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VERTEBRAL OSTEOMYELITIS IS CHARACTERISED BY INCREASED RANK/OPG AND RANKL/OPG EXPRESSION RATIOS IN VERTEBRAL BODIES AND INTERVERTEBRAL DISCS

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Abstract

Vertebral osteomyelitis (VO) is an infection of the spine mainly caused by bacterial pathogens. The pathogenesis leading to destruction of intervertebral discs (IVDs) and adjacent vertebral bodies (VBs) is poorly described. The present study aimed at investigating the connection between infection and bone/disc metabolism in VO patients.

14 patients with VO (infection group) and 14 patients with burst fractures of the spine (fracture group; control) were included prospectively. Tissue biopsies from affected IVDs and adjacent VBs were analysed by RT-qPCR for mRNA-expression levels of 18 target genes including chemokines, adipokines and genes involved in bone metabolism.

Most importantly, the receptor activator of NF-κB/osteoprotegerin (*RANK*/*OPG*) expression ratio was drastically elevated in both VBs and IVDs of the infection group. In parallel, expression of genes of the prostaglandin-E2-dependent prostanoid system was induced. Such genes regulate tissue degradation processes *via* the triad OPG/RANK/RANKL as well as *via* the chemokines IL-8 and CCL-20, whose expression was also found to be increased upon infection. The gene expression of the adipokine leptin, which promotes inflammatory tissue degradation, was higher in IVD tissue of the infection group, whereas the transcription of omentin and resistin genes, whose functions are largely unknown in the context of infectious diseases, was lower in infected VBs.

In summary, similar expression patterns of pro-inflammatory cytokines and pro-osteoclastogenic factors were identified in VBs and IVDs of patients suffering from VO. This suggests that common immuno-metabolic pathways are involved in the mechanisms leading to tissue degradation in VBs and IVDs during VO.

Keywords: Vertebral osteomyelitis, signalling molecules-cytokines, adipokines, cells/tissues-intervertebral disc, infection-*in vivo*, spine-vertebral body, osteoimmunity, RANK/RANKL/OPG.

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List of Abbreviations

ADAMTS	a disintegrin and metalloprotease with thrombospondin motifs
ADIPOQ	adiponectin
AF	annulus fibrosus
AO	Arbeitsgemeinschaft für Osteosynthesefragen
BMI	body mass index
CCI	Charlson comorbidity index
CCL20	chemokine (C-C motif) ligand 20
COMI	Core outcome measurement index
COX2	cyclooxygenase 2
CRP	C-reactive protein
CT	computed tomography
CXCL	C-X-C motif chemokine ligand
DEXA	dual-energy X-ray absorptiometry
EQ-5D	EuroQoL in 5 dimensions
ELISA	enzyme-linked immunosorbent assay
GUSB	glucuronidase beta
IL	interleukin
ITLN1	intelectin 1 (omentin)
IVD	intervertebral disc
LEPR	leptin
MALDI-TOF	matrix-assisted laser desorption/ ionisation-time of flight
MMP	matrix metalloproteinases
mPEGS1	microsomal prostaglandin E synthase 1
NAMPT	nicotinamide phosphoribosyltransferse (visfatin)
NP	nucleus pulposus
NSAIDs	non-steroidal anti-inflammatory drugs
ODI	Oswestry disability index
OPG	osteoprotegerin
PCT	procalcitonin
PGE2	prostaglandin E2
PPAR-γ	peroxisome proliferator-activated receptor gamma
PROM	patient-reported outcome measures
QoL	quality of life
qPCR	quantitative polymerase chain reaction
RANK	receptor activator of nuclear factor κB
RANKL	RANK ligand
RETN	resistin
RT-PCR	real-time quantitative polymerase chain reaction
S. aureus	*Staphylococcus aureus*
TLRs	Toll-like-receptors
TNF-α	tumour necrosis factor alpha
VB	vertebral body
VO	vertebral osteomyelitis

Introduction

Pyogenic VO is defined as an infection of the IVD and adjacent VB and is frequently accompanied by destruction of bone tissue. VO is the most frequent manifestation of haematogenous osteomyelitis in adults, with an estimated incidence of 2.4 cases per 100,000 people in European countries (Gouliouris *et al*., 2010; Grammatico *et al*., 2008). The most common causative pathogen is *S. aureus*, followed by *Escherichia coli* and *Pseudomonas aeruginosa* (Doutchi *et al*., 2015). Several studies have investigated the clinical (Loibl *et al*., 2014; Mylona *et al*., 2009), microbiological (Jean *et al*., 2017; Kim *et al*., 2015), histological (Iwata *et al*., 2019) and radiological features (Foreman *et al*., 2017; Kouijzer *et al*., 2018) of VO. Nevertheless, the interplay between infection, immune responses and bone/tissue metabolism remains poorly understood. Bone homeostasis and the relation between osteoblasts, osteoclasts and osteocytes are regulated by the RANK/RANKL/OPG signalling pathway (Wada *et al*., 2006). RANK and its ligand (RANKL) were first identified in the context of the immune system (Anderson *et al*., 1997). More than 20 years later, their axis is accepted to be at the interface between bone metabolism and the immune system, thereby forming the basis for the concept of osteoimmunology (Nakashima and Takayanagi, 2009; Okamoto *et al*., 2017). OPG is a soluble decoy receptor for RANKL, with high affinity. It is expressed by mature osteoblasts and it binds to RANKL, preventing the interaction with its cell surface receptor RANK and inhibiting the production of osteoclasts (Kong *et al*., 2000). The physiological ratio of RANK/RANKL/OPG is sensitively balanced. An increase in RANKL leads to bone resorption through increased osteoclast formation, function and extended osteoclast survival. On the other hand, OPG plays a bone-protecting role. The RANKL/OPG ratio has been described as a key determinant of the degree to which osteoclast-mediated bone resorption occurs (Dougall, 2012; Kostenuik, 2005). Bacterial pathogens can stimulate osteoclastogenesis in animal models by inducing RANKL and PGE2, which can potentiate bone resorption, resulting in bone destruction (Meghji *et al*., 1998; Okahashi *et al*., 2003; Somayaji *et al*., 2008). RANKL production by osteoblasts is increased and OPG production decreased following *in vitro S. aureus* infection of both a mouse pre-osteoblastic cell line and human osteoblasts (Widaa *et al*., 2012; Young *et al*., 2011). Furthermore, *S. aureus* interaction with osteoblasts stimulates the expression of many chemokines (*e.g.* CXCL8, CXCL10), cytokines (*e.g.* IL1β, IL18, TNF-α, IL6, IL12), COX2 and important growth factors (Josse *et al*., 2015). IL8 as well as the T- and B-cell-attracting cytokine CCL20, both induced by the action of PGE2 on T cells (Boniface *et al*., 2009; Caristi *et al*., 2005; Chizzolini *et al*., 2008), enhance osteoclastogenesis through modulation of cytokine production by human primary osteoblasts (Pathak *et al*., 2015).

In contrast to the well-vascularised VB, the IVD, consisting of the NP, AF and cartilage-like endplate, is an avascular, immune-privileged organ. This may be a reason why comprehensive exploration of the immunological mechanisms in the infected

Table 1. Primer sequences for 18 target genes and the reference *GUSB* used for qPCR.

Gene	Gene symbol	Sequence (5′ to 3′)
Glucuronidase beta	*GUSB*	GAAAATACGTGGTTGGAGAGCTCATT
		CCGAGTGAAGATCCCCTTTTTA
RANK	*TNFRSF11A*	ATGCGGTTTGCAGTTCTTCTC
		ACTCCTTATCTCCACTTAGG
RANKL	*TNFSF11*	CACTATTAATGCCACCGAC
		GGGTATGAGAACTTGGGATT
OPG	*TNFRSF11B*	GCTTGAAACATAGGAGCTG
		GTTTACTTTGGTGCCAGG
Leptin	*LEPR*	CAAGCTGTGCCCATCCAAAA
		GGATCACGTTTCTGGAAGGC
Resistin	*RETN*	TCGCCGGCTCCCTAATATTT
		ACTGGCAGTGACATGTGGT
Adiponectin	*ADIPOQ*	GAGATCCAGGTCTTATTGGTC
		CTTTCCTGCCTTGGATTCC
Nicotinamide phosphoribosyltransferase (visfatin)	*NAMPT*	GAGTTCAACATCCTCCTGGC
		TCACGGCATTCAAAGTAGGA
Intelectin 1 (omentin)	*ITLN1*	ACTGCGGGATTTGTTCAGTT
		GTATCCTCCTCCACCAATGC
COX2 /prostaglandin-endoperoxide synthase-2	*PTGS2*	CTCCTGTGCCTGATGATTGC
		TCTAGCCAGAGTTTCACCGT
Microsomal prostaglandin E synthase-1	*PTGES (mPGES1)*	GAGACACGGAGGCCCCCAGTATTG
		GCCCGCAGCTTCCCCAGGTAG
Peroxisome proliferator activated receptor gamma (PPAR-g)	*PPARG*	GGGGTTCTCATATCCGAGGG
		GGGCGGTCTCCACTGAGAATAA
CCL1	*CXCL1*	AAGTGTGAACGTGAAGTCC
		GGATTTGTCACTGTTCAGCA
CCL5	*CXCL5*	GGAAGGAAATTTGTCTTGATCC
		TTTCCTTGTTTCCACCGTC
CCL8	*CXCL8 (IL8)*	GGCAGCCTTCCTGATTTCTGCAGC
		GTTTTCCTTGGGGTCCAGACAGAGC
CCL10	*CXCL10*	TTCAAGGAGTACCTCTCTCTAG
		CTGGATTCAGACATCTCTTCTC
CCL20	*CCL20 (MIP-3a)*	TGCTGTACCAAGAGTTTGCTC
		CGCACACAGACAACTTTTTCTTT
MMP13	*MMP13*	CTGCATCCTCAGCAGGTTG
		GTCTCGGATAGTCTTTATCC
ADAMTS4	*ADAMTS4*	CAAGGTCCCATGTGCAACGT
		CATCTGCCACCACCAGTGTCT

