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Effect of amino acid substitutions in alpha-gliadin peptides on their immunogenicity in coeliac disease

Beatriz Fernandes Côrte-Real

Dissertação

Mestrado em Biologia Humana e Ambiente

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Tese orientada por: Professor Doutor Paul John Ciclitira

Professora Doutora Deodália Dias

2015

FOREWORD

The work created in this thesis was elaborated at the department of Gastroenterology at St. Thomas Hospital London in collaboration with Kings College London.

It was co-oriented by Doctor Tanja Suligoj and Master Nika Japelj.

Results obtained in this work, were presented at the 16th International Coeliac Disease Symposium in Prague, held between 21 and 24 of June 2015, in a form of a poster. The poster was entitled ***'Abrogation of coeliac immunogenicity of gluten peptides by amino acid point substitutions'*** (Attachments I).

The Portuguese texts present in this document do not obey the new writing accord.

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SUMÁRIO

A doença celíaca, ou intolerância ao glúten, é uma enteropatia crónica do intestino delgado mediada pelo sistema imunitário, que é activada pela presença de glúten na dieta em indivíduos geneticamente predispostos a ela.

Na sua forma clássica, manifesta-se como uma inflamação na mucosa do intestino delgado que prejudica a arquitectura das vilosidades. Isto conduz à má absorção e, por sua vez, à perda de peso, diarreia e/ou deficiências de nutrientes, levando ao aparecimento de anemia e osteoporose. As complicações a longo prazo incluem aumento do risco de desenvolvimento de doenças autoimunes e malignidade.

A doença celíaca é tratada com uma dieta muito rigorosa isenta de glúten ao longo da vida do doente, o que envolve a eliminação de trigo, centeio e cevada da alimentação destes indivíduos. Alguns pacientes também são intolerantes à aveia. A prevalência é maior na Europa e na América do Norte, onde estima-se que afete 1% da população.

A maioria das pessoas que sofrem de intolerância ao glúten são positivas para o antígeno leucocitário humano (ALH) DQ2, enquanto que a minoria (5%) são ALH-DQ8 positivo. Moléculas ALH-DQ2 e DQ8 são um pré-requisito para a ligação e reconhecimento de peptídos de glúten. Estas moléculas favorecem resíduos de aminoácidos carregados negativamente em certas posições dos péptidos.

Proteínas de glúten que integram gliadinas e gluteninas são tóxicas para doentes celíacos. O tecido transglutaminase, uma molécula encontrada em vários tipos de tecidos e órgãos responsável por catalisar diferentes tipos de reacções, tem a capacidade de desaminar resíduos alvo de glutamina em péptidos de glúten, o que leva à conversão de glutamina neutra em ácido glutâmico de carga negativa. Péptidos desaminados de glúten são melhores imunogénicos para as células T que se ligam com uma maior afinidade a moléculas de ALH-DQ2 e DQ8 no ligando peptídeo alterado. Por sua vez, estas moléculas apresentam os péptidos de glúten às células T.

Em resposta à apresentação do antigénio, células T especificas CD4+ começam a proliferar e a segregar o interferão-γ (IFN-γ). O último induz o aumento do número de células T citotóxicas na lâmina própria. A doença celíaca é considerada como sendo uma enteropatia mediada por células T específicas de glúten.

Celíacos não tratados têm um risco aumentado de desenvolverem doenças malignas e auto-imunes. Uma dieta rigorosa sem glúten, é então fundamental para a sua saúde.

No entanto, a adesão a uma dieta isenta de glúten apresenta um grande desafio, já que

a dieta é muito restritiva. O glúten está presente em muitos alimentos processados (tais como sopas, molhos, doces, batatas fritas e pão) e alimentos sem glúten são menos agradáveis ao palato e mais caros, resultando numa insatisfação e desvantagem social dos pacientes com este tipo de intolerância. Estes factores, por sua vez, levam a uma baixa adesão a este tipo de dieta, que é essencial para o manejo clínico da doença.

Com este trabalho tivemos o objectivo de testar a potencial toxicidade de dois péptidos de glúten (DQ2.5-glia α3 simbolizado por α2a e DQ8-glia αI simbolizado por α3I) encontrados no trigo e das suas versões modificadas (α2c e α3II, respectivamente) em pacientes diagnosticados com a doença celíaca.

Para tal recorreu-se ao uso de técnicas de cultura celular, nomeadamente de cultura de células T. Estas células foram isoladas a partir de biopsias do intestino de pacientes diagnosticados como celíacos, e foram mantidas em cultura durante várias semanas (entre duas e quatro semanas). Após estimuladas com glúten ao longo das semanas e do número de células por cultura atingir um valor satisfatório ($\approx 100x10^4$ células), estas foram submetidas a um ensaio de proliferação na presença dos péptidos a testar e onde também se recorreu ao uso de radioactividade. A incorporação da radioactividade pelas células foi medida, os índices de estimulação (SI) calculados e as conclusões retiradas.

Biopsias recolhidas de vinte e quatro pacientes foram usadas para cultura de células T, submetidas a um ensaio de proliferação e os SI foram calculados. Destas vinte e quatro, apenas seis produziram resultados positivos e de possível conclusão. Das seis amostras apenas uma mostrou a potencial toxicidade de dois dos péptidos testados, o α2a e o α2c, apresentando valores de SI superiores a dois. Os restantes péptidos testados com esta cultura tiveram SI<2, o que representa falta de toxicidade. Para as restantes culturas de células, apenas uma apresentou SI>2 para o péptido α2a, apresentando SI<2 para os restantes péptidos. As outras quatro culturas tiveram sempre resultados negativos independentemente do péptido testado.

Os nossos resultados mostram que um dos péptidos testados, que resulta da substituição de vários aminoácidos de um péptido conhecido como tóxico, poderá também ele ser tóxico, levando à conclusão que estas substituições poderão não eliminar a 100% a toxicidade encontrada no péptido original. Apesar destes resultados, as conclusões tiradas estão longe de ser definitivas e novos testes e ensaios têm de ser realizados de modo a comprovar-se o que neste estudo se concluiu.

Palavras-chave: Doença celíaca, epítopos de glúten, toxicidade e estratégias terapêuticas

ABSTRACT

Coeliac disease (CD) is a chronic small intestinal immune-mediated enteropathy triggered by dietary gluten in genetically predisposed individuals.

Most CD sufferers are positive for human leucocyte antigen (HLA) DQ2, whereas a minority (5%) are HLA-DQ8 positive. HLA-DQ2 and -DQ8 molecules are a prerequisite for binding of gluten peptides; majority of gluten proteins that comprise gliadins and glutenins are CD-toxic. Tissue transglutaminase (tTG) deamidates target glutamine residues in gluten peptides, which leads to conversion of neutral glutamine to negatively charged glutamic acid. Deamidated gluten peptides are better immunogens for T cells as they bind with higher affinity to HLA-DQ2 and -DQ8 molecules on antigen presenting cells (APC).

The aim of this study was to test the potential toxicity of two different wheat epitopes and their variants (labelled as α2a, α2c, α3I and α3II) for patients diagnosed with CD.

Peptides were tested using small intestinal gluten sensitive T cells. Biopsies from twenty-four CD patients were collected, T cells isolated and stimulation indices (SI) obtained. The cells were stimulated with gluten every week. After, they were submitted to proliferation assays using the different peptides. Radioactivity was injected to each assay and the amount incorporated in the cells measured and expressed as SI.

From the twenty-four samples, only six developed positive results. In one assay, α2a and α2c peptides resulted as CD-toxic. The other tested peptides had SI below 2, which indicate they were no immunogenic. For the other cell lines, one of them also responded with SI above 2 for the peptide α2a but not for the remaining peptides. All the other four cell lines responded with SI below two for every peptide tested.

Our results show that the substitutions made in the core of the epitope DQ2.5-glia α3 (α2a) induces toxicity in some patients, which contradicts results from previous studies. On the other hand, the substitutions made to epitope DQ8-glia α1 appeared to eliminate the CD-toxicity of this epitope.

Despite these results, we can not take very strong conclusions and further tests and assays need to be made in order to completely ensure that our findings are correct.

Key words: Coeliac disease, gluten epitopes, toxicity, immunogenicity and therapeutic strategies

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LIST OF ABBREVIATIONS

%- Percentage

> - Higher

< - Lower

ºC- Celsius

µCi- Micro Curie

µm- Micrometre

µL- Microliter

g- Gram

L- Litter

mCi- Milli Curie

mL- Millilitre

mg- Milligram

M- Molar

α- Alpha

β- Beta

γ- Gamma

ω- Omega

δ- Delta

^{3}H- Tritiated thymidine

A

aa- Amino acid

ALH-Antigénio Leucocitário Humano

APC- Antigen Presenting Cells

APL- Altered Peptide Ligand

ASM- Autologous Serum Medium

B

BL- Blank

C

$CaCl_2$- Calcium Chloride

CD- Coeliac Disease

CO_2- Carbon Dioxide

Cpm- Counts Per Minute

CV-Coefficient of Variation

D

DMSO-Dimethyl Sulphoxide

E

ECH- Enterocyte Cell Height

EMA- Anti-endomysial Antibodies

G

GFD- Gluten Free Diet

GLU- Gluten

GWAS- Genome wide association studies

Gy- Gray

H

HLA- Human Leucocyte Antigen

HMWG- High Molecular Weight Glutenin

HCl- Hydrogen Chloride

I

IEL-Intra-Epithelial Lymphocytes

INF γ- Interferon Gamma

IL-2- Interleukin 2

L

LMWG- Low Molecular Weight Glutenin

M

MHC-Major Histocompatibility Complex

N

NaOH- Sodium Hidroxyde

NK- Natural Killer

O

O_2- Oxygen

OC- Organ Culture

OCM- Organ Culture Medium

P

PBMC- Peripheral Blood Mononuclear Cell

PBS- Phosphate Buffered Saline

Psi- Pound-force Per Square Inch

PT Gluten- Peptic-Tryptic Digest of Industrial Gluten

R

rpm- Revolutions per minute

RPMI- Rosewell Park Memorial Institute

S

SD- Standard Deviation

SI- Stimulation Index

SIL- Small Intestinal Lymphocytes

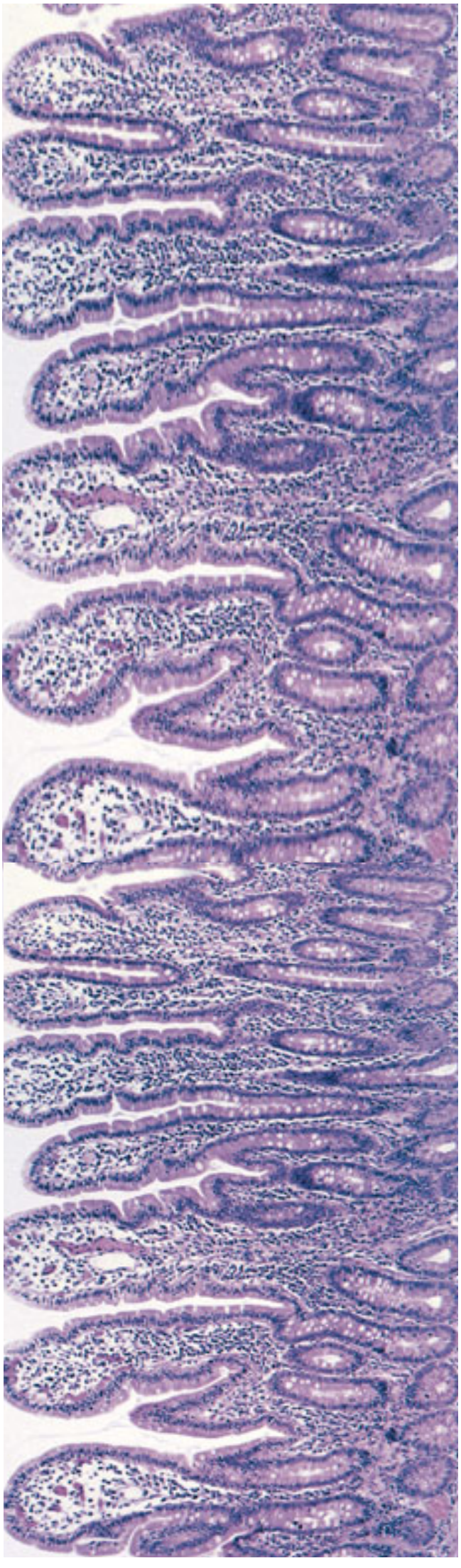
SNP- Single nucleotide polymorphism

T

TCR- T Cell Receptor

tTG-Tissue Transglutaminase

1. INTRODUCTION



Coeliac disease (CD) is an inflammatory autoimmune disorder that affects at least 1% of the adult population in many countries. It is characterised by small intestinal damage with loss of absorptive villi and hyperplasia of the crypts, leading to malabsorption. In addition to nutrient deficiencies, prolonged CD is associated with an increased risk for malignancy, especially intestinal T cell lymphoma (Dieterich *et al.,* 1997; Fraser & Ciclitira, 2001; Lichtwark *et al.,* 2014; Nasr *et al*., 2012). The disease is also known as sprue, coeliac sprue, gluten sensitive enteropathy and gluten intolerance (Ludvigsson *et al.,* 2013).

CD is precipitated and triggered by ingestion and exposure to gluten, a generic term to collectively describe all the cereal storage proteins in wheat, barley and rye, that are toxic for the individual, that is genetically predisposed to CD and that carries genes encoding HLA-DQ2 or HLA-DQ8 molecules (Dewar *et al.,* 2012; Fraser & Ciclitira, 2001; Ludvigsson *et al.,* 2013; Nasr *et al*., 2012). While the disease is primarily an intestinal disorder that is histologically characterised by a raised number of intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy, there is an increasing support for a broader concept of the condition being a systemic inflammatory disease (Lichtwark *et al.,* 2014).

