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der Universität zu Köln
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The potential of cytokines as diagnostic markers for vertebral osteomyelitis

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Die in dieser Arbeit angegebenen Experimente und Ergebnisse sind Teil der CyProSpon-Studie. Die Promovierenden dieser Studie Frau Julia Brinkmann, Frau Julia Schenk und Herr Jan Scharrenberg haben die Rekrutierung der Patientinnen und Patienten und die Probengewinnung auf der Krankenstation der Klinik Orthopädie und Unfallchirurgie, Uniklinik Köln durchgeführt.

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Glossary

AUC	Area under the curve
BMI	Body mass index
CoNS	Coagulase-negative staphylococci
CRP	C-reactive protein
CSF	colony-stimulating factor
CT	Computed tomography
CyProSpon study	Cytokine Profiles in Spondylodiscitis study
d	Days
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
FGF	Fibroblast growth factor
HIV	Human immunodeficiency virus
ID	Identification
IFN	Interferon
IL	Interleukin
IQR	interquartile range
L	Liter
MCP	Monocyte chemoattractant protein
mg	Milligram
MIP	Macrophage inflammatory protein
mL	Milliliter
MRSA	Methicillin-resistant Staphylococcus aureus
MRI	Magnetic resonance imaging
M. tuberculosis	*Mycobacterium tuberculosis*
OP	Operation
P. acnes	*Propionibacterium acnes*
PCT	Procalcitonin
PET	Positron emission tomography
pg	Picogram
ROC	Receiver operating characteristic
S. aureus	*Staphylococcus aureus*
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VO	Vertebral osteomyelitis

1. Summary

The aim of this study was to improve the diagnostic options for vertebral osteomyelitis (VO). The disease VO describes an infectious condition of the spine starting in the bone marrow of the vertebral bodies, which then expands to the intervertebral discs. A diagnosis in the initial course of a VO is often not accurate because there is a lack of specific and minimally-invasive diagnostic methods for an early and reliable detection of VO cases. Only with an advanced status of the disease presenting with irreversible tissue damage, medical imaging shows to be a successful diagnostic method. The present laboratory parameters such as CRP, ESR, and leucocytes are not specific, and blood cultures do not always detect the pathogen successfully. In spite of diagnostic delays that act to the disadvantage of the patients outcome, this study presents the potential application of cytokines for diagnosing VO. Cytokines play an essential role in inflammation by modulation of immune responses, and their concentration is often increased in various infectious diseases. Cytokines as biomarkers may be easily implemented and represent a minimally-invasive method for an early diagnosis. Accordingly, the aim of this study was to determine if cytokines can discriminate between VO patients, who present with an infection of the spine and the diagnosis of *osteochondrosis intervertebralis,* which presents without an infection of the spine. The hypothesis of this study was that a selection of cytokines are sensitive and specific diagnostic biomarkers to distinguish infectious from non-infectious diseases of the spine.

In total, 36 patients were included in this single-center case-control study. The patient group included 16 patients with the diagnosis of VO and 20 patients with the diagnosis of osteochondrosis intervertebralis who functioned as the control group. The exclusion criteria included other infections external to the spine, malignancies, and autoimmune diseases. All included patients underwent a partial operative stabilization of the spine, which was carried out at the Department of Orthopedic and Trauma Surgery of the University Hospital Cologne. In this study, the levels of 27 cytokines and CRP were measured at different timepoints throughout the course of the diseases to investigate the use for diagnosis of VO. During the hospital stay and follow-up, blood samples were drawn once before surgery (-20-0d pre-OP) and four times after surgery (3-5d post-OP, 6-11d post-OP, 40-56d post-OP, and 63-142d post-OP). The measurement of the cytokines was done using a multiplex assay. For the statistics, p-values were used to show the significance of the effects, and ROC curves with AUCs were used to investigate the predictive potential. Also, influences on the cytokine levels, specifically on the type of pathogen causing VO, were examined.

The cytokines IL-6, IL-8, IL-12 (p70), and VEGF showed to have the strongest potential for diagnosing VO. Compared to the control group without infection of the spine, these cytokines and also CRP showed an AUC above 0.85 at the pre-OP timepoint. IL-6 scored the highest AUC of 0.98 followed by CRP with an AUC of 0.92. Especially, the combination of all

candidate cytokines scored the highest predictive potential for diagnosing VO (AUC: 0.99). Considering the pathogen causing VO, the diagnostic potential of cytokines decreased in patients with low-virulent pathogens. Only the discrimination between VO patients with high-virulent bacteria and patients with *osteochondrosis intervertebralis* is reliable. Additionally, considering the follow-up measurements, the results implicate that IL-6 and IL-12 (p70) are suitable to monitor the therapeutical success. In conclusion, this study showed that the cytokines IL-6, IL-8, IL-12 (p70), and VEGF can be used as biomarkers to detect VO and that further research would be of great advantage.

2. Zusammenfassung

Thema dieser Arbeit ist die Verbesserung der Diagnosemöglichkeiten einer vertebralen Osteomyelitis (VO). Eine VO wird durch einen Zustand der Wirbelsäule charakterisiert, der mit einer Infektion im Knochenmark der Wirbelkörper beginnt, welche sich dann zu den Bandscheiben ausbreitet. Die Diagnose in der initialen Erkrankungsphase ist oftmals nicht akkurat, denn es besteht ein Mangel an spezifischen und minimal-invasiven diagnostischen Mitteln. Die medizinische Bildgebung ermöglicht erst dann eine Diagnose, wenn die Erkrankung ein fortgeschrittenes Stadium erreicht hat und bereits irreversible Schäden vorhanden sind. Auch verfügbare Laborparameter, wie zum Beispiel CRP, ESR und Leukozyten sind unspezifisch und Blutkulturen sind oftmals negativ. Diese Bedingungen verkompliziert eine frühzeitige Diagnosestellung. Diagnostische Verzögerungen wirken sich aber regelmäßig zum Nachteil für Patienten aus. In dieser Studie wird das diagnostische Potenzial von Zytokinen untersucht. Zytokine spielen eine bedeutende Rolle bei immunologischen Reaktionen in Entzündungsprozessen und bei infektiösen Erkrankungen werden häufig hohe Zytokinkonzentrationen gemessen. Es ist entsprechend das Ziel dieser Studie herauszufinden, ob man mit der Hilfe von Zytokinen zwischen einer VO, die sich mit einer entzündeten Wirbelsäule präsentiert und einer *Osteochondrosis intervertebalis*, die sich ohne Entzündung präsentiert, unterscheiden kann. Zytokine als Biomarker bieten das Potenzial für ein einfaches und minimal-invasives diagnostisches Mittel, welches eine frühzeitige Diagnose einer VO ermöglicht. Die Arbeitshypothese dieser Studie umfasst die Annahme, dass eine Auswahl an Zytokinen als spezifischer und sensitiver Laborparameter eingesetzt werden kann, um zwischen einer infektiösen und einer nicht-infektiösen Wirbelsäulenerkrankung zu unterscheiden.

Die Studie wurde in der Klinik für Orthopädie und Unfallchirurgie der Uniklinik Köln als monozentrische Fall-Kontroll-Studie durchgeführt. Insgesamt waren 36 Patienten an der Studie beteiligt. Davon gehörten 16 Patienten zu der VO-Gruppe und 20 Patienten litten an einer Osteochondrosis intervertebralis. Die Patientengruppe mit Osteochondrosis intervertebralis diente dabei als Kontrollgruppe. Zu den Ausschlusskriterien zählten andere Infektionen, Krebs und Autoimmunerkrankungen. Bei allen Patienten wurde eine stabilisierende Operation der Wirbelsäule vorgenommen. In diesem Zusammenhang wurden 27 Zytokine und CRP zu unterschiedlichen Zeitpunkten im Krankheitsverlauf gemessen: einmal vor der Operation (-20-0 Tage prä-OP) und viermal nach der Operation (3-5, 6-11, 40-56 und 63-142 Tage post-OP). Die Zytokinkonzentrationen wurden mittels eines Multiplex-Assays gemessen. Zytokin- und CRP-Konzentrationen wurden statistisch analysiert. Hier zeigten P-Werte die Signifikanz und ROC-Kurven mit den AUCs das Potenzial, die Diagnose VO vorherzusagen. Zusätzlich wurden die Zytokinkonzentrationen unter dem Einfluss von hoch- und niedrig-virulenten VO Erregern analysiert.

Die Zytokine IL-6, IL-8, IL-12 (p70) und VEGF zeigten das größte Potenzial, um VO zu diagnostizieren. Im Vergleich zu der Kontrollgruppe zeigten diese Zytokine sowie CRP einen AUC über 0.85 zum Zeitpunkt vor der Operation. IL-6 erzielte den höchsten Wert (AUC: 0.98), gefolgt von CRP (AUC: 0.92). Insbesondere die Kombination aus allen signifikanten Zytokinen zeigte das höchste Potenzial, die Diagnose VO vorherzusagen (AUC: 0.99). Eine Verschlechterung des diagnostischen Potenzials zeigte sich bei der Differenzierung unterschiedlicher Krankheitserreger: VO Patienten mit schwach virulente Erregern wurden weniger zuverlässig diagnostiziert. Allein die Unterscheidung zwischen VO Patienten mit hoch virulenten Erregern und Patienten mit *Osteochondrosis intervertebalis* war verlässlich. Zusammenfassend zeigt diese Studie, dass sich IL-6, IL-8, IL-12 (p70) und VEGF als Biomarker für eine VO eignen. Darüber hinaus belegten die Ergebnisse der post-operativen Werte, dass IL-6 und IL-12 (p70) geeignete Parameter für die Überwachung des Therapieerfolgs sind. Insgesamt ergibt sich hier ein guter Ausgangspunkt für weitere Forschung.

3. Introduction

3.1. Vertebral osteomyelitis

Inflammatory diseases of the spine can be divided into two categories, namely infectious diseases, such as VO, and non-infectious diseases, such as rheumatic diseases including rheumatic arthritis or ankylosing spondylitis. VO is a form of osteomyelitis, which is an infection of the bone marrow. Depending on the pathology, the terms VO, spondylitis or spondylodiscitis are used in the literature. Spondylodiscitis refers to an infection that originates in the intervertebral disc and spreads into the vertebral body. This is different in VO; here, the infection starts in the vertebral body and then expands to the intervertebral disc. In spondylitis, which is a form of osteomyelitis, the infection also originates in the vertebral body, but the term also includes the non-infectious disease ankylosing spondylitis. This study uses the term VO. However, at the time of diagnosis usually both structures of the spine are affected, and the primary focus of infection is not obvious anymore [1-3].

VO is a rare disease predominantly found in adults older than 50 years, but all ages can be affected. Men are 1.9-3 times more frequently affected than women [4-6]. The incidence is at 0.4-2.74 per 100.000 people per year [7-10]. In addition, men with underlying medical conditions are more likely to be affected by the disease. In several studies the most common underlying illness was diabetes mellitus with 24-35% of patients being affected [11-15]. Over the last years, the incidence increased due to a higher awareness of the disease, improved diagnosis, and possibly due to an increased occurrence of risk factors caused by demographic circumstances in the population [16,17].

