third of subjects negative for IAA[82]. A

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very similar result was obtained analysing the ABIS cohort[105]. These two publications enhance the hypothesis that insulin is a predominant antigen in most T1D patients, and that oxPTM may intensify the autoimmune reactivity. To explain the involvement of oxPTM-INS in T1D pathogenesis, we hypothesize two main mechanisms: the first mechanism involves reactive oxidants released by leukocytes during insulitis in insulin modification, the second mechanism involves an altered redox state within beta-cells under stress[82,105]. Some of the oxPTM are predominantly related to immune system activation, such as HOCl production by myeloperoxidase during inflammatory burst[123], therefore acting on the secreted insulin. Other oxPTM may come from oxidants produced in the beta-cell (•OH) in response to pro-inflammatory or metabolic stress[124]. The concept of oxPTM applied to self-proteins that can trigger autoimmune response and enhance the reactivity in T1D patients may be extended to other proteins such as (pro)insulin, or C-peptide[121].

In conclusion, oxPTM-INS-Ab may identify children progressing to T1D with a higher sensitivity and specificity than the current standard autoantibody markers, such as GADA and IA-2A. Furthermore, in combination with the current standard biomarkers, oxPTM-INS-Ab lead to the identification of 100% of children progressing to T1D. To confirm the predictive potential of oxPTM-INS-Ab and confirm these findings, additional studies with larger cohorts are required.

5.4 OxPTM-INS reactivity: plasma vs serum

As aforementioned, the predictive value of oxPTM-INS-Ab needs to be validated in larger cohorts. All the previous data about oxPTM-INS reactivity were obtained analysing serum samples[82,105], however the availability of samples in large cohort studies often vary between serum and plasma. It was therefore important to compare the reactivity in plasma and sera from the same patients. The results of this comparison showed a very high correlation between serum samples and plasma reactivity (figure 35B). The importance of this part of the study is understandable if we think that both plasma and serum derived from whole blood,

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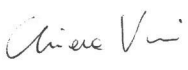
but the collection procedures are different, as well as the influence on the coagulation cascade and all related proteins[125]. In some cases, the difference is not significant even if the different collection procedures may alter metabolite profiles and some molecular pathways that have not be fully characterized[125]. The use of the wrong biological fluid can lead to improper results. For this reason, I have analysed plasma and serum from the same patients obtained from the same blood donation. The results obtained allowed us to analyse either plasma or sera from T1D patients opening a new scenario and a considerable number of opportunities to validate our clinical test.

5.5 OxPTM-INS: epitope mapping

The pathogenesis of T1D is not well characterised, with the epitopes responsible for the autoimmunity and the first autoantibodies produced during disease currently unknown. The process of epitope mapping is necessary to identify the binding site on the oxPTM-INS sequence that is responsible for the reactivity, and to understand if in this case the epitopes are linear, conformational and/or discontinuous. Based on the mass spectrometry analysis and on previous publications[82,97,103], there is a high probability that the neoepitopes are conformational epitopes. The modifications mapped by mass spectrometry and the difficulties to visualize oxPTM-INS in SDS-PAGE may be an indication of the kind of neoepitopes forming. Indeed, starting from our results, it is possible to suppose that the newly formed neoepitopes after oxPTM are conformational. Technologies that can be used for the epitope mapping are varying and some of them can be combined to obtain a higher resolution. Of all the techniques available, including BIAcore, X-ray crystallography and mutagenesis, I have decided to use the 2D gel technique associated with a western blot. These 2 techniques help to get a good separation, based on pH and size, among all the oxPTM-INS fragments and to quick analyse the T1D serum samples reactivity to the different fragments. This double separation is commonly used to identify small



proteins and peptides that appear in the gel as spots. The comparison between the insulin 2D gel and the oxPTM-INS 2D gel provided some different reactive protein spots present in the oxPTM-INS but not in the native insulin 2D gel (figure 36). The appearance of several protein spots per modification can be explained with the possibility of the presence of more than one epitope responsible for the autoreactivity. The epitope mapping owes its importance to involvement in the development of therapeutic monoclonal antibodies, stable diagnostic and prognostic assays, the understanding of the mechanism of the disease and allowing the determination of specific mechanism of interaction antigen-antibody in pathologies but also in monoclonal antibody. However, there are a considerable number of antibodies targeting conformational epitopes and consequently more publications investigating this kind of epitopes, the difficulties of mapping conformational neoepitopes are always more than mapping only the amino acids sequence as indeed they are only formed in the native structure of the protein[97]. Insulin and oxPTM-Ins are likely part of this group of epitopes. The epitope mapping was challenging mainly because all that techniques used had to be converted and revised to work without denaturing native insulin and its modifications. Due to this particular and very complicated situation and to all the new settings that had to be tested I was able to identify some interesting protein spots using the 2D gel-western blot, but I could not map the amino acid sequence. Indeed, the amino acid sequence of these neoepitopes, presented in the different protein spots, may be completely independent or may overlap by a few amino acids. In order to understand which is the case, this epitope mapping study needs to be elaborated and repeated using a bigger group of T1D serum samples before proceeding to the mass spectrometry analysis of the most promising protein spots.

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CHAPTER 6

INFLAMMATORY ARTHRITIS: INTRODUCTION

6.1 Background inflammatory arthritis

Inflammatory arthritis (IA) is a group of progressive chronic diseases characterized by inflammation and swelling of the joints, stiffness, pain, restricted motions and reduced physical strength. Many of the subsets of inflammatory arthritis are considered to be autoimmune diseases. IA is estimated to effect around 3% of the world's population. The most common types of IA are rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS). Figure 37 shows a comparative illustration that highlights the main differences between these three types of IA. The prevalence of each of these subtypes worldwide is as follows: about 1% for RA[126] and 1.9 % for spondyloarthritis (SpA). It should be noted that SpA includes AS and PsA and a few other arthritic conditions[127]. The prognosis for patients with these types of IA is improving after the introduction of new disease-modifying anti-rheumatic drugs (DMARDs) and new biological agents, such as tumour necrosis factor (TNF) inhibitors[128]. There is much supporting evidence for the benefits of diagnosing and treating IA at the early stages of disease[129, 130]. This "window of opportunity" hypothesis for therapeutic intervention is based on the existence of a time frame within which there is a potential for a greater response to therapy, resulting in sustained benefits both socio-economically and for the patient. There is increasing evidence for the beneficial effects of early DMARD therapy over delayed treatment in patients who present with arthritis of recent onset. Unfortunately, the aetiology of IA is not clear, several studies suggest that genetics play a key role but they also show that genes alone do not determine the development of these diseases and that environmental factors trigger an abnormal immune response[131, 132, 133].

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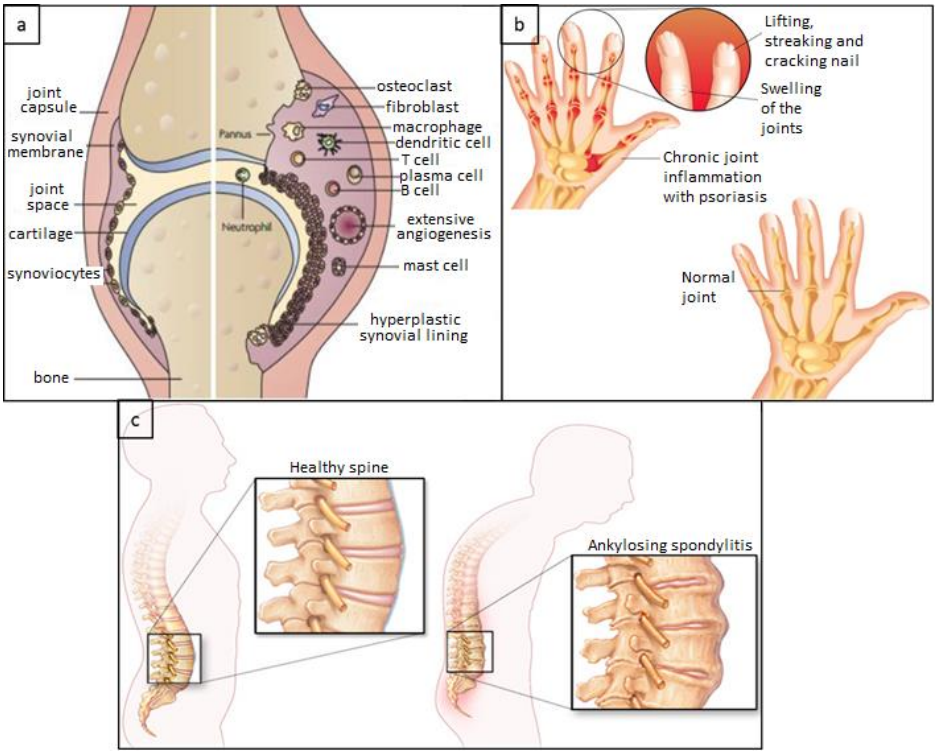

Figure 37. Comparison of healthy and rheumatic joints in the three most common types of IA. Figure A shows a comparison between healthy knee and a knee from a RA patient[134]. Figure B shoes swelling and psoriasis in hand from PsA patient[135]. Figure C shows a comparison between healthy and ankylosing spondylitis spinal column[136].

6.2 Rheumatoid arthritis

RA is a chronic inflammatory disease associated with articular damage, leading to morbidity and increased mortality[137, 134]. In RA the synovium becomes inflamed resulting in an influx of immune cells such as macrophages, plasma cells, dendritic cells, T lymphocytes and mast cells, and the activation of resident cells such as synovial fibroblasts. Type II collagen (CII), is the main constituent of articular cartilage, thus it is a prominent target for post-translational chemical modification by ROS in inflamed joints. In the serum and synovial fluid of RA patients, autoantibodies have been found to be present, towards both native and citrullinated CII[138, 139] and more recently against oxPTM-CII[9, 140].

As with many autoimmunity diseases RA is associated with the presence of auto-antibodies in serum and synovial fluid. The first autoantibody described in RA was

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rheumatoid factor (RF) leading to the discovery of subsequent autoantibodies that have been associated with RA. Together with RF, anti-citrullinated protein antibodies (ACPA) show consistency and prognostic value. RF is an antibody directed to the Fc portion of IgG, bound together to create an immune complex that contributes to the development of the disease[141]. Under inflammatory conditions, arginine amino acid can be enzymatically converted into citrulline in proteins. If protein shape and structure are significantly altered, the immune system may be able to recognize these as antigens, leading to an autoimmune response[142]. ACPAs are directed to peptides and proteins that are citrullinated. They are present in over 60% of patients with rheumatoid arthritis and are used as a diagnostic biomarker for RA[143]. Compared to RF, they show higher specificity in RA patients. Conversely, autoantibodies to native CII have been reported since the 1970s[144] and have low sensitivity and specificity. It remains unclear how clinically significant autoantibodies to CII are[145]. It is already known that autoantibodies to native and denatured CII are detected in 10%-30% of serum samples from RA patients[146,147] and they are also present in synovial fluids[148].

6.3 Spondyloarthritis

The term SpA is used to identify a group of inflammatory chronic diseases characterised by chronic back pain and arthritic inflammation. It contains a group of diseases involving the joint and enthesis. SpA prevalence ranges is from 0.3% to 1.9%[127, 149, 150, 151]. The most common diseases in this group are AS, PsA, inflammatory bowel disease (IBD) and reactive arthritis (ReA). All these different subgroups of SpA seem to have a common genetic component, a predisposition to the HLA-B27[152] genotype.

SpA is characterized by inflammation of sacroiliac joints and/or joints between the vertebral bodies. The term, sacroiliitis refers to the inflammation of the lower sacroiliac synovial joint whereas the term spondylitis is used to identify inflammation of the interface between the vertebral bodies. When the inflammation results in osteoproliferation and fusion of the joints the disease is referred to as ankylosing[152].

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Based on symptoms, SpA may be divided in two subgroups, as clarified in figure 38: axial SpA (axSpA) that is characterised by lower back pain, and peripheral SpA that is characterised by swelling of the arms and legs. AxSpA are then subdivided into AS, that is defined by radiographic changes, and axSpA, without radiographic changes. Peripheral SpA include ReA, PsA and IBD[153].

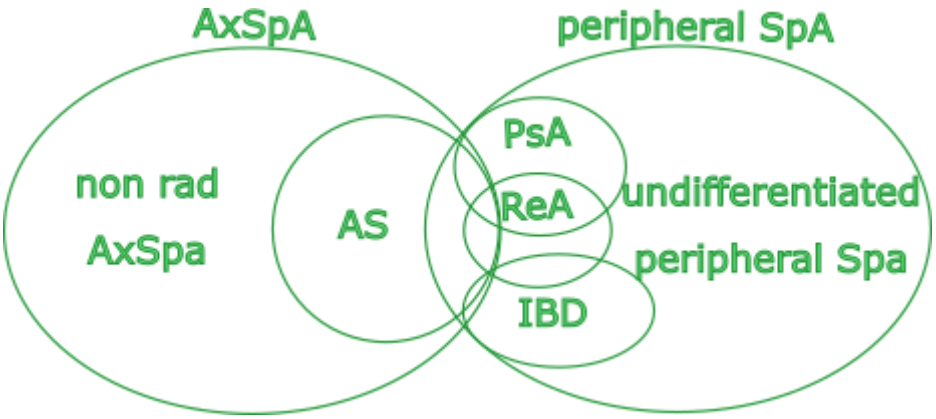

Figure 38. Classification of SpA. Based on symptoms, SpA may be divided in two subgroups, axSpA and peripheral SpA. AxSpA are then subdivided into AS and axSpA, without radiographic changes. Peripheral SpA include ReA, PsA and IBD. [Adapted from Raychaudhuri et al. Journal of Autoimmunity].

Diagnostic tests for this group of diseases are not currently available despite the high prevalence. The well-defined classification criteria were developed to help clinicians achieve the correct diagnosis. Although there is not a specific treatment for SpA, several publications have proposed recommendations and combinations of drugs with the aim of alleviating symptoms [154]. For example, the Assessment of SpondyloArthritis international Society (ASAS) suggested a combination of pharmacological therapy and physiotherapy treatment as the most successful treatment for AS, which is also applicable to patients with other subgroups of SpA[154]. The first stages of axSpA are usually treated with non-steroidal anti-inflammatory drugs (NSAIDs). In cases of insufficient response or when collateral effects are observed, treatment with anti-TNF blockers can be used as suggested in several studies[155, 156]. DMARDs can also be used as treatment for SpA which often helps to relieve symptoms and in some cases prevent joint damage[157].

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Genome-wide association studies (GWAS) have identified several genes associated with SpA, particularly with AS. In a large study, 44% of the genetic predisposition was attributable to variants in the MHC referring mainly HLA-B27, but also HLA-B40, HLA-B51, HLA-B7, HLA-A2, and HLA-DPB1. The remaining 38% of predispositions are non-MHC variants or not yet identified[158]. The haplotype HLA-B27 is very common in the Caucasian population and studies have shown its influence in the involvement in the development of SpA when it is combined with "other factors" that are not very well characterised[159].

The correlation to HLA-B27 varies among different geographic and ethnic groups but overall, the prevalence of SpA is higher in populations with a higher prevalence of HLA-B27. The correlation between SpA and HLA-B27 differs between the different subgroups of SpA (table 12); AS has the clearest association[160, 161].

Table 12. HLA-B27 prevalence in different kind of SpA[161].

DISEASE	PERCENTAGE OF PATIENTS HLA-B27 POSITIVE
AxSpA (including AS)	• Caucasians: 92% • African-Americans:50%
ReA	60-80%
Enteropathic arthropathy or SpA associated with IBD	60%
PsA	40-50%
Undifferentiated SpA	20-25%

SpA=spondyloarthritis, AxSpa=axial spondyloarthritis, AS=ankylosing spondylitis, ReA=reactive arthritis, IBD=Inflammatory bowel disease.

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6.3.1 Axial Spondyloarthritis

Axial SpA (axSpA) is a form of SpA and includes different conditions characterised by chronic inflammation and arthritis involving the spine and the sacroiliac joints. AxSPA results in back pain, fatigue, stiffness and may lead to ankyloses[155]. Based on symptoms, axSpA are subdivided into two groups: 1) ankylosing spondylitis, whereby bone damage in the spine/sacroiliac joints have been already developed and; 2) non-radiographic axial SpA (nr-axSpA) which describe patients who appear negative upon radiographic analysis. Diseases grouped as nr-axSpA may be early or middle stage of AS or they may not develop any structural bones damage in the axial skeleton[159, 162]. The age of the disease onset is usually between 15 and 30 years. Slightly more patients with AS are female than male, with a female to male ratio approximately of 2–3:1. However, the sex correlated frequency in nr-axSpA patients is equal[163]. The incidence of axSpA may be underestimated due to unreported cases. In addition to this, more data are available for the prevalence of AS than there are for axSpA as a whole. The reported prevalence of axSpA is between 0.32% and 1.4%[151, 164, 165]. Most studies of pathogenesis, especially those of genetics, have in the past focused on AS, which constitutes a more homogeneous group than that of all patients with axSpA. The bone damage (erosion, sclerosis, joint fixation) is irreversible and may be progressive, whereas AxSpA in general may be associated with systemic inflammation and therefore, patients may present with eye, skin and gut complications[166]. The ASAS criteria for axSpA were developed to support the diagnosis. A patient would be diagnosed with axSpA if the individual presents with sacroiliitis upon imaging in addition to at least one other feature listed in the figure below. Alternatively, a diagnosis for axSpA would require HLA-B27 positive status in addition to at least two other features as clarified in figure 39.

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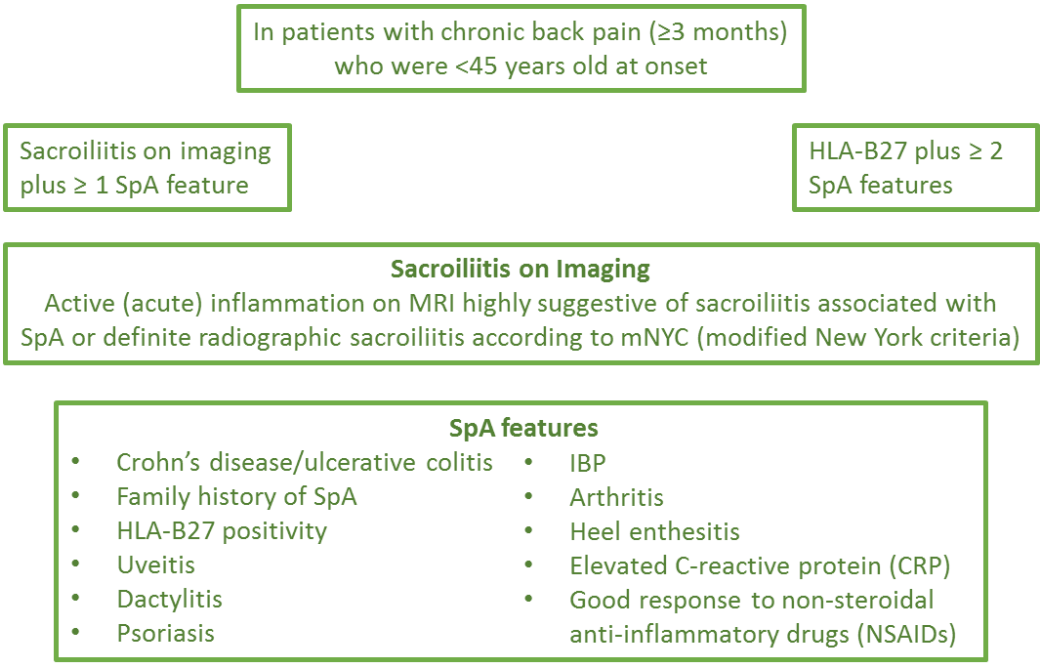

Figure 39. Diagnostic criteria of axSpA. The Assessment of SpondyloArthritis international Society criteria for axSpA were developed to support the diagnosis. A patient would be diagnosed with axSpA if the individual presents with sacroiliitis in addition to at least one other feature listed or HLA-B27 positivity and at least two other features. [Adapted from Rudwaleit, et al. Annals of the Rheumatic Diseases].

Non-radiographic axSpA

After the "Modified New York Criteria" the term nr-axSpA is used to classify the early disease stage of ankylosing spondylitis, as well as less severe forms of ankylosing spondylitis or, in general, forms of axSpA with no radiographic changes visible[159]. Studies by Groupe Français d'Etude Génétique des Spondylarthropathies has demonstrated that the presence of radiographic sacroiliitis increases with increasing disease duration. Moreover, about 60% of patients will require 10 or more years of active disease to present with radiographic changes. This study underlined the importance of this new classification and how it can help decide upon the type of treatment and management of the disease[167]. Patients characterised by the presence of an AS

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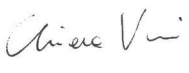
clinical picture but negative for radiographic sacroiliitis would today be referred to as nr-AxSpA. This diagnosis is based on magnetic resonance imaging (MRI) and/or specific clinical manifestations. Although there is no clear explanation for this phenomenon, the existence of protective factors against new pathologic bone formation should be questioned. Due to the new classification, the epidemiology of nr-AxSpA is still being investigated. However, it has been shown that a high percentage of patients with nr-AxSpA will progress to AS after many years of disease, and this progression is observed in about 10% of patients over 2 years of follow-up[168]. On the other hand, not all patients follow the same characteristics of progression. Some patients will have nr-AxSpA for their entire lives without presenting with damage detectable by radiography and the disease would still progress to AS[167]. Identification of the differences between AS and nr-axSpA are important mainly because these may influence the treatment strategy. Some TNF-inhibitors that have been approved as medications for AS may help towards treating the nr-symptoms of axSpA[169].

Ankylosing Spondylitis

The modified New York criteria for diagnosing AS were devised in 1984[155]. A patient must then present with radiographic sacroiliitis and at least one of the following features: inflammatory lower back pain and stiffness (for a minimum of three months duration) that improves with exercise but not relieved by rest; limited motion in the lumbar area; limitation of chest expansion[162].

As previously explained for SpA, AS is a chronic progressive condition that cannot currently be cured. It is managed primarily with physiotherapy, occupational therapy, hydrotherapy, NSAIDs, and anti-TNF therapy[170]. AS has an association with HLA-B27 and mainly involves the spine and the sacroiliac joints[152]. It is considered an immune-mediated inflammatory condition where different subsets of T cells play a critical role to induce the inflammatory proliferative cascades associated with pannus formation and joint destruction[171]. Recent studies have investigated the involvement of several immuno-modulatory pathways in AS, such



as IL-17, already associated to distinct autoimmune diseases[172]. In contrast with RA, AS involves both inflammatory erosive osteopenia and bony overgrowth. The chronic inflammation in the joints is linked to an excess of pro-inflammatory cytokines[173], free radicals[174], and MMPs[175]. The high influx of metabolically active immune cells into inflamed joints consumes increased amounts of oxygen in association with respiratory bursts, resulting in the generation of reactive oxidants which contribute towards acute and chronic inflammation[15, 176].

6.3.2 Peripheral SpA

Peripheral spondyloarthritis is a group of SpA characterised by symptoms localised predominantly or entirely on the periphery of the body. These features include arthritis, enthesitis, and dactylitis, with peripheral manifestations of musculoskeletal involvement also often present. In this subgroup are patients who have PsA, ReA, SpA related to IBD, but also a subset of patients who present symptoms that do not meet the definitions of axSpA[177, 178]. There are no general guidelines to distinguish among the different diseases grouped as peripheral spondyloarthritis. The most well identifiable peripheral spondyloarthritis is PsA, even if in some cases the characteristic skin manifestations do occur after the rheumatic symptoms[179, 180].

Psoriatic Arthritis

PsA is a form of chronic arthritis associated with psoriasis[181] that is associated with a high body mass index and obesity[182, 183], the presence of viruses, microtrauma and biomechanical stress[184] that stimulate the innate immune response and cause persistent inflammation. PsA affects women and men equally and the age of the onset is usually between 30 and 50 years[185]. The incidence of the condition is estimated to be approximately 6 cases per 100,000 per year. In PsA, the skin and joints are characterized by a prominent lymphocytic infiltrate of activated $CD4^+$, $CD8^+$ T cells and with increased infiltration of neutrophils[181, 186]. T helper (Th) 17 cells are increased in the circulation of PsA patients and their production of

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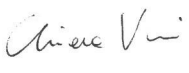
interleukin (IL)-17 acts on the different cell types of the skin, further stimulating the production of pro-inflammatory antimicrobial peptides, chemokines and angiogenic factors along with the additional recruitment of inflammatory cells[187, 188]. Cytokines, such as TNF and IL-1β, increase the production of MMPs from macrophages that contribute to cartilage degradation[189, 190]. Cutaneous psoriasis usually precedes the onset of arthritis in PsA in 70% of cases, whereas in 15% of cases the arthritis precedes psoriasis and in a further 15% of cases, psoriasis is present at the time of arthritis onset, but it not detected by the clinician[179, 180]. There are no diagnostic criteria or laboratory tests specific to PsA. A diagnosis of PsA is usually made on the basis of an inflammatory arthritis accompanying psoriatic skin and/or nail disease in a characteristic distribution, accompanied by enthesitis and/or tenosynovitis commonly in the absence of RF. The Classification for Psoriatic Arthritis (CASPAR) criteria were devised in 2006 to aid the classification of participants enrolled in research studies[191]. Patients at the early stages of PsA are treated with non-steroidal anti-inflammatory drugs, simple analgesia, physiotherapy and occupational therapy. Advanced stages of the disease require more intensive therapy with synthetic and biologic disease modifying anti-rheumatic drugs[192]. Since diagnostic tests nor a cure are available, 47% of diagnosed cases are reported to have peripheral radiographic erosions, and 56% require the use of DMARDs after two years [181].