1.1 Pathogenesis

1.1.1 Gluten

Some storage proteins found in wheat, barley and rye have been shown to cause intestinal inflammation in patients with CD. In some patients there were also found intolerance to oats, however it is only found in, approximately 50% of individuals with CD (Ciclitira, 2003; Dieterich *et al.,* 1997; Fraser & Ciclitira, 2001; Molberg *et al.,* 1998).

Wheat gluten proteins can be divided into two major groups: the glutenins and the prolamins (Fraser & Ciclitira, 2001). Prolamins, the aqueous alcohol soluble fraction of storage proteins, are one group responsible for triggering the disease (Wieser *et al.,* 2014). Wheat, barley and rye, being closely related, all contain prolamins with a high composition of glutamine and proline, whereas the prolamins of oats and more distantly related cereals contain less glutamine and proline and more alanine and leucine (Fraser & Ciclitira, 2001). Glutenins comprise subunits, which are connected with intramolecular disulphide bonds, to form polymers. They are insoluble in neutral aqueous solution or saline solution but can be extracted with a mixture of aqueous alcohol (such as 60% ethanol or 50% propanol) plus reducing agents (such as dithiothreitol) and disaggregating compounds (such as urea)

(Wieser *et al.,* 2014). The high levels of peptides from each of these groups in proline (10-15%) and glutamine (30-35%) residues contributes to their resistance against proteolysis in human gut, allowing them to reach the whole of the small intestine and crossing the epithelial barrier into the lamina propria, that lies bellow the epithelial barrier (Shan *et al.,* 2002).

Depending on the cereal, prolamins may be called gliadins from wheat, hordeins from barley, secalins from rye and avenins from oats (Bai *et al.*, 2013). The gliadins may be subdivided according to their relative electrophoretic mobility into α, β, γ and ω sub fractions; or according to their N-terminal amino acid (aa) sequence into α, β or ω sub fractions (Fraser & Ciclitira, 2001; Tighe & Ciclitira, 1995). CD toxicity has been shown to reside with all four classes of gliadins, although α-gliadins appear to have the greatest *in vivo* effects. Glutenins can be divided into two groups, high molecular weight (HMWG) and low molecular weight (LMWG). The HMWG contains proteins that are important for the baking quality of the flour (Bai *et al.*, 2013). The toxicity of this fraction was, previously, very hard to evaluate since the extraction and purification of glutenins was hard to achieve (Dewar *et al.,* 2006).

A total of thirty-four T cell epitopes derived from both of these protein groups have been identified and tested as toxic for CD patients, at this time. The majority of these epitopes are associated with binding to HLA-DQ2.5 followed by HLA-DQ8. The peptides tested in this work contain two different T cell epitopes previously determined to be CD toxic, the DQ2.5-glia α3 (FRPEQPYPQ) and the DQ8-glia α1 (EGSFQPSQE) (Sollid *et al.,* 2012). Vader *et al.,* (2002) were the first research group to demonstrate that the epitope DQ2.5-glia α3 (ie Glia α20 according to the previous nomenclature) was toxic for CD patients. Their experiments were undertaken with children diagnosed with CD from whom gluten specific T cell lines and clones were generated. They were able to characterize different new and toxic T cell epitopes, and the DQ2.5-glia α3 was among them (Vader *et al.,* 2002). Van de Wal *et. al* discovered the DQ8-glia α1 epitope earlier in 1998. In this case only one patient, that expressed both HLA-DQ2 and DQ8, was used from whom gluten specific T cell clones were generated. With this study they were able to identify and demonstrate that gluten sensitive T cell epitopes are not located only in the N-terminal of gliadins but also in the C-terminal, since the epitopes discovered with this research are all located in that region (van de Wal *et al.,* 1998)

Besides these two epitopes, point nucleotide alterations in the core of each of them were made in order to detect potential lack of CD toxicity. This approach is based in previous experiments that showed that alteration of certain aa at particular positions could decrease and even eliminate T cell responses (Ellis *et al.,* 2003; Japelj, 2013; Mitea *et al.*, 2010). For example, the study made by Japelj in 2013, showed that point substitutions of a proline to

serine residue at position 67 of α2-gliadin, decreased the T cell stimulation and, when more than one substitution was introduced to the core of that particular epitope, changing the key aas responsible for T cell receptor (TCR) binding, abrogated T cell responses.

1.1.2 Immune responses

Immune activation occurs after the ingestion of CD toxic cereals, when CD toxic peptides are presented, in connection with the major histocompatibility complex (MHC) class II molecules, HLA-DQ2/8 (Fraser & Ciclitira, 2001).

1.1.2.1 Adaptive response

The adaptive immune response plays a central role in the pathogenesis of CD. It provides a link between the main genetic factors and gluten (Wieser *et al.,* 2014).

Tranglutaminases are one of the contributors for the aberrant adaptive immune response. They are enzymes that catalyse the acyl transfer of a glutamine side chain to a primary amine (Fig.1). Tissue transglutaminase (tTG), or transglutaminase 2, is a ubiquitous cytoplasmic enzyme, which is found mainly in respiratory and gut epithelial cells. It's a monomeric protein comprise 687 aa composed by four different domains (Wieser *et al.,* 2014).

tTG plays a critical role in biological processes and it is important in the prevention of tissue damage, by catalysing protein cross-linkage, causing formation of iso-peptide bonds between glutamine and lysine residues. If the pH is low (pH<7, can occur when there is an inflammation), or there are no primary lysine residues available, tTG catalyses deamidation of proteins with glutamine residues to glutamic acid, making it negatively charged (Fig. 1) (Fraser & Ciclitira, 2001; Koning *et al*., 2005; Molberg *et al.,* 1998; Tjon *et al.,* 2010).

The enzyme is not a requirement for T cell stimulation but deamidation of glutamine residues to glutamic acid (Fig. 1), with negatively charged aas, favours the binding to the DQ molecules (Fraser & Ciclitira, 2001; Wieser *et al.,* 2014).

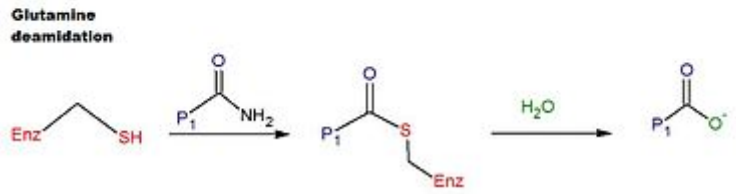


Figure 1. Deamidation reaction catalysed by tTG (Wikipédia. Tissue Transglutaminase)

Activation of T cells leads to interferon-gamma (INF-γ) production and, consequently, higher presentation of gluten peptides to gluten sensitive T cells (Koning *et al*., 2005; Tjon *et al.,* 2010). The final result is a significant T cell response, with more INF-γ inducing tissue damage and increasing release of tTG (Koning *et al*., 2005; Tjon *et al.,* 2010).

1.1.2.2 Innate response

The innate immune response collaborates with the adaptive response to induce a pro-inflammatory Th1 response, to increase the number of intra-epithelial lymphocytes (IELs). This favours a cytolytic attack on the epithelium. Certain gluten epitopes are not recognized by the adaptive immune system but are able to activate an innate response. However, the mechanisms involved remain unknown (Wieser *et al.,* 2014).

An increase in IEls is one of the main features of CD. They represent an abundant and heterogeneous population of T cells that reside between the intestinal epithelial cells at the basolateral side of the epithelium. Their most important role is to promote immune protection by preventing the entry and spread of pathogens and avoiding excessive inflammatory reactions that can damage the intestinal epithelium (Wieser *et al.,* 2014). They are composed of the antigen experience of memory effector T cell subtype CD8+ and natural killer (NK) cells (Wieser *et al.,* 2014). Even though, IELs have regulatory functions that contribute to the repair and healing of the epithelium, they also contribute to inflammatory and tissue destructive reactions such as the ones occuring in CD. They are the main producer of INF-γ in active CD. They are enriched in cytolytic proteins where their expression is associated with increased epithelial apoptosis (Wieser *et al.,* 2014). A count of 20-25 IELs per 100 enterocytes is

estimated to be the borderline between normal and CD-damaged biopsies, with <20 being generally accepted as normal (Wieser *et al.,* 2014).

1.1.3 Genetic factors

Susceptibility to gluten sensitivity appears to be, at least in part, genetically determined. It is known that HLA-DQ2/8 explains 40% of hereditability to CD; the 60% remaining is explained by an unknown number of non-HLA genes. The incidence of CD in first degree relatives of an affected individual, has been estimated at 10%-15%; while monozygotic twin data indicate a 75%-80% disease concordance, and 30%-50% concordance in HLA identified CD- affected siblings (Wieser *et al.,* 2014).

1.1.3.1 HLA genes

The influence of HLA genes have been well characterised both in family and population studies. The predisposition to the disease is closely associated with the inheritance of two alleles at the HLA-DQ loci that encode for the α and β chains of a specific HLA class II molecules. These alleles are found in >90% of individuals with CD (Tighe & Ciclitira, 1995). These HLA-DQ alleles are located at the loci within the MHC on chromosome 6 (Wieser *et al.,* 2014). They are usually inherited together with alleles occurring at neighbouring loci on what is termed an extended haplotype. This common inheritance of several alleles has created a difficulty in the identification of the primary susceptibility alleles (Tighe & Ciclitira, 1995).

All patients with CD have been found to express either HLA-DQ2 or HLA-DQ8 class II molecules. HLA class II molecules are glycoproteins, with α and β chains, located on the surface of cells membranes of antigen presenting cells (APC). They are responsible for biding exogenous proteins and presenting them to CD4+ T cells (Bergseng, 2007; Ciclitira, 2003; Wieser *et al.,* 2014). The large majority of CD patients are DQ2 positive (90-95%); the remainder are DQ8 positive. Two common DQ2 isoforms have been found: DQ2.5 and DQ2.2; most DQ2 patients have the DQ2.5 isoform which is encoded by DQA1*05 (α-chain) and DQB1*0501 (β-chain). The DQ2.2 heterodimer is encoded by the DQA1*0201 and the DQB1*0202 alleles. The DQ8

heterodimer is formed by α and β chains encoded by DQA1*03 and DQB1*0302, respectively (Kooy-Winkelaar *et al.,* 2011).

The HLA-DQ2.5 genotype is associated with a high risk for CD, followed by DQ8 and DQ2.2. The different between the risk of DQ2.5 and DQ2.2 correlates with the different ability of these HLA molecules to form stable complexes with many gluten peptides (Fallang *et al.,* 2009).

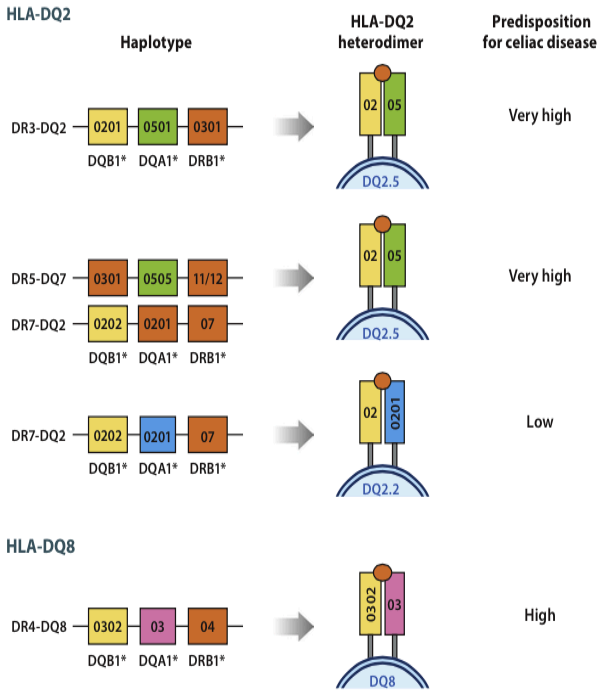


Figure 2. Human leukocyte antigen (HLA) associations in CD (Wieser *et al.,* 2014)

1.1.3.2 Non-HLA genes

Most individuals who express DQ2 or DQ8 never develop CD; therefore, even thought they are necessary they are insufficient for the development of CD. A number of new loci, including many immunological candidates have been identified. Fourty genomic regions harbouring more than sixty candidate genes have been described (Hunt *et al.,* 2008; Tjon *et al.,* 2010; Trynka *et al.,* 2010). Most of these loci contain immune related genes (Gutierrez-Achury *et al*., 2011).

Most non-HLA genes related to CD are shared with other immune-related diseases such as type 1 diabetes and autoimmune thyroiditis. Genome-wide association studies (GWAS) have started to uncover genetic components contributing

to CD but the challenge is to find the primary target of the genetic association to uncover the functional consequences of the true causal risk variant (Kumar et al., 2012; Wieser *et al.,* 2014)

1.1.4 Peptide binding

1.1.4.1 HLA DQ2/8-peptide binding

HLA molecules have a characteristic-binding groove, which differs in size, shape and position between HLA class II alleles, and which can be used to predict the sequence of peptides needed to fit into it (Bergseng, 2007; Ciclitira, 2003; Fraser & Ciclitira, 2001). As mentioned before, HLA-DQ2 and HLA-DQ8 play a key role in the pathogenesis of the disease by presenting peptides to antigen specific T cells, which promulgate the observed inflammatory response (Ciclitira, 2003; Fraser *et al.,* 2003; Tighe & Ciclitira, 1995).