Risk factors for VO are infections elsewhere in the body and septic diseases [18]. Moreover, an immunocompromised status or a consuming illness such as human immunodeficiency virus (HIV), malignancies or graft rejection in transplantation and their therapy with immunosuppressive medication and chronic steroid use increase the risk for VO. Smoking and intravenous drug abuse can also increase the risk of developing VO. Notably, the increasing number of older patients with comorbidities such as diabetes mellitus, cardiac diseases, liver diseases and end-stage kidney diseases increase the occurrence of VO [12,19,20]. Also direct inoculation can cause the disease. In fact, 14-30% of a study population had a surgical intervention in a spinal segment and later developed VO in that region [17,21,22].

The most common cause of VO is the hematogenous spread of an endogenous infection. It involves the vascular system, where pathogens are spread via the venous, arterial and lymphogenous system [2,8,23]. Here, it is essential to understand the vascular supply of the spine. Batson et al. describe the spread of cancer as well as infectious pathogens via the valveless paravertebral plexus (the Batson plexus), which allows a retrograde flow of blood during the time of increased intra-thoracic and intra-abdominal pressure [24,25]. The most frequent path of

hematogenous spread is the arterial bacterial transmission [8,16], where pathogens from primary infections such as endocarditis [26,27], generalized infections like sepsis or dental infections can be transported to a spinal level [18,28]. In a large study by Park et al. with 345 patients, the urinary tract was the most common source (25.7%), followed by skin and subcutaneous tissue infection (22.8%) [14]. Notably, exogenous or iatrogenic causes of VO are surgeries of the spine, orthopedic injections or other invasive procedures like central vein catheter and port-systems [2,13]. The current data available consistently indicate that VO occurs most commonly in the lumbar spine, followed by the thoracic and the cervical spine [5,8,12,17,19,22]. Seldomly, more than one spinal level is affected [29-31].

The intervertebral disc in adults is nearly avascular unlike the intervertebral bodies of children. For adults, this results in reduced local immune defense and easier infarction [8,32] with a rapid progress of destruction, loss of function and decreased disc heights, which later leads to complications in facet joints, spondylolisthesis and osteochondrosis [33]. VO can follow an acute, subacute or chronic course depending on the pathogen involved. Adhesion of a microorganism to the extracellular matrix, overcoming the hosts immune response, and forming a biofilm characterize the beginning of VO. Osteoblasts are then actively integrated in the process of internalization of bacteria. The interaction between the host and the pathogen initiates a local inflammation process through secretion of pro-inflammatory cytokines, which leads to a recruitment of inflammatory cells and a resorption of bone tissue [34]. Penetration of the infection into the intervertebral disc, paravertebral soft tissue, spinal canal, and surrounding tissue can lead to paravertebral, psoas or epidural abscess formation [3,14,19,22,35]. Moreover, a meningitis can occur [23].

The prognosis of VO is largely dependent on the length of time between the occurrence of first symptoms and the diagnosis [21,23,36]. Since the symptoms in the beginning of the diseases are often missing or unspecific, early diagnosis is a problem. It is crucial to prevent the development of abscesses and severe courses through early diagnosis and therapy [12,16,37]. In addition, patients with neurological symptoms or hospital-related infection had a worse outcome and a higher risk for relapse than patients without these risk factors [23]. The mortality rate is about 0% to 16.8% [7,8,10,23,33]. Grammatico et al. describe a rate of 3% in a large study population that had to stay in the intensive care unit (ICU) for a mean duration of 11 days [7]. The recurrence rate ranges from 0% to 14% [2,23]. Higher risks for a relapse were found in patients with methicillin-resistant *Staphylococcus aureus* (MRSA) infection, undrained paravertebral or psoas abscesses and end-stage kidney diseases [14,38].

3.1.1. Pathogens

There are three types of pathogens that can cause VO, which include 1) bacteria with a pyogenic course, 2) fungi, brucellar or tuberculosis with a granulomatous course, and 3)

parasites [39]. A mono-infection with *Staphylococcus (S.) aureus* is responsible for up to 60% of all VO cases [8,12,14,17]. Park et al. found that 43.3% of the study population with *S. aureus* had the methicillin-resistant variant, which is always a nosocomial-associated infection [12,14]. In general, *S. aureus* is widely spread in the adult population (25-30%), with the epithelial surfaces such as the nose and the skin as the main location for constant or temporary colonization. If physical barriers are overcome by, for example, invasive interventions and if an adaptation to the intracellular environment is achieved, it is then possible to infect and damage bony tissues [40]. Here, small colony variants with mutants and a number of virulence gene expressions play a role, which all contribute differently to the evolutionary success of the pathogen and the severity of the disease [41-43]. Consequently, there are high-invasive and low-invasive strains with varying cytotoxicity. However, Tuchscherr et al. described a similar gene pattern of *S. aureus* isolates, which are involved in sepsis and osteomyelitis [44]. A large number of virulence factors makes *S. aureus* a successful pathogen. These include adherence proteins displayed on the bacterial surface such as staphylococcal protein A and exoproteins secreted by *S. aureus* such as Panton-Valentine leucocidin and coagulase. These factors interact with osteoclasts, osteoblasts, and RANK-L to induce bone destruction and bone loss [45].

In some regions, such as developing countries and Russia, high rates (9-46%) of *Mycobacterium (M.) tuberculosis* provoke VO [5]. Grammatico et al. describe a rate of 56% in Ile de France, compared to a rate of 22% in the other regions of France [7]. Patients with *M. tuberculosis* are usually younger, and up to 60% of HIV-positive patients have skeletal tuberculosis, where the most common form is the spinal tuberculosis [5,46].

Furthermore, a variety of other infectious agents is also frequently seen in VO; for example, gram-negative bacilli with the most frequent candidate *Escherichia (E.) coli* (8.8-21.7%), streptococci (5.9-16.3%), *Staphylococcus (S.) epidermidis* as well as other coagulase-negative staphylococci (CoNS) (2.9-7%), and enterobacteria (2.9-6%) [7,14,17,18,23]. Seldomly anaerobic bacteria (3-4%) such as *Propionibacterium (P.) acnes* and fungi (1-2%) are found [5,47]. *E. coli* is often seen in patients with a urinary tract infection [48]. The gram-negative *Pseudomonas aeruginosa* is known to be more present in intravenous drug abusers. Streptococci and enterococci are associated with endocarditis and diabetes mellitus [5]. Patients with previous surgery had more often CoNS such as *S. epidermidis* or other skin flora-associated bacteria [22]. The seldom occurring fungal-associated VO is more commonly seen in immunosuppressed patients [5]. Notably, a high number of causative microorganisms remain unidentified. The percentage of those cases varies widely but in most studies, it is about 34-50% [5,7,18,49].

The causative pathogens *P. acnes* and CoNS-like *S. epidermidis* are considered to be the low-virulent candidates for VO [50,51]. These organisms can cause a chronic, slow-growing, and low-grade infection of the spine. They can also play a role as causative pathogens in

conditions such as degenerative disc disease or sciatica [52-55]. Notably, a lack of infectious signs and symptoms is related to this type of infection, which makes early diagnosis difficult [51,56,57]. Fungi are also considered low-virulent candidates [58].

3.1.2. Diagnosis

The diagnosis of VO is often difficult and delayed because of its heterogeneous clinical presentation and its slow progress from weeks to several months [2,4,6,12,33,39,59]. This can delay treatment with antibiotics and surgical intervention, which can lead to increased morbidity and mortality [60]. Neurologic symptoms and fever can often lead to earlier diagnosis [12,60]. The differential diseases of VO are *osteochondrosis intervertebralis*, osteoporosis, disc herniation, pathological fracture, and cancer-related destruction [1,2,8]. The first suggested diagnosis is often a degenerative spinal disease [60]. It is helpful to follow a strict protocol for a fast diagnostic process [61]. There are four important pillars on which diagnosis of a VO case rests: 1) the anamnesis, where questions about symptoms like fever and pain, previous infections and other predominant factors are asked; 2) the physical examination; 3) the laboratory diagnostic including blood cultures and biopsies; and 4) the use of imaging techniques [2,5,16,62,63].

3.1.2.1. Symptoms and physical examination

The disease often involves an atypical, chronic course with unspecific symptoms such as nightly back pain, which are present in 86-99% of the patients [12,14,22,59]. During physical examination, percussion and compression need to be performed on the spine [3,62]. In the inspection, a kyphosis can be noted because of destruction of the vertebral bodies in an advanced stage [64]. Increased tension in muscles can be palpated along the spine, and active range of motion can be restricted and painful [29,65,66].

Temperatures over 37.5°C also help during the clinical examination [5]. Some patients describe a febrile illness weeks before the onset of the first back pain [8]. Patients sometimes present with systemic b symptoms, namely, fever, night sweat, and weight loss. The percentage of patients with fever is about 14-70% [6,9,11,12,14,17,22].
Neurological symptoms are reported for 16.8-32% of the patients [14,17,22,29,59] including sensory deficits, motor weakness, paralysis or sphincter dysfunction. Conditions such as epidural abscesses, infectious deformity of the spine or pathological fractures contribute to compression of the nervous tissue [8,23,47].

3.1.2.2. Laboratory diagnostics

The most frequent parameters used for laboratory examination are leucocyte count, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) with a high sensitivity and a

low specificity for all parameters, especially with an acute VO [4,5,47,67,68]. Leukocytosis is present in about 58% of the patients [37]. Leucocyte, CRP and ESR often show pathological values in acute cases, but in subacute or chronic cases there might be no or only a slight alteration [3]. The use of procalcitonin (PCT) alone, a biomarker for bacterial infection, has low sensitivity for detecting VO [18,69]. Additional tests are carried out to rule out brucellosis and tuberculosis [70].

3.1.2.3. Pathogen identification

Identification of the causing pathogen is described as the most important step in the diagnostic process after which a specific antibiotic therapy can be implemented [8,16,47]. Successful detection of the pathogen depends on prior antibiotic treatment, fever, the causing organism, mode of infection, and the concentration in the bloodstream [39]. To identify pathogens, at least two pairs of blood cultures - aerobic and anaerobic - have to be taken from every patient. In ideal constellations, two pairs of blood culture are obtained every day until the causative pathogen is identified. If the status of a patient allows this, already ongoing antibiotic therapy should be discontinued for several days to increase the detection rate [2]. Finding the source of infection is also important. For example, urinary tract infection can be a source of the hematogenous spread. Therefore, it is useful in these cases to take urine cultures from midstream or from a urinary catheter [3].

Due to low detection rates (58-78%) of the pathogen in blood cultures, invasively derived biopsies or tissue samples are needed [2,12,27]. Potential procedures are percutaneous computed tomography (CT)-guided fine needle biopsies or open biopsies during surgeries [8,63]. Biopsies are examined through microbiological culture to identify the pathogen and through histopathological examination to distinguish between a pyogenic and a granulomatous infection or tumorous alterations [16,67,71]. The quantity of the material obtained from intraoperative tissue samples is larger than that from percutaneous biopsies, which allows for better histopathological examination. Therefore, open biopsies yield higher detection rates [2,20,39]. However, if there is no indication for surgery, a CT-guided fine needle biopsy is the best option [29]. A new technique is to inject and aspirate a saline solution during the biopsy, which increases the detection rate by up to 91.6% [72]. Detecting the pathogen through biopsies is more successful in patients without previous antibiotic therapy (80%) than in patients who have undergone antibiotic treatment (48%) [37]. If the patient develops an abscess, the drained material can also act as a sample for a microbiological examination [36].

