Effect of combined Low-Dose Radon- and Hyperthermia Treatment (LDRnHT) of patients with ankylosing spondylitis on serum levels of cytokines and bone metabolism markers: a pilot study

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Abstract: Ankylosing Spondylitis (AS) is a rheumatic disease and almost 50% of the affected patients suffer from osteoporosis. The ratio of Receptor Activator of Nuclear Factor κB Ligand (RANKL) and Osteoprotegerin (OPG) has become an important marker to assess the status of systemic bone metabolism and has been shown to be dysregulated in AS patients. Combined Low-Dose Radon- and Hyperthermia Therapy (LDRnHT) causes pain reduction in patients with AS and induces the cytokine transforming growth factor-β1 (TGF-β1). TGF-β1 acts as an anti-inflammatory cytokine and influences the OPG/RANK/RANKL system.We therefore performed a study on 33 AS patients to investigate the effect of LDRnHT on serum levels of bone metabolism markers and cytokines involved in chronic inflammatory disorders. It is shown that TGF-β1, TNF-α, IL-6, OPG and RANKL levels significantly increased after LDRnHT and that the ratio of OPG over RANKL is significantly elevated.

Keywords: radon; OPG; RANKL; RANK; inflammatory cytokines; TGF-β1; ankylosing spondylitis.

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1 Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease, mainly characterised by tissue destruction in axial joints and bilateral sacroiliitis, sometimes also affecting peripheral joints and extra-articular organs such as lung and heart valves. Chronic inflammatory processes may cause bone resorption, which frequently results in secondary osteoporosis, a typical extra-articular symptom that severely affects the spine in about 41% and the femur in 46% of AS patients (Sivri et al., 1996; Meirelles et al., 1999; Capaci et al., 2003) and predisposes the patient to a high risk of fracture (Marwick, 2000; Borman et al., 2001; Walsh et al., 2005; Jacobs and Fehlings, 2008). Of note, osteoporosis is further aggravated by functional and pain-related disuse bone atrophy as well as by frequently employed glucocorticoid medication (Sambrook and Lane, 2001). Combined low-dose radon/hyperthermia therapy (LDRnHT) has been reported to cause functional improvement and pain reduction in patients with AS (van Tubergen et al., 2001; Falkenbach et al., 2005) or rheumatoid arthritis (Becker, 2004; Erickson, 2006; Franke et al., 2007). According to a previous study, pain alleviation in AS correlates to an elevation in post-treatment serum levels of the anti-inflammatory cytokine TGF-β (Shehata et al., 2006). Apart from its role as an immune-modulator, TGF-β1 also plays an essential role in bone metabolism, particularly by acting as differentiation factor for monocytes/osteoclasts and via stimulation of osteoblast- and down-regulation of osteoclast activity (Fox and Lovibond, 2005). TGF-β1 is released from bone matrix during active bone resorption and exerts its effects in concert with other cytokines and hormones like TNF-α, INF-γ, IL-4 and oestrogens by influencing the OPG/RANKL/RANK system, which is crucial in the control of the biological activity balance of the osteoblast-osteoclast interplay. Receptor activator of nuclear factor κB-ligand (RANKL) is a potent stimulator of osteoclast-mediated bone resorption and promotes osteolysis. RANKL is mainly produced by osteoblasts and bone marrow stromal cells, but is also released from lymphocytes, monocytes, macrophages, megakaryocytes, fibroblasts, synoviocytes and endothelial cells (Gravallese, 2002; Hofbauer, 2006). It acts by binding to receptor activator of nuclear factor-κB (RANK) on osteoclasts. Osteoprotegerin (OPG) is the functional antagonist of RANKL as it acts as a soluble RANKL decoy receptor that, upon

