LUND UNIVERSITY

Transglutaminase and peptidylarginine deiminase in the pathogenesis of autoimmune diseases

Roth, Bodil

2008

Link to publication

Citation for published version (APA):
Roth, B. (2008). *Transglutaminase and peptidylarginine deiminase in the pathogenesis of autoimmune diseases*. [Doctoral Thesis (compilation), Faculty of Medicine]. Chronic Inflammatory and Degenerative Diseases Research Unit.

Total number of authors:
1

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Transglutaminase and peptidylarginine deiminase in the pathogenesis of autoimmune diseases

Bodil Roth

ISSN: 1652-8220
ISBN: 978-91-85897-62-9
Lund University
Faculty of Medicine
Doctorial Dissertation Series 2008: 9
Printed in Lund, Sweden, by Media-Tryck, 2008
© Bodil Roth

"Everything should be made as simple as possible,
but not one bit simpler."
Albert Einstein

To my family

Table of contents

1. List of papers **7**

2. Abbreviations **8**

3. Background **10**

Autoimmune diseases 10
Coeliac disease 12
Transglutaminases 13
Rheumatoid arthritis 17
Sjögren's syndrome 18
Peptidylarginine deiminases 19

4. Aims **22**

5. Subjects **23**

Study I 23
Study II 23
Study III 23
Study IV 23

6. Methods **25**

ELISA 25
Endomysial autoantibody immunofluorescence (EmA) (Study I, II, III, IV) 26
Electrophoresis 26
Scion Image for Windows 27
RBA based on hr-TG2 (Study II) 27
Statistics 28

7. Results **29**

Study I 29
Study II 30
Study III 32
Study IV 32

8. Discussion **33**

The effect of calcium 33
The effect of zinc 34
The structure of the neoantigen 34
The effect of antibodies on the activity of TG2 in CD 37
Anti-PAD in RA 37
Epitope spreading 38
Antibodies in Sjögren's syndrome 39
Antibodies in neurodegenerative diseases 39

9. Conclusions **40**

10. General discussion **41**

11. Summary **43**

12. Sammanfattning på svenska **45**

13. Acknowledgements **48**
14. References **49**
15. Appendix (Papers I-IV) **57**

1. List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals (I-IV). They are reprinted with the permission of the respective publisher.

I. Roth EB, Sjöberg K, Stenberg P. Biochemical and immuno-pathological aspects of tissue transglutaminase in celiac disease. Autoimmunity 2003; 36:221-226.

II. Agardh D, Roth EB, Lernmark Å, Stenberg P. Calcium activation of tissue transglutaminase in radioligand binding and enzyme-linked autoantibody immunoassays in childhood celiac disease. Clinica Chimica Acta 2005; 358:95-103.

III. Roth EB, Stenberg P, Book C, Sjöberg K. Antibodies against transglutaminase, peptidylarginine deiminase and citrulline in rheumatoid arthritis – new pathways to epitope spreading. Clin Exp Rheumatol. 2006; 24:12-18

IV. Roth EB, Theander E, Londos E, Sandberg-Wollheim M, Larsson Å, Sjöberg K, Stenberg P. Pathogenesis of autoimmune diseases: Antibodies against transglutaminase, peptidylarginine deiminase and protein-bound citrulline in primary Sjögren syndrome, multiple sclerosis and Alzheimer's disease. Manuscript.

2. Abbreviations

ACR American College of Rheumatology

AD Alzheimer's disease

AECC American European Consensus Criteria

ANA anti-nuclear antibody

Anti-CP anti-citrulline protein

APC antigen presenting cell

CCP cyclic citrulline-containing peptide

CD coeliac disease

ECM extracellular matrix

EDTA ethylenediaminotetraacetic acid

ELISA enzyme-linked immunosorbant assay

EmA endomysial antibodies

ESPGHAN European Society for Paediatric Gastroenterology, Hepatology and Nutrition

FXIII factor XIII

GAD glutamic acid decarboxylase

Gp Guinea pig

GTP guanosine triphosphate

HLA human leukocyte antigen

Hr human recombinant

MBP myelin basic protein

MDTC monodansylthiacadaverine

MS multiple sclerosis

MTX methotrexate

PAD peptidylarginine deiminase

pSS primary Sjögren's Syndrome

RA rheumatoid arthritis

RBA radioligand binding assay

RF rheumatoid factor

SS Sjögren's Syndrome

TBS Tris buffer saline

TG transglutaminase

TG2 transglutaminase type 2

TGF-β1 transforming growth factor–β1

T_H-cells T helper cells

SLE systemic lupus erythematosus

3. Background

Autoimmune diseases

One of the most intriguing issues in immunology is how the immune system can distinguish between "self" and "non-self". Occasionally, tolerance to self-antigens breaks down, causing autoimmune diseases, i.e., conditions where an inappropriate immune response results in damage to an individual's organs, tissues or cells. These conditions can affect almost any part of the body, resulting in a wide panorama of manifestations. As a group, they comprise approximately 80 different diseases [1]. Based on American epidemiological studies [2], these illnesses afflict 5-8 % of the population, corresponding to a prevalence of the same magnitude as of cancer or cardio-vascular disease. Thus, it can be estimated that more than one hundred million people are affected globally. Moreover, the incidence is rising. In general, women are more frequently affected than men. For example, for Sjögren's syndrome (SS), thyreoiditis and systemic lupus erythematosus (SLE), more than 80 % of the patients are females. In fact, autoimmune diseases are a leading cause of death among young and middle-aged American women. The mechanism causing this gender difference is not known, although the influence of oestrogen has been discussed. The chronic, often debilitating character of these conditions further emphasizes the vast dimensions of the health problem. Medication, if available, is mainly symptomatic, curative treatment is missing, and prevention is even more elusive.

Autoimmune disorders fall into two general types: those that affect multiple organs (systemic autoimmune diseases, such as SLE) or one single organ (localized, such as Hashimoto's disease). However, the distinctions often become blurred as the effects of localized autoimmune disorders frequently extend beyond the primary target, indirectly affecting other organs and systems. Autoimmune diseases have many common features and are increasingly recognized as a group of related illnesses that should be studied collectively as well as individually [2].

The aetiology of autoimmune diseases is multifactorial and probably comprises genetic and environmental as well as lifestyle factors. Both T-cells, B-cells, and auto-antibodies might be involved, but for most of these disorders the pathogenesis is completely unknown. Furthermore, understanding of autoimmune diseases is hampered by the fact that some level

of autoimmunity is present in all normal persons in the form of naturally occurring autoantibodies and self-reactive T- and B-cells. On the other hand, clinically relevant autoimmune diseases develop only in susceptible cases. During their differentiation, the cells recognising the HLA-receptor are allowed to mature further (positive selection) and, in the next step, self-reactive B- and T-cells are eliminated by a mechanism called negative selection. When they occasionally escape into the blood, the central tolerance is complemented by peripheral mechanisms involving anergy, immunological ignorance and active regulation. A special subset of the so-called regulatory T-cells orchestrates the regulatory control, counteracting the T_H-cells. If this balance is disturbed, e.g. when the suppressive influences fail, the risk of autoimmune diseases increases.

Autoimmune diseases tend to cluster in families. Moreover, an individual with one autoimmune disease is more likely to acquire another, which indicates that common mechanisms are involved in disease susceptibility [3]. Studies of the prevalence of autoimmune diseases in monozygotic twins show that genetic as well as environmental factors (such as infection) are necessary to develop the disorder [4]. The genetic factor has been estimated at approximately 30 % (range 20-60 %). In most cases, the human leukocyte antigens (HLA) have been in focus.

Of the environmental factors, exposure to drugs, metals and infectious agents are considered to be tentative triggers of autoimmune diseases. For example, procainamide [5] and hydralazine [6] can induce an SLE-like syndrome in genetically susceptible subjects, and intake of drugs against tuberculosis, such as isoniazide and p-aminosalicylic acid [7], has been associated with the development of antibodies against coagulation factor XIII (FXIII). In animal studies, the possible role of exposure to various metals has been investigated. While most metals inhibit cell proliferation, mercury, gold and silver can induce lymphocyte proliferation and subsequent autoimmunity.
Zinc is required for the activity of approximately 300 enzyme systems, some of which play important roles in the immune system [8]. Zinc depletion is known to increase the risk of infection [9, 10]

Of the infectious agents, group A beta-haemolytic streptococci have a central role in the development of rheumatoid heart disease [11]. Moreover, Epstein-Barr virus has been implicated in SLE [12] and rheumatoid arthritis (RA) [13, 14]. A mechanism often called on to

explain the association of infection with autoimmune disease is ”molecular mimicry,” that is, antigens (epitopes) of a micro-organism closely resemble self-antigens [15, 16]. The induction of an immune response to the microbial antigen thus results in a cross-reaction with self-antigens [17]. Although epitope-specific cross-reactivity between microbes and self-tissues has been shown in some animal models [18, 19], molecular mimicry has not been clearly demonstrated in human diseases [20].

Another possibility is that micro-organisms expose self-antigens to the immune system by directly damaging tissues during an active infection. This mechanism has been referred to as the "bystander effect" [21]. However, whether pathogens mimic self-antigens, release sequestered self-antigens, or both, is difficult to determine.

As an example of the influence of lifestyle factors, smoking has been associated with an increased risk of RA [22] and SLE [23] while the risk of ulcerative colitis might be reduced among smokers [24]. The mechanism for the effects of smoking in this context is not known. Interestingly, water-soluble components of cigarette smoke inhibit plasma FXIII [25]. Furthermore, stress, physical as well as mental, affects the immune system [26]. Hard physical exercise reduces resistence against infections [27], and in vitro studies have shown that sorrow-stricken people have a reduced proliferation of lymphocytes [28].

Coeliac disease

The condition which we now know as coeliac disease (CD) was first described in detail by Samuel Gee in 1887 in London. Almost 60 years ago, the Dutch paediatrician Wim Dicke recognized for the first time gluten as the trigger for CD [29]. A few years later, the histological changes in jejunum were described [30]. During the 1950s, new biopsy techniques were invented when the gastric biopsy tube and the intestinal biopsy capsule were introduced [31, 32]. New diagnostic tools arrived when different antibodies against reticulin [33], gliadin [34] and endomysium (EmA) [35, 36] could be detected in patients with CD. In 1985, Bruce at al. [37] reported an increased transglutaminase activity in duodenal biopsies taken from patients with CD as compared with normal subjects. Finally, in 1997 Dietrich et al showed that the antigen of EmA is transglutaminase type 2 (TG2) [38].

CD is an acquired and permanent enteropathy, induced by gluten and related cereal proteins. The pathological lesion is characterized by a flattened small intestinal mucosa with a lymphocytic infiltrate, increased epithelial cell proliferation with crypt hyperplasia, and reduced enterocyte differentiation [39]. As a consequence, absorptive function is impaired. Patients frequently experience symptoms of lethargy and diarrhoea and may develop anaemia, osteoporosis, and even central nervous defects, such as ataxia. The pathological changes and symptoms are generally resolved with withdrawal of gluten from the diet. Moreover, patients with CD have an increased risk of developing intestinal non-Hodgkin lymphoma [40, 41].

The genetic risk factors for CD have been well characterised. More than 95% of coeliac patients share two HLA haplotypes, mainly DQ2 and, to some extent, DQ8 [42]. These HLA haplotypes have been found in 20–30% of the general population in Northern Europe while the prevalence of CD in a general Western population is close to 1% and is somewhat higher in certain European populations [43]. First-degree relatives of CD patients carry a tenfold risk of developing CD compared to the general population [3, 44].

Transglutaminases

The term transglutaminase (TG) was coined by Waelsch and his co-workers in 1959 [45] to describe the transamidating activity observed in Guinea pig (gp)-liver. TGs form a large family of intracellular and extracellular enzymes that catalyze a calcium-dependent post-translational modification of proteins. At the genomic level, eight members of the transglutaminase family (E.C. 2.3.2.13) have been identified [46] (Table 1). Of these, six isozymes have been isolated and characterized as calcium-dependent thiol enzymes. In addition, a TG-like protein is found in red blood cells [47]. Since the position of the active site cysteine is replaced by an alanine residue, this protein, called erythrocyte membrane protein band 4.2, has no enzyme activity. Instead it constitutes a major component of the red blood cell membrane.

