SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

CERTAIN ASPECTS OF PATHOGENESIS, ORGAN MANIFESTATIONS AND CLINICAL COURSE OF PRIMARY SJÖGREN'S SYNDROME

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The Examination takes place at the Discussion Room of Department of Infectious Diseases and Pediatric Immunology, Faculty of Medicine, University of Debrecen at 12 AM, 13th of October, 2014.

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The PhD Defense takes place at the lecture hall of Augusta Center, Faculty of Medicine, University of Debrecen at 2 PM, 13th of October, 2014.

1. INTRODUCTION

Primary Sjögren's syndrome (pSS) is one of the most common systemic autoimmune diseases. The Division of Clinical Immunology of the University of Debrecen is one of the largest tertiary referral centers in Hungary for systemic autoimmune diseases where more than 1100 pSS patients are followed up regularly under the leadership of my supervisor Prof. Dr. Margit Zeher.

The pathogenesis of the disease is still not fully understood, but there is no doubt that it is a multifactorial process, in which autoantibody production, antigen-specific autoreactive T cells and consecutive autoimmune cascades damage the target tissues.

My work focused on certain factors (endocrine dysfunctions, sex hormones and vitamines) which may potentially influence the disease pathogenesis and the clinical course. The long-term retrospective analysis of our large patient population allowed me to assess the clinical and laboratory characteristics of pSS thoroughly, and to determine risk groups with unfavorable prognostic factors. The practical use of our research is the determination of targeted diagnostic protocols and therapeutic approaches.

1.1. Epidemiology, definition, subgroups

The prevalence of pSS is 1-3%, the yearly incidence rate 3-6 per 100,000 persons. It develops mostly in females during the fourth and fifth decades of life (the ratio of male to female is 1:9). Based on the definition of American-European Consensus Conference (AECC), pSS is an autoimmune epithelitis characterized by the lymphocytic infiltration of exocrine glads and other epithelial srtuctures. There are two clinical groups of primary Sjögren's syndrome, based on the absence or presence of extraglandular manifestations (EGMs).

1.2. Etiology and pathomechanism

PSS has a multifactorial origin and both intrinsic (organizational) and extrinsic (environmental) factors play role in the disease development. In susceptible individuals, trigger factors (hormonal changes, external environmental factors, viral infection) lead to the appearence of autoantigens on epithelial surface, which results in the "homing" process of autoimmune inflammation generating cells and the lymphocytic infiltration of certain tissues (e.g. exocrine

glands). The autoimmune inflammation leads to the destruction of the involved tissues and consequential loss of function.

The susceptibility genes (such as human leukocyte antigens [HLA-B8 - DW3, -DR3, -DQA1*0501], genes encoding chemokines, cytokines and transcription factors) increase the risk of the development of pSS and can influence the clinical picture and the immunological profile, as well. Autoimmune processes can be triggered by infectious agents (EBV, CMV, HTLV-1, HHV-6, -8, Candida, Tropheryma, Streptococcus) via conserved molecular structures (PAMP = pathogen-associated molecular patterns), molecular mimicry and activation of epithelial cells.

The homing process is directed by the adhesion molecules (e.g. E-cadherin) displayed on the surface of immune-competent cells and endothelium, and by the chemokines and their receptors (e.g. CXCL family members). In pSS, the typical histological picture of minor salivary gland biopsies includes infiltration of mononuclear cells, formation of ectopic germinal centers, lymphoepithelial lesions and salivary gland destruction. The infiltrating lymphocytes are predominantly CD4+ T-cells and B-cells, plasma cells, macrophages, natural killer (NK) and dendritic (DC) cells.

Epithelial cells induce homing process by the expression of adhesion molecules and integrins, lead to antigen presentation and T-cell activation by the expression of costimulatory molecules (HLA-DR, -B7, CD40), produce chemokines, cytokines, B-cell activating factor (BAFF), moreover, trigger their own destruction by apoptosis regulator BAX protein production. Cell surface autoantigens appearing during apoptosis lead to the break of immune tolerance.

The dysfunction of dendritic (both myeloid and plasmacytoid) cells leads to the loss of peripheral tolerance by contributing to the persistence of autoreactive T cells, production of pro-inflammatory cytokines, enhanced antigen presentation and activation of inflammatory cells.

During the activation of infiltrating CD4 + T cells and DCs, proinflammatory cytokines, such as interleukin (IL) -1, -6, -7, -10, interferon (IFN)-γ and tumor necrosis factor (TNF)-α are released. In early stages, T-helper (Th) 2, later, Th1 cytokines dominates.

The alteration in the proportion and function of regulatory T cells [CD4+ CD25+ Tregs, IL-10 producing T regulative type 1 (Tr1), transforming growth factor (TGF)-beta producing Th3] may also play an important role in the disease pathogenesis.

IFN-α, by stimulating the BAFF production of epithelial, DC and T cells, leads to the generation of autoreactive B cells, and autoantibody producing plasma cells.

The overexpression of B cell lymphoma apoptosis regulator gene (bcl)-2, BAFF and APRIL (a proliferation-inducing ligand) provides the apoptosis resistance of cells and prolonge their survival.

The characteristic autoantibodies are anti-Ro/SS-A and anti-La/SS-B, which are directed against the complex ribonucleoprotein antigens physiologically located in cytoplasm. Their presence increases the production of apoptosis-inducing TNF-α thereby autoantigen expression and autoantibody production as well. Antibodies against alpha-fodrin and M3 muscarinic receptors can also occure. The latter has parasympatholytic effects and inhibits the transport of aquaporin channels to the apical cell surface which leads to the dysfunction of exocrine glands.

The role of sex hormones in the pathogenesis of pSS arises from female dominance and a number of experimental observations (presence of estrogen receptors in the salivary glands, sialoadenitis of ovarectomized mice, B-cell lymphoma in estrogen-deficient mice, estrogen deficiency-dependent apoptosis, autoimmune exocrinopathy, and prevention of glandular epithelial apoptosis and development of sialoadenitis by estrogen substitution).

1.3. Clinical symptoms and diagnosis

The disease affects primarily the exocrine glands, leading to decreased lachrymal and salivary secretion, which can be accompanied by bilateral asymmetric parotidomegaly, dermatitis sicca, vaginitis sicca, tracheitis and bronchitis sicca, chronic pancreatitis, and athropic gastritis. The autoimmune inflammation of exocrine glands leads to structural damages and glandular dysfunctions. Besides the characteristic glandular symptoms, certain systemic symptoms, denoted as extraglandular manifestations (EGMs), can also develop in a subset of patients. Chronic fatigue, mild fever, weight loss and muscle pain can be parts of general symptoms.