ligation to RANKL, abrogates the interaction with RANK and consequently inhibits maturation and activation of osteoclasts and their precursors (Hsu et al., 1999; Bolon et al., 2002; Gravallese, 2002; Sipos et al., 2008). OPG is produced by osteoblasts and bone marrow stromal cells, but also by dendritic and smooth muscle cells. The relative concentrations of OPG and RANKL determine the status of bone metabolism and thus, the OPG/RANKL ratio has become an important marker to assess the prevailing metabolic bone turnover situation. An increased OPG/RANKL ratio indicates an anabolic and a decreased ratio a katabolic bone state (Hofbauer and Schoppet, 2001). Both OPG and RANKL exert their functions in concert with other cytokines and hormones including TGF-β,TNF-α, M-CSF, IL-1β, IL-6, -7, -11, -15, -17, -18, PTH, PTH-rP, corticosteroids and PGE2, as well as mechanical strain (Hofbauer, 2006). In AS patients, elevated serum levels of RANKL and RANKL/OPG ratios significantly correlate with the manifestation of osteoporosis (Franck et al., 2004; Karst et al., 2004; Kim et al., 2006).

The finding that LDRnHT causes an increase in anti-inflammatory cytokine TGF-β1 in AS patients (Shehata et al., 2006) and the fact that this cytokine is able to down-regulate RANKL and to up-regulate OPG (Thirunavukkarasu et al., 2001; Karst et al., 2004; Fox and Lovibond, 2005; Hofbauer, 2006) led us to establish the hypothesis that LDRnHT promotes bone remodelling by elevating the OPG/RANKL ratio in AS. Therefore, we performed a pilot study on 33 patients with diagnosed AS to establish a basal setting for a planned randomised controlled trial to investigate the effect of therapeutic LDRnHT on serum levels of TGF-β1, OPG, RANKL, the OPG/RANKL ratio as well as on other prominent key players in inflammation and bone metabolism that have also been selected as targets for therapeutic intervention in rheumatoid arthritis, like TNF-α, IL-6 and IL-17 (Bessis and Boissier, 2001; Nishimoto and Kishimoto, 2006; Woo et al., 2007). The observed changes support the hypothesis that LDRnHT is effective to modulate the osteo-immune system by elevating TGF-β1, TNF-α and IL-6 levels as well as the OPG/RANKL ratio.

2 Patients and methods

The study was designed as pilot study (Lancaster et al., 2004) and was approved by the ethics committee of the state of Salzburg, Austria. Written informed consent was obtained from all patients. Inclusion criteria were: age between 20 and 75 years, diagnosed spondylitis ankylosans based on the modified New York Criteria for the Diagnosis of ankylosing spondylitis (Goie The et al., 1985), physician prescribed LDRnHT and previously given written consent. Exclusion criteria were: therapy with glucocorticoids, bisphosphonates, parathyroid hormone, vitamin-D or strontium renalate four weeks prior or during the study, treatment with TNF-α blockers or other biologicals eight weeks prior or during the study as well as hormone replacement therapy in post-menopausal women. A total of 33 patients (8 women, 25 men) median aged 53 (ranging from 21 to 74 years) were enrolled without performing new radiographic examinations before enrolment. The median duration of disease was 28 years (ranging from 10 to 58). Fourteen patients received NSAIDs. Data prior and post-LDRnHT were compared. LDRnHT was performed in the thermal tunnels of Boeckstein-Bad Gastein with a total

administration of ten treatments during three weeks. Each treatment was of 90 min at 37–40.5°C ambient temperature, 70–95% air humidity and radon (^{222}Rn) activity of ~4.5 nCi/L. Standardised questionnaires for the evaluation of BASDAI, BASFI and BASMI scores were completed by the patients.

2.1 Blood serum preparation

Forearm venous blood samples were drawn immediately before the first and immediately after the last treatment. Blood was collected in monovettes (Becton Dickinson, NJ, USA), incubated for 15 min at room temperature and centrifuged for 15 min. Serum was stored in aliquots at –20°C prior to analysis.