In case of negative cultures, also for patients taking antibiotics, a polymerase chain reaction (PCR) test can be an option for identifying the pathogen. However, it has to be taken into consideration that there is a higher chance for contamination [47,73].

3.1.2.4. Medical imaging

Imaging techniques are very important tools for diagnosing VO in addition to the clinical and laboratory examinations. Radiography, CT scans, magnetic resonance imaging (MRI), scintigraphy, and positron emission tomography (PET) can be used [1] but MRI is considered the gold standard for diagnosing VO [63,74,75]. An inflammatory hyperemia of an affected segment and an increased bone turnover and destruction are signs for the existence of VO [76].

Because of its high availability, conventional radiography is almost always performed in the process of diagnosing acute or chronic back pain [2,12]. X-ray has a sensitivity of 82% and a specificity of 57% in diagnosing VO [77]. A disadvantage is the poor soft tissue contrast [76]. In the early stage of VO, either a normal picture or structural changes such as decreased disc spaces, bone erosion or sclerosis at the intervertebral bodies can be seen [9,60,77,78].

CT is an X-ray-based cross-sectional imaging technique that produces a three-dimensional image [79]. A possible presence of VO can be identified through early signs, which include a decrease of disc height, end plate irregularities or erosive changes as well as effacement of the paravertebral fat and a hypodense intervertebral disc. CT-imaging is the best possible method to identify epidural, paravertebral or other abscesses, phlegmons, and changes affecting the spinal cord, nerve roots or dural sac - especially when used with contrast medium [60]. Through CT images, degenerative changes, fractures or metastatic diseases can often be differentiated from infectious diseases in back pain patients. Destruction of the bone and consequential instability is best observed in CT images [76]. While inferior to an MRI, a CT is still a good option if MRI is contraindicated due to, for example, a cardiac pacemaker. CT-imaging is also used for CT-guided fine needle biopsies, drainage of abscesses, and for planning surgeries [2,75,80].

As mentioned above, the MRI is the imaging technique of choice because of an accuracy of 94% for diagnosing VO; its sensitivity is 96% and its specificity is 92% [39,47,60,74,77,78]. The MRI uses electromagnetic waves, and with the production of a magnetic field and the emitted signals from different proton densities of certain tissues in the body, a three-dimensional image is created [79]. For visualizing the early signs of VO, the contrast medium gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) should be used [23,78]. One advantage of the MRI in comparison to X-ray-based techniques is the lack of ionizing radiation. On the other hand, one disadvantage of the MRI is that it cannot be used for patients with a pacemaker or other implants [75]. For diagnosing VO, the standard protocol includes T1- and T2-weighted sequences, which allow the evaluation of bone edema and the degree of inflammation [2,74,76,78]. In bone edema, the affected vertebral body appears hypointense in T1 and hyperintense in T2 [78,81]. A differential presentation of soft tissue changes and paraspinal or epidural changes can be achieved using MRI. Also a loss of disc height and erosion of the vertebral body can be shown [81]. MRI is a useful diagnostic tool for patients with neurological symptoms because it

provides a clearer picture for epidural and spinal cord involvement [67]. Figure 1 presents exemplarily two MRI scans of patients with VO. Furthermore, a tuberculous infection can be differentiated from another infection with an MRI [78]. Nevertheless, in early VO phases, MRI is not a good tool because there might be no changes yet or the picture may appear similar to malignancies, ankylosing spondylitis or MODIC type 1 *osteochondrosis intervertebralis* [77,82].

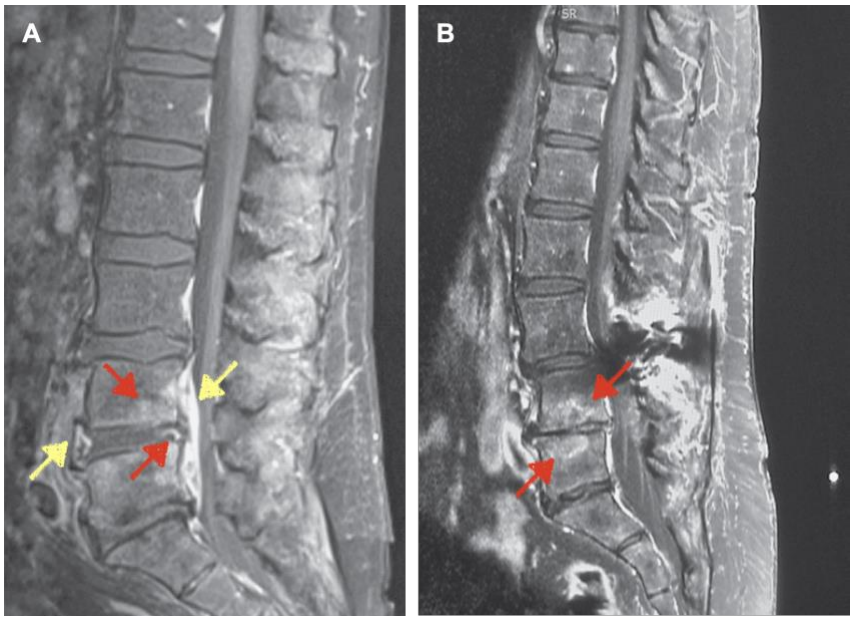


Figure 1: MRI scans of the lumbar spine from VO patients; T1-weighted sequence, gadolinium-enhanced, and fat-suppressed

A shows the MRI scan of a patient with symptoms since two weeks. The scan shows edema in the bone marrow and intervertebral disc (red arrows) as wells as abscesses prevertebral and in the epidural space (yellow arrows).

B shows the MRI scan of a patient with an exacerbation of chronic lumbar pain. It is a typical image for a patient with VO presenting with bone edema of the vertebral bodies (red arrows). The MRI scan suggests that this condition started with an *osteochondrosis intervertebralis*, which is shown by the degenerative changes.

Scans from Zimmerli [67].

Scintigraphy, a form of nuclear imaging technique, can also be used for diagnosing VO. The sensitivity of this method is about 90% and the specificity averages 78% [77,83]. For radionucleotide imaging, a gallium-67 scintigraphy can also be performed, which increases the specificity to 100% [77]. Bone scintigraphy is performed by using ^{99m}Tc- diphosphonate, where the uptake depends on blood flow and new bone formation. It is performed to differentiate between an infection and other diseases of the spine. There are three phases showing different aspects, namely the perfusion phase after the intravenous injection of the radionucleotide,

immediately followed by the blood-pool phase, and finally the skeletal phase two to four hours after the injection. The last phase is the interesting phase for osteomyelitis [60,74,84].

Another diagnostic tool for VO is a PET with ^{18}F-Fluordesoxyglucose (^{18}FDG/PET) [60]. This technique allows the detection of the exact location of infection, thus making it possible to differentiate between infectious diseases of the spine, degenerative changes, and malignancies [2,75,85]. In comparison to MRI, this technique can also show early changes of VO [49]. Other advantages are that it can be an option for patients with contraindications for CT- and MRI-contrast medium and that it has lower rates of radiation. Nevertheless, it is an expensive method, and it is limited in availability, which means it is mostly used in specialized centers [63,75].

3.1.3. Therapy

The main goals for the therapy of VO are elimination of the causative pathogen and infection, pain reduction, maintenance of the stability of the spinal column, and improvement of neurological deficits [2,63,86]. Commonly, a patient with a suspected spinal infection is hospitalized [3]. Two approaches need to be individualized for every patient in the treatment of the disease; 1) the conservative approach, which includes immobilization, analgesia, and antimicrobial therapy - mostly antibiotics; and 2) the surgical approach, which includes debridement of the infected tissue, restoration of spinal stability, decompression of nervous tissue, and drainage of abscesses [2,63,86,87]. The study of Zadran et al. indicates no significant differences between the outcomes of surgical and conservative treatment methods [10]. The severity of the disease and the status of a patient determines a suitable treatment strategy [62,88]. Notably, there is no agreement on a gold standard method although all treatment strategies include antibiotic, antifungal or antiparasitic medication that correspond to the causative pathogen [1,2,63].

Markers for inflammation and medical imaging can help monitor the success of a treatment in VO patients [39,70]. A normalization of CRP or a reduction in ESR by at least 25% indicate a recovery [16]. For medical imaging, X-ray is an accessible method to show progressive improvements of the spinal segments [3]. A more expensive method, which provides a clearer picture of the local changes, is a ^{18}FDG/PET [61]. MRI proved unsuitable as a follow-up marker due to a poor correlation with clinical response. This indicates that symptom monitoring can be more applicable than medical imaging [47,60,67].

3.1.3.1. Conservative approach

About 18-51% of the patient population of several studies were treated with only conservative methods [10,21,62,89,90]. There are less obvious indications for the conservative

approach [30]. However, the indications that should be considered are a clinical condition with the absence of neurological deficits, maintained stability of the spine, and the absence of larger abscesses [2,87,91]. A conservative treatment is mostly implemented in elderly VO patients with a debilitated general condition and multi-morbidity due to higher peri-operative risks [92]. Conservative treatment consists of pharmacological treatment using antimicrobial treatment and pain medication, immobilization, and physiotherapy under continuous control of the clinical status of the patient [3,62,63].

The administration of a specific antimicrobial medication is crucial for the therapy of VO. Hence, there should be a close consultation with an infectiologist for making a suitable decision on antimicrobial treatment. A suitable medication depends on the causative pathogen, which are mostly bacteria in VO, and on the antibiogram [2,15,37,47,67]. In severe cases, broad-spectrum antibiotics are used in the beginning of the disease because the detection of the pathogen takes time and can even fail entirely. For this reason, an empiric antibiotic medication for the most commonly found pathogen is used with the intention to prevent an acute progression of the disease and severe complications [2,37,93]. The empiric therapy is changed to a calculated therapy as soon as the microbiological examinations detect the causative pathogen [30,94,95]. If detection of the pathogen is not possible, a combined therapy with two antibiotics covering *S. aureus* and gram-negative organisms is implemented [16,47]. For oral therapy, quinolones and clindamycin are potential therapeutics [94]. In case of MRSA, oxazolidinone (Linezolid) is the antibiotic of choice [47,95]. Due to low bioavailability and low ability to penetrate into the bone tissue, the choice of the antibiotic agent and the question if oral application is viable are to be considered [63,94,96]. The duration of antibiotic therapy varies but a duration of six weeks in uncomplicated cases is mostly recommended [14,67,70,97,98]. Another specific therapeutic regime is required if the pathogen is *M. tuberculosis* or a fungal pathogen [99-101].

In addition to the antibiotic treatment, an immobilization of the affected segment is important in the conservative approach. Accordingly, a declining orthosis is applied to immobilize, stabilize, and provide spinal alignment. The reclining position helps to shift the stress away from the infectious parts in the ventral column to the dorsal column of the spine. Additionally, the rotation of the spine is limited through the orthosis [2,3]. If the status of the patient allows, the patient can be mobilized and there is the possibility for physical exercise to help regain functionality. Mobilization reduces the risk of deep vein thrombosis, pulmonary embolism, pneumonia, decubitus ulcers, pseudoarthrosis, and chronic pain [1,87].

Indicated conservative treatment is successful in up to 90% of the cases [89]. If conservative treatment fails or a relapse occurs, a surgical approach needs to be considered [30,93,102].

