OPHTHALMOLOGICAL SIGNS AND LYSOSOMAL ENZYME ACTIVITIES IN PATIENTS WITH POLYSYSTEMIC DISEASES

Ph.D. Thesis

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Publications

List of full papers directly related to the subjects of the Thesis:

1. **Sohar N**, Sohar I, Hammer H: Lysosomal enzyme activities: New potential markers for Sjögren's syndrome. *Clin Biochem* 2005, 38; 1120-1126.
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2. Altorjay A, Paal B, **Sohar N**, Kiss J, Szanto I, Sohar I: Significance and prognostic value of lysosomal enzyme activities measured in surgically operated adenocarcinomas of the gastroesophageal junction and squamous cell carcinomas of the lower third of esophagus. *World J Gastroenterol* 2005, 11; 5751-5756.
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3. **Sohar N,** Hammer H, Sohar I: Lysosomal peptidases and glycosidases in rheumatoid arthritis. *Biol Chem* 2002, 383; 865-869.
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5. **Sohár N**, Pető T, Hammer H, Philip G: Szisztémás lupus erythematosus diagnosztikus problémája a szemészeti gyakorlatban. *Allergológia és Klinikai Immunológia* 2005, 8; 203-206.
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2. **Sohár N**., Tóth-Molnár E., Skribek Á., Sohár I, Hammer H: Lizoszómális enzimaktivitások és szemészeti tünetek különböző poliszisztémás betegségben szenvedő betegekben. Magyar Szemorvostársaság Kongresszusa, Szeged, 2005, Szemészet, 140. I. Supplementum 1-116, pp. 34.
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1. ABBREVIATIONS

ACA	anti cardiolipin anticoagulant
AGU	α-glucosidase
AMAN	α-mannosidase
AMC	7-amino-4-methylcoumarin
ANA	antinuclear antibodies
BGA	β-galactosidase
CATH B	cathepsin B
CATH H	cathepsin H
CATH D	cathepsin D
CD	cluster differential
DFP	diisopropyl fluorophosphate
DPP I	dipeptidyl-peptidase I
DPP II	dipeptidyl-peptidase II
FITC	fluorescein isothyocyanate
GCU	β-glucuronidase
HEX	β-hexosaminidase
HLA-DR	human leukocyte antigen
KCS	keratoconjunctivitis sicca
LA	lupus anticoagulant
MHC	major histocompatibility
4-MU	4-methylumbelliferyl
PMNL	polymorphonuclear leukocytes
RA	rheumatoid arthritis
RF	rheumatoid factor
SLE	systemic lupus erythematosus
SS	Sjögren`s syndrome
TPP I	tripeptidyl-peptidase I

2. INTRODUCTION

Sjögren`s syndrome (SS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) are members of the family of systemic autoimmune diseases, in which the immune response is activated to recognize a limited variety of ubiquitously expressed autoantigens, and during which several target tissues may become damaged. This tissue destruction and functional decline are due to autoreactive T cells that escape self-tolerance (1, 2). It has recently been proposed that the genetic factors involving autoimmune diseases might be shared among these (3).

Patients with rheumatic diseases are characterized by the presence of a variety of antinuclear antibodies (ANA) in their sera. Anti-SSA antibodies are clinically important anti-nuclear antibodies in patients with systemic rheumatic diseases. These antibodies are found in ~ 60%, in ~ 30%, and in 5-8% of patients with SS, SLE, and RA, respectively (4, 5). Several studies have shown that particular profiles of autoantibodies are more closely associated with extended HLA-DR haplotypes than with clinical manifestations. They suggest that many older women diagnosed as having SLE probably have primary SS. The diagnosis is based largely on the presence of ANA and arthralgias not associated with visceral systemic manifestation, with no inquiry about sicca symptoms (6-9).

It has been widely accepted that both organ-specific and systemic autoimmune diseases may often become associated with autoimmune thyroid disorders. An increased frequency of thyroid autoimmunity was found in a few larger cohorts among SLE, RA, and SS (10).

Features of SS may be found in almost every autoimmune rheumatic disease, including RA (11), SLE, scleroderma and others. The development of secondary SS associated with RA occurs on a different genetic background (HLA-DR4) (6, 12)).

Early diagnosis and appropriate treatment are essential for optimal management of SS, and affect the patients` general well being and quality of life. At present, there are no specific diagnostic tests for SS and no universally accepted diagnostic criteria (12, 13)).

Many outcome parameters have to be recorded in order to assess the response for the treatment in clinical studies of RA. Only few laboratory markers, such as C-reactive protein or erythrocyte sedimentation rate correlate well with the inflammatory activity of RA. Since both parameters are frequently elevated in case of other conditions e.g.

infections, there is currently no surrogate marker that specifically reflects the degree of joint inflammation. This is of importance, since the interobserver variability in assessing the clinical status of RA patients can be considerable (14).

Most autoantibodies are not specific for SLE and might be produced non-specifically as a result of polyclonal B cell activation. An ideal test would be specific, sensitive and have a high positive predictive value, and it should reflect disease activity, correlate with organ involvement, or predict relapse. No test or test panel can currently perform all these tasks because some of the clinical features of SLE are not antibody mediated (15). Considering the importance of ophthalmologic signs in polysytemic amutoimmune diseases, it is instructive in understanding how autoimmune disfunction may contribute to ophthalmologic diseases.

Lysosomal enzymes seem to play an important role in many physiological and pathological processes. Alterations in their activity have been observed in a great variety of disorders, like inflammation process (16, 17) or neoplasias (18-22). The altered activities of enzymes may lead to changes in the structure of cell suface glycoconjugates accompanied by alterations in carbohydrate metabolism leading to carcinogenesis, tumor growth and metastasis (18). Metastatic melanoma patients with high levels of cathepsin B and cathepsin H experienced significantly shorter overall survival rates than the patients with low levels of these enzymes (23). One study published in 2002 that the activity of several enzymes involved in ganglioside and sulfatide catabolism was analyzed in leukocytes and skin fibroblasts derived from individuals with Alzheimer`s disease and Down`s syndrome (24).

Previous studies have also demonstrated increased activities of lysosomal hydrolaseses in the serum of patients with RA, SLE, dermatomyositis or psoriasis (25).

We measured the activity of lysosomal enzyme activities in patients with adenocarcinomas of the gastroesophageal junction and the squamous cell carcinomas of the lower third of the esophagus (26). Our results and other studies have called our attention to compare the progression of the well known ophthalmic observations in polysystemic diseases to the lysosomal enzyme activities in leukocytes which were used for the diagnosis of other type of diseases.

3. CONCEPTUAL BACKGROUND

3.1 Sjögren`s syndrome

Sjögren's syndrome (SS), one of the most common autoimmune diseases, is characterized by progressive lymphocytic and plasma cell infiltration of the salivary and lacrimal glands leading to xerostomia and xerophthalmia, respectively (2, 27). Other tissues and organs may also be affected with multiple extraglandular manifestations such as arthritis, cutaneous vasculitis, renal tubular acidosis, and chronic pulmonary disease (28-30).

Previous studies have used a variety of classifications for SS (31). The 'European Community criteria' were developed in 1993 (28), and revised by the ’American-European Consensus Group` for the standardization of research studies (32, 33). These criteria depend on the demonstration of dry eyes and mouth and on focal inflammation of the salivary gland and the presence of autoantibodies in the serum. To meet this classification, each patient must have either the presence of an abnormal biopsy, or anti-Ro and/or anti-La antibodies (32-35).

Primary SS (occurring in the absence of another autoimmune condition or manifest organic disease) predominantly affects women in the fourth and fifth decades of life, though it may occur in male or female individuals of all ages (12, 36, 37, 38). Constitutional and environmental etiologies have been proposed, but the factors triggering SS are unknown (27, 29, 30, 34). Constitutional factors include hormones and multiple genes, and the environmental factors are mainly viral. Some of the clinical manifestations of SS are caused by tissue-infiltrating cells. The majority of T cells in the lymphocytic infiltrate are CD4+ and CD45RO+. The serologic changes most commonly seen in SS are cryoglobulinemia, hyperglobulinaemia, and B cell stimulation which can lead to non-Hodgkin`s lymphoma (37). The presence of autoantibodies: rheumatoid factor (RF), antinuclear antibodies (ANA), and anti-SSA/Ro and anti-SSB/La is associated with early disease duration, higher frequency of extraglandular manifestation, and more intense lymphocytic infiltration of minor salivary glands (39).

The most common form of ocular involvement is immune mediated noninfectious inflammation of the lacrimal glands and ocular surface resulting in decreased tear

production and inflammatory changes on the ocular surface, known as SS associated keratoconjunctivitis sicca (KCS). Some common signs of KCS are conjunctival injection, mucous strands, filamentary keratitis, tear film debris, and a punctate keratopathy. Advanced KCS may lead to serious complications, including keratitis, symblepharon, pannus, corneal thinning, and ceration with perforation (30, 40).

Topical and symptomatic treatments are available for the patients with SS. Dry eyes can be treated topically with artifitial tears and lubricating ointment. Occlusion of the lacrimal ducts to preserve the residual lachrymal film can also be effective. Plastic or collagen plugs can be inserted in the ducts, or the ducts can be cauterized. Cyclosporin, as 1% eye drops, was found relieving xerophthalmia (30, 41, 42). Treatments for systemic disease, such as moderate to high dose steroids, methotrexate, cyclosporine, cyclophosphamide, and plasmapheresis have been used with variable success (30).

New and emerging treatment options are using androgens to suppress glandular inflammation and muscarinic M3 agonists, i.e., cevimeline and pilocarpine (30, 41, 42).

3.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease primarily affecting the synovial membrane and leading to joint damage and joint destruction (43). Apart from the potentially destructive joint manifestations of the disease, it is also characterized by systemic features (44-46). The American Rheumatism Association revised the criteria for the diagnosis of the RA in 1987 for the purpose of classification in research studies (47-50).

Extraarticular manifestations such as rheumatoid nodules, pericarditis, pleuritis, Felty`s syndrome and various manifestations of vasculitis may occur with considerable frequency and underline the systemic nature of the condition (44-46, 51). Extra-articular manifestations are thought to be particularly frequent in severe, active disease. The incidence of the disease is two to three times greater in women than in men. RA affects about 0.3 to 1.5% of the population worldwide, has a significant long-term morbidity, and is associated with early mortality (45, 46, 52).