IVD remains challenging. On the other hand, the degenerated IVD has been studied in detail. In degenerative discs, tissue-degrading enzymes such as MMPs, ADAMTSs and TLRs are induced (Bachmeier *et al.*, 2009; Kepler *et al.*, 2013; Klawitter *et al.*, 2014; Millward-Sadler *et al.*, 2009). Interestingly, the RANK/RANKL/OPG system has also been shown in *ex vivo* studies of human IVD tissue to be involved in IVD degeneration (Sano *et al.*, 2019; Takegami *et al.*, 2017). To the authors' knowledge, there have been no studies investigating the role of the RANK/RANKL/OPG system in conditions of VO. For this reason, mRNA expression patterns were investigated in tissue samples from IVDs and adjacent VBs of patients suffering from VO and vertebral fractures (control patients). Candidate genes were selected to provide an overview of basic bone metabolism and inflammation. Moreover, genes previously implicated in bacterial infection were analysed. The study hypothesis was that the RANK/RANKL/OPG axis as well as key immune-regulatory (adipo-) cytokines and proteinases are affected by VO.

Materials and Methods

Patients

The study prospectively included patients suffering from clinically and radiologically confirmed native pyogenic VO who were treated at the University

Hospital Regensburg, Germany between 2013 and 2015 (infection group). Patients suffering postoperative, implant-associated infection, tuberculosis, chronic inflammatory diseases and solid tumours were not included in the study. Patient-related data (gender, age, BMI) and treatment details (surgical strategy, duration of hospitalisation, treatment with NSAIDs and antibiotics) were recorded and the CCI (Charlson *et al*., 1987) was assessed for each patient in the infection group based on the medical history. PROM before surgery and at least 12 months postoperatively were recorded: for the evaluation of the QoL outcomes, the well-established COMI (Mannion *et al*., 2009) and the ODI (Fairbank and Pynsent, 2000) were used. Laboratory parameters (CRP, leukocyte count, PCT) were measured as part of routine controls and documented at 5 different time points: (i) admission to the hospital, (ii) the week before surgery, (iii) the day before surgery, (iv) discharge, (v) last follow-up. In patients with two-stage surgical procedures, the corpectomy (second surgery) was chosen as a reference point.

As a control (fracture) group, patients that received stabilisation of the anterior spinal column for the treatment of burst fractures at the University Hospital Regensburg, Germany between 2013 and 2015 were prospectively included in the study.

Ethics statement

This study was carried out in accordance with the Declaration of Helsinki and approved by the ethics committee at the University of Regensburg (Institutional Review Board number 12-101-0218). Written informed consent was obtained from all individual participants included in the study.

Tissue samples

Tissue samples from the VB and IVD were collected from all patients in both groups either by CT-guided biopsy or open surgical biopsy. Intraoperatively, the area of interest at the infected VB and IVD was identified and samples were obtained as part of the necessary debridement of the infected segment. In fracture patients, the fractured VB was removed (partial/complete), together with the affected IVD, before the anterior column reconstruction, using an iliac crest bone graft (mono-segmental) or an expandable vertebral body replacement (bi-segmental). Biopsies of VB and IVD were harvested intra-operatively, placed in 500 µL RNAlater solution (Ambion) and stored at 4° C overnight to allow the solution to thoroughly penetrate into the tissue. Next, they were stored at – 20° C until processing.

Microbiology

In patients of the infection group, pathogens were detected in blood cultures (n = 5), in CT-guided biopsies of either the VB (n = 1) or IVD (n = 2), or in intraoperative open biopsies of either the VB (n = 11) or IVD (n = 11). Cultured isolates were identified using MALDI-TOF mass spectrometry using a Microflex LT device and BioTyper software (Bruker Daltonik, Bremen, Germany). In one case, culture was negative; however, a specific pSa-442 PCR (Reischl *et al*., 2000) detected *S. aureus* in the VB as well as in the IVD biopsy.

mRNA isolation and cDNA synthesis

Total RNA was isolated from preserved tissue samples using the RNAqueous Total RNA Isolation kit (Ambion). Tissue samples were disrupted and homogenised in the Lysis/Binding solution provided with the kit by using a 5 mm stainless steel bead and a TissueLyser (Qiagen) for 6-8 min at 30 Hz. Isolated RNA was treated using the TURBO DNA-free kit (Invitrogen) to remove genomic DNA. Purified RNA was quantified using a NanoDrop 1000 and the quality analysed using an Agilent Bioanalyzer 2100. Approximately 300 ng of total RNA per sample were used for reverse transcription using the iScript Advanced cDNA Synthesis Kit (Bio-Rad).

qRT-PCR

Absolute quantification of transcripts was done by qPCR using a TaqMan ABI 7900HT system (Applied Biosystems) and SYBR Green I. Amplification was carried out in duplicates over 45 cycles of 10 s at 95° C, 15 s at 55-61° C and 15 s at 72° C. In each qPCR run, an external standard curve was generated using a 5 Log-spanning serial dilution of the pGEM®-T Easy plasmid vector (Web ref. 1) harbouring one copy of the respective target sequence per plasmid molecule. qPCR was performed in a reaction volume of 10 µL containing 5 µL of LightCycler 480 SYBR Green I Master (Roche), 0.4 µmol/L of each primer (Table 1) and 2 µL of the cDNA sample in a 5-fold dilution. Calculated copy numbers of the target genes were normalised to 10^5 copies of the reference gene *GUSB*. A second reference gene (beta-2-microglobulin) was used to cross-check and to verify the obtained results. Specificity of products was assessed by high-resolution melting curves.

Statistical analysis

Statistical analysis was carried out using R version 3.6.2 (R Core Team, Vienna, Austria). Figures were designed using the R packages ‘ggplot2’, ‘ggpubr’ and ‘corrplot’. Variables were tested for normal distribution using the Shapiro-Wilk test. Groups were compared using Mann-Whitney U tests. Spearman’s rank coefficient tests were used to evaluate the degree of correlation of gene expression levels in VB and IVD and to evaluate the degree of correlation of gene expression levels and clinical parameters, separately for the infection group and for the fracture group. Differences in PROM between groups and time points were evaluated using the independent and dependent paired t-test, respectively. $p < 0.05$ was considered statistically significant.

Table 2. Demographic, treatment details and PROMs of the infection group (n = 14) compared to the fracture group (n = 14). Variables are given in absolute number or mean ± standard deviations. Significance was assessed by *chi*-squared test for frequencies of anti-inflammatory drug and preoperative antibiotic intake and deaths and by independent t-test for the hospitalisation time and the PROM results. p values indicated in bold are statistically significant.

	Parameter	Infection group (n = 14)	Fracture group (n = 14)	p
Demographics	Age (years)	67.1 ± 11.7 (44.0-81.0)	42.7 ± 13.5 (22.0-68.0)	**0.000**
	Sex (male)	n = 9	n = 12	0.284
	BMI (kg/m²)	30.5 ± 6.0	23.7 ± 3.4	**0.002**
Treatment	Hospitalisation (d)	28.4 ± 12.8	19.3 ± 8.1	**0.043**
	Antiinflammatory drugs preoperative (yes)	n = 10	n = 14	**0.034**
	Antibiotics preoperative (yes)	n = 10	n = 0	**0.001**
	Deaths during follow-up (yes)	n = 2	n = 0	0.150
PROM	ODI preoperative	59.7 ± 21.5	82.7 ± 10.3	**0.030**
	ODI follow-up	20.8 ± 18.4	26.7 ± 13.5	0.424
	COMI preoperative	6.8 ± 2.0	8.4 ± 2.4	0.213
	COMI follow-up	2.3 ± 2.3	1.7 ± 1.6	0.875

Results

Demographics and treatment details

28 patients were included in the study, with 14 patients in the infection group (infection group) and 14 patients in the control group (fracture group). Intraoperative tissue samples from the affected VB and IVD were collected from all patients after written informed consent was obtained. 14 patients (5 females, 9 males) with clinical and radiological proof of VO in whom a causative pathogen was identified were evaluated. In the fracture group, all tissue samples needed for mRNA isolation were collected from the VB (n = 11) and IVD (n = 11) by intraoperative open biopsy.

The mean age of patients in the infection group was 67.1 ± 11.7 (44-81) years. The mean BMI was 30.5 ± 6.1 kg/m². The median CCI was 3.5 (0-8). VO was located in the cervical spine in 3 cases, the thoracic spine in 4 cases, the lumbar spine in 6 cases and 1 patient suffered from multifocal VO of the cervical, thoracic and lumbar spine. The mean CRP value at admission (i) was 84.0 ± 62.9 mg/L and reached 129.3 ± 74.1 mg/L in the week before surgery (ii). The day before surgery (iii), 87.6 ± 60.4 mg/L CRP was measured. The CRP value at discharge (iv) was 40.5 ± 44.8 mg/L and decreased to 9.0 ± 9.6 mg/L at the last follow-up (v). CRP-values at discharge (iv) and at the last follow-up (v) were significantly lower compared to the values at admission (i; $p < 0.001$ and $p = 0.020$, respectively). Both values (iv and v) were also significantly lower compared to the highest CRP values (the week before surgery, ii) ($p = 0.010$ and $p = 0.010$, respectively). Furthermore, treatment success was monitored using the QoL analysis and patients in the infection group were followed for 18.2 ± 5.3 months. The mean ODI and COMI improved significantly from preoperative values to the follow-up ($p = 0.003$ and $p = 0.001$, respectively; Table 2).