3.1.3.2. Surgical approach

About 49-82% of the patient population are treated surgically [10,21,62,89,90]. Indications for the surgical approach are sepsis, neurologic impairments, bone involvement with signs of instability, large infection-related masses, and an unclear cause. In addition, failure of conservative treatment, unmanageable pain, and the lack of compliance of a patient can require surgical treatment [1,2,4,10,86,87]. The therapeutic aim of surgical treatment is decompression of a narrowed spinal canal, reconstruction, and stabilization of deformed and destructed vertebral bodies as well as open biopsies for further examination of the pathogen. Furthermore, the purpose of the spinal surgeries is to stop the progress of infection, to allow healing, and to provide functionality of the spine and shorten the hospital stay [3,62,86,87]. The timing of surgery, ideally within 24 hours after hospital admission, is important for a good outcome concerning mortality, neurologic status, and post-operative complications [103].

Invasive procedures for VO patients range from minimally invasive methods, such as CT-guided drainage of abscesses and percutaneous transpedicular discotomies to open surgeries [39,62,80,87,104]. Depending on the condition of a patient, the different steps of surgical intervention can sometimes be separated into two stages. In the first surgical procedure, a debridement and a decompression are carried out and tissue samples can be taken. An advantage of the two-stage surgery is the possibility of recovery after the first surgery. In a second surgical procedure, a mechanical stabilization of the spine is carried out. [2,86,87,105]. There is no preferred procedure. The choice depends on the defect of the spine and the executing surgeons [1,87,106]. Open surgeries can be done via ventral, dorsal, dorsoventral or ventrodorsal access. The routes of surgical access depend on the general condition of a patient, comorbidities, the degree of bony destruction, neurological involvement, and the localization of the inflammation [62,86,103]. A decompression of nervous tissue alone, without stabilization of the spinal segment, shows no benefit because it results in decreased stability, hyperkyphosis and worse neurological status. Thus, stabilization is fundamentally important in VO patients [4,89,102]. Procedures for stabilization are fusion cages, bone grafts, locking plate systems, and screw-rod-systems [86,87]. The latter reconstruct and replace affected bone tissue and intervertebral discs with titan cages or bone implants. Different kinds of fusions of the adjacent vertebral bodies, such as PLIF (posterior intervertebral fusion), TLIF (transforaminal intervertebral fusion) or ALIF (anterior intervertebral fusion) can be performed [62,107]. The risk for an implant-associated infection can be diminished by accurate debridement and local antibiotics [1,87]. If an implant-associated infection occurs, the implants must be removed and examined microbiologically [108].

For fungal VO, surgical debridement proves to be more effective as medical treatment alone [99,109]. For all other types of infection, an advantage of surgical treatment in comparison to conservative treatment can be faster mobilization of the patients, higher quality of life, higher

satisfaction with the results, and a shortened hospital stay [87,92,102]. However, as mentioned above, both approaches show similar results in the long-term follow-up [10,98].

3.2. Osteochondrosis intervertebralis

Osteochondrosis intervertebralis involves degenerative changes of the vertebral bodies and the intervertebral discs. These progressive changes can be caused by age, genetics, metabolic diseases, and physiological stress such as frequent heavy lifting, vibrations (for example for truck drivers), scoliosis or traumas. The degenerative changes usually occur in the lower levels of the lumbar spine (L4-S1) where most load-bearing takes place [110]. Recent studies report a low-grade infection with low-virulent bacteria like *P. acnes* as a contributing factor, especially for the infectious type of the disease described below [53,111-116]. However, an unbalanced intervertebral disc microbiome could also play a role [117]. Moreover, intervertebral disc degeneration can involve other conditions such as disc herniations, spinal stenosis, and facet joint arthritis [118,119].

Notably, degenerative changes can very often be visible in MRIs of persons without any back pain or other symptoms [120]. Hence, there is a low correlation between visible degenerative changes and their symptoms [119,121]. However, osteochondrosis can present with back pain and thus plays an important role as a differential diagnosis for VO. The differentiation between the two diseases is easiest with an MRI [110]. In an MRI, so-called MODIC changes show alterations in the vertebral end-plate and bone marrow. The classification by Modic and Ross categorizes osteochondrosis into three different types according to the MRI findings with T1- and T2-weigthed images. Type 1 changes are hyperintense in the T2-weigthed image and show an inflammation and edema of the bone marrow; Type 2 changes are hyperintense in the T1-weighted image and show a fatty degeneration; Type 3 changes are hypointense in both T1- and T2-weighted images, and show sclerotization. Notably, the inflammatory condition of the MODIC type 1 involves similar characteristics such as MRI findings in the beginning of the disease and elevated inflammatory biomarkers compared to VO patients [122-124]. An example of a MRI scan from a patient with MODIC type 1 is shown in Figure 2.

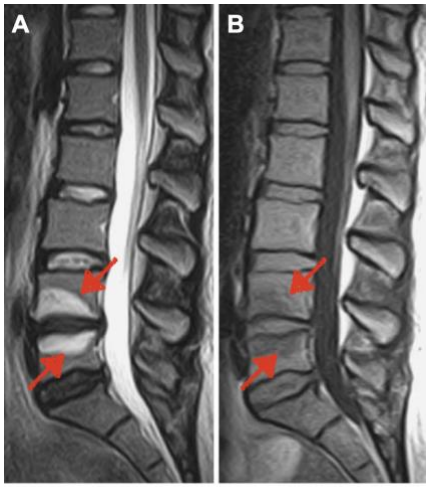


Figure 2: MRI scans of a patient with MODIC type 1

A shows the T2-weighted sequence and **B** shows the T1-weighted sequence. The scans show edema in the bone marrow (red arrows).
Scans from Albert et al. [125].

Osteochondrosis intervertebralis patients can be treated with physical therapy, analgesics, and surgery. Surgical interventions consist of different techniques such as discotomies, interbody fusions, and newer cellular transplantation therapy [126,127].

In this study, *osteochondrosis intervertebralis* patients acted as the control group. Comparison with this group of patients was beneficial because this disease is a non-infectious condition with less or no increased inflammatory biomarkers, and the same surgical techniques are being used as for the VO group. Under those circumstances, the inflammatory biomarkers can be evaluated in a non-infectious and an infectious group.

3.3. Cytokines

Cytokines are small hormone-like endogenous proteins, which are produced and secreted by T- and B-leukocytes and other immune-regulatory cells such as macrophages, fibroblasts, mast cells and bone marrow stromal cells [128,129]. They can be released via an autocrine, paracrine, juxtacrine or endocrine pathway and have a multitude of pleiotropic functions, which depend on different factors such as the responding cell and receptor. Their effects include communication with the help of signals for the coordination of the immune responses, inflammation, tissue repair, and development of components of the immune system [130-133]. Cytokine responses are initiated by an interaction with a receptor and the initialized pathways provoke a gene activation, which then leads to a biological function and networking mechanism. A downregulation of cytokine production can also be provoked through these pathways [131].

In general, pro-inflammatory and anti-inflammatory mechanisms can be involved. Pro-inflammatory cytokines such as TNF-α, IL-1ß, IL-12, IFN-γ, and IL-6 induce an inflammatory process in case of an infection. Anti-inflammatory cytokines such as IL-4, IL-10, IL-13, and TGF-ß are important for homeostasis of the immune-response in infectious diseases by triggering a negative modulation of pro-inflammatory cytokines [134-137]. Imbalances in the secretion of pro- and anti-inflammatory proteins can lead to chronic infectious diseases and severe sepsis [135,136,138].

Cytokines can be classified in different ways, for example, by grouping them either based on their structure, their function or their receptor-specific signal transduction [131,133]. The classification by function is most common, but functions can also overlap within the groups. Originally, cytokines were categorized along their biological effects into groups, which are interferon (IFN), interleukin (IL-1 to IL-23), tumor necrosis factor (TNF), colony-stimulation factors (G-CSF, GM-CSF), growth factors (PDGF-bb, VEGF, FGF basic), transforming growth factors (TGF), virokines, and chemokines (RANTES, MCP-1α, MIP-1ß, IP-10) [131].

In the following, four cytokines are exemplarily presented briefly. IL-6 is produced by B-lymphocytes and T-lymphocytes, mononuclear phagocytes, keratinocytes, hepatocytes, fibroblasts, mast cells, endothelium, and bone marrow stromal cells [132,139,140]. If IL-6 binds the IL-6 receptor (IL-6R) with its signal-transducing component glycoprotein 130, a signalling pathway typically starts an intracellular cascade by activating the JAK/STAT (Janus kinase/signal transducer and activator of transcription), which results in gene expression for the stimulation to perform the pleiotropic functions of IL-6 [132,141-143]. Functions of IL-6 include the induction of antibody-forming plasma cell maturation, haematopoiesis, and bone modulation through altering osteoblast-activity [132,139,140,142,144-146]. The IL-6 cytokine acts mainly in a pro-inflammatory way and is one of the mediators in the acute-phase reaction as well as in the fever reaction [132,140,147,148]. Studies show that the range of fever temperatures and the concentration of IL-6 in serum correlate with the severity of infection and the patient outcome [132]. Thus, the clinical use of IL-6 acts as a good prognostic marker and also as a monitoring marker for the therapeutic effect of an antibiotic treatment [148-150]. A dysregulation of IL-6 plays a role in chronic infection, autoimmunity, and oncogenesis [151,152].

IL-8, also known as CXCL8, is identified as a chemotactic cytokine and is foremost produced by macrophages. IL-8 binds to the receptors called CXCR1 and CXCR2, which results in an intracellular signal cascade to enforce the functions of IL-8. Leukocytes, especially neutrophil granulocytes, express these specific receptors and, in this case, IL-8 operates in a pro-inflammatory way and acts chemotactically. Neutrophil granulocytes are upregulated to cause an increased adhesion on the endothelium and extracellular matrix. Equally, degranulation of neutrophils, release of myeloperoxidase, and formation of oxygen radicals

take place through the influence of IL-8 [132,143,153]. Additionally, it has been shown that IL-8 has an effect on angiogenesis, regulating and enhancing the development of blood vessels [154-156].

IL-12 (p70) is the biologically active form of IL-12, consisting of two peptides called IL-12 (p35) and IL-12 (p40). This cytokine resembles IL-23 and IL-27 with regards to its subunits, receptors, and signalling pathways [132,157]. IL-12 is secreted by monocytes, macrophages, and especially dendritic cells [132,140]. As a result of an interaction with pathogens like bacteria, viruses, and parasites, an increased secretion and activation of IL-12 occurs. The main function of IL-12 is to stimulate natural killer cells and induce their proliferation, functioning and cytotoxicity [132,157]. Under the influence of IL-12, an induction of IFN-γ secretion in natural killer cells and T-cells takes place and a differentiation of naive T-cells towards T helper cells occurs in the early immune response [132,157,158]. Other activities attributed to IL-12 include the secretion of various cytokines such as TNF-α and GM-CSF [132,158].

Vascular endothelial growth factor (VEGF) includes a family of proteins and they provoke intracellular signals through specific VEGF-receptors. This cytokine is important for regulating angiogenesis and lymphangiogenesis by building new vessels through promoting cell migration and division of endothelial cells [159-161]. It especially reacts in case of hypoxic stress, whereby VEGF is secreted by many different cells and shows a high specificity for endothelial cells [160,162]. In addition, macrophages and monocytes are stimulated [160,163]. During human embryonic development, VEGF attributes to vasculogenesis. VEGF can be increased in many different cancer types and therefore, this fact can be used for targeted therapy in those malignancies [159-161].