Although the pathogenesis of RA is unknown, several reports provide evidence that both genetic and environmental factors determine host susceptibility to RA (46, 51, 52). The development and severity of the disease appear to be linked to class II major histocompatibility (MHC) genes DR4 and DR1, which are expressed on T lymphocytes and macrophages (53-55). Several environmental stimuli such as infections, vaccine inoculations, and emotional trauma have been implicated as inciting factors. The infective agents could be bacterial candidates like mycoplasma, mycobacteria, and various enteric organisms (43). Tempting, but inconclusive evidence of a viral trigger is supported by the reactivity of synovial lymphocytes to antigens of a specific virus over time and, in some cases, isolation of viral DNA and RNA from such lymphocytes (46, 54, 55).

Rheumatoid arthritis is associated with numerous ophthalmic signs and symptoms. The most significant complications include KCS, keratitis, sclerokeratitis, scleritis, scleromalacia perforance, and uveitis. Rapid detection and early treatment decreases the ocular complications and increases the overall quality of the life of patients (43, 46, 56).

The initial treatment of eye disease in case of RA is directed at the dry eye (artifitial tear drops and ointments) and associated symptoms (46). None of the existing treatments can be considered to be curative or definitive therapies for the problems involving the eye. Oral nonsteroidal antiinflammatory drugs and, if inflammation is not controlled, systemic oral or intravenous corticosteroid may be instituted. Both cyclophosphamide or methotrexate have been shown to be beneficial (57-59). The results of the current analyses indicate that therapy with infliximab plus methotrexate affords articular protection not associated with clinically manifest improvement in inflammation (60).

RA patients with longer duration of the disease do not respond well to treatment as compared with patients with early disease. This has implications for new tests to detect the disease as early as possible (58, 59, 61).

3.3 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic generalized inflammatory connective tissue disease with characteristic autoantibodies primarily affecting the skin, musculoskeletal system, serous membranes, kidneys, central nervous system (CNS), and cells of the blood. Acute exacerbations of disease activity are followed by periods of remission. About 80%-90% of SLE patients are women (62-64).

The diagnosis can be definitively established if 4 of the 11 updated criteria of the American College of Rheumatology are met, but ocular lesions are not included. Preliminary criteria were established in 1982 and revised in 1997 (65).

SLE may derive from a dysfunction in immunoregulation and may be triggered by environmental agents such as microbes or chemicals (66). A definite genetic predisposition to SLE exists, there is a significantly higher incidence of HLA-DR2 and HLA-DR3 in patients with SLE. In genetically predisposed individuals, postnatal environmental factors probably play a critical role in inducing the disease (63, 64, 67).

Autoantibodies to a number of nuclear and cytoplasmic constituents may be present and may be the result of a generalized polyclonal B cell hyperactivity (68, 69). Elevated antinuclear antibodies (ANA) can be seen in other systemic diseases, too. ANAs include antinative double-stranded DNA (anti-ds DNA) that is very specific for SLE, anti-single-stranded DNA (anti-ss DNA) that is commonly found but is nonspecific, and the anti-ribo-nucleoproteins, the anti-Ro/SSA and anti-La/SSB antibodies. Autoantibodies to cytoplasmic antigens include a group against phospholipids, mainly the „lupus anticoagulant" (LA) and anticardiolipin antibody (ACA). These autoantibodies are thrombogenic and have been associated with recurrent arterial and venous thromboses, recurrent abortions, thrombocytopenia, and most of the neurologic complications of lupus (70, 71). Most autoantibodies increase during active periods of the disease, but few prospective data are currently available to justify treatment on the basis of rising titres (15).

Systemic lupus erythematosus can affect many ocular and adnexal structures. Ocular manifestations tend to occur in patients who have active systemic disease and should alert the clinician to the likely presence of extraocular disease activity (63).

KCS, with or without xerostomia and retinopathy are the most common ocular manifestations (72). Involving the retina, cotton-wool spots and retinal hemorrhages are

the most frequently reported findings, but retinal edema, hard exudates, microaneurysms, and vascular tortuosity have also been noted. Lupus retinopathy can occur as an independent manifestation of the underlying disease process (63, 73). Central retinal vein occlusion has also been reported in patients with SLE, but appears to be less common than arterial occlusive disease (74). Most occluded vessels have either a mild perivasculitis or no evidence of active inflammation. Lupus choroidopathy results in multifocal serous detachments of the retina and underlying retinal pigment epithelium (72, 73, 75, 76).

Central nervous system lupus may be directly visualized clinically in lupus patients with multifocal retinal artery occlusions, this is the so-called `bland-vasculopathy`. Direct involvement of the optic nerve can occur as acute retrobulbar neuritis, acute anterior optic neuritis, and anterior ischemic optic neuropathy. Both optic neuropathy and retinal occlusive disease can result in optic atrophy (63, 77, 78).

The drugs used to treat this systemic disease have potential ocular side effects. Corticosteroids may induce cataracts and glaucoma, chloroquine or hydroxychloroquine may cause reversible macular pigment mottling and a bulls-eye pattern of pigmentary maculopathy in its later phases (79, 80). Non-steroidal anti-inflammatory agents and antimalarials are also used for treatment of the nonspecific manifestations of the disease. Despite the side effects, systemic corticosteroids are used orally and intravenously for the treatment of organ involvement. Immunosuppressive drugs, azathioprine, cyclophosphamide, methotrexate, and cyclosporine A have been employed when life-threatening complications of lupus are unresponsive to corticosteroids. Efforts are being made to identify more selective, less toxic agents (81). Since the ocular complications of SLE are generally associated with active disease elsewhere in the body, control of the systemic disease may lead to resolution of ocular manifestations (63, 79, 80).

3.4 Lysosomal enzymes

Human polymorphonuclear leukocytes (PMNL) play a fundamental role in many inflammatory diseases (82, 83). Their proinflammatory activity is exerted trough the release of different chemical mediators, such as preformed mediators, including several lysosomal enzymes and de novo synthesized mediators, like oxygen-free radicals. The activation of human PMNL can be induced by either immunologic or non-immunologic stimuli that activate these cells through different signal transduction mechanisms (82, 83).

Lysosomes are cytoplasmic organelles. Typically, they contain over fifty hydrolytic enzymes. Lysosomal enzymes act only within a relatively narrow acid pH range between 3.5 and 6.5. (84 85).

Lysosomal enzymes are ubiquitous, biologically active molecules, which can degrade macromolecules such as proteins, carbohydrates, nucleic acids, lipids, and their conjugates. It is well known that they have been implicated as having a role in tissue injury and repair, inflammation, and phagocytosis. All components of the connective tissue can be the target of metabolic disorders affecting either their biosynthesis or degradation (86).

Lysosomal enzymes are common in human body fluids, such as serum, urine, and tears. Some studies have also revealed the lysosomal enzyme activities in aqueous humor and subretinal fluid (87-89), but so far no study has investigated the specific activities of lysosomal enzymes from the leukocytes of patients with SS, RA, and SLE.

Specific activity of some lysosomal enzymes have been demonstrated in the anterior segment of the eye, in ciliary body, corneal epithelium, stormal keratocytes, endothelium, as well as lens epithelium (90-94).

The interaction with extracellular matrix strongly influences the functional response of the acinar cell to neural stimuli. Therefore, the increased release of proteolytic enzymes in the lacrimal gland may degrade the extracellular matrix, thus leading to decreased secretion (95). Studies in SS patients have further demonstrated increased levels of serum salivary isoamylase (96) and prolactin (97, 98), the latter one upregulates cathepsin B and cathepsin D expression in the minor salivary glands (99). The salivary glands of SS patients have been found to have increased expression of matrix

metalloproteinases (100). However, no study up to now has compared the activity changes of lysosomal glycosidases and proteases from the leukocytes of patients with varying stages of this disease.

Proteolytic enzymes are responsible for the degradation of cartilage and bone components in RA. Therefore these enzymes could have a potential as disease-specific surrogate markers of RA (101-104). Cysteine proteinases, for example cathepsin B (CATH B), is a candidate surrogate marker since it is capable of degrading cartilage and bone matrix components and of activating metalloproteinases (101, 102). Higher activity of CATH B was found in patients with RA in their synovial fluids (102, 103, 105-107). Cathepsin H (CATH H) is another lysosomal cysteine protease that simillarly to CATH B has also exo- and endopeptidase activity. CATH H was shown to cleave several proteins (108), but the endopeptidase activity is limited. Collagen, laminin, for instance, were not degraded by cathepsin H. Dipeptidyl-peptidase I (DPP I) (old name was cathepsin C) differs from most other relatives of papain in family C1 in its large molecular size, oligometric structure and requirement of haloid ions for activity (108). It can hydrolyze a dipeptide from the unsubstituted N-termini of protein substrate. The N-terminal amino acid can not be basic amino acid. This fact and the potentiation by sulfhydryl reagents can distinguish it from the serine enzyme dipeptidyl-peptidase II (DPP II). DPP I has no endopeptidase activity, it is responsable for the activation of the proenzymes of several serine proteinases. Mutations of the DPP I gene are responsible for Papillon-Lefebre syndrome (109) that is an autosomal recessive disorder characterised by palmoplantar hyperkeratosis and severe early onset periodontitis. Recently, however, a study showed that cathepsins B, H and L lysosomal cysteine proteinases have not played a role in RA (110). Cathepsin K published as a specific enzyme with collagenolytic activity (111).

A lysosomal serine protease, tripeptidyl-peptidase I (TPP I) may play a role in the terminal stages of intracellular collagen degradation, that very likely depends on DPP II for the further reduction of the liberated dipeptides and free amino acids (112). TPP I activity has been known for four decades, but the active serine center has been proved recently (113, 114). The deficiency of the enzyme can cause blindness, epilepsy and brain damage in children at age of 6-10 years (115). DPP II can also release N terminal dipeptides from oligopeptides and the N-terminal residue could be any amino acids, but

acidic ones are not favorable. DPP II prefers action on tripeptides generated by TPP I and the generated dipeptides can cross the lysosomal membrane (112).

Cathepsin D (CATH D) is an aspartate endopeptidase with a pH optimum between pH 3.5 and 5.0 depending on the substrate and assay condition (116). Cathepsin D prefers to digesting proteins (possible also collagen) instead of synthetic small peptide substrate (117).

Lysosomal-associated membrane proteins are transmembrane lysosomal glycoproteins which are detectable at the cell surface of lymphocytes in patients with SLE. Undigested oligosaccharides not only accumulate in the lysosomes, but also leak into the cytoplasm, into the circulation and into the extracellular space and might trigger SLE onset in patients with predisposition to SLE (117, 118).

The activity of selected lysosomal hydrolases, ß-galactosidase (BGA), α-mannosidase (AMAN) was determined in the blood serum of patients with SLE. An increased activity was found, and may be responsible for the desintegration of the connective tissue ground substance in SLE (119-124).

