UNIVERSITÀ DEGLI STUDI DI TRIESTE

UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXIV CICLO DEL DOTTORATO DI RICERCA IN

SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

THE INVESTIGATION OF THE ORIGIN'S SITE AND THE PATHOGENETIC ROLE OF ANTI- EPIDERMAL TRANSGLUTAMINASE 3 ANTIBODIES IN DERMATITIS HERPETIFORMIS

Settore scientifico-disciplinare: MED/38

DOTTORANDA
BARTOLOMEA GAITA

COORDINATORE
PROF. PAOLO GASPARINI

SUPERVISORE DI TESI
PROF. TARCISIO NOT

ANNO ACCADEMICO 2020/2021

Abstract

Dermatitis herpetiformis (DH) is an inflammatory skin disease characterized by granular IgA deposits in the papillary dermis and represents an extra-intestinal manifestation of coeliac disease (CD). DH and CD are autoimmune diseases, both share a similar genetic HLA background, have serum IgA antibodies and benefit clinical improvement with a gluten free dietary treatment. The epidermal transglutaminase (TG-3) has been recognised as the main autoantigen in DH disease.

In this study the presence and the production of TG-3 protein at intestinal and peripheral level are demonstrated. This evidence induced us to investigate the origin's site of TG-3 antibodies in both areas.

To carry out a project, the antibody-Libraries display technologies are used. Antibody-Libraries from intestinal and peripheral B lymphocytes of three DH and three control patients were constructed and examined by using the phage and yeast-display antibody methods. The combination of these technologies allowed to isolate and characterize anti-TG-3 antibodies, suggesting that both, intestinal and peripheral level, could be the primary site of antibodies production. The present study also focused on investigating the pathogenetic role of anti-TG-3 antibodies in DH disease. Human keratinocytes, representing the inflammatory site of DH, are proposed as a suitable in vitro model. The TG-3 protein is detected in the intracellular compartment but not in the plasma membrane, suggesting that the antigen-antibody binding site does not occur on keratinocytes. Therefore, this in-vitro model does not allow to investigate the pathogenetic role of anti-tg3 antibodies.

Riassunto

La Dermatite Erpetiforme (DE) è una malattia infiammatoria cutanea caratterizzata da vescicole papulari pruriginose e da depositi granulari di IgA in corrispondenza delle papille dermiche o lungo la giunzione derma-epiderma. La malattia è considerata la manifestazione cutanea della celiachia, condividendo con quest'ultima l'origine autoimmune, la predisposizione genetica, la presenza di autoanticorpi IgA a livello sierico e la remissione clinica in seguito all'eliminazione del glutine dalla dieta.

La transglutaminasi 3 o epidermica (TG-3 or eTG) rappresenta l'antigene verso cui si sviluppa la reazione autoimmunitaria.

Il primo scopo di questo studio è indagare l'origine degli autoanticorpi contro la TG-3, e a tal fine è stata utilizzata la tecnologia delle librerie anticorpali. La fisiologica presenza e produzione dell'antigene riscontrata sia a livello intestinale che periferico, ha suggerito, la possibile genesi degli anticorpi anti TG-3 in entrambi i distretti.

Librerie anticorpali, costruite da linfociti B intestinali e periferici di tre pazienti con DE e di tre soggetti di controllo, sono state analizzate mediante il metodo del phage-display e dell'yeast-display. L'approccio utilizzato ha permesso di isolare anticorpi anti TG-3 da entrambi i distretti.

Il presente studio si è focalizzato, inoltre, sull'indagine del ruolo patogenetico degli anticorpi anti-TG-3 sulla pelle. A tal fine è stato ideato un modello in vitro, costituito da una linea cellulare primaria di cheratinociti umani. Gli esperimenti di immunofluorescenza e di monitoraggio in Real-Time dopo stimolazione con anticorpi anti-TG-3, hanno escluso la presenza dell'antigene sulla membrana plasmatica. Ne consegue che in questo distretto cellulare non può avvenire il legame tra l'antigene e il suo anticorpo. I risultati osservati e il modello proposto non hanno permesso, pertanto, di indagare ulteriormente il ruolo patologico di questi anticorpi.

Contents

1. INTRODUCTION

1.1 Dermatitis herpetiformis (DH)

Dermatitis herpetiformis (DH) is a severe, inflammatory and autoimmune skin disease described as an extraintestinal manifestation of coeliac disease (CD).

The hallmarks of this pathology are represented by the presence of diffuse skin rush and itchy papular vesicles (Figure 1. a-b), symmetrically located on the surface extensor of elbows, knees, forearms , shoulders as well as on back, scalp and buttocks[1] (Figure 1. c).

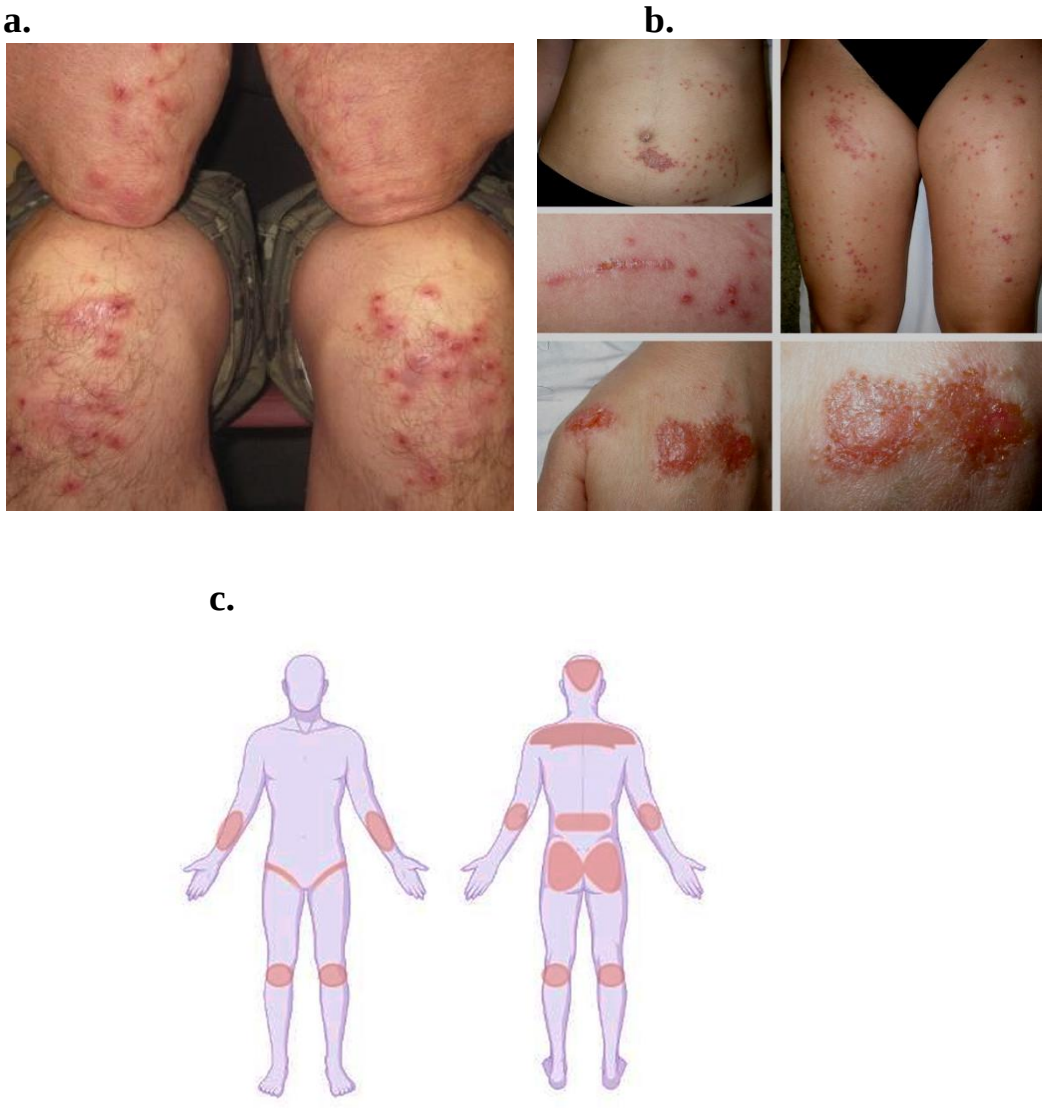


Fig. 1. Typical clinical picture of DH with blister and papules on the elbows and knees[2] (a), erythematous and vesiculous lesions in a body of DH patients[3] (b). Body representation of the regions in which DH occurs (c).

The term "herpetiform" refers to the clustered and serpiginous appearance of the lesions, similar to those found in herpes virus infections, but does not indicate a causal relationship with herpes viruses.[4]

DH was first described as a clinical pathology by the doctor Luis Duhring in 1884 and four years before Samuel Gee published a first clear description of CD symptoms, suggesting that gluten free dietary (GFD) treatment might be of benefit. [4,5]

However, it was only in the late 1960s that DH was related to gluten intolerance. In fact, CD is often associated with extra-intestinal manifestations and among these, DH is considered the specific skin manifestation[6].

The results of Van der Meer's studies in the 60s, have led the description of the granular deposits of Immunoglobulins type A (IgA) in the papillary dermis of DH patients [7], a survey that currently represents the immunological sign of DH.

The finding of Sárdy et al. in 2002[8] has demonstrated that epidermal transglutaminase (e-TG or TG-3) is present in skin and colocalizes with typical IgA aggregates. Since this evidence reported, TG-3 is regarded as the main autoantigen of DH. As Dieterich et al.[9] had previously shown that tissue transglutaminase (t-TG or TG-2) is the autoantigen in CD and colocalizes with IgA deposits in mucosa of the small intestine.[7]

DH is a relatively rare disease affecting predominantly Caucasians population, mostly in northern Europe, compared to African, American or Asian populations. [10]

In Europe and USA, the prevalence of DH ranges from 11.2 to 75.3 per 100.000 subjects and the higher incidence was recorded in Sweden and in Finland. [11,12]

In Asia, including Chinese and Japanese populations, the association between DH and CD appears to be weaker than Western countries, probably because the rarity of CD in these populations may have led to overlook the diagnosis of DH and consequently underestimate the prevalence of illness, although DH shows similar clinical symptoms and immunopathological features.[13] Overall, the ratio of CD to DH indicates that DH is the most common extraintestinal manifestation of CD [14] and it affects around 20- 25% of celiac patients.[15]

DH can appear at any age, but typically during adulthood, mostly between the third and the fourth decade of life, with a mean age at diagnosis of 40 years. It is more common in men than in women, with a male to female ratio of 2:1. [12,16]

1.2 Immunopathogenesis

The pathogenesis of DH is dominated by the production of IgA auto-antibodies directed against TG-3 enzyme.[17] The abundant presence of anti-TG-3 antibodies forms granular deposits of Immunoglobulins A (IgAs) along the papillary dermis in the dermal-epidermal junctions.[7,18]

It has been shown that TG-3 in the IgA deposits of patients with DH is present in an active form, and is therefore able to bind soluble fibrinogen, attracting cells of the immune system, such as T lymphocytes and polymorphonuclear leukocytes, at the level of the dermal papillae.[19]

The immune cells trigger an inflammatory process and produce inflammatory cytokines and proteases that cause the formation of subepidermal vesicles and, therefore, the onset of typical skin symptoms and manifestations.

It has been studied and verified that IgA deposits do not co-localize with other transglutaminases expressed in the skin, such as TG-2 and/or keratinocytic transglutaminase (k-TG or TG-1).[8]

The appearance of skin manifestations only in some sites (knees, elbows and buttocks), could be explained by the frequent bending to which these extensor surfaces are subjected. It is possible that these mechanical forces cause the direct activation of TG-3 present in the deposits at the dermal level and therefore these events are the triggering force of the subsequent formation of vesicles.[20]

Furthermore, the co-localization of IgA deposits and TG-3 was not found in skin biopsies of patients suffering from other skin disorders also characterized by IgA deposits, highlighting that this manifestation is a specific marker of dermatitis herpetiformis.[8,18] DH patients also have elevated IgA autoantibodies levels against TG-3, TG-2 and anti-endomysium (EMA), in both serum and intestinal mucosa levels, confirming the pathogenic relation with CD.[17]

According to the mechanism of CD-immunopathogenesis, hypothesized by Reunala et al.[20], at the level of the intestinal mucosa the gliadin peptides are modified by TG-2 and subsequently recognized by Antigen Presenting Cells (APC) presenting HLA DQ2 / DQ8. The consequent immune reaction is triggered causing the production of IgA against TG-2 and against gliadin peptides by activated B lymphocytes / plasma cells.

The presence of antibodies against TG-2, TG-3 and gliadins in the serum, could lead to hypothesize an epitope spreading phenomenon, i.e. an autoreactive response to endogenous epitopes that are distinct and not cross-reactive with respect to the epitopes that cause the disease[21].

Similarly to TG-2, the TG-3 (and TG-6 in gluten ataxia) can specifically deamidate gluten and is able to complex with gluten peptides through a thioester bound, although less efficient than TG-2. However, the specificities of the enzymes are different and TG-3 was shown not to be able to form an iso-peptide binding complex[22].

The pathophysiology of CD is induced by exogenous gluten. This represents a unique autoimmune disease, to which DH is related, that involves an external trigger (dietary gluten) that leads HLA recognition, innate and adaptive immune responses to gluten peptides via autoantigens, chronic inflammation in the intestinal epithelium, intestinal permeability, gradual villous atrophy and small bowel mucosal injury.[23,24]

In DH patients, the typical CD gastrointestinal symptoms are present, some patients show normal small bowel villous architecture and minor celiac-type enteropathy [1], but generally, the majority of patients with DH, show some degree of villous atrophy and intestinal changes, similar to those found in CD.[6,25]

It has been reported that CD with classic enteropathy may evolve over time into DH on a gluten-containing diet (GCD)[26], and Warren et al.[27] also reported that a prevalent lymphocytic infiltrate probably corresponds to a later stage of the disease.

This observation suggests that the pathogenesis of CD can extend and be attributed to the spread of the adaptive immune response to tissues other than intestinal mucosa, affecting various systems and organs, including manifestations on the skin (as in the case of DH), musculoskeletal and central nervous system.[24]

CD and DH are genetically predisposed, sharing the same genetic human leukocyte antigen (HLA) class II background, closely linked to DQ2 (more common) or DQ8 haplotypes, with the more common histocompatibility alleles DQ Al*0501 and Bl*0201. The genetic locus is on the short arm of chromosome 6.[15]

These different disease phenotypes can occur in the same families, even in monozygous twins and in case of genetic predisposition, first degree relatives have an increased risk of developing CD or DH. [11]

1.3 Diagnosis and Treatment

Diagnosis of DH is established on clinical symptoms, serologic antibodies, predisposing genetic factors, histologic and direct immunofluorescence (DIF) on skin biopsy at lesional and perilesional level, demonstrating granular IgA deposits in the dermal papillae of uninvolved skin (Figure 1.1). The gold-standard method to verify DH diagnosis is DIF examination[28] and the site of the biopsy, to perform the DIF test, is therefore somewhat discriminating. The perilesional site (just adjacent to an affected area) is preferred because biopsies taken from the blisters can give false negatives results, as IgA can be removed from inflammatory cells. [12,29]

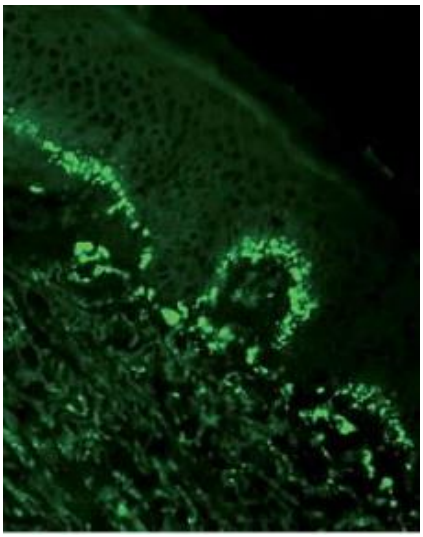


Fig. 1.1 DIF examination of uninvolved skin of DH patient. It shows a granular IgA deposit at the dermo-epidermal junction.[20]

The pattern most found in positive DH specimens show the formation of inflammatory infiltrate microvesicles with neutrophils and few eosinophils accumulation at the ends of the perivascular dermal papillae.[15]

Only this pattern would not allow to differentiate DH from other linear IgA bullous dermatosis diseases, not related with CD, and can causes difficulties in diagnosis.[30]

However, the presence of granular IgA-deposits in the papillary dermis is the diagnostic criterion that differentiates DH from other dermatitis that have a homogeneous (non-granular) accumulation of IgA along the entire dermal-epidermal junction.[15] The examination of skin biopsy can be accompanied by serological tests for

the anti TG-2, EMA and anti TG-3 antibodies. These tests are generally recommended to monitor the course of the disease after the introduction of the GFD. Nevertheless, the IgA deposits can persist for a long time in the epidermis, even when the itching and rash symptoms are disappeared after the GFD. [1,26]

As in the case of celiac disease, the only useful treatment to resolve the long-term DH symptoms is the introduction of a strict GFD. However, the diet takes time to relieve the skin symptoms. To reduce skin manifestations in a shorter time, the use of dapsone is the treatment of choice for this persistent disease.[29,31]

Dapsone is a drug with anti-inflammatory and antibacterial properties, which inhibits the chemoattraction of neutrophils and therefore skin lesions and inflammation. It should be used with close monitoring of the patient's clinical status, due to possible side effects. [10] After a few months from the start of a GFD, drug treatment is usually stopped and the serum level of antibodies progressively decreases, thus demonstrating the close relationship to gluten.[20] Blisters are often eroded and crusted, and lesions can resolve to leave post-inflammatory hyperpigmentation. If not diagnosed, DH can lead to an increased risk of developing more severe diseases such as, for example, neurological problems (ataxia), malabsorption syndrome and low bone mineral density. [29]

1.4 Transglutaminases and overview on TG-3

The etiology of CD and DH remain not fully understood. However, TG-2 and TG-3 are the predominant autoantigens respectively in the intestine and in the skin. For this reason, serum antibodies against these transglutaminase are used in the serological screening and follow up of dietary compliance.[15] Transglutaminases (TGs) form a family of calcium dependent enzymes that catalyse the formation of protein network by introducing isopeptide bonds and these are specialized in the cross-linking of protein involved in different physiological situations.[8] These specifically catalyse a Ca^{2+} transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and various primary amines or between lysine and glutamine residues. TGs also carry out the deamidation reaction in which a water molecule replaces an amine

on a glutamine residue which after this modification becomes glutamic acid.[32] The primary purpose of TG-mediated modifications is the formation of stable protein structures, such as cornified cell envelope (CCE) in differentiation of keratinocytes, dermal-epidermal junctions, a hair fiber, bones, or a fibrin clot. [33] Some TGs can function as atypical GTPases and/or ATPases, protein kinases and may also be associated with cell signalling and cell-matrix interactions or adhesion.[32] In humans, depending on the distribution in organs and tissues, nine different TGs have been identified which show a high conservation enzymatic domain. They are TG- 1 to 7, Factor XIII and Band 4.2.[8] TG1, TG-3, TG5 and TG6 are widely expressed in epidermal keratinocytes playing an important role in cells differentiation, cornification and skin development, carrying out the cross-linking process of proteins to form CCE in the skin epidermis, as well as to strengthen hair fiber[34] (Table 1).