3.3.1. Cytokine measurement

The quantification of cytokines can be realized using different matrices of the body such as blood, tissue biopsies, cell cultures, cavity fluids, urine, saliva or tears. Many different test methods are available, for example PCR or advanced biosensors. In addition, cytokines can be identified through the enzyme-linked immunosorbent assay (ELISA), which offer many clinically relevant benefits. It detects molecules using antibodies in a quantitative way by showing an enzymatic colour change. There are different types of ELISA. For example, direct ELISA, which only uses one type of antibody with a conjugated enzyme that binds specifically to the antigen. The antibody is directly coated on the well. By adding a substrate, a measurable colour change develops. Additionally, the competitive ELISA exists, which uses a synthetic antigen that mimics the sample antigen. This creates a competitive situation between both types of antigens when binding to the site of the primary antibody that is pre-coated on the well. After the enzymatic colour change reaction, the antigen can be detected inversely to the sample antigen concentration.

The most used method is a “sandwich”-ELISA, which uses two antibodies. With this type of ELISA, the surface of a 96-well plate is coated with antibodies, which are specific for the substance to be determined. After the addition of the sample, the antigen binds to the specific antibody. Then, the supernatant and unspecific antibodies can be removed in a washing step and only antigen-antibody-complexes remain on the plate. After this step, the second antibody, which is attached to an enzyme, binds to another binding side of the antigen to form an antibody-antigen-antibody-complex, which resembles a “sandwich”. After another washing and removal of unbound secondary antibodies, a chemical substance that reacts with the enzyme is added to produce a colour change. The colour change can be detected photometrically proportional to the antigen in the sample [164].

The method used in this study is a multiplex assay, which ensures easy quantification of many cytokines simultaneously. Other advantages of this method are the need of only a small amount of sample material and that there is a quick test result within approximately four hours. This method includes specific antibodies against the substance to be identified, which are bound to magnetic beads. After addition of the sample, the specific antigens in the sample bind to the antibodies on the beads. This takes place instead of binding of antibodies to a plate as occurs with the other types of ELISA mentioned. The supernatant and unbound molecules are then removed by several washing steps. After washing, second antibodies, which are biotinylated are added, and antibody-antigen-antibody-complexes are built. The detection of these complexes is possible after the addition of streptavidin phycoerytin conjugate (Fluorecent SA-PE) - a fluorescent indicator - that binds to the biotin on the secondary antibodies. Two lasers detect the beads with the phycoerytin. A red laser with a 635 nm wavelength detects the fluorescence of the beads while a green laser with a 532 nm wavelength quantifies the antigens bound indicated by the fluorescence intensity. The data is directly analysed through the machine. To visualize the results, a software generates a standard curve for each cytokine. The y-axis shows the fluorescence intensity (FI), and the x-axis shows the cytokine concentration (pg/ml). Outliers are also visualized on the curve [165]. An overview of the multiplex assay method is shown in Figure 3.

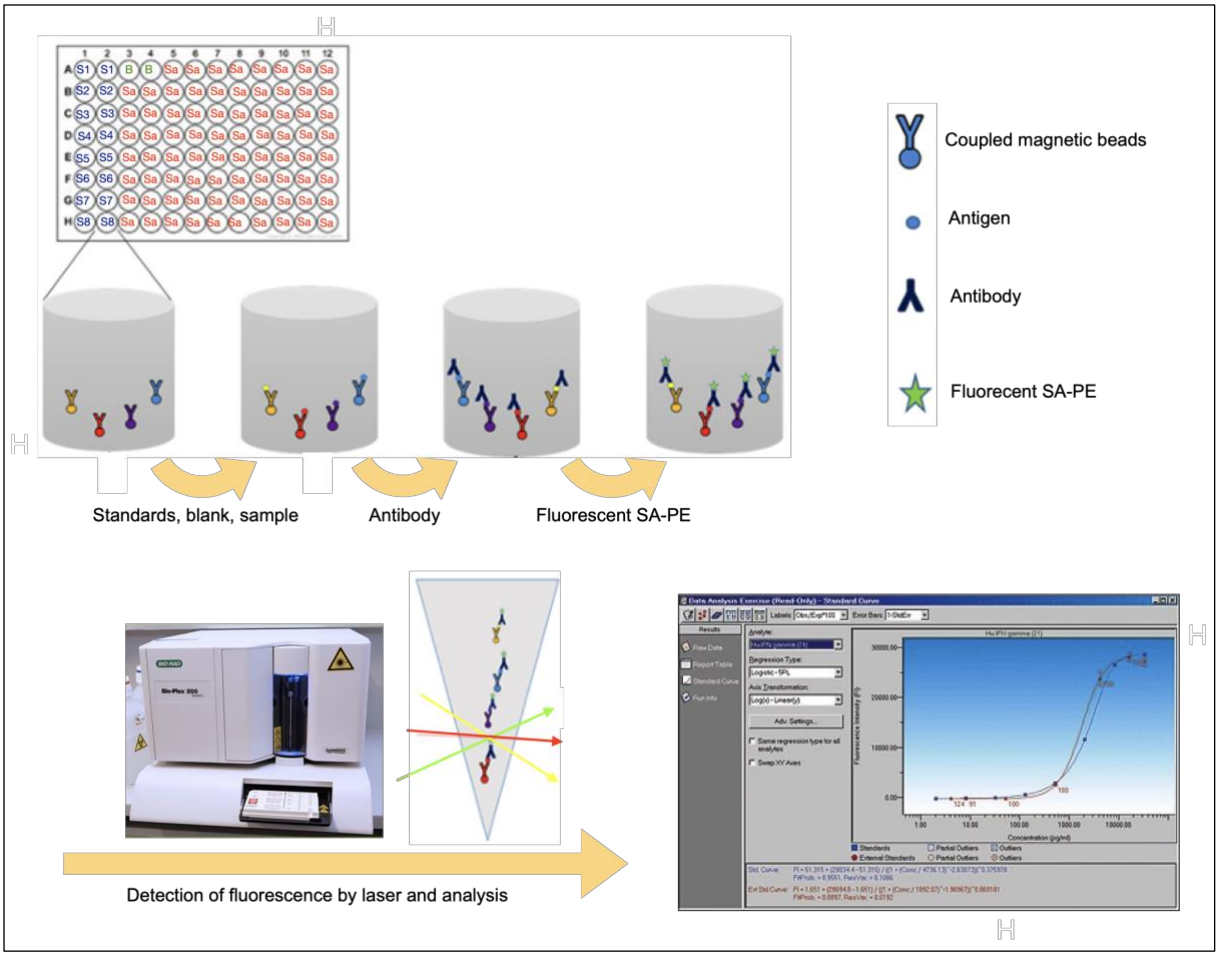


Figure 3: Principle of a multiplex cytokine assay

The plate layout includes Standards (S1-S8), a blank (B), and 39 samples (Sa) in technical duplicate. Antibodies are added. For detection with lasers, a fluorescent indicator (Fluorescent SA-PE) is added and the data are analysed. A software generates a standard curve for each cytokine.

3.4. Aim of the CyProSpon study

Currently, no specific clinical or laboratory methods are available to differentiate a degenerative spine disease from VO. Medical imaging of the disease is only successful when tissue damage is already present, which often correlates with an irreversible condition. Laboratory parameters such as CRP, ESR, and leucocytes are not specific, and blood cultures cannot always lead to detection of the pathogen. Often, the surgical intervention with its risks for complications is the only way to diagnose VO. Accordingly, one aim of the CyProSpon study (Cytokine Profiles in Spondylodiscitis study) was to develop a minimally-invasive diagnostic biomarker to distinguish infectious from non-infectious diseases of the spine. The potential of cytokines has already been studied as diagnostic biomarkers for various infectious diseases. This study established the possibility of cytokine profiling to discriminate between

VO and degenerative diseases of the spine. The levels of CRP and 27 cytokines were analyzed before surgery and four times after surgery for the VO group and the *osteochondrosis intervertebralis* group. In addition, the type of pathogen for the VO patients, which possibly influences the cytokine levels, were taken into consideration. The potential of those new biomarkers lies in a minimally-invasive method for an easy approach and early diagnosis.

4. Materials and Methods

4.1. Study design

The CyProSpon study was performed as a collaboration between the Center for Molecular Medicine Cologne (CMMC), the Orthopedic Department, and the Internal Medicine I Department at the University Hospital of Cologne. The study was designed as a single-center case-control study. The active recruitment phase took place from 2015 till 2017, and the experimental phase took place until 2020. The present study is compliant with the Helsinki guidelines (seventh revision, 2013) and the German guidelines for good clinical and laboratory practice (GCP-V, Federal Ministry of Justice and Consumer Protection, Berlin, Germany, last update 2012). It was authorized by the Ethics Committee of the University of Cologne (Trial protocol: Uni-Köln 9-2014 and revised on 22.08.2016). The CyProSpon study is registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT02554227). The patients included were informed about the study and gave their written consent. All patient data and stored samples were anonymized.

4.2. Study Participants

All participants in this study were patients in the Department of Orthopedic and Trauma Surgery, University Hospital of Cologne, Germany, and had an indication for a surgical stabilization of segments of the spine by removing intervertebral disc(s) in the lumbar and/or thoracic vertebrae. Altogether, 98 patients with VO were reviewed but 82 patients did not meet the inclusion criteria. The criteria for selection and inclusion in the study were both genders, age between 40 and 85 years, existence of informed consent, and legal competence of the patient. Patients with *osteochondrosis intervertebralis* MODIC type 1 with bone marrow edema, diagnosed via MRI, were excluded. Other exclusion criteria were the existence of autoimmune diseases, cancer, and other acute infections external to the spine. In total, 36 patients were included in this study, which include 16 patients with VO (n=16, patient ID 1-16) and 20 patients with *osteochondrosis intervertebralis* (n=20, patient ID 21-40). This information is presented in Figure 4. The diagnosis of VO patients was confirmed by microbiological results, clinical findings, and medical imaging. A total of 15 patients from the VO group received medical imaging by an MRI and one patient (patient ID 2) by a CT scan due to a cardiac pacemaker, which represents a contraindication for MRI. The patients diagnosed with *osteochondrosis intervertebralis* served as the control group.

