Lorenzo Altamore

PhD

Ciclo XXXI

UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN
SCIENZE CHIMICHE

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COORDINATORE Prof. PIERO BAGLIONI

Use of peptide mimetics of proteins for characterization of immune response in different pathological conditions

Settore Scientifico Disciplinare CHIM/06

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ABSTRACT

Proteins and peptides play a key role in almost all biological processes. All these functions are regulated through specific interactions between proteins and their ligands (e.g. peptides or other small molecules or ions) or other proteins; dysregulations of these mechanisms commonly lead to pathological conditions. An exhaustive comprehension of these interactions is fundamental in order to clarify their involvement in the onset of several diseases and to develop novel therapeutic strategies. Moreover, proteins and peptides are the most common and important antigenic target in the majority of pathologies. Protein fragments and peptides represent portions which interact with antibodies: these portions are known as active sites or epitopes. To correctly reproduce them, the synthesis of mimetic molecules is considered a powerful approach. Mimetic peptides represent only the epitope or the active site of the respective protein, but they are much easier to synthetize, to handle and to eventually chemically modify, besides they are generally more proteolytically stable.

The principal aim of this PhD project is to highlight how powerful and highly versatile the use of modified and non-modified peptide and protein antigenic probes as mimetics of specific target proteins can be for the study of many different pathological conditions and, furthermore, of several aspects of these pathologies which were never or poorly investigated before. In this PhD thesis the advantages and the versatility of the use of the aforementioned antigenic probes will be show on different types of diseases, from autoimmune to immuno-mediated and only genetic-related ones. Both B and T immune responses were considered, besides several kind of environmental triggers, such as viral or bacterial infections but also side effects of drugs. Following the so called Chemical Reverse Approach, specific peptide and protein probes were

used as mimetics of putative epitope fractions to reveal the presence of specific B- or T-cell responses which can clarify several aspects of the pathology, such as the role of environmental factors or post-translational modifications in its onset.

Some aspects of five different pathological conditions have been studied to demonstrate the highly versatile role of synthetic modified and non-modified peptide and protein fragments as antigenic probes for both B and T immune response characterization. The most important aspect this work wanted to investigate was the possible correlation between the pathogenesis of these diseases and several environmental factors, such as viral or bacterial infection and adverse reactions related to reactive small molecules. Using synthetic modified and non-modified peptide and protein fragments as antigenic probes, the possible role of viral and bacterial infections as triggers for different kind of pathologies was then investigated on well-known autoimmune diseases, such as Type 1 diabetes (T1D), Latent Autoimmune Diabetes in Adults (LADA), psoriasis (PsO) and psoriatic arthritis (PsA), and supposed totally genetic-related diseases, such as Rett syndrome. Furthermore, the adverse B immune response highlighted after therapeutic treatments was also studied on immune-mediated diseases such as Fabry Disease (FD). Finally, a specific T cell response against modified endogenous protein characteristic of allergic reactions to antibiotics was also investigated.

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GENERAL INTRODUCTION

1. THE IMMUNE SYSTEM

The complex set of different kind of protections referred to as “immune system” is composed by numerous physical, biological and chemical barriers and mechanisms whose purpose is to avoid the penetration, the infection and the subsequent damages caused by several types of exogenous pathogens, e.g. viruses or bacteria. Biochemical protections can be humoral and/or cell-mediated and are regulated by different organs (spleen, bone marrow, lymph nodes etc.), tissues or circulating cells.

The very first stumbling block against pathogens penetration is mainly physical and is represented by all the anatomical barriers such as the skin or the mucous membranes.

The second line of defense is called “innate immune system” and is composed by several generic and aspecific response mechanisms against the vast majority of exogenous factors, thanks to its ability to recognize common non-self molecular profiles, e.g. carbohydrates moieties belonging to the cell wall of pathogens. It is always operational and immediate, but not persistent. Two kind of cells referred to as leukocytes or white blood cells belong to the innate immune system: myeloid cells or phagocytes, which can be further divided into macrophages, neutrophils, monocytes and dendritic cells (DC), are able to incorporate and destroy pathogens besides to produce and release inflammatory mediators, such as cytokines, which play a key role in the stimulation on other kind of defense responses against the threat; lymphoid cells or Natural Killer cells (NK cells) are able to induce apoptosis in the infected cells. Furthermore, the so called “complement cascade system” is composed by several biochemical

reactions between 26 different specific plasmatic proteins which cooperate with phagocytes in order to detect and destroy the pathogen.

Finally, the last and most specific defense mechanism which belongs to the immune system is the adaptive immune system. Differently from the previously described one, the adaptive immune system is a prerogative of vertebrates and is characterized by a slower but much higher specific response against a given pathogen. It can produce an immunological memory after the first contact with the exogenous agent in order to increase its defensive efficacy in case of subsequent encounters with the same pathogen. The clonal expansion of a specific subset of leukocytes, B and T lymphocytes (cell-mediated response), produced by stem cells in the bone marrow, composes the adaptive immune system. T lymphocytes (or T cells) expose T cell receptors (TCRs) on their surface and can recognize the pathogen only with the assistance of the so called "antigen presenting cells" (APC), such as DC. T cells carry out their action by producing and releasing cytokines ($CD4^+$ T cells) in order to enhance the immune response or by destroying infected cells ($CD8^+$ T cells). Differently, B lymphocytes (or B cells) are characterized by B cell receptors (BCRs) and are able to recognize the pathogen by detecting linear or conformational portions of it, called epitopes. They produce molecular structures referred to as antibodies which specifically detect and interact with pathogenic antigens (Ags), subsequently remaining in the immune system for several years leading to the immunological memory described before. [1]

1.1 Recognition of non-self antigens

The role of the immune system is to defend the organism by all the potentially infectious exogenous factors. These factors are referred to as "non-self", as not

belonging to the organism. For this reason, the immune system, in particular the adaptive one, has to be able to correctly distinguish self- from non-self. The adaptive immune system is partially supported in this recognition by the so called Major Histocompatibility Complex (MHC), or Human Leukocyte Antigen (HLA) in humans, a set of polymorphic genes which encode for several cell-surface proteins or peptides. These molecules can be classified as MHC class I and II. MHC class I molecules are short peptides (8-9 amino acid residues) exposed on the cell surface and presented in order to indicate a possible intracellular infection to $CD8^+$ T cells and leading eventually to the apoptosis. MHC class II molecules are longer than those of class I (15-30 amino acid residues) and are expressed by APCs. After a proteolytic cut of a non-self antigen, its epitope fragment is presented by these molecules to the $CD4^+$ T cells in order to activate B cells and to produce specific antibodies.

On the other hand, B cells are able to distinguish self- and non-self without the assistance of MHC proteins, thanks to the specificity of their BCRs. The interaction between B cells and the corresponding non-self antigen plus the additional signal from $CD4^+$ leads to the production of specific antibodies.

1.2 Antibodies

Structurally, antibodies (Abs) or immunoglobulins (Igs) are quaternary proteins composed by two identical light chains (about 25 kDa) coupled to two identical heavy chains (about 55 kDa) through disulfide bridges. Some types of them are characterized by polymeric structures. The complete structure shows a characteristic “Y” shape (Fig. 1).

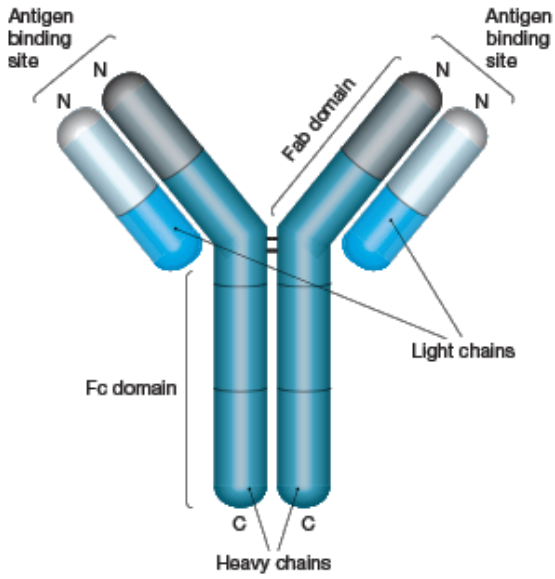


Figure 1 - The Ab structure

The two N-terminal portions of the Ab are called fragment antigen binding (Fab) sites or Fab domains; they are responsible for the interaction with the specific antigen and are able to bind one epitope each one. The terminal part of the Fab domains is referred to as paratope and is the portion of the Ab which directly and specifically interact with the epitope of the corresponding antigen. The reversible Ag-Ab interaction is non-covalent and its corresponding Ka value represents the affinity of the Ab to the Ag.

The epitope fraction of the antigen can be linear (or continuous or sequential) if it is represented by a given amino acid sequence made of residues subsequent one to each other; or conformational (or discontinuous) if it is composed by amino acid residues more or less distant in the overall sequence of the antigen but which result spatially close one to each other because of the secondary structure of the antigen.

Antigens commonly show several different types of epitopes. This characteristic determines the classification of two different types of antibodies: polyclonal antibodies (pAbs) are defined as produced by different B cell lines and are represented by a collection of immunoglobulins which each specifically interact with one of the available epitope of the considered antigen; monoclonal antibodies (mAbs) are secreted by identical B cells that are all clones of a unique parent cell and react against a specific antigen identifying a unique epitope.

The C-terminal fraction of the Ab is called fragment crystallizable (Fc) or Fc domain and is involved in the interactions with the receptors belonging to the effector cells of the immune system, such as phagocytes, allowing them to correctly recognize and destroy the Ags.

The N-terminal fractions of both the light and the heavy chains are characterized by a variable domain that differentiate the various types of Abs, while the C-terminal portion maintain an identical sequence. 5 subsets of antibodies are commonly described nowadays in mammals: IgG, IgM, IgD, IgA and IgE, which differ for chemical and biological properties (Fig. 2).

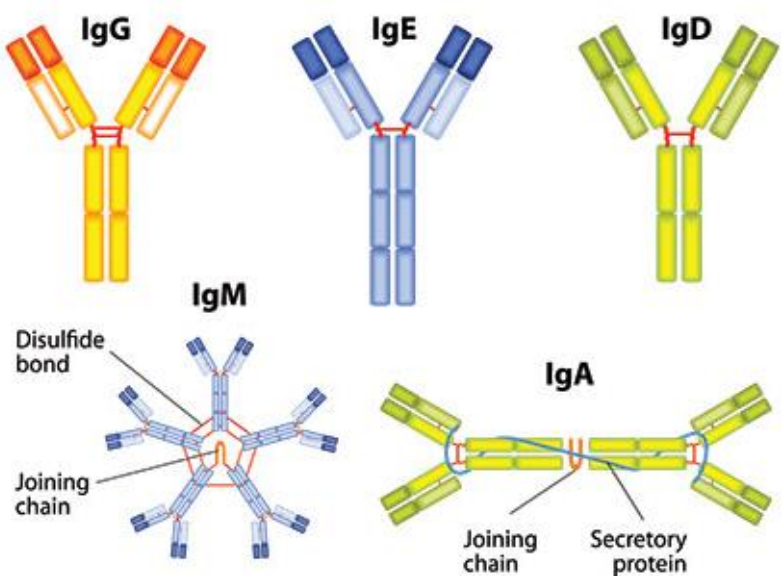


Figure 2 - The 5 types of antibodies

IgM is the very first Ab class produced by the immune system following the infection. Because of their pentameric structure, they show a peerless ability to bind several antigen molecules but they are also less stable than Igs belonging to the other classes. For this reason, during the secondary immune response, the most stable IgG become the major Igs class produced by the immune system, about 75% of the circulating Igs, becoming the class of antibodies most present in serum.

Finally, IgA are characteristic of mucosal tissues and saliva, IgE are principally found in the skin and IgD are the least abundant class of antibodies in the organism.

2. AUTOIMMUNE DISEASES

Autoimmune diseases are defined as a very heterogeneous set of chronic pathological conditions not necessarily related one to each other but sharing a common aspect: they are characterized by loss of the ability of the immune system to correctly differentiate self- from non-self, leading to an immune autoreactivity against self-Ags. The immune system starts to produce autoantibodies (autoAbs), coupled to the appearance of activated lymphoid cells against the organism itself, provoking several kind of damages to organs and tissues and/or the dysregulation of numerous endogenous biochemical processes.

Witebsky et al. defined some key features in order to classify pathological conditions as autoimmune. [2] These characteristics include the confirmed presence of autoAbs or pathogenic T cells, clinical evidences and comparable results obtained in animal models. Autoimmune diseases are estimated to affect about the 8% of the world population, [3] in particular women. [4] The major part of the biological and molecular mechanisms the autoimmune diseases are bade on still remain unclear, as well as their triggering causes. For this reason, autoimmune diseases are nowadays classified as multifactorial diseases, including both genetic and environmental factors as possibly involved in their pathogenesis. [5] The most common therapeutic approaches to this kind of pathologies are represented by the administration of immunosuppressants which reduce the clinical symptoms but are not efficient at all in order to counteract the onset and the development of the disease, in addition to an increase of the risk of infective complications.

Autoimmune diseases are generally divided into two categories: organ-specific and non organ-specific, or systemic. [6] Organ-specific ones show an

autoimmune response directed against multiple antigens specific of one organ, including hormonal receptors or intracellular enzymes (e.g., pancreatic β-cells in type 1 diabetes). Systemic diseases are instead characterized by a wide distributed autoimmune reaction against several organs and in particular involving, as autoantigens, molecules which are largely distributed in the organism. Three main phases are commonly described for the development of autoimmune diseases (Fig. 3):

- The initiation phase, during which the disease is triggered by a combination of genetic and environmental factors;
- The propagation phase is characterized by a progressive phlogosis and tissue damages, coupled to the release of inflammatory mediators and cytokines. First symptoms are detected in patients during this phase, due to an accumulation of effector T cells in tissues;
- The resolution phase consists in the activation of intrinsic and extrinsic cellular mechanisms in order to limit the effector responses and to restore the equilibrium between effectors and regulator T cells. Patients in this phase of the disease commonly show relapses and remissions. A key role in the resolution seems to be played by the Tregs. Tregs are regulator lymphocytes which are able to inhibit effector T cells. The loss of Tregs production and/or maintenance leads to a chronic pathological condition.

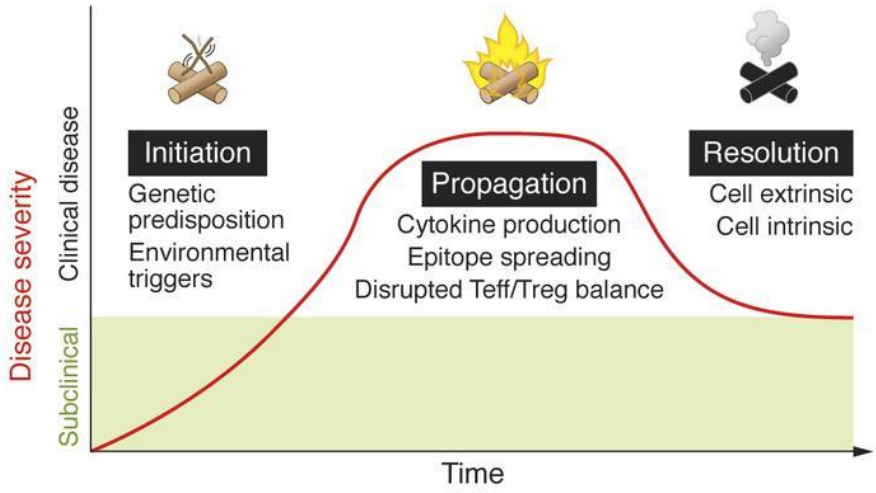


Figure 3 - Principal phases of autoimmune diseases *[7]*

2.1 The role of genetics in different pathologies

The description of the general and typical features of autoimmune diseases is quite recent. The first trigger evaluated and investigated for their pathogenesis was genetics; the complex set of possible types of mutation which can occurs on the human genome allow to approximatively justify the aberrant autoimmune response and its subsequent damages. Each autoimmune disease known and described nowadays is related to a specific but often complicated genetic condition which involves numerous factors. Indeed, autoimmune diseases are known to be potentially hereditary.

Several examples can be reported. In case of type 1 diabetes (T1D) and its latent version (LADA) and psoriasis (PsO), a well-defined genetic constitution is necessary for their pathogenesis and numerous susceptibility genes related to them have been described. Furthermore, the existence of specific, diagnostic and prognostic autoantibodies in T1D has been demonstrated and the nature

and the role of the autoantigen in PsO has already been hypothesized and partially investigated.

Genetic predisposition is fundamental in order to explain the pathogenesis mechanisms also for non-autoimmune diseases or pathologies not yet defined as such. For example, the key role of a monogenetic dominant mutation in the pathogenesis of Rett syndrome and of several inherited mutations on a well-defined gene in Fabry Disease have been already demonstrated.

2.2 Environmental triggers

Despite the role of genetics as trigger of many kind of diseases is undoubted, numerous pathologies, in particular in case of autoimmune ones, show a phenotype and clinical symptoms so heterogeneous and complex to make genetics itself not sufficient to justify them exhaustively. For this reason, also the possible role of exogenous factors such as environmental factors began to be investigated. The etiology of autoimmune diseases still remains unclear but a combination of genetic and environmental factors is nowadays currently accepted as necessary for their pathogenesis. Viral and bacterial infections have been strongly investigated in the last years. One of the most supported hypothesis describes the autoimmunity as induced by more than one infection among the many contracted since childhood. [8] Indeed, for example, numerous viral infections, such as the Epstein-Barr [9] and the hepatitis A [10] infection, but also bacteria such as *Yersinia enterocolitica* [11] and *Staphylococcus aureus* [12] have been related to the rheumatoid arthritis condition.

Three main molecular mechanisms are currently associated to the role of infections in the onset of autoimmunity: epitope spreading, bystander activation and molecular mimicry.

a. In case of the epitope spreading, the infection itself but also the defensive action of the immune system against the pathogen in the infected tissues cause tissue damages, leading to the release of endogenous antigens. Commonly, these antigens are structurally slightly modified and for this reason recognized as non-self by the APC cells. Furthermore, this secondary immune response is directed against all the endogenous substrates which share structural features with the antigenic one. For example, in case of rheumatoid arthritis, an Arg residue is substituted with a citrulline one in the target protein, leading to an immune response against all the citrullinated substrates. [13]
b. The bystander phenomenon is observed in case of stimulation of toll-like receptors (TLR) by the bacterial infection, leading to the production of inflammatory mediators and cytokines which cause tissue damages and the release of endogenous antigens. A T-cell immune response is described against both microbial and tissue antigens. [14]
c. If the epitope fraction of exogenous antigens is characterized by structural or molecular similarities compared with self-molecules, a molecular mimicry mechanism occurs. [15] Antibodies which interact specifically with the epitope portion of the exogenous antigen are in this case not able to correctly distinguish self- from non-self because of their homology, recognizing neo-epitopes in endogenous molecules and cross-reacting with them. Several autoimmune diseases have been correlated to this particular trigger mechanism, such as primary biliary cholangitis, [16] systemic erythematous lupus, numerous chronic pathological conditions of the respiratory tract [17] and T1D. In the latter case, a molecular mimicry has been hypothesized between the endogenous enzyme human glutamic acid decarboxylase (*h*GAD),

involved in the regulation of glucose metabolism, and a nonstructural protein called P2c found in the capsid of the Coxsackie virus B4. [18]

3. ANTIGENS: NATIVE VS. MODIFIED

The adverse immune reaction against self-antigens can occur because of two different kind of triggers. In the first case, the previously described molecular mimicry mechanism leads to the inability of the immune system to correctly distinguish self- from non-self because of structural or molecular similarities between them. Therapeutic approaches for these diseases are generally directed to clinical symptoms only, because of the constant and continuous production of the target endogenous molecule by the organism. Alternatively, immunosuppressants are used in the most serious conditions. Diseases like T1Dp and PsO belong to this category, being characterized by native self-antigens such as the endogenous enzyme *h*GAD and the antimicrobial peptide LL37. Differently, other kind of pathologies show an autoimmune response against self-antigens triggered by specific aberrant post-translational modifications promoted on these endogenous molecules by pathogens or as consequence of the presence of reactive molecules (e.g. drugs). In fact, largely used drugs such as antibiotics have been reported to be responsible of the formation of covalent interactions with endogenous proteins, leading to immune responses and allergic reactions against these drug-protein complexes.

Moreover, several bacterial infections, such as the *Haemophilus influenzae* ones already described in the pathogenesis of multiple sclerosis, [19] can lead to well-defined post-translational modifications on self- and non-self proteins which can be then related to the onset of different kind of pathologies.

In these cases, a deep comprehension of the molecular mechanisms and of the role of pathogens or reactive species in the onset of these diseases is critical in order to develop functional and targeted therapeutic strategies against the specific trigger factor. To investigate the pathogenesis and the development mechanisms of several kind of diseases, the ability to synthetically reproduce both native and modified protein or peptide antigens is fundamental.

4. SYNTHETIC PEPTIDE AND PROTEIN PROBES AS PROTEIN MIMETICS

Proteins and peptides play a key role in almost all biological processes, from the catalysis of biochemical reactions to the maintenance of structural integrity of cells and tissues. They regulate all these functions through specific interactions between proteins and their ligands, such as peptides, other small molecules and ions, or other proteins; several pathological conditions are related to dysregulations of these mechanisms. In order to clarify their involvement in the onset of many kind of diseases and to develop novel therapeutic strategies, an exhaustive comprehension of these interactions is fundamental in order to. Furthermore, proteins and peptides are the most common and important antigenic target in the major part of pathologies. Considering the big structure of proteins, only portions of them effectively act as binding or interaction sites for ligands or antibodies. These portions are respectively referred to as active sites or epitopes.

In order to correctly reproduce the aforementioned portions, the synthesis of mimetic molecules is considered a powerful approach. Despite the recombinant strategies, which consist in the transformation of selected bacterial cells

engineered with plasmids for the expression of entire native or modified proteins, still remain one of the most used approaches for the synthesis of antigenic proteins, recently the use of mimetic peptide and protein probes also aroused interest. Mimetic peptides basically represent only the epitope or the active site of the respective protein, but they are much easier to synthetize, to handle and to eventually chemically modify. Furthermore, they commonly show a greater proteolytic stability compared with the corresponding entire protein.

Three features of the parental protein structure are considered in order to synthetize a mimetic peptide of the binding site of a protein: structure, sequence and function. Using the so called combinatorial approach, only the function of the protein is important: indeed, large populations of peptides are synthesized and tested as ligands or inhibitors of the complementary protein. [20] On the contrary, the so called epitope mapping method is based on the reproduction of the entire protein amino acid sequence using short and overlapping peptides, in order to screen them as binding sequences for the complementary protein. [21] Obviously, this approach only allow the identification of linear or sequential binding sites or epitopes, omitting the informations about the folding. Finally, structure-based design approach consists in the design and the synthesis of peptides which are mimetics of the protein active site or epitope but which also correctly maintain the 3D informations and characteristics of the native protein, commonly taking advantage of specific chemical modifications. [22] This method allows to reproduce and mimic also conformational or discontinuous epitopes. Two main secondary structures are reported to be present in protein 3D folding: α-helices and β-sheets. α-helices mostly contribute to about the 62% of the protein-protein interactions, while β-sheets are principally involved in protein aggregation. For these reasons, peptide mimetics of these two secondary structures are an attractive target for drug development. Nowadays, the

Merrifield's approach for the solid phase peptide synthesis still remains the most used in order to produce synthetic peptides. Through this chemical method, compared with the recombinant one, a greater number of amino acid residues, including D- and non-proteinogenic amino acids, and synthetic building blocks can be insert in the sequence in order to confer to the peptide several specific features, such as an increase of the proteolytic stability or the affinity with the corresponding protein.

On the other hand, the expression of protein fragments mimetics of target proteins by taking advantage of the transformation of selected bacterial cells engineered with plasmids also provides powerful tools for the study of the possible role of the aforementioned proteins in the onset of several kind of pathological conditions.

The principal aim of this PhD project is to highlight how powerful and versatile the use of modified and non-modified peptide and proteins antigenic probes as mimetics of specific target proteins can be for the study of many different pathological conditions and, furthermore, of several aspects of these pathologies which were never or poorly investigated before. One of the most important ones is the possible role of several kind of environmental factors in the pathogenesis of these diseases. In this PhD thesis the advantages and the high versatility of use of the aforementioned antigenic probes will be showed on different types of diseases, from autoimmune to immuno-mediated and only genetic-related ones, considering both B and T immune responses, besides several kind of environmental triggers, such as viral or bacterial infections but also side effects of drugs.

5. THE "CHEMICAL REVERSE APPROACH"

The Interdepartmental Laboratory of Peptide and Protein Chemistry and Biology of the University of Florence (PeptLab@UniFi) recently developed a novel approach referred to as "Chemical Reverse Approach" (CRA). [23] This strategy is based on the screening of synthetic antigenic peptide and protein probes depending on the circulating autoantibodies founded in patients' sera (Reverse), followed by an optimization of the chemical properties of these probes (Chemical). Figure 4 shows a complete scheme of this methodology. In this sense, autoantibodies can be used as biomarkers to diagnose and sometimes to prognosticate the corresponding autoimmune disease. A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". [24]

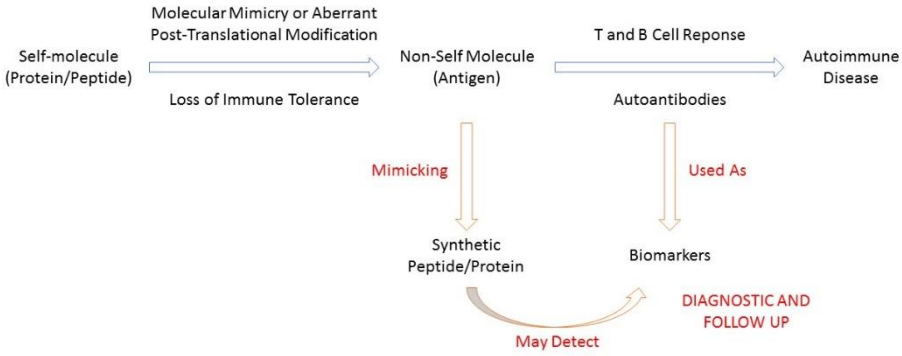


Figure 4 - The Chemical Reverse Approach

CHAPTER ONE: STUDY OF A POSSIBLE CORRELATION BETWEEN TYPE 1 DIABETES AND VIRAL INFECTIONS

1. INTRODUCTION

1.1 Diabetes: etiology and immunology

Three types of diabetes mellitus are nowadays known and classified: type 1 diabetes mellitus (T1D), type 2 diabetes mellitus (T2D) and Latent Autoimmune Diabetes in Adults (LADA). LADA was only recently differentiated by T2D because of they both affect the adult age. Only T1D and LADA show autoimmune mechanisms, the first one from the early stages of life and the second one after decades. [25] Commonly, each type of diabetes, in its advanced phase, is characterized by several health complications such as kidney failure, heart disease, ketoacidosis, stroke and blindness, in addition to clinical signs such as excessive thirst, urination and hunger (Fig. 5).

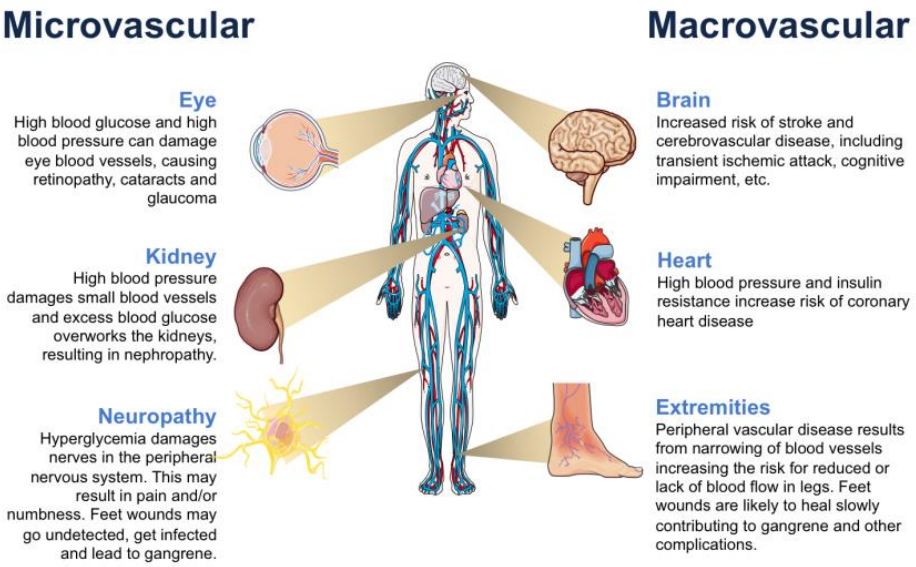


Figure 5 - Complications of diabetes *[26]*

All these symptoms are consequence of hyperglycemia which is caused by a not regular functioning of the insulin system. This condition results from different mechanisms in the two types of diabetes: in case of T2D, the non-autoimmune one, usually associated with obesity or older age, insulin is produced but an insulin resistance is observed; an autoimmune mechanism is instead highlighted in T1D and LADA. This condition occurs principally in genetically susceptible individuals, in a certain extent probably triggered also by environmental factors. [27] So called autoantibodies, produced by the body's own immune system, are identified to be responsible of damages against β-cells in the pancreatic islet of Langerhans; this damages provoke a reduction or a complete elimination of the insulin production, leading to a deficiency of it.

In 2015, about 415 million people were estimated to be diabetic, in an age range between 20-79 years. About 5.0 million deaths were attributed to diabetes,

corresponding to a global health cost of 673 billion US dollars. These numbers raised up in the following years.

1.2 The genetics of T1D

It is now well established that a specific genetic constitution is required for the pathogenesis of diabetes. Different susceptibility genes have been highlighted to be connected to the development of this disease:

a. *RARE MONOGENIC FORMS*

   Usually accompanied by several other autoimmune conditions, these monogenic forms represent a small part of T1D cases; [28]

b. *HLA GENES*

   The HLA region on chromosome 6p21 (or IDDM1) is known to be a susceptible locus for many autoimmune diseases, including T1D; [29]

c. *THE INSULIN GENE*

   Also the IDDM2 locus on chromosome 11, containing the insulin gene region, has been reported to be responsible for a genetic predisposition to T1D. [30] This is not at all surprising because of the known nature of insulin as one of the most important autoantigens in T1D.

d. *PTPN22*

   Responsible for encoding the lymphoid protein tyrosine phosphatase, this member of the T1D susceptibility gene set has been recently highlighted. [31]

e. *IL2RA*

   Variations in the interleukin (IL)-2 receptor-α gene region have been related to a genetic risk factor implicated in T1D pathogenesis. In several

autoimmune conditions such as multiple sclerosis (MS) and T1D, increased levels of soluble IL2RA have been highlighted in circulation.

1.3 The role of environmental factors in T1D

Despite the complete pathogenesis mechanism of T1D still remain unclear, there are evidences about the autoimmune nature of the disease. Indeed, many autoantibodies characteristic of T1D, such as autoantibodies against β-cells or against the endogenous enzyme *h*GAD65, whose role will be discussed later, are commonly detected after the onset of the pathology and by now considered some of the most important markers for this disease, not only in the diagnostic but sometimes also in the prognostic phase. [32] These informations suggest that a sequence of inciting events precedes the hyperglycemia for months or years. As described before, because genetics itself is not sufficient to explain these events exhaustively, also the role of different environmental factors, such as viral and bacterial infections, in the pathogenesis of autoimmune diseases became a very investigated subject, despite in most cases no direct evidences have been still reported.

1.3.1 Viral infections

Different kind of viruses, in particular Rotaviruses and Enteroviruses, have been associated with T1D but a causal correlation has not been proven yet. These genera are two of the most common infectious agents of the human beings during the early age period; some of the hypotheses about their relationship with the pathogenesis of T1D are based on the concept of “molecular mimicry”, because of some similarities found between viral and endogenous proteins. [33]

In case of Enteroviruses, many studies designate them, and in particular one of their subgroups named Coxsackie viruses, as primary viral candidates that can cause the onset of T1D. [34]

Coxsackie viruses are cytolytic viruses belonging to the Picornaviridae family. 61 non-polio Enteroviruses are known, of which 23 of them are Coxsackie A and 6 are Coxsackie B. The most recent classification of Enterovirus rigorously divides them in Polioviruses, human Enteroviruses A (including the major part of Coxsackie A), human Enteroviruses B (including Coxsackie B), human Enteroviruses C (including some other Coxsackie A). Two Coxsackie (A4 and A6) have still not been assigned to any class. Coxsackie A are responsible of diseases such as the Hand Foot Mouth Disease, the hemorrhagic conjunctivitis and herpangina.

A homology between the nonstructural protein 2c found in the capsid of the Coxsackie virus B4 (CVB4 P2c) and portions of both the two isoforms, the *h*GAD65 and the *h*GAD67, of the endogenous enzyme human glutamic acid decarboxylase (*h*GAD), one of the major autoantigens in T1D, led to the postulation of a mechanistic hypothesis about viral mimicry in the etiology of T1D [18] [35]. In particular, the sequone PEVKEK, which can be identified in all the three aforementioned portions, was proposed as putative epitope responsible for the cross-reactivity of the antibodies between the viral and the enzymatic sequences (Fig. 6).

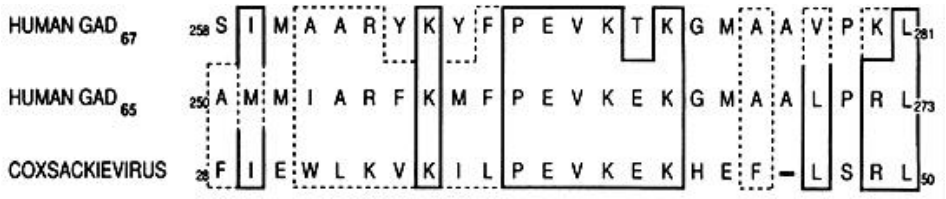


Figure 6 - Similarities between *h*GAD67(258-281), *h*GAD65(250-273) and CVB4 P2c(28-50) *[18]*

Figure 7 shows the position of this sequone in the 3D complete structure of the *h*GAD65 protein, made by two identical subunits. Sequone PEVKEK is highlighted in red.

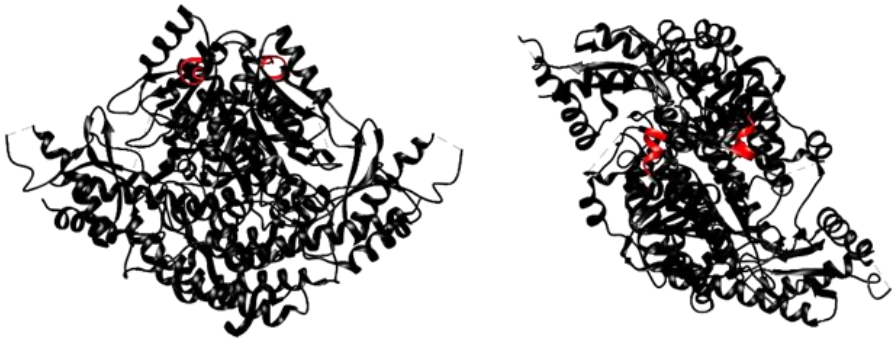
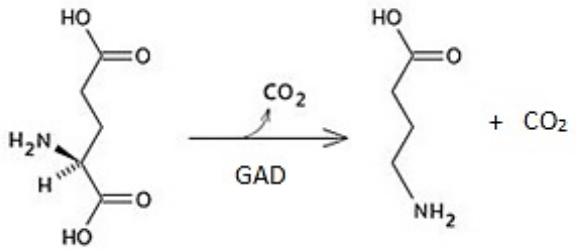


Figure 7 - 3D structure of *h*GAD65 (PDB: 2OKK)

*h*GAD65 is the major autoantigen in T1D, although autoantibodies against *h*GAD67 are occasionally observed. [36] The anti-*h*GAD autoantibodies are considered nowadays the most important biomarker for T1D not only in the diagnostic phase but also in the prognostic one. Moreover, anti-*h*GAD autoantibodies are also found both in the rare neurological disease called Stiff

Person Syndrome (SPS) and the autoimmune polyendocrine syndrome type 1. [37] [38]

*h*GAD is a fundamental enzyme present in all eukaryotes and in many prokaryotes. Biologically the enzyme *h*GAD deals with the production of the neurotransmitter molecule γ-aminobutyric acid (GABA) through a decarboxylation reaction starting from glutamic acid (Scheme 1).



Scheme 1 - γ-aminobutyric acid production

In mammals, there are two isoforms of *h*GAD, depending on their molecular weight: *h*GAD67 (67kDa) and *h*GAD65 (65kDa) (Fig. 8). They are expressed by two different genes, respectively GAD1 and GAD2, prevalently in pancreas and cerebral regions. [39] *h*GAD67 is characterized by a uniform distribution inside the cell, differently *h*GAD65 is localized exclusively in nerve terminals and at the membrane level. This reflects a functional difference between the two isoforms: *h*GAD67 synthesizes GABA for neuron activity unrelated to neurotransmission, such as the synaptogenesis; *h*GAD65 synthesizes GABA specifically for neurotransmission, consequently being necessary only in nerve terminals. [40] The two isoforms have been divided into three functional domains according to

their linear sequence: an amino N-terminal domain, a middle PLP-binding domain containing the active catalytic site and a carboxyl C-terminal domain. The isoforms show a general homology of the overall sequence, particularly focused in the PLP-binding and the C-terminal domains (74%), while differ (25%) for the N-terminal domain, mainly in the first 100 aa. [37] This latter portion contains the membrane-binding sequence that properly anchors *h*GAD65 to synaptic vesicles, ensuring an efficient GABA biosynthesis and packaging into them. Both the isoforms are regulated through diametrically opposite phosphorylation reactions: *h*GAD65 is activated by phosphorylation, performed by protein kinase C, while *h*GAD67 is inhibited by it, performed by protein kinase A. Finally, the pyridoxal-5-phosphate (PLP) act as a common regulator for them. [41]

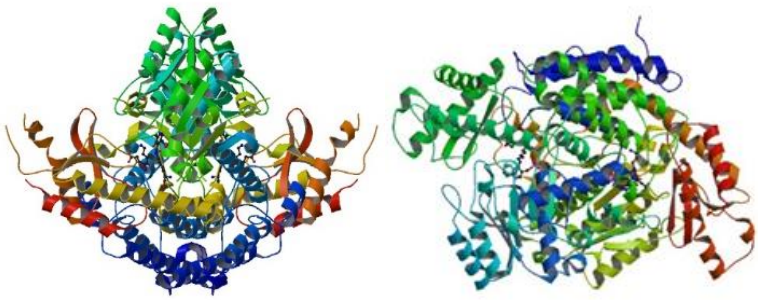


Figure 8 - *h*GAD65 and *h*GAD67 (PDB: 2OKK and 2OKJ)

GABA is principally an inhibitory neurotransmitter in the Central Nervous System of mammals. In humans is also responsible of muscle tone. However, there are evidences about the role of GABA as secondary regulator of glucose metabolism. [42] [43] Some hypotheses suggest that GABA could act as paracrine signaling

molecule between β-cells and the other endocrine cells of the islet of Langerhans. In this sense, GABA has been proposed to play a role in the inhibition of glucagon release. [42] [43]

For this reason, an immune damage against the *h*GAD enzyme, hypothetically due to the showed homology of its amino acid sequence compared with the CVB4 P2c one, and a subsequent dysregulation of glucose metabolism has been suggested to be a possible trigger for the onset of T1D. Different studies argued both in favor [44] and against [45] this hypothesized pathogenesis mechanism.