4. AIMS OF THE INVESTIGATIONS

Based on previous studies, we hypothetized that

- the lysosomal glycosidase and protease activities in leukocytes may provide information on the role of tissue damage by enzymes in polysystemic autoimmune disease,
- proteinases and glycosidases from leukocytes might represent potential markers of the disease activity.

In order to get answer for the hypotheses above, patients with primary SS, RA, and SLE were chosen for our investigations.

The aims of this study were:

- the ophthalmic examinations of patients in the three groups of autoimmune diseases and controls,
- to measure the activities of lysosomal glycosidases and proteases in leukocytes of the patients in the three groups of autoimmune diseases and controls,
- to find a potential correlation between the ophthalmic signs and activity values of lysosomal enzymes in patients of the three autoimmune diseases,
- to determine whether activities of enzymes in leukocytes might be markers for monitoring the progression of SS, RA, and /or SLE.

5. PATIENTS AND METHODS

5.1. Patients

Thirty-eight patients with primary SS (1 male, 37 female) with a mean age of 53 years; thirty persons (5 males and 25 females), mean age 56 years, who were registered as RA patients; thirty-seven SLE patients (1 male and 36 females) with a mean age of 45 years, and 36 healthy subjects (12 males and 24 females) with a mean age of 50 years were selected randomly for this study.

The control group was without any autoimmune or other documented disease. For the purposes of analysis, patients with SS, RA and SLE were subdivided into 3 groups based on the duration of the illness: patients were diagnosed 1) less than 5 years, 2) 5-10 years, and 3) more than 10 years prior to enrollment. In cases of SS, the first group had nine, the second group had seven, and the third group had twentytwo patients. In cases of RA: fourteen, ten, and six patients were in the 1^{st}, 2^{nd}, and 3^{rd} groups, respectively. Sixteen SLE patients were in the first, ten in the second, and eleven in the third group.

5.2. Ophthalmic examinations

Patients with SS, RA, and SLE and the control subjects underwent standard ophthalmologic examinations including visual acuity tests using Kettesy`s decimal visual chart, intraocular pressure with applanation tonometer, slit-lamp biomicroscopic examination of the anterior segment of the eye using HOYA H-100 (7905, Japan) and Haag-Streit (Liebefeld-Bern, Switzerland) slit-lamps, and ophthalmoscopic examination with Welch-Allyn 11620 direct ophthalmoscope (Skaneateles Falls, NY, USA) and 90 D Ocular lense (060123, Bellevue, WA, USA). The patients` perimetry was carried out by Perimeter/TAP-CC (56600, Okulus, Germany) and Goldmann perimeter (S 9406636, Haag-Streit, Bern, Switzerland). Additionally, all subjects were evaluated for KCS using the Schirmer I test (without any anesthetics), break-up time test (BUT), and rose bengal score measurements (28, 30). The ocular tests were considered positive as follows:

wetting of ≤5 mm of the paper strip in 5 minutes on the Schirmer I test, BUT ≤10 seconds, and rose bengal score of ≥4 on the von Bijsterweld scoring system.

5.3. Sample preparation for enzymatic assay

Blood samples were taken using Vacutainer® cell preparation tubes containing sodium citrate. For leukocyte purification, a heparin-dextran solution was added to the blood according to Johnson et al. (127). After incubation at room temperature for 1–2 hours, the upper layer was removed and centrifuged at 870 x *g* for 10 minutes. The pellet was resuspended in 1 mL normal saline plus 3 mL distilled water and centrifuged at 515 x *g* for 8 minutes. The pellet was free of red blood cells, and was resuspended in 4 mL normal saline and centrifuged at 515 x *g* for 8 minutes. After repetition of the last step, the leukocytes were homogenized in 0.15 M NaCl in 0.1% Triton-X 100 with a "Brinkman" Polytron homogenizer. The homogenized material was then centrifuged, and the supernatant was used for the enzyme assays and protein determination.

5.4. Measurement of enzyme activities

Glycosidase activities were measured with the use of 4-methylumbelliferyl (4-MU) substrates (128, 129). Protease assays with 7-amino-4-methylcoumarin (AMC) substrates or FITC-hemoglobin were carried out as described previously (105, 114, 128). Samples of 5, 10 or 25 µL were diluted 2-, 4-, and 8-fold with 0.15 M NaCl-0.1% Triton-X 100. Duplicates from each dilution were transferred to 96-well microtiter plates. The enzyme reaction was initiated by addition of a solution containing a substrate in buffer, and the plates were incubated at 37 °C for 30–180 min. Assays involving 4-MU and AMC substrates were stopped by adding 100 µL 0.5 M glycine-NaOH buffer at pH 10.5, or 100 µL 0.1 M monochloroacetic acid in 0.1 M acetic acid at pH 4.3. The amount of 4-MU or AMC liberated at the assay is proportional to the enzyme activity; the unit for both assays is nmol/h/mg protein. The substrates were obtained from Sigma (St. Louis, MO, USA), except for the DPP I substrate (H-Gly-Arg-AMC) and the cathepsin L

inhibitor (Z-Phe-Phe-diazomethylketone) for the CATH B assay, which were obtained from Bachem Bioscience Inc. (King of Prussia, PA, USA).

The emission (λ_{ex} 360 nm, λ_{em} 460 nm) was measured with a Cytofluor II, Fluorescence multi-well plate reader (CytoFluor 4000, PerSeptive Biosystems, Inc., Framingham, MA, USA). The activities were normalized to the protein content in the sample measured according to the method of Lowry et al. (130).

5.5. Statistical analysis

Data are presented as mean±SE. Statistical significance was assessed by the Student *t* test and the Kruskal-Wallis one-way ANOVA on ranks. The results were considered significant, if $p \leq 0.05$ (*means significant values on the graphs).

5.6. Ethics

This study was conducted in accordance with the Declaration of Helsinki. This medical research was subject to ethical standards that promote respect for all human beings and protect their health and rights. It conformed to generally accepted scientific principles based on a thorough knowledge of scientific literature, other relevant sources of information, and on adequate laboratory experimentation. The experimental protocol was approved by the ethical review committee of the University of Szeged. The right of research subjects to safeguard their integrity was always respected.

6. RESULTS

6.1. Sjögren`s syndrome (SS)

6.1.1 Ophthalmological signs of patients with SS

The ophthalmological signs in the 38 patients diagnosed with SS are demonstrated in Table 1.

Table1. Ophthalmic signs of the patients diagnosed with SS

Patients	Duration*	KCS	Tear production	Tear break up time	Conjunctival degeneration	Corneal degeneration	Keratitis filiformis	Cataract	Cupping of the optic disc	Atrophy of the optic disc	Visual field defect	Degeneration of the macula	Angiopathy	Maculopathy
1	1	yes	**<5mm**	4sec		yes								
2	1	yes	5mm<	10sec						yes				
3	1	yes	**<5mm**	3sec			yes			yes				
4	1	yes	5mm<	8sec			yes							
5	1	yes	**<5mm**	2sec		yes					yes			
6	1	yes	5mm<	9sec						yes	yes			
7	1	yes	5mm<	10sec			yes							
8	1	yes	5mm<	1sec						yes				
9	1	yes	**<5mm**	5sec						yes	yes			
10	2	yes	5mm<	5sec						yes	yes			
11	2	yes	5mm<	6sec										
12	2	yes	5mm<	5sec										
13	2	yes	5mm<	10sec						yes				
14	2	yes	5mm<	2sec				yes			yes			
15	2	yes	**<5mm**	0sec			yes			yes				
16	2	yes	**<5mm**	2sec										
17	3	yes	5mm<	10sec					yes	yes	yes			
18	3	yes	**<5mm**	2sec	yes		yes		yes	yes				yes
19	3	yes	5mm<	10sec			yes	yes		yes			yes	
20	3	yes	**<5mm**	1sec						yes	yes			
21	3	yes	5mm<	10sec										
22	3	yes	**<5mm**	2sec					yes	yes				
23	3	yes	**<5mm**	4sec			yes		yes	yes	yes			yes
24	3	yes	**<5mm**	7sec		yes								
25	3	yes	**<5mm**	0sec					yes	yes		yes		
26	3	yes	5mm<	10sec						yes	yes		yes	yes
27	3	yes	**<5mm**	3sec			yes							
28	3	yes	5mm<	8sec										
29	3	yes	5mm<	6sec					yes	yes	yes			
30	3	yes	**<5mm**	5sec				yes						
31	3	yes	5mm<	6sec						yes				
32	3	yes	5mm<	8sec			yes			yes				
33	3	yes	**<5mm**	0sec					yes	yes				
34	3	yes	**<5mm**	5sec						yes				
35	3	yes	5mm<	2sec						yes				
36	3	yes	**<5mm**	2sec		yes								
37	3	yes	5mm<	6sec			yes			yes				
38	3	yes	**<5mm**	1sec			yes	yes	yes	yes				

* Group 1, patients who were sick for less than 5 years.
Group 2, patients who were sick for more than 5, but less than 10 years.
Group 3, patients who were sick for more than 10years.

Filamentary keratitis (n=7) and a decreased tear production (n=12) were more marked in the third group of SS patients (who were sick for more 10 years), than that of in the second group (5-10 years) (n=1 and n=2, respectively). Cupping of the optic disc was only found in the third group (≥10 years).

We also found that all of the SS patients had KCS and one patient had conjunctival degeneration (pinguecula), too. The next observed ophthalmological signs did not differ with the duration of the disease: corneal degeneration (4 patients), cataract (4 patients), atrophy of the optic nerve (26 patients), and visual field defects (12 patients). In two cases, we found maculopathy, and one patient exhibited macular degeneration.

6.1.2 Lysosomal enzyme activities in leukocytes of patients with SS

The values of lysosomal enzyme activities in the leukocytes from the SS patients and the controls are shown in Table 2. In all SS patients, significantly increased activities of the glycosidases BGA, AMAN, GCU, HEX; and the proteases CATH B, DPP I, TPP I, and CATH D were found as compared with the controls.

Figure 1 shows the specific enzyme activities in the three groups of SS patients, normalized to the control activities, namely, the average values of the enzyme activities were divided by the average values of the controls. In the first group (SS was recognized less than 5 years prior to our investigations), the activities of BGA, AMAN, GCU, HEX, CATH B and DPP I were increased significantly as compared to the controls. In the second group (5-10 years of sickness), all of the measured lysosomal enzyme activities were elevated as compared both to the controls and to the first SS group, except for two enzymes: CATH B, which showed elevated values only when compared to the control group and AGU, which activity was not higher than that of the controls. In the third group (≥10 years), the enzyme activities were less than in the second group, but the activities of AMAN, GCU, and HEX remained significantly elevated compared to the control values.