Protein and synonyms	Coding Gene	Functions	Human Disease
Keratinocyte TG / TG-1	TGM 1	CCE formation, wound healing	Lamellar ichthyosis
Tissue TG/ TG-2	TGM 2	Apoptosis, wound healing, matrix stabilization, cell differentiation	Inflammation, a mediator in inflammatory response to UV irradiation, associated with non-skin human disorder (CD)
Epidermal TG/ TG-3	**TGM 3**	CCE formation, hair fiber stabilization	**DH**, uncombable hair syndrome
TG-X/ TG-5	TGM 5	CCE formation, Epidermal differentiation	Skin peeling syndrome, hyperkeratosis in ichthyosis and psoriasis
TG-Y/TG-3-like/ TG-6	TGM 6	Late stage CCE and hair follicle formation	No skin defects, associated with other syndrome (ataxia)

Table 1. Distribution of TGM genes in the skin, related functions and human diseases.[33]

The TG-3 is of particular interest because recognized as the autoantigen involved in the development of DH. This enzyme is encoded by TGM3-protein coding gene located on the chromosome 20q11–12. The protein is composed of 693 amino acids and has a molecular mass of 76.632 Da. It consists of two polypeptide chains, which are synthesized as a precursor or zymogene form of a single polypeptide, activated by proteolytic cleavage. The activation may be catalysed by cathepsin L (CTSL) or Cathepsina S (CSS) proteins.[35,36]

TG-3 protein is localized at the cytosolic level and in humans it was initially discovered in the hair fiber and hair follicle. Later its expression was found in mucosa, oesophagus, kidneys, lung tissues, testes skeletal muscles and skin[33], where it is constitutively expressed in suprabasal layers of the stratified keratinocytes[8] (Figure 1.2).

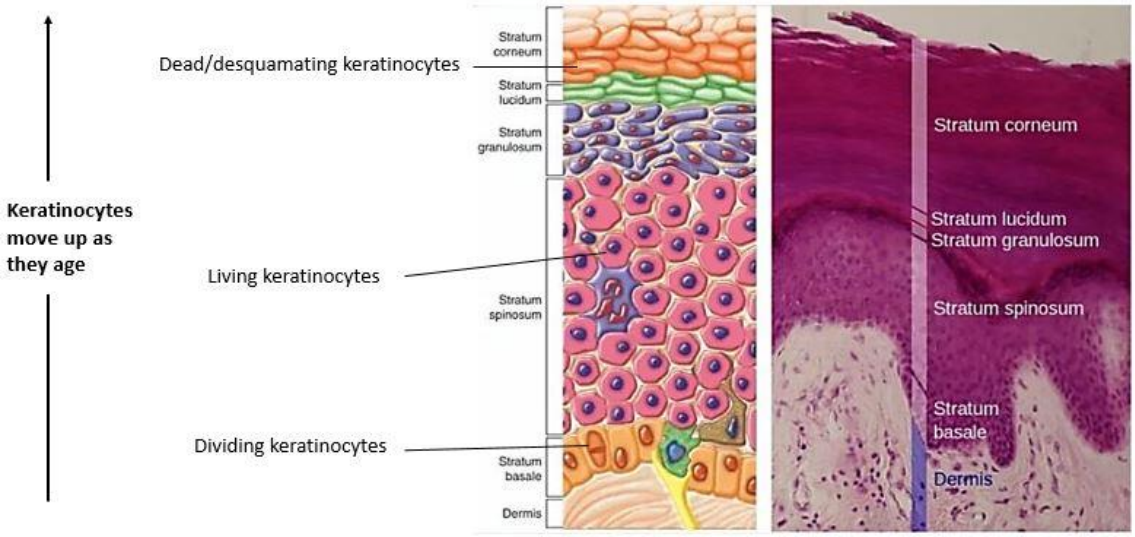


Fig. 1.2 Representation of layers, anatomy and structure of skin with aging levels of keratinocytes.

The protein produced as zymogen form consists of 4 domains:

- N-terminal β-domain containing 9 β-sheets interspersed with 3-α helices and includes the amino acids from 1 to 134 position,
- the catalytic core containing 15 β-strands interspersed with 15 α-helices and includes amino acids from Asn 135 to Gly 472,
- a first β-barrel domain comprising amino acids from 473 to 592 position,
- a second β-barrel domain comprising amino acids from 593 to 692 position.

The active site is hidden in a narrow slit comprising by two β-strands of the catalytic core and the C-terminal portion of the first-barrel domain.[36] The activation of zymogen requires a specific cleavage at Ser 469,[35] converting it into the enzyme that can be activated by Ca^{2+}. The two fragments of about 30 kDa and 47 kDa are held together by non-covalent bonds. In the zymogen form, TG-3 binds a calcium ion, which is maintained even after the proteolytic cleavage and is thought to be required to ensure its stability. However, the binding of two other calcium ions is required for enzymatic activation, in particular the binding of the third calcium ion produces a protein conformational change, which leads to the opening of a channel leading to the catalytic site.[36]

The proteolytic cleavage allows the binding of a magnesium ion to keep the enzyme inactive. As intracellular calcium levels rise, a calcium ion replaces the magnesium ion, allowing it to switch from an inactive to an active enzyme.

TG-3 activity is also regulated by the presence of GTP and GDP, which bind the catalytic core and the first β-barrel domain. The binding of GTP is associated with the replacement of the calcium ion by the magnesium ion with consequent inactivation of the enzyme.[36,37] Contrariwise, the hydrolysis of GTP into GDP allows the enzyme to return to its active conformation.

1.5 Antibody-libraries technologies: Phage and Yeast display

The immune system represents a biological exception in which the reorganization of DNA sequences is used to regulate the expression of genes.

This is in fact, an extended case of genomic content change in which recombination creates a vast antibody repertoire in the lymphocytes, combining the recombination of antibody and somatic gene segments hypermutation.[38]

The antigenic specificity of antibodies in humans is mainly governed by six complementarity determining regions (CDRs), each approximately 10 amino acids (aa) long. CDR1, CDR2 and CDR3 are on the heavy and light chain of the antibody. During B cell differentiation, these six sequences are randomized through V (D) J recombination. Upon participation in an immune response, CDR regions can further mutate, producing high affinity antibodies for specific antigens. Among the CDRs, CDR3H is the most highly variable and generally contributes to antigen (Ag) specificity.[39]

The perception of the different and useful human applications of antibodies has led scientists to try to mimic the diversity achieved within B cells performing antibody libraries in vitro. The antibodies display technology was first described by Smith in 1985[40] and further developed by other groups. The first successful experiment of B-cell antibody repertoire cloning, recombinantly and in combinatorial libraries, was carried out by McCafferty et his team in 1989 and marked the explosion of monoclonal antibody (mAb) technology.[41] About 1 year later another hallmark for in vitro antibody discovery has occurred, namely the development of display technologies for isolation and characterization of recombinant antibody fragments from these ab-libraries.[42]

The antibody display technologies provided a way for antibody selection and screening, based on binding to the Ag and allowed to identify hundreds of different antibodies

against a specific target, opening a new field for different types of antibody libraries without the need for immunization.

The in-vitro antibodies (Figure 1.3) can be produced in the form of:

- single chain variable fragment (scFv): consisting of variable domains of heavy (V_H) and light (V_L) chain, joined by a flexible linker of 15-20 ammino acids [43],
- antigen binding fragment (Fab): consisting of heavy (CH) and light (CL) chain polypeptides assembled with a disulphide bond.

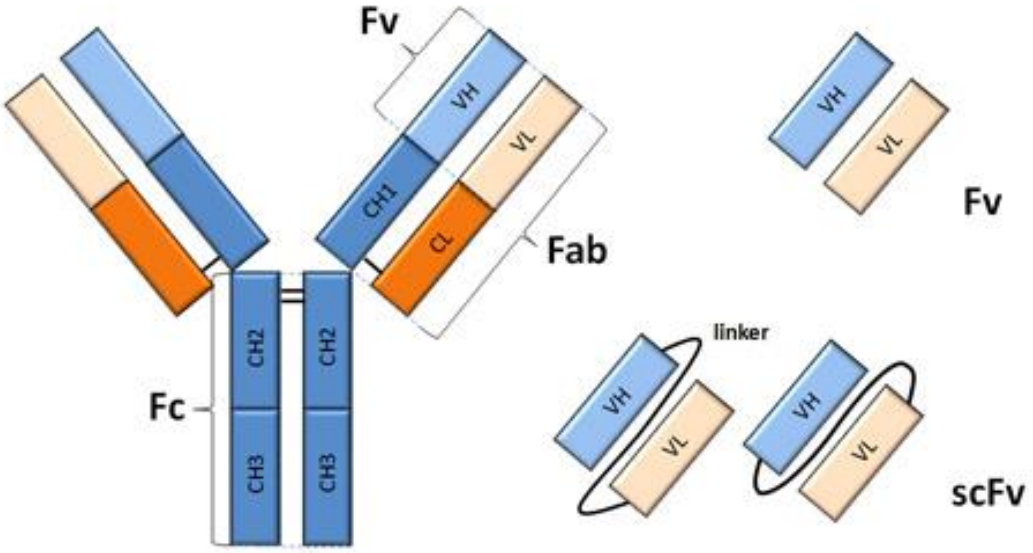


Fig. 1.3 A typical Ig molecule is composed of two heavy chains (blue) and two light chains (orange), linked by disulphide bonds. Fab are linked to the crystallizable fragment (Fc) by the hinge region. The variable fragments (Fv) joined by flexible linker make up the scFv.

The mAbs can be constructed using genetic engineering procedures by cloning and expressing the V_H-V_L immunoglobulin genes of entire response repertoires immune antibody in different vectors and cells type.

The starting material is peripheral B lymphocytes, isolated from blood or lymphoid tissues (such as the intestine). From these the RNA is extracted, reverse-transcribed into cDNA and the immunoglobulin variable-region genes are amplified.

Furthermore, by introducing hypervariable regions into the genes, with high levels of randomness by means of mutagenesis, it is possible to isolate and select in vitro

recombinant human monoclonal antibodies directed towards a wide variety of Ags that have different molecular weights, conformational structures and origins.[39]

Evolution has allowed the development of several and different strategies for antibody-libraries generation. It based on differences for the design and the means of construction, but in any case all focused on the antigen-affinity, size and diversity of the library.[38]

In this thesis the antibody-library technologies used were the phage and yeast display.

Phage display antibody libraries use bacteria cell. This technology is based on genetic engineering of bacteriophages (viruses that infect bacteria) and repeated cycles of selection on the target Ag of the antibodies displayed on phages.[44]

The V_H and V_L antibody-domains PCR products, representing the antibody repertoire, are ligated into a phage display vector and expressed as a scFv fused to a capsid protein of a filamentous bacteriophage of Escherichia coli.[45]

Due to its design, the connection between the expression of scFv on the outside and the genetic information inside the phage is possible. This technique permits very large libraries generation and both, genetic and functional analyses of the mAb selected, thus facilitating studies on mechanisms of the human immune system. [37]

The limitations of this procedure may be due to phage display which may not recover all antigen-specific mAbs present in a given antibody library [45], and it is cumbersome to fine-tune the process of selecting and isolating antibody with specific properties.

In this case, the selection is a random process, up to the screening of single phages clones, there is little or no evidence of the quality of the ongoing selections. Furthermore, it is also limited by the possibility of quantitatively following enrichment during the selection cycles.[46]

Yeast-display use eukaryotic cell of yeast, in particular EBY100- Saccharomyces cerevisiae strain, likewise the plasmid encoding the antibody is carried and it allows the expression of much more copies of antibody on cell surface. Furthermore, the rigidity of the cell wall allows a stable maintenance of the protein exposure.

This system takes advantage of the association of Aga1 and Aga2 proteins to display a recombinant scFv on the yeast cell surface. The scFv is cloned into yeast vector in frame with the Aga2 gene and the expression of both the Aga2 and the Aga1 proteins, in the

EBY100 yeast strain, is regulated by the *Gal1* promoter detectable in presence of galactose (Figure 1.4). Upon induction with galactose, the Aga1 and the Aga2 fusion protein associate within the secretory pathway, and the epitope-tagged scFv antibody is displayed on the cell surface.[47]

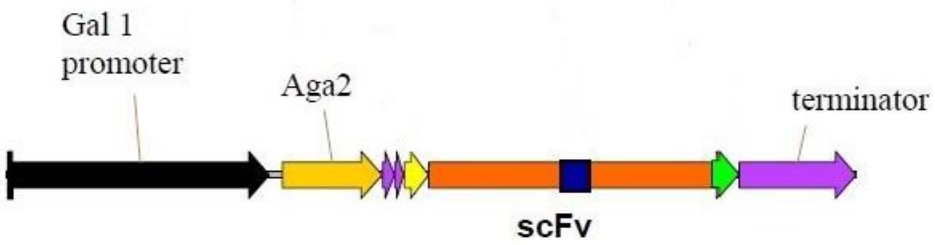


Fig. 1.4 The *Gal1* promoter regulates the expression of cloned scFv in frame with the *Aga2* gene

The analyses require the Flow Cytometry. The selection parameters can be accurately controlled by cytometry-based analysis and sorting flow.[46]

Comparing the phage and the yeast-display technologies, it is possible to understand the two main advantages of eukaryotic respect to prokaryotic-based display systems:

1. using the unicellular eukaryote cells, the expression of eukaryotic proteins is favourited because the antibodies can be correctly folded and modified to be closer to their native structure in mammals, avoiding the misfolding issues encountered in phage display.
2. using the Fluorescent-Activated Cell Sorter (FACS) is possible to customize the selection towards high affinity antibodies that bind specifically labelled target Ags.

The combination of the two display technologies takes advantage of both platforms by starting with a large antibody library and selection outputs obtained from phage antibody display, followed by the precise sorting and enrichment of the clones specifically recognizing the target of interest with yeast antibody display.[46]

2. PURPOSE OF THE THESIS

DH is characterized by the presence of serum IgA anti-TG3 antibodies, which disappear in GFD. Since DH and CD are closely related, it is reasonable hypothesize a similar origin and diffusion of specific antibodies. In CD it has been demonstrated that anti TG-2 antibodies are synthesized by intestinal and not peripheral B lymphocytes, and the spill over from this site justifies their presence in serum.[48,49]

This project aims to investigate whether in DH IgA anti TG-3 antibodies are produced at intestinal and/or peripheral level.

Using PCR gene amplification and ELISA assay, TG-3 protein production has been investigated in serum and in small intestinal biopsy samples. In addition, ab-libraries in a scFv form, from intestinal and peripheral B-lymphocytes of all recruited patients, were constructed and tested using phage and yeast display methods.

In proliferating keratinocytes of papillary dermis of DH patients granular IgA against TG-3 are typical. Therefore, this study has the further aim of investigating the pathogenetic role of anti TG-3 antibodies in DH disease. Human primary keratinocytes cells have been hypothesized as a suitable cellular model to study the pathogenetic effects of anti TG-3 antibodies.

3. MATERIAL AND METHODS

3.1 Patients' cohort

The study includes three paediatric patients with diagnosis of DH, according to the regular clinical practice, based on typical clinical picture and demonstration of granular IgA deposits in the papillary dermis. These are celiac patients and at the time of the inclusion in the study were in gluten containing diet (GCD).

The control patients are three and suffers from CD, functional dyspepsia and gastritis. All the control patients were tested negative for serum anti TG-3 antibodies and reported regular GCD. All patients of this study are diagnosed, followed and referred to the Burlo Garofolo Hospital in Trieste, Italy.

The clinical data, the serum levels of IgA anti-TG2, anti-TG-3antibodies, EMA and the genetic HLA- class II background are reported in Table 2.

The cut-off value of IgA Anti-TG3 antibodies concentration of 1.4 µg/ml has been described by Ziberna et al.[50] This novel quantitative ELISA test is currently used at Burlo Garofolo Hospital to detect serum IgA anti-TG3 Abs in patients with DH.

Written informed consent was obtained from the parents of the children enrolled.

Table 2.

CASE	AGE, SEX, ETHNICITY	FINAL DIAGNOSIS	IgA anti-TG2 serum level > 7 U/ml	IgA anti-TG3 serum level >1,4 µg/ml	IgA EMA serum level	INTESTINAL Histological aspects- Marsh Classification	SKYN Direct Immunofluorescence Results	HLA Typing
Patient DH- 1	17y, Male, White	Celiac, Dermatitis Herpetiformis	128 U/ml	9,6 µg/ml	POSITIVE	Villous atrophy- M 3a	Neutrophilic papillitis on elbow, Microabscesses at the papillary dermal, Granular IgA deposits on the upper dermis	HLA DQ 2.5/x
Patient DH- 2	14y, Male, White	Celiac, Dermatitis Herpetiformis	229 U/ml	9,5 µg/ml	POSITIVE	Villous atrophy M 3b	Granular IgA deposits on the upper dermis	HLA DQ 2.5/x
Patient DH- 3	16y, Male, White	Celiac, Dermatitis Herpetiformis	280 U/ml	7,6 µg/ml	POSITIVE	Villous atrophy M 3a	Granular IgA deposits on the upper dermis	HLA DQ 2.5/x
Patient C-1	4y, Male, White	Celiac	13 U/ml	0,28 µg/ml	POSITIVE	Villous atrophy M 3a	NONE	HLA DQ 2.5/x
Patient C-2	16y, Male, White	Functional Dispepsia	0,3 U/ml	0,6 µg/ml	NEGATIVE	NONE	NONE	HLA DQ7
Patient C-3	9y, Male, White	Gastritis	0,1 U/ml	0,4 µg/ml	NEGATIVE	NONE	NONE	HLA DQB1

3.2 PCR TGM-3 gene amplification

The expression of TGM-3 gene, in the intestinal biopsies from one DH and one control patient, is detected using Polymerase Chain Reaction (PCR). The cDNA from human oesophagus sample was used as positive control.

1µg of total RNA of each distal duodenum sample was used to synthesize the first strand cDNA, which was used as template for PCR amplification.

The RNA extraction and retro-transcription protocols are described in the "construction of antibody library" section.

To design specific primers pairs to amplify the TG-3 sequence region an online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used, submitting the TG-3 cDNA FASTA template downloaded from open-source database Ensembl (https://www.ensembl.org/index.html).

The primers Sequence (5'->3') designed and used are:

- Forward primer: AGCGCATCACACAGACAAGT
- Reverse primer: AGTGGAAACACAGCCTTCGT

Primer pairs are specific to template, no other targets were found in selected database: Refseq mRNA, referred to Homo Sapiens Organism.

The PCR reaction was optimized using the Q5 Hot Start High-Fidelity DNA products (New England BioLabs) and requires the following components:

- cDNA (5ng/µl)- 3µl
- Q5 HS Buffer 5x- 10µl
- dNTPs 10Mm- 1µl
- Primer forward 10µm- 2.5µl
- Primer reverse 10µm- 2.5µl
- Q5 HS DNA polymerase- 0.5µl
- H2O to 50µl

Conditions of amplification:

	Temperature (C)	Time (sec)	Cycles
Initial denaturation	98°	30	
Denaturation	98°	5	X 30
Annealing	59°	10	
Extension	72°	30	
Final Extension	72°	120	
Hold	4°		

- The PCR reaction product was analysed by electrophoresis on a 3% agarose gel.

3.3 Enzyme-linked immunosorbent assay (ELISA)

The sera and culture biopsy of DH patient-3 and control patient C-3 were analysed by sandwich ELISA for human epidermal transglutaminase 3 (TGM3 elisa kit- MyBiosource) following the protocol provided.