Many studies have been promoted in order to clarify the role of Coxsackie viruses in the pathogenesis of T1D; unfortunately, many conflicting results have been obtained. In 1971, a team followed the diabetes incidence after an outbreak of CVB4 infection on the isolated Pribilof Islands. During a subsequent period of five years, no appreciable differences in terms of incidence of T1D between the CVB4-infected and non-infected individuals have been reported. [46] On the other hand, a CVB4 strain has been isolated from a child with recent-onset T1D, [47] some functional data about enhanced T-cell responses to CVB4 proteins in children with T1D have been reported [48] and the evidence the presence of enterovirus in pancreatic islets of a significant number of recent-onset T1D patients compared with controls has been described, [49] suggesting that T1D is a consequence of selective viral infection of β-cells.

It is therefore evident the controversial nature of the role of enteroviruses in relation to T1D onset, in particular as regard the putative trigger event: a direct damage on β-cells in pancreatic islets or a molecular mimicry and a subsequent autoimmune damage leading to a dysregulation of the glucose metabolism. Anyway, cumulatively, all these data strongly suggest the involvement of CVB4 in at least a subset of T1D cases.

1.3.2 Bacterial infections

The relationship between bacteria and the onset of T1D is object of several studies. Some evidences highlighted the role of the intestinal bacterial flora in T1D development in animal model; [50] furthermore, the treatment with antibiotics has been shown to be able to prevents diabetes. [51] Because of the apparent lack of capacity of the intestine wall to perfectly separates luminal bacteria and the immune system in T1D patients compare with controls, the so called “leaky gut” phenotype has been hypothesized to be capable to improve the contact between bacterial antigens and the immune system. According to this hypothesis, evidences show that antibiotics and probiotics are able to alter T1D development. [52]

1.3.3 Other environmental triggers

Several other factors have been reported to be potential causal triggers of T1D, such as cow’s milk and wheat proteins [53] [54]. Among these, some other molecules commonly taken with food and/or produced by the body, such as vitamin D, might affect T1D development. Vitamin D (Fig. 9) is endogenously produced by the organism after the exposure of the skin to the sunlight. Its connection with T1D has been hypothesized because of the seasonal incidence of the disease, which is inversely proportional with the increase in hours of sunshine per month. [55] This relationship seems to be due to the ability of vitamin D to inhibits dendritic cells (DC) differentiation and their immune activation.

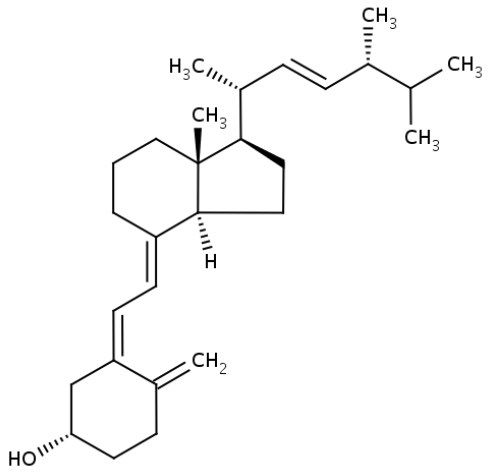


Figure 9 - Vitamin D structure

1.4 Pathogenesis of T1D: timeline

Different timelines have been hypothesized in order to best represent the putative clinical phases of the pathogenesis of T1D. One of the most commonly cited still remains the "linear β-cells decline hypothesis" (Fig. 10) presented by Eisenbarth in 1986. [56] In case of a specific genetic predisposition, as described before, some environmental agents can induce autoimmunity against the pancreatic islets causing a linear decay in β-cell mass and some subsequent prediabetic stages, such as direct reduction and eventually elimination of insulin production, with consequent hyperglycemia and the complete loss of the insulin-related C-peptide, one of the most commonly used clinical marker for insulin monitoring. Indeed, the 31 amino acids C-peptide is apparently free of biological functions but is one of the constituents of the pro-insulin and for this reason its concentration in blood can be directly correlated to the insulin ones. [57]

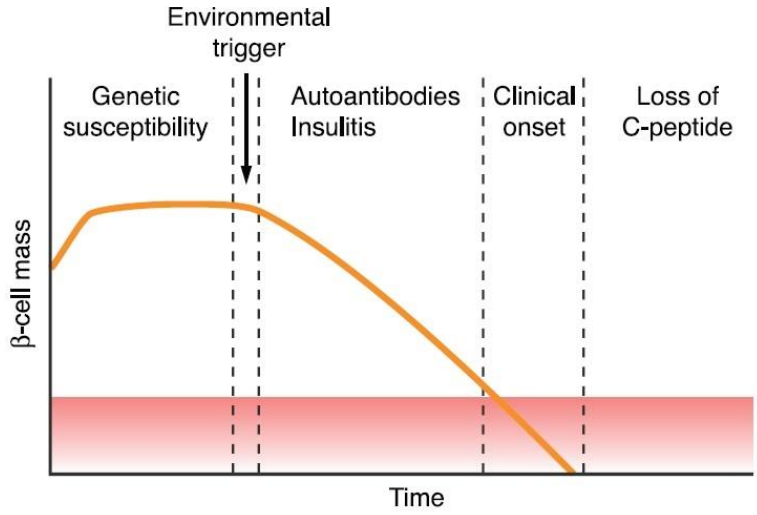


Figure 10 - The "linear β-cells decline hypothesis" *[56]*

1.5 General overview on immunization assays

Typically, two sites are involved in the interaction between an immunoglobulin and the related antigen: the paratope (or antigen-binding site), the portion of the antibody which specifically recognizes and binds the antigen, and the epitope (or antigenic determinant), the corresponding portion of the antigen detected by the immune system and bound by the immunoglobulin. [58] Epitopes are represented usually by small functional groups or portions of the antigen molecule that can induce an immune response and specifically interact with antibodies. As described before, epitopes can be linear (or continuous or sequential) as a results of the primary structure only, or conformational (or discontinuous) as a results of the secondary three-dimensional structure of the protein / peptide.

Performing immunization assays on several animal models, two types of specific antibodies can be produced against one antigen: polyclonal antibodies (pAbs) are produced by different B cell lines and are represented by a collection of immunoglobulins which each specifically interact with one of the available epitope of the considered antigen; monoclonal antibodies (mAbs) are secreted by identical immune cells that are all clones of a unique parent cell and they react against a specific antigen identifying a unique epitope. A polyclonal antibodies antiserum is then characterized by several antibodies against all the different epitopes of the immunogenic antigen used. Compared to the corresponding monoclonal ones, these antibodies are characterized by a broader spectrum of action but also a less sensitivity to antigen changes. For this reason, polyclonal antibodies are commonly used in preliminary tests working with unknown antigens or in order to try to identify a possible interaction with a putative antigen. Monoclonal and polyclonal antibodies can be greatly produced starting from synthetic peptide sequences which mimic specific portions (recognized or putative epitopes) of an antigenic native protein. These peptide-derived antibodies are powerful tools in biological assays such as SP-ELISA experiments and can be easily produced from any peptide of choice. In order to make small peptides immunogenic, a conjugation with carrier proteins such as Bovine Serum Albumin (BSA) or Keyhole Limpet Hemocyanin (KLH) is necessary; this conjugation improves the immune presentation of this small molecules, inducing an immune reaction [59] (Fig. 11).

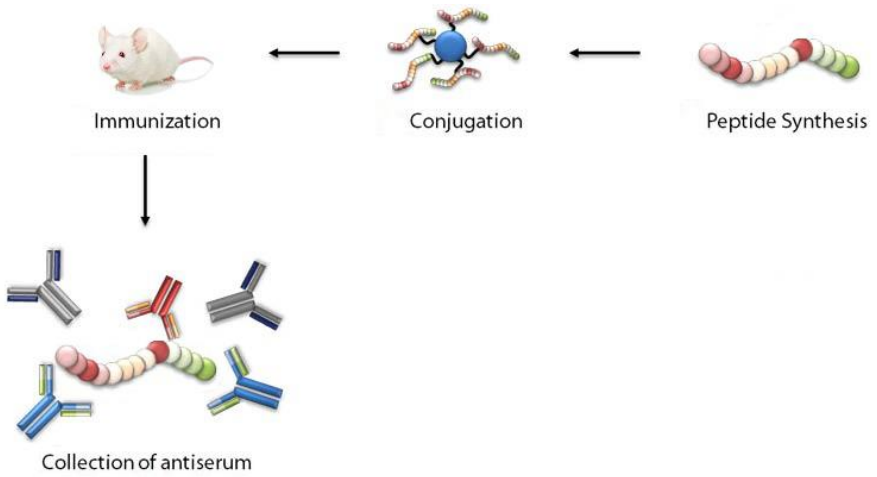


Figure 11 - Generic immunization assay on NMRI mice obtaining polyclonal antibodies *[59]*

1.6 Objectives of the project

The molecular mimicry mechanism still remains one of the most plausible trigger event hypothesized to lead to the onset of T1D. The similarity between CVB4P2c(28-50), *h*GAD65(250-273) and *h*GAD67(258-281) could play a key role in this putative mechanism but this has not yet been demonstrated. According to the literature, we propose the syntheses of these three sequences in order to use them in immunoaffinity tests such as ELISA on T1D, LADA and NBD patients' sera, in collaboration with Professor Lapolla from UOC Diabetologia e Dietetica of the Department of Medicine, University of Padova. The aim of this part of the project is to determine, following the Chemical Reverse Approach strategy, the presence or the absence in diabetic patients' sera, compared with controls, of specific antibodies cross-reactive against at least the viral sequence and one of the endogenous sequences. The presence of this kind of antibodies would be a further step towards understanding the role of CVB4 in the pathogenesis of T1D.

Furthermore, N-terminal cysteine-bearing Ac-CVB4P2c(28-50)-NH_2, Ac-*h*GAD65(250-273)-NH_2 and Ac-*h*GAD67(258-281)-NH_2 were synthetized and conjugated to the carrier maleimide-activated KLH protein (Fig. 12).

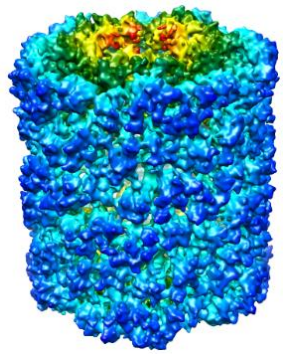
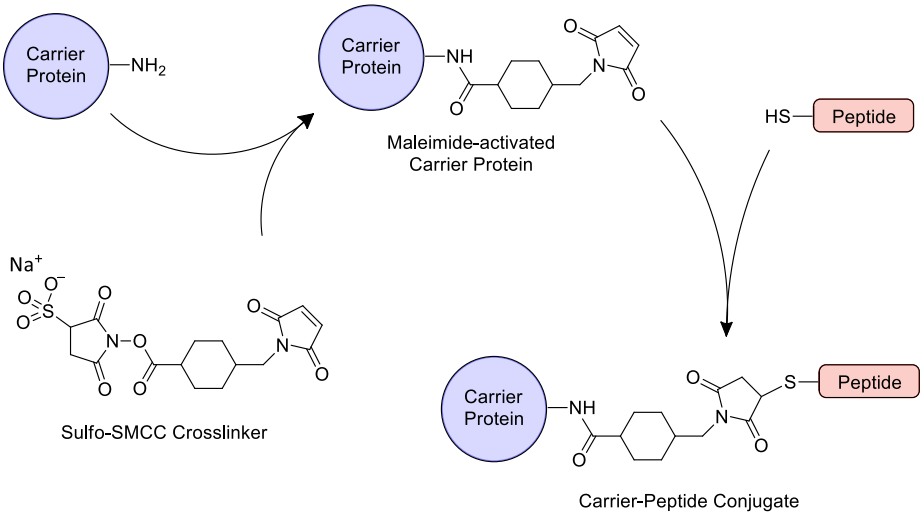


Figure 12 - 3D structure of the Keyhole Limpet Hemocyanin (PDB: EMDB-1569)

The activation of KLH using sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) crosslinker and its conjugation to generic cysteine-containing peptide are reported in the following Scheme 2.



Scheme 2 - Maleimide-activated KLH production and conjugation with a cys-peptide

These peptide-KLH conjugates were then sent to the research group of Professor Hansen and Professor Houen in the Statens Serum Institut of Copenaghen in order to perform some active immunization assays exposing 12 Naval Medical Research Institute (NMRI) mice in a controlled way to our antigens. In this research, immunization aims at the production of specific polyclonal antibodies against our synthetic peptides, in order to preliminary verify the potential cross-reactivity of these antibodies against the native enzymatic target *h*GAD due to the sequone PEVKEK that it shares with the viral protein CVB4 P2c, using the recombinant *h*GAD65 as a coated antigen.

2. RESULTS AND DISCUSSION

2.1 Synthesis of Ac-CVB4P2c(28-50)-NH_2, Ac-*h*GAD65(250-273)-NH_2 and Ac-*h*GAD67(258-281)-NH_2

The syntheses of the three sequences, selected according to what reported by Kaufman et al. and previously showed in Figure 6, to use them as probes for specific autoantibody characterization in T1D and LADA, were performed using the Fmoc/tBu Solid Phase Peptide Synthesis (SPPS) strategy assisted by microwaves and performed with the automatic synthesizer Liberty Blue (CEM). All the syntheses were carried out at the Interdepartmental Laboratory of Peptide & Protein Chemistry & Biology (PeptLab) in the Chemistry Department "Ugo Schiff" of the University of Florence, Italy, following the procedure reported in the General procedure for SPPS assisted by microwaves.

Chemically synthesized peptides carry positively and negatively charged amino- and carboxy- termini, respectively. As these positions are generally not charged in vivo, they are commonly modified by N-terminal acetylation and C-terminal amidation, which remove the respective charges in order to mimic natural peptides or the portion of a native protein, eventually aberrantly modified, object of the study.

All the peptides were then synthetized starting from a Fmoc-RinkAmide resin type (0.48 mmol/g) (Fig. 13), characterized by a linker between the resin grain and the first amino acid of the peptide chain which leave as amide the C-terminus when removed with the cleavage reaction (Scheme 3).

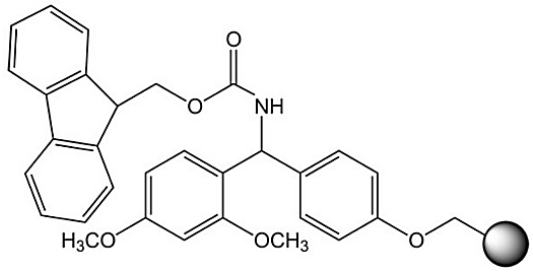
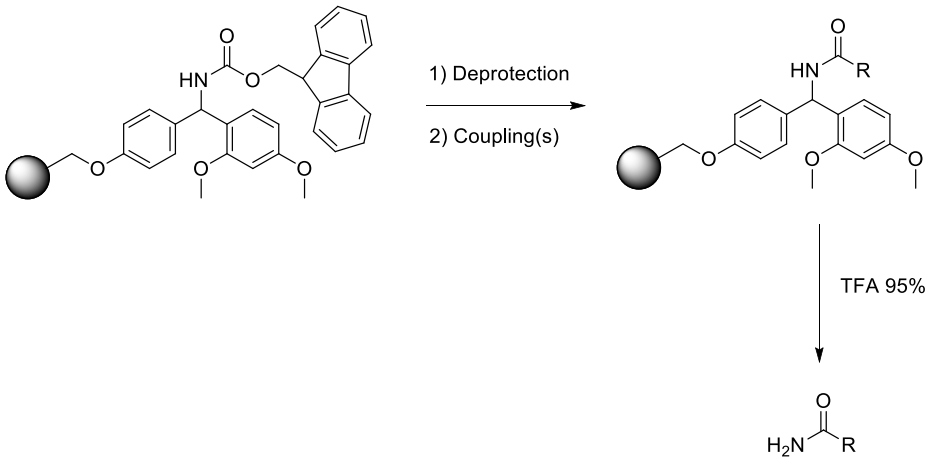


Figure 13 - Fmoc-RinkAmide resin linker

The acetylation reaction was performed when the products were still attached to the resin and protected on the side chains of the amino acids using acetic anhydride.



Scheme 3 - General mechanism of a cleavage reaction from a RinkAmide resin

Each coupling reaction was performed using 2.5 ml of a 0.2 M amino acid solution in N,N-dimethylformamide (DMF), 1 ml of a 0.5 M activator solution (N,N'-diisopropylcarbodiimide, DIC) in DMF and 0.5 ml of a 1 M activator base solution (ethyl cyano(hydroxyimino)acetate, OxymaPure) in DMF.

The cleavage reactions from the respective resin and the deprotection of all the side chains of the amino acids were carried out by exposing the peptide-resin to a TFA 95% solution (TFA/H_2O/TIS 95:2.5:2.5) for 3h at RT, as described in the General procedure for cleavage and micro-cleavage. H_2O and triisopropylsilane (TIS) were used as scavengers in order to avoid secondary reactions during the cleavage phase. The three obtained products were therefore:

- **Ac-*h*GAD65(250-273)-NH_2**: Ac-$_{250}$AMMIARFKMFPEVKEKGMAALPRL$_{273}$-NH_2
- **Ac-*h*GAD67(258-281)-NH_2**: Ac-$_{258}$SIMAARYKYFPEVKTKGMAAVPKL$_{281}$-NH_2
- **Ac-CVB4P2c(28-50)-NH_2**: Ac-$_{28}$FIEWLKVKILPEVKEKHEFLSRL$_{50}$-NH_2

All the crude peptides were purified through 2 purification steps, as described in the General procedure for peptide purification and characterization: a pre-purification or desalting step and a semi-preparative purification using a RP-HPLC. They were finally characterized via ESI-MS.

In the following Table 1 all the details about the characterization of the products are reported.

PEPTIDE	PURITY DEGREE	GRADIENT (0.6 ml/min)	Rt (min)	ESI-MS (m/z) $(M+2H)^{2+}$ Found (Calculated)
Ac-*h*GAD65(250-273)-NH_2	>99%	30%-70% B	3.47[a]	1404 (1403)
Ac-*h*GAD67(258-281)-NH_2	>99%	25%-65% B	1.97[a]	1371 (1370)
Ac-CVB4P2c(28-50)-NH_2	>99%	30%-70% B	4.53[a]	1462 (1461)

Solvents used: A 0.1% TFA in H_2O; B 0.1% TFA in CH_3CN 84%

a: Phenomenex Jupiter C18 (5 lm, 2504.6 mm), 0.6 mL/min

Table 1 - Characterization of the pure products

2.2 Acetylation reaction on the N-terminus

As described in the General procedure for acetylation reaction, all the acetylation reactions on the amino group in N-terminus position of the synthesized peptides were performed covering the peptide-resin with the minimum volume of DMF, adding 100 µL of Ac_2O each 200 mg of resin and leaving the mixture stirring for 10' at RT. The solution was filtered and replaced with a fresh one, for another 10'. The resin was finally washed 3 times with dichloromethane (DCM) and the acetylation checked by Kaiser test.

2.3 SP-ELISA experiments

The three synthetic antigenic peptides were tested in Solid Phase Enzyme-Linked ImmunoSorbent Assays (SP-ELISA) using T1D, LADA and NBD patients' sera, in collaboration with Professor Lapolla from the U.O.C. di Diabetologia e Dietetica of the Department of Medicine, University of Padova, in order to verify the presence in them of cross-reactive antibodies against the selected sequences and to understand the role of viral infections in the pathogenesis of T1D.

All the SP-ELISA experiments were performed in collaboration with Ms. Giuditta Celli and Dr. Feliciana Real in the Department NeuroFarBa, Section of Pharmaceutical Sciences and Nutraceutics of the University of Florence., following the General procedure for SP-ELISA experiments.

Our three sequences were tested on 8 T1D sera, 13 LADA sera and 13 NBA sera. Data distributions for anti-Ac-*h*GAD65(250-273)-NH_2, anti-Ac-*h*GAD67(258-281)-NH_2 and anti-Ac-CVB4P2c(28-50)-NH_2 IgG and IgM antibody titers (Fig. 14a, 14b and 14c) are reported hereafter.

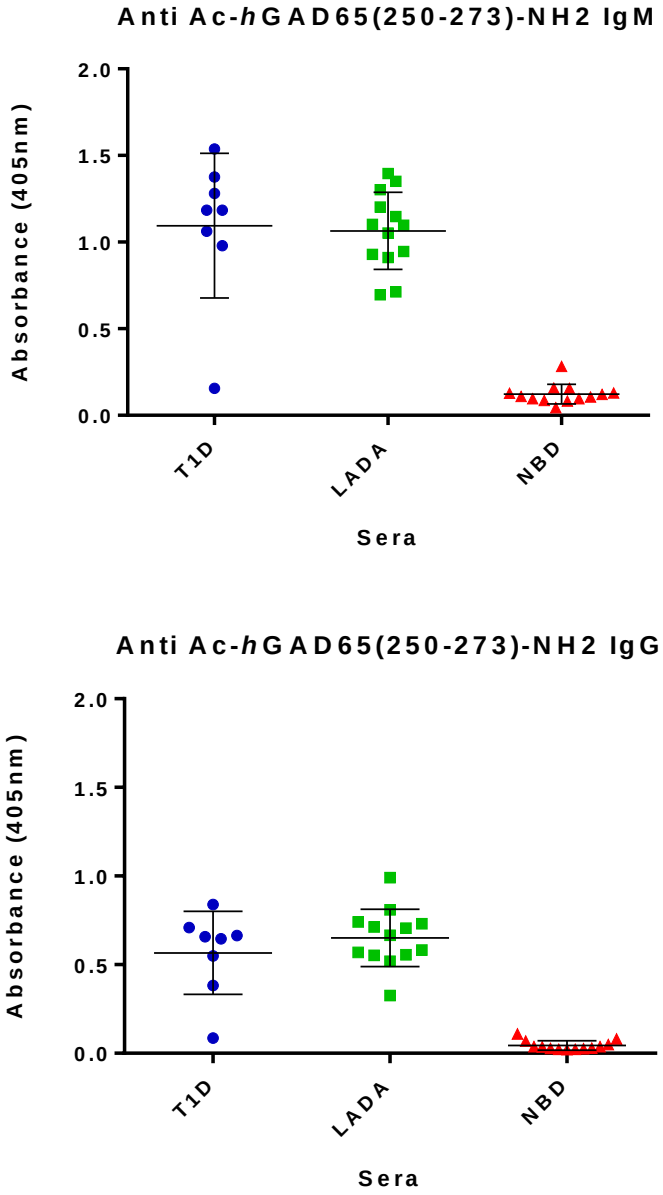


Figure 14a - Anti-Ac-*h*GAD65(250-273)-NH_2 IgM and IgG titers on T1D, LADA and NBD patients' sera

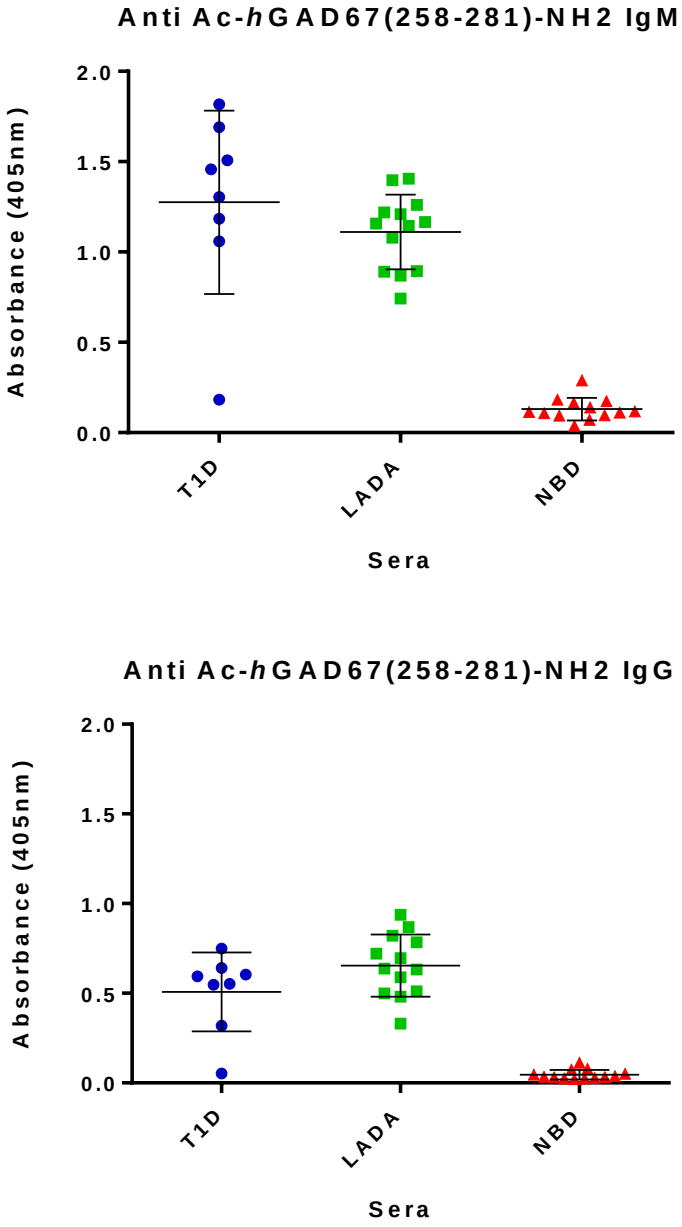


Figure 14b - Anti-Ac-*h*GAD67(258-281)-NH_2 IgM and IgG titers on T1D, LADA and NBD patients' sera

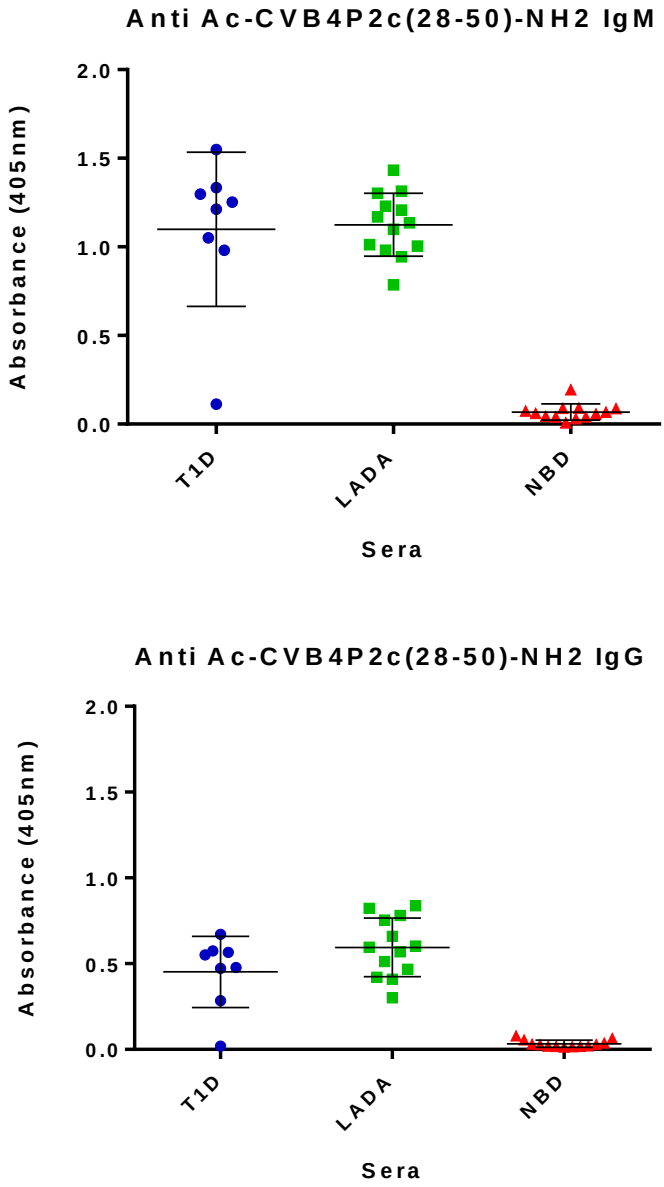


Figure 14c - Anti-Ac-CVB4P2c(28-50)-NH_2 IgM and IgG titers on T1D, LADA and NBD patients' sera

From the data reported in the previous graphics it appears that both the T1D and LADA patients' sera types showed high titers of antibodies, especially IgM, against all the three tested peptides.

Statistically, data distribution was evaluated using a D'Agostino-Parson test. The results of these tests are reported in the following Table 2.

	P value	**Passed normality test (alpha=0.05)?**
anti-Ac-*h*GAD65(250-273)-NH_2 IgM	<0.0001	No
anti-Ac-*h*GAD65(250-273)-NH_2 IgG	<0.0001	No

	P value	**Passed normality test (alpha=0.05)?**
anti-Ac-*h*GAD67(258-281)-NH_2 IgM	<0.0001	No
anti-Ac-*h*GAD67(258-281)-NH_2 IgG	<0.0001	No

	P value	**Passed normality test (alpha=0.05)?**
anti-Ac-CVB4 P2c(28-50)-NH_2 IgM	<0.0001	No
anti-Ac-CVB4 P2c(28-50)-NH_2 IgG	<0.0001	No

Table 2 - D'agostino-Parson test results

In all the three cases, both for IgM and IgG antibodies, data distribution is not Gaussian. For this reason, data were subsequently analyzed using a non-parametric test (Mann-Whitney).

Each serum was tested two times on different days. Each showed data derived from an average between the two measures, respectively called IgM1/IgM2 and IgG1/IgG2. In the following Table 3 are reported the Mann-Whitney analyses which compare the two measurements. In all cases they result not significantly different, highlighting a negligible error between them.

	P value	**Significantly different? (P<0.05)**
anti-Ac-*h*GAD65(250-273)-NH_2 IgM1 vs IgM2	0.8948	No
anti-Ac-*h*GAD65(250-273)-NH_2 IgG1 vs IgG2	0.4810	No
anti-Ac-*h*GAD67(258-281)-NH_2 IgM1 vs IgM2	0.8527	No
anti-Ac-*h*GAD67(258-281)-NH_2 IgG1 vs IgG2	0.9141	No
anti-Ac-CVB4 P2c(28-50)-NH_2 IgM1 vs IgM2	0.8527	No
anti-Ac-CVB4 P2c(28-50)-NH_2 IgG1 vs IgG2	0.9141	No

Table 3 – Mann-Whitney comparison between the two measurements

Finally, the titers obtained from T1D and LADA sera were compared with the NBD ones, in order to verify the eventual presence of a significant difference between the two kinds of patients and the NBD (Table 4).

	P value	Significantly different? (P<0.05)
anti-Ac-*h*GAD65(250-273)-NH_2 IgM		
T1D vs NBD	<0.0001	Yes
LADA vs NBD	<0.0001	Yes
anti-Ac-*h*GAD65(250-273)-NH_2 IgG		
T1D vs NBD	<0.0001	Yes
LADA vs NBD	<0.0001	Yes
anti-Ac-*h*GAD67(258-281)-NH_2 IgM		
T1D vs NBD	<0.0001	Yes
LADA vs NBD	<0.0001	Yes
anti-Ac-*h*GAD67(258-281)-NH_2 IgG		
T1D vs NBD	<0.0001	Yes
LADA vs NBD	<0.0001	Yes
anti-Ac-CVB4 P2c(28-50)-NH_2 IgM		
T1D vs NBD	<0.0001	Yes
LADA vs NBD	<0.0001	Yes
anti-Ac-CVB4 P2c(28-50)-NH_2 IgG		
T1D vs NBD	0.0009	Yes
LADA vs NBD	<0.0001	Yes

Table 4 – Mann-Whitney comparison between T1D/LADA and NBD

The showed results highlight some important considerations:

- All the three tested peptides are apparently able to detect specific antibodies in both T1D and LADA patients' sera and to discriminate them from NBD sera;
- The results obtained testing the three selected sequences are comparable to each other, both considering IgM or IgG antibody titers; this could be one of the first demonstrations of the hypothesized cross-reactivity mentioned before and therefore of the role of the Coxsackie virus B4 infection in the pathogenesis of T1D;
- Both in case of IgM and IgG antibodies, each one of our peptide probes are able to correctly discriminate T1D and LADA sera from NBD. IgM titers are, in all the cases, higher than IgG ones; this could be due to a reminiscence or a persistence of the infection in the organism.

2.4 Conjugation with KLH carrier protein and immunization assays on NMRI mice

The 3 selected peptide sequences were also synthesized with an additional Cys residue on the N-terminal position to allow the conjugation of these peptides with the KLH carrier protein:

- **Ac-Cys-*h*GAD65(250-273)-NH_2**: Ac-C-$_{250}$AMMIARFKMFPEVKEKGMAALPRL$_{273}$-NH_2
- **Ac-Cys-*h*GAD67(258-281)-NH_2**: Ac-C-$_{258}$SIMAARYKYFPEVKTKGMAAVPKL$_{281}$-NH_2
- **Ac-Cys-CVB4P2c(28-50)-NH_2**: Ac-C-$_{28}$FIEWLKVKILPEVKEKHEFLSRL$_{50}$-NH_2

The conjugation reaction was performed with the carrier protein maleimide-activated KLH as described in the General procedure for peptide-KLH conjugation.

The three peptide-KLH conjugates were then shipped to the Statens Serum Institut of Copenaghen where the research group of Professor Hansen and Professor Houen performed immunization assays of NMRI mice and Mr. Niccolò Valdarnini performed the ELISA experiments using the obtained antisera. In order to prepare antisera from our peptides, 12 NMRI mice were immunized using our three conjugates:

a. KLH[Ac-[Cys^{249}]*h*GAD65(250-273)-NH_2]
b. KLH[Ac-[Cys^{257}]*h*GAD67(258-281)-NH_2]
c. KLH[Ac-[Cys^{27}]CVB4 P2c(28-50)-NH_2]

The content of anti-peptide antibodies in the obtained antisera was determined by ELISA technique. The pre-immunization sera of the mice were used as controls and were collected prior to the initial immunization. The immunization was carried out by injecting at four separate subcutaneous sites (two inguinal, two axillary) 0.25 mg of the corresponding peptide-KLH conjugate emulsified with Freund's complete adjuvant (FCA). A second injection with 0.25 mg of the same antigen emulsified with Freund's incomplete adjuvant (FIA) was administered 14 days after the initial immunization. Subsequent booster injections with 0.25 mg of again the same peptide-KLH conjugate emulsified with Freund's incomplete adjuvant (FIA) were performed every 4 weeks collecting the serum 10 days after each booster injection.

Each ml of Freund's complete adjuvant (FCA) contains 1 mg of Mycobacterium tuberculosis, heat killed and dried, 0.85 ml paraffin oil and 0.15 ml mannide monooleate. It is commonly used to create water-in-oil emulsion of antigens in order to stimulate high and long-lasting antibody responses which can be attributed to the slow release of the antigen. Differently from the complete form, the incomplete one (FIA) lacks the mycobacterial components.

The following graphics show the results obtained from the 3 groups of 4 mice each one, immunized separately with our three different peptide-KLH conjugates. Sera were tested in ELISA, coating Ac-*h*GAD65(250-273)-NH_2, Ac-*h*GAD67(258-281)-NH_2 and Ac-CVB4P2c(28-50)-NH_2 separately, each time after all the 5 booster injections. In case of mice immunized with KLH[Ac-[Cys^{27}]CVB4 P2c(28-50)-NH_2] (Group C), 4 booster injection were considered sufficient to determine the progress of the immune response. Preliminary results are reported in the following Figure 15a, 15b, 15c and 15d.