2.2 Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs were performed according to the manufacturer's instructions. OPG and RANKL determination kit was purchased from Biovendor-Laboratorni (Modrice, Czech Republic). IL-17 and TGF-β1 kits were from eBioscience (San Diego, USA). TNF-α and IL-6 kits were from ImmunoTools (Friesoythe, Germany).

Limits of quantitation according to the manufacturer specifications were 0.4 pmol/L (OPG), 10 pmol/L (RANKL), 60 pg/mL (TGF-β1), 3 pg/mL (TNF-α), 4 pg/mL (IL-17) and 4.0 pg/mL (IL-6), respectively. Values beyond assay sensitivity were assumed equal to limit of quantitation according to the manufacturers specifications.

Fold changes were calculated as c_a/c_b where c_a and c_b are the measured concentrations after (c_a) and before (c_b) LDRnHT, respectively.

2.3 Statistical analysis

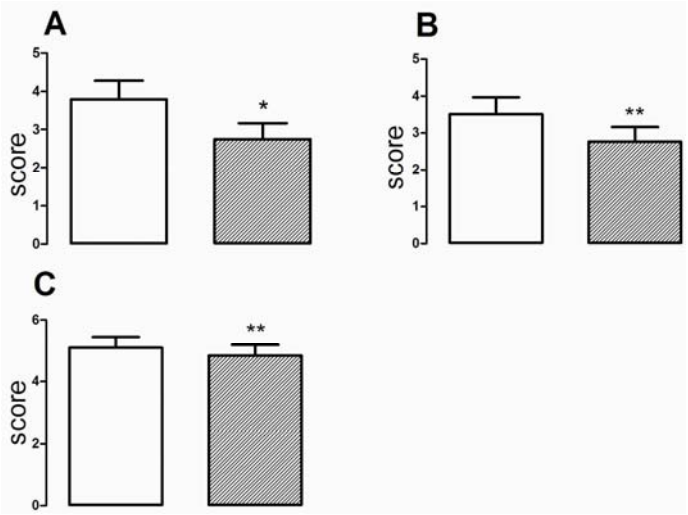
Data were evaluated for normal distribution prior to statistical analysis. Data are expressed as mean ± SEM. *P*-values <0.05 were considered significant. Statistical analysis was performed using a student's paired *t*-test or, where no normal distribution was present, by Wilcoxon signed rank test. All tests were performed using GraphPad Prism software (GraphPad Software, Inc.).

3 Results

3.1 Decrease of disease-associated symptom scores

For clinical assessment of LDRnHT efficacy, disease-associated symptom score indices were calculated from standardised and validated questionnaires completed by physician and the patients. Mean scores of disease activity (BASDAI) significantly decreased from 3.8 ± 0.9 to 2.7 ± 0.4 (p = 0.0145), functional restriction (BASFI) from 3.5 ± 0.4 to 2.8 ± 0.4 (p = 0.015) and spine mobility (BASMI) from 5.1 ± 0.4 to 4.8 ± 0.4 (p = 0.0046) after treatment (Figure 1).

Figure 1 Changes of disease associated symptom scores means ± SEMs prior to (white bars) and post (filled bars) LDRnHT (A) BASDAI, (B) BASFI and (C) BASMI scores (N = 21; two-tailed paired *t*-test)



3.2 Increased levels of circulating TGF-β1 in its active form

Mean levels of active TGF-β1 in peripheral blood significantly increased by 1.53 ± 0.26 fold (p = 0.0224) from 360.4 ± 27.4 to 431.5 ± 33.85 pg/mL (Figures 2A and 3). Individual concentrations ranged from 60 pg/mL to 944.2 pg/mL.

