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

Comorbidities in patients with idiopathic inflammatory myopathies

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 02nd of February, 2024 at 13:00

Introduction

1. Idiopathic inflammatory myopathies

Idiopathic inflammatory myopathies (IIM) are immune-mediated chronic inflammatory diseases, mostly affecting the proximal muscles, causing progressive and symmetric muscle weakness and muscle pain. Pain and weakness can often lead to immobility. There may also be characteristic skin lesions (dematomyositis), as well as extramuscular and extraskeletal involvement, such as pruritus, pulmonary involvement. The course of the disease can be various: monophasic, chronic progressive, relapsing, and remitting forms.

Both innate and acquired immune processes and non-immune mechanisms play an important role in the pathomechanism.

Several forms of the disease can be distinguished. Based on clinical, immunopathological and demographic features, the following subgroups are known polymyositis, necrotizing autoimmune myopathy, dermatomyositis, amyopathic dermatomyositis, juvenile dermatomyositis/polymyositis, inclusion body myositis. Recently, serological classification of the disease based on the presence of myositis-specific or myositis-associated autoantibodies in the serum of patients has been increasingly used.

To establish the diagnosis of IIM, we use the EULAR/ACR classification criteria published in 2017. Taking into account various factors, a scoring system helps to make the correct diagnosis; based on age, clinical signs such as muscle weakness, skin symptoms, dysphagia or oesophageal dysmotility, laboratory tests (anti-Jo-1 antibody, CK, LDH, GOT, GPT) and, if done, histopathological features of muscle biopsy.

Our primary aim in the treatment of the disease is to prevent acute muscle damage, joint contractures, and relapses, thereby improving the quality of life of patients. In addition to medication, we must not forget the important role of physiotherapy, which are necessary to increase muscle strength and maintain the range of motion of the joints. Several studies have shown the benefits of aerobic gymnastics combined with resistance training.

2. The association between the osteoporosis and idiopathic inflammatory myopathies

Osteoporosis is a common metabolic skeletal disorder characterized by decreased bone mass and deteriorated bone structure, leading to increased fracture rate. Since the average age and the proportion of elderly persons in the population is increasing continuously, osteoporosis and consecutive fractures have become a global public health problem with enormous ocioeconomic consequences. It is widely known that rheumatoid arthritis (RA) is one of the most important causes of secondary osteoporosis. Osteoporotic bone fractures are of crucial importance in the functioning and quality of life of patients. The pathogenesis of bone loss in autoimmune disorders is multifactorial: the increased serum and tissue levels of pro-inflammatory mediators, dietary factors (decreased calcium /Ca/ and vitamin D3 intake), decreased muscle mass/strength and functional capacity, immobilization, deficient intestinal Ca absorption, reduced levels of sexual steroids, avoidance of sunlight and use of sunscreens and, last, but not least, glucocorticoid (GC) use. The chronic GC exposure leads to increased bone resorption and reduced bone formation. In patients with RA, systemic osteoporosis coincides with local bone resorption, as a typical consequence of inflammatory synovitis. The pathogenesis of bone fracture in patients with IIM is multifactorial. The cutaneous manifestations of DM are extremely photosensitive; thus, most of the patients are educated to avoid of UVA and UVB and to use sun protection factors, topical sunscreens, which can lead to inadequate vitamin D3 supply. Immobility is also an important cause of bone loss, because decreased mechanotransduction increases osteoclast function and decreases osteoblast activity, thereby shifting bone metabolism from formation to resorption. In the long term, this leads to damage to the microstructure of the bone and bone loss and ultimately to increased fragility, resulting in increased morbidity and mortality. Inflammatory cytokines like IL-1, TNF-α, and IL-6 are elevated in the sera of IIM patients, which may stimulate osteoclastogenesis while decreasing osteoblastogenesis. Vertebral fractures are important, but under recognized

manifestations of osteoporosis. Most of them are asymptomatic, which makes their recognition more difficult, consequently, they might remain unnoticed for years.

The diagnosis of osteoporosis is based on the DXA examination, but nowadays there are more risk assessments to evaluate the fracture risk. The most widely accepted and used method in clinical practice is the Fracture Risk Assessment Tool (FRAX®) developed and validated by Kanis et al., more than 10 years ago, which estimate the 10-year probability of osteoporotic fractures. The FRAX score takes into account the relevant risk factors for a bone fracture, e.g., the presence of RA, but not myositis. During the calculation we can consider the patients' BMD or T-score results, but we can use the FRAX without these datas as well. In the original assessment the use of glucocorticoid is applied as a yes/no option, but in 2011 Kanis et al. developed the calculation with the steroid dose correction.

3. Pruritus

In the last decade, our knowledge about the molecular mechanisms of acute itch was greatly expanded; however, the development of chronic itch (>6 weeks) is not fully understood. The pruriceptive fibers of the skin are involved in the generation of itch, which, in response to endogenous and exogenous itch-inducing mediators, generate an action potential in the nerve endings of the skin (which is also involved in pain sensation) and then induce itching sensations through the activation of appropriate receptors via neurons in the dorsal root ganglia.

More recent research has shown that itching is not always triggered at the level of the nerve endings but can also be triggered by mediators produced by other cells in the skin (keratinocytes, immune cells).