Group A – Mice immunized with KLH[Ac-[Cys^{249}]*h*GAD65(250-273)-NH_2]	**Group B – Mice immunized with KLH[Ac-[Cys^{257}]*h*GAD67(258-281)-NH_2]**	**Group C – Mice immunized with KLH[Ac-[Cys^{27}]CVB4P2c(28-50)-NH_2]**
Mouse A1	Mouse B1	Mouse C1
Mouse A2	Mouse B2	Mouse C2
Mouse A3	Mouse B3	Mouse C3
Mouse A4	Mouse B4	Mouse C4

Figure 15a – Groups of immunized mice

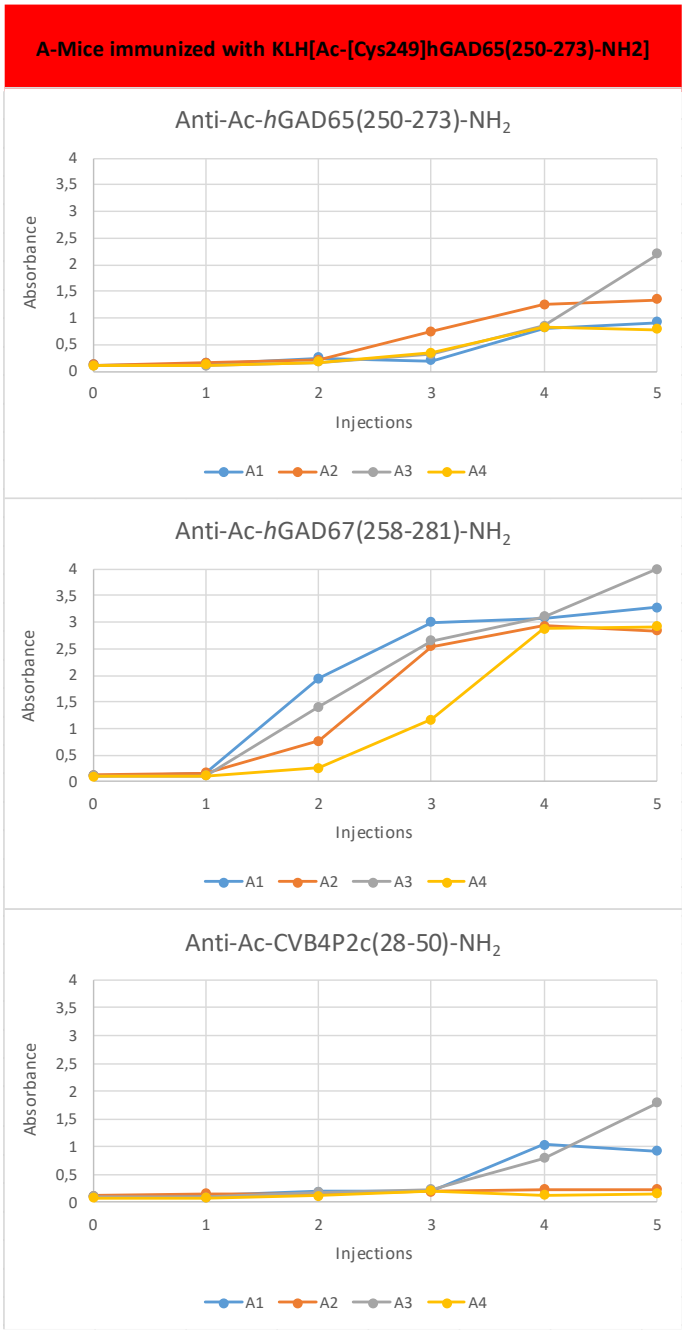


Figure 15b – Results obtained from Group A, mice immunized with KLH[Ac-[Cys249]*h*GAD65(250-273)-NH_2]

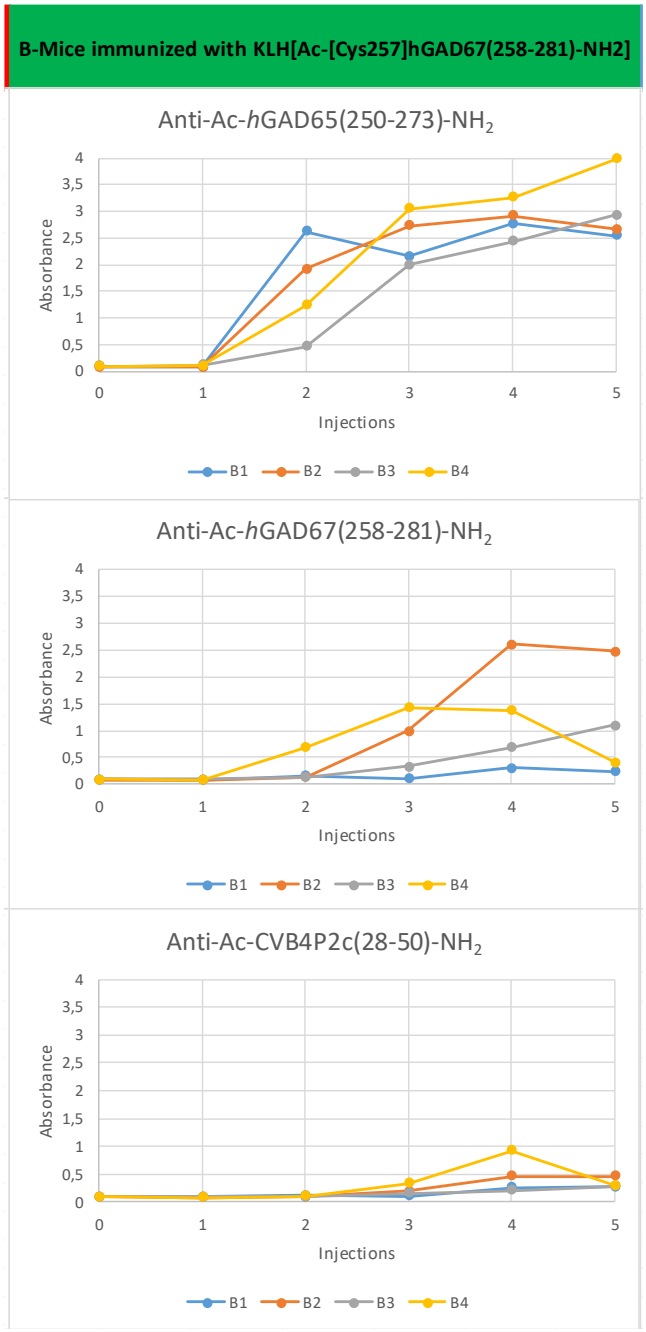


Figure 15c – Results obtained from Group B, mice immunized with KLH[Ac-[Cys249]*h*GAD67(258-281)-NH_2]

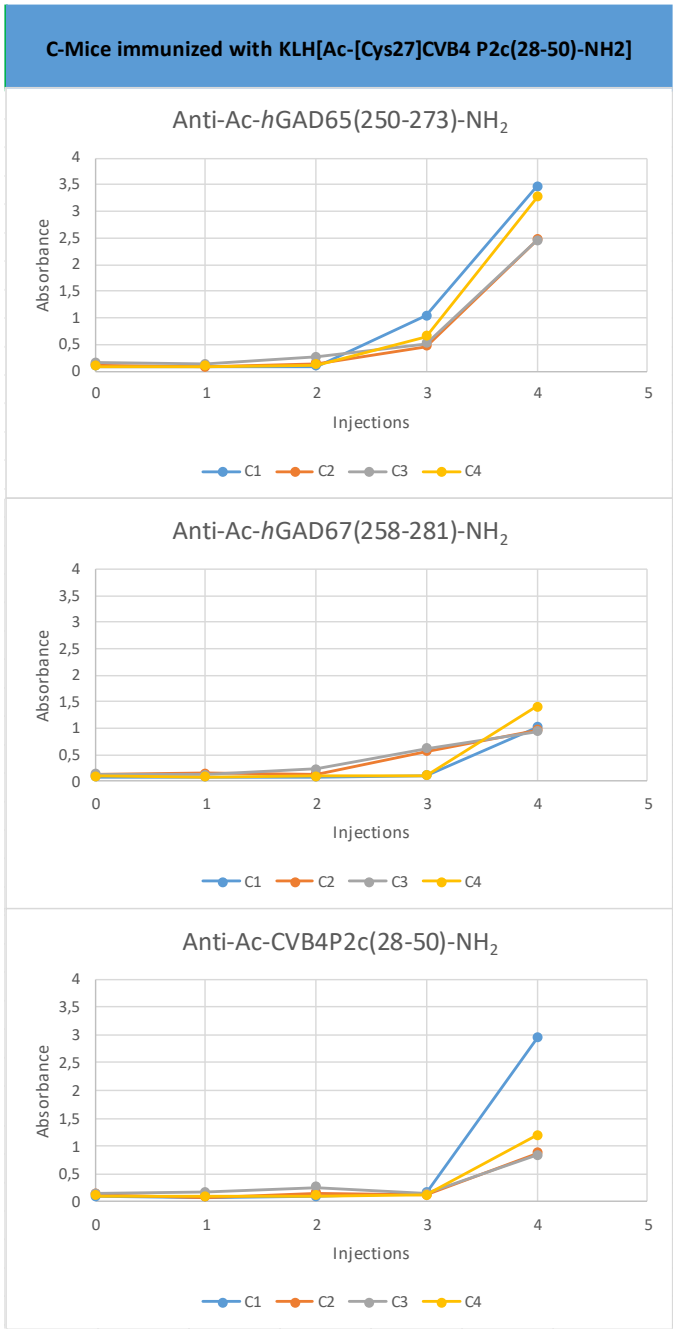


Figure 15d – Results obtained from Group B, mice immunized with KLH[Ac-[Cys249]CVB4 P2c(28-50)-NH_2]

Graphics show a general increase of antibodies concentration, for each immunized mouse, against all the three peptides.

Group A mice, immunized with KLH[Ac-[Cys^{249}]*h*GAD65(250-273)-NH_2], and Group B mice, immunized with KLH[Ac-[Cys^{257}]*h*GAD67(258-281)-NH_2], showed high antibody titers against Ac-*h*GAD65(250-273) and Ac-*h*GAD67(258-281) while a low response against Ac-CVB4 P2c(25-50) peptide.

Group C mice, immunized with KLH[Ac-[Cys^{27}]CVB4 P2c(28-50)-NH_2], after the 3rd booster injection, shows an increase of titers against all the three antigens, especially Ac-*h*GAD65(250-273).

These preliminary data indicate the existence of a cross-reactivity showed by specific polyclonal anti-Ac-CVB4P2c(28-50)-NH_2 antibodies against our antigenic sequences derived from *h*GAD, representing an important step forward in the demonstration of the role of the Coxsackie virus B4 in the pathogenesis of T1D and LADA.

3. CONCLUSIONS

The role of environmental factors in the onset autoimmune diseases is an important topic nowadays. An increasing number of this kind of pathologies is indicated to be related to viral or bacterial infections. In particular, the correlation between Coxsackie virus infection and the onset of T1D has been insistently proposed and strongly investigated in the last decades. Despite this, it was not demonstrated to date and the molecular mechanisms which connect the infection with the development of anti-*h*GAD autoantibodies still remain unclear. Even more so in case of LADA patients, only recently distinguished from T2D ones.

Our novel strategy, based on the Chemical Reverse Approach, proposes to use the SPPS technique assisted by microwaves in order to synthesize antigenic peptide probes which mimic native proteins and to use them to deepen the interactions with specific autoantibodies and to characterize them. We also investigate the role of these peptide fragments as putative epitopes, in order to understand the molecular mechanisms these interactions are based on.

In this part of the work we proposed the synthesis, the characterization and the screening on T1D, LADA and NBD patients' sera of 3 antigen peptide probes which mimic respectively of the natives *h*GAD65 (Ac-hGAD65(250-273)-NH_2), *h*GAD67 (Ac-hGAD67(258-281)-NH_2) and CVB4 P2c (Ac-CVB4P2c(28-50)-NH_2). Using these probes, we were able to distinguish T1D and LADA patients from NBD, preliminary demonstrating the specific interaction of our products with autoantibodies characteristic of this pathological conditions and, furthermore, the nature of these fragments as possible epitopes involved in these interactions. Finally, the shown cross-reactivity of the T1D and LADA specific autoantibodies against all the 3 peptides in the 96% of the tested sera represents an important step forward in order to demonstrate the role of Coxsackie virus as mimetic of *h*GAD in the pathogenesis of T1D.

The results obtained on the animal model are further promising and seems to confirm the SP-ELISA ones.

CHAPTER TWO: SYNTHESIS OF A NATIVE ANTIMICROBIAL PEPTIDE FOR AUTOANTIBODY CHARACTERIZATION IN PSORIASIS AND ITS RELATED DISEASES

1. INTRODUCTION

1.1 Epidemiology of psoriasis

The human skin role is to act mainly as the first physical, but also biochemical and immunological barrier against many pathogenic insults. The physical protection is performed by the waterproof protein keratin contained in the stratum corneum; the biochemical protection is carried out by lipids, acids, hydrolytic enzymes and antimicrobial peptides (AMP). Finally, the immunological protection (skin immune system) is allowed by the presence of the humoral and cellular constituents of the immune system. [60] Normally, skin inflammatory responses occur rapidly and in a self-limiting way. Related diseases can occur in case of a dysregulation of any of the factors involved in these reactions. The inflammation can become chronic if this pathologic condition is not solved.

Psoriasis (PsO) is a common skin disease. It is characterized by an immune-mediated inflammatory condition that can degenerate affecting also joints. PsO was classified and recognized as a single disease in 1841. [61] In 2012, about the 2-3% of the world population was reported to be affected by PsO, in particular American and Canadian population. About 70-80% of patients show a mild form of the pathology, easily managed with common topical therapies. In 2013 the Executive Board of WHO proposed to classified PsO as a major global health

problem. [62] People in the range of age between 15-30 years and 50-60 years are the most at risk. [63] PsO is also sometimes associated with an increased risk of mortality and a high degree of morbidity. [64] Moreover, a relationship between this pathological condition and mental health diseases or has been reported. [65]

1.2 Psoriatic Arthritis

PsO is known to possibly degenerate in other kind of inflammations such as psoriatic arthritis (PsA). Among the 0.04% and the 1.2% of healthy patients can be affected by PsA, while between 25% and 34% of PsO patients can show its symptoms, confirming the connection between the two conditions. Finally, PsA is known to be able to further degenerate in an erosive and polyarticular disease, characterized by joint destruction which lead to a total loss of their functionality. Many clinical features of PsA are typical of the spondyloarthropathies family; for this reason, PsA was included in this group of diseases which comprehend also reactive arthritis, undifferentiated spondiloarthritis and so on.

PsA was described for the first time in 1973 by Moll and Wright, [66] who indicated 5 clinical subtypes of this pathology: symmetrical polyarthritis, arthritis mutilans, distal interphalangeal arthritis, spondylitis and asymmetrical oligoarthritis. Because of the difficulty to identify milder forms of PsA using these criteria and because of the subsequent classification of both distal interphalangeal arthritis and arthritis mutilans as separate diseases, [67] many other sets of classification and diagnostic criteria have been proposed in the following years. In 2006, a new classification method was indicated by the Classification Criteria for Psoriatic Arthritis (CASPAR) study group [68] and is still one of the most used today. Anyway, PsA can only be diagnosed based on the

phenotypical evidences because of the lack of any specific biomarkers, still unknown. [69]

In addition to the evident correlation with PsO, PsA is described as a polygenic autoimmune $CD8^+$- and T cell derived cytokines-mediated disease. Genetics plays a key role in the pathogenesis of this condition, as demonstrated by its strong heritability, but it is now clear how the onset of this complex illness is also strongly linked to environmental factors and immune mechanisms. [70]

1.3 Phenotypical manifestations of PsO

PsO and its related diseases are characterized by a huge spectrum of different phenotypical features. Generally, 5 main types of PsO are described: plaque PsO, or vulgaris, one of the most common (about 90% of all PsO cases); guttate PsO, or eruptive; inverse PsO, or intertriginous or flexural; pustular PsO, which can degenerate in palmoplantar pustulosis; erythrodermic PsO, the rarest one. Besides the inflammation, the typical symptoms and manifestations of PsO comprises a thickening of the skin layer, papillomatosis, hypogranulosis and new vessel formation which lead to an excessive vascularity of the lesions.

1.4 Different players in PsO pathogenesis

The high phenotypical variability of PsO suggests that different factors can play a key role in its pathogenesis and onset, such as the genetic predisposition, some environmental factors and several immunological aspects.

1.4.1 The genetics of PsO

The role of a specific genetic predisposition in the pathogenesis of PsO is well established. [71] Many loci and genes have been described to be strongly related to the onset of this pathology. The most representative are reported below.

a. PSORS1

   Psoriasis susceptibility 1 is the most important determinant known for PsO. It is represented by a 220-kb region of the MHC on the chromosome 6. [72] The role of PSORS1 in the onset of PsO was described for the first time around 1970. [73] This locus was associated to the major risk for PsO; anyway, 9 other loci outside of the MHC were subsequently gradually identified (from PSORS2 to PSORS10) to be related with this disease; [74]

b. IL-23/Th17

   Several studies showed the key role of a chronic inflammation Th17 cell-mediated and induced by the cytokine IL-23 in the pathogenesis of PsO.

1.4.2 Environmental factors

Several studies, especially on mono- and dizygotic twins, highlighted a multifactorial etiology of this disease, implicating also the involvement of some environmental factors, listed below. [75] [76] Anyway, the relationship between them and the onset of PsO still remains unclear.

a. Bacterial infections, in particular promoted by streptococci, were hypothesized to be related to some forms of PsO, such as guttate PsO; [77]
b. Traumatic physical events such as tattoos or surgery are known to be possibly responsible of chronic inflammation events; [78]
c. Specific medicines, smoke or alcohol.

1.4.3 Immunological aspects

Many different kinds of cells, and often several endogenous bioactive molecules directly or indirectly related to them, are involved in various biological mechanisms related to the triggering of an inflammatory event in case of their dysregulation. Some studies even advance the hypothesis about to describe PsO as a prolonged lesions-healing process. [79]

a. Dendritic cells (DC) are commonly considered as the connection between the innate and the adaptive immune system. They are known to act as antigen presenting cells inducing the respective T-cell response. [60] A correlation between both myeloid DC and plasmacytoid DC and the pathogenesis of PsO has been reported. [80] In particular, myeloid DC are described to be present in huge quantities in psoriatic skins. [81]
b. Langerhans cells (LC) are responsible of the expression of several molecules related with different roles. [82] In case of healthy skin, they result in an immature form. Their relationship with PsO still remain unclear.
c. Lymphocytes; in healthy conditions the level of dermal $CD4^+$ is higher compared to the in circulation one [83] and intraepidermal lymphocytes are estimated to be about 2% of the overall lymphocyte composition in

the skin. In psoriatic conditions, both compartments are characterized by an evidently increased level of $CD2^+$, $CD3^+$, $CD5^+$, CLA^+, while dermis and epidermis show abnormal levels respectively of $CD4^+$ and cytotoxic $CD8^+$. [84]

d. Keratinocytes (KC) are the major cellular component of the skin. They are responsible about the mechanical protection and the complete regulation of the immune response of the epidermis. [85] They are classified in the native immune system and they are characterized by a rapid but nonspecific mechanism of action. Like DC, they can act as antigen presenting cells. Depending on the differentiation or activation degree of KC, their role in psoriatic conditions compared with healthy ones can change. In healthy skins, a complete differentiation can be observed and KC provide for the expression of keratins, which are fundamental for the mechanical integrity of the epidermis and in order to counteract desiccation and infections (through the stimulation of the innate immune response). [86] In psoriatic skin, KC are not able to complete their differentiation leading to a dysregulation of their growth pathways. This causes a fast over-proliferation and an abnormally rapid maturation of these cells. Furthermore, differently to the normal condition, the incomplete differentiation results in a retention of the nuclei by the KC in the stratum corneum (Fig. 16).

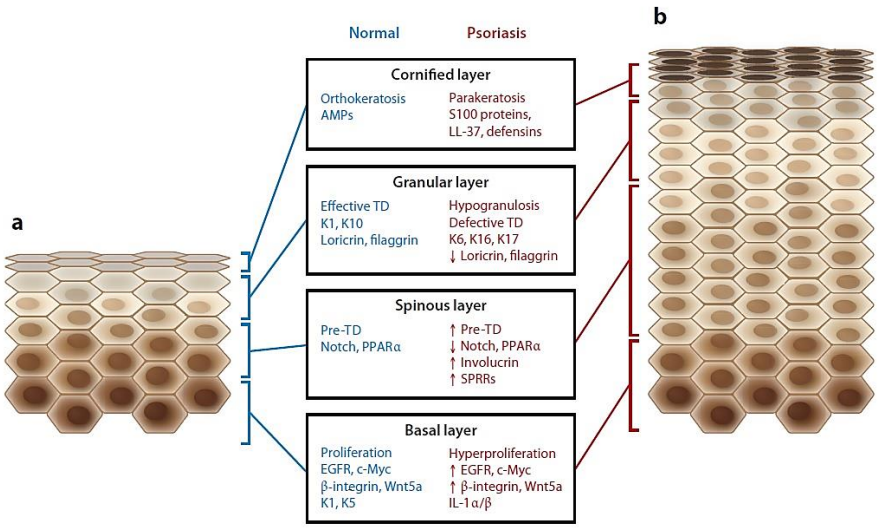


Figure 16 - Normal skin KC (a) compared with PsO skin KC (b) *[87]*

Finally, KC with IL-17A and IL-22 are responsible of the induction of AMPs [88] which were found in abnormal high levels in psoriatic skins but decreasing after antipsoriatic treatments. [89] The family of AMPs includes cationic cathelicidins (e.g., LL37), defensins and S100 proteins. [90] Recently, also myeloid DC have been reported to be related to a new activation pathways in PsO which involves LL37. According to this pathways, a self-DNA/LL37 complex is formed leading to an activation and a maturation of myeloid DC. This complex has been described in psoriatic skin and correlated with advanced stages of the pathology. [91]

Figure 17 shows a complete resume of all the adaptive and innate players associated with PsO.

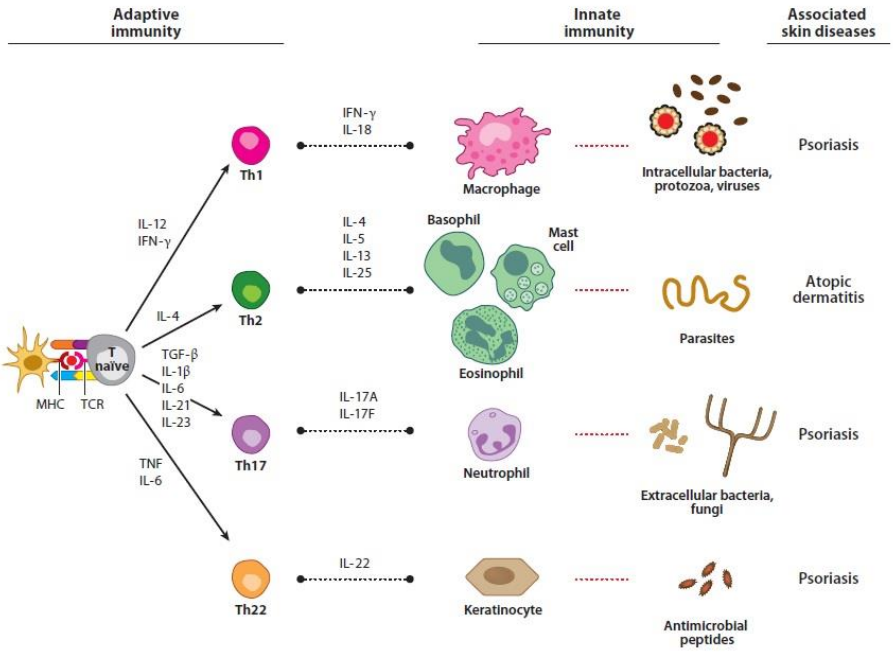


Figure 17 - Adaptive and innate immunity related to PsO *[92]*

1.5 Antimicrobial peptides

The human skin plays a fundamental protective role not only as a physical barrier, but also biochemically and immunologically. It is considered as the first line of defense against several type of environmental factors. The biochemical response is promoted by a family of small proteins or peptides (<100 amino acid residues) called antimicrobial peptides (AMPs) which form prevalently two main secondary structures: β-sheets in case of Cys-rich AMPs (because of the formation of disulfide bridges) and α-helices (the most common one) in case of Trp- and Pro-rich AMPs. [93] They are classified in the innate immune system and are able to detect and destroy many different pathogens with non-specific mechanisms but correctly differentiating between exogenous microorganisms

and endogenous cells. AMPs are believed to be one of the oldest defensive mechanisms developed during evolution and they are known to be able to act not only against pathogens like fungal, protozoal and bacteria, but also against viral infections. [94] [95] All the molecules belonging to the AMPs family show an amphipathic character which allow a disruptive interaction between them and the lipid membranes of bacteria cells, leading to their apoptosis. AMPs are generally cationic molecules; for this reason, their antimicrobial action is mainly directed against Gram(-) bacteria, but also functioning as a protection for neutral mammalian cell membranes. The dynamic expression of AMPs foresees both a constant production in the skin as a preventive protection mechanism and an increase of their biosynthesis induced by exogenous pathogens or by pro-inflammatory events. [96] Indeed, they were commonly correlated to several inflammatory pathologies such as PsO or many kinds of dermatitis. [97] Supporting this association, psoriatic skins are characterized by a surprisingly low incidence of infections because of an over-expression and a subsequent up-regulation of AMPs.

The following Table 5 summarize the main characteristics of the different AMPs classes. The 3 major ones (defensins, cathelicidins and the S100 protein family) are shortly described below.

AMP	Activity
hBD-2	Killing activity preferentially against Gram-negative bacteria like *E. coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Propionibacterium acnes* Induced by HPV Chemotactic activity on T cells and dendritic cells Involved in wound repair
hBD-3	Broad spectrum against Gram-negative and Gram-positive bacteria including MRSA, vancomycin-resistant *Enterococcus faecium* (VRE) Induced by HPV Chemotactic activity on dendritic cells, T cells and monocytes Promotion of wound healing
RNase 7	Antimicrobial activity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*P. acnes*, *S. aureus*, MRSA) and yeast (*Candida albicans*) Ribonucleolytic activity
Psoriasin (S100A7)	Active against *E. coli*, *P. acnes* (enhanced activity in synergy with LL-37) Chemotactic for CD4+ T lymphocytes and neutrophils
Human cathelicidin LL-37	Antimicrobial (e.g. against *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, *Klebsiella pneumoniae*, *P. acnes*), antifungal (*C. albicans*) and antiviral (HIV-1) activity Chemotactic activity for neutrophils, monocytes and T cells Mediator of inflammation Promotion of re-epithelialization of healing skin and angiogenesis
Dermcidin	Active against *E. coli*, *E. faecalis*, *S. aureus*, and *C. albicans*

Table 5 - Summary of the main characteristics of AMPs *[98]*

a. Defensins are considered one of the most studied group of AMPs. These small molecules are characterized by the presence of a high number of Cys residues and a β-hairpin, allowed by disulfide bonds. [99] The defensins family is composed by 3 subfamilies, the α-, β-, and circular θ-defensins. This class of AMPs was described for the first time at the end of the 20th century. [100] They show a huge spectrum of activity against several pathogens, such as E. coli, P. aeruginosa and C. albicans. Defensins are also reported to be related to the stimulation of KC in order to induce the production of many pro-inflammatory molecules. [101] Furthermore, defensins showed also a cytotoxic effect on cancer cells. [102]
b. S100 protein family is composed by Ca^{2+} binding proteins such as psoriasin (or S100A7). It was described for the first time in psoriatic skin [103]; in healthy conditions S100A7 is normally barely expressed in

epithelial cells but its level is reported to growth in case of infections or other triggers. [104] It is particularly active against E. coli.

c. Cathelicidins; Some studies described this class of AMPs for the first time between the end of the 20th and the beginning of the 21th century [105] as antimicrobial peptides which derived by an enzymatic cleavage from an inactive precursor. It is a pro-form characterized by an N-terminal cathelin domain and a C-terminal portion which shows antimicrobial activity. [106] Human beings show only one type of cathelicidin (*h*CAP18), precursor of the antimicrobial peptide *h*LL37.

1.6 The *h*LL37 peptide

The human genome includes a single cathelicidin gene called CAMP on the chromosome 3p21.3, [107] which expresses the only human cathelicidin *h*CAP18 in epithelial cells. *h*CAP18 is a 170-residue protein composed by a 30-residue signal region on the N-terminus, a 103-residue cathelin domain (corresponding to the pro-region) and the 37-residue antimicrobial LL37 (the C-terminus) (Fig. 18). The signal region and the cathelin domain form together the conserved region and the entire *h*CAP18 is usually indicated as the pro-form of the bioactive *h*LL37.

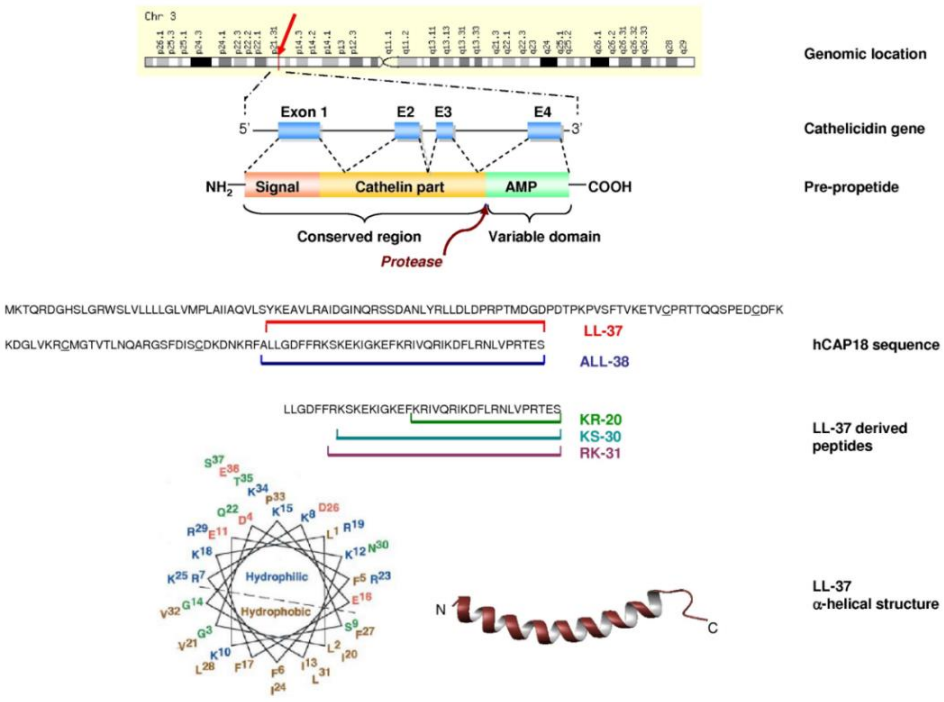


Figure 18 - The *h*LL37 production and structure *[108]*

Vitamin D is reported to be an important endogenous factor of the induction of *h*CAP18 expression [109] as well as a promoter of antimicrobial defense in the epidermis. [110] After the expression and the secretion of the entire protein, performed by eccrine glands, the 37-residue C-terminal portion of *h*CAP18 is extracellularly cleaved by a serine protease belonging to the kallikrein family [111]. After the initial release of *h*LL37, the enzyme continues to perform proteolytic cuts producing shorter fragments with an adjuvant role in the antimicrobial activity. The evident function of the pro-form is to maintain the antimicrobial part inactive until needed; however, because of the not negligible conservation state of the conserved region after the cleavage of *h*LL37, the effective role of the pro-form is still under in depth investigation. [112] Indeed,

*h*LL37 shows its biological activity only after the proteolytic cut from the conserved region; this is considered an important regulatory mechanism of its release.

The *h*LL37 amino acid sequence is characterized by more alkaline residues (5 Arg, 6 Lys) than acid ones (3 Glu, 2 Asp). This determines an evident positive charge of the peptide at physiological conditions which makes the antimicrobial action against Gram(-) bacteria easier. As all the typical AMPs, also *h*LL37 shows an amphipathic character which allows the interaction both with aqueous solutions and the lipid membrane of bacteria. [113]

*h*LL37 is reported to be unfolded in aqueous solutions, assuming instead an α-helix conformation at physiological levels of certain anions. The α-helix is in fact allowed by several intramolecular weak interactions (e.g., salt bridges) spaced i+3 or i+4. [114] Moreover, this secondary structure involves a one-side positioning of the major part of the hydrophobic residues of the sequence (Fig. 18), conferring it the ability to interact with and penetrate the phospholipid bilayer of cell membrane of bacteria in order to create transmembrane pores and induce cell death (Fig. 19). [115] Because of these features, the antimicrobial activity of *h*LL37 is defined as medium-dependent. Differently from bacterial ones, cell membranes typical of mammals are characterized by an association with cholesterol, which protects them from the pore-forming effect of *h*LL37, except for in case of abnormally high concentrations of the peptide. [116]

When the weak interactions, such as H and electrostatic ones in addition to salt bridges, became excessive, an oligomerization of *h*LL37 is observed. This phenomenon reduces the ability of the peptide to interact with the cell membranes, partially hiding its hydrophobic part and increasing the rigidity of the structure (Fig. 19).

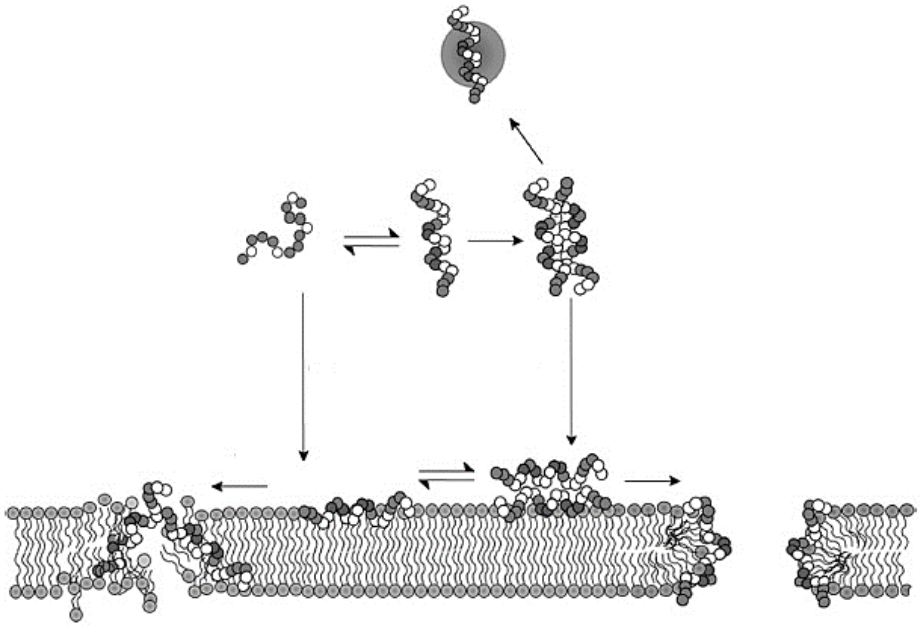


Figure 19 - Antibacterial *h*LL37 function *[117]*

Gram(-) bacteria are characterized also by an external lipopolysaccharide cell wall, composed by an oligosaccharide moiety bound to lipid A. Taking advantage of electrostatic interactions, *h*LL37 approaches and efficiently crosses the lipopolysaccharide monolayer and is than translocated to the cytoplasmic membrane. [118] Indeed, susceptibility of bacteria to *h*LL37 can be drastically reduced through lipid A modifications. [119]

Besides its antimicrobial and anti-inflammatory action, *h*LL37 is associated to several others functions related to the immunomodulation and the induction of the secretion of pro-inflammatory signal molecules; for this reason, it is recently being described as a *factotum* peptide. [120] Furthermore, its ability to create

pores in cell membranes has been hypothesized to be associated with release mechanisms of molecules or ions through that membranes. [121]

The following Figure 20 summarizes all the pro- and anti- inflammatory activities associated to *h*LL37.

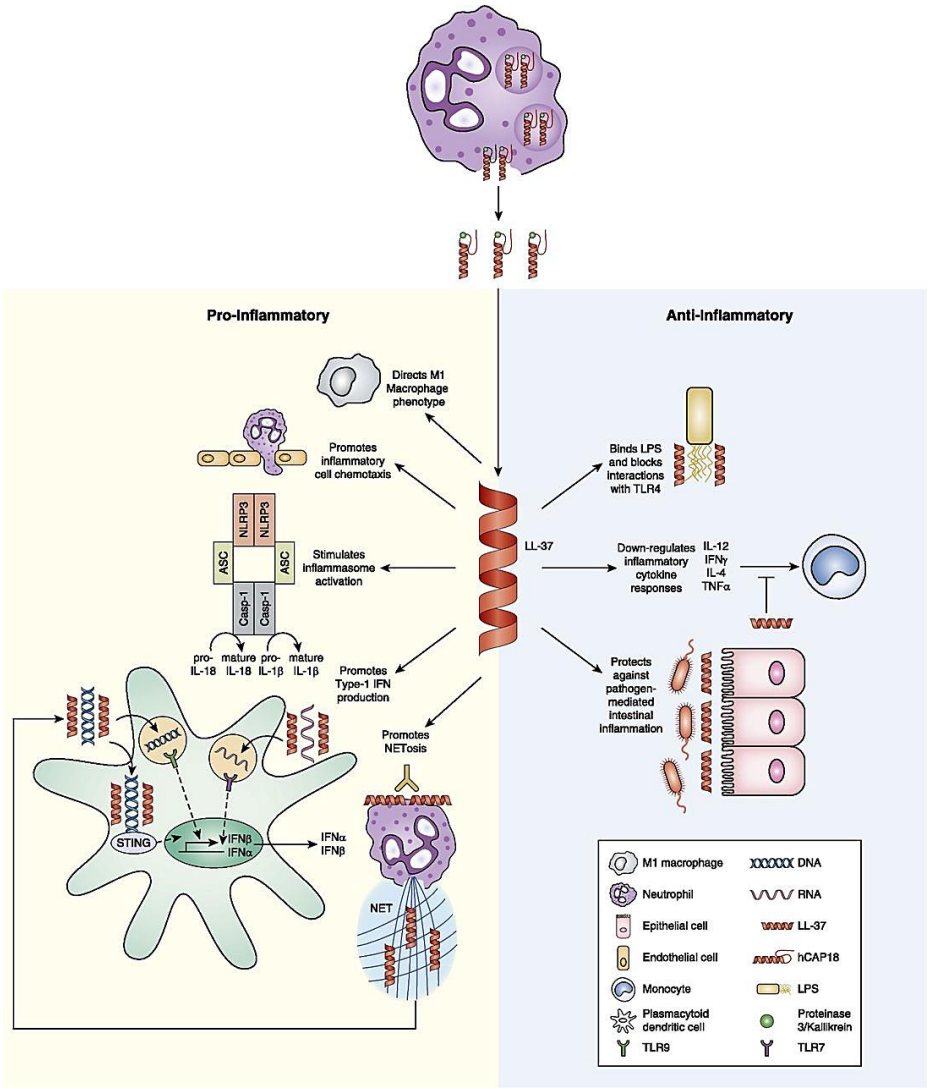


Figure 20 - Summary of *h*LL37 abilities *[122]*

*h*LL37 is over-expressed in PsO plaques. [123] Moreover, *h*LL37 is described to generate complexes with self-DNA transforming it into a powerful trigger in the stimulation of DC involved in inflammation mechanisms. [91] For these reasons, this peptide has been suggested to play a key role as a putative autoantigen both in PsO and its related diseases pathogenesis. [124] On the other side, epidermal *h*LL37 is also reported to bind cytosolic DNA promoting an anti-inflammatory effect. Which effect between the pro- and the anti-inflammatory is predominant still remain unclear.