The culture biopsy came from one intestinal (distal duodenum) fragment, cultured for 72 hours at 37 °C and stimulated with a peptic-tryptic digest of gliadin (PT-gliadin) (Antiendomysium biopsy kit- Eurospital) following the manufacturer's instructions.

The culture biopsy and sera samples were collected and stored at -20 °C until analysis. All samples were tested 1:10 diluted in a sample-dilution solution.

It was not possible to test the samples of all the patients enrolled due to unavailability of the necessary specimens.

3.4 Construction of antibody library

Antibody-libraries were constructed from B-lymphocytes from peripheral blood and distal duodenal biopsy of recruited patients. The biopsy materials were obtained from patients undergoing intestinal biopsy to confirm their diagnoses. Biological samples were frozen in Trizol reagent (Invitrogen) and kept at -80°C until RNA extraction, to preserve the integrity of the nucleic acids. RNA extraction was performed using AllPrep

RNA mini kit (Qiagen) which allows RNA to bind to the membrane, any contaminants are efficiently washed away, and the RNA is eluted in RNAse-free water.

RNA Extraction from intestinal biopsy tissue

Lysis and Homogenization:

- Thaw the sample in Trizol preserved, transfer it to Buffer RLT (Qiagen), proceed to disruptions with a pestle, and homogenize with syringe and needle,
- Proceed with separation, washing and RNA elution following the protocol provided by Qiagen kit.

RNA Extraction from Peripheral Blood Lymphocytes (PBLs)

Samples of human PBLs are purified by density gradient centrifugation on Ficoll Paque PLUS (VWR) following the protocol described below:

- Dilute the blood sample stored in the presence of 1:1 anticoagulant in phosphate buffered saline (PBS),
- Place in a Falcon 1 Volume of Ficoll and 2 volumes of the previously diluted sample
- Centrifuge at 850 xg per 30 minutes
- Transfer the peripheral lymphocyte phase (PBL) to a test tube
- Add 10-15 ml of PBS
- Centrifuge at 300x g per 10 minutes
- Remove the supernatant, re-suspend the pellet in 1 ml of Trizol reagent and store at -80°C for several months.

To extract the RNA:

- the thawing sample is centrifuged at 400x g and supernatant is discarded, the pellet is resuspended in 1ml of RLT buffer and proceed following the protocol previously described.

The extracted RNA is stored at -80°C to prevent degradation or used immediately for reverse transcription reaction.

[Note: Blood samples should be processed as soon as these are collected from the donor. Prolonged storage can result in the isolation of degraded RNA]

Retrotrascription of cDNA

The reaction was performed by SuperScript IV Reverse Transcriptase kit (Thermofisher Scientific) following the protocol described below.

Add the following components to the sample:

- 1 µl of Random hexamers 50µM
- 1µl of 10 mM dNTP mix (each 10 mM)
- Template RNA (10pg- 5µg) up to 11µl
- Nuclease free water to 13 µl

Incubate 5 minutes to 65°C and then incubate on ice for at least 1'. Add the following components to the reaction tube:

- 5x SuperScript IV Buffer
- 10Mm DTT
- SuperScript IV Reverse Transcriptase 200 U/µL
- Nuclease free water to 20µl

Briefly mix and centrifuge the reaction mixture, incubate at 55° C for 10 minutes and inactivate the reaction by incubation at 80° C for 10 minutes. Use immediately or store at -20° C.

Antibody Library: Phage display technology

Bacteria Media and required reagents:

- Escherichia coli DH5αF' strain and pDAN5 [51] phage display vector,
- DH5αF' Electrocompetent cells (New England BioLabs),
- Q5 Hot Start High-Fidelity DNA amplification kit (New England BioLabs),
- Gel Extraction kit (Qiagen),
- DNA Clean columns Kit (Zymo Research),
- Qubit dsDNA HS (High Sensitivity) assay kit (0.2-100 ng) (ThermoFisher),
- NEB Restriction endonucleases products: CutSmart buffer, BSSHII, NheI (New England BioLabs),
- T4 DNA Ligase (New England BioLabs),
- Borate acid (Mf = 0,749M) + Ethylenediaminetetraacetic acid (Mf = 0,0255M) dissolved in distilled H2O,

- LB (liquid broth) bacteria media: 10 g bacto-tryptone, 5 g bacto-yeast, and 5 g NaCl to 1 L of distilled H_2O and autoclave. Add glucose 1x, Ampicillin 1x and Kanamycin 1x antibiotics,
- LB Agar plates: 1.5% bacto-agar to LB media. Make up to 1 L with distilled H_2O and autoclave. Add glucose 1x, Ampicillin 1x and Kanamycin 1x antibiotics,
- PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 in 1 L H2O, final pH 7.4,
- PBS-Tween 20%: add 1 mL of Tween-20 per liter of PBS1x,
- PEG-NaCl- Solution for precipitation of phages: 20% (w/v) polyethylene glycol (PEG) 6000 and 2.5 M NaCl. The solution is filtered and stored at 4°C,
- 1M Sulphuric Acid (H2SO4): 55.6 mL 97% sulfuric acid dilute up to 1 L H_2O,
- TMB (3,3',5,5'-tetramethylbenzidine) ready-to-use, premixed solution for colorimetric HRP-based ELISA detection
- MaxiSorp-ELISA 96-well plates and MaxiSorp-Immuno Tubes for Ag-immobilization,
- Helper phage M13KO7,
- Antigen human TG-3: commercial protein (Zedira)
- Anti-M13 HRP phage antibody (Euroclone),
- All Primer sequences are reported in the below Table 3.

Amplification of cDNA

1st PCR

The first PCR was optimized for the amplification of V_H, V_K and V_λ domains from B-cell cDNA.

V_H domain needs 7 different amplifications that share the same IgA forward primer to amplify the chosen antibody class but with different reverse primers (VH-4/5/6/10/12/14/22) for amplification of all gene families.

In the case of V_K and V_λ domains the same forward primer mix, depending on the type of light chain is required: V_K-mix made up of the equimolar mixture of VK-1/2/4/3/5 forward and V_λ-mix made up of the equimolar mixture of Vλ1/2/7 forward and different reverse primers to amplify the various families [51].

Set up the reaction as follows:

- Bcell cDNA (5ng/µl)- 5µl
- Q5 HS Buffer 5x- 10µl
- dNTPs 10Mm- 1µl
- Primer for 10µm- 2.5µl
- Primer rev 10µm- 2.5µl
- Q5 HS DNA polymerase- 0.5µl
- H2O to 50µl

Conditions of amplification:

	Temperature (C)	**Time (sec)**	**Cycles**
Initial denaturation	98°	30	
Denaturation	98°	5	X 25
Annealing	59°	10	
Extension	72°	20	
Final Extension	72°	120	
Hold	4°		

- Check the PCR products on TBE 3% agarose gel
- Combine same volume of each domain amplification to obtain three V gene family mix VH, VK, Vλ
- Check them on TBE 3% agarose gel.

The samples can be used directly as template for the 2nd PCR or if the control gel-run shows non-specific bands use the gel extracted product as template.

2nd PCR

This reaction adds overlapping a linker for the introduction of cutting sites for some restriction enzymes useful for cloning into vector regions. The primers required for VH domain are VH-mix forward and VHPTL reverse, the primers for VK/Vλ domains are VLPTL forward and VLPT-1 reverse.

Set up reactions as follows:

- mixPCR VH/VK/Vλ (5ng/µl)- 5µl
- Q5 HS Buffer 5x- 10µl
- dNTPs 10Mm- 1µl

- Primer for 10µm- 2.5µl
- Primer rev 10µm- 2.5µl
- Q5 HS DNA polymerase- 0.5µl
- H2O to 50µl

Conditions of amplification:

	Temperature (C)	**Time (sec)**	**Cycles**
Initial denaturation	98°	30	
Denaturation	98°	5	X 20
Annealing	61°	10	
Extension	72°	20	
Final Extension	72°	120	
Hold	4°		

- Check the PCR product on agarose TBE gel 3% and purify the sample by extraction of gel band (Gel Extraction kit-Qiagen) following the protocol provided. The gel extracted product is used as template for the 3rd PCR

3rd PCR (assembling)

The third PCR allows, thanks to the linker complementarity, to assemble heavy chains and light chains, reproducing in vitro the process that takes place in vivo.

The final formed structure is called Single chain variable fragment (scFv)

Set up reactions as follows:

- 2nd PCR VH/ VK/ VΛ
- Q5 HS Buffer 5x- 10µl
- dNTPs 10Mm- 1µl
- Primer VHPT-1 forward 10µm- 2.5µl
- Primer VLPT-2 reverse 10µm- 2.5µl
- Q5 HS DNA polymerase- 0.5µl
- H2O to 50µl

In this case the Primers are added at the end of the eighth amplification cycle

Conditions of amplification:

	Temperature (C)	Time (sec)	Cycles
Initial denaturation	98°	30	
Denaturation	98°	5	X 33
Annealing	65°	10	
Extension	72°	20	
Final Extension	72°	120	
Hold	4°		

- Check the PCR products on agarose TBE gel 3%
- Purify the sample by extraction of gel band
- Quantify the sample by reading with the Qubit 2.0 Fluorometer instrument (Invitrogen) following the protocol of the Qubit dsDNA HS (High Sensitivity- 0.2-100 ng) assay kit.

Cutting with enzyme restrictions

This reaction cuts the assembling product in order to create the sites for vector cloning. The protocols have been optimized using the NEB Restriction endonucleases products (New England BioLabs).

BssHII restriction enzyme recognizes the sequence G^CGCGC, while NheI recognizes the sequence G^CTAGC.

Set up reactions as follows:

- gel extracted scFv
- CutSmart Buffer 10X
- NheI 10U/µg
- H2O to volume

Incubate for 1h at 37 °C, to perform inactivation of the enzyme incubate for 20' to 65°C

- Add BsshII 10U/µg

Incubate 1h at 50 °C, to perform inactivation of the enzyme incubate for 20' to 65°C

- Check on TBE gel 3% the efficiency of the cut by loading the undigested sample, the first and second digestion
- Purify the sample by extraction of gel band
- Quantify the sample by reading with the Qubit 2.0 Fluorometer instrument

Ligation

The ligation is performed to introduce the insert of interest in the vector previously cut following the protocol described above. To facilitate an efficient ligation between insert and vector, a ratio of 3:1 is used, but also considering the respective dimensions (the pDAN5-vector consists of about 5000 bp, while the insert of 850 bp).

The ligation is carried out using the T4 DNA Ligase reagents, following the conditions reported: incubate the sample for 16H at 16°C and then 20' at 65°C to inactivate the enzyme.

Precipitation of the ligation

The ligation is purified using DNA Clean columns, following the protocol provided.

Electroporation

After thawing the electro-competent Escherichia coli DH5αF' cells, we proceeded with the electroporation, a process to introduce the plasmid into the cells.

The passage of the electric current allows the opening of pores on the cell membrane that facilitate the entry of the DNA of interest:

- Place 3µl of precipitated ligate in 1 aliquot of 90µl of competent cells. For each ligation to do three electroporation reaction,
- Keep on ice for 1minute and subsequently place the cells with the ligate in the cold 2-mm cuvette. Be sure to eliminate any air bubbles present,
- Electroporate at 220 Volts for 2 mm (400µl) cuvettes,
- Immediately add 1ml of SOC medium to the couvette, to resort the cells, then transfer to culture tubes and incubate at 37°C in agitation for 1 hour,
- Plate onto a selective plate and incubate overnight (O/N) at 37°C.

3.5 Selection of Phage Antibody-Libraries

First selection

DAY 1:

- Inoculate 10-20 µl of the collection library (VH+VK and VH+Vλ) in 10 ml of LB medium + 1x ampicillin + 1x glucose in a 50 ml Falcon to start from an initial OD (600 nm) = 0.05
- Grow in a shaking incubator at 37°C until OD (600 nm) = 0.5
- Infect with helper- phage stock solution. Helper phage is used at an MOI 100:1 (phage: cell) and incubate for 1 hour at 37° C in a standing 37°C incubator,
- After infection, centrifuge the bacteria at 3200xg for 20 min and remove supernatant,
- Resuspend the pellet in 50 ml of LB + Ampicillin1x + Kanamicin1x,
- Grow in a 30°C shaking incubator, 250 rpm for 16 hours,
- Coating in immunotubes (one for each library): 10 µg/ml of TG-3-Ag diluted in 500 µl of PBS 1x each, and left O/N at 4°C.

DAY 2:

On Day 2, the produced phages are purified by PEG precipitation

- Centrifuge the culture at 7500x g for 20 minutes at 10°C,
- Recover 40 ml of the supernatant and transfer them to a clean 50 ml Falcon and add 10 ml of sterile PEG-NaCl (ratio PEG: supernatant 1: 4) to allow PEG-precipitation,
- Stir the solution vigorously and incubate for 1 hour at 4°C on ice (continue to stir PEG/Phage solution throughout the incubation at 15 – 20 minutes intervals),
- Centrifuge at 5200 x g per 30minutes,
- Remove the supernatant, resuspend the pellet in 1 ml of PBS 1x and transfer to a 1,5 ml tube,
- Centrifuge at 13000x g per 5 minutes (this step is essential to remove any remaining bacteria from the phage solution),
- Saturate 600µl of 1:1 phages in 4% milk,
- At the same time, empty the immunotubes and saturate them completely with 2% milk,
- Incubate 1 hour at RT,
- Inoculate 1 colony of Dh5αf', plated the day before, in 5 ml of LB + Ampicillin 1x and grow at 37°C until OD (600 nm) =0.5
- After saturation, empty the immunotubes and add 500µl of the milk-saturated phages,
- Incubate in rotation for 30’ and stand for 1 hour and 30 minutes at RT,
- Empty the immunotubes and perform 5 washes with PBS Tween 0.1% and then 5 more with PBS 1x
- Incubate 500µl of DH5αF' cells per 45' at 37°C by stirring occasionally

The bacteria express the pilo, therefore the phages with the expressed Scfv on their surface. Plate in a LB + Ampicillin 1x + glucose 1x plates and grow O/N at 37° C:

・200 µl DH5αF' to control the absence of contamination,

・500 µl of phage infected DH5αF' cells,

・a serial dilution to calculate the title of the 1st selection

DAY 3

- Calculate the title of the first selection,
- Collect the first selection in 1 ml of LB, add sterile glycerol to the maximum final concentration of 20% and store the glycerol stock solution at -80°C.

Second selection

- Thaw a 50 µl aliquot of the first selection
- Repeat the steps of day 1 and 2, but increase the tightness of the washes
- After incubation, empty the immunotubes and perform 10 washes with PBS 1x - Tween 0.1% and 10 with PBS 1x
- Add 2 ml PBS 1x - Tween 0.1% and leave in rotation for 30min at room temperature
- Empty the immunotubes and perform 5 washes with PBS 1x - Tween 0.1% and 5 with PBS 1x
- Place 500 µl DH5αF' at OD600 = 0.5 in the immunotubes
- Incubate 45min to 37° C by stirring occasionally

Plate in a LB + Ampicillin 1x+ glucose 1x plates and grow O/N at 37° C

・200 µl DH5αF' to control the absence of contamination

・500 µl of phage-infected DH5αF' cells

・two dilutions to calculate the title of the 2nd selection

Phage-ELISA test

DAY 1

- Calculate the title of the second selection and collect it in 1 ml LB, add sterile glycerol to the maximum final concentration of 20%, store the glycerol stock solution at -80°C.
- Prepare a master plate (one for each type of light antibody chain) in a 96-well microtiter plate by placing 120 µl of LB + ampicillin 1x + glucose 1x + 1 colony from the dilution plates of the 2nd selection.

- Grow at 37 ºC by shaking.
- Prepare the replicate plates by placing 100 µl LB + Ampicillin 1x + glucose 1x and 20µl of the corresponding master plate well, starting with OD600 = 0.05
- Inoculate separately 1 colony of positive and negative control into 1 ml of LB + ampicillin 1x + glucose 1x
- Continue to grow the over day master plate, subsequent keep the master plate at 4°C
- Grow the replicate plate while stirring at 37°C to OD600 = 0.5
- Infect with 1 µl helper phage + 49 µl LB per well so that the phage: bacteria MOI ratio is 100: 1
- Infect positive and negative controls with 10 µl helper phage (respecting MOI 100:1 ratio)
- Incubate 45 minutes to 37° C
- Centrifuge replicate plates at 423x g for 20 minutes
- Remove the supernatant
- Resuspend pellets in 140 µl of LB + ampicillin 1x + kanamycin 1x
- Centrifuge control samples at 478x g per 15min
- Remove the supernatant and resuspend pellets in 1 ml LB + Ampicillin 1x + Kanamycin 1x
- Grow O/N by shaking at 30° C
- Coating of ELISA-plate: 5 µg/ml of TG-3-antigen diluted in PBS 1x and keep O/N at 4°C

DAY 2

- Empty the coating plate and saturate it with 120 µl per well of milk 2%
- Incubate at room temperature for 45 minutes
- Centrifuge replicate plates at 500xg for 20 minutes
- Centrifuge control samples at 500x g per 15 minutes
- Remove 2% milk from the ELISA plate
- Incubate with 50 µl per well of primary antibody given by the supernatant (phages) diluted 1:1 in 4% milk. Use 50 µl of LB as the blank control
- Incubate for 1 hour and 30 minutes at room temperature
- Empty the plate and wash 3 times with PBS 1x - Tween 0.1% and 3 with PBS 1x

- Incubate with 100 µl per well with a secondary antibody (anti M13-HRP) diluted 1:2000 in 2% milk per 1 hour at RT
- Empty the plate and wash 3 times with PBS 1x - Tween 0.1% and 3 with PBS 1x
- Develop with 65 µl of TMB (3,3',5,5' tetramethylbenzidine dihydrochloride) per well
- Block the reaction after 10- 15min adding 1M sulphuric acid (H2SO4) 35 µl per well
- Read at 450 nm

Phage-Elisa of Positive Clones

Positive clones should be confirmed:

- Smear the corresponding clone from the master plate on a plate LB + Ampicillin 1x + glucose 1x and let grow O/N at 37° C
- Repeat the procedure of the Phage-ELISA test following the protocol previously described

If the clones are reconfirmed, prepare the sterile glycerol stock aliquot to store at -80° C.

To determine how many different antibodies have been selected, fingerprinting and Sanger Sequencing are performed.

Fingerprinting

PCR

This procedure consists in the amplification of the variable regions of the light and heavy chains followed by digestion with the restriction enzyme BstNI. This procedure permits to analyse the pattern of bands produced by cutting.

The protocols were optimized using the Ex Taq PCR reagents (TaKara) and BstNI restriction endonuclease (New England BioLabs).

Set up reaction as follows:

- Inoculate a colony of positive clones in 50 µl of LB, 1 µl is used as template
- Ex Taq Buffer 10X- 2µl
- dNTP mix (2.5 mM each)- 1.6µl
- MgCl2 50 mM- 0.6µl

- Primer VHPT2 forward 10 µM- 0.5 µl
- Primer VLPT2 back 10 µM- 0.5 µl
- ExTaq 5 U/µl- 0.1 µl
- H2O- 13.7µl

Condition of amplification

	Temperature (C)	**Time (sec)**	**Cycles**
Initial denaturation	94°	300	
Denaturation	94°	30	X 25
Annealing	60°	30	
Extension	72°	45	
Final Extension	72°	600	
Hold	4°		

- Check 3µl of PCR product on agarose TBE 3% gel

Fingerprinting Digestion

Prepare a fingerprinting-Mastermix combining:

- NEB cutSmart Buffer 10x- 3µL
- BstNI enzyme 10 U/µL- 0.2 µL
- H2O- 11.6µL
- add 15 µL to each PCR previously prepared
- Digest samples at 60° C for 3 h.
- Load 15-20 µL on a TBE 3% agarose gel, run and analyse the different patterns to identify unique clones.