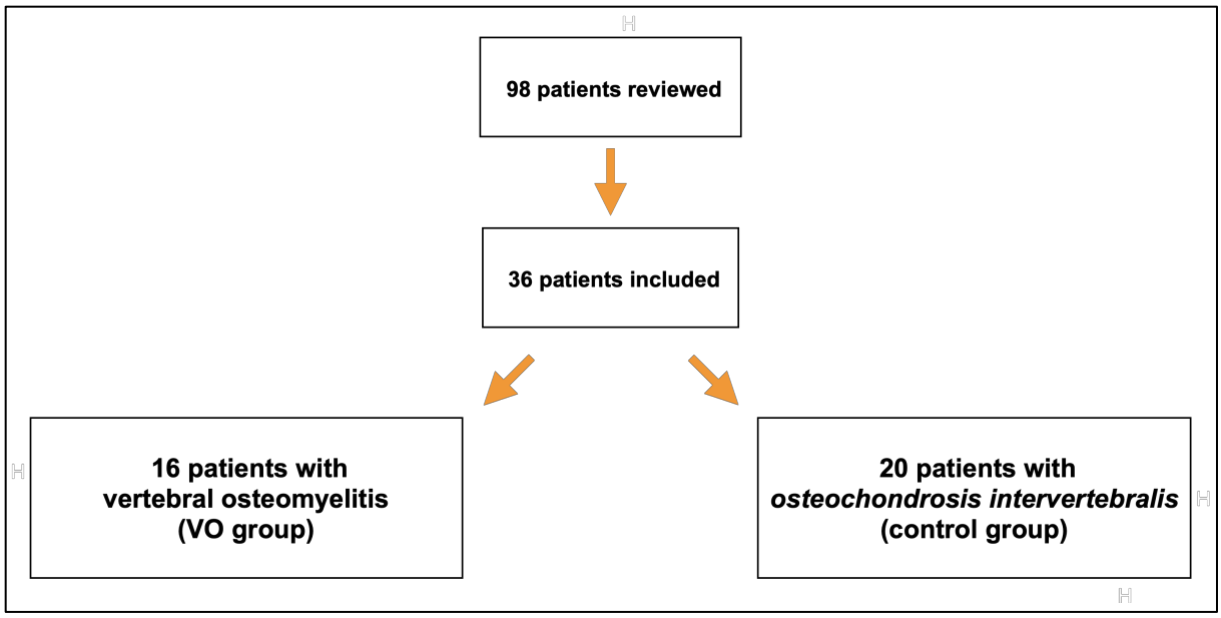


Figure 4: Overview of the number of vertebral osteomyelitis and control patients

The figure shows that the recruiting phase involved the review of 98 VO patients. In total, 82 VO patients did not meet the inclusion criteria. 36 patients were included and divided into a VO group of 16 patients and a control group of 20 patients.

All relevant data of the patients were documented. At the timepoint of inclusion, the mean age was 68.8 in the VO group versus 65.8 in the control group. In the VO group, 62.5% of the patients were men and in the control group, 45% were men, as shown in Table 1.

Table 1: Epidemiological data of the vertebral osteomyelitis and control patients

	All patients	Vertebral osteomyelitis	Control
Number of patients	36	16	20
Male	19 (52.8%)	10 (62.5%)	9 (45.0%)
Female	17 (47.2%)	6 (37.5%)	11 (55.0%)
Mean age (years)	67.1	68.8	65.8

Epidemiological data shown including sex and mean age for the study groups.

This table was adapted from one as a SOP from AG Mahabir-Brenner.

Further relevant data were collected as shown in Table 2a and Table 2b, which included body mass index, comorbidities, diagnosis, symptoms, and diagnostic procedures. Most of the patients had lifestyle diseases such as arterial hypertension, diabetes mellitus or hypothyroidism. Also, data such as nicotine and/or alcohol abuse, analgesic support before and after surgery, type of surgery and implant material as well as complications during surgery and in the follow-up phase were noted.

During their surgical interventions, all patients received intravenous general anesthesia with intubation. For the patients of the control group, Cefazolin (1x 2g) was used as peri-operative antibiosis. Pre-operative antibiosis in VO patients was discontinued at least three days before surgery, except for three patients (patient ID 4, patient ID 10 and patient ID 16).

Table 2a: Demographic data and comorbidities of each participating patient in the VO group

Patient ID	Age in years	Gender	BMI (kg/m^2)	Comorbidities
1	76	m	23.4	s/p NPP L2/ L3 left side
2	79	m	24.2	AH, type 2 DM, mitral-/tricuspid regurgitation, CHD, implantations of defibrillator, s/p sigma diverticulitis, s/p partly gastric resection, s/p glomus tumor
3	58	f	35.3*	AH, s/p CMV-infection, s/p thrombophlebitis, paroxysmal atrial fibrillation, obstructive sleep apnea syndrome, hypothyroidism, obesity, hepatic steatosis, renal insufficiency, hyperbilirubinemia, s/p cholecystectomy, s/p hysterectomy
4	66	m	31.3*	AH, type 2 DM, diabetic foot syndrome, diabetic nephropathy, atrial septal aneurysm, s/p hepatitis E infection, type C-gastritis, obstructive sleep apnea syndrome, s/p borreliosis
5	71	m	29.4	AH, s/p urosepsis, s/p acute renal failure, paroxysmal atrial fibrillation, middle grade aortic stenosis and low grade insufficiency of aortic valve, hypothyroidism, incomplete disc herniation in thoracic/lumbar spine
6	53	f	23.7	None
7	68	f	27.9	AH, s/p osteomyelitis in childhood, s/p gastric ulcer
8	75	f	30.9*	AH, s/p urosepsis, s/p thrombophlebitis, CHD, s/p TKR
9	63	m	22.4	Bradycardia, pacemaker
10	71	m	30*	AH, s/p hepatitis A infection, s/p TKR, s/p shoulder surgery
11	54	m	31.6*	s/p VO 07/16 with S. epidermidis, s/p decompression of lumbar spine, s/p deep vein thrombosis right leg, s/p fracture of the left femur and left lower leg
12	72	f	32.4*	AH, s/p ovarian cancer, urethral splint, s/p transient ischemic attack, paroxysmal atrial fibrillation
13	59	m	28.7	AH, s/p deep vein thrombosis
14	77	m	23.4	AH,s/p cholecystectomy, benign prostate hyperplasia, s/p aneurysm rupture
15	85	m	29.4	AH, atrial fibrillation, hypothyroidism, s/p decompression of lumbar spine
16	73	f	17.7	AH, DM, s/p dorsal spondylodesis

Patients with obesity, BMI ≥ 30 kg/m2 are marked (*).

VO: vertebral osteomyelitis patients, m: male, f: female, s/p: status post, L: lumbar, AH: arterial hypertension, CHD: coronary heart disease, DM: diabetes mellitus, TKR: total knee replacement, NPP: Nucleus pulposus prolapse, CMV: Cytomegalovirus.
Table adapted from AG Mahabir-Brenner.

Table 2b: Demographic data and comorbidities of each participating patient in the control group

Patient ID	Age in years	Gender	BMI (kg/m²)	Comorbidities
21	80	f	27.2	AH, DM, hypothyroidism, CHD, inner ear hearing loss left side
22	75	f	28.2	AH, s/p multiple decompression of lumbar spine
23	70	f	36*	AH, CHD, metabolic syndrome
24	74	f	31.6*	AH, s/p pacemaker, s/p meningioma
25	78	f	23.6	AH, s/p Colonic carcinoma
26	54	m	21.8	s/p nucleotomy L4/L5, s/p decompression L5/S1, CHD, s/p coronary bypass-surgery, PAD, s/p A. iliaca communis stenting
27	57	f	28.7	AH, s/p facet joint cyst removal and foraminotomy L5 left
28	58	m	34.1*	AH, atrial fibrillation
29	66	m	25.1	AH, gastro-esophageal reflux disease, vertebral instability L4/5, stenosis of neuroforamina L4 right side, s/p decompression surgery of spinal stenosis L4/5 left side,
30	63	f	27.3	AH, PAD, multiple femoropopliteal bypass surgery in both legs
31	52	m	28.4	None
32	63	f	33.1*	AH, hypothyroidism
33	72	m	24	AH
34	59	f	28.9	type 2 DM, s/p sigma diverticulitis, s/p gastritis, esophageal varices, nodular goiter, alcohol-toxic liver cirrhosis
35	77	m	29.1	AH, DM type 2, CHD, s/p dual coronary bypass surgery, PAD, bypass surgery in both legs, spondylodesis L3-5
36	72	f	25.9	osteoporosis, s/p compression fracture Th11, Th12, L3, incomplete fracture Th10
37	60	m	31.6*	None
38	72	f	31.2*	AH, type 2 DM, dyslipoproteinaemia, breast cancer, vitamin D deficiency
39	53	m	35.3*	AH
40	61	m	24.2	AH, decompression L2/3, decompression and lumbar fusion L4/S1, dyslipoproteinaemia, hypothyroidism, CHD, s/p pacemaker implantation

Patients with obesity, BMI ≥ 30 kg/m² are marked (*).

C: control patients, m: male, f: female, s/p: status post, L: lumbar, Th: thoracic, AH: arterial hypertension, CHD: coronary heart disease, DM: diabetes mellitus, PAD: peripheral artery disease.

Table adapted from AG Mahabir-Brenner.

To determine the pathogen status of VO patients, blood cultures were taken before surgery. During the surgical procedure, tissue samples from the intervertebral discs were collected and stored. The materials were analyzed microbiologically and pathologically, and causative pathogens were identified using blood sample and tissue biopsies (Table 3). To detect low-virulent agents such as *Propionibacterium spp.,* at least two samples were analyzed. To summarize, 24% *S. aureus*, 24% *S. epidermidis,* and 24% *E. coli* were found in the samples. There were two pathogens simultaneously (*S. aureus* and *E. coli*) in one patient (patient ID 14). Adequate antibiotic treatment for patients with VO depended on the microbiological findings and bacterial resistance. None of the patients from the control group suffered from VO or other complications during the follow-ups.

Table 3: Microbiological sample results and antibiotic treatment for each patient suffering from VO

Patient ID	Causative pathogen	Method of microbiological detection	Specific antibiotic treatment
1	*S. epidermidis*	Biopsy	Vancomycin
2	*S. epidermidis*	Biopsy	Flucloxacillin, Rifampicin
3	*S. aureus* (MRSA)	Biopsy	Vancomycin, Fosfomycin
4	*Streptococcus dysgalactiae*	Blood culture	Penicillin G
5	*E. coli*	Blood culture	Ceftriaxon
6	*S. epidermidis*	Biopsy, blood culture	Vancomycin, Rifampicin
7	*E. coli*	Biopsy	Ciprofloxacin
8	*S. aureus* (MSSA)	Biopsy	Flucloxacillin
9	*S. epidermidis*	Biopsy	Clindamycin
10	*Parvimonas micra*	Biopsy	Penicillin G
11	*Propionibacterium acnes*	Biopsy	Clindamycin
12	*Streptococcus dysgalactiae*	Biopsy, blood culture	Penicillin G
13	*S. aureus* (MSSA)	Biopsy, blood culture	Vancomycin, Rifampicin
14	*S. aureus* (MSSA), *E. coli*	Biopsy, blood culture	Flucloxacillin, Rifampicin, Ciprofloxacin
15	*E. coli*	Biopsy	Meropenem
16	*S. lugdunensis*	Biopsy	Cotrimoxazol, Rifampicin

VO: vertebral osteomyelitis, S.: Staphylococcus, MRSA: methicillin-resistant *S. aureus*, MSSA: methicillin-sensitive *S. aureus*, *E. coli*: *Escherichia coli.*

Table adapted from AG Mahabir-Brenner.