1.7 Objectives of the project

The involvement of *h*LL37 is now widely accepted in the pathogenesis and onset of PsO and its related diseases because of the over-expression of this AMP in psoriatic skins, but its role as autoantigen still remain unclear. Moreover, its antimicrobial nature also strongly suggests the implication of environmental factors, in particular bacterial infections, in the triggering of this pathology. Some studies investigated and reported the T-cell response against LL37 in PsO. [124]

Following the Chemical Reverse Approach strategy, in this part of the project we decided to synthetize, purify and characterize the native *h*LL37 sequence through the SPPS technique assisted by microwaves in order to use it as an antigenic probe to perform ELISA experiments on PsO, PsA and similar arthritis diseases, including rheumatoid arthritis and RA, patients' sera. Sera were collected by the research group of Professor Selmi of the Division of Rheumatology and Clinical Immunology Humanitas Research of the Hospital Rozzano in Milan. Furthermore, we synthetized and characterized four 12-residue and one 13-residue fragments derived from the entire *h*LL37 peptide in

order to perform an epitope-mapping of the peptide and to better understand its role as epitope for eventual specific autoantibodies.

2. RESULTS AND DISCUSSION

2.1 Synthesis, purification and characterization of the native *h*LL37

The synthesis procedure of the native *h*LL37 through the Fmoc/tBu SPPS strategy was entirely performed at the Interdepartmental Laboratory of Peptide & Protein Chemistry & Biology (PeptLab) in the Chemistry Department "Ugo Schiff" of the University of Florence, Italy, following the General procedure for SPPS assisted by microwaves. The complete *h*LL37 sequence is:

- **Human LL37** (or *h*CAP18(134-170)):
  $_{1}$LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES$_{37}$

The synthesis was performed starting from a Cl-MPA ProTide (LL) resin type (0.15 mmol/g) (Fig. 21). Each coupling was performed using 2.5 ml of a 0.2 M amino acid solution in DMF, 1 ml of a 0.5 M activator solution (N,N'-diisopropylcarbodiimide, DIC) In DMF and 0.5 ml of a 1 M activator base solution (ethyl cyano(hydroxyimino)acetate, OxymaPure) in DMF.

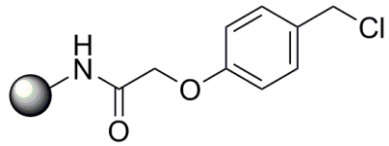


Figure 21 – Cl-MPA ProTide (LL) resin

Each deprotection step was carried out using a 20% piperidine in DMF solution; the resin was washed with DMF after each coupling and deprotection steps.

The cleavage of the peptide from the resin and the deprotection of all the side chains of the amino acids were carried out by exposing the resin to a TFA 95% solution (TFA/H_2O/TIS 95:2.5:2.5) for 3h at RT, as described in the General procedure for cleavage and micro-cleavage. H_2O and triisopropylsilane (TIS) were used as scavengers in order to avoid secondary reactions during the cleavage phase. The crude peptide was purified through 2 purification steps: a pre-purification or desalting step and a semi-preparative purification using a RP-HPLC, following the General procedure for peptide purification and characterization. It was finally characterized via ESI-MS. All the details about the characterization of the product are reported in Table 6.

PEPTIDE	**PURITY DEGREE**	**GRADIENT (0.6 ml/min)**	**Rt (min)**	**ESI-MS (m/z) $(M+3H)^{3+}$ Found (calculated)**
***h*LL37**	>99%	30%-70% B	2.63[a]	1499 (1499)

Solvents used: A 0.1% TFA in H_2O; B 0.1% TFA in CH_3CN 84%

a: Phenomenex Jupiter C18 (5 lm, 2504.6 mm), 0.6 mL/min

Table 6 - *h*LL37 characterization

2.2 SP-ELISA experiments

The obtained *h*LL37 peptide was tested in Solid Phase Enzyme-Linked ImmunoSorbent Assays (SP-ELISA) initially using PsA, RA and NBD patients' sera, obtained in collaboration with Professor Selmi of the Division of Rheumatology and Clinical Immunology Humanitas Research of the Hospital Rozzano in Milan, in order to verify the presence in them of specific autoantibodies against this sequence and to investigate the role of this antimicrobial endogenous peptide as autoantigen in the pathogenesis of PsO and related diseases.

All the SP-ELISA experiments were performed in collaboration with Ms. Lucrezia Riley and Dr. Feliciana Real in the Department NeuroFarBa, Section of Pharmaceutical Sciences and Nutraceutics of the University of Florence, following the General procedure for SP-ELISA experiments.

The *h*LL37 sequence was tested on 35 PsA sera, 10 RA sera and 34 NBD sera. What is to underline is that all the experiments were performed blindly, i.e. we didn't have any kind of information about the obtained sera, except for the code ICHx assigned to each one of them. Data distribution for anti-*h*LL37 IgM antibody titers (Fig. 22) are reported hereafter. Anti-*h*LL37 IgG antibody titers were negligible and are then not reported here.

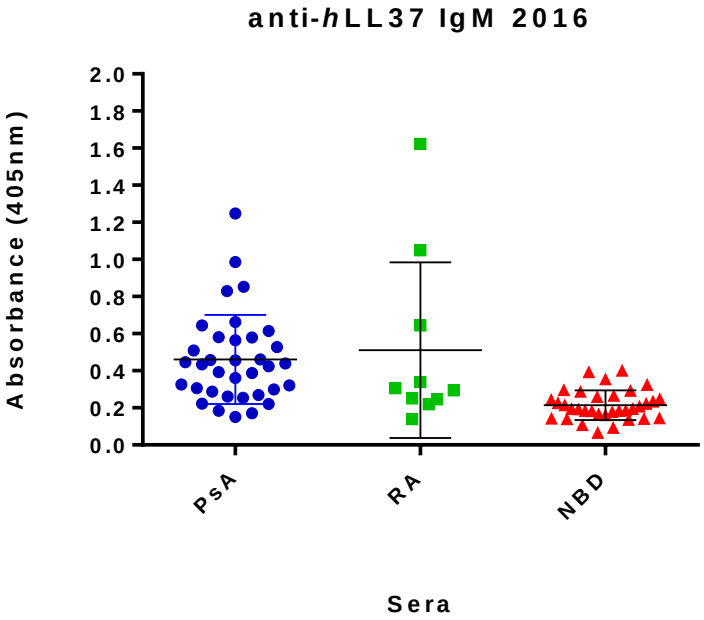


Figure 22 - anti-*h*LL37 IgM titers (2016)

From data reported in the previous graphic it appears that using the native sequence of *h*LL37 on both PsA and RA patients' sera we were able to identify higher titers of anti-*h*LL37 IgM antibodies compared with NBD. Furthermore, the 3 RA sera which show higher titers were found to belong to patients in whom the disease has been going on for longer.

Statistically, data were analyzed using a D'Agostino-Parson test to verify their distribution. The results of this test are reported in the following Table 7.

	P value	**Passed normality test (alpha=0.05)?**
anti-*h*LL37 IgM	<0.0001	No

Table 7 - D'Agostino-Parson test results

Data distribution is not Gaussian. For this reason, data were subsequently analyzed using a non-parametric test (Mann-Whitney). Each serum was tested two times in different days. Each showed data derived from an average between the two measures, respectively indicated as IgM1 and IgM2.

In the following Table 8 is reported the Mann-Whitney analysis comparing the two repetitions of the measurement. They result significantly different; this parameter indicates a non-optimal reproducibility of the obtained data.

	P value	**Significantly different? (P<0.05)**
anti-*h*LL37 IgM1 vs. IgM2	0.0126	Yes

Table 8 - Mann-Whitney comparison between the two measurements

Finally, titers obtained from PsA and RA patients' sera were respectively compared with the NBD ones, in order to verify the presence of a significant difference between the two kinds of patients and NBD (Table 9).

	P value	**Significantly different? (P<0.05)**
anti-*h*LL37 IgM		
PsA vs NBD	<0.0001	Yes
RA vs NBD	0.0061	Yes

Table 9 - Mann-Whitney comparison between PsA/RA and NBD

The showed results highlight some important considerations:

- The *h*LL37 sequence is able to recognize specific IgM autoantibodies in PsA and RA patients' sera, suggesting its involvement as autoantigen in these diseases. Most importantly, this peptide is able to discriminate them from NBD sera;
- In addition to the autoimmune T response to *h*LL37 already described in the literature, our experiments highlighted also the presence of specific autoantibodies in PsO related diseases;

3. CONCLUSIONS

The T-cell autoimmune response against *h*LL37 have been already reported, while its role as autoantigen in PsO, PsA and related diseases is partially still under investigation. Following the concept of the Chemical Reverse Approach to use of synthetic antigenic peptide probes as antigens in immunoenzymatic assays, we were able to investigate also the presence of specific autoantibodies in autoimmune or autoimmune-related diseases.

We then performed the synthesis for the endogenous peptide *h*LL37 in order to use it as antigenic probe in SP-ELISA experiments to search for specific autoantibodies in PsO, PsA and RA patients' sera. Our preliminary results are strongly promising and supported by our ability to correctly differentiate the majority of PsA and RA sera from NBD before to obtain any kind of information about them; moreover, all the super-positive sera were found to belong to patients in whom the disease is definitely established.

A further confirmation of these results could demonstrate the critical role of the endogenous antimicrobial peptide *h*LL37 as autoantigen in PsO and its related diseases such as PsA and RA. Moreover, the antimicrobial nature of this peptide could be a key feature to in depth study the role of environmental factors (e.g. bacterial infections) in the pathogenesis of these diseases.

CHAPTER THREE: SYNTHESIS AND CHARACTERIZATION OF A NOVEL PEPTIDE EPITOPE FROM α-GALACTOSIDASE A INVOLVED IN FABRY DISEASE

1. INTRODUCTION

1.1 Pathophysiology of Fabry Disease

1.1.1 Lysosomal Storage Diseases

The group of Lysosomal Storage Diseases (LSDs) is composed by about 50 rare metabolic pathologies characterized by a deficiency of a specific endogenous lysosomal enzyme primarily involved in the degradation of a well-defined lysosomal substrate. [125] LSDs were firstly described at the end of 1800; [126] nowadays four main diseases belonging to this group are commonly reported: Mucopolysaccharidoses (MPS), Gaucher Disease (GD), Pompe Disease (PD) and Fabry Disease (FD). They are considered one of the most studied groups of pathologies, characterized by an estimated frequency of 1/7500 live births. [127]

Eukaryotic cells catabolize endogenous and exogenous macromolecules through the lysosomal system, an intra-cellular pathway which degrades these big molecules and handles their monomeric metabolites. [128] Over 50 different lysosomal acid hydrolase enzymes have been described to be contained in each lysosome. These enzymes catalyze the hydrolytic digestion of several kind of biomolecules (e.g., DNA, lipids etc.) working only in a pH range between 3.5 and

5.5, in contrast with the neutral pH typical of the external cytosol. This characteristic is fundamental as security control in case of damage of the lysosomal membrane, in order to avoid unwanted degradations of the cytosol content. [129] The acid conditions required in the organelle are maintained through a proton pump mechanism which transfers cytosolic H^+ inside the lysosome. [130]

A dysregulation, caused by genetic defections or mutations, of any function related to a lysosomal component lead to a total lack, or only partial, degradation of the cited macromolecules and a subsequent accumulation of them in the organelle. [131] This accumulation is described to be the trigger for LSDs (Fig. 23).

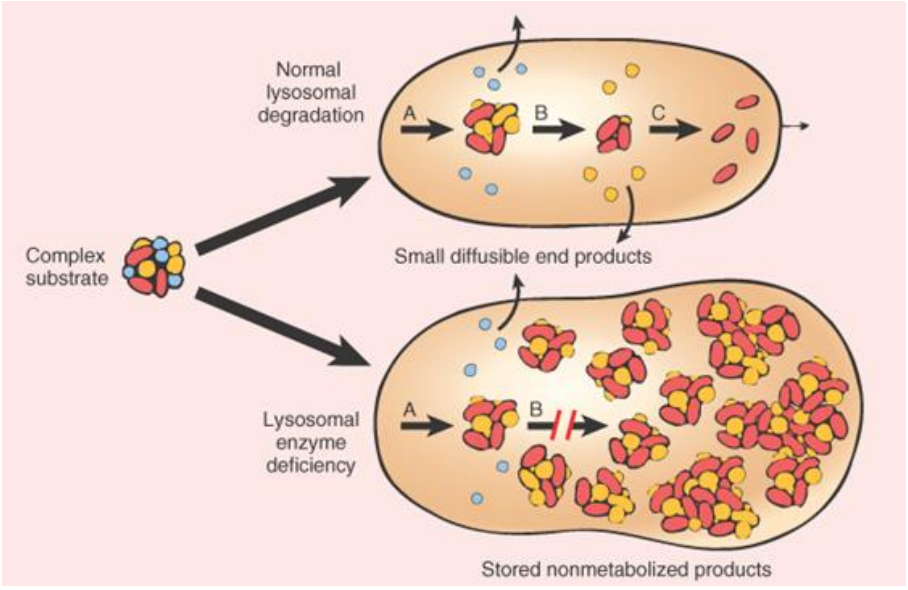


Figure 23 - Lysosomal Storage Diseases trigger

LSDs show a huge and various clinical phenotype related to several factors, including the chemical nature of the accumulated substance, the entity of the accumulation etc. Commonly, LSDs are classified depending on the chemical features of the stored molecule.

Because of their low therapeutic efficiency, many of the common treatments for LSDs, such as plasma infusions, enzyme intravenous injections and cell implantations, have been replaced by more recent therapy techniques. In particular, the Enzyme Replacement Therapy (ERT), the Substrate Reduction Therapy (SRT), the Chemical Chaperone Therapy (CCT), the Gene Therapy (GT), the Residual Enzyme Activation (REA) and the Gene Promoter Activation (GPA) are the most commonly used nowadays.

ERT is defined as a biomedical treatment based on the concept of compensating for the deficiency or lack of the lysosomal enzyme by intravenously injecting the same enzyme, produced by recombinant technique, which then results to be able to reach the intra-lysosomal environment. [132]

1.1.2 The function of the α-Galactosidase A enzyme

The α-Galactosidase A enzyme (α-Gal A) (Fig. 24) is an about 100 kDa homodimeric glycoprotein composed by 429 amino acid residues, including a 31 amino acid signal sequence which is removed prior to the transfer of the enzyme into the organelle. It is encoded by the 1.45 kb α-Gal A gene on the Xq22.1 region of the X chromosome. The homodimeric structure is made up of two monomeric 46 kDa polypeptide chains; they are synthetized as 50 kDa precursors, which are converted to the 46 kDa mature form and then undergo a series of modifications, such as N-linked post translational carbohydrate attachment and cleavage of the

signal sequence, performed in several districts of the organism, before to penetrate the lysosome through the mannose-6-phosphate (M6P) pathway.

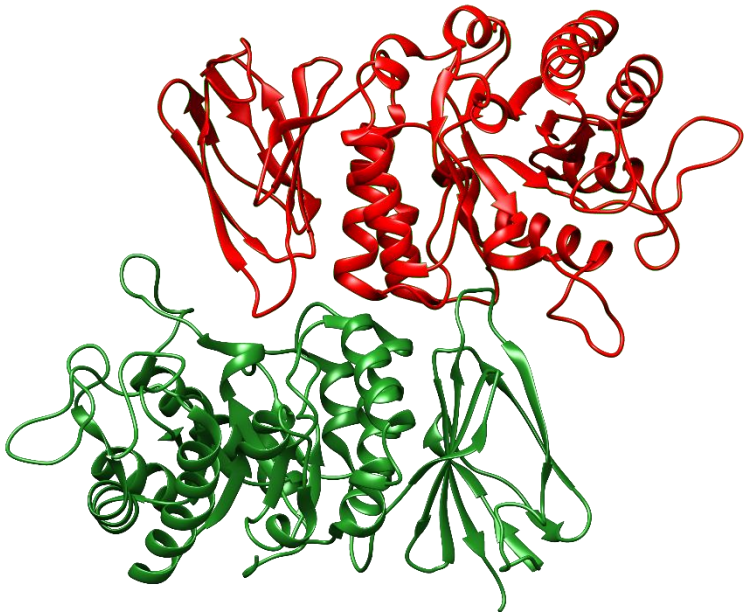
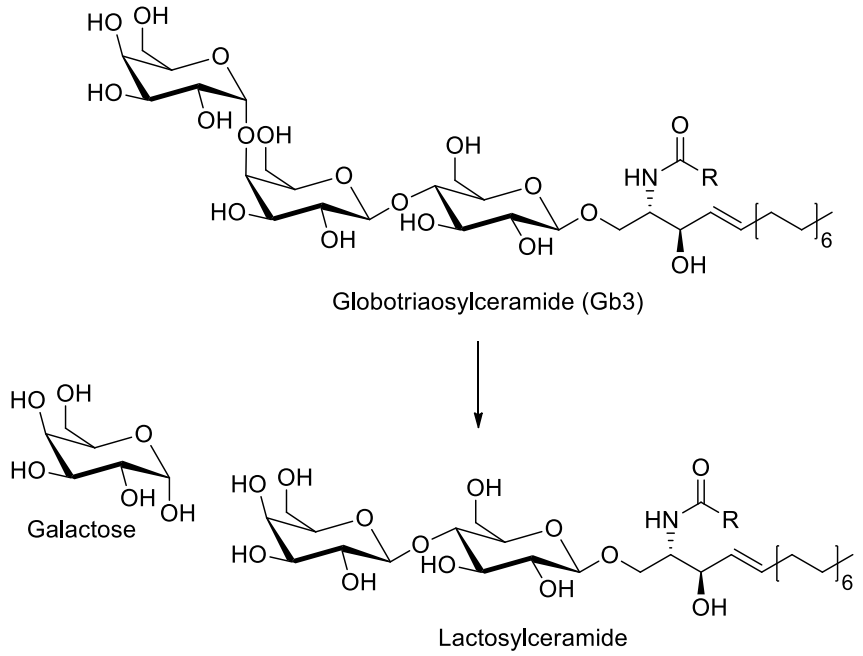


Figure 24 - 3D structure of the homodimeric human α-Gal A (PDB: 1R46)

Each monomer is constituted by two domains, one of which is represented by an $(\alpha/\beta)_8$ barrel motif which include the active site. [133] Moreover, each monomer is characterized by five disulfide bridges and four Asn residues available for possible N-glycosylation modification. [134] Finally, two Asp residues are supposed to be involved in the catalytic mechanism. The two active sites seem not to cooperate with each other, being reported to be about 50 Å distant also in the active form of the enzyme.

The human α-Gal A is defined as a ceramide-trihexosidase enzyme which catalyzes the hydrolytic degradation of the α-galactosyl fraction of several

oligosaccharides, glycoproteins and, especially, glycosphingolipids in the lisosome. [135] The conversion of the glycosphingolipid globotriaosylceramide (Gb3) in galactose and lactosylceramide (Gb2) is considered the major natural mechanism of action for α-Gal A (Scheme 4) and Gb3 is reported to be the most important lysosomal accumulated product in Fabry Disease.



Scheme 4 - The major natural mechanism of action of α-Gal A

1.2 Triggers in FD

FD is the second most common LSD after GD. It was described for the first time by Anderson and Fabry in 1898 [136] as an "angiokeratoma corporis diffusum", or red-purple maculopapular skin lesions typical of this pathology. FD is now defined as a heterogeneous, genetically hereditary, progressive, x-linked

pathology. It is triggered by an accumulation of the endogenous glycosphingolipid Gb3 in lysosomal space because of a deficiency of the α-Gal A enzyme. This accumulation causes high levels of the substrate in blood vessels, skin, hearth and kidney, leading to organ dysfunctions and subsequently a cascade of many different clinical symptoms, associated to FD, such as angiokeratoma, pain, cardiac arrhythmias, transient ischemic attacks and stroke, impaired renal function, gastrointestinal disturbances and neuropathies. Patients affected by FD commonly face premature death around 15-20 years old, later in males compared to females. [137]

1.2.1 Genetic factors

FD is genetically strongly related to several inherited mutations on the α-Gal A gene in the Xq22.1 region of the X-chromosome. 429 mutations have been described for this gene connected to the pathogenesis of FD. 306 are point mutations (missense, nonsense and splice sites), 115 are short-length rearrangements and 8 are gross rearrangements. [138] The α-Gal A gene is composed by seven exons, of which exons 3, 5 and 6 bring about a half of the 306 described point mutations. In depth studies about the 3D structure of the enzyme are fundamental in order to better understand its molecular mechanism and its correlation with the different mutations. [139] In particular, missense mutations determine the smallest possible structural modifications on the enzyme sequence by replace only one amino acid residue. These modifications have been divided in three groups:

a. Mutations that implies the substitution of one amino acid residue belonging to the active site of the enzyme and/or fundamental for the 3D structure of the molecule;

b. Mutations which replace one amino acid residue far from the active site but however directly implicated in the folding and the stability of the enzyme;
c. Mutations which don't belong to either of the previous categories but negatively affect the biologic function of the enzyme in any case.

Because of the existence of these mutations, a complete and exhaustive comprehension of the structure-activity relationship for α-Gal A could lead to the development of novel and perfected therapy techniques for FD.

1.3 Treatment: Enzyme Replacement Therapy

ERT was conceptually proposed as a treatment strategy by De Duve in 1964. Recently, molecular biology innovations allowed the production of recombinant enzymes which can be widely used in ERT. Specific Chinese hamster ovary (CHO) cells and human fibroblasts have been described to be able to overexpress the recombinant α-Gal A. [140]

Initially, the enzyme was isolated and purified from intestinal and placental tissues or from human plasma. After a complete sequencing and structural characterization of this protein, the recombinant technique was optimized. Two principal pharmaceutical formulations are currently commercially available: "agalsidase alfa" (Replagal®, Shire) and "agalsidase beta" (Fabrazyme®, Genzyme) (Fig. 25). The first one is produced in human fibroblasts, the second one in CHO cells. There is no difference between the two enzymes apart for the N-linked glycosylation pattern, due to the different kind of cells used for the expression.

Figure 25 - Agalsidase alfa from Shire Pharmaceuticals Ireland Limited and beta from Genzyme Ireland Ltd

1.3.1 Complications of ERT

An important limitation of the common treatment techniques for LSDs, in particular ERT, is represented by the frequent adverse immune reactions observed in male patients after these therapies. Moreover, the consequences of these responses on the onset of the pathology still remain unclear. [141] Both the formulations of the recombinant α-Gal A showed before are responsible for the formation of specific IgE antibodies, commonly related to symptoms typical of allergic reactions, and IgG antibodies, which directly interact with the protein by inhibiting its catabolic function and cancelling or drastically reducing its therapeutic effect. [142] Female patients are not reported to develop appreciable titers of anti-α-Gal A antibodies, while about the 95% of males commonly show the described adverse immune responses already after few months from the treatment. At the moment, immuno-modulating or immuno-

suppressive approaches are the only strategies able to temporary or variably stem the antibody formation. [143]

1.4 Objectives of the project

Because of its evident efficiency, ERT is now considered the major therapeutic strategy for FD. Despite its not negligible advantages, the adverse immune reactions against the recombinant α-Gal A and the subsequent from mild to severe side effects described in the majority of the male patients represent an important limitation of this approach.

The molecular mechanisms the interaction between the showed antibodies and the enzyme is based on still remain unclear. For this reason, an in depth structural study of α-Gal A in order to extend the comprehension of its role as antigen and subsequently to develop alternative or complementary therapeutic strategies to ERT is fundamental.

In this part of the project, performed in collaboration with the research group of Professor Przybylski from the Steinbeis Center for Biopolymer Analysis and Biomedical Mass Spectrometry in Germany, the identification, the synthesis and the structural-affinity characterization of a novel peptide derived from the native α-Gal A is proposed. The putative nature of this part of the α-Gal A as epitope specifically recognized by antibodies characteristic of FD makes it an excellent candidate for innovative therapeutic strategies in the perspective to avoid the adverse immune reaction or to develop some alternative approaches, such as plasmapheresis. Several techniques were used, such as MS, SPPS and immune-analytical characterization of antigen-antibody interactions via SPR.

2. RESULTS AND DISCUSSION

2.1 Identification of the putative epitope fraction of α-Gal A

The characterization of the primary structure of the α-Gal A was performed by the research group of Professor Przybylski through the Proteolytic Excision and Extraction Mass Spectrometry (ProtEx-MS) technique, which consists of an in solution proteolytic digestion of the commercial recombinant protein and a subsequent peptide mapping by ESI-MS. After an affinity purification step of the obtained fragments through a sepharose-immobilized commercial anti-α-Gal A monoclonal antibody and subsequent MS identification and SPR biosensor affinity determination, the α-Gal(309-332) sequence was identified as best candidate to represent the putative epitope in the native enzyme (Fig. 26).

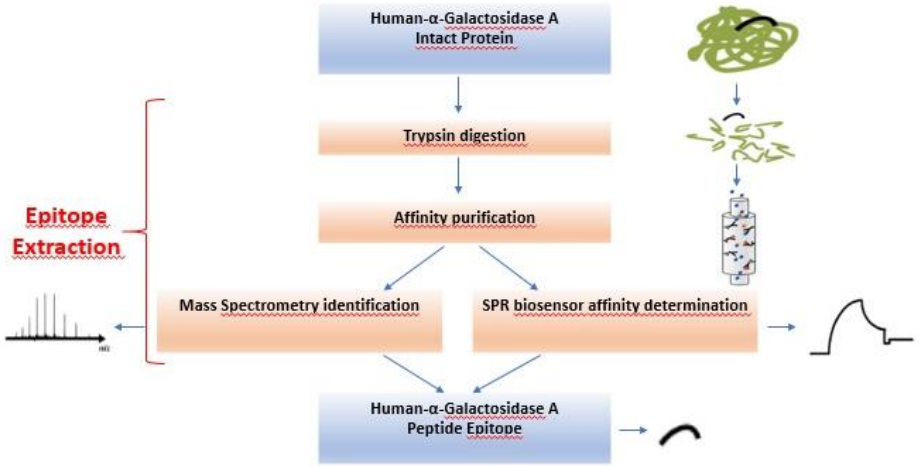
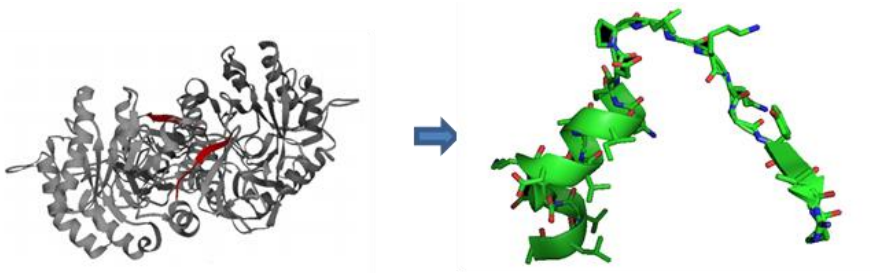


Figure 26 - Identification of the putative epitope fraction of α-Gal A

Figure 27 shows the selected peptide inside the native protein amino acid sequence (a) and its position in the 3D structure of the enzyme plus its peptide folding (b).

```
..1 MQLRNPELHL GCALALRFLA LVSWDIPGAR ALDNGLARTP TMGWLHWERF .50
.51 MCNLDCQEEP DSCISEKLFM EMAELMVSEG WKDAGYEYLC IDDCWMAPQR 100
101 DSEGRLQADP QRFPHGIRQL ANYVHSKGLK LGIYADVGNK TCAGFPGSFG 150
151 YYDIDAQTFA DWGVDLLKFD GCYCDSLENL ADGYKHMSLA LNRTGRSIVY 200
201 SCEWPLYMWP FQKPNYTEIR QYCNHWRNFA DIDDSWKSIK SILDWTSFNQ 250
251 ERIVDVAGPG GWNDPDMLVI GNFGLSWNQQ VTQMALWAIM AAPLFMSNDL 300
301 RHISPQAKAL LQDKDVIAIN QDPLGKQGYQ LRQGDNFEVW ERPLSGLAWA 350
351 VAMINRQEIG GPRSYTIAVA SLGKGVACNP ACFITQLLPV KRKLGFYEWT 400
401 SRLRSHINPT GTVLLQLENT MQMSLKDLL
```

(a)



(b)

Figure 27 - (a) α-Gal(309-332) in the native α-Gal A complete sequence (b) α-Gal(309-332) position (highlighted in red) in the α-Gal A 3D structure and the α-Gal(309-332) peptide folding

2.2 Synthesis, purification and characterization of α-Gal(309-322) and its derived overlapped and sequential fragments

The synthesis procedure for the entire epitope peptide sequence α-Gal(309-332) was performed using the Fmoc/tBu Solid Phase Peptide Synthesis (SPPS) strategy

assisted by microwaves, performed with the automatic synthesizer Liberty Blue (CEM). We decided to perform also the synthesis processes for three 12-aa overlapping peptides (α-Gal(309-320), α-Gal(315-326), α-Gal(321-332)) and three 8-aa sequential peptides (α-Gal(309-316), α-Gal(317-324), α-Gal(325-332)) deriving from the selected α-Gal(309-332) in order to perform an epitope mapping. All the syntheses were performed at the Interdepartmental Laboratory of Peptide & Protein Chemistry & Biology (PeptLab) in the Chemistry Department "Ugo Schiff" of the University of Florence, Italy, following the General procedure for SPPS assisted by microwaves, in collaboration with Mr. Fabio Borri. Fmoc-Arg(Pbf)-Wang resin (0.27 mmol/g), Fmoc-Asn(Trt)-Wang resin (0.6 mmol/g), Fmoc-Lys(Boc)-Wang resin (0.7 mmol/g), Fmoc-Val-Wang resin (0.74 mmol/g) and Fmoc-Leu-Wang resin (0.65 mmol/g) from Matrix Innovation (Quebec, CND) were used.

Each coupling was performed using 2.5 ml of a 0.2 M amino acid solution in DMF, 1 ml of a 0.5 M activator solution (N,N'-diisopropylcarbodiimide, DIC) in DMF and 0.5 ml of a 1 M activator base solution (ethyl cyano(hydroxyimino)acetate, OxymaPure) in DMF.

The cleavage reactions from the respective resin and the deprotection of all the side chains of the amino acids were carried out by exposing the resin to a TFA 95% solution (TFA/H_2O/TIS 95:2.5:2.5) for 3h at RT, following the General procedure for cleavage and micro-cleavage. H_2O and triisopropylsilane (TIS) were used as scavengers in order to avoid secondary reactions during the cleavage phase. The seven obtained products are represented in Figure 28.

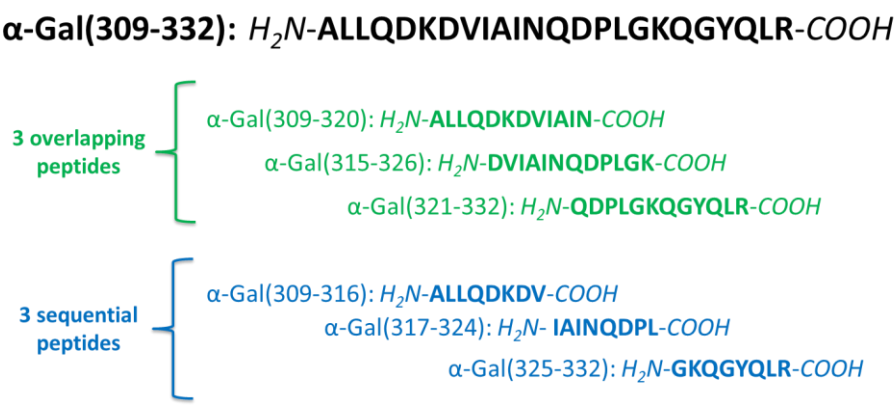


Figure 28 - The α-Gal(309-332) sequence and the 3 overlapping and 3 sequential fragments

All the crude peptides were purified through 2 purification steps: a pre-purification or desalting step and a semi-preparative purification using a RP-HPLC, following the General procedure for peptide purification and characterization. They were finally characterized via ESI-MS.

In the following Table 10 all the details about the characterization of the products are reported.

PEPTIDE	PURITY DEGREE	GRADIENT (0.6 ml/min)	Rt (min)	ESI-MS (m/z) Found (Calculated)
α-Gal(309-332)	>95%	20%-40% B	4.51[a]	$[M+4H]^{4+}$: 875 (875)
α-Gal(309-320)	>95%	20%-100% B	2.65 [a]	$[M+2H]^{2+}$: 657 (657)
α-Gal(315-326)	>95%	20%-100% B	2.47 [a]	$[M+2H]^{2+}$: 642 (642)
α-Gal(321-332)	>95%	20%-100% B	1.30 [a]	$[M+2H]^{2+}$: 702 (702)
α-Gal(309-316)	>95%	20%-100% B	1.32 [a]	$[M+2H]^{2+}$: 451 (451)
α-Gal(317-324)	>95%	20%-100% B	2.40 [a]	$[M+H]^{+}$: 883 (883)
α-Gal(325-332)	>95%	20%-100% B	0.63 [a]	$[M+2H]^{2+}$: 475 (475)

Solvents used: A 0.1% TFA in H_2O; B 0.1% TFA in CH_3CN 84%

a: Phenomenex Jupiter C18 (5 lm, 2504.6 mm), 0.6 mL/min

Table 10 - Characterization of the pure products

2.3 Structural-affinity characterization

All the experiments to evaluate the affinity of the synthesized products against the commercial monoclonal antibody were performed at the Steinbeis Center for Biopolymer Analysis and Biomedical Mass Spectrometry using SPR biosensor-MS combination technology.

The following Table 11 reports all the dissociation constants (K_D) values obtained for all the synthetized products, compared to the recombinant α-Gal A.

PRODUCT	K_D (M)
Recombinant α-Gal A	$16x10^{-9}$
α-Gal(309-332)	$39x10^{-9}$
α-Gal(309-320)	$15x10^{-5}$
α-Gal(315-326)	$43x10^{-6}$
α-Gal(321-332)	$71x10^{-7}$
α-Gal(309-316)	$82x10^{-5}$
α-Gal(317-324)	$16x10^{-4}$
α-Gal(325-332)	$49x10^{-5}$

Table 11 - K_D determination of synthetic peptide sequences deriving from α-Gal A compared with the full-length recombinant enzyme, against an anti-α-Gal A monoclonal antibody, by SPR biosensor

Furthermore, Figure 29 shows the comparison between the fitted sensograms obtained using both the fragment α-Gal(309-332) and recombinant α-Gal A.

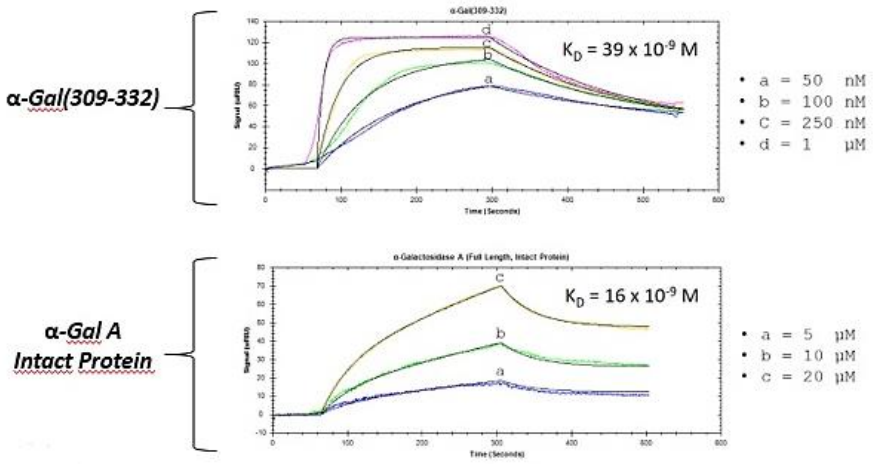


Figure 29 - Comparison between the α-Gal(309-332) and the α-Gal A fitted SPR sensograms

α-Gal (309-332) was found to be the only product among all those synthesized characterized by a high nanomolar affinity, comparable to the full-length enzyme. For this reason, is was considered a novel and important molecular basis for further structural and affinity characterization of α-Gal A peptide epitope.

All the reported results have been published. [144]

3. CONCLUSIONS

LSDs represent one of the major challenge for biomedical research. Moreover, the adverse immune reaction described in FD male patients after the ERT, one of the most common treatment strategies used, make the investigation of alternative or complementary therapeutic strategies to ERT a fundamental goal. In order to better understand the molecular mechanisms underlying the interaction between the endogenous α-Gal A enzyme and the specific IgE and IgG antibodies highlighted in patients after 6 months from the treatment, an in

depth structural-affinity study of the enzyme was necessary. In this part of the project, the epitope of the human α-Gal A was identified using a commercial monoclonal anti-α-Gal A antibody. The full-length epitope sequence and 3 deriving overlapping peptides plus 3 deriving sequential peptides were synthetized, purified and characterized in order to perform structural-affinity characterization through SPR biosensor-MS combination technology.

Our results showed a comparable high nanomolar binding affinity between the commercial recombinant α-Gal A and the full-length α-Gal(309-332) fragment, confirming its role as epitope in the native protein. On the contrary, all the overlapping and sequential synthetic fragments deriving from α-Gal(309-332) showed substantially lower micromolar and millimolar affinities, suggesting the missing of fundamental structural characteristics. Therefore, the epitope minimization in order to use a shorter synthetic peptide fragment compared to α-Gal(309-332) did not produce satisfying results, but it significantly contributed to elucidate the importance of the structural features of the full-length epitope.

CHAPTER FOUR: EXPRESSION OF A GLUCOSYLATED BACTERIAL PROTEIN FOR IMMUNOAFFINITY STUDIES ON SERA FROM PATIENTS AFFECTED BY RETT SYNDROME

1. INTRODUCTION

1.1 Clinical manifestations and stages of Rett syndrome

Rett syndrome is a neurological disease of the development, described for the first time by the Austrian pediatrician Andreas Rett in 1966. It affects almost exclusively female subjects because it is directly related to a monogenetic dominant mutation on the Xq28 region of the X chromosome. [145] [146]

Rett syndrome is the second most common cause of mental retardation related to a specific genetic condition [147] (1 on 10000 live female birth in US). It is characterized by a wide clinical variability, but 4 principal phases of the progression of this pathology can be distinguished [148]:

a. **Early Onset Phase**. This phase is characterized by a stall or a complete stop of the development. In some cases, symptoms could be very weakly evident and parents and/or health care providers may not notice them at first.
b. **Rapid Destructive Phase**. The child loses skills (regresses) quickly, starting usually from purposeful hand movements and speech. Breathing problems and hands movements such as clasping, clapping or tapping also tend to start during this stage. Moreover, first symptoms of autism

are reported to appear in this stage, besides a slowing of growth of the cranial circumference.

c. **Plateau Phase**. The child's regression slows and other problems may seem to lessen, or there may even be improvement in some areas. Despite this, seizures and movement problems are still common at this stage. Many Rett syndrome patients spend most of their lives in this stage.
d. **Late Motor Deterioration Phase**. During this phase, patients may show stiffness or loss of muscle tone, up to immobility in severe cases. Scoliosis (an abnormal curvature of the spine) may be present and even become severe enough to require bracing or surgery. Some hands movements defections and breathing problems seem to decrease.