4. The association between the pruritus and the systemic dermatological diseases, including dermatomyositis

The literature suggests that the following molecules may play an important role in the most common pruritus associated skin diseases: IL-31 and TSL in atopic dermatitis, IL-17, IL-22 and PPAR-γ in psoriasis, IL-33 in contact dermatitis and IL-31 in dermatomyositis. In dermatomyositis, questionnaires suggest that most of patients experience itching, which significantly affects their quality of life, and DM produces more pruritus than cutaneous lupus erythematosus and is worse than that reported for psoriasis, atopic dermatitis, Darier's disease, and vitiligo.

The clinical management of itching conditions is one of the biggest challenges of daily dermatological practice even today. The key molecules playing potential roles in the pathogenesis of acute and chronic itch in inflammatory skin diseases are endothelin-1 (ET-1), interleukin 31 (IL-31), interleukin 6 (IL-6), interleukin 17 (IL-17), interleukin 33 (IL-33), tumor necrosis factor α (TNF-α), thymic stromal lymphopoietin (TSLP), and peroxisome proliferator activator γ (PPAR-γ) that can activate directly or indirectly pruritic nerve endings. TNF-α was shown to demonstrate a critical role in acute and chronic itch in mice and targeting this molecule may be beneficial for itch treatment. Furthermore, in psoriasis, the PPAR-γ agonists have been recently shown to diminish pruritus.

The ion channels involved in the initial depolarization of sensory neurons are considered amplifiers of pruriception. The best studied of these ion channels mostly belong to the transient receptor potential (TRP) family of ion channels.

However, in DM, the connection between the sensation of itch and the roles of the TRP ion channels, TNF-α, PPAR-γ, and other pruriceptors was poorly examined yet and data from primary human skin samples are scarce.

AIMS

During our present cross-sectional study, we examined patients from our National Myositis Center, and we intended to answer the following questions:

The examination of the risk of fractures and prevalence of vertebral fractures:

1. What is the prevalence of low BMD, vertebral fracture, and high fracture risk in our patients with inflammatory myositis and RA?

2. Which factors are associated with higher fracture rates in myositis and RA patients?

3. How do the vertebral fractures influence the physical function and quality of life of patients?

The examination of dermatomyositis associated pruritus:

1. Is there a connection between pruritus and cutaneous disease activity of dermatomyositis?

2. Can previously identified DM associated cytokines and inflammatory mediators contribute to the development of itch?

3. Which pruritogenic gene expression pattern is associated with pruritus and disease activity in lesional DM skin compared to non-lesional DM skin?

Methods

1. The examination of the risk of fractures and prevalence of vertebral fractures

This scientific cross-sectional study was conducted on our own initiative, in 52 consecutive patients with myositis and 43 patients with RA under the care of the National Myositis Center, in the Division of Clinical Immunology, Faculty of Medicine, at the University of Debrecen between January 2017–June 2018. This study meets and follows all ethical standards of medicine. Informed consent was obtained from all the subjects. The eligibility criteria were the diagnosis of probable, or definitive idiopathic inflammatory myopathy (IIM) based on the Bohan and Peter criteria, and rheumatoid arthritis according to the 2010 American College of Rheumatology-European League Against Rheumatism (ACR-EULAR) classification criteria. The patients with confounders of bone health were excluded from the study: if the patient took any drug affecting bone mineral density (including bisphosphonates, thiazide diuretics, anticoagulants, anticonvulsants, glitazones, etc.) except for vitamin D3 and Ca, but including secondary osteoporosis and those patients suffering from malignancies.

In total, 121 patients were included at the start of the study, and finally 26 individuals were excluded based on the presence of exclusion criteria, or missing BMD and/ or FRAX data.

1.1 Laboratory test

Laboratory tests included the measurements of calcium, alkaline phosphatase, C-reactive protein (CRP), thyroid-stimulating hormone, serum total 25-OH Vitamin D levels and bone turnover markers (BTM): (parathyroid hormone, osteocalcin /OC/, beta-cross laps, C-terminal telopeptides of type-I collagen /CTX-I/).

1.2 Determination of bone mineral density

We measured the BMD of the lumbar spine (L1–4 vertebrae) and the left femoral neck by AP-DXA. The scan was performed with a DPX Pro bone densitometer (GELunar Radiation Corporation, Madison, WI, USA), according to the manufacturer's protocol. In patients with a history of a previous hip fracture, hip replacement surgery, or severe joint destruction, we measured bone mineral density in the right femoral neck. Osteoporosis was diagnosed according to the criteria proposed by the World Health Organization Study Group, when the BMD was 2.5 or more standard deviations below the young-adult mean, and osteopenia was diagnosed when the BMD was between -1 and -2.5.

1.3 Applied questionnaires

The FRAX, Health Assessment Questionnaire (HAQ) and Short Form 36 (SF-36) questionnaires were completed by a personal interviewer. The web-based algorithm at http://www.shef.ac.uk/FRAX® was applied as the FRAX® algorithm (version 3.6) adapted for Hungary. Special risk factors (age, sex, weight, height, previous fracture, parental hip fracture, current smoking, GCs, RA, secondary osteoporosis, alcohol 3 or more units/day, femoral neck BMD) were recorded into this calculator, for every single patient. The output was a 10-year probability of hip fracture and a 10-year probability of a major osteoporotic fracture (clinical spine, forearm, hip, or shoulder fracture). We measured quality of life including mental health with the SF-36 questionnaire validated for use in Hungary. The assessment of the patients' physical function was performed using the HAQ questionnaire.