Table 4a: Affected segment, relevant diagnosis, and surgical treatment for each patient suffering from VO

ID	Affected segment and relevant diagnosis	Surgical treatment
1	L2/3 with psoas abscess; s/p nucleotomy L2/3	d0: debridement L2/3; d16: PLIF L2/3
2	Th 8/9	d0: debridement Th8/9, laminectomy Th8, DIS Th7-10
3	Th 5/6	d0: VIS Th4-7; d10: PLIF Th4-7
4	L 5/6 with psoas abscess	d0: PLIF L5/S1
5	L1/2, L5/6	d0: PLIF L1/2, TLIF L5/S1
6	L4/5; s/p nucleotomy and decompression L4/5	d0: debridement and decompression L4/5, DIS L4/5; d7: ALIF L4/5
7	L1/2	d0: DIS Th12-L3; d12: ALIF L1/2
8	L3/4, L4/5 with epidural abscess	d0: debridement of epidural abscess L3/4, debridement L3/4 and L4/5, DIS L3-5; d13: corporektomy
9	T12/L1, L1/2	d0: decompression, nucleotomy and debridement Th12-L2, PLIF Th12-L2
10	L4/5	d0: decompression of psoas abscess, PLIF L4/5
11	L4/5, L5/S1; s/p decompression and removal of hematoma L5/S1	d0: TLIF L4/5, L5/S1
12	L2/3, L4/5, L5/S1 with psoas abscess	d0: DIS L2-S1, PLIF L4/5, L5/S1; d13: wound revision, debridement psoas abscess
13	T11-L1	d0: decompression Th12/L1, DIS T11-L1; d8: debridement and decompression, VIS T12/L1
14	L3/4, instability and screw pull-out L3/4	d0: DIS L3/4; d12: VIS L3/4; d58: metal removement L3/4 and DIS L1-L3; d69: ALIF L3/4
15	L 2/3, L3/4, impairment of wound healing	d0: DIS L1-3, PLIF L2/3; d:7, d:13, d 20, d55 local debridement and wound revision
16	L2-S1, infectious screw pull out S1; s/p PLIF L2-S1	d0: DIS metal removement and spondylodesis L2-ilium

VO: vertebral osteomyelitis, s/p: status post, L: lumbar, Th: thoracic S: sacral, d0: day of the surgery, PLIF: posterior lumbar interbody fusion, TLIF: transversal lumbar interbody fusion, ALIF: anterior lumbar interbody fusion, DIS: dorsal instrumented spondylodesis, VIS: ventral instrumented spondylodesis

Table 4b: Affected segment, relevant diagnosis, and surgical treatment for each patient suffering from *osteochondrosis intervertebralis*

ID	Affected segment and relevant diagnosis	Surgical treatment
21	Osteochondrosis L4/5, L5/S1; ventrolisthesis L4/5	d0: PLIF L4/5 with PTC cage
22	Osteochondrosis and connection instability L5/S1	d0: TLIF L5/S1, decompression L5/S1, decompression L2/3
23	Spinal canal stenosis and spondylolisthesis L3-L5; MODIC type 2	d0: PLIF L3-L5
24	Osteochondrosis L3-S1, anterolisthesis L3/4; MODIC type 2	d0: decompression, facet denervation L3/4, PLIF L3/4
25	Spondylolisthesis L3/4, L4/5	d0: decompression of nerve root L3-L5, TLIF L3/4, L4/5
26	Osteochondrosis L4-S1	d0: decompression and neurolysis L5 TLIF L4/5, L5/S1
27	Degenerative instability L4/5	d0: PLIF L4/5
28	Spondylolisthesis L5/S1; MODIC type 2	d0: TLIF L5/S1
29	High grade spondylathrosis L4/5, instability L4/5; s/p decompression; MODIC type 2	d0: TLIF L4/5
30	Osteochondrosis L3-S1, de novo scoliosis with spondylolisthesis L3/L5; MODIC type 2	d0: PLIF L3-5
31	Anterolisthesis L4/S1; MODIC type 2	d0: PLIF L5/S1
32	Spondylolisthesis L4/5, osteochondrosis L5/S1; MODIC type 2	d0: TLIF L4/5
33	Spondylolisthesis L4/5, spinal canal stenosis, Re-NPP	d0: TLIF L4/5
34	Anterolisthesis L4/5, NPP L4/5; MODIC type 2	d0: neurolyse L4/5, TLIF L4/5 and L5/S1
35	Spinal canal stenosis and connection instability L2/3; MODIC type 2	d0: PLIF 2/3 and posterolateral fusion L3-L5
36	Anterolisthesis L4/5, osteochondrosis L5/S1; MODIC type 2	d0: decompression and neurolyse L4/5, denervation of vertebral arche joint L4/5, PLIF L4/5
37	Spondylathrosis L4/5, osteochondrosis L3- S1; MODIC type 2	d0: TLIF L4/5
38	Pseudospondylolisthesis L3/4, spondylathrosis L4/5; MODIC type 2	d0: decompression L3/4 and L4/5, TLIF L4/5
39	NPP L4/5, facet joint arthrosis; MODIC type 2	d0: TLIF L4/5 and L5/S1
40	NPP L3/4 osteochondrosis L4/5, connection instability L2/3, L3/4; MODIC type 2	d0: PLIF L2-S1

L: lumbar, S: sacral, NPP: Nucleus pulposus prolapse, d0: day of the surgery, PLIF: posterior lumbar interbody fusion, TLIF: transversal lumbar interbody fusion, PTC: cage as a combination of PEEK and titan (PEEK: polyetheretherketon)

The diagnosis and surgical treatment for each patient are shown in Table 4a and Table 4b. Most of the VO patients suffered from monosegmental VO (n=9). The other patients showed two (n=6), and one patient had more affected segments (patient ID 12). The lumbar segments were more often affected (n=14) than the thoracic segments (n=4). In most cases, the surgical intervention included a one-stage stabilization of the affected segment (n=6) and in nine cases a stabilization of two or more segments was performed. One patient (patient ID 8) received a corporectomy because it was not possible to stabilize all infected vertebral bodies. Surgery involved debridement of infected tissue and biopsies for microbiological examination of intervertebral bodies.

The patients in the control group suffered from lumbar osteochondrosis caused by spondylolisthesis (n=12), a connection instability after previous spine-surgery (n=3), degenerative spine-instability (n=1), spondylarthrosis (n=3), or facet joint arthrosis (n=1).

4.3 Blood draws and timepoints

Blood samples were drawn in the morning at five timepoints during stationary stay and follow-up at the Department of Orthopedic and Trauma Surgery, University Hospital Cologne. The five timepoints were once before surgery (pre-OP) and four times after surgery at 3-5d post-OP, 6-11d post-OP, 40-56d post-OP, and 63-142d post-OP. The timepoints for the patients are shown in Figure 5. It was not possible to draw blood from two patients (patient ID 14, patient ID 15) at the timepoint 3-5d post-OP, from five patients (patient ID 1, patient ID 2, patient ID 15, patient ID 16, patient ID 40) at the timepoint 40-56d post-OP, and from one patient (patient ID 29) at the timepoint 63-142d.

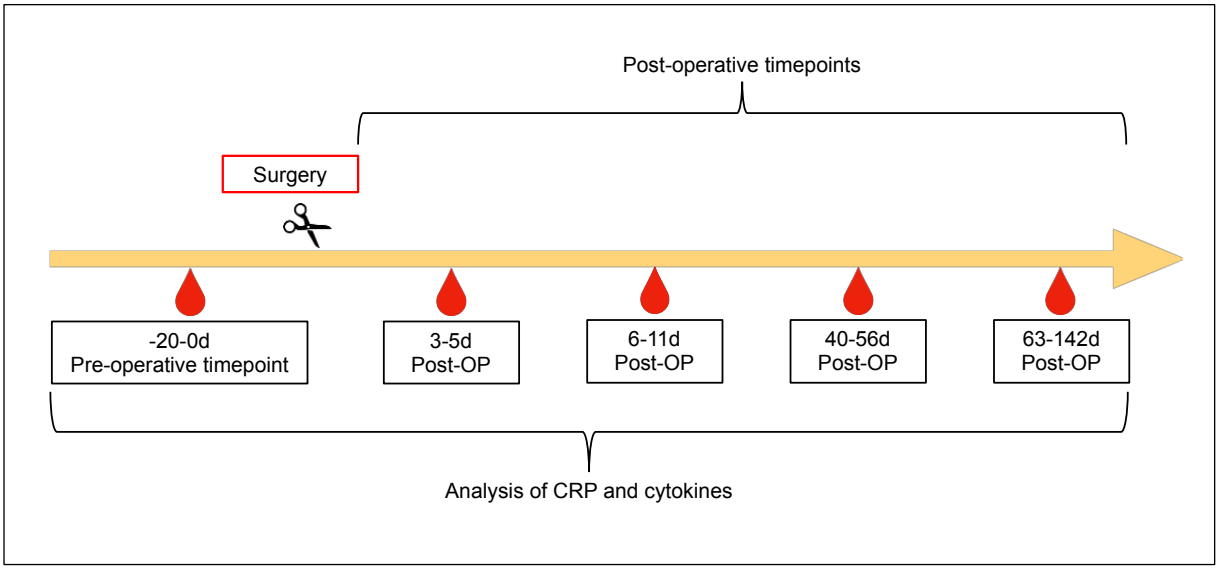


Figure 5: Timepoints of blood draws for all study participants

d: days, Pre-OP: before surgery, Post-OP: after surgery, CRP: C-reactive protein

The blood draws were performed according to the standard procedure in supine position. Blood was taken from the peripheral veins of the lower arm, the back of the hand or from a central venous catheter after overnight fast. In cases of puncture of the peripheral veins, stasis was maintained for a maximum time of two minutes. Serum gel monovettes (S-Monovette® Serum Gel 4.7 mL, Sarstedt, Nümbrecht, Germany) were used for the collection of blood samples for the measurement of cytokines and lithium-heparin monovettes (S-Monovette®, Lithium-Heparin, 4.7 mL, Sarstedt) were used for the measurement of CRP.

4.4. CRP level determination

The level of CRP was determined by the Central Laboratory of the University Hospital of Cologne. Plasma from Lithium-Heparin monovettes (S-Monovette®, Plasma-Gel 4.7mL, Sarstedt, Nümbrecht, Germany) was used and processed via latex agglutination assay according to the manufacturer's instructions (C-Reactive Protein Gen.3, cobas®, Roche Diagnostics, Mannheim, Germany). Plasma was diluted 1:100 and added on a slide. Human CRP agglutinates with the latex reagent, which is pre-coated with monoclonal anti-CRP-antibodies. After ten minutes of incubation, clear agglutination was observed and measured turbidimetrically using the analytic system Cobas® C702 (Roche Diagnostics). CRP values above 5 mg/L were considered pathological. Levels below 3 mg/L were considered clinically irrelevant and were adjusted to 0 mg/L.

4.5. Cytokine analysis

4.5.1. Preparation and storage of serum for cytokine analysis

For collection and processing of samples, all tubes were labelled accordingly. Serum gel monovettes (S-Monovette® Serum Gel, Sarstedt) were used for the blood draws. The monovettes were then stored for 30 to 45 minutes in an upright position at room temperature. Then the blood samples were centrifuged at 3461 x g for five minutes at room temperature (EBA 20 Centrifuge, Hettich Lab Technology, Tuttlingen, Germany) and the serum samples were aliquoted. The storage tubes (NuncTM, CryoTubeTM, 1.8 mL, ThermoFisher Scientific, Waltham, USA) were labelled with the patient's ID, timepoint, date of blood drawing, processing method, and volume. Afterwards, the samples were stored at -80°C until they were analyzed. Haemolyzed samples were avoided for the measurement and the haemolysis grade (grade 0 - 3) was documented for each sample. Visual examples for the haemolysis grades are shown in Figure 6.