TG	Synonyms	Chromosome location	Function	kDa	Distribution
FXIII	Fibrin stabilizing factor	6q24-25	Blood clotting and wound healing	83	Cytosol, extracellular
Band 4.2	Erythrocyte membrane protein	15q15.2	Structural protein in erythrocytes - no activity	72	Membrane
TG1	Keratinocyte TG	14q11.2	Cornified envelope assembly in surface epithelia	90	Cytosol, membrane
TG2	Tissue TG	20q11-12	Matrix assembly, adhesion, cell death/differentiation	80	Cytosol, nucleus, membrane, cell surface, extracellular
TG3	Epidermal TG	20q11-12	Cornified envelope assembly in surface epithelia	77	Cytosol
TG4	Prostate TG	3q21-22	Semen coagulation in rodents	77	Unknown
TG5	TG X	15q15.2	Epidermal differentiation	81	Nuclear matrix, cytoskeleton
TG6	TG Y	20q11	Unknown	Unknown	Unknown
TG7	TG Z	15q15.2	Unknown	80	Unknown

Table 1. Characteristics of the various members of the transglutaminase family

Transglutaminase-catalyzed reactions

TGs require calcium for their transamidating activity. Zinc competes with calcium for the enzyme metal-binding sites and serves as a most potent inhibitor of the activation of TGs [48]. Once activated, TGs can catalyse an intermolecular crosslinking between protein-bound lysine and glutamine residues, forming ε-(γ-glutamyl)lysyl bridges. TGs display strict specificity in recognition of glutamine protein substrates but a lower specificity for the amine group. Synthetic, low molecular weight amines such as monodansylthiacadaverine (MDTC) [49], and certain carbonyl-containing compounds such as β-phenylpropionylthiocholine, can also serve as substrates [50]. Moreover, in the absence of a suitable amine with nucleophilic capacity, water may act as the second substrate with the consequent deamidation of protein-bound glutamine residues [51, 52]. Both the transamidation and the deamidation reaction proceed via a common intermediate, a thioester between the active site cysteine thiol group of the enzyme and the carbonyl group of the first substrate (Figure 1).

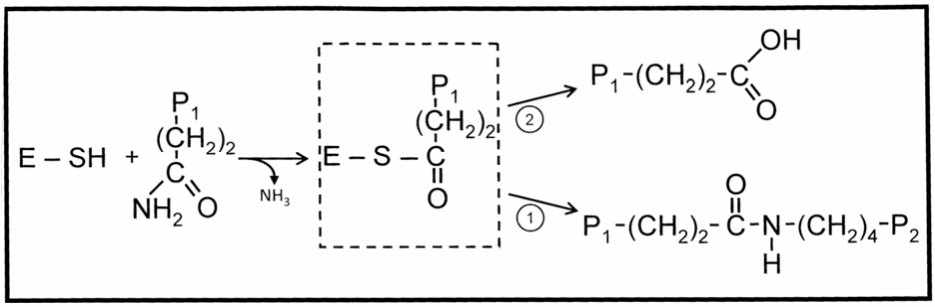


Figure 1: TG-catalyzed reactions*. The thiol group of the activated enzyme forms a thioester with a γ-carbonyl group of protein-bound glutamine and ammonia is released. An ε-amino group of protein-bound lysin nucleophilically attacks the thioester forming the pseudo-peptide bond between P_1 and P_2 (reaction ① = transamidation). In the absence of the amine, water serves as the second substrate causing a hydrolysis of the glutamine residue (reaction ② =deamidation). In the transamidation, the first step is the rate-limiting while the second step in general is considered being rate-limiting in deamidation.*

D

In addition to catalyzing calcium-dependent cross-linking reactions, TG2 can bind and hydrolyze guanosine triphosphate (GTP) [53]. Such GTPase activity of TG2 is independent of the crosslinking activity, and both activities are regulated in an allosteric way [54]. GTP binding by TG2 inhibits calcium binding and crosslinking activity, whereas calcium binding inhibits GTP binding [53, 55]. It is worth noting that hydrolysis of GTP by TG2 is strictly an intracellular function, while the crosslinking reaction can take place both in intracellular and extracellular compartments [56].

Physiological functions

TG2 is the most widely distributed form of TGs. Due to its multifunctional enzymatic activity and to its localization both in cell compartments (cytosol, plasma membrane and nucleus) [57, 58] and in the extracellular matrix (ECM) [59], a number of physiological functions have been proposed for TG2, but so far no definite evidence is at hand. Cell proliferation, cell migration, receptor-mediated endocytosis, ECM reorganization, wound healing and apoptosis [60, 61] are some areas where TG2 has been proposed as playing a role. Interestingly, TG2 knockout mice show no obvious developmental or histological abnormalities of major organs, including the intestines [62]. However, pseudosubstrates of the amine type, such as MDTC or 5-dibenzylaminopentylamine [49], functioning as inhibitors of transamidation, are comparatively toxic. It is possible that other TGs substitute for TG2 in the knockout mice.

There is widespread evidence for up-regulation of the TG2 gene during cell death and it has been suggested that TG2 is important in the stabilization of the apoptotic cell by intracellular crosslinking [60]. Interestingly, more recent work has suggested that increased expression of TG2 prolongs cell survival by preventing apoptosis via a GTP binding mechanism in dying cells [61]. TG2 expression is also involved in the production of active transforming growth factor β-1 (TGF-β1) in various biological contexts [63-65]. Indeed, another role of TG2 in apoptosis may be where macrophages perform the recognition, binding and internalization of apoptotic cells without causing an inflammatory response, thanks to the release of TGF-β1 [66, 67]. Both up-regulation of the enzyme in cells, its ability to induce calcium-mediated intracellular crosslinking and its ability to immobilize on fibronectin to support cell adhesion in an integrin-independent manner, are likely to be related to its proposed role in wound healing and may also be significant in preventing leakage and lysis of dying cells, thus maintaining their structural integrity [68]. In cultured cells, TG2 may exert both pro- and anti-apoptotic effects depending upon the type of cell, the kind of death stimuli, the intracellular localization of the enzyme and the type of activity (cross-linking or GTP) which is switched on[61].

Pathological aspects

TG2 has also been associated with a series of pathological conditions. As early as 1966, Laki et al [69], using the plasma cell tumour YPC-1 in a mouse model, reported a correlation between

the extent of metastasis and TG-activity of the affected organ. Although the approaches nowadays are more sophisticated, the involvement of TG2 in cancer metastasis is still an exciting and promising field of research [70].

TG2 may contribute to the remodelling of cellular architecture in the development of lens fibre cells, and there is evidence that the enzyme may also play a role in cataract formation [71, 72]. The hallmark of transglutaminase activity, ε-(γ-glutamyl)lysine crosslinks, has been identified in polymers isolated from human cataract.

Moreover, examination of kidney biopsies from diabetic patients demonstrated an increase in both the TG2 enzyme and its crosslink product [73, 74]. Cellular export of TG2 may therefore be a factor in the perpetuation of diabetic nephropathy by crosslinking and stabilisation of the ECM, while intracellular activation may lead to cell death contributing to tubular atrophy [75]. Recently, TGs have been implicated in nephrogenic systemic fibrosis, which occurs after gadolinium contrast exposure in patients with renal failure [76].

.

Finally, TG2, the major transglutaminase in the mammalian nervous system, is localized predominantly in neurons [77, 78]. Recent data indicates that one or more TGs are associated with neuro-degenerative disorders, such as Alzheimer's (AD) [79], Parkinson's [80] and Huntington's diseases [81], and multiple sclerosis (MS) [82].

To my knowledge, no reports prior to 1997 include the association of TG2 with autoimmune diseases. The report by Dieterich et al [38] that TG2 is the antigen of the biomarker EmA in CD therefore constitutes a milestone in TG science.

.

Rheumatoid arthritis

According to the Arthritis Foundation, RA is the second most common type of arthritis. This autoimmune disease, of which the exact aetiology is not known, leads to destruction of cartilage and bone and eventually to disability in the patient. According to the criteria of the American College of Rheumatology (ACR), the diagnosis of RA is based on a pattern of symptoms such as the distribution of the inflamed joints, the blood and X-ray findings.

The prevalence of RA is relatively constant in many populations worldwide at 0.5-1.0 % [83]

and has a female-to-male ratio of 3:1 [84]. The gender difference diminishes in older age groups, supporting the idea of oestrogen involvement in the pathogenesis [85].

Various blood autoantibodies are often present in RA. Rheumatoid factor (RF), an IgM antibody directed against the Fc fragment of IgG, is present in the sera of more than 75% of patients [86]. Unfortunately, this biomarker is not very specific for RA. In contrast, the autoantibody against protein-bound citrulline, an amino-acid generated post-translationally by the action of peptidylarginine deiminase (PAD) on peptide bound arginine, displays high specificity [87]. Anti-citrulline protein (anti-CP) is present in 60-80% of patients with RA and can be detected long before clinical symptoms of RA have appeared [88, 89]. Another antibody, called the antinuclear antibody (ANA), is also frequently found in patients with RA [90].

Besides environmental influences, such as infectious agents, smoking and oral contraceptives, genetic factors are believed to be responsible for approximately 60% of the risk of developing RA [91].The main genetic risk factor of RA is the HLA DRB1 alleles, and the strongest susceptibility factor so far has been the HLA DRB1*0404 allele. Moreover, the gene coding for one of the PAD isoenzymes may be significant. Although disputed by European investigators [92], a Japanese [93] and a Korean [94] study have shown that the functional haplotype of PAD4, is associated with RA in Asian populations [95]. Moreover, a meta-analysis including six replication studies, one from Japan and five from Europe and North America, showed a positive association between PAD4 and RA not only in the Japanese population but also in populations of European descent [96].

Sjögren's syndrome

Primary Sjögren's Syndrome (pSS) is the second most common autoimmune rheumatic disease. Defined strictly as an autoimmune disease according to the American European Consensus Criteria (AECC) [97], the prevalence of pSS is around 0.2 to 0.4 % [98, 99]. It is characterized by inflammation of the exocrine glands, leading to impaired function. Although exocrine involvement is a defining feature, SS is considered a systemic disorder.

Primary SS, has a female-to-male ratio of 9:1 [100], together with primary biliary cirrhosis the greatest of any autoimmune disorder [101].

Affected individuals have an increased tendency to develop additional autoimmune diseases, such as hypothyroidism. The pathogenesis is completely unknown and consequently the treatment is symptomatic.

Peptidylarginine deiminases

The enzyme activity was first detected in guinea-pig hair follicles [102]. All of these enzymes convert arginine residues to citrulline in proteins (Figure 2).

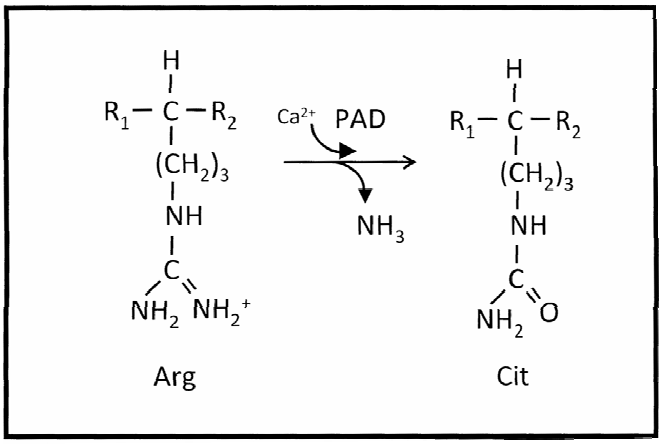


Figure 2: PAD-catalyzed reaction

Several types of PADs (EC.3.5.3.15) have been found in mammalian tissue, including the epidermal type (PAD 1), muscle type (PAD2), hair follicle type (PAD3), another isoenzyme expressed in several types of blood cells (PAD4; previously called PAD5)[103] and finally, germ cells type (PAD6)[104] (Table 2). All these thiol enzymes rely strongly on the presence of calcium for activity.

PAD	Chromosome location	Proposed functions	Size aa	Location
PAD1	1q36.13	Cornification of epidermis		Epidermis, Uterus
PAD2	1q36.13	Apoptosis	631	Brain, Spinal cord, Uterus, Sweat glands, Salivary glands, Skeletal muscles, Macrophages, Bone marrow, Pancreas
PAD3	1q36.13	Growth of the hair fiber, terminal differentiation		Hair follicles
PAD4/5	1q36.13	Apoptosis	631	Blood cells
PAD6	1q36.13	Female fertility		Germ cells

Table 2: Characteristics of the various members of the PAD family

The PADs catalyze the deimination of the guanidino groups of peptide-bound arginine residues to citrulline. As it is not incorporated into proteins during translation, citrulline is a non-standard amino-acid. In general, citrullination decreases the net positive charge of the protein, causing loss of potential ionic bonds. These changes affect intramolecular bonds, leading to partial unfolding of the protein, but intermolecular interactions with other molecules can also be altered.

Physiological functions

PAD1 is mainly expressed in epidermis and uterus. During terminal differentiation of keratinocytes, filaggrin and keratins are citrinullated, which may reduce the flexibility of the keratin cytoskeleton and stimulate the cornification of epidermis [103].