1.4 Evaluation of thoracolumbal spine X-ray

To assess the prevalence of vertebral fractures patients underwent a bidirectional (antero-posterior and lateral) X-ray imaging of the thoracic (Th) and lumbar (L) spine on separate cassettes for each picture. To decrease probability of potential selection bias, all the patients were individually instructed to undergo X-ray examinations, on personally scheduled dates, and finally 40 myositis and 35 RA patients were able to complete the study. On standard radiographs the Genant's semi-quantitative assessment was used to evaluate vertebral fractures: vertebral shape (wedge, concave, or crush) and decreases in anterior, posterior, and/or middle vertebral height (grade 0: no reduction; grade 1: minimal fracture, 20–25% height decrease; grade 2: moderate fracture, 25–40% height decrease; and grade 3: severe fracture, greater than 40% height decrease).

1.5 Statistical analysis

Statistical analysis was performed with version 26 of the SPSS software package (IBM Corp., Armonk, NY, USA). The normality of the distributions in case of continuous variables was tested using the Shapiro-Wilk test. Normally distributed continuous variables were described by mean and standard deviation values (SD). Categorical variables were described using frequencies (case number) and percentages. For comparing the groups, we used independent samples t-test, or Mann-Whitney test depending on the distribution. The connection of two scalar variables was characterized by Spearman's correlation, while in case of binary variables we used Fisher's exact test. To identify the risk factors of vertebral fracture, we applied stepwise discriminant analysis (Wilks), also. The multivariate general linear model was applied to determine the factors that influence HAQ and SF36 results. P values of less than 0.05 were regarded as statistically significant.

2. The examination of dermatomyositis associated pruritus

This scientific cross-sectional study was conducted on our initiative, in 17 consecutive patients with DM under the care of the National Myositis Center, in the Division of Clinical Immunology, Faculty of Medicine, at the University of Debrecen, Hungary between February 2017 and September 2021. Written informed consent was obtained from all the subjects. The eligibility criteria were the diagnosis of probable or definitive dermatomyositis based on Bohan and Peter and/or 2017 EULAR/ACR classification criteria with active skin symptoms. Cancer-associated dermatomyositis or patients with other conditions associated with itch (cholestatic liver diseases, infections, chronic renal failure, and other systemic skin diseases) were excluded.

The global disease activity of DM was evaluated with the physician global visual analog scale (VAS), and the cutaneous activity was determined by the Cutaneous Dermatomyositis Disease Area and Severity Index (CDASI), which is a validated disease severity score of DM. CDASI activity score ranges from 0 to 100, and the damage score ranges from 0 to 32. Muscle weakness was assessed with a manual muscle test (MMT).

The severity of pruritus was assessed with a 5-D itch scale (ranging from 5 to 25). The 5-D itch scores were divided into three groups for analysis: no, or mild pruritus (score 5–10), moderate pruritus (score 11–15), and severe pruritus (score 16–25).

Laboratory tests included the serum level of creatine kinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST/GOT), glutamate–pyruvate transaminase (ALT/GPT), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and creatinine.

2.1. Skin biopsy

From the 17 DM patients, a lesional (consisting of erythematous patches) and a non-lesional site 4–4 mm punch biopsies were performed. The active lesional samples were collected from the upper region of the back in 14 patients, from the upper arm in two patients, and from the lateral upper thigh in one patient. Non-lesional samples were taken from the same anatomical

region without DM involvement. Then punches were cut into two sections, one for RT-qPCR (tissue homogenization, total RNA isolation, and quantitative real-time PCR) and the other one for immunohistochemistry.

2.2. RT-qPCR

Tissue homogenization was performed with 400Hz, 60 s × 4 times; one zirconium bead/tube in 800 µl of TRIzol reagent (Life Technologies Corporation, Foster City, CA, USA) using BeadBug homogenizer (Benchmark Scientific, Sayreville, NJ, USA). Total RNA was isolated according to the manufacturer's instructions and digested with recombinant RNase-free DNase-1 (Life Technologies) according to the manufacturer's protocol. After isolation, 1 µg of total RNA was reverse transcribed into cDNA using the high-capacity cDNA kit (Life Technologies, USA) following the manufacturer's protocol. Quantitative real-time PCR was performed on a LightCycler 384 well sequence detection system (Roche) by using a 5' nuclease assay. PCR amplification of the genes of interest (TRPV1, TRPV2, TRPV3, TRPV4, TRPM2, TRPM3, TRPC1, TRPC6, TRPA1, TNF-α, PPAR-γ, IL-6, and IL-33) was performed using specific TaqMan primers and probes using the TaqMan Gene Expression Master Mix Protocol (Life Technologies, USA).

2.3 Immunohistochemistry

The immunohistochemical investigation of IL-6 (Novus Biologicals, Littleton, USA), IL-33 (Boster Biological Technology, CA, USA), TNF-α (Sigma-Aldrich, MO, USA), and PPAR-γ (LifeSpan BioSciences, WA, USA) was performed on formalin fixed paraffin-embedded skin

samples taken from 15 patients diagnosed with dermatomyositis. The immunoreaction was completed on lesional and non-lesional and healthy skin areas originating from the same patient. Serial 4μm thick sections were cut from paraffin blocks, and hereon heat-induced antigen retrieval was performed on slides. Endogenous peroxidase activity was blocked with 3% H2O2 for 10 min. After blocking, tissue sections were incubated at room temperature with primary antibodies diluted in antibody diluent solution (DAKO, Glostrup, Denmark). Sections were then incubated with the EnVision FLEX Labeled polymer-HRP anti-rabbit and anti-mouse System (DAKO, Glostrup, Denmark) at room temperature for 30 min with 3,3'-diaminobenzidine (DAB) visualization techniques. Cell nuclei were counterstained with hematoxylin, and tissue sections were finally mounted in a permanent mounting medium (Histolab, Göteborg, Sweden). Normal skin sections served as positive controls for IL-6, IL-33, and PPAR-γ detection. The colon sample diagnosed as ulcerative colitis was used to validate TNF-α expression. Negative controls were obtained by omitting the primary antibody in all cases. The analysis of expressions was performed semi quantitatively with ImageJ 1.48v software (NIH). Statistical analysis and "dot plot" diagrams were performed. Every dot represents the staining intensity of one patient's skin sample.