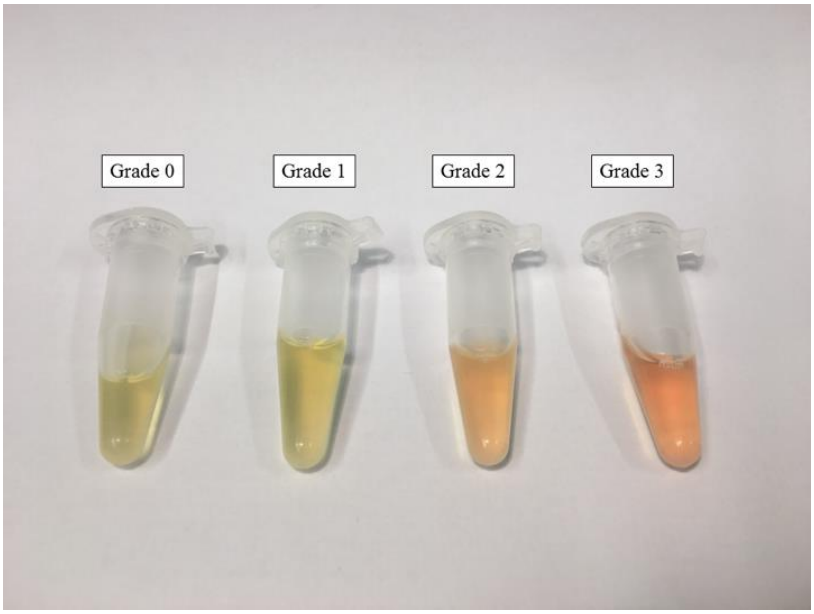


Figure 6: Haemolysis of serum samples grade 0 - 3
Grade 0 shows no haemolysis and grade 3 haemolysis was avoided for measurement. Picture from AG Mahabir-Brenner.

4.5.2. Multiplex cytokine assay

For the quantitative determination of cytokines in the current study, a multiplex ELISA was performed using the Bio-Plex multiplex immunoassays (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the Bio-Plex Pro™ Human Cytokine 27-Plex panel (Bio-Rad) according to the manufacturer's instructions. The levels of 27 cytokines were measured, namely IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-bb, RANTES, TNF-α, and VEGF.

A list of materials, which were used in this study for the cytokine measurements is shown in Table 5. Regarding the net price, one test kit from the manufacturer Bio-Rad was approximately 280€ per cytokine, although varying for the specific cytokine. The price for a larger test kit in order to measure more cytokines is lower. The prices for the additional materials used for this study add up to about 139€.

Table 5: Materials that were used in this study for the cytokine assay

Material	Manufacturer
Detection antibody diluent HB	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Standard diluent HB	Bio-Rad
Sample diluent HB	Bio-Rad
Assay buffer	Bio-Rad
Wash buffer	Bio-Rad
Steptavidin-PE	Bio-Rad
Double distilled water	From the tap
Multichannel pipette	Eppendorf, Hamburg, Germany
96-well microplate	Bio-Rad
Safe-lock tubes 1.5 ml	Eppendorf, Hamburg, Germany
Pipettes	Eppendorf, Hamburg, Germany
Pipette tips	VWR, Darmstadt, Germany
Sealing tape	Bio-Rad
Handheld magnetic washer	Bio-Rad
Bio-Plex 200 System	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Bio-Plex Pro Human Cytokine Assay	Bio-Rad
Centrifuge, model EBA 20	Hettich Lab Technology, Tuttlingen, Germany
Multiwell plate shaker	Sigma-Aldrich Chemistries GmbH, Taufkirchen, Germany
Bio-Plex 200 washing station	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Vortex (Vornado™ Vortex Mixer, 115V)	Benchmark Scientific, Edison, USA

In the following, each step for the cytokine measurement is presented. An example of a plate plan is shown in Figure 7. Before the measurements, the Bio-Plex Manager software protocol was prepared using the plate layout and standard values from the assay kit.

	A	B	C	D	E	F	G	H	I	J	K	L
1	Standard	Standard	Blank	Blank	8.1	8.1	16.1	16.1	28.1	28.1	36.1	36.1
2	Standard	Standard	1.1	1.1	9.1	9.1	21.1	21.1	29.1	29.1	37.1	37.1
3	Standard	Standard	2.1	2.1	10.1	10.1	22.1	22.1	30.1	30.1	38.1	38.1
4	Standard	Standard	3.1	3.1	11.1	11.1	23.1	23.1	31.1	31.1	39.1	39.1
5	Standard	Standard	4.1	4.1	12.1	12.1	24.1	24.1	32.1	32.1	40.1	40.1
6	Standard	Standard	5.1	5.1	13.1	13.1	25.1	25.1	33.1	33.1	1.5	1.5
7	Standard	Standard	6.1	6.1	14.1	14.1	26.1	26.1	34.1	34.1	2.5	2.5
8	Standard	Standard	7.1	7.1	15.1	15.1	27.1	27.1	35.1	35.1	3.5	3.5

Figure 7: Example of a plate plan

This plate plan was for the timepoint 1 (-12/0) pre-OP. Every plate layout includes standards, a blank, samples of the VO group (yellow) and samples of the control group (red) in duplicates.

The following steps for measuring cytokines can be divided into the preparation phase and the actual test phase. For the preparation phase, the Bio-Plex system was started, and with the use of the Bio-Plex calibration kit, warmed up and the equipment was calibrated. The assay buffer (Bio-Plex Assay buffer), the wash buffer (Bio-Plex Wash buffer) as well as the standard diluent (Bio-Plex sample diluent) were brought to room temperature and the other items from the kit were kept on ice until used. Then, the wash station for the plate was primed using the wash buffer (Bio-Plex Wash buffer).

A 1:4 standard dilution series was created. At first, 128 µL of the standard diluent (Bio-Plex sample diluent) was pipetted into a first tube (S1). For the other tubes (S2-8) and blank tube, 150 µL of the standard diluent were used. Then, the tubes were mixed using the tube mixer (Vornado™ Vortex Mixer, 115V, Benchmark Scientific, Edison, USA) for five seconds and the tubes were placed on ice for 30 minutes of incubation. After incubation, standard series were produced using diluted standards and standard diluent according to Figure 8. The pipette tips were changed between each dilution.

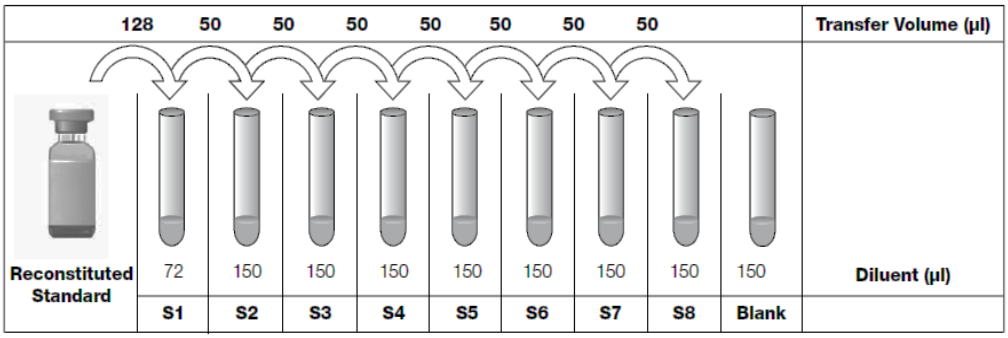


Figure 8: Preparation of standard series
According to the manufacturer's instructions (Bio-Rad).

The frozen serum samples were kept on ice and thawed slowly. After thawing of the samples, they were centrifuged and diluted to a 1:4 dilution with the sample diluent (Bio-Plex sample diluent). Then, they were pipetted on to a 96-well plate according to the plate plan. The solution of coupled beads was vortexed for 30 seconds using a tube mixer (Vornado™ Vortex Mixer, 115V, Benchmark Scientific, Edinson, USA). A volume of 575 µL coupled magnetic beads (Bio-Plex, coupled magnetic beads (10x) Group I 27-Plex) were diluted with the assay buffer in a separate tube.

Next, the actual test phase started whereby diluted beads were shaken for 20 seconds and 50 µL of the diluted beads were added to each well. Then, the plates were washed two times using BioPlex 200 Washing station (Bio-Plex Pro™ Wash Station), where 100 µL of the wash buffer were used for each washing step.

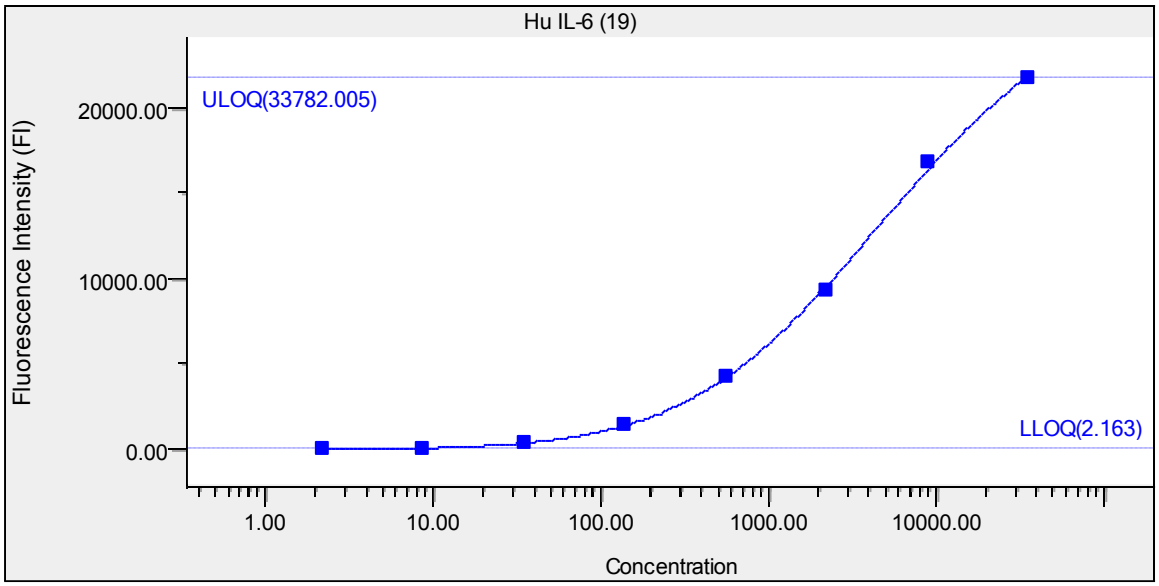
A volume of 50 µL of the serum samples, standards and blanks were added to each well on a 96-well plate (Bio-Plex 96-well microwell plate) according to the plate plan. The plates were covered with sealing tape to protect from light and were incubated on the shaker (IKA® MTS 2/4 multi-well plate shaker, Sigma-Aldrich Chemistries GmbH, Taufkirchen) for 30 minutes at 850 rpm. After incubation, the 96-well plate was washed three times with a 100 µL wash buffer for each well and washing step.

Then, the detection antibodies (Bio-Plex detection antibodies (10x) Group I 27-Plex) were prepared as follows. A volume of 300 µL of detection antibodies was diluted with 2700 µL detection antibody diluent, and 25 µL of antibody solution were added to each well. The plate was covered with an aluminum sealing tape, protected from light, and incubated on the shaker (IKA® MTS 2/4 multi-well plate shaker, Sