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Steroids in NeuroEndrocrine Immunology and Therapy of Rheumatic Diseases

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Abstracts

Session 1: *Sex hormones and aromatases in chronic inflammation and cancer*

1

The steroid cascade in NEI – an overview

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Cholesterol is the starting point of synthesis of steroid hormones and vitamin D. Thus, its uptake, storage, transport, and conversion into respective downstream hormones is of outstanding importance. Cholesterol is taken up into the cell by the cholesterol uptake receptor SR-BI. In adrenal glands, it is stored as cholesterol ester in vesicles that gives the organ its characteristic yellow color. Cholesterol ester is degraded by lipases on demand. Cholesterol ester is transported into mitochondria where it is converted into pregnenolone, the starting point of steroidogenesis. Pregnenolone is converted into a multitude of downstream hormones with the major pathways leading to mineralocorticoids, glucocorticoids, and adrenal androgens. In chronic inflammatory diseases, these pathways are altered so that the major pathway to glucocorticoids is up-regulated at the expense of adrenal androgens. Nevertheless, the amount of secreted glucocorticoids is inadequate in relation to systemic inflammation leading to relative insufficiency. In the animal model of experimental arthritis, we recently demonstrated mitochondrial defects in the chronic phase of the disease which most probably contributes to inadequate low levels of glucocorticoids in relation to inflammation. This presentation highlights defects and alterations in steroidogenesis. These alterations belong to an adaptive program positively selected for short-lived inflammatory episodes but not for chronic life-long inflammation.

2

Gender-dependent regulation and roles of the p200-family cytosolic DNA sensors: implications for sex bias in autoimmunity

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The development of certain autoimmune diseases in patients and mouse models exhibit a gender bias. Although studies implicated factors such as the X-chromosomal gene dosage effect and sex hormones (such as estrogen) in gender bias in the development of autoimmune diseases, the molecular mechanisms remain unclear. Development of autoimmune diseases and their progression involve immune dysregulation at the interface between the innate and adaptive immune systems. Accumulating evidence indicates that a defective clearance of cellular debris, which can activate innate immune responses, contributes to a loss of self-tolerance, autoantibody production (against nuclear antigens and DNA), and the formation of immune complexes (ICs). Several clinical manifestations of autoimmune diseases are believed to be the result of autoantibody and immune complex deposition in tissues and organs; thus, leading to secondary inflammatory responses and organ damage. Evidence indicates that activation of toll-like receptors (TLR)-dependent and independent innate immune responses, which result in increased production of type I interferon-α/β (IFN-α/β) and an increased expression of the IFN-inducible genes ("IFN-signature") contribute to the development of disease phenotype in certain autoimmune diseases (which include systemic lupus erythematosus or SLE). We have identified a mutually-positive regulatory feedback loop between IFN-α/β and the estrogen receptor-α (ERα) in immune cells. Further, our studies revealed that the expression of certain IFN-inducible p200-family proteins (such as human IFI16 and murine p202 and Aim2) that act as innate immune sensors for cytosolic DNA is differentially regulated by the sex hormones. Upon sensing the cytosolic DNA, the p200-family proteins either assemble an inflammasome or induce expression of type I IFNs through an activation of the STING/TBK1/IRF3 axis. Activation of the DNA-responsive Aim2 inflammasome promotes secretion of pro-inflammatory cytokines (such as IL-1β and IL-18). Further, increased levels of estrogen and IFN-inducible p202 protein in immune cells potentiate the production of type I IFNs and induce expression of the B-cell activating factor (BAFF) and Unc93b1 (a transporter of certain TLRs). In conclusion, our studies identified the molecular mechanisms through which the p200-family innate immune sensors for the cytosolic DNA contribute to sex bias in the development of certain autoimmune diseases.

3

Glucocorticoids, sex and life and death

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Glucocorticoids are necessary for life after birth and regulate numerous biological processes in man, including glucose homeostasis, protein catabolism, skeletal growth, respiratory function, inflammation, development, behavior and apoptosis. They are also one of the most prescribed classes of drugs in the world particularly for diseases involving inflammation. Interestingly, males and females exhibit distinct differences in the prevalence of many major diseases, including autoimmune disease, hepatocellular carcinoma, diabetes, and osteoporosis, which all have important inflammatory components in their etiology. These gender-specific diseases are largely considered to reflect the actions of sex hormones on the susceptibility to inflammatory stimuli. However, inflammation is recognized to reflect a balance between pro- and anti-inflammatory signals and glucocorticoids are the primary physiological anti-inflammatory hormone in mammals. Synthetic derivatives of these hormones are extensively prescribed as anti-inflammatory agents, irrespective of patient gender. We explored the possibility the sexually dimorphic actions of glucocorticoid regulation of gene expression may contribute to the dimorphic basis of inflammatory disease by evaluating the rat liver, a classic glucocorticoid-responsive organ. Surprisingly, glucocorticoid administration expanded the profile of hepatic sexually dimorphic genes. Pathway analysis identified sex-specific glucocorticoid-regulated gene expression in several canonical pathways involved in susceptibility to progression of diseases with gender differences in prevalence. These gender specific actions of glucocorticoids in liver were substantiated *in vivo* using a sepsis model of systemic inflammation.

4.

Estrogens metabolism and autoimmunity

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All players of the immune response including B cells, T cells, antigen-presenting cells and macrophages have different capacities to take up, to metabolize estrogens (called intracrinology) and to be modulated (1). Estrogen metabolism depends on transport into cells, desulfation of sulfated estrogens, sulfatation of non-sulfated estrogens, androgen aromatization, estrogen conversion to downstream hydroxylated or methylated estrogens. Furthermore, up- or downregulation of estrogen receptors alpha and beta (in cells or on the cell surface), of co-activators, and of co-repressors might well depend on involved cells and microenvironmental conditions such as accompanying hypoxia, local growth factors and proinflammatory cytokines. In addition, it has been demonstrated, but in one animal model, that 17β–estradiol accelerates immune-complex glomerulonephritis but may ameliorate focal sialadenitis, renal vasculitis, and periarticular inflammation (2). These data might suggest that different pathologies might even be present in the same human individual so that estrogens and/or their peripheral metabolites may have beneficial effects on one aspect of the disease but a different influence on other mechanisms. The different response most probably on involved cell types, concentrations and notably on peripheral estrogen metabolism rate and even by use of animal models to test hypothesis (3). Very recent studies also showed that the small change of estrogen or progesterone levels during therapy with oral contraceptives or hormone replacement therapy has variable power to increase the risk or severity of B cell-related autoimmune diseases (i.e. systemic lupus erythematosus or anticardiolipin syndrome), with more evident effects on overt disease. Moreover, estrogens can even stimulate several immune mechanisms at postmenopausal levels due to their bimodal role described (3). Interestingly, there are important similarities between chronic inflammatory diseases and inflammatory reactions in certain types of cancer such as breast cancer or prostate cancer. In inflammatory tissue of patients with RA, estrogen precursors are particularly converted into 16-hydroxylated estrogens, which are pro-proliferative and covalently bound to the estrogen receptor type alpha, whereas, generation of anti-proliferative 2-hydroxylated estrogens is blocked (4). Conversion can happen in synovial macrophages and fibroblasts. Similarly, macrophages in breast cancer tissue can convert precursor hormones to 16-hydroxylated estrogens with

a similar increase of estrogenic effects (5). Similar to precursor 17β-estradiol, the converted 16-hydroxylated estrogens can stimulate important growth factors such as TGF-β, basic fibroblast growth factor, keratinocyte growth factor, and angiogenesis all involved in both chronic inflammation (immune response) and cancer. As matter of fact, this is in agreement with the growth-supporting role of 17β-estradiol during pregnancy. In conclusion, in both chronic immune/inflammatory disease and cancer, in which overshooting growth responses play a decisive pathogenic role, the 17β-estradiol-stimulated increase of these growth factors is most probably crucial.

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5

Aromatase and endometriosis: estrogens play a role

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Endometriosis is an estrogen dependent inflammatory disease defined by the growth of endometrial stroma and glands outside of the uterus. In endometriosis, estrogens promote the growth and invasion of endometriotic tissue and prostaglandins play a crucial role in the mediation of pain, inflammation, and infertility. The proliferation and inflammation processes in endometriotic lesions are supported by stradiol (E2), the biologically active form of estrogen. It has been observed a significant correlation between local estrogen content of endometriotic lesions and the expression levels of the steroidogenic enzyme aromatase cytochrome P450. Since the late 1990s, several studies using either PCR or immunohistochemistry demonstrated the expression of aromatase P450 in both eutopic and ectopic endometrium from patients with endometriosis, but not in eutopic endometrium from disease-free women and in endometriosis-free peritoneal tissue. The primary substrate for aromatase activity in endometriotic tissue is androstenedion (adrenal and ovarian) that is converted in estrone, which is further converted to the more active E2. Aromatase is regulated at the levels of transcriptional expression, protein expression, and enzyme activity in endometriosis. It is involved in a positive feedback loop that favors expression of key steroidogenic genes. Estrogen stimulates expression of the COX-2 enzyme, resulting in elevated levels of prostaglandin E2 (PGE2), which is a potent stimulator of aromatase activity in endometriosis. This leads to continuous local production of E2 and PGE2 in endometriotic tissue. Over the last 10 years, aromatase inhibitors have been proposed for the treatment of endometriosis both in post-menopausal women and in subjects of reproductive age.

6

Aromatase and regulation of estrogens: androgens ratio in synovial tissue inflammation: a common pathway in both sexes

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Sex hormones play an active role in inflammatory response, with androgens being anti-inflammatory whereas estrogens are mostly proinflammatory, even if they can also act anti-inflammatory dependent on different criteria (1). Clinical evidences point out the influence of sex hormones on chronic inflammatory diseases: anti-inflammatory adrenal hormones are low in patients with autoimmune diseases compared to controls, and incidence of autoimmune diseases is much higher in women compared to men.

During chronic synovial inflammation, sex hormone metabolism is altered in inflamed joints, with low level of androgens and high levels of estrone in the synovial fluid of rheumatoid arthritis (RA) patients compared to controls (2). Of interest, no differences were observed between male and female patients, thus suggesting that local hormone metabolism during inflammation is not dependent on gender. Aromatase is the key enzyme for the conversion of androgens into estrogens, and it is expressed by differentiated synovial macrophages (3). Proinflammatory cytokines such as TNF, IL-1β and IL-6 have been found to stimulate aromatase activity in peripheral macrophages, therefore the inflammatory milieu during arthritis can stimulate androgen to estrogen conversion and sustain the inflammatory process in the synovium. Moreover, gonadal hormones such as dehydroepiandrosterone (DHEA), testosterone precursor, and testosterone inhibit aromatase activity: if the concentration of androgens is low, aromatase is expressed while if their concentration is high, aromatase is repressed (4). Considering that local androgen levels are very low in synovial fluid of RA patients compared to controls, this unbalanced hormonal concentration can also contribute to high aromatase activity in synovial tissue during arthritis.

Due to the key role of aromatase activity and sex hormone imbalance in the synovial tissue during arthritis, a clinical relevance for aromatase inhibitor (AI) in arthritis can be suggested.

However, clinical evidences show an increased occurrence of joint pain in patients undergoing AI therapy because of estrogen receptor positive breast cancer (5). Even if this evidence could seem to contradict aromatase effect on joint inflammation, it is important to point out that systemic aromatase blockade can influence mechanisms independent of sex hormone levels in the joint, such as melatonin pathway, thereby causing joint pain (6).

Another very promising hormone involved in aromatase activity during arthritis is vitamin D (Vit D). This steroid hormone was demonstrated to downregulate aromatase expression in human breast cancer cells and also in human RA macrophages (7). Moreover, Vit D decreases pro-inflammatory cytokine in human activated macrophages, thus suggesting also a direct anti-inflammatory role on cytokine production.

Taken together, these evidences suggest a key role of aromatase and sex hormone balance in the synovial tissue during chronic inflammation, and point out the importance of vitamin D as possible new tool for modulating aromatase pathway in arthritis.

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Selected Presentations on the topic

7

To compare the efficacy of norethisterone acetate (NETA; group N) or letrozole combined with NETA (group L) in treating endometriotic ovarian cysts

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Objective. To compare the efficacy of norethisterone acetate (NETA; group N) or letrozole combined with NETA (group L) in treating endometriotic ovarian cysts.
Study design. This patient-preference study included 20 patients in group N and group L, respectively. The primary aim of the study was to compare the volume of the endometriomas during and after treatment. The secondary outcome was the evaluation of the changes in pain symptoms during and after treatment.
Results. After 6-month of treatment, the volume of the endometriomas significantly decreased compared with baseline in both study groups; it was smaller in group L than in group N (p=0.026). The rate of satisfied patients at 6-month of treatment was similar between the study groups (p=0.451). No significant difference was reported between the two study groups in the amelioration of pain symptoms and in the incidence of adverse events.
Conclusions. Letrozole combined with NETA is more efficacious than NETA alone in reducing the volume of endometriotic cysts but in none of the 40 patients included in the study the endometriomas disappeared. However the efficacy of aromatase inhibitors should be balanced with the need to administer long-term treatment and the incidence of adverse events.

8

Pregnancy in women with monogenic and non-hereditary autoinflammatory syndromes: results from a retrospective multi-centric study

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Italy Autoinflammatory diseases (AIDs) are a group of inherited and acquired multifactorial syndromes sharing dysregulation of the innate immunity which includes familial Mediterranean fever (FMF); tumor necrosis factor receptor-associated periodic syndrome (TRAPS); Cryopyrin-associated periodic syndromes (CAPS), Familial Cold Autoinflammatory Syndrome (FCAS), Muckle Well Syndrome (MWS), Blau's syndrome (BS); Schnitzler syndrome (SS); Adult Onset Still disease (AOSD); Mevalonate Kinase Deficiency (MKD). Despite the childbearing age of many patients, few data about the reciprocal relationship between AIDs and pregnancy are available to date, due to the low incidence of AIDs and the low female-to-male ratio. All patients affected with AIDs who experienced at least one pregnancy and seen in three Italian referral centers (Rheumatology Unit, Department of Medicine, University of Padova; Rheumatology Unit, Policlinico Le Scotte, University of Siena; Rheumatology Unit, University of Ferrara) were included in the study. Data regarding fetal and maternal outcomes as well as disease activity during pregnancy were retrospective collected. Pregnancies were also sub-divided into two groups according to the time of the disease onset: group 1 after the disease onset and group 2 before the disease onset. 17 women with AIDs reported 30 pregnancies: 2 in patients with MKD, 9 with TRAPS, 8 with CAPS, 4 with FCAS and 4 with MWS, 3 with BS, 5 with AOSD and 3 with SS. There were 16 pregnancies in group 1 and 14 in group 2. Mean maternal age at conception was 27±6 years in group 1 and 30±4 years in group 2. Median disease duration at delivery was 13±7 years in group 1 and 15±3 years in group 2. We recorded the following pregnancy complications (group 1 vs group 2): gestational hypertension 37,5% vs 14.3%; preeclampsia 18% vs 0; abortions 12,5% vs 30%; preterm delivery 12,5% vs 0. No cases of disease worsening were observed during pregnancy in the group 1. By contrast, we recorder 2 cases (12,5%) of complete remission and 2 cases (12,5%) of partial remission. Conclusions. Patients with pregnancy after the disease onset experienced a frequency of gestational hypertension and pre-eclampsia higher than women who developed the disease after pregnancy. The disease course remained unaffected during pregnancy and postpartum.