Nowadays, this pathology is known to be potentially reversible. Despite this, no treatment is still able to regress or stop the neurological degeneration [149], both in case of medicines or behavioral and speech therapies.

Besides the classic variant of the Rett syndrome, 5 other more have been described [147]:

a. Congenital, a very rare one.
b. Forme Fruste, the most common group of atypical variants characterized by a pattern of quite discrete neurodevelopmental deviations which nevertheless become compatible with Rett syndrome with increasing age.
c. Preserved Speech, this subgroup is considered to represent an entity linked to both Rett syndrome and autistic disorders.
d. Late Regression, occurring late at preschool or early school age.
e. Convulsion and Early Onset.

The high complexity and eterogenicity of the Rett syndrome phenotype suggest that this pathology could be not only a neurological disease related to a specific genetic condition, but rather a multisystemic disease. Several phenotypic aspects typical of the Rett syndrome, such as the reduction of the blood flow in peripheral blood vessels called “Raynaud’s phenomenon”, have been highlighted also in a relevant number of well-established autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis. [150] Furthermore, Rett patients are commonly characterized by a dysregulation of the redox system, suggesting again the partially autoimmune character of the disease. In fact, oxidative stress is reported to be typical of many autoimmune diseases. [151]

1.2 The role of post-translational modifications in the pathogenesis of Rett syndrome

In the last few years the literature has been greatly enriched of many publications which investigated the possible involvement of different environmental factors, such as viral and bacterial infections, in the pathogenesis of autoimmune diseases. Post-translational modifications (PTM) are one of the mechanisms most commonly referred to as putatively triggers of autoimmune responses and often associated to these kind of infections. [152] PTM occur aberrantly on native peptides and proteins, which are covalently modified by smaller molecules, such as carbohydrates or drugs. Two different subsequent conditions can be observed: the modified substrate itself is recognized as non-self and attacked by the immune system; or proteolytic enzymes differently act on the modified substrate leading to the formation of cryptic self-antigens characterized by neo-epitopes which are recognized by T and B lymphocytes. In these cases, B lymphocytes are referred to as autoantibodies. [153]

1.1.1 The N-glycosylation

The N-glycosylation (Fig. 30) is one of the most in depth studied PTM. It occurs between a carbohydrate, characterized by a N-acetylglucosamine function as first residue, and the side chain of Asn, through the formation of a N-glycosidic bond. [154]

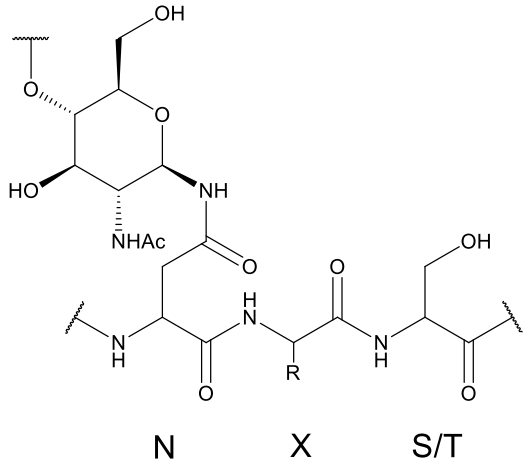


Figure 30 - N-glycosylation

Any Asn residue in the consensus sequence Asn-X-Ser/Thr can be N-glycosylated. Pro is not tolerated in the X position and Asn cannot be replaced by Gln. Any amino acid is allowed in the subsequent and previous positions. [155]

In oxidative stress conditions high levels of N-glycosylation on several endogenous proteins have been highlighted and a correlation between different kind of diseases and this condition is increasingly reported in literature. [156]

In particular, an aberrant modification consisting in a reduction of the N-glycosylation pattern of the protein NPP-5 has been reported to clinically precede the Rett syndrome. [157]

1.1.2 The N-glucosylation

In 2003 an unusual N-glucosylation has been highlighted on the bacterial protein adhesin HMW1 from the non-typable *Haemophilus influenzae* (NTHi). In this particular case, an Asn residue is N-glucosylated by a single glucose molecule. [158] Subsequently, the key role of the bacterial cytoplasmic glycotransferase HMW1C in this modification has been demonstrated. [159] The human proteome lacks of this mono-glucosylation on a specific amino acid. For this reason, a possible connection between this bacterial infection and the development of pathologies characterized by glucosylated antigens, such as multiple sclerosis, has been hypothesized [160] and subsequently demonstrated. [19] Because of the number of features which Rett syndrome has in common with these diseases, as described before, we decided to use the same modified protein antigen, the N-glucosylated HMW1, for the identification and the characterization of putatively specific autoantibodies in Rett syndrome patients' sera.

1.3 The NTHi's HMW1 and HMW1C

The *Haemophilus influenzae* is a Gram(-) bacteria which belongs to the γ-proteobacteria class. It affects only human beings. The different bacterial strains are divided in: capsulated, characterized by the presence of a polysaccharide capsule like a covering layer or envelope around the cell wall (serotypes a-f); and

non-capsulated (non-typable). Non-typable *Haemophilus influenzae* (NTHi) are commensal bacteria of human airways; they are responsible of several kinds of mucosal infections such as otitis, sinusitis, bronchitis and pneumonia. [161] [162] Many of this kind of infections are very common, in particular in the first stages of life. Nearly 80% of non-typable strains are able to express the protein adhesin HMW1 (Fig. 31), which promotes their adhesion to the respiratory epithelial cells, facilitating the colonization.

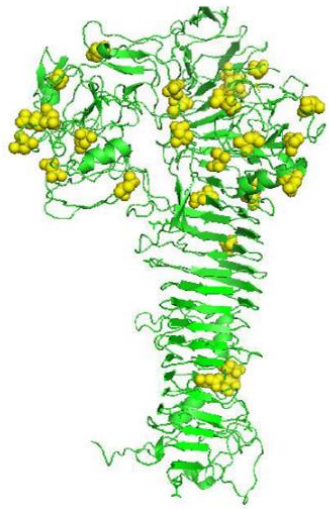


Figure 31 - HMW1

HMW1 is synthetized as pre-protein and it undergoes a cut in the secretion apparatus performed by a type 1 signal peptidase. Subsequently, it is exposed on the external membrane of the bacteria cell through an interaction with the HMW1B translocators and stabilized by non-covalent interactions. Part of the protein is released in the supernatant of the culture (Fig. 32). [163]

HMW1 is fundamental for the adhesion of the bacteria on the host [164] [165] [166] and the N-glucosilation plays a key role about the stability and the transport of the protein on the cell surface. HMW1 is modified by a cytoplasmic N-glycosyltransferase called HMW1C [158] which catalyzes the covalent attachment of mono- or di-hexoses on Asn residues in the consensus sequence NX(S/T). [159] In depth studies performed using protein digestion and MS analyses revealed the presence of 31 available sites on the protein for this modification. [159]

The N-glycosyltransferase HMW1C is considered as founder of a new family of enzymes characterized by unique skills different from the classic oligosaccharyltransferases. Indeed, in the cytoplasm HMW1C can transfer the sugar moiety directly on the Asn residue of the target protein without needing a lipid linker. [159] This enzyme is characterized by several homologues found in other kind of Gram(-) bacteria, such as *E. coli enterotoxigenic, Yersinia pseudotuberculosis, Y. enterocolitica, Y. pestis, H. ducreyi, Mannheimia spp., Xanthomonas spp., Burkholderia spp e Actinobacillus pleuropneumoniae*. [167]

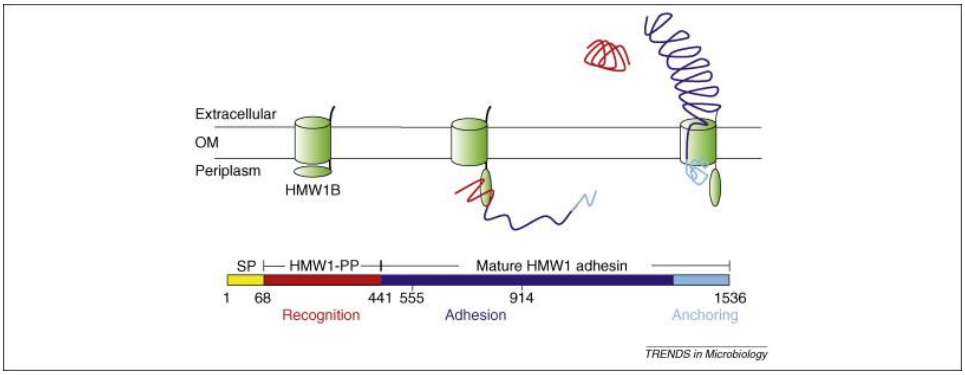


Figure 32 - Exposure and secretion of HMW1 *[168]*

The latter (ApHMW1C) showed the ability to N-glucosylate HMW1 in vitro and in vivo. [169] Structural modelling analyses, glucosylation assays and site-direct mutagenesis assays have shown that this particular homologous is characterized by the same structure and substrate specificity of *Haemophilus influenzae*'s HMW1C. [170] [155] Because of the insolubility in water of the recombinant *Haemophilus influenzae*'s HMW1C at high concentrations and because of its refractory nature to the crystallization, ApHMW1C has been used as alternative. Further structural informations about this new class of enzymes have been obtained performing crystallography analyses on ApHMW1C. [170] Data indicate that these enzymes are characterized by GT-B folding on the C-terminus and an ADD domain on the N-terminus. They have been classified in the GT-41 family and they act through an inverting catalysis mechanism, forming β-glycosidic bonds without the support of metal ions. [155] They are specific for activated carbohydrates such as UDP-Glc and UDP-Gal, with a particular affinity for the first one. [171]

1.4 Objectives of the project

The complex phenotype of Rett syndrome suggests that also environmental factors could be involved in the pathogenesis of this particular disease. Furthermore, the several clinical similarities between Rett syndrome and well-established autoimmune diseases prompted us to investigate the possible role of post-translational modifications in its development. Because of our knowledge about the role of the N-glucosylated bacterial protein adhesin HMW1 from *Haemophilus influenzae* in multiple sclerosis and our expertise about the expression of specific modified protein probes, we decided, following the Chemical Reverse Approach concept, to investigate the possible role of this

particular bacterium in the pathogenesis of Rett syndrome using our protein probe HMW1ct, both in its glucosylated and non glucosylated form. We used these two antigens in order to perform ELISA experiments on Rett syndrome and Pervasive Development Disorder patients' sera, comparing them with Normal Blood Donors. Pervasive Development Disorder (PDD) is a condition recently associated to the preliminary phase of Rett syndrome. All the sera were selected by Professor Hayek from the "Child Neuropsychiatry Unit" of the University Hospital of Siena.

2. RESULTS AND DISCUSSION

2.1 Expression of the protein antigen and the enzyme

The Interdepartmental Laboratory of Peptide and Protein Chemistry and Biology (PeptLab) of the University of Florence developed a recombinant protein probe in order to investigate the presence of cross-reactive antibodies against epitopes represented by N-glucosylation moieties on specific sequences of the bacterial protein HMW1 in patients' sera. [19] This protein probe is composed by the C-terminus domain of HMW1 (1205-1536), the so called HMW1ct (Fig. 33). The HMW1ct fragment was chosen because it is well expressed, soluble and stably folded. [169] On the N-terminus of this fragment, a 6-His tag was added in order to further improve the solubility and to ease the purification steps; moreover, a Trp residue was added because of its absorption in the UV region. HMW1ct is characterized by 12 Asn residues available as N-glucosylation sites. Through a co-expression technique of both HMW1ct and ApHMW1C, the protein antigen characterized by 8 N-glucosylation motifs was obtained. Furthermore, two additional genes for the resistance to carbenicillin and kanamycin antibiotics were added to the plasmids used for the expression. This characteristic allows to

grow up the selected modified bacterial cells in culture soils rich of those antibiotics, avoiding the development of different kind of bacterial colonies.

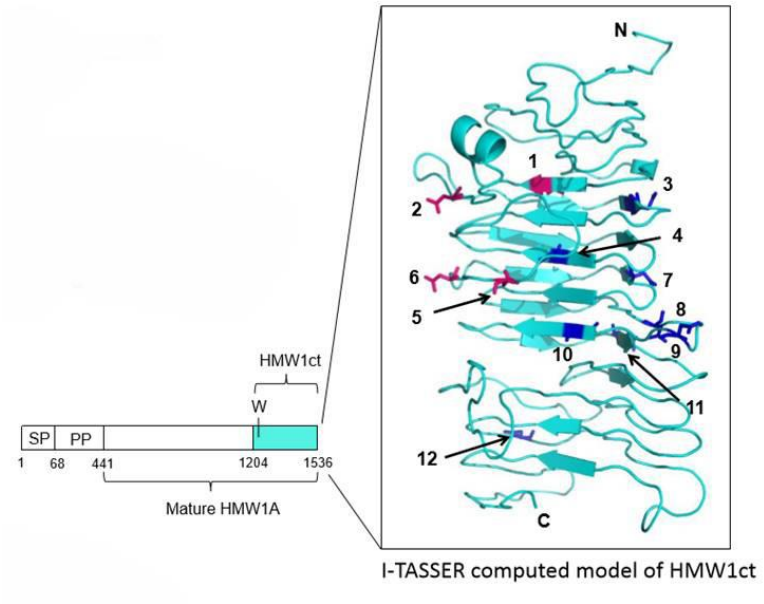
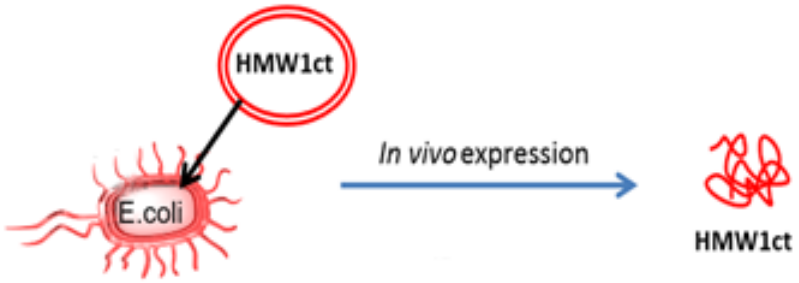


Figure 33 - HMW1ct *[19]*

The expression of the protein probe HMW1 was performed starting from transformed bacterial cells of *E. coli* BL12 engineered with a plasmid coding for the protein sequence HMW1ct (Fig. 34a). In case instead of expression of the N-hyperglucosylated protein probe HMW1ct(Glc), the *E. coli* BL12 cells were engineered also with a plasmid coding for the enzyme ApHMW1C (Fig 34b). These plasmids also contain, respectively, the genes for the resistance to carbenicillin and both carbenicillin and kanamycin antibiotics.



(a)

(b)

Figure 34 – (a) In vivo expression of HMW1ct; (b) In vivo expression of HMW1ct(Glc) and ApHMW1C

All phases of cell growth and expression were performed in sterile ambient using containers and materials sterilized at 121°C for 40'. Principal steps are described below, while details are reported in the General procedure for protein expression:

a. **Primary culture**: a 5 µl aliquot of the mother solution of cells was put in the center of a plate containing the solid culture soil with the antibiotics. The aliquot was distributed all over the plate homogeneously. The plate

was incubated overnight at 37°C. The culture soil provides to the cells all the nutrients and all the growth factors they need.

b. **Pre-culture**: one isolated bacterial colony was taken from the plate and transferred in 5 ml of liquid culture soil with the antibiotics. It was incubated under stirring overnight at 37°C, becoming opalescent. The entire solution was then added to 1 L of the same liquid culture soil with the antibiotics and incubated again at 37°C for about 3 h. Cell growth was monitored measuring the optical density (OD) of the solution through an UV instrument (600 nm) and using the only liquid culture soil as blank. Cell scission happens about every 20 minutes, finally reaching a maximum density of about $3x10^9$ cells/ml (Fig. 35). Protein expression has to be triggered in the moment of maximum cell growth, corresponding to an OD value equal to 0.6. Over this value, a stationary phase of the cell growth is observed, followed by a senescence phase or cell death.

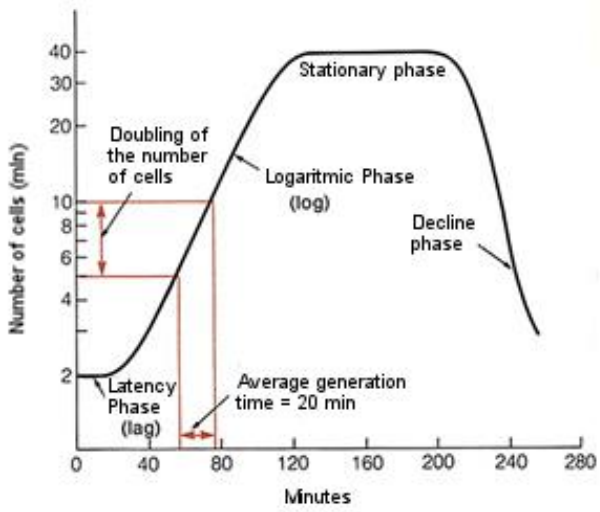


Figure 35 - Bacterial growth

c. **Induction of the transcription**: once an OD value equal to 0.6 was reached, 1 ml of isopropyl-β-D-1-thiogalactopyranoside (IPTG) 1 mM was added to the solution, which was then incubated overnight at 16°C. IPTG is a lactose analogue which is not metabolized by β-galactosidase and which induces gene expression under the control of operon Lac in *E. coli* (Fig. 36). Moreover, this bacterium natively possesses a sufficient UDP-Glc concentration to allow the N-glucosylation of the protein fragment in case of co-expression with the enzyme.

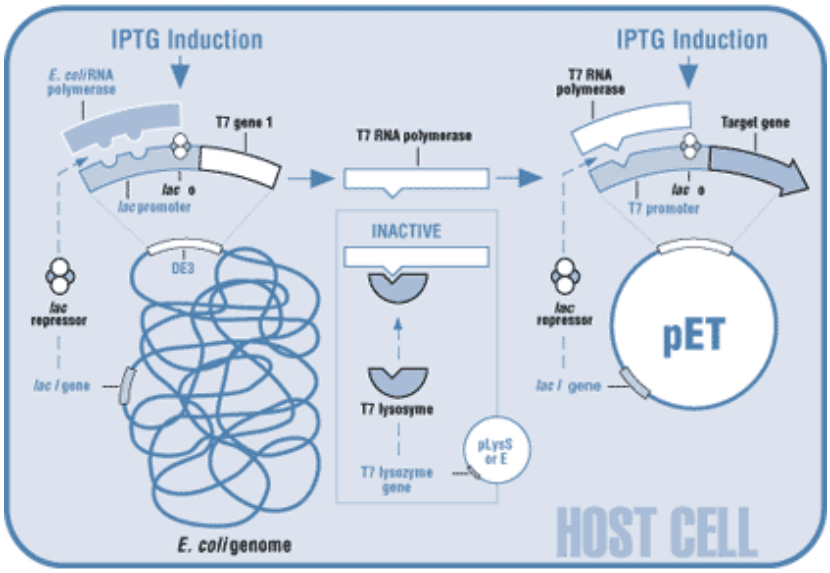


Figure 36 - Induction of the transcription *[172]*

d. **Pellet**: the solution was finally centrifuged at 4000 rpm for 30' at 4°C. The supernatant was eliminated, the precipitate suspended in 40 ml of

lysis buffer and centrifuged again. Only the precipitate was stocked at -20°C.

2.2 Cell lysis and protein purification

Cell lysis was carried out by suspending the pellet in 30 ml of lysis buffer and adding a protease inhibitor (cocktail Set III EDTA free, 10µL each g of cells). Cell membrane was lysed using an immersion sonicator, performing 3 cycles 1 minutes each one, with an amplitude of 40%. The solution was then centrifuged for 110 min at 35000 rpm and the supernatant containing the product(s) was recovered. The purification of the antigen was carried out taking advantage of the ability of the protein to interact reversibly with a specific ligand blocked on a solid matrix, such as the coordination interaction between the imidazole ring of His residues on the protein and bivalent metal ions (e.g. Ni^{2+}). [173] This technique is called IMAC, or Immobilized Metal Ion Affinity Chromatography (Fig. 37). [174]

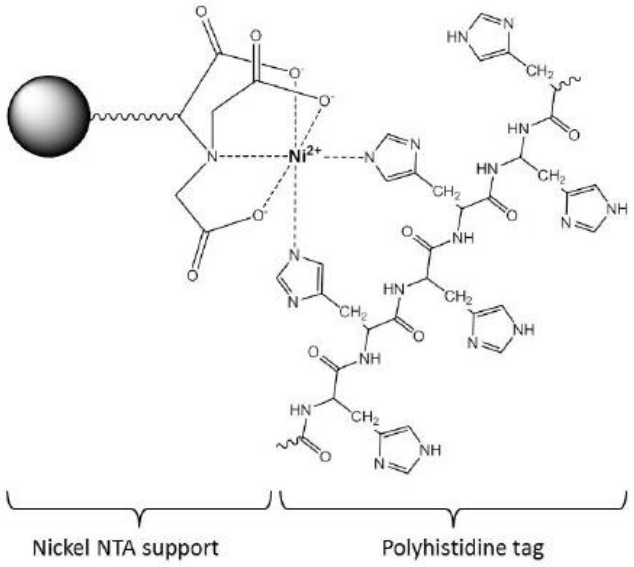


Figure 37 - Example of the complex formed between the poly-histidine tag of HMW1ct and a Ni^{2+}-derivatized support during the IMAC *[175]*

Because of the presence of the His tag on the N-terminus, HMW1ct and HMW1ct(Glc) show a higher affinity for the matrix compared with the other protein fractions. The protein antigen was purified using an Äkta FPLC (Fast Protein Liquid Chromatography) system. In case of expression of HMW1ct, only one purification step was performed using a Hi Trap-His column (45 mg of capacity); this column is composed by resin beads derivatized with Ni^{2+} which allow the purification through the IMAC technique (Fig. 38). [176]

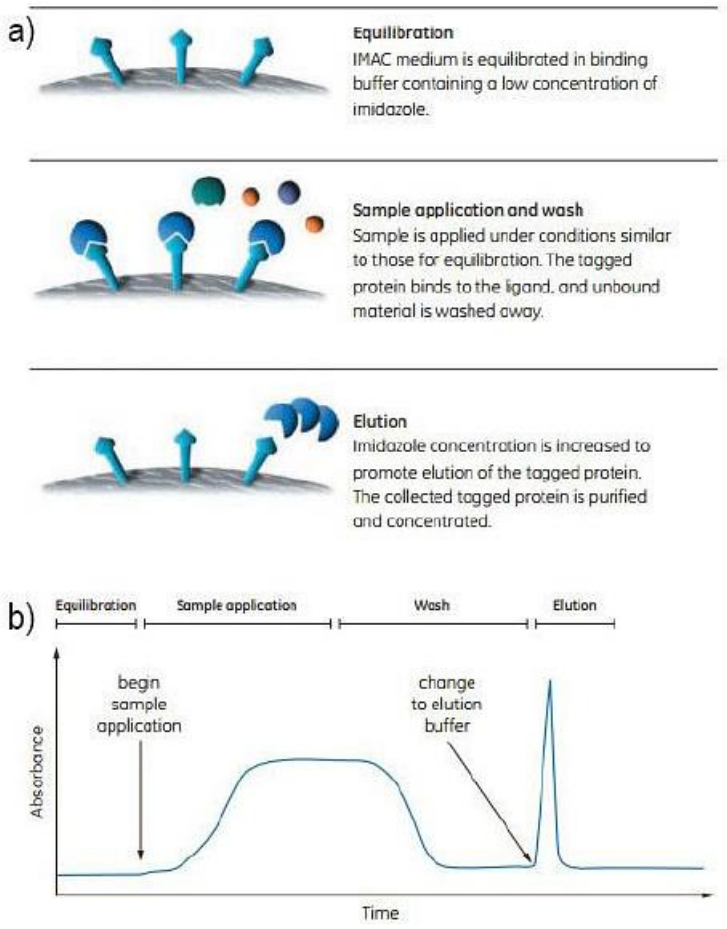


Figure 38 - IMAC purification *[175]*

In case of expression of HMW1ct(Glc) also a subsequent purification step is necessary using a Hi Trap Q-FF column (5 ml of capacity) in order to separate the protein HMW1(Glc) from the enzyme ApHMW1C through the ion exchange technique. ApHMW1C doesn't have the His tag but it forms a complex in solution with HMW1ct(Glc), remaining with it on the resin. The ion exchange technique allows to separate different protein substrates characterized by different

superficial charges, taking advantage of the reversible electrostatic interactions between charge macromolecules and a matrix with opposite charge. ApHMW1C interact stronger with the column than HMW1ct(Glc), resulting in a higher retention time (Fig. 39).

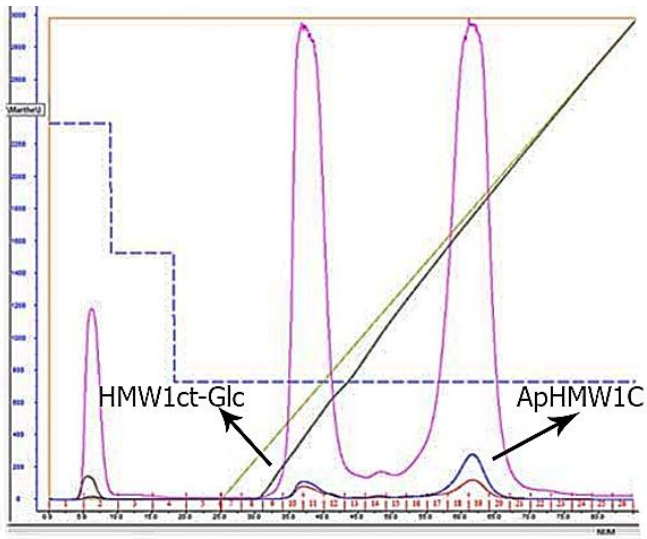


Figure 39 - Ion exchange chromatography to separate HMW1ct(Glc) from ApHMW1C

The Hi Trap-His column was conditioned with buffer A1 (binding buffer for Hi Trap-His). The supernatant obtained from the cell lysis was then injected and eluted using a gradient from 0% to 100% of buffer B1 (elution buffer for Hi Trap-His). The UV detector was set to 280 nm and 215 nm. All the fractions obtained were analyzed using Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis technique (SDS-PAGE) (Fig. 40).

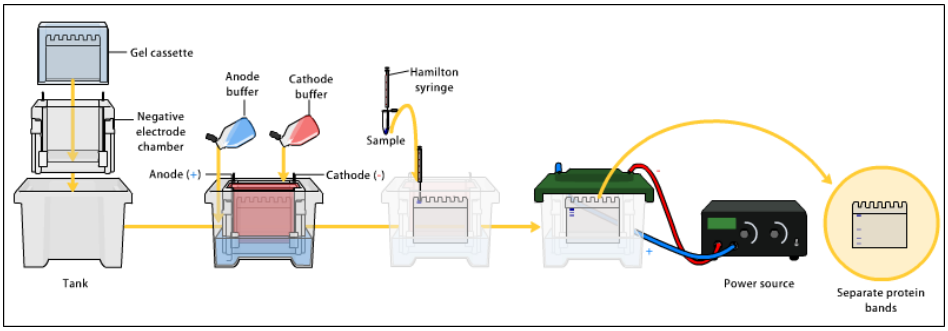


Figure 40 – SDS-PAGE

In case of expression of HMW1ct(Glc) a buffer exchange was performed on the selected fractions obtained from the IMAC in order to substitute the elution buffer B1 with the buffer A2 (binding buffer for Hi Trap Q-FF). Falcon Amicon Ultra Centrifugal Filters were used, characterized by a pore dimension MWCO<30 kDa. The Hi Trap Q-FF column was then conditioned with buffer A2, the fractions injected and eluted using a gradient from 0% to 100% of buffer B2 (elution buffer for Hi Trap Q-FF). The UV detector was set to 280 nm and 215 nm. All the fractions obtained were analyzed using SDS-PAGE technique.

Finally, a further buffer exchange was carried out in order to stock both the protein antigens in PBS buffer (pH 7.4) at -20°C. The concentration of the PBS-protein sample was obtained using an UV spectrometer set up between 320 nm and 240 nm, in order to be able to visualize the typical protein absorption peak at 280 nm. The concentration was calculated through the Lambert-Beer law. All the details about these protocols are reported in the General procedure for protein expression, protein purification and SDS-PAGE.

2.3 SP-ELISA experiments

Both HMW1ct(Glc) and HMW1ct were used as antigens in SP-ELISA experiments on Rett syndrome and PDD patients' sera, a condition recently associated to the preliminary phase of Rett pathology, following the General procedure for SP-ELISA experiments. NBD sera were used as negative controls. Preliminary data are reported in Figure 41a and 41b.

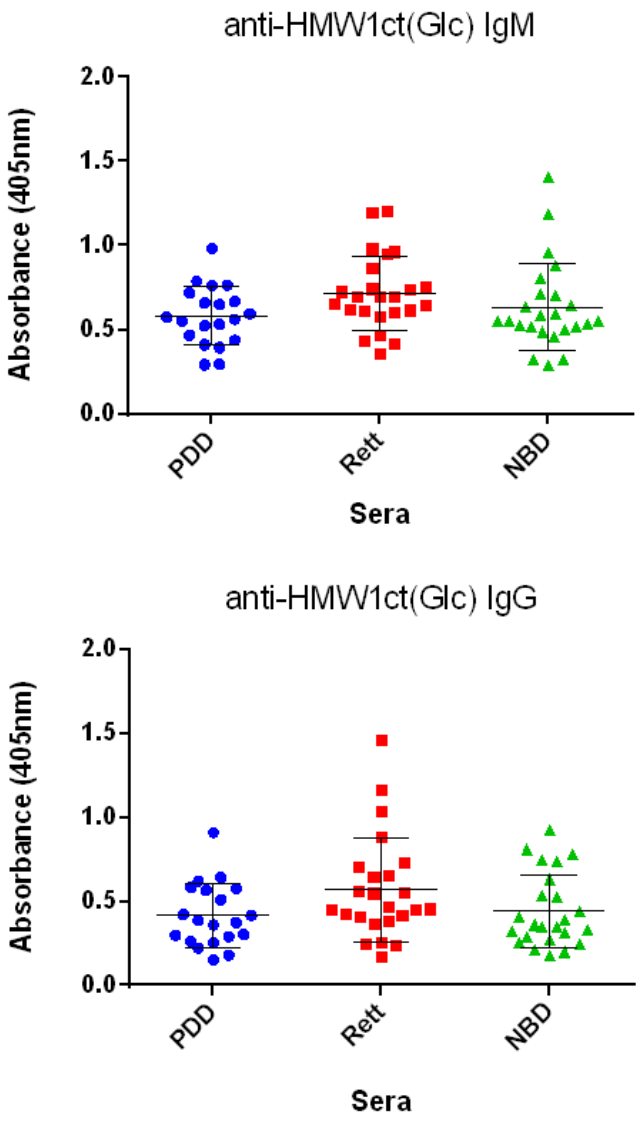


Figure 41a – Anti-HMW1ct(Glc) IgM and IgG titers on PDD, Rett and NBD patients' sera

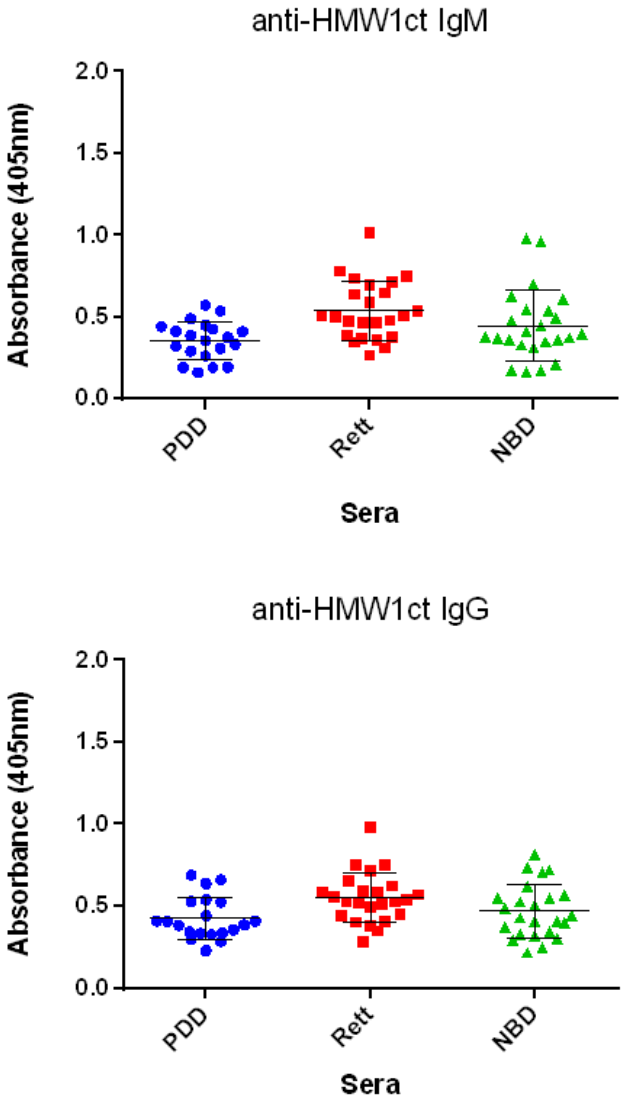


Figure 41b - Anti-HMW1ct IgM and IgG titers on PDD, Rett and NBD patients' sera

The analysis of the data highlights that HMW1ct(Glc) and HMW1ct are not able to significantly discriminate the three categories of sera examined (PDD, Rett, NBD); furthermore, titers obtained with both the antigens are comparable each other. This effect has been hypothesized to be due to the possible recognition, by the antibodies present in the tested sera, of epitopes related not only to the N-glucosylation sites on the protein but also to the structure of the protein itself.

2.4 Inhibition tests

In order to verify the aforementioned hypothesis, we selected two of the tested sera belonging to Rett patients and characterized by an anti-HMW1ct(Glc) IgG titer >1 and an anti-HMW1ct IgG titer <1. We performed inhibition tests on these two samples using both the two antigens as inhibitors following the General procedure for inhibition assays. Results are reported in Figure 42.

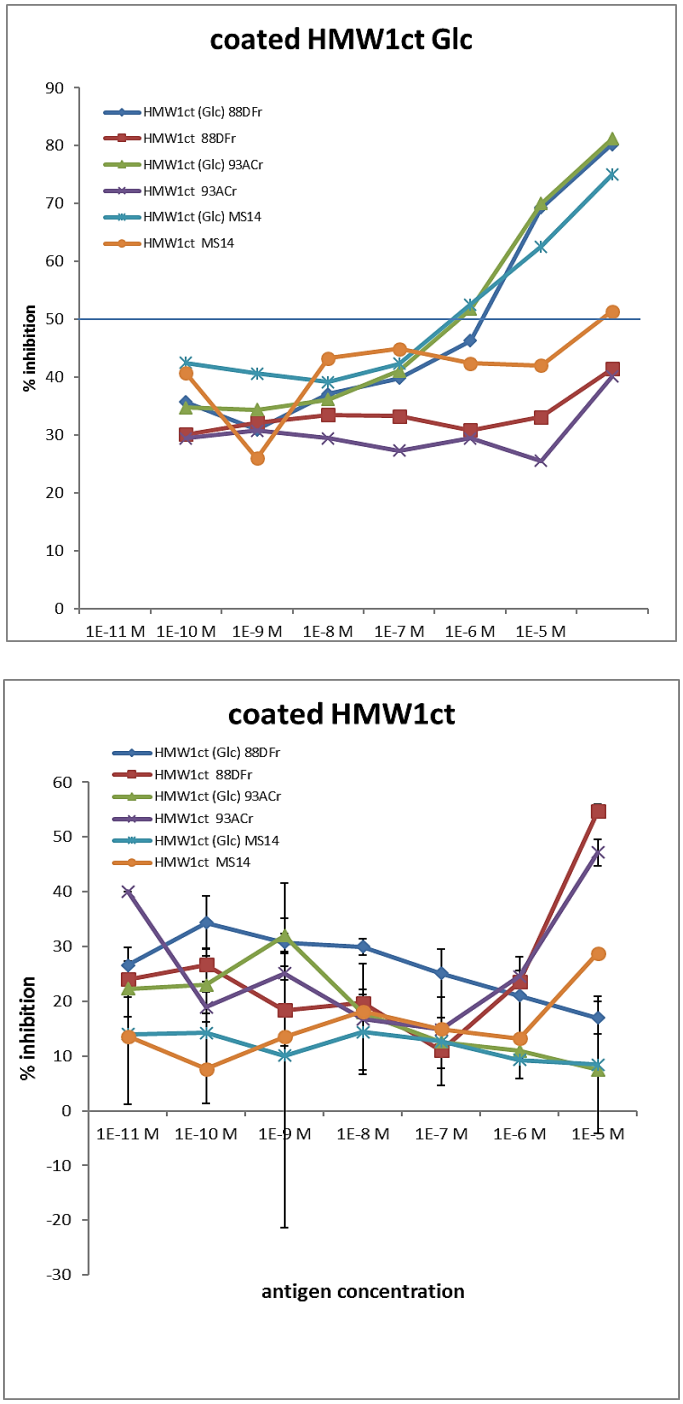


Figure 42 - Preliminary results of the inhibition tests

Coating HMW1ct we were not able to inhibit the signal, either using HMW1ct or HMW1ct(Glc) as inhibitors. Coating instead HMw1ct(Glc) we were able to inhibit the signal using the same HMW1ct(Glc) as inhibitor, confirming the presence of a specific interaction between the antibodies characteristic of Rett syndrome and the N-glucosylation motifs of the bacterial protein HMW1ct(Glc).

3. CONCLUSIONS

Rett syndrome has been considered and classified as a genetic disease related exclusively to a specific mutation on the Xq28 region of the X chromosome. This disease is however characterized by a very complex phenotype, suggesting the involvement of other kind of factors in its pathogenesis and development. The hypothesis that genetics alone is not sufficient to describe all the variants is increasingly accepted. Because of the reported similarities between Rett syndrome and well-established autoimmune diseases such as multiple sclerosis, we decided to investigate the possible role of the N-glucosylated bacterial protein HMW1 from *Haemophilus influenzae* in the onset of Rett syndrome. The antigenic role of HMW1(Glc) in multiple sclerosis have been already demonstrated. [19]

Using the Chemical Reverse Approach strategy, we used the antigenic protein probe HMW1ct, both in its glucosylated and non-glucosylated forms, as antigen in order to perform SP-ELISA tests on Rett syndrome and PDD patients' sera, compared with NBD. Our preliminary results highlight that HMW1ct(Glc) and HMW1ct are not able to significantly discriminate the three categories of sera examined; furthermore, titers obtained with both the antigens are comparable. For this reason, we decided to perform inhibition assays on two of the tested Rett sera characterized by an anti-HMW1ct(Glc) IgG titer >1 and an anti-HMW1ct

IgG titer <1. We were able to inhibit the signal only using HMW1(Glc), suggesting the presence of a specific interaction between antibodies characteristic of Rett syndrome and the N-glucosylation motifs of the bacterial protein HMW1ct(Glc).

CHAPTER FIVE: PENICILLOYLATED PEPTIDES FOR THE STUDY OF ALLERGIC RESPONSES TO ANTIBIOTICS

1. INTRODUCTION

1.1 β-lactam antibiotics: origin and characteristics

Penicillins are one of the most common classes of antibiotic molecules used for the treatment of a wide range of infections. These antibacterial agents can be natural, deriving from *Penicillum chrysogenum* mold and discovered for the first time by Alexander Fleming in 1928, or synthetic. All the molecules belonging to the penicillin family are characterized by three common functional groups: a thiazoldine ring, a β-lactam ring and a side chain (Fig. 43). Nowadays, other antibiotics are characterized by the β-lactam core, like cephalosporins, carbapenamines and monobactams.

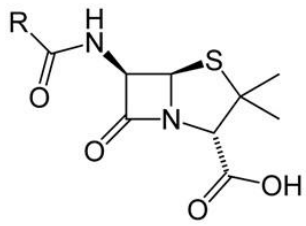


Figure 43 – The penicillin core

Generally, five categories of penicillin antibiotics are recognized: natural penicillins, β-lactamase-resistant penicillins, aminopenicillins, extended spectrum penicillins and aminopenicillin/ β-lactamase inhibitor combinations. [177]

The antibacterial mechanism which characterize this class of compounds is to bind the enzymes used to produce the proteins which compose the cell wall itself, leading to an inhibition of the cell wall synthesis.

Natural penicillins, such as penicillin G and penicillin V, are effective against aerobic gram-positive bacteria, such as streptococci and enterococci. Gram-negative bacteria represent one of the most common examples of natural resistance to penicillins; in this kind of bacteria, the penetration through the cell wall of generic penicillin molecules is prevented by an additional external membrane which protects the cell. In some other cases, such as for most of the *staphylococcus* bacteria, organisms produce specific enzymes which attack and destroy the β-lactam structure by opening the β-lactam ring. [177] These enzymes are known as β-lactamase. In order to prevent this effect, a β-lactamase inhibitor such as clavulanic acid (Fig. 44) is normally added to the formulation of these antibiotics, increasing their effectiveness against these kind of organisms. [178]

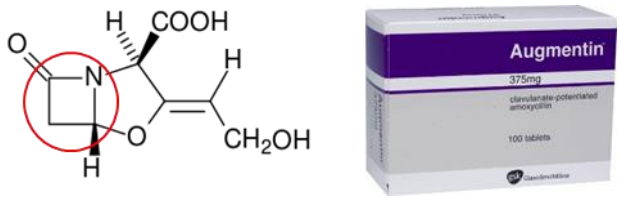


Figure 44 - Clavulanic acid

Nowadays, also synthetic penicillins are commonly produced. Aminopenicillins (e.g., amoxicillin AX) (Fig. 45) and extended spectrum penicillins belong to this class. Chemically modifying the general β-lactam core, an increase of the activity spectrum has been reached, leading to an antibiotic effect also against some gram-negative bacteria.

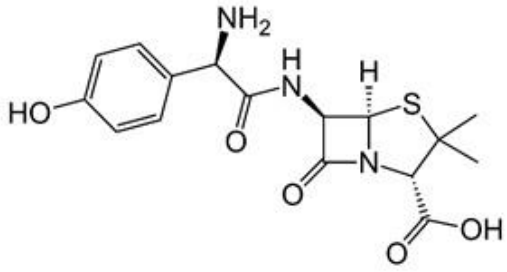


Figure 45 - Amoxicillin (AX)

1.2 Allergic reaction to β-lactam antibiotics

Penicillins are the widest and the safest class of antibiotics used. Despite this, about 0.7-10% of patients manifest adverse responses to them, from adverse side effects to allergic reactions. In particular, these latter are the most common but also the most misunderstood adverse responses to penicillins. [179] They have been classified by the type of reaction, characterized by different mediators and symptoms (Table 12).

Type	*Time of Onset*	*Mediators*	*Characteristics*	*Skin Testing Indicated*
Type I	<1 h	IgE	Anaphylaxis and/or angioedema, respiratory distress, hypotension	Yes
Type II	>72 h	IgG, IgM complement	Fever, arthralgia, splenomegaly, lymphadenopathy. Typically self-limiting and resolves completely within days to weeks of stopping penicillin	No
Type III	>72 h	IgG, IgM, complement	Autoimmune responses producing local ischemia and/or necrosis as a result of complement activation	No
Type IV	>72 h	T lymphocytes	Contact dermatitis	No
Idiopathic	>72 h	Varies	Maculopapular rash (most common reaction) Stevens-Johnson syndrome (rare)	No

Table 12 - Classification of allergic reactions to penicillins *[177]*

Benzylpenicillin and penicillin G (respectively BP and PG) are known to cause allergic reaction in patients with a prevalence of 1.9%. In agreement with these data, human T lymphocytes specific to PG have been found to be involved in this kind of hypersensitivity reactions. Nevertheless, PG is not immunogenic by itself. [180]

1.3 Haptenation of native proteins

Recently, several mechanisms behind the adverse reactions to penicillins have been investigated. One of the most importantly related to allergic reactions to antibiotics is the “haptenation”. The reactive form of the drug act like an hapten spontaneously modifying a target protein; this hapten can covalently bind several kind of protein substrates, leading to a sufficiently big complex to be immunogenic and to provoke an immune response. [181] Indeed, this strategy is commonly used in immunological studies in order to understand, for example, the role of different kind of small molecules, not immunogenic by themselves, in many pathological conditions e.g., the conjugation of peptides hypothetically involved in the pathogenesis of some autoimmune diseases to carrier proteins

like KLH or BSA to make them "visible" to the autoantibodies characteristic of the pathology.

After the haptenation, the resulting complex can be exposed through MHC by DCs or antigen presenting cells, in order to be presented to TCRs. The MHC is composed by several cell surface proteins which play a fundamental role in the recognition of foreign molecules by the acquired immune system in vertebrates. Antigens are bind by the MHC molecules and presented on the cell surface in order to be recognized by the appropriate T-cells. [182]

1.4 Reactivity of β-lactams on the Human Serum Albumin

Chemically, the β-lactam ring is the most interesting reactive part of penicillins. Its electrophilic character allows a nucleophilic attack which leads to an opening of the structure. Amino groups characteristic of side chains of Lys residues are commonly reported to be able to perform this reaction. [183]

β-lactam-protein adducts have been already detected before, including PG conjugates with human serum albumin (HSA). [184] In fact, haptenation of HSA performed by penicillins has been reported *in vivo* in patients' sera and penicillin binding sites represented by Lys residues have been identified. Moreover, the synthesis of HSA-penicillin bioconjugates haptenated on several available Lys residues in vitro confirmed this mechanism (Fig. 46). [185]

In this context, not only the drug itself but also its complex with the target protein could represent the epitope for antigen recognition by the TCR. Following this concept, the synthesis of PG-HSA bioconjugates and the synthesis of all the haptenated peptide mimetics of the modification sites considered putative epitopes, in order to use them as antigens in biological in vitro tests, is

considered the best way to study exhaustively this kind of allergic responses. Furthermore, the existence of native $CD4^+$ T-cells specific for PG-HSA conjugates have been demonstrated also in healthy donors, suggesting that most people can be sensitized to penicillin but only a few individuals can mount an allergic response due to additional factors, providing necessary co-stimulation signals for T-cells activation. These additional factors are still poorly described. [186]

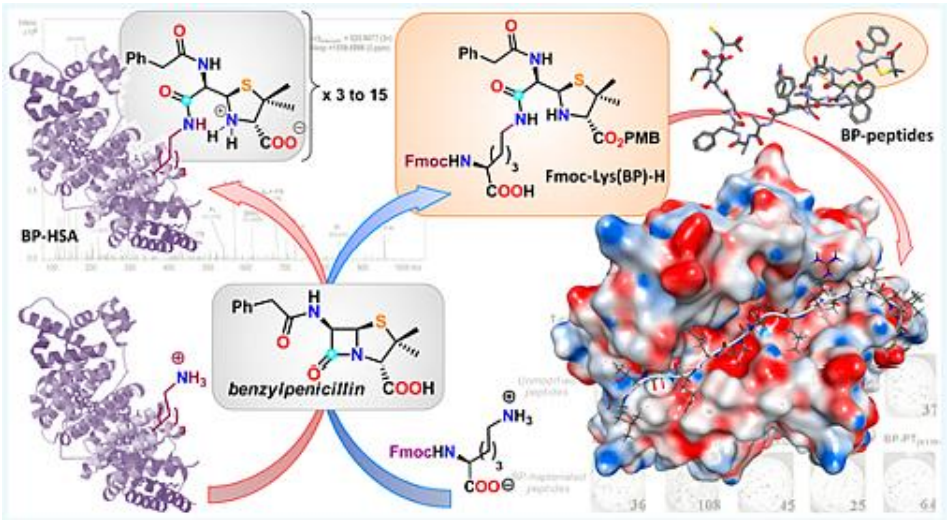


Figure 46 - The hypothesized mechanism of the haptenation of HSA by β-lactam substrates *[180]*

Previous studies focused on the detection of all the possible interaction sites between PG and HSA, selecting them among all the 59 lysine residues available on the protein. [180] This strategy planned to synthesize the PG-HSA bioconjugates in a non-sitospecific way and in weakly alkaline and physiological pH conditions. The MALDI-TOF technology allowed to confirm the covalent nature of the PG-HSA bond. After a triptic digestion of these bioconjugates and a subsequent analysis of the obtained fragments via nanoLC-MS/MS, numerous

binding sites have been detected. In particular, 51 benzopenicilloylated Lys residues have been reported deriving from PG-HSA bioconjugates synthetized in weak alkaline conditions and 21 deriving from PG-HSA bioconjugates synthetized in physiological conditions. Among these, 10 were selected for a more in-depth study because of their common detection also *in vivo* in allergic patients' sera: K137, K159, K190, K195, K199, K212, K351, K432, K436, and K525. These residues are shown in Figure 47 in both the HSA chains A and B. Lys residues involved in haptenation by penicillins are highlighted in red.

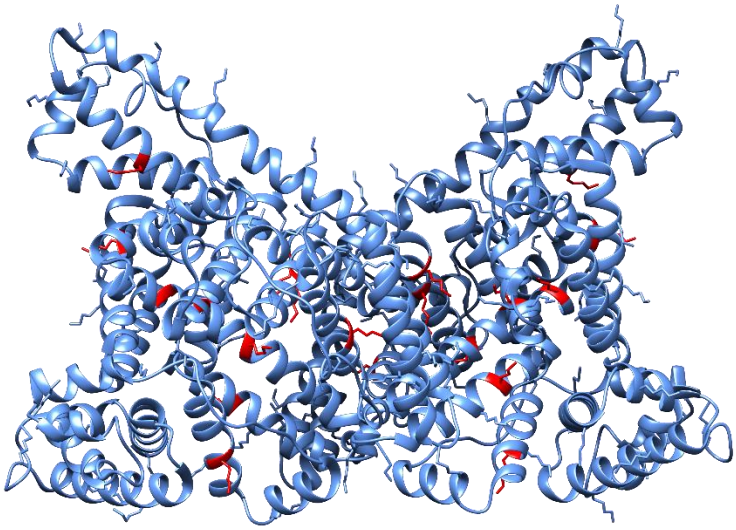


Figure 47 – The most important Lys residue for haptenation by penicillins highlighted in the HSA 3D structure (both chains A and B) (PDB: 1BM0)

Through two in silico approaches called MHC CLASS II BINDING PREDICTION TOOL and STANDARD COMPUTATIONAL ALANIN SCAN MUTAGENESIS PROTOCOL, all the 180 putative 15-mer PG-peptide mimetics of the HSA

sequences around the ten selected Lys residues were identified. It has been demonstrated previously that PG specific T-cells can be activated by synthetic peptides containing a PG-modified Lys residue in one of the positions indicated above if these peptides are MHC Class II ligands on the surface receptor HLA-DR. Furthermore, differently from other natural peptide ligands for MHC Class II, characterized by sequences composed from 13 to 25 aminoacids, peptide ligands specific for HLA-DR receptor are prevalently characterized by sequences made of 15 amino acids. Among all the 180 putative 15-mer PG-peptides, 12 potentially immunogenic sequences have been then selected, considering the 7 more frequent Caucasian HLA-DRB1 alleles, the orientation of these chains and their consequent binding affinity to HLA-DR receptor. These potentially immunogenic sequences are reported in Table 13.

modified lysines	peptide sequences	peptides
K137	LKK^{*}_{137}YLYEIARRHPYF	$LF_{[K137]}$
K159	PELLFFAK^{*}_{159}RYKAAFT	$PT_{[K159]}$
K190	LRDEGK^{*}_{190}ASSAKQRLK	$LK_{[K190]}$
K195	LRDEGKASSAK^{*}_{195}QRLK	$LK_{[K195]}$
	SSAK^{*}_{195}QRLKCASLQKF	$SF_{[K195]}$
K199	LRDEGKASSAKQRLK^{*}_{199}	$LK_{[K199]}$
	SSAKQRLK^{*}_{199}CASLQKF	$SF_{[K199]}$
K212	FGERAFK^{*}_{212}AWAVARLS	$FS_{[K212]}$
K351	VVLLLRLAK^{*}_{351}TYETTL	$VL_{[K351]}$
K432	TPTLVEVSRNLGK^{*}_{432}VG	$TG_{[K432]}$
K436	VEVSRNLGKVGSK^{*}_{436}CC	$VC_{[K436]}$
K525	KERQIKK^{*}_{525}QTALVELV	$KV_{[K525]}$

Table 13 -12 selected potentially immunogenic peptides derived from PG-HSA bioconjugates triptic digestion *[180]*

1.5 Objectives of the project

The use of peptides or modified peptides as mimetic of the putative antigenic determinant of a target protein in pathogenic conditions is an increasingly used technique for the study, the identification and the characterization of different types of immune or adverse responses in many kind of diseases, as well as for the study of chemical and biological mechanisms the pathogeneses of these diseases are based on.

Because of the role of the haptenation performed by β-lactam structures on the HSA in allergic reactions to antibiotics, we decided to synthetize some of the modified peptides showed in Table 13 to use them as mimetic probes of the haptenated HSA in IFN-γ ELISpot experiments. According to the literature about the supposed mechanisms triggering the allergic reactions to β-lactam antibiotics, we propose the optimization of synthesis and purification strategies of a collection of 10 haptenated and non-haptenated peptides (in collaboration with the group of Professor Joseph from BioCIS, Université Paris-Sud, CNRS, Université Paris-Saclay, Chatenay-Malabry, France) deriving from PG-HSA bioconjugate, in particular focusing on K159, K212 and K525 residues over the 12 reported in Table 13, in order to perform in vitro IFN-γ ELISpot experiments on specific T-cells lines from allergic patients, using a co-cultural approach (in collaboration with the group of Professor Pallardy, INSERM UMR 996, Université Paris-Sud, Université Paris-Saclay, Chatenay-Malabry, France).

A complete scheme of the entire rational procedure of this part of the project is reported in Figure 48.

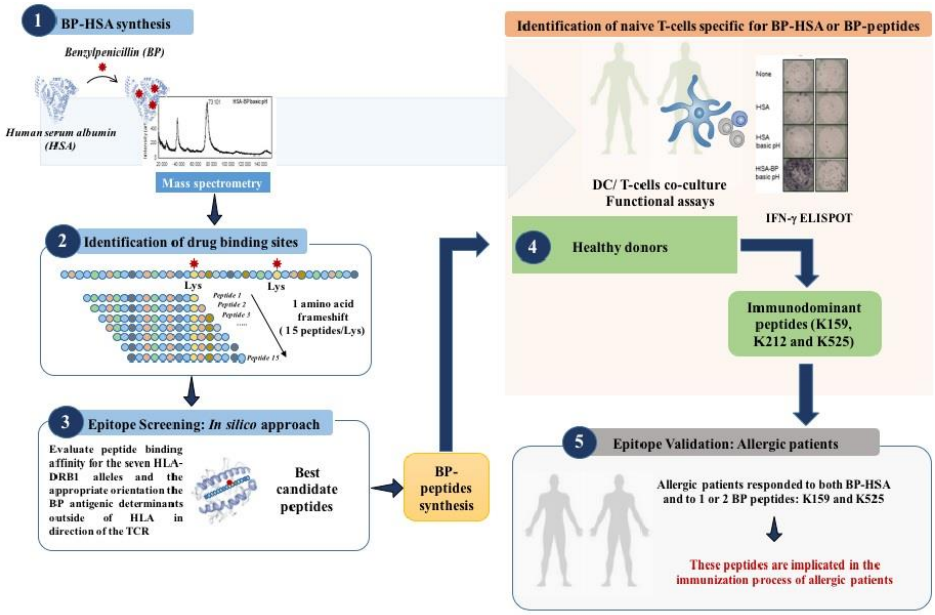


Figure 48 - A complete scheme of the "penicilloylated peptides for the study of allergic responses to antibiotics" project

Moreover, due to the extremely lipophilic character of this kind of peptides, we investigated different solubilization strategies of these products, according to the different toxicity limits of the most common organic solvents tolerated by the cells used in biological experiments. We aim to identify the optimal organic solvent to be added to the primary solvent (PBS buffer) in the correct percentage in order to be able to solubilize all the peptides of the collection remaining below the level of toxicity for the cells.

2. RESULTS AND DISCUSSION

2.1 The immunodominant peptides

A previous study [181] preliminary evaluated the capacity of all the 12 selected PG-haptenated peptides reported in Table 13 to prime $CD4^+$ T-cells *in vitro* from fourteen healthy donors. Peptide $PT_{[K159]}$(PG) was preferentially recognized by the major part of the donors. Lys159 was previously identified as one of the positions occupied by PG in PG-HSA bioconjugates synthetized *in vitro* and on HSA purified from patients' sera. Moreover, four other peptides shown high frequencies of positivity: $LF_{[K137]}$(PG), $FS_{[K212]}$(PG), $VL_{[K351]}$(PG) and $KV_{[K525]}$(PG). Both Lys525 and Lys 212 were described to be preferential sites for this kind of modifications. [179] At last, the two peptides named $SF\text{-}2_{[K199]}$(PG) and $SF_{[K195]}$(PG) are characterized by the same amino acid sequence but with PG on different Lys residue and they were differently recognized in tested donors. Indeed, $SF\text{-}2_{[K199]}$(PG) was recognized by T-cells from two donors over the fourteen tested while $SF_{[K195]}$(PG) was never recognized at all. This observation strongly suggests that the position of the PG modification on these peptides is a primary requisite for T-cells recognition.

In conclusion, the three peptides named $PT_{[K159]}$(PG), $FS_{[K212]}$(PG) and $KV_{[K525]}$ (PG) showed in these preliminary experiments the highest frequency of positivity in the tested healthy donors and were indicated as "immunodominant". The three immunodominant peptides were preliminary tested also on T-cells derived from allergic patients, selected on the basis of clinical manifestations to AX or PG. In that case, five allergic patients responded to two of the three tested peptides, $PT_{[K159]}$(PG) and $KV_{[K525]}$(PG), indicating that they are probably implicated in the immunization process of allergic patients to penicillins.

Based on these preliminary data, the three immunodominant peptide sequences were selected to be synthetized in different versions (Table 14).

SYNTHESIZED PEPTIDES
Ac-PT$_{[K159]}$(PG)-NH$_2$: Ac-PELLFFAK*$_{159}$(PG)RYKAAFT-NH$_2$
H-PT$_{[K159]}$(PG)-OH: H-PELLFFAK*$_{159}$(PG)RYKAAFT-OH
Ac-FS$_{[K212]}$(PG)-NH$_2$: Ac-FGERAFK*$_{212}$(PG)AWAVARLS-NH$_2$
Ac-KV$_{[K525]}$(PG)-NH$_2$: Ac-KERQIKK*$_{525}$(PG)QTALVELV-NH$_2$
Ac-PT$_{[K159]}$(AX)-NH$_2$: Ac-PELLFFAK*$_{159}$(AX)RYKAAFT-NH$_2$
H-PT$_{[K159]}$(AX)-NH$_2$: H-PELLFFAK*$_{159}$(AX)RYKAAFT-OH
Ac-PT-NH$_2$ Non-Hapt: Ac-PELLFFAKRYKAAFT-NH$_2$
H-PT-OH Non-Hapt: H-PELLFFAKRYKAAFT-OH
Ac-FS-NH$_2$ Non-Hapt: Ac-FGERAFKAWAVARLS-NH$_2$
Ac-KV-NH$_2$ Non-Hapt: Ac-KERQIKKQTALVELV-NH$_2$

Table 14 - Haptenated and non-haptenated synthesized peptides

2.2 Synthesis and characterization of haptenated and non-haptenated peptides

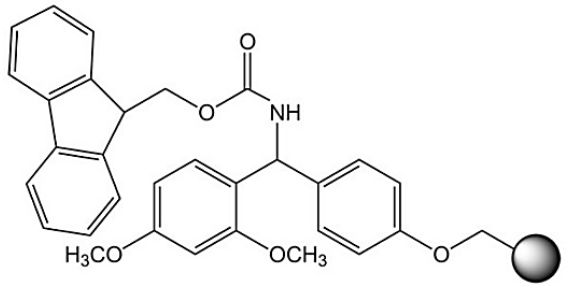
The syntheses of the haptenated peptides were made in collaboration with the research group of Professor Joseph from BioCIS, Université Paris-Sud, CNRS, Université Paris-Saclay, Chatenay-Malabry, France, while the biological tests on different T-cell lines were performed by the research group of Professor Pallardy, INSERM UMR 996, Université Paris-Sud, Université Paris-Saclay, Chatenay-Malabry, France. The syntheses of non-haptenated peptides were completely performed at the Interdepartmental Laboratory of Peptide & Protein Chemistry & Biology (PeptLab) in the Chemistry Department "Ugo Schiff" of the University of Florence, Italy. Both the haptenated and non-haptenated peptide syntheses were performed following the General procedure for SPPS assisted by microwaves. The non-haptenated peptides were synthetized in order to use them as negative controls in biological tests.

The use of peptides as mimetic of more complex structures, such as proteins, is a useful and increasingly used technique for the study of different aspects of many pathological conditions, in particular to investigate the role of aberrant post-translational modifications on protein substrates in the pathogenesis of these diseases.

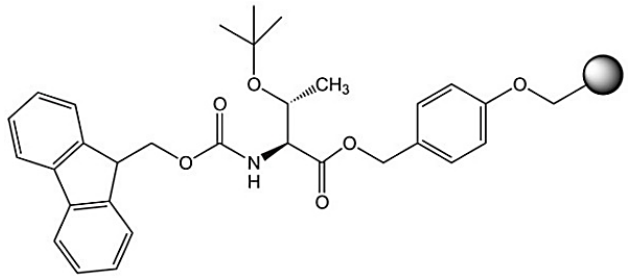
Chemically synthesized mimetic peptides carry positively and negatively charged amino and carboxy termini, respectively. Because these termini are generally not charged in vivo, they are commonly modified by N-terminal acetylation and C-terminal amidation, which remove the respective charges to mimic the portion of a native protein, eventually aberrantly modified, object of the study. In our case, only the $PT_{[K159]}$(PG) peptide was synthetized both in its protected and unprotected versions on the terminal positions, in order to verify not only the

role of the haptenation but also the role of the amino acid sequence who bring the modification in the process of T-cells recognition.

All the C-terminal amidated peptides were synthetized starting from a Fmoc-RinkAmide resin type (0.48 mmol/g or 0.59 mmol/g) (Fig. 49a), characterized by a linker between the resin grain and the first amino acid of the peptide chain which leaves an amide bond on that position after the cleavage reaction, as previously reported in Scheme 3. The acetylation reaction was performed when the products were still attached to the resin and protected on their side chains, using acetic anhydride.



(a)



(b)

Figure 49 – (a) Fmoc-RinkAmide resin and (b) Fmoc-Thr(tBu) Wang resin linkers

The synthesis of the unprotected peptides on both the terminal positions were performed using Wang type resins, commercially available with one amino acid previously loaded on them and a linker between the resin grain and this amino acid which leaves the carboxylic group unmodified after the cleavage reaction. In particular, according to the amino acid sequence of the $PT_{[K159]}$ peptide, a Fmoc-Thr(tBu) Wang resin 0.7 mmol/g was chosen (Fig. 49b). Furthermore, also the $PT_{[K159]}$(AX) was synthetized, both in its protected and unprotected versions on the terminal positions, in order to investigate the role of a different haptenation using one of the most common β-lactam antibiotic nowadays.

The synthesis of all the haptenated peptides was performed following the so called "building block strategy". This strategy the is based on the insertion of a carefully designed modified amino acid in the peptide sequence through classical peptide synthesis steps. In our case, the synthetic building blocks were composed by the selected amino acid (Lys) covalently bind to the hapten (PG or AX). Otherwise, modified peptides can be synthetized following the so called "convergent strategy", involving the direct insertion of the modification on the dedicated side chain after the complete synthesis of the peptide, taking advantage of the protecting groups chemistry. The synthesis of the two building blocks Lys(PG) and Lys(AX), performed by the group of Professor Joseph from BioCIS, Université Paris-Sud, CNRS, Université Paris-Saclay, was previously reported in literature. [180] These modified amino acids were introduced in the synthesized sequences as protected Fmoc-L-Lys(PG)-OH and protected Fmoc-L-Lys(AX)-OH (Fig. 50), according to the Fmoc/tBu SPPS strategy.

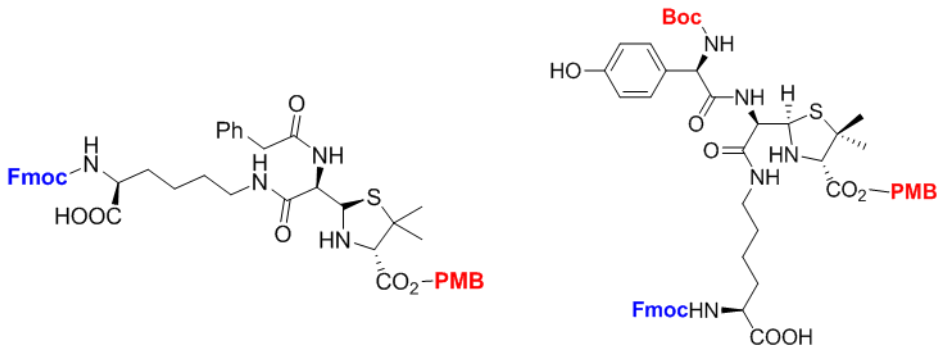


Figure 50 - Fmoc-L-Lys(PG)-OH and Fmoc-L-Lys(AX)-OH

All the syntheses were performed through the automatic solid phase peptide synthesis (SPPS) approach assisted by microwaves and in particular using the Fmoc/tBu strategy. The automatic synthesizer used was the Liberty Blue (CEM). Each coupling was performed using 2.5 ml of a 0.2 M amino acid solution in DMF, 1 ml of a 0.5 M activator solution (N,N'-diisopropylcarbodiimide, DIC) in DMF and 0.5 ml of a 1 M activator base solution (ethyl cyano(hydroxyimino)acetate, OxymaPure) in DMF.

All the cleavage reactions from the respective resin and the deprotection of all the side chains of the amino acids were performed in one step exposing the resin to a TFA 95% solution (TFA/H_2O/TIS 95:2.5:2.5) for 3h at RT. H_2O and triisopropylsilane (TIS) were used as scavengers in order to avoid secondary reactions during the cleavage phase.

All the crude peptides were purified through 2 purification steps, one of pre-purification or desalting step and one of semi-preparative purification using a RP-HPLC, following the General procedure for peptide purification and characterization. Finally, they were characterized via ESI-MS.

Due to the extremely lipophilic character of these peptides, a 60%-40% H_2O (+0.1% TFA)-CH_3CN 84% (+0.1% TFA) plus 2/3 drops of MeOH solution was required to solubilize them in order to perform the desalting step.

In the following Table 15 are reported all the details about the characterization, performed by ESI-MS, for all the synthesized products.

PEPTIDE	PURITY DEGREE	GRADIENT (0.6 ml/min)	Rt (min)	ESI-MS (m/z) $(M+2H)^{2+}$ Found (calculated)
Ac-PT(PG)-NH_2	85% (diastereoisomers)	30%-70% B	5.40 + 5.52[a]	1089 (1088)
H-PT(PG)-OH	>99%	30%-70% B	3.73 [a]	1069 (1068)
Ac-FS(PG)-NH_2	90%	30%-70% B	4.93 [a]	1043 (1042)
Ac-KV(PG)-NH_2	85% (diastereoisomers)	30%-70% B	3.27 + 3.35 [a]	1080 (1079)
Ac-PT(AX)-NH_2	>99%	30%-70% B	4.05 [a]	1104 (1105)
H-PT(AX)-NH_2	90%	30%-70% B	4.48 [a]	1084 (1084)
Ac-PT-NH_2 Non-Hapt	90%	20%-60% B	5.42 [a]	923 (922)
H-PT-OH Non-Hapt	90%	20%-60% B	4.07 [a]	902 (901)
Ac-FS-NH_2 Non-Hapt	85%	20%-60% B	4.33 [a]	876 (875)
Ac-KV-NH_2 Non-Hapt	90%	20%-60% B	2.92 [a]	913 (912)

Solvents used: A 0.1% TFA in H_2O; B 0.1% TFA in CH_3CN 84%

a: Phenomenex Jupiter C18 (5 lm, 2504.6 mm), 0.6 mL/min

Table 15 - Products characterization

2.2.1 Peculiarities of the synthesis of amoxicillinated peptides

The synthesis of H-PT$_{[K159]}$(AX)-OH on the Fmoc-Thr(tBu) Wang resing characterized by a loading of 0.7 mmol/g showed an incomplete coupling of the used building block Lys(AX) (Fig. 51). This was hypothesized to be due to steric reasons. Indeed, the amoxicillin substrate is bigger than the penicillin one and apparently this leads to an incomplete coupling of the Lys(AX) on resins with a loading higher than 0.5 mmol/g.

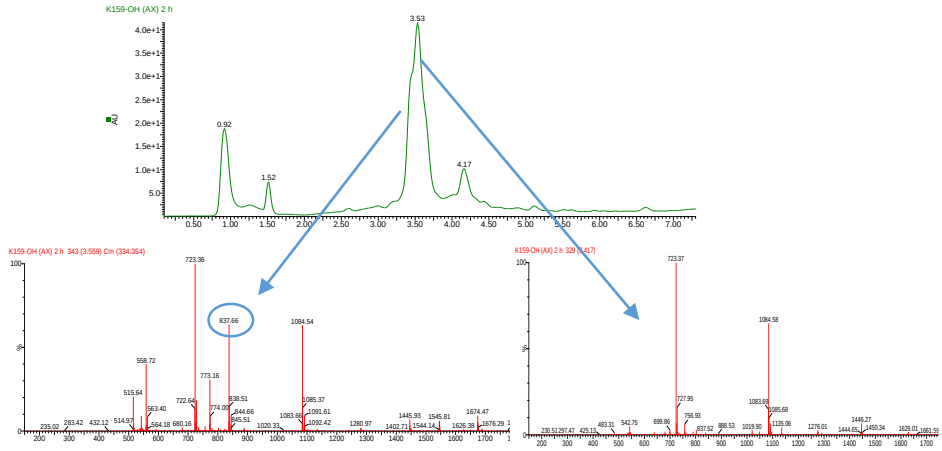


Figure 51 - The incomplete coupling of Lys(AX) during the synthesis of H-PT$_{[K159]}$(AX)-OH on Fmoc-Thr(tBu) Wang resin 0.7 mmol/g

Furthermore, the high chemical-physical similarity of the two products makes the separation of them relatively difficult via semi-preparative RP-HPLC. In fact, by synthesizing 15-mer peptides containing the building block Lys(AX) but using lower loading resins (<0.5 mmol/g), the disappearing in the ESI-MS analysis of the peak relative to the deletion sequence lacking Lys(AX) was highlighted.

2.3 Acetylation reaction of the N-terminus

Following the General procedure for the acetylation reaction, all the acetylation reactions of the amino group in N-terminus position were performed covering the resin with the minimum volume of DMF, adding 100 µL of Ac_2O each 200 mg of resin and leaving the mixture stirring for 10' at RT. The solution was filtered and replaced with a fresh one, for another 10'. The resin was washed 3 times with DCM and the acetylation checked by Kaiser test.

2.4 Peculiarities of the cleavage from the resin for haptenated peptides

As reported by Meng *et al.,* [179] the β-lactam core can bind an amino group, like in Lys(PG) or Lys(AX) case, forming two different adduct, the (5R, 6R) and the (5R, 6S), diastereoisomers among them. Moreover, an epimerization reaction can occur converting one to each other. This epimerization was described in acid conditions but aa consequence of the catalytic effect of HSA itself on the rearrangement of these structures. [187]

The SPPS through the Fmoc/tBu strategy is followed by a cleavage step of the synthetized peptide chains from the resin, which also involves the deprotection of all the amino acid side chains from their respective protecting groups. Following the concept of "orthogonality" of the protecting groups, this step take place consequently in opposite conditions with respect of the deprotection of the N-terminal Fmoc group (piperidine 20% in DMF). Indeed, the cleavage and the deprotection of all the side chains of the amino acids are performed exposing the resin to a TFA 95% solution for 3h at RT.

In this conditions, the epimerization reaction of the penicillin or amoxicillin substrates seems to take place randomly and not depending on the amino acid sequence of the modified peptide. As previously showed in Table 15, we observed this phenomenon two times, in particular after the cleavage of peptides **Ac-PT$_{[K159]}$(PG)-NH$_2$** and **Ac-KV$_{[K525]}$(PG)-NH$_2$**. The epimerization reaction appears evident already afterwards less than 1h in TFA ambient and the two diastereoisomers that derive from that are easily identifiable via ESI-MS. Unfortunately, they are definitely not separable through the two purification steps we performed on each crude peptide. In Figure 52 is reported the ESI-MS analysis of the pure **Ac-PT$_{[K159]}$(PG)-NH$_2$**; the two diastereoisomers are represented by two distinguishable peaks with exactly the same molecular mass.

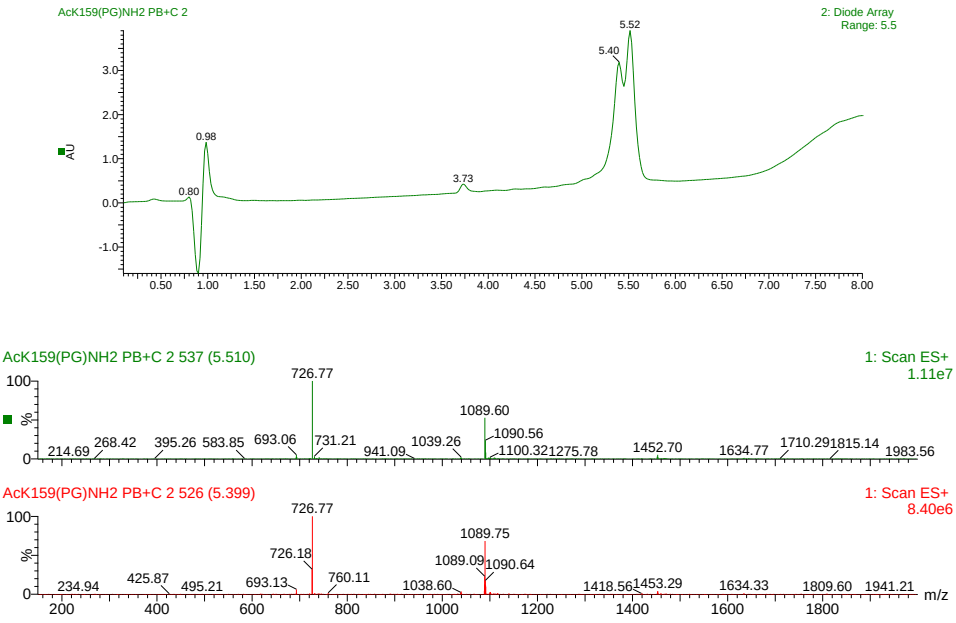


Figure 52 - UV spectra derived from ESI-MS analysis of pure Ac-PT$_{[K159]}$(PG)-NH$_2$, MW: 2176 g/mol. $[M+2H]^{+2}$ and $[M+3H]^{+3}$ are reported

2.5 Schematic procedure of IFN-γ ELISpot experiments

2.5.1 Cellular stimulation

The synthesized products showed before were finally used to perform IFN-γ ELISpot experiments in order to verify their possible interaction with specific T-cell lines belonging to patients which show allergic reactions to penicillins. The identification of this interaction would confirm the role of these peptides and the portions of HSA they are mimicking as epitopes in the adverse reaction to antibiotics. Figure 53 shows in detail the entire followed procedure for different cells isolation from blood samples and cellular stimulation with different antigens, performed by the group of Professor Pallardy, INSERM UMR 996, Université Paris-Sud, Université Paris-Saclay, Chatenay-Malabry, France, with our haptenated and non-haptenated peptides.

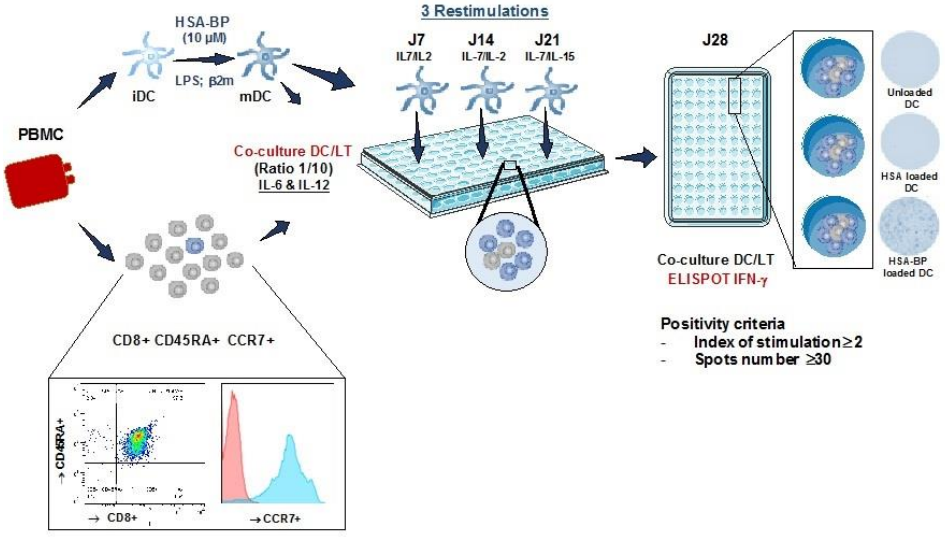


Figure 53 - Schematic cells isolation and cellular stimulation protocols for IFN-γ ELISpot experiments

In order to obtain Dendritic Cells (DC) or Antigen Presenting Cells, starting from patients' blood samples, Peripheral Blood Mononuclear Cells (PBMC) were separated by centrifugation. This step is necessary because of the lack of a sufficient concentration of DC into blood to perform the IFN-γ ELISpot experiments. PBMC are able to express $CD14^+$, white blood cells or monocytes. $CD14^+$ were then magnetically separate from the rest adding a $CD14^+$ specific magnetic-labeled antibody and using a magnetically activated column. After washing, the magnet was removed and the $CD14^+$ separately eluted. The magnetic-labeled antibody biodegrades. $CD14^+$ were then incubated, using AIM5 as medium, with two different cytokines, the Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin-4 (IL-4), in order to induce the differentiation into DC. Differently, $CD4^+$ cells are commonly present in blood and can be easily isolated using a $CD4^+$ specific magnetic-labeled antibody.

In case of bioconjugated antigens such as PG-HSA or protein antigens such as KLH (used as negative control), a pre-incubation with the obtained DC is necessary in order to permit the assimilation of these kind of antigens by them. During this pre-incubation step, immatureDC (iDC) assimilate the antigen becoming matureDC (mDC), able to present the antigen to T-cells. On the contrary, peptide antigens do not require this step, being DC already able to present them to T-cells. Different $CD4^+$ T-cell lines, 32 in our case, were then stimulated adding the antigen(s) to be tested and iDC, in case of peptide antigens, or mDC, in case of bioconjugate or protein antigens, into the wells of the plates, creating the so called "co-culture". 3 more further steps of stimulation on the T-cell lines were performed using new DC and new antigen(s) aliquots respectively on day 7, day 14 and day 21 from the 1^{st} stimulation.

2.5.2 IFN-γ ELISpot experiments

The IFN-γ ELISpot protocol provides for 6 fundamental steps:

a) Coating of the cytokine (IFN-γ) specific antibody
b) Incubation of T-cells ($CD4^+$) and DC (co-cultural approach)
c) IFN-γ capture by the immobilized antibody
d) Addition of the secondary antibody
e) Addition of the substrate
f) Revelation

a) COATING OF THE CYTOKINE (IFN-γ) SPECIFIC ANTIBODY

The monoclonal IFN-γ specific antibody was immobilized on ethanol treated PVDF membrane plates.

b) INCUBATION OF T-CELLS ($CD4^+$) AND DC (CO-CULTURAL APPROACH)

After the coating of the Ab, a blocking step of the free remained sites in the wells was performed using BSA 1% in PBS for 2h. Antigen(s), T-cells and DC were prepared in the meantime.

b.1. Preparation of peptide antigens

Peptides were dissolved in 10% DMSO in PBS 500 µM and diluted 1:100, obtaining final DMSO concentration less than 0.1%, the limit tolerated by cells. In the first phase, haptenated peptides were tested as 3-peptide or 6-peptide pools, testing in parallel the corresponding non-haptenated peptide pool. Only in case of positive results with one or more T-cell lines, the peptides which made up the pool will be tested separately.

However, mixing the 3-peptide or 6-peptide solutions containing the 0.1% of DMSO in order to obtain the pool leads to concentrations of DMSO which exceed the tolerance limit for the cells. This procedure is the reason why we decided to perform more complete solubility tests on these peptides in order to reduce the initial percentage of DMSO and, consequently, respecting the tolerance limit after the creation of the pool. Solubility tests results are reported below.

b.2. Preparation of T-cells

After the stimulation phase previously described, an aliquot of each one of the 32 T-cell lines was centrifugated, washed and suspended in AIM5.

b.3. Preparation of DC

A count step to verify the number of living cells present in the sample is fundamental in order to add to each well about 5000 living DC. DC were then suspended in AIM5.

Plates were then washed and 3 wells were assigned to each T-cell line following this scheme:

- 1st well: 100 µl DC solution + 15 µl T-cells solution
- 2nd well: 15 µl of non-haptenated peptide pool solution + 100 µl DC solution + 15 µl T-cells solution
- 3rd well: 15 µl of haptenated peptides pool solution + 100 µl DC solution + 15 µl T cells solution

Plates were then incubated overnight at RT.

c) IFN-γ CAPTURE BY THE IMMOBILIZED ANTIBODY

During the overnight incubation period, the DC present the antigens to $CD4^+$. In case of one or more T-cell lines are reactive to the presented antigens, cytokines are produced and captured by the immobilized specific antibody.

d) ADDITION OF THE SECONDARY ANTIBODY

After washing the plates, another blocking step was performed. Then, the ExtrAvidin-alkaline phosphatase-labelled secondary antibody solution was added and incubated 1h at RT. If cytokines have been produced and captured by the primary antibody, this secondary antibody will be able to bind them.

e) ADDITION OF THE SUBSTRATE

Plates were washed and the substrate solution was added. After 5 minutes, spots will be also easily identified to the naked eye.

f) REVELATION

The number of the spots was finally evaluated through a plates reader coupled to a specific software.

Figure 54 shows the preliminary results obtained with one of the immunodominant sequences highlighted by Azoury et al., in particular peptides Ac-$PT_{[K159]}$(PG)-NH_2, H-$PT_{[K159]}$(PG)-OH and their respective non-haptenated forms Ac-PT-NH_2 and H-PT-OH as negative controls. The presence of a greater number of spots in the wells containing the haptenated antigens is easily appreciable, supporting the theory about the role of the haptenation on this

specific HSA-derived sequences in the onset of allergic reactions to β-lactam antibiotics.

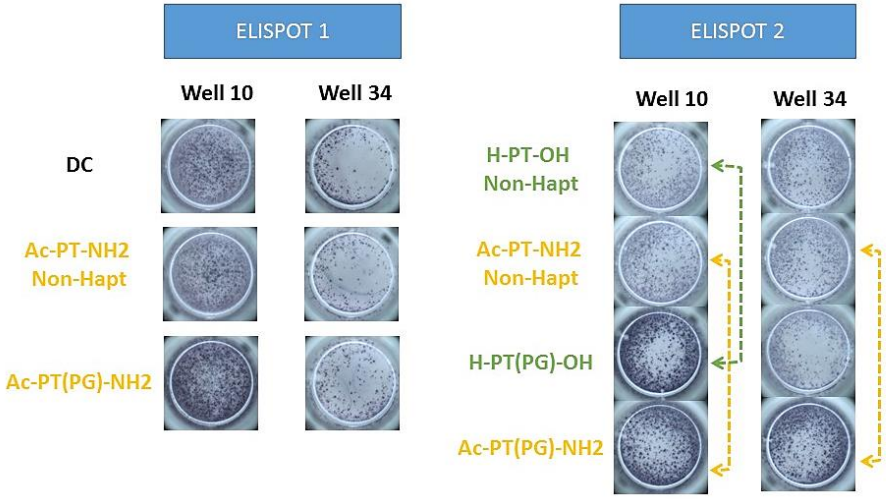


Figure 54 - Preliminary ELISpot results

2.6 Solubility tests

We performed solubility tests on all the synthesized peptides in order to reduce the initial percentage of DMSO necessary for their solubilization and, consequently, respecting the tolerance limit for the cells after the creation of the 3-peptide or 6-peptide pool. The obtained results are reported in Table 16a, 16b and 16c.

Lys	NAME	SEQUENCE	RESULTS
K159	Ac-PT$_{[K159]}$(**PG)-**NH$_2$	Ac-PELLFFAK*RYKAAFT-NH$_2$	DMSO **3%** in PBS buffer: Poorly Soluble DMSO **5%** in PBS buffer: Poorly Soluble
K159	H-PT$_{[K159]}$(**PG)-**OH	H-PELLFFAK*RYKAAFT-OH	DMSO **3%** in PBS buffer: Not Soluble DMSO **5%** in PBS buffer: Not Soluble
K525	Ac-KV$_{[K525]}$**(PG)**-NH$_2$	Ac-KERQIKK*QTALVELV-NH$_2$	DMSO **3%** in PBS buffer: Soluble DMSO **5%** in PBS buffer: Soluble
K212	Ac-FS$_{[K212]}$**(PG)-**NH$_2$	Ac-FGERAFK*AWAVARLS-NH$_2$	DMSO **3%** in PBS buffer: Not Soluble DMSO **5%** in PBS buffer: Not Soluble

Table 16a - Solubility test results on PG haptenated peptides

Lys	NAME	SEQUENCE	RESULTS
K159	Ac-PT$_{[K159]}$**(AX)-**NH$_2$	Ac-PELLFFAK*RYKAAFT-NH$_2$	DMSO **3%** in PBS buffer: Poorly Soluble DMSO **5%** in PBS buffer: Poorly Soluble
K159	H-PT$_{[K159]}$**(AX)-**OH	H-PELLFFAK*RYKAAFT-OH	DMSO **3%** in PBS buffer: Not Soluble DMSO **5%** in PBS buffer: Not Soluble

Table 16b – Solubility test results on AX haptenated peptides

Lys	NAME	SEQUENCE	RESULTS
K159	Ac-PT-NH_2	Ac-PELLFFAKRYKAAFT-NH_2	DMSO **3%** in PBS buffer: Soluble DMSO **5%** in PBS buffer: Soluble
K159	H-PT-OH	H-PELLFFAKRYKAAFT-OH	DMSO **3%** in PBS buffer: Poorly Soluble DMSO **5%** in PBS buffer: Poorly Soluble
K525	Ac-KV-NH_2	Ac-KERQIKKQTALVELV-NH_2	DMSO **3%** in PBS buffer: Soluble DMSO **5%** in PBS buffer: Soluble
K212	Ac-FS-NH_2	Ac-FGERAFKAWAVARLS-NH_2	DMSO **3%** in PBS buffer: Soluble DMSO **5%** in PBS buffer: Soluble

Table 16c - Solubility test results on non-haptenated peptides

3. CONCLUSIONS

The in depth study of allergic reactions to penicillins is fundamental principally because of the large use of this kind of antibiotics in everyday life. Our novel approach proposes to investigate the role of the post-translational modification performed by these molecules on the HSA using mimetic synthetic peptide probes properly modified with penicillin G or amoxicillin. Compared with proteins, peptides are indeed easier to synthetize, stock, handle and modified.

All the products showed before were synthetized, purified and obtained with a purity degree between 85% and 99% in the Interdepartmental Laboratory of

Peptide & Protein Chemistry & Biology (PeptLab) in the Chemistry Department "Ugo Schiff" of the University of Florence, Italy. As indicated before, the synthesis of haptenated peptides using Lys(AX) building block have to be necessarily performed on resins characterized by a loading <0.5 mmol/g. During the cleavage step of all kind of haptenated peptides (both penicillinated and amoxicillinated ones) an epimerization reaction could occur randomly. Following the synthetic strategy described before, in particular the "building block" strategy coupled to the Fmoc/tBu strategy to synthetize these modified peptides, the showed epimerization reaction turns out to be inevitable when it happens.

Preliminary IFN-γ ELISpot results obtained using some of our haptenated peptides showed positive results with 2 of the 32 T-cell lines tested, compared with the respective non-haptenated ones. These results are very promising in order to better understand the molecular mechanisms on which this pathologic condition is based on and the role of the selected fragments as putative epitopes. They also represent a confirmation of the power and the versatility of this approach and of the use of peptides as antigen probes for the study of many different diseases.

The solubility tests we performed on all the haptenated and non-haptenated products highlight that we would be able to reduce the DMSO percentage necessary for the solubilization of non-haptenated ones except for the H-PT-OH product; on the contrary, only Ac-$KV_{[K525]}$(PG)-NH_2 over all the haptenated products shows an acceptable solubility in PBS solutions containing less than 10% of DMSO. These results suggest to use these peptides singularly instead of mixed in pools for biological tests, in order to respect the tolerance limit of toxic organic solvents for the cells.

CONCLUSIONS

Peptides are commonly described as very versatile and powerful compounds. Furthermore, endogenous and exogenous peptides and proteins are considered one of the most common and important role player in most known diseases. These features allow them to be largely used both in therapeutic and diagnostic approaches. Moreover, peptide synthesis can be efficiently performed through many different strategies depending on the different nature of the substrate, increasing the versatility of their use.

The aim of this PhD work is to demonstrate the advantages of the use of peptides in the study of pathological conditions. Five different diseases have been selected: two well-known autoimmune diseases, such as Type 1 Diabetes and Psoriasis, two genetic diseases, such as Fabry Disease and Rett syndrome, and one allergic condition, such as the adverse reaction to antibiotics. The different nature of the base mechanisms which characterize those pathologies is fundamental in order to demonstrate the versatility of the potentially diagnostic and therapeutic use of peptides.

The main concept of this work is the so called “Chemical Reverse Approach”, which is based on the synthesis of modified and non-modified peptides and proteins to screen them as synthetic antigenic probes depending on the circulating autoantibodies founded in patients’ sera (Reverse), followed by an optimization of the chemical properties of these probes (Chemical).

After a general introduction about the immune system, the nature of the different types of antigens and the use of peptides and protein fragments as mimetics of proteins, this manuscript presents five diseases: Type 1 Diabetes, Psoriasis, Fabry Disease, Rett syndrome, and the allergic reaction to antibiotics.

The first chapter describes Type 1 Diabetes. We successfully synthesized two different versions of three peptide fragments, two derived from the human Glutamic Acid Decarboxylase 65 and 67 and one derived from the viral Protein 2c belonging to the Coxsackie virus B4. The first version was used as antigenic probe to recognize autoantibodies specific of this condition performing ELISA experiments on diabetic patients' sera compared with Normal Blood Donors; the second one was used to create conjugates with the carrier protein KLH in order to immunize mice. The CVB4 is indeed commonly related with the pathogenesis of T1D but their relationship has never been demonstrated. Our results highlighted how the homologies in the amino acid sequences of these three fragments play a key role in the supposed mimetic mechanism according to which autoantibodies are not able to properly differentiate the viral sequence from the enzymatic ones, because of their epitope nature. This leads to a dysregulation of glucose metabolism.

The second chapter deals with Psoriasis. We successfully optimized the synthesis of the human LL37 antimicrobial peptide which is supposed to be involved in the pathogenesis of this disease and some related conditions, such as Psoriatic Arthritis and Rheumatoid Arthritis. Our preliminary results are very promising and show how using the LL37 peptide sequence as probe in ELISA experiments is possible to correctly differentiate the major part of PsA and RA patients' sera from NBD.

The third chapter describes Lysosomal Storage Diseases and in particular Fabry Disease. Using mass spectrometry and SPR technologies, we and our co-workers successfully identified the putative epitope of the endogenous enzyme α-Galactosidase A, which is directly involved in the pathogenesis and the development of the disease. Furthermore, the epitope fraction has been correctly synthesized and screened using SPR, compared with shorter fragments

derived from it and the complete recombinant α-Galactosidase A. The very similar affinity for the commercial monoclonal anti-α-Gal A showed by our synthetic epitope and the recombinant enzyme is a very relevant result to demonstrate the ability of peptides to mimic proteins and to develop alternative or complementary therapeutic approaches to the Enzyme Replacement Therapy.

The fourth chapter deals with Rett syndrome. Because of the still unclear nature of this disease and its similarities with pathological conditions strongly related with bacterial infections, such as Multiple Sclerosis and the non-typable *Haemophilus influenzae*'s infection, we decided to use a well-known glucosylated protein probes derived from the bacterial protein HMW1, called HMW1ct, as probe to recognize putative autoantibodies in Rett syndrome and related conditions, such as Pervasive Development Disorder, in order to clarify the pathogenesis mechanisms of these diseases. We successfully express the modified and non-modified protein probes and used them in ELISA experiments on Rett syndrome and PDD patients' sera, compared with NBD. Our results represent a first indication of the possible presence of specific antibodies directed against the bacterial protein in these diseases.

The fifth chapter investigates the active role of β-lactam antibiotics in the modification of endogenous protein substrates which leads to allergic reaction to these molecules. We and our co-workers successfully identified and synthesized several peptide probes derived from the modified Human Serum Albumin. These peptides have been used as antigenic probes in ELISpot experiments on T-cell lines from patients who show allergic symptoms. Our results show the key role of some of these sequences in the interaction with the T-cell lines, highlighting their nature as epitopes.

In conclusion, this manuscript reports the synthesis and the screening of many different peptide and protein fragments. Considering different types of pathological conditions, characterized by different pathogenesis and development mechanisms, this work shows how peptides can be used to study several aspects of these diseases, sometimes providing completely innovative perspective and new informations about how they work and which kind of players are involved. Moreover, this manuscript also aims to demonstrate the ability of peptides to correctly mimic proteins. Peptides are indeed more easily synthesizable, storable and manageable than proteins. A deep demonstration of the possibility to use them as mimetic of bigger substrates in diagnosis, prognosis and therapeutic approaches is fundamental in order to develop novel research and clinical approaches.

EXPERIMENTAL PART

1. MATERIAL AND METHODS

All the chemicals were purchased from Sigma-Aldrich (Milan, Italy), Carlo Erba (Milan, Italy) and used without further purification. N,N-dimethylformamide (DMF) for peptide synthesis and CH_3CN for peptide HPLC purification and characterization were from Carlo Erba or Sigma-Aldrich. OxymaPure, N,N'-Diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA) and triisopropylsilane (TIS) for peptide synthesis were from Iris Biotech GmbH (Marktredwitz, Germany) and Sigma-Aldrich. KLH carrier protein was from Sigma-Aldrich. All the protected amino acids were from Sigma-Aldrich and Carbosynth (Compton, UK) and resins were purchased from Iris Biotech GmbH, Sigma-Aldrich and CEM Corporation (Cologno al Serio, Italy). The following side-chain protected amino acids were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(OtBu)-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(OtBu)-OH, Fmoc-Val-OH. Microwave assisted micro-cleavages were performed with a microwave apparatus CEM DiscoverTM single-mode MW reactor equipped with Explorer-48 autosampler (CEM Corporation). Solid Phase Peptide Syntheses were performed using the automatic peptide synthesizers CEM LibertyBlue (CEM Corporation). Spot Liquid Chromatography Flash (SLCF) was performed using a Li-Chroprep C18 column on an Armen Instrument working at 20 mL/min. The solvent systems used were: A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). Semi-preparative purifications via RP-HPLC were performed by a Phenomenex Jupiter C18 (250 × 4.6 mm) column at 25 °C using a Waters 2669 instrument working at 4 mL/min,

with the respective linear gradients. The solvent systems used were: A (0.1% TFA in H_2O) and B (0.1 % TFA in CH_3CN). Analytical RP-HPLC-MS system is a 2695 Alliance Chromatography (Waters) with a SUPELCO C18 (100x3 mm, 2,7µm) at 30 °C, working at 0.6 mL/min, with the respective linear gradients, coupled to a single quadrupole ESI-MS (Micromass). The solvent systems used were: A (0.1% TFA in H_2O) and B (0.1 % TFA in CH_3CN). Peptides were lyophilized using an Edwards Modulyo or a 5Pascal Lio5P lyophilizer.

2. GENERAL PROCEDURE FOR SOLID PHASE PEPTIDE SYNTHESIS ASSISTED BY MICROWAVES

All the coupling reactions were performed using the LibertyBlue automatic peptide synthesizer (CEM), previously described in Materials and Methods, using a Fmoc/tBu protection scheme. Each amino acid coupling step was characterized by two main steps which are reported in Table 17, coupled to the initial resin-swelling step:

Synthetic step	Reagents and Solvents	MW protocol and reaction times
Swelling	DMF	120 sec
Fmoc-deprotection	20 % piperidine in DMF	1° cycle: 15 sec, 75°C, 155W 2° cycle: 30 sec, 90°C, 30W
Coupling	**AA** in DMF (0.2M) **OximaPure** in DMF (1M) **DIC** in DMF (0.5M)	**Single (Standard) coupling:** a) 15 sec, 75°C, 170W b) 110 sec, 90°C, 30W **Double Arg coupling:** a) 1500 sec, 25°C, 0W b) 120 sec, 75°C, 30W **50° coupling (His/Cys):** a) 120 sec, 25°C, 0W b) 140 sec, 50°C, 35W

Table 17 - General coupling and swelling steps details

Each step was followed by 3 washes with 4 ml of DMF.

2.1 General procedure for cleavage and micro-cleavage

All the cleavage from the resin and side chain deprotection reactions were performed using a well-defined cleavage mixture (TFA/TIS/H2O, 95:2.5:2.5

v/v/v). The peptide-resin was treated for 3 h with the mixture (1 mL each 100 mg of resin) at RT under stirring. The resin was filtered off and the solution is concentrated under N_2 flow. The peptide was precipitated, using cold Et_2O, centrifuged and lyophilized.

Micro-cleavages were performed assisted by microwaves in order to reduce reaction times. The peptide-resin was treated with the already mentioned cleavage mixture. Micro-cleavage reactions were performed through a CEM DiscoverTM single-mode (CEM Corporation) treating the resin at 45°C using 15W for 15 min. The resin was then filtered off and the solution is concentrated under N_2 flow. The peptide was finally precipitated, using cold Et_2O, centrifuged and analyzed by HPLC-MS.

2.2 General procedure for peptide purification and characterization

The first purification step (desalting step) for peptides was performed by Spot Liquid Chromatography Flash (SLCF) in order to remove salts and impurities. It was used an RP-C18 LiChroprep columns on an automatic Armen Instrument. Three main steps were carried out before the injection of the product: column washings with CH_3CN (3 column volumes); column conditioning with H_2O (3 column volumes); dissolving the peptide in H_2O or a mixture of H_2O/CH_3CN, depending on its solubility (1 column volume). Peptides dissolved in water were absorbed on the solid phase and eluted with different H_2O/CH_3CN gradients, depending on their chemical properties. Fractions are checked by analytical RP-HPLC ESI-MS; product-containing homogeneous ones are pooled together and lyophilized.

The second purification step was carried out by semi-preparative RP-HPLC. The column was conditioned with the selected H_2O/CH_3CN gradient, depending on the chemical properties of the peptide, for 15 minutes at 4 ml/min. Maximum 20 mg of peptide were dissolved in 2 ml of H_2O or H_2O/CH_3CN mixture, injected and eluted with the chosen gradient. Fractions were checked by analytical RP-HPLC ESI-MS and product-containing homogeneous ones are pooled together and lyophilized. All the peptides were obtained with a purity ≥ 95% to be used for ELISA experiments.

3. GENERAL PROCEDURE FOR ACETYLATION REACTION

All the acetylation reactions of the amino group in N-terminus position were performed covering the resin with the minimum volume of DMF, adding 100 µL of Ac_2O each 200 mg of resin and leaving the mixture stirring for 10' at RT. The solution was then filtered and replaced with a fresh one, for another 10'. After removing again the solution, the resin was finally washed 3 times with DCM and the acetylation checked by Kaiser test.

4. GENERAL PROCEDURE FOR KAISER TEST

Kaiser test was performed always on small amounts of peptide-resin, which were placed in a test tube. Three drops for each of the following solutions were then added: ninhydrine (5 g) in ethanol (100 mL); phenol (80 g) in ethanol (20 mL); KCN (2 mL of 1 mM aqueous solution) in pyridine (98 mL). The tube was heated at 100 °C in a sand bath for 5 min. Four different results can be observed: [188]

- **Colorless or faint blue color**: complete coupling or acetylation;
- **Dark blue solution but beads are colorless**: nearly complete coupling or acetylation, eventually extend coupling or cap again unreacted chains;
- **Solution is light blue but beads are dark blue**: coupling or acetylation incomplete, perform the reaction again;
- **Solution is intense blue and all beads are blue**: totally failed coupling or acetylation, perform the reaction again.

5. GENERAL PROCEDURE FOR SP-ELISA EXPERIMENTS

The general protocol we used for ELISA experiments provides for a 3-days procedure.

- DAY ONE: COATING. A 1 mg/ml solution of the synthetic peptide in H2O was prepared. A 1:100 dilution was then carried out using a properly selected Coating Buffer, depending on the isoelectric point of the peptide (Carbonate Buffer, 12 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6; or PBS Buffer, 80 g NaCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, 2.0 g KCl, 10 L H_2O, pH 7.2), then 100 µl/well of the solution were dispensed in a 96-well NUNC Maxisorp plate, in order to have about 1 µg of the antigen in each well. Plates were finally incubated at 4°C overnight.
- DAY TWO: ADDITION OF THE SERA. Plates were washed 3 times with a Washing Buffer solution (0.9% NaCl, 0.01% Tween 20) and blocked 1h at RT with 100 µl/well of Fetal Bovine Serum (FBS) Buffer (10% FBS in Washing Buffer). Meantime, 1:100 solutions of each serum in FBS Buffer were prepared. The FBS blocking solution was then

removed from the plate and 100 µl/well of the aforementioned sera solutions were dispensed. Blank were always added for first in their respective wells in all the plates, and were obtained using just FBS Buffer. Plates were finally incubated at 4°C overnight.

- DAY THREE: ADDITION OF THE SECONDARY AB AND REVELATION. Plates were washed 3 times with the Washing Buffer solution. Then 100 µl/well of secondary Ab solution were added (anti-*h*IgG: 1:8000 in FBS Buffer, anti-*h*IgM 1:200 in FBS Buffer) and plates were incubated 3h at RT. Plates were then washed 3 times with the Washing Buffer solution and 100 µl/well of the Substrate Solution (1mg/ml p-nitrophenylphosphate in Substrate Buffer: 1M diethanolamine, 1 mM $MgCl_2$, pH 9.8) were dispensed. Plates were incubated for 15-40 min at RT and then absorbance at 405 nm of each well was read with a spectrophotometer.

Absorbance value for each serum was calculated as (mean absorbance of triplicate) – (mean absorbance of blank triplicate).

6. EXPERIMENTAL PART: CHAPTER ONE

6.1 General procedure for peptide-Maleimide Activated KLH conjugation

The commercial vial, containing 5 mg of Maleimide Activated KLH lyophilized from 10 mM sodium phosphate buffer, pH 6.6, with 0.115 mM NaCl, 1 mM EDTA, and 40 mM sucrose as stabilizer, was open to release the vacuum. The content was then reconstituted using 1 ml of MilliQ H_2O in order to obtain a 5 mg/ml solution of maleimide activated KLH in 20 mM sodium phosphate buffer with 230

mM NaCl, 2 mM EDTA, and 80 mM sucrose, pH 6.6. The solution was used immediately. The commercial conjugation buffer was reconstituted with 10 ml of water to obtain a solution of 20 mM sodium phosphate buffer with 100 mM EDTA and 80 mM sucrose, pH 6.6.

4 mg of the cysteine-containing peptide (about 1500 Da) were dissolved in 0.5 ml of conjugation buffer or alternatively in water, depending on the solubility of the product. 50 µl of the peptide solution were retained and stored at 4°C in order to determine the conjugation efficiency. The peptide solution was immediately mixed with the reconstituted Maleimide Activated KLH solution. After a quick degassing step using a gentle N_2 stream for 2 minutes, the solution was maintained under stirring at 4°C overnight. 100 µl of the conjugation solution were then taken to determine the coupling efficiency through the Ellman's test.

6.2 General procedure for Ellman's test

A 0.1 M sodium phosphate, 0.15 M NaCl and 0.1 M EDTA, pH 7.2 solution (conjugation buffer) was prepared and 200 µl of it dispensed into each well to be used of a 96-well NUNC Maxisorp plate. 10 µl of the peptide solution before conjugation were added to the appropriate wells in duplicate. 10 µl of the reaction mixture after the conjugation were added to a different set of wells. 20 µl of the 1 mg/ml Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), which reacts with sulfhydryls to form a highly colored chromophore having an absorbance maximum at 412 nm) were added to each well including one containing only the buffer (220 µl) to use as a blank. The plate was incubated 15 minutes at room temperature and then the absorbance measured at 410 nm.

7. EXPERIMENTAL PART: CHAPTER TWO

7.1 General procedure for protein expression

Protein fragment HMW1ct and the enzyme ApHMW1C were expressed in *E. coli* BL21 cells engineered with plasmid pET-45a (+) (Novagen), encoding for the fragment HMW1ct and equipped with the gene for carbenicillin resistance and plasmid pET-24a (+) (Novagen), encoding for the glucosyltransferase enzyme ApHMW1C and equipped with the gene for kanamicin resistance.

Plasmids were inserted inside the cells through a thermic shock. Cells growth was monitored measuring the optical density (OD) with an UV instrument (Amersham Biosciences, Little Chalfont, UK). Cells cultures were prepared using Luria-Bertani (LB) culture soils; the LB medium (SOC) liquid soil was composed by 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl dissolved in 1 L of H_2O MilliQ, while the LB Agar Plates soil was composed by 2 g of tryptone, 1 g of yeast extract, 2 g of NaCl and 3 g of Agar dissolved in 200 ml of H_2O MilliQ. 25 µL of carbenicillin, in case of expression of HMW1ct, and also 100 µL of kanamycin, in case of expression of HMW1ct(Glc), were added before the solidification. Lysis buffer (pH 7.5) was composed by 5.96 g of HEPES (50 mM), 2.92 g of NaCl (100 mM) and 50 ml of glycerol (10%) dissolved in 0.5 L of H_2O MilliQ.

The expression of the protein probe in BL21 E. coli bacterial cells was performed through the following steps:

a. Cells were coated putting in the center of the plate with LB Agar Plates soil, containing the antibiotic(s), 5 µL of mother solution and distributing them uniformly. The plate was incubated overnight at 37°C in order to allow the growth of the bacterial colonies;

b. The pre-culture phase was performed picking up one single circular and isolated colony and putting it in 5 ml of LB medium (SOC) liquid soil containing the antibiotic(s). The solution was incubated again overnight at 37°C under stirring;
c. The inoculation was occurred transferring the pre-culture solution in 1 L of the same LB medium (SOC) liquid soil containing 250 µL of carbenicillin and, eventually, 500 µL of kanamycin. The solution was incubated for about 3 h under stirring at 37 °C. The bacterial growth was quantified using an UV instrument and measuring the optical density (OD, 600 nm). The same LB medium (SOC) liquid soil was used as blank;
d. When the OD value was about 0.6, the induction of the expression was performed adding 1 ml of isopropyl-β-D-1-thiogalactopyranoside (IPTG). The solution was incubated overnight at 16°C under slight stirring.

Cells were recovered through centrifugation at 4000 rpm for 30 min at 4°C. The supernatant was eliminated and the pellet suspended in 20 ml of lysis buffer. It was centrifuged again and only the pellet stored at -20°C.

7.2 General procedure for protein purification

The pellet was suspended in 30 ml of lysis buffer adding 10 µL/g of cells of a protease inhibitor (cocktail Set III EDTA-free). The lysis of the cell membrane was carried out using an immersion sonicator performing 3 cycles 1 minute each one with 40% of amplitude. The lysis solution was then centrifuged for 110 min at 35000 rpm and the supernatant containing the product(s) was recovered. The purification was performed using an Äkta FPLC system.

During the first purification step a Hi Trap-His column was used with the binding buffer A1 for Hi Trap-His (20 mM Tris buffer, 0.5 M NaCl, 30 mM imidazole, pH 7.4) and the elution buffer B1 for Hi Trap-His (20 mM Tris buffer, 0.5 M NaCl, 0.5 M imidazole, pH 7.4).

The conditioning of the column was performed using buffer A1 for 10 minutes. The supernatant containing the products was then injected and eluted with a gradient from 0% to 100% of buffer B1. The UV detector was set to 280 nm and 215 nm. All the fraction obtained were analyzed through Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis technique (SDS-PAGE).

The separation of HMW1ct(Glc) from ApHMW1C was obtained in the second purification step through the ion exchange technique. A Hi Trap Q-FF column was used. A buffer exchange in order to substitute buffer B1 with binding buffer A2 (20 mM Tris buffer, 20 mM NaCl, pH 8) for Hi Trap Q-FF was performed using Falcon Amicon Ultra Centrifugal Filters characterized by a pore size MWCO<30 kDa. The Hi Trap Q-FF column was then conditioned with buffer A2 for 10 minutes. The sample was injected and eluted using a gradient from 0% to 100% of elution buffer B2 (20 mM Tris buffer, 1 M NaCl, pH 8) for Hi Trap Q-FF. The UV detector was set to 280 nm and 215 nm. All the fraction obtained were analyzed through Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis technique (SDS-PAGE).

Both HMW1ct and HMW1ct(Glc) were stocked in PBS buffer (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 e 0.24 g of KH_2PO_4 dissolved in 1 L of H_2O Milli-Q) at -20°C. Their concentration was calculated using the Lambert-Beer law after an absorption measure performed using an UV spectrometer set to a range from 320 and 240 nm.

7.3 General procedure for SDS-PAGE

The SDS-PAGE gel was prepared by depositing between two glasses the running gel 16% solution, composed by 1.6 ml H_2O MilliQ, 4.27 ml 30% acrylamide, 2 ml 1.5M tris buffer pH 8.8, 80 µl 10% SDS, 80 µl 10% ammonium persulfate (APS), 10 µl tetramethylethylenediamine (TEMED). After the polymerization, the stacking gel 4% solution (1.8 ml H_2O MilliQ, 0.4 ml 30% acrylamide, 0.750 ml 0.5M tris buffer pH 6.8, 30 µl 10% SDS, 30 µl 10% APS, 6 µl TEMED) was deposited above the previous one inserting the comb for the formation of the wells. After the polymerization, the gel was positioned inside the SDS-PAGE apparatus and the tank buffer 1x (100 mL of Tris buffer/Glycine/SDS (10x) in 1 L) was added. 10 µL of each sample were combined with 5 µL of loading buffer 5x (200mM of Tris-Cl (pH 6,8), 400mM of DTT, 8% of SDS, 0,4% of bromophenol blue and 40% of glycerol), treated to 100°C for a few minutes and centrifuged. Each sample was then loaded in the dedicate well. The commercial marker PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa, was used as reference. The electrophoresis was performed for 120 min at 100 mV and subsequently the gel subjected to stain using a Comassie solution (25 ml H_2O MilliQ, 20 ml MeOH, 5 ml AcOH, 0.05 g Comassie blue dye) for 30 min. In order to remove the excess dye, the stained gel aws treated overnight with a destain solution (700 ml H_2O MilliQ, 200 ml MeOH, 100 ml AcOH) under slight stirring.

7.4 General procedure for inhibition assays

a. Coating: all the wells in the plate were functionalized with the antigen solution in coating buffer characterized by a concentration of 1 mg/ml. Each well was filled with 100 µl of the antigen solution ad the plate incubated overnight at 4°C. Each serum was tested both with

HMW1ct(Glc) and HMW1ct as antigens in triplicate. Each experiment was performed two times and the average of those results was reported;

b. Washing: plates were washed 3 times with the washing buffer solution;
c. Blocking: the aspecific binding sites were blocked with 100 µl of FBS buffer at RT for 2 h;
d. Addition of the sera: sera were diluted 1:200 in FBS buffer. They were mixed with different solutions characterized by increasing concentrations of antigen (1E-11, 1E-10, 1E-9, 1E-8, 1E-7, 1E-6, 1E.5). 100 µl/well of each serum+antigen solution were added to the corresponding 3 wells. The plate was incubated for 1 h at RT;
e. Washing: plates were washed 3 times with the washing buffer solution;
f. Addition of the secondary antibody: 100 µl/well of secondary antibody solution were added to the respective plate which was then incubated for 3 h at RT.
g. Washing: plates were washed 3 times with the washing buffer solution;
h. Addition of the substrate solution: 100 µl/well were added. P-NPP is a chromogen on which the alkaline phosphatase, conjugated with the secondary antibody, acts. The enzyme reacts specifically with this substrate, converting it into a yellow colored product. The intensity of the coloring of each well qualitatively indicate the concentration of IgM or IgG antibodies in the examined sample;
i. Reaction blocking: the reaction was blocked adding 50 µl/well of NaOH 1 M;
j. Reading: the absorbance values were registered through a Tecan-Sunrise spectrophotometer working at 405 nm.