The mean age of the patients in the fracture group was 42.7 ± 13.5 (22-68) years. The mean BMI was 23.7 ± 3.4 kg/m². The most frequently seen fracture morphology was the AO type A2 fracture (n = 6), followed by A3 (n = 4) and A4 (n = 4). In 4 cases an additional flexion-distraction injury (AO type B2) was documented. Fractures were found at the thoraco-lumbar transition in 13 cases (Th11: n = 6; Th12/L1: n = 1; L1: n = 6). The cervical spine was affected in 1 case. The mean time between the trauma and the stabilisation surgery for the anterior column with biopsy was 7.4 d (range 1-18 d). Results of the ODI and COMI values of patients in the fracture group are listed in Table 2.

Therapy with anti-inflammatory drugs and antibiotics

All patients in the infection group received antibiotic treatment during hospitalisation. Treatment was started before surgery for 12 patients (Table 2). The most frequently pre-operatively used antibiotics (and combinations thereof) were rifampicin in 7 cases (mean: 14.6 d; range: 5-31 d), flucloxacillin in 3 cases (mean: 13.7 d; range: 6-23 d), cefazolin in 2 cases (mean: 8.5 d; range: 4-13 d) and meropenem in 2 cases (mean: 31.5 d; range: 8-55 d). Ciprofloxacin (18 d), linezolid (4 d), levofloxacin (9 d) or metronidazol (5 d) ware used in 1 case each.

In the infection group, 10 out of 14 patients took anti-inflammatory drugs at the time of biopsy, most frequently metamizol (n = 5) and ibuprofen (n = 5) (Table 2). All patients in the fracture group received anti-inflammatory drugs preoperatively, most frequently metamizol (n = 10) and ibuprofen (n = 10).

Causative pathogens and severity of VO

S. aureus was identified as the causative pathogen in 8 patients. *Staphylococcus epidermidis* was found in 2 cases. In 1 case each *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Propionibacterium avidum* and

Achromobacter spp. were identified. Blood cultures were positive in 5 cases (all *S. aureus*). 4 patients were diagnosed with (concomitant) sepsis. In 5 cases a psoas and/or an epidural abscess were found. 1 patient was diagnosed with endocarditis.

Most VO-associated complications were seen in patients with *S. aureus* infection: 4 out of 5 epidural abscesses, 4 out of 5 psoas abscesses and 1 out of 1 endocarditis were found in these patients. Patients infected with *S. aureus* had also a significant higher CRP value at admission (122.5 ± 56.9 mg/L) than the other patients in the infection group (32.6 ± 13.9 mg/L) ($p = 0.03$).

Transcriptional changes at infected sites in VO

To provide an overview of changes in basic bone metabolism and tissue degradation in a VO setting, mRNA expression of *RANK*, *RANKL*, *OPG* and proteases were measured (Fig. 1).

OPG mRNA expression was significantly lower in VB ($3.3 \times 10^3 \pm 3.4 \times 10^3$) and showed a trend towards lower expression in IVD ($7.2 \times 10^3 \pm 7.1 \times 10^3$) tissue samples in the infection group compared to the fracture group (VB: $2.6 \times 10^4 \pm 2.4 \times 10^4$, $p = 0.003$; IVD: $7.9 \times 10^4 \pm 9.5 \times 10^4$, $p = 0.097$). In the infection group, *RANK* expression in the VB was significantly higher ($8.4 \times 10^3 \pm 7.4 \times 10^3$) and with a trend towards a higher expression in IVDs ($6.4 \times 10^3 \pm 5.9 \times 10^3$) than in the fracture group (VB: $2.9 \times 10^3 \pm 2.6 \times 10^3$, $p = 0.034$; IVD: $9.2 \times 10^2 \pm 8.5 \times 10^2$, $p = 0.097$). There was no significant difference in *RANKL* mRNA expression in VBs (infection: $1.7 \times 10^4 \pm 2.8 \times 10^4$; fracture: $1.6 \times 10^4 \pm 1.8 \times 10^4$) and IVDs (infection: $1.7 \times 10^4 \pm 1.6 \times 10^4$; fracture: $1.0 \times 10^4 \pm 9.2 \times 10^3$) between both groups (Fig. 1).

mPEGS1 was significantly upregulated in IVDs ($5.9 \times 10^4 \pm 7.8 \times 10^4$, $p = 0.026$) and with a tendency towards upregulation in VBs ($4.1 \times 10^4 \pm 7.4 \times 10^4$, $p = 0.068$) of the infection group compared to the fracture group (IVD: $5.3 \times 10^3 \pm 3.2 \times 10^3$; VBs: $1.5 \times 10^4 \pm 3.2 \times 10^4$) (Fig. 1). *COX2* mRNA expression showed a trend towards a higher expression in VBs ($8.0 \times 10^3 \pm 8.3 \times 10^3$) and IVDs ($9.3 \times 10^3 \pm 1.8 \times 10^4$) of the infection group compared to the fracture group (VB: $5.7 \times 10^3 \pm 1.4 \times 10^4$, $p = 0.064$; IVD: $2.8 \times 10^3 \pm 1.2 \times 10^3$, $p = 0.566$).

Among the proteases, only *ADAMTS4* was found to be upregulated in VBs of the infection group (infection: $1.3 \times 10^5 \pm 2.5 \times 10^5$; fracture: $3.1 \times 10^4 \pm 4.3 \times 10^4$; $p = 0.022$). *PPAR-γ* was significantly downregulated in VBs of the infection group (infection: $7.3 \times 10^3 \pm 6.4 \times 10^3$; fracture: $1.4 \times 10^4 \pm 8.7 \times 10^3$; $p = 0.007$), whereas no significant difference was seen in the IVDs (infection: $6.4 \times 10^3 \pm 5.5 \times 10^3$; fracture: $1.1 \times 10^4 \pm 3.6 \times 10^3$; $p = 0.138$). The protease *MMP13* showed a trend towards a higher mRNA expression in VBs ($1.6 \times 10^6 \pm 3.5 \times 10^6$) and IVDs ($8.2 \times 10^5 \pm 9.4 \times 10^5$) in the infection group compared

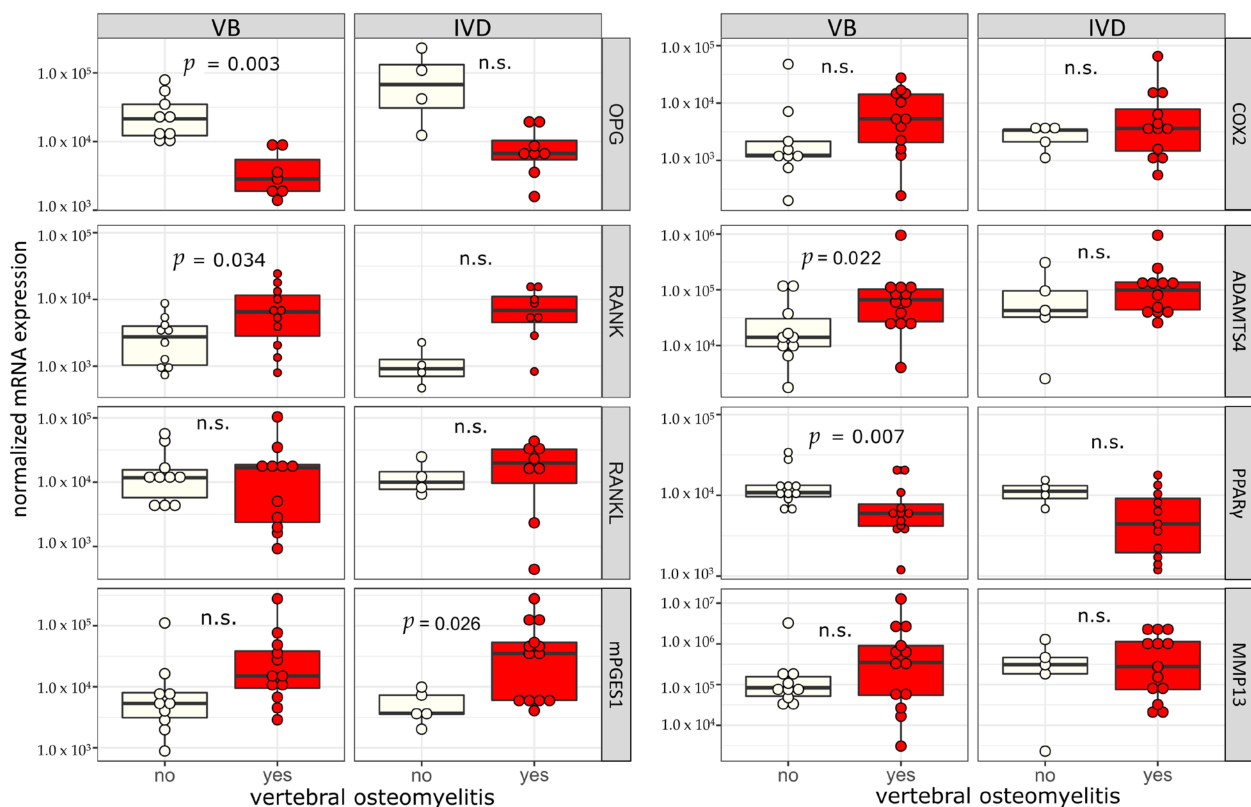

Fig. 1. Comparison between mRNA expressions of (bone) metabolic genes in fracture (beige) and infection group (red) in VBs and IVDs. The infection group is indicated by "yes" (VO), the fracture group by "no" (no VO). Depicted are copy numbers of each target gene normalised to 10^5 copies of *GUSB*. Boxes represent the interquartile range with indicated median. Significance was assessed by Mann-Whitney U test. n.s: statistically not significant.