9

Oestrogens accelerate lupus-like glomerulonephritis in NZB/WF1 mice

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Background. Systemic lupus erythematosus (SLE) is a systemic disease involving many organ systems and glomerulonephritis (GLN) is one of the most frequent manifestations. Serum levels of oestrogens, androgens, prolactin and other adrenal hormones are different in SLE patients compared with healthy subjects and, conversely, changes in SLE activity have been observed in physiological conditions, such as pregnancy, characterized by fluctuations in hormone serum levels.
Aim. The aim of the study was to evaluate the effect of oestrogens in the progression of lupus GLN.
Materials and methods. Female NZB/WF1 mice, a murine model which spontaneously develops a lupus-like GLN at about 5 months of age, were subdivided into 2 groups, 8 each: pellets containing 17-β-estradiol, and releasing it at a daily dose of 18μg for 90 days, were subcutaneously implanted in the ear by microscissors in the mice of group 1 at week 19, whereas 200μl of PBS were injected in the same site and at the same week in group 2, as controls. All mice were bred until natural death occurred. Urine samples were weekly collected; blood samples were collected before the implantation of pellets or PBS injection and every 4 weeks, thereafter. Proteinuria levels were evaluated by multistix reagent strips (Siemens), whereas circulating levels of anti-dsDNA, anti-C1q antibodies, and BLyS will be evaluated by standardized home-made ELISA tests.
Results. Proteinuria-free survival rate (<300mg/dl) was significantly lower in group 1 than in control group (p=0.046). At the 30th week 62.5% of group 1 compared with 25% of group 2 developed proteinuria levels ≥300mg/dl (p=0.046). At 33rd week 100% mice of group 1 compared with 50% group 2 mice developed proteinuria levels ≥300mg/dl (p=0.046). Mean proteinuria free survival rate (weeks±SD) was significantly lower in group 1 mice than in control group (30.0±2.0 vs. 32.4±2.3, p=0.046). Mice survival rate was significantly lower in group 1 than in control group (p=0.032). At 30th week of age 50% of group 1 compared with 12.5% of group 2 were dead (p=0.032), and when the last mouse of group 1 (week 34) dead only 75% of group 2 were dead (p=0.032). Mean survival (weeks±SD) was significantly lower in group 1 mice than in group 2 (30.9±2.5 vs. 33.6±2.1, p=0.034). Data on anti-dsDNA and anti-C1q antibody levels as well as BLyS serum levels will be presented at the congress.
Conclusions. Oestrogens seem to accelerate lupus-like GLN in NZB/WF1 mice.

10

Reduced risk for rheumatoid arthritis in women mediated by a *CYB5A* gene polymorphism which increases androgen synthesis

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Rheumatoid arthritis (RA) exhibits a clear sex bias with roughly three times more affected women than men. The underlying mechanisms remain elusive despite intense research efforts. RA is characterized by decreased androgen levels, which was the first hormonal abnormality described. Previous studies showed that steroidogenesis is shifted towards endogenous glucocorticoids at the expense of anti-inflammatory androgens. The key step governing androgen synthesis is the 17,20-lyase activity of a *CYP17A1*-encoded dual function enzyme, whereas its 17α-hydroxylase is required for androgen and glucocorticoid synthesis alike. Only 17,20-lyase activity depends on the presence of a critical cofactor, cytochrome b5 A, encoded by the *CYB5A* gene. Therefore, we included the *CYB5A* gene in a screening for RA-associated single nucleotide polymorphisms (SNP) in genes for steroidogenic enzymes.
The data sets of two genome-wide association studies (GWAS) on RA were screened for SNPs in or near the *CYPB5A* gene. Candidate SNPs in *CYPB5A* were studied in an independent case-control study population of Slovak origin (n=521 cases, n=321 controls). Functional analyses were done in synovial fibroblasts lines from knee samples of RA patients by quantitative RT-PCR, steroid conversion was measured using radiolabeled substrates, and cytochrome b5–expression was detected by immunohistochemistry.
We identified the RA-associated SNPs rs1790834 in the NARAC/EIRA (p=0.0073, OR=0.83) and rs1790858 in the WTCCC (p=0.0095, OR=0.44) cohort, respectively. The intronic SNP rs1790834 in the *CYB5A* gene was confirmed in our case-control study. The minor allele reduced RA risk selectively in women (p=0.0041, OR=0.63, 95%CI [0.46-0.86]). The protective effect was confined to rheumatoid factor-positive (OR=0.53, 95%CI [0.37-0.75]) and anti-cyclic citrullinated peptide-positive (OR=0.58, 95%CI [0.41-0.83]) cases, respectively. The protective allele doubles *CYB5A* mRNA-expression, leading to two- to threefold activation of steroid 17,20-lyase activity and resulting in accumulation of androgens in fibroblast cultures. Furthermore, increased mRNA-expression was accompanied by a higher density of cytochrome b5-positive cells in synovial tissue. In conclusion, *CYB5A* is the first RA susceptibility gene shown to be involved in androgen synthesis. Our functional analysis of SNP rs1790834 indicates that it contributes to the sex bias observed in RA.

11

Association between low sex hormone levels and antibody (Ab) presence in women with premature ovarian insufficiency (POI) and autoimmune diseases (AID)

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Significant difference in gender specific prevalence of AID after the age of 35 could be related to sex hormone effect on immune system. Many studies confirmed coexistence of AID and POI. Etiology of POI is considered to be hromozomic dearrangement in 2.5%, immunologic diseases in 45% and idiopathic in 52% cases. AIM: to define the association of low sex hormon levels and antibodies in POI with coexistence AID.
Methods. 180 women with idiopathic POI (loss of menstrual cycle before age of 40, FSH>40IU, E2<50 pmol/l). Women with iatrogenic POI were excluded. Patients were divided in two groups: POI Ab(+), 82 women, with presence of at least one antibody (antimicrosomal, antithyreoglobulin, antiparietal, anti DNA, antinuclear, antimitochondrial, antiphospholipid, antineutrophil, anticardiolipin or antiovarian), and POI Ab(-), 98 women without any of these Ab. The groups were equal and comparable in age, BMI, time of menarche, last menstruation and the length of anovulatory period. The blood was taken for FSH, LH, E2, T, DHEAS, SHBG, TSH, T4, PTH and ACTH.
Results. Existence of AID in POI At (+), vs. POI At(-), group was 46% vs. 6%, respectively $p<0.05$. AID in POI At (+) group: Hashimoto thyreoiditis 38%, SLE 5%, RA 4%, Sy Sjögren 4%, M.Adissoni 2.5%, Antiphospholipid sy. 1%. AID in POI At (-) group: Gluten enteropathy 3%, Hashimoto thyreoididtis 2%, M. Adissoni and Collitis ulcerosa 1%. Differences in E2 (estradiol) level in POI Ab(-), vs. POI Ab(+), group were 35,3±85.3/ 19,2±34,7 pmol/l, ($p=0.22$). Differences in Testosteron level in POI Ab(-) vs. POI Ab(+) group were 2.34±11.21/0.63±0.94 nmol/l, ($p<0.05$). Differences in TSH level in POI Ab (+) vs. POI Ab(-) group, were 2.3±3.9/0.6±0.9 IU/l, ($p<0.001$). There were no significant differences in level of FSH, LH, PTH, T4, PRL, SHBG and DHEAS between the two groups.
Conclusion. AID were found in significantly higher percent of POI Ab(+) patient. Lover level of E2, and statistical significant lower level of testosterone found in POI Ab(+) group, confirmed the coexistence of lower sex steroid hormone levels and AID in POI Ab(+) group. Looking for tissue specific Ab should be a relevant diagnostic tool in women in POI. It could distinguish a group of women with potential benefit of immunomodulating therapy in goal to preserve ovarian failure. Correction of sex steroid hormone levels, as therapy for POI, modulate autoimmune response and could potentially affect the appearance of other AID.

12

Androgen in postmenopausal systemic sclerosis patients

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Introduction. Systemic sclerosis (SSc) is a chronic connective tissue disease characterized by immunological abnormalities, vascular lesions and fibrosis of the skin and internal organs. SSc is occurring more frequently in women suggesting that sex hormones may play an important role in disease pathogenesis. It is well-known that prolactin, sex hormones and adrenal androgens have multiple immunomodulatory functions. A few studies showed low dehydroepiandrosteron sulfate (DHEAS) and testosterone levels in SSc patients and correlation with disease severity.
The aim of this study was to investigate the serum levels of estradiol, testosterone, DHEAs and androstendione in postmenopausal SSc patients and to examine the possible correlation with autoantibodies in SSc.
Methods. Twenty-seven post-menopausal SSc patients who fulfiled the preliminary American College of Rheumatology (ACR) criteria for the classification of SSc and twenty-seven healhty women were enroled in the study. They matched for age and post-menopausal duration and none of them had received any hormone replacement therapy. Serum levels of estradiol, testosterone, androstendione and DHEAs were measured in both groups. Serum levels of anticentromeric antibodies (ACA) were measured only in SSc patients.
Results. Serum levels of testosterone (0,80±0,62 nmol/L v. 1,64±1,02 nmol/l, $p<0,001$), DHEAs (1,16±1,00 μmol/L v. 2,00±1,08 μmol/L, $p=0,008$) and androstendione (3,26±3,08 nmol/L v. 5,79±2,82 nmol/L, $p=0,004$) were significantly lower in SSc patients compared to controls. There wasn't a significant difference in serum level of estradiol between groups ($p=0,250$). Serum levels of androstendione negatively correlated with ACA antibodies ($r=-0,434$, $p=0,024$).
Conclusion. Circulating testosterone, anrdostendione i DHEAs levels are decreased in post-menopausal patients with SSc compared with healthy post-menopausal women. Our study contributed in recent cognitions on altered hormonal status in SSc patients. Correlation between androgen compounds androstendione and ACA support a protective anti-inflammatory role of androgen steroids in SSc.

Session 2: *The Vitamin D endocrine system: a new player in NEIRD and rheumatic diseases*

13

Diet, sun and life style as determinants of vitamin D status

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Vitamin D3 is synthesized in the skin under ultraviolet radiation or absorbed from the intestine after ingestion of vitamin D containing food or supplements. Subsequently, it is hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D) and in the kidney to 1,25-dihydroxyvitamin D (1,25(OH)2D), the active metabolite that stimulates calcium absorption.
Vitamin D status can be easily assessed by the measurement of the serum concentration of 25(OH)D. Vitamin D deficiency (serum 25(OH)D <25 nmol/l) and insufficiency (serum 25(OH)D 25-50 nmol/l) are common in European countries and elsewhere especially in risk groups, such as young children, pregnant women, older persons, non-western immigrants, and patients with malabsorption. Sunshine exposure only is effective during 6 months of the year, except in (sub) tropical countries. In autumn and winter most ultraviolet radiation is absorbed in the atmosphere. The seasonal variation in serum 25(OH)D is about 25 nmol/l. Vitamin D synthesis in the skin is decreased by skin pigmentation and by the use of sunblock. It decreases with age, but the capacity is enormous. Serum 25(OH)D is lower in obese persons than in non-obese, due to lower sunshine exposure, lower body surface/volume ratio, and storage in fat tissue. Lifestyle variables such as clothing negatively influence vitamin D synthesis, and vitamin D deficiency is particularly common in the Middle East. The only food that is naturally rich in vitamin D is fatty fish. Eating fish two times per week significantly increases serum 25(OH)D. Dairy products contain some vitamin D. Margarine is fortified with vitamin D in most European countries. Milk is fortified in some countries and this has a large influence on serum 25(OH)D as is known from the USA. Milk and yoghurt contain much calcium and this has a vitamin D sparing effect. A high calcium intake suppresses parathyroid function and this increases the half life of serum 25(OH)D. The consumption of cod liver oil can also improve vitamin D status considerably, similar to vitamin D supplements. Improvement of vitamin D status in the population can best be achieved by a combination of sunshine exposure, food fortification and the use of vitamin D supplements.

14

D hormone, a full steroidal hormone: recent evidences including synergisms with glucocorticoids

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Recently, vitamin D deficiency is receiving an increased worldwide attention for its involvement in increasing risk for several chronic diseases including many cancers, infectious diseases, type-1 diabetes and notably autoimmune rheumatic diseases (1). The final active metabolite of vitamin D (1,25(OH)D3) is considered a steroid hormone for its origin from cholesterol (D-hormone), and like glucocorticoids exerts immunomodulatory activities (GC) (2). Pathysiopathological investigations confirm that severe hypovitaminosis D, in genetically predisposed subjects, can impair self tolerance and immune responses (like in deficiency on glucocorticoids) by compromising the regulation of dendritic cells, regulatory T-lymphocytes (Tregs), Th1 cells and B cell function (3). However, cross-sectional studies have shown that deficient serum levels of vitamin D (25(OH)D) (<20 ng/mL) are present in a significant percentage, not only in patients with autoimmune diseases such as multiple sclerosis (MS), type-1 diabetes, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), but also in healthy subjects (3). As a matter of fact, several data suggest synergistic actions between glucocorticoids and vitamin D on immune/inflammatory reactions, for example asthmatic patients with a higher serum level of 25(OH)D experienced more significant reduction in asthma symptoms score and steroid-sparing effect of SIT (4). Steroid-sparing effect correlated with 25(OH)D serum level at baseline and after 3 months effectively supporting synergistic actions. Interestingly, in other studies treatment with 1,25(OH)2D3 potentiated the inhibitory effect of dexametasone on IL-17A and TNFalpha production by memory T cells sorted by FACS from patients with early RA (5). Furthermore, combination of 1alpha,25-dihydroxyvitamin D(3) with dexamethasone enhances cell cycle arrest and apoptosis, with a role for nuclear receptor cross-talk and Erk/Akt signaling (6). Another prominent endocrine role for 1,25(OH)D3 was recently discovered in peripheral estrogen metabolism and in estrogen-related cell proliferative activities. 1,25(OH) D3 decreases the expression of aromatase, the enzyme that generally catalyzes the peripheral synthesis of estrogens from androgens, especially in cancer tissues where its intracrine activity is significantly increased, such as in breast and prostate cancer, (7). Similar inhibitory effects by 1,25(OH)D3 have been very recently reported on cultures of human macrophages with consequent induction of a reduced synthesis of cytokines (8).

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15

Severe asthma, immune regulation and vitamin D

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Asthma is a chronic inflammatory disease of the conducting airways. The heterogeneity of disease is highlighted by the highly variable responses to treatment observed in different patient cohorts. Vitamin D insufficiency is highly prevalent worldwide, with estimates in the UK of over 60% insufficiency in the summer and autumn months and almost 90% insufficiency in winter and spring. It is associated with increased severity and poor control of asthma, including in pediatric cohorts. My laboratory has focussed on studies in moderate to severe asthma patients who fail to respond to corticosteroids, the primary treatment for asthma. Whilst these patients comprise only 5-10% of all asthmatics, they utilise 50% or more of healthcare resources. We have described changes in regulatory T cell numbers and function, as well as in $CD4^+$ T cell responses in these asthma patients. The steroid enhancing properties of vitamin D in human asthma, particularly in steroid refractory asthma, and the immunological basis of these effects will be discussed, as will the broader effects of vitamin D in maintaining respiratory health. Ongoing studies combine *in vitro* laboratory observations, with ex vivo correlates in different patient cohorts, and *in vivo* studies in patients following steroid and/or vitamin D treatment.

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16

Why do T cells express the vitamin D receptor?

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Diseases where T cell derived IFN-γ and IL-17 is pathologic like inflammatory bowel disease are suppressed by active vitamin D ($1,25(OH)_2D_3$). Paradoxically, infectious diseases that require these same responses for protection are unaffected by $1,25(OH)_2D_3$ treatments. Vitamin D receptor (VDR) knockout (KO) $CD8^+$ T cells, but not wildtype (WT) $CD8^+$ T cells, induced colitis in Rag KO recipients. In addition, co-transfer of VDR KO $CD8^+$ T cells with naïve $CD4^+$ T cells accelerated colitis development. The more severe colitis was associated with rapidly proliferating VDR KO $CD8^+$ cells and increased IFN-γ and IL-17 in the gut. Naïve $CD8^+$ VDR KO T cells proliferated more rapidly than WT $CD8^+$ T cells *in vivo* and *in vitro*. The increased proliferation of VDR KO $CD8^+$ cells was due in part to the higher production and response of the VDR KO cells to IL-2. Vitamin D is critical in the control of $CD8^+$ T cell proliferation. T cells express low levels of the vitamin D receptor until 48h post-stimulation. In addition, $CD8^+$ T cells produce the 1-alpha hydroxylase that converts $25(OH)D_3$ into $1,25(OH)_2D_3$ but the enzyme is not induced before 48h of stimulation. T cell regulation by vitamin D is a late event. Therefore the data support a new model where vitamin D is required to shut off the T cell response. The inability to signal through the VDR results in the generation of pathogenic $CD8^+$ T cells from rapidly proliferating cells that contribute to the development of inflammation in the gut.

17

Vitamin D and infections

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In the old days patients with tuberculosis were instructed to be exposed to the sun (sent to sanatorium). Eventually it was found that vitamin D secretes cathelicitidines which specifically kill mycobacteria. It is believed that common cold and pneumonias care more prevalent in winters because in part during the winter we are less exposed to sun and have lower levels of vitamin D.
During the years the association of low vitamin D was related to the incidences of HBV, HCV, EBV, HIV, upper respiratory viruses and enteric infections, sepsis, pneumonia, clostridium, gonorrhea, H1N1, influenza and vaginosis and otitis media.
Eventually several interventional studies were carried out and proved the concept; the high dose therapy with Vitamin D may be beneficial in many of the above infections.
Vitamin D may be acting as a "panacea antibiotic" and should be used as an adjuvant therapy in diverse infections. The mechanisms by which the Vitamin D is acting to counteract infections will be delineated.

18

Anti-inflammatory properties of vitamin D receptor agonists

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1α, 25-dihydroxyvitamin D3 [$1,25(OH)_2D_3$], the biologically active form of vitamin D, is a secosteroid hormone essential for bone and mineral homeostasis. In addition, this hormone regulates growth and differentiation of many cell types, and has pronounced immune regulatory and anti-inflammatory properties. Vitamin D receptor (VDR) agonists are real immune modulators, able to promote innate immunity and to regulate adaptive immune responses, typically leading to anti-inflammatory effects. In addition to exerting direct effects on T cell activation, VDR agonists markedly modulate the phenotype and function of antigen-presenting cells, in particular dendritic cells, inducing them to acquire tolerogenic properties that favor the induction of regulatory rather than effector T cells. Current therapeutic indications include osteoporosis, secondary hyperparathyroidism and psoriasis, but the anti-proliferative, pro-differentiative, anti-bacterial, immune modulatory and anti-inflammatory properties of VDR agonists could be exploited in a variety of additional clinical conditions. In particular, the pleiotropic anti-inflammatory effects induced by VDR agonists could turn out to be beneficial in different pathologies associated with chronic inflammatory responses.

Selected Presentations on the topic

19

The effects on T-cell phenotype in patients with systemic lupus erythematosus treated with two different regimens of vitamin D supplementation: an update after a year of therapy

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Background. Recent studies highlight that Vitamin D (VD) may have an immunomodulatory action on T cells, inhibiting Th1 and Th17 response and enhancing Th2 and Treg function. After repeated antigenic presentation, T cells differentiate into highly experienced memory T cells (CD45RA+CCR7-) with a peripheral expansion of the CD28-T cells, a subpopulation with peculiar effector activities (high γ-IFN production).
Objectives. To verify the effect of VD supplementation on the circulating levels of effector memory T cells (CD45RA±CCR7-), Treg cells (CD25highCD127low),CD28-T cells, and on cytokines production.
Methods. 34 female SLE pts (median age=32.5, range 10-90thpercentile: 24-40.7) years; median duration of disease=7 (1.3-16.7) years), SLEDAI=2(0-4) were enrolled. 17 pts were supplemented with an intensive regimen (IR) of colecalciferol (300.000UI for the first month and 50.000UI/monthly as maintenance), whereas 17 pts were supplemented with a standard regimen (SR) (25.000UI monthly). Phenotypic analysis of peripheral T lymphocyte was evaluated by flow-cytometry at baseline and after every 6 months of treatment. The same technique was used to quantify the intracytoplasmatic production of IL-17, IL-4 and γ-IFN from peripheral blood mononuclear cells (PBMC) of 12 pts, stimulated *in vitro* for 5 hours with PMA+ionomycin (5+500 ng/ml).
Results. After 12 months of treatment, a significant decrease in the number of Treg cells was observed (from 8.4 (5.7-13.5) to 6.7 (4.7-14.6)% of $CD4^+$T cells; p=0.04) in the entire cohort of pts. Within Treg subtype, an increase in peripheral induced (CCR7-) Treg cells was seen, along an increase in the total amount of circulating CD4+CD45RA+CCR7-T cells. Moreover, a reduction of the $CD8^+$CD28-T cells in SLE pts was demonstrated only in the SR group (Table I). In the IR group of pts evaluated for cytokines production (n=6), a reduction of γ-IFN/IL-4 (from 9.7 (0.8-18.3) to 6 (1.6-15.1); p=0.04) among $CD8^+$T cells was detected.
Conclusions. VD may promote the enhancement of peripheral induced Treg cells and the production of Th2 cytokines. Further studies will be necessary to understand the role of highly experienced memory T cells and of CD28-T cells, whose number seems to be inversely correlated with their functional capacity.

Table I.

Cell phenotype	IR		
	T0	T12	*p*
TREG CCR7-(%TREG)	30.9 (15.4-43.1)	34.8 (22.3-63.2)	0.01
T CD4+CD45RA +CCR7-(cell/µl)	6 (2.8-12.5)	9.2 (3.5-33.3)	<0.01
TCD8+CD28-(%CD8+)	24.9 (11.1-49.7)	24.7 (9.7-51.8)	ns
Cell phenotype	**SR**		
	T0	T12	*p*
TREG CCR7-(%TREG)	25.3 (17.3-42.4)	34.7 (19.5-55.5)	0,03
T CD4+CD45RA+CCR7-(cell/µl)	7.7 (2.4-20.9)	16.9 (10.4-51.3)	<0.01
TCD8+CD28-(%CD8+)	26.7 (13.7-63.7)	19 (7.4-34.6)	0,01

20

Vitamin D serum level correlates with nailfold microangiopathy extent in systemic sclerosis patients

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Background. Vitamin D is involved in both innate and adaptive immunity (1). In several autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and undifferentiated connective tissue disease, low 25-hydroxyvitamin D [25(OH)D3] serum concentrations correlate with disease activity (1). Systemic sclerosis (SSc) is a severe connective tissue disease characterized by vascular, immune and fibrotic changes in several internal organs, with a progressive sequence.

Aim. The aim of the study was to assess whether 25(OH)D3 serum concentrations might be associated with capillaroscopic microvascular markers and clinical features of SSc.

Methods. 117 SSc patients were enrolled (mean age 67±12SD years; 83% female; mean disease duration from onset of Raynaud phenomenon 13±13 years). All patients were evaluated by nailfold videocapillaroscopy (NVC) to score and classify the severity of the microangiopathy (both NVC patterns ("early", "active" and "late") and microangiopathy evolution score [MES] were assessed), as previously reported (2,3). 25(OH)D3 serum levels were evaluated by radioimmunoassay, and they were classified as normal (>30 ng/ml), insufficient (30 <25(OH)D3 <10 ng/ml) or deficient (<10 ng/ml) (4). Clinical features of the disease were assessed using Medsger's severity scale (score 0-4) (5). Statistical analysis was performed by non parametric tests.

Results. 25(OH)D3 resulted significantly lower in patients with "late" NVC pattern of microangiopathy in comparison with patients showing either "active" or "early" pattern (17.1±12.4 vs 18.2±13.3 vs 20.2±7.4, $p<0.005$). A negative correlations was found between 25(OH)D3 concentrations and both MES (r=-0,49, $p<0.003$) and peripheral vascular disease according to Medsger scale (r=-0.24, $p<0.01$). There was no significant relationship between serum 25(OH)D3 and other clinical features of SSc, including skin, lung, gastrointestinal, renal, heart and joint involvement, assessed using the Medsger's severity scale. Any statistical significant difference between skin subsets of SSc or gender was not found.

Conclusion. Our data demonstrate a negative correlation between 25(OH)D3 serum levels and severity of peripheral microvascular/vascular clinical involvement, in SSc patients.

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21

Hypovitaminosis D predicts more aggressive evolution and lower response to treatment in early rheumatoid arthritis after 12 months of follow-up

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It has been suggested that the vitamin D active form (1,25 (OH)2D3) has immunoregulatory activities, regulating both the innate and adaptive immune responses. Recently, vitamin D has been studied as potential player in the pathogenesis of Rheumatoid Arthritis (RA). Indeed, some recent studies showed a negative association between serum vitamin D levels and RA activity. Aim of this study was to evaluate the correlation between serum vitamin D levels and disease activity assessed with clinimetric, biochemical and ultrasound (US) parameters at baseline and after a follow-up of 12 months in early RA patients. We recruited 37 consecutive patients affected by early RA and naïve for treatment among patients referring to the Early Arthritis Clinic. Hypovitaminosis D was diagnosed for 25(OH) vitamin D value <20ng/ml. CRP, ESR, Rheumatoid Factor (RF) and anti-citrullinated peptide antibody (ACPA) levels were also measured. Swollen and tender joint counts, Disease Activity Score with 28 and 44 joints assessment (DAS28 and DAS44) scores, Simplified Disease Activity Index (SDAI) and Clinical Disease Activity Index (CDAI) were assessed at baseline and after 12 months of treatment. Moreover all patients underwent US assessment for synovitis and power Doppler evaluation. At baseline patients with normal levels of vitamin D did not show any significant difference in clinimetric, serological and US parameters compared to patients with low levels of vitamin D. After a 12 months follow-up patients with sufficient vitamin D levels at baseline had a significant lower disease's activity and a higher prevalence of remission compared to the group with hypovitaminosis (68% vs 16%, respectively; $p<0.001$) according to DAS28, DAS44, CDAI and SDAI scores ($p<0.001$ for all indexes). Furthermore, normal vitamin D levels were also associated with higher percentage of responders to RA treatment (EULAR criteria) compared with vitamin D insufficiency (100% vs 75%, $p<0.001$). The percentage of patients that didn't present a reduction of the US synovitis score was higher in the hypovitaminosis patients group. In the present study, early RA subjects with normal vitamin D levels showed both a greater reduction of disease activity and higher prevalence of response to treatment and of remission compared to patients with hypovitaminosis D after 12 months of follow-up. Our results provide further support to the immunomodulatory role of vitamin D in inflammatory arthritis and indicate that baseline hypovitaminosis D may predict a more aggressive evolution of the disease and a worse response to treatment. Thus, evaluation of vitamin D serum levels and its possible supplementation should become part of the clinical practice, especially in patients naïve to treatment.

Session 3: *Glucocorticoids today: the most updated reports*

22

Target therapies in RA: the place for GC

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Though considered a 'historic' therapeutic in the management of rheumatoid arthritis (RA), glucocorticoids (GC) remain at the core of most current therapeutic algorithms and guidelines. Their broad immune modulatory effector function mediates effective therapeutic benefits in RA manifest in improvement in signs and symptoms, reduced erosive progression (*i.e.* articular damage) and improved metrics of quality of life. Their effects on common co-morbidities *e.g.* in the vasculature, bone and brain are less clear reflecting the interaction between target mediated adverse events and the potentially beneficial effects of relief from inflammation in those extra-articular organs thus affected. Remarkably little is known of the optimal use of GC in RA – recent studies have demonstrated for example that the appropriate choice of timing of delivery can alter the benefit profile considerably. Moreover their role as critical co-therapeutics with conventional and also with biologic disease modifying anti-rheumatic drugs (DMARD) is relatively little studied – they have tended to be used as an adjunct rather than focus of trial design in the last decade. There is now however evidence that they can enhance the effect of initial methotrexate administration. In several treat to target and strategy trials their use is also in part contributory to the beneficial effects in some study arms over others. I shall discuss the current use of GC in RA management and allude to future areas in which their use, and that of future GC like entities may develop over time.

23

Membrane receptors of GCs: origin and functional activity

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Glucocorticoids (GC) are the most common used drugs in the treatment of a wide range of rheumatic and other inflammatory diseases. They exert their anti-inflammatory and immunosuppressive effects primarily via so called genomic mechanisms, thus mediated by the cytosolic glucocorticoid receptor (cGR). However, rapid effects of GC exist, which are mediated by specific and unspecific non-genomic mechanisms. The membrane-bound glucocorticoid receptor (mGR) has been suggested to play an important role by the mediation of specific non-genomic GC-action. It has already been shown, that mGRs are up-regulated on monocytes of patients with inflammatory diseases, like rheumatoid arthritis, and that the frequency of mGR expression positively correlates with the disease activity. Furthermore, modified mGR expression has been shown for other inflammatory diseases like systemic lupus erythematosus, ankylosing spondylitis or after vaccination. Indeed, the clinical relevance of mGR is assumed, but the origin and functional activity remained unexplained and has been in focus of our study.
We analyzed the origin of mGR protein with the help of RNA-interference technology. Therefore, we performed a transient (via siRNA) and a stable (shRNA) GR knockdown in mGR positive HEK293T cells. The knockdown efficacy was verified on mRNA level by RT-qPCR, the reduction of GR protein was investigated by immunoblot analysis and mGR expression was analysed with high-sensitive immunofluorescent staining. These experiments demonstrated that, in contrast to a transient reduction, a stable knockdown of GR mRNA diminished mGR expression.
For functional analysis, LPS stimulated mGR positive human CD14+ monocytes were treated with dexamethasone bound to BSA (Dex-BSA). Due to the size this substance is membrane-impermeable and thus Dex-BSA selectively activates mGR. In order to identify rapid GC effects, PepChip™ array technique was used. We were able to show a Dex-BSA specific induction of rapid (de)phosphorylation processes mediated by different kinases. A number of MAP-kinases, including p38 MAPK, were identified to show an increased phosphorylation according to Dex-BSA treatment.
A genome analysis via microarray also revealed that Dex-BSA altered gene expression. A functional classification into biological processes of identified genes via Panther database demonstrated that processes of signal transduction and metabolism are most affected by selective mGR activation.
Altogether, we were able to demonstrate that there is only one gene encoding for both, the cGR as well as for the mGR. Furthermore, an activation of mGR by membrane-impermeable Dex-BSA revealed that external signals were transferred in the cell via rapid (de)phosphorylation processes by the mean of kinases. Additionally, it has been shown that an activation of the mGR results in an altered gene expression. This is the evidence of the functional activity of the mGR. However, these effects need to be further investigated in order to identify mGR signalling pathways and their target genes more in detail. Nevertheless, the human mGR represents an interesting target for optimised treatment strategies in case of rheumatic or other inflammatory diseases.

24

Glucocorticoid metabolism and inflammation: crucial interactions

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The anti-inflammatory actions of endogenous and exogenous glucocorticoids are well established. Until now, most attention has been focused on the level of glucocorticoids in the circulation. However, the level of glucocorticoids within cells and tissues is also regulated by local glucocorticoid metabolism. The most important enzymes regulating local glucocorticoid metabolism are the 11b-hydroxysteroid dehydrogenases (11b-HSDs). The 11b-HSD type 1 enzyme converts inactive cortisone to active cortisol whereas the 11b-HSD type 2 enzyme converts cortisol to cortisone. 11b-HSD1 is expressed in a range of tissues including bone and synovium. Expression *in vitro* and *in vivo* appears to increase in response to proinflammatory cytokines such as TNF-α and IL-1b. In rheumatoid arthritis 11b-HSD1 activity appears to also correlate with the level of inflammation but it is possible that the relative level of expression is lower than that expected for the degree of inflammation. Global deletion of 11b-HSD1 expression in mice results in an exaggerated inflammatory response to experimental arthritis and an abnormal bone phenotype. The inactivating enzyme 11b-HSD2 has also been identified in macrophages within the rheumatoid synovium along with peripheral blood mononuclear cells in individuals with rheumatoid arthritis. Expression of this enzyme outside of the classical mineralocorticoid receptor positive tissues is highly unusual but would be expected to confer glucocorticoid resistance to immune cells where expressed. The exact function of 11b-HSD2 within the joint has not been fully explored. Current studies are exploring the specific role of 11b-HSD1 and 11b-HSD2 in inflammatory disease in humans and in particular whether an abnormal expression of these enzymes can predispose to the development or persistence of inflammation.

25

GC and microRNA: a new discovered cross talk

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Glucocorticoids (GCs) are widely used in the treatment of inflammatory diseases and in hematological malignancies such as multiple myeloma (MM). However, the development of resistance to GCs limits their clinical utility and understanding the mechanisms of GC resistance may provide additional therapeutic approaches for improved clinical outcomes. We have examined the mechanisms of GC resistance in models of multiple myeloma (MM) and the loss of glucocorticoid receptor-alpha (GR) expression is frequently the basis for resistance. We have previously developed cell lines from a MM patient who had been treated with GCs and become resistant to that treatment. We have reported that the response to GCs is dependent on an active GR which is present in the GC sensitive cell line (MM.1S) and down-regulated in the GC resistant derivatives MM.1Re and MM.1RL. We determined that regulation of GR expression occurs at a post-transcriptional level and further examined the involvement of microRNAs (miRNAs) in this process. MiRNAs predominantly bind sites in the 3' untranslated region (UTR) of coding genes to mediate post-transcriptional gene silencing. Here we observed that luciferase reporters containing the 3'-UTR of GR are significantly repressed in MM.1R cells when compared to MM.1S cells. To identify specific miRNAs involved in regulation of GR, we sequenced the small RNA population in MM.1S, MM.1Re and MM.1RL cells using next generation sequencing. We identified candidate miRNAs through potential binding sites in the GR 3' UTR and through differential expression in GC-sensitive versus –resistant MM cells. We manipulated the expression of candidate miRNAs through over-

expression of candidate miRNAs mimics predicted to target GR. We identified miR-130b as a miRNA that is consistently up-regulated in MM.1R cells and able to repress endogenous GR protein levels in MM.1S and also repress a luciferase reporter containing the 3'-UTR of GR-alpha. In addition we show that GC treatment of MM.1S transfected with hsa-miR-130b mimics induces resistance to GC actions, reduces the levels of GC inducible gene GILZ, a downstream target of GR, and inhibits apoptosis. We conclude that differential expression of miRNAs play a role in the regulation of GR expression contributing to GC resistance.

26

Anti-cytokine therapy affecting pain-related central nervous system activity

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Cytokine blocking agents have substantially improved our therapeutic armamentarium to treat inflammatory diseases such as rheumatoid arthritis (RA). Although inflammation in diseases like RA depends on the expression of a multitude of cytokines, chemokines and growth factors, only few of them have emerged as major therapeutic targets. In particular, neutralization of tumor necrosis factor α (TNF-α) achieves very profound and fast amelioration of some clinical symptoms of arthritis, which appears to precede its anti-inflammatory effect. There has always been a gap in explaining of how TNF-α blockade can so rapidly affect the patients' disease state, considering that RA is a very chronic condition. We thus hypothesized that blockade of TNF-α acts through the central nervous system (CNS) before directly affecting joint inflammation. By use of functional magnetic resonance imaging (fMRI), we demonstrate that neutralization of TNF-α blocks nociceptive CNS activity in the thalamus and somatosensoric cortex but also the activation of the limbic system as early as 24 hours after the onset of treatment. Moreover, arthritic mice overexpressing human TNF-α showed an altered pain behavior and a more intensive, widespread and prolonged brain activity upon nociceptive stimuli as compared to wild-type mice. Similar to humans, these changes as well as the rewiring of CNS activity resulting in tight clustering in the thalamus were rapidly reversed after neutralization of TNF-α. These results suggest that neutralization of TNF-α affects nociceptive brain activity in the context of arthritis, long before it achieves anti-inflammatory effects in the joints.

27

Circadian CLOCK-mediated HPA axis and gene-specific regulation of peripheral glucocorticoid receptor transcriptional activity by acetylation

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Circulating cortisol concentrations fluctuate diurnally under the control of the "master" circadian CLOCK system located in the hypothalamus, while we recently reported that the peripheral "slave" circadian CLOCK system regulates the transcriptional activity of the glucocorticoid receptor (GR) by acetylating it at local target tissues. To examine Clock-mediated GR acetylation and circadian changes in the sensitivity of peripheral target tissues to glucocorticoids (GCs) in humans, we examined the acetylation of the GR and the mRNA expression of the GR, Clock, Bmal1, and 8 known GC-responsive (4 transactivated and 4 transrepressed) genes in peripheral blood mononuclear cells (PBMCs) obtained from 10 healthy subjects at 8 am and at 8 pm. GR acetylation levels were higher in the morning than in the evening, in synchrony with fluctuations of Clock and Bmal1 mRNAs and the levels of circulating ACTH and cortisol. The mRNA expression of certain genes regulated positively (GILZ and tristetraprolin) or negatively (interferon gamma, IL-1alpha and IL-12 p40) by GCs demonstrated the expected concomitant diurnal fluctuations, while, surprisingly, those of other such genes (Annexin A1, dual phosphatase 1 and TNFalpha) did not fluctuate. To further examine details of the acetylation-mediated regulation of GC-responsive genes, we obtained PBMCs from 6 additional healthy subjects and cultured them for 24 h *ex vivo* in the absence or presence of added hydrocortisone, assuming continuing oscillation of the cellular CLOCK system. In these cells, hydrocortisone-induced mRNA expression of the GC-responsive genes previously found to not correlate with circulating cortisol *in vivo* demonstrated circadian fluctuation, mirroring the levels of GR acetylation and Clock mRNA expression, while that of the genes that correlated with circulating cortisol *in vivo* did not show fluctuation ex vivo. Knockdown of Clock by its siRNA in cultured PBMCs abolished this gene-specific fluctuation of GR transcriptional activity. These results suggest that the transcriptional activity of the human GR is moderated in a gene-specific fashion through circadian GR acetylation by the peripheral CLOCK, counteracting the transcriptional effect of circulating cortisol. Coordinated regulation of GC action at target tissues by circulating cortisol and peripheral CLOCK-mediated epigenetic modulation of the GR appears to be essential for the maintenance of GC homeostasis in man. Uncoupling of such coordination might lead to increased exposure of tissues to glucocorticoids and pathologies related to functional hypercortisolism.

Selected Presentations on the topic

28

Does adrenocortical androgenic and glucocorticoid imbalance occur before onset of rheumatoid arthritis (pre-RA) in women: results of a controlled cohort study

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Background. Polymorphic variations of the adrenal cortex may influence intrinsic susceptibility to neuroendocrine-immune (NEI) disease risk, including rheumatoid arthritis (RA).
Objective. The aim of this study was twofold, initially to identify literature evidence of adrenocortical polymorphisms, and additionally to analyze baseline serum levels of DHEAS (zona reticularis androgenic marker) and cortisol (zona fasciculata glucocorticoid) steroids in women, before onset of rheumatoid arthritis (pre-RA) in a controlled cohort study.
Study Methods. PubMed literature search included hypo- and hyper-function/plasia of the adrenal cortex, its hypothalamic-pituitary (HP) control, genetic mechanisms (SF-1 and DAX-1), zonal steroidogenic molecular and morphologic polymorphism, adrenarche, and aging. Analysis involved the 1974 CLUE 1 cohort, which enrolled 12,381 Caucasian women of Washington County, MD, from whom 36 baseline pre-RA developed ACR-positive RA, after 3 to 18 (median 12) yrs. Four CN were closely matched to each pre-RA. Pearson bivariate correlations and scatterplots of baseline DHEAS and cortisol levels were analyzed, using z-scores specific to laboratory batch assays. Multivariate regression analysis (MRA) estimated the ability of cortisol to independently predict the dependent DHEAS outcome values in the study groups (pre-RA vs CN) and by other subject stratifications.
Results. Complex heritability was associated with a greater variability in serum adrenocortical androgens (AA), including DHEA, its sulfate (DHEAS), during development (adrenarche) and aging, than cortisol. Interactions of steroidogenic factor (SF-1) and DAX-1 (*i.e.*, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) genes are believed to influence hyperglucocorticoid synthesis in rats, and DAX-1-deficient mice may be predisposed to adrenal failure in aging.
Baseline serum cortisol and DHEAS z-scores correlated negatively (r=-0.725) in 12 pre-RA who had pre-menopausal onset, but positively (r=0.448) in 23 cases with post-menopausal onset (p<0.001). MRA of the pre-RA hormonal data confirmed the opposite correlations between pre- vs post-menopausal onsets (p=0.009). Opposite correlations (p=0.002) were also observed between the 16 younger cases who entered the cohort under age 44 years (r=-0.501) vs 20 older pre-RA (r=0.512), but did not occur between their respective matched 64 younger (r=0.055) and 80 older (r=-0.100) control subjects (p=0.363).
Conclusions. A minority subgroup of women may have adrenocortical androgenic and glucocorticoid imbalance before onset of RA, which deserves further study.

Session 4: *Glucocorticoids in rheumatoid arthritis*

29

Disease-modifying effects of glucocorticoids in the treatment of rheumatoid arthritis: new evidence

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The so called CAMERA (Computer Assisted Management in Early Rheumatoid Arthritis) II study has recently been performed, in which all patients were treated with the computer guided monthly tight control scheme, aiming for remission, with increasing dosages of methotrexate (MTX), but randomized to addition of 10mg prednisone/daily or of placebo for study duration of 24 months. All patients had early RA according to the ACR 1987 criteria, with disease duration less than one year, DMARD and GC naïve. Treatment was started with MTX 10 mg/week, if necessary increasing every month with 5 mg up till 30 mg/week; if no remission was reached adalimumab 40 mg/2 weeks was added. In addition either 10 mg prednisone or placebo was added. When remission was reached the treatment was reduced again. 235 patients were randomized 60% women; mean age 54 years; 68% rheumatoid factor positive; mean ESR 35; mean 16 tender and 15 swollen joints. Primary outcome after two years was the absolute erosion score; this primary outcome was met in favor of the GC group. Importantly, after two years 70% of the patients treated with tight control MTX had no erosions, in the patients treated with additional prednisone this percentage increased to 82. As expected, clinical variables, as well as CRP and ESR, improved during the first 6 months more in the prednisone than in the placebo group; after 6 months this was similar in all groups. The definition of remission that was used in the CAMERA II study was: zero swollen joints and 2 out of 3: tender joints 3 or less, ESR 20 or less, VAS general health (0-100) 20 or less. Remission was reached in the prednisone group in 72%, versus 61% in placebo group; the start of the remission was earlier in the prednisone group (6 versus 11 months). If oral MTX was not well tolerated, or not effective enough, MTX was given subcutaneous; if remission was not reached adalumimab was added as an additional step. In the prednisone group only 26 patients needed sc MTX versus 60 in the placebo group; the difference in use of biologicals was even more impressive: only 16 in the prednisone group versus 42 in the placebo group. A detailed register of adverse events was kept at each monthly visit; there was no increase in infections in the prednisone group, no increase in cardiovascular events, diabetes mellitus, hypertension or fractures. There were significantly less gastrointestinal adverse events in the prednisone group, especially nausea (51 in the prednisone group versus 152 in the placebo group), and remarkably less liver function disturbances (ALAT above upper limit of normal): 87 in the placebo group versus 30 in the prednisone group. GC seemed to improve the gastrointestinal tolerance of MTX.

30

Bone safety by low-dose glucocorticoids in rheumatic diseases

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At high doses glucocorticoids produce predictable loss of bone via direct deleterious effects on osteoblasts, osteocytes, and osteoclasts. At lower doses, the evidence is less robust, but it continues to support toxicity to bone, in a dose dependent fashion. In contrast to their toxic effect to bone, glucocorticoids also suppress the pro-inflammatory cytokines that contribute to bone loss and these effects potentially partially counter their negative effects on bone. The majority of data on the glucocorticoid effects on bone come from observational data; many studies examining data other than in RA. The association of glucocorticoids with bone loss using this data is prone to bias, such as confounding by indication and diagnostic detection bias. Several randomized controlled trials of glucocorticoids also have examined this issue and the findings have been less conclusive of significant bone issues. However, these RCTs are underpowered to examine this question. A variety of therapeutic agents are approved for prevention and treatment of glucocorticoid induced osteoporosis. The timing of initiation, sequencing, and long-term safety of these drugs is a subject of debate. Despite international guidelines, many RA patients on glucocorticoids do not receive bone protective agents.

31

Two decades of experience with the COBRA study

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The lofty title of this presentation is based on the fact that the design of the COBRA (Dutch: COmbinatietherapie Bij RA) study started in 1990, recruitment in 1992. The data were published in 1997, and many spinoff and follow up studies followed.
This presentation gives a bird's eye overview of the original trial results, the long-term follow up, and results of replications and variations of the treatment regimen.
The COBRA regimen comprises the combination of oral prednisolone, methotrexate and sulfasalazine:

1. an oral prednisolone pulse in a schedule that starts with 60 mg/d in the first week, with weekly steps down (week 2-6: 40; 30; 20; 15; 10 mg/d) to 7,5 mg/d in week 7, after which the dose is kept constant until month 6;
2. methotrexate, 7,5 mg/w;
3. sulfasalazine, 2 g/d (1g/d in the first 2 weeks).

In the original trial, prednisolone was tapered to 0 in 6 weeks starting at month 6, and methotrexate in 4 weeks after month 9. Drugs were reinstituted if a flare occurred.
COBRA was found to be superior to sulfasalazine monotherapy in signs and symptoms in the first 6 months, and in damage progression and physical function in the first 12 months and beyond (1). In addition, COBRA had less side effects and it dominated the control arm in terms of direct and indirect costs (2, 3).
Two follow up studies (at 5 and 11 years) with patients treated according to physician preference suggest the initial structural benefit was maintained without an increase in toxicity (4, 5).
The BeSt (Dutch: BehandelStrategieën bij RA) study compared 4 regimens: sequential monotherapy, step-up, COBRA, and initial high dose methotrexate with infliximab. Both in the initial trial and in subsequent follow up studies, the latter two arms were superior in terms of clinical and radiological results, with differences between groups gradually becoming less prominent. (6, 7). Importantly, COBRA was indistinguishable from high dose methotrexate and inflximab in terms of clinical and radiological results, at a fraction of the price of treatment (8).
Despite these impressive results, implementation has been sluggish, no doubt partially due to relentless marketing of biological agents. However, in an implementation study we found and addressed several barriers, mostly on the physician side (9-11). For the patients we produced information materials and support, and for the physicians a collection of supporting literature as well as templates for prescription freely available in Dutch and English (see: www.cobratherapy.nl).
In other centers, René Westhovens in Leuven, Belgium was one of the original trial center coordinators who has published on the efficacy of COBRA in routine practice (12). In Bristol, UK John Kirwan has implemented COBRA in the routine care of patients that meet the inclusion criteria of the trial (personal communication). Many centers now apply modified step-down schedules in their routine care, but until recently evidence for their efficacy was lacking. We recently published the half-year results of the 'COBRA-light' trial, that suggests a modified step-down regime (starting with 30 mg of prednisolone) combined with high-dose methotrexate has similar clinical efficacy compared to the full COBRA schedule (13). Analysis of the 1-year results (that include radiographs) is currently underway.
On the more intensive side, we performed a small pilot study that suggested the efficacy of COBRA could be much enhanced by increasing the methotrexate and adding hydroxychloroquine (14). Unfortunately we were unable to date to secure funding for a larger study.
In summary, the COBRA regimen has proved to be highly effective in the short and long run, with a very acceptable safety profile. Its equivalence to initial high-dose methotrexate plus infliximab has no doubt helped to put a hold on the steady advance of biological therapy in early disease. Implementation has been sluggish, but appears to be gathering speed. Current studies are aimed at optimization.

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32

The efficacy, effectiveness, and safety of 3mg/day prednisone for initial and long-term management of rheumatoid arthritis

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Objectives. Prednisone treatment in 308 patients with rheumatoid arthritis (RA) of one academic rheumatologist was analyzed from 1980-2004 for initial dose, long-term effectiveness, and adverse events. In the early 2000s, a randomized, double-blind, placebo-controlled, withdrawal clinical trial was conducted of prednisone <5 mg/day versus placebo.
Patients and Methods. A database of all patient visits included medications, adverse events, and patient self-report multidimensional health assessment questionnaire (MDHAQ) scores for physical function, pain, and routine assessment of patient index data (RAPID3), at each visit. Data were analyzed in 5-year periods, 1980-84, 1985-99, 1990-94, 1995-99, and 2000-04. The randomized, double-blind, placebo-controlled, withdrawal clinical trial was conducted in patients with stable clinical status over 12 weeks while taking 1-4mg prednisone/day in three phases: a) "equivalence" - 1-mg tablets taken for 12 weeks, to ascertain efficacy versus the patient's usual prednisone tablets prior to randomization; b) "transfer" - substitution of a 1-mg prednisone or identical placebo tablet at a rate of a single 1-mg tablet every 4 weeks (over 0-12 weeks) to the same number as baseline prednisone; c) "comparison" - observation over 24 subsequent weeks taking the same number of either placebo or prednisone tablets as at baseline. The primary outcome was withdrawal due to patient-reported lack of efficacy versus continuation in the trial for 24 weeks.
Results. Mean initial prednisone doses were 10.3, 6.5, 5.1, 4.1 and 3.6 mg/day in 1980-84, 1985-99, 1990-94, 1995-99, and 2000-04. The proportions of patients whose initial prednisone doses were >5 mg/day were 49%, 16%, 7%, 7% and 3% of patients, 5 mg/day in 51%, 80%, 70%, 26% and 10%, and <5 mg/day in 0, 4%, 23%, 67% and 86% in the respective 5 year periods. Most patients received early concomitant methotrexate after 1990. Patients treated with >5 mg/day had higher MDHAQ scores, reflecting poorer clinical status. MDHAQ scores were improved similarly in patients treated with <5 or >5 mg/day, maintained over >8 years. Primary adverse events were skin-thinning and bruising. New hypertension, diabetes and cataracts were seen in <10%. In the randomized trial, 31 patients were randomized, 15 to prednisone and 16 to placebo, with 3 administrative discontinuations. In "intent-to-treat" analyses, 3/15 prednisone and 11/16 placebo participants withdrew (p=0.03). Among participants eligible for the primary outcome of withdrawal for lack of efficacy, 3/13 prednisone versus 11/15 placebo participants withdrew (p=0.02). No meaningful adverse events were reported, as anticipated.
Conclusion. The data suggest that many patients with RA might be treated effectively with initial and long-term prednisone <5 mg/day. The efficacy of 3 mg prednisone/day was documented in a small clinical trial, with statistically significant results suggesting robust treatment effects.

Session 5: *Glucocorticoids and biological therapies*

33

Glucocorticoid combination with biologics in JA: recommendations and guidelines

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Glucocorticoids have few indications in juvenile idiopathic arthritis given that their several side effects including growth arrest or retardation may outweigh any benefits on articular disease. Moderate or high-dose systemic corticosteroid therapy are reserved for patients with systemic JIA whose disease is not controlled by non-steroidal anti-inflammatory drugs although the recent introduction of other effective therapies, such as interlukin-1 and interleukin-6 inhibitors, has greatly reduced their indication. In the other juvenile idiopathic arthritis categories a course of low-dose prednisone may be considered for reducing pain and stiffness in patients with severe polyarthritis unresponsive to other therapies or while awaiting the full therapeutic effect of a recently initiated second-line agent. Intra-articular steroid injections with triamcinolone hexacetonide are frequently needed at disease onset or during disease course especially to prevent deformities secondary to joint contracture

34

Glucocorticoid sparing effect of TNF inhibitors in RA

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Tumour necrosis factor inhibitors (TNFi) heralded the highly successful biological DMARD era in the management of rheumatoid arthritis (RA). Glucocorticoids (GC) however remain a commonly used adjunctive medication. Whilst effective and shown to be safe in moderate doses, GCs are nonetheless associated with safety concerns, particularly in certain populations such as the elderly and those with co-morbidity. The success of TNFi has enabled the evaluation of concomitant medication, including the use of GC. This talk will review the literature on GC use with TNFi in both trials and real-life practice; discuss whether initiation of TNFi has been associated with decreased GC utilisation and the potential benefits of this. Guidance on how this may be undertaken will also be discussed.

35

Effects of glucocorticoids on bone in RA, the BeSt study

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Until recently, the majority of RA-patients was treated with DMARD-monotherapy, resulting in joint erosions and cartilage loss in many patients. A two-fold increase in the prevalence of osteoporosis, defined as a T-score <-2.5, was found in a large Norwegian study in RA-patients versus healthy controls (Haugeberg G, *Arthritis Rheum* 2000). In line with that, the risk of a having a vertebral fracture was doubled in RA-patients (Orstavik RE, *Arch Int Med* 2004). Nowadays it is thought that in patients with systemic inflammation, bone resorption is upregulated, modulated by changes in RANKl/OPG, while the inhibition of the Wnt-pathway induces depressed bone formation (Schett G, *Ann Rheum Dis* 2010). Theoretically, adequate suppression of systemic inflammation in RA might have bone sparing effects. This has been investigated in the BeSt study, a novel study design comparing four different treatment strategies in which treatment adjustments were made continuously when low disease activity, defined as disease activity score (DAS) <2.4, was not reached in patients with recent-onset RA (Goekoop-Ruiterman YP, *Ann Int Med* 2007). The treatment strategies were: group 1. sequential monotherapy starting with methotrexate (MTX), group 2. step-up combination therapy starting also with MTX, group 3. initial combination therapy with MTX, sulphasalazine and quickly tapered high dose of prednisone, and group 4. initial combination therapy with MTX and tumor necrosis factor alpha (TNF-α) inhibitor infliximab. After 2 years of treat to target therapy, BMD decreased at the hips by -1.1% (group 1), -0.2% (group 2), -0.2% (group 3) and -0.6% (group 4). At the lumbar spine, the bone loss was -0.4%, -1.6%, -0.5% and -1.0%, respectively (Guler-Yuksel M, *Ann Int Dis* 2009). We concluded that generalized bone loss was limited in all 4 groups of patients and, that the generalized bone loss was not higher in patients initially treated with high dose prednisone. In addition, radiological joint damage was low in all 4 groups. This clearly suggests that the negative effect of glucocorticoids (GC) on bone should be outweighted against the strong anti-inflammatory effects of GC on bone (Vis *et al*., Osteoporosis Int 2013). Unfortunately, since the BEST-study was a treat to target study, several treatment options were prescribed to patients who do not have low disease activity: it was not a randomized controlled trial comparing the effects of prednisone versus placebo. However, it has recently been demonstrated that prednisone 10 mg per day in early RA-patients treated with MTX, has a positive effect not only on disease activity, but also on radiological joint damage (Bakker MF, *Ann Int Med* 2012). In conclusion, the BEST-study was among the first modern treat to target studies that showed that the use of GC may not be harmful to the bone.

36

The combination glucocorticoid-adalimumab-methotrexate as induction therapy in early aggressive rheumatoid arthritis

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Early remission is the treatment goal in rheumatoid arthritis (RA). Clinical trials indicate that it can be more commonly obtained by combination therapies either including different traditional DMARDs or DMARDs with a biologic agent or DMARDs with high-dose, short-term glucocorticoids (COBRA regimen). Combination therapies can be effective using a "tight control" step-up strategy and also as starting treatment to take more advantage of the window of opportunity.
In patients with early aggressive RA, combination therapy with biologic agents and methotrexate (MTX) leads to higher remission rates when compared with mono-therapy regimens. Moreover, it has been clearly demonstrated that a short-term aggressive treatment with high dose prednisone associated to traditional DMARDs may lead to long-term benefits and also to a very high remission rate in a pilot study.
We designed a prospective controlled study in order to evaluate the effect on remission rate and remission duration of an intensive induction treatment including high-dose prednisone (COBRA-like) in addition to both MTX and Adalimumab. Two hundred and forty-four subjects with early (>6 weeks, <1 year), MTX-naive RA were enrolled from 20 Italian tertiary referral centers. All patients had active, aggressive disease with >8 swollen and tender joints, CRP >15 mg/L or ESR ≥28 mm/h, and ≥1 joint erosion or positive test for rheumatoid factor or anti-citrullinated peptide antibodies. Patients were randomly selected to receive either prednisone (60 mg/day tapered to 6.25 mg in 6 weeks and to 0 in 6 months) or placebo in association with MTX 20 mg/week and Adalimumab 40mg eow. Remission rate at 12 months was the primary outcome. Those patients who achieved remission at the end of month 12, were followed up for another 12 months-period while receiving only MTX as maintenance therapy. Persistence of remission after Adalimumab discontinuation was a secondary objective of the study.
Enrollment was accomplished at the end of 2012 and a complete evaluation of the primary endpoint will become available in February 2014. An interim analysis at 6 months was carried out (in a blind way, as safety check) on the first 122 patients enrolled. The analysis showed a frequency of withdrawal for severe adverse events (mostly due to MTX-related toxicity) <10%, whilst the overall remission rate exceeded 60%.
To date, no clinical studies have been reported on high-dose glucocorticoids in combination with biologic agents as treatment strategy for induction of remission and maintenance. Our preliminary report suggests that this approach might be effective with an acceptable safety profile.

37

Anti-TNF therapy improves the hypothalamic-pituitary-adrenal axis

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TNF-α, a potent cytokine produced by monocytes, macrophages, B and T cells, and fibroblasts plays a key role in RA and can change the balance of T regulatory cells and control acute immunological responses, and interferes with neuro-endocrine axes. RA patients with high TNF levels have cortisol and ACTH levels that are insufficient in relation to inflammation. The reason for this is unknown, but continuous TNF and interleukin (IL)-6 stimulation of the hypothalamus induces a hypothalamic-pituitary adaptation that annuls organ responses: cytokine levels remain high, whereas hormone concentrations are at most normal. The lower levels of adrenal hormones can be attributed to TNF's direct inhibitory effect on the expression of the steroidogenic acute regulatory protein and the ACTH-stimulated expression of the steroidogenic enzymes P450ssc, P450c21, and P450c11 in

adrenocortical cells. A recent study has demonstrated a rapid increase in ACTH levels in patients with prednisolone-naïve RA after anti-TNF antibody injections; in relation to serum TNF levels, ACTH and cortisol levels continuously increased during the 12 weeks of anti-TNF treatment. Furthermore, the decrease in the serum cortisol to ACTH ratio suggested the sensitisation of ACTH secretion was associated with a relative increase in ACTH. Long-term therapy with anti-TNF sensitises the pituitary gland and improves adrenal androgen secretion in patients with prednisolone-naïve RA, which indicates the normalisation of the HPA axis and must therefore be considered evidence of the additional anti-inflammatory effect of anti-TNF treatment in RA patients.
We have investigated the role of HPA axis hormones as predictors of immediate clinical improvement during anti-TNF antibody therapy, and found that the improvement in RA responders may be related to an increase in serum cortisol levels because TNF inhibits the adrenal conversion of 17OHP to cortisol. These findings indicate that the rapid benefit of anti-TNF agents in some patients is probably due to the resoration of P450c21, and P450c11 in adrenocortical cells.
In conclusion, long-term anti-TNF therapy restores the hormonal pathway, leading to the normalisation of hormone levels/ratios that is associated with a rapid clinical improvement of RA.

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38

Do biologic drugs induce a reduction in steroid burden in systemic lupus erythematosus?

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Different patterns of disease activity were identified in SLE patients including relapsing remitting, long quiescent and chronic active pattern.
Clinical quiescent disease can be observed in approximately 30-40% of cases and chronic active and relapsing remitting disease in the other 60-70%. The persistence of disease activity has some important consequences: first of all it leads to organ damage which we know to predict more damage and death in SLE. One of the major determinants of damage is use of corticosteroids, especially when they are administerd at high dosage. It has been shown that the Hazard ratio for organ damage progressively increases according to the progressive increase of cumulative average dose of prednisone. Thus, to stop corticosteroids or to reduce their dosage are unmet needs in SLE.
Belimumab is a fully human monoclonal antibody which selectively targets and inhibits soluble B lymphocyte stimulator (BLyS), also called B activating Factor (BAFF). Inhibition of BLyS can result in autoreactive B-cell apoptosis. Belimumab obtained the registration for lupus by Food and Drugs Administration and by European Medicine Agency.
In phase III randomized control trials, BLISS-52 which enrolled 865 patients, and BLISS-76 which enrolled 819 patients, patients with mild-to-moderate disease activity who were stable in their baseline treatment for at least 6 months were randomized to receive placebo, Belimumab 1 mg/kg or Belimumab 10 mg/kg plus the standard of care.
Both studies met their primary end-points showing a higher frequency of clinical response in patients treated with Belimumab in addition to standard of care compared with those treated with standard of care alone. Belimumab plus standard of care was generally well tolerated, with a safety profile comparable to that of placebo plus standard of care.
Notably, in BLISS 52 a significantly higher response was observed at each visit until week 52, starting at week 16 whith a dose related response. In addition, the probability of developing a flare was also reduced in patients treated with Belimumab; however, as far as severe flares are concerned, Belimumab 10 mg was superior over Belimumab 1 mg and standard of care. At the same time Belimumab had a steroid sparing effect; in fact the proportion of patients with at least 50% reduction in prednisone dose were significantly greater with belimumab 10 mg at every visit from visit 24 to 52, again with a dose dependent response.
However, the magnitude of the results of the two BLISS studies at 52 weeks and on the lack of effect of Belimumab at 76 weeks in BLISS 76 has been questioned. In this regard, it has to be mentioned that prednisone tapering during Belimumab treatment may restore residual disease activity. Hence a delicate balance is orchestrated between lowered steroid dosage and dampening of disease activity.

39

Pregnancy and biological therapy: the role of GC combination

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Biological drugs are used for an ever increasing proportion of rheumatic patients. Most biological agents are monoclonal antibodies of the IgG1 class. IgG is actively transferred through the placenta by Fc receptors on the trophoblast. Fetal exposure to IgG is very low during organogenesis, but placental transfer starts at the beginning of the 2nd trimester and increases until term when maternal and fetal serum levels are equal or higher in cord serum. Biological agents that contain an Fc-part are also transferred actively through the placenta.
Among the TNF inhibitors infliximab, adalimumab, and golimumab show increasing transplacental passage throughout pregnancy. Transplacental passage of etanercept, a fusion protein directed against the TNF receptor, is much less than for the complete antibodies, and is minimal for the pegylated Fab fragment certolizumab which lacks the Fc part of immunoglobulin. Published experience for infliximab, adalimumab, etanercept and certolizumab have not shown an increase in miscarriage or congenital malformations. No human data on golimumab during pregnancy have been published. It appears that TNF inhibitors are not teratogen and may be used pre-conceptional and in the first trimester, in the 2nd and 3rd trimester only when severity of maternal disease requires it.
The B cell inhibitor rituximab appears not to be a strong human teratogen. However, 2nd and 3rd trimester exposure causes B cell depletion in the fetus with unknown long-term effects in the child. Abatacept and tocilizumab should be discontinued three months before conception because experience in human pregnancy is up to date very limited. Because of its short half-life, prophylactic discontinuation of anakinra before a planned pregnancy is not necessary, but it should not be continued during pregnancy.
Combination of biological drugs with corticosteroids (Cs) may occur in women planning a pregnancy since methotrexate has to be discontinued before pregnancy. Doses of corticosteroids >7.5 mg increase the risk of side effects in pregnant patients. Exposure of the fetus to high doses of Cs in the 2nd and 3rd trimester increases the risk for intrauterine growth restriction and premature delivery. A combination of TNF inhibitors and Cs can suppress the immune function of the child though this has not been studied in detail.

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40

Glucocorticoids and chronotherapy in RA

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Glucocorticoids are usually given after the patient affected by arthritis (*i.e.* rheumatoid arthritis (RA)) awakes in the morning. At a time clinical symptoms such as gelling and stiffness are already at a maximum, but this is not the optimal moment during the day. The question arises why night-time glucocorticoids can be more beneficial than morning glucocorticoids.
In fact, recent data of a double blind placebo-controlled randomized study in hundreds of patients with RA demonstrated a more marked and significant effect on morning stiffness and serum IL-6 when glucocorticoids are given at 2 am in the night (1). Again the question appears why immunosuppressive treatment with glucocorticoids can inhibit proinflammatory sequelae better when given at an early time point at 2 am (2). These observations are very important in understanding antiinflammatory counter-regulation of immune responses. It is has been demonstrated that glucocorticoids induce the transcription of the inhibitor of kappa B (IκBα) gene, which results in an increased rate of IkBα protein synthesis and inhibition of proinflammatory NF-κB effects. Other studies have shown that glucocorticoids can interfere with the transcriptional activation potential of DNA-bound NF-κB complexes leading to antiinflammatory effects (3). These effects appear very early in the turning on phase of a proinflammatory response (early stimulation of immune cells). The turning-on phase of a proinflammatory reaction is much more vulnerable to immunosuppressants as compared to the turning-off phase. Therefore, the regulation of an important proinflammatory factor such as TNF increase, must occur very early because otherwise an overwhelming secretion of this harmful cytokine would occur. In view of the circadian rhythms and the impact of the timing of immunosuppressive administration on efficacy, there is great interest to explore timed-release forms of cytokine neutralizers (on the basis of small molecules) or cronobiological administration of antiproliferative drugs such as methotrexate as therapeutic modality in RA (1). Reformulating old drugs such as glucocorticoids (nocturnal hormone) in new circadian drug delivery forms has recently optimized the clinical efficacy and improved immunosuppressant activities in RA (4). In a 12-week, multicentre, randomized, double-blind trial, 288 patients with active RA were randomly assigned to either a modified-release prednisone tablet (releasing prednisone at 2am) or to an immediate-release prednisone tablet. The modified-release tablet was taken at bedtime (10pm) and prednisone was released with a delay of 4 h after ingestion. This treatment was compared with morning administration of immediate-release prednisone as an active comparator showing a significant superiority (4). In addition, no worsening of adrenal impairment was observed on treatment with nighttime-release prednisone in patients with low responsiveness to CRH testing before the treatment with modified-release prednisone, and no change of adrenocortical function was observed over 12 months (5). In conclusion, since immunosuppressive effects of glucocorticoids are fast, it becomes understandable why night-time availability of glucocorticoids has a stronger immunosuppressive effect compared to treatment in the morning.

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Posters on the topics

P01

Systemic metabolic signaling in acute and chronic gastrointestinal inflammation of inflammatory bowel diseases

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Background. Acute and chronic intestinal inflammation stimulates innate and adaptive immune systems, thereby increasing energy demand of activated immune cells. Energy regulation by systemically released mediators is of critical importance for homeostasis. We asked how systemic metabolic mediators are affected during intestinal inflammation.
Methods. A total of 123 patients suffering from Crohn's disease (CD), 76 patients with ulcerative colitis (UC), and 21 healthy controls were recruited. Patients receiving systemic steroids or therapy regimens including biologicals (anti-TNF) were excluded from the study. Serum levels of IL-6, CRP, insulin, glucose, free fatty acid, and RBP-4 were measured by ELISA and RIA.
Results. Intestinal inflammation was accompanied by elevated systemic inflammatory parameters such as IL-6 and CRP in UC and CD and, concomitantly, with elevated insulin levels and increased insulin/glucose ratio in patients with UC. This indicates insulin resistance in liver, muscle, and fat. In addition, intestinal inflammation was associated with elevated levels of circulating free fatty acids in UC and CD, indicating an activation of the organism's appeal for energy-rich substrates (energy appeal reaction). RBP-4 serum levels were also high in acute and chronic intestinal inflammation in UC and CD, which can support insulin resistance.
Conclusions. The organism's "energy appeal reaction" in response to acute and chronic inflammation provides free energy in the circulation, which is needed by inflammatory cells. A major mechanism of the re-direction program is insulin resistance. New therapeutic strategies might be developed in the future, directly impacting on the storage and utilization of energy-rich fuels.

P02

No difference in DKK-1 protein content in synovial tissue, synovial fluid, and plasma samples of rheumatoid arthritis and osteoarthritis patients

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Objective. In arthritic diseases, the dysbalance between Wnt mediated bone formation and TNF/RANK ligand mediated bone resorption has been described. In rheumatoid arthritis (RA), the inflammatory situation with high TNF concentrations increases the expression of the Wnt antagonist Dickkopf (DKK-1) leading to bone erosions. In contrast, active Wnt signaling promotes osteoblastogenesis and osteophyte formation in osteoarthritis (OA)*. It is also known that inflammatory processes in experimental arthritis are strongly affected by the sympathetic nervous system (SNS): In the early acute phase of experimental arthritis the SNS acts proinflammatory, whereas in the late chronic phase the SNS is anti-inflammatory. It was the aim of this study to analyze DKK-1 content in human OA and RA synovial tissue samples with or without sympathetic influence.
Methods. Synovial tissue, synovial fluid, and blood plasma samples were obtained from patients with rheumatoid arthritis (RA, n=10) and osteoarthritis (OA, n=10). Synovial fluid and blood plasma DKK-1 concentration was determined immediately after taking of samples using ELISA technique. Release of DKK-1 from synovial tissue was determined by tissue superfusion with or without noradrenergic stimulation (noradrenaline 10-9 to 10-5 M) for 6 hours.
Results. DKK-1 concentration in OA synovial tissue superfusates without noradrenergic stimulation was significantly higher compared to RA. There were no differences in DKK-1 levels between RA and OA in synovial fluid and blood plasma samples. In OA superfusates treated with 10-5 M noradrenaline, DKK-1 concentration was significantly increased which was not observed in RA. Conclusion. In summary, this study presents that DKK-1 release from synovial tissue is higher in OA compared to RA. In addition, a sympathetic influence via noradrenaline at high concentrations might increase DKK-1 release in OA synovial tissue. These unexpected findings might depend on relatively low inflammatory levels in our RA patients with longstanding chronic inflammation or concomitant immunosuppressive medication.

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P03

Apremilast, an oral phosphodiesterase 4 inhibitor, in patients with psoriatic arthritis: results of a phase 3, randomized, controlled trial

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Purpose. Apremilast, an oral phosphodiesterase 4 inhibitor, works intracellularly to modulate a network of pro- and anti-inflammatory mediators, including those implicated in the etiopathogenesis of psoriatic arthritis (PsA). This phase 3 study (PALACE 1) compared the efficacy and safety of apremilast with placebo (PBO) in subjects with active PsA despite previous DMARDs and biologics.
Materials and Methods. Subjects were randomized 1:1:1 to PBO, apremilast 20 mg BID, or apremilast 30 mg BID stratified by baseline DMARD use. At wk 16, subjects with <20% reduction from baseline in swollen and tender joint counts were re-randomized to apremilast 20 mg BID or 30 mg BID (PBO group), or remained on their initial dose (apremilast groups). All subjects then continued assigned treatment through wk 24. Stable concurrent treatment with MTX, sulfasalazine, leflunomide or a combination was allowed.
Results. 504 subjects were randomized and comparable across treatment groups for demographics, disease characteristics, and prior/concurrent therapy. Of note, 23.6% of patients had prior biologic exposure, including 9.3% considered biologic failures. At baseline, 64.9% were taking DMARDs (54.2%, MTX). At wk 16, a significantly greater proportion of subjects treated with apremilast 20 mg BID (31.3%; p=0.0140) and 30 mg BID (41.0%; p<0.0001) achieved an ACR20 vs PBO (19.4%) (Table). In subjects receiving apremilast 30 mg BID, higher ACR20 responses were seen in subjects receiving apremilast monotherapy and in biologic-naïve subjects compared with the overall population response. At wk 24, apremilast was associated with significant differences vs PBO in ACR20, ACR50, ACR70, HAQ-DI, SF-36 Physical Function scores, DAS-28, and EULAR response. In general, response rates were higher with apremilast 30 mg BID. Apremilast was generally well tolerated. Adverse events (AEs) occurring in ≥5% of any treatment group were diarrhea (PBO, 2.4%; apremilast 20 mg BID, 11.3%; and apremilast 30 mg BID, 19.0%), nausea (6.5%, 9.5%, and 18.5%), headache (4.8%, 10.1%, and 10.7%), and upper respiratory tract infection (3.6%, 6.0%, and 4.2%). The majority (>95%) of AEs were mild or moderate; discontinuations due to AEs were similar across all treatment arms (5-7%). Serious AEs occurred in 7 (PBO), 8 (apremilast 20 mg BID), and 9 (apremilast 30 mg BID) subjects. No opportunistic infections (including TB) or lymphoma were observed, and there was not a greater risk of cardiovascular events.
Conclusion. Apremilast significantly improved signs and symptoms of PsA and resulted in statistically and clinically meaningful improvements in physical function. Apremilast was generally well tolerated and no new safety or laboratory signals were detected.

Table. Primary and select secondary end points

	PBO (n=165)	Apremilast 20 mg BID (n=163)	Apremilast 30 mg BID (n=161)
ACR20 at wk 16, %	19.4	31.3*	41.0†
APR alone (n=172)	10.5	31.5*	50.8†
APR+DMARDs (n=317)	24.1	31.2‡	35.0‡
ACR20 at wk 16	23.7	31.2‡	43.3*
Biologic-naïve subjects (n=363)			
APR alone (n=89)	11.5	24.1‡	58.8*
APR+DMARDs (n=274)	27.2	33.3‡	37.2‡
Select secondary end points at wk 24			
ACR50, %	4.2	15.3*	19.9†
ACR70, %	0.6	5.5*	11.2†
HAQ-DI, LS mean change from baseline (SE)	-0.077 (0.037)	-0.212 (0.037)*	-0.260 (0.037)*
DAS-28, LS mean improvement from baseline (SE)	0.20 (0.087)	0.66 (0.087)*	0.91 (0.087)†
Good or moderate EULAR response achievement, %	29.1	45.4*	51.6†
SF-36 Physical Function, LS mean change from baseline (SE)	1.46 (0.671)	3.50 (0.675)*	5.06 (0.674)†

Efficacy analyses were conducted using the per-protocol population (N=489); last observation carried forward was used for missing data.
*p<0.05; †p≤0.0001;‡p=non-significant vs. PBO.

P04

Lower serum dehydroepiandrosterone and androstenedione levels in pre-rheumatoid arthritis versus normal control women: correlations with lower serum cortisol levels

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Background. Rheumatoid arthritis (RA) is a leading cause of disability, occurring two or three times more frequently in women than men, and increasing in incidence during adult aging. Low serum adrenal androgens, including androstenedione (Δ4A), dehydroepiandrosterone (DHEA), and its sulfate (DHEAS), have previously been reported in female RA patients. As yet, no study has been performed on androstenedione and DHEA levels before onset of RA (pre-RA).
Objective. This study investigates a broad panel of adrenal steroids, including glucocorticoids and androgens, and their enzymatic pathways, to determine if differences occur between women who later develop RA versus matched controls.
Study Method. "Operation CLUE" is a nested case-control cohort study which enrolled 12,381 females of Washington Co., Maryland, in 1974. The pre-RA cases had later onset of American College of Rheumatology (ACR) criteria-positive RA (from 1977–1992). Four controls (CN) were matched to each pre-RA case on gender, race, and cohort entry age. A comprehensive panel of adrenocortical steroids was assayed on the baseline 1974 stored sera. Levels were standardized by menopausal status and compared in 36 female pre-RA vs 144 CN, by t-tests and age-adjusted partial correlations.
Results. Mean androstenedione levels were lower in total pre-RA vs CN subjects (p=0.015). When analyses were restricted to women with cortisol levels less than the population mean, the preceding Δ4A difference was magnified in these subjects (p=0.005). Also, in subjects having lower cortisol, the mean DHEA level was lower in pre-RA vs CN women (p=0.012). The enzyme leading to Δ4A production, 17,20 lyase, also tended (p=0.053) to be lower in pre-RA than CN among those subjects having lower mean cortisol values. The pre-RA women who had lower Δ4A levels tended (p=0.097) to develop clinical RA sooner after entry into the cohort than the remaining of pre-RA cases.
Conclusions. Study data indicate that women who later developed clinical RA had combined lower baseline cortisol and adrenal androgens (AAs), DHEA and Δ4A, than matched cohort women. Physiologically, cortisol levels remain constant during aging, but AAs progressively diminish. Adrenal function may also decline more rapidly with aging in a subset of pre-RA women having combined lower cortisol and AA levels, than occurs in a control population. Women with relative adrenal insufficiency may have lesser control of inflammatory pathways involved in the multifactorial development of RA.

P05

Combined interactions between dexamethasone and CTLA4-Ig (Abatacept) on activated cultured human macrophages

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Background. In clinical practice, the combination of glucocorticoids and CTLA4-Ig allows to obtain larger clinical improvement in rheumatoid arthritis (RA) patients compared to CTLA4-Ig monotherapy. *In vitro* studies showed that CTLA4-Ig binds to CD86 on human macrophages and that, besides its effect on T cells, induces reverse signaling on macrophages upon the binding [1-3].
Aim. The aim was to investigate the anti-inflammarory effects of dexamethasone (DEX) alone or combined with CTLA4-Ig on cultured human macrophages.
Methods. THP-1 cells, activated into macrophages (PMA 0.05 μg/ml; 24 hrs), were cultured for 48 hrs with DEX (10-7 M) alone, or combined with CTLA4-Ig (500 μg/ml). Cells untreated and treated with CTLA4-Ig alone, were used as controls. CD86 expression was evaluated by immunofluorescence (IF) and by flowcytometry (FACS). In addition, qRTPCR for IL-1β, TNF-α and IL-6 gene expression was performed at 1, 3 hrs after treatments.
Results. Qualitative IF of CD86 demonstrated a decrease of the untreated macrophages positivity after DEX treatment alone and, more prominently after DEX plus CTLA4-Ig-combined treatment. Quantitative FACS revealed 25.4% of CD86 positivity in untreated cells. The CD86 expression on cells treated with DEX alone was reduced, as well as in CTLA4-Ig-treated cells, by 78% and 57%, respectively, compared to untreated cells. DEX plus CTLA4-Ig induced an evident CD86 decrease by 97%. qRTPCR showed in macrophages treated with DEX

alone or plus CTLA4-Ig-combined treatment, after 1 hr, a reduction for the expression of all assayed cytokines. CTLA4-Ig alone reduced IL-1β, TNF-α and IL-6 expression at 3 hrs, but not at 1 hr from treatment. At 3 hrs from DEX and DEX plus CTLA4-Ig treatment, cells still showed a reduction of IL-1β and IL-6 gene expression.
Conclusions. Both DEX and DEX plus CTLA4-Ig treatments, induce a reduction in CD86 expression and an anti-inflammatory effect on human macrophages, by decreasing cytokine gene expression. The results of the combined treatments, seems mainly due to the CTLA4-Ig/CD86 binding and partially related to the genomic effects of DEX and might explain the improved clinical conditions in RA patients treated with CTLA4-Ig and glucocorticoids (1,4).

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P06

A favorable response with rituximab therapy in a lupus patient with digital gangrene

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We report a 38-year-old female patient who had digital ischemic lesions of hands and systemic lupus erythematosus (SLE) including arthritis (Jaccoud arthritis), photosensitivity, oral ulcers, renal disease, and positive ANA, RNP antibody and anti-beta 2 glycoprotein 1-IgA isotype. She had high disease activity of lupus. There was no history of obstetric morbidity and other vascular events.
Laboratory findings: white blood cell: 5.6 $x10^9$/L, Hb:11.9 g/dL, platelet :276 $x10^9$/L, Hct:35.4 %, eritrosit sedimentation rate:120 mm/h, C-reactive protein: 80mg/L, eser proteinuria and microscopic hematuria on urinalysis. Kreatinin and hepatic enzymes were within normal values.
Autoimmune serology showed ANA (1/3200 titer, ++++, speckled pattern), U1RNP (+++), p-ANCA (+), anti-beta2 GP1-IgA isotype (43,5 U/mL; normal: <5 U/mL). There was hypocomplementemia (C3:0,647 g/L, C4 0,0758 g/L; normal : 0.9-2g/L and 0.1-0.4 g/L, respectively) ds-DNA and Sm antibodies and rheumatoid factor were negative. Microbial analyses were negative. Electrocardiographic, echocardiographic and Computer Tomography (CT) of the thorax were unremarkable.
Initial treatment was started with glucocorticoid (1mg/kg/day), aspirin (300 mg/day), intravenous prostaglandin (2 mcg/kg/min) and intravenous cyclophosphamide (500 mg/m2). Digital ischemia showed a serious progression to the digital gangrene over one month in spite of follow up under treatment with glucocorticoid, immunosuppressant, antiaggregant and potent vasodilatator agents. Rituximab (RTX) with 2 infusions of 1g at 14-day intervals was added to the treatment regimen. After 6 months, second cycle of RTX therapy was given. During the following, a complete recovery of digital lesions and the regression of active disease signs were observed, and acute phase responses receded. The patient is still clinical remission with glucocorticoid (10mg/day), aspirin (100mg/day), azathioprine (2.5mg/kg/day) and hydroxychloroquine (200mg/day) for the last 2 years. As a conclusion in patients with progressive digital ischemic lesions and systemic lupus erythematosus under traditional immunosuppressive agents, RTX can be an option.

P07

17β-estradiol and 5α-dihydrotestosterone influence integrin expression levels and influence IL-6 production in an integrin β1-dependent manner in synovial fibroblasts from RA and OA donors

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Background. In rheumatoid arthritis (RA), synovial fibroblasts (SF) secrete large amounts of IL-6, IL-8 and matrix metalloproteinases (MMPs) which are crucial for cartilage destruction. The production of pro-inflammatory factors is in part mediated by enhanced constitutive integrin signaling since expression of these adhesion molecules is increased in RA. Furthermore, anti-inflammatory androgens are reduced in RA while pro-inflammatory estrogen metabolites are increased. Since sex hormones modulate integrin levels and function in a variety of cell types, this study demonstrates the influence of sex hormones on integrin expression and cytokine production under basal and inflammatory conditions in SFs.
Methods. Integrin levels were determined by flow cytometry. Cytokines were detected by sandwich ELISA.
Results. 5α-dihydrotestosterone (DHT) and 17β-estradiol (E2) marginally influenced levels of integrin subunits α1, α3, α5 and αv and dose-dependently decreased integrin β1 levels. A decrease in β1 integrin by DHT correlated with decreased IL-6 production in RA while decreased β1 by estrogen correlated with reduced IL-6 levels in OA. Furthermore, DHT and E2 decreased IL-1β stimulated IL-6 and IL-8 production only when SFs were pre-incubated with respective sex hormones. The effects of DHT and E2 were enhanced under COX-2 inhibition.
Conclusion. DHT and E2 elicit ant-inflammatory effects by downregulating IL-6 production in SFs. This occurs partly via an integrin β1 dependent pathway under basal conditions. Under pro-inflammatory conditions, E2 and DHT might act via a COX-2 metabolite since a) pre-incubation with DHT and E2 is necessary to elicit anti-inflammatory effects and b) effects are enhanced by COX-2 inhibitor nimesulide.

P08

The synthetic cannabinoid and CB_1/CB_2 agonist WIN55212,2 decreases the production of inflammatory mediators in rheumatoid arthritis synovial fibroblasts by activating TRPV1 and non-cannabinoid receptor targets

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Background. In rheumatoid arthritis (RA), synovial fibroblasts (SF) secrete large amounts of IL-6, IL-8 and matrix metalloproteinases (MMPs) which are crucial for cartilage destruction. RASFs are sensitive to the action of cannabinoids and they express cannabinoid receptors type I and II (CB_1 and CB_2) and the transient receptor potential channels type vanilloid (TRPV1) and ankyrin (TRPA1). The synthetic cannabinoid WIN55212,2 demonstrated strong anti-inflammatory effects in monocytes and synovial fibroblasts only in high concentrations in a non-cannabinoid receptor dependent manner. In this study we assessed the ability of WIN55212,2 to modulate cytokine and MMP-3 production over a wide concentration range under normoxic and hypoxic conditions in synovial fibroblasts from RA and OA donors.
Methods. MMP-3, IL-6 and IL-8 were determined by ELISA.
Results. Under normoxic conditions and IL-1β as inducer of cytokine production, WIN55212,2 did not significantly modulate IL-6 and IL-8 production in concentrations below 2μM, while higher concentrations completely inhibited cytokine production. This was not dependent on activation of either CB_1 or CB_2. Under hypoxic (1% O_2) conditions and TNF as inducer of cytokine production, WIN55212,2 (10^{-6}M to 10^{-12}M) dose-dependently inhibited IL-6, IL-8 and MMP-3 production. In RASFs but not OASFs, the effects of WIN were attenuated by capsazepine, a TRPV1 antagonist. Furthermore, fetal calf serum content in culture media strongly influenced the efficacy of WIN55212,2.
Conclusion. The synthetic cannabinoid WIN55212,2 exhibits anti-inflammatory effects in synovial fibroblasts independent of CB_1 and CB_2. Our results indicate a TRPV1 dependent mechanism that might be coupled to cellular energy status since decreasing serum content and hypoxia augment the effects of WIN55212,2 on production of IL-6, IL-8 and MMP-3.

P09

The endocannabinoid-like fatty acid amides palmitoylethanolamine and oleoylethanolamine exert anti-inflammatory effects in synovial fibroblasts from RA and OA donors

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Background. In rheumatoid arthritis (RA), synovial fibroblasts (SF) secrete large amounts of IL-6, IL-8 and matrix metalloproteinases (MMPs) which are crucial for cartilage destruction. While the endocannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG) are abundant in RA and OA synovial tissue and fluid, the related fatty acid amides palmitoylethanolamine (PEA) and oleoylethanolamine (OEA) are only found in synovial tissue from healthy individuals. Although AEA and 2-AG are considered to be anti-inflammatory, OEA and PEA are

important for the resolution of inflammation. Therefore, in this study we assessed the ability of PEA and OEA to modulate cytokine and MMP-3 production under normoxic and hypoxic conditions in synovial fibroblasts from RA and OA donors.
Methods. MMP-3, IL-6 and IL-8 were determined by ELISA.
Results. Under normoxic conditions and IL-1β as inducer of cytokine and MMP-3 production, PEA and OEA significantly reduced IL-6 and IL-8 production in RASFs but only slightly in OASFs. Under hypoxic (1% O_2) conditions and TNF as inducer of cytokine and MMP-3 production, OEA (10^{-5}M to 10^{-11}M) dose-dependently inhibited IL-6, IL-8 and MMP-3 production only with concomitant COX-2 but not FAAH inhibition. In analogy to that, PEA decreased IL-6, IL-8 and MMP-3 which was also enhanced by COX-2 inhibition.
Conclusion. The endocannabinoid-like compounds PEA and OEA exhibit anti-inflammatory effects in synovial fibroblasts which was enhanced by COX-2 inhibition. Since both compounds do not bind COX-2 directly, our results indicate an indirect mechanism possibly involving degradation of PEA and OEA by FAAH and subsequent oxygenation by COX-2.

P10

Osteoclast progenitors, fibroblast-like cells and draining lymph node cells induce catecholaminergic-to-cholinergic transition of sympathetic nerve fibers under healthy conditions but not in highly inflamed arthritic tissue

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Sympathetic nerve fibers innervate bone and tissue adjacent to joints. They play an important role in bone and tissue homeostasis. Under certain conditions, sympathetic nerve fibers can change their phenotype from catecholaminergic to cholinergic (see innervation of sweat gland and periosteum). This can be important because anti-inflammatory effects of acetylcholine have been described which is mainly mediated by the alpha7-nicotinic acetylcholine receptor. We asked whether this transition could also occur in the joint during collagen-induced arthritis (CIA) in mice or during rheumatoid arthritis (RA) and osteoarthritis (OA) in humans. Arthritic limbs from 30 immunized C57Bl/6J mice were collected at distinct time points covering all stages of the disease. Sections of mouse limbs and synovial tissue samples obtained from 30 OA and 12 RA patients were stained for tyrosine hydroxylase (TH, noradrenergic fibers), and for vesicular acetylcholine transporter (VAChT, cholinergic fibers). For co-culture experiments, sympathetic ganglia were obtained from newborn mice and double-stained for TH and VAChT after a co-culture period of two to three days with osteoclast progenitors attained from the femoral and tibial bonemarrow as well as lymphocytes obtained from the draining lymph nodes and fibroblast-like cells isolated from the paws of adult mice. In mouse joint area, an increase in the ratio of cholinergic to catecholaminergic nerve fibers appeared at day 35 after immunization. Most of the nerve fibers were located in joint-adjacent skin or muscle tissue, and only very few were detected in synovial tissue or near erosions. In human tissue sections, we were able to show cholinergic fibers in the synovial tissue of four OA patients but in none of the RA patients. Co-cultures of sympathetic ganglia and osteoclast progenitors as well as lymphocytes and fibroblast-like cells obtained from healthy mice showed more catecholaminergic-to-cholinergic transition when compared to experiments with the respective cells from arthritic mice. In men and mice, catecholaminergic-to-cholinergic transition is possible in less inflamed tissue of the joint but not in highly inflamed arthritic tissue.

P11

The spatial energy expenditure configuration and possible applications in an experimental model of arthritis

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Background. An autoimmune response with differentiation and proliferation of immune cells and the subsequent tissue-directed inflammatory process in the symptomatic phase of the disease are very energy-demanding. As recent calculations demonstrate, the activated immune system needs approximately 20% of the basal metabolic rate. During long standing inflammatory diseases like rheumatoid arthritis, a reallocation of energy-rich fuels to the activated immune system is necessary in order to nourish the inflammatory process. Energy consumption and, thus, ATP generation can be measured by studying the consumption of oxygen. The energy expenditure in different organs at different time points has never been investigated during immunization. We want to find out if, and how the energy expenditure in different organs changes during the course of experimental arthritis.
Methods and Results. A new technique termed "spatial energy expenditure configuration (SEEC)" was developed to demonstrate bodily areas of high energy demand. SEEC is based on removal of tissue during the course of arthritis, and subsequent determination of oxygen consumption. For that purpose, small weighed pieces of the respective organ with a size of 4 mm are placed in 24-well multidishes with integrated oxygen sensors, which allows for non-invasive detection of oxygen consumption *in vitro*. SEEC was established in healthy control animals, arthritic animals and animals that underwent prior sympathectomy. We determined the oxygen consumption in spleen, thymus, draining lymph nodes, liver, kidney, brain and knee joints during the course of experimental arthritis for 70 days. In draining lymph nodes of arthritic DBA/1J mice we observed a marked increase in oxygen consumption during the course of arthritis (200%). Sympathectomy prior to immunization increases energy consumption in draining lymph nodes, which is most probably a sign of retention of leucocytes in the lymph node. C57BL/6 mice deficient for the important adipose triglyceride lipase revealed an increased oxygen consumption in the liver. This might be due to a lack of lipolysis activity, and therfore increased gluconeogenetic activity in the liver for the generation of energy rich fuels in form of glucose. ATGL-deficient arthritic animals also showed higher energy demand in lymph nodes, adrenals and gut.
Conclusions. The SEEC technique enables us to identify locations of high energy demand that are involved in the initiation and continuation of the autoimmune process in an animal model of arthritis. We identified the draining lymph nodes as target organ of the sympathetic nervous system, which will be further investigated. The technique will be applied to other chronic inflammatory disease models in order to detect further participating organs.

P12

Limited correlations between sex hormones and nailfold microangiopathy extent, peripheral blood perfusion and finger dermal thickness in a small cohort of systemic sclerosis patients

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Background. 17beta-estradiol (E) has been shown to induce a significant increase of fibronectin, collagen type I, and laminin synthesis in both normal and systemic sclerosis (SSc) fibroblasts *in vitro* (1, 2). Furthermore, dehydroepiandrosterone sulphate (DHEAS) was found decreased in SSc patients in comparison with healthy subjects, whereas prolactin (PRL) was detected higher (3, 4).
Aim. Clinical study to investigate possible correlations between sex hormone serum levels and nailfold microangiopathy extent, peripheral blood perfusion (PBP) and finger dermal thickness (FDT) in a small cohort of SSc patients.
Methods. Twenty-three post-menopausal SSc patients (leRoy 2001 or ACR criteria) were enrolled (mean±SD age 60±7, disease duration 8±5 years). Serum levels of E, total (Tt) and free testosterone (Tf), DHEAS, and PRL were evaluated by standard methods. At the same time nailfold microangiopathy extent was assessed by videocapillaroscopy (NVC), in order to detect the pattern of microvascular damage ("Early", "Active", "Late") and the microangiopathy evolution score (MES) (5). In addition, PBP was quantified by both laser Doppler flowmetry and laser speckle contrast analysis at the level of the fingertips (6). FDT was measured by high frequency ultrasound, as previously reported (7).
Results. PRL was significantly higher in SSc patients with "Active" and "Late" pattern of microangiopathy in comparison with "Early" pattern (p=0.01). DHEAS, Tt and Tf were found progressively lower in patients with "Early", "Active" and "Late" pattern of microangiopathy, but this was not statistically significant. A not statistically significant progressive increase of E was detected in patients with "Early", "Active" and "Late" pattern of microangiopathy. Furthermore, no statistically significant correlation was found between investigated hormones and MES, PBP, and FDT.
Conclusion. With the exclusion of PRL, which was found significantly higher in SSc patients with more advanced patterns of nailfold microangiopatrhy, the study demonstrates no significant correlations between sex hormones and nailfold microvascular damage extent, PBP and FDT. However, the small cohort of patients included into the study limited the possible significance of the analyzed parameters.

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P13

NeuroEndocrine-Immunology in orthogeriatric patients: preliminary report on the acute modification of urinary cortisol and serum BDNF levels in Alzheimer's disease after osteoporotic hip fracture

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Introduction. Osteoporotic hip fracture needs a specific clinical approach and treatment. When AD patients present an osteoporotic hip fracture, the clinical management becomes complex. Data are scanty about neuroendocrine-immune modifications in AD patients. Glucocorticoids on cerebral cells (*e.g.*, neurons and astrocytes) are relevant for brain functioning (with resulting damaging effects in AD) and cortisol levels during the stress reaction (fracture and surgery) have important metabolic effects. Conversely, Brain Derived Neurotrophic Factor (BDNF) promotes neuronal maintenance, survival and synaptic plasticity (with resulting protective effects in AD).
Objectives. The aim of the study was to investigate cortisol and BDNF levels in orthogeriatric patients with AD, with respect to controls.
Methods. We enrolled: AD patients after surgery for hip fracture (n=5) (A), untreated AD patients (n=6) (B), healthy elderly (n=8) (C) and young (n=6) (D) controls. AD diagnosis was carried out by NINCDS-ADRDA criteria; cognitive evaluation included Mini Mental State Examination. The serum BDNF levels were measured through ELISA (Promega). The urinary cortisol was collected from 8 am to 8 pm (diurnal cortisol, Fd) and from 8 pm to 8 am (nocturnal cortisol, Fn).
Results. After the stress condition (hip fracture with surgical intervention), AD patients showed a marked increase of Fd and Fn (457.5+174.0 and 203.8+144.3), when compared to untreated AD patients (120.5+26.2 and 74.5+70.7), elderly (115.3+40.7 and 64.4+47.1) and young controls (93.4+43.8 and 59.1+45.1 mcg) (Fd A vs. B $p<0.01$, A vs. C $p<0.001$, A vs. D $p<0.001$). The serum BDNF levels were reduced in AD patients after surgery for hip fracture (A, 8.4+2.4), with respect to untreated AD patients (B, 9.6+2.4), elderly (C, 11.5+2.5) and young controls (D, 16.1+2.1 pg/ml) (BDNF A vs. B n.s., A vs. C $p<0.05$, A vs. D $p<0.001$).
≠ The osteoporotic hip fracture and the related surgery in AD induce a marked alteration of the neuroendocrine-immune parameters (BDNF and cortisol), synergistically involved in the clinical course of AD patients. Further studies are necessary in order to evaluate the effects of these modifications on cerebral (worsening of cognition and appearance of delirium) and general (alteration of glucose metabolism, risk of infection) functions.

P14

A prospective study in premenopausal women with Systemic Lupus Erythematosus supplemented with two different regimes of vitamin D: efficacy and safety at 12 months of follow-up

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Background. Systemic Lupus Erythematosus (SLE) patients (pts) are at risk for low vitamin D (VitD) levels because of lack of sun exposure. The current strategies of VitD supplementation do not seem to be sufficient not only for the prophylaxis of osteoporosis, but perhaps also to bring out the immunomodulatory effects of VitD that were highlighted by some *in vitro* studies. Few prospective studies are available on the effects of VitD supplementation SLE pts.
Objectives. To evaluate at 12 months of follow-up (T12) the efficacy, safety and the effects on SLE disease activity of an oral Cholecalcipherol supplementation given with 2 different regimens in a cohort of SLE pts.
Methods. 34 premenopausal SLE women were enrolled. A group of 18 pts (group S) were given "standard" regimen of supplementation (Cholecalcipherol 25.000UI once/month).The other 16 pts (group I) were given an "intensive" regimen (Cholecalcipherol 300.000UI bolus, then 50.000UI once/month). The circulating levels of 25-OH VitD were dosed every 3 months with a chemiluminescence assay kindly performed by the manufacturer (DiaSorin S.p.A., Italy).
Results. At baseline (T0) there was no significant difference in VitD levels in the 2 groups. After 3, 6, 9 and 12 months "group I" showed significantly higher VitD levels (median at T12: 32.0 vs. 24.8 p=0,04). There were no significant differences upon season of enrollment. At T0 there was no difference in the proportion of sufficient pts (>30 ng/ml) between groups (S:50%, I:56%), while at T12 sufficient pts were 28% in S and 75% in I (p=0.02). No significant variations in the levels of calcium, phosphorus and PTH were observed. No cases of PTH suppression. There were 3 cases of transitory mild hypercalciuria (2 in I, 1 in S). The pts had clinically quiescent disease (median SLEDAI 2 in S, 4 in I), but serologically active disease (positive anti-DNA and/or complement consumption in nearly 50% of the pts). No statistically significant variation in the titers of anti ds-DNA and in the levels of C3, C4, CH50 was observed at T12 in both groups.
Conclusions. Intensive supplementation with VitD has a safe profile as the standard regimen but it is able to induce sufficient levels in a larger number of pts. No particular effects on serological SLE parameters was noted, probably due to the stable remission state of the pts. More data will come from the second year of the study in which patients will switch to the other group of supplementation.