Musculosceletal manifestation can be a symmetric, non-erosive, non-deformative polyarthritis, which affects dominantly the small joints, or mild myositis even without elevated serum muscle enzymes or alteration in electromyogram. Latter is characterized by focal lymphocytic infiltration in muscle biopsy. Raynaud’s phenomenon (RP) is often present in patients positive for anti-SS-A or anti-SS-B autoantibodies, beside other EGM. Cutan vasculitis may develop in limited or systemic necrotizing form. Small and medium-sized blood vessels affected dominantly, mononeuritis multiplex or polyneuropathy may manifest with the presence

of anti-SS-A, RF or cryoglobulinemia. Clinically, it is characterized by purpura, recurrent urticaria and ulceration; histologically, lymphocyte or neutrophil infiltration is typical.

Motility disorders of the esophagus and gastro-esophageal reflux disease (GERD) is partly due to the reduced saliva production and gastric acid neutralizing capacity. Indigestion and chronic gastritis are common features, which can be accompanied by presence of antibodies against parietal cells (APA), reduced levels of serum vitamin B12, gastrin and pepsinogen. Because of the gastric irritation, chronic cough, sore throat and dysphonia may occur. Based on our observations, celiac disease is more common in pSS than in the general population. The common points in the pathomechanism of PBC and pSS are the presence of periductal lymphocytic infiltration, autoimmune epithelitis and autoantibodies (anti-SS-A, SS-B, antimitochondrial antibody = AMA). Serum amylase levels may call the attention to chronic pancreatitis.

The renal involvement occurs mainly subclinical. The most common forms are tubulointerstitial nephritis and distal tubular acidosis. Its histological picture ranges from interstitial lymphocytic infiltration to fibrosis and tubular atrophy. In untreated cases, nephrolithiasis and chronic kidney failure may develop. Glomerulonephritis is a severe EGM, often associates with cryoglobulinemia or hypocomplementemia. Histologically, mesangioproliferative or membranous form appears. Autoimmune interstitial cystitis may also develope with frequent urination, perineal or lower abdominal pain.

The main central nervous system manifestations are aseptic meningitis, increased tendency to convulsion, encephalopathy, anxiety, depression and memory disorders. The frequent peripheral sensorimotor polyneuropathy is due to the medium-sized vessel vasculitis. The neuritis affecting trigeminal and optic nerve and the thin fiber neuropathy is rare.

Leukopenia and anemia can be a component of autoimmune disease or even a consequence of the treatment. The increased risk of non-Hodgkin's lymphoma (NHL) can be explained by chronic antigen stimulation, persistent, excessive B cell activation and resistance to apoptosis. The lymphoproliferative disease (LPD) is more common in the presence of persistent parotidomegaly, lymphadenopathy, splenomegaly, mixed cryoglobulinemia and hypocomplementemia.

At present, the diagnosis is based on 2002 American-European Consensus Group Criteria AECC). This includes both subjective and objective criteria of eye and mouth symptoms,

histological alterations in glandular biopsy (focal lymphocytic sialoadenitis), and the positivity for anti-Ro/SS-A and/or anti-La/SS-B antibody.

2. OBJECTIVES

The etiologic factors and processess involved in the pathogenesis of primary Sjögren's syndrome is only partially understood. The variety of clinical course of the disease suggests the possibility of different prognostic groups.

The main goal of our work was to determine the factors influencing the disease development, the clinical course and the outcome in a large number of patients, who were followed-up closely and regularly, with the following objectives:

1. By assuming a common pathomechanism (autoimmune epithelitis), we assessed the autoimmune thyroiditis associated cases of pSS. We defined different forms of thyroiditis with respect to:
    - the frequency and formation time compared to the development of pSS
    - the characteristic result of functional tests, the organ-specific antibodies and the histological evaluation
2. By determining the sex differences in circumstances of disease development, clinical course and immunoserological profile, we investigated the potential effects of sex hormonal background on immunological processes.
3. We determined the clinical characteristics of a large patient population with a special emphasis on sex, age and time of diagnosis in order to asssess the following:
    - the main types and freaquencies of extraglandular manifestations and associated diseases
    - the characteristic features and importance of immunoserological parameters
4. We revealed warning signs of pSS by assessing the exact time of development of certain clinical and laboratory conditions.
5. We determined the effects of clinical and immunoserological characteristics pSS patients on survival and mortality to discriminate high-risk subgroups.
6. We estimated mortality risk of the total group of patients and compared it to the total Hungarian population matched by sex and age.
7. We compared the mortality rates of subgroups of male and female with glandular and extraglandular manifestations.
8. We examined the immunomodulatory effects of fat-soluble vitamins in pSS.

3. PATIENTS AND METHODS

3.1. Patients

Patients were recruited from the Autoimmune Outpatient Clinic of the Division of Clinical Immunology of University of Debrecen, where they received regular follow-up and treatment. Before 2002, Fox RI criteria were used for establishing the diagnosis; after 2002, AECC were used; cases diagnosed before 2002 were revised according to AECC. Informed written consent was obtained from the subjects, and the study has been approved by the Ethics Committee of University of Debrecen. All experiments carried out were in compliance with the Helsinki Declaration.

3.1.1. Thyroiditis study

479 patients (449 female and 30 male; mean age 57.8 years, range 24–88) were enrolled in the study. During the study, the patients' treatments included artificial tears and intermittent nonsteroidal antiinflammatory drugs (NSAIDs) and, or low-dose corticosteroids.

3.1.2. Investigation of sex differences

In this retrospective study, 492 patients with pSS were involved (432 women and 60 men, female to male ratio 7:1). Our results were based on the data processed between 2000 and 2005. At the time of the diagnosis, the mean age of women with pSS was 46.85 (46.85±9.25) years while the men's was 47.83 (47.83±8.35) years. The mean time of the follow-up period was 8.8 years.

3.1.3. Long-term follow-up study on clinical and immunoserological features affecting disease outcome

In the present study, we collected all the patients who were diagnosed and followed-up regularly with primary Sjögren's syndrome between 1975 and 2010 at our center. From the whole group of these 1094 patients, we gained a random sample by using a systemic sampling method. After arranging their name in alphabetical order, we selected every second patient for the analyses. Consequently, the final population of our retrospective study consisted of 547 (487

women and 60 men, gender ratio: 8 to 1) patients with pSS. The mean follow-up period was 11.4 ± 6.2 years with a range 2 to 37 years.

3.1.4. Examination of the immunomodulatory effects of A-, D- and E-vitamins

Twenty-five [22 females and 3 males; mean age 56.4 (9.4) years] patients with pSS were enrolled in this study. Among patients with pSS, 18 had EGMs, whereas seven had only sicca symptoms. The distribution of EGMs of pSS patients were as follows: thyroiditis n=1; pulmonary involvement n = 2; myositis/myalgia n=3; polyneuropathy n = 4; vasculitis n=7; RP n = 9; and polyarthritis n = 12. The exclusion criteria included treatment with immunosuppressive/immunomodulant agents. A cohort of age- and sex-matched healthy individuals served as controls (n = 15). No patients or controls enrolled in this study had ongoing infections, either viral or bacterial. No subjects received vitamin A, D or E supplementation during the study period and within 6 months before enrolment. No patients or controls had fat malabsorption due to liver and gall bladder diseases, pancreatic diseases ‘maldigestion’ or celiac disease ‘malabsorption’.

3.2. Methods

3.2.1. Testing and diagnosis of thyroid diseases

Serum TSH (normal range: 0.4–4.2 mU/L), FT3 (normal range: 3.5–6.2 pmol/L), and FT4 (normal range: 9–23.2 pmol/L) levels were measured with radio-immune assay (RIA) by using Liaison FT3, FT4, and TSH kits (Di-aSorin) and read on a LIAISON analyzer (Di-aSorin). The criteria for overt hypothyroidism were an elevated serum thyrotropin (TSH) concentration and low serum free triiodothyronine (FT3) and free thyroxine (FT4) levels. The criteria for subclinical hypothyroidism were an elevated serum TSH concentration and normal serum FT3 and FT4 concentrations. The criteria for subclinical hyperthyroidism were a subnormal serum TSH and normal serum FT3 and FT4 concentrations. The criteria for overt hyperthyroidism were a subnormal serum TSH and elevated serum FT3 and FT4 levels. Seropositive with euthyroid status was defined as non-specific thyreoiditis.

Serum anti-thyroglobulin (TgAb) and anti-thyroid-peroxidase (TPOAb) autoantibody titers were measured with enzyme-linked immunosorbent assay (ELISA) (Hycor Biomedical

GmbH) and read with an ETI-MAX 3000 analyzer (Di-aSorin). Autoantibody titers were considered elevated if the TgAb antibody reading was >225 U/mL, the TPOAb was >35 U/mL, and the anti-SS-A and anti-SS-B was >10 U/mL. TSHRAb was measured using Western blot technique. Recombinant human TSHR, purified with affinity chromatography served as antigen. TSHRAb values of >5U/mL were considered positive.

Thyroid ultrasound was performed on 95 patients with thyroid dysfunction. In HT, the typical ultrasonographic appearance is diffuse glandular enlargement with a homogeneous, but coarsened parenchymal echo texture, generally more hypoechoic than normal thyroid parenchyma. Fibrotic septations may produce a pseudolobulated appearance of the parenchyma. In GD, the borders are shaded; the structure is diffusely echo-poor due to the presence of edema. Glandular enlargement and highly echogenic bands and spots due to fibrosis have also been described.

Fine needle aspiration (FNA) cytology had been performed as part of routine care on 42 consenting patients who presented with focal abnormalities such as thyroid nodules or cysts. The clinic's practice was to perform thyroid surgery and subsequent histopathological examination for patients with thyroid nodules greater than 3 cm, thyroid enlargement with substernal extension, or positive FNA for malignancy. The FNA findings of HT were diffuse lymphocytic and plasma cell infiltration, occasionally ectopic germinal center formation, parenchymal atrophy, small round thyroid follicles, multinuclear giant cells, and the destruction of the basement membrane. The thyrocytes were polygonal, mostly with oxiphyl, granular cytoplasm and a large hyperchromatic nucleus. In GD, follicular hyperplasia, multifocal lymphocytic infiltration and rare lymphoid ectopic germinal centers were found. The majority of infiltrating lymphocytes were T cells, while B cells were much less common than in HT.

The Mann-Whitney test was used to analyze the data. All computations were performed using the statistical package SPSS for Windows version XP (SPSS Inc., Chicago, IL). Since the data distributions were not normal for most parameters, the median, minimum, and maximum values are reported. P Values less than 0.05 were considered statistically significant.

3.2.2. Investigation of sex differences in pSS

All the patients had sicca symptoms, which included keratoconjunctivitis sicca, xerostomy, enlarged parotis, submandibular, or sublingual glands. For functional measurements of exocrine glands, we used Schirmer's test and measured break-up-time (BUT) by supravital

staining of the cornea; also, for salivary function assessment sialometry was performed. In most of the cases, histological analyses of salivary gland biopsies were also performed.

Extraglandular manifestations, including arthritis, Raynaud's phenomenon, vasculitis, polyneuropathy, lymphadenopathy, myositis, pleuritis, and pericarditis were assessed and recorded; specific autoantibodies in the sera samples and histological diagnoses were evaluated. When polyarthritis was suspected, targeted imaging tests were carried out and inflammatory markers (ESR, CRP), rheumatoid factor (RF), anti-cyclic citrullinated peptide levels (CCP) was determined. In case of Raynaud's phenomenon, capillarmicroscopy was applied. In the presence of palpable purpura and ulcers, additional investigations, assessment of complement, cryoglobulin and anti-neutrophil cytoplasmic antibody (ANCA) levels and targeted biopsy was done to determine vasculitis. Polyneuropathies were identified by electroneurography (ENG) and n. suralis biopsy. The diagnosis of lymphadenopathy involved imaging tests, targeted biopsy sampling, and studies on immunoglobulins, paraproteins and cellular parameters. In myositis, electromyography (EMG) and histological tests; in serositis, (pleuritis, pericarditis), chest X-ray and echocardiography confirmed the diagnosis.

ANF positivity was detected by indirect immunofluorescence on HEp-2 cells. Autoantibody titers were assessed by enzyme-linked immunosorbent assay (ELISA) technique according to the manufacturer's instructions: anti-Ro/SS-A, anti-La/SS-B, ENA, anti-TG, anti-TPO (Hycor Biomedical GmbH), ANF, anti-dsDNA (Bio Systems), ANCA (Orgentech Diagnostica GmbH), and RF (Dialab Produktion). Anti-CCP autoantibodies were detected in serum samples using the Immunoscan- RA CCP2 ELISA test (Euro Diagnostica) according to the manufacturer's instructions. The autoantibody titers were considered elevated, if anti- SS-A and anti-SS-B >10 U/ml, ENA >8 U/ml, anti- TG >225 U/ml, anti-TPO >35 U/ml, anti-dsDNA >20 U/ml, RF >9 U/ml, anti-CCP >25 U/ml, and ANF staining was homogeneous or granular.

Prevalence of EGMs and immunoserological findings were compared with statistical analyses usually by Fischer's exact test and chi2 test of SPSS 13.0 software. Results under $p=0.05$ were considered as statistically significant.

3.2.3. Long-term follow-up study on clinical and immunoserological features affecting disease outcome

During follow-up visits, EGMs, associated diseases and immunoserological characteristics were recorded. The methods for determination of glandular and certain EGMs was

described earlier. The quantitative measurement of autoantibodies (anti- ENA, anti-SS-A/-SS-B, anti-CCP, anti-TG, anti-TPO, ANA, RF, and anti-DNA) was performed with enzyme-linked immunosorbent assay (ELISA) technique as described earlier. Immunoglobulin levels and complement activity were determined with turbidimetry and nephelometry techniques and haemolysis test in sheep red blood cell suspension, respectively.

Microscopic colitis (MC) is characterized by chronic watery diarrhea, a normal or near-normal gross appearance of the colonic mucosa. Beside clinical picture, the diagnosis of MC based the analyzed colonic biopsy specimens (specific histological picture includes crypt architecture distortion, inflammation, surface degeneration, presence and thickness of a sub-epithelial collagen band, intraepithelial lymphocytes, excess of eosinophils or neutrophils and the presence of apoptosis). Detection of lymphoproliferative disorders (LPD) is based on the presence of lymphadenomegaly and B-symptoms (fever, weight loss, night sweats) in addition to the results of imaging tests, targeted biopsy sampling and laboratory tests (hypersedimentatio, leukocytosis, immunoglobulins, paraproteins, β2-microglobulin, flow cytometry). In antiphospholipid syndrome (APS), the laboratory criteria are the presence of lupus anticoagulant (LA), IgG/IgM anti-cardiolipin (a-CL), anti-glycoprotein β2- (B2GPI) antibodies, the clinical signs are arterial and venous thrombosis and habitual abortions. The diagnosis of autoimmune hepatitis (AIH) can be confirmed by the presence of anti-smooth muscle (SMA) and antinuclear (ANA) antibodies, and the histological findings (marginal or bridging necrosis, portal/periportal lymphocytic infiltration, lymphoid follicle formation and cirrhosis). The diagnosis of sarcoidosis is based on the histological signs of non-caseating, epithelioid cell granulomas. Immune thrombocytopenic purpura (ITP) was characterized by purpura, thrombocytopenia, prolonged bleeding time, antibodies against platelet membrane glycoproteins (GPIIb/IIIa, GPIb/IX complexes) and megakariocytosist in bone marrow biopsy specimens.

The presence of each element and its changes in time were monitored in the whole study population. Data were evaluated by comparing well-defined subgroups (women/men, glandular/EGM, presence/absence of associated diseases, presence/absence of immunoserological differences, and patients alive at the end of the study/deceased over time). The SPSS version 20.0 was used for statistical analysis. To analyze the distribution of the data, Kolmogorov-Smirnov test was used. In cases of normal distribution, we determined mean ± standard deviation (SD) values and used two-sample t-test for statistical evaluation of the experimental data. In cases of

non-normal distribution, median, minimum, and maximum values were calculated, and Mann-Whitney test was used. Survival time and rate were assessed using Kaplan-Meier estimator. Chi-square test and Fisher's exact test were used to discriminate between patient groups; we used Cox-regression model to predict poor outcome of the disease. For comparison among patient and control groups, standardized mortality ratios (SMRs) were calculated. Differences were considered statistically significant at $p < 0.05$.

3.2.4. Examination of the immunomodulatory effects of of A-, D- and E-vitamins in pSS

Serum A and E as well as plasma 25(OH)2D3 vitamin levels were determined at the Department of Clinical Biochemistry and Molecular Pathology Laboratory of the University of Debrecen. Samples were taken after fasting overnight and analysed by HPLC using a Jasco HPLC system and Bio-Rad reagent kit. For detection and quantification, we used a diode array detector; for the detection of 25(OH)2D3 vitamin, wavelength was set at 265 nm, whereas for vitamins A and E the wavelength was set at 340 nm, which was switched to 295nm at the 4th min of the run.

In order to determine lymphocyte subpopulations (NK, NK-T, total T, CD4+ and CD8+ T and B cells) as well as activated T cells isolated from heparinized blood samples, monoclonal antibodies against cell surface markers CD3, -4, -8, -19 and -56 (BD Biosciences and Immunotech) were used. The expression of T-lymphocyte activation markers, HLA-DR and CD69, were also determined on CD3+ cells (BD Biosciences). The other antibodies used in this study were as follows: CD45RA-FITC/CD4-RPE (AbD Serotec) and CD45RO-FITC/CD4-RPE (Dako). The same was applied for CD8. Samples were processed according to the Coulter Q-PREP protocol and system. For the method of intracellular staining of CD4+ T-cell subsets, the following monoclonal antibodies were used: FITC-labelled anti-IFN-γ, PE-labelled anti-IL-4, PE-conjugated anti-IL- 10 (all from BD Biosciences) or PE-labelled anti-IL-17 (R& D Systems). Based on intracytoplasmic staining, the phenotypes within CD4+ cells were determined as follows: Th1 cells, CD4+IFNγ+IL-4-; Th2 cells, CD4+IFNγ-IL-4+; Tr1 cells, CD4+IL10+; and Th17 cells, CD4+IL17+. Cell surface (CD4, CD25) and intracellular (Foxp3) staining was carried out on freshly isolated PBMCs from heparinized blood by using an intracellular staining kit (eBioscience) according to the manufacturer's instructions. Lymphocytes were gated on the basis

of their forward and side scatter properties by using FACS Calibur flow cytometry (Becton Dickinson). Data were analysed using CellQuest software (Becton Dickinson).

Serum levels of IL-1, -2, -4, -6, -10, TNF-α, TGF-β and IFN-γ were measured by the corresponding BD OptEIA ELISA kits (BD Biosciences) according to the manufacturer's instructions. As part of the routine diagnostic evaluation, anti-SSA and -SSB autoantibodies were determined by indirect immunofluorescence (IIF) technique.

To analyse the data, the Kolgomorov–Smirnov test was used. In cases of normal distribution, we determined mean (SD) values and used a two-sample t-test for statistical evaluation of the experimental data. In the case of distributions other than normal, median, minimum and maximum values were calculated, and Mann–Whitney U-test was used. When the strength of the linear relationship between two variables was evaluated, Pearson's correlation coefficient was used, whereas in cases of non-normal distribution, Spearman's correlation coefficient was applied. The general linear model-repeated measures ANOVA analysis was used to evaluate the significance of changes in parameters over time. Differences were considered statistically significant at $p<0.05$.

4. RESULTS

4.1. Thyroid diseases in primary Sjögren's syndrome

Of the 479 patients with pSS, 95 (21.25%) met the diagnostic criteria for either HT, GD, nonspecific thyroiditis or nodular thyroid disease. Of the patients with thyroid disease, 30 patients (31.6%) had HT, 18 (18.8%) had GD, 10 (10.6%) had nonspecific thyroiditis, and 37 (39%) had nodular thyroid disease. Twenty-one of the last group had thyroid cysts and 16 had solitary thyroid nodules.

Almost all pSS patients having HT with overt hypothyroidism and subclinical hypothyroidism experienced fatigue and tiredness and about 70% (n=21) of this group had problems with concentration. Cold intolerance, dry skin, cold skin, bradycardia, and hair loss were common (57-74%). All of the HT patients (n = 30, 100%) had muscle and joint complaints, which were presumably the EGMs of the basic autoimmune disease. Only four patients with HT (13.33%) had constipation and weight gain and this was moderate. Myalgia and arthralgia was found almost in all patients. Hypotension was noted in more than half of the patients with HT (56.67%). The most common physical differences were the enlarged, sometimes nodular thyroid glands (n=24, 80%).

Twenty-eight of the thirty patients had positive tests for TgAb or TPOAb. In two patients with HT and goiter the HT diagnosis was made by the additional finding of a positive FNA for HT. TSHRAb tests were negative (<5U/mL) in all patients with HT and pSS. Positive tests for TPOAb and negative tests for TgAb were found in 14 of 27 patients with HT and pSS. In eight patients with HT (26.67%), the condition developed before the appearance of pSS. The mean period between the onset of the two diseases was 4.1 years. In 15 patients (50%), HT developed an average of 5.5 years after the onset of pSS. Simultaneous or nearsimultaneous appearance of HT and pSS occurred in seven patients (23.33%). Sixteen patients with HT had overt hypothyroidism and 13 had subclinical hypothyroidism. In patients with pSS and HT the onset of HT was most likely in their fifth decade, as in patients without pSS.

GD was diagnosed in 18 of the 479 (3.76%) of patients with pSS. Seven patients had overt hyperthyroidism and 11 had subclinical hyperthyroidism. The main symptoms of GD patients included fatigue (n=18, 100%), muscle weakness (n=15, 83.33%), weight loss (n=15, 83.33%), irritability (n=15, 83.33%), and heat intolerance with excessive sweating. Hair loss (n = 9, 50%) and diarrhea (n = 8, 44.44%) occurred in 40-50% of patients. GD did not start

simultaneously with pSS in any of the patients. In six of the patients with GD (33.33%), GD developed before the clinical appearance of pSS and in the 12 remaining patients with GD (66.66%) the development of GD occurred, on average, slightly more than 2 years after the onset of pSS. The peak age range for the onset of GD in patients with pSS and GD was between 55 and 64 years. Positive tests for TSHRAb (>5 U/mL) were obtained in 12 patients with GD.

4.2. Investigation of sex differences in pSS

The leading EGMs in both genders were polyarthritis, although its prevalence was higher in men than in women (68% vs. 42%). The second most frequent EGM in women was Raynaud's phenomenon (30%), while in men it was just the fifth (4.7%), similar to pulmonary fibrosis. Vasculitis (cutan vasculitis and polyneuropathy) was the third most frequent EGM in women (24%), while in men it was the second (15%). Interestingly, we found twice as frequently lymphadenopathy in men (10%) than in women (5%) but concerning myositis or kidney manifestations there were no differences between the two genders (8% vs. 7%, and 5% vs. 4%). We found a significant difference between men and women in the prevalence of polyarthritis ($p=0.0002$) and Raynaud's phenomenon ($p< 0.0001$).

Moreover, we registered Sjögren's syndrome associated diseases too. Depression and mood disorders in women were nearly four times more likely to occur than in man (33% vs. 23%). LPDs and sarcoidosis occurred in both sexes similarly small percentage (1-2%). Chronic pancreatitis and a nephrolithiasis developed in males and females with the same frequency (3% and 8%). Autoimmune thyroiditis and monoclonal gammopathies occurred only in female patients with a frequency of 7% for both condition). Collagen colitis (male: 5%, female: 10%) and autoimmune liver diseases (male: 1%, female: 2%), were presented twice as frequently in women than in men. Autoimmune thyroiditis was found to be specific to women, because no cases in men were found in contrast to the 7% occurrence in women. According to our observations, chronic pancreatitis represented in 3–3% both in men and women.

The most frequent antibodies were anti-SS-A, anti-SS/B, ANF, and ENA, while in some cases other specific autoantibodies such as dsDNA, ANCA, CCP, anti-TG, and anti-TPO associated with EGM were detected. In 25% of men with pSS, no detectable serum autoantibodies were found. There was a higher titer of ANF ($p=0.0002$) in women compared to

men, while anti-CCP occurred more frequently in men than in women (p=0.001). Cryoglobulinemia was detected in 3% of females and 7% of males.

4.3. Long-term follow-up study on clinical and immunoserological features affecting disease outcome

The mean ages at the time of pSS diagnosis were as follows: male: 47.55 ± 12.051 years, female: 49.99 ± 11.366 years. The mean follow-up period was 11.4 ± 6.2 years with a range from 2 to 37 years. There was no significant difference in the mean age at the time of pSS diagnosis and in the follow-up period between males and females. The mean age at the time of diagnosis of the 51 deceased patients was significantly higher than for the 496 patients still alive at the end of the study (55.35±12.038 years vs. 49.14±11.249 years, $p<0.001$, respectively), while there were no significant differences in follow-up periods between the two groups. Interestingly, we statistically confirmed that when at least one EGM was present, pSS was diagnosed 3.5 years earlier on average than in the glandular subgroup. We did not observe significant difference between the sex ratios and the presence of EGMs in patient groups stratified by age.

The three leading EGMs each affecting more than one quarter of patients were polyarthritis (48.1%), Raynaud's phenomenon (39.9%), and vasculitis (25%). The frequency of lymphadenopathy, myositis, pulmonary fibrosis, renal manifestations, and serositis ranged between 5.3% and 9.3%. With an incidence of 13.9%, thyroiditis was the most common associated disease. The occurrence of other associations (microscopic colitis, LPDs, APS, autoimmune liver diseases, sarcoidosis, and ITP) was lower than 4% in our study.

The onset of certain EGMs (e.g., polyarthritis, Raynaud's phenomenon, lymphadenopathy and pulmonary fibrosis) might precede the establishment of pSS diagnosis even with 5-9 years. APS and autoimmune liver diseases often preceded the onset of pSS, while sarcoidosis was characterized by later manifestation. Raynaud's phenomenon and serositis were manifested typically in the early phases of the autoimmune disease; a great proportion of cases were already present when the diagnosis of pSS is established. Vasculitis and renal manifestations usually developed after the diagnosis of pSS. The pSS predominantly occurred in perimenopause; additionally, in most cases, the investigated EGMs and associated diseases also developed in this time interval. As an exception, in some cases (lymphadenopathy, lung fibrosis, renal

manifestations, microscopic colitis, thyroiditis, and LPDs), incidence peaked between 40 and 49 years of age.

Approximately, 55%–75% of pSS patients were positive for ENA, anti-SS-A, ANA, and anti-SS-B or had hypergammaglobulinemia. RF positivity and hypocomplementemia occurred in more than 20% of cases, while more than 10% of patients were anti-TPO and anti-DNA positive. Other tested parameters (anti-TG, anti-CCP, and cryoglobulin) were positive in 6–9 percent of cases. Significant gender differences were found for eight investigated factors, six of which were predominant in women (Raynaud's phenomenon, thyroiditis, anti-SS-B, anti-DNA, anti-TG and anti-TPO), and two in men (polyarthritis, RF).

During the follow-up period, 51 patients (46 women and 5 men) died. Mortality of the whole patient population was almost 9%, with no significant differences between genders. Cardiovascular events (myocardial infarction, pulmonary embolism, and stroke) were the leading causes of death, being followed by solid tumours (bronchial, colorectal, and bladder carcinoma, as well as invasive ductal breast cancer and malignant melanoma). We compared our patients' data with sex- and age-adjusted data of general Hungarian population, based on the report of the Hungarian Central Statistical Office from 2007. According to our observations, pSS hardly affected the trend of cause of death in our patient population.

Calculated mortality per 1,000 individuals per year was as follows: in the Hungarian population adjusted for age and gender ratios of the whole pSS population 7.821 (based on the data of the Hungarian Central Statistical Office from 2001); in the whole pSS population 10.360; among female patients 10.495; among male patients 9.259. Additionally, calculated mortality per 1,000 individuals in the EGM subgroup (11.887) was two-and-a-half-fold higher than that in the glandular group (4.752). Standardized mortality ratios (SMRs) were also assessed in the whole pSS population, and separately for women and men, and for glandular and EGM subgroups as well, based on the data of the Hungarian Central Statistical Office from 2001 (whole patient population: 1.32; female patients: 1.49; male patients: 0.65; patients with EGMs: 1.62; patients without EGMs: 0.51).

Median survival time in the whole population was 33.71 years. Patients with pSS, complicated from the time of diagnosis with EGM or associated diseases, could be characterized with significantly worse survival ratios. This was also valid in the case of early occurrence of polyarthritis, vasculitis, and LPD. Late occurrence of cryoglobulinemia (even years after the

diagnosis of pSS) also impaired survival ratios significantly. In the above listed cases, estimated median survival time is obviously shortened, compared to patients with uncomplicated pSS, at the higher extent in the presence of LPD and vasculitis. Mortality risk in subgroups with significantly worse survival ratios increased 1.085–10.716-fold. An older age at the time of pSS diagnosis also increased the risk for death, numerically by 8.5 percent per year.The presence of vasculitis before the diagnosis of pSS resulted in the highest risk, while the lowest risk was associated with a younger age at onset of pSS.

4.4. Examination of the immunomodulatory effects of A-, D- and E-vitamins in pSS

Vitamin A concentrations in the plasma of pSS patients were similar to those found in healthy individuals. While comparing patient subsets, vitamin A levels in patients with EGMs were significantly decreased compared with those without EGMs (2.33 ± 0.46 vs 2.99 ± 0.43 mmol/l, respectively; $p = 0.005$).

The vitamin D levels were found to be similar in both the overall pSS patient population and controls (79.96 ± 37.87 vs 71.57 ± 23.01 nmol/l, respectively; $p = 0.408$). Also, no significant differences were found in vitamin D levels between patients with and without EGMs (82.41 ± 42.81 vs 73.00 ± 19.16 nmol/l, respectively; $p = 0.478$).

Vitamin E levels were significantly increased in pSS patients when compared with healthy individuals (41.41 ± 8.96 vs 33.68 ± 6.20 mmol/l, respectively; $p = 0.004$). Both subgroups of patients have significantly elevated levels of vitamin E compared with controls (pSS without EGMs vs control: 40.66 ± 6.44 mmol/l vs 33.68 ± 6.20 mmol/l, respectively; $p = 0.029$; pSS with EGMs vs control: 41.70 ± 9.92 vs 33.68 ± 6.20 mmol/l, respectively; $p = 0.010$).

Concerning vitamin A, we observed positive correlation with the percentages of both the NK cell ($R= 0.425$; $p = 0.038$) and Th17 cell ($R= 0.486$; $p = 0.025$) in patients with pSS. A positive correlation was observed between the vitamin E levels and the percentages of NK cells in the disease ($R= 0.416$; $p = 0.043$). Furthermore, positive correlation was found between the percentages of Th1 cells ($R= 0.445$; $p = 0.049$) and Th1/Th2 ratio ($R= 0.457$; $p = 0.043$) with vitamin E levels in pSS. In healthy individuals, we found no correlation between the plasma vitamins and the investigated cellular parameters. We found positive correlation between plasma vitamin E and serum IL-10 levels ($R= 0.717$; $p = 0.003$) in healthy individuals. Schirmer's test and sialometry results (objective measurements of sicca syndrome) were correlated with the

measured peripheral immune parameters. A significant negative correlation was observed between vitamin A levels and Schirmer's test values in patients ($R=-0.486$; $p = 0.035$).

5. DISCUSSION

5.1. Thyroid diseases in primary Sjögren's syndrome

As a result of contradictory findings in the literature, which were probably due to relatively small patient cohorts, we investigated almost 500 patients with pSS with regard to several types of thyroid disease. Our patient cohort was almost three times larger than the largest previously investigated pSS population. Besides the evaluation of the prevalence of thyroid diseases in pSS, we also investigated the time relation between pSS and two autoimmune thyroid diseases (HT and GD).

We evaluated all patients with pSS and found a higher prevalence of HT (6.26%) compared to the general population (1-3%). In 50% of the patients, the diagnosis of HT was secondary to pSS and followed it by 5.5 years on the average. Previous studies of HT in the general population indicated that the peak prevalence was between age 30 and 50 and the female to male ratio was 10:1–15:1. In the present study we noted that when HT occurs in pSS there is also a female preponderance and a similar age predisposition. Concerning GD, the clinical features, endocrinological, and immunoserological status does not significantly differ between patients with or without pSS.

Primary SS is a general disorder of immune homeostasis, leading to systemic manifestations. The disproportional immune activation and pathological immune responses, affecting both the cellular and humoral components of the immune system, contribute to the tissue injury in various organs. The major organs targeted by pSS are the exocrine glands, leading to sialadenitis and autoimmune epithelitis. We hypothesize that the thyroid, a tissue of epithelial origin, may also be affected by an autoimmune disturbance in pSS that is directed towards epithelial structures.

In summary, autoimmune thyroid disease occurs with increased frequency in patients with pSS and is likely to follow the onset of pSS. Therefore, periodic thyroid function tests should be performed in patients with pSS so that appropriate treatment can be started as early as possible.

5.2. Investigation of sex differences in pSS

Primary SS affects predominantly middle-aged women (pSS usually develops in women around menopause). The mean female:male ratio of genders is approximately 9:1. The female dominance and the late onset of pSS have been explained by the key role of sex steroids in the

pathogenesis. Recent findings confirm the influence of sex hormones on autoimmune diseases, resulting in the higher prevalence of these disorders in women than in men, also signified by the onset, usually after puberty and the variability of clinical symptoms, often associated with parts of the menstrual cycle and gravidity. Metabolites of estrogen especially hydroxylated forms increase the rate of B-cell differentiation, leading to increased autoantibody secretion and activation of T-cells leading to proinflammation cytokine production. The association of androgen oxidation pathway and autoimmunity with the protective role of male sexual hormones was suggested in pSS. More specifically, the driving factor behind pSS was assumed to be the lack of androgens. It has been shown that patients with pSS have low concentrations of circulating dehydroepiandrosterone sulfate compared to age-matched healthy controls. In the regulation of the neuroendocrine system, there are sexual hormones with stimulating effects such as prolactine; also, others have suppressive effects such as progesterone and androgens while estrogen harbors both features.

In the present study, we assessed the influence of gender differences in the development and characteristics of pSS. We found polyarthritis to be the most frequent EGM symptom in both genders, although the prevalence was higher in men than in women (68% vs. 42%). Raynaud's phenomenon presented in higher proportion in women, which assumes that sexual hormones have vascular effects. Vasculitis and polyneuropathy was the third EGM in women with 24%, while in men it reached second place with 15%. Interestingly, we could not find autoimmune thyroiditis, pneumonitis in men with pSS, which suggests that the immunopathological pathways in the thyroids or lungs are partly driven by female hormones. Although lymphadenopathy was found to be twice as frequent in men as in women, mono- or polyclonal gammopathy developed exclusively in women. The prevalence of B-cell type lymphomas or sarcoidosis was found to be similar in both genders. Primary biliary cirrhosis and autoimmune colitis were twice more frequent in women than in men, theoretically raising the possibility that a hormonal background may be part of the pathogenesis. The most frequent antibodies were anti-SS-A, anti-SS/B, ANF, and ENA, while in some cases other specific autoantibodies (such as dsDNA, ANCA, CCP, anti-TG, and anti-TPO) associated with EGMs were detected. Similar to our results, another study also found differences in EGMs in the serological profile. Our data correspond with their findings that both the frequency of Raynaud's phenomenon and levels of ANA and anti-SS-A were reduced in men. We found an increased number of polyarthritis associated with pSS in contrast to

results from this Greek group, which could be due to geographical and ethnic differences. Our results on thyroiditis, RF and cryoglobulinaemia correlated with Spanish observations.

Taking our findings together, we conclude that pSS is more frequent in women, yet it is also important to consider this disease in the male population. Since the prevalence of polyarthritis, vasculitis, and lymphadenopathy are more frequent in male pSS, it is important to focus on the EGMs in proper disease management. Also, by pinpointing significant differences between the two genders, novel therapeutic regimes can be designed in the future treatment of male pSS.

5.3. Long-term follow-up study on clinical and immunoserological features affecting disease outcome

In our retrospective study, we reported on research outcomes of 547 patients with pSS from one Hungarian clinical immunology centre. No other research on pSS patients with similar ethnical characteristics has been conducted in any East-Central European centre before. Compared to earlier publications, our pSS population was the third largest, but it was the first, when considering the patient proportion related to the whole population of the given countries. The spectrum of our study (the number of factors potentially influencing the clinical outcome and mortality) was broad, and we investigated more aspects than prior studies.The value of our observations is increased by the long follow-up period (11.4 ± 6.2 years, ranging from 2 to 37 years). In Greek publications, the proportion of female patients was high (women to men ratio = 16-22 : 1); in British and Chinese studies it follows internationally accepted rates, while, in Hungary, the proportion of men is higher than usual (women to men ratio = 8:1). The mean age of our patients at the time of pSS diagnosis is in line with the British values. In Southern Europe, the disease sets on 2–5 years later, while in China 8–10 years earlier.

The influence of the gender, the presence of EGMs and associated diseases on the time of pSS diagnosis, and the typical time intervals for the manifestation of clinical features were investigated only by our working group, leading to new findings. The diagnosis of pSS is established circa 2.5 years earlier in men, while in the presence of at least one EGM, it is made 3.5 years earlier, irrespective of the gender. In accordance with this finding, it is also noted that, in women, pSS begins more often with tolerable sicca syndromes, explaining the delayed seek for help, while in men, a severe EGM may occur as the first symptom. In our study, we evaluated

which EGMs and associated diseases, in which age groups, at what typical time intervals, and in what proportion occurred among our patients.

The three leading EGMs were polyarthritis, Raynaud's phenomenon, and vasculitis. The frequency of other EGMs ranged between 5% and 10%. When comparing our results to the literature data, we can conclude that, in the Hungarian population, the order of frequency for EGM is similar to what is seen in British people; the frequency of polyarthritis and vasculitis correlates with Chinese data, while that of Raynaud's phenomenon and pulmonary fibrosis corresponds to Greek data. Lymphadenopathy and renal manifestations occurred in a lower proportion among our patients; the incidence of myositis was higher than in the literature, while serositis developed in an approximately similar proportion.

With a few exceptions, EGMs tend to precede the onset of systemic autoimmune disease, as if anticipating it.The newly defined characteristic manifestation time intervals of each EGM draw attention to the importance of cooperation with related professions in conditions predicting pSS. Significant gender differences were found for two EGMs: polyarthritis with the predominance inmales and Raynaud's phenomenon predominating in females.The presence of EGMs enabled an earlier establishment of the diagnosis.

During many decades of care activity, we concluded that certain associated diseases worsen the course of pSS; therefore, we decided to analyse them with the method applied for EGM. The most common associated disease in our patients was thyroiditis, while incidence of other associated diseases was below 3%-4%. The percent incidence of thyroiditis in the Hungarian and British population was approximately the same, and in Chinese people it was more than twofold as compared to Hungarians, while in the Greek population no such investigation was performed. The incidence of LPD in Hungarian pSS patients was in line with Greek values. Autoimmune liver diseases occurred half as frequently in our patients, when compared to Greek, British, and Chinese data. Apart from our working group, APS was evaluated only by Chinese researchers, who found a two to three fold higher prevalence, compared to the Hungarian population. Our data regarding association with microscopic colitis, sarcoidosis, and ITP appear for the first time in the literature.

Differences in the prevalence of EGMs and associated diseases modifying the clinical picture, as compared to literature data, may be explained by the influence of different genetic, life style, and geographical factors. Our results regarding the distribution of associated diseases by

gender and age group and their influence on survival and mortality ratios are new, and no similar analysis has been performed earlier.

Significant gender differences were found for several serological factors. Anti-TG and anti-TPO positivity prevailed in females, while RF showed male predominance, in accordance with the observed clinical differences, and with the dominance of thyroiditis in women and polyarthritis in men. Cryoglobulinemia can be considered a highly relevant immunoserological abnormality, the emergence of which in the follow-up period of pSS significantly impairs survival ratios and increases mortality risk.

Summarizing our results, we concluded that pSS is composed of subgroups displaying a different clinical picture and mortality risk. During our work, we identified clinical and immunoserological features characterizing Hungarian patients. Based on significantly worse survival ratios and the concomitantly increasing mortality risk, pSS subgroups with polyarthritis, vasculitis, LPD, or cryoglobulinemia should be clinically classified as severe pSS. Consequently, we recommend the use of targeted diagnostic protocols for identifying patients with severe pSS. Moreover, close observation of cases associated with polyarthritis, vasculitis, LPDs, or cryoglobulinemia is also essential.

5.4. Examination of the immunomodulatory effects of A-, D- and E-vitamins in pSS

Disproportionate levels of vitamins A, D and E have been implicated in both autoimmune animal models and human autoimmune conditions. Since the fat-soluble vitamins (except vitamin D) and their immunoregulatory functions in patients with pSS have not been described previously, we aimed to measure levels of vitamins A, E and 25(OH)2D3 and assess their immunoregulatory role in these patients. The tissue or cellular accretion of these fat-soluble vitamins from plasma mainly depends on their plasma levels. In addition, intracellular metabolism, or disease states may also regulate the tissue or cellular uptake of these vitamins. The absorption of fat-soluble vitamins depends on many factors, of which the functional integrity of the intestinal mucosa is of key importance. Fat-soluble vitamins can influence the absorption of each other; e.g. carotenoids significantly impair alpha-tocopherol absorption, which can be a possible explanation of the inverse tendency between vitamin A and E levels in our results. The altered metabolism of these vitamins can also explain the increased vitamin E, and decreased vitamin A levels in pSS patients with EGMs.

The liver is a storage pool of fat-soluble vitamins from which, in case of increased needs, vitamin E can be released in higher quantities. The hepatic alpha-tocopherol transfer protein (α-TTP) stimulates the secretion and transfer of vitamin E from hepatocytes to circulating lipoproteins; therefore elevated α-TTP, due to a general systemic inflammatory response, may lead to an increased vitamin E level in the plasma. Vitamin A decrease can be caused by inappropriate intestinal absorption, leading to the deficiency of micronutrients, such as magnesium and zinc, having a strong influence on retinol-binding proteins (RBPs), causing disproportionate vitamin A levels.

Concerning vitamin A plasma levels in pSS patients with EGMs, which is indicative of a systemic, more severe disease course, we found significantly decreased vitamin A levels compared with patients with milder forms of the disease. We assume that reduced vitamin A levels and immunomodulatory activity may play an important role in the development of a generalized, more pronounced course of the disease. We found positive correlation between NK and Th17 cell percentages and vitamin A levels, which reinforces our hypothesis that vitamin A operates as a regulator of immune processes, therefore its reduction may contribute to disease propagation, or the development of more severe manifestations. Moreover, vitamin A has an important role in maintaining the functional integrity of epithelial and mucosal surfaces and in the production of mucous secretions. Disturbances in vitamin homoeostasis may contribute to the development of sicca syndrome in pSS, underscored by our correlational findings between plasma vitamin A levels and Schirmer's test, an objective indicator of secretory disorders.

Vitamin E levels were significantly increased in both subgroups of pSS patients, compared with those found in healthy individuals. Interestingly, we observed a positive correlation between NK, Th1 cells and the plasma levels of vitamin E, also the Th1/Th2 ratio showed positive correlation with vitamin E levels, indicating an ongoing immunoregulatory abnormality in patients, at least partly driven by disproportionate fat-soluble vitamin levels.

6. SUMMARY

Based on our analysis on a large patient cohort, we found significantly higher frequency of Hashimoto's thyroiditis (HT) among pSS patients compared to the general population. In most cases, the diagnosis of HT was secondary to pSS and followed it by 5.5 years on the average, and interestingly, we observed also a female preponderance and a similar age predisposition. The thyroid, a tissue of epithelial origin, may also be affected by an autoimmune disturbance in pSS that is directed towards epithelial structures. Therefore, periodic thyroid function tests and assessment of organ-specific autoantibodies should be performed in patients with pSS in order to start the appropriate treatment as early as possible.

Although, pSS is more frequent in women, it is also important to consider this disease in the male population. Based on our observations, polyarthritis (with a higher number of anti-CCP antibody positivity) and various vasculitis symptoms were more frequent in men, while Raynaud's phenomenon, autoimmune thyroiditis, pneumonitis and collagen colitis developed tipically in women. Behind the the different disease characteristics, we assume the immunomodulating effects of sexual hormones.

Regarding our study on the disease course and prognosis of pSS, we concluded that the disease is composed of subgroups displaying a different clinical picture and mortality risk. Based on significantly worse survival ratios and the concomitantly increasing mortality risk, pSS subgroups with polyarthritis, vasculitis, LPD, or cryoglobulinemia should be clinically classified as severe pSS and followed-up carefully. The three leading extraglandular manifestations (EGMs) were polyarthritis, Raynaud's phenomenon and vasculitis. Anti-TG and anti-TPO positivity prevailed in females, while RF showed male predominance, in accordance with the observed clinical differences, and with the dominance of thyroiditis in women and polyarthritis in men.

Our laboratory investigations revealed positive correlation betweens NK and Th17 cell percentages and vitamin A levels, which suggests that vitamin A operates as a regulator of immune processes, therefore its reduction may contribute to disease propagation, or the development of more severe manifestations. Additionally, disturbances in vitamin homoeostasis may contribute to the development of sicca syndrome in pSS, underscored by our correlational findings between plasma vitamin A levels and Schirmer's test, an objective indicator of secretory

disorders. Vitamin E levels were significantly increased in both subgroups of pSS patients, compared with those found in healthy individuals. Interestingly, we observed a positive correlation between NK, Th1 cells and the plasma levels of vitamin E, also the Th1/Th2 ratio showed positive correlation with vitamin E levels, indicating an ongoing immunoregulatory abnormality in patients, at least partly driven by disproportionate fat-soluble vitamin levels.

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List of publications related to the dissertation

1. **Horváth, I.F.**, Szántó, A., Papp, G., Zeher, M.: Clinical course, prognosis, and cause of death in primary Sjögren's syndrome.
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