The combined α and β chains bind immunogenic gluten epitopes in a 'peptide-groove' and present them to T cells in the lamina propria. The N-terminal domains of the heterodimers combine to form the groove that has a five-turn α-helix from the α-chain that runs parallel to a longer but kinked α-helix from the β-chain forming the side wall of the groove (Kim *et al.,* 2004). In this groove, antigen peptide is bound for presentation to the TCR. Within this groove, pockets are sited to accept amino acid side chains of the antigen peptide to enable binding (Ciclitira, 2003). Alterations in the aa sequence of peptides by aa substitutions within this biding groove, predominantly at the location of these pockets, can affect binding affinity to a particular HLA molecule (Ciclitira, 2003; Tighe & Ciclitira, 1995).

The peptide binding of HLA-DQ2.5 and DQ2.2 are similar but DQ2.5 has the capacity to hold immunogenic peptides for much longer compared to DQ2.2. This fact also explains the higher disease risk associated with HLA-DQ2.5 (Fallang *et al.,* 2009; van de Wal *et al.,* 1997). As in HLA-DQ2, the HLA-DQ8 favours the binding of negatively charged peptides. In additon functional binding studies suggest anchor positions P1 and P9 for glutamic acid and P4 for hydrophobic residues. In contrast to DQ2, that requires only one glutamic acid residue, DQ8 requires two (Wieser *et al.,* 2014). The glutamate introduced by tTG is usually in positions P4, P6 or P7 in HLA-DQ2.5 restricted epitopes and, as mentioned, at position P1 and/or P9 in HLA-DQ8 restricted

epitopes. These glutamatic acid residues serve as anchor residues important for binding of the peptides and both HLA-DQ2.5 and DQ8 prefer negatively charged residues at these anchors sites. This positioning of deamidated glutamine residues is strongly related to the positioning of proline residues, which is particularly strict in the case of DQ2.5 epitopes, as DQ2.5 only accepts proline at certain position in the peptide-binding groove (Kim *et al.,* 2004). This results in a dominant presence of proline at P1, P6 and P8 and leads to the modification by tTG of the glutamine residues at P4 and P6, respectively. Such positioning of proline residues is less strict in the case of the DQ8 epitopes (Sollid *et al.,* 2012).

1.1.4.2 TCR-peptide binding

HLA-DQ2 and DQ8 heterodimers bind to gluten peptides in their peptide groove and then present them to T cells (Fig. 3) (Wieser *et al.,* 2014). CD4+ T cells express a TCR, which is responsible for binding to the HLA-peptide complex. This receptor comprises of a α, β, γ or δ chains than contain three domains: one extracellular Ig-like, a trans membrane and a cytoplasmic tail (Bergseng, 2007). TCRs possess unique antigen specificities determined by the structures of the antigen-biding site formed by the α and β chains, and which are activated by contact with a peptide/MHC complex. APCs that have bound the antigens in the intestinal tissue can travel to the mesenteric lymph node, where antigen presentation and priming of naïve CD+ T cells take place (Qiao *et al.,* 2012; Wieser *et al.,* 2014).

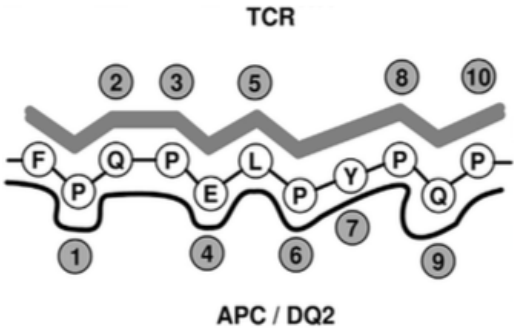


Figure 3. Binding sites of APC and TCR for an epitope of α-2 gliadin (Wieser *et al.,* 2012)

Following activation, gluten sensitive CD4+ T cells migrate to the lamina propria. In the tissue, primed CD4+ T cells are either reactivated by local resident APCs presenting gluten peptides or remain dormant as memory T cells (Wieser *et al.,* 2014).

1.1.5 Methods for investigating coeliac disease

There are a variety of different methods, including *in vivo* and *in vitro* methodology, that help investigate the pathogenesis of CD.

1.1.5.1 *In vitro* methods

1.1.5.1.1 Organ Culture of intestinal tissue

The most reliable of the *in vitro* methods is the organ culture of small intestinal tissue obtained from CD patients. This tissue samples are taken from the small intestine and incubated with culture medium under special conditions at 37°C. With this method the biopsies survive up to 24 hours. It is used to evaluate peptide toxicity for CD. The biopsies are incubated with gluten and gluten fractions; then histological changes of the biopsies are evaluated after the incubation period (Browning & Trier, 1969; Ciclitira *et al.,* 2005; Lindfors *et al.,* 2012).

One of the benefits of this method is the fact that various features characteristic for CD can be reproduced in biopsies from treated patients, thus allowing researchers to find mechanisms related to development of CD (Falchuk *et al.,* 1972). Because intestinal biopsies contain enterocytes and lamina propria, this model is useful in determining both innate and adaptive responses.

The main disadvantage of the method is that it is not a high-throughput method and the tissue lacks circulation, a nervous system and connection to lymphatic organs (Wieser *et al.,* 2014).

1.1.5.1.2 T cell lines and clones

T cell lines and clones of the small intestinal mucosa have been used to measure immunogenic effects of proteins and peptides and to test potential novel treatments (Wieser *et al.,* 2014). CD4+ T cells play the most important role in the adaptive immune response in CD pathology. To isolate the T cells, biopsies from the small intestinal are first incubated in the presence of an antigen toxic (gluten fractions or gluten peptides) for CD patients. Multiple restimulations are required to cultivate gluten sensitive T cells *in vitro* (Lindfors *et al.,* 2012; Wieser & Koehler, 2008).

Following activation with the antigen, interleukin 2 (IL-2) receptor (CD25) increases in number. If IL-2 is later added as stimulant, only lines that are specific for the antigen will grow. Irradiated peripheral blood mononuclear cells (PBMCs) are used as APC, helping gluten-sensitive T cells to proliferate (Lindfors *et al.,* 2012; Wieser & Koehler, 2008). Proliferation of T cells in culture when stimulated with antigen can be determined in T cell proliferation assays. In this assay, T cells are incubated with the antigen, APCs and tritiated thymidine (^{3}H). The ^{3}H is incorporated into the nuclei of the cells that are dividing and this amount is then measured by a scintillation counter (Lindfors *et al.,* 2012; Wieser & Koehler, 2008).

T cells tests have been used widely to compare the level of immunogenic effects and they are the first approach used when testing toxic epitopes in CD. However, immunogenicity in T cells do not always correspond to the CD toxicity demonstrated *in vivo* or in OC tests, that is why there is always the need to apply other methods, to confirm the results obtained with T cells (Hooper *et al.,* 2012; Maynard *et al.,* 2012).

1.1.5.2 *In vivo* methods

Small intestinal *in vivo* gluten challenge is used for purposes of evaluating CD toxicity and help diagnosing patients and it can be a short-term challenge or a long-term challenge. The problem with *in vivo* studies and methods is that they can be highly invasive and very time consuming (Ciclitira *et al.,* 2005).

1.1.5.2.1 Long-term Challenge

In long-term challenge patients who are eating food-containing gluten for 2 to 6 weeks are submitted to an endoscopy where biopsies of the small intestine are collected. These biopsies are then used to assess histological changes (Ciclitira *et al.,* 2005).

1.1.5.2.2 Short-term Challenge

In short-term challenge, gluten fractions or peptides are applied directly to the small intestinal mucosa. Later, biopsies from the duodenum are collected in interval periods to check for how long and at which dose the histological changes (such as villus height, crypt depth, enterocyte cell height or intra-epithelial cell counts) are dependent (Ciclitira *et al.,* 2005).

1.2 Symptoms and Diagnosis

The effects of abnormal interaction between the immune system and gluten may be expressed not only in gut (CD) and skin (dermatitis herpetiformis), but also in the mouth (recurrent aphthae), kidneys (IgA nephropathy), joints (some arthrithes) and brain (Ferguson *et al.,* 1993).

The symptoms can be subdivided into two groups: the intestinal disorders and the extra intestinal features caused mainly by malabsortion of essential nutrients. When only extra intestinal symptoms happen, the diagnosis with CD is harder and less likely to occur (Wieser *et al.,* 2014). Small intestinal damage (Fig. 4), diarrhoea, anaemia, osteoporosis, infertility, neurological and/or skin conditions, including dermatitis herpetiformis are the more common symptoms in CD patients (Silano *et al.,* 2008). CD is also characterized by flattening of the mucosa that can vary from mild through partial villous atrophy to total absence of villi (Fig. 4 B).

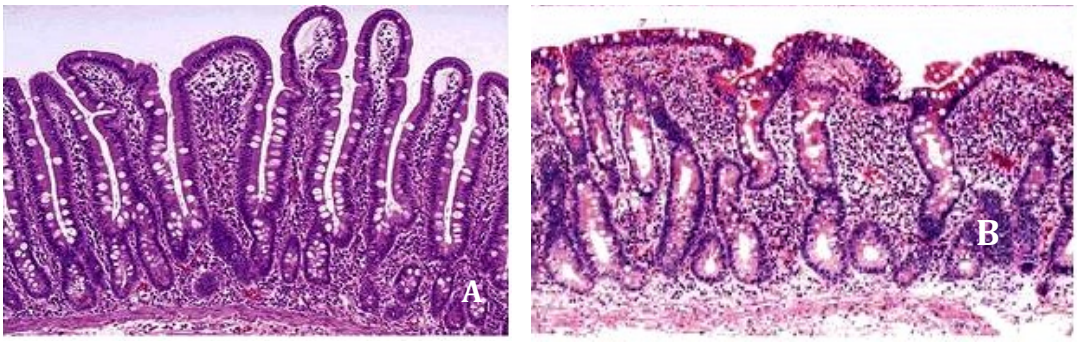


Figure 4. Small intestine villus (A- Normal; B- Absence of villous due to gluten sensitivity) (CeliAct. Villous Atrophy)

CD is one of the most under diagnosed pathologies due to a complex diagnosis that requires a high level of clinical suspicion. Some CD-associated complications, such as osteoporosis and growth retardation, can become permanent if the disease is not diagnosed in time. Through the years the methods for diagnosis have changed due to a better understanding of CD and the availability of new, more specific and sensitive tests (Wieser *et al.,* 2014). Some groups believe that duodenal biopsies are essential for a correct diagnosis. In these cases, up to five biopsies are collected during diagnostic procedures and villous atrophy is measured. The biopsies should be collected when the patients are undergoing a gluten containing diet to achieve better and more precise results. Later, when the patient is already under a gluten free diet (GFD), new biopsies are collected to verify if there is an improvement in the mucosa (Wieser *et al.,* 2014).

Several serological screening tests are available for the detection of CD. Tests for antibodies from serum or plasma samples are currently often apply. Anti-endomysial antibody (EMA) of IgA class enables the most reliable serological testing with diagnostic sensitivity of 90% and diagnostic specificity of 99% (Stern & Ce, 2000). Due to the requirement of specific biological materials for these tests (including monkey oesophagus and umbilical cord), demanding execution of the protocol and interpretation of the results, antibodies to tTg are more commonly use in clinical practice (Cataldo *et al.,* 1998).

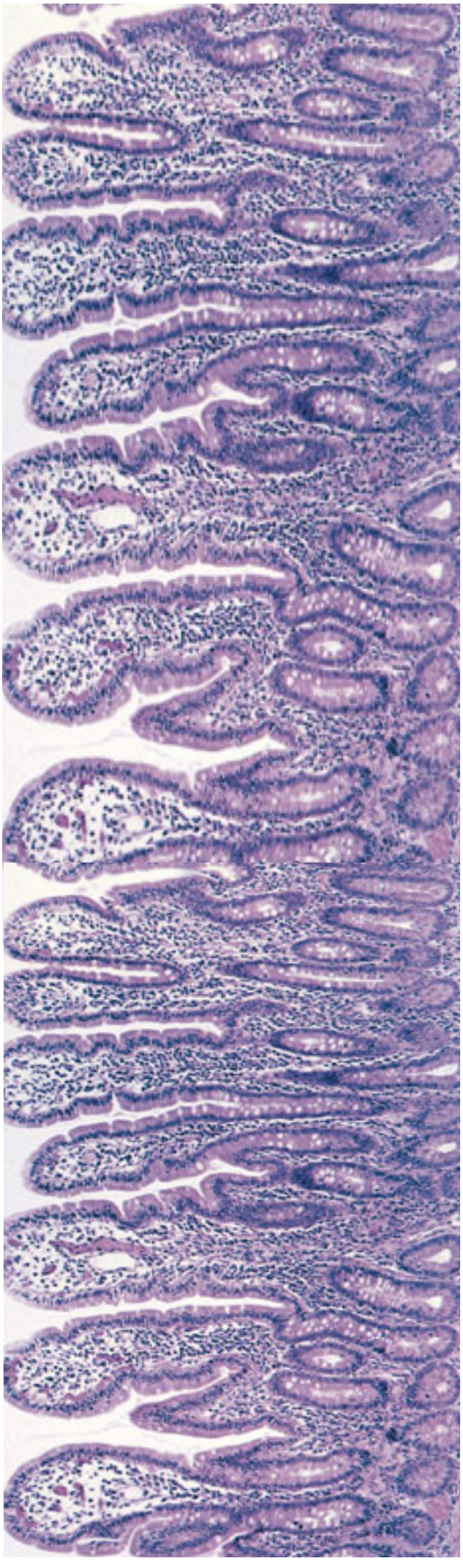
1.3 Treatment

Once a diagnosis of CD has been made, the patient needs to be established on a GFD. All foods, which contain wheat or wheat flour, as well as barley and rye, must be avoided. There

are a number of foods that contain hidden gluten, including soy sauce, mustard, mayonnaise and beer, which contain barley prolamin. Gluten free flour, bread, biscuits, pasta and snacks are available and should be eaten has a replacement for the gluten containing products in a GFD for patients with CD. In patients who do not respond to a strict GFD, or who relapse on a GFD, steroids can be used (Fraser & Ciclitira, 2001).