Sanger Sequencing of Individual Clones

The scFv sequences of the binding clones, with a different pattern, can be determined by sequencing of the PCR products.

Set up Sanger sequence pre-PCR reaction as follows:

- Inoculate a colony of positive clones in 50 µl of LB, 1 µl is used as template
- Ex Taq Buffer 10X- 2µl
- dNTP mix (2.5 mM each)- 1µl
- Primer VH-seq forward 10 µM- 0.5 µl
- Primer M13-revseq reverse 10 µM- 0.5 µl
- ExTaq 5 U/µl- 0.1 µl
- H2O- 15.9µl

Condition of amplification:

	Temperature (C)	Time (sec)	Cycles
Initial denaturation	94°	300	
Denaturation	94°	30	X 25
Annealing	60°	30	
Extension	72°	45	
Final Extension	72°	600	
Hold	4°		

- Check 3µl of PCR product on agarose TBE 3% gel and purify the pre-PCR using MinElute PCR Purification kit (Qiagen) following the protocol provided.

Sanger Sequence PCR reaction has been set on BigDye Terminator Sequencing kit (Applied Biosystems).

Set up the reaction as follows:

- DNA template pre-PCR purified- 1µl
- BigDye Buffer 5x- 2µl
- BigDye Terminator Mix- 0.8µl
- Primer VH-seq forward/ M13-revseq reverse 10 µM- 0.32 µl
- H2O- 5.88µl

Condition of amplification:

	Temperature (C)	Time (sec)	Cycles
Initial denaturation	96°	60	
Denaturation	96°	10	X 25
Annealing	50°	5	
Extension	60°	240	
Hold	4°		

- Purify the Sanger sequence PCR using Big Dye Xterminator purification kit (Applied Biosystems)

- Proceed with Sanger Sequencing

- Analyse electropherograms using online database IMGT (http://www.imgt.org/) and compare the obtained sequences in order to verify the variability.

3.6 Analysis and Selection of Yeast Antibody- Libraries

Yeast media and required reagents

- S. cerevisiae strain EBY100 and pDNL6[52] yeast display vector,
- YPD (Yeast Extract-Peptone-Dextrose): 10 g Yeast Extract, 20 g Peptone (Bacto), 20g Dextrose. Bring up to 1L of distilled H2O. Filter sterilize with 0.22µm units and add Kanamycin 1x and Tetracycline 1x antibiotics. Store at 4°C,
- YPD Agar plates: add 20g/L of agar to YPD media. Bring up to 1 L with distilled H2O, autoclave, add Kanamycin 1x and Tetracycline 1x antibiotics and 66 mL of glucose 1x. Store plates at 4°C,
- SD/CAA Selective Growth media: 5 g/L casamino acids, 20 g/L dextrose, 1.7 g/L YNB (Yeast Nitrogen Base) w/o ammonium sulphate and amino acids, 5.3 g/L ammonium sulphate, bring up to 1L of distilled H2O, filter sterilize with 0.22µm filter units, add Kanamycin 1x and Tetracicline1x antibiotics. Store a 4°C,
- SD/CAA Agar plates: add 15g/L of agar to SD-CAA solution, bring the volume to 1 L with H2O, autoclave to sterilize. Store plates at 4°C,
- SGR/CAA Selective Induction media: 5g Casamino Acids, 7g Yeast Nitrogen Base, 20g Galactose, 20g Raffinose, 1 g Dextrose, bring up to 1L of distilled H2O, filter sterilize with 0.22 µm filter units, add Kanamycin 1x and Tetracycline 1x antibiotics. Store a 4°C,
- Yeast Washing Buffer: PBS 1x supplemented with 2 mM EDTA and 0.5% BSA. Store a 4°C,
- Yeast transformation commercial kit (Sigma- Alderich),
- Biotinylated Ag, 150 Nm: human TG-3 commercial protein (Zedira) biotinylated using a commercial kit (EZ-Link™ Sulfo NHS-SS Biotinylation Kit- ThermoFisher Scientific) following the instructions of the manufacturer,
- Mouse anti-SV5 PE-Cyanine7 labeled antibody (ThermoFischer Scientific). Anti-SV5 antibody bind the peptide-sequence GKPIPNPLLGLDST of scFv displayed,
- Streptavidin-Alexa Fluor 647 conjugate (ThermoFischer Scientific),
- Plasmid DNA is prepared using a commercial Miniprep kit (Qiagen Miniprep Kit Qiagen), following the instructions of the manufacturer.

[Note: The adding of Kanamycin and Tetracycline antibiotics is suggested to all the yeast media solutions to prevent bacteria contamination. The yeast, in fact, is unaffected by the antibiotics]

From the final phage display selection output a scFv plasmid is produced, re-ligated into yeast vector pDNL6 and transformed into EBY100 yeast cells.

- Use 100 µL of the glycerol stock bacteria from last selection phage output and grow O/N at 37°C in 1 mL of LB+Glu 1x +Amp 1x to obtain plasmid DNA, using a Miniprep kit and following the instructions provided.

scFv region is amplified by specific primers pDantopDNL6-forward and pDantopDNL6-reverse, digested with BsshII and NheI restriction enzymes and it is used for subsequent homologous recombination in a linearized yeast display vector pDNL6 (~6000 bp).

Set up the scFv amplification as follows:

- 5 ng of the plasmid miniprep as template
- Q5 HS Buffer 5x- 10µl
- dNTPs 10Mm- 1µl
- Primer For 10µm- 2.5µl
- Primer Rev 10µm- 2.5µl
- Q5 HS DNA polymerase- 0.5µl
- H2O to 50µl

Conditions of amplification:

	Temperature (C)	**Time (sec)**	**Cycles**
Initial denaturation	98°	30	
Denaturation	98°	5	X 20
Annealing	60°	10	
Extension	72°	20	
Final Extension	72°	120	
Hold	4°		

- Check the PCR product on a 1.5% agarose gel.
- Purify the sample by extraction of gel band
- Quantify the sample by reading with the Qubit 2.0 Fluorometer instrument and Qubit dsDNA HS assay kit.

Transformation into Yeast cells

The generation of the yeast library is possible thanks to homologous recombination between scFv produced and linearized yeast vector. Both include the homology nucleotides present at the extremity. The plasmide is introduced into yeast competent cells by chemical transformation.

Yeast competent cells

One single colony of EBY100 yeast strain, from a YPD plate, is required to prepare the yeast culture.

- Inoculate it into a 10 mL YPD culture medium + Kanamycin 1x + Tetracycline 1x and let grow O/N at 30°C with shaking at 250 rpm
- When the culture reach OD600 > 2 dilute it into 100 mL YPD medium+ Kanamycin 1x + Tetracycline 1x
- Grow the culture with shaking for 5–6 h at 30° C until it is reached an OD600 around 1.5
- Centrifuge the yeast culture for 10’ at 3000x g. Discard the supernatant and wash the harvest cells with 50 mL sterile H2O. Centrifuge again in the same conditions.
- Discard the supernatant and resuspend in 1 mL of Yeast Transformation Buffer. The competent cells are ready, use immediately or store at 4°C for a few hours.

Transformation protocol

100 µL of yeast competent cells are required for each yeast transformation, which can be performed in a 1.5 mL tube, adding:

- 500 ng of digested vector and 1.5 µg of scFv PCR product
- 5 µg of salmon testes DNA
- 600 µL of Yeast Plate Buffer and vortex

Incubate for 30 minutes at 30°C with shaking

- Add DMSO, 10% of the total volume

Heat shock for 15 minutes at 42°C in a water bath

- Spin 5 seconds and remove the supernatant

- Resuspend in 1 mL of SD/CAA and plate a dilution on SD/CAA plates to calculate the size of the library
- Dilute the rest of the transformed cells into 25 mL selective SD/CAA media + Kanamycin 1x + Tetracycline 1x and let grow 2–3 days at 30°C, until colonies appear on the plate.

Induction and staining of the Yeast Antibody Display Library

In order to analyse and perform rounds of sorting, it is necessary to induce the scFvs display on the yeast surface.

The induction of a promoter responding to the presence of galactose in the suitable media.

Dilute the yeast transformed culture to start from at OD600= 0.5 (1x10^6 cells/mL) and prepare a 10 mL culture in SG/R-CAA, grow at 20°C with shaking O/N.

Check the success of the induction of the yeast library using the mouse anti-SV5-PE antibody only:

- wash 100 µL of the induced cells culture adding 1mL of Yeast Wash Buffer,
- spin down at 10,000x g for 30 seconds and resuspend the pellet cells in PBS 1x
- add anti-SV5-PE antibody according to the protocol provided.

Incubate on ice for at least 30 minutes, repeat the wash and finally resuspend in PBS 1x, analyse the expression by flow cytofluorimetry.

At least 30% of the population should be SV5-positive. The induced library is now ready for Ag staining and subsequent FACS selection:

- Wash 100 µL of the induced cells culture adding 1 mL of Yeast Wash Buffer
- Spin down at 10,000x g for 30 seconds and resuspend the pellet cells in PBS 1x
- Add 150 nM of biotinylated TG-3 target Ag

Incubate 30 minutes at RT with rotation

- Wash with 1 mL of Yeast Wash Buffer, spin and eliminate supernatant
- Resuspend the yeast cells with 100 µL of PBS solution containing diluted anti-SV5-PE and diluted Streptavidin-Alexa Fluor 647 conjugate, according to the protocol, for 30 minutes in the dark at 4°C with rotation

- Wash the cells with Yeast Wash Buffer and resuspend population of stained cells in 1 mL of PBS 1x.
- Keep the cells on ice in the dark until analysis and sorting.

Analysis and selection of the yeast population by FACS

The purpose of the first analyses is to ensure the functioning of the system, the presence of the scFv construct, the staining and to compare the populations stained with and without Ag. The negative control is required to check for nonspecific binders. The sort gates are placed to select the more diversity positive clones. (Figure 2)

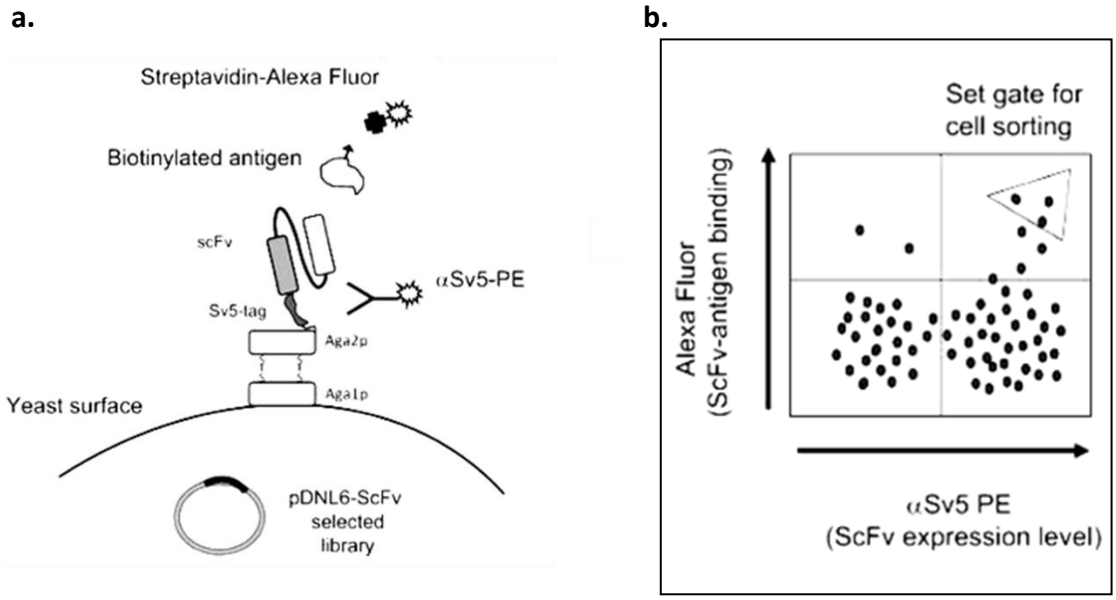


Fig. 2. Representation of scfv- displayed on the yeast cell surface and specific binding of the various tags. The ab-expression can be detected using a fluorescently conjugated anti- SV5 ab, the bound Ag can be detected by utilizing fluorescently conjugated streptavidin (**a**). The identification, by flow cytometry, of the clones expressed and positive for Ag-binding are doubly positive for fluorescence signals (Alexa fluor and PE), The selection of the interested clones is based on the setting of the gate for the cell sorting (**b**).

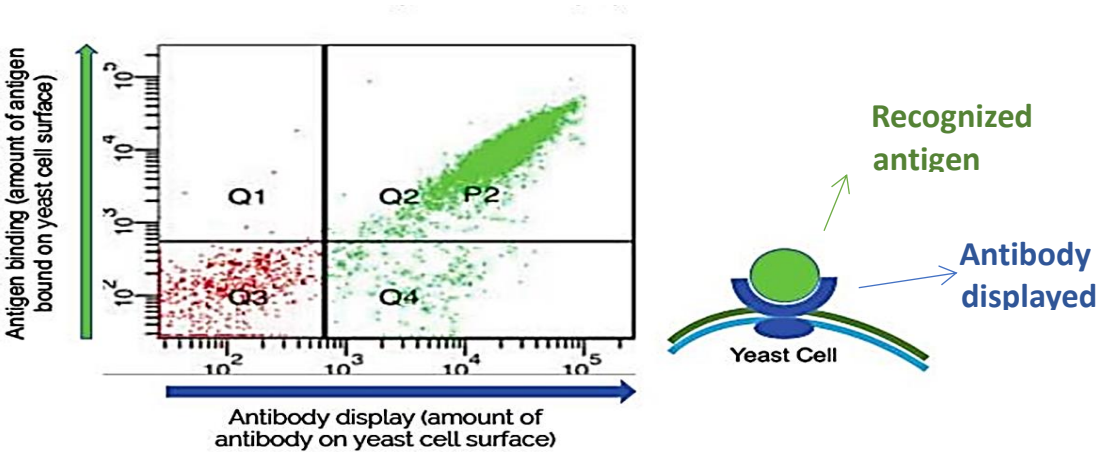
Usually, between 10,000 and 20,000 events are sorted directly into 1 mL of SD-CAA media and subsequent transferred in 10 mL of SD-CAA media and grow at 30°C with 250 rpm shaking for 2 days. For each DH sample are performed 2 rounds of sorting, following the same induction, staining and selection protocol already described. For each Control sample are performed only one negative sorting. From the culture of last sort analysed the glycerol stock solutions have been prepared adding sterile glycerol up to a maximum concentration of 20% and stored at -80°C.

The interpretation of the flow cytometry graphs of the yeast ab-display, that will be shown below, is fundamentally important (Figure 2.1).

Logarithmic scale for sideward scatter (SS) and for forward scatter (FS) was used.

Sample data were acquired using BD FACSAria™ Fusion flow cytometer (BD Biosciences, 656700) and MACSQuant Analyzer (Milteny Biotec), these were analysed with FlowJo (BD Biosciences) or FlowLogic (Invai Technologies) software.

a.



b.

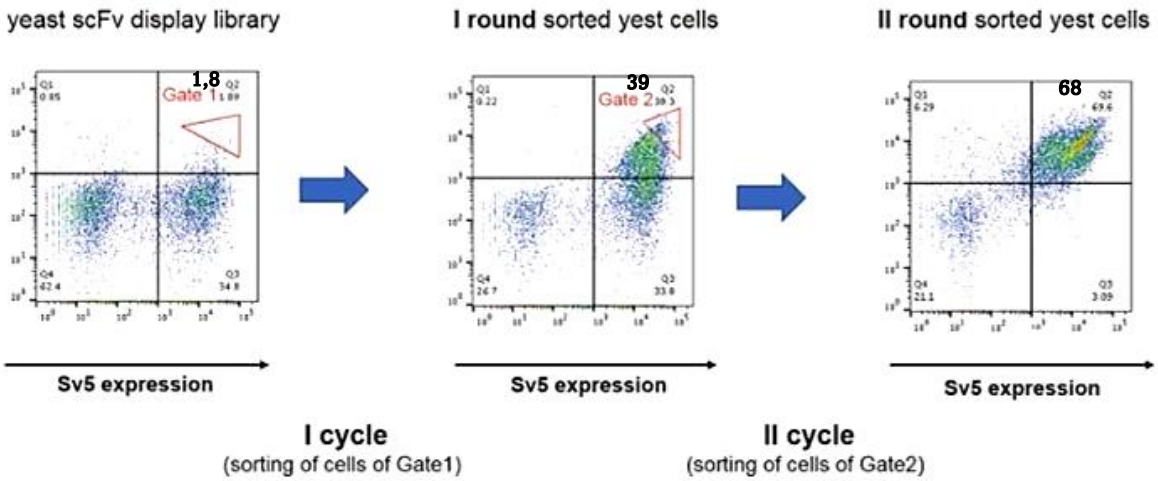


Fig. 2.1 In general, FACS or flow cytometry plots is divided into 4 areas. (**a**) On the x-axis is reported the expression level of scFv, on y-axis is reported the binding to antigen. The presence of yeast population and cells without scFv expression is shown in Q3 area. The yeast cells expressing scFv will be shown in Q4 area and to consider valid an analysis it is necessary that at least 30% of the population is PE-positive (Q3 area), while the cells expressing scFv binding Ag will be shown in the Q2/P2 quadrant. (**b**) Illustration of the analysis and sorting flow chart. Starting from the first analysis of the polyclonal library transformed into yeast cells and the selection of clones within the set gates and subsequent analysis. Positive binding clones were enriched with rounds of FACS and the red triangles represent the gates used to sort the cells for each round. In each quadrant a number is indicated that represents the percentage of cells presenting the characteristic of the area (positivity for PE and / or Alexa Fluor). The percentage of cells in Q2 (double positive) increases after each sorting cycle (1,8% -> 39% -> 68%) indicating the enrichment of the Ag-binding scFv clones.

Analysis and Sanger Sequencing of individual clones

The polyclonal phage scFv display selection outputs selected on target Ag were used as starting material for making yeast scFv display libraries and sorting cycles of the TG-3 Ag positive clones were performed. From these last selection outputs random single clones, plated on SD-CAA plates, were tested by flow cytometry for binding affinity to TG-3 Ag, and full length of CDRH3s, which represent the Ag-binding domains of the antibody, were identified by Sanger Sequencing.

[Note: The settings of flow cytometry analysis remained unchanged. These tests were carried out on a flow cytometer not combined with a cell sorter]

- An aliquot of 30µL, from the glycerol stock solutions, is diluted in 3mL of SD-CAA media and grow at 30°C with 250 rpm shaking for 3-4 hours,
- Check the OD600 and plate a dilution on SD/CAA plates to have 10^2/10^3 cells. Let grow 2–3 days at 30°C until colonies appear on the plates.

  [Note: OD600= 1 corresponds to 1 x 10^7 cells]
- Inoculate separately random colony in 3mL of SD-CAA media and grow at 30°C with 250 rpm shaking O/N,
- Proceed with induction, staining and flow cytofluorimetry analysis of monoclonal antibodies yeast-displayed following the protocol previously described.

The scFv sequences and corresponding CDRH3 of the binding mAbs are determined by yeast colony miniprep, by using Miniprep kit, and subsequent transformation into competent bacteria cells, followed by Sanger sequencing of the PCR products as already described.

[Note: lysis of yeast cells is hardest, mechanical lysis or tissue-lyser instrument may be required]

Table 3. Primers sequence: The primers were designed to be compatible with the phagemid vector pDan5 [51] and the pDNL6 yeast display vector [52].

	Ig-A forward	GGGGCTGGTCGGGGATGC
VH reverse	**VH-5 reverse**	TTATCCTCGAGCGGTACCCAGGTACAGCTGCAGCAGTCA
	VH-6 reverse	TTATCCTCGAGCGGTACCCAGGTGCAGCTACAGCAGTGGG
	VH-10 reverse	TTATCCTCGAGCGGTACCGAGGTGCAGCTGKTGGAGWCY
	VH-12 reverse	TTATCCTCGAGCGGACCCAGGTCCAGCTKGTRCAGTCTGG
	VH-14 reverse	TTATCCTCGAGCGGTACCCAGRTCACCTTGAAGGAGTCTG
	VH-22 reverse	TTATCCTCGAGCGGTACCCAGGTGCAGCTGGTGSARTCTGG
VK for MIX-forward	**VK-1 forward**	GAAGTTATGGTCGACCCTCCGGATTTGATTTCCACCTTGGTCC
	VK-2/4 forward	GAAGTTATGGTCGACCCTCCGGATTTGATCTCCASCTTGGTCC
	VK-3 forward	GAAGTTATGGTCGACCCTCCGGATTTGATATCCACTTTGGTCC
	VK-5 forward	GAAGTTATGGTCGACCCTCCGGATTTAATCTCCAGTCGTGTCC
VK reverse	**VK-1 reverse**	AGCAAGCGGCGCGCATGCCGACATCCRGDTGACCCAGTCTCC
	VK-2 reverse	AGCAAGCGGCGCGCATGCCGAAATTGTRWTGACRCAGTCTCC
	VK-9 reverse	AGCAAGCGGCGCGCATGCCGATATTGTGMTGACBCAGWCTCC
	VK-12 reverse	AGCAAGCGGCGCGCATGCCGAAACGACACTCACGCAGTCTC
Vλ for MIX-forward	**Vλ-1/2 forward**	GAAGTTATGGTCGACCCTCCGGATAGGACGGTSASCTTGGTCC
	Vλ-7 forward	GAAGTTATGGTCGACCCTCCGGAGAGGACGGTCAGCTGGGTGC
Vλ reverse	**Vλ-1 reverse**	AGCAAGCGGCGCGCATGCCCAGTCTGTSBTGACGCAGCCGCC
	Vλ-3 reverse	AGCAAGCGGCGCGCATGCCTCCTATGWGCTGACWCAGCCAC
	Vλ-4 reverse	AGCAAGCGGCGCGCATGCCCAGCCTGTGCTGACTCARYC
	Vλ-7/8 reverse	AGCAAGCGGCGCGCATGCCCAGDCTGTGGTGACYCAGGAGCC
	Vλ-9 reverse	AGCAAGCGGCGCGCATGCCCAGCCWGKGCTGACTCAGCCMCC

	Vλ-11 reverse	AGCAAGCGGCGCGCATGCCTCCTCTGAGCTGASTCAGGASCC
	Vλ-13 reverse	AGCAAGCGGCGCGCATGCCCAGTCTGYYCTGAYTCAGCCT
	Vλ-15 reverse	AGCAAGCGGCGCGCATGCCAATTTTATGCTGACTCAGCCCC
	Vλ-38 reverse	AGCAAGCGGCGCGCATGCCTCCTATGAGCTGAYRCAGCYACC
VH for MIX-forward	**VH-1/2 forward**	GATTGGTTTGCCGCTAGCTGAGGAGACRGTGACCAGGGTG
	VH3 forward	GATTGGTTTGCCGCTAGCTGAAGAGACGGTGACCATTGT
	VH4/5 forward	GATTGGTTTGCCGCTAGCTGAGGAGACGGTGACCAGGGTT
	VH-6 forward	GATTGGTTTGCCGCTAGCTGAGGAGACGGTGACCGTGGTCC
	VHPTL reverse	GGAGGGTCGACCATAACTTCGTATAATGTATACTATACGAAGTTATCCTCGAGCGGTA
	VLPTL forward	ACCGCTCGAGGATAACTTCGTATAGTATACATTATACGAAGTTATGGTCGACCCTCC
	VLPT-1 reverse	CGCTGGATTGTTATTACTCGCAGCAAGCGGCGCGCATGCC
	VHPT-1 forward	CCAGGCCCAGCAGTGGGTTTGGGATTGGTTTGCCGCTA
	VLPT-2 reverse	TACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTC
	VH-seq forward	CAACTTTCAACAGTAGCGGC
	M13-revseq reverse	AGCGGATAACAATTTCACACA
	pDantopDNL$_6$ forward	TCTGGTGGTGGTGGTTCTGCTAGAGGCGCGGCAGCAAGCGGCGCGCATGCC
	pDantopDNL$_6$ reverse	ATCCAGGCCCAGCAGTGGGTTTGGGATTGGTTTGCC

3.7 Cellular experiments

Normal Human Epidermal Keratinocytes (NHEK) from pooled donors produced at Promo-Cell's cell culture facility were used. The cells were isolated from adult normal human tissue from different locations, e.g. face, breast, abdomen and thighs.

The NHEK were cultured in an incubator with an atmosphere of 5% CO2 at 37°C in keratinocyte serum-free medium (K-SFM- PromoCell) without antibiotics or antimycotics.

The medium, refreshed every 48±72 hours, was supplemented with supplements (Promo-Cell): Bovine Pituitary Extract (0.004 g/ml), Epidermal Growth Factor (0.125 ng/ml), recombinant human Insulin (5 µg/ml), recombinant human Transferrin (10 µg/ml), Epinephrine (0.39 µg/ml) and CaCl2 (0.06 mM), necessary for the optimal growth of human keratinocytes.

To monitor the production of TG-3 in relation to cellular senescence, the keratinocytes were treated with calcio chloride ($CaCl_2$), which induces cellular aging and involves the binding of transcription factors Sp1 and Ets at the level of the binding sites on the promoter of the TGM3 gene.[53,54]

$CaCl_2$ home-made solution (1mM) was added to medium. The treated cells were incubated with $CaCl_2$ up to 10 days to increase cellular calcium-inducible differentiation.

Reagents required:

- Filaggrin-2 Polyclonal Antibody- Rabbit / IgG (ThermoFischer Scientific), used as positive intracellular protein control,
- Human epidermal transglutaminase (TG-3) polyclonal antibody- Rabbit / IgG (Zedira),
- Goat anti-rabbit IgG polyclonal Secondary antibody labeled with Alexa Fluor 488 (Invitrogen),
- Fixation and Permeabilization Kit (Invitrogen),
- PBS and PBS Wash Buffer: sterile PBS 1x (Euroclone) + Bovine Serum Albumin (BSA) 0.2%,
- NHEK DetachKit2 containing a lower concentration of Trypsin/EDTA (0.025%/0.01%) (PromoCell),

- PCA chamber slides (Sarsted),
- Vectashield mounting medium with DAPI (Vector Laboratories),
- Cover slip for microscope slide (Sarsted).

Immunofluorescence (IF) in vitro experiments were performed on PCA chamber slides plating cells at a density of 5 x 10^3 cells per well (0.8 cm2) and let them attach overnight. The cells were cultured until reaching 80% confluence on the flask and subsequently detached, using the NHEK Detach Kit, following the instructions provided, and plated on PCA chambers.

Fixation and cells permeabilization

- Aspirate liquid, wash 2 times in PBS Wash Buffer and proceed with a fixation and permeabilization in the dark at room temperature, following the manufacturer's protocol

- Rinse 3 times in PBS Wash Buffer

- Proceed with IF staining.

[Note: Fixation is a preliminary step in order to preserve cells morphology while maintaining antigenicity and ensuring free access of the antibody to its immobilized Ag, in this study the IF TG-3 staining was performed on cells with and without prior permeabilization procedure]

Immunofluorescence staining

- Prepare working dilution of antibodies in PBS 1x:
    - Filaggrin antibody 1:20 dilution
    - TG-3 antibody 1:100 dilution
    - Secondary antibody 1:500 dilution

- Incubate different specimens in 100 µL of prepared antibody dilutions to cover specimens, and incubate at 4°C overnight

- Aspirate and rinse 1 time in PBS Wash Buffer

- Incubate with 100 µL of secondary antibody to cover each specimen, 45 minutes in the dark at RT

- Aspirate and rinse three times in PBS Wash Buffer

- Apply one drop of Vectashield mounting medium with DAPI for cell nuclei staining and mount the coverslip slides in each specimen
- Examine specimens immediately at the appropriate excitation wavelength of fluorophore otherwise store slides flat at 4°C protected from light.

The images were acquired using an Axioplan 2 fluorescence microscope (Carl Zeiss, Oberkochen) and processed by AxioVision software (Carl Zeiss, Oberkochen).

3.8 Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants of NHEK treated as indicated, with and without $CaCl_2$, were analysed by sandwich ELISA for human epidermal transglutaminase 3 (TGM3 Elisa kit-MyBiosource) following the protocol provided.
The supernatants were collected in sterility, centrifuged at 350x g for 5 minutes, to eliminate any dead cells.
Data were analyzed using GraphPad Prism 5.0 biochemical statistical package (GraphPad Software, Inc., San Diego, CA). Comparison between measurements was performed using Student t test. Level of $p < 0.05$ was considered statistically significant.

3.9 xCELLigence Real-Time Cell Analysis (RTCA)

The xCELLigence RTCA system (Agilent) allows for label-free and dynamic monitoring of cellular phenotypic changes. The instrument uses specially plates containing interdigitated gold non-invasive microelectrodes integrated on the bottom of culture E-Plates (Agilent) to continuous monitor the cultured cells using electrical impedance measurement, which is displayed as cell index (CI) value (Figure 2.2). It is possible to distinguish quantitative information about perturbations of cell viability, cell arrest proliferation, cell adhesion changes and cell death[55].

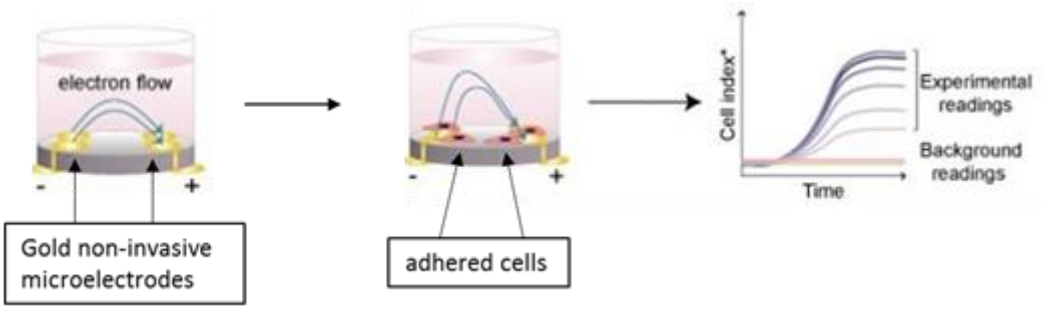


Fig. 2.2 If the cells do not adhere to the gold microelectrodes, they do not cause an impedance change directly. Adherent target cells prevent the flow of electric current between the electrodes. These impedance values, plotted as a cell index parameter, increases during cell proliferation, stabilizes as cell confluence and decrease if cells die or changing their adherence.

The cell index determination assay, for continuous monitoring of viability under different stimulations by xCELLigence, were used for NHEK cells. xCELLigence instrument and E-Plate 16 (Agilent) were used.

The confluence NHEK cells subculture was detached using the NHEK DetachKit2 (PromoCell) following the instructions provided.

4.5 x10^3 cells were splitted in each E-Plate 16 well and 200 µL of growth medium was added. The E-Plate was put in the instrument inside the 37°C cells incubator and the cell monitoring step started.

The monitoring lasted 95:51 hours for a total of 97 sweep status. Stimuli were administered in duplicate, with and without the refreshing media, at the 43rd sweep status, when the cells viability was stable.

The stimulations, listed below, were administered at two different concentrations:

1 and **5**µg/mL for TG-3-antibodies, and at **1**µg/mL for the positive and negative control stimuli.

1. Human epidermal transglutaminase (TG-3) polyclonal antibody- Rabbit / IgG (Zedira),
2. Human epidermal transglutaminase (TG-3) monoclonal antibody- ExpiCHO expression system/ IgA (UniTS)
3. Lipopolysaccharide (LPS) from Escherichia coli-B4 (sigmaAldrich)- positive control
4. IgG1 monoclonal antibody against non-human antigens (DaKo)- negative control

The growth medium and NHEK cells incubations without any stimulation were necessary to check the assay conditions.

4. RESULTS

4.1 TGM-3 gene PCR

The presence of the TGM-3 gene from duodenal biopsies' cDNA of DH- 3 and C-3 patients was tested using Polymerase Chain Reaction (PCR), to investigate the expression of the TG-3 Ag also at the intestinal level. cDNA from Esophagus biopsy sample was used as positive control. The design of target primers, on primer-BLAST online tool, provided a length of 174 bp for the predicted PCR product.

The Figure 3 shows the tool output (A) and the agarose gel electrophoresis picture of the PCR amplification (B). The PCR product is 174 bp for each sample tested, like the positive sample size.

(A)

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	AGCGCATCACACAGACAAGT	20	60.25	50.00
Reverse primer	AGTGGAAACACAGCCTTCGT	20	59.82	50.00

Products on target templates

>NM_003245.4 Homo sapiens transglutaminase 3 (TGM3), mRNA

```
product length = 174
Forward primer  1    AGCGCATCACACAGACAAGT  20
Template        117  ....................  136

Reverse primer  1    AGTGGAAACACAGCCTTCGT  20
Template        290  ....................  271
```

(B)

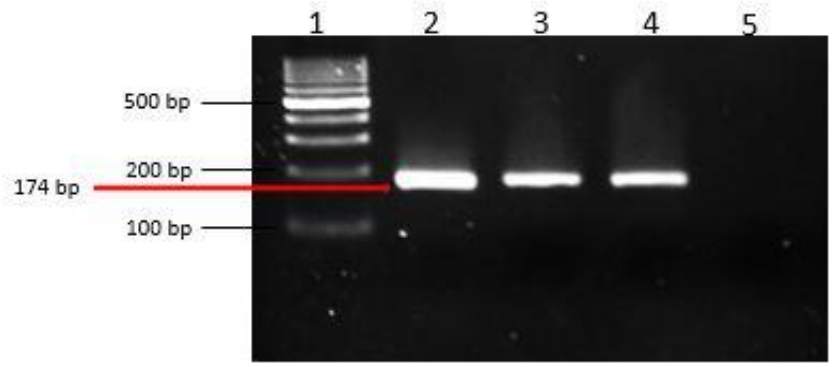


Fig. 3. (A) The primers specificity check provides information on the matched organism, gene and product length of the PCR amplification. **(B)** Lane 1: 100-base pair ladder molecular weight; lane 2: positive control from esophagus cDNA; lane 3: DH-3 cDNA from duodenal biopsy; lane 4: C- 3 cDNA from duodenal biopsy; lane 5: negative (water) control.

4.2 ELISA human TG-3

The presence and concentration of human TG-3 protein, in serum and in supernatant from cultured and stimulated intestinal biopsies, was determined by sandwich-ELISA assay (Figure 3.1). The protein was determined in both samples and in both type of patients, DH and Control.

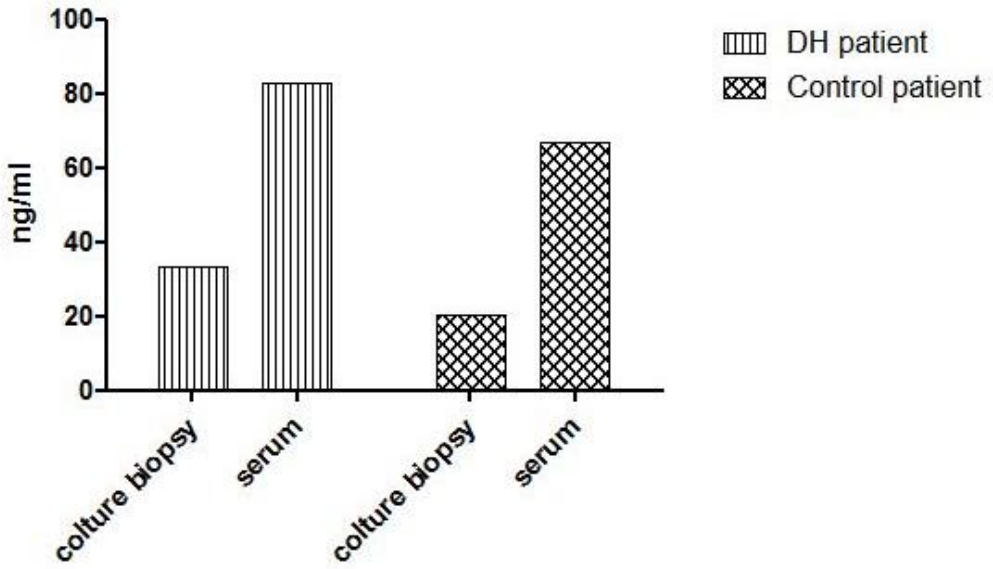


Fig. 3.1 The culture supernatant of duodenal biopsies and sera of DH- 3 patient and C-3 control patient are tested and represented in a bar graph. The protein concentration is expressed in ng/ml.

4.3 Antibody Library

4.3.1 Phage display Ab-Libraries construction

The antibody-scFv libraries were constructed from either peripheral blood B lymphocytes (PBL) and intestinal B Lymphocytes (IBL) from three DH patients and three control patients. This methodology involves the extraction of RNA from B lymphocytes isolated, the extracted RNA was checked on 1% TBE gel and subsequently reverse transcribed to produce the cDNA, from that VH and VL chains were amplified by PCR

using a set of oligonucleotides that recognize all human V genes. For the VH chains, the 3' primer was specific for IgA Abs.

The amplified VH and VL segments were assembled by PCR and cloned into the phagemid vector pDAN5 to obtain the primary libraries.

The libraries ranged in size from 1×10^6 to 1×10^7, and randomly picked clones were shown to contain full-length scFv (850 bp).

4.3.2 Phage display Ab-Libraries selection

Abs were affinity selected on commercial human TG-3 Ag. According to phage- display method, the patient's Ab repertoire is expressed on phage surface and fused to the coat protein of a filamentous phage. Each phage carries a single Ab and can be selected by undergoing the ab-phage library to repeated cycles of binding, washing and eluting on the target Ag. After each selection cycle, the eluted phages are tested in ELISA against the Ag used for the selection, to investigate their affinity to the target.

TG-3 positive clones were reconfirmed in ELISA and analyzed by BstNI fingerprinting. Clones showing variability and different pattern of bands, produced by enzymatic cleavage, were sequenced to determine the number of different mAbs. After two cycles of selection, only IBL ab- library of DH- 1 patient showed a positive clone to TG-3, whereas no positive clones were obtained from others IBL and PBL libraries. This result is shown Table 4.

The results of the Sanger sequencing with the analysis of the V-gene family, carried out on the IMGT online database, and the amino acid sequence of the complementarity determine regions (CDR3) are shown in the Table 5.

As expected, the control patients did not show TG-3 positive clones.

Patient	Source	Epidermal Transglutaminase (TG-3)		
		Tested clones	Positive clones	Different clones
DH- 1	IBL	48	1	1
DH- 1	PBL	48	0	/
DH- 2	IBL	48	0	/
DH- 2	PBL	48	0	/
DH- 3	IBL	48	0	/
DH- 3	PBL	48	0	/

Table 4. Results from the 2nd cycle selection.
IBL: Intestinal B Lymphocytes, PBL: Peripheral B Lymphocytes

v-H domain	**CDR3**	**v- K/ λ domain**	**CDR3**
vH-3	PASLSFYMHGNYMDV	vλ-6	QSYDGTNHDVV

Table 5. V-gene family and CDR3 amino acids sequence of positive TG-3 IBL clone found after last phage selection.

4.3.3 Yeast display Ab-libraries transformation and selection

The bacteria plasmid DNA, from the last phage-selection output, was used to amplify the scFv, with the described primers, and transformed into EBY100 yeast competent strain. Through homologous recombination into pDNL6 vector, yeast antibody library was obtained, analysed and enriched by cell sorting against labelled TG-3 antigen, at 150 nM concentration. In this way, specific binding clones were identified. The preliminary tests of unstained yeast cells and staining with secondary reagents only allowed the setting of the analysis tools and instruments. (Figure 3.2 (A))

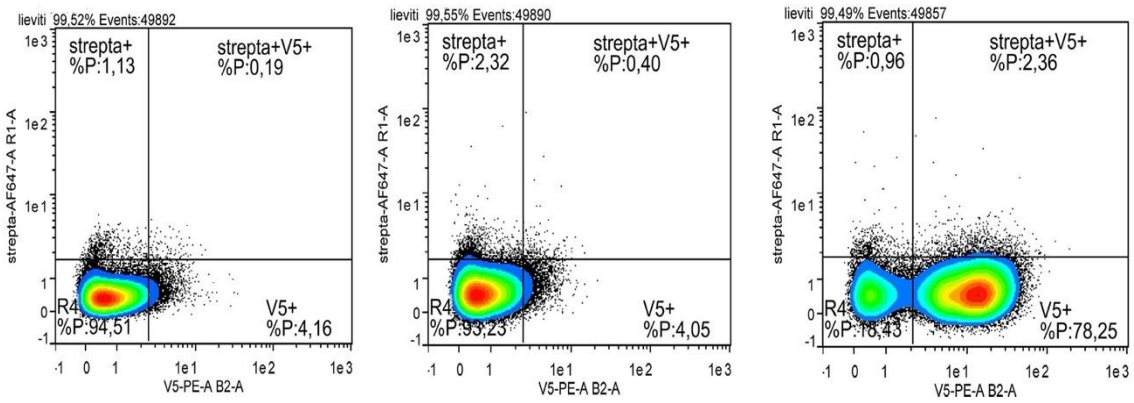
Set up the system, the first step was the analysis of the yeast libraries and the setting of the selection area for the population to be sorted. The constructed, transformed and analyzed ab-libraries refer to VK and Vλ assembled to VH domains antibody gene families of the IBL and PBL levels. (Figure 3.2 (B))

The sorted population was diluted in SD-CAA media, let grow and induced for subsequential sorting steps. The initial percentage of TG-3 positive and sorted clones was low. After only one round of sort the percentage increased but the population expressing the scFv compared to the positive one was still unequal, for this reason, it was decided to perform two rounds of sorting for each DH sample (Figure 3.2 (C/D/E)). The binding population increases significantly after one or two rounds. The TG-3 positive population was enriched in ab-libraries of both intestinal and peripheral origin. In the latest analysis of the PBL ab-library, VH + VK domains of patient DH-3 (Figure 3.2 (E)), the percentage of positive TG-3 clones was lower (1.70%) than in the previous analysis (9,28%). This result is explained by the high percentage of non-induced yeast population (Q4 quadrant).

However, the entire population scFv-expressing was positive for the target antigen. This demonstrates the optimal selection efficiency of the 2nd sorting round.

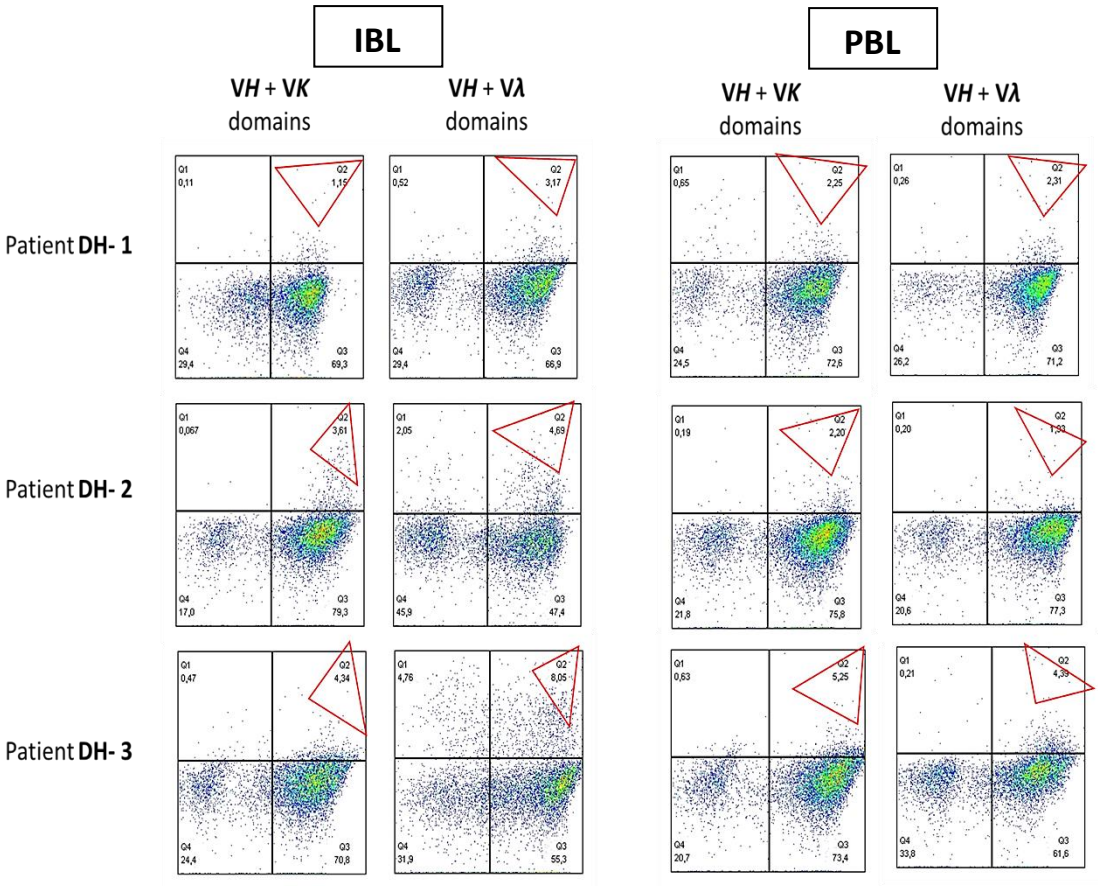
For each Control sample was performed one negative sorting, selecting all yeast cells expressing scFV. The analysis after this selection showed whole yeast cell population expresses scFv and no bound clones for the target (Figure 3.2 (F)).

(A)

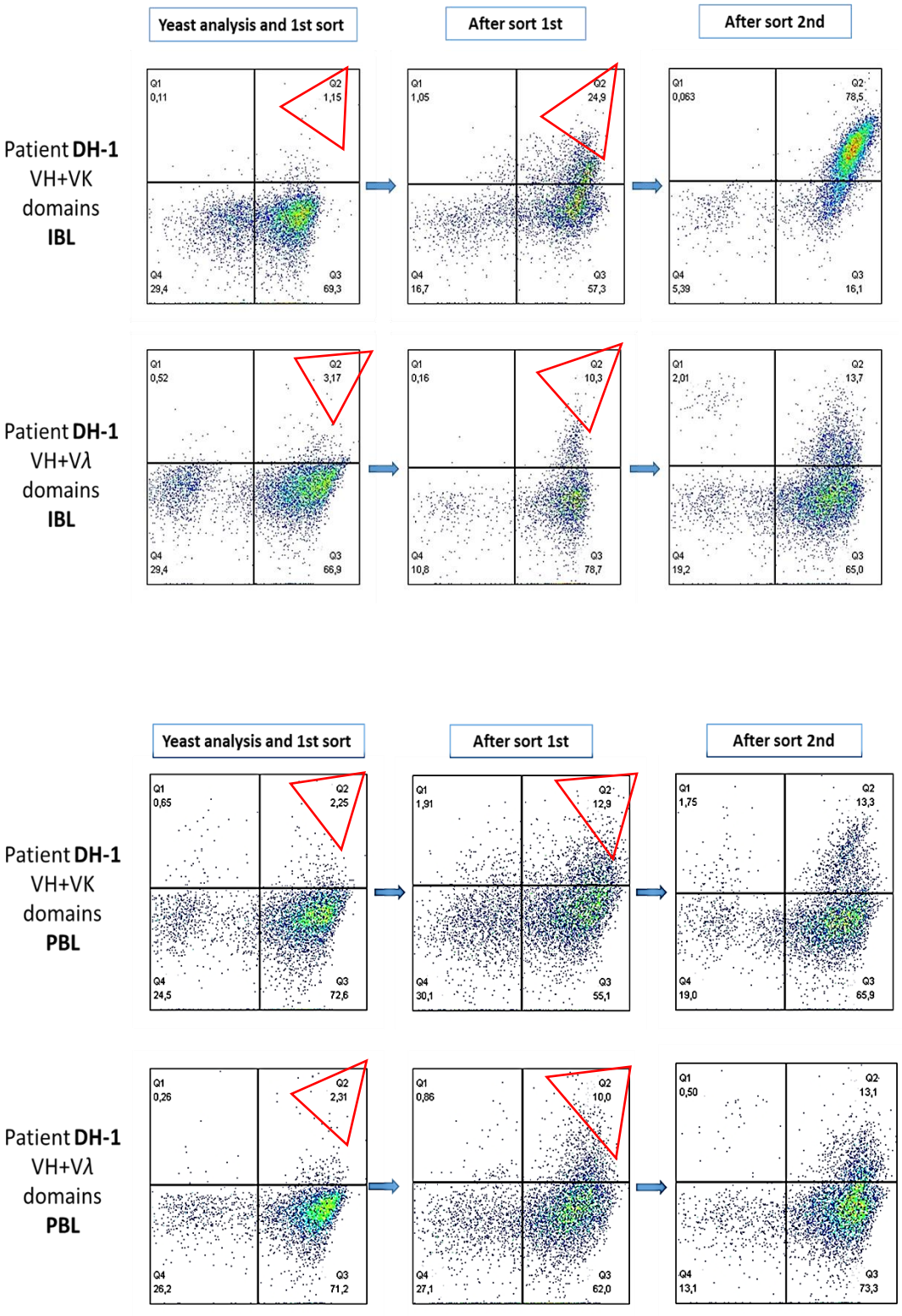

lieviti 99,52% Events:49892
strepta+ %P:1,13
strepta+V5+ %P:0,19
R4 %P:94,51
V5+ %P:4,16
strepta-AF647-A R1-A
V5-PE-A B2-A
lieviti 99,55% Events:49890
strepta+ %P:2,32
strepta+V5+ %P:0,40
R4 %P:93,23
V5+ %P:4,05
lieviti 99,49% Events:49857
strepta+ %P:0,96
strepta+V5+ %P:2,36
R4 %P:18,43
V5+ %P:78,25

(1) (2) **(3)**

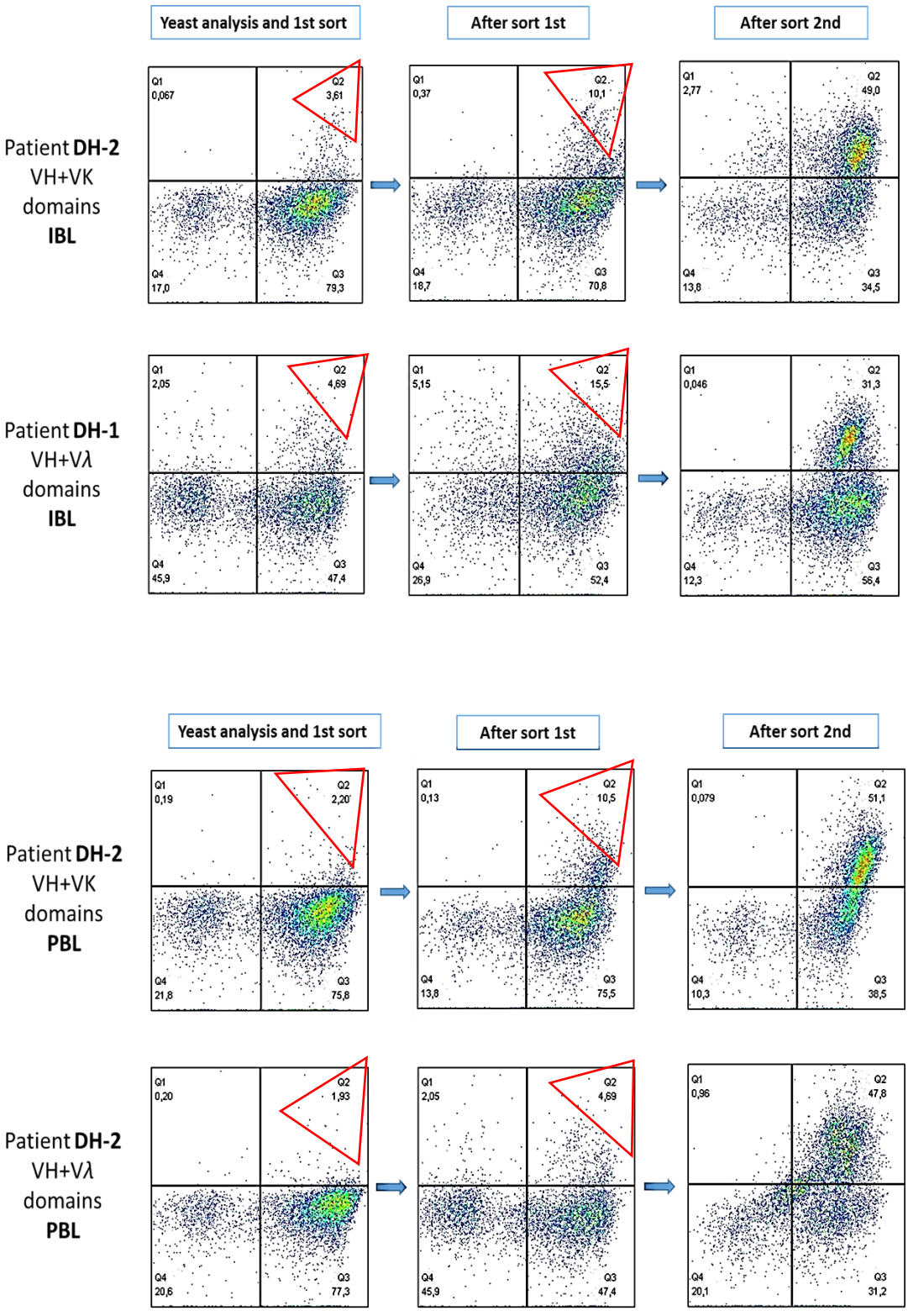
(B)


IBL
PBL
VH + VK domains
VH + Vλ domains
VH + VK domains
VH + Vλ domains
Patient DH- 1
Patient DH- 2
Patient DH- 3

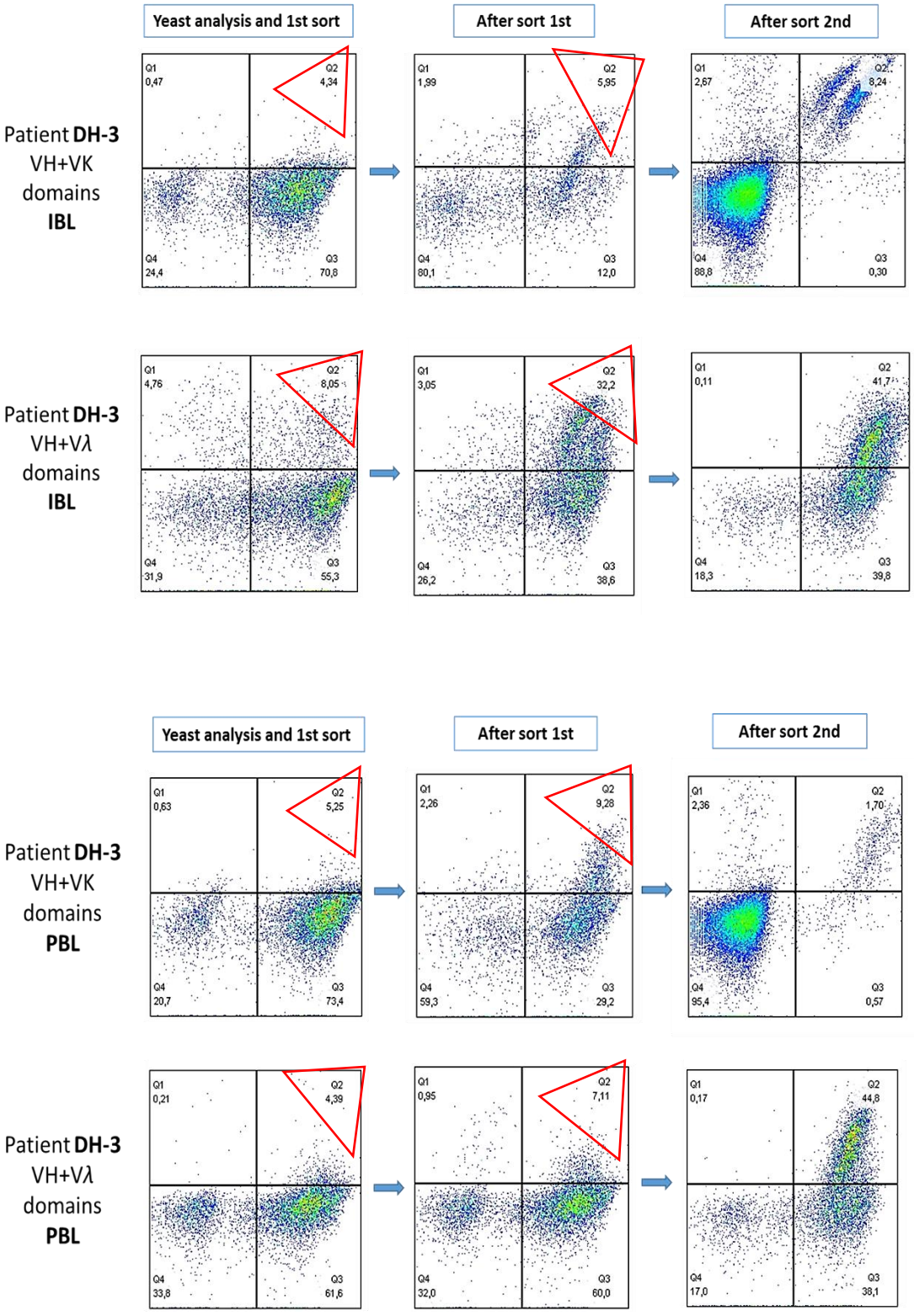
(C)