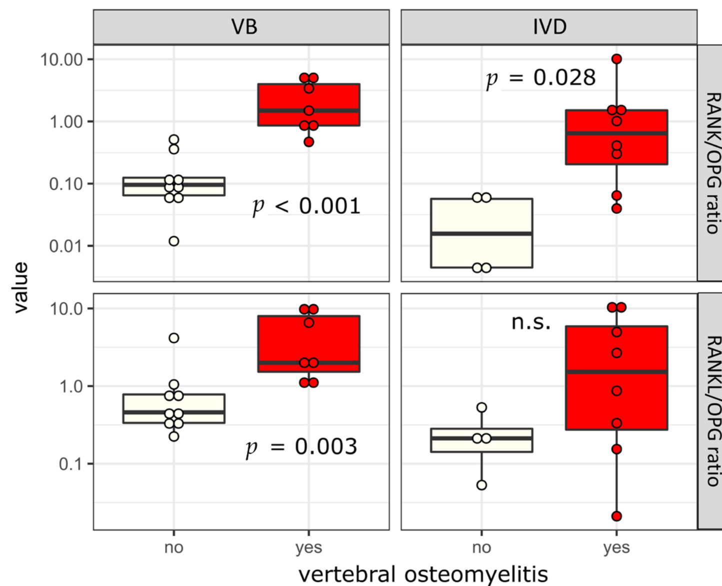

Fig. 2. Comparison between the calculated *RANK/OPG* and *RANKL/OPG* expression ratios in fracture (beige) and infection group (red) in VBs and IVDs. The infection group is indicated by "yes" (VO), the fracture group by "no" (no VO). Significance was assessed by Mann-Whitney U tests. n.s: statistically not significant.

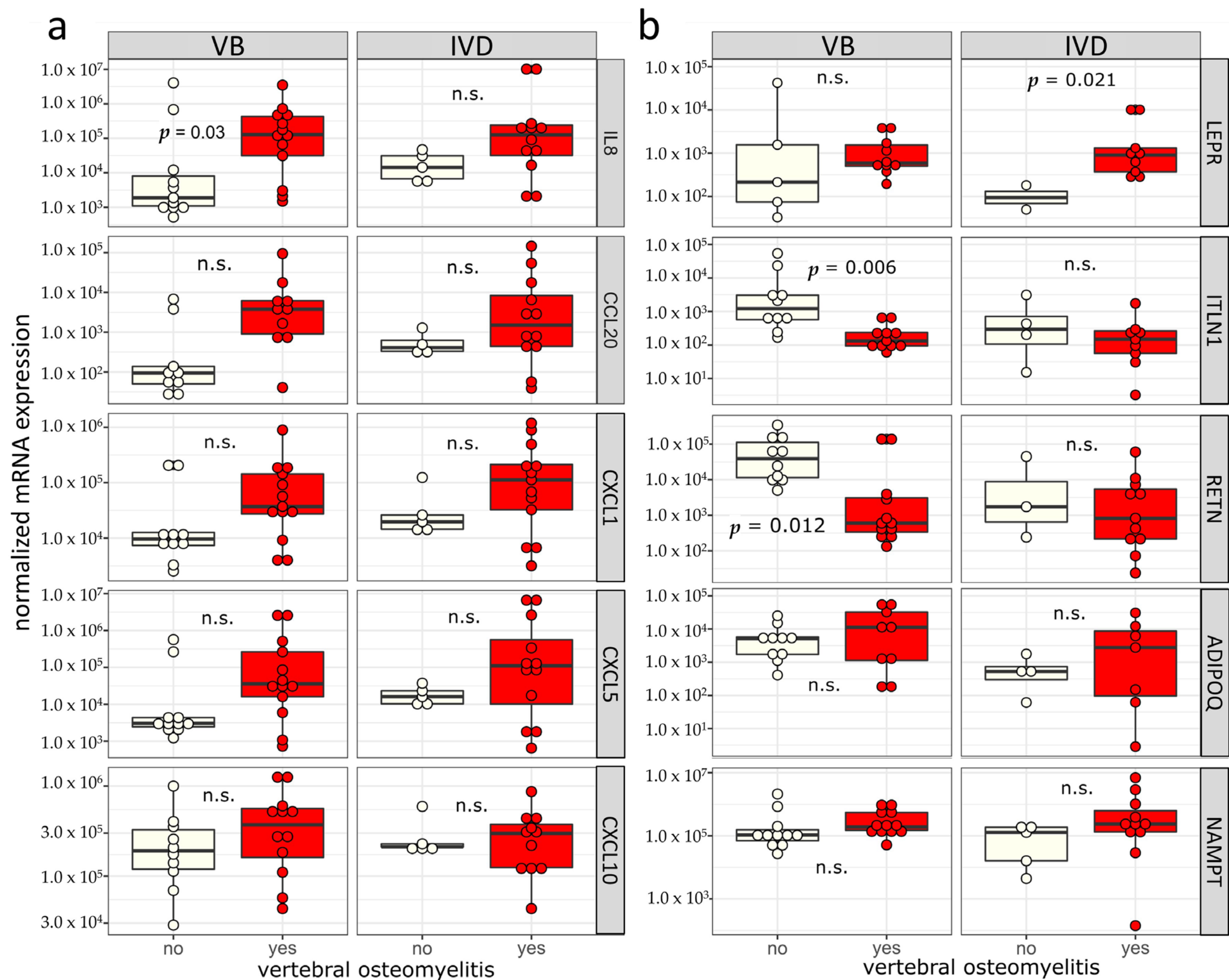

Fig. 3. (a) Comparison between the mRNA expressions of cytokines in the fracture (beige) and infection group (red) in VBs and IVDs. (b) Comparison between the mRNA expression of pro- and anti-inflammatory adipokines in VBs and IVDs. The infection group is indicated by "yes" (VO), the fracture group by "no" (no VO). Depicted are copy numbers of each target gene normalised to 10^5 copies of *GUSB*. Boxes represent the interquartile range with indicated median. Significance was assessed by Mann-Whitney U tests. n.s: statistically not significant.

to the fracture group (VB: $3.7 \times 10^5 \pm 9.6 \times 10^5$, $p = 0.252$; IVD: $4.5 \times 10^5 \pm 5.0 \times 10^5$, $p = 0.849$).

The mean *RANK*/*OPG* ratio was significantly higher in both compartments in the infection group (VB: 2.5 ± 2.0, $p < 0.001$; IVD: 1.9 ± 3.4, $p = 0.028$) compared to the fracture group (VB: 0.2 ± 0.2; IVD: 0.0 ± 0.0; Fig. 2). Similarly, the mean *RANKL*/*OPG* ratio was significantly higher in VBs (4.6 ± 4.0) and also with a trend towards a higher expression in IVDs (3.7 ± 4.4) of the infection group compared to the fracture group (VB: 0.9 ± 1.2, $p = 0.003$; IVD: 0.3 ± 0.2, $p = 0.214$).

To assess the role of osteoclastogenesis-enhancing chemokines, the mRNA expressions of *IL8* and *CCL20* were measured (Brylka and Schinke, 2019; Pathak *et al*., 2015). Furthermore, the mRNA expressions of the neutrophil-attracting chemokines *CXCL1* and *CXCL5* were measured (Fig. 3**a**). Neutrophils are, on the one hand, key elements in the cellular defence against bacterial infection and, on the other hand, they play a critical role in bacteria-induced apoptosis and/or in phagocytosis-induced cell death (Asensi *et al*., 2017).

Compared to tissues samples from fracture patients, *IL8* expression was significantly higher (infection: $4.6 \times 10^5 \pm 9.4 \times 10^5$; fracture: $4.3 \times 10^5 \pm 1.2 \times 10^6$; $p = 0.03$) and *CCL20* showed a trend towards a higher expression (infection: $1.0 \times 10^4 \pm 2.6 \times 10^4$; fracture: $1.0 \times 10^3 \pm 2.2 \times 10^3$; $p = 0.181$) in VB tissue in the infection group. Chemokine expression in IVDs as well as *CXCL1*, *CXCL5* and *CXCL10* expression in VBs and IVDs showed only a trend towards a higher expression in the infection group compared to the fracture group (Fig. 3**a**).

Changes in the transcription profile of the pro-inflammatory adipokines *LEPR*, *ADIPOQ* and *NAMPT* as well as of the adipokines *ITLN1* and *RETN*, whose functions in infectious diseases are scarcely described, were evaluated to shed light into their role in inflammatory processes potentially contributing to tissue degradation in VO. From the adipokine group, the pro-inflammatory *LEPR* showed a significantly higher expression in IVD tissue samples in the infection group (infection: $2.3 \times 10^3 \pm 3.9 \times 10^3$; fracture: $4.6 \times 10 \pm 7.8 \times 10$; $p = 0.021$), whereas *ITLN1* and *RETN* were significantly lower expressed in VB tissue of the infection group (*ITLN1*: $2.0 \times 10^2 \pm 2.2 \times 10^2$; $p = 0.006$; *RETN*: $2.2 \times 10^4 \pm 5.2 \times 10^4$; $p = 0.012$) compared to the fracture group (*ITLN1*: $8.0 \times 10^3 \pm 1.7 \times 10^4$; *RETN*: $7.6 \times 10^4 \pm 1.1 \times 10^5$) (Fig. 3**b**). The pro-inflammatory *ADIPOQ* and *NAMPT* showed trends towards a higher expression in IVD and VB of the infection group compared to the fracture group (all $p \geq 0.05$; Fig. 3**b**).