The defining feature is the expectation that the intestinal lesion improves with a strict GFD, excluding all forms of gluten from the diet. However, from a nutritional point of view, a GFD does not guarantee adequate nutrient intake and some nutrient deficiencies have been described after a treatment with a long term GFD. In some cases, there is an unbalance intake of carbohydrates, protein, fat and a limited intake of certain nutrients (Mustalahti, *et al.,* 2010; Schuppan *et al.,* 2009). Due to this and because a lifelong strict GFD is hard to sustain (restricted availability and higher cost), there is an urgent need to develop safe and effective alternatives. Several studies have been made in order to achieve therapeutic agents capable of competing and replace a GFD (Wieser *et al.,* 2014). Studies such as aa substitutions in gliadin peptides made by Ellis *et al.,* 2003 and Japelj, 2013 or the studies made by Zevallos *et al.,* 2012 & 2014, on the lack of gluten toxicity in quinoa, are examples of approaches that have been made in order to find and develop new ways to treat CD.

2. PURPOSE OF THESIS



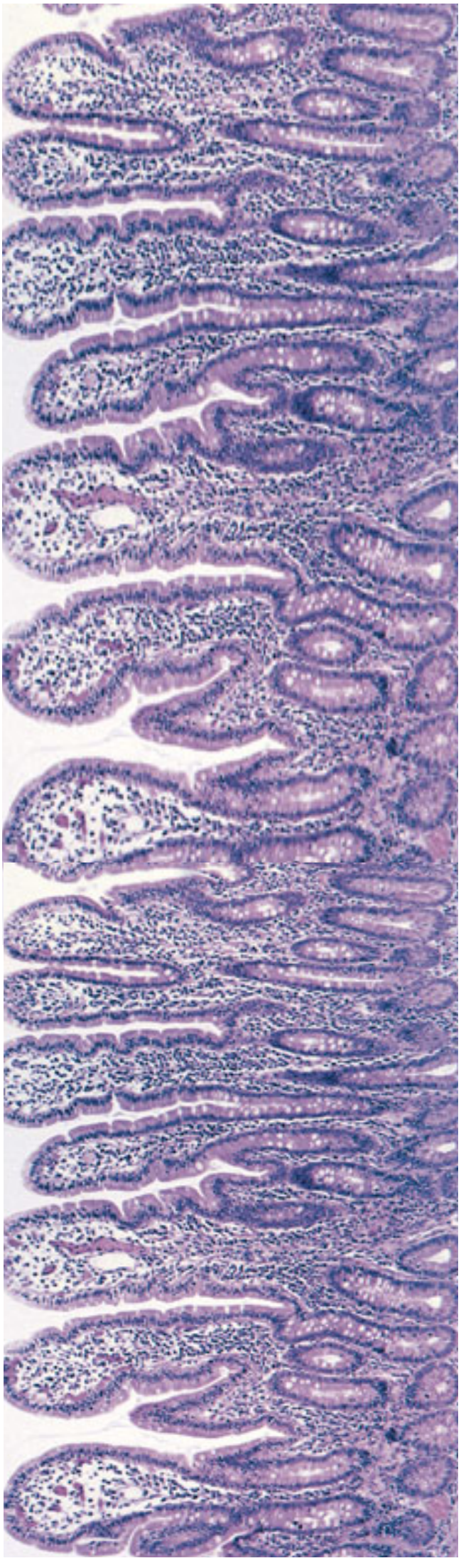
This work aims to test the toxicity of different epitopes found in wheat, which may or may not be toxic for patients diagnosed with CD.

As known, α gliadins are the most immunogenic peptides found in wheat, since they harbour several major CD triggering epitopes. The elimination of these epitopes could be an important step towards the elimination of CD toxicity of these gliadins.

Four α gliadin epitopes were tested using T cell proliferation assays to evaluate their toxicity. Of these epitopes, DQ2.5-glia α3 (labelled as α2a) and DQ8-glia α1 (labelled as α3I) have already been tested by different research groups, and were found to be toxic in the majority of cases. The other two (labelled as α2c and α3II) are the product of multiple aa substitutions in the core of the aa sequence of the previous epitopes.

We hope, with this study, to establish a relationship between the interactions of CD toxic epitopes, involving the substitution of aas in the core of the epitopes and the potential loss of their toxicity to CD patients finding, this way, a potential new approach to treat CD.

3. PATIENTS AND MATERIALS



3.1 Patients

Biopsies of the small intestine were taken from patients with CD or from potential coeliac patients, during the routine work-up for the diagnosis and routine procedures. One to two 50 mL tubes of blood were also collected at the same time, in order to obtain PBMCs, essential to the experiment.

The subjects had to read, understand and sign an informed consent form (Attachments II & III) explaining the aims of the project and their potential role on it.

This project was approved by the St. Thomas Hospital Research Ethics Committee (reference numbers 05/Q0207/167). All volunteers were diagnosed according to the British Society of Gastroenterology guidelines (British Society of Gastroenterology. Guidelines on the diagnosis and management of adult coeliac disease).

From six participant volunteers, out of twenty-four, we were able to obtain gluten sensitive T cell lines (Table 1). Of these volunteers, two were males and four were females. Only four patients were able to provide their DQ status. If possibly the patients chosen to participate in this trial were CD patients that were eating gluten at the time of the procedures or that were on a GFD not for long.

As previous mentioned, the biopsies were collected during routine procedures, and placed in bijou tubes containing 100µl of organ culture medium (OCM) and transported to the laboratory as soon as possible. T cells obtaining from these biopsies were cultured two to three weeks before tested in proliferation assays.

Table 1. General information about the patients used in T cell trial (Sex- M for male and F for female; Age; Time on a GFD; DQ status; Time of T cells in culture)

Patients	Sex	Age at time of the biopsy	DQ status	GFD	Weeks of T cell lines in culture
A	M	51	Unknown	6 months	3 weeks
B	M	33	DQ2+	0	2 weeks
C	F	35	DQ2+	0	2 weeks
D	F	34	DQ2+	2 years	3 weeks
E	F	20	DQ2+	4 years	2 weeks
F	F	22	Unknown	0	2 weeks

3.2 Reagents, Chemicals, Solvents

- Albumin from human serum (A4327, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Amphotericin B (P11-001, PPA, UK)
- $CaCl_2$ (C-1016, Sigma, UK)
- Dimethyl Sulphoxide Hybri-Max (D2650, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Double Processed Tissue Culture Water (W3500, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Heat inactivated Fetal Bovine Serum (70259OF, Gibco, Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK)

- Heparin (Mucous) Injection B. P. 1000Units/ml, heparin sodium (PL0043/0041R, Leo Laboratories Ltd, Longwick Road, Princes Risborough, Buckinghamshire, HP27 9RR, UK)
- Hepes buffer 1M (HO887, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Hydrocloric acid (around 36%, AA-38744, BDH Chemicals, UK)
- IL-2, Human, Recombinant (17908-10KU, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Industrial gluten (Vital wheat gluten, NW676, Roquette UK Ltd, Sallow Road Weldon Industrial Estate, Corby Northants NN17 5JX, UK)
- Lectin from *Phaseolus vulgaris* (red kidney bean) (L2769-2MG, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Lymphocyte Separation Medium Light Sensitive (LSM 1077, PAA, UK)
- Pepsin Agarose from porcine gastric mucosa (P0609-10KU, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Phosphate buffered saline tablets (P4417, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Plasmocin 50mg (ant-mpt, InvivoGen, 5rue Jean Rodier F-31400 Toulouse, France)
- RPMI 1640 with L-glutamine (E15-840, PAA, PAALaboratories Ltd, Termare Clore, Houndstone Business Park, Yeovil, Somerset BA22 8YG UK)
- Sodium hydroxide 1.0 M (38215, Sigma, UK)
- Thymidine, [6-^{3}H], 5mCi (NET355, Perkin Elmer, PerkinElmer Life & Analytical Sciences, Chalfont Road, Seer Green, Bucks, HP9 2FX, UK)
- Transglutaminase from guinea pig liver (T5398-10UN, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)
- Trypsin from Bovine Pancreas Agarose (T1763-50UN, Sigma, Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK)

3.3. Solutions

3.3.1. Autologous serium medium (ASM)- 50mL

- 44 mL of RPMI
- 5 mL of patient's heat inactivated serum
- 500 µL of Hepes buffer 1M
- 500 µL of Amphotericin 0,25 g/L
- 50 µL of Plasmocin 25 g/L

3.3.2. Organ culture medium (OCM)- 10mL

- 8 mL of RPMI
- 1,5 mL of Heat inactivated Fetal Bovine Serum
- 100 µL of Hepes buffer 1M
- 100 µL of Amphotericin 0,25 g/L
- 10 µL of Plasmocin 25 g/L

3.3.3. tTG mix solution

- 135 µL of $CaCl_2$ (2mM in distilled water)
- 10 µL of Peptic-tryptic digested gluten (10 g/L in distilled water)
- 125 µL of transglutaminase from guinea pig liver (200 µL/mL in phosphate buffered saline (PBS))

3.3.4. Stock solution of IL-2 (1000U/mL)

- 10 mg of Albumin from Human Serum
- 10 mL of PBS
- IL-2

3.3.5. Working concentration of IL-2 (100U/mL)

- 1 mL of IL-2 (1000U/mL)
- 9 mL of RPMI

3.4. Materials and equipment

3.4.1. Plastics

- Bijou tube 7 mL (129A, Sterilin, Ltd)
- Blowtorch
- Cryo 1ºC Freezing Container (5100-0001, Thermo Scientific Nalgene, USA)
- Disposable scalpels
- Electronic pipette (4-000-205-E, Drummond Scientific Company, Broomall, PA, USA)
- Macropipettes (1mL, 10mL)
- Microliter glass syringe (Hamilton Syringe 500µl 750LT, ESSLAB Ltd)
- Micropipettes (0,5-10 µL, 20-200 µL, 100-1000 µL)
- NALGENE Cryo 1ºC Freezing container
- Nunc 1,8mL Cryotube (368632, Nunc, A/S, Kamstrupvej 90,DK-4000, Roskilde, Denmark)
- Organ culture dish (353037, BD Falcon)

- Plastic Pasteur pipettes
- Polypropylene conical tubes (15 mL, 50 mL)
- 1 mL and 3 mL syringe
- 24-well flat bottomed plat (353047, BD Falcon)
- 70 µm Cell strainer (352350, BD Falcon)
- 96-well U bottomed plate (353077, BD Falcon)

3.4.2. Radioactivity work

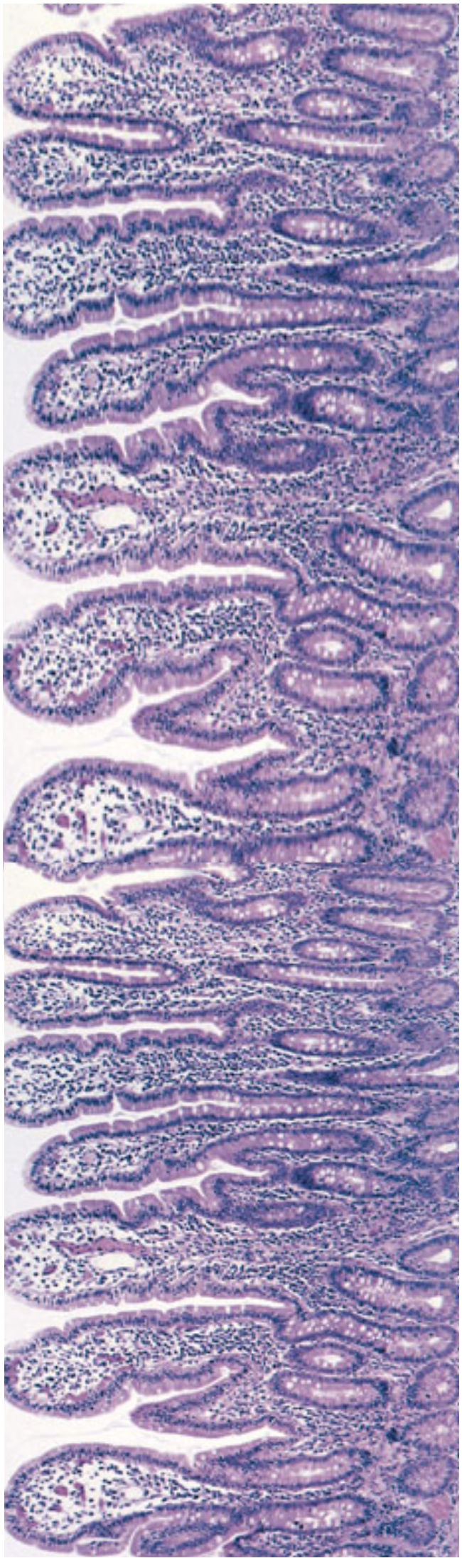
- Harvester 96 (Mach, Tomtee, 706 Harboview, Orange, CT 06477)
- Heat sealer (Wallac 1295-012, Perkin Elmer)
- Liquid scintillation counter (Wallac 1450 Microbeta plus, Perkin Elmer)
- Neubauer counting chamber (1110000, LO-Laboroptik GmbH)
- Sample bag, for microbeta (1450-432, Perkin Elmer, UK)
- Filtermat A, for microbeta (1450-421, Perkin Elmer, UK)
- Forceps