2.4 Statistical analysis

Statistical analysis was performed using the SPSS 28 statistical software. The normality of distributions in the case of continuous variables was tested using the Shapiro–Wilk test. Data with normal distribution were presented as mean and standard deviation (SD) with minimum and maximum values, and data with non-normal distribution as the median and interquartile range (IQR). Categorical variables were described using frequency (case number) and percentage. Wilcoxon exact test was applied for gene expression changes. Kendall's tau was used with calculations based on concordant and discordant pairs. For the pairwise comparison,

Hochberg's correction was applied. For comparing the immunohistochemistry staining intensity, we used an independent sample t-test. In different itching subgroups, Kruskal–Wallis exact and Jonckheere–Terpstra exact tests were applied. During statistical analysis, a p-value of < 0.05 was regarded as statistically significant.

Results

1. The examination of the risk of fractures and prevalence of vertebral fractures

One hundred and twenty-one Caucasian patients participated in the study and fulfilled the inclusion criteria, while 26 patients were excluded based on the presence of exclusion criteria, or missing BMD, or FRAX results. The final myositis group consisted of 52 patients (9 males and 43 females, with a mean age of 57.46 years), while the RA group consisted of 43 patients (2 males and 41 females, with a mean age of 58.58 years). There were no significant differences between the two groups in the basic clinical data and laboratory parameters (including the mean BMD and total 25-OH Vitamin D level). The proportion of patients receiving oral Ca and vitamin D substitution did not differ significantly between the two groups (34 vs. 29 patients). We could not find any significant differences between the two groups in terms of other factors included in the FRAX® tool (previous fracture, parental hip fracture, smoking, glucocorticoids, alcohol consumption) except the presence of rheumatoid arthritis. In the myositis and RA groups normal BMD was found in 27 and 53.5%, respectively, whilst osteopenia was found in 60 and 39.5% of the patients, respectively, and osteoporosis found in 13.5 and 7% of the patients, respectively, and the difference in frequency of osteoporosis found to be statistically significant between the two groups (Fisher's exact test, $p = 0.045$).

The fracture risk assessment was calculated first without applying the BMD values. Regarding the other major and femoral neck fractures the fracture risk in RA patients was significantly higher than in myositis patients (15.58% vs. 9.68 and 6.23% vs 3.06%; $p = 0.008$ and $p = 0.022$). As a second step, the fracture risk calculation was repeated, and this time with the BMD values taken into account, with the earlier significant difference in the fracture probability disappearing (13.25% vs. 9.44 and 3.57% vs. 2.77%; $p = 0.053$ and $p = 0.811$). During the third step, the fracture risk assessment was performed after adjustment to the dose of glucocorticoids according to Kanis et al. With this correction the magnitude of the difference

further decreased: the risk of major osteoporotic and hip fractures were found to be 9.96% vs 9.54% ($p = 0.884$) and 2.46% vs. 2.87% ($p = 0.128$).

As previously mentioned, 75 patients underwent bidirectional vertebral X-ray examinations, 40 myositis patients (8 males and 32 females, mean age 60.97 years) and 35 RA patients (all female, mean age 59.71 years). Patients with myositis had significantly longer disease duration (13 vs. 7 years, $p = 0.021$) and higher cumulative steroid dose (17.6 g vs. 4.1 g, $p = 0.009$) (Table 2). Overall, 194 vertebral fractures were discovered in 54 patients (115 fractures in 30 myositis and 79 fractures in 24 RA patients), with these patients representing 75% of the myositis group and 68% the RA group, and the difference was not statistically significant. As a next step the myositis and RA patients were divided into two groups according to the presence of vertebral fractures. The mean age of the fractured patients was significantly higher in both groups using Mann-Whitney test (62.83vs. 55.4; $p = 0.034$ in the myositis group, and 63.25 vs. 52,0; $p = 0.022$ in the RA group), which was similarly found with stepwise discriminant analysis in myositis ($p = 0.042$), but age was not an independent predictor of vertebral fractures in the RA group. In addition, significantly lower lumbar and femur neck BMD were seen in fractured patients in the RA group (1.0 g/cm2 vs 1.19 g/cm and 0.83 g/cm2 vs 0.94 g/cm2; $p = 0.008$ and $p = 0.01$). The correlation of lower lumbar BMD and fractures in RA was further confirmed by stepwise discriminant analysis ($p = 0.001$), but with this method femur-neck BMD and age of the RA patients were not profound independent significant factors, according to their interdependency. The mean total 25-OH Vitamin D levels showed no correlation with the presence of vertebral fractures.