Fig. 4 provides a graphical summary of hypothetical regulatory pathways leading to tissue degradation in bacterial VO, considering the local transcriptional changes identified in the present study.

Correlation between mRNA expression levels and preoperative clinical parameters

To identify potential concordant or discordant expression patterns between the above-mentioned genes and preoperative clinical parameters 1 d before surgery (iii), correlations in the infection group were visually analysed using a correlogram by tissue type (Fig. 5).

A positive correlation between the VB mRNA expression of *PPAR-γ* and the expression of *ADIPOQ* and *CXCL10* as well as the CRP value at the time of surgery (iii) was found (Fig. 5, bone). Similarly,

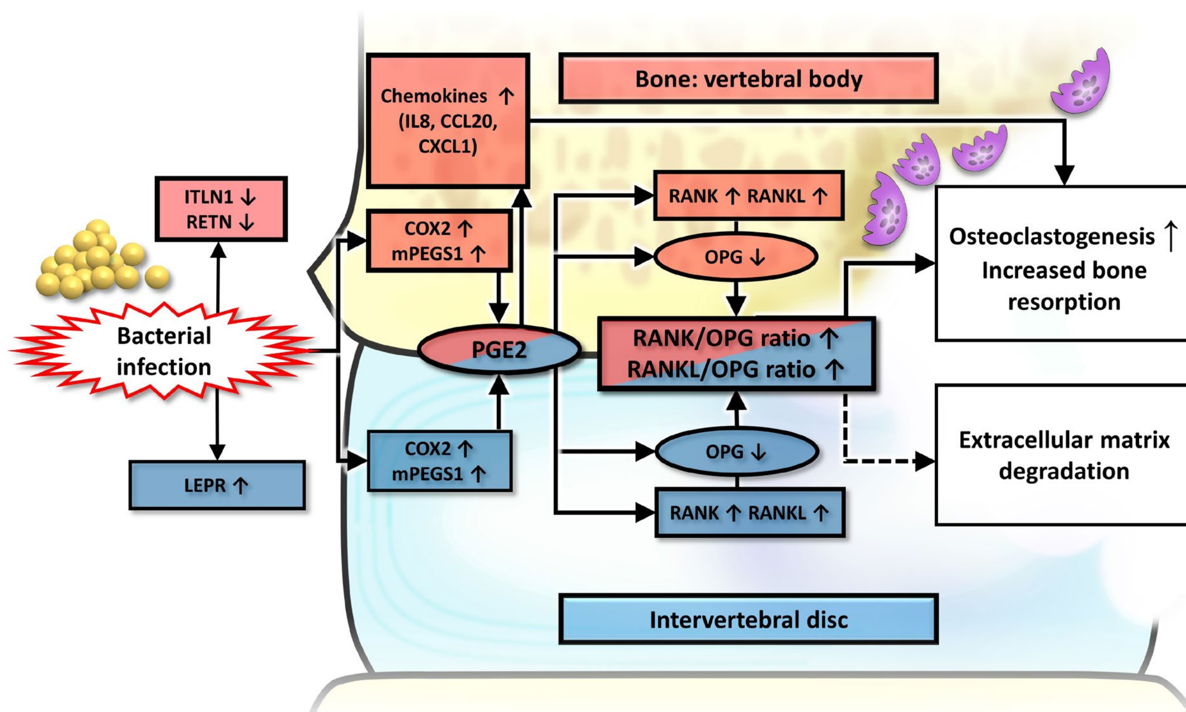

Fig. 4. Graphical summary of hypothetical regulatory pathways leading to tissue degradation in bacterial VO, considering local transcriptional changes identified in the present study. The mRNA regulations are illustrated separately for the VB (red) and IVD (blue).

a positive correlation between *COX2* and *CCL20* expression in VBs was seen (Fig. 5, bone). In the IVDs of the infection group a positive correlation between *mPGES-1* as well as *COX2* expression and *IL8*, *CXCL1*, *CXCL5* and *CCL20* expression was detected (Fig. 4, disc). In infected IVD tissue, the mRNA expression levels of the T-cell- and macrophage-attracting chemokine *CXCL10* and of *OPG* showed negative correlations with the expression of *COX2*, *mPEGS1*, *NAMPT*, *CXCL1*, *CXCL5*, *IL8* and *CCL20* (Fig. 5, disc). Among others, the PCT value at the time of surgery (iii) was negatively correlated with the expression of *ADAMTS4*, *RANK*, *RANKL* and *MMP13* of infected IVDs (Fig. 5, disc).

Case-specific alterations to mRNA expressions

To evaluate whether mRNA expressions were altered in the VB and IVD depending on the donor, Spearman correlation coefficients were calculated for the expression levels in VB and IVD separately for the infection and fracture groups.

In the infection group, *MMP13* mRNA expression showed a statistically significant positive Spearman correlation between VB and IVD samples ($\rho = 0.827$; $p = 0.002$). Also, *CXCL1* mRNA expression in VBs significantly correlated with the expression in IVD samples ($\rho = 0.736$; $p = 0.01$). Out of the adipokines, only the mRNA expression of *NAMPT* in VBs and IVDs showed a statistically significant positive correlation ($\rho = 0.697$; $p = 0.025$). Lastly, *RANKL* mRNA was also found to have a strong and significant correlation between VB and IVD tissue in the infection group ($\rho = 0.704$; $p = 0.034$). None of the other tested genes showed a relevant or statistically significant correlation.

In the fracture group, no significant positive correlations in VBs and IVDs were detected. However, *ADAMTS4*, *CCL20* and *LEPR* mRNA expressions revealed a strong and negative correlation between IVDs and VBs ($\rho = -0.800$; $\rho = -0.800$ and $\rho = -0.738$; all $p > 0.05$). A positive but not statistically significant, correlation was seen in the mRNA expression of *CXCL5* in IVDs and VBs ($\rho = 0.8$; $p = 0.2$).

Discussion

14 patients with confirmed VO and 14 control patients with burst fractures of the thoraco-lumbar spine were prospectively included in the present study to compare the mRNA expression in tissue samples from IVDs and adjacent VBs. The induction of the proinflammatory (adipo-)cytokines *IL8*, *CCL20* and *LEPR* was detected in infected IVDs and VBs, as well as mRNA expression regulation of genes involved in bone and disc metabolism, such as *mPEGS1*, *ADAMTS4* and *RANK*/*RANKL*/*OPG*. An increase in the *RANKL*/*OPG* and the *RANK*/*OPG* ratios was

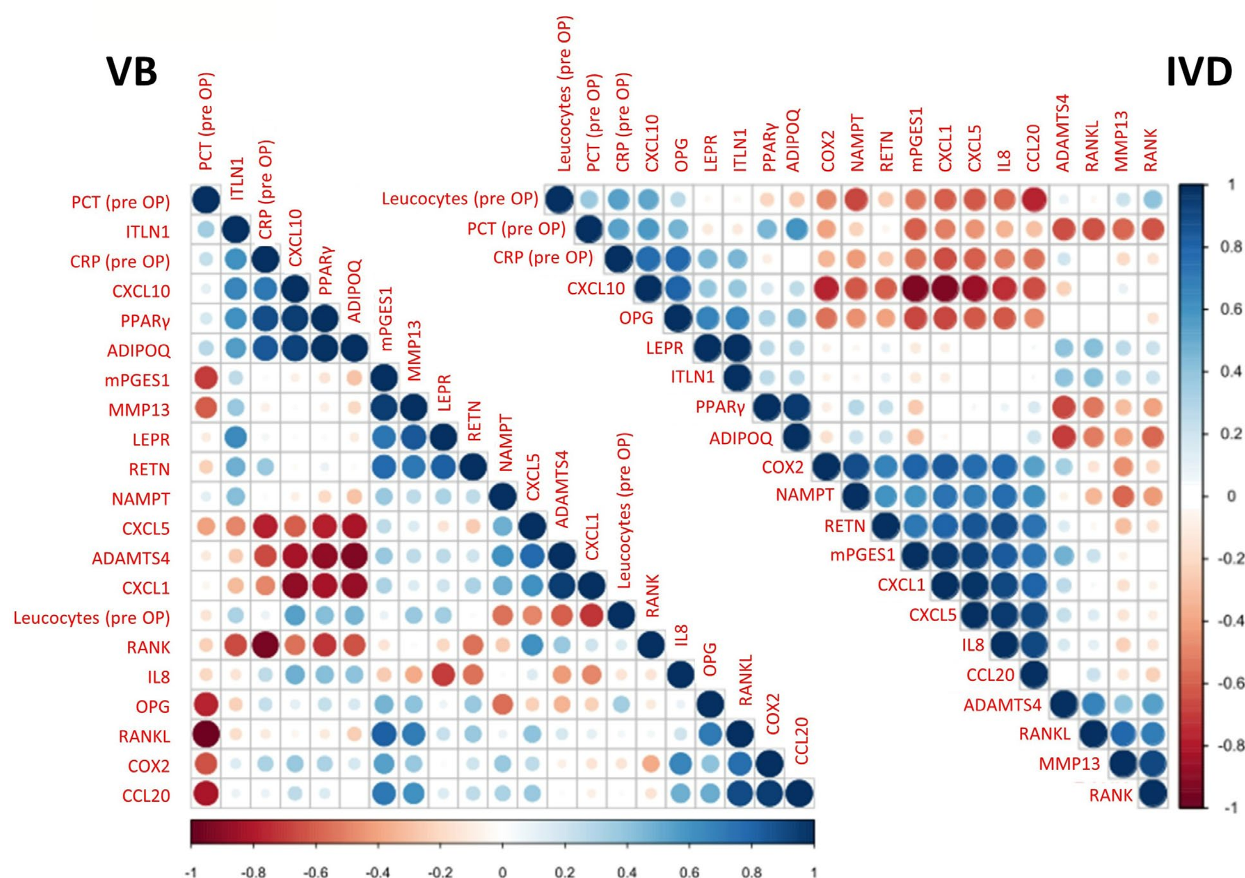

Fig. 5. Correlogram illustrating the correlations between mRNA expression levels and preoperative clinical parameters by tissue type (bone, lower left; disc, upper right). Positive correlations are depicted in blue, negative correlations in red. Colour intensity and size of the circle are proportional to the correlation coefficients.