3.4.3. Non-consumables

- Centrifuge (Sorvall RT7, Thermo Fisher Scientific)
- Freezer (-20°C and -56°C)
- Fridge
- Gammacell 1000 Elite (GC-1000, Nordion International Inc., Kanata, Ontario, Canada)
- Gas Jacketed Incubator (BB 6220, Hearaeus, Switzerland)
- Laminar flow hood
- Magnetic stirrer hotplate (SM3, Stuart Scientific, UK)
- Microscope (CK, Olympus, Japan)
- Modular incubator chamber (3886047, Billups-Rothenberg Inc, P. O. BOX 977, Del Mar California 92014 CA)

- Scales (GR-200, A&D Instruments Ltd, Japan)
- Vortex (K-550-GE, Scientific Industries Inc, Bohemia, NY, USA)
- Water bath (JB1, Grant Instruments Ltd, Barrington, Cambridge, CB2 5QZ, England)
- Water-Jacketed CO_2 Incubator (51201069, Napco 6000, US)

4. EXPERIMENTAL WORK



4.1. Antigen preparation

- 1 g of industrial gluten is dissolved in 50 mL 0.1M HCl and pH adjusted to 2
- This mixture is incubated with 4000U pepsin at 37ºC for 2 hours
- The mixture is centrifuged for 20 minutes at 2500rpm (800 xg). The supernatant's pH is adjusted to 7,8 with 1M NaOH
- 25U of trypsin is then added and incubated ate 37ºC for 2 hours
- The solution centrifuged for 20 minutes at 2500rpm (800 xg). The supernatant's pH is adjusted to 7 with 1M HCl and air dried in a glass petri dish at room temperature
- The gluten fractions are deamidated with the tTG (see tTG mix solution) prior to incubation with APC

4.2. tTG mix

4.2.1. Gluten solution

- Digested gluten is dissolved in distilled water to make 10 g/L and irradiated for 45 minutes

4.2.2. $CaCl_2$ solution

- $CaCl_2$ is mixed with distilled water to make a 2mM solution

4.2.3. tTG solution

- 5 PBS tablets are dissolved in 1 L distilled water and irradiated. To that mixture, tTG is added to a final concentration of 0,5U/mL. Smaller aliquots are made and frozen to -56ºC
- All 3 solutions (gluten, $CaCl_2$ and tTG) are mix in ratio (see reference 3.3.3), incubate for 4hours at 37ºC and irradiated for 30 minutes

4.3. Isolating Peripheral Blood Mononuclear Cells (PBMCs) from blood

- Blood samples are centrifuged at 2000rpm (500 xg) for 15 minutes, to separate the plasma and the cells
- After removing supernatant (plasma), the cell layer is mixed and divided into 10 mL aliquots in 50 mL centrifuge tubes
- In each aliquot, 28 mL of RPMI is added and mixed with the cell layer
- To separate white cells from the others, a lymphoprep solution needs to be added. 10 mL of lymphoprep was carefully underlayered, while slowly lifting the pipette
- This mixture is centrifuged at 3000rpm (1200 xg) for 20 minutes.
- The white cells, which are lying on the RPMI layer, are collected to 50 mL tubes and centrifuged again at 2000rpm (500 xg) for 15 minutes.
- The supernatant is remove slowly but in only one turn and the cell pellet is resuspended in 2 to 5 mL of ASM
- To count the PBMCs, 20 µL of this mix is taken and stained with the same volume of tryptan blue and a haemocytometer chamber is used to help counting (Fig. 5)

Equation 1. Equation to calculate the number of cells from standard haemocytometer chamber

Nº cells= 2(1:1 dilution of trypton blue) x 10^4 (count 10 squares) x The original volume of liquid from which the sample was removed (mL) x nº of cells per square

- $4x10^6$ cells are irradiated (22 Gy) and placed into one well of a 24 well plate to combine with the SIL fraction the following day as a support for the cells
- The remain cells are frozen down (see freezing PBMCs for liquid nitrogen) and later used as APC for the T cells in the restimulation and proliferation assays

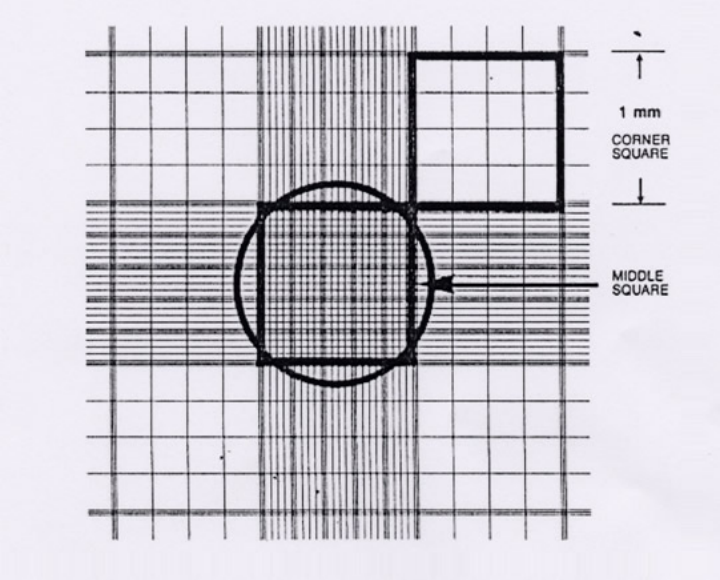


Figure 5. Standard Haemocytometer chamber to count PBMCs and T cells (University of Vermont- Basic Cell Culture-I)

4.4. Obtaining autologous plasma for cultured medium

- Following the separation of cells and plasma, this last one is placed in 15 mL tubes and put in a water bath at 56ºC for 35 minutes, so this way the complement is inactivated
- The heat inactivated plasma is centrifuged at 2000rpm (500 xg) for 15 minutes
- The supernatant is collected and used to serve as autologous serum medium

4.5. Freezing PBMCs for liquid nitrogen storage

- After counting the PBMCs, suitable amount of empty cryotubes are placed on ice along with a 10 mL tube containing 20% dimethyl sulphoxide (DMSO) in ASM
- After 20 minutes, the cells are added to the ice
- The cells stay on ice for another 20 minutes

- 20% DMSO is then added to cells, drop-wise and gently mixing after each drop. The volume of 20% DMSO depends on the volume of ASM in which PBMCs are resuspended (V_{DMSO}: V_{ASM}= 1:1)
- The cryotubes contained around $7x10^6$ cells. The tubes are placed in a chamber with iso-propanol and frozen to -50ºC overnight.
- The following day cryotubes are transferred to liquid nitrogen for long-term storage.

4.6. Small intestinal organ culture for T cell work

- Gluten (5 mg/mL) is solubilised in OCM and placed in the incubator to warm up
- To prepare organ culture dish (Fig. 6) in advance a sterile grid is placed over centre well and 1,5 mL of sterile water is added to outer well. The dish is kept in the incubator at 37ºC
- During the diagnostic procedures biopsies were taken
- Following biopsies for routine histology, the ones collected for research were placed in bijou tubes with 100 µl of organ culture medium
- In the laboratory, the biopsies were placed on the grid
- The orientation of the biopsies has to be with the serosal side down (redder side with red dot) and mucosal up. Biopsies should be touching (cell contact) to obtain an improved yield of cells, but should not be covering each other
- Organ culture medium with the digested gluten is injected into the central well to get capillary effect that covers the biopsies. It is important that the tissue is not completely submerged in organ culture medium
- Dishes are placed into the organ culture chamber. In this chamber, gas mixture of 95% O_2/5% CO_2 is flowed
- Then the chamber is sealed and the pressure of 2 psi has to be reached
- After this the chamber is placed in the incubator at 37ºC overnight

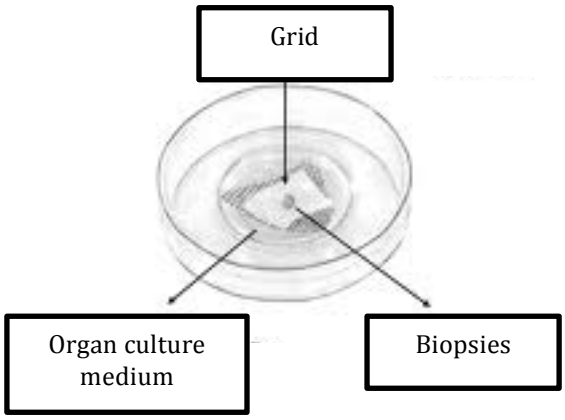


Figure 6. Schematic image of the organ culture dish with grid and biopsies (Image adapted from Encrypted- Organ Culture Dish)

4.7. Isolating T cells from in vitro gluten-challenged duodenal biopsies

- To isolate T cells, biopsies are chopped into small pieces with scalpel blades for 20-35 minutes. The whole time biopsies need to be in ASM
- After the 20 minutes the shopped biopsies are passed through a 70 μm cell strainer and washed with ASM
- The pieces from the strainer (debris fraction) is collected separately from the cells that passed trough the strainer (SIL fraction)
- Irradiated PBMCs from the day before are added to the SIL fraction to provide more cell to cell contact
- Cells were washed twice in 10 mL ASM warmed to 37ºC and centrifuged at 1500rpm (300 xg) for 7 minutes
- Cell pellets are resuspended in 4 mL ASM and divided into 2 wells on a 24-well plate

4.8. Thawing of PBMCs

- RPMI, squeezing pipettes and 15mL centrifuge tubes need to be cooled down on ice first
- Vials with PBMCs are taken from liquid nitrogen storage and placed under tepid water until the suspension melt
- RPMI is then added drop by drop to a volume of 10 mL whilst constant agitation
- Thawed cells are immediately centrifuge at 1500rpm (300 xg) for 7 minutes, resuspended in another 10 mL ice-cold medium and spun down once again
- The pellet is then resuspended in 1 mL ASM and the cells counted
- PBMCs are irradiated (22Gy) to prevent their proliferation

4.9. Maintenance of T cell lines in culture

- After T cells have been isolated every 2-3 days, depending on colour change of medium and cell growth rate, medium is refreshed by removing half the volume of culture supernatant and replacing it with fresh autologous serum medium
- Concurrently, to every mL of ASM a 100 μL of IL-2 is added to serve as T cell growth factor
- If cells in well are overcrowded, the suspension is split in two wells

4.10. T cell proliferation assay

- Autologous PBMCs are used as antigen presenting cells
- They are thawed, counted with a haemocytometer and irradiated (22 Gy). Then the PBMCs are suspended in ASM to reach the right concentration ($5{,}5x10^4$ per 50 μL ASM)
- In a 96 well U-bottom plate, 50 μL per well of this suspension is given. Depending on the size of the peptide the incubation time at 37ºC varied. Whole proteins need 18 hours of incubation, mean while for

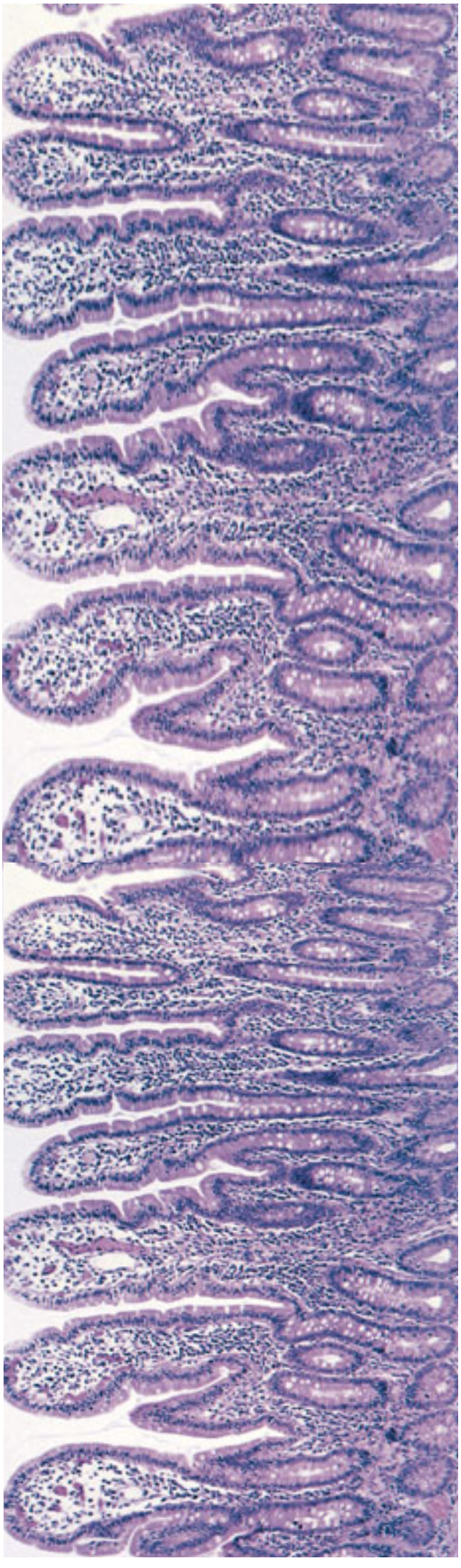
small peptides 4 hours is sufficient for the PBMCs to present antigen on their surface

- The final concentration of complex antigen has to be 100 μg/mL, for small peptides is 10 μg/mL
- Peptic-tryptic (PT) digested gluten is used as a positive control and ASM without any peptides (medium only) as a blank
- Small tested peptides were purchased commercially and have 95% purity. They are marked as peptide α2a, α2c, α3I and α3II for simplicity of peptide labelling in text and tables
- Tested peptides harbour T cell epitopes or their counterparts with amino acid substitutions
- The T cells to be tested for antigen reactivity are counted using haemocytometer. Cells are subsequently centrifuge at 1500rpm (300 xg) for 7 minutes, resuspended in ASM so they can reach the concentration of $5x10^4$ cell/100 μL/well and add to the pre-pulsed APC
- After 48 hours of incubation, ^3H is added (1 μCi/well in 20 μL ASM) and plate is incubated for further 18 hours
- Plates are harvested with tomtec cell harvester. The level of thymidine incorporation is measured using a wallac 1450 microbeta plus liquid scintillation counter
- The SI are calculated for each antigen by dividing the mean counts per minute (cpm) for the T cells plus APC plus test antigen by the cpm of T cells plus APC alone (Equation 2)
- Stimulation indexes equal or greater than 2 are considered to be positive, meaning it is toxic for CD patients
- When the coefficient of variation (cv) was 0,2 or more, the value which deviated the most was excluded and the SI calculated again

Equation 2. Equation to calculate the SI

$$SI = \frac{Mean\ cpm\ for\ T\ cells\ plus\ APC\ plus\ antigen}{Mean\ cpm\ for\ T\ cells\ plus\ APC}$$

5. PEPTIDES AND EPITOPES



In this study we tested the immunogenicity of four different α-gliadin peptides sequences (Table 2), analysing the potential effect that aa alterations in the original epitopes would have on their toxicity to CD patients.