PAD2 is the most widely expressed type of PADs and is observed in skeletal muscle, brain, spleen, macrophages and secretory glands. Thus far, two natural substrates for PAD2 are known, myelin basic protein (MBP) and vimentin [105].

PAD3 shows co-expression and co-localization with its natural substrate trichohyalin, which is a major structural protein of inner root sheath cells of hair follicles. Citrinullation of trichohyalin opens its α-helix structure and renders it available for efficient crosslinking to keratin by TG3 [106].

PAD4 is expressed mainly in granulocytes and monocytes, and can therefore be detected in a variety of tissues. This is also the only PAD that resides in the cell nucleus. Citrinullation is often observed during terminal differentiation, a process closely related to apoptosis [93, 103].

PAD6 is an enzyme that is uniquely expressed in male and female germ cells. Inactivation of the PAD6 gene in mice leads to female infertility whereas male fertility is not affected [104].

Pathological aspects

PAD enzymes and their products, citrinullated proteins, might play a role in several human diseases such as psoriasis, RA, AD, and MS.

The keratinocytes in the psoriatic plaques do not contain citrinullated keratin, which is essential for the normal cornification process of the epidermis. Whether the absence of citrinullation is associated with a defective PAD1 is unknown.

Evidence suggests that PAD4 plays an essential role in the pathogenesis of RA [107]. The most specific family of RA autoantibodies is directed against citrinullated proteins. These autoantibodies are produced locally in the inflamed synovium [105]. Normally, PAD enzymes are present intracellularly, either in the cytosol or in the nucleoplasm. However, when cells are dying, as a result of extensive oxidative stress in the inflamed synovium, PAD enzymes might leak out of the cell, become activated and induce citrinullation of extracellular proteins, such as fibrin [108].

PAD2 and PAD4 are the only PAD isotypes which can be detected in the synovium of patients with RA. Both isotypes are probably involved in the citrullination of fibrin [109].

4. Aims

The overall aim of this thesis was an elucidation of the involvement of TG and PAD in the pathogenesis of autoimmune diseases.

The specific aims were:

- Understanding the reasons for the divergent reports on the effect of calcium on the affinity between CD-antibodies and TG2 in radioligand binding assay (RBA) and enzyme-linked immunosorbant assay (ELISA).
- Determination of the impact of zinc ions on the affinity between CD-antibodies and TG2
- An answer to the issue of CD-antibodies affecting the enzyme activity of TG2
- An elucidation of the occurrence and correlation of antibodies against citrulline and PAD, TG2, and Factor XIII in sera from RA-patients
- An evaluation of the occurrence of antibodies against PAD, TG2, and citrulline in patients with pSS, MS, and AD.

5. Subjects

Study I

Serum samples were collected from seven adult patients with untreated CD, verified by biopsy. Three patients with gastrointestinal symptoms but with normal small bowel biopsy and negative EmA titres served as controls. The samples were collected during 1999–2001.

Study II

Serum samples were obtained from 86 children who were investigated consecutively with intestinal biopsy at the Department of Paediatrics, Malmö University Hospital, during the years 2000–2003. A total of 51 children were considered to have active CD according to the revised ESPGHAN criteria [110]. The remaining 35 children were included as disease controls.

Study III

Rheumatoid arthritis

All patients attending the Rheumatological Unit of Malmö University Hospital during 1995-2002, diagnosed with RA according to the criteria by the ACR 1987, were consecutively registered and systematically monitored. A total of 184 patients were enrolled in the study. At the time of sampling, 71 patients had initiated treatment with methotrexate (MTX).

Blood donors

Blood samples were collected in March 1998 from 59 blood donors and served as controls.

Study IV

Primary Sjögren's syndrome

Seventy-eight patients with pSS consecutively attending the outpatient clinic at the Department of Rheumatology, Malmö University Hospital, Malmö, Sweden for routine visits were included. All patients fulfilled the AECC for pSS.

Multiple sclerosis

Blood samples from 85 patients fulfilling the "McDonald Criteria" [111] for MS were collected consecutively during 1999-2001 at the Department of Neurology of Lund University Hospital.

Alzheimer's disease

Seventy-nine patients with dementia were investigated. The patients attended the Neuropsychiatric Clinic, Malmö University Hospital, Malmö, Sweden and were evaluated with a detailed clinical investigation of cognitive function during 1999-2003. Only patients with AD showing mild or moderate disease and with a complete investigation were selected to participate in the study.

Blood donors

Blood samples were collected from 100 blood donors in April 2007 when the donors were attending for regular blood donation at Malmö University Hospital.

6. Methods

Enzyme-linked immunosorbant assay (ELISA)

For all ELISAs, cut-off was calculated as mean + 2 S.D. of controls (blood donors) except in the case of commercial kits.

ELISA of IgG and IgA antibodies against transglutaminases (Study I, II, III)

For the analysis of anti-TG2, a guinea pig liver TG2 (gp-TG2, Sigma; 1 µg/well) (study I, II, III) and a human recombinant TG (hr-TG2, N-Zyme; 0.5 µg/well) (study III) were used. Freshly prepared solutions of the antigens in Tris buffer saline (TBS) containing 5.0 mM $CaCl_2$ were added to microtitre plates (CovaLink, Nunc). After incubation the plates were washed and blocked. Diluted sera from patients with CD or RA and healthy blood donors were added to each well in duplicate followed by incubation, and then washed. Peroxidase-conjugated antihuman IgG (DAKO) or IgA (DAKO) were added to each well. After incubation and washing, the plates were developed according to standard procedure. Absorbance was estimated at 490 nm in a microplate-reader (Emax, Molecular Devices).

ELISA of IgG and IgA antibodies against hr-TG2, commercial kit (Study II, IV)

Study II: This analysis was performed in our laboratory with the Eu-tTGR IgA umana (Eurospital) kit in accordance with the manufacturer's manual. As antigen, this method uses hr-TG2. The manufacturer is not willing to disclose any information about calcium addition. The cut-off level was defined according to the manufacturer's manual as 7 AU/ml.

Study IV: This analysis was performed in our laboratory with a commercial kit, Celikey® Varelisa (Phadia), according to the manufacturer's manual. As antigen, this method utilizes an hr-TG. The cut-off for IgA- and IgG anti-rhTG2 was defined according to the manufacturer's manual as > 8, and > 10 AU/ml respectively.

ELISA of IgG antibodies against peptidylarginine deiminase (Study III, IV)

For the analysis of PAD-antibodies, a rabbit skeletal muscle PAD (Sigma) was used as antigen. Microtitre plates (Maxisorp Nunc) were coated with 1 μg PAD/ well (Study III) or with 0.2 μg PAD/well (Study IV), freshly diluted in 100 μl of 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS) and 5.0 mM $CaCl_2$. After incubation, the plates were washed and blocked. Sera from blood donors (Study III, IV) and from patients with RA (Study III) pSS, MS and AD (Study IV) were diluted and added in duplicate followed by incubation. After the washing procedure, peroxidase-conjugated antihuman IgG (DAKO) was added to each well. The plates were developed according to standard procedure. Absorbance was estimated at 490 nm in a microplate-reader (E max).

ELISA of IgG antibodies against peptide-bound citrulline (Study III, IV)

The analysis of anti-cyclic citrulline-containing peptide (CCP) was performed in our laboratory with the Immunoscan RA Mark2 kit (Euro-Diagnostica) in accordance with the manufacturer's manual. As antigen, this method utilizes a synthetic CCP.

Endomysial autoantibody immunofluorescence (Study I, II, III, IV)

All EmA were analyzed at the Department of Clinical Microbiology and Immunology, Lund University Hospital, Lund, Sweden, by indirect immunofluorescence [35, 36]. EmA was detected with fluorescein isothiocyanate conjugated goat antihuman IgA antibodies. Results were expressed as the highest dilution factor giving a positive fluorescence pattern in microscope. All sera manifesting fluorescence titre 1:10 or higher were considered to be positive.

Electrophoresis

Agarose gel electrophoresis was performed according to Laurell [112] at pH 8.6 and in the presence of EDTA.

Crossed immuno-electrophoresis

Crossed immuno-electrophoresis for the characterization of TG2 antibodies was carried out at pH 8.6 (barbital buffer 50mM containing 2.0mM EDTA) as outlined by Ganrot [113]. Serum (10%) from a 55-year-old female with CD and an IgA anti-TG2 level of 2740 arbitrary units was included in the gel during the second step.

Activity staining

Staining for TG activity was performed according to Stenberg and Stenflo [114]. The technique is based on the transglutaminase-catalysed incorporation of a fluorescent amine, MDTC, (Larodan Fine Chemicals, Malmo, Sweden) into casein.

Scion Image for Windows

Scion Image for Windows is a freeware program for processing and analysing images.
In this study, we used the program to count the dark pixels appearing in a designated area of a picture taken in UV-light of the agarose gel processed with crossed immuno-electrophoresis and activity staining.
The picture of the gel was scanned, transferred to the PC and inverted to enable Scion Image to measure the area of each selected part of the graph. Numerical results in area units (pixels) were given.

RBA based on hr-TG2 (Study II)

Human TG2 was synthesized by in vitro transcription and translation as described elsewhere [115]. TG2 cDNA (kindly provided by George Eisenbarth) subcloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) was used and the protein was labelled using the TNT SP6 coupled reticulocyte lysate system (Promega, Madison, WI, USA) in the presence of ^{35}S-methionine (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The in vitro transcription and translation were carried out in an EGTA-containing buffer. Before TG2 antigen was added to the micro-titre wells, it was freshly diluted in TBS containing $CaCl_2$ or EDTA. When analyzing IgG antibodies, ^{35}S-TG2 was added together with human serum into a U-bottom 96-well micro-titre plate and incubated. Protein A Sepharose (Sigma, St.Louis, MO, USA) was used to separate the free ^{35}S-TG2 from the antibody bound form. IgA antibodies were

analyzed similarly. Finally, the plate was washed and the radioactivity measured in a MicroBeta Counter (Wallac, Turku, Finland). Autoantibody levels were expressed as relative units (RU) in reference to positive and negative sera with a cut-off level representing the 99.9^{th} percentile of healthy control subjects

Statistics

Study I

Two-tailed, paired Student's t-tests were used for the comparisons between the ELISA results in the presence and absence of calcium and zinc.

Study II

Statistical differences between antibody frequencies were evaluated with the Chi-squared test or Fisher's exact test when appropriate. Antibody levels were expressed as median (range) and differences between the assays were tested with the Wilcoxon Signed Rank tested for significance. To measure change in antibody levels between CD children and disease controls, the Mann–Whitney U-test was used for comparison. Linear regression examined whether increase in TG2 antibody levels was related to severity of mucosa damage, and antibody levels were expressed as mean FSE. P-values < 0.05 were considered significant.

Study III

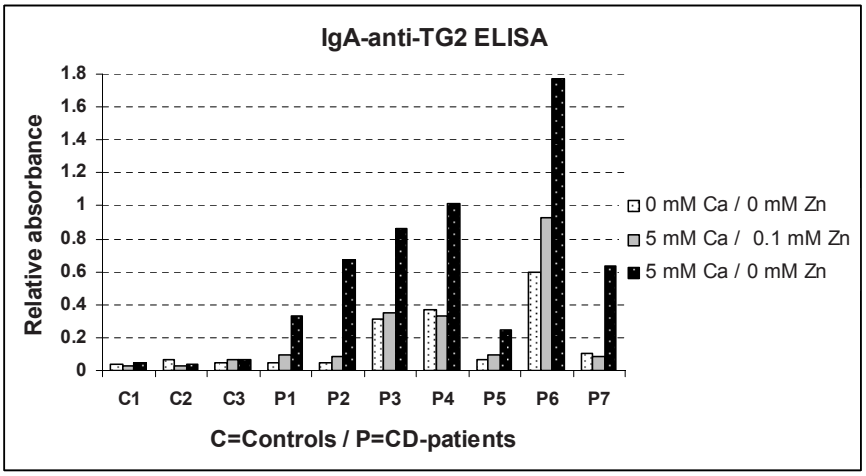
The statistical significance of differences was determined by the χ^2-test and 2-tailed Student's t-test. Due to skewed distribution of the values, logarithmic transformation was carried out before analysis. Spearman rank correlation was used to evaluate correlation. P-values < 0.05 were considered significant.

7. Results

Study I

The effect of calcium and zinc

Antibodies from CD-patients displayed a much stronger (4-14) affinity when exposed to the activated conformation of gp-TG2 than of the EDTA-treated enzyme. Furthermore, zinc was shown to erase the calcium-induced increase of affinity between the CD-antibodies and TG2. Low concentration of zinc decreased the antibody binding to the same level as for the inactive enzyme (Figure 3).



.