Yeast analysis and 1st sort
After sort 1st
After sort 2nd
Patient DH-1 VH+VK domains IBL
Q1 0,11
Q2 1,15
Q4 29,4
Q3 69,3
Q1 1,05
Q2 24,9
Q4 16,7
Q3 57,3
Q1 0,063
Q2 78,5
Q4 5,39
Q3 16,1
Patient DH-1 VH+Vλ domains IBL
Q1 0,52
Q2 3,17
Q4 29,4
Q3 66,9
Q1 0,16
Q2 10,3
Q4 10,8
Q3 78,7
Q1 2,01
Q2 13,7
Q4 19,2
Q3 65,0
Yeast analysis and 1st sort
After sort 1st
After sort 2nd
Patient DH-1 VH+VK domains PBL
Q1 0,65
Q2 2,25
Q4 24,5
Q3 72,6
Q1 1,91
Q2 12,9
Q4 30,1
Q3 55,1
Q1 1,75
Q2 13,3
Q4 19,0
Q3 65,9
Patient DH-1 VH+Vλ domains PBL
Q1 0,26
Q2 2,31
Q4 26,2
Q3 71,2
Q1 0,86
Q2 10,0
Q4 27,1
Q3 62,0
Q1 0,50
Q2 13,1
Q4 13,1
Q3 73,3

(D)


Yeast analysis and 1st sort
After sort 1st
After sort 2nd
Patient DH-2 VH+VK domains IBL
Q1 0,067
Q2 3,61
Q4 17,0
Q3 79,3
Q1 0,37
Q2 10,1
Q4 18,7
Q3 70,8
Q1 2,77
Q2 49,0
Q4 13,8
Q3 34,5
Patient DH-1 VH+Vλ domains IBL
Q1 2,05
Q2 4,69
Q4 45,9
Q3 47,4
Q1 5,15
Q2 15,5
Q4 26,9
Q3 52,4
Q1 0,046
Q2 31,3
Q4 12,3
Q3 56,4
Yeast analysis and 1st sort
After sort 1st
After sort 2nd
Patient DH-2 VH+VK domains PBL
Q1 0,19
Q2 2,20
Q4 21,8
Q3 75,8
Q1 0,13
Q2 10,5
Q4 13,8
Q3 75,5
Q1 0,079
Q2 51,1
Q4 10,3
Q3 38,5
Patient DH-2 VH+Vλ domains PBL
Q1 0,20
Q2 1,93
Q4 20,6
Q3 77,3
Q1 2,05
Q2 4,69
Q4 45,9
Q3 47,4
Q1 0,96
Q2 47,8
Q4 20,1
Q3 31,2

(E)


Yeast analysis and 1st sort
After sort 1st
After sort 2nd
Patient DH-3 VH+VK domains IBL
Q1 0,47
Q2 4,34
Q4 24,4
Q3 70,8
Q1 1,99
Q2 5,95
Q4 80,1
Q3 12,0
Q1 2,67
Q2 8,24
Q4 88,8
Q3 0,30
Patient DH-3 VH+Vλ domains IBL
Q1 4,76
Q2 8,05
Q4 31,9
Q3 55,3
Q1 3,05
Q2 32,2
Q4 26,2
Q3 38,6
Q1 0,11
Q2 41,7
Q4 18,3
Q3 39,8
Yeast analysis and 1st sort
After sort 1st
After sort 2nd
Patient DH-3 VH+VK domains PBL
Q1 0,63
Q2 5,25
Q4 20,7
Q3 73,4
Q1 2,26
Q2 9,28
Q4 59,3
Q3 29,2
Q1 2,36
Q2 1,70
Q4 95,4
Q3 0,57
Patient DH-3 VH+Vλ domains PBL
Q1 0,21
Q2 4,39
Q4 33,8
Q3 61,6
Q1 0,95
Q2 7,11
Q4 32,0
Q3 60,0
Q1 0,17
Q2 44,8
Q4 17,0
Q3 38,1

(F)

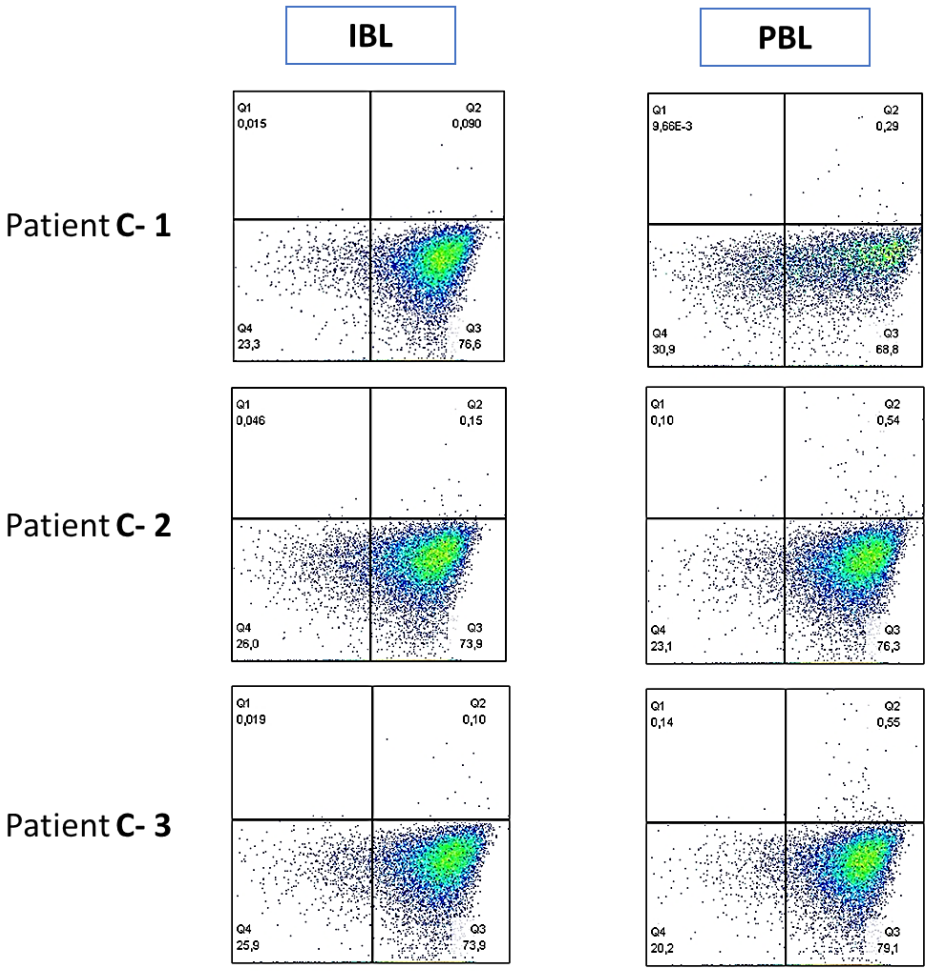


Fig. 3.2 (A) Characterizing the system testing the unstained yeast cells population (1), streptavidin-Alexa Fluor 647-conjugate stained yeast cells (2), Streptavidin-Alexa Fluor 647-conjugate and anti-SV5-PE-conjugate stained yeast cells (3) to setting the instrument. **(B)** FACS plots representing the first analysis of each polyclonal ab-library of both intestinal and peripheral origin, of each DH-patient. The red triangle has been arbitrarily set to select and sort the definitely positive clones. **(C)** FACS plots analysis and sorting rounds of patient DH-1 ab-libraries. **(D)** FACS plots analysis and sorting rounds of patient DH-2 ab-libraries. **(E)** FACS plots analysis and sorting rounds of patient DH-3 ab-libraries. **(F)** FACS plots after negative sort of three control ab-libraries of both levels (IBL and PBL). In each plot, the double positive clones (anti-SV5 and anti-TG3) are represented in Q2

4.3.4 Analysis and Sanger Sequencing of individual clones

Single IBL clones of DH-1 patient, from last FACS selections, were tested by flow cytometry for their TG-3 binding activity (Figure 3.3) in order to verify the specificity of the sorter selection and to compare the CDR3 sequence and VH- gene family used in Abs biosynthesis. In CD, the use of the human Ab VH families appears limited, indicating a possible preferential use of VH-5 gene family in the autoimmune response to TG-2.48 In DH the same study has not been yet investigated.

The amino acids sequences and V domains are reported in Table 6. These same mAbs were also tested, by flow cytometry, for TG-2 antigen binding activity, to exclude cross reactivity with the CD-autoantigen. The analysis shows at least 30% of scFv expression and no bound clones for the TG-2 target (Data not shown).

Clone **1.** Clone **2.** Clone **3.**

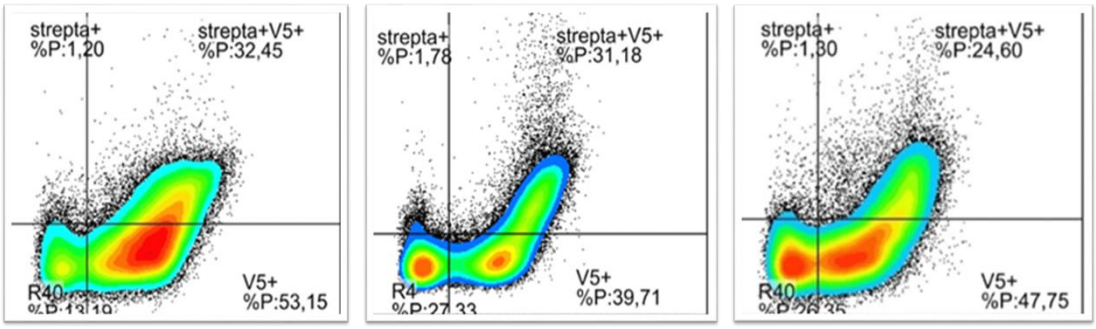


Fig. 3.3 Flow cytometry plots of individual clones. Random clones analyzed (1. 2. 3) show at least 30% of scFv expression (V5+ indicated signal) and marked positivity to the TG-3 target antigen (strepta+V5+ indicated signal).

Clone	v-H domain	CDR3	v- κ/ λ domain	CDR3
1	vH-1	DSSGDCRSTPCFPKE	vλ-6	QSYDSSSWV
2	vH-2	TGYYDSSGYHTYVY	vλ-6	QSYDSTNQV
3	vH-6	HTTSVYFDS	vλ-2	SSYTSSSTYV

Table. 6 V-gene family and CDR3 amino acids sequences of randomly chosen positive TG-3 IBL clones from DH-1 patient, after last sort selection.

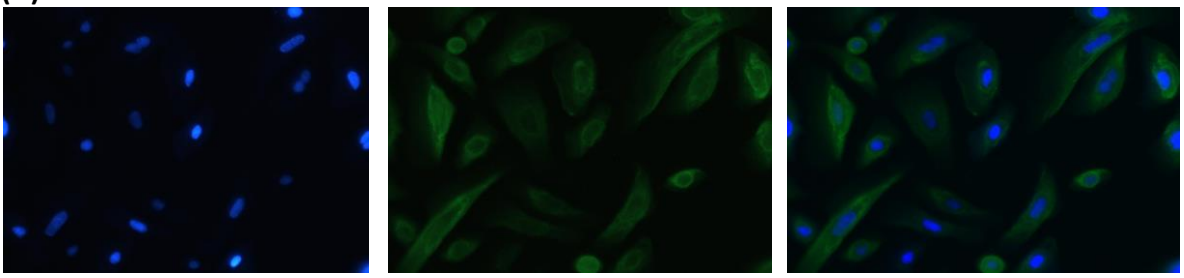
4.4 Immunofluorescence assay

Indirect immunofluorescence assay was performed on cultured NHEK to examine TG-3 protein expression. Using a rabbit polyclonal antibody against human TG-3 protein, a weak but distinct fluorescence staining pattern with individual keratinocytes in the non-permeabilized cells and a clearer and distinct staining in the permeabilized ones were found (Figure 3.4).

The filaggrin protein staining represents the positive control to validate the experiment. The experiments are conducted on young cells (Figure 3.4 A-B-C panels) cultured for 10 days, and on senescent cells (Figure 3.4 D panel) by supplementing the culture medium with CaCl2 (1M) for 8 days.

DAPI Green Merge

(A)



(B)

(C)

(D)

Fig. 3.4 (A) Immunofluorescence staining of permeabilized cultured NHEK cells show the expression of filaggrin as a cytosolic marker of keratinocytes. **(B)** Immunofluorescence staining of not-permeabilized NHEK cells and cultured w/o $CaCl_2$ supplements, show a weak fluorescent signal of the human TG-3 expression. **(C)** Immunofluorescence staining of permeabilized NHEK cells and cultured w/o $CaCl_2$ supplements, show a fluorescent signal of the human TG-3 expression. **(D)** Immunofluorescence staining of permeabilized NHEK cells and cultured with $CaCl_2$ supplements, show a distinct fluorescent pattern of the human TG-3 expression.
Nucleus staining with blu DAPI, IgG secondary antibody staining with green Alexa Fluor 488, and merge image (DAPI+ AlexaFluor 488) are reported.

4.5 ELISA human-TG-3

The production and extracellular release of the human TG-3 protein by cultured NHEK cells was confirmed by the sandwich-ELISA assay (Figure 3.5).

Quantification of TG-3 protein reported that cultured NHEK with $CaCl_2$ produce higher level of TG-3 protein than cultured NHEK without $CaCl_2$.

Values of all measurements were expressed as mean ± SD.

The Student t test has indicated that this difference is statistically significant (p= 0,0001).

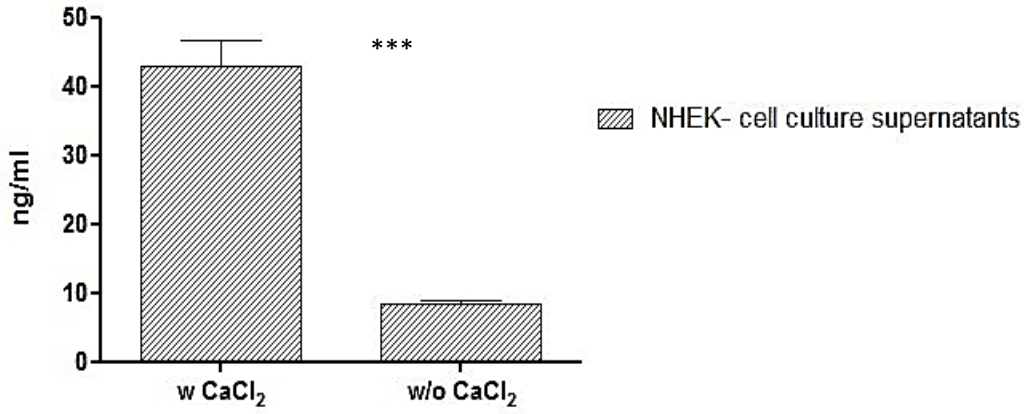


Fig. 3.5 NHEK cells were cultured in normal condition for 10 days and with $CaCl_2$ (1mM) supplements for 8 days. The medium alone samples were used as a negative control. The protein concentration is expressed in ng/ml.

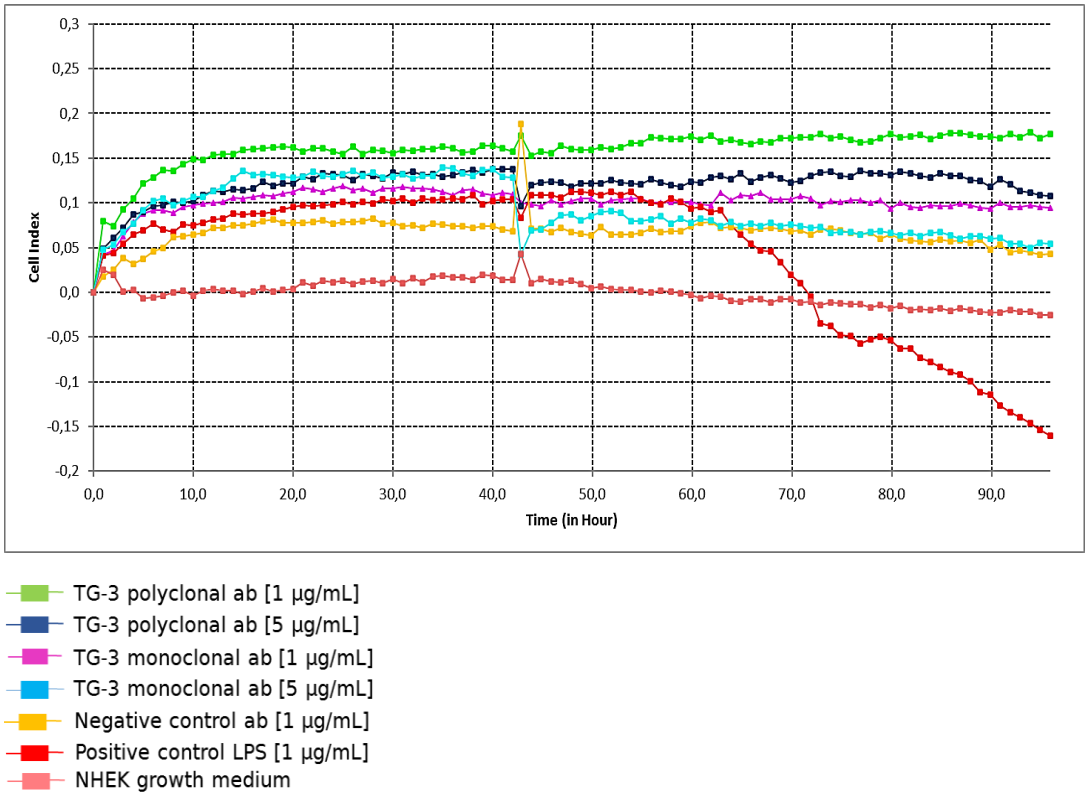
4.6 xCELLigence cellular

An impedance-based test (xCELLigence system) was used to find out information on antigen-antibody interactions. NHEK cells were subjected to different stimuli, and at different concentrations (1 and 5µg/mL) of TG-3 monoclonal and polyclonal antibodies, to monitor their susceptibility. This system mimics the exact continuous cellular biological responses in real-time settings. Briefly, impedance to an electric current occurs when the cells adhere to the electrodes, conversely, the electrical impedance is reduced as the cells detach after killing.

Figure 3.6 shows the NHEK xCELLigence plot for all stimuli **(A)** and well graphs representative of each individual stimulus **(B)**.

The moment of stimulation with the various molecules occurred at the 43rd hour of monitoring and causes an alteration of the cellular balance represented in the plot by an upward spike. The addition of TG-3 antibodies stimuli to a monolayer of attached and stabilized cells, did not affect the impedance measurements, however, changes were observed with the LPS stimulus (red line), as expected, that translate into morphology and detachment events. This label-free approach demonstrated that the TG-3 antibody stimulations did not cause toxicity, conformational or cell adhesion changes compared to positive LPS stimulus. Therefore, the TG-3 presence and the antigen-antibody binding site cannot occur in the plasma membrane of keratinocytes.