The peptide α2a harbours the DQ2.5-glia α3 epitope while the peptide α2c harbours an altered version of it, with four different aa substitutions (Table 3). On the other hand, the peptide α3I harbours a different epitope, the DQ8-glia α1 while the peptide α3II have in his sequence the modified version of this last one which includes three aa substitutions (Table 4). The first peptide (α2a) is located in the N-terminal of α-gliadin while α3I is located at the C-terminal of the same peptide.

The peptides were synthesized and employed in T cell proliferation assays using gluten sensitive T cell lines.

Table 2. Peptides' labels and their amino acid (aa) sequences. Amino acid substitutions in known immunogenic peptides are marked in red

Peptide label	aa Sequence
α2a	QPFRPEQPYPQPQPQ
α2c	QPFPPKQSYSQPQPQ
α3I	LGEGSFQPSQENP
α3II	LGKGSFRPSQKNP

Table 3. Amino acid comparison sequence between α2a peptide and its altered version (α2c) and the epitope they harbour

Peptides	**aa Sequence**														
α2a *	Q	P	F	R	P	E	Q	P	Y	P	Q	P	Q	P	Q
α2c	Q	P	F	P	P	K	Q	S	Y	S	Q	P	Q	P	Q

Epitope DQ2.5-glia α3

Amino acid substitutions

* Peptide found in the N-terminal of α-gliadin, between positions 92 and 105.

Table 4. Amino acid comparison sequence between α3I peptide and its altered version (α3II) and the epitope they harbour

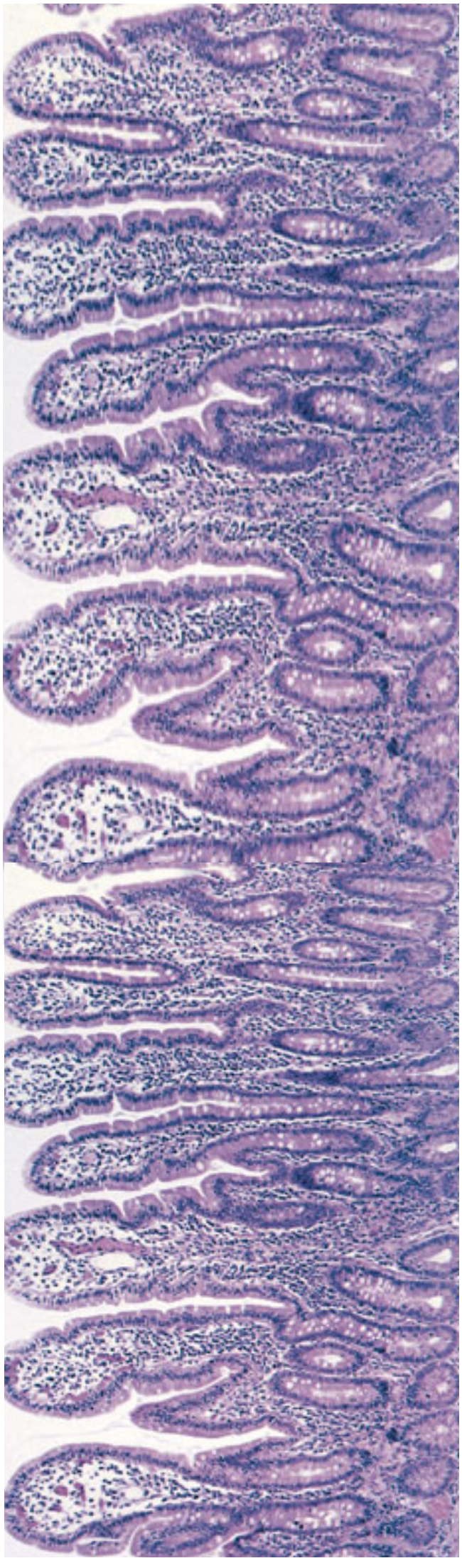
Peptides	**aa Sequence**											
α3I *	L	G	E	G	S	F	Q	P	S	Q	E	N	P
α3II	L	G	K	G	S	F	R	P	S	Q	K	N	P

Epitope DQ8-glia α1

Amino acid substitutions

* Peptide found in the C-terminal of α-gliadin, between positions 206 and 218.

6. RESULTS



6.1. T cell work

We isolated and cultured small intestinal T cell lines from twenty-four different CD patients. These lines were used in proliferation assays using antigens such as peptic-tryptic (PT) digested and tTG treated gluten (GLU), four different α-gliadin peptides (Table 2) and culturing them with medium only.

Since PT-digested gluten served as a positive control, only the cell lines where the SI from GLU were equal or greater than 2 were considered gluten specific. From all twenty-four T cell lines tested, only six (25% of total isolated T cell lines) showed gluten specificity (Table 5). For the gluten specific T cell lines the SI for gluten ranged between 2,1 and 4,5. Of these six cell lines, 33,3% of cases (2 out of 6) showed toxicity for the peptide α2a and 16,67% of cases (1 out of 6) showed toxicity to the peptide α2c. The remaining peptides have shown negative SI in 100% of cases (6 out of 6).

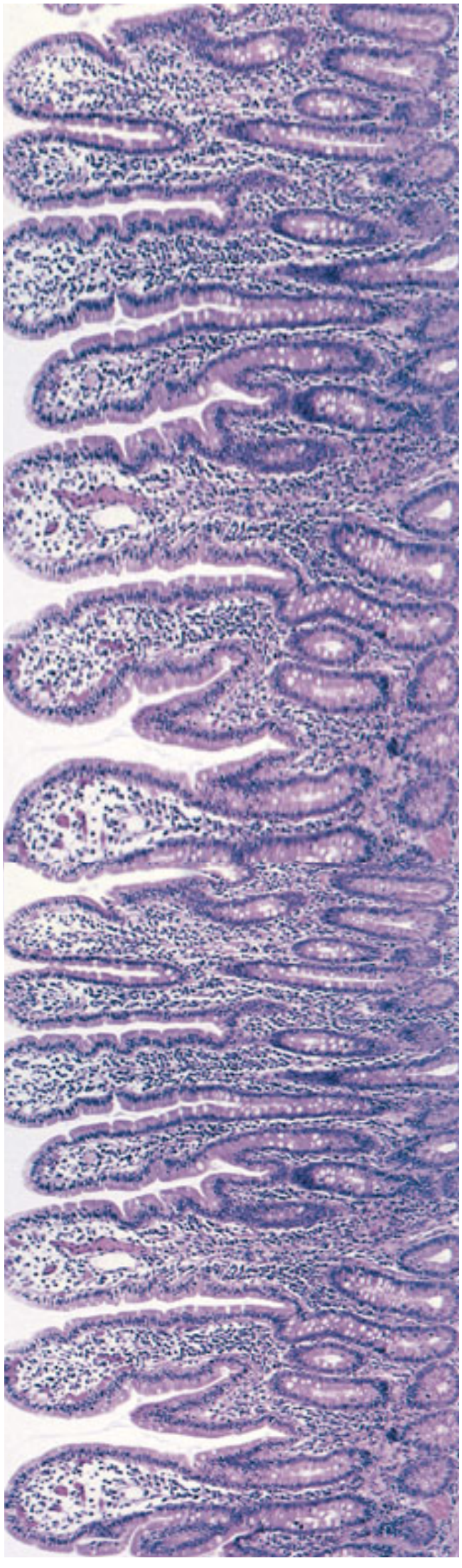
The peptide α2a triggered response in two gluten specific T cell lines. Cell lines A and E had SI values of 2,5 and 3,2 respectively. The cell line A also tested positive for the modified version of this last peptide, the α2c (SI=2,2) but, on other hand, cell line E, and all other gluten specific T cell lines, showed abrogation of toxicity when those aa substitutions were introduced (Table 5).

The peptides α3I and α3II tested negative in all gluten specific T cell lines. In these cases the SI ranged between 0,6 and 1,6 (Table 5).

Table 5. Stimulation indexes values from the proliferation assays of 6 gluten-specific T cell lines (A, B, C, D, E and F) tested with blank (medium only), gluten (GLU) and four different α-gliadin peptides (α2a, α2c, α3I and α3II). Positive SI (SI≥2) are marked in bold.

Patient	Blank	GLU	α2a	α2c	α3I	α3II
A	1	**4**	**2,49**	**2,23**	1,58	1,45
B	1	**2,16**	0,91	0,99	0,95	1,12
C	1	**3,01**	0,83	0,67	0,54	0,82
D	1	**4,42**	0,85	0,80	0,69	0,57
E	1	**3,47**	**3,16**	0,56	0,71	0,60
F	1	**4,46**	1,84	1,29	1,13	1,33

7. DISCUSSION



7.1. Raising gluten-specific T cell lines

For the elaboration of this type of experiment, gluten digested with pepsin and trypsin was used as a stimulation antigen for the biopsies and also used to help stimulation and proliferation of T cells, after gluten exposure to tTG. A total gluten fraction was used for this purpose since it is assumed that exist immunogenic determinants also in HMW and LMW glutenins and the use of only gliadins fractions would not stimulate specific cells. It is assumed that with each stimulation the number of gluten specific T cells increases and, therefore, the specificity of the cell line is ensured. To confirm the gluten specificity of the cell lines, every experiment is undertaken with not only the peptides we want to test, but also blank (medium only) as a baseline and digested gluten as a positive control.

The cell lines should be restimulated using PBMCs, as APCs, and digested gluten, as antigen, at least once. The reason behind this is the fact that, cell lines that were tested in proliferation assays before being restimulated, never shown to be gluten specific and so were incapable of providing results.

Sometimes, even after the cell lines had been restimulated with gluten more than once, they may lack specificity. The reason behind that could be the fact that the industrial gluten used is out of date and therefore enable to stimulate the gluten sensitive cells. Other reasons are, for example, the IL-2 that is used as a culture supplement, as it is a growth factor for T cells. If the concentration of IL-2 is not correct, T cells may not proliferate or other cells, which are not gluten specific, can also be stimulated.

One of the most important mixtures in this type of T cell proliferation work is the tTG mix. Like mentioned previously, this mixture is made with the use of digested gluten, a solution of $CaCl_2$ and industrial tTG. If any of these solutions are not correctly working (sometimes it's hard to dissolve the calcium which is essential for the function of the enzyme or even the tTG could have some industrial problems and might not be working properly) the work would be compromised and we would not be able to raise gluten-specific T cell lines.

Other reason, also crucial for the presentation of the antigen to the T cells, is the optimised radiation of PBMCs. PBMCs that are not correctly irradiated with the right dose, may not only be unable to present gluten in their surface as they will also proliferate. The correct dose to stop PBMCs to proliferate is 22 Gy. Every year, irradiation time has to be prolonged due to our cesium source losing some of its activity. Due to that, not only the correct dose is important, but also the time that the PBMCs are exposed to this irradiation is crucial for them to present antigen and stop dividing.

Knowing that, six T cell lines were shown to be gluten specific and used to test the selected epitopes.

7.2. T cell epitopes

Certain peptides derived from α-gliadins induce strong T cell responses in the large majority of patients. A crucial step towards the elimination of gluten toxicity would be the deletion of T cell stimulatory properties of α-gliadins sequences (Mitea *et al*., 2010).

It is known that four different HLA-DQ2.5 restricted α-gliadin epitopes have been identified: HLA-DQ2.5-glia α1a, DQ2.5-glia α1b, DQ2.5-glia α2 and DQ2.5-glia α3. One of our peptides harbours the DQ2.5-glia α3 epitope while peptide contains a variant. Then we also worked with a different peptide harbouring a different HLA-restricted element, having in their sequence the DQ8-glia α1 epitope and another harbouring its variant (Sollid *et al*., 2012).

Our peptides were chosen based on the hypotheses that one or more nucleotide substitutions would lead to CD patients. Knowing this, some alterations (aa substitutions) were made based on mutations that occur naturally.

The main alterations found in α2c and α3II peptides in our study were mainly proline to serine and glutamic acid to serine. In peptide α2c a substitution of an arginine to a proline was made and in the α3II peptide, one of the glutamine was substituted for an arginine (Tables 2 & 3).

In this study, the four peptides corresponding to residues 92 to 105 (α2a and α2c) and 206 to 218 (α3I and α3II) of α-gliadin were synthesised, isolated and tested in T cell proliferation assays. Peptides α2a and α3I differ from each other in their length and on the epitope they harbour. α2a is a 15mer peptide that have present in his sequence the epitope DQ2.5-glia α3 (previously named glia α20) whereas α3I has 13 aas and harbours a completely different epitope from a different DQ element, the DQ8-glia α1. These two epitopes are located in different ends of the α-gliadin peptide. The α2a is an epitope located in the N-terminal of α-gliadin, where the amount of proline residues is much higher, and the α3I is located in the C-terminal of the α-gliadin.

Our results showed that the peptide α2a had, in two of the tested cell lines, SI>2, indicating that it was CD toxic. As mentioned, these results were only obtained in two gluten-specific T cell lines, the other four showed SI lower than 2. Contrary, the peptide α3I never tested positive with our gluten- specific T cells, since the stimulation indexes obtained were always lower than 2 for all of the tested lines. This data shows that not all T cell lines are the

same nor react in the same way. Each cell line responds differently towards different peptides and they can be sensitive to one certain toxic epitope and fail to respond to others or the opposite.

Our results obtained with the peptide α2a are in concordance with previous studies (Camarca *et al*., 2009; Salentjn, *et al*., 2013; Sollid *et al*., 2012; Vader *et al*., 2002) that also reported on this particular epitope being CD toxic. They, however, undertook T cell proliferation assays using T cell clones from CD patients, which is a different technique than ours. This shows that both methodologies work and are suitable to test the potential toxicity of different gluten peptides.

On the other hand, we observed that the peptide α3I is not CD toxic as oppose to Van de Wal *et al*. (1998) study. Our results show that this peptide is not CD toxic in any of the gluten sensitive T cell lines tested when, for other groups, the epitope present in this peptide triggered T cell proliferation in T cell clones. As mentioned before, not all cell lines respond the same way for the same epitopes so, if one epitope is toxic to some patients it may not be to others. That would explain the fact that, for this particular epitope, our cell lines fail to respond but, when tested in other groups, it showed toxicity. Also, the majority of our patients, as the majority of all CD patients (95%) are HLA-DQ2+. Since the peptide α3I has in their sequence a HLA-DQ8 epitope, it would be required for the patients to also express this DQ element in order to recognize it and be stimulated by it. So these results, which were obtained previous to the patients DQ status, are the ones potentially expected.

7.3. Effect of amino acid alteration in the toxic gluten epitopes and the impact on T cell proliferation

Aa alterations in genes, also known as mutations, are common events that occur in different specimens. These mutations can take different forms and be express in different ways. The most common types of mutations are the ones that occur with a single nucleotide change known as SNPs (Single Nucleotide Polymorphism). This nucleotide alteration can lead to an alteration of the aa expressed and, therefore, to a modification of the protein or their behaviour towards physiological events.

Peptides α2c and α3II are the products of multiple aa substitutions in the core of the epitope that each of them harbour. The substitutions that are more common between this peptides and their original version are proline to serine and glutamic acid to lysine. Both of

these amino acids differ from each other in one nucleotide. Proline is synthesized by CCA, CCC, CCU or CCG codons, whereas serine is synthesise by UCA, UCC, UCU, UCG codons. A single alteration of a C for a U, in the beginning of each codon, would be required to transform these amino acids. On the other hand, glutamic acid or glutamine (whereas it was previously deamidated by tTG or no) requires GAA, GAG, GAC or GAU to be synthesised. Lysine would need the presence of AAA, AAG, AAC or AAU. In this case a modification of a G to an A, also in the beginning of the codon, would be sufficient to produce a different aa. Besides these modifications there is the presence of other two, which only occur one time and one in each epitope. α2c also as an alteration of an arginine to a proline, that is also the product of one nucleotide alteration (C→G) and α3II has the change of a glutamine to an arginine. This one is generated due to a different type of mutation known as inversion mutation. In this case one nucleotide is not altered to other but the order of them is modified.

All of these mutations are naturally occurring and can be harmless. But some can change the complete behaviour of the protein in the organism. The modifications were made with this principal and these peptides were also tested in T cell proliferation assays.

The results we obtained with the proliferation assays for α2c peptide showed, in one of the tested cell lines, SI above 2. As mentioned before, this indicates that the particular peptide is toxic for patients with CD. Our findings do not corroborate with what was found by other groups. Mitea *et al.* (2010) had found that, when the arginine, present at P2 in gliadin epitope DQ2.5-glia α3, was substituted by a proline, the T cell stimulatory capacity was destroyed. This substitution was tested in assays where the capacity of epitopes for binding to HLA-DQ2 was measured and in T cell proliferation assays using a different methodology than ours. The same group also tested the elimination of T cell stimulation when, an altered version of DQ2.5-glia α3 epitope with a proline to serine substitution at P8 was introduced to T cell proliferation assays. They found that this substitution alone was sufficient to eliminate toxicity of this epitope.

α2c peptide is the product of not only one but four aa substitutions: two proline to serine at P6 and P8, one arginine to proline at P2 and one glutamic acid to lysine at P4. As known there are two factors important to form strength interactions between peptide-HLA complexes with TCR: physical proximity and chemical (charge) complementarities. Also, it is known that hydrogen bonds occur mainly at positions 2, 4, 6 and 9 and that the ones at position 2 and 4 are the most important for binding to HLA-DQ2. Proline is one of the most important aa for high TCR binding. A pro residue at positions P1, P6 and P8 helps tTG deamidation of the peptides leading to an increase and better anchor to HLA. However,

proline is the only natural occurring aa whose amide hydrogen bond is lost because of participation in the secondary amide in the polypeptide backbone, as consequence of that, it can not form hydrogen bonds. Serine is charged the same as proline but the side chain is smaller. This fact leads to modification of TCR contact and to a weaker signal. But, even if serine it is not a good ligand, their hydroxyl group can participate in hydrogen bonds as donor or acceptors and can form these bonds with HLA-DQ2 molecules. As known, HLA molecules prefer negatively charged residues at anchor positions since they are stronger binders. Lysine is a positive charged aa and, unlike glutamic acid, its atoms can only serve as hydrogen donors and not as hydrogen acceptors, which means, that it cannot form hydrogen bonds with HLA.

Due to all that, the expectation would be that the peptide α2c would no longer promote T cell proliferation and, therefore, would not present toxicity. As mentioned, this was not the case for one of the tested cell lines. A potential reason could be that the proline introduced at p2 promotes binding when all these alterations are made in the core of the epitope. Since there are no previous published findings that have tested all these modifications in this epitope, at once, using T cell proliferation assays or other techniques, that could explain the toxicity found. But since only one out of six (16,67% of cases) gluten specific T cell lines in this study showed proliferation, the interaction between the peptide-HLA complex and TCR could be a weak interaction that does not occur with all CD patients. In addiction, Mitea and her team (2010) produce their results using T cell clones while we used T cell lines from different CD patients. Maybe, due to something, the method used by them could not detect the toxicity while ours could. Despite all this, more testing should be done in order to increase the number of responses and data and to prove the results obtained are indeed real.

For the last peptide tested, α3II, the stimulation indexes obtained in all tested T cell lines were lower than 2, meaning that this peptide did not showed potential toxicity for CD patients. This peptide, that harbours the altered version of the DQ8-glia α1 epitope, has three substitutions: two glutamic acid to lysine at P1 and P9 and one glutamine to arginine at P5. Studies made by Mitea and her team (2010) showed that a single substitution of a glutamine to an arginine at P5 was sufficient to stop the binding of peptide to HLA-DQ2 and to destroy the T cell stimulatory properties. Since none of our results showed SI>2 we could start to conclude that this peptide was non-toxic and that our results were the ones expected. However, since all the results obtained with all 6 gluten sensitive T cell lines for the immunogenic peptide (α3I) were also negative it is difficult to make definitive conclutions.

It is known that the glutamine residues at positions P1 and P9 of DQ8-glia α1 anchor on the basis of the peptide-binding motif. The one at P9 is pointing towards the TCR so it has a direct role and effect in the T cell recognition. Their conversion to glutamic acid by tTG, results in an increase of the affinity for DQ8 and, therefore, resulting in a higher coeliac response. The modifications of these residues by others that not glutamic acid would decrease this binding and eliminate or, at least, lower the peptide's toxicity. So despite the fact that, like in the previous case, there is no other research that tested this particular epitope with all the aa substitutions found in it, it is expected that the toxicity be eliminated and the results obtained in this research are in concordance with that.

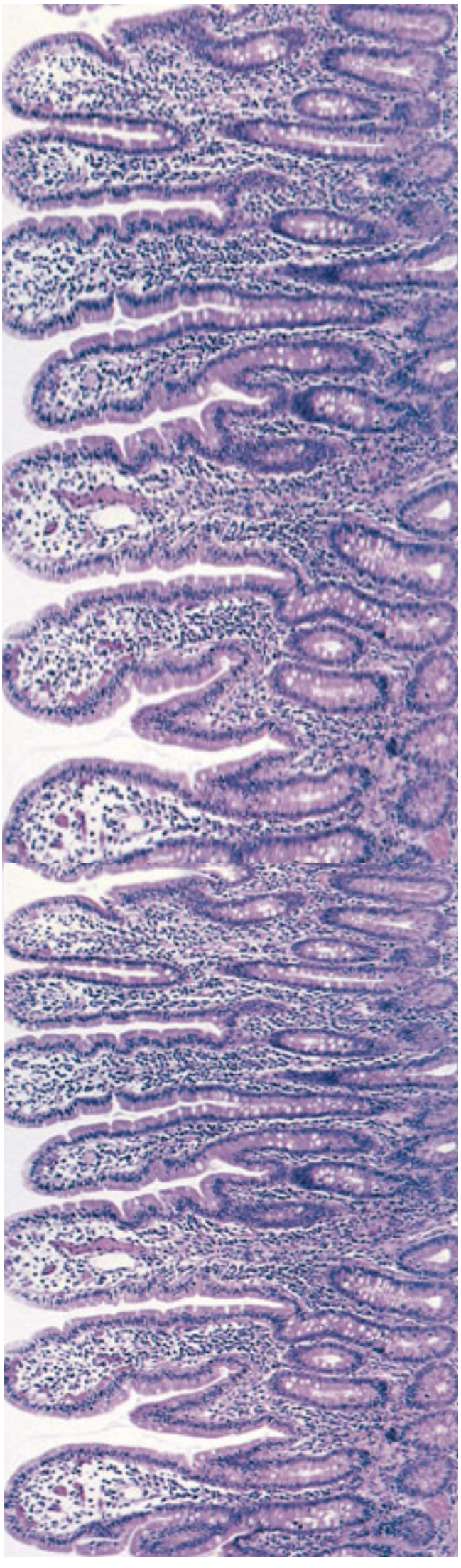
One reason that could justify the absence of positive results with this peptide is, like mentioned before, the DQ element of the patients used. Four of them are DQ2+ so it's expected that they do not respond to peptides harbouring DQ8 elements. Our supposition is that the other two patients that were not able to provide the DQ status are also HLA-DQ2 and, therefore, enable to react to this particular epitope.

Like with the previous peptide, more tests should be done to prove the elimination of toxicity, but for this particular peptide the use of HLA-DQ8 patients is required. If after several testing the peptide still prove to be non-toxic new approaches such as *in vitro* OC testing and *in vivo* feeding studies should be done to 100% confirm safety for CD patients.

As shown here, HLA-DQ-peptide binding is one of the crucial roles in CD pathogenesis. Blocking the presentation of CD-toxic peptides could lead to a control of disease expression. That way it would be essential to develop a peptide that could still have a strong affinity to HLA-DQ molecules but hinders contact with TCR. That could be achieving by changing the peptides structure making it bulkier or smaller on the TCR side, which would not allow recognition and/or binding. Other approach could be the delivery of a drug to the target place. This drugs would need to be taken at the same time as gluten products would be ingested and compete with HLA-DQ binding. The biggest problem with this approach is the *in vivo* stability of peptides as drugs and their rapid inactivation by gastrointestinal enzymes.

As mentioned, before any of our tested epitopes can be used in any potential treatment approach for CD, more tests regarding T cell proliferation assays should be done and further and more specific research such as *in vitro* OC and *in vivo* feeding studies. Only after these different research steps, the peptides may be considered safe for CD patients and used in approaches like the ones mention previously.

8. CONCLUSIONS AND FUTURE PERSPECTIVES



We have undertake T cell assays using, not only CD toxic epitopes, but also modified versions of these motifs where several aa substitutions had been made. We have demonstrate that, potentially, some substitutions are not viable for blocking T cell response but, if the correct alterations in the right positions are made, T cell proliferation is reduced or even eliminated. Our results also show that T cell proliferation assays using biopsies from CD patients are crucial to determine and characterize the potential toxicity of certain gluten peptides.

We have also demonstrated that gluten-specific T cells from different patients can recognise different gluten epitopes and that not all epitopes are found to be toxic for every gluten-specific T cell line. So, even if one gluten-specific T cell line fail to respond to one gluten epitope, it does not mean that other will or vice-versa.

We can conclude from our results, more testing should be undertaken in order to built a stronger and more solid conclusion. In addiction different tests should later be apply to prove potential lack of CD toxicity in the peptides tested. In our study, only α-gliadins were tested. Due to the similar properties and mechanisms of binding, similar approaches could be done for other gliadins to try to make more peptides for immunomodulation.