Figure 3: *The effect of calcium and zinc on the affinity between TG2 and antibodies from healthy controls (C1-C3) and coeliacs (P1-P7). (Relative absorbance in an ELISA)*

Enzyme stability

For this purpose we used agarose gel electrophoresis [112] combined with the activity staining procedure developed by Stenberg and Stenflo for assessment of TG transamidating activity [114]. The commercial gp-TG2 was shown to be very unstable. When dissolved in Tris pH 7.4, most of the enzyme activity faded after 24 hours at -20°C. Human serum stabilised the enzyme, probably thanks to the presence of protease inhibitors of serum. We also showed that when calcium was added to the buffer, the degradation of TG2-activity proceeded much faster than in the EDTA containing buffer.

The effect of CD-antibodies on TG2 activity

To elucidate whether antibodies from a CD patient were able to extinguish the TG transamidating activity we performed a cross-immune electrophoresis. Serum from a patient with a high level of IgA TG2 antibodies was incorporated in to the agarose gel and in the second step of the electrophoresis, bands containing TG-activity were transferred to the serum containing gel. An immune precipitate developed and when staining for enzyme activity, the enzyme showed full activity as verified by the new technique based on Scion Imaging (Figure 4).

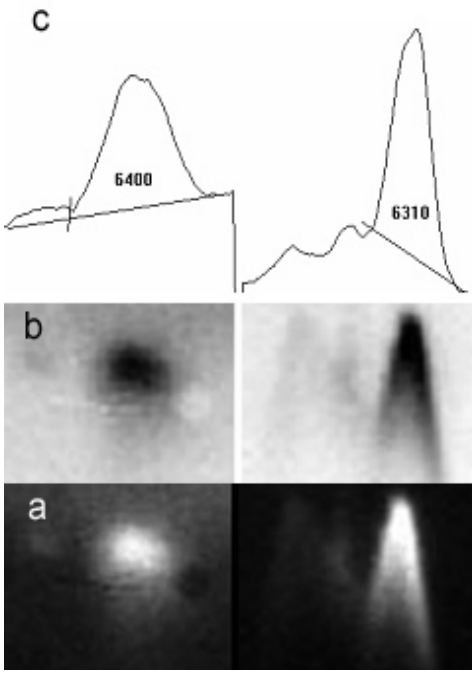


Figure 4: *a; A crossed immuno-electrophoretogram stained for TG activity. Left; normal serum; right serum from a patient with CD. b; Inverted. c; Scion Image.*

Study II

With our new knowledge based on the ELISA with gp-TG2 in adults, we wanted to further investigate in childhood CD the influence of calcium on the affinity between CD-antibodies

and hr-TG2 using ELISA and an RBA. Overall, detection of IgA-TG2 in sera from children with CD did not differ between the RBA and the ELISA based on gp-TG2 or with the commercial Eu-tTGR IgA umana ELISA test (based on hr-TG2). A high positive correlation was found between gp-Ca-ELISA and the Eu-tTGR IgA umana ELISA and also for Ca-RBA and Eu-tTGR IgA umana ELISA.

Calcium had no influence on the IgA-TG2 RBA. Fifty of the 51 children with CD and one of the 35 controls were positive. In the EDTA-based ELISA, 47 of the 51 children were positive as well as one of the 35 controls. Calcium-addition to the ELISA increased the sensitivity to 50/51 but then another three of the disease controls were also positive. In the case of Eu-tTGR IgA umana ELISA test, the manufacturer is not willing to disclose any information about possible calcium addition.

In the IgG-TG2 RBA, addition of calcium significantly reduced the affinity between hr-TG2 and CD antibodies. In contrast, calcium addition to the IgG-TG2 ELISA increased the affinity extensively.

The affinity of IgA-TG2 increased with the grade of severity of intestinal mucosal damage in both assays. In contrast, affinity of IgG-TG2 showed no significant linear increase for either of the RBA or ELISA methods.

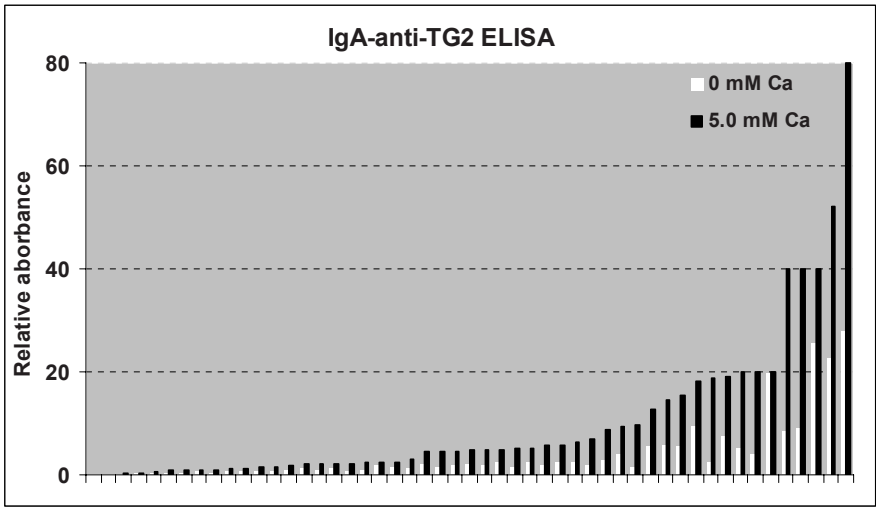


Figure 5: *The effect of calcium expressed as relative absorbance in an ELISA, on affinity between TG2 and antibodies from 51 children with CD.*

Hr-TG2 was prepared by in vitro transcription and translation in a rabbit reticulocyte lysate system (Promega). When analysed with agarose gel electrophoresis/activity staining, the lysate of the Promega TNT SP6-coupled reticulocyte system contained TG enzyme activity in the area corresponding to gp-liver and human erythrocyte TG2.

Study III

Sera from 184 patients with RA, 113 without MTX treatment, were analysed for antibodies against PAD, CCP, gp-TG2, hr-TG2 and FXIII.

Compared to controls, RA patients showed increased levels of IgG anti-PAD, and IgG anti-CCP, in line with other reports. Furthermore, there was a good correlation between the prevalence of antibodies against PAD and CCP, IgA and IgG anti-gp-TG2.

Moreover, RA patients showed significantly increased frequencies of IgA and IgG anti-TG2 and IgA anti-hr-FXIII compared to controls. In the MTX treated group, five types of autoantibodies were significantly reduced in serum, and the correlations between different autoantibodies were less pronounced.

Study IV

The prevalence of measured antibodies in the patient groups was very modest. Of the 242 patients, only 17 cases displayed autoantibodies and none had more than one type. Moreover, among the blood donors, five of the 100 carried autoantibodies. Of the 78 patients with pSS, two had antibodies against PAD and four against CCP.

8. Discussion

The effect of calcium

After the discovery by Dieterich et al [38] several investigators, many with a clinical focus, became acquainted with TG2. This enzyme requires calcium for enzyme activity. In order to understand the pathogenesis of CD, ELISAs were performed in the absence and presence of calcium during coating of the plates. Interestingly, the results were contradictory. While some groups found an increased affinity between CD-antibodies and calcium-treated TG2, others did not. Moreover, in RBA based on hr-TG2 expressed in a lysate of rabbit reticulocytes, addition of calcium rather reduced the affinity.

TG2 is a very unstable molecule. If isolated from gp-liver, contaminating proteases can easily degrade the protein [116]. When activated by calcium, the reactive thiol group of the active site cysteine might oxidize. For scientists not familiar with these potential pitfalls, it is easy to overlook the practical aspects. In Paper I, this is illustrated by a rapid loss of TG-activity in solutions stored in a freezer, a common practice in this context. Obviously, if the enzyme structure is not native, addition of calcium would hardly affect the conformation in a natural way. Consequently, no change of affinity between enzyme and antibodies would be found. But when good laboratory practice is applied, our studies clearly show that presence of calcium during the coating of the ELISA plates dramatically increases the affinity between TG2 and CD antibodies (See figure 5).

The interesting effect of calcium on the RBA had a more intriguing explanation when we were able to show presence of native TG2 originating from the lysate of rabbit reticulocytes used in the commercial kit. This kit contains an efficient calcium chelating agent (EGTA) and is stored frozen until used. The transcription/translation is performed without the presence of calcium. In spite of the fragility of the enzyme, this environment seems to allow part of the TG2 originating from the rabbit cells to remain native, respond to calcium activation, and compete successfully with the radio-labelled human recombinant TG2. This kit is also used to express other human recombinant proteins. Clearly, there is a risk that other native components of the rabbit reticulocytes might contaminate the intended products.

The effect of zinc

Zinc is an important component of approximately 300 enzyme systems, many of which are involved in the immune defence [8]. In 1974-75, studies at Northwestern University showed that zinc very efficiently inhibited the calcium-induced activation of TGs, such as factor XIII and TG2 [117]. The human body contains 2-3 grams of zinc, unevenly distributed, and with remarkably high concentrations in the prostate gland [118], the pancreatic beta cells [119], and in parts of the eye [120]. Like all trace metals, zinc is bound to various proteins, functioning as carriers. The bindings are reversible and, logically, the metal will bind to the structure that offers the highest affinity for the time being. For example, zinc is rapidly redistributed in the intestines of animals exposed to an infectious agent [121]. The homeostasis of zinc is maintained mainly in the gastrointestinal tract [122]. Hyperzincaemia is a rare condition while zinc deficiency is a severe threat against public health in developing countries [10, 123].

At physiological concentrations zinc inhibits the calcium-induced increase of affinity between CD-antibodies and TG2 (See figure 3). The clinical significance of this zinc effect is not known, but a pilot study on the effect of zinc-fortified flour to cases with newly diagnosed CD is under way.

The structure of the neoantigen

In CD, antibodies are displayed against epitopes of both TG2 and gliadin peptides. Recently, it has been shown that a deamidation of glutamine residues of gliadin peptides increases the affinity to CD-antibodies [124]. However, ingestion of totally deamidated gluten peptides does not induce CD [125], nor does intake of free glutamine, neither of which are substrates to TGs. The dual immune response against TG2 and peptide-bound glutamine indicates that the neoantigen is a complex between enzyme and substrate. The structure of this complex is still controversial. If inappropriately activated, TG2 can catalyze the cross-linking of many peptides, including the incorporation of glutamine-containing peptides such as gliadin, into the enzyme itself.

Initially, a complex between TG2 and gliadin, formed by an ε-(γ-glutamine)lysine bridge, was proposed to constitute the autoantigen [126]. Based on our knowledge of the kinetics of TG-catalyzed reactions, we have suggested another mechanism [116]. In transamidation reactions catalyzed by TGs, the first step, that is the formation of the thioester intermediate, is normally

rate-limiting, and the intermediate will be a short-lived victim for a nucleophilic attack by the second substrate, the base form of a primary amine. However, in the absence of the amine substrate, or at a lowered pH – such as the situation at a site of inflammation – when the amine will be protonized, water will function as the second substrate, and the result will be a deamidation. In CD, this hydrolysis of specific, but not all, glutamine residues proceeds in an ordered way, so that the preferred glutamine residue is deamidated first, then the second most favourable, and so on.

Logically, the deamidation of the final glutamine residue will be the slowest. The negative charges of the glutamate residues formed during the initial deamidations of the gliadin might repel the water molecule from performing the final, nucleophilic attack on the thioester. Assuming that the second step of this reaction is still rate-limiting, the concentration of the thioester between TG2 and the previously deamidated gliadin will peak during the final hydrolysis. Therefore, as an attractive alternative we have proposed that the autoantigen in CD is comprised of the thioester intermediate between TG2 and partly deamidated gliadin. Indeed, our ideas are supported by in vitro data by Fleckenstein et al [127], who found comparatively high concentrations of the thioester at neutral pH in a TG2 catalyzed hydrolysis of low concentrations of two glutamine containing peptides. A reduced pH would probably further have favoured the formation of the thioester (Figure 6).

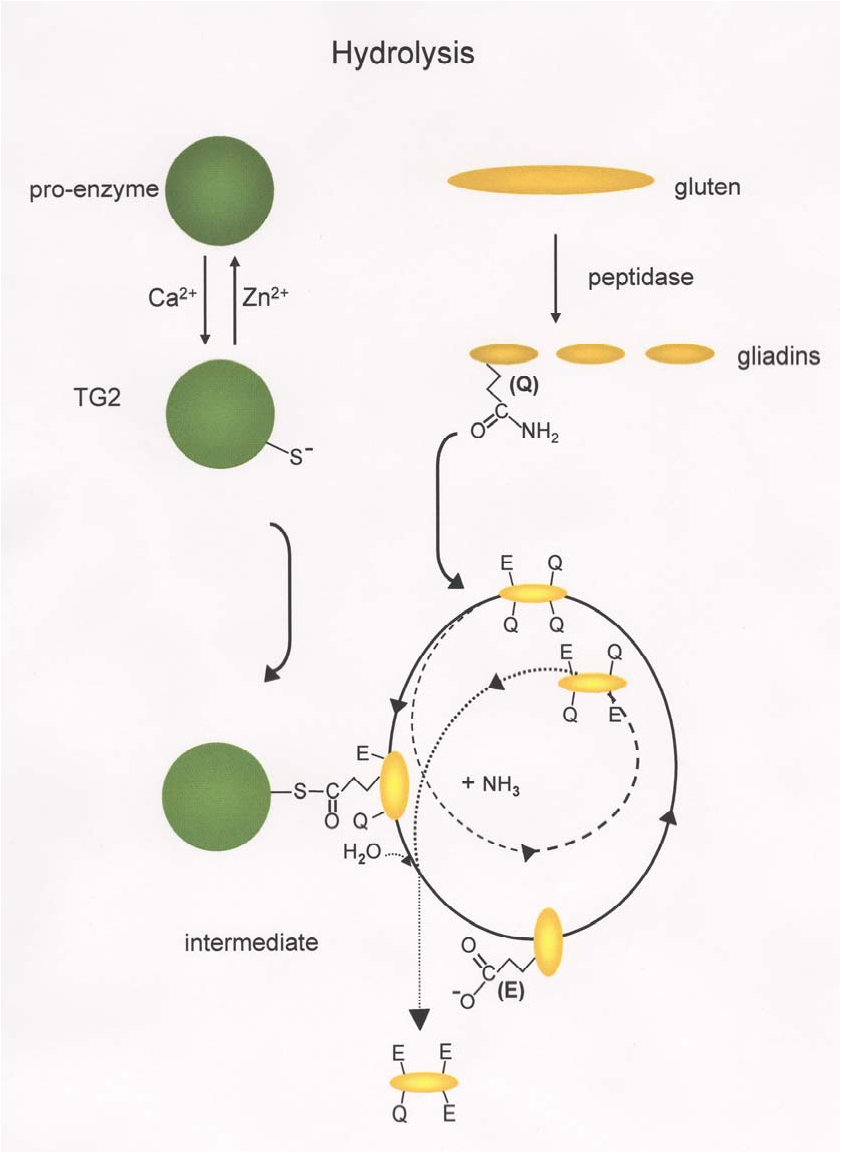


Figure 6: TG2-catalyzed deamidation of specific glutamine residues (Q) of gliadin in CD. *The deamidation proceeds in an ordered way. The activated TG2 forms a thioester complex with the glutamine residue. In the absence of the ordinary second substrate (the primary amine), water functions as the nucleophile in the second step. Initially, the preferred glutamine residue is deamidated, then the second most favourable, and so on. Logically, the deamidation of the final glutamine residue will be the slowest. The negative charges of the glutamate residues formed during the initial deamidations might repel the water molecule from performing the final, nucleophilic attack on the thioester. Assuming that the second step of this reaction is still rate-limiting, the concentration of the thioester between TG2 and the previously deamidated gliadin will peak during the final hydrolysis.*

The effect of antibodies on the activity of TG2 in CD

In order to elucidate the pathogenesis, the effect of CD-antibodies on the TG2-activity is another important issue. Not surprisingly, depending on the conditions and considering the fragile character of TG2, various groups have come to different conclusions [128, 129]. The results of the pilot study as described in Paper I and illustrated in Figure 4 did not indicate any inhibitory effect of the CD-antibodies on TG2-activity. However, we used gp-liver TG and calcium-free conditions during the crossed immune-electrophoresis. On the other hand, we clearly observed TG-activity in the immune-complex between TG2 and CD-antibodies.

Anti-PAD in RA

The occurrence of antibodies against citrulline in RA offered an opportunity to test our hypothesis of an enzyme-substrate complex being the autoantigen in autoimmune diseases. The family of enzymes orchestrating the post-translational deimination (citrullination), the peptidylarginine deiminases, has many similarities to the TGs (Table 3). However, there is no obvious reason to believe that PADs, similarly to TGs, would be able to incorporate a substrate into the enzyme. Our findings, supported by similar results by another two groups, clearly show that the prevalence of anti-PAD is significantly increased in RA-patients. Thus, as in another common autoimmune disease, antibodies are observed against a calcium-dependent thiol enzyme and its modified substrate.

Feature	TG2	PAD4
Thiol-enzyme	+	+
Calcium-dependent	+	+
Active post-translationally	+	+
Release of ammonia	+	+
Zinc inhibits enzyme activity	+	+
Change of substrate charge	+	+
Antibodies (RA;CD) against the modified substrate	+	+
Antibodies (RA;CD) against the enzyme	+	+
Several isoforms	+	+
Widely distributed	+	+

Table 3: Common features for TG2 and PAD 4

Furthermore, the levels of antibodies against TGs were also increased. In synovium, fibrin is one of the suggested substrates for PAD. In the inflamed joint, another two enzymes using fibrin/fibrinogen as substrates, FXIII and TG2, are present, probably originating from monocytes and macrophages respectively [130].

Since a part of our RA-cohort was treated with MTX, we had an opportunity to test if this immunosuppressive antagonist of folic acid affected the formation of antibodies in RA. Not surprisingly, the patients treated with MTX displayed lower levels of anti-PAD than the untreated group. In the study by Nissinen at al [131], 88 % of newly diagnosed non-treated RA-patients displayed elevated levels of anti-PAD. Three years later the frequency had declined to 70 %. Studies of other patients with a long (20 year) duration of RA revealed only anti-PAD among 20 %. Unfortunately, medication is not included in the report, but certainly, treatment by an immuno-suppressive such as MTX can be an explanation. Furthermore, the MTX-treated RA patients carried lower levels of antibodies against TGs, but not anti-CD, than the untreated group. Some autoimmune diseases, for example inflammatory bowel disease, tend to decrease their inflammatory activity during the natural course of disease and this phenomenon might also contribute to the decreasing occurrence of autoantibodies over the years.

Epitope spreading

In the RA cohort not treated with immuno-suppression, significant correlations were observed between the expressions of the various antibodies. This is a common situation in autoimmune diseases and is sometimes referred to as epitope spreading, where immune responses develop to new epitopes, distinct from and non-cross reactive with the primary epitope causing the disease. For example, 116 different antibodies have been described in connection with SLE [132]. The mechanisms for epitope spreading remain to be explained. Similar to the situation in CD, complexes between modified substrates and inappropriately activated post-translational enzymes, such as PADs and TGs, might explain the appearance against antibodies directed towards epitopes in both the enzyme and the substrate part of the molecule.

Antibodies in Sjögren's syndrome

In SS, the second most common rheumatic disease, anti-CP is not expressed. Therefore the hypothesis of an enzyme-substrate complex being the autoantigen in autoimmune diseases is challenged by findings of an increased prevalence of anti-PAD [131]. In order to analyze this situation, we tested the prevalence of antibodies in a larger cohort of well-defined cases with primary SS. However, we were not able to reproduce the data showing an increased prevalence of anti-PAD in pSS. The reasons for this difference in results between our study and the one by Nissinen et al are not obvious. One explanation might be that the diagnosis of pSS in the Finnish group was based on old criteria while we used the updated version of AECC. After our studies on pSS, our conclusion is that the hypothesis of an enzyme-substrate complex being the autoantigen in autoimmune diseases is still valid.

Antibodies in neurodegenerative diseases

Both TGs and PADs have been implicated in neuro-degenerative diseases such as Huntington and Parkinson [80, 81] and also in MS and AD [79, 82]. We were able to study cohorts of AD and MS with respect to antibodies against PAD2 and TGs. However, we did not observe increased levels of antibodies against these enzymes in the two patient groups studied.

9. Conclusions

- Calcium increases the affinity between antibodies from CD-patients and gp-TG2 in an ELISA. The fragile character of commercial gp-TG2 may explain the divergent results from different reports. In RBA, calcium has no effect on IgA-anti-TG2, or the opposite effect on IgG-anti-TG2, or on the antibody affinity to hr-TG2. This contradiction can be explained by the presence of native rabbit-TG2 (originating from the kit used for translation and transcription of the human enzyme), which responds to calcium activation, and which competes successfully with the radio-labelled human recombinant TG2.
- Physiological concentration of zinc decreases the affinity between CD-antibodies and gp-TG2 in an ELISA.
- Antibodies from a CD patient did not seem to influence or decrease TG2 activity.
- Serum samples from RA-patients contained antibodies against PAD and two kinds of TGs, and showed good correlation between several different autoantibodies.
- Treatment with MTX significantly reduced the expression of five out of seven investigated autoantibodies (IgG-anti-PAD, IgA and IgG-anti-hr-TG2, IgG-anti-gp-TG2 and IgA-anti-hr-FXIII.)
- In patients with pSS, MS, and AD, no increased occurrence of antibodies against PAD, CCP or TG2 could be shown.

10. General discussion

Due to their chronic character, high prevalence and, regretfully, lack of cure, autoimmune diseases represent a major threat to public health. Most of these disorders are being investigated individually but, doubtless in order to find a possible main thread and understand the pathogenesis, it is also important to study them collectively.

Although the human genome only codes for 20 primary amino-acids, post-translational modifications can enrich the variety of nature. In fact, a majority of the human proteins are believed to be enzymatically modified in this way, for instance via glycosylation, phosphorylation, acylation or alkylation [133]. Transamidation catalyzed by TGs and citrullination by PADs are other examples. It is probable that in most cases the products display new conformations. Moreover, during specific conditions post-translational modifications can create new self-antigens and cause autoimmune responses. For example, apoptosis is accompanied by intense post-translational modifications of intra-cellular proteins. Some of these modified proteins have been shown to be antigenic in certain autoimmune diseases such as SLE [134]. TG2 is one of many calcium- dependent enzymes which are up-regulated during apoptosis and where an influx of calcium activates the enzyme intra-cellularly. Hypothetically, by catalyzing crosslinking of intra-cellular proteins, the activated TG2 stabilizes the dying cell, thus preventing inflammation and exposition of possible antigens. Regulation of TG2 activity intra-cellularly is probably a sophisticated interplay between the low concentration of calcium, the presence of GTP and the inhibitory effect of zinc. Indeed, increased apoptosis in vivo may be a direct consequence of a decrease in intracellular zinc concentration.

The situation becomes even more intriguing if the enzymatic reaction is atypical, such as the TG2-catalyzed deamidation of specific glutamine residues in gliadins. Then focus will be on the inappropriate activation of the enzyme, and again zinc might play a leading role due to its ability to antagonize the calcium activation of TG2. In type-1 diabetes, another calcium-dependent thiol enzyme, glutamic acid decarboxylase (GAD), is auto-immunogenic. Unfortunately, it is not known if zinc affects the activity of GAD. Recently, Wenzlau et al [119] have shown that a pancreas-specific zinc efflux transporter (ZnT8) is a major autoantigen in type-1 diabetes. Interestingly, 12 of 39 non-diabetics, TG2-autoantibody positive individuals with CD related to type-1 diabetic patients, showed antibodies against ZnT8 [119]. Indeed, zinc

appears to be a key factor in the pathogenesis of CD. Due to malabsorption caused by atrophy of the villi in untreated CD, deficiency of vitamins and trace metals such as zinc, is common. Thus, after triggering the activation of TG2 due to a stress-mediated rearrangement of intestinal zinc, a reduced absorption of the metal might induce a vicious circle in CD. Figure 7 illustrate the theoretical processes involved.

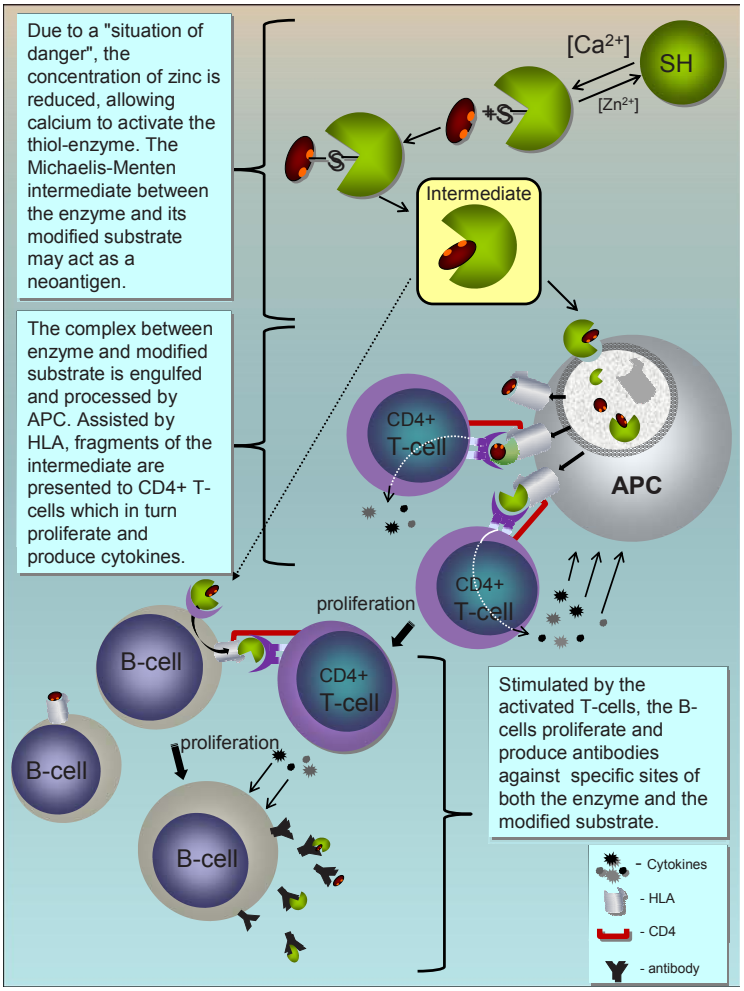


Figure 7: Theoretical mechanism for the triggering of autoimmune diseases

11. Summary

Autoimmune diseases such as rheumatoid arthritis (RA), coeliac disease (CD) and Sjögren's syndrome (SS), are conditions where an inappropriate immune response results in damage to an individual's organs, tissues or cells. As a group they comprise approximately 80 different diseases, afflicting more than one hundred million people globally. The incidence is rising and women are more frequently affected than men. The debilitating character of these chronic conditions further emphasizes the magnitude of the health problem. Moreover, curative medication is missing and treatment is mainly symptomatic.

The aetiology of autoimmune diseases is multifactorial and probably includes genetic, environmental and lifestyle factors. However, for most of these disorders the pathogenesis is completely unknown. In 1997, German investigators reported that a ubiquitous, calcium-dependent enzyme, transglutaminase type 2 (TG2), might be a major factor during the development of CD, a T-cell driven autoimmune condition with a prevalence of one per cent. In CD, serum antibodies are present against both TG2 and gluten. The aim of these studies was to characterize the involvement of TG2 in CD. Furthermore, we have investigated the possible involvement of TG2 and peptidylarginine deiminase (PAD), an enzyme with many similarities with TG2, in RA, SS, multiple sclerosis (MS) and Alzheimer's disease (AD).

Based on good laboratory practice, our results show that calcium increases the affinity between the fragile TG2 and CD-antibodies from both infants and adults, indicating that the enzyme is activated when becoming antigenic. Physiological concentrations of zinc, a potent inhibitor of the calcium-activation of transglutaminases, erase the calcium-induced increase of affinity. This zinc effect might be of clinical importance. CD-antibodies do not inhibit TG2-activity. With radio-bound immuno-assays, based on recombinant radio-labelled human proteins such as TG2, produced in a lysate of rabbit reticulocytes, native proteins might contaminate the product and jeopardize the results.

Similarly, in RA we observed serum antibodies against another calcium-dependent thiol enzyme, PAD, as well as against the new biomarker of RA, citrulline, which is the product formed after PAD-catalyzed deimination of peptide-bound arginine residues. Thus, in two major autoimmune diseases, CD and RA, serum antibodies were observed against both calcium-dependent thiol-enzymes and against their modified substrates. RA-serum also

comprised IgA- and IgG-antibodies against TG2 and factor XIII. Samples from RA-patients being treated with methotrexate displayed reduced levels of antibodies. Most of the patients displayed more than one type of antibody, a possible indication of epitope spreading.

Although patients with SS in general do not carry antibodies against citrulline, a report in the literature describes a high prevalence of antibodies against the citrullinating enzyme, PAD. Such a situation argues against our hypothesis of an enzyme-substrate complex being the neoantigen in autoimmune diseases. However, applying modern criteria when diagnosing primary SS in 78 cases, our study did not reveal an increased prevalence of anti-PAD. We also tested serum from patients with MS and AD. Although attractive from a theoretical point of view, PAD and TG2 do not seem to be involved directly in autoimmune mechanisms in those neuro-degenerative diseases.

To summarise, our results indicate that a complex between an enzyme and its modified substrate constitute the neoantigen in major autoimmune diseases. Events such as stress, infections and inflammations leading to an inappropriate activation of the enzyme involved might then be the trigger of the pathogenesis.

12. Sammanfattning på svenska

Bakgrund – Vid autoimmun sjukdom skadar individens immunsystem kroppsegna organ, vävnader eller celler. Autoimmuna sjukdomar omfattar ett 80-tal olika åkommor och i stort sett alla kroppsdelar kan drabbas. Förekomsten är 5-8 % vilket betyder att mer än 100 miljoner människor är drabbade globalt, det vill säga samma omfattning som för cancer eller hjärt-kärlsjukdom. Autoimmuna tillstånd är ofta kroniska, drabbar i allmänhet kvinnor i högre grad än män, leder ibland till invaliditet eller andra svåra handikapp och saknar botande behandling.

Orsakerna till uppkomsten av autoimmun sjukdom är mångfasetterade, och såväl arv, miljö, livsstil som obalans i immunsystemet anses vara inblandade. De närmare mekanismerna är inte kända. År 1997 rapporterade dock en tysk forskargrupp att ett kalciumberoende enzym, transglutaminas typ 2 (TG2), kan spela en viktig roll vid uppkomsten av glutenintolerans (coeliaki, CD), en kronisk tunntarmssjukdom som drabbar cirka 1 % av befolkningen. CD har därefter blivit en modellsjukdom för att förstå mekanismerna för autoimmunitet. Vid CD utvecklas antikroppar mot såväl gluten i födan som mot TG2. Med hjälp av TG2 omvandlas en speciell aminosyra i gluten, glutamin, till glutaminsyra, en mycket ovanlig TG2-styrd reaktion.

Syfte – Den övergripande målsättningen för denna avhandling har varit att förstå de närmare mekanismerna för uppkomsten av CD och därefter testa om liknande processer orsakar andra inflammatoriska sjukdomar såsom reumatoid artrit (RA), Sjögrens syndrom (SS), multipel skleros (MS) och Alzheimers sjukdom (AD).

För att förstå mekanismerna vid utvecklingen av CD har den specifika målsättningen med delarbete I och II varit att studera om kalcium och zink påverkar bindningen mellan TG2 och antikroppar från personer med CD. Dessutom har vi studerat om antikroppar vid CD hämmar enzymaktivitet hos TG2. Våra resultat baserade på ELISA visar att kalcium flerfaldigt ökar bindningen mellan intakt TG2 and CD-antikroppar. Motsägande resultat från andra forskargrupper kan förklaras av den mycket labila karaktären hos enzymet. När en radioaktiv metod (RBA) används, baserad på isotopmärkt, rekombinant humant TG2 (rh-TG2), för att mäta bindningen kan den negativa eller uteblivna kalciumeffekten förklaras av en förbisedd förorening av kanin-TG2, som finns i de celler som används vid tillverkningen av rh-TG2.

Vi har också kunnat visa att mycket låga koncentrationer av zink hämmar den kalciuminducerade ökningen av bindning mellan TG2 och CD-antikroppar.

Vid CD bildar TG2 och redan tidigare modifierat gluten ett komplex där TG2 utgör autoantigenet.

Målsättningen med delarbete III har varit att undersöka om personer med RA har antikroppar mot såväl ett enzym som dess modifierade substrat, d.v.s. samma situation som vid CD. Sedan tidigare är det känt att serum från RA-patienter innehåller antikroppar mot citrullin i proteinbunden form (anti-CP). I vår studie kunde vi nu visa att RA-patienter också har antikroppar mot det enzym, peptidylarginindeiminas (PAD), som styr omvandlingen av arginin till citrullin. RA-patienter har också förhöjda nivåer av antikroppar mot TG2 och faktor XIII, en koagulationsfaktor. Behandling med metotrexat, ett läkemedel som hämmar immunförsvaret, minskar antikroppsutvecklingen. En klar korrelation finns mellan frekvensen av de olika antikropparna.

Syftet med delarbete IV har varit att reproducera ett arbete som visar att patienter med SS har antikroppar mot PAD men inte mot detta enzyms modifierade substrat, peptidbundet citrullin. Dessutom ville vi studera förekomsten av antikroppar mot PAD, TG2 och citrullin hos personer med MS och AD. Våra resultat visar att personer med SS saknar anti-CP och antikroppar mot PAD. Personer med AD eller MS har inte heller förhöjda nivåer av antikroppar mot PAD, TG2 eller peptidbundet citrullin.

Slutsatser – Sammantaget leder denna avhandling till följande hypotes för uppkomsten av CD:

- En stressreaktion, t ex i form av en inflammation, leder till en omfördelning av zink och därmed sänkt zinkkoncentration i tarmslemhinnan.
- Den reducerade zinknivån ökar risken för kalciumaktivering av transglutaminas i tarmslemhinnan och/eller i makrofager som lockats till platsen för inflammationen.
- Det aktiverade TG2 omvandlar specifika glutaminrester till glutaminsyra i gluten, tillförd via födan.
- Intermediatet mellan TG2 och gluten kan förväntas vara relativt långlivat då det sista steget i denna ovanliga reaktion är hastighetsbestämmande.

- Det genetiska bidraget till mekanismerna är att fragment av komplexet mellan enzym och substrat presenteras för T-lymfocyter med hjälp av HLA DQ2/DQ8 som immunförsvaret använder för att identifiera främmande proteiner.
- T-lymfocyterna aktiveras och producerar cytokiner, d.v.s. immunsystemets signalämnen.
- Cytokinproduktionen leder till att B-lymfocyter stimuleras att producera antikroppar mot såväl gluten som TG2.
- Immunaktiveringen leder till inflammation och aktivering av enzymer som kan omvandla ytstrukturer på vävnader, vilket medför skada på tarmluddet i tarmslemhinnan.
- Det påföljande nedsatta näringsupptaget leder till en reducerad kroppspool av zink, varför transglutaminas i nya reaktioner lättare kan aktiveras. Den onda cirkeln är därmed sluten.

Även vid andra autoimmuna sjukdomar skulle autoantigenet kunna bestå av ett komplex mellan ett enzym och dess omvandlade substrat. Vid RA finns till exempel en likartad bild med antikroppar mot såväl ett kalciumberoende enzym (PAD) som dess modifierade substrat (citrullin) i proteinbunden form.

13. Acknowledgements

To all co-authors, colleagues, family and friends, who have contributed to this thesis, professional as well as personal, for the encouragement you have given, I would like to express my sincere gratitude. Without you, I would not have been able to start and complete this thesis.

In particular, I wish to acknowledge:

My tutors

Klas Sjöberg, to whom I am most grateful for tutorship, mentorship and true friendship but also for long discussions though not always strictly scientific. Your commitment, energy and never-ending source of knowledge have been invaluable.

Pål Stenberg, my second tutor and supervisor, to whom I am most thankful for giving me the opportunity to accomplish this thesis and for making tremendous efforts to transform the text into recognizable language. Your enthusiasm and encouragement throughout the years have been outstanding.

My co-authors

Daniel Agardh, Åke Lernmark, Christina Book, Elisabeth Londos, Magnhild Sandberg-Wollheim, Åke Larsson and Elke Theander for most enjoyable and fruitful collaboration.

My colleagues and friends

at the Hospital Pharmacy, for your support and compassion.

Lars Söderberg, for your kindness and technical assistance in a wide range of issues.

Christina Bengtsson for superb secretarial assistance and for your loyal friendship.

Rikard Lehmann for the generous donation of the strikingly symbolic cover picture.

All my friends, not mentioned by name, for making my spare time enjoyable and meaningful and for always being there for me.

My family

Andreas, Jonathan and Alex, my sons, for providing me with an inexhaustible source of joy and happiness.

Inga and Rune, my parents, for your concern and love over the years.

Magnus and Anna-Karin, my brother and sister, for your love, care and admirable capacity to help whenever I needed it. Let's make a "Kålleslav"!

The studies were supported by the Anna&Edvin Berger´s Foundation.