Finally, we investigated the influence of vertebral fractures on these patients' physical function and quality of life using HAQ and SF-36 questionnaires. It was found that the decrease in physical function and quality of life was proportional to the number of vertebral fractures if we analyzed the two groups together. In addition, female gender was significantly associated with poor SF-36 results ($p = 0.015$). The worsening of physical function was more pronounced

in the myositis group compared to the RA group (R = 0.457; p = 0.008 vs. R = 0.376; p = 0.041). Surprisingly, we could not detect any significant correlations regarding the SF-36 data of patients with RA, but in myositis patients and in the total patient group the number of bone fractures was strongly associated with poor SF-36 results. Furthermore, general linear model analysis showed that in the RA group the history of previous fractures (p = 0.024), and its cooccurrence with steroid treatment (p = 0.032) were significant factors for poor SF-36 results. The same results were found, if we examined separately the mental and the physical components of the questionnaire.

2. The examination of dermatomyositis associated pruritus

A total of 17 patients (six men and 11 women) participated in the study. The demographic, basic clinical, laboratory parameters, and relevant organ involvements. The mean age at the biopsy was 58.82 ± 9.78 years (43–76), and the disease duration was 46 months (20–78). Every patient received the standard treatment for DM, including glucocorticoids and/or immunosuppressive agents. The global disease activity was mild-moderate in most patients (mean: 2.85 ± 0.99 /range 1–4/), whereas the mean CDASI activity score was 26.24 ± 12.64 (8–49). Evaluating the pruritus sensation, the patient's median 5-D itching score was 8 (5–14). A total of 59% (10/17) of the patients experienced mild itch, 23.5% (4/17) moderate, and 17.65% (3/17) severe. The 5-D itch score correlated with CDASI activity (Kendall's tau-b = 0.571; p = 0.003), but not with CDASI damage score (p = 0.21). We could not detect a significant connection between the Global VAS and the 5-D itch score (Kendall's tau-p = 0.27).

In 17 patients with active dermatomyositis, targeted gene expressions of pruritogenic mediators or receptors were evaluated in lesional and non-lesional skin samples. The mRNA

levels of TNF-α were significantly higher in lesional skin samples than in non-lesional skin samples (Wilcoxon exact; p = 0.009). Furthermore, we could detect significantly different

results of lesional TNF-α mRNA levels (normalized to levels of non-lesional samples) in the subgroups of patients with different itch intensities (Kruskal–Wallis exact; $p = 0.038$); however, the normalized TNF-α levels did not correlate with 5-D itch score (Kendall's tau-b=0.127; $p > 0.1$).

The mRNA levels of IL-6 in lesional DM skin samples were not significantly different in comparison with the non-lesional levels (Wilcoxon exact; $p > 0.1$). However, lesional IL-6 mRNA levels correlated positively with 5-D itch and CDASI activity score (Kendall's tau-b = 0.585; $p = 0.008$ and 0.45; $p = 0.013$, respectively). The normalized mRNA expressions of the PPAR-γ were numerically decreased in DM skin; however, this was not statistically significant (Wilcoxon exact; $p > 0.1$). Furthermore, the normalized mRNA levels of PPAR-γ did not show a significant correlation with pruritus (Kendall's tau-b = −0.362; $p = 0.058$), and similarly, the levels were not different in the different itching intensity categories (Jonckheere–Terpstra exact; $p = 0.053$). Interestingly, the mRNA expressions of IL-33 were not significantly different between lesional and non-lesional skin samples.

The mRNA levels of TRP family members (TRPV1, TRPV2, TRPV3, TRPV4, TRPM2, TRPM3, TRPC1, TRPC6, and TRPA1) were not significantly different in the lesional and non-lesional samples, and we could detect correlations neither between any of the TRP channels and the 5-D itch score nor between any TRP channel and the CDASI activity score. In contrast, we could detect a significant positive correlation between the normalized mRNA levels of TRPV4 and CDASI damage score (Hochberg's correction; Kendall's tau-b = 0.626; $p < 0.001$).

In view of gene expression data, immunohistochemistry analysis was performed staining for TNF-α, PPAR-γ, IL-6, and IL-33. Histological analysis revealed subepidermal and perivascular inflammatory infiltrates in lesional samples of DM skin. The protein expression of the TNF-α was localized mainly in the cytoplasm of keratinocytes, dendritic, and endothelial cells; however, the semi-quantitative expressions of the staining were similar in the lesional and

non-lesional slides (independent sample t-test, $p > 0.1$). PPAR-γ was detected with various expressions in the dendritic and endothelial cells, in the epidermis, but there was no significant difference between the lesional and non-lesional samples. Regarding IL-6, we could detect strong cytoplasmic reaction in the epidermis, endothelial, and dendritic cells; furthermore, IL-6 was stained in the sweat glands and sebaceous glands; however, the staining expression was not different in the lesional and non-lesional areas. We detected a positive reaction in the nucleus of endothelial cells with IL-33, but there was no significant difference between lesional and non-lesional DM skin.

Discussion

1. The examination of the risk of fractures and prevalence of vertebral fractures

To our knowledge this is the first study, which investigates and compares bone fracture risks in IIM and RA, and the first work that correlates BMD, FRAX and vertebral fracture data of myositis patients with rheumatoid arthritis patients.

Basically, fracture risk assessed without taking BMD into consideration showed a greater risk of fracture in patients with rheumatoid arthritis than in myositis patients. If the BMD data were applied as well, it showed there was no longer any significant differences between the values of the two groups. This might support the argument for the lower BMD - which is more frequent in patients with myositis - counterbalances the "confounding" effect of RA as a risk factor in the FRAX tool. With an adjustment in FRAX according to the dose of glucocorticoids, the remaining non-significant differences were further decreased.