6.4 Potential autoantigens: type II collagen

Collagen is the main structural protein in the human body. Its expression profile is distributed in many parts of the body and plays a major functional role in many structures and organs. For example, in bones, muscles, skin and tendons, collagen forms a scaffold to provide strength and structure[138].

There are multiple forms of collagen, type II collagen (CII) is a fibrillar collagen found predominantly in cartilage and vitreous humour. The protein is approximately 140kDa in size and it consists of three identical chains. Type II



collagen is the main component of the articular cartilage that comprises up to 50% of all cartilage proteins. The molecular component of a collagen fiber, consisting of three polypeptide chains coiled around each other known as tropocollagen (Figure 40). The amino acid sequence of CII follows a regular and distinctive arrangement with proline or hydroxyproline and glycine (Gly) accounting for over 30% and 33% of the sequence respectively, and often follows the pattern Gly-X-Y, with X being proline and Y proline or hydroxyproline predominantly[193].

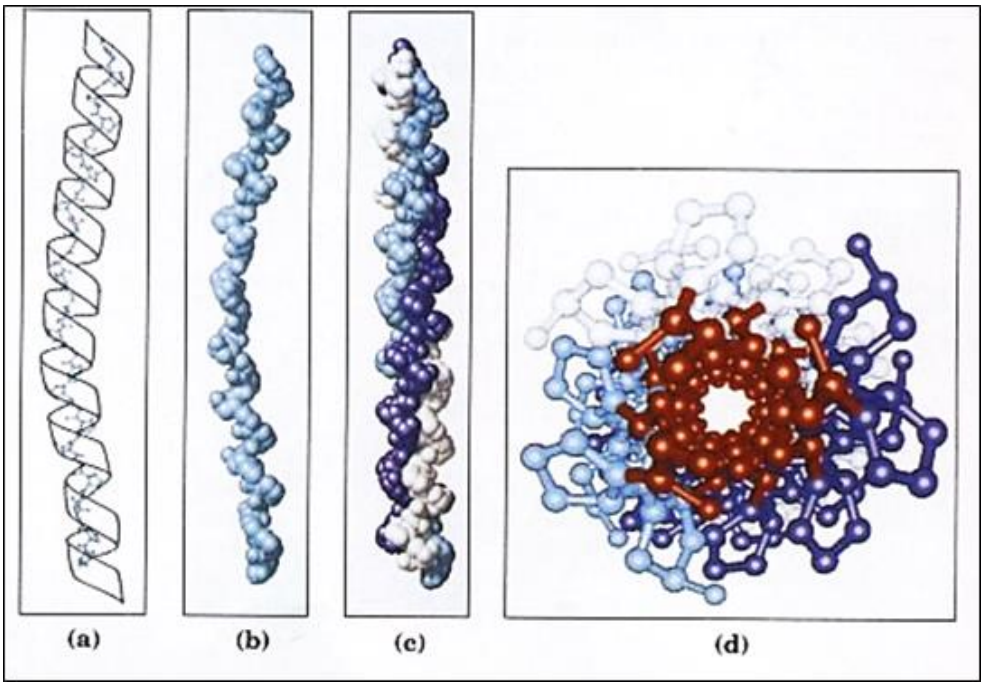


Figure 40. Type II collagen structure. **(a)** The repeating tripeptide sequence in the left-handed helical structure. **(b)** Space-filling model of the collagen helix. **(c)** Three helices wrapped around one another: tropocollagen. **(d)** The three-stranded collagen super helix shown from one end, glycine residues are shown in red[194].

Structural proteins such as type II collagen are good targets for ROS because of their long half-life. Inflammatory processes induce ROS that can modify CII and other structural proteins. These modifications can result in aggregation or fragmentation and may also involve the formation of collagen cross-links[195]. Moreover, even in the absence of hyperglycemia, non-enzymatic glycation has been reported in RA.

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6.4.1 OxPTM-CII and inflammatory arthritis

The discovery and validation of anti-citrullinated protein antibodies in RA has highlighted the relevance of post-translational modifications in autoimmune diseases and more specifically, in RA. The idea of generating novel autoantibodies against neoepitopes after PTM modification of proteins were based on such findings. The damage present in RA patients has been associated with the action of excess inflammatory cytokines and MPP[4]. The inflammatory cells infiltrate the inflamed synovial membrane and consume increased amounts of oxygen, generating of reactive oxidants[196]. In the inflamed joint, increased ROS damage of protein was detected[15]. Several studies have demonstrated the relevance of oxPTM-CII and shown that ROS presented in inflamed joints of RA patients can generate CII neoantigens[9, 16, 4, 197]. OxPTM-CII have been previously characterized through SDS-PAGE and three-dimensional fluorescence showing aggregation or fragmentation depending on the modification. Glycated CII has a slightly higher molecular weight and the glycation by ribose also causes fragmentation of the CII molecule. Furthermore, hydroxyl radical, hypochlorous acid and peroxynitrite modification causes a loss of intact CII resulting in both fragmentation and formation of higher molecular weight aggregates[4]. The previously published three-dimensional fluorescence profiles of CII and modified CII (figure 41) show an increase in the relative fluorescence intensity for chlorinated CII. The highest fluorescence was detected for glycated CII, as a result of pentosidine formation[4]. The differences between the native molecule and the modified CII were also highlighted by the increased light scattering due to the increasing levels of fragmentation and aggregation as demonstrated by the three-dimensional fluorescence[4].

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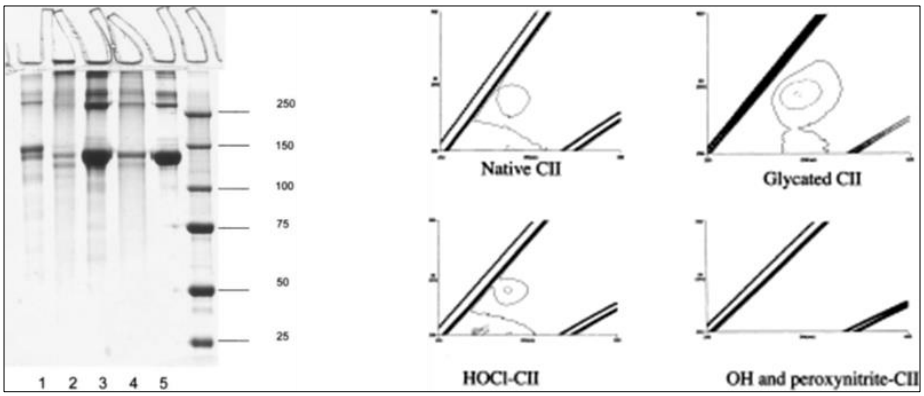

Figure 41. OxPTM-CII modifications. On the left SDS–PAGE analysis of chemically modified CII. Lane 1, Glycation; lane 2, hydroxyl radical modification; lane 3, hypochlorous acid modification; lane 4, peroxynitrite modification; lane 5, native CII. On the right three dimensional fluorescence[4].

When modified, as just described by conditions found within the inflamed joint, the modified CII acts as an autoantigen in RA patients stimulating autoimmunity. Such autoimmunity has been detected in serum samples from RA patients, and binding to oxPTM-CII was indeed increased in 14 of 31 RA sera[4]. Serum samples have also shown strong binding to various fragments and aggregates of oxPTM-CII, as well as strong binding to fragmented CII present in RA synovial fluid[9]. In patients with longstanding RA, autoreactivity to oxPTM-CII changed longitudinally whereby 92.9% of serum samples from RA patients (of early stage) bind to anti-ROS-II with sensitivity and specificity[9]. It was also shown that autoantibodies to OXPTM-CII have the potential to become diagnostic biomarkers of RA with a sensitivity and specificity of binding respectively of 98% and 92%[9].

As consequence of these findings, it is therefore apparent that the antigen ROS-CII exists in the inflamed joint. It was hypothesized that these neoantigens could be found exclusively in the inflamed joint, where CII has been oxPTM by the high levels of ROS. To target the neoantigen, Hughes et al produced single chain variable fragments (scFv) specific to oxPTM-CII from a semisynthetic phage display human library. The *in vitro* techniques showed the scFv bound specifically to damaged cartilage, but not healthy cartilage. The antibodies were also tested *in vivo* using optical imaging techniques. Models of monoarthritis were used to

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assess the specific localisation in the one inflamed joint and successful localisation was observed. The production of scFv specific to oxPTM-CII gives potential for targeting therapeutics specifically to the arthritic joint. Hughes et al proved this concept by fusing vIL-10 to one of the oxPTM-CII scFv and administering systemically in arthritic mice. The study found the antibody localised in the arthritic joint and reduced inflammation more effectively than vIL-10 fused to a non-specific scFv[198,199].

6.5 Aim of IA project

My group has previously reported in few studies an autoimmune reactivity against oxPTM-CII in RA patients. They have also proved that it is possible to target the oxPTM-CII present in the inflamed joint of arthritis mice.

My aim was to perform a study to identify the presence of anti-oxPTM-CII reactivity in inflammatory arthritis patients and to explore the reactivity to oxPTM-CII in axSpA. Therefore, as for the oxPTM-INS, part of the project was dedicated to identify and map, within the CII sequences, the neoepitopes responsible for the autoreactivity.

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CHAPTER 7

INFLAMMATORY ARTHRITIS: METHODS

7.1 Chemical modifications of CII.

Chemical oxPTM were performed *in vitro* using ROS. For CII modifications the protocol by Nissim et al[4, 9] was followed. Following modification, protein was tested by SDS-PAGE electrophoresis to detect degradation through a concentration range and ELISA to check the binding to oxPTM proteins. Bovine CII in PBS (1mg/ml) was chemically modified using different concentration of the following systems:

1) Non-enzymatic glycosylation with 2M D-Ribose at 37°C for three days (GLYC-CII);

2) 1mM sodium hypochlorite (HOCl-CII);

The human serum albumin (HSA) (A3782, SIGMA) and bovine serum albumin (BSA) (A7030, SIGMA) were modified, treated in parallel and used as antigens control.

7.1.1 Monitoring CII modifications

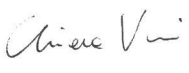
Changes in molecular weight and structure (fragmentation, aggregation, etc.) induced by ROS modifications were evaluated using SDS-PAGE. CII modifications were monitored using an 8% gel. To be sure that modifications are repetitive and consistent binding of these proteins was checked using an ELISA analysis, the primary antibody used was a single chain variable fragment (scFv) antibody directed specifically to native and oxPTM-CII named 6-11D and previously developed in house. This scFv was raised using a semisynthetic phage display library and was expressed in Escherichia Coli HB2151 as previously described by Hughes et al[199]. Every batch of oxPTM-CII was checked as described above.

7.2 Preparation of antibody fragments

E. Coli previously transformed with the plasmid of interest were cultured overnight using 5mL of 2xTY (16g tryptone, 10g yeast extract, 5g NaCl, pH 7.4), 100µg/mL ampicillin and 1%glucose. Cultures were incubated overnight at 37˚C and 125 rpm. The following day the culture was diluted 1:100 in 2xTY + 100ug/mL ampicillin + 0.1% glucose. Bacteria were grown to reach the optical density (OD) between 0.7 and 0.9 and then induced with 1mM IPTG (isopropyl β-D-thiogalactoside) and incubated overnight at 30˚C and 125 rpm. Cultures were then centrifuged at 5000rpm (4000xg) for 25 minutes at 4°C. Supernatant was collected and filtered meanwhile the pellet was resuspended in 1/20 of the original volume of 30mM Tris pH 7.0 + 20% sucrose + 1mM EDTA + 1:100 bacterial protease inhibitors and left for 20 minutes on ice. The resuspended pellet was centrifuged at 6000rpm for 40 minutes was required and the periplasmic fraction supernatant was collected. The pellet was resuspended in 1/20 of the original volume of 5mM MgSO4, incubated for 20 minutes on ice and then spun at 6000 rpm 30 minutes. The osmotic shock supernatant was collected and added to the periplasmic fraction.

7.2.1 Purification using a protein A resin

Protein A-Sepharose resin (17078001, GE Healthcare) can be used to purify antibody fragments encoded by VH segments from the VH3 family. The Protein A-Sepharose CL-4B was pre-swelled by adding 1g of powder in 10mL PBS and storing 1 hour at room temperature and then added to a chromatography column. The sample was allowed to flow through the column and hence the resin to allow binding of the antibody to the protein A-Sepharose. The column was washed with 5 column volumes of: PBS; PBS + 0.5M NaCl; 0.2M glycine pH 6; 0.2M glycine pH 5. After washing the protein was eluted with 3 column volumes of 0.2M glycine pH 3. Fractions were collected into tubes containing 0.2mL 1M Tris pH9 and then analysed by spectrophotometry and SDS-PAGE. After purification antibodies were checked by SDS-PAGE, ELISA and Western blotting.



7.3 SDS-PAGE

An 8% Native gel was used to check CII modifications (table 13). The resolving gel was cast following a standard protocol and samples were prepared using the Laemmli buffer (240mM Tris- HCl pH6.8; 40% Glycerol; 8% SDS; 0.04 % Bromophenol Blue; 10% β-mercaptoethanol). Just before pouring the mixed media, 10% ammonium persulfate and TEMED were added. The resolving gel was added and the mixture was then covered with dH_2O and left until solidified.

Table13. Recipe for preparation of 2 SDS polyacrylamide 8% running gel.

H2O	4.77ml
1.5 M Tris-HCl, pH 8.8	2.5ml
Acrylamide/Bis-acrylamide	2.63ml
10%(w/v) SDS	100ul
10%(w/v) ammonium persulfate (APS)	50ul
TEMED	5ul

After the gel was cast, the water was removed, and the stacking gel was added on the top of the gel. Stacking gel was made according to table 14.

Table 14. Recipe for preparation of 2 SDS polyacrylamide stacking gel.

H2O	3.075ml
0.5 M Tris-HCl, pH 6.8	500ul
Acrylamide/Bis-acrylamide	670ul
10%(w/v) SDS	50ul
10%(w/v) ammonium persulfate (APS)	30ul
TEMED	5ul

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A comb was placed on the top of the gel before it was cast. The gel was left to polymerise for 15-30 minutes. The apparatus was assembled and filled with 1x running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH8.3) and subjected to electrophoresis under constant voltage of 120 - 150 V.

The staining was conducted using Coomassie Blue (0.1%w/v Coomassie Blue, 45% methanol and 10% acetic acid). It was left in the staining solution for 30 minutes and then the gel was transferred for 4 hours to the destaining solution (40% methanol and 10% acetic acid).

7.4 Western blotting

An 8% SDS-PAGE was run and electroblotted with 10µg oxPTM-CII, native CII, oxPTM-BSA and BSA for 1 hour and 30minutes at 80V. The membranes were blocked with 5% milk and then incubated overnight at 4°C with the primary antibody:

1) 0.5µg/mL of scFv 6-11D in 5% Marvel–PBS.
2) 1:500 dilution of serum samples in 5% Marvel–PBS (pre-incubated during blocking).

The following day membranes were incubated 1hour with 1:1000 dilution of anti-human IgG-HRP. Membranes were then washed and developed with Luminata Forte Western HRP substrate, a film was exposed for the required time in a hypercassette in a dark room, then developed in the film processor SRX 101A Konica Minolta.

7.5 Patients in cohort 3: San Giovanni di Dio study

Serum samples analysed were obtained from Rheumatology Unit of San Giovanni di Dio Hospital, Florence (Italy) between July 2015 and April 2016. The cohort analysed includes 98 patients with axSpA, 60 samples from early rheumatoid arthritis (ERA) patients, 49 from undifferentiated arthritis (UA) and 60 from PsA patients; as controls, 19 samples from patients with fibromyalgia (FM) and 70 healthy controls (HC) were used (Table 1). Patients with axSpA were defined

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according to Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and ASAS[200]. All patients were treated with different biological drugs (infliximab, adalimumab, ethanercept, certolizumab pegol, golimumab, secukinumabek, ustekinumab or abatacept) after failing on non-biological DMARDs. Out of the 67 patients with axSpA, 10 patients had axSpA in association with IBD; 7 with axSpA and oligoarthritis and 10 patients had axSpA with enthesitis.

7.6 Patients in cohort 4: Glasgow study

Serum samples analysed in the cohort 4 were obtained from University of Glasgow. This cohort included 144 patients with AS, 15 samples from PsA patients, 35 patients with psoriasis (PS), 8 patients with Crohn's disease (CD) and 1 with ulcerative colitis (UC); 42 healthy controls were also used. Most of the patients included in this cohort were screened for BASDAI (only AS patients), CRP and ESR. Most of the patients included in the cohort were treated with different biological and non-biological drugs but the responding to treatment was reaching the 100% only in the CD group.

7.7 Immunoprecipitation

Autoantibodies from the most reactive samples from patients with axSpA, PsA and UA were purified using Sure Beads protein A magnetic beads (1614013, BioRad). Serum sample and 100µl of beads were incubated on rotation for 1 hour at room temperature, in a final volume of 250µl. After washing, 5µg of CII, oxPTM-CII, trypsinised CII and trypsinised oxPTM-CII were added and incubated on rotation for additional 1 hour at room temperature. The elution was performed by adding 40µl of Laemmli buffer and incubating for 10 minutes at 70˚C. Trypsinization of CII and oxPTM-CII was done for 30 minutes using 2mg/mL of trypsin at room temperature. Trypsin was then inactivated by a 10 minute incubation at 70˚C.

7.7.1 Immunoprecipitation: western blotting

Samples eluted from immunoprecipitation with Sure Beads protein A magnetic beads were loaded on 8% SDS-PAGE. As a probe for detecting the immune-

precipitated CII and oxPTM-CII we used a recombinant antibodies developed in house. After blocking with 5% milk in PBS-T, membranes were incubated overnight at 4˚C with 0.5µg/mL of recombinant antibody. Membranes were washed 3 times with PBS-T and then incubated for 1 hour with monoclonal anti-poly-Histidine conjugated with horseradish peroxidase, specific to the Histidine tag attached to the recombinant antibody. Membranes were developed using Luminata Forte chemiluminescent substrate in the film processor SRX 101A Konica Minolta.

7.8 ELISA

The ELISA was used to certify the consistency of oxPTM-CII and then to analyse reactivity to native and modified CII of sera from patients with IA. The ELISA optimized[4, 9] follows this protocol: a 96 well NUNC assay plate was coated and incubated overnight at 4°C with 100 µl per well of modified and native CII in PBS. The day after, coating proteins were removed and wash plates washed 3 times with 0.1% Tween PBS and then blocking it using 2% milk in 0.1% Tween PBS for 2 hours. Following washing, 100 µl per well of primary antibody were added and incubated for 2 hours. The primary antibody was:

1) A dilution 1:200 of serum samples in 2% milk in 0.1% Tween PBS.
2) A serial dilution of scFv 6-11D in 2% milk in 0.1% Tween PBS, starting from the concentration of 5ug per well.

A 1:1000 dilution of anti-human IgG-HRP in in 2% milk in 0.1% Tween PBS was added as a secondary antibody and incubated for 2 hours at room temperature. The ELISA plates were washed and 100µl/well of a 1:100 dilution of 10mg/mL TMB (3, 3', 5, 5'-tetramethylbenzidine) in 0.1M sodium acetate pH 6 plus 2µl/10mL of H2O2 was added. The reaction was stopped using 20% sulphuric acid. The optical density (OD) was measured at 450 nm using a Thermofisher multiskan fc plate reader. In the analysis of the cohort 3 we have used samples from ERA patients[4, 9]. As a positive control a group of healthy subjects and a small group of patients with FM were used as negative controls.

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FM is characterized by chronic widespread pain and a raised pain response to pressure[201, 202]. The cause of fibromyalgia is unknown; however, it is believed to involve a combination of genetic and environmental factors with half the risk attributed to each[203, 204]. Most publications indicate that there are some abnormalities within the hypothalamic-pituitary-adrenal axis including elevated activity of corticotrophin-releasing-hormone and substance P that may not only affect the PHA axis, but other endocrine and immune processes with elevated levels of cytokines in the serum of FM patients[205, 206].

7.8.1 Competitive ELISA

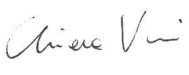
The competitive ELISA was conducted using a 2 hours pre-incubation between serum samples and native or HOCl-CII or HOCl-CIII as an antigen control. The primary antibody was incubated with native, HOCl-CII or HOCl-CIII, antibody-antigen complexes were then added to the ELISA plate pre-coated with native CII and oxPTM-CII. Unbound antibodies were removed washing the plate, as in the standard protocol, just before adding the secondary antibody (anti-human IgG) that is specific for the human serum samples and HRP-conjugated. The developing of the plate was done following the standard protocol.

7.9 Statistical analysis.

Data analysis was performed using Prism Software version 6.01 (GraphPad, San Diego, CA, USA). BSA and oxPTM-BSA values were used as background controls. The Mann-Whitney test was used to test and compare the antibody binding. To determine predictive discrimination between axSpA and healthy control groups, we used the 97.5th percentile of the healthy individuals as cut-off point absorbance units to construct a contingency table of positive binders to oxPTM-CII and tested it by Fishers Exact Test.

7.10 Two dimensions electrophoresis

As previously described proteins loaded in 2D Gel strips, move across the pH gradient and, when they reach the region of the gel that matches their isoelectric



point, they stop moving. After the separation by isoelectric point, they can be analysed based on their mass.

First dimension: IsoElectroFocusing

System used: Ettan IGPhor Isoelectrofocusing system (GE Healthcare).

The first step was the rehydration of Immobiline Dry Strip pH 3-10NL (GE Healthcare) using 25-30µg of protein and 100µl of rehydration buffer prepared as described below and adding 7mg/2.5mL of DTT just before use. Rehydration buffer:

- 3g Urea
- 0.125g Chaps
- 80µl IPG buffer
- 20µl Bromophenol Blue 0.1%
- 4mL dH2O

The rehydration required an incubation at room temperature at least for 10 hours. The strip was positioned with the gel side down and the pointed end of the strip directed toward the pointed end of the strip holder. DryStrip cover fluid was applied to cover the IPG strip to assure a complete wetting. After the rehydration strips were processed isoelectrofocusing following this protocol:

9. Step-on-hold 100V 30minutes
10. Gradient 300V 30minutes
11. Step-on-hold 300V 10minutes
12. Gradient 1000V 1hour
13. Gradient 3000V 1hour and 30minutes
14. Gradient 4000V 1hour and 30minutes
15. Gradient 5000V 2hours
16. Step-on-hold 5000V 1hour

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Second dimension: SDS-PAGE

After the IEF, each strip was equilibrated 15 minutes in equilibration buffer plus 10mg/mL DTT and 15 minutes in denaturant equilibration buffer plus 25mg/mL Iodacetamide. Equilibration buffer:

- 36g Urea (final concentration 6M)
- 5mL TRIS-HCl 1.5M PH 8.8 (final concentration 75mM)
- 2g SDS or 20mL SDS 10% (final concentration 2%)
- 2mL Bromophenol Blue 0.1% (final concentration 0.002%)
- H2O up to 100mL

Strips were then loaded on an 8% SDS-PAGE gel, the running gel was made as previously described, the stacking gel was made based on the strip. The IGP strip was sealed in place using a 0.5% agarose gel. The gel was then subjected to electrophoresis under constant voltage of 120V. Once the gel finished running it was removed and used for a western blot.

The 2D gel western blot was performed using the whole protein of CII and oxPTM-CII and also the trypsinised CII and oxPTM-CII (trypsinization: incubations for 30 minutes with 2mg/mL of trypsin at room temperature).

The western blot was performed as previously described using as 1-11E and 3-11E scFv as primary antibodies in a concentration of 20µg per membrane.

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CHAPTER 8

INFLAMMATORY ARTHRITIS: RESULTS

8.1 Single chain fragment variable (scFv) antibodies

The scFv weigh ~25kDa and consists only of the heavy chain (VH) and light chain (VL) regions of an immunoglobulin. VH and VL are connected by a short flexible polypeptide linker that allows the association of the VH and VL to form the antigen binding site (figure 42).

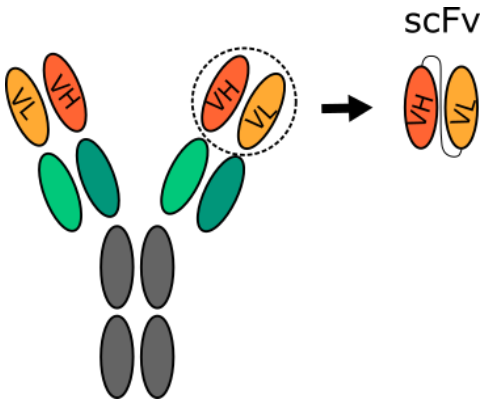

Figure 42. Structure of intact antibody and scFv.

Antigen-specific antibody fragments *in vitro*, such as scFv, can be expressed in E. coli and can be produced in high quantities for any application. The small size and single-gene qualities of scFv make them suitable for molecular biology-dependent techniques [207, 208].

8.1.1 ScFv production

ScFv were produced in E. Coli strain HB2151 and purified using protein A sepharose resin. To check the functionality of the scFv a western blot (figure 43) and an ELISA (figure 44) were performed. As shown in figure 43, the main band for all the scFv tested was displayed between 25 and 30 kDa. In addition, other minor bands were observed at a lower molecular weight.