14. References

1 Fox PC. Autoimmune diseases and Sjogren's syndrome: an autoimmune exocrinopathy. Ann N Y Acad Sci 2007; 1098: 15-21

2 Committee TADC. Progress in Autoimmune Disease Research: A Report to Congress. U.S.Department of Health and Human Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases. (Access at www.niaid.nih.gov/publications/pdf/ADCCFinal.pdf). 2005

3 Not T, Tommasini A, Tonini G, Buratti E, Pocecco M, Tortul C, Valussi M, Crichiutti G, Berti I, Trevisiol C, Azzoni E, Neri E, Torre G, Martelossi S, Soban M, Lenhardt A, Cattin L, Ventura A. Undiagnosed coeliac disease and risk of autoimmune disorders in subjects with Type I diabetes mellitus. Diabetologia 2001; 44: 151-5

4 Rose NR. Mechanisms of autoimmunity. Semin Liver Dis 2002; 22: 387-94

5 Artinian B, Reesal M. Procainamide-induced SLE and lymphoreticular disorders. Can Med Assoc J 1974; 110: 314 passim

6 Jonsen A, Bengtsson AA, Nived O, Truedsson L, Sturfelt G. Gene-environment interactions in the aetiology of systemic lupus erythematosus. Autoimmunity 2007; 40: 613-7

7 Lewis JH. Hemorrhagic disease associated with inhibitors of fibrin cross-linkage. Ann N Y Acad Sci 1972; 202: 213-9

8 Selinus O, Alloway B, Centeno J, Finkelman R, Fuge R, Lindh U, Smedley P. Essiential of Medical Geology; Impact of the Natural Enviroment on Public Health. Book 2005

9 Rink L, Gabriel P. Extracellular and immunological actions of zinc. Biometals 2001; 14: 367-83

10 Black RE. Zinc deficiency, infectious disease and mortality in the developing world. J Nutr 2003; 133: 1485S-9S

11 Guilherme L, Ramasawmy R, Kalil J. Rheumatic fever and rheumatic heart disease: genetics and pathogenesis. Scand J Immunol 2007; 66: 199-207

12 McClain MT, Harley JB, James JA. The role of Epstein-Barr virus in systemic lupus erythematosus. Front Biosci 2001; 6: E137-47

13 Yokochi T, Yanagawa A, Kimura Y, Mizushima Y. High titer of antibody to the Epstein-Barr virus membrane antigen in sera from patients with rheumatoid arthritis and systemic lupus erythematosus. J Rheumatol 1989; 16: 1029-32

14 Toussirot E, Roudier J. Pathophysiological links between rheumatoid arthritis and the Epstein-Barr virus: an update. Joint Bone Spine 2007; 74: 418-26

15 Fujinami RS. Virus-induced autoimmunity through molecular mimicry. Ann N Y Acad Sci 1988; 540: 210-7

16 Liang B, Mamula MJ. Molecular mimicry and the role of B lymphocytes in the processing of autoantigens. Cell Mol Life Sci 2000; 57: 561-8

17 Wucherpfennig KW. Structural basis of molecular mimicry. J Autoimmun 2001; 16: 293-302

18 Fairweather D, Lawson CM, Chapman AJ, Brown CM, Booth TW, Papadimitriou JM, Shellam GR. Wild isolates of murine cytomegalovirus induce myocarditis and antibodies that cross-react with virus and cardiac myosin. Immunology 1998; 94: 263-70

19 Olson JK, Ercolini AM, Miller SD. A virus-induced molecular mimicry model of multiple sclerosis. Curr Top Microbiol Immunol 2005; 296: 39-53

20 Rose NR, Mackay IR. Molecular mimicry: a critical look at exemplary instances in human diseases. Cell Mol Life Sci 2000; 57: 542-51

21 Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat Med 1998; 4: 781-5

22 Hutchinson D, Shepstone L, Moots R, Lear JT, Lynch MP. Heavy cigarette smoking is strongly associated with rheumatoid arthritis (RA), particularly in patients without a family history of RA. Ann Rheum Dis 2001; 60: 223-7

23 Hardy CJ, Palmer BP, Muir KR, Sutton AJ, Powell RJ. Smoking history, alcohol consumption, and systemic lupus erythematosus: a case-control study. Ann Rheum Dis 1998; 57: 451-5

24 Benoni C, Nilsson A, Nived O. Smoking and inflammatory bowel disease: comparison with systemic lupus erythematosus. A case-control study. Scand J Gastroenterol 1990; 25: 751-5

25 Galanakis DK, Laurent P, Janoff A. Cigarette smoke contains anticoagulants against fibrin aggregation and factor XIIIa in plasma. Science 1982; 217: 642-5

26 Cohen H, Ziv Y, Cardon M, Kaplan Z, Matar MA, Gidron Y, Schwartz M, Kipnis J. Maladaptation to mental stress mitigated by the adaptive immune system via depletion of naturally occurring regulatory CD4+CD25+ cells. J Neurobiol 2006; 66: 552-63

27 Gleeson M, Bishop NC. The T cell and NK cell immune response to exercise. Ann Transplant 2005; 10: 43-8

28 Bartrop RW, Luckhurst E, Lazarus L, Kiloh LG, Penny R. Depressed lymphocyte function after bereavement. Lancet 1977; 1: 834-6

29 Dicke WK, Weijers HA, Van De Kamer JH. Coeliac disease. II. The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. Acta Paediatr 1953; 42: 34-42

30 Paulley JW. Observation on the aetiology of idiopathic steatorrhoea; jejunal and lymph-node biopsies. Br Med J 1954; 2: 1318-21

31 Shiner M. Jejunal-biopsy tube. Lancet 1956; 270: 85

32 Crosby WH, Kugler HW. Intraluminal biopsy of the small intestine; the intestinal biopsy capsule. Am J Dig Dis 1957; 2: 236-41

33 Seah PP, Fry L, Rossiter MA, Hoffbrand AV, Holborow EJ. Anti-reticulin antibodies in childhood coeliac disease. Lancet 1971; 2: 681-2

34 Unsworth DJ, Kieffer M, Holborow EJ, Coombs RR, Walker-Smith JA. IgA anti-gliadin antibodies in coeliac disease. Clin Exp Immunol 1981; 46: 286-93

35 Chorzelski TP, Sulej J, Tchorzewska H, Jablonska S, Beutner EH, Kumar V. IgA class endomysium antibodies in dermatitis herpetiformis and coeliac disease. Ann N Y Acad Sci 1983; 420: 325-34

36 Chorzelski TP, Beutner EH, Sulej J, Tchorzewska H, Jablonska S, Kumar V, Kapuscinska A. IgA anti-endomysium antibody. A new immunological marker of dermatitis herpetiformis and coeliac disease. Br J Dermatol 1984; 111: 395-402

37 Bruce SE, Bjarnason I, Peters TJ. Human jejunal transglutaminase: demonstration of activity, enzyme kinetics and substrate specificity with special relation to gliadin and coeliac disease. Clin Sci (Lond) 1985; 68: 573-9

38 Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, Schuppan D. Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat Med 1997; 3: 797-801

39 Marsh MN. Coeliac Disease volume Anderson, C.M. Chapter 1/The evolution of a successful treatment for coeliac disease. Oxford: Blackwell Scientific Publications, 1992

40 Green PH, Jabri B. Coeliac disease. Lancet 2003; 362: 383-91

41 Smedby KE, Akerman M, Hildebrand H, Glimelius B, Ekbom A, Askling J. Malignant lymphomas in coeliac disease: evidence of increased risks for lymphoma types other than enteropathy-type T cell lymphoma. Gut 2005; 54: 54-9

42 Lundin KE, Gjertsen HA, Scott H, Sollid LM, Thorsby E. Function of DQ2 and DQ8 as HLA susceptibility molecules in celiac disease. Hum Immunol 1994; 41: 24-7

43 Schuppan D. Current concepts of celiac disease pathogenesis. Gastroenterology 2000; 119: 234-42

44 Petaros P, Martelossi S, Tommasini A, Torre G, Caradonna M, Ventura A. Prevalence of autoimmune disorders in relatives of patients with celiac disease. Dig Dis Sci 2002; 47: 1427-31

45 Mycek MJ, Clarke DD, Neidle A, Waelsch H. Amine incorporation into insulin as catalyzed by transglutaminase. Arch Biochem Biophys 1959; 84: 528-40

46 Beninati S, Piacentini M. The transglutaminase family: an overview: minireview article. Amino Acids 2004; 26: 367-72

47 Korsgren C, Lawler J, Lambert S, Speicher D, Cohen CM. Complete amino acid sequence and homologies of human erythrocyte membrane protein band 4.2. Proc Natl Acad Sci U S A 1990; 87: 613-7

48 Credo RB, Stenberg, P., Tong, Y.S., Lorand, L. Inhibition of Fibrinoligase and Transglutaminase by Zinc Ions. Fed. Proc. San Fransisco, 1976

49 Lorand L, Parameswaran KN, Stenberg P, Tong YS, Velasco PT, Jonsson NA, Mikiver L, Moses P. Specificity of guinea pig liver transglutaminase for amine substrates. Biochemistry 1979; 18: 1756-65

50 Ljunggren C, Hoffmann KJ, Stenberg P, Nilsson JL. Fibrin-stabilizing factor inhibitors. 10. Thiol esters as potent enzyme inhibitors. J Med Chem 1973; 16: 1186-8

51 Curtis CG, Stenberg P, Brown KL, Baron A, Chen K, Gray A, Simpson I, Lorand L. Kinetics of transamidating enzymes. Production of thiol in the reactions of thiol esters with fibrinoligase. Biochemistry 1974; 13: 3257-62

52 Folk JE, Finlayson JS. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. Adv Protein Chem 1977; 31: 1-133

53 Achyuthan KE, Greenberg CS. Identification of a guanosine triphosphate-binding site on guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity. J Biol Chem 1987; 262: 1901-6

54 Begg GE, Carrington L, Stokes PH, Matthews JM, Wouters MA, Husain A, Lorand L, Iismaa SE, Graham RM. Mechanism of allosteric regulation of transglutaminase 2 by GTP. Proc Natl Acad Sci U S A 2006; 103: 19683-8

55 Bergamini CM. GTP modulates calcium binding and cation-induced conformational changes in erythrocyte transglutaminase. FEBS Lett 1988; 239: 255-8

56 Caputo I, D'Amato A, Troncone R, Auricchio S, Esposito C. Transglutaminase 2 in celiac disease: minireview article. Amino Acids 2004; 26: 381-6

57 Martinez J, Chalupowicz DG, Roush RK, Sheth A, Barsigian C. Transglutaminase-mediated processing of fibronectin by endothelial cell monolayers. Biochemistry 1994; 33: 2538-45

58 Lesort M, Attanavanich K, Zhang J, Johnson GV. Distinct nuclear localization and activity of tissue transglutaminase. J Biol Chem 1998; 273: 11991-4

59 Upchurch HF, Conway E, Patterson MK, Jr., Maxwell MD. Localization of cellular transglutaminase on the extracellular matrix after wounding: characteristics of the matrix bound enzyme. J Cell Physiol 1991; 149: 375-82

60 Fesus L, Madi A, Balajthy Z, Nemes Z, Szondy Z. Transglutaminase induction by various cell death and apoptosis pathways. Experientia 1996; 52: 942-9

61 Fesus L, Szondy Z. Transglutaminase 2 in the balance of cell death and survival. FEBS Lett 2005; 579: 3297-302

62 De Laurenzi V, Melino G. Gene disruption of tissue transglutaminase. Mol Cell Biol 2001; 21: 148-55

63 Kojima S, Nara K, Rifkin DB. Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells. J Cell Biol 1993; 121: 439-48

64 Verderio E, Gaudry C, Gross S, Smith C, Downes S, Griffin M. Regulation of cell surface tissue transglutaminase: effects on matrix storage of latent transforming growth factor-beta binding protein-1. J Histochem Cytochem 1999; 47: 1417-32

65 Rosenthal AK, Gohr CM, Henry LA, Le M. Participation of transglutaminase in the activation of latent transforming growth factor beta1 in aging articular cartilage. Arthritis Rheum 2000; 43: 1729-33

66 Szondy Z, Sarang Z, Molnar P, Nemeth T, Piacentini M, Mastroberardino PG, Falasca L, Aeschlimann D, Kovacs J, Kiss I, Szegezdi E, Lakos G, Rajnavolgyi E, Birckbichler PJ, Melino G, Fesus L. Transglutaminase 2-/- mice reveal a phagocytosis-associated crosstalk between macrophages and apoptotic cells. Proc Natl Acad Sci U S A 2003; 100: 7812-7

67 Falasca L, Iadevaia V, Ciccosanti F, Melino G, Serafino A, Piacentini M. Transglutaminase type II is a key element in the regulation of the anti-inflammatory response elicited by apoptotic cell engulfment. J Immunol 2005; 174: 7330-40

68 Griffin M, Casadio R, Bergamini CM. Transglutaminases: nature's biological glues. Biochem J 2002; 368: 377-96

69 Laki K, Tyler HM, Yancey ST. Clot forming and clot stabilizing enzymes from the mouse tumor YPC-1. Biochem Biophys Res Commun 1966; 22: 776-81