(A)



(B)

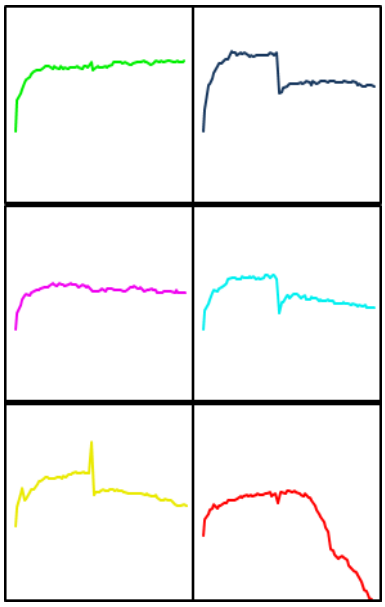


Fig. 3.6 (A) NHEK xCELLigence plot shows total cell monitoring for the entire duration of the experiment, 95:51 hours, until total cell detachment (negative cell index value) under the positive LPS stimulus shown in red. Time 0 denotes the time of cell addition to the plate which was first scanned. The upward spike (43rd hour) represents the stimulation step that causes alteration of cellular conditions.
(B) The trend of cell conditions under stimuli of the TG-3 antibodies at different concentrations does not cause significant changes. The cell index values are comparable to that of the negative stimulus shown in yellow.

5. DISCUSSION and CONCLUSION

DH is considered the skin manifestation of CD. The relationship between intestinal mucosa and systemic immune responses is not well understood, and the origin of circulating antibodies against TG-3 antigen, in DH, has not been investigated thoroughly. In this study, TG-3 protein has been found in the gut and confirmed peripherally, in keratinocytes. Current evidence shows the presence of TG-3 antibody-secreting plasma cells in the intestine of DH patients.[56,57] All these data suggested to investigate the origin's site of anti- TG3 antibodies by means of two different ab-display library technologies from IBL and PBL source.

No TG-3 binding clones from IBL or PBL ab-libraries of Control patients have been found, confirming the high specificity of the phage and yeast display technologies.

Phage- and yeast-display antibody selections have been able to detect anti TG-3 antibody-secreting plasma cells at intestinal level in DH patients. However, phage-display detected these intestinal plasma cells only in one of the three enrolled patients. In addition, yeast-display technology, after two cycles of selections, has been able to show scFv TG-3 binders at intestinal and also at peripheral level of all DH patients.

According to the current results, the yeast-display method can be considered more sensitive, allowing the expression of thousands of copies of the antibody on each cell and the accurate control of the selection parameters by flow cytometry-based analysis and sorting. However, the phage-display remains the elective method to generate a larger ab-libraries and to identify the individual binding clones from the first selection.

This result shows that the failure of selections in phage-display was not attributable to the lack of such Abs in libraries, but to the low sensitivity of the system in isolation and detection of scFv.

In CD it has been demonstrated that anti-TG2 antibodies are produced by intestinal B lymphocytes and not by the peripheral ones. However, the intestinal origin of anti TG-2 antibodies has been investigated and demonstrated by using phage and not yeast-display approach. Considering that overall great homology between the human TG-2 and TG-3, autoantibody cross reactivity and epitope spreading has been investigated. In this study, mAb cross reactivity to TG-2 antigen binding activity has been excluded, ruling out the epitope spreading as a mechanism that antibodies do not cross-react

between TG-2 and TG-3. TG-3 protein is able to form thioester linked complexes with gluten peptide and this evidence suggests the direct TG-3 involvement in DH pathology.[22]

The VH gene sequences of the identified TG-3 antibodies do not show the use of the VH-5 family typical of the immune system to construct Abs in CD (to which DH is related). The involvement of specific VH-1/2/6 gene families (different from CD's VH-family) could be a specific immunological feature in the gluten-dependent autoimmunity field involving the skin. Nevertheless, to have a complete characterization of all identified antibodies it would be necessary a deep sequencing of all antibodies from the last FACS selection.

An important criticism to this approach is that the VH-VL combination occurring in vivo are not usually recreated in vitro with ab-libraries, in fact, VH and VL genes are randomly paired. Nevertheless, previous studies and findings suggest that phage Ab libraries can act as surrogates for the humoral response.[48]

Keratinocytes, representing the inflammatory site where DH occurs, could be a suitable in vitro model to study the pathogenetic role of anti TG-3 antibodies. The TG-3 protein has been quantified on keratinocytes culture supernatant and detected in the intracellular compartment but not in the plasma membrane.

However, referring to the studies carried out in CD on intestinal epithelial cells to detect the interaction with extracellular membrane and anti TG-2 antibodies, further cellular assays have been performed. In CD, it has been demonstrated the active role of anti-TG2 antibodies in the pathogenesis, compromising the function of intestinal barrier, inhibiting the differentiation of, causing cytoskeleton rearrangements and therefore morphological and tissue alterations.[58,59] Similarly, in keratinocytes the anti-TG-3 antibodies might somehow intercept the antigen and alter cellular homeostasis.

The use of a real-time assay (xCelligence cellular system) was suitable for monitoring the biological response of keratinocytes to the TG-3 antibody stimulation. Notable, the label-free approach to preserve the physiological condition of the keratinocytes allowed to demonstrate that the antibody stimulations did not cause toxicity, conformational or cell adhesion changes compared to positive LPS stimulus. This finding rules out both the presence of the protein at the membrane level and the antigen-antibody binding site in

the plasma membrane of keratinocytes. Therefore, keratinocyte in-vitro model did not allow to investigate the pathogenetic role of anti TG-3 antibodies.

The pathogenesis of DH is not due to the antigen-antibody direct interaction. It is reasonable to speculate on the immunocomplex theory that is not new in DH, several studies on this topic have been conducted since 1980.[60] The circulating IgA in the form of immunocomplexes with the TG-3 antigen, form aggregates in dividing keratinocytes of papillary dermis.[8,61,62] These dermal deposits are often associated with C-3 of Complement to support the C-5 and alternative pathway activation. The C-5a activated fraction is highly chemotactic for neutrophils, macrophages and inflammatory cytokines necessary to initiate the fibrinolytic process. These immune cells result in typical DH blisters and in lymphocytic infiltrate, probably corresponding to a later stage of the disease.[27] The circulating immunocomplexes can explain the long persistence of IgA deposits and DH cutaneous manifestation also under GFD. Long-lasting IgA and IgG-immunocomplexes are also observed in other immunocomplex-diseases in remission for years, like systemic lupus erythematosus.[61]

In conclusion, this study confirms that the autoantigen in dermatitis herpetiformis is the epidermal transglutaminase. The research has confirmed that TG-3 autoantibodies are produced at intestinal level. Moreover, for the first time, it has been demonstrated that the origin's site is also peripheral. Studies on the binding activities of mAbs and a deep sequencing is required, as future work, to have a complete characterization of all identified TG-3 antibodies. The keratinocyte in-vitro model did not allow to investigate the pathogenetic role of anti TG-3 antibodies. Nevertheless, these study results have demonstrated that the pathogenesis of DH is not related to the antigen-antibody direct interaction on the keratinocytes plasma membrane.

There are still many aspects to be clarified regarding the pathogenesis of DH, including what triggers the production of anti-TG-3 and why the breaking of the immune tolerance in patients with DH is directed against TG-3 as well as TG-2.

Bibliography

1. Reunala, T., Salmi, T., Hervonen, K., Kaukinen, K. & Collin, P. Dermatitis Herpetiformis: A Common Extraintestinal Manifestation of Coeliac Disease. *Nutrients* **10**, 602 (2018).
2. Salmi, T. T. Dermatitis herpetiformis. *Clin. Exp. Dermatol.* **44**, 728–731 (2019).
3. Caproni, M., Bonciolini, V., D'Errico, A., Antiga, E. & Fabbri, P. Celiac Disease and Dermatologic Manifestations: Many Skin Clue to Unfold Gluten-Sensitive Enteropathy. *Gastroenterol. Res. Pract.* **2012**, 952753 (2012).
4. Duhring, L. A. Dermatitis Herpetiformis. *JAMA* **250**, 212–216 (1983).
5. Losowsky, M. S. A history of coeliac disease. *Dig. Dis. Basel Switz.* **26**, 112–120 (2008).
6. Marks, J., Shuster, S. & Watson, A. J. Small-bowel changes in dermatitis herpetiformis. *Lancet Lond. Engl.* **2**, 1280–1282 (1966).
7. Meer, J. B. GRANULAR DEPOSITS OF IMMUNOGLOBULINS IN THE SKIN OF PATIENTS WITH DERMATITIS HERPETIFORMIS. AN IMMUNOFLUORESCENT STUDY. *Br. J. Dermatol.* **81**, 493–503 (1969).
8. Sárdy, M., Kárpáti, S., Merkl, B., Paulsson, M. & Smyth, N. Epidermal Transglutaminase (TGase 3) Is the Autoantigen of Dermatitis Herpetiformis. *J. Exp. Med.* **195**, 747–757 (2002).
9. Dieterich, W. *et al.* Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat. Med.* **3**, 797–801 (1997).
10. Antiga, E. *et al.* Dermatitis Herpetiformis: Novel Perspectives. *Front. Immunol.* **10**, 1290 (2019).
11. Reunala, T. Incidence of familial dermatitis herpetiformis. *Br. J. Dermatol.* **134**, 394–398 (1996).
12. Gawkrodger, D. J. *et al.* Dermatitis herpetiformis: diagnosis, diet and demography. *Gut* **25**, 151–157 (1984).
13. Zhang, F. *et al.* Dermatitis herpetiformis in China: a report of 22 cases: Dermatitis herpetiformis in China. *J. Eur. Acad. Dermatol. Venereol.* **26**, 903–907 (2012).
14. Losurdo, G. *et al.* Extra-intestinal manifestations of non-celiac gluten sensitivity: An expanding paradigm. *World J. Gastroenterol.* **24**, 1521–1530 (2018).
15. Collin, P. & Reunala, T. Recognition and Management of the Cutaneous Manifestations of Celiac Disease: A Guide for Dermatologists. *Am. J. Clin. Dermatol.* **4**, 13–20 (2003).
16. Graziano, M. & Rossi, M. An update on the cutaneous manifestations of coeliac disease and non-coeliac gluten sensitivity. *Int. Rev. Immunol.* **37**, 291–300 (2018).
17. Dieterich, W. *et al.* Antibodies to Tissue Transglutaminase as Serologic Markers in Patients with Dermatitis Herpetiformis. *J. Invest. Dermatol.* **113**, 133–136 (1999).
18. Antiga, E. *et al.* Granular Deposits of IgA in the Skin of Coeliac Patients Without Dermatitis Herpetiformis: A Prospective Multicentric Analysis. *Acta Derm. Venereol.* **101**, adv00382 (2021).

19. Taylor, T. B., Schmidt, L. A., Meyer, L. J. & Zone, J. J. Transglutaminase 3 Present in the IgA Aggregates in Dermatitis Herpetiformis Skin Is Enzymatically Active and Binds Soluble Fibrinogen. *J. Invest. Dermatol.* **135**, 623–625 (2015).

20. Reunala, T., Salmi, T. & Hervonen, K. Dermatitis Herpetiformis: Pathognomonic Transglutaminase IgA Deposits in the Skin and Excellent Prognosis on a Gluten-free Diet. *Acta Derm. Venereol.* **95**, 917–922 (2015).

21. Vanderlugt, C. J. & Miller, S. D. Epitope spreading. *Curr. Opin. Immunol.* **8**, 831–836 (1996).

22. Stamnaes, J., Dorum, S., Fleckenstein, B., Aeschlimann, D. & Sollid, L. M. Gluten T cell epitope targeting by TG3 and TG6; implications for dermatitis herpetiformis and gluten ataxia. *Amino Acids* **39**, 1183–1191 (2010).

23. Spurldand, A. *et al.* Dermatitis herpetiformis and celiac disease are both primarily associated with the HLA-DQ (a1*0501, p1*02) or the HLA-DQ (a1"03, pl*0302) heterodimers. 6.

24. Leffler, D. A., Green, P. H. R. & Fasano, A. Extraintestinal manifestations of coeliac disease. *Nat. Rev. Gastroenterol. Hepatol.* **12**, 561–571 (2015).

25. Hervonen, K. *et al.* Concordance of Dermatitis Herpetiformis and Celiac Disease in Monozygous Twins. *J. Invest. Dermatol.* **115**, 990–993 (2000).

26. Kurppa, K. *et al.* Changing Phenotype of Celiac Disease After Long-term Gluten Exposure. *J. Pediatr. Gastroenterol. Nutr.* **47**, 500–503 (2008).

27. Warren, S. J. P. & Cockerell, C. J. Characterization of a Subgroup of Patients With Dermatitis Herpetiformis With Nonclassical Histologic Features.: *Am. J. Dermatopathol.* **24**, 305–308 (2002).

28. Caproni, M., Antiga, E., Melani, L., Fabbri, P. & Immunopathology, T. I. G. for C. Guidelines for the diagnosis and treatment of dermatitis herpetiformis. *J. Eur. Acad. Dermatol. Venereol.* **23**, 633–638 (2009).

29. Bolotin, D. & Petronic-Rosic, V. Dermatitis herpetiformis: Part I. Epidemiology, pathogenesis, and clinical presentation. *J. Am. Acad. Dermatol.* **64**, 1017–1024 (2011).

30. Chorzelski, T. P., Jabłońska, S. & Maciejowska, E. Linear IgA bullous dermatosis of adults. *Clin. Dermatol.* **9**, 383–392 (1991).

31. Clarindo, M. V. *et al.* Dermatitis herpetiformis: pathophysiology, clinical presentation, diagnosis and treatment. *An. Bras. Dermatol.* **89**, 865–877 (2014).

32. Raghunath, M. *et al.* Cross-linking of the dermo-epidermal junction of skin regenerating from keratinocyte autografts. Anchoring fibrils are a target for tissue transglutaminase. https://www.jci.org/articles/view/118901/pdf (1996) doi:10.1172/JCI118901.

33. Chermnykh, E. S., Alpeeva, E. V. & Vorotelyak, E. A. Transglutaminase 3: The Involvement in Epithelial Differentiation and Cancer. *Cells* **9**, 1996 (2020).

34. Candi, E., Schmidt, R. & Melino, G. The cornified envelope: a model of cell death in the skin. *Nat. Rev. Mol. Cell Biol.* **6**, 328–340 (2005).

35. Kim, I. G., Gorman, J. J., Park, S. C., Chung, S. I. & Steinert, P. M. The deduced sequence of the novel protransglutaminase E (TGase3) of human and mouse. *J. Biol. Chem.* **268**, 12682–12690 (1993).

36. Ahvazi, B. Three-dimensional structure of the human transglutaminase 3 enzyme: binding of calcium ions changes structure for activation. *EMBO J.* **21**, 2055–2067 (2002).

37. Hitomi, K. *et al.* Analysis of epidermal-type transglutaminase (transglutaminase 3) in human stratified epithelia and cultured keratinocytes using monoclonal antibodies. *J. Dermatol. Sci.* **32**, 95–103 (2003).

38. Ministro, J., Manuel, A. M. & Goncalves, J. Therapeutic Antibody Engineering and Selection Strategies. in *Current Applications of Pharmaceutical Biotechnology* (eds. Silva, A. C., Moreira, J. N., Lobo, J. M. S. & Almeida, H.) vol. 171 55–86 (Springer International Publishing, 2019).

39. Adams, R. M., Mora, T., Walczak, A. M. & Kinney, J. B. Measuring the sequence-affinity landscape of antibodies with massively parallel titration curves. *eLife* **5**, e23156.

40. Smith, G. P. Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface. *Science* **228**, 1315–1317 (1985).

41. Orlandi, R., Gussow, D. H., Jones, P. T. & Winter, G. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci.* **86**, 3833–3837 (1989).

42. McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552–554 (1990).

43. Hoogenboom, H. R., Marks, J. D., Riffiths, A. D. G. & Winter, G. Building Antibodies from their Genes. *Immunol. Rev.* **130**, 41–68 (1992).

44. Lerner, R. A. Combinatorial antibody libraries: new advances, new immunological insights. *Nat. Rev. Immunol.* **16**, 498–508 (2016).

45. Hammers, C. M. & Stanley, J. R. Antibody Phage Display: Technique and Applications. *J. Invest. Dermatol.* **134**, 1–5 (2014).

46. *Human Monoclonal Antibodies: Methods and Protocols*. vol. 1904 (Springer New York, 2019).

47. Boder, E. T. & Wittrup, K. D. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15**, 553–557 (1997).

48. Marzari, R. *et al.* Molecular Dissection of the Tissue Transglutaminase Autoantibody Response in Celiac Disease. *J. Immunol.* **166**, 4170–4176 (2001).

49. Borrelli, M. *et al.* Intestinal anti-transglutaminase 2 immunoglobulin A deposits in children at risk for coeliac disease (CD): data from the PreventCD study. *Clin. Exp. Immunol.* **191**, 311–317 (2018).

50. Ziberna, F. *et al.* A novel quantitative ELISA as accurate and reproducible tool to detect epidermal transglutaminase antibodies in patients with Dermatitis Herpetiformis. *J. Eur. Acad. Dermatol. Venereol.* **35**, (2021).

51. Sblattero, D. & Bradbury, A. Exploiting recombination in single bacteria to make large phage antibody libraries. *Nat. Biotechnol.* **18**, 75–80 (2000).

52. Ferrara, F. *et al.* Using Phage and Yeast Display to Select Hundreds of Monoclonal Antibodies: Application to Antigen 85, a Tuberculosis Biomarker. *PLOS ONE* **7**, e49535 (2012).

53. Kim, C. D. *et al.* Characterization of Ets-binding sequence of human transglutaminase 3 gene promoter. *Exp. Dermatol.* **13**, 529–534 (2004).

54. Min, M. *et al.* Role of keratin 24 in human epidermal keratinocytes. *PLOS ONE* **12**, e0174626 (2017).

55. *Mammalian Cell Viability*. vol. 740 (Humana Press, 2011).

56. Hietikko, M. *et al.* Ex vivo Culture of Duodenal Biopsies from Patients with Dermatitis Herpetiformis Indicates that Transglutaminase 3 Antibody Production Occurs in the Gut. *Acta Derm. Venereol.* **98**, 366–372 (2018).

57. Sankari, H. *et al.* Intestinal TG3- and TG2-Specific Plasma Cell Responses in Dermatitis Herpetiformis Patients Undergoing a Gluten Challenge. *Nutrients* **12**, 467 (2020).

58. Barone, M. V. *et al.* Humoral Immune Response to Tissue Transglutaminase Is Related to Epithelial Cell Proliferation in Celiac Disease. *Gastroenterology* **132**, 1245–1253 (2007).

59. Paolella, G. *et al.* Celiac Anti-Type 2 Transglutaminase Antibodies Induce Phosphoproteome Modification in Intestinal Epithelial Caco-2 Cells. *PLOS ONE* **8**, e84403 (2013).

60. Hall, R. P., Lawley, T. J., Heck, J. A. & Katz, S. I. IgA-containing circulating immune complexes in dermatitis herpetiformis, Henoch-Schönlein purpura, systemic lupus erythematosus and other diseases. *Clin. Exp. Immunol.* **40**, 431–437 (1980).

61. Görög, A. *et al.* Circulating Transglutaminase 3-Immunoglobulin A Immune Complexes in Dermatitis Herpetiformis. *J. Invest. Dermatol.* **136**, 1729–1731 (2016).

62. Cannistraci, C. *et al.* Co-localization of IgA and TG3 on healthy skin of coeliac patients. *J. Eur. Acad. Dermatol. Venereol.* **0**, 070209222700168-??? (2007).