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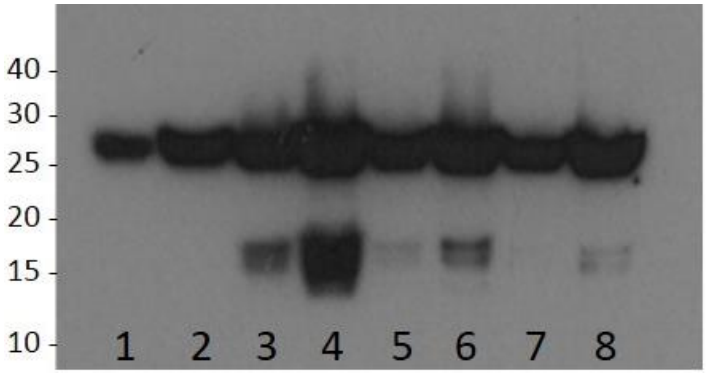

Figure 43. ScFv western blotting. Western blot to check the functionality of 3 scfv specific for oxPTM-CII (1-11E, 3-11D, 6-11D) and 1 scFv negative for oxPTM-CII (C7). Lane 1=1ug of C7, Lane 2=3ug of C7, Lane 3=1ug of 1-11E, Lane 4=3ug of 1-11E, Lane 5=1ug of 3-11E, Lane 6=1ug of 3-11E, Lane 7=1ug of 6-11D, Lane 8=3ug of 6-11D.

To confirm the results in the western blot, the scFv were also checked in ELISA. Figure 44 shows that 1-11E, 3-11E and 6-11D were binding glycated and chlorinated CII and 6-11D bound also to CII, as expected. The negative control scFv, C7, was not binding CII nor oxPTM-CII. None of the antibodies could bind to the antigen control (BSA) that has been subtracted from the CII reactivity as background.

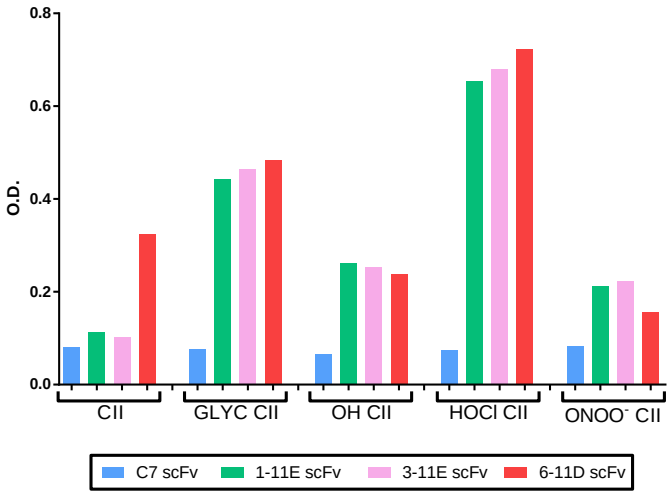

Figure 44. ScFv ELISA. ELISA to check the binding to CII and oxPTM-CII of 3 scfv specific for oxPTM-CII (1-11E, 3-11D, 6-11D) and 1 scFv negative for oxPTM-CII (C7).

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8.2 Monitoring oxPTM-CII

8.2.1 SDS and tricine PAGE analysis of CII and oxPTM-CII

Modification of CII were performed as previously described[4] and checked by SDS-PAGE, tricine PAGE and ELISA. Figure 45A shows th result from the SDS-PAGE. The gel showed one single band in lane 1 representing CII, glycation led to a shift to a higher molecular weight, oxidation, chlorination and peroxynitration led to the presence of a smear of protein in lanes 3, 4 and 5. Figure 45B shows the tricine PAGE. The two PAGE analysis in figure 45A and 45B are very similar, but the tricine gel had a higher definition with more clear additional bands for glycated CII (lane 2) and a very dark smear of protein for the chlorinated CII (lane 3). The native CII, in lane 1, showed a clear band as in the SDS-PAGE with few weaker bands with a higher molecular weight.

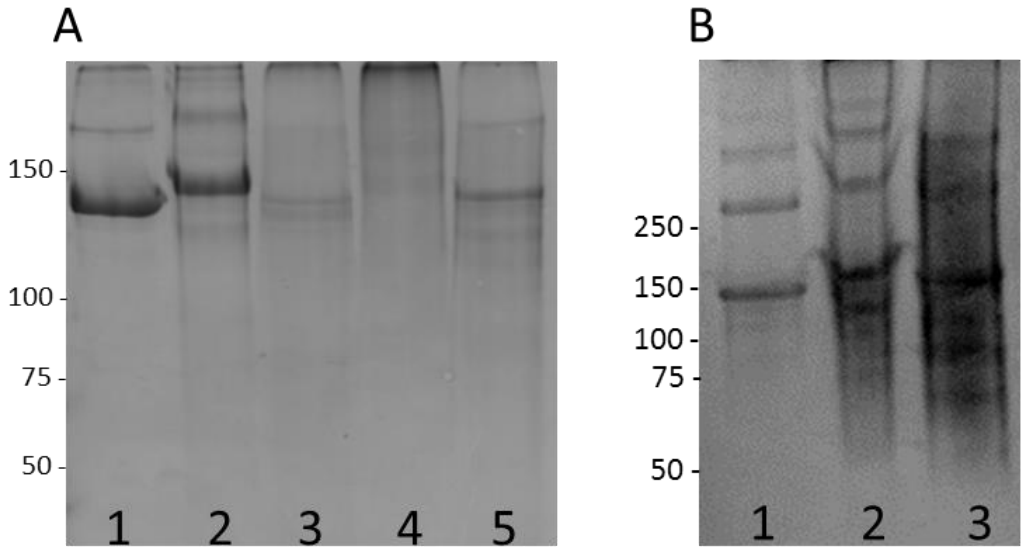

Figure 45. SDS and tricine PAGE analysis of CII and oxPTM-CII. Native CII (lane 1) showed an electrophoretic band that migrated to the region of 140 kDa protein marker; glycation by ribose (lane 2) presented a small shift to a higher molecular weight; oxidised (lane 3), chlorinated (lane 4) and peroxynitrated (lane 5) CII resulted in a smear of protein, indicating fragmentation.

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8.2.2 Western blot

Binding to native and modified CII was also detected by western blot to check the specificity of the binding. In the western blot analysis, some of the most reactive ERA serum samples used previously[9] (figure 46A) and scFv specific for CII and oxPTM-CII[198] (figure 46B) were used as primary antibodies. In figure 46A the binding to CII is not detected, while there was evident binding to two bands of glycated CII at about 100 and 75kDa. There was also strong binding to several bands between 130 and 60 kDa in the chlorinated CII. In figure 46B, the binding of a recombinant antibody specific to CII and oxPTM-CII is shown. The western showed binding directed to one single band for the native CII, whereas binding to glycated and chlorinated CII was stronger and directed to a several bands with a slower mobility compared to the native protein.

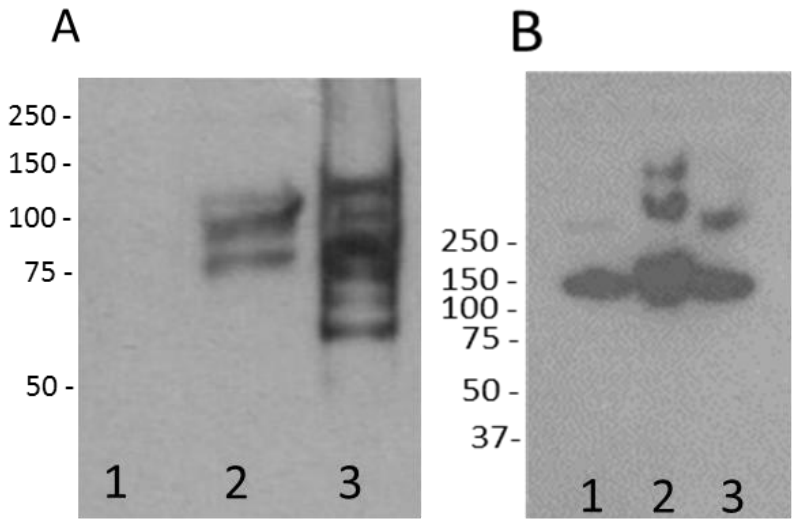

Figure 46. Binding to native and oxPTM-CII, as detected by western blot. Figure A shows binding of some of the most reactive ERA samples[9]; figure B shows binding of one of the synthetic antibodies produced in house specific for CII and oxPTM-CII. In both figures binding to native CII is shown in the lane 1, binding to glycated CII in the lane 2 and binding to chlorinated CII in lane 3.

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8.2.3 ELISA

CII and oxPTM-CII were also checked in ELISA using positive recombinant antibodies and positive samples previously used[9, 198]. A representative ELISA analysis using positive recombinant antibodies is shown in the figure 47. This ELISA with the serial dilution of 1-11E (as example of all the scFv) highlighted the specificity of these antibodies. The binding increases constantly and consistently when the concentration of scFv increases.

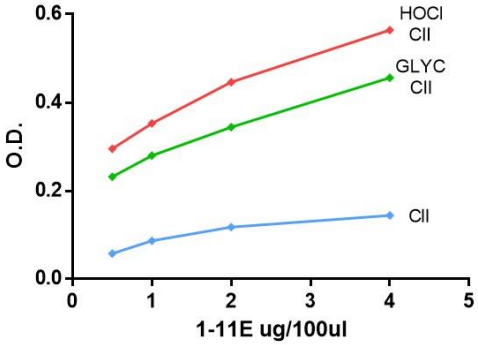

Figure 47. Binding to CII and oxPTM-CII of serial dilution of scFv detected by ELISA. This ELISA has performed using serial dilution of scFv to highlight its specificity (1-11E was used as example of specific scFv antibodies).

Figure 48 shows the reactivity of RA samples to oxPTM-CII, binding to glycated and chlorinated CII was stronger compare to the binding to native CII ($p<0.0001$), confirming data previously published[4, 9].

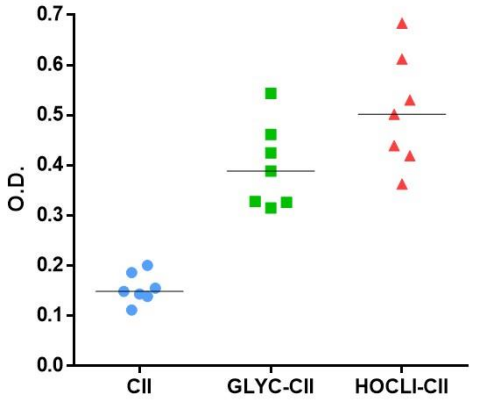

Figure 48. RA samples reactivity to native and oxPTM-CII detected by ELISA. Binding to oxPTM-CII was stronger compare to native protein.

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8.3 Longitudinal stability study

8.3.1 SDS-PAGE analysis

To be sure that the modifications are stable over time, the oxPTM-CII were analysed at different time points following *in vitro* modification. Figure 49 shows the comparison between fresh and 1-month old modifications. The figure shows an SDS-PAGE stained with Coomassie blue. Native and oxPTM-CII showed a very high similarity between fresh and 1-month old modifications, indicating stability of the modifications through time.

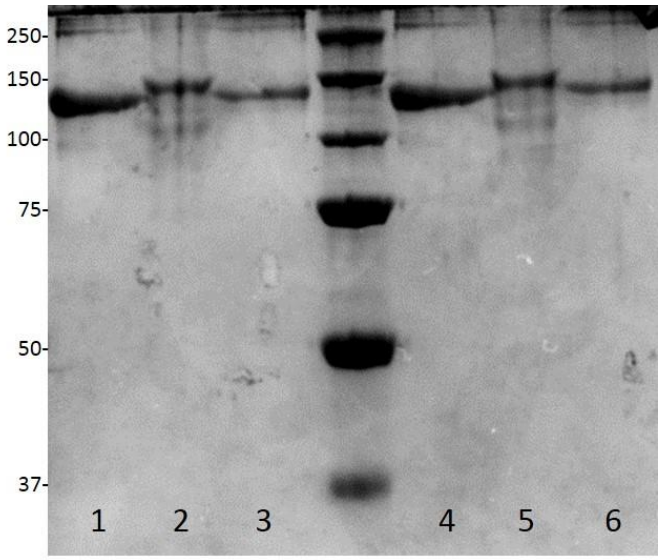

Figure 49. SDS-PAGE of CII and oxPTM-CII over time. Lanes 1, 2 and 3 were loaded with fresh modifications, native CII, glycated CII and chlorinated CII respectively; lanes 4, 5, and 6 were loaded with 1-month old modifications in the same order reported for the fresh modifications.

8.3.2 ELISA

Fresh and 1-month old modifications were also checked by ELISA to determine any changes in binding capacity over time. Serial dilution of the antibody 1-11E and some of the most reactive samples from different cohorts of RA patients were used as positive controls to analyse the modifications. The results of these ELISA analysis are shown in figure 50A and 50B, the differences between old and fresh modifications are not significant.

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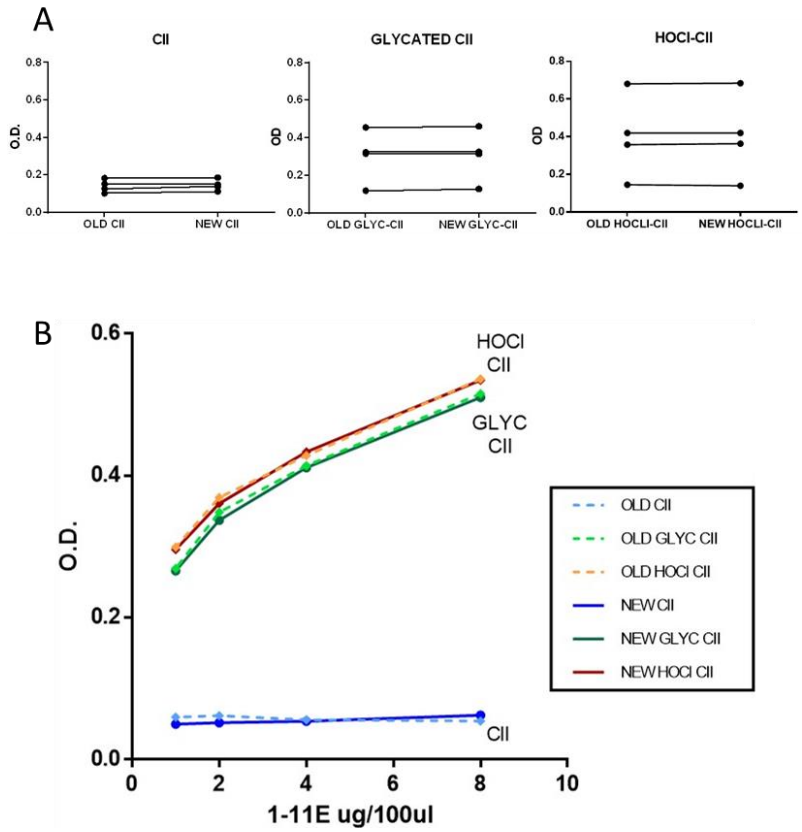

Figure 50. Comparison of fresh and 1-month old modifications by ELISA. Some of the most reactive samples from different cohort of patients with RA were used to check if there were any difference in the binding to old and new modified insulin (A). Serial dilutions of the recombinant antibody specific for oxPTM-CII, 1-11E, were used (B).

8.4 Patients analysis cohort 3: San Giovanni di Dio study

To study the autoreactivity to oxPTM-CII in patients with inflammatory autoimmune arthritis, binding of serum samples from patients with axSPA, ERA, UA, PsA to CII and oxPTM-CII, either glycated and chlorinated, was evaluated. As negative controls, healthy subjects and FM patients were analysed. The characteristics of the patients are summarised in table 15. The average age of the patients was comparable among the different disease groups, whereas the duration of the diseases since diagnosis was higher for axSpA and PsA. axSpA and PsA patients were treated with several biological drugs and most of the patients (axSpA 90% and PsA 100%) were responding to the therapy. ASDAS of all the axSpA

patients was between 1.1 and 1.3 which means that the disease was controlled by the treatment and CRP was very low. ERA and UA samples were collected at an early stage of the diseases and the patients were not under treatment.

Table 15. Characteristics of patients in cohort 3.

VARIABLE	axSpa	ERA	PsA	UA	FM	HC
N. patients	98	60	60	49	19	70
Gender (F/M)	0.6	2	1.4	1.9	1.4	0.4
Age (y)	52 (23-80)	50 (28-63)	56 (31-76)	60 (33-84)	51 (40-64)	47 (21-58)
Duration of disease (y)	6 (1-12)	N/A	6 (3-15)	<1	4 (3-11)	-
BASDAI	15.14 (1.4-30)	-	-	-	-	-
ASDAS	1.51 (1.1-3.8)	-	-	-	-	-
ACR20	-	-	15.57 (10.4-24.6)	-	-	-
CDAI	-	-	-	18.3 (6-62)	-	-
Treatment	ADA=28, ETA=20, IFX=9, GOLI=7, CERTO=2, SEK=18	No	ETA=21, ADA=16, GOLI=11, IFX=4, USTE=2	No	N/A	-
Respond (%)	90%	-	100%	-	N/A	-

BASDAI= Bath Ankylosing Spondylitis Disease Activity Index, ASDAS= Ankylosing Spondylitis Disease Activity Score, ACR20=American College of Rheumatology, CDAI=Crohn's Disease Activity Index, ADA=adalimumab, ETA=ethanercept, IFX=infliximab, GOLI=golimumab, CENTRO=certolizumab pegol, SEK=secukinumabek, USTE=ustekinumab.

CCP in axSpA

To investigate the level of CCP positivity in axSpA and the potential correlation between oxPTM-CII and CCP in axSpA patients, CCP levels were analysed in a representative group of 44 axSpA samples (figure 51). The binding to CCP was very low, with a rate of positivity about 2%. Due to the low positivity rate to CCP any correlation analysis was not performed.

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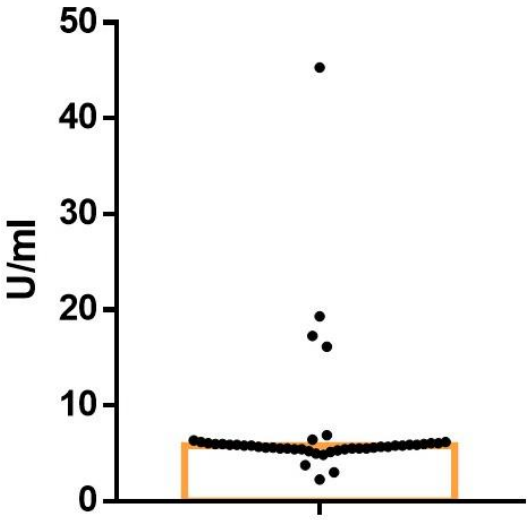

Figure 51. CCP analysis of a representative group of axSpA samples. Samples are negative if lower than 20 U/ml; samples are weakly positive between 20 and 39 U/ml; positivity is between 40 and 59 U/ml; samples are strongly positive if higher than 60 U/ml.

HLA in axSpA

Table 16 shows the prevalence of the HLA genotypes in axSpA patients in cohort 4. The HLA haplotypes most represented in the axSpA group are: HLA B35, HLA DBR1 01, 11 and HLA DBQ 05. These common haplotypes are present in the 53% of the patients. In general, the HLA haplotypes most represented in axSpA patients are HLA B27, which is frequently associated with the development of axSpA[209], and HLA DRB1 01, 04, 10 and 14, which are frequently associated with rheumatoid arthritis[210]. In this cohort the haplotype listed above are present in 17% of the patients for HLA B27 and 35% for the HLA DRB 01, 04, 10 and 14.

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Table 16. Prevalence of the HLA genotypes in the axSPA patients in the cohort 3.

HLA	PATIENTS (%)
B35	**53%**
B27	**17%**
CW06	**14%**
CW07	**25%**
DRB1 01	**35%**
DBR1 01,11	**53%**
DBR1 01,04,10,14	**35%**
DBR1 07,08	**47%**
DBQ 03	**29%**
DBQ 05	**53%**

8.4.1 OxPTM-CII autoreactivity in cohort 3

The autoreactivity of these patients has been tested and the results are presented in figures 52A, B, C and D. Figure 52A shows the binding of serum samples from patients with axSpA in comparison to peripheral SpA (UA and PsA), FM patients and HC. Binding of serum samples to oxPTM-CII from patients with peripheral SpA, including UA and PsA, was significantly lower compared to the binding of axSpA serum samples ($p<0.0001$), with 35% and 33% of positivity to UA and PsA, respectively (97.5th percentile). Binding of axSpA to oxPTM-CII was significantly higher than binding to native CII ($p<0.0001$) with a percentage of autoreactivity of 37% to CII and 74% to at least one form of oxPTM-CII.

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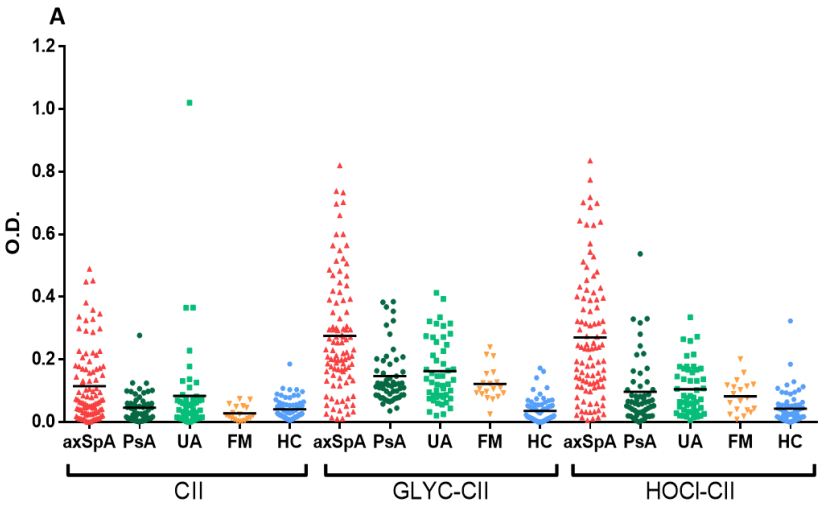

Figure 52A. Autoreactivity to CII and oxPTM-CII in cohort 3. Binding of axSpA, PsA, EA, FM and healthy subjects (HC) serum samples to CII and oxPTM-CII. Reactivity to CII and oxPTM-CII was low in samples from healthy individuals and patients with FM. Binding to oxPTM-CII was significantly higher than to native CII in samples from axSpA patients ($p<0.0001$). Reactivity to oxPTM-CII was reported in 74% of axSpA samples, in 33% of PsA samples and in 35% of UA samples (cut off 97.5th percentile).

There was not a significant difference in anti-oxPTM-CII antibody reactivity between samples from patients with axSpA and patients with axSpA associated with oligoarthritis or IBD or enthesitis ($p<0.05$) as shown in the figure 52B.

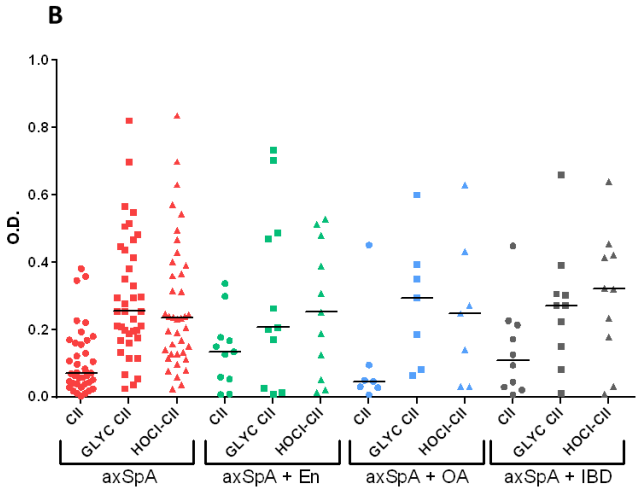

Figure 52B. Autoreactivity to CII and oxPTM-CII in cohort 3. Binding to CII and oxPTM-CII of axSPA samples compare to samples from patients in associations with enthesitis (En), oligoarthritis (OA) and/or IBD. The difference between patients with axSpA and patients with axSpA associated with OA or IBD or EN was not significant.

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As a positive control we have analysed a group of ERA patients and compared this group with the results of the axSpA samples. Figure 52C shows the binding of ERA sera was specific to oxPTM-CII. Positive binding was detected in 91.7% of ERA samples, compared to the 74% of axSpA samples.

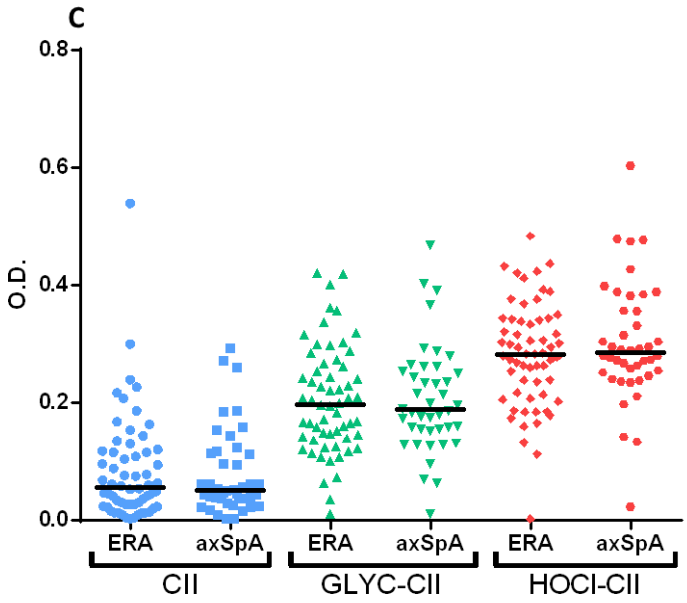

Figure 52C. Autoreactivity to CII and oxPTM-CII in cohort 3. Binding of axSpA and ERA serum samples to CII, oxPTM-CII. There are no significant differences between reactivity to CII and oxPTM-CII in samples from axSpA and ERA patients.