70 Mehta K, Fok JY, Mangala LS. Tissue transglutaminase: from biological glue to cell survival cues. Front Biosci 2006; 11: 173-85

71 Lorand L, Hsu LK, Siefring GE, Jr., Rafferty NS. Lens transglutaminase and cataract formation. Proc Natl Acad Sci U S A 1981; 78: 1356-60

72 Samuelsson B, Stenberg P, Pandolfi M. Localization and characteristics of transglutaminase in the rabbit and human eye. Graefes Arch Clin Exp Ophthalmol 1982; 218: 233-6

73 Skill NJ, Griffin M, El Nahas AM, Sanai T, Haylor JL, Fisher M, Jamie MF, Mould NN, Johnson TS. Increases in renal epsilon-(gamma-glutamyl)-lysine crosslinks result from compartment-specific changes in tissue transglutaminase in early experimental diabetic nephropathy: pathologic implications. Lab Invest 2001; 81: 705-16

74 Johnson TS, El-Koraie AF, Skill NJ, Baddour NM, El Nahas AM, Njloma M, Adam AG, Griffin M. Tissue transglutaminase and the progression of human renal scarring. J Am Soc Nephrol 2003; 14: 2052-62

75 Skill NJ, Johnson TS, Coutts IG, Saint RE, Fisher M, Huang L, El Nahas AM, Collighan RJ, Griffin M. Inhibition of transglutaminase activity reduces extracellular matrix accumulation induced by high glucose levels in proximal tubular epithelial cells. J Biol Chem 2004; 279: 47754-62

76 Parsons AC, Yosipovitch G, Sheehan DJ, Sangueza OP, Greenberg CS, Sane DC. Transglutaminases: the missing link in nephrogenic systemic fibrosis. Am J Dermatopathol 2007; 29: 433-6

77 Perry MJ, Haynes LW. Localization and activity of transglutaminase, a retinoid-inducible protein, in developing rat spinal cord. Int J Dev Neurosci 1993; 11: 325-37

78 Kim SY, Grant P, Lee JH, Pant HC, Steinert PM. Differential expression of multiple transglutaminases in human brain. Increased expression and cross-linking by transglutaminases 1 and 2 in Alzheimer's disease. J Biol Chem 1999; 274: 30715-21

79 Appelt DM, Kopen GC, Boyne LJ, Balin BJ. Localization of transglutaminase in hippocampal neurons: implications for Alzheimer's disease. J Histochem Cytochem 1996; 44: 1421-7

80 Junn E, Ronchetti RD, Quezado MM, Kim SY, Mouradian MM. Tissue transglutaminase-induced aggregation of alpha-synuclein: Implications for Lewy body formation in Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci U S A 2003; 100: 2047-52

81 Kahlem P, Green H, Djian P. Transglutaminase action imitates Huntington's disease: selective polymerization of Huntingtin containing expanded polyglutamine. Mol Cell 1998; 1: 595-601

82 van Strien M, Drukarch B, Bol J, Brevé J, van Dam A-M. Tissue Transglutaminase in Multiple Sclerosis: A Novel Therapeutic Target? 9th International Conference on Transglutaminases and Protein Cross Linking 2007: 83

83 Silman AJ, Pearson JE. Epidemiology and genetics of rheumatoid arthritis. Arthritis Res 2002; 4 Suppl 3: S265-72

84 Silman AJ, Hochberg MC. Rheumatoid arthritis. In Epidemiology of the Rheumatic Diseases. Oxford: Oxford University Press, 2001

85 Doran MF, Crowson CS, O'Fallon WM, Gabriel SE. The effect of oral contraceptives and estrogen replacement therapy on the risk of rheumatoid arthritis: a population based study. J Rheumatol 2004; 31: 207-13

86 Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, Saigo K, Morinobu A, Koshiba M, Kuntz KM, Kamae I, Kumagai S. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med 2007; 146: 797-808

87 van Venrooij WJ, Pruijn GJ. Citrullination: a small change for a protein with great consequences for rheumatoid arthritis. Arthritis Res 2000; 2: 249-51

88 Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum 2003; 48: 2741-9

89 van Venrooij WJ, Zendman AJ, Pruijn GJ. Autoantibodies to citrullinated antigens in (early) rheumatoid arthritis. Autoimmun Rev 2006; 6: 37-41

90 Slater CA, Davis RB, Shmerling RH. Antinuclear antibody testing. A study of clinical utility. Arch Intern Med 1996; 156: 1421-5

91 MacGregor AJ, Snieder H, Rigby AS, Koskenvuo M, Kaprio J, Aho K, Silman AJ. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. Arthritis Rheum 2000; 43: 30-7

92 Poor G, Nagy ZB, Schmidt Z, Brozik M, Meretey K, Gergely P, Jr. Genetic background of anticyclic citrullinated peptide autoantibody production in Hungarian patients with rheumatoid arthritis. Ann N Y Acad Sci 2007; 1110: 23-32

93 Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M, Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 2003; 34: 395-402

94 Kang CP, Lee HS, Ju H, Cho H, Kang C, Bae SC. A functional haplotype of the PADI4 gene associated with increased rheumatoid arthritis susceptibility in Koreans. Arthritis Rheum 2006; 54: 90-6

95 Suzuki A, Yamada R, Yamamoto K. Citrullination by peptidylarginine deiminase in rheumatoid arthritis. Ann N Y Acad Sci 2007; 1108: 323-39

96 Iwamoto T, Ikari K, Nakamura T, Kuwahara M, Toyama Y, Tomatsu T, Momohara S, Kamatani N. Association between PADI4 and rheumatoid arthritis: a meta-analysis. Rheumatology (Oxford) 2006; 45: 804-7

97 Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS, Pillemer SR, Talal N, Weisman MH. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002; 61: 554-8

98 Bowman SJ, Ibrahim GH, Holmes G, Hamburger J, Ainsworth JR. Estimating the prevalence among Caucasian women of primary Sjogren's syndrome in two general practices in Birmingham, UK. Scand J Rheumatol 2004; 33: 39-43

99 Trontzas P, Andrianakos A, Miyakis S, Pantelidou K, Vafiadou E, Garantziotou V, Voudouris C. Seronegative spondyloarthropathies in Greece: a population-based study of prevalence, clinical pattern, and management. The ESORDIG study. Clin Rheumatol 2005; 24: 583-9

100 Pedersen AM, Nauntofte B. Primary Sjogren's syndrome: oral aspects on pathogenesis, diagnostic criteria, clinical features and approaches for therapy. Expert Opin Pharmacother 2001; 2: 1415-36

101 Jones DE. Pathogenesis of primary biliary cirrhosis. Gut 2007; 56: 1615-24

102 Rogers GE, Harding HW, Llewellyn-Smith IJ. The origin of citrulline-containing proteins in the hair follicle and the chemical nature of trichohyalin, an intracellular precursor. Biochim Biophys Acta 1977; 495: 159-75

103 Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. Bioessays 2003; 25: 1106-18

104 Esposito G, Vitale AM, Leijten FP, Strik AM, Koonen-Reemst AM, Yurttas P, Robben TJ, Coonrod S, Gossen JA. Peptidylarginine deiminase (PAD) 6 is essential for oocyte cytoskeletal sheet formation and female fertility. Mol Cell Endocrinol 2007; 273: 25-31

105 Vossenaar ER, Radstake TR, van der Heijden A, van Mansum MA, Dieteren C, de Rooij DJ, Barrera P, Zendman AJ, van Venrooij WJ. Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. Ann Rheum Dis 2004; 63: 373-81

106 Tarcsa E, Marekov LN, Mei G, Melino G, Lee SC, Steinert PM. Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. J Biol Chem 1996; 271: 30709-16

107 Takizawa Y, Sawada T, Suzuki A, Yamada R, Inoue T, Yamamoto K. Peptidylarginine deiminase 4 (PADI4) identified as a conformation-dependent autoantigen in rheumatoid arthritis. Scand J Rheumatol 2005; 34: 212-5

108 Chang X, Yamada R, Suzuki A, Sawada T, Yoshino S, Tokuhiro S, Yamamoto K. Localization of peptidylarginine deiminase 4 (PADI4) and citrullinated protein in synovial tissue of rheumatoid arthritis. Rheumatology (Oxford) 2005; 44: 40-50

109 Foulquier C, Sebbag M, Clavel C, Chapuy-Regaud S, Al Badine R, Mechin MC, Vincent C, Nachat R, Yamada M, Takahara H, Simon M, Guerrin M, Serre G. Peptidyl arginine deiminase type 2 (PAD-2) and PAD-4 but not PAD-1, PAD-3, and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation. Arthritis Rheum 2007; 56: 3541-53

110 Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. Arch. Dis. Child. 1990; 65: 909-11

111 McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weinshenker BY, Wolinsky JS. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 2001; 50: 121-7

112 Jeppson JO, Laurell CB, Franzen B. Agarose gel electrophoresis. Clin Chem 1979; 25: 629-38

113 Ganrot PO. Crossed immunoelectrophoresis. Scand J Clin Lab Invest Suppl 1972; 124: 39-47

114 Stenberg P, Stenflo J. A rapid and specific fluorescent activity staining procedure for transamidating enzymes. Anal Biochem 1979; 93: 445-52

115 Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, Karlsen AE, Boel E, Michelsen B, Lernmark A. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 1994; 37: 344-50

116 Roth EB, Sjoberg K, Stenberg P. Biochemical and immuno-pathological aspects of tissue transglutaminase in coeliac disease. Autoimmunity 2003; 36: 221-6

117 Stenberg P. The effect of metal ions on transamidating enzymes. Fed. Int. Pharm. Warsaw, 1976

118 Jonsson M, Linse S, Frohm B, Lundwall A, Malm J. Semenogelins I and II bind zinc and regulate the activity of prostate-specific antigen. Biochem J 2005; 387: 447-53

119 Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, Hutton JC. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 2007; 104: 17040-5

120 Grahn BH, Paterson PG, Gottschall-Pass KT, Zhang Z. Zinc and the eye. J Am Coll Nutr 2001; 20: 106-18

121 Srinivas U, Ohlsson T, Hallstadius L, Hansson L, Abdulla M, Strand SE, Jeppsson B. Organ sequestration of (65)Zn during experimental sepsis. Clin Nutr 1989; 8: 263-7

122 Krebs NF. Overview of zinc absorption and excretion in the human gastrointestinal tract. J Nutr 2000; 130: 1374S-7S

123 Prasad AS. Zinc deficiency. Bmj 2003; 326: 409-10

124 Sjostrom H, Lundin KE, Molberg O, Korner R, McAdam SN, Anthonsen D, Quarsten H, Noren O, Roepstorff P, Thorsby E, Sollid LM. Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. Scand J Immunol 1998; 48: 111-5

125 Crooke MA. Discussion on some problems of steatorrhea and reduced stature. Proceedings of the Royal Society of Medicine 1956; 49: 1009-13

126 Molberg O, McAdam SN, Sollid LM. Role of tissue transglutaminase in celiac disease. J Pediatr Gastroenterol Nutr 2000; 30: 232-40

127 Fleckenstein B, Molberg O, Qiao SW, Schmid DG, von der Mulbe F, Elgstoen K, Jung G, Sollid LM. Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. Role of enzyme specificity and pH influence on the transamidation versus deamidation process. J Biol Chem 2002; 277: 34109-16

128 Sulkanen S, Halttunen T, Laurila K, Kolho KL, Korponay-Szabo IR, Sarnesto A, Savilahti E, Collin P, Maki M. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology 1998; 115: 1322-8

129 Nakachi K, Swift G, Wilmot D, Chapman C, Baker S, Powell M, Furmaniak J, Rees Smith B. Antibodies to tissue transglutaminase: comparison of ELISA and immunoprecipitation assay in the presence and in the absence of calcium ions. Clin Chim Acta 2001; 304: 75-84

130 Wollheim FA, Stenberg, P, Marsal, L. Characterisation of transglutaminases in plasma and synovial fluid from patients with arthritis. 15th International Congress of Rheumatology. Paris, 1981

131 Nissinen R, Paimela L, Julkunen H, Tienari PJ, Leirisalo-Repo M, Palosuo T, Vaarala O. Peptidylarginine deiminase, the arginine to citrulline converting enzyme, is frequently recognized by sera of patients with rheumatoid arthritis, systemic lupus erythematosus and primary Sjogren syndrome. Scand J Rheumatol 2003; 32: 337-42

132 Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. Semin Arthritis Rheum 2004; 34: 501-37

133 Doyle HA, Mamula MJ. Post-translational protein modifications in antigen recognition and autoimmunity. Trends Immunol 2001; 22: 443-9

134 Utz PJ, Anderson P. Posttranslational protein modifications, apoptosis, and the bypass of tolerance to autoantigens. Arthritis Rheum 1998; 41: 1152-60