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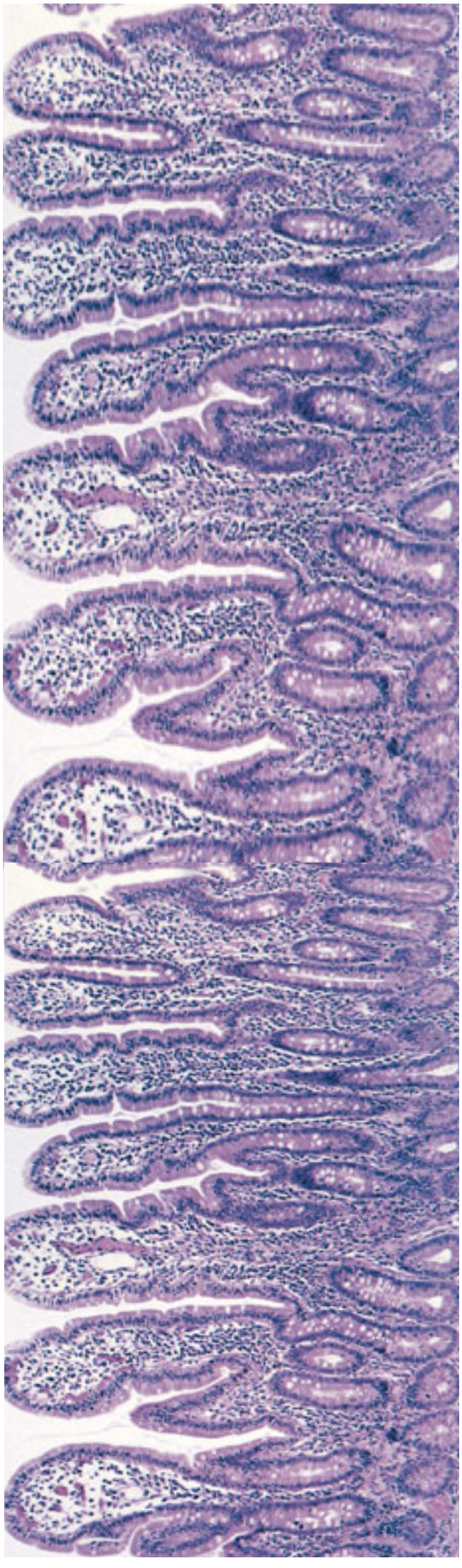

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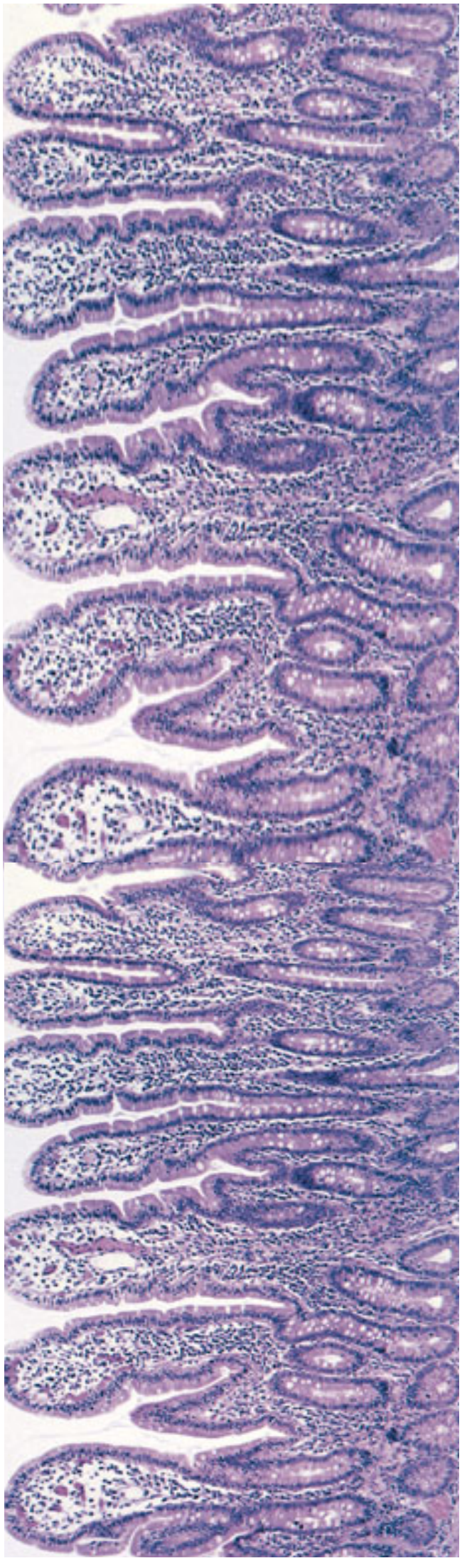
10. ATTACHMENTS



Attachment I

Poster presented at the 16th ICDS in Prague

Abrogation of coeliac immunogenicity of gluten peptides by amino acid point substitutions

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[2]Rutgers, The State University of NJ, Waksman Institute of Microbiology, Piscataway, New Jersey

1. Background and aims:

A gluten-free diet is the only available treatment for individuals with coeliac disease (CD). This significantly reduces their quality of life, such that new approaches are sought. Naturally existing variants of gliadins and glutenins have been identified that might be less immunogenic in CD. We tested selected variants of α-gliadin peptides in T-cell proliferation assays to evaluate their CD immunogenicity. Peptides with lower immunogenic profiles could form the basis of new dietary strategies.

2. Methods:

- Gluten specific polyclonal T-cell lines (TCL) were generated from individuals with CD.
- Candidate peptides were tested in proliferation assays using radioactive labelled thymidine to measure proliferation.
- We tested six α-gliadin peptides synthesised as 15 or 16mers. The first set of tested peptides harboured the overlapping T-cell epitopes DQ2.5-glia-α1a, DQ2.5-glia-α2 and part of DQ2.5-glia-α1b and naturally occurring variants that differed in a few amino acids (AA). A second set of α-gliadin peptides with similar AA substitutions harboured the DQ2.5-glia-α3 epitope.

Peptide label	Amino acid sequences
	Epitopes DQ2.5-glia-α1a, DQ2.5-glia-α2
Peptide 3	Q L Q P F P Q P E L P Y P Q P E
Peptide 5	Q L Q P F P Q P E L S Y P Q P E
Peptide 6	Q L Q P F P Q P K L S Y P Q P E
Peptide 9	Q L Q P F P K P K L P Y P K P Q
	Epitope DQ2.5-glia-α3
Peptide α2a	Q P F R P E Q P Y P Q P Q P Q
Peptide α2c	Q P F P P K Q S Y S Q P Q P Q

Figure 1: Amino acid sequences for tested α-gliadin peptides

- A stimulation index ≥2 was considered as positive.

3. Results

- The known immunogenic peptide α2a (QPFRPEQPYPQPQPQ) and its variant peptide α2c (QPFPPKQSYSQPQPQ) both induced positive responses in 1 out of 3 gluten-sensitive T-cell lines.
- A-gliadin has shown retained immunogenicity after introduction of amino acid substitutions (Patient A).

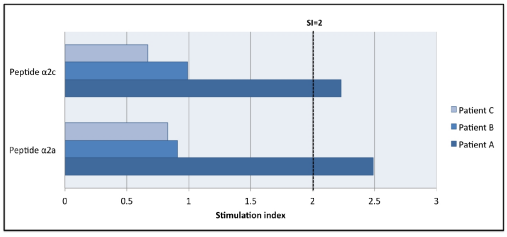

Figure 2: Presented gluten sensitive T-cell lines were obtained from small intestinal biopsies from 3 coeliac patients. In the figure stimulation indices are presented as a response on 2 different α-gliadin peptides

- The immunodominant peptide 3 (QLQPFPQPELPYPQPE) triggered positive T-cell responses in 4 out of 10 gluten-sensitive T-cell lines.
- Its point substituted variant peptide 5 (QLQPFPQPELSYPQPE) triggered positive T-cell responses in 2 out of 6 gluten-sensitive T-cell lines.
- Peptide 6 (QLQPFPQPKLSYPQPE) with two amino acid substitutions and peptide 9 (QLQPFPKPKLPYPKPQ) with three amino acid substitutions in the immunodominant gluten peptide did not stimulate the tested gluten- sensitive T-cell lines (n=8 and n=5 respectively).

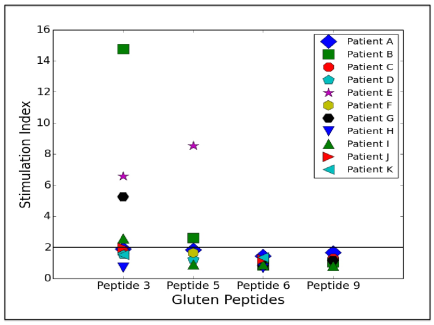

Figure 3: Presented gluten sensitive T-cell lines were obtained from small intestinal biopsies from 11 coeliac patients. In the figure stimulation indices are presented as a response on 4 different α-gliadin peptides

4. Discussion

- The heterogenic responses of different cell lines to the same epitope indicate the importance of testing gluten peptides with polyclonal T-cell lines.
- Our results confirm the importance of elimination of proline and glutamine residues in α-gliadin structure to decrease their immunogenicity
- Our results demonstrate that with carefully selected single point amino acid substitution in the core epitope of α-gliadin, immunogenicity of peptide can be decreased.
- We have shown that introduction of two carefully selected amino acid substitutions in α-gliadin peptides response of gluten-sensitive T-cell lines can be abrogated.

5. Conclusion

- Our results have implications in utilizing naturally occurring gluten proteins that lack CD toxic epitopes for new dietary strategies.
- These candidate peptides need additional assessment using small intestinal biopsy organ culture and oral testing to confirm their non-toxicity for individuals with CD.

Attachment II

Consent Form signed by all patients

Paul J Ciclitira
Professor of Gastroenterology

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LREC Study Number: 05/Q0702/167
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: In Vitro Characterisation of Gluten Fractions

Name of Researcher: Paul J Ciclitira, Professor of Gastroenterology

Please initial box

1. I confirm that I have read and understand the information sheet (Version 2 dated 10.12.08) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [company name] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to a sample of my donated tissue being kept for future research ☐
5. I agree to take part in the above study. ☐

Name of Patient		Date	Signature

Attachment III

Patient information sheet

10.12.08

PATIENT INFORMATION SHEET

In Vitro Characterisation of the gluten fractions that exacerbate coeliac disease

Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being undertaken and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. Please ask us for a copy, or if you wish, a copy may be obtained from CERES, PO Box 1365, London N16 0BW.

The Purpose of the study

Coeliac disease requires treatment with a life long gluten-free diet in which individuals need to avoid wheat, rye, barley and possibly oats. Characterisation, that is, learning the details of those fractions of those cereals that exacerbate the disease, will enable us to develop improved methods for measuring these proteins in foods for individuals with coeliac disease and also to develop non-toxic variants of these peptides. This will allow these peptides to introduced into other cereals such as maize, plants which are gluten-free, which should allow up to develop an improved flour which will have baking and nutritional qualities similar to wheat flour but which is non toxic for patients with coeliac disease.

We have been involved over a number of years investigating which fractions of gluten exacerbate coeliac disease. We wish to extend this study with new laboratory techniques understand better those parts of wheat gluten cause this disease. This information is important, as it will allow us to devise fractions of wheat that do not exacerbate the condition and therefore develop improved treatment for this condition. The study will involve you being asked to donate 50 ml of blood, that is 10 teaspoons and also you to agree additional small intestinal biopsies at the time of your endoscopy.

Why have I been chosen?

You have been asked whether you would like to participate because you are due to have an endoscopy for the investigation and management of your coeliac disease or related condition.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you

decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen to me if I take part?

During the course of your endoscopy you will be asked to give 50 ml of blood, that is approximately 10 teaspoons and in addition will have up to four additional small intestinal biopsies taken at the endoscopy. This should provide no additional risk or injury to yourself.

What do I have to do?

You will participate in the study having signed the consent form. Additional blood samples and biopsies will be taken at the time of your endoscopy.

What is the procedure that is being tested?

The samples we take will be used in the laboratory to access the toxicity of different gluten fractions. This will provide information in order to facilitate investigation of coeliac disease and to develop improved assays for measuring gluten in food for individuals with coeliac disease and novel therapies for this condition and also, the alternatives for diagnosis or treatment. This is not part of the diagnosis of your condition or treatment method. The study will provide additional information on the management of coeliac disease, for patients such as yourself.

What are the side effects of taking part?

There are no side effects for taking the additional blood samples. There is a minimal risk of haemorrhage, damage to the bowel, which occurs in less than one in 15,000 endoscopic procedures.

What are the possible disadvantages and risks for taking part?

There should be no risks for providing an additional blood sample. There is a minimal risk of one in less that 15,000 procedures of experiencing some gastrointestinal haemorrhage or damage to the bowel having small intestinal biopsies taken.

What are the possible benefits of taking part?

There may not be any clinical benefit to you due to taking part in this study. However, the information will enable us to learn more of the nature of fractions of gluten that exacerbate coeliac disease. This will help develop better assays for measuring gluten in gluten-free food and to devise improved treatment strategy for this condition, including cereals with baking and nutritional qualities of wheat but which do not exacerbate the condition.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

What happens when the research study stops?

This will make no difference at all to your treatment. Some of the samples that we collected from you will be stored, with a code number, not your name. The samples may be used for future research, but only if that research has received approval from the St Thomas' Research Ethics committee.

What happens if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms may be available to you.

Will my taking part in this study be kept confidential?

Information collected about you during the course of the research would be strictly confidential.

What will happen to the results of the research?

The results of the research will be submitted for publication in a pier-review journals. If you wish to have a copy of any published work it could be sent to you. You will not be identified in any reports or publications.

Who is organising a funding of the research?

The study is funded by sources for the European Union and the technical department of the University of the German Government. The researchers conducting the study will not be paid for including you in the study.

Who as reviewed the study?

10.12.08

This study has been reviewed by St Thomas' Hospital Research Ethics Committee.

Contact for further information

Should you require information, you should contact Principal Investigator, Professor Paul J Ciclitira whose address is given below, or through Professor Ciclitira's secretary at St Thomas' Hospital.

Professor P J Ciclitira
The Rayne Institute
4th Floor Lambeth Wing
St Thomas' Hospital
London SE1 7EH

TEL: 0207 620 2597
FAX: 0207 261 0667

What is next?

You will be given a copy of this information sheet to keep. Should you wish to take part in this study you will be asked to sign three copies of the consent form: one copy for you to keep, one copy is of researcher, and the last is to be kept with the hospital notes.

Thank you for considering to take part in this study.