ABBREVIATIONS

Ab = antibody

Abs = absorbance

Ag = antigen

APC = antigen presenting cells

BCR = B-Cell receptor

DCM = dichloromethane

DMF = N,N-dimetilformamide

DNA = deoxyribonucleic acid

ESI-MS = electrospray ionization mass spectrometry

Fab = fragment antigen binding

FBS = fetal bovine serum

Fc = fragment crystallizable

Fmoc = fluorenylmethyloxycarbonyl protecting group

FPLC = Fast protein liquid chromatography

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA = human leukocyte antigen

HMW1 = adhesin (high-molecular-weight proteins)

HMW1C = cytoplasmic N-glycosyltransferase

HMW1ct = adhesin C-terminal portion

HPLC = high performance liquid chromatography

Ig = immunoglobuline

IL-23 = Interleukin-23

IMAC = immobilized metal ion affinity chromatography

IPTG = Isopropyl β-D-1-thiogalactopyranoside

LB = Luria Bertani

MHC = Major Histocompatibility Complex

OD = optical density

RNA = ribonucleic acid

SDS-PAGE = sodium dodecyl sulphate - polyacrylamide gel electrophoresis

MS = multiple sclerosis

NK = natural killer

CNS = central nervous system

SPE = solid phase extraction

SP-ELISA = solid phase enzyme linked immunosorbent assay

SPPS = solid phase peptide synthesis

TCR = T-cell receptor

TEMED = N,N,N',N'-Tetramethylethylenediamine

TFA = trifluoroacetic acid

TGS = Tris-Glycine-SDS

TIS = triisoproprylsilane

TLR = toll-like receptor

Treg = T regulator limphocytes

PTM = post-translational modifications

T1D = type 1 diabetes

T2D = type 2 diabetes

LADA = latent autoimmune diabetes in adults

*h*GAD = human glutamic acid decarboxylase

PsO = psoriasis

PsA = psoriatic arthritis

RA = rheumatoid arthritis

FD = Fabry disease

ERT = enzyme replacement therapy

A-Gal A = human α-galactosidase A

PDD = pervasive development disorder

PG = penicillin G

AX = amoxicillin

DC = dendritic cells

LC = Langerhans cells

KC = keratinocytes

pAbs = polyclonal antibodies

mAbs = monoclonal antibodies

CVB4 = Coxsackie virus B4

GABA = γ-aminobutyric acid

NMRI = Naval Medical Research Institute

NBD = normal blood donors

KLH = keyhole limpet hemocyanin

Glc = glucose

AMP = antimicrobial peptides

*h*CAP18 = human cathelicidin

OxymaPure = ethyl (hydroxyimino)cyanoacetate

DIC = diisopropyl carbodiimide

LSD = lysosomal storage disorders

MPS = mucopolysaccharidoses

GD = Gaucher disease

PD = Pompe disease

SRT = substrate reduction therapy

CCT = chemical chaperone therapy

GT = gene therapy

REA = residual enzyme therapy

GPA = gene promoter therapy

CHO = Chinese hamster ovary

BP = benzylpenicillin

HSA = human serum albumin

REFERENCES

[1] R. J. POLJAK, «Structure of antibodies and their complexes with antigens,» *Molecular immunology,* vol. 28, n. 12, pp. 1341-1345, 1991.

[2] E. WITEBSKY et al., «Chronic thyroiditis and autoimmunization,» *Journal of the American Medical Association,* vol. 164, n. 13, pp. 1439-1447, 1957.

[3] G. S. COOPER et al., «Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases,» *Journal of autoimmunity,* vol. 33, n. 3-4, pp. 197-207, 2009.

[4] D. L. JACOBSON et al., «Epidemiology and estimated population burden of selected autoimmune diseases in the United States,» *Clinical immunology and immunopathology,* vol. 84, n. 3, pp. 223-243, 1997.

[5] J. H. CHO et al., «Genomics and the multifactorial nature of human autoimmune disease,» *New England Journal of Medicine,* vol. 365, n. 17, pp. 1612-1623, 2011.

[6] S. IKEHARA et al., «Organ-specific and systemic autoimmune diseases originate from defects in hematopoietic stem cells,» *Proceedings of the National Academy of Sciences,* vol. 87, n. 21, pp. 8341-8344, 1990.

[7] M. D. ROSENBLUM et al., «Mechanisms of human autoimmunity,» *The Journal of clinical investigation,* vol. 125, n. 6, pp. 2228-2233, 2015.

[8] S. KIVITY et al., «Infections and autoimmunity–friends or foes?,» *Trends in immunology,* vol. 30, n. 8, pp. 409-414, 2009.

[9] F. PRATESI et al., «Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti–citrullinated protein antibodies in rheumatoid arthritis,» *Arthritis & Rheumatism,* vol. 54, n. 3, pp. 733-741, 2006.

[10] S. VENTO et al., «Is there a role for viruses in triggering autoimmune hepatitis?,» *Autoimmunity reviews,* vol. 3, n. 1, pp. 61-69, 2004.

[11] K. BECH et al., «Cell-Mediated Immunity to Yersinia enterocolitica Serotype 3 in Patients with Thyroid Diseases,» *Allergy,* vol. 33, n. 2, pp. 82-88, 1978.

[12] Z.-Q. LIU et al., «Staphylococcal peptidoglycans induce arthritis,» *Arthritis Research & Therapy,* vol. 3, n. 6, p. 375, 2001.

[13] A. FLOREANI et al., «Environmental basis of autoimmunity,» *Clinical reviews in allergy & immunology,* vol. 50, n. 3, pp. 287-300, 2016.

[14] D. R. GETTS et al., «Virus infection, antiviral immunity, and autoimmunity,» *Immunological reviews,* vol. 255, n. 1, pp. 197-209, 2013.

[15] M. F. CUSICK et al., «Molecular mimicry as a mechanism of autoimmune disease,» *Clinical reviews in allergy & immunology,* vol. 42, n. 1, pp. 102-111, 2012.

[16] A. K. BURROUGHS et al., «Molecular mimicry in liver disease,» *Nature,* vol. 358, n. 6385, p. 377, 1992.

[17] G. S. G. SHERBET, «Bacterial infections and the pathogenesis of autoimmune conditions bacterial infections and the pathogenesis of autoimmune conditions,» *British Journal of Medical Practitioners,* vol. 2, n. 1, pp. 6-13, 2009.

[18] D. L. KAUFMAN et al., «Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus,» *The Journal of clinical investigation,* vol. 89, n. 1, pp. 283-292, 1992.

[19] M. T. WALVOORT et al., «Antibodies from multiple sclerosis patients preferentially recognize hyperglucosylated adhesin of non-typeable Haemophilus influenzae,» *Scientific reports,* vol. 6, p. 39430, 2016.

[20] R. A. HOUGHTEN et al., «Mixture-based synthetic combinatorial libraries,» *Journal of medicinal chemistry,* vol. 42, n. 19, pp. 3743-3778, 1999.

[21] R. FRANK, «The SPOT-synthesis technique: synthetic peptide arrays on membrane supports—principles and applications,» *Journal of immunological methods,* vol. 267, n. 1, pp. 13-26, 2002.

[22] J. EICHLER, «Peptides as protein binding site mimetics,» *Current opinion in chemical biology,* vol. 12, n. 6, pp. 707-713, 2008.

[23] A. M. PAPINI, «The use of post-translationally modified peptides for detection of biomarkers of immune-mediated diseases,» *Journal of peptide science: an official publication of the European Peptide Society,* vol. 15, n. 10, pp. 621-628, 2009.

[24] BIOMARKERS DEFINITIONS WORKING GROUP et al., «Biomarkers and surrogate endpoints: preferred definitions and conceptual framework,» *Clinical Pharmacology & Therapeutics,* vol. 69, n. 3, pp. 89-95, 2001.

[25] R. D. G. LESLIE et al., «Diabetes classification: grey zones, sound and smoke: Action LADA 1,» *Diabetes/metabolism research and reviews,* vol. 24, n. 7, pp. 511-519, 2008.

[26] V. P. Singh et al., «Advanced Glycation end products and diabetic complications,» *The Korean Journal of Physiology & Pharmacology,* vol. 1, n. 1, p. 18, 2014.

[27] M. A. ATKINSON et al., «Type 1 diabetes: new perspectives on disease pathogenesis and treatment,» *The Lancet,* vol. 358, n. 9277, pp. 221-229, 2001.

[28] R. BACCHETTA et al., «Defective regulatory and effector T cell functions in patients with FOXP3 mutations,» *The Journal of clinical investigation,* vol. 116, n. 6, pp. 1713-1722, 2006.

[29] J. NERUP et al., «HL-A antigens and diabetes mellitus.,» *The Lancet,* vol. 304, n. 7885, pp. 864-866, 1974.

[30] G. I. BELL et al., «A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes melliitus,» *Diabetes,* vol. 33, n. 2, pp. 176-183, 1984.

[31] N. BOTTINI et al., «A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes,» *Nature genetics,* vol. 36, n. 4, p. 337, 2004.

[32] J. TUOMILEHTO et al., «Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease,» *The Lancet,* vol. 343, n. 8910, pp. 1383-1385, 1994.

[33] M. C. HONEYMAN et al., «T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA-2: potential for mimicry with rotavirus and other environmental agents,» *Molecular Medicine,* vol. 4, n. 4, p. 231, 1998.

[34] C. M. FILIPPI et al., «Viral trigger for type 1 diabetes: pros and cons,» *Diabetes,* vol. 57, n. 11, pp. 2863-2871, 2008.

[35] M. LÖNNROT et al., «Antibody cross-reactivity induced by the homologous regions in glutamic acid decarboxylase (GAD65) and 2C protein of coxsackievirus B4,» *Clinical & Experimental Immunology,* vol. 104, n. 3, pp. 398-405, 1996.

[36] B. JAYAKRISHNAN et al., «An analysis of the cross-reactivity of autoantibodies to GAD65 and GAD67 in diabetes,» *PLoS One,* vol. 6, n. 4, p. e18411, 2011.

[37] S. AGCA et al., «Characterization of continuous B-cell epitopes in the N-terminus of glutamate decarboxylase67 using monoclonal antibodies,» *Journal of Peptide Science,* vol. 20, n. 12, pp. 928-934, 2014.

[38] F. e. a. ALI, «Stiff-person syndrome (SPS) and anti-GAD-related CNS degenerations: protean additions to the autoimmune central neuropathies,» *Journal of autoimmunity,* vol. 37, n. 2, pp. 79-87, 2011.

[39] M. G. ERLANDER et al., «Two genes encode distinct glutamate decarboxylases,» *Neuron,* vol. 7, n. 1, pp. 91-100, 1991.

[40] D. L. KAUFMAN et al., «Two forms of the γ-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions,» *Journal of neurochemistry,* vol. 56, n. 2, pp. 720-723, 1991.

[41] J. WEI et al., «Protein phosphorylation of human brain glutamic acid decarboxylase (GAD) 65 and GAD67 and its physiological implications,» *Biochemistry,* vol. 43, n. 20, pp. 6182-6189, 2004.

[42] Y. SHI et al., «Increased expression of GAD65 and GABA in pancreatic β-cells impairs first-phase insulin secretion,» *American Journal of Physiology-Endocrinology And Metabolism,* vol. 279, n. 3, pp. E684-E694, 2000.

[43] A. WENDT et al., «Glucose inhibition of glucagon secretion from rat α-cells is mediated by GABA released from neighboring β-cells,» *Diabetes,* vol. 53, n. 4, pp. 1038-1045, 2004.

[44] M. A. ATKINSON et al., «Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes,» *The Journal of clinical investigation ,* vol. 94, n. 5, pp. 2125-2129, 1994.

[45] M. S. HORWITZ et al., «Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry,» *Nature medicine,* vol. 4, n. 7, p. 781, 1998.

[46] S. E. DIPPE et al., «Lack of causal association between Coxsackie B4 virus infection and diabetes,» *The Lancet,* vol. 305, n. 7920, pp. 1314-1317, 1975.

[47] J.-W. YOON et al., «Virus-induced diabetes mellitus: isolation of a virus from the pancreas of a child with diabetic ketoacidosis,» *New England Journal of Medicine,* vol. 300, n. 21, pp. 1173-1179, 1979.

[48] S. JUHELA et al., «T-cell responses to enterovirus antigens in children with type 1 diabetes,» *Diabetes,* vol. 49, n. 8, pp. 1308-1313, 2000.

[49] S. J. RICHARDSON et al., «The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes,» *Diabetologia,* vol. 52, n. 6, pp. 1143-1151, 2009.

[50] J.-F. BACH, «The effect of infections on susceptibility to autoimmune and allergic diseases,» *New England journal of medicine,* vol. 347, n. 12, pp. 911-920, 2002.

[51] S. BRUGMAN et al., «Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes?,» *Diabetologia,* vol. 49, n. 9, pp. 2105-2108, 2006.

[52] O. VAARALA et al., «The “perfect storm” for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity,» *Diabetes,* vol. 57, n. 10, pp. 2555-2562, 2008.

[53] J. KARJALAINEN et al., «A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus,» *New England journal of medicine,* vol. 327, n. 5, pp. 302-307, 1992.

[54] A. J. MACFARLANE et al., «A type 1 diabetes-related protein from wheat (Triticum aestivum) cDNA clone of a wheat storage globulin, Glb1, linked to islet damage,» *Journal of Biological Chemistry,* vol. 278, n. 1, pp. 54-63, 2003.

[55] S. B. MOHR et al., «The association between ultraviolet B irradiance, vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide,» *Diabetologia,* vol. 51, n. 8, pp. 1391-1398, 2008.

[56] G. S. EISENBARTH, «Type I diabetes mellitus,» *New England Journal of Medicine,* vol. 314, n. 21, pp. 1360-1368, 1986.

[57] D. F. STEINER et al., «Insulin biosynthesis: evidence for a precursor,» *Science,* vol. 157, n. 3789, pp. 697-700, 1967.

[58] R. GOLDSBY et al., Immunology (5th ed.), WH Freeman and Company, 2003, pp. 57-75.

[59] A. SWAMINATHAN et al., «Keyhole limpet haemocyanin–a model antigen for human immunotoxicological studies,» *British journal of clinical pharmacology,* vol. 78, n. 5, pp. 1135-1142, 2014.

[60] F. O. NESTLE et al., «Skin immune sentinels in health and disease,» *Nature Reviews Immunology,* vol. 9, n. 10, p. 679, 2009.

[61] B. W. Sch¨on MP, «Psoriasis,» *N. Engl. J. Med.,* vol. 352, p. 1899–12, 2005.

[62] WHO, «World psoriasis day—document EB133.R2, agenda item 6.2.,» 2013.

[63] S. P. RAYCHAUDHURI et al., «A comparative study of pediatric onset psoriasis with adult onset psoriasis,» *Pediatric dermatology,* vol. 17, n. 3, pp. 174-178, 2000.

[64] J. M. GELFAND et al., «The risk of mortality in patients with psoriasis: results from a population-based study,» *Archives of dermatology,* vol. 143, n. 12, pp. 1493-1499, 2007.

[65] C. OLIVIER et al., «The risk of depression, anxiety, and suicidality in patients with psoriasis: a population-based cohort study,» *Archives of dermatology,* vol. 146, n. 8, pp. 891-895, 2010.

[66] J. M. H. MOLL et al., «Psoriatic arthritis. In: Seminars in arthritis and rheumatism,» *WB Saunders,* pp. 55-78, 1973.

[67] C. SALVARANI et al., «Isolated peripheral enthesitis and/or dactylitis: a subset of psoriatic arthritis,» *The Journal of rheumatology,* vol. 24, n. 6, pp. 1106-1110, 1997.

[68] W. TAYLOR et al., «Classification criteria for psoriatic arthritis: development of new criteria from a large international study,» *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology,* vol. 54, n. 8, pp. 2665-2673, 2006.

[69] A. P. ANANDARAJAH et al., «The diagnosis and treatment of early psoriatic arthritis,» *Nature Reviews Rheumatology,* vol. 5, n. 11, p. 634, 2009.

[70] O. B. PEDERSEN et al., «On the heritability of psoriatic arthritis. Disease concordance among monozygotic and dizygotic twins,» *Annals of the rheumatic diseases,* vol. 67, n. 10, pp. 1417-1421, 2008.

[71] W. WATSON et al., «The genetics of psoriasis,» *Archives of dermatology,* vol. 105, n. 2, pp. 197-207, 1972.

[72] R. C. TREMBATH et al., «Identification of a major susceptibility locus on chromosome 6p and evidence for further disease loci revealed by a two stage genome-wide search in psoriasis,» *Human molecular genetics,* vol. 6, n. 5, pp. 813-820, 1997.

[73] T. J. RUSSELL et al., «Histocompatibility (HL-A) antigens associated with psoriasis,» *New England Journal of Medicine,* vol. 287, n. 15, pp. 738-740, 1970.

[74] C. D. VEAL et al., «Identification of a novel psoriasis susceptibility locus at 1p and evidence of epistasis between PSORS1 and candidate loci,» *Journal of medical genetics,* vol. 38, n. 1, pp. 7-13, 2001.

[75] F. BRANDRUP et al., «Psoriasis in an unselected series of twins,» *Archives of dermatology,* vol. 114, n. 6, pp. 874-878, 1978.

[76] E. M. FARBER et al., «Natural history of psoriasis in 61 twin pairs,» *Archives of dermatology,* vol. 109, n. 2, pp. 207-211, 1974.

[77] H. VALDIMARSSON et al., «Psoriasis–as an autoimmune disease caused by molecular mimicry,» *Trends in immunology,* vol. 30, n. 10, pp. 494-501, 2009.

[78] S. P. RAYCHAUDHURI et al., «Revisiting the Koebner phenomenon: role of NGF and its receptor system in the pathogenesis of psoriasis,» *The American journal of pathology,* vol. 172, n. 4, pp. 961-971, 2008.

[79] M. D. HERTLE et al., «Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis,» *The Journal of clinical investigation,* vol. 89, n. 6, pp. 1892-1901, 1992.

[80] C.-C. CHU et al., «Harnessing dendritic cells in inflammatory skin diseases,» *Seminars in immunology,* pp. 28-41, 2011.

[81] F. O. NESTLE et al., «Characterization of dermal dendritic cells in psoriasis. Autostimulation of T lymphocytes and induction of Th1 type cytokines,» *The Journal of clinical investigation,* vol. 94, n. 1, pp. 202-209, 1994.

[82] M. MERAD et al., «Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells,» *Nature Reviews Immunology,* vol. 8, n. 12, p. 935, 2008.

[83] R. A. CLARK et al., «The vast majority of CLA+ T cells are resident in normal skin,» *The Journal of Immunology,* vol. 176, n. 7, pp. 4431-4439, 2006.

[84] C. ALBANESI et al., «Resident skin cells in psoriasis: a special look at the pathogenetic functions of keratinocytes,» *Clinics in dermatology,* vol. 25, n. 6, pp. 581-588, 2007.

[85] J. N. BARKER et al., «Keratinocytes as initiators of inflammation,» *The Lancet,* vol. 337, n. 8735, pp. 211-214, 1991.

[86] T. BANNO et al., «Unique keratinocyte-specific effects of interferon-gamma that protect skin from viruses, identified using transcriptional profiling,» *Antiviral therapy,* vol. 8, n. 6, pp. 541-554, 2003.

[87] G. K. PERERA et al., «Psoriasis,» *Annu. Rev. Pathol. Mech. Dis.,* vol. 7, pp. 385-422, 2012.

[88] J.-M. SCHRÖDER, «The role of keratinocytes in defense against infection,» *Current opinion in infectious diseases,* vol. 23, n. 2, pp. 106-110, 2010.

[89] T. GAMBICHLER et al., «Expression of antimicrobial peptides and proteins in etanercept-treated psoriasis patients,» *Regulatory peptides,* vol. 167, n. 2-3, pp. 163-166, 2011.

[90] M. BANDO et al., «Interleukin-1α regulates antimicrobial peptide expression in human keratinocytes,» *Immunology and cell biology,* vol. 85, n. 7, pp. 532-537, 2007.

[91] D. GANGULY et al., «Self-RNA–antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8,» *Journal of Experimental Medicine,* vol. 206, n. 9, pp. 1983-1994, 2009.

[92] K. P. GAYATHRI et al., «Psoriasis,» *Annual Review of Pathology: Mechanisms of Disease,* vol. 7, pp. 385-422, 2012.

[93] M. R. YEAMAN et al., «Mechanisms of antimicrobial peptide action and resistance,» *Pharmacological reviews,* vol. 55, n. 1, pp. 27-55, 2003.

[94] W. ERHART et al., «Induction of human β-defensins and psoriasin in vulvovaginal human papillomavirus–associated lesions,» *Journal of Infectious Diseases,* vol. 204, n. 3, pp. 391-399, 2011.

[95] V. NIZET et al., «Innate antimicrobial peptide protects the skin from invasive bacterial infection,» *Nature,* vol. 414, n. 6862, p. 454, 2001.

[96] O. E. SØRENSEN et al., «Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors,» *The Journal of Immunology,* vol. 170, n. 11, pp. 5583-5589, 2003.

[97] J. HARDER et al., «Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury,» *Journal of Investigative Dermatology,* vol. 130, n. 5, pp. 1355-1364, 2010.

[98] M. MARCINKIEWICZ et al., «The role of antimicrobial peptides in chronic inflammatory skin diseases,» *Advances in Dermatology and Allergology/Postępy Dermatologii i Alergologii,* vol. 1, n. 6, p. 33, 2016.

[99] S. H. WHITE et al., «Structure, function, and membrane integration of defensins,» *Current opinion in structural biology,* vol. 5, n. 4, pp. 521-527, 1995.

[100] J. HARDER et al., «A peptide antibiotic from human skin,» *Nature,* vol. 387, n. 6636, p. 861, 1997.

[101] F. NIYONSABA et al., «Antimicrobial peptides human β-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines,» *Journal of Investigative Dermatology,* vol. 127, n. 3, pp. 594-604, 2007.

[102] J. J. OPPENHEIM et al., «Roles of antimicrobial peptides such as defensins in innate and adaptive immunity,» *Annals of the rheumatic diseases,* vol. 62, n. suppl. 2, pp. ii17-ii21., 2003.

[103] P. MADSEN et al., «Molecular cloning, occurrence, and expression of a novel partially secreted protein "psoriasin" that is highly up-regulated in psoriatic skin,» *Journal of Investigative Dermatology,* vol. 97, n. 4, pp. 701-712, 1991.

[104] S. ALOWAMI et al., «Psoriasin (S100A7) expression is altered during skin tumorigenesis,» *BMC dermatology,* vol. 1, n. 1, p. 3, 2003.

[105] M. ZANETTI et al., «Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain,» *FEBS letters,* vol. 1, n. 1-5, p. 374, 1995.

[106] O. E. SØRENSEN et al., «Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3,» *Blood,* vol. 97, n. 12, pp. 3951-3959, 2001.

[107] G. H. GUDMUNDSSON et al., «The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes,» *European Journal of Biochemistry,* vol. 238, n. 2, pp. 325-332, 1996.

[108] M. SEIL et al., «Spotlight on human LL-37, an immunomodulatory peptide with promising cell-penetrating properties,» *Pharmaceuticals,* vol. 3, n. 11, pp. 3435-3460, 2010.

[109] T.-T. WANG et al., «Cutting edge: 1, 25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression,» *The Journal of Immunology,* vol. 173, n. 5, pp. 2909-2912, 2004.

[110] J. SCHAUBER et al., «The vitamin D pathway: a new target for control of the skin's immune response?,» *Experimental dermatology,* vol. 17, n. 8, pp. 633-639, 2008.

[111] K. YAMASAKI et al., «Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin,» *The FASEB journal,* vol. 20, n. 12, pp. 2068-2080, 2006.

[112] M. PAZGIER et al., «Structural and functional analysis of the pro-domain of human cathelicidin, LL-37,» *Biochemistry,* vol. 52, n. 9, pp. 1547-1558, 2013.

[113] Z. OREN et al., «Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity,» *Biochemical Journal,* vol. 341, n. Pt.3, p. 501, 1999.

[114] J. JOHANSSON et al., «Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37,» *Journal of Biological Chemistry,* vol. 273, n. 6, pp. 3718-3724, 1998.

[115] C.-C. LEE et al., «Transmembrane pores formed by human antimicrobial peptide LL-37,» *Biophysical journal,* vol. 100, n. 7, pp. 1688-1696, 2011.

[116] B. DING et al., «Physiologically-relevant modes of membrane interactions by the human antimicrobial peptide, LL-37, revealed by SFG experiment,» *Scientific reports,* vol. 3, p. 1854, 2013.

[117] D. XHINDOLI et al., «The human cathelicidin LL-37—A pore-forming antibacterial peptide and host-cell modulator,» *Biochimica et Biophysica Acta (BBA)-Biomembranes,* vol. 1858, n. 3, pp. 546-566, 2016.

[118] F. NEVILLE et al., «In situ characterization of lipid A interaction with antimicrobial peptides using surface X-ray scattering,» *Biochimica et Biophysica Acta (BBA)-Biomembranes,* vol. 1758, n. 2, pp. 232-240, 2006.

[119] L. A. LEWIS et al., «Phosphoethanolamine substitution of lipid A and resistance of Neisseria gonorrhoeae to cationic antimicrobial peptides and complement-mediated killing by normal human serum,» *Infection and immunity,* vol. 77, n. 3, pp. 1112-1120, 2009.

[120] D. VANDAMME et al., «A comprehensive summary of LL-37, the factotum human cathelicidin peptide,» *Cellular immunology,* vol. 280, n. 1, pp. 22-35, 2012.

[121] A. ELSSNER et al., «A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1β processing and release,» *The Journal of Immunology,* vol. 172, n. 8, pp. 4987-4994, 2004.

[122] J. M. KAHLENBERG et al., «Little Peptide, Big Effects: The Role of LL-37 in Inflammation and Autoimmune Disease,» *Journal of Immunology,* vol. 191, n. 10, pp. 4895-4901, 2013.

[123] P. Y. ONG et al., «Endogenous antimicrobial peptides and skin infections in atopic dermatitis,» *New England Journal of Medicine,* vol. 347, n. 15, pp. 1151-1160, 2002.

[124] R. LANDE et al., «The antimicrobial peptide LL37 is a T-cell autoantigen in psoriasis,» *Nature communications,* vol. 5, p. 5621, 2014.

[125] C. R. SCRIVER et al., «The metabolic and molecular bases of inherited disease,» *McGraw Hill. Inc, New York,* pp. 3367-3398, 1995.

[126] M. BECK et al., «Fabry disease: perspectives from 5 years of FOS,» *PharmaGenesis,* 2006.

[127] P. J. MEIKLE et al., «Prevalence of lysosomal storage disorders,» *Jama,* vol. 281, n. 3, pp. 249-254, 1999.

[128] B. WINCHESTER et al., «The molecular basis of lysosomal storage diseases and their treatment.,» *Biochemical Society Transactions,* vol. 28, n. 2, pp. 150-154, 2000.

[129] J. A. MINDELL, «Lysosomal acidification mechanisms,» *Annual review of physiology,* vol. 74, pp. 69-86, 2012.

[130] M. GRABE et al., «The mechanochemistry of V-ATPase proton pumps,» *Biophysical journal,* vol. 78, n. 6, pp. 2798-2813, 2000.

[131] F. M. PLATT et al., «Lysosomal storage disorders: The cellular impact of lysosomal dysfunction,» *J. Cell. Biol.,* vol. 199, n. 5, pp. 723-734, 2012.

[132] S. ORTOLANO et al., «Treatment of lysosomal storage diseases: recent patents and future strategies,» *Recent patents on endocrine, metabolic & immune drug discovery,* vol. 8, n. 1, pp. 9-25, 2014.

[133] S. C. GARMAN et al., «The molecular defect leading to Fabry disease: structure of human α-galactosidase,» *Journal of molecular biology,* vol. 337, n. 2, pp. 319-335, 2004.

[134] Y. A. IOANNOU et al., «Human α-galactosidase A: glycosylation site 3 is essential for enzyme solubility,» *Biochemical Journal,* vol. 332, n. 3, pp. 789-797, 1998.

[135] S. GARMAN, «Structure-function relationships in α-Galactosidase A,» *Acta Paediatr. Suppl.,* vol. 96, pp. 6-16, 2007.

[136] H. FABRY, «Angiokeratoma corporis diffusum–Fabry disease: historical review from the original description to the introduction of enzyme replacement therapy,» *Acta Paediatrica,* vol. 91, pp. 3-5, 2002.

[137] K. D. MACDERMOT et al., «Anderson-Fabry disease: clinical manifestations and impact of disease in a cohort of 98 hemizygous males,» *Journal of medical genetics,* vol. 38, n. 11, pp. 750-760, 2001.

[138] E. Schaefer et al., «Gal A Genotype and phenotype in Fabry disease: analysis of the Fabry Outcome Survey,» *Acta Paediatr. Suppl.,* vol. 94, pp. 87-92, 2005.

[139] F. Matsuzawa et al., «Fabry disease: correlation between structural changes in α-Galactosidase, and clinical and biochemical phenotypes,» *Hum. Genet.,* vol. 117, n. 4, pp. 317-28, 2005.

[140] Y. A. IOANNOU et al., «Overexpression of human alpha-galactosidase A results in its intracellular aggregation, crystallization in lysosomes, and selective secretion,» *The Journal of cell biology,* vol. 119, n. 5, pp. 1137-1150, 1992.

[141] S. PORTER, «Human immune response to recombinant human proteins,» *Journal of pharmaceutical sciences,* vol. 90, n. 1, pp. 1-11, 2001.

[142] G. E. LINTHORST et al., «Enzyme therapy for Fabry disease: neutralizing antibodies toward agalsidase alpha and beta,» *Kidney international,* vol. 66, n. 4, pp. 1589-1595, 2004.

[143] S. G. BANUGARIA et al., «Persistence of high sustained antibodies to enzyme replacement therapy despite extensive immunomodulatory therapy in an infant with Pompe disease: need for agents to target antibody-secreting plasma cells,» *Molecular genetics and metabolism,* vol. 105, n. 4, pp. 677-680, 2012.

[144] Z. KUKACKA et al., «Antibody Epitope of Human α-Galactosidase A Revealed by Affinity Mass Spectrometry: A Basis for Reversing Immunoreactivity in Enzyme Replacement Therapy of Fabry Disease,» *ChemMedChem,* vol. 13, n. 9, pp. 909-915, 2018.

[145] S. NAIDU, «Rett syndrome: a disorder affecting early brain growth,» *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society,* vol. 42, n. 1, pp. 3-10, 1997.

[146] N. SIRIANNI et al., «Rett syndrome: confirmation of X-linked dominant inheritance, and localization of the gene to Xq28,» *American journal of human genetics,* vol. 63, n. 5, p. 1552, 1998.

[147] B. HAGBERG, «Clinical manifestations and stages of Rett syndrome,» *Ment. Retard. Dev. Disabil. Res. Rev.,* vol. 8, n. 2, pp. 61-65, 2002.

[148] B. HAGBERG et al., «Rett Syndrome: A suggested staging system for describing impairment profile with increasing age towards adolescence,» *Am. J. Hum. Genet.,* vol. 25, n. S1, pp. 47-59, 1986.

[149] S. L. WILLIAMSON et al., «Rett syndrome: new clinical and molecular insights,» *European Journal of Human Genetics,* vol. 14, n. 8, p. 896, 2006.

[150] J. L. NEUL et al., «Rett syndrome: revised diagnostic criteria and nomenclature,» *Annals of neurology,* vol. 68, n. 8, pp. 944-950, 2010.

[151] G. DI DALMAZI et al., «Reactive oxygen species in organ-specific autoimmunity,» *Autoimmunity Highlights,* vol. 7, n. 1, p. 11, 2016.

[152] H. A. DOYLE et al., «Post-translational protein modifications in antigen recognition and autoimmunity,» *Trends in immunology,* vol. 22, n. 8, pp. 443-449., 2001.

[153] H. A. DOYLE et al., «Autoantigenesis: the evolution of protein modifications in autoimmune disease,» *Current opinion in immunology,* vol. 24, n. 1, pp. 112-118, 2012.

[154] R. G. SPIRO, «Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds,» *Glycobiology,* vol. 12, n. 4, pp. 43R-56R, 2002.

[155] F. SCHWARZ et al., «Cytoplasmic N-glycosyltransferase of Actinobacillus pleuropneumoniae is an inverting enzyme and recognizes the NXS/T consensus sequence,» *Journal of Biological Chemistry,* vol. M111, p. 277160, 2011.

[156] J. H. LIN et al., «Endoplasmic reticulum stress in disease pathogenesis,» *Annu. Rev. pathmechdis. Mech. Dis.,* vol. 3, pp. 399-425, 2008.

[157] Y. OHE et al., «Characterization of nucleotide pyrophosphatase-5 as an oligomannosidic glycoprotein in rat brain,» *Biochemical and biophysical research communications,* vol. 308, n. 4, pp. 719-725, 2003.

[158] S. GRASS et al., «The Haemophilus influenzae HMW1 adhesin is glycosylated in a process that requires HMW1C and

phosphoglucomutase, an enzyme involved in lipooligosaccharide biosynthesis,» *Molecular microbiology,* vol. 48, n. 3, pp. 737-751, 2003.

[159] J. GROSS et al., «The Haemophilus influenzae HMW1 adhesin is a glycoprotein with an unusual N-linked carbohydrate modification,» *Journal of Biological Chemistry,* vol. 283, n. 38, pp. 26010-26015, 2008.

[160] G. PACINI et al., «Epitope mapping of anti-myelin oligodendrocyte glycoprotein (MOG) antibodies in a mouse model of multiple sclerosis: microwave-assisted synthesis of the peptide antigens and ELISA screening,» *Journal of Peptide Science,* vol. 22, n. 1, p. 5, 2016.

[161] D. C. TURK, «The pathogenicity of Haemophilus influenzae,» *Journal of medical microbiology,* vol. 18, n. 1, pp. 1-16, 1984.

[162] K. J. STAPLES et al., «Relationships between mucosal antibodies, non-typeable Haemophilus influenzae (NTHi) infection and airway inflammation in COPD,» *PloS one,* vol. 11, n. 11, p. e0167250, 2016.

[163] I. J. W. ST GEME et al., «Secretion of the Haemophilus influenzae HMW1 and HMW2 adhesins involves a periplasmic intermediate and requires the HMWB and HMWC proteins,» *Molecular microbiology,* vol. 27, n. 3, pp. 617-630, 1998.

[164] S. J. BARENKAMP et al., «Cloning, expression, and DNA sequence analysis of genes encoding nontypeable Haemophilus influenzae high-molecular-weight surface-exposed proteins related to filamentous hemagglutinin of Bordetella pertussis,» *Infection and immunity,* vol. 60, n. 4, pp. 1302-1313, 1992.

[165] J. W. ST GEME et al., «High-molecular-weight proteins of nontypable Haemophilus influenzae mediate attachment to human epithelial cells,» *Proceedings of the National Academy of Sciences,* vol. 90, n. 7, pp. 2875-2879, 1993.

[166] J. W. S. GEME et al., «Prevalence and Distribution of the hmwand hia Genes and the HMW and Hia Adhesins among Genetically Diverse Strains of Nontypeable Haemophilus influenzae,» *Infection and immunity,* vol. 66, n. 1, pp. 364-368, 1998.

[167] S. GRASS et al., «The Haemophilus influenzae HMW1C protein is a glycosyltransferase that transfers hexose residues to asparagine sites in the HMW1 adhesin,» *PLoS pathogens,* vol. 6, n. 5, p. e1000919, 2010.

[168] J. W. S. GEME III et al., «A prototype two-partner secretion pathway: the Haemophilus influenzae HMW1 and HMW2 adhesin systems,» *Trends in microbiology,* vol. 17, n. 8, pp. 355-360, 2009.

[169] K.-J. CHOI et al., «The Actinobacillus pleuropneumoniae HMW1C-like glycosyltransferase mediates N-linked glycosylation of the Haemophilus influenzae HMW1 adhesin,» *PLoS One,* vol. 5, n. 12, p. e15888, 2010.

[170] F. KAWAI et al., «Structural insights into the glycosyltransferase activity of the Actinobacillus pleuropneumoniae HMW1C-like protein,» *Journal of Biological Chemistry,* vol. M111, p. 237602, 2011.

[171] A. NAEGELI et al., «Substrate specificity of cytoplasmic N-glycosyltransferase,» *Journal of biological chemistry,* vol. M114, p. 579326, 2014.

[172] Novagen, pET System Manual 11th Edition, p. 34.

[173] R. C. F. CHEUNG et al., «Immobilized metal ion affinity chromatography: a review on its applications,» *Applied microbiology and biotechnology,* vol. 96, n. 6, pp. 1411-1420, 2012.

[174] G. Healthcare, Strategies for Protein Purification Handbook.

[175] S. MAGDELDIN et al., Affinity chromatography: Principles and applications, Affinity Chromatography. InTech, 2012.

[176] L. C. Andersson et al., «Improved Purification of Histidine-tagged Proteins with Ni Sepharose High Performance,» *GE Healthcare,* pp. 11-0008-47 A, 2007.

[177] E. L. MILLER, «The penicillins: a review and update,» *Journal of Midwifery & Women's Health,* vol. 47, n. 6, pp. 426-434, 2002.

[178] N. L. LEE et al., «β-lactam antibiotic and β-lactamase inhibitor combinations,» *Jama,* vol. 285, n. 4, pp. 386-388, 2001.

[179] X. MENG et al., «Direct evidence for the formation of diastereoisomeric benzylpenicilloyl haptens from benzylpenicillin and benzylpenicillenic acid in patients,» *Journal of Pharmacology and Experimental Therapeutics,* vol. 338, n. 3, pp. 841-849, 2011.

[180] N. SCORNET et al., «Bioinspired Design and Oriented Synthesis of Immunogenic Site-Specifically Penicilloylated Peptides,» *Bioconjugate chemistry,* vol. 27, n. 11, pp. 2629-2645, 2016.

[181] M. E. AZOURY et al., «Identification of T-cell epitopes from benzylpenicillin conjugated to human serum albumin and implication in penicillin allergy,» *Allergy,* 2018.

[182] C. A. JANEWAY JR et al., Immunobiology: The Immune System in Health and Disease. 5th edition, Garland Science, 2001.

[183] E. PADOVAN et al., «Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy,» *European journal of immunology,* vol. 27, n. 6, pp. 1303-1307, 1997.

[184] D. GARZON et al., «Mass spectrometric strategies for the identification and characterization of human serum albumin covalently adducted by amoxicillin: ex vivo studies,» *Chemical research in toxicology,* vol. 27, n. 9, pp. 1566-1574, 2014.

[185] A. ARIZA et al., «Hypersensitivity reactions to β-lactams: relevance of hapten-protein conjugates,» *Journal of investigational allergology & clinical immunology,* 2015.

[186] C. NHIM et al., «Identification and frequency of circulating CD4+ T lymphocytes specific to benzylpenicillin in healthy donors,» *Allergy,* vol. 68, n. 7, pp. 899-905, 2013.

[187] B. SMITH et al., «Catalytic effect of serum albumin on the O-rearrangement of N-sulfooxy-2-acetylaminofluorene, a potential hepatocarcinogen in the rat, to nonmutagenic sulfuric acid esters of Oamidofluorenols,» *Biochem. Pharmacol.,* vol. 38, p. 3987–3994, 1989.

[188] G. B. FIELDS, Methods in Enzymology, Volume 289 Solid-Phase Peptide Synthesis, Academic Pres, 1997.

[189] V. M. LUNDGREN et al., «GAD antibody positivity predicts type 2 diabetes in an adult population,» *Diabetes,* vol. 59, n. 2, pp. 416-422, 2010.