Figure 2 Changes of serum cytokines, OPG and RANKL concentrations and the OPG/RANKL ratio prior to (white bars) and post (filled bars) LDRnHT (A) TGFβ1, (B) IL6, (C) TNFα, (D) IL17, (E) OPG, (F) RANKL and (G) ratio of OPG/RANKL (N = 33; A, C, D, E, F – two-tailed; G – one-tailed paired *t*-test and B – Wilcoxon signed rank test, respectively)

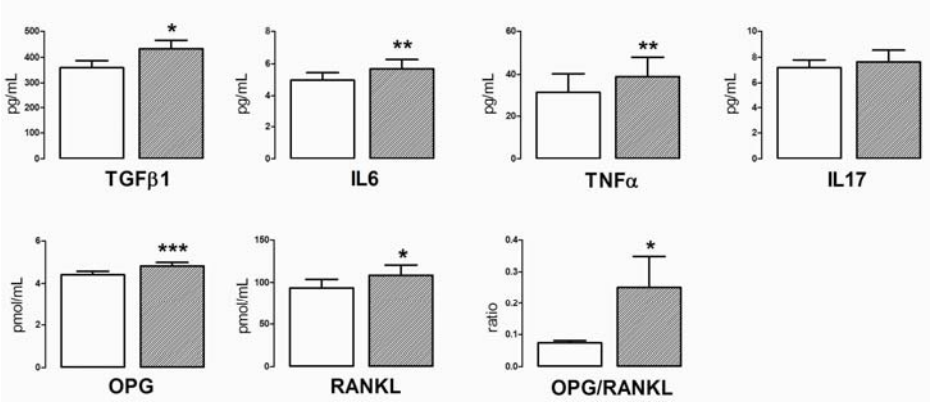
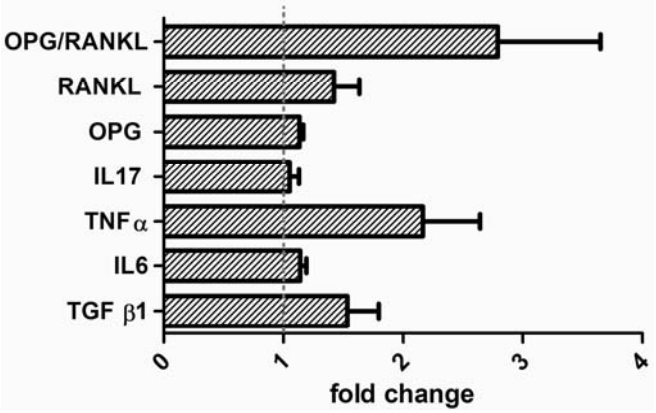


Figure 3 Post-treatment fold changes compared to baseline values of TGFβ1, IL-6, TNFα, IL-17, OPG, RANKL and the OPG/RANKL ratio (Mean ± SEM)



3.3 Changes of inflammatory markers TNF-α, IL-6 and IL-17

Mean serum concentrations of TNF-α significantly increased by 2.16 ± 0.48 fold (p = 0.0013) from 31.6 ± 8.7 to 39 ± 9.1 pg/mL (Figures 2C and 3). Individual concentrations ranged from 3.0 pg/mL to 179.8 pg/mL. IL-6 concentrations significantly rose by 1.14 ± 0.05 fold (p = 0.0024) from 5.0 ± 0.5 to 5.7 ± 0.6 pg/mL (Figures 2B and 3). Individual concentrations ranged from 4.0 pg/mL to 18.1 pg/mL. IL-17 levels were not significantly elevated by 1.05 ± 0.08 fold (p = 0.4477) from 7.3 ± 0.6 to 7.7 ± 0.9 pg/mL (Figures 2D and 3). Individual concentrations ranged from 4.0 to 29.1 pg/mL, respectively.

3.4 Elevation of serum OPG, RANKL and OPG/RANKL ratio

The mean serum OPG concentration significantly increased by 1.12 ± 0.03 fold (p = 0.0006) from 4.4 ± 0.18 pmol/L to 4.8 ± 0.17 pmol/L after therapy (Figures 2E and 3). Individual concentrations ranged from 1.2 pg/mL to 7.0 pg/mL. Mean changes of serum RANKL levels significantly elevated by 1.42 ± 0.21 fold (p = 0.0339) from 93.7 ± 10.3 to 108.8 ± 12 pmol/L (Figures 2F and 3). Individual concentrations ranged from 10.0 pg/mL to 398.6 pg/mL. The determination of the OPG/RANKL ratio revealed a significant increase by 2.79 ± 0.85 fold (p = 0.0364; one-tailed paired t-test) from 0.07 ± 0.007 to 0.25 ± 0.1 (Figure 2G and 3).

4 Discussion

This study confirms the previous observation showing that LDRnHT leads to a significant improvement of BASDAI, BASFI and BASMI scores and to an up-regulation of circulating TGF-β1 in its active form (Figure1) (Shehata et al., 2006). TGF-β1 is secreted in a latent complex (Lawrence et al., 1985; Annes et al., 2003), which is cleaved in its active form in response to oxidative stress (Barcellos-Hoff, 1996; Barcellos-Hoff and Dix, 1996). Alpha-particles have been previously shown to initiate the production

of reactive oxygen species (ROS; superoxide anions and hydrogen peroxide) in human cells (Narayanan et al., 1997) and increase in ROS correlate with enhanced secretion of TGF-β1, TNF-α, IL-1 and IL-8 (Morgan, 2003a; Morgan, 2003b). In line with this, increased activities of superoxide dismutase and catalase have been measured in the blood from patients with osteoarthritis after radon treatment (Yamaoka et al., 2004). TGF-β1 exerts anti-inflammatory effects by favouring the differentiation of regulatory T cells and down-regulating the activity of effector T cells and phagocytes (Chen et al., 2003) but has also been shown to induce TNF-α secretion from human T cells (Gunnlaugsdottir et al., 2005). In addition, TGF-β is a major regulator of extracellular matrix remodelling by stimulating collagen deposition (Verrecchia and Mauviel, 2004) and has a fundamental role in the control of bone metabolism by acting on the OPG/RANKL/RANK system where it plays a dual role. On the one hand TGF-β is essential to promote the differentiation of monocyte precursors to mature osteoclasts by maintaining the cells' responsiveness to the osteoclastogenic cytokine RANKL. On the other hand TGF-β is released from bone matrix during active resorption and inhibits this process by repression of RANKL and induction of OPG (Karst et al., 2004; Fox and Lovibond, 2005). Beside TGF-β, the OPG/RANKL/RANK system is tightly regulated by different cytokines and hormones. Particularly those associated with chronic inflammatory disorders seem to enhance bone resorption. IL-1, IL-11, IL-17, parathyroid hormone, PGE2 and glucocorticoids augment RANKL and down-regulate OPG expression, while IL-4 and 17β-estradiol decrease RANKL expression. OPG is up-regulated by IL-1, IL-13, IL-18, BMP-2, 17β-estradiol, leptin and mechanical strain (Hofbauer and Heufelder, 2001). Moreover, TGF-β1 in context with IL-6 seems to play a pivotal role in the differentiation of the Th17 lineage, a T helper cell subset secreting IL-17 originally discovered in murine models of chronic inflammation and, recently also identified to play an important role in human rheumatic disorders. Kotake et al. (2001) reported that RANKL and IL-17 contribute to osteoclastic bone resorption in patients with rheumatoid arthritis (Chabaud et al., 1999; Miossec, 2003; Lubberts et al., 2005; Lubberts, 2008).