The overlapping binding is shown in figure 53. In the axSpA group 73 samples were positive for native and modified CII, and only 36 out of 73 were positive for both modifications and negative for native CII. In the ERA group of patients, the positivity to oxPTM-CII was very high (91.7%) and all the positive samples were reacting to chlorinated CII and overlapping mainly with glycated CII. Indeed about half of the samples were positive for both modifications. In PsA and UA groups positivity to CII was very low, binding is mainly to glycated CII and overlapped with chlorinated CII in about half of the samples.

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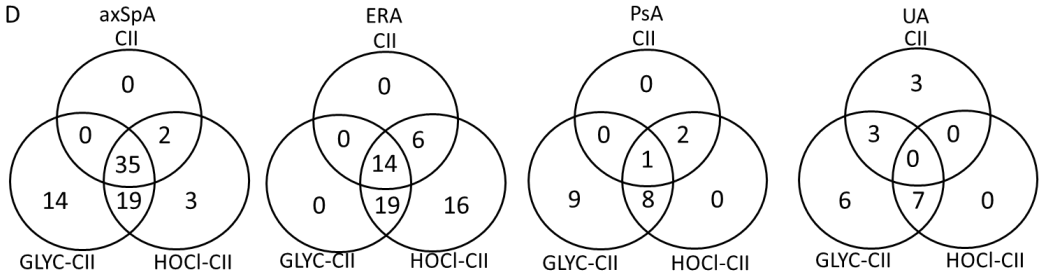

Figure 53. Overlapping binding of axSpA, ERA, PsA and UA serum samples to CII, glycated and chlorinated CII.

8.4.2 Serum binding specificity

To assess the serum binding specificity and confirm the results just described 3 classes of experiments were run: comparison of oxPTM-CII binding to an oxPTM antigen control; competitive ELISA and western blot.

The binding of axSpA samples to an antigen control was tested. Due to the similarity in the sequence and structure, CIII was chosen as control. CII and CIII were modified in parallel and, as shown in figure 54, there is no significant difference between the binding to the native proteins whereas the difference is significant between oxPTM-CII and oxPTM-CIII for both modification, glycation ($p<0.0001$) and chlorination ($p<0.0006$).

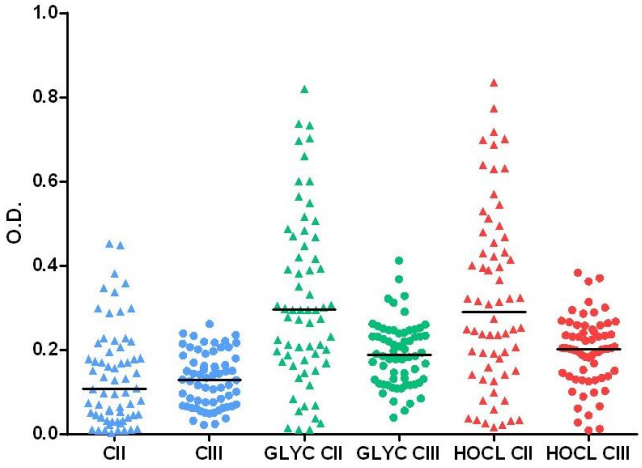

Figure 54. Binding of axSpA serum samples to CII, oxPTM-CII, CIII and oxPTM-CIII. Binding of axSpA samples to an antigen control was tested showing significant difference between binding to oxPTM-CII and oxPTM-CIII for both modification, glycation ($p<0.0001$) and chlorination ($p<0.0006$).

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The most reactive samples from the axSpA, PsA, ERA and UA were also used to confirm the specificity of the binding in competitive ELISA. The competition was performed using an excess of CII, oxPTM-CII and oxPTM-CIII. Figure 55A shows that pre-incubation of serum samples with oxPTM-CII before binding to oxPTM-CII in the ELISA resulted in a significant reduction of the reactivity for all the groups of patients.

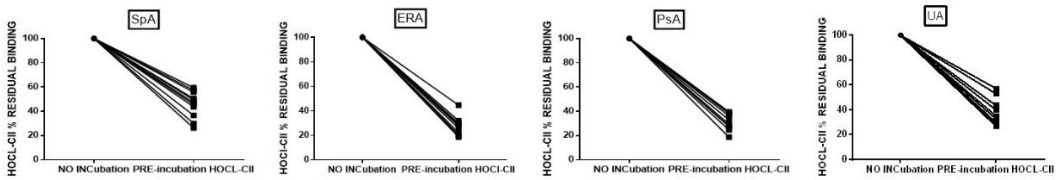

Figure 55A. Competitive ELISA. As competitor chlorinated CII as an example of oxPTM-CII was used. Pre-incubation of serum samples with oxPTM-CII before binding to oxPTM-CII in the ELISA resulted in a significant reduction of the reactivity for all the groups of patients.

When the competitor was native CII, the binding to oxPTM-CII was not significantly reduced in ERA and axSpA patients and slightly reduced in PsA and UA samples (figure 55B).

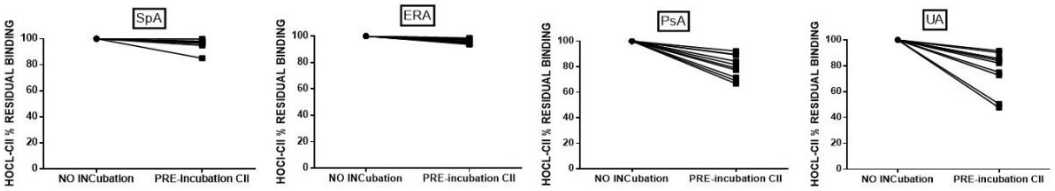

Figure 55B. Competitive ELISA. As competitor native CII was used. The binding to oxPTM-CII was not significantly reduced in ERA and axSpA patients and slightly reduced in PsA and UA samples.

An oxPTM antigen control, oxPTM-CIII, was also used as competitor in the ELISA to confirm that the reduction shown in figure 55A is specifically due to the modifications of CII. The results of the competition with the antigen control are shown in figure 55C.

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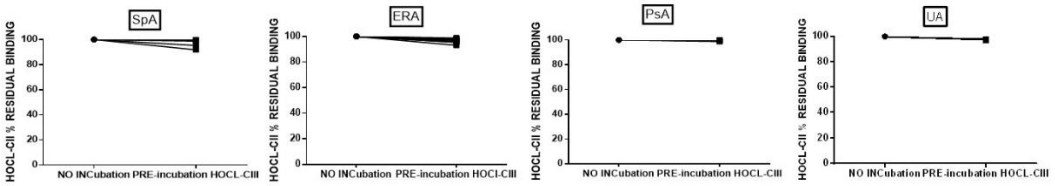

Figure 55C. Competitive ELISA. As competitor chlorinated CIII as a modified control antigen was used. The binding to oxPTM-CII was not significantly reduced in all the groups of patients.

Serum binding specificity was also assessed by western blot. The analysis was performed using a range of the most reactive axSpA, ERA and PsA serum samples tested in ELISA and to reduce background, antibodies in the serum samples were captured on protein A beads. Figure 56 shows the binding of the autoantibodies from the serum samples to native and chlorinated CII before and after incubation with trypsin. The digestion with trypsin was used to try to expose the epitope before the western blot. There was no binding to the serum samples non incubated with antigen, neither to samples incubated with CII, regardless the patiens sera used (axSpA, ERA or PsA); axSpA bound a chlorinated CII fragment about 50kDa. After incubation with trypsin, axSpA samples bound a native CII fragment strongly and at about 50kDa; after trypsinization of chlorinated CII the binding is to a smear of protein from 100kDa to 40kDa and 2 extra band at about 30kDa and 25kDa. The pattern of binding to trypsinised CII and chlorinated CII, before and after incubation with trypsin, of ERA and PsA samples was very similar. Both groups of samples showed a binding to bands at 70 kDa, 50 kDa, 30 kDa and 25 kDa with a background binding to a smear of protein along the whole lane. The difference between the 2 groups is the intensity of the binding: ERA samples showed a strong binding to trypsinised chlorinated CII, less strong to chlorinated CII before trypsinization and weak to native trypsinised CII. PsA samples showed a strong binding to trypsinised chlorinated CII but weak binding to chlorinated CII before trypsinization and native trypsinised CII.

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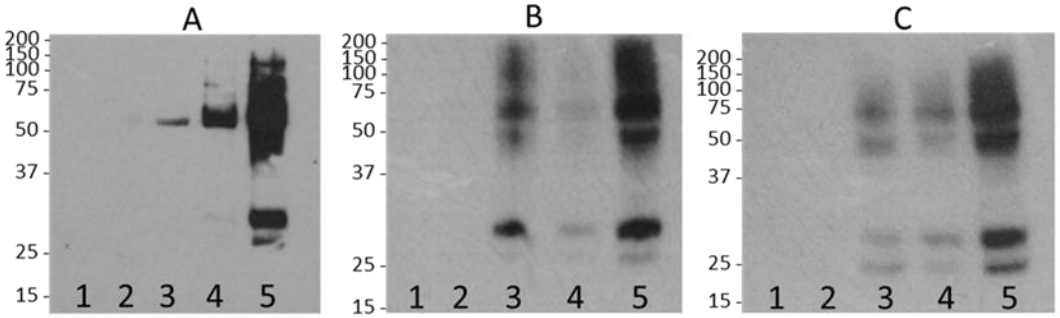

Figure 56. Binding to CII and oxPTM-CII, before and after trypsinization detected by western blotting. Serum samples tested are from patients with axSpA (A), ERA (B) and PsA (C) patients detected by immunoprecipitation and western blot. Gels were loaded in the same way, lane 1 was loaded with the serum samples after immunoprecipitation, and lane 2 was loaded with antibodies in the serum samples plus CII and lane 3 plus chlorinated CII, in lane 4 and 5 instead of CII and chlorinated CII antibodies in the serum samples were respectively incubated with trypsinised CII and chlorinated CII.

8.4.3 OxPTM-CII reactivity correlation to HLA haplotypes

HLA was genotyped to investigate the correlation between the HLA haplotypes commonly associated with axSpA and the reactivity to oxPTM-CII. The statistical analysis was performed analysing HLA-B27 and the shared epitopes HLA-DRB1 01, 04, 10 and 14. The results of the HLA correlation are shown in table 17 and in figure 57. The association between the HLA and oxPTM-CII was calculated using chi-squared test, odds ratios and kappa agreement. There is no statistical evidence of an association between either comparison, the chi-squared test shows no evidence to reject the null hypotheses that the two characteristics are independent, the odds ratios, although elevated, are not statistically significant and the kappa agreement showed a very little agreement between HLA haplotypes and modified CII.

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Table 17. Statistical calculation of correlation between the HLA haplotype (HLA-B27 and shared epitope HLA-BRB1 01, 04, 10, 14) and CII or oxPTM-CII reactivity.

	HLA-B27		HLA-DBR1 01, 04, 10, 14	
		p-value		p-value
Chi-Squared test for independence	0.3408	0.559	0.0156	0.901
Odds Ratio (95% CI)	1.53 (0.37, 6.35)	0.560	1.14 (0.14, 9.29)	0.901
Kappa Statistic (95% CI)	9.1 (-21.6, 39.9)	0.280	2.5 (-37.2, 42.3)	0.450

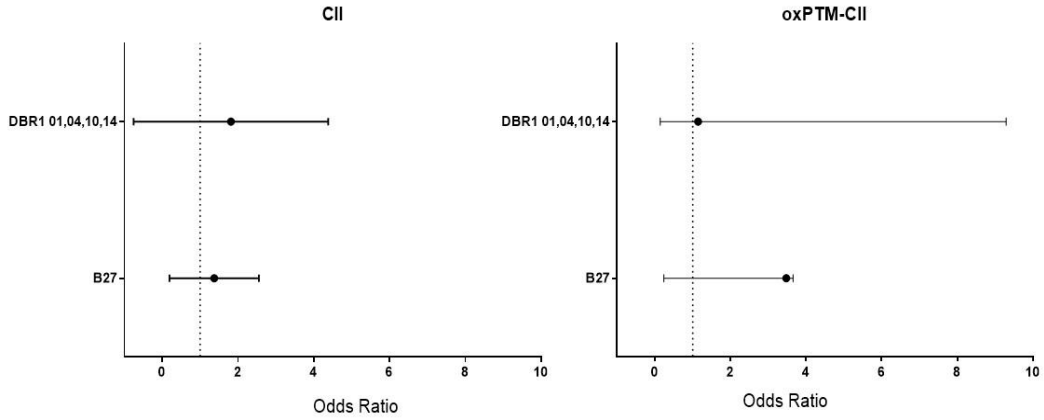

Figure 57. Correlation between the HLA haplotype (HLA-B27 and shared epitope HLA-BRB1 01, 04, 10, 14) and CII or oxPTM-CII reactivity. There is no statistical evidence of an association.

8.5 Patients analysis cohort 4: Glasgow study

In this cohort I had the opportunity to study the autoreactivity to oxPTM-CII in patients with inflammatory autoimmune arthritis. Binding of serum samples from patients with AS, PsA, PS, CD and UD to CII and oxPTM-CII was evaluated. As a negative control, healthy subjects were analysed. The characteristics of the patients are summarised in table 18. The different groups of patients were not comparable in terms of number of patients and sex, but the age of patients was very similar among the groups and the duration of diseases was similar for AS and PS and for PsA and CD. The average age of the AS patients was 55 years and the average of the duration of the disease was 23 years. The group of AS patients

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included patients not treated, patients treated with NSAID and patients treated with biologic drugs.

Table 18. Characteristics of patients in cohort 4.

variable	AS	PsA	PS	CD	UC
n. patients	144	15	35	8	1
Gender (F/M)	0.32	1.5	0.84	1	1
Age (y)	55.22 (27-82)	58.4 (38-84)	46.35 (27-73)	43.38 (24-68)	74
Duration of disease (y)	23.15 (1-51)	10.17 (1-25)	20.22 (1-51)	15.36 (3-33)	27
BASDAI	4.33 (0.14-9.38)	-	-	-	-
CRP	8.23 (0.2-66)	5.3 (1-15)	-	33 (0.5-77)	2
ESR	14.98 (2-71)	10.9 (2-29)	-	-	9
Treatment	NSAID=70, BIO=36, NIL=19, OTHER=19	MTX=8, NIL=4, NAPR=2, LEFL=1	ADA=10, MTX=11, FUMA=2, CISP= 4, OTHER=8	AZAT=4, ADA=1, ASACOL=1 6MERT=2	-
Responders	77.8%	66.7%	77%	100%	-

BASDAI= Bath Ankylosing Spondylitis Disease Activity Index, CRP= C-reactive protein, ESR= erythrocyte sedimentation rate, ADA=adalimumab, MTX=methotrexate, FUMA=fumaderm, CISP=ciclosporin, AZAT=azathioprine, 6MERT=6mercaptoturine, NAPR=naproxen, LEFL=leflunomide, NSAID=non-steroidal anti-inflammatory drug, BIO=biologics, NIL=untreated.

8.5.1 OxPTM-CII autoreactivity in cohort 4

The autoreactivity of these patients to CII and oxPTM-CII have been tested and the results are presented in the figure 58A, B and C. Figure 58A shows the binding of serum samples from patients with AS in comparison to PsA, PS, CD, UC and HC. Binding of AS serum samples to oxPTM-CII was significantly higher than binding to native CII ($p<0.0001$) with a percentage of autoreactivity of 30% to CII and 67.5% to at least one form of oxPTM-CII. The difference between the AS and the other inflammatory diseases PsA, PS and CD was not significant even though the number of patients included in the different disease-groups was very different. The percentage of reactivity to at least one oxPTM-CII was 53% in PsA group, 26% in PS group and in 37.5% in CD group (cut off 97.5th percentile).

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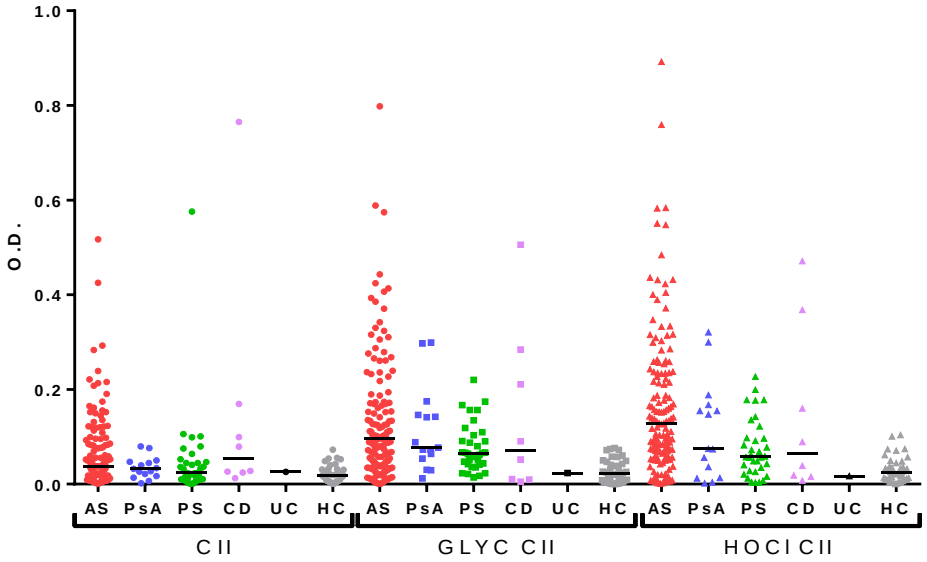

Figure 58A. Autoreactivity to CII and oxPTM-CII in cohort 4. Binding of AS, PsA, PS, CD, UD and healthy subjects (HC) serum samples to CII, oxPTM-CII. Reactivity to CII and oxPTM-CII was low in samples from healthy individuals. Binding to oxPTM-CII was significantly higher than to native CII in samples from AS patients ($p<0.0001$). Reactivity to oxPTM-CII was reported in 67.5% of AS samples, in 53% of PsA samples, 26% PS and in 37.5% of CD samples (cut off 97.5th percentile).

The overlapping binding is shown in figure 58B. In the AS group 17.4% of the samples were positive for native and modified CII while 12.5% were positive for both oxPTM, glycation and chlorination. In PsA and PS groups, the positivity to the native CII was very low while reactivity to oxPTM-CII was overlapping in 33% of PsA patients and 11% of PS patients. CD and UC have not been included in the analysis because of the small number of patients and the high percentage of negativity.

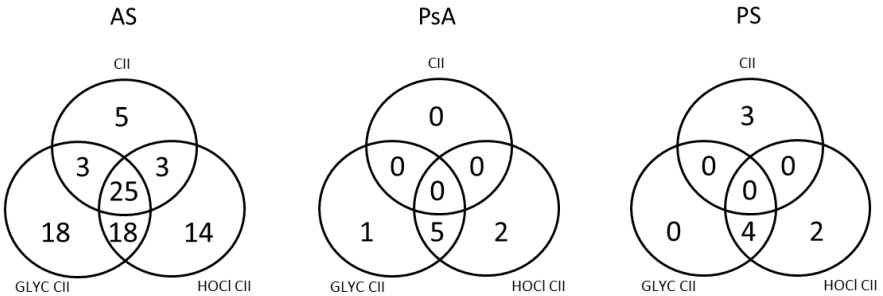

Figure 58B. Autoreactivity to CII and oxPTM-CII in cohort 4. Overlapping binding of AS, PsA and PS patients included in the cohort 4.

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The AS patients were grouped, based on the therapy they were following, in 3 different groups: NIL were patients without treatments, NSAID were patients using non-steroidal anti-inflammatory drugs and BIO were biologic treatment. Negative samples were used as control. Figure 58C shows a generally high reactivity to oxPTM-CII compared to native CII. The most interesting result is that there is a significant difference between patients treated and not treated, regardless the kind of treatment.

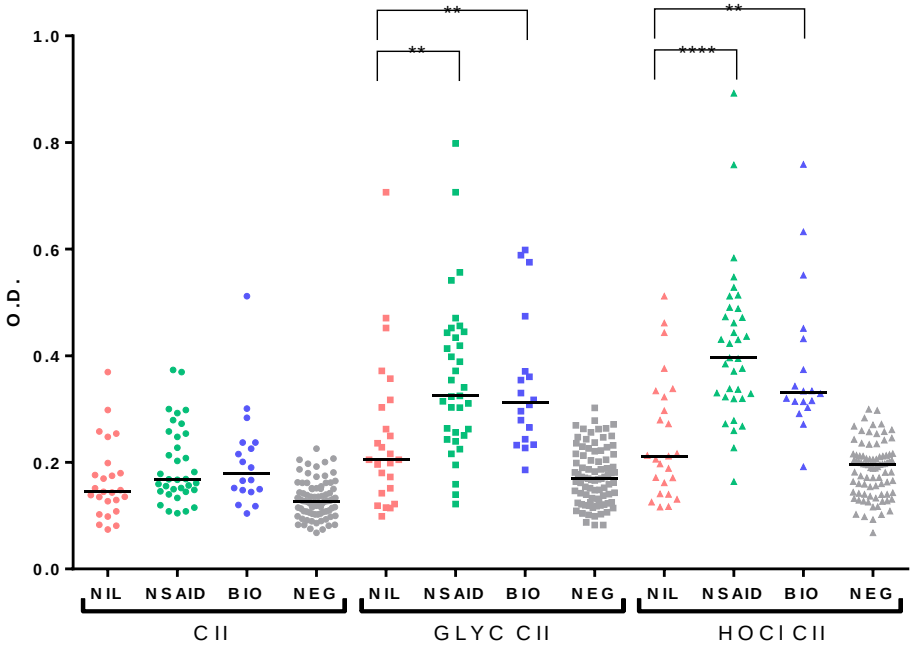

Figure 58C. Autoreactivity to CII and oxPTM-CII in cohort 4. Binding of AS patients grouped based on the therapy they are following. NIL=no treatment, NSAID=non-steroidal anti-inflammatory drug, BIO=biologic treatment, NEG=samples negative to oxPTM-CII. Reactivity of samples from patients treated with NSAID and BIO were significantly ($p<0.005$) more reactive than NIL, NEG.

8.6 CII and oxPTM-CII epitope mapping

The interaction showed in inflammatory arthritis patients to oxPTM-CII have a great interest from diagnostic, therapeutic and monitoring points of view. These autoantibodies and the epitopes responsible for the binding may be used for

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diagnostic assays, may become target for specific treatments and also may be linked to processes that correlates with specific stage of the diseases.

CII and oxPTM-CII were run in a 2D gel to obtain a high-resolution separation of the whole protein and its modifications, glycation and chlorination. The first dimension was an IEF using the IPG4 system, the second dimension was a standard SDS-PAGE in which the stacking gel was replaced by the IEF strip. The gels were stained with Coomassie blue and Silver stain kit. Due to the detection limit of these two staining techniques the stained gels are not available. To map the epitope/epitopes responsible for the autoreactivity in autoimmune diseases, mainly rheumatoid arthritis and axial spondiloarthritis, a western blot (after 2D gel) was necessary. CII size is about 140 kDa and present a very repetitive structure, due to these characteristics the 2D gel was run using the whole molecules of native and oxPTM protein but also after incubation with trypsin. The availability of specific scFv antibodies (1-11E and 3-11E) helped in the setting of the western blot. The results are shown in figure 59, 60 and 61. Figure 59A and B show the 2D gel run with the whole CII and chlorinated CII and using as scFv 1-11E antibody for detection.

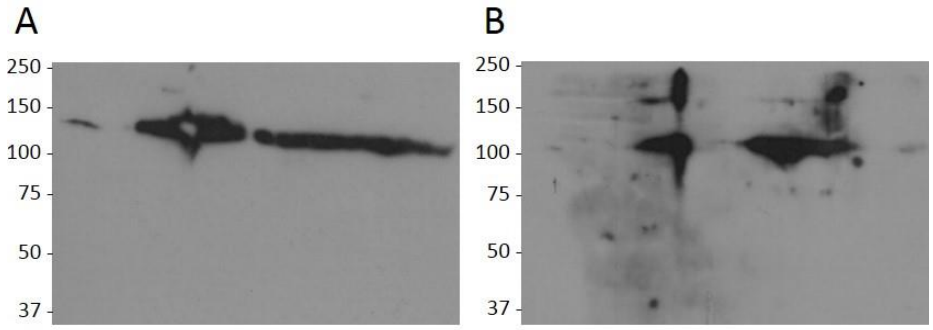

Figure 59. 2D gel-western blot of CII and oxPTM-CII. Figure A shows the 2D gel-western blot of native CII and the figure B shows the 2D gel-western blot of chlorinated CII (B) using 1-11E scFv to detect the oxPTM-CII.

Figure 60A shows the western blot of the native CII and figure 60B shows the western blot of the chlorinated CII both after incubation with trypsin. The

recombinant antibody used was 1-11E. Comparing these two figures it was possible to highlight few protein spots present in chlorinated CII western blot and not in the western blot of the native CII. The regions differently expressed are displayed in red circles.

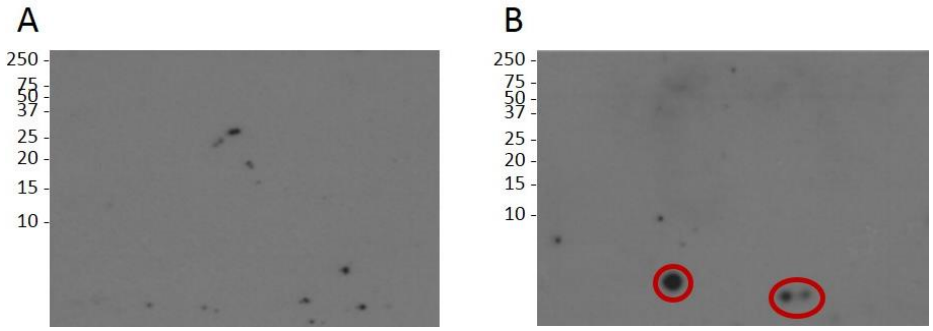

Figure 60. 2D gel-western blot of trypsinised CII and oxPTM-CII (1-11E). Figure A shows the 2D gel-western blot of trypsinised native CII, figure B shows the 2D gel-western blot of trypsinised chlorinated CII using 1-11E scFv to detect CII and oxPTM-CII. The red circles highlight the regions differently present in oxPTM-CII in comparison with native CII.