Table 2. Lysosomal enzyme activities in the 3 groups of patients with SS

	All patients					0-5yrs[&]				5-10yrs[&]				10yrs<[&]				control	
	average*	SE †	p	SS/ctr ‡		average*	SE †	p	SS/ctr ‡	average*	SE †	p	SS/ctr ‡	average*	SE †	p	SS/ctr ‡	average*	SE †
AGU	12,3	± 1,09	0,124	1,14		11,2	± 1,59	0,407	1,04	11,9	± 2,10	0,266	1,10	13,0	± 1,71	0,093	1,20	10,8	± 0,75
BGA	439	± 95,1	**0,023**	1,83		466	± 231	**0,029**	1,94	526	± 201	**0,005**	2,19	389	± 125	0,070	1,62	241	± 15,2
AMAN	145	± 25,8	**0,003**	2,15		124	± 43,6	**0,011**	1,84	186	± 57,8	**0,000**	2,76	135	± 37,0	**0,014**	2,00	67,4	± 5,77
GCU	372	± 81,3	**0,009**	2,19		338	± 158	**0,019**	1,99	598	± 255	**0,001**	3,51	284	± 73,3	**0,029**	1,66	170	± 9,60
HEX	120	± 25,3	**0,009**	2,08		125	± 56,8	**0,012**	2,16	172	± 71,7	**0,002**	2,97	95,2	± 26,1	**0,040**	1,65	57,7	± 4,04
DPP I	2602	± 508	**0,006**	2,09		2356	± 862	**0,012**	1,89	4154	± 1441	**0,000**	3,34	1997	± 571	0,056	1,61	1244	± 112
CATH H	50,2	± 9,56	0,116	1,41		51,9	± 19,9	0,185	1,46	67,2	± 20,7	**0,042**	1,89	41,7	± 13,0	0,329	1,18	35,5	± 7,56
DPP II	35,7	± 7,31	0,064	1,53		31,4	± 10,5	0,168	1,34	52,9	± 25,2	**0,022**	2,26	29,7	± 6,38	0,165	1,27	23,4	± 3,19
TPP I	285,0	± 47,6	**0,018**	1,68		278	± 107,5	0,071	1,64	356	± 116	**0,009**	2,10	255	± 59,1	0,066	1,51	169	± 25,4
CATH B	27,0	± 2,54	**0,032**	1,28		30,9	± 7,01	**0,027**	1,46	29,4	± 6,00	**0,041**	1,38	24,4	± 2,75	0,154	1,15	21,2	± 1,77
CATH D	395	± 31,3	**0,020**	1,28		393	± 77,1	0,103	1,27	427	± 63,7	**0,029**	1,38	382	± 41,7	0,065	1,24	309	± 26,7

* Unit of enzyme activity: nmol substrate/h/mg
† SE: Standard Error
‡ ss/ctr: Values are normalized to the control
& duration of the disease

Figure 1. Relative enzyme activities of leukocytes in patients with SS concerning the duration of the illness

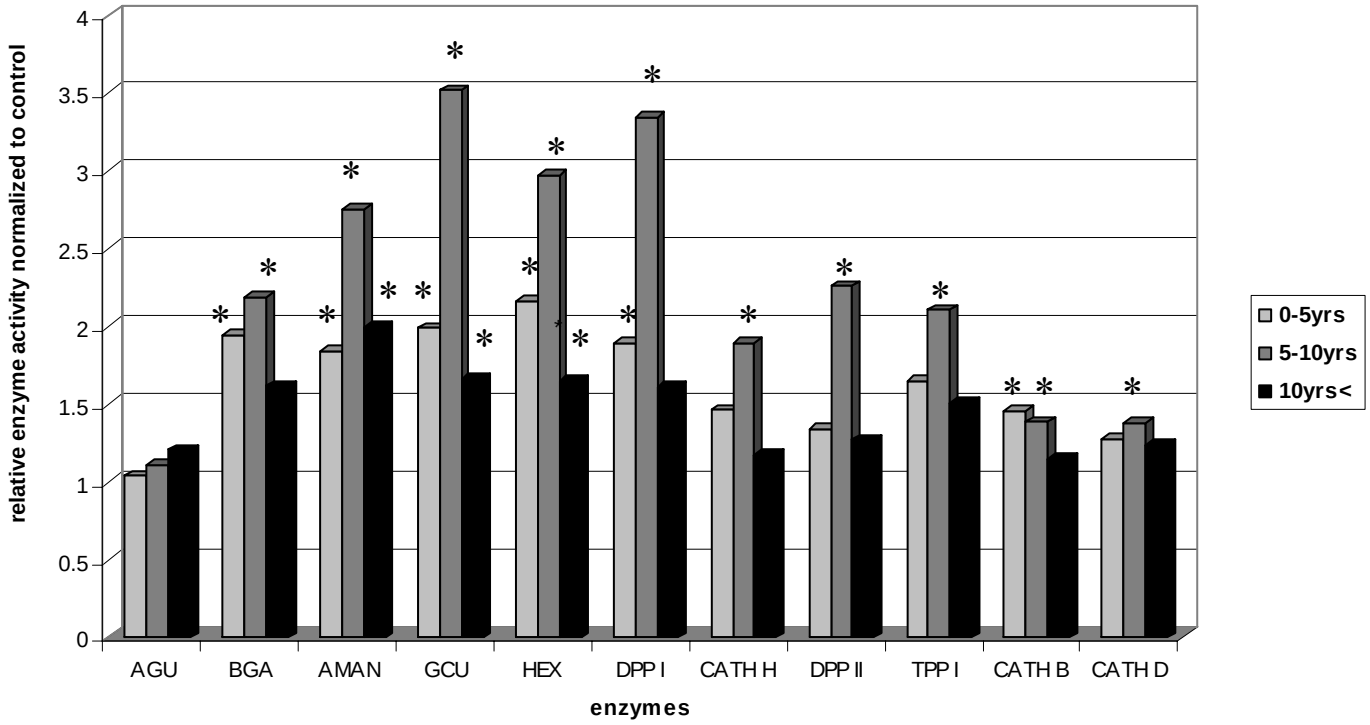
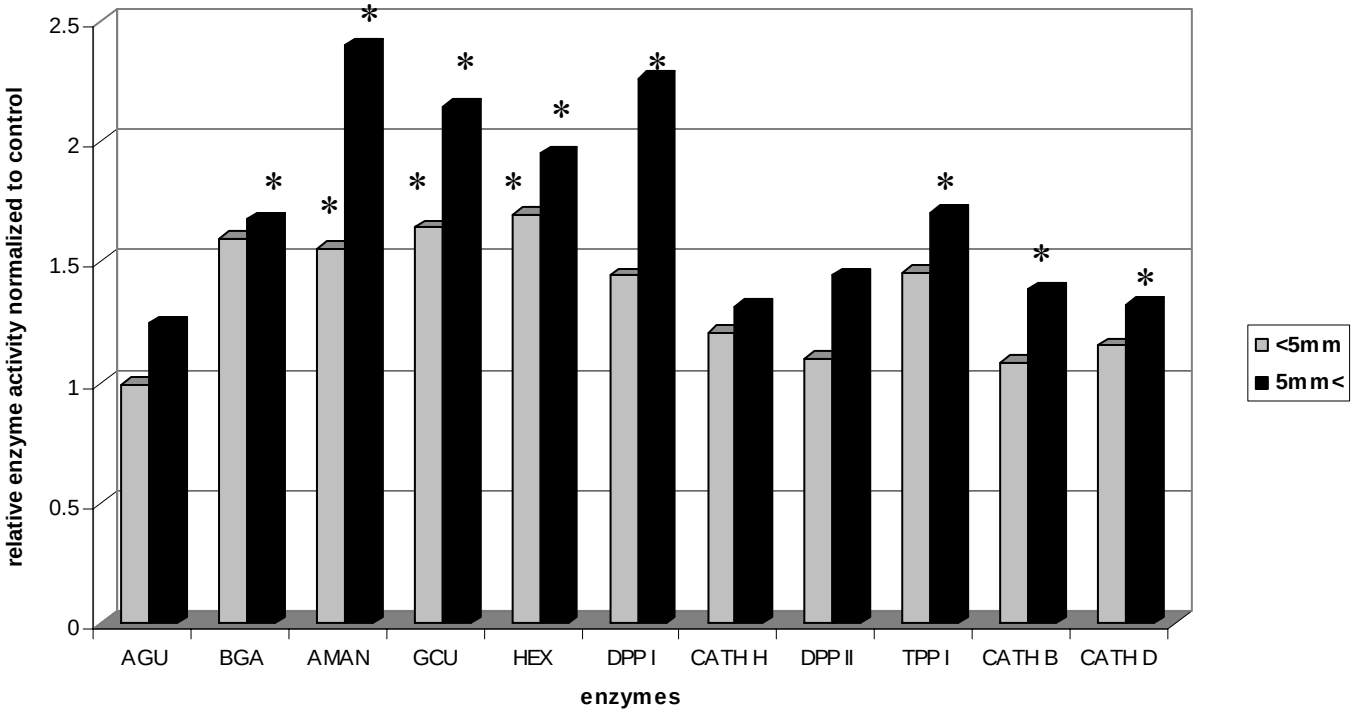


Figure 2. Lysosomal enzyme activities in patients with SS related to the tear production



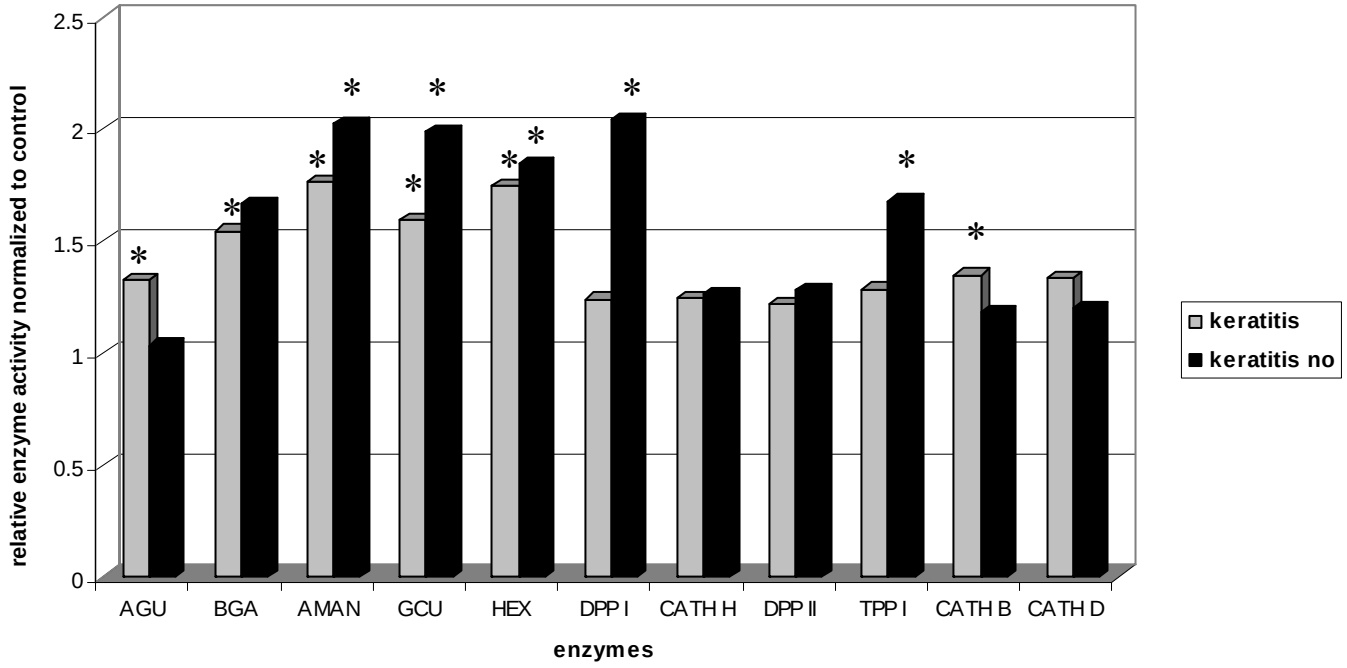
6.1.3 Association between lysosomal enzyme activities and ophthalmologial signs of patients with SS