identified in VBs and IVDs of VO patients compared to fracture patients. Results suggested hypothetical regulatory pathways leading to tissue degradation in bacterial VO. These observations provided a first step to disclose a potential common link between pathogen defence, dysregulated inflammation and tissue degradation in human VBs and IVDs under VO conditions. This finding has not been shown before and might provide new insights into the pathomechanism of pyogenic VO.

The *RANK*/*RANKL*/*OPG* axis is a central part of the molecular mechanisms of bone destruction in osteomyelitis (Mbalaviele *et al*., 2017). On the other hand, the mechanisms leading to the destruction of the IVD still have to be clarified. Significant bone loss in osteomyelitis can be explained by an increase in *RANKL* activity, which is, after binding to its signalling receptor *RANK*, likely to trigger osteoclast-induced bone resorption and bone destruction (Lio *et al*., 2012; Montonen *et al*., 2006; Wada *et al*., 2006). It must be noted that *RANKL* is not only produced by resident bone cells, but also by activated T lymphocytes (Anderson *et al*., 1997; Horwood *et al*., 1998). Remarkably, the inflammatory cytokines IL1β and TNF can stimulate *RANKL* production by osteoblasts and T-cells and concomitantly depress *OPG* expression in osteoblasts (Walsh and Choi, 2014; Young *et al*., 2011).

RANKL/*OPG* ratio is considered an index of osteoclastogenic stimulus (Montonen *et al*., 2006). In 2013, Marriott described how bacterial infection leads to inflammatory bone loss resulting from increased formation and/or activity of bone-resorbing osteoclasts (Marriott, 2013). Similarly, Sanchez demonstrated that biofilm components from clinical wound isolates of *S. aureus* induce *RANKL* production and increase the *RANKL*/*OPG* ratio in human osteoblasts *in vitro* under normal growth or osteogenic conditions (Sanchez *et al*., 2013). In the present translational study, an increase in *RANKL*/*OPG* and *RANK*/*OPG* ratios was identified in VBs and IVDs of VO patients compared to fracture patients. The increased ratios originated primarily from a decreased *OPG* mRNA expression, accompanied by a trend towards increased *RANK* and *RANKL* expression, both in VB and IVD tissue.

The present study compared two distinct spinal pathologies of VO and vertebral fractures. During surgical treatment, tissue from VBs and IVDs were resected, as part of the standard surgical procedure. To the authors' knowledge, there is no comparable information concerning bone metabolism and the *RANK*/*RANKL*/*OPG* system in vertebral fractures. However, Wang *et al*. (2013) reported a reduced serum RANKL/OPG ratio in a fracture group compared to a healthy control group during the first 4 weeks of intertrochanteric fractures healing in elderly patients.

Influencing variables, especially NSAIDs *via* the regulation of *COX2* and *PGE2*, may interfere with infection-induced regulation of gene expression. Therefore, potential influences of anti-inflammatory drugs on the prostanoid and *RANK*/*RANKL*/*OPG* system must be considered (Kotake *et al*., 2010; Pilbeam *et al*., 1997). In the present study, all patients in the fracture group and the majority (10 out 14) of patients in the infection group received anti-inflammatory medication pre- and post-operatively as part of the standard pain treatment. No patients with known chronic inflammatory diseases were included. This homogeneity diminished relevant drug-related gene expression differences between both groups.

The regulation of *RANKL* and *OPG*, mediated by *COX2* and *mPEGS1*, could represent a direct connective mechanism by which bacterial infection results in bone destruction: *COX2* and *mPEGS1*, both elevated in VBs and IVDs of the infection group are involved in PGE2 biosynthesis. Liu *et al*. (2005), using murine cells, reported that PGE2 stimulates osteoclastogenesis through the stimulation of *RANKL* production, inhibition of *OPG* secretion by osteoblasts and up-regulation of *RANK* expression in osteoclasts. Suda *et al*. (2004) studied the role of PGE2 in the suppression of *OPG* mRNA expression and showed how this regulation induces osteoclast formation. They suggested two parallel events in osteoblasts: direct enhancement of *RANKL* expression and suppression of OPG production mediated by PGE2. Additionally, they highlighted that the suppression of OPG production in osteoblasts seems to be more important than the induction of *RANKL* expression for the stimulation of osteoclastogenesis (Suda *et al*., 2004). These assumptions support the present study's findings of reduced *OPG* mRNA expressions, without significantly elevated *RANKL* mRNA expressions.

RANKL has been described as a target for therapeutic approaches in osteodestructive diseases. Sakrurai *et al*. (2003) inoculated mice with *Streptococcus pyogenes*, resulting in a high incidence of septic arthritis. They found that an increase in the amount of RANKL was evident in the joints of infected mice and that blocking RANKL with OPG prevented bone destruction. Similarly, Verdrengh *et al*. (2010) showed that inhibition of *RANKL* signalling in *S. aureus*-inoculated mice could prevent bone loss. Keeping this in mind, the pathological imbalance in *RANKL*/*OPG* mRNA expression in the present study suggested a potential therapeutic approach to counteract bone loss in VO by inhibition of *RANKL* as a basis for further experimental studies. Using an anti-RANKL antibody in an animal model might be a potential experimental set up to evaluate the role of the RANK/RANKL/OPG pathway in osteomyelitis (de Castro *et al*., 2019).

Circulating OPG and soluble RANKL serum levels have been discussed and suggested as potential biomarkers in different contexts (Dovio *et al*., 2005; Kraj *et al*., 2005; Moschen *et al*., 2005). Levels of active RANKL were found to be markedly increased in patients affected by fibrous dysplasia of the bone and highly correlated with disease burden (de Castro *et*

al., 2019). Circulating OPG or soluble RANKL levels were not measured in the present study and the relation of the serum levels to local gene expressions in IVD and VB was not clear. However, this might be the subject for further clinical studies with larger cohorts shedding light on the potential of serum RANKL/OPG ratios for osteomyelitis monitoring.

Adipokines belong to a large group of cytokine-like molecules that are produced not only by adipocytes, but largely by immune cells and resident tissue cells. In certain circumstances they can amplify inflammation, immune response and tissue damage (Ouchi *et al*., 2011). *LEPR* expression was significantly elevated in IVD tissue from the infection group. Leptin is a proinflammatory cytokine that is produced by inflammatory cells and its mRNA levels can be increased by several inflammatory stimuli, including interleukins (Faggioni *et al*., 2001). Iliopoulus *et al*. (2007) demonstrated that leptin is also able to induce the synthesis of relevant MMPs involved in cartilage damage, such as MMP13. Similarly, the present study's results showed a significant positive correlation between *LEPR* and *MMP13* mRNA expression in VB tissue of the infection group, suggesting a role of *LEPR* at the interface between inflammatory response and tissue degradation. Moreover, similar to RANK/RANKL, leptin is known as a regulator of bone homeostasis: it has been revealed that leptin regulates bone mass through a hypothalamic relay, using two neural mediators, both acting on osteoblasts (Karsenty, 2006).

A trend towards a higher *NAMPT* expression was observed in VBs and IVDs in the infection group. Visfatin has been proven to be involved in impaired bone remodelling through induction of proinflammatory factors and dysregulated MMP/tissue inhibitor of MMP balance during mesenchymal stem cell differentiation (Tsiklauri *et al*., 2018). Results suggested that adipokines may represent the interface between the inflammation mediated by cytokines and the destruction of VBs and IVDs mediated by *RANKL* and *MMP13*, among others. Moreover, the type and level of adipokines might be considered as a potential circulating biomarkers' signature to monitor VO and support treatment decisions.

A further hint for a common pathomechanism of tissue degradation can be seen in the synchronous transcriptional activation of key regulatory genes in VBs and IVDs: *MMP13*, *CXCL1*, *NAMPT* and *RANKL* mRNA expression showed significant positive correlations in the infection group, whereas in the fracture group no statistically significant correlations of genes' expression in VBs and IVDs were found.

Limitations

One main limitation of the study was the demographic differences between the VO and the fracture group. Age-related changes in the mRNA expression of inflammatory mRNA in the older VO population due to degeneration cannot be ruled out. Although patients with documented history of osteoporosis were excluded, DEXA measurements in VO or fracture patients were not conducted. Second, the study presented a limited sample size of 14 patients in each group. Therefore, statistical evaluation must be interpreted with caution. Third, the experiments only extend to mRNA but not protein levels, which were not verified by *e.g*. immunohistochemistry or Western blotting. Therefore, a potential discrepancy between the mRNA expression of a gene and the according protein level cannot be ruled out. Further studies should include ELISA to quantify secreted cytokines.