Figure 61A shows the western blot of native CII, whereas figure 61B shows the western blot of chlorinated CII, both using the recombinant antibody 3-11E. CII and chlorinated CII were both incubated with trypsin. The protein spots present in chlorinated CII and not in the native CII western blot were highlighted comparing these two figures, the protein spots differently expressed are displayed in red circles.

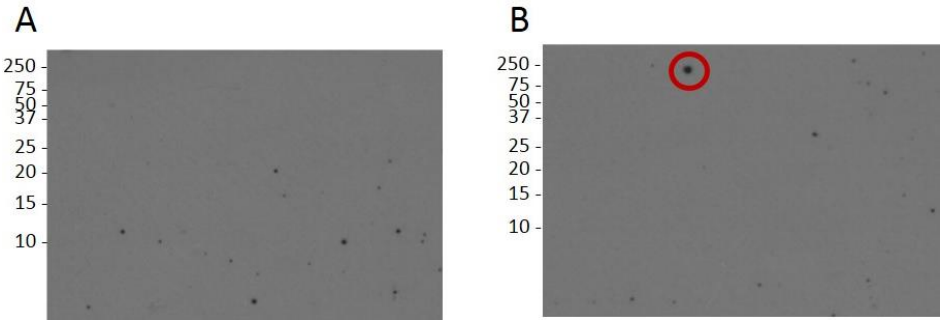

Figure 61. 2D gel-western blot of trypsinised CII and oxPTM-CII (3-11E). Figure A shows the 2D gel-western blot of trypsinised native CII, figure B shows the 2D gel-western blot of trypsinised chlorinated CII using 3-11E scFv to detect CII and oxPTM-CII. The red circles highlight the regions differently present in oxPTM-CII in comparison with native CII.

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8.7 Summary of the results

In this second part of the project I have confirmed that CII is modified by ROS *in vitro* and that the production of synthetic antibodies specific to oxPTM-CII is a stable process for our group[198]. As previously published, oxPTM-CII have a clinical role in the development of RA[4, 9]. The analysis of cohort 3 allowed us to analyse several arthritic diseases: axSpA, ERA, PsA, UA and FM. I have shown that binding of serum samples from patients with axSpA to oxPTM-CII was significantly higher than binding to native CII ($p<0.0001$) with 37% and 74% binders to native CII and oxPTM-CII, respectively. Binding of serum samples to oxPTM-CII from patients with peripheral SpA, including UA and PsA, was significantly lower compared to binding of axSpA serum samples ($p<0.0001$), with 35% and 33% of binders respectively. Furthermore, the reactivity in axSpA patients is comparable to the reactivity in ERA patients and, also in this case, the reactivity was independent from HLA haplotype, ACPA reactivity and clinical index. In addition, there was no significant difference in anti-oxPTM-CII reactivity in samples from SpA patient with axial SpA (axSpA) versus samples from patients with axSpA associated with oligoarthritis (axSpA-OA), inflammatory bowel disease (axSpA-IBD) or patients with enthesitis (axSpA-En). I have also shown in 3 different ways (comparison to an antigen control, competitive ELISA and western blot) that the reactivity to oxPTM-CII shown in ELISA is specific. Cohort 4 was including patients with AS in comparison to PsA, PS, CD, UC and HC. Binding of AS serum samples to oxPTM-CII was significantly higher than binding to native CII ($p<0.0001$) with a percentage of autoreactivity of 30% to CII and 67.5% to at least one oxPTM-CII. The difference between the AS and the other inflammatory diseases PsA, PS and CD was not significant. The AS patients were then grouped, based on the therapy they were following. The difference between patients treated and not treated, regardless the kind of treatment, was significant. Even if between the 2 groups of treatments there is no significant difference, the reactivity is slightly higher in patients treated with biological drugs. As done for oxPTM-INS, also in this second part of the project I have started mapping the neoantigenic epitopes generated by the oxidative

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stress in patients. In this case I had the opportunity to work using synthetic antibodies but due to the structure and sequence of the CII I could do the mapping only using fragmented CII/oxPTM-CII. The preliminary results generated using the 2D gel technique were promising with the individuations of some spots candidate for the sequencing.

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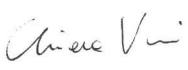
CHAPTER 9

INFLAMMATORY ARTHRITIS: DISCUSSION

Inflammatory arthritis includes progressive chronic diseases characterised by inflammation and swelling of the joints, stiffness and pain. IA is estimated to effect around 3% of the world's population. The most common types of IA are rheumatoid arthritis (RA), psoriatic arthritis (PsA) and ankylosing spondylitis (AS). The prevalence of each of these subtypes worldwide is as follows: about 1% for RA[126] and 1.9 % for spondyloarthritis (SpA)[164]. RA is a chronic inflammatory autoimmune disease associated with articular inflammation and cartilage damage[137, 134]. Indeed, proteins are susceptible to different kind of PTM that have important effect on their function and structure. These PTM include glycation, glycosylation, phosphorylation, and oxidation[16]. The specific conversion of aspartic acid to isoaspartic acid and arginine to citrulline have been deeply studied and it has been found that these amino acids conversion are involved in autoimmunity leading to the formation of autoantibodies against them in rheumatic diseases[211]. In the inflamed joint the high influx of metabolically active immune cells increases the formation of ROS, which results in protein oxidation[15]. Generation of neoepitopes after PTM and oxPTM may contribute to the epitope spreading in autoimmunity. The main extracellular matrix component of articular cartilage is type II collagen which is therefore a prominent target for oxidative post translational modifications by ROS, which results in the formation of oxPTM-CII. Several studies have demonstrated the relevance of oxPTM-CII and shown that ROS presented in the inflamed joints of RA patients can generate CII neoantigens[9, 16, 4, 197]. Antibodies to oxPTM-CII have been detected in serum samples from RA patients[4]. In patients with longstanding RA, autoreactivity to oxPTM-CII changed longitudinally whereby 92.9% of serum samples from RA patients (of early stage) bind to oxPTM-CII with high sensitivity (92%) and specificity (98%)[9].

Based on this data about autoimmune reactivity to oxPTM-CII in rheumatoid arthritis, the hypothesis was that oxPTM-CII play a key role in inflammatory



arthritic diseases. The aim of this second part of the study was to establish the hypothesis and investigate the role of oxPTM-CII-Ab in SpA. Spondylitis arthritis is a group of chronic diseases characterised by chronic back pain and arthritic inflammation, all the subgroups seem to have a common genetic component, predisposition to the HLA-B27[152]. Based on the joints involved in symptoms, SpA are divided in axSpA and peripheral SpA. AxSpA is characterized by inflammation of sacroiliac joints and/or joints between the vertebral bodies[153]. Cartilage of the axial skeleton is an important target for the disease process in axSpA[212]. Several studies have demonstrated the involvement of CII metabolism in axSpA pathology, in particular several markers of degradation and the activity of MMP have been found in axSpA patients, indicating the involvement of degradation pathways[213,214]. Whether or not the degradation of CII is also due to the high presence of ROS and oxPTM-CII is still not proven, but we can hypothesise that other inflammatory factors, such as MMP, present in axSpA inflammation are associated with ROS.

9.1 OxPTM-CII characterization

The hypothesis of this study was that ROS released during chronic inflammation can modify macromolecules, such as CII. *In vitro* oxPTM-CII were previously used and tested in our group[4,9]. I have started the study confirming the data previously published, reproducing and validating the protocols for the *in vitro* oxPTM, both glycation and chlorination. Having discovered that oxPTM-INS has a reduced stability over time (4.2), the stability of oxPTM-CII was also assessed.

To check that oxPTM-CII was always modified in a comparable way every batch was tested using several techniques: monitoring changes by SDS-PAGE and tricine PAGE gels that were stained with Coomassie blue and the ELISA using serum samples from RA patients and recombinant anti-oxPTM-CII that was developed in our group[9].

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All the observation done in this preliminary part of the study correlate with studies previously published by our group.

9.2 OxPTM-CII stability

In order to respect the criteria of consistency and reproducibility, especially in the analysis of larger cohorts of patients, it became necessary to analyse the stability of modified CII. For this reason I have compared fresh modifications and 1 month-old modifications showing that modified collagen was stable (figures 49 and 50). OxPTM-CII was tested at different time point in SDS-PAGE and ELISA using both, positive serum samples previously used[9] and serial dilution of recombinant antibodies specific to oxPTM-CII. OxPTM-CII result stable after 1 month from the *in vitro* modifications. The high stability showed by oxPTM-CII, especially after the similar experiment conducted on insulin, was surprising. The stability of the oxPTM-CII is probably due to the size and the structure of the native molecule. Indeed, the CII consists of three chains coiled around each other and the amino acid sequence is regular and repetitive[193] whilst insulin is a very small protein. The difference in stability between the two proteins may also be related the endogenous half-life, structural proteins such as CII have a long half-life[215] while insulin half-life is measured in terms of minutes[216].

9.3 OxPTM-CII reactivity: cohort 3

The elevated level of ROS production during joint inflammation in RA leads to an increase of oxPTM. This may be the origin and the cause of the high prevalence of reactivity to oxPTM-CII compared to the native protein, suggesting a potential role anti-oxPTM-CII as biomarker in RA[9]. The role of oxPTM-CII in other inflammatory arthritis diseases has not been evaluated yet. This second part of the study has been designed to investigate the involvement of oxPTM-CII in SpA pathogenesis and development.

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The main strength of this study was number of patients recruited per group and the cohort being well characterized. The limitations of this study were represented by the long duration of the diseases and the small number of patients not under treatment. Hence, I did not have access to patients at onset and before any treatment. Unfortunately, due to the late diagnosis of axSpA and the weak and nonspecific initial symptoms the recruitment of patients in an early stage or at diagnosis is very difficult. Additionally, patients were already treated with biologics that can influence the patients' immune haemostasis by, for example, immunosuppression[217–219].

The analysis of cohort of patients with IA, including SpA, was performed in collaboration with the hospital san Giovanni di Dio in Florence. Binding of axSpA to oxPTM-CII was significantly higher than binding to native CII and the percentage of autoreactivity was 74% to at least one form of oxPTM-CII indicating, for the first time, that PTM are playing a role in development of autoantibodies in axSpA patients (figure 52A). In opposition to a recent study that has shown association between CII seromarker pattern in axSpA with disease activity and HLA B27[214] we have not found any significant correlation between oxPTM-CII reactivity and disease activity, HLA genotypes, or secondary arthritic manifestation such as enthetitis, IBD or OA. AxSpA samples were also characterised for CCP to analyse every possible correlation but, as shown before by several studies[220], the binding to CCP was very low (2% positivity). This possible implication and association between anti-oxPTM-CII reactivity and cartilage oxidation/damage as a result of the joints inflammation is similar to what we observed in RA patients[4,9]. This similarity has been already reported[221,222]. The rational of our hypothesis relies on the fact that inflamed joints are populated by metabolic active immune cells indeed, both diseases involve joints inflammation and damage of cartilage, MMP high activity and the increased activity of chondrocytes[214,223].

In cohort 3 the binding of the positive controls (ERA patients) was detected in 91.7% (figure 54C) of the samples, confirming the data previously published[9] and the potential of oxPTM-CII-Ab to become a biomarker for RA. In our experiments

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we have observed a big difference between axSpA and RA: in RA oxPTM-CII reactivity was observed in very early stages and before any treatment while in SpA we observed it after long duration of the disease which implies a different pathobiology. The serum binding specificity was validated, and the results just described were confirmed with 3 different classes of experiments: the comparison of the reactivity to oxPTM-CII with the reactivity to oxPTM modified collagen type III and BSA; competitive ELISA using as competitors CII, oxPTM-CII and another oxPTM antigen and western blot. All these different experiments were confirming the specificity of axSpA serum samples to oxPTM-CII making these findings stronger.

This data shows for the first time the presence of oxPTM-CII-Ab in patients with axSpA. This data also proved the specificity of this reactivity in comparison with other diseases and other oxPTM antigens. Antibodies to oxPTM-CII might be a potential serological test for longstanding axSpA. Hence, it might be possible to use this test in critical diagnosis to stratify patients that have axSpA from PsA and UA before the comparison of bone spinal lesions. From previous publications about RA[4,9] it is possible to suppose that the imbalanced production of ROS taking place during joints inflammation plays a role in the axSpA pathogenesis even though there are no other evidence except from the analysis of this cohort and there are not publications about PTM in this group of diseases. This first study needs to be validated in a larger, possibly in a longitudinal cohort to establish when anti-oxPTM-CII-Ab are present at the onset, early stages or at later stage of disease when inflammation is substantial and patients are under immunosuppressive drugs.

9.4 OxPTM-CII reactivity: cohort 4

The absence of correlation with disease activity, HLA genotypes or other features in the analysis of the cohort 3 requires the analysis of a new cohort in order to understand the role of oxPTM-CII-Ab in axSpA.

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AxSpA included mainly AS patients that are defined by radiographic changes[224], for this reason to validate the results of the cohort 3 we have analysed another cohort of patients in collaboration with the University of Glasgow. The strength of this analysis is the dimension of the cohort, the number of patients with AS recruited was large and the group was well characterised with information about treatments and state of the diseases available. The limitations of this study were represented by the long duration of the diseases and the small number of the PsA, PS, CD and UC patients. The biggest limitation was the impossibility of a longitudinal analysis.

The reactivity of these AS patients to oxPTM-CII was significantly higher than binding to native CII with a percentage of reactivity slightly lower but still similar to the cohort 3 (cohort 3 figure 52A, cohort 4 figure 58A). The difference between the AS and the other inflammatory diseases PsA, PS and CD was not significant with a very high percentage of reactivity in PsA patients, even though the number of patients included in the different disease-groups was very different with an average difference of 100 patients. The information available about the patients included in this cohort were permitting an analysis of the reactivity in correlation with the kind of treatment used in the AS group of patients. Patients were treated with NSAID, DMARD and biologics such as anti-tumour necrosis factor (TNF) blockers and anti-IL17. Interestingly, this analysis was revealing a significant difference in oxPTM-CII reactivity between the naïve patients for treatment and patients treated either with NSAID/DMARDs and biologic treatments. These new findings in the AS group of patients introduces the new hypothesis of the development of oxPTM-CII-Ab at later stage of disease when inflammation is consistent and after treatment. One of the hypothesis that can justify this result is that chronic inflammation and hence longitudinal treatment with immunosuppressive may reflect a possible role of biological drugs in modify immune response against oxPTM-CII neoepitopes. This hypothesis is supported by the standard adverse effect of biological therapies and monoclonal antibodies founded in clinic. These adverse effects are related to immunosuppression,

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immunostimulation and hypersensitivity [225] and potentially to immune response to self-antigens and neoepitopes[226]. In regard to this interesting data, further studies are needed, in a larger cohorts and with longitudinal follow up, with the aim to assess the reactivity before and after therapy. It may also be that the analysis of oxPTM-CII-Ab give information about the response to treatment, in order to understand the potential use of oxPTM-CII-Ab to discriminate patients who respond/do not respond to therapy more studies are required.

9.5 OxPTM-CII: epitope mapping

As previously explained regarding the mapping of the oxPTM-INS, the identification of the binding site on the autoantigen has a very high importance connected with all the fields in clinics: diagnosis, prognosis and therapy. The process of epitope mapping can follow several directions, I have decide to use the same approach used for the oxPTM-INS mapping for the high resolution and the use of this technique to separate small proteins and peptides. For oxPTM-CII, I could use specific recombinant antibodies[199] instead of serum samples, simplifying the setup of the western blot.

Firstly, I have tried to perform the 2d gel-western blot using the whole molecules of CII and oxPTM-CII and one of the specific recombinant antibody. The mapping using the whole molecule of CII and oxPTM-CII was not giving us good results, I could not identify protein spots for further analysis. The resolution problems may be due to the big size of CII (140kDa) and to the repetitive nature of the amino acid sequence that may easily result in repetition of the immunogenic epitope sequence. For this reason, before the 2d gel analysis, the CII and the oxPTM-CII were both digested, this was helping to obtain smaller fragments that could be easily separated. The analysis conducted on the digested proteins was working very nicely and it was possible to use two of the specific recombinant antibodies. This new analysis was letting us identifying spots differently recognised between CII and oxPTM-CII that may be good candidates for further analysis. The presence

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of different spots may be due to either to the presence of several neoepitopes and to the presence of repetition of the epitope sequence. From the comparison of the western blot done using two different antibodies we can also suppose that different antibodies recognise different epitopes on the oxPTM-CII sequence. This new idea implies the necessity of a detailed check of the reactive serum samples in order to understand if the phenomena is happening only using the recombinant antibodies or it is also happening in patients. In order to understand which is the case, the epitope mapping needs to be elaborated and repeated using a group of reactive serum samples from patients ERA and axSpA before proceeding to the mass spectrometry analysis.

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CHAPTER 10

CONCLUSIONS

Post-translational modifications are physiological mechanisms in the human body. PTM may change the primary and tertiary structure of proteins, biological functions and interaction. Each of the PTM may play a critical role in the generation immunogenic or tolerogenic self-peptides. Several studies illustrate that PTM can create new self-antigens by altering immunologic processing and presentation[16]. The number of autoimmune diseases associated with post-translationally modified autoantigens continually increases. Our laboratory has focused on understanding the role of oxPTM of key proteins in autoimmunity. This study was investigating the role of oxPTM-INS in T1D and oxPTM-CII in axSpA, both recognised as potential autoantigens from preliminary studies[4,9,82]. The study was divided into two different sections describing the role of the two modified proteins, oxPTM-INS and oxPTM-CII, in the development of autoimmune diseases.

The conditions for the *in vitro* oxPTM of insulin and CII have been already published[4,82] and my study confirmed all the data previously shown. The biggest difference between the two proteins was the size. Due to the small size, insulin necessitated native conditions in the monitoring and epitope mapping experiments. In part, this is due to the fragmentation derived from the oxPTM and probably also because the neoepitopes generated with the oxPTM are conformational and consequently not visible in denaturing conditions.

On the other hand one of the big limitations of working with CII is due to the big size and the characteristic repetitive structure. The denaturing conditions were caused no issue in the visualization of the oxPTM-CII, but the modifications on the 3 long chains of CII were not easy to map using a 2d gel and necessitate of an additional digestion.

The reactivity of serum samples from patients was tested to both oxPTM proteins. T1D is an autoimmune disease well studied and characterised, this was helping in the collection of samples. Even if the ABIS cohort was including a small number of

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children who progress to T1D before the onset, the meaning of this reactivity is very clear and it confirmed data previously published by our group[82]. The conclusion of the first part of the study is that immune reactivity to oxPTM-INS-Ab is present before the clinical onset of type 1 diabetes and levels of oxPTM-INS-Ab can identify children progressing to type 1 diabetes revealing an important predictive potential.

The reactivity of serum samples from patients with different inflammatory arthritis to oxPTM-CII was tested. In this case I had the opportunity to test two different cohorts of patients that were including a large number of samples. The high reactivity to oxPTM-CII in ERA was confirmed, but I have also shown for the first time a high and specific reactivity in axSpA patients indicating oxPTM-CII neoepitope can be formed after long standing inflammation and that this can breach tolerance. The study could be completed by testing SpA patients at early stages and before treatment with immunosuppressive drugs and follow them longitudinally.

The study involving oxPTM-INS and T1D requires additional studies to confirm the predictive potential of oxPTM-INS-Ab in type 1 diabetes. Despite the additional studies required, this study can be considered a success who may help the clinic in the prediction of T1D.

The results of oxPTM-CII and IA were not implying that antibodies to oxPTM-CII can be used as diagnostic biomarker, but may shed light on the possible mechanism that correlate long standing inflammation with a breach of immune tolerance. For the first time we have reported reactivity to an oxPTM autoantigen in axSpA, and we have confirmed this reactivity in two large groups of patients. Unfortunately, most of the patients were longstanding and under treatment and the reason of reactivity to oxPTM-CII in axSpA patients is not still completely clear.

In conclusion, it is becoming more evident that PTM are play an important role in autoimmunity. The results of my study suggest that ROS released during chronic inflammation are able to post-translationally modify proteins and that this may

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lead to the formation of neoepitopes and to a consequently immune response. T1D is the perfect example demonstrating that this process is happening before the clinical onset and can be investigating to generate new diagnostic assays and target therapies.

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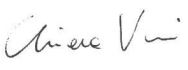

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ABSTRACTS SUBMITTED TO INTERNATIONAL CONFERENCE

The 10th International Congress on Autoimmunity 2016

"Involvement of post-translational modification by reactive oxygen species of neuronal-proteins in the pathophysiology of multiple sclerosis." Chiara Vinci, Jens Kuhle, Gavin Giovannoni and Ahuva Nissim

INTRODUCTION. Reactive oxidants (ROS) physiologically play a role in human health but under inflammatory conditions its levels are abnormally high. During inflammation, the influx of ROS results in chemical post-translational modification of normal proteins which leads to the formation neoepitopes. These modified proteins could be recognised by the immune system and leads to the production of auto-antibodies to ROS-modified proteins as described in inflammatory autoimmune diseases such as Rheumatoid Arthritis (RA) and Multiple Sclerosis (MS).

HYPOTHESIS. ROS present in MS patients are able to modify neuronal-proteins and in turn triggers the production of auto-antibodies against ROS-modified neuronal-proteins.

As an initial target, we chose to study Myelin Oligodendrocyte Glycoprotein (MOG) which is a glycoprotein important in the myelination of nerves in the central nervous system and it is already known as an auto-antigen in MS.

METHODS. ROS-modified MOG was generated by incubation with various ROS and modifications and were visualised by SDS-PAGE. Serum samples of MS patients (n=30 were tested by ELISA for their ability to bind different ROS-MOG. Human serum albumin (HSA) was also modified and used as a negative control. Sera from patients with other neurological diseases (n=30) and healthy controls (n=15) were also examined.

RESULTS. Autoantibodies to native MOG was increased in MS patients in comparison to healthy controls. No significant binding to HSA or ROS-HSA is shown. Autoantibodies to ROS-MOG was detected in X patients.

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CONCLUSION AND FUTURE STUDY. Our preliminary results suggest the presence of auto-antibodies against ROS-MOG in a group of MS patients. We are going to examine more patients as well as stratifying the samples into different disease stages. Furthermore we will be examining other neuronal proteins to determine the major ROS-modified auto-antigen in MS.

"Targeting of viral interleukin-10 with an antibody fragment specific to damaged arthritic cartilage improves its therapeutic potency." Ahuva Nissim, Chris Hughes, Bjarne Faurholm, Ngee Han Lim, Angelica Sette, Chiara Vinci, Louise Topping

INTRODUCTION. Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by synovial inflammation which results in a progressive joint damage. The current systemic treatment with disease modifying antirheumatic drugs (DMARDs) is associated with side effects, since such treatment does not deliver pharmacologically active molecules solely to the site of disease activity in the joints.

OBJECTIVE. The aim of this project is to specifically target an anti-inflammatory cytokine to the inflamed joint which will results in high local concentrations and low systemic concentrations, increasing efficacy whilst minimizing side-effects. We chose collagen type II (CII) modified by reactive oxygen species (ROS), namely anti-ROS-CII, as a target, as it is uniquely present in damage cartilage as a result of the inflammation process. As a targeting unit we used a single chain fragment variable (scFv) specific to ROS-CII.

METHODS. We have fused the viral interleukin-10 (vIL-10), a major anti-inflammatory cytokine, to anti-ROS-CII scFv (1-11E) to create 1-11E/vIL-10 fusion via a matrix-metalloproteinase (MMP) cleavable linker, so that the anti-inflammatory cytokine is released in the inflamed knee in which MMPs are upregulated. The specific binding of 1-11E/vIL-10 to ROS-CII was determined by ELISA, Western blotting and by immunostaining of arthritic cartilage, while vIL-10 bioactivity was evaluated in vitro using an MC-9 cell proliferation assay. We also

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assessed 1-11E/vIL-10 in vivo localization and also its therapeutic efficacy, by employing the mouse model of antigen-induced arthritis (AIA).

RESULTS. We were able to demonstrate the specific binding of 1-11E/vIL-10 to damaged arthritic cartilage. Interestingly, the in vitro IL-10 activity in the fusion protein was observed only after cleavage with MMP-1. We observed that 1-11E/vIL-10 systemically administered to arthritic mice, localised specifically to the arthritic knee, with peak accumulation observed after 3 days. Moreover, 1-11E/vIL-10 reduced inflammation significantly quicker than vIL-10 fused to the control anti-hen egg lysozyme scFv (C7/vIL10).

CONCLUSION. Here we describe that vIL-10 fused to the human antibody fragment specific for damaged arthritic cartilage is a valid targeted anti-inflammatory therapy in the treatment of mouse model of arthritis. Our results further support the hypothesis that targeting bio-therapeutics to arthritic joints may be extended to include anti-inflammatory cytokines which lack efficacy when administered systemically.

Protein & Antibody Engineering Summit Europe (PEGS) 2017

"Antibodies to post-translationally modified insulin in type 1 diabetes." Rocky Strollo, Chiara Vinci, Mayda H. Arshad, David Perrett, Claudio Tiberti, Francesco Chiarelli, Nicola Napoli, Paolo Pozzilli, Ahuva Nissim.

Insulin is the most specific beta-cell antigen and a potential primary auto-antigen in type 1 diabetes. Insulin autoantibodies (IAA) are the earliest marker of beta-cell autoimmunity, however, only slightly more than 50% of children and even fewer adults newly-diagnosed with type 1 diabetes are IAA positive.

We investigated if autoimmunity in T1D is associated with insulin that is post-translational modified by reactive oxidant (oxPTM-INS)

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oxPTM-INS was analysed by mass spectrometry. Binding to native and oxPTM-INS was evaluated by ELISA and Western Blotting.

Mass spectrometry of oxPTM-INS identified chlorination of Tyr16 and Tyr26; oxidation of His5, Cys6 and Phe24 and glycation of Lys29 and Phe1 in B-chain. Significant higher binding to oxPTM-INS vs native-insulin was observed in type 1 diabetic subjects ($p<0.0001$, 84% sensitivity and 99% specificity) and was directed toward oxPTM-INS fragments with slower mobility than native insulin.

Conclusion: oxPTM-INS is a potential autoantigen in new-onset type 1 diabetics.

<u>The European League Against Rheumatism (EULAR) 2017</u>

"Antibodies to type II collagen: a novel tool for the diagnosis of spondyloarthritis?" C. Vinci, M. Infantino, L. M. Topping, R. Strollo, V. Grossi, M. Manfredi, F. Bandinelli, F. Li Gobbi, A. Damiani, Pozzilli, M. Benucci, A. Nissim.

Spondyloarthritis (SpA) are a group of inflammatory joint diseases which cause chronic, progressive, axial inflammation of the spine and the sacroiliac joints. Diagnosic criteria for SpA are clinical symptoms, radiology, MRI or ultrasound and following ASAS (Assessment of SpondyloArthritis international Society) criteria. SpA is similar to other inflammatory joint diseases such as rheumatoid arthritis (RA) and psoriatic arthritis (PsA), nevertheless they show considerably different pathologies.

The aim of our study is to test whether a novel assay originally developed for RA can be used for SpA diagnosis. We have previously shown that antibodies to oxidative post-translationally modified collagen type II (oxPTM-CII) are present especially in RA patients.

Our study intends to investigate the reactivity to oxPTM-CII in SpA patients compared to early undifferentiated arthritis (EA) and PsA patients.

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OxPTM-CII were generated using various reactive oxidants and were analysed by SDS-PAGE. Binding to native and oxPTM-CII was evaluated by ELISA and Western Blotting. We used a cohort of sera from 67 patients with SpA, 54 patients with PsA and 49 patients with EA. As controls, 19 patients with fibromyalgia (FM) and 70 healthy subjects (HC) were used. The specificity of the binding was further assessed by competitive ELISA and western blot.

We detected stronger reactivity of SpA sera compared to PsA and even EA samples. Specific binding to oxPTM-CII was observed in the 71% of SpA sera compared to 26% in PsA and 25% in EA. There was minimal binding in samples from FM and HC. A selection of the most reactive samples were evaluated by western blot and competitive ELISA to assess the serum binding specificity to oxPTM-CII.

For the first time it has been demonstrated that anti-ROS-CII may be a potential biomarker for SpA diagnosis.

13th World Congress on Inflammation 2017

"Antibodies to post-translationally modified insulin in type 1 diabetes."

Rocky Strollo, Chiara Vinci, Mayda H. Arshad, David Perrett, Claudio Tiberti, Francesco Chiarelli, Nicola Napoli, Paolo Pozzilli, Ahuva Nissim.

Insulin is the most specific beta-cell antigen and a potential primary auto-antigen in type 1 diabetes. Insulin autoantibodies (IAA) are the earliest marker of beta-cell autoimmunity, however, only slightly more than 50% of children and even fewer adults newly-diagnosed with type 1 diabetes are IAA positive.

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Conclusion: oxPTM-INS is a potential autoantigen in new-onset type 1 diabetics.

The 11th International Congress on Autoimmunity 2018

"Oxidative posttranslationally modified neoantigens as biomarker for autoimmune diseases"

C. Vinci, A. Nissim

Our studies have been mostly focused on studying the mechanisms that lead to the formation of disease and tissue-specific neoepitopes in autoimmune diseases. We have discovered that auto reactivity in autoimmune diseases is against oxidative post-translationally modified neoantigens (oxPTM). We have also showed that autoantibodies to oxPTM collagen type II (oxPTM-CII) neoantigens are biomarkers that can be utilised for early diagnosis and for stratification of patients with rheumatoid arthritis. Similarly oxPTM-CII antibodies are present in spondylitis arthritis. Additional example for the involvement of oxidative posttranslational modification in autoimmune disease is type 1 diabetes (T1D). We have discovered that oxPTM insulin is a neoantigen in T1D. Hence, autoantibodies to oxPTM-insulin are biomarkers for early diagnosis of T1D. More recently, we revealed that reactivity to oxPTM-insulin could predict development of T1D, even in children who were negative for gold standard serological tests. In conclusion, oxidative posttranslational modification play a role in autoimmune disease pathogenesis and can be used to identify disease tissue specific biomarkers for early diagnosis and prediction of the disease.

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The European League Against Rheumatism (EULAR) 2018

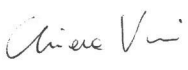
"Antibodies to post-translationally modified collagen II in spondyloarthritis"

C. Vinci

Background: Spondyloarthritis (SpA) are a group of rheumatic diseases with either predominantly axial inflammatory symptoms of the spine and sacroiliac joints, or predominantly peripheral arthritis. The most common axial SpA (axSpA) are non-radiographic axSpA and in particular ankylosing spondylitis. The current gold standard diagnostic criteria for axSpA are clinical symptoms, radiology, MRI or ultrasound according to Assessment of SpondyloArthritis international Society (ASAS) criteria [1]. We have previously showed that antibodies to oxidative post-translationally modified collagen type II (oxPTM-CII) are present and specific in RA patients whether ACPA positive or negative.

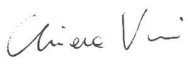
Objectives: The aim of the current study was to test the presence of antibody to oxidized collagen type II (CII) in axSpA, based on the hypothesis that spinal inflammation in axial SpA results in oxidative post-translational modification (oxPTM) of axial joints cartilage matrix proteins such as CII with the consequently formation of neoepitopes and a secondary humoral autoimmune response.

Methods: CII was oxidized by exposing CII to ribose and hypochlorous acid. Levels of antibodies specific to native CII and CII post-translationally modified by oxidants (oxPTM-CII) was assessed by enzyme-linked immunosorbent assays (ELISA) in serum samples obtained from patients with axSpA (n=67) in remission and axSpA patients (n= 14) non in remission. Reactivity in axSpA was compared to reactivity in samples from patients with predominantly peripheral arthritis such as psoriatic arthritis (PsA, n=54)), undifferentiated arthritis (UA, n=49) and early rheumatoid arthritis (ERA, n=60). As a control we used fibromyalgia (FM, n=19) and healthy subjects (HC, n=70). The specificity of the binding was further assessed by competitive ELISA and western blot.



Results: Stronger binding to oxPTM-CII was observed in serum samples from axSpA patients, the positivity was 72% for patients in remission and 86% for patients not in remission (86%) compare to positivity in PsA group (33%), UA group (35%) and FM group (16%), ($p<0.0001$). Interestingly, binding of axSpA samples was similar to binding of serum samples from ERA (95%). Binding to ROS-CII was directed to a range of ROS-CII fragments between 25 and 150 kDa.

Conclusions: Formation of oxPTM-CII neoantigens in the inflamed axial joints results in an immune response that elicits antibodies specific to oxPTM-CII. Once established in future studies, antibodies to oxPTM-CII may be developed as potential biomarker for axSpA.



PUBLICATIONS AND PAPERS SUBMITTED

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Rocky Strollo, Chiara Vinci, Mayda H. Arshad, David Perrett, Claudio Tiberti, Francesco Chiarelli, Nicola Napoli, Paolo Pozzilli*, Ahuva Nissim*.

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ARTICLE

Antibodies to post-translationally modified insulin in type 1 diabetes

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Abstract
Aim/hypothesis Insulin is the most specific beta cell antigen and a potential primary autoantigen in type 1 diabetes. Insulin autoantibodies (IAAs) are the earliest marker of beta cell autoimmunity; however, only slightly more than 50% of children and even fewer adults newly diagnosed with type 1 diabetes are IAA positive. The aim of this investigation was to determine if oxidative post-translational modification (oxPTM) of insulin by reactive oxidants associated with islet inflammation generates neoepitopes that stimulate an immune response in individuals with type 1 diabetes.
Methods oxPTM of insulin was generated using ribose and various reactive oxygen species. Modifications were analysed by SDS-PAGE, three-dimensional fluorescence and MS. Autoreactivity to oxPTM insulin (oxPTM-INS) was observed by ELISA and western blotting, using sera from participants with type 1 or type 2 diabetes and healthy controls as probes. IAA was measured using the gold-standard radiobinding assay (RBA).
Results MS of oxPTM-INS identified chlorination of Tyr16 and Tyr26; oxidation of His5, Cys7 and Phe24; and glycation of Lys29 and Phe1 in chain B. Significantly higher binding to oxPTM-INS vs native insulin was observed in participants with type 1 diabetes, with 84% sensitivity compared with 61% sensitivity for RBA. oxPTM-INS autoantibodies and IAA co-existed in 50% of those with type 1 diabetes. Importantly 34% of those with diabetes who were IAA negative were oxPTM-INS positive. Altogether, 95% of participants with type 1 diabetes presented with autoimmunity to insulin by RBA, oxPTM-INS or both. Binding to oxPTM-INS was directed towards oxPTM-INS fragments with slower mobility than native insulin.

Paolo Pozzilli and Ahuva Nissim contributed equally to this study.

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Conclusion/interpretation These data suggest that oxPTM-INS is a potential autoantigen in individuals with new-onset type 1 diabetes.

Keywords Insulin autoantibodies · Oxidative stress · Post-translational modification · Reactive oxygen species · Type 1 diabetes

Abbreviations

Em_{max}	Maximum emission
Ex_{max}	Maximum excitation
HEL	Hen egg lysozyme
HOCl	Hypochlorous acid
HOCl-INS	Insulin modified by hypochlorous acid
IAA	Insulin autoantibody
IMDIAB	Immunotherapy Diabetes
$^{\bullet}OH$	Hydroxyl radical
$^{\bullet}OH$-INS	Insulin modified by the hydroxyl radical
oxPTM	Oxidative post-translational modification
oxPTM-INS	Oxidative post-translationally modified insulin
PTM	Post-translational modification
RBA	Radiobinding assay
ROS	Reactive oxygen species

Introduction

Oxidative stress might be a critical player in the pathogenesis of type 1 diabetes. Hyperglycaemia and a large influx of metabolically active immune cells infiltrating the inflamed islets might result in the formation of high levels of reactive oxygen species (ROS) [1, 2]. The key ROS that are known to be produced are the superoxide radical, hydrogen peroxide, the hydroxyl radical ($^{\bullet}OH$), hypochlorous acid (HOCl), nitric oxide, peroxynitrite and oxidants derived from glycation as a result of hyperglycaemia. High levels of ROS may lead to oxidative post-translational modification (oxPTM) of beta cell self-proteins and the formation of neoepitopes. Neoepitopes are epitopes that have not been previously presented to the immune system [3, 4] and therefore escape immune tolerance and generate autoimmunity. Other intracellular events, such as increases in ROS and endoplasmic reticulum stress, impair beta cell autophagic activity [5]. This leads to apoptosis, inflammation, altered processing of beta cell antigens and accumulation of extracellular matrix, which are in turn both targets for ROS and further oxPTM.

Although this mechanism is well established in other autoimmune diseases such as coeliac disease and rheumatoid arthritis [6, 7], there is scant evidence available for type 1 diabetes [8–12]. We have previously shown that patients with type 1 diabetes had increased levels of autoantibodies against collagen type II modified by ROS, and that this response was under the genetic control of the *HLA-DRB1*04* shared epitope alleles [13]. Although the effect of ROS in inducing autoimmunity towards beta-cell-specific antigens remains largely unknown, this role for ROS is supported by the evidence that antibodies against oxidised GAD are present in diabetes [11]. Insulin is the most specific antigen for pancreatic beta cells and a potential primary autoantigen in type 1 diabetes. Insulin autoantibodies (IAAs) have been reported since 1983 [14] and represent the earliest marker of beta cell autoimmunity [15]. However, only slightly more than half of children and even fewer adults with recent-onset type 1 diabetes are IAA positive [16, 17].

It is an enigma why tolerance to insulin malfunctions in type 1 diabetes. Our hypothesis is that oxPTM by oxidants associated with islet inflammation has a role in breaching tolerance to insulin in type 1 diabetes. To address this hypothesis, we have tested reactivity to native and oxPTM insulin (oxPTM-INS) in individuals with newly diagnosed type 1 diabetes, and compared the results with those from control participants using both cross-sectional and longitudinal designs.

Methods

Participants Serum samples were obtained from participating centres of the Immunotherapy Diabetes (IMDIAB) group. A population of young individuals diagnosed with type 1 diabetes according to ADA criteria was studied. Serum samples from participants with type 1 diabetes (n=116) were collected at diagnosis, before the start of insulin therapy. For 69 patients, a second serum sample was obtained at the following time points after the start of insulin therapy: 3 months (19 samples); 3–6 months (16 samples); 6–9 months (19 samples); and 12 months (15 samples). Sera from 64 individuals with type 2 diabetes and 113 healthy individuals (45 schoolchildren and 68 adults) were used as controls. Features of the studied cohorts are reported in Table 1. This project was approved by the ethical committee at the University Campus Bio-Medico within the framework of the IMDIAB investigators' type 1 diabetes study, with informed consent signed by participants or their parents.

In vitro chemical modifications Human recombinant insulin (1 mg/ml) in PBS was incubated overnight at 37°C with the following agents: (1) 2 mol/l ribose (Sigma, Gillingham, UK); (2) 9 mmol/l HOCl (BDH, Oxford, UK); (3) 4.5 mmol/l $CuCl_2$ (Sigma) and 9 mmol/l hydrogen peroxide (Sigma), which was used to produce $^{\bullet}OH$ by the Fenton reaction. Hen egg lysozyme (HEL; Sigma) was also modified as above and used as the control antigen. Modification of insulin was monitored by 20% reducing SDS-PAGE and 20% native-PAGE, followed by staining with Coomassie blue (Sigma).

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Table 1 Clinical and biochemical features of the studied populations

Characteristic	Type 1 diabetes		Type 2 diabetes (n=64)	No diabetes	
	At diagnosis (n=116)	After insulin[a] (n=69)		Children (n=45)	Adults (n=68)
Age, years	13.9±0.8	14.8±1.0	59.5±3.4	14.2±0.6	38.0±1.4
Ratio of male:female	0.85	0.97	1	0.33	0.66
Blood glucose, mmol/l	21.4±0.7	8.4±0.7	8.6±0.4	4.2±0.1	
HbA_{1c}, % (mmol/mol)	11.0±0.3 (96±2.9)	6.8±0.3 (51±3.1)	7.4±0.3 (57±2.9)	5.4±0.1 (39±4.02)	
BMI, kg/m^2	18.7±0.4	18.7±0.5	31.8±2.1	21.4±0.8	

Data are means±SE, unless otherwise stated

[a] See "Methods" for disease duration

Three-dimensional (3D) fluorescence 3D scanning fluorescence spectra were obtained using a Hitachi F-4500 spectrofluorometer (Tokyo, Japan). Samples were briefly centrifuged prior to scanning to remove aggregated material. Simultaneous excitation (200–800 nm) and emission (200–800 nm) spectra were recorded.

MS Samples were desalted with StageTip C18 and analysed by MALDI-TOF in positive, linear mode, mass range m/z from 2,000 to 25,000. An aliquot of the samples was reduced with DTT, digested with Glu-C (Calbiochem, Merck, Darmstadt, Germany), desalted using StageTip C18 and submitted to MALDI-TOF (MS) and MALDI-TOF-TOF (MS/MS) analysis and nano-electrospray ionisation tandem MS.

Detection of autoantibodies to oxPTM-INS An ELISA was performed using native insulin and oxPTM-INS or control native HEL and oxPTM-HEL as targets. Briefly, ELISA plates (Nunc, London, UK) were coated with 10 μg/ml modified or native protein in 0.05 M carbonate/bicarbonate buffer (pH 9.6) at 4°C overnight. Plates were then washed three times with PBS. After blocking for 2 h with 5% BSA in 0.5% Tween-PBS, 100 μl of 1:200-diluted serum samples in 5% BSA in 0.5% Tween-PBS were added to each well, followed by 2 h incubation at room temperature. Plates were then washed with PBS plus 0.1% Tween, followed by three washes with PBS. Anti-human IgG–HRP conjugated antibodies (Sigma) were then added at 1:1,000 dilution in 5% BSA in 0.5% Tween-PBS for another 2 h incubation. The ELISA plates were washed, and 100 μg/ml 3,3′,5,5′-tetramethylbenzidine substrate (Sigma) in 100 mmol/l sodium acetate (pH 6.0) was added. Subsequently, the reaction was stopped with 1 mol/l sulphuric acid. Absorbance was measured at 450 nm using a GENios plate reader and Magellan software (Tecan, Reading, UK).

The ELISA absorbance values obtained for HEL and oxPTM-HEL were used as background controls that were subtracted from the absorbance values of native insulin and oxPTM-INS, respectively. In addition, to control assay fluctuation, binding to insulin, oxPTM-INS, HEL and oxPTM-HEL was tested for each individual sample on the same plate. Each assay included known positive or negative reference control samples. Longitudinal samples obtained from the same individuals (before and after insulin treatment) were tested on the same plate. Levels of oxPTM-INS antibodies above the 99th percentile of the healthy individuals were defined as the ELISA cut-off.

A competitive ELISA was performed to assess the binding specificity of serum to oxPTM-INS. The competitive ELISA was carried out in a similar manner as above, except that the serum samples were pre-incubated for 2 h or overnight, with and without 10 μg/ml native insulin or oxPTM-INS as the competitor, before adding the serum samples to the coated ELISA plate.

Radiobinding assay (RBA) for detection of IAA IAAs were measured by RBA using a modified radioimmunoprecipitation assay initially described by Williams and coworkers [18]. Briefly, 20 μl serum was incubated for 2 days at 4°C in the presence of human 125-I insulin. Immune complexes were then precipitated by using 50% protein A/G-Sepharose. After several washings, bound 125-I insulin was measured in a beta counter. Results were expressed as an index defined as follows: (sample cpm – negative standard control cpm) / (positive standard control cpm – negative standard control cpm). Serum samples with an index >0.002 were considered to be positive. The assay limit of positivity was calculated according to >99th percentile values of 150 healthy control sera. This assay achieved 46% sensitivity and 100% specificity at the Islet Autoantibody Standardization Proficiency Workshop in 2012.

Western blot PAGE was run as described above and proteins were transferred to nitrocellulose using standard protocols. Membranes were blocked in 5% dry milk powder (Marvel) for 2 h, followed by overnight incubation at 4°C with serum samples diluted 1:1,000 in 5% dry milk powder. After washing twice for 5 min in 0.5% Tween-PBS and for 5 min in PBS, membranes were incubated for 1 h with anti-IgG HRP

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(Sigma), diluted 1:1,000 in 5% dry milk powder. Membranes were then washed twice for 10 min in 0.5% Tween-PBS and for 10 min in PBS before incubating with 1 ml Luminata Forte Western HRP (Sigma). Bands were detected using the FluorChem E System (ProteinSimple, Santa Clara, CA, USA).

Statistical analysis Statistical analyses were performed using Prism (GraphPad, San Diego, CA, USA) and SPSS (SPSS, Chicago, IL, USA). Differences in antibody levels between the groups were tested using the Mann–Whitney *U* (Wilcoxon) test, and data are presented as medians±SE. Longitudinal changes in antibody binding were evaluated using the Wilcoxon paired test. Pearson's correlation test was used to assess the association between reactivity to oxPTM-INS and metabolic control. To determine diagnostic discrimination between participants with type 1 diabetes, type 2 diabetes and healthy controls, we used the 99th percentile of the healthy individuals as the cut-off point for absorbance units to construct a contingency table of positive autoantibodies against the clinical diagnosis (type 1 diabetes vs healthy control; type 1 diabetes vs type 2 diabetes) and tested it using Fisher's exact test. The ELISA intra-assay CV was calculated as the SD of a set of triplicate measurements divided by the mean of the serum samples. The inter-assay CV was calculated similarly for samples assessed in different experiments performed by different operators.

Results

PAGE analysis of native and oxPTM-INS Using SDS-PAGE analysis, ROS-modified insulin (oxPTM-INS) and native insulin migrated to the region of 5 kDa with no significant differences in mobility, although a smear above the major 5 kDa band was observed for oxPTM-INS (Fig. 1a, SDS-PAGE). In non-denaturing native-PAGE, differences emerged between native and modified insulin. After glycation by ribose, a clear shift in the position of the insulin band appeared, together with two additional bands, all with slower mobility. Exposure of insulin to the $^{\bullet}$OH-generating system and HOCl induced the appearance of additional and slower mobility bands, in conjunction with a smear of protein through the entire line, suggesting fragmentation (Fig. 1b).

3D-fluorescence profile of native and oxPTM-INS To determine the effect of ROS modifications on the structural changes of insulin, we performed a 3D-fluorescence profile study of both native and oxPTM-INS (Fig. 1c–f). Intrinsic fluorescence was detectable in native insulin, with a maximum excitation (Ex_{max}) of 279 nm and maximum emission (Em_{max}) of 318 nm, possibly attributed to tyrosine residues. Ex_{max} and Em_{max} shifted to 326 and 452 nm, respectively, after modification of insulin with ribose, and to 336 and 431 nm, respectively, after modification with HOCl, suggesting changes in insulin structure. Modification with $^{\bullet}$OH resulted in the loss of fluorescence and an increase in light scattering, suggesting aggregation of the native molecules.

MS analysis of native and oxPTM-INS Native and modified insulin were analysed by high-resolution MS. Native insulin showed a peak at m/z 5,808 Da, corresponding to the molecular mass of insulin. After glycation by ribose, four additional peaks appeared with mass differences of 132 Da, indicating the addition of ribose molecules to insulin and corresponding to the mono-, di-, tri- and tetra-glycated insulin forms. Exposure to HOCl induced the development of additional peaks at lower m/z with respect to the native form, indicating the formation of degradation products induced by the reaction with the oxidant. A peak at m/z 5,824 Da (+16) was shown for insulin modified by $^{\bullet}$OH ($^{\bullet}$OH-INS); the mass difference between native insulin and $^{\bullet}$OH-INS was 16 Da, which corresponds to one oxidation site (Fig. 2a–d). MS/MS analysis suggested that the main oxidative changes involved Phe1 and Lys29 (glycation); His5, Cys7 and Phe24 ($^{\bullet}$OH and HOCl); and Tyr16 and Try26 (HOCl) in chain B (Fig. 2e, Table 2).

Antibody binding to oxPTM-INS in patients with type 1 diabetes and control individuals Native insulin and oxPTM-INS were used as targets in ELISA to assess antibody binding to native insulin vs oxPTM-INS in sera from individuals with type 1 diabetes. As controls, we used samples from a group of healthy schoolchildren and a group of adults. To avoid interference with antibodies against exogenous insulin, samples from patients with type 1 diabetes (mean±SE age 13.90±0.77 years) were taken at diagnosis, before the patients started insulin therapy (Table 1). Binding to insulin modified by HOCl (HOCl-INS) and $^{\bullet}$OH-INS was significantly higher than binding to native insulin. Reactivity to native insulin and oxPTM-INS was detected in 30% and 84%, respectively, of patients with type 1 diabetes, with median absorbances of 0.043±0.012, 0.183±0.012 and 0.231±0.013 for binding to native insulin, HOCl-INS and $^{\bullet}$OH-INS, respectively ($p<0.0001$, Fig. 3a). Binding to glycated insulin was significantly lower compared with native insulin (absorbance 0.007±0.006, $p<0.0001$, data not shown). Antibodies to the control antigens HEL and oxPTM-HEL were present in only 5% and 3.5% of participants with type 1 diabetes, respectively (Electronic supplementary material [ESM] Table 1). Binding of HOCl-INS and $^{\bullet}$OH-INS was higher in sera from participants with type 1 diabetes than in that from participants with type 2 diabetes and healthy individuals ($p<0.0001$). The sensitivity and specificity of oxPTM-INS reactivity in patients with type 1 diabetes were 84% and 99%, respectively,

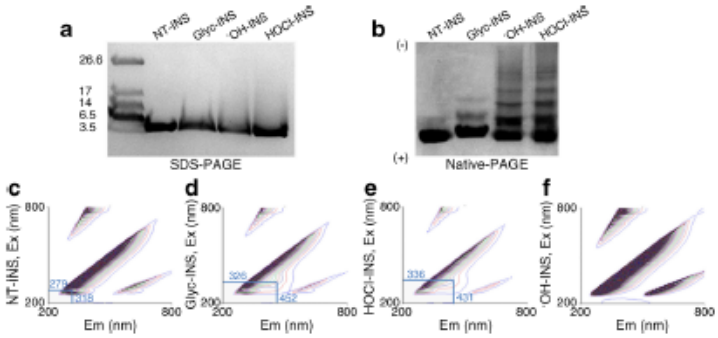

Fig. 1 Analysis of native insulin (NT-INS) and oxPTM-INS. (**a**) SDS-PAGE and (**b**) native-PAGE analysis of oxPTM-INS: glycation by ribose (Glyc-INS), modification by $^{\bullet}$OH ($^{\bullet}$OH-INS) and HOCl (HOCl-INS). There were no clear changes in molecular mass between NT-INS and oxPTM-INS. The position of the molecular mass markers (in kDa) is shown in (**a**). Native-PAGE demonstrated a clear reduction in the mobility of insulin after glycation by ribose, as shown by two bands with slower mobility. Exposure of insulin to the HOCl- and $^{\bullet}$OH-generating systems induced the appearance of additional and slower mobility bands, and a smear of protein through the entire line suggesting fragmentation. (**c**–**f**) 3D fluorescence profile of NT-INS and oxPTM-INS. Modification of insulin with ribose (**d**) or HOCl (**e**) resulted in a substantial shift in the Em_{max} and Ex_{max} wavelengths (indicated by blue lines and numbers) compared with NT-INS. (**f**) Modification with $^{\bullet}$OH resulted in loss of the native fluorescence and increased light scattering, suggesting aggregation of the native molecules

compared with healthy schoolchildren, and 66% and 99%, respectively, compared with patients with type 2 diabetes.

To exclude the possibility that serum reactivity was due to metabolic alteration at the time of the clinical onset of diabetes, binding in 69 patients with type 1 diabetes was evaluated longitudinally after insulin treatment (Fig. 3c–e). Insulin therapy induced a slight increase in binding to native insulin (median absorbance 0.040±0.014 vs 0.083±0.026; $p<0.0001$) and HOCl-INS (median absorbance 0.187±0.019 vs 0.262±0.024; $p=0.007$), but not to $^{\bullet}$OH-INS (data not shown). Overall, binding to oxPTM-INS remained significantly higher than to native insulin ($p<0.0001$). Intra-assay CV of triplicates was <8% (mean 4%, $n=10$). Inter-assay CVs were <10% (mean 6%) and <13% (mean 6%) for native insulin and oxPTM-INS ($n=12$), respectively.

Comparison between oxPTM-INS ELISA and RBA At diagnosis, reactivity to oxPTM-INS ($^{\bullet}$OH-INS and/or HOCl-INS) and IAA in RBA was detected in 84% and 61% of participants with type 1 diabetes, respectively (Fig. 3a, b). The sensitivity of oxPTM-INS antibodies was 84% vs 61% for IAA measured by RBA ($p=0.0001$), while specificity was 99% for both assays. oxPTM-INS antibodies by ELISA and IAA by RBA coexisted in 50% of individuals with type 1 diabetes, but oxPTM-INS antibodies were able to detect an additional 34% of participants who were IAA negative. Overall, 82.7% of participants who were positive on RBA were also oxPTM-INS positive on ELISA, while 17.3% who were positive on RBA were oxPTM negative on ELISA. The combined measurement of IAA by RBA and oxPTM-INS antibody ELISA raised the detection of insulin autoimmunity to 95% of participants with new-onset type 1 diabetes.

Antibody binding specificity to oxPTM-INS by competitive ELISA and western blot A competitive displacement assay was performed to evaluate serum binding specificities to oxPTM-INS by pre-incubating sera with either native insulin or oxPTM-INS (Fig. 4). When type 1 diabetes sera were pre-incubated with an excess amount of native insulin, no significant displacement occurred and the competitive assay showed only a 25% lower absorbance compared with the direct binding oxPTM-INS assay. In contrast, pre-incubation of sera with an excess of oxPTM-INS ($^{\bullet}$OH-INS or HOCl-INS) led to a strong reduction in binding (88% mean reduction in absorbance), indicating that antibody specificities are mainly to oxPTM-INS. Western blot analysis was then performed on a range of serum samples that exhibited the strongest binding in ELISAs. Weak binding to a fragment corresponding to native insulin was evident, but this was lost after glycation. Binding to $^{\bullet}$OH-INS and HOCl-INS was stronger and directed towards a diffused fragment that had slower mobility than native insulin. Serum samples from healthy controls did not bind to any of the insulin formats (Fig. 5).

Relationship of antibody binding to oxPTM-INS with clinical features Binding of samples from individuals with type 1 diabetes to native insulin and oxPTM-INS was unrelated to indices of metabolic control, such as fasting plasma glucose and HbA_{1c}, either at diagnosis or after metabolic compensation ($-0.03<\rho<0.16$, $p>0.132$), or to fasting C-peptide, insulin requirements or age ($-0.07<\rho<-0.01$, $p>0.916$).

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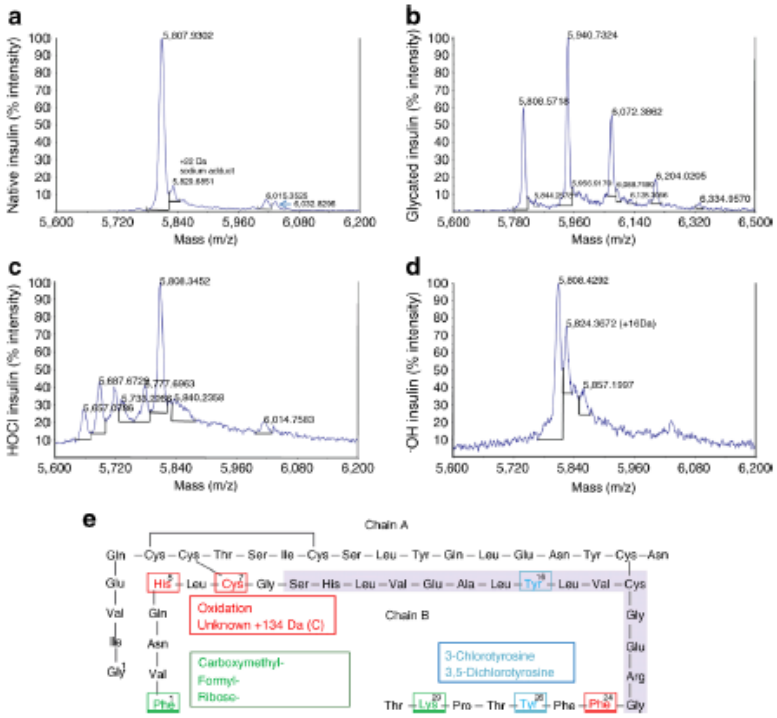

Fig. 2 Analysis of native insulin and oxPTM-INS by MS. (**a**–**d**) MALDI-TOF analysis of native insulin and oxPTM-INS. (**a**) Native insulin showed a peak at m/z 5,808 Da. (**b**) After glycation by ribose, four additional peaks appeared with mass differences of 132 Da and corresponding to the mono-, di-, tri- and tetra-glycated insulin forms. (**c**) Exposure to HOCl induced the development of additional peaks at lower m/z than the native form, indicating the formation of degradation products induced by the reaction with the oxidant. (**d**) A peak at m/z 5,824 Da (+16) was shown for insulin modified by °OH; the mass difference between native insulin and °OH-INS was 16 Da, which corresponds to one oxidation. (**e**) MS/MS analyses of oxPTM-INS showed that the main oxidative changes induced by °OH and HOCl involved His5, Cys7 and Phe24 in chain B (red). Moreover, treatment with HOCl induced the generation of chlorine species on Tyr16 and Tyr26 (blue), while the main modifications by ribose involved N-terminal Phe1 and Lys29 in the chain B (green). The shaded area shows the immunodominant epitope B:9–23

Discussion

This study strengthens the support for the concept of insulin as the prominent autoantigen in type 1 diabetes and suggests that oxPTM neo-antigenic epitopes are involved in insulin reactivity in the large majority of children diagnosed with type 1 diabetes. We have demonstrated, for the first time, that autoreactivity to oxPTM-INS in individuals with newly diagnosed type 1 diabetes is significantly more prevalent than IAAs measured by the gold-standard RBA. Altogether, oxPTM-INS antibodies and IAAs by RBA were detected in 95% of patients with newly diagnosed type 1 diabetes.

Our data indicate that insulin chain B is the main site of oxPTM. MS mapped oxPTM to chlorination of Tyr16 and Tyr26; oxidation of His5, Cys7 and Phe24; and glycation of Lys29 and Phe1 in chain B. Our observations correlate with previous studies showing that glycation of insulin involves N-terminal Phe1 and Lys29 [19, 20] in chain B. Similarly, the oxidation in a more central insulin sequence correlates with a previous report implicating Tyr16 and Tyr26, Phe24 and Cys19 as the preferred sites of changes [20]. It is interesting to note that one of the observed modifications involving chlorination of Tyr16 of chain B (Tyr B16) is within the region 9–23 of chain B (B:9–23), a known dominant epitope in autoimmune diabetes [21], with Tyr16 being crucial in immunoreactivity. Substitution of Tyr in position 16 to Ala (Tyr B16:Ala) has been shown to abrogate T cell reactivity [22, 23], and administration of a modified B:9–23 (Tyr B16:Ala) peptide has been reported to suppress expression of IAA and prevent diabetes in the NOD mouse [24]; conversely, it is possible that Tyr16 modification by HOCl

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Table 2 MS/MS analysis of insulin chain B. The table shows the main oxPTM involving the peptides B:1–13 (FVNQHLCGSHLVE), B:14–21 (ALYLVCGE) and B:21–30 (ERGFFYTPKT). No changes were detected in chain A

MH+ found	Sequence
Native insulin	
1482.7268	-.FVNQHLCGSHLVE.A
1514.6863	-.FVNQHLCGSHLVE.A+dioxidation (C)
867.43	ALYLVCGE
1116.5885	E.RGFFYTPKT.G
Glycated insulin	
1482.6830	-.FVNQHLCGSHLVE.A
1510.6870	-.FVNQHLCGSHLVE.A+formyl (N-terminal)
1540.68	-.FVNQHLCGSHLVE.A+carboxymethyl (N-terminal)
1614.73	-.FVNQHLCGSHLVE.A+D-ribose (N-terminal)
1116.5745	E.RGFFYTPKT.G
1144.5599	E.RGFFYTPKT.R+formyl (K)
1174.5616	E.RGFFYTPKT.R+carboxymethyl (K)
1248.61	E.RGFFYTPKT.R+D-ribose
1194.5848	E.RGFFYTPKT.R+D-ribose − $3H_2O$
$^{\bullet}$OH-INS	
1482.6886	-.FVNQHLCGSHLVE.A
1498.6687	-.FVNQHLCGSHLVE.A+oxidation (HW)
1514.6658	-.FVNQHLCGSHLVE.A+oxidation (C); oxidation (HW)
1514.6863	-.FVNQHLCGSHLVE.A+dioxidation (C)
1530.68	-.FVNQHLCGSHLVE.A+trioxidation (C)
1116.5701	E.RGFFYTPKT.G
1132.5	RGFFYTPKT+oxidation (F)
HOCl-INS	
1482.7009	-.FVNQHLCGSHLVE.A
1530.6876	-.FVNQHLCGSHLVE.A+oxidation (C); oxidation (F); oxidation (HW)
1530.6876	-.FVNQHLCGSHLVE.A+trioxidation (C)
867.43	ALYLVCGE
901.39	ALYLVCGE +3-chlorotyrosine
1116.5859	E.RGFFYTPKT.G
1150.5411	RGFFYTPKT +3-chlorotyrosine
1184.4957	RGFFYTPKT +3,5-dichlorotyrosine

might enhance immunogenicity of this insulin epitope. It is also possible that the other oxidative changes induced by $^{\bullet}$OH and HOCl generate neoepitopes as a result of structural changes or by exposing native hidden amino acid sequences.

oxPTM by ROS appears to play a key role in the pathogenesis of several human autoimmune diseases [13, 25–27]. This is of particular relevance to type 1 diabetes, where islet inflammation and the ensuing hyperglycaemia are substantial sources of ROS production [2, 5]. In this regard, beta cells are very susceptible to oxidative damage. Levels of oxygen radical scavenger enzymes are physiologically lower in beta cells compared with other cell types [28], and exposure to cytokines during insulitis might further disrupt the intracellular redox state by enhancing ROS production. The high prevalence of reactivity to oxPTM-INS in the present study indicates that the imbalance of the redox state takes place in a large proportion of patients with type 1 diabetes, and is therefore potentially relevant to disease pathogenesis. Hyperglycaemia can unbalance the redox status later in the disease and contribute to a vicious cycle by providing additional neoepitopes to which the immune system is not tolerant. Indeed, insulin glycation has been detected both in the pancreas in vivo [29] and in the serum of individuals with diabetes [30]. Interestingly, we did not observe increased reactivity to glycated insulin in our study. This is in contrast to the increased autoreactivity to glycated collagen type II that we have previously observed [7, 13]. Although this might imply that imbalance of the redox state and hence ROS production is more

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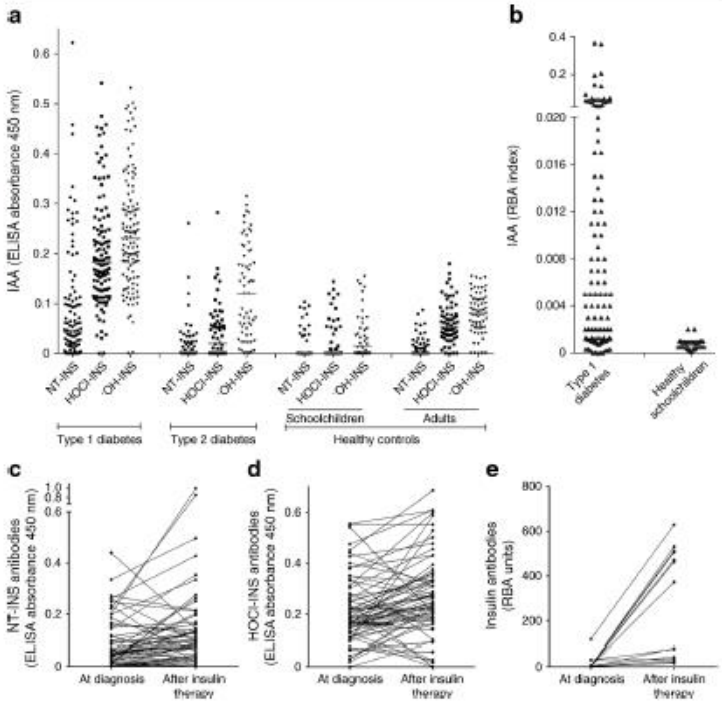

Fig. 3 Binding of type 1 diabetes serum to native insulin (NT-INS) and oxPTM-INS. (**a**) ELISA binding to insulin by serum samples from patients with type 1 diabetes, patients with type 2 diabetes and healthy controls. Reactivity to NT-INS and oxPTM-INS was significantly higher in samples from those with type 1 diabetes compared with type 2 diabetes and healthy controls ($p<0.001$). Binding to oxPTM-INS was significantly higher than to NT-INS in samples from participants with type 1 diabetes ($p<0.0001$). (**b**) Levels of IAA were assessed by RBA in the same group of patients with type 1 diabetes and healthy schoolchildren. (**c**–**e**) Longitudinal changes in antibody binding to (**c**) NT-INS ($p<0.0001$) and (**d**) HOCl-INS ($p=0.007$) by ELISA and (**e**) insulin antibodies assessed by RBA ($p<0.0001$), before and after insulin therapy

likely inducing the formation of insulin neoepitopes, the interplay between hyperglycaemia (and the substantial contribution of glycoxidation to the formation of $^{\bullet}OH$) and ROS in inducing oxPTM-INS should be investigated in a separate study.

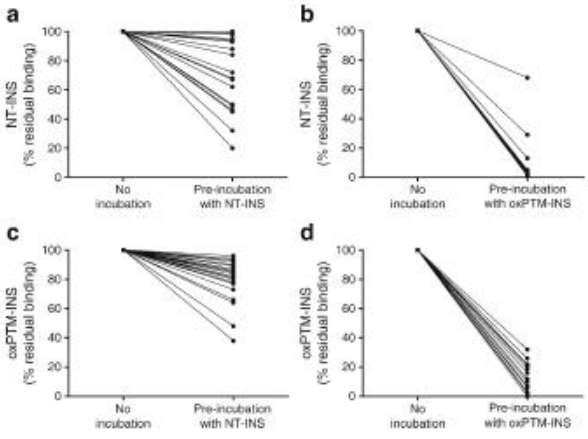

Fig. 4 Serum binding specificity to oxPTM-INS. Pre-incubation of type 1 diabetes serum samples with oxPTM-INS, but not with native insulin (NT-INS), strongly inhibited binding to oxPTM-INS, indicating the presence of antigen-binding sites specific to oxPTM-INS. Data are shown for insulin modified by $^{\bullet}OH$, as an example of oxPTM-INS

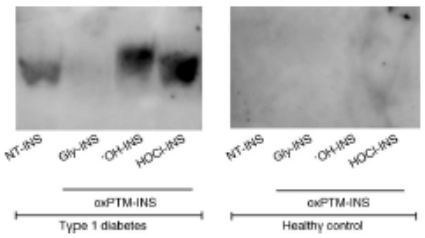

Fig. 5 Binding to native insulin (NT-INS) and oxPTM-INS, as detected by western blot. Binding to NT-INS and a stronger-intensity binding to a smear of a smaller mobility fragment of ˙OH-INS and HOCl-INS was observed. No binding to Gly-INS was seen

In this study, we developed an ELISA for detecting autoreactivity to oxPTM-INS. Historically, ELISAs have shown lower performance than liquid-phase assays such as RBA in detecting IAAs [31]. Discrepancies between the techniques have been related to differences in binding affinity and conformational changes induced by the binding of insulin to the plastic in ELISA [32]. However, while being the reference method for IAAs [31], development of an RBA method for oxPTM-INS might not be straightforward, as fragments resulting from oxPTM might lack the amino acids required for radiolabelling, and a complex labelling approach might need to be developed. Furthermore, reactivity in RBA might not be straightforward, as insulin sites for radiolabelling are also potential sites for oxidation. Future studies should address the feasibility of RBA for detecting oxPTM-INS antibodies.

In summary, the present study provides proof of concept for a role of oxPTM in islet autoimmunity, and suggests anti-oxPTM-INS autoreactivity as a novel immune response in type 1 diabetes. Additional studies with larger cohorts (cross-sectional, longitudinal and at-risk individuals) are required to further study the role of oxPTM in the pathogenesis of type 1 diabetes and to confirm the potential of anti-oxPTM-INS as a diagnostic biomarker in humans with type 1 diabetes. It would be of interest to evaluate whether oxPTM-INS antibodies are also present in patients diagnosed as adults, where IAA are usually rarely detected, and in patients who are classified as autoantibody negative to the other existing markers (GADA, IA-2A, ZnT8A). If proven relevant to the disease pathogenesis, oxPTM might mark diseased-tissue pathways, providing previously unknown targets for the development of drugs, biomarkers and imaging techniques.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement RS was responsible for the conception and design of the study, data acquisition, analysis and interpretation of data, and writing the manuscript and revising it critically for important intellectual content. CV and MHA were responsible for data acquisition and revised the manuscript critically for important intellectual content. DP, CT, FC and NN made substantial contributions to data acquisition and revised the manuscript critically for important intellectual content. PP made a substantial contribution to the conception and design of the study, and critically revised the manuscript for important intellectual content. AN made substantial contributions to the conception and design of the study, analysis and interpretation of data, and writing the manuscript, and revised the manuscript critically for important intellectual content. All authors approved the final version. RS and AN are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Antibodies to post-translationally modified insulin as a novel biomarker for prediction of type 1 diabetes in children.

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ARTICLE

Antibodies to post-translationally modified insulin as a novel biomarker for prediction of type 1 diabetes in children

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Abstract
Aims/hypothesis We have shown that autoimmunity to insulin in type 1 diabetes may result from neoepitopes induced by oxidative post-translational modifications (oxPTM). Antibodies specific to oxPTM-insulin (oxPTM-INS-Ab) are present in most newly diagnosed individuals with type 1 diabetes and are more common than autoantibodies to native insulin. In this study, we investigated whether oxPTM-INS-Ab are present before clinical onset of type 1 diabetes, and evaluated the ability of oxPTM-INS-Ab to identify children progressing to type 1 diabetes.
Methods We used serum samples collected longitudinally from the 'All Babies in Southeast Sweden (ABIS)' cohort tested for the gold standard islet autoantibodies to insulin (IAA), GAD (GADA), tyrosine phosphatase 2 (IA-2A) and zinc transporter 8 (ZnT8A). We studied 23 children who progressed to type 1 diabetes (progr-T1D) and 63 children who did not progress to type 1 diabetes (NP) after a median follow-up of 10.8 years (interquartile range 7.7–12.8). Of the latter group, 32 were positive for one or more islet autoantibodies (NP-AAB$^+$). oxPTM-INS-Ab to insulin modified by *OH or HOCl were measured by our developed ELISA platform.
Results Antibodies to at least one oxPTM-INS were present in 91.3% of progr-T1D children. oxPTM-INS-Ab co-existed with GADA, IA-2A, IAA or ZnT8A in 65.2%, 56.5%, 38.9% and 33.3% progr-T1D children, respectively. In addition, oxPTM-INS-Ab were present in 17.4%, 26.1%, 38.9% and 41.6% of progr-T1D children who were negative for GADA, IA-2A, IAA and ZnT8A, respectively. *OH-INS-Ab were more common in progr-T1D children than in NP-AAB$^+$ children (82.6% vs 19%; $p < 0.001$) and allowed discrimination between progr-T1D and NP-AAB$^+$ children with 74% sensitivity and 91% specificity. None of the NP-AAB$^-$ children were positive for oxPTM-INS-Ab.
Conclusions/interpretation oxPTM-INS-Ab are present before the clinical onset of type 1 diabetes and can identify children progressing to type 1 diabetes.

Keywords Biomarker · Insulin · Insulin autoantibodies · Islet autoantibodies · Oxidative stress · Post-translational modifications · Type 1 diabetes

Paolo Pozzilli, Johnny Ludvigsson and Ahuva Nissim share senior authorship.

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Abbreviations

ABIS	All Babies in Southeast Sweden
GADA	GAD autoantibodies
GLY-INS	Glycated insulin
HEL	Hen egg lysozyme
HOCl-INS	HOCl-modified insulin
IA-2A	Tyrosine phosphatase autoantibodies

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IAA	Insulin autoantibodies
$^{\bullet}$OH-INS	$^{\bullet}$OH-modified insulin
NT-INS	Native insulin
oxPTM	Oxidative PTM
oxPTM-HEL	Oxidative post-translationally modified HEL
oxPTM-INS	Oxidative post-translationally modified insulin
oxPTM-INS-Ab	Antibodies to oxPTM-INS
progr-T1D	Progressing to type 1 diabetes;
NP-AAB^{+}	Autoantibody-positive, non-progressing to type 1 diabetes
NP-AAB^{-}	Autoantibody-negative, non-progressing to type 1 diabetes
PTM	Post-translational modification
RBA	Radiobinding assay
ZnT8A	Zinc transporter 8 autoantibodies

Introduction

Type 1 diabetes is characterised by insulin deficiency and hyperglycaemia due to extensive destruction of insulin-producing beta cells. The autoimmune nature of the disease is suggested by the presence of a pool of circulating autoantibodies against beta cell proteins even years before the clinical onset, such as autoantibodies to insulin (IAA), GAD (GADA), tyrosine phosphatase (IA-2A) and zinc transporter 8 (ZnT8A) [1].

The mechanism underlying the breach of immune tolerance to beta cell antigens is still unclear. Neoepitopes that are post-translationally modified from the native antigens have been recently described in type 1 diabetes [2–7]. Being different from the native proteins, the post-translational modification (PTM) of self-antigens may be recognised as foreign and result in breakdown of tolerance [8–10]. Oxidative stress is a key feature of many autoimmune diseases and results in an excess of reactive oxidants able to generate oxidative PTM (oxPTM) [4, 11]. Products of oxidative stress are increased in type 1 diabetes [12] and also in individuals at risk [13]. During insulitis, the beta cells that are under stress and the high influx of metabolically active immune cells generate large quantities of reactive oxidants, including the superoxide radical, hydroxyl radical ($^{\bullet}$OH), hypochlorous acid (HOCl) and peroxynitrate [14]. We have shown that autoimmunity to insulin may result from neoepitopes induced by oxPTM [11]. We found that antibodies specific to oxidative post-translationally modified insulin (oxPTM-INS) are present in the majority of newly diagnosed individuals with type 1 diabetes and are significantly more abundant than autoantibodies to native insulin (NT-INS) [11].

Islet autoantibodies (IAA, GADA, IA-2A and ZnT8A) represent the most robust approach to identify individuals at risk and to predict progression to clinical disease in those genetically at risk [15] and in the general population [16]. Children with any two autoantibodies may have a risk of 80% for developing type 1 diabetes during childhood or adolescence [15]. However, multiple antibody testing is required for the best prediction. An additional problem is that radiobinding assays (RBAs), the gold standard for islet autoantibodies, have expensive regulatory requirements. These elements introduce additional complexity that may limit the implementation of autoantibody screening. Finally, a significant number of individuals test negative to these markers [17]. Therefore, the development of alternative technologies remains an unmet need. To address this we have developed an ELISA that detects auto-reactivity to oxPTM-INS. We showed that the assay detecting antibodies to oxPTM-INS (oxPTM-INS-Ab) is highly accurate (84% sensitivity, 99% specificity), may detect over 30% of individuals who are negative to the RBA for IAA and, when combined with IAA, identifies 95% of individuals with newly diagnosed type 1 diabetes [11]. However, the predictive potential of oxPTM-INS-Ab is not known yet.

We hypothesise that oxPTM-INS-Ab are present before the clinical onset of the disease. To test this hypothesis, considering the high prevalence of oxPTM-INS reactivity in newly diagnosed type 1 diabetes, we evaluated the ability of this novel autoantibody specificity to identify children progressing to clinical disease. We used serum samples from the ABIS (All Babies in Southeast Sweden) study, a large prospective study in which unselected children from the general population born during 1997–1999 have been followed prospectively with regular evaluation of islet autoantibodies for the development of type 1 diabetes [18, 19].

Methods

Participants and serum samples Serum samples from the ABIS study were obtained and analysed in a blinded fashion for detection of oxPTM-INS-Ab. The ABIS study is a prospective population-based follow-up study which included 17,055 unselected children born between 1 October 1997 and 1 October 1999 in southeast Sweden [18]. Of the screened children, 116 developed type 1 diabetes during the follow-up. In the present study, we tested 51 samples from the 23 children progressing to type 1 diabetes (progr-T1D), collected longitudinally before diagnosis at three different time points (at the ages of 5, 7 and 11 years). Samples were selected where sufficient serum and autoantibody data were available for this study. Only one time point was available from seven progr-T1D children before the collection at the second time point. As controls, we used samples from 63 children of similar age and sex who did not progress to type 1 diabetes over time, including 64 samples from 32 autoantibody-positive children (autoantibody-positive, non-progressing to type 1 diabetes [NP-AAB^{+}]) and 31 samples from

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autoantibody-negative children (autoantibody-negative, non-progressing to type 1 diabetes [NP-AAB$^-$]). NP-AAB$^+$ children were defined as positive to at least one islet-antibody marker (IAA, GADA, IA-2A or ZnT8A). Informed consent was obtained from parents prior to the collection of blood. The study was approved by the Research Ethics Committees of the Medical Faculties of Linköping University, Linköping and Lund University, Lund, Sweden and by the Ethics Committee of the Università Campus Bio-Medico di Roma.

ELISA for detection of oxPTM-INS-Ab Insulin was chemically modified as previously described to generate oxPTM-INS modified by HOCl, $^{\bullet}$OH and glycation using ribose [11]. Hen egg lysozyme (HEL; Sigma-Aldrich, Milan, Italy) was similarly modified and used as a control antigen. oxPTM-INS encompasses glycated (GLY-INS), $^{\bullet}$OH-modified ($^{\bullet}$OH-INS) and HOCl-modified insulin (HOCl-INS). An ELISA was performed using NT-INS, oxPTM-INS, control native HEL or control oxidative post-translationally modified HEL (oxPTM-HEL) as targets. Development and calibration of the ELISA is described in our previous publication [11]. ELISA plates (Nunc, London, UK) were coated with 10 μg/ml of modified or native protein in 0.05 mol/l carbonate/bicarbonate buffer (pH 9.6) at 4°C overnight. Plates were then washed three times with PBS. After blocking for 2 h with 5% BSA in 0.5% Tween PBS, 100 μl of 1:200-diluted serum samples in 5% BSA in 0.5% Tween PBS were added to each well, followed by 2 h incubation at room temperature. Plates were then washed with PBS plus 0.1% Tween, followed by three washes with PBS. Anti-human IgG-horseradish peroxidase-conjugated antibodies (Sigma-Aldrich) were then added at 1:1000 dilution in 5% BSA in 0.5% Tween PBS for another 2 h incubation. The ELISA plates were washed, and 100 μg/ml 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich) in 100 mmol/l sodium acetate (pH 6.0), was added. Subsequently, the reaction was stopped with 1 mol/l sulphuric acid. The absorbance was measured at 450 nm using a GENios plate reader and Magellan software (Tecan, Reading, UK). The ELISA absorbance values obtained for HEL and oxPTM-HEL were used as background controls that were subtracted from the absorbance values of NT-INS and oxPTM-INS, respectively. In addition, to account for assay fluctuation, binding to insulin, oxPTM-INS, HEL and oxPTM-HEL was tested for each individual sample on the same plate. Each assay included known positive or negative reference control samples. Longitudinal samples obtained from the same individuals were tested on the same plate. Levels of oxPTM-INS-Ab above the 99th percentile of 88 healthy individuals were defined as ELISA cut-off. Intra-assay CV was <8% (n = 10). Inter-assay CVs were <10% and <13% for NT-INS and oxPTM-INS-Ab (n = 12), respectively.

Islet autoantibodies Islet autoantibodies were measured by RBA. IAA were measured according to the method of Williams et al [20], with some modifications [21]. GADA and IA-2A were measured as previously described by Wahlberg et al [19] and ZnT8A (variants ZnT8RA, ZnT8QA and ZnT8WA) were measured as described by Vaziri-Sani et al [22]. Thresholds have been defined as equivalent to the 98th percentile for GADA, IA-2A and ZnT8A, and the 95th percentile for IAA [23]. In the 2005 Diabetes Autoantibody Standardization Programme (DASP), the RBAs for GADA, IA-2A and IAA achieved 76%, 72% and 28% sensitivity, respectively, with 96%, 100% and 100% specificity. In the 2013 Islet Autoantibody Standardization Programme (IASP), the RBA for ZnT8AR, ZnT8AQ and ZnT8WA achieved 50%, 18% and 36% sensitivity, respectively, with 99%, 100% and 93% specificity.

HLA genotyping HLA typing and subtyping was performed as previously described [24]. The HLA genotyping revealed the haplotypes *HLA-DQB1*, *-DQA1* and *-DRB1* and these were categorised as susceptibility-associated (S), neutral (N) and protective (P), according to Hermann et al [24]. Susceptibility-associated haplotypes included *DR4-DQ8* (*DRB1*0401/2/4/5-DQB1*0302*) and *DR3-DQ2* (*DQA1*05-DQB1*02*), protective haplotypes included *DR2-DQ6* (*DQB1*0602*), *DR11/12/1303-DQ7* (*DQA1*05-DQB1*0301*), *DR7-DQ3* (*DQA1*0201-DQB1*0303*), *DR14-DQ5* (*DQB1*0503*), *DR403-DQ8* (*DRB1*0403-DQB1*0302*) and *DR1301-DQ6* (*DQB1*0603*). Other haplotypes were defined as neutral.

Statistical analysis Statistical analyses were performed using Prism Software version 6.01 (GraphPad, San Diego, CA, USA). Differences in antibody levels between groups were tested by the Mann–Whitney test. Longitudinal changes in antibody binding were evaluated by the Wilcoxon paired test. To determine predictive discrimination between progr-T1D and control groups, we used the 99th percentile of the healthy individuals as cut-off point absorbance units to construct a contingency table of positive oxPTM-INS-Ab against clinical diagnosis and tested it by Fisher's Exact Test.

Results

Features of the studied population The characteristics of the study population are shown in Table 1. At the earliest time point studied, the progr-T1D, NP-AAB$^+$ and NP-AAB$^-$ groups were comparable in terms of sex, while age was slightly higher in the NP-AAB$^+$ group (p = 0.04). The majority of progr-T1D children (n = 18, 78%) had multiple positive islet autoantibodies; two were single-positive. Two children were negative for IAA, GADA and IA-2A; one child was negative for GADA and IA-2A (IAA was not assessed). Only 25% (n = 8) of NP-AAB$^+$ children had multiple positive islet autoantibodies. HLA typing was available in 43 children; HLA susceptible phenotypes were more common in progr-T1D

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Table 1 Characteristics of the study population

Characteristics	Progr-T1D ($N = 23$)	Children not progressing to type 1 diabetes	
		NP-AAB$^+$ ($N = 32$)	NP-AAB$^-$ ($N = 31$)
Age at baseline, years	6.17 ± 1.49	7.61 ± 2.42	7.13 ± 2.07
Sex, male	15 (65)	21 (66)	17 (55)
Multiple autoantibodies (≥2)	18 (78)	8 (25)	NA

Data are presented as means ± SD or *n* (%)

The three groups were comparable in terms of sex, although age was slightly higher in the NP-AAB$^+$ group than in the progr-T1D group ($p = 0.04$). Eighteen (78%) of progr-T1D and 8 (25%) of NP-AAB$^+$ children had multiple positive islet autoantibodies

than in NP-AAB$^+$ and NP-AAB$^-$ children ($p < 0.007$), while the prevalence of protective phenotypes was similar between the three groups ($p > 0.07$) (Table 2). In the progr-T1D group, median follow-up from the first sample analysed to diagnosis was 5.1 years (interquartile range 3.2–7.7). After a median follow-up of 10.8 years (interquartile range 7.7–12.8), all children in the control groups, either NP-AAB$^+$ or NP-AAB$^-$, remained free from diabetes (last assessment performed in December 2016).

Cross-sectional evaluation of oxPTM-INS-Ab prevalence Binding and prevalence of oxPTM-INS-Ab were compared between progr-T1D, NP-AAB$^+$ and NP-AAB$^-$ children at the earliest time point available. In the progr-T1D group, binding to HOCl-INS and *OH-INS was significantly higher than binding to NT-INS ($p < 0.001$; Fig. 1a). Serum samples from 21 (91.3%) progr-T1D children bound to at least one oxPTM-INS (*OH-INS or HOCl-INS) and 17 (73.9%) children were found to be double-positive to both *OH-INS and HOCl-INS. Binding to HOCl-INS and *OH-INS was higher in sera from progr-T1D children compared with either NP-AAB$^+$ or NP-AAB$^-$ children ($p < 0.001$; Fig. 1a). oxPTM-INS-Ab were more common in progr-T1D children than in NP-AAB$^+$ children, with respective reactivity to *OH-INS in 82.6% (19/23) vs 19% (6/32) ($p < 0.0001$) and reactivity to HOCl-INS in 82.6% (19/23) vs 40.6% (13/32) ($p = 0.0024$) (Fig. 1b–d). *OH-INS-Ab allowed discrimination between progr-T1D and NP-AAB$^+$ children with 74% sensitivity and 91% specificity. The overlap between NT-INS and oxPTM-INS-Ab is shown in Fig. 1b–d. Binding to NT-INS was more common in progr-T1D children than in NT-AAB$^+$ and NT-AAB$^-$ children ($p < 0.001$). Binding to both NT-INS and oxPTM-INS occurred in 14 (60%) progr-T1D children, while 7 (30%) displayed binding only to oxPTM-INS. In the NP-AAB$^+$ children, 6 (19%) displayed binding to both NT-INS and oxPTM-INS, while 7 (22%) displayed binding only to oxPTM-INS. None of the NP-AAB$^-$ children were positive for oxPTM-INS-Ab.

Table 2 HLA-genotypes prevalence in the study population

HLA susceptibility category	Progr-T1D ($N = 17$)	Children not progressing to type 1 diabetes	
		NP-AAB$^+$ ($N = 16$)	NP-AAB$^-$ ($N = 10$)
SS	8 (47)	3 (18.75)	0 (0)
SN	6 (35)	3 (18.75)	1 (10)
SP	3 (18)	4 (25)	4 (40)
NN	0 (0)	2 (12.5)	2 (20)
NP	0 (0)	2 (12.5)	2 (20)
PP	0 (0)	2 (12.5)	1 (10)

Data are presented as *n* (%)

HLA haplotypes were available in 43 children and were categorized into susceptibility-associated (S), neutral (N) and protective (P) groups according to Hermann et al. [24]. Susceptibility-associated haplotypes included *DR4-DQ8* (*DRB1*0401/2/4/5-DQB1*0302*) and *DR3-DQ2* (*DQA1*05-DQB1*02*), protective haplotypes included *DR2-DQ6* (*DQB1*0602*), *DR11/12/1303-DQ7* (*DQA1*05-DQB1*0301*), *DR7-DQ3* (*DQA1*0201-DQB1*0303*), *DR14-DQ5* (*DQB1*0503*), *DR403-DQ8* (*DRB1*0403-DQB1*0302*) and *DR1301-DQ6* (*DQB1*0603*). Other haplotypes were defined as neutral

Longitudinal changes of oxPTM-INS-Ab in progr-T1D children Binding to oxPTM-INS (either *OH-INS or HOCl-INS) did not change significantly over time (median follow-up 3 years [range 3–6 years]; $p = 0.725$; Fig. 2a). Seroconversion occurred in four children: two became positive at a later stage while two became negative. When evaluated according to time before diabetes onset, reactivity to oxPTM-INS appeared as early as 11 years before disease onset (median time to diabetes onset 6 years [range 2–11 years]; Fig. 2b).

Distribution of oxPTM-INS-Ab and islet autoantibodies Figure 3 shows the degree of overlap between oxPTM-INS-Ab (*OH-INS-Ab) and other islet autoantibodies in progr-T1D and NP-AAB$^+$ children. IAA data were available in 18 progr-T1D and 27 NP-AAB$^+$ children and ZnT8A data were available in 12 progr-T1D and 10 NP-AAB$^+$ children. While binding and prevalence of oxPTM-INS-Ab were significantly higher in progr-T1D children than in NP-AAB$^+$ children ($p < 0.001$), both titres and prevalence of GADA, IAA and ZnT8A were similar between the two groups ($p > 0.257$). IA-2A was the most specific

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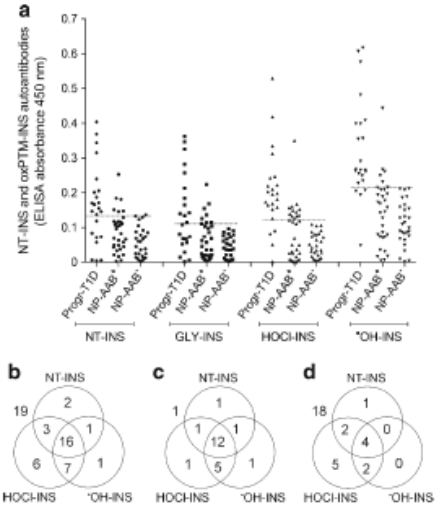

Fig. 1 Cross-sectional evaluation of antibody binding to oxPTM-INS in study population. (**a**) Reactivity to NT-INS and oxPTM-INS was significantly higher in samples from progr-T1D children compared with non-progressing children, regardless of whether they were NP-AAB$^+$ or NP-AAB$^-$ to the standard islet autoantibody markers ($p < 0.001$). Binding to oxPTM-INS modified by HOCl and °OH was significantly higher than to NT-INS in progr-T1D children ($p < 0.0001$). Data on the earliest time point available are reported. Values above the dashed lines were defined as positive for antibodies to NT-INS and oxPTM-INS modified by glycation (GLY), HOCl or °OH, respectively (99th percentile of a group of 88 healthy control children). (**b**–**d**) Overlapping prevalence of antibodies to NT-INS and oxPTM-INS in all children positive for at least one islet autoantibody (**b**) and in progr-T1D (**c**) and NP-AAB$^+$ children (**d**). Values outside the circles are children negative for the antibody evaluated in the diagram

marker among autoantibodies evaluated, being significantly higher in the progr-T1D group than in the NP-AAB$^+$ group ($p < 0.001$). In the progr-T1D group, 19 (82.6%) children were positive to °OH-INS-Ab, 18 (78.3%) to GADA, 16 (69.5%) to IA-2A, 10 (55.5%) to IAA and 6 (50%) to ZnT8A (Fig. 3e–h). oxPTM-INS-Ab co-existed with GADA, IA-2A, IAA or ZnT8A in 15/23 (65.2%), 13/23 (56.5%), 7/18 (38.9%) and 4/12 (33.3%) progr-T1D children (Fig. 3e–h). In addition, oxPTM-INS-Ab detected 4/23 (17.4%), 6/23 (26.1%), 7/18 (38.9%) and 5/12 (41.6%) progr-T1D children who were negative for GADA, IA-2A, IAA and ZnT8A, respectively (Fig. 3e–h). Two progr-T1D children were negative for GADA, IA-2A and IAA (ZnT8A not assessed) but showed positive for oxPTM-INS-Ab. The assessment of oxPTM-INS-Ab in combination with IA-2A and IAA led to the identification of 100% progr-T1D children with the lowest percentage of false-positive results among the other possible antibody combinations (Fig. 4). In the NP-AAB$^+$ group, oxPTM-INS-Ab reactivity was similar in those who were positive to one or more standard islet autoantibodies ($p > 0.148$).

Discussion

We have recently shown that oxPTM-INS-Ab are very common in newly diagnosed type 1 diabetes, being detected in 84% of individuals [11]. In this study, we found that oxPTM-INS auto-reactivity is present before diabetes diagnosis in over 90% of individuals. oxPTM-INS-Ab could discriminate between prog-T1D children and those who did not progress to type 1 diabetes regardless of positivity to other islet autoantibodies. Therefore, these data may suggest a potential role for oxPTM-INS-Ab as a predictive biomarker of type 1 diabetes.

The main strength of our study is the prospective evaluation of oxPTM-INS-Ab in a well-characterised cohort of children from the general population, tested for the standard diabetes autoantibodies. The uniqueness of the ABIS study resides in the possibility of applying its findings to the general population, since participants were not selected according to predetermined diabetes risk (genetic or familial). An additional strength was that the long follow-up in some progr-T1D children allowed us to discover whether oxPTM-INS-Ab develop very early in the natural history of the disease. Our study has limitations. The earliest time point tested in the progr-T1D children was at 5 years of age. Data from birth cohorts suggest that peak age of seroconversion in high-risk individuals is around 2 years of age [25]. Therefore, we cannot exclude

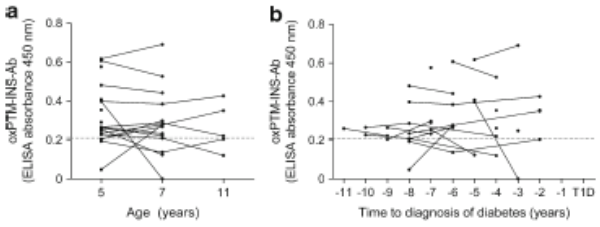

Fig. 2 Longitudinal changes of oxPTM-INS-Ab in prog-T1D children according to age (**a**) and time before diagnosis of type 1 diabetes (**b**). Values above the dashed lines were defined as positive for antibodies to oxPTM-INS; °OH-INS is shown as an example for oxPTM-INS-Ab

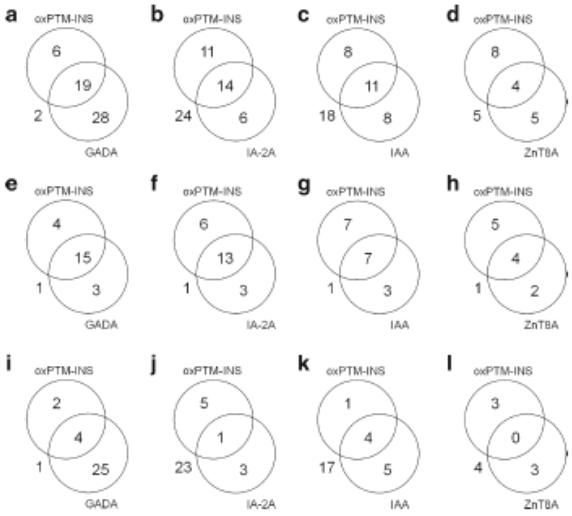

Fig. 3 Overlapping prevalence of oxPTM-INS-Ab, GADA, IA-2A, IAA and ZnT8A. Data are shown for the whole study population of children positive to at least one islet autoantibody (**a**–**d**) and for progr-T1D (**e**–**h**) and NP-AAB$^+$ children (**i**–**l**). Data for IAA and ZnT8A were available in 45 and 22 children, respectively. ZnT8A included positivity to one or more ZnT8RA, ZnT8AWA and ZnT8QA variants. ^{7}OH-INS is shown as example for oxPTM-INS-Ab. Values outside the circles are children negative to the antibody evaluated in the diagram

the possibility that children defined as negative for the standard islet autoantibodies have not previously been seropositive for one or more islet autoantibodies. A second limitation is that the determination of ZnT8A was performed on a limited number of children, as ZnT8A assays became available only after the follow-up for many children in the ABIS study.

Our findings shed further light on type 1 diabetes pathogenesis. Detection of oxPTM-INS reactivity before clinical onset of

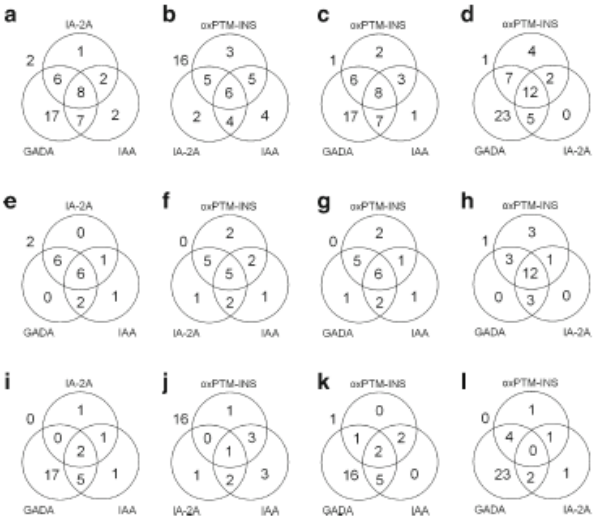

Fig. 4 Overlapping prevalence of oxPTM-INS-Ab, GADA, IA-2A and IAA evaluated with three standard islet autoantibodies or with oxPTM-INS-Ab substituted for GADA, IA-2A or IAA. Data are shown for the whole study population of children positive to at least one islet autoantibody (**a**–**d**) and for progr-T1D (**e**–**h**) and NP-AAB$^+$ children (**i**–**l**). IAA was not available in five progr-T1D and five NP-AAB$^+$ children; therefore these children are not included in the diagrams assessing the overlapping prevalence of IAA. ^{7}OH-INS is shown as example for oxPTM-INS-Ab. Values outside the circles are children negative to the antibodies evaluated in the diagram

type 1 diabetes is consistent with evidence that the unbalanced redox state takes place early in the natural history of the disease. Individuals with a short disease duration have an early impairment in antioxidant capacity and over threefold increased levels of lipid peroxidation regardless of blood glucose control [12]. An impaired oxidation status anticipates dysglycaemia; it has been shown that plasma malondialdehyde and erythrocyte malondialdehyde, two markers of oxidative stress, are abnormal in euglycaemic individuals at increased risk for type 1 diabetes compared with individuals not at risk [13]. These data, together with our findings, may support the involvement of oxidative stress in type 1 diabetes pathogenesis.

To our knowledge, this is the first study investigating antibody reactivity to oxPTM/PTM of a beta cell antigen as a predictive biomarker of type 1 diabetes. Therefore, a direct comparison with prediction potential of other modified antigens is not possible. Our data are consistent with previous finding by our group in newly diagnosed individuals with type 1 diabetes. Similar to our previous study [11], oxPTM-INS-Ab identified over 80% of individuals with the disease and were able to detect more than one-third of those who tested negative to the IAA assay. Additional neoepitopes derived from PTM of beta cell autoantigens have been described in humans and in animal models of type 1 diabetes, including antibody response to oxidised GAD [26] and T cell reactivity to GAD citrullination [7], C-peptide deamination [5] and hybrid fused peptides [2, 27]. The response to modified antigens may also involve proteins that are not proper to beta cells [4, 28], especially in the presence of defined genetic background. An example is the identification of antibodies to oxPTM-collagen type II in a large proportion of individuals carrying *HLA-DRB1*04* [4]. In this regard, the extracellular matrix surrounding beta cells [29], or other tissues attacked by autoimmune response in type 1 diabetes (thyroid, gut, joints, etc) [30], may become additional potential targets of oxPTM [4, 31]. Often such PTM forms induce a more pronounced immune reactivity than the native antigen.

Our results may also have implications for disease prediction and staging. Consistent with previous findings [15, 32], we found low predictive accuracy of GADA and IAA when analysed as a single test, while IA-2A showed a highly specific association with type 1 diabetes progression [15]. A main finding of our study is the predictive accuracy of oxPTM-INS-Ab. Of note, oxPTM-INS-Ab identified individuals with preclinical disease otherwise classified as antibody-negative or single-positive. As highlighted by a recent statement by the JDRF, Endocrine Society and ADA, islet autoimmunity (as defined by the presence of two or more islet autoantibodies) represents the earliest stage of type 1 diabetes and identifies a target population for prevention trials and future preventive strategies [33]. If confirmed in larger studies, oxPTM-INS-Ab may be adopted as an additional biomarker to further redefine disease taxonomy, allowing better prediction and therefore better stratification into eligibility trials.

In conclusion, we showed that immune reactivity to oxPTM-INS is present before clinical onset of type 1 diabetes and that measurement of oxPTM-INS-Ab may identify children likely to progress to type 1 diabetes. This is the first evidence suggesting that oxPTM of a beta cell autoantigen precedes diabetes onset in humans and that auto-reactivity to oxPTM may act as a predictive biomarker of the disease. Additional studies with larger cohorts are required to confirm the predictive potential of oxPTM-INS-Ab in type 1 diabetes.

Data availability The data are available on request from the authors.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement RS, PP, JL and AN conceived the study. CV contributed to acquisition and analysis of data. NN contributed to data acquisition. RS analysed the data and wrote the first draft. All authors critically revised the manuscript for intellectual content. All authors have seen and approved the final draft. RS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Antibodies to post-translationally modified insulin improve prediction of type 1 diabetes in children

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UNDER SUBMISSION

Targeting Microvesicles to the Arthritic Joint using Antibodies Specific to Damaged Cartilage

L. M. Topping, H. I. Rhys, J. Lopez-Tremoleda, M. B. Voisin, C. Vinci, H. L. Law, M. Perretti, L. V. Norling, H. Azevedo, A. Nissim.

UNDER SUBMISSION

Antibodies to type II collagen: a novel tool for the diagnosis of spondyloarthritis?

C. Vinci, M. Infantino, A. Tindell, L. M. Topping, R. Strollo, H. Amital, S. Gertel, V. Grossi, M. Manfredi, F. Bandinelli, F. Li Gobbi, A. Damiani, P. Pozzilli, C. Goodyear, M. Benucci, A. Nissim.

UNDER SUBMISSION