Figure 2 shows the relation between tear production and lysosomal enzyme activities. The Schirmer I test, performed according to previous studies (27, 29) was the most accurate among all of the published functional tests. Enzyme activities increased to a greater degree in subjects with SS whose Schirmer I test was >5 mm in 5 min (n = 18) than in those patients whose Schirmer I test was ≤5 mm in 5 min (n = 20). DPP I, AMAN, GCU, and HEX activities were significantly increased in case of subjects whose tear production was ≤5 mm in 5 min, as compared to the control group. Direct correlation has been found between the activity of CATH B and D and the Schirmer I test (data not shown). Additional studies should be conducted to determine the reason for this relation.

Excessive corneal dryness may lead to the appearance of mucous threads attached to the cornea, a condition known as filamentary keratitis (131). Lysosomal enzyme elevations versus controls were lower in patients with SS who had filamentary keratitis (n = 27) compared with those subjects who did not (n = 11) with the exception of AGU, CATH B, and CAH D, which were mildly elevated (Figure 3).

The specific enzyme activities and some of the ophthalmologic findings depended on the duration of SS, but they were independent on the severity of the disease.

Figure 3. Lysosomal enzyme activities in patients with SS related to the presence of keratitis



6.2. Rheumatoid arthritis (RA)

6.2.1 Ophthalmological signs of patients with RA

The ophthalmological signs of the 30 patients diagnosed with RA are shown in Table 3. We found that 16 patients had KCS which is the most frequent ophthalmological symptom of RA. Distorsion of the episcleral blood vessels, cataract, angiopathy, atrophy of the optic disc and visual defect were found at 5, 9, 8, 10, and 5 patients, respectively. Keratitis, corneal degeneration, corneal ulcer and maculopathy were found at three, while pseudoexfoliation and conjunctival degeneration were found at two patients.

Keratitis (22%) were more common in the second group (5-10 years), while corneal degeneration occurred mainly both in the second (5-10 years) and in the third group (≥10 years) (10%-10%).

Table 3. Ophthalmic signs of the patients diagnosed with RA

Patients	Duration*	Kerato-conjunctivitis sicca	Episcleral vessel-distension	Keratitis	Corneal degeneration	Corneal ulcer	Conjunctival degeneration	Cataract	Pseudo-exfoliation	Angiopathy	Atrophy of the optic disc	Perimetry	Maculopathy
1	1	yes	yes							yes			
2	1		yes					yes	yes				
3	1	yes											
4	1	yes											
5	1	yes									yes	yes	
6	1												
7	1					yes		yes					
8	1										yes		
9	1												
10	1	yes								yes			
11	1												
12	1		yes								yes	yes	
13	1				yes	yes							yes
14	1	yes						yes		yes			
15	2			yes									
16	2	yes	yes							yes	yes		
17	2	yes	yes	yes			yes		yes		yes		yes
18	2	yes			yes			yes			yes	yes	
19	2	yes								yes			
20	2	yes											
21	2	yes									yes		
22	2	yes											
23	2	yes											yes
24	2							yes					
25	3							yes		yes	yes		
26	3	yes									yes	yes	
27	3			yes		yes		yes					
28	3						yes			yes		yes	
29	3	yes			yes			yes					
30	3							yes		yes	yes		

* Group 1, patients who were sick for less than 5 years.
Group 2, patients who were sick for more than 5, but less than 10 years.
Group 3, patients who were sick for more than 10years.

Table 4. Lysosomal enzyme activities in the 3 groups of patients with RA

			All patients					**0-5yrs** [&]					**5-10yrs** [&]					**10yrs<** [&]			**control**		
	average*		SE [#]	p	ra/ctr	average*		SE [#]	p	ra/ctr [‡]	average*		SE [#]	p	ra/ctr [‡]	average*		SE [#]	p	ra/ctr [‡]	average*		SE [#]
AGU	20.0	±	2.00	**0.000**	1.85	17.0	±	2.68	**0.002**	1.57	20.8	±	3.68	**0.000**	1.93	25.7	±	4.49	**0.000**	2.38	10.8	±	0.75
BGA	219	±	19.0	0.186	0.91	175	±	27.6	**0.016**	0.73	269	±	33.9	0.203	1.12	240	±	24.4	0.487	0.99	241	±	15.2
AMAN	84.3	±	9.46	0.057	1.25	58.3	±	15.0	0.247	0.87	92.8	±	6.16	**0.020**	1.38	124	±	20.3	**0.001**	1.83	67.4	±	5.77
GCU	196	±	16.6	0.087	1.15	155	±	21.1	0.219	0.91	227	±	33.5	**0.014**	1.33	240	±	21.9	**0.004**	1.41	170	±	9.60
HEX	73.7	±	7.43	**0.027**	1.28	57.0	±	9.57	0.466	0.99	85.5	±	15.1	**0.007**	1.48	92.8	±	10.6	**0.001**	1.61	57.7	±	4.04
DPP I	1844	±	222	**0.007**	1.48	1536	±	332	0.144	1.23	1952	±	381	**0.009**	1.57	2383	±	475	**0.001**	1.92	1244	±	112
CATH H	36.9	±	4.69	0.441	1.04	30.0	±	5.07	0.333	0.85	34.2	±	6.89	0.466	0.96	57.4	±	15.2	0.135	1.62	35.5	±	7.56
DPP II	23.6	±	3.16	0.483	1.01	18.1	±	4.43	0.183	0.77	25.8	±	5.16	0.363	1.10	32.9	±	7.57	0.134	1.40	23.4	±	3.19
TPP I	165	±	14.0	0.440	0.97	142	±	22.9	0.264	0.84	175	±	24.6	0.457	1.03	201	±	12.2	0.309	1.19	169	±	25.4
CATH B	133	±	37.4	**0.001**	6.29	148	±	49.9	**0.000**	6.99	90.8	±	56.1	**0.010**	4.28	169	±	122	**0.001**	8.00	21.2	±	1.77
CATH D	460	±	43.8	**0.002**	1.49	438	±	58.6	**0.015**	1.42	494	±	100	**0.007**	1.60	448	±	49.7	**0.025**	1.45	309	±	26.7

* Unit of enzyme activity: nmol substrate/h/mg
† SE: Standard Error
‡ ra/ctr: Values are normalized to the control
& duration of the disease

6.2.2 Lysosomal enzyme activities in leukocytes of patients with RA

The lysosomal enzyme activities in leukocytes from 30 patients diagnosed with RA and 36 controls are shown in Table 4. Significantly increased activities were found in case of AGU, HEX, DPP I, CATH B and CATH D calculated with the results of all patients in the three groups, while BGA, AMAN, CATH H, DPP II and TPP I did not show any significant changes.

Figure 4 shows the specific enzyme activities in the 3 separated groups of RA patients normalized (See *6.1.2*) to the control activities. Significantly decreased lysosomal enzyme activities were found in case of BGA in the first group (who were diagnosed with RA less than five years before our investigations); but in case of AMAN, GCU, HEX, CATH H, DPP II, and TPP I the decrease was not significant. The enhancement in the enzyme activities was the highest in the third group (who were sick for more 10 years) with the exception of BGA. CATH B showed the greatest enzyme activity increase in all of the 3 groups of patients. The activities of CATH B were 7 times, 4 times, and 8 times higher in the first, second, and third group, respectively than in the control group.

Figure 4. Relative enzyme activities of leukocytes in patients with RA concerning the duration of the illness

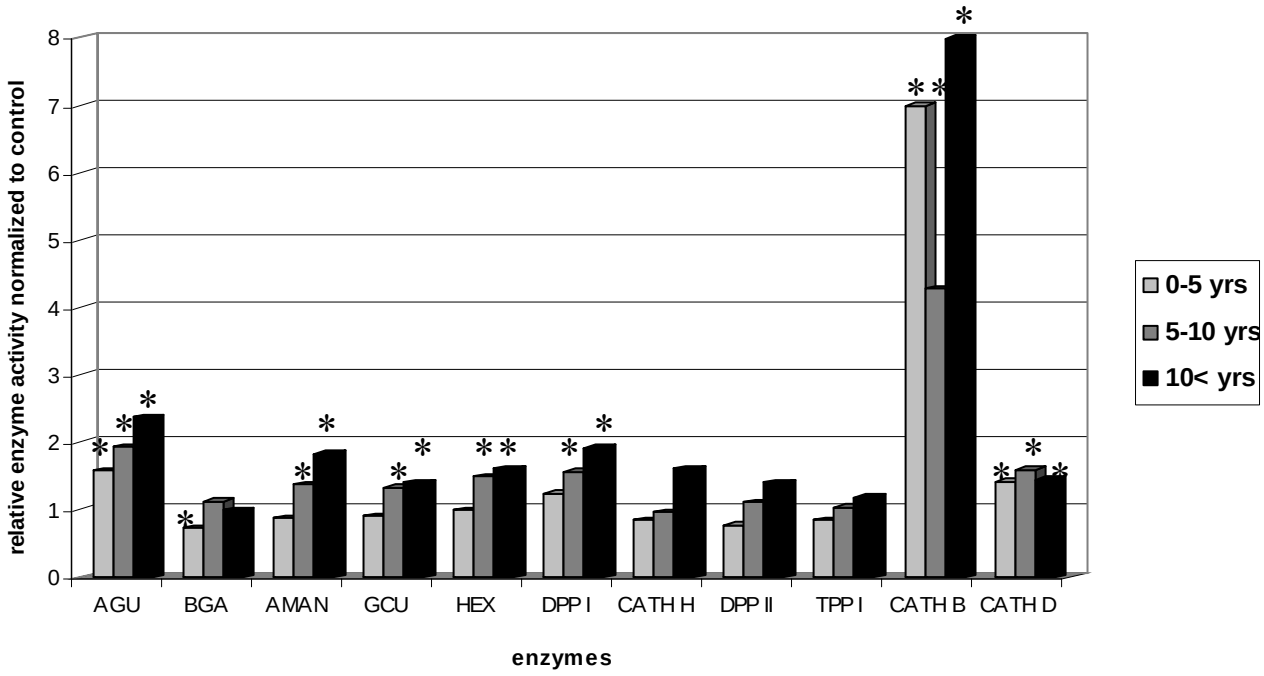
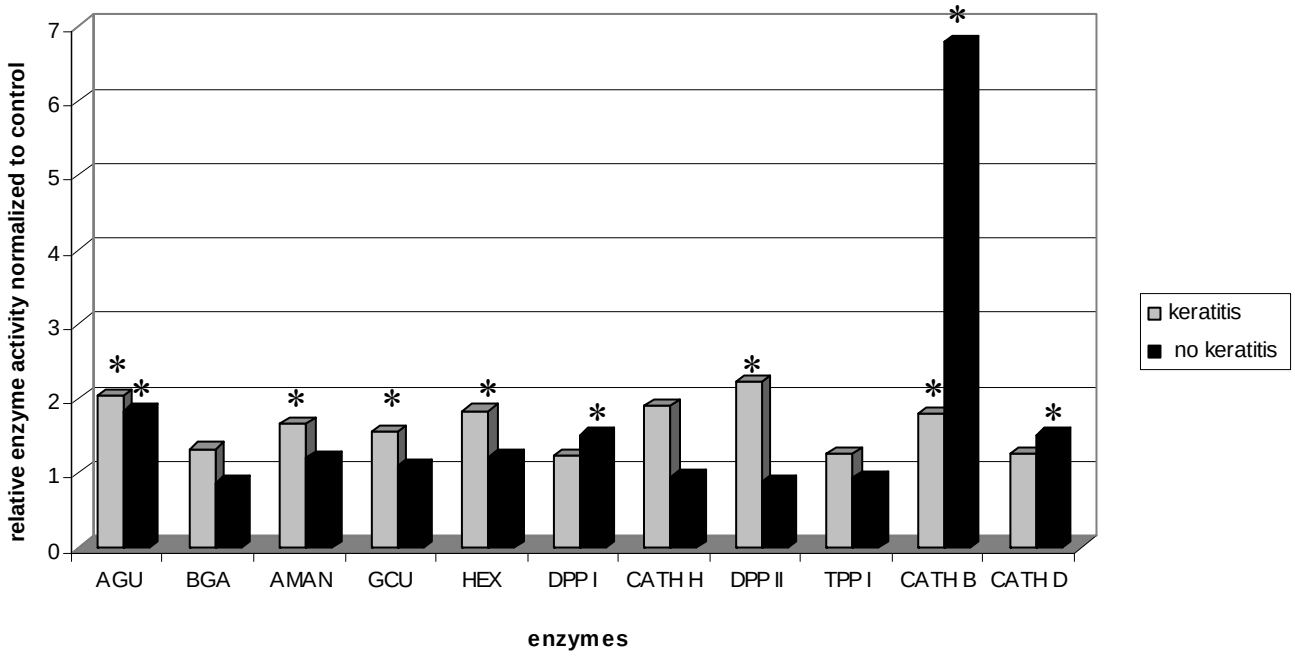


Figure 5. Lysosomal enzyme activities in patients with RA related to the presence of keratitis



6.2.3 Association between lysosomal enzyme activities and ophthalmologial signs of patients with RA

Figure 5 demonstrates the correlation between the presence of keratitis and the specific enzyme activities. All of the enzymes showed higher elevation among those patients who had keratitis, except for DPP I, CATH B and CATH D, while those patients who had any kind of corneal degeneration showed high elevation of CATH B (Figure 6).

Figure 6. Lysosomal enzyme activities related to the presence of corneal degeneration

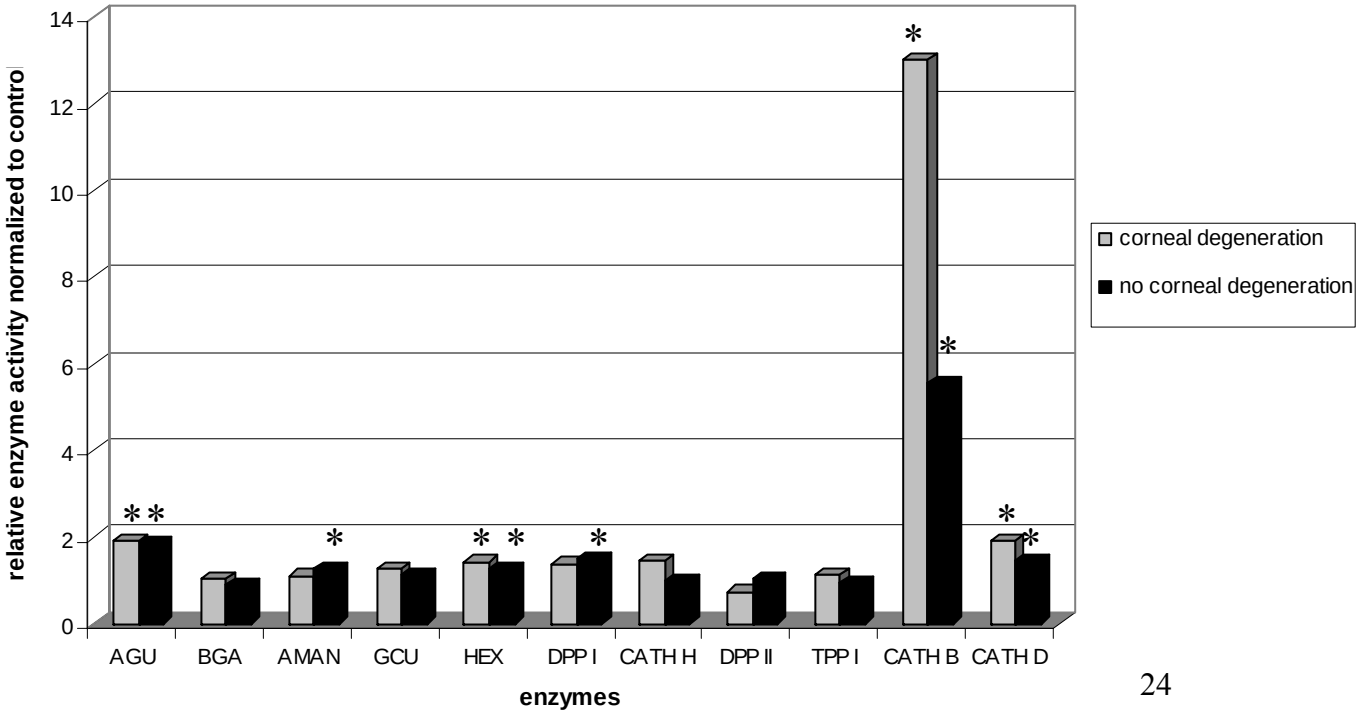


Table 5. Ophthalmic signs of the patients diagnosed with SLE

Patients	Duration*	KCS	Corneal degeneration	Conjunctival degeneration	Cataract	Angio-pathy	Atrophy of the optic disc	Perimetry
1	1	yes				yes		
2	1	yes						
3	1	yes					yes	
4	1		yes					
5	1	yes						
6	1	yes						
7	1	yes						
8	1	yes						
9	1	yes		yes				
10	1				yes	yes		
11	1	yes						
12	1					yes		
13	1		yes					
14	1	yes						
15	1						yes	
16	1	yes			yes			
17	2					yes		
18	2	yes		yes			yes	yes
19	2	yes				yes		
20	2		yes	yes		yes		
21	2						yes	yes
22	2	yes						
23	2	yes					yes	
24	2							
25	2				yes			
26	2							yes
27	3							
28	3				yes	yes		
29	3							
30	3	yes				yes		
31	3				yes	yes	yes	
32	3				yes			
33	3		yes	yes			yes	
34	3	yes						
35	3			yes		yes		yes
36	3							
37	3	yes						

* Group 1, patients who were sick for less than 5 years.
Group 2, patients who were sick for more than 5, but less than 10 years.
Group 3, patients who were sick for more than 10years.

Table 6. Lysosomal enzyme activities in the 3 groups of patients with SLE

	All patients					0-5yrs[&]				5-10yrs[&]				10yrs<[&]				control	
	average*	SE †	p	SS/ctr ‡		average*	SE †	p	SS/ctr ‡	average*	SE †	p	SS/ctr ‡	average*	SE †	p	SS/ctr ‡	average*	SE †
AGU	28.1	2.89	**0.000**	2.6		28.5	5.50	**0.000**	2.77	22.4	3.45	**0.000**	2.08	28.9	5.03	**0.000**	2.68	10.8	0.75
BGA	375.4	21.6	**0.000**	1.55914		404.9	29.5	**0.000**	1.48	400.2	52.2	**0.000**	1.66	351.7	43.0	**0.002**	1.46	241	15.2
AMAN	176.0	20.4	**0.000**	2.61119		137.4	34.6	**0.000**	2.59	130.0	25.1	**0.000**	1.93	211.3	41.3	**0.000**	3.13	67.4	5.77
GCU	350.8	29.9	**0.000**	2.05933		348.9	49.3	**0.000**	1.99	343.3	55.8	**0.000**	2.02	348.3	58.5	**0.000**	2.04	170	9.60
HEX	122.3	10.2	**0.000**	2.11743		106.2	16.7	**0.000**	2.03	119.6	17.2	**0.000**	2.07	123.3	21.4	**0.000**	2.14	57.7	4.04
DPP I	3638.6	470	**0.000**	2.92537		3204.9	727	**0.000**	2.53	3955.9	893	**0.000**	3.18	3764.8	913	**0.000**	3.03	1244	112
CATH H	27.4	3.70	0.169	0.77301		26.9	5.31	0.238	0.76	36.6	9.46	0.496	1.03	20.7	3.67	0.147	0.58	35.5	7.56
DPP II	40.4	4.20	**0.001**	1.72611		42.1	5.62	**0.031**	0.49	46.2	8.33	**0.001**	1.97	40.6	8.85	**0.013**	1.73	23.4	3.19
TPP I	313.8	24.6	**0.000**	1.85411		271.4	46.5	**0.000**	2.05	286.5	34.9	**0.007**	1.69	277.9	37.1	**0.018**	1.64	169	25.4
CATH B	46.8	6.41	**0.000**	2.20764		44.3	12.0	**0.000**	2.50	41.5	8.90	**0.000**	1.96	39.7	10.5	**0.003**	1.87	21.2	1.77
CATH D	1.0	0.12	**0.000**	3.23723		1139.5	0.17	**0.000**	3.29	1023.0	0.31	**0.000**	3.31	874.9	0.16	**0.000**	2.83	309	26.7

* Unit of enzyme activity: nmol substrate/h/mg
† SE: Standard Error
‡ sle/ctr: Values are normalized to the control
& duration of the disease

6.3. Systemic lupus erythematosus (SLE)

6.3.1 Ophthalmological signs of patients with SLE

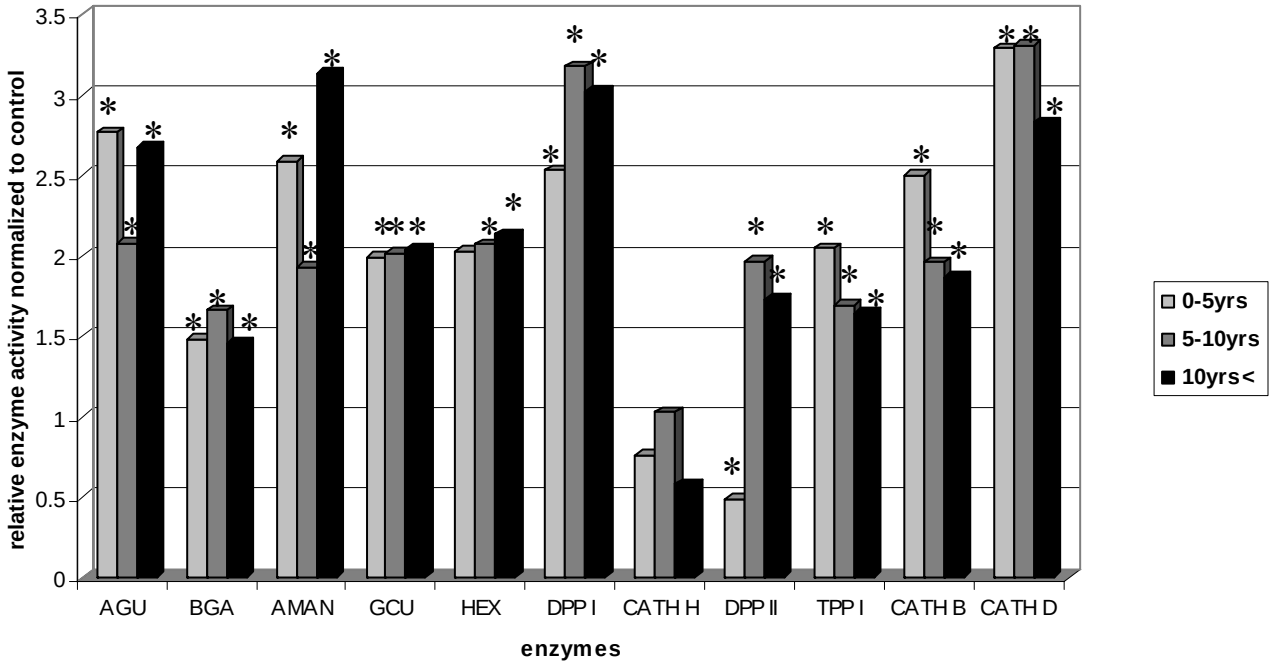
Table 5 presents the ophthalmological signs of the 37 patients investigated in this study. We found that 18 patients had KCS, 4 and 5 had corneal- and conjunctival degeneration, respectively, 6 had cataract, 10 had angiopathy of the fundus, 7 had atrophy of the optic nerve, 4 had visual field defect, and 1 had retinal branch artery occlusion.

Both optic neuropathy and retinal occlusive disease can result in optic atrophy. We investigated the occurance of the optic nerve atrophy in the three groups of patients. The number of patients with atrophy of the optic nerve was less (30%) in the third group (≥10 years) than in the second one (5-10 years) (40%).

6.3.2 Lysosomal enzyme activities in leukocytes of patients with SLE

The lysosomal enzyme activities in the leukocytes from the 37 SLE patients and the controls are shown in Table 6. Significantly increased activities of the glycosidases and proteases were found in all SLE patients as compared to the controls with the exception of CATH H.

Figure 7. Relative enzyme activities of leukocytes in patients with SLE concerning the duration of the illness

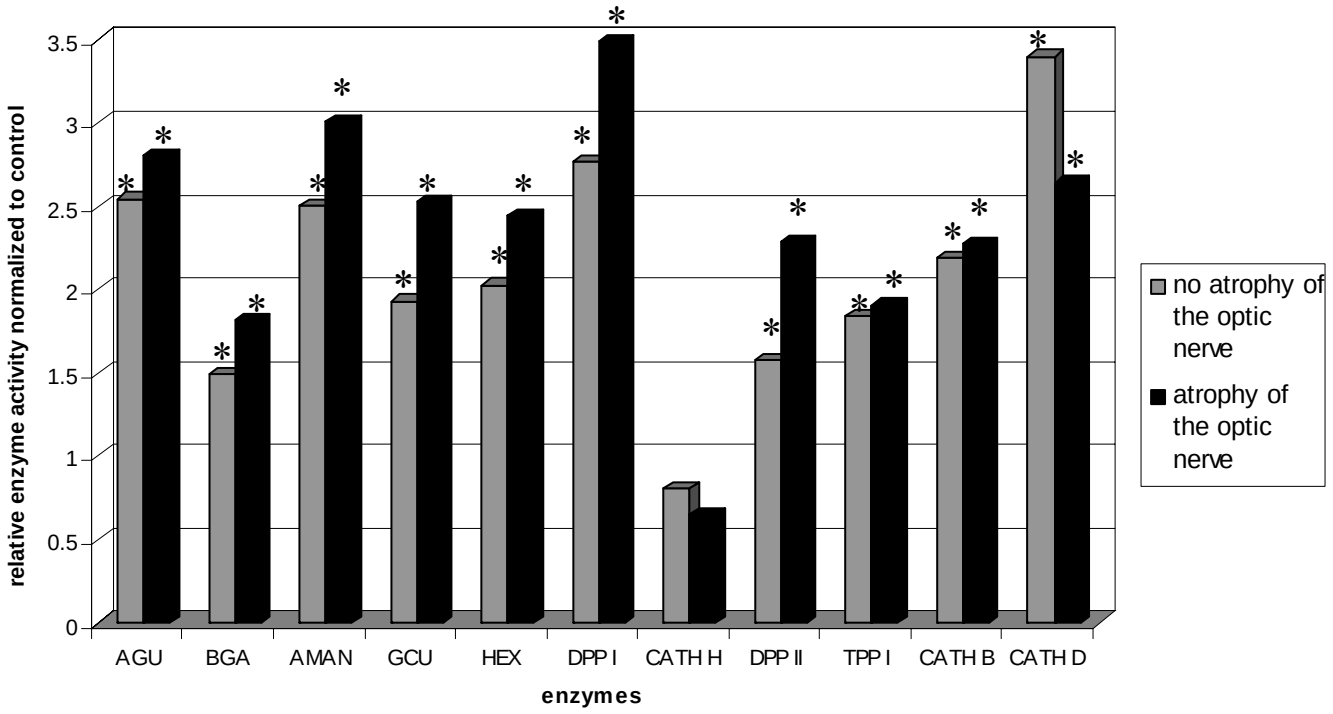


In all of the SLE patients, significantly increased activities of the glycosidases (AGU, BGA, AMAN, GCU, HEX), and the proteinases (CATH C, DPP I, TPP I, CATH B, CATH D) were found, as compared with controls ($p \leq 0.05$). Figure 7 shows the specific enzyme acivities in the three groups of patients, normalized (See *6.1.2.*) to the control activities. In cases of AGU and AMAN, the enzyme activities decreased in the second group (SLE was diagnosed more than 5, but less than 10 years prior to our investigations), and than increased again in the third group (≥10 years). In cases of GCU and HEX, the same amount of enzyme level elevation was shown in all three groups. TPP I and CATH B had the highest enzyme activities in the first group of patients, who were diagnosed less than 5 years before our investigations. BGA, CATH C, DPP I and II, and CATH D showed the highest elevation in the second group of SLE patients.

6.3.3 Association between lysosomal enzyme activities and ophthalmologial signs of patients with SLE

Figure 8 presents the correlation between the presence of atrophy of the optic nerve and the specific enzyme activities. All of the enzymes showed higher elevation among those patients who had optic nerve atrophy, except for CATH H and CATH D.

Figure 8. Lysosomal enzyme activities related to the presence of the atrophy of the optic nerve (disc)



6.4. Comparison of the results of the three polysystemic autoimmune diseases

Significant changes were found in specific lysosomal enzyme activities in leukocytes from patients of the three groups of investigated polysytemic autoimmune diseases compared to the healthy controls (Figures 1, 4, and 7). We found, that in the cases of patients with SS, the highest enzyme activity elevations were observed in the second period of SS (5-10 years). Whereas, most of the enzyme activities were lower in the third group of SS patients ($\geq$ 10 years) than in the second group.

In occurrence of RA, contrary to the results of SS, the increase in the enzyme activities was the highest in the third group (who were sick for more than 10 years) with the exception of BGA.

In case of patients with SLE, we found that the activity changes of lysosomal enzymes during the progression of the disease showed a high variety, although similar pattern in the elevation of activities of BGA, DPP I, and CATH H has been observed as in patients with SS.

7. DISCUSSION

7.1 Ophthalmological signs in patients of the three polysystemic autoimmune diseases

Many ophthalmological signs were seen even in the first groups of patients diagnosed with SS, RA, or SLE who were sick less than 5 years. Different signs were characteristic for the investigated three polysystemic autoimmune diseases, except for KCS which was the most frequent ophthalmological symptom in each disease. Most of the observed ophthalmological signs differ with the duration of the disease. Interestingly, less patients had corneal degeneration, cataract, atrophy of the optic nerve, and visual field defects in case of SS and atrophy of the optic nerve in case of RA and SLE in the third group ($\geq$10 years) than in the second one (5-10 years). The complexity of the autoimmune diseases and the different treatments that patients received make hard to explain this contradiction.

7.2 Changes in lysosomal enzyme activities

Lysosomal enzymes can degrade proteins, carbohydrates, nucleic acids, and lipids (132) and activate proenzymes to enzymes or prohormones to hormones by limited proteolysis. They have been implicated as playing a role in tissue injury and repair, inflammation and phagocytosis. All components of the connective tissue can be the target of metabolic disorders affecting either their biosynthesis or degradation (86). Therefore, lysosomes and lysosomal enzymes participate in pathologic processes such as inflammation (133), degeneration (83), and cancer (18-22).

Changes in lysosomal enzyme activities may result in impairment of phagocytic and endocytic activities, inadequate extracellular matrix turnover, and remodeling, which suggest that lysosomal enzyme activities might be involved in the pathogenesis of autoimmune diseases (82, 83, 134). Therefore, we supposed that activities of lysosomal enzymes might also show correlation with ophthalmic signs of the disease. In our study,

the activities of most of the investigated lysosomal enzymes in the leukocytes from patients with polysystemic autoimmune diseases (SS, RA, SLE) were elevated as compared with the healthy control group. The highest enzyme activity elevations were observed in subjects with SS of 5-10 years' duration. The activities of most of the enzymes assessed were also increased in patients with SS of >10 years' duration, but the elevation was less than in the previous group. Such a decrease in lysosomal enzyme activity after 10 years of the diagnosis of the disease may be explained by the slower anabolic pathway, including slower lysosomal proenzyme synthesis. The proenzymes are activated by themself or by other lysosomal enzymes with limitated proteolysis and play a role in the digestion of macromolecules in the lysosome. On the other hand, the slower ATP synthesis results in decreased energy level in the cell that is required for the proton pump maintaining the proper pH in the lysosomes. Similar processes have been described in starvation (135) and cancer (18-22).

Relatively little has been published on acid glycosidases in the pathogenesis of SS, and their roles in this disease is worthy of further study. We found that acid glycosidases, except for AGU, showed 2- to 3-fold higher elevation of activities than that of the control values. These were the greatest increases among the lysosomal enzymes measured in the leukocytes of individuals with SS. These enzymes play a role in catabolism of glycoproteins to monosaccharides and amino acids. The normal breakdown must be complete to avoid lysosomal storage diseases that can occur even when fragments as small as dimers are left undigested (136). Degradation of the oligosaccharide portion is accomplished by exoglycosidases, which act only from the nonreducing end of the carbohydrate chains to release sugar monomers as product.

In addition to previously described cytosolic or extracellular proteases, our results demonstrate that lysosomal proteases also play a role in the development of SS. All of the six enzymes studied were extremely hyperactivited in subjects with SS of 5-10 years' duration. The role of CATH B (105) and D are relatively well-known (117), but enhancements in the activity of the cysteine proteases DPP I (137) and CATH H are rarely seen. Recent studies of other research groups, however, have shown that DPP I is responsible for more than just proteolytic degradation in the lysosome. Loss-of-function mutations in DPP I result in early onset periodontitis and palmoplantar keratosis,

ostensibly resulting from effects on the integrity of the tissues surrounding the teeth and in the processing of proteins such as keratins (138). Furthermore, DPP I–deficient mice were found to have inactive chymotrypsin-like serine proteases in the granules of immune (cytotoxic T lymphocytes, natural killer cells) and inflammatory (neutrophils, mast cells) cells that are primarily involved in the defense of the organism (114, 136).

We experienced that the proteases with the most elevated activities in SS are DPP I and TPP I (Table 2). Previous study showed that TPP I plays a role in neuron metabolism (113), but together with DPP II is important in the terminal stage of intracellular degradation. Indeed, most of the tripeptide substrates of DPP II are likely generated by TPP I (112). The complementary specificities displayed by DPP II and TPP I support the theory of synergistic action by these exopeptidases. This action was proved previously in the breakdown of a model collagen A chain, poly(Gly-Pro-Ala) (132).

Some studies attempted to prove the role of lysosomal enzymes in RA using serum samples for the examination, but they have not found elevated enzyme activities (109, 124). We investigated the specific activities of lysosomal enzymes in leukocytes of RA patients and controls (Table 4) and found that the activities of two lysosomal cysteine proteases (CATH B and DPP I), two glycosidases (AGU and HEX), and an aspartic protease (CATH D) were elevated, but these elevations can not give information about the role of these enzymes in the progress of the disease. AGU, AMAN, GCU, HEX (glycosidases), and CATH D showed moderate increase of the activity. In contrary, the elevation of DPP I and CATH B activity was much more emphasized. They were the highest in the group of patients that were sick for more than 10 years. DPP II and TPP I and the cysteine protease CATH H activities did not change during the observation period, as we predicted by the substrate specificity.

Previous results of other research groups did not prove direct connection between lysosomal enzyme activities in leukocytes and activities in synovial fluid (102). The lysosomal enzyme activity elevation could have resulted from the increase of the number of leukocytes in blood, but this possibility was eliminated by the determination of the specific activity of the enzymes as normalized to the protein content (130).

Most autoantibodies increase during active period of SLE, but few prospective data are currently available to justify treatment on the basis of rising titers (15). Most of

the autoantibodies are not specific for SLE and might be produced non-specifically as a result of polyclonal B cell activation (68, 69). An ideal test is needed which detects (1) only those who have the disease, (2) all of the patients with it, (3) a high positive predictive value, and (4) high negative predictive value. Results of assays may reflect the activity of the disease, correlate with organ involvement, or predict relapse, allowing pre-emptive treatment (70, 71). All of the lysosomal enzymes showed significant changes, but there was no pattern like in cases of SS, and RA patients.

There was only one previous study which measured the lysosomal enzyme activities in leukocytes of patients with SLE (124). They investigated only GCU and found that the activity of GCU was decreased as compared to normal subjects. They also found that the severity of clinical SLE activity did not affect the degree of suppression of this enzyme. In contrast to the previous study (124), our investigations demonstrated that in cases of patients with SLE, all of the lysosomal enzymes being investigated showed significant increase except for CATH H. BGA, CATH C, and DPP I. showed the highest enzyme activity increase in the second group, which was similar in case of SS patients. The elevation of enzyme level in cases of GCU and HEX was similar in the three groups of patients. TPP I, CATH B and CATH D had the highest enzyme activities in the first group of patients, who were diagnosed less than 5 years before the investigation. The other enzymes did not show any characteristic pattern.

7.3 Relation between ophthalmic findings and elevated lysosomal enzyme activities

The reduction in tear secretion noted in subjects with SS of $\geq$10 years' duration was consistent with the aforementioned results, as the increased release of proteolytic and glycolytic enzymes in the lacrimal glands could cause degradation of the glandular extracellular matrix, thus leading to this effect (Figure 2).

Filamentary keratitis, a severe ophthalmologic sign in SS patients (131), was also seen mostly in subjects with SS of $\geq$10 years' duration. However, enzyme activities were higher among patients who did not have this ophthalmic disorder, except in the cases of AGU, CATH B, and CATH D (Figure 3). Thus, lysosomal enzyme activities in the

leukocytes of subjects with SS appeared to follow the state of the disease in the first 10 years. The relatively lower elevations of activities in patients with SS of ≥10 years' duration as compared to those who were sick for 5-10 years were not indicative of recovery from the disease, as evidenced by the relatively higher incidence of keratitis in this group (Table 1). Rather, it suggests that enzyme activity cannot increase any further because of the slower protein synthesis in this stage of the disease; a similar phenomenon is seen with late-stage starvation (135) and cancer (20-22). In our study, the mean age in the first group (ill for <5 years) was 50 years, and in the second (5-10 yesrs) and third group (>10 years), it was 57 years of age. The findigs of Florakis et al. (29) showed that the cupping and atrophy of the optic disc are a consequence of the disease and are not correlated with the age of the patients. We found atrophy of the optic disc only in the third group. Since there is not much difference between the mean age of the groups, we concluded in agreement with Florakis et al. (29) that this symptom is not related to the age of the patients.

In every individual with RA, we found significant correlation between the presence of keratitis and elevation of specific activities of the measured glycosidases, but BGA and DPP II. On the other hand, CATH B also showed elevated activity, but the elevation did not correlate with the observed keratitis (Figure 5). CATH B and CATH D showed significant elevation among those patients who had any other kind of corneal degeneration (Figure 6). Since only three patients had keratitis and also three ones had corneal degeneration, a study of larger populations is needed to confirm the prognostic importance and clinical relevance of this factor in RA.

KCS is the most common ocular manifestation of SLE (72), but visual morbidity is usually due to retinal and neuro-ophthalmic manifestations of the disease. Ocular manifestations of lupus are significant in that they reflect extraocular involvement of the disease (63). Their presence should alert the clinician to the likely presence of extraocular disease activity. Less patients had atrophy of the optic nerve (30%) in the third group (≥10 years) than in the second one (5-10 years) (40%). All of the enzymes showed higher elevation among those patients who had optic nerve atrophy, except for CATH H and CATH D. This means, that all of the glycosidases and the proteases, but CATH H and CATH D could show the progression of the disease in case of SLE.

8. SUMMARY AND CONCLUSIONS

- Significantly increased lysosomal enzyme activities (proteases and glycosidases) were found in leukocytes from patients who had been suffering from SS for more than 5 years, but less than 10 years.
- The changes in lysosomal enzyme activities indicate that these enzymes might play a role in SS-associated tissue injury. The activities of glycosidases, which are important in glycoprotein breakdown, and proteases, DPP I and TPP I, which play a role in proteolytic and in collagen degradation, were elevated.
- In the latter stages of SS (>10 years), the presence of ophthalmic signs may be a consequence of the long-lasting tissue injury caused by elevated lysosomal enzymes.
- We can conclude from our results that elevated levels of two glycosidases (AGU and HEX) and some lysosomal proteases (DPP I, CATH B and CATH D), but not TPP I or DPP II, are associated with RA. Lysosomal cysteine and aspartate peptidases may be responsible for the elevated level of protein destruction in RA.
- All of the enzymes showed higher elevation among those RA patients who had keratitis, except for DPP I, CATH B and CATH D, while those patients who had any kind of corneal degeneration showed high elevation of CATH B.
- Since CATH B showed the highest enzyme activity increase in the three groups of RA patients investigated, it may provide a reliable marker for monitoring the progression of RA.
- In cases of patients with SLE, there were almost the same number of patients with atrophy of the optic nerve in the second (5-10 years) and in the third group (≥10 years) and higher elevation of enzyme activities was found among these cases. All of the glycosidases and the proteases, but CATH H and CATH D show the progression of the disease.
- We suggest that the measurement of lysosomal enzyme activities in leukocytes and ophtalmic observations will be of great value for further

evaluation of these enzymes as a prognostic marker in polysystemic autoimmune diseases and tissue damage.

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