Taking into account the high prevalence of osteoporosis/osteopenia in the myositis group, it seems logical to consider incorporating a factor that modifies the FRAX tool and allows for a more reliable risk calculation in patients with myositis. Of course, this requires studies with a larger patient population and with bone fracture endpoints. In addition, it would generate a necessity for multiple, disease dependent modifying factor development according to other systemic musculoskeletal diseases (lupus, Sjögren's syndrome, vasculitis, etc).

We showed that the fractured patients were significantly older in myositis, but had lower lumbar BMD levels in RA. The occurrence of vertebral fractures in both myositis and rheumatoid arthritis were very common and seriously affected the patients' physical function and quality of life, especially in those with multiple fractures. It is interesting to observe that this effect was more pronounced in females and in myositis patients with regard to the HAQ results, and, surprisingly, the fractures did not significantly modify the health status of the RA

patients. This latter phenomenon could be explained by the frequent joint damage and secondary fibromyalgia seen in RA, which might bias the results of the questionnaire.

There were only a few studies in recent years aiming at the evaluation of bone health in patients with IIMs. In the different cohort studies, the prevalence of osteoporosis was between 13-27%, while the prevalence of osteopenia was between 7-75%. The presence of vertebral fractures was found between 11-75% of the patients. In a Brazilian case-control study, it was proved that osteoporosis was more frequent in female DM/PM patients than in controls measured by DXA in both lumbar spine and the femoral neck. Moreover, a high prevalence of fractures was found in patients in comparison to healthy subjects (17.9 vs. 5.1%, $p = 0.040$; OR = 3.92; CI 95%:1.07–14.33). In a large population-based retrospective analysis from Taiwan, the authors found that patients with DM/PM were 2.99 times more likely to develop osteoporosis than those without DM/PM. After a 13-year follow-up period, the cumulative incidence for osteoporosis in the DM/PM cohort was 5.35% higher than the incidence for the comparisons. Interestingly, the osteoporosis risk was independent of corticosteroids and immunosuppressant treatment. However, some essential data was lacking, including detailed demographic information on smoking habits, alcohol consumption, body mass index, socioeconomic status, physical activity, vitamin D deficiency, calcium/vitamin D supplements, and bone-strengthening medication. Data from a single center study from Hong Kong revealed that female gender, low serum albumin levels at onset, high Myositis Disease Activity Assessment Visual Analogue Scales (MYOACT) score, and high cumulative prednisolone dose were associated with lower BMD results. Similarly, during a long-term follow-up in a single UK center, patients with long-term prednisolone doses of more than 5 mg had a significantly shorter time to develop osteoporosis/osteopenia ($p<0.0001$) than those with less than 5 mg. In a more recent study by Gupta et al., in a relatively young cohort, asymptomatic vertebral fractures were present in nearly half of the patients. This was much higher than it was found from lupus patients from the same center, without ethnic and environmental differences; thus,

it seems plausible that a higher fracture rate is due to disease-specific factors. In their myositis population the median age and disease duration were shorter than in our population, and only patients with myositis were investigated. Despite the longer duration of the disease in our population the prevalence of fractures was not more frequent, therefore it is logical to speculate that the majority of fractures occur in the early phase of the disease, when the administration of higher corticosteroid doses is more frequent. The only available longitudinal data were recently published by this group. They found in the original, but a smaller patient population that the fracture rate increased from 46 to 61.29% after 3 years. In addition, those patients who had previous vertebral fractures had a higher risk of developing a new fracture when compared with those with no vertebral fractures (76.5% vs. 14.28%, RR: 5.35). The number of fractures correlated significantly with age, T scores at the L4 level, and lower third of radius on DXA, myositis damage index (MDI), and modified MDI, where osteoporotic fracture item in MDI was removed. Neither conventional nor disease related variables differed between progressors and nonprogressors. In the Indian study the most affected bones were the 11^{th} and 12^{th} thoracic vertebrae (30.4%), while in our population the 7^{th} and 8^{th} thoracic and 5^{th} lumbar vertebrae. This might be the consequence of different age or ethnicity of the two populations.

The possible limitations of this study should be acknowledged. This work was a single center study from a national myositis unit in Hungary, and the number of participants in the study was relatively low. The lower number of patients with vertebral X-ray examinations could be a cause for selection bias, and due to the cross-sectional nature of the investigation the calculated and the real fracture risks were not comparable.

Therefore, it can be concluded that both the prevalence and the risk of osteoporosis and fractures in patients with IIM are higher than in healthy individuals and that fractures significantly affect the quality of life. The results showed a good concordance with data of groups from different regions of the world, suggesting that the high fracture prevalence is a global myositis-dependent feature. Even in younger patients, asymptomatic fractures might

present in the early phase of the disease, and this could increase the risk of development of further fractures. Based on the results of our study, a national patient educational material, and a patient advisory card has been constructed, with an aim to increasing the patients' awareness and adherence to preventive pharmacological and non-pharmacological antiporotic treatments.

2. The examination of dermatomyositis associated pruritus

In the present study, targeted expressions of mediators and receptors, playing a crucial role in itch sensation, were assessed in a Hungarian cohort of DM patients. We can summarize our recent study as follows: (1) we confirmed that itch sensation was significantly associated with the cutaneous disease activity of DM determined by CDASI; (2) the mRNA expressions and immunohistochemistry analysis of the majority of examined pruritogenic mediators and receptors were not different in the lesional and non-lesional skin samples; and (3) TNF-α and IL-6 might contribute to the development of itch in patients with DM.

Dermatomyositis is one of the most obvious pruritus-associated conditions among systemic autoimmune diseases, involving the skin. In a study involving 191 DM patients, 90.6% of the participants had at least mild itch, and more than 50% had moderate or severe itch based on a visual analog scale. In addition, in another cohort, 84.6% of DM patients experienced pruritus, and pruritus produced an effect on daily living in the majority of the patients, even though the muscle disease was not active at the time of the survey.

Literature data about the pathomechanism of itch in DM are limited, especially investigations on primary human samples are scarce. It seems that cutaneous disease activity and consequent inflammation of the skin are associated with itch sensation, arguing for the direct relationship between disease pathogenesis and pruritus.

Our results demonstrated that the itch sensation of the patients was associated with the affected area and severity of cutaneous manifestations of DM (CDASI score) and not with the global disease activity or with CDASI damage score. These results are in line with previous literature reports and suggest that itch associates dominantly with cutaneous disease activity and not with global disease activity (including muscle, pulmonary, cardiac, and other scores) or cutaneous damage.

Tumor necrosis factor α is a key molecule in the pathogenesis of many systemic inflammatory diseases, and a wide range of well-known molecules are on the market for treatment, including TNF inhibitors. Our results, regarding the elevated TNF-α mRNA levels in affected DM skin, are arguing for an important role of the molecule in the pathogenesis of the diseases and consequently in pruritus. Furthermore, the levels of TNF- α were different in skin samples of patients with different itches; however, a direct association between normalized TNF- α levels and itching score was not detected. Elevated levels of expressions of TNF- α have been found in the serum and muscle fibers of patients with DM, which led to some pilot studies with TNF inhibitors for the treatment of DM. However, the effectiveness of these agents is unclear. In some studies, TNF- α inhibitors produced contradictory results and, in some cases, even worsened cutaneous disease. Furthermore, there are case reports demonstrating DM development under anti-TNF treatment for pre-existing conditions. In contrast, in a large cohort, 60 refractory juvenile DM patients had received at least 3 months of adalimumab/infliximab treatment and compared to baseline; there were improvements at 6 and 12 months in skin disease, including calcinosis.

The abovementioned literature data and our results might indicate that there could be some unknown factors determining disease subtypes with different pathomechanisms and thus the treatment effectivity of TNF inhibitors in skin-dominant DM patients. There are discrepancies between normalized mRNA levels and protein expressions of TNF-α in the skin samples. These

might indicate a post-transcriptional regulation of TNF-α in dermatomyositis, alterations in protein degradation by the ubiquitin system, or other unknown mechanisms.

IL-6 is a proinflammatory cytokine, whose expression in the serum of DM patients was significantly higher than that of normal controls. We could not detect elevated mRNA levels of IL-6 in lesional samples; however, normalized IL-6 mRNA levels were associated with 5-D itch and CDASI activity score, which argue for a significant role of IL-6 in inflammation and in DM-related itch sensation. Furthermore, the serum levels of IL-6 were significantly correlated with the disease activity of DM patients, indicating an important role of IL-6 in disease pathogenesis. The elevated levels of IL-6 were detected in patients with severe uremic pruritus in comparison with uremic patients without pruritus. Interestingly, in a recently published phase 2 trial, an IL-6 inhibitor tocilizumab was safe and well-tolerated but did not meet the primary or secondary efficacy outcomes in refractory DM and PM patients; however, only eight DM patients received tocilizumab, and the effect of the drug on pruritus was not presented.

There is evidence indicating the involvement of PPAR-γ receptors in the pathogenesis of skin diseases. The antidiabetic agents, glitazones, are agonists of the nuclear receptor PPAR-γ, which have anti-inflammatory and immunomodulatory properties. They are attributed to the downregulation of inflammatory interleukins (IL-1b, IL-2, and IL-6) and TNF-α. The advantageous property of PPAR-γ agonists on itching in various dermatological disorders has been reported by many clinical studies. PPAR-γ agonists, which are used in the treatment of diabetes mellitus, have been recently shown to diminish pruritus not only in animal models but also in patients suffering from psoriasis. We believe that PPAR-γ might play a negative regulator role in DM-related pruritus, but further experiments with higher patient numbers are required to determine this function in itching pathogenesis.

One of the key molecules, which could participate in DM associated itch, is IL-31. It was found to be upregulated in the DM skin and correlated with pruritus. Furthermore, small fiber neuropathies are thought to be a major cause of neuropathic itch. However, to our knowledge, there was no data in the literature about the role of the TRP family in DM-related pruritus. Based on our data, it seems that there is no direct connection between the itch sensation or cutaneous disease activity and the amount of different TRP channels in the affected skin since the mRNA levels were associated neither with any marker of disease activity nor with a 5-D itch score. In contrast, we could detect that the normalized expression of TRPV4 mRNA was associated with the CDASI damage score which might indicate that this channel participates in regenerating processes following cutaneous inflammation. This hypothesis is strengthened by the fact that TRPV4 activation accelerates barrier recovery, and the formation of intercellular junctions between keratinocytes since this channel is co-localized to adherent junction proteins such as E-cadherin and b-catenin.

The limitations of this study should be acknowledged. This study was a single-center study from a national myositis unit in Hungary; the number of participants was relatively low. The lower number of patients with mRNA examinations could be a cause for selection bias, and the determination of protein expression was performed using a semi-quantitative method. Furthermore, the patients involved in the study had background immunosuppressive medication, which might affect the results.

It can be concluded that itching sensation is a common and probably underestimated condition of DM patients, which is associated with cutaneous disease activity. It seems that the members of the TRP family are not contributing directly to DM associated itch, but TRPV4 might participate in skin regeneration processes. Our experiments argue for a determining role of TNF-α and IL-6 in DM-associated pruritus, and further experiments are required to study the role of PPAR-γ. We believe that our findings may help the development of new treatments for

DM associated itch, but further investigations are necessary to control this excruciating symptom.

Summary

In the present work we ascertained and compared fracture risk, the prevalence of vertebral fractures and their effect on patients' function and quality of life in patients with inflammatory myopathies and rheumatoid arthritis. Beside we investigated targeted gene expressions in active dermatomyositis patients to discover the specific molecular pathway in development of DM associated pruritus. We attempted to find correlations between the examined molecules, the disease activity and the severity of itch.

In our first study 52 patients with myositis and 43 patients with rheumatoid arthritis participated. We confirmed the higher prevalence of osteoporosis in patients with myositis. The risk of fracture, applying the FRAX calculation, was similar between the myositis and RA population if we took into account the BMD results and the steroid dose adjustment. The prevalence of vertebral fractures was common in both population (75-68%), which affected the patients' physical function and quality of life significantly. The fractures correlated with older age in myositis and lower BMD results in RA.

In our second study we examined 17 dermatomyositis patients with active skin symptoms. We confirmed the association between the severity of pruritus and the severity of DM associated skin lesions. The expression of TNF-α was significantly higher in lesional DM skin samples compared with non-lesional samples, and the normalized level of TNF-α mRNA expression correlated positively with the severity of pruritus. The lesional IL-6 mRNA levels correlated with the CDASI activity score and the pruritus. The expressions of the TRP ion channels were not different between the lesional and non-lesional skin samples, however the level of the mRNA expression of the normalized TRV4 correlated positively with CDASI damage score.

It can be concluded that the osteoporosis and the risk of fractures in myositis are very common, and they produce major burden of disease. Therefore, it is important to apply all the possible methods to maintain the bone heath from the diagnosis of myositis. The

dermatomyositis associated itch is affected by the disease activity. The TNF-α and the IL-6 might play a determining role, which may help to develope new treatments to control this excruciating symptom.

New scientific achievements

1. The prevalence of osteoporosis and the risk of fracture is very common in patients with idiopathic inflammatory myopathies, similarly to rheumatoid arthritis.
2. The calculation of the risk of fracture in patients with IIM we should applicate both the DXA results and the steroid dose adjustment to avoid the underestimation of the real risk.
3. The occurence of the vertebral fractures is also very common both in myositis and rheumatoid arthritis and they affect seriously the patients' quality of life.
4. The most significant factors which associated with fractures was the age in myositis and the lower BMD results in rheumatoid arthritis.
5. The pruritus of the patients with dermatomyositis is associated with the cutaneous disease activity.
6. The mRNA expressions of the TRP family members were not different in the lesional and non-lesional DM skin samples, thus probably they don't have determinig role in the pathomechanism of DM.
7. The TNF- α and the IL-6 might play an important role in the dermatomyositis associated itch according to their mRNA expression and their association with pruritus.

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Registry number: DEENK/432/2023.PL
Subject: PhD Publication List

Candidate: Anett Vincze
Doctoral School: Gyula Petrányi Doctoral School of Allergy and Clinical Immunology

List of publications related to the dissertation

1. **Vincze, A.**, Lisztes, E., Szabó, K., Béldi, T., Nagy-Vincze, M., Pór, Á., Varga, J., Dankó, K., Bíró, T., Tóth, I. B., Griger, Z.: Pruritogenic molecules in the skin of patients with dermatomyositis. *Front. Med. 10*, 1168359, 2023.
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   IF: 3.9 (2022)

2. **Vincze, A.**, Gaál, J., Griger, Z.: Bone Health in Idiopathic Inflammatory Myopathies: diagnosis and Management.
   Curr. Rheumatol. Rep. 23 (7), 1-11, 2021.
   DOI: http://dx.doi.org/10.1007/s11926-021-01016-8
   IF: 4.686

3. **Vincze, A.**, Bodoki, L., Szabó, K., Nagy-Vincze, M., Szalmás, O., Varga, J., Dankó, K., Gaál, J., Griger, Z.: The risk of fracture and prevalence of osteoporosis is elevated in patients with idiopathic inflammatory myopathies: cross-sectional study from a single Hungarian center.
   BMC Musculoskelet. Disord. 21 (1), 1-8, 2020.
   DOI: http://dx.doi.org/10.1186/s12891-020-03448-2
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List of other publications

4. Nagy-Vincze, M., Béldi, T., Szabó, K., **Vincze, A.**, Miltényi-Szabó, B., Varga, Z., Varga, J., Griger, Z.: Incidence, features and outcome of disease relapse after Covid-19 vaccination in patients with idiopathic inflammatory myopathies.
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6. Szabó, K., Bodoki, L., Nagy-Vincze, M., Béldi, T., **Vincze, A.**, Zilahi, E., Varga, J., Szűcs, G., Dankó, K., Griger, Z.: Clinical, Serological, and Genetic Characteristics of a Hungarian Myositis-Scleroderma Overlap Cohort.
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7. Szabó, K., **Vincze, A.**, Nagy-Vincze, M., Dankó, K., Griger, Z.: Multiplex tüdőtályoggal társuló súlyos polymyositis esete.
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