It must be highlighted that the proposed mechanisms of interplay are only based on the current findings of mRNA alterations and the literature. To confirm these mechanisms, pathway analysis and inhibition models are needed.

Conclusion

The mRNA expression of various cytokines and adipokines as well as of the *RANK*/*RANKL*/*OPG* system in VB and IVD biopsies in pyogenic VO patients is dysregulated from the physiological balance favouring catabolism and inflammation. The regulation of the *RANK*/*RANKL*/*OPG* system seems to be a key pathomechanism in the destruction of bone and disc tissue in human pyogenic VO. The elevation of *COX2* and *mPEGS1* mRNA suggested a central role of PGE2 as a mediator of this mechanism, leading to bacteria-induced osteoclastogenesis. The elevation of mRNA expressions of osteoclastogenesis-enhancing chemokines *IL8* and *CCL20* in infected tissue supported this interpretation. A shift in the adipokine profile in VO most likely regulates the pro- and anti-inflammatory pathways that eventually favour degradation of both VB and IVD. For the first time, the present study identified common mechanisms in the pathological destruction of VBs and IVDs in VO patients and thereby provided potential future research approaches to develop tailored diagnostic and therapeutic methods targeting the RANK/RANKL/OPG axis and related mediators.

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References

Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe

MC, DuBose RF, Cosman D, Galibert L (1997) A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature **390**: 175-179.

Asensi V, Collazos J, Celada A, Valle Garay E (2017) Role of neutrophils apoptosis in osteomyelitis pathogenesis. Clin Microbiol **06**. DOI: 10.4172/2327-5073.1000e139.

Bachmeier BE, Nerlich A, Mittermaier N, Weiler C, Lumenta C, Wuertz K, Boos N (2009) Matrix metalloproteinase expression levels suggest distinct enzyme roles during lumbar disc herniation and degeneration. Eur Spine J **18**: 1573-1586.

Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, McKenzie BS, Kastelein RA, Cua DJ, de Waal Malefyt R (2009) Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. J Exp Med **206**: 535-548.

Brylka LJ, Schinke T (2019) Chemokines in physiological and pathological bone remodeling. Front Immunol **10**: 2182. DOI: 10.3389/fimmu.2019.02182.

Caristi S, Piraino G, Cucinotta M, Valenti A, Loddo S, Teti D (2005) Prostaglandin E2 induces interleukin-8 gene transcription by activating C/EBP homologous protein in human T lymphocytes. J Biol Chem **280**: 14433-14442.

Charlson ME, Pompei P, Ales KL, MacKenzie CR (1987) A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. J Chronic Dis **40**: 373-383.

Chizzolini C, Chicheportiche R, Alvarez M, de Rham C, Roux-Lombard P, Ferrari-Lacraz S, Dayer J-M (2008) Prostaglandin E2 synergistically with interleukin-23 favors human Th17 expansion. Blood **112**: 3696-3703.

de Castro LF, Burke AB, Wang HD, Tsai J, Florenzano P, Pan KS, Bhattacharyya N, Boyce AM, Gafni RI, Molinolo AA, Robey PG, Collins MT (2019) Activation of RANK/RANKL/OPG pathway is involved in the pathophysiology of fibrous dysplasia and associated with disease burden. J Bone Miner Res **34**: 290-294.

Dougall WC (2012) Molecular pathways: osteoclast-dependent and osteoclast-independent roles of the RANKL/RANK/OPG pathway in tumorigenesis and metastasis. Clin Cancer Res **18**: 326-335.

Doutchi M, Seng P, Menard A, Meddeb L, Adetchessi T, Fuentes S, Dufour H, Stein A (2015) Changing trends in the epidemiology of vertebral osteomyelitis in Marseille, France. New Microbes New Infect **7**: 1-7.

Dovio A, Data V, Angeli A (2005) Circulating osteoprotegerin and soluble RANKL: do they have a future in clinical practice? J Endocrinol Invest **28**: 14-22.

Faggioni R, Feingold KR, Grunfeld C (2001) Leptin regulation of the immune response and the immunodeficiency of malnutrition. FASEB J **15**: 2565-2571.

Fairbank JC, Pynsent PB (2000) The Oswestry disability index. Spine (Phila Pa 1976) **25**: 2940-2952.

Foreman SC, Schwaiger BJ, Gempt J, Jungmann PM, Kehl V, Delbridge C, Wantia N, Zimmer C, Kirschke JS (2017) MR and CT imaging to optimize CT-guided biopsies in suspected spondylodiscitis. World Neurosurg **99**: 726-734.e7.

Gouliouris T, Aliyu SH, Brown NM (2010) Spondylodiscitis: update on diagnosis and management. J Antimicrob Chemother **65 Suppl 3**: iii11-24.

Grammatico L, Baron S, Rusch E, Lepage B, Surer N, Desenclos JC, Besnier JM (2008) Epidemiology of vertebral osteomyelitis (VO) in France: analysis of hospital-discharge data 2002-2003. Epidemiol Infect **136**: 653-660.

Horwood NJ, Elliott J, Martin TJ, Gillespie MT (1998) Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. Endocrinology **139**: 4743-4746.

Iliopoulos D, Malizos KN, Tsezou A (2007) Epigenetic regulation of leptin affects MMP13 expression in osteoarthritic chondrocytes: possible molecular target for osteoarthritis therapeutic intervention. Ann Rheum Dis **66**: 1616-1621.

Iwata E, Scarborough M, Bowden G, McNally M, Tanaka Y, Athanasou NA (2019) The role of histology in the diagnosis of spondylodiscitis: correlation with clinical and microbiological findings. Bone Joint J **101-B**: 246-252.

Jean M, Irisson JO, Gras G, Bouchand F, Simo D, Duran C, Perronne C, Mulleman D, Bernard L, Dinh A (2017) Diagnostic delay of pyogenic vertebral osteomyelitis and its associated factors. Scand J Rheumatol **46**: 64-68.

Josse J, Velard F, Gangloff SC (2015) *Staphylococcus aureus vs*. osteoblast: relationship and consequences in osteomyelitis. Front Cell Infect Microbiol **5**: 85. DOI: 10.3389/fcimb.2015.00085.

Karsenty G (2006) Convergence between bone and energy homeostases: leptin regulation of bone mass. Cell Metab **4**: 341-348.

Kepler CK, Ponnappan RK, Tannoury CA, Risbud MV, Anderson DG (2013) The molecular basis of intervertebral disc degeneration. Spine J **13**: 318-330.

Kim CJ, Kang SJ, Choe PG, Park WB, Jang HC, Jung SI, Song KH, Kim ES, Kim HB, Oh MD, Park KH, Kim NJ (2015) Which tissues are best for microbiological diagnosis in patients with pyogenic vertebral osteomyelitis undergoing needle biopsy? Clin Microbiol Infect **21**: 931-935.

Klawitter M, Hakozaki M, Kobayashi H, Krupkova O, Quero L, Ospelt C, Gay S, Hausmann O, Liebscher T, Meier U, Sekiguchi M, Konno S, Boos N, Ferguson SJ, Wuertz K (2014) Expression and regulation of toll-like receptors (TLRs) in human intervertebral disc cells. Eur Spine J **23**: 1878-1891.

Kong YY, Boyle WJ, Penninger JM (2000) Osteoprotegerin ligand: a regulator of immune

responses and bone physiology. Immunol Today **21**: 495-502.

Kostenuik PJ (2005) Osteoprotegerin and RANKL regulate bone resorption, density, geometry and strength. Curr Opin in Pharmacol **5**: 618-625.

Kotake S, Yago T, Kawamoto M, Nanke Y (2010) Effects of NSAIDs on differentiation and function of human and murine osteoclasts – crucial 'human osteoclastology.' Pharmaceuticals (Basel) **3**: 1394-1410.

Kouijzer IJE, Scheper H, de Rooy JWJ, Bloem JL, Janssen MJR, van den Hoven L, Hosman AJF, Visser LG, Oyen WJG, Bleeker-Rovers CP, de Geus-Oei L-F (2018) The diagnostic value of 18F-FDG-PET/CT and MRI in suspected vertebral osteomyelitis – a prospective study. Eur J Nucl Med Mol Imaging **45**: 798-805.

Kraj M, Owczarska K, Sokołowska U, Centkowski P, Pogłód R, Kruk B (2005) Correlation of osteoprotegerin and sRANKL concentrations in serum and bone marrow of multiple myeloma patients. Arch Immunol Ther Exp (Warsz) **53**: 454-464.

Lio P, Paoletti N, Moni M, Atwell K, Merelli E, Viceconti M (2012) Modelling osteomyelitis. BMC Bioinformatics **13 Suppl 14**: S12. DOI: 10.1186/1471-2105-13-S14-S12.

Liu X-H, Kirschenbaum A, Yao S, Levine AC (2005) Cross-talk between the interleukin-6 and prostaglandin E(2) signaling systems results in enhancement of osteoclastogenesis through effects on the osteoprotegerin/receptor activator of nuclear factor-κB (RANK) ligand/RANK system. Endocrinology **146**: 1991-1998.

Loibl M, Stoyanov L, Doenitz C, Brawanski A, Wiggermann P, Krutsch W, Nerlich M, Oszwald M, Neumann C, Salzberger B, Hanses F (2014) Outcome-related co-factors in 105 cases of vertebral osteomyelitis in a tertiary care hospital. Infection **42**: 503-510.