In the pathophysiology of chronic inflammatory disorders, a dominant role has been assigned to TNF-α, as it is a key player in the maintenance of chronic inflammation and augments osteoclast formation and release of osteoclast-precursors from the bone marrow into the blood stream via NFATc1 dependent mechanisms (Takayanagi, 2005). Treatment of patients with TNF-α blocking agents has been demonstrated to ameliorate inflammation (Vis et al., 2006), arrested spine and hip bone loss but not metacarpal cortical hand bone loss (Vis et al., 2006) and decrease the RANKL/OPG ratio (Ziolkowska et al., 2002; Catrina et al., 2006). However, a recent study revealed that levels of OPG, RANKL and C-telopeptide remained unchanged whereas bone-specific alkaline phosphatase and osteocalcin significantly increased after treatment with TNF-α blocking agent etanercept. Interestingly in this study serum levels of TGF-β were significantly decreased after treatment (Woo et al., 2007). These studies indicate an interdependency of these cytokines and point to differential regulation of the OPG/RANKL/RANK system and other biochemical bone turnover markers by TNF-α and TGF-β. TGF-β1 antagonises TNF-α in many biological aspects critical for the pathophysiology of chronic rheumatic diseases. However, the network and hierarchical order of the osteo-immune system is complex and are still poorly understood. Thus, manipulating one player in this interwoven network is likely to result in unpredictable net effects on the parameters of interest. In AS patients, elevated serum levels of RANKL and RANKL/OPG ratios have been demonstrated to correlate significantly with the

manifestation of osteoporosis (Kim et al., 2006). The incidence of osteoporosis is high in AS patients and patients with active disease are especially at risk for developing osteoporosis (Borman et al., 2001; Walsh et al., 2005; Jacobs and Fehlings, 2008).

In our study the OPG/RANKL ratio significantly increased after LDRnHT (Figure 2G) suggesting a shift of bone metabolism towards bone restoring processes despite significantly increased concentrations of TNF-α and IL-6 (Figure 2B and C), which are in favour of enhanced bone resorption. TNF-α also contributes to Cox-2 activation and leads to increased PGE_2 production (Pruzanski et al., 1998; Fehrenbacher et al., 2005) that contribute to pain in patients with chronic inflammatory disorders. As prostaglandins are local factors with half-life times of only a few seconds, serum levels of PGE2 do not necessarily reflect their concentration at the site of inflammation and therefore PGE2 is likely to be an inadequate biochemical indicator for systemic effects in the current setting. Obviously, to assess how these elevating levels of TNF-α, IL-6 and TGF-β1 interact, if they compensate for or synergise with each other, further analysis at later time points after treatment is required to better define the real impact on bone metabolism and chronic inflammation in AS patients.

Although it is tempting to assume that elevating serum OPG/RANKL ratios reflect a systemic stimulation of anabolic bone remodelling, it has to be determined whether or not LDRnHT also arrests focal or systemic bone loss or ultimately leads to enhanced bone density and improvement of osteoporosis. Thus, beside bone metabolism marker- and pro-inflammatory cytokine measurements, radiological assessment and bone densitometry analysis will further evaluate the long-term benefit of this kind of therapy and its place among the different treatment options for the disease.

The data obtained in this pilot study now allow to design a randomised controlled trial to further investigate the biological responses of low-dose radon and/or hyperthermia treatment of AS on the human osteo-immune system as they clearly demonstrate systemic responses to LDRnHT, which to date have been only poorly investigated in terms of objective biological parameters.

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Abbreviations

AS: Ankylosing Spondylitis
BASDAI: Bath Ankylosing Spondylitis Activity Index
BASFI: Bath Ankylosing Spondylitis Functional Index
BASMI: Bath Ankylosing Spondylitis Metrology Index
Cox-2: Cycloxygenase-2
INF-γ: Interferon-γ
IL: Interleukin
LDRnHT: Low-Dose Radon- and Hyperthermia Treatment
M-CSF: Macrophage-Colony Stimulating Factor
NSAID: Non-Steroidal Anti-Inflammatory Drug
OPG: Osteoprotegerin
PTH: Parathyroid Hormone
PTHrp: Parathyroid Hormone-related Protein
PGE_2: Prostaglandin E_2
RANK: Receptor Activator of Nuclear Factor κB
RANKL: Receptor Activator of Nuclear Factor κB-Ligand
TGF-β1: Transforming Growth Factor β1
TNF-α: Tumor Necrosis Factor α