Mannion AF, Porchet F, Kleinstück FS, Lattig F, Jeszenszky D, Bartanusz V, Dvorak J, Grob D (2009) The quality of spine surgery from the patient's perspective. Part 1: the core outcome measures index in clinical practice. Eur Spine J **18 Suppl 3**: 367-373.

Marriott I (2013) Apoptosis-associated uncoupling of bone formation and resorption in osteomyelitis. Front Cell Infect Microbiol **3**: 101. DOI: 10.3389/fcimb.2013.00101.

Mbalaviele G, Novack DV, Schett G, Teitelbaum SL (2017) Inflammatory osteolysis: a conspiracy against bone. J Clin Invest **127**: 2030-2039.

Meghji S, Crean SJ, Hill PA, Sheikh M, Nair SP, Heron K, Henderson B, Mawer EB, Harris M (1998) Surface-associated protein from *Staphylococcus aureus* stimulates osteoclastogenesis: possible role in *S. aureus*-induced bone pathology. Br J Rheumatol **37**: 1095-1101.

Millward-Sadler SJ, Costello PW, Freemont AJ, Hoyland JA (2009) Regulation of catabolic gene expression in normal and degenerate human intervertebral disc cells: implications for the pathogenesis of intervertebral disc degeneration. Arthritis Res Ther **11**: R65. DOI: 10.1186/ar2693.

Montonen M, Li T-F, Lukinmaa P-L, Sakai E, Hukkanen M, Sukura A, Konttinen YT (2006) RANKL and cathepsin K in diffuse sclerosing osteomyelitis of the mandible. J Oral Pathol Med **35**: 620-625.

Moschen AR, Kaser A, Stadlmann S, Millonig G, Kaser S, Mühllechner P, Habior A, Graziadei I, Vogel W, Tilg H (2005) The RANKL/OPG system and bone mineral density in patients with chronic liver disease. J Hepatol **43**: 973-983.

Mylona E, Samarkos M, Kakalou E, Fanourgiakis P, Skoutelis A (2009) Pyogenic vertebral osteomyelitis: a systematic review of clinical characteristics. Semin Arthritis and Rheum **39**: 10-17.

Nakashima T, Takayanagi H (2009) Osteoimmunology: crosstalk between the immune and bone systems. J Clin Immunol **29**: 555-567.

Okahashi N, Sakurai A, Nakagawa I, Fujiwara T, Kawabata S, Amano A, Hamada S (2003) Infection by *Streptococcus pyogenes* induces the receptor activator of NF-κB ligand expression in mouse osteoblastic cells. Infect Immun **71**: 948-955.

Okamoto K, Nakashima T, Shinohara M, Negishi-Koga T, Komatsu N, Terashima A, Sawa S, Nitta T, Takayanagi H (2017) Osteoimmunology: the conceptual framework unifying the immune and skeletal systems. Physiol Rev **97**: 1295-1349.

Ouchi N, Parker JL, Lugus JJ, Walsh K (2011) Adipokines in inflammation and metabolic disease. Nat Rev Immunol **11**: 85-97.

Pathak JL, Bakker AD, Verschueren P, Lems WF, Luyten FP, Klein-Nulend J, Bravenboer N (2015) CXCL8 and CCL20 enhance osteoclastogenesis via modulation of cytokine production by human primary osteoblasts. PLoS One **10**: e0131041. DOI: 10.1371/journal.pone.0131041.

Pilbeam CC, Fall PM, Alander CB, Raisz LG (1997) Differential effects of nonsteroidal anti-inflammatory drugs on constitutive and inducible prostaglandin G/H synthase in cultured bone cells. J Bone Mineral Res **12**: 1198-1203.

Reischl U, Linde HJ, Metz M, Leppmeier B, Lehn N (2000) Rapid identification of methicillin-resistant *Staphylococcus aureus* and simultaneous species confirmation using real-time fluorescence PCR. J Clin Microbiol **38**: 2429-2433.

Sakurai A, Okahashi N, Nakagawa I, Kawabata S, Amano A, Ooshima T, Hamada S (2003) *Streptococcus pyogenes* infection induces septic arthritis with increased production of the receptor activator of the NF-κB ligand. Infect Immun **71**: 6019-6026.

Sanchez CJ, Ward CL, Romano DR, Hurtgen BJ, Hardy SK, Woodbury RL, Trevino AV, Rathbone CR, Wenke JC (2013) *Staphylococcus aureus* biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption *in vitro*. BMC Musculoskelet Disord **14**: 187. DOI: 10.1186/1471-2474-14-187.

Sano T, Akeda K, Yamada J, Takegami N, Sudo T, Sudo A (2019) Expression of the RANK/RANKL/

OPG system in the human intervertebral disc: implication for the pathogenesis of intervertebral disc degeneration. BMC Musculoskelet Disord **20**: 225. DOI: 10.1186/s12891-019-2609-x.

Somayaji SN, Ritchie S, Sahraei M, Marriott I, Hudson MC (2008) *Staphylococcus aureus* induces expression of receptor activator of NF-κB ligand and prostaglandin E2 in infected murine osteoblasts. Infect Immun **76**: 5120-5126.

Suda K, Udagawa N, Sato N, Takami M, Itoh K, Woo J-T, Takahashi N, Nagai K (2004) Suppression of osteoprotegerin expression by prostaglandin E2 is crucially involved in lipopolysaccharide-induced osteoclast formation. J Immunol **172**: 2504-2510.

Takegami N, Akeda K, Yamada J, Sano T, Murata K, Huang J, Masuda K, Sudo A (2017) RANK/RANKL/OPG system in the intervertebral disc. Arthritis Res Ther **19**: 121. DOI: 10.1186/s13075-017-1332-y.

Tsiklauri L, Werner J, Kampschulte M, Frommer KW, Berninger L, Irrgang M, Glenske K, Hose D, El Khassawna T, Pons-Kühnemann J, Rehart S, Wenisch S, Müller-Ladner U, Neumann E (2018) Visfatin alters the cytokine and matrix-degrading enzyme profile during osteogenic and adipogenic MSC differentiation. Osteoarthritis Cartilage **26**: 1225-1235.

Verdrengh M, Bokarewa M, Ohlsson C, Stolina M, Tarkowski A (2010) RANKL-targeted therapy inhibits bone resorption in experimental *Staphylococcus aureus*-induced arthritis. Bone **46**: 752-758.

Wada T, Nakashima T, Hiroshi N, Penninger JM (2006) RANKL–RANK signaling in osteoclastogenesis and bone disease. Trends Mol Med **12**: 17-25.

Walsh MC, Choi Y (2014) Biology of the RANKL-RANK-OPG system in immunity, bone, and beyond. Front Immunol **5**: 511. DOI: 10.3389/fimmu.2014.00511.

Wang X-F, Zhang Y-K, Yu Z-S, Zhou J-L (2013) The role of the serum RANKL/OPG ratio in the healing of intertrochanteric fractures in elderly patients. Mol Med Rep **7**: 1169-1172.

Widaa A, Claro T, Foster TJ, O'Brien FJ, Kerrigan SW (2012) *Staphylococcus aureus* protein A plays a critical role in mediating bone destruction and bone loss in osteomyelitis. PLoS One **7**e40586. DOI: 10.1371/journal.pone.0040586.

Young AB, Cooley ID, Chauhan VS, Marriott I (2011) Causative agents of osteomyelitis induce death domain-containing TNF-related apoptosis-inducing ligand receptor expression on osteoblasts. Bone **48**: 857-863.

Web Reference

1. https://www.promega.de/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol/ [20.10.2021]

Discussion with Reviewer

Reviewer: While the VB is a well-vascularised tissue, the IVD has much less vascularisation. Would you have expected different expression profiles of the various cytokines, adipokines?

Authors: The physiological vascularisation of the IVD and VB differs distinctly. However, during inflammation, neoangiogenesis is triggered in the IVD, where numerous cytokines have been detected, at least in a degenerative setting (Liu *et al.*, 2021, additional reference). More importantly, gene expression, as opposed to soluble mediators, was measured, indicating that the presence of resident cells that can be transactivated by infection is probably more crucial than their supply by blood and lymph vessels. Regarding adipokines, disc cells express adipokines, *e.g.* leptin (Zhao *et al.*, 2008, additional reference), and the role of adipokines in disc degeneration has been shown (Sharma, 2018, additional reference). Therefore, the expression of adipokines does not seem to depend principally on the extent of vascularisation. For this reason, the same set of target genes was chosen to be evaluated in IVDs and VBs. For adipokines and cytokines, roughly similar expression profiles were expected and those profiles were shown for the first time in VO. The similar expression pattern of *RANK*/*RANKL*/*OPG* in the IVD and VB tissue was most unexpected. Although this system, which is mainly linked to bone metabolism, has been hypothesised to be also involved in IVD degeneration (Sano *et al.*, 2019), it was unexpected to find comparable expressions in VBs and IVDs in VO patients.

Additional References

Liu C, Liang G, Deng Z, Tan J, Zheng Q, Lyu F-J (2021) The upregulation of COX2 in human degenerated nucleus pulposus: the association of inflammation with intervertebral disc degeneration. Mediators Inflamm **2021**: 2933199. DOI 10.1155/2021/2933199.

Sharma A (2018) The role of adipokines in intervertebral disc degeneration. Med Sci (Basel) **6**: E34. DOI: 10.3390/medsci6020034.

Zhao C-Q, Liu D, Li H, Jiang L-S, Dai L-Y (2008) Expression of leptin and its functional receptor on disc cells: contribution to cell proliferation. Spine **33**: E858. DOI: 10.1097/BRS.0b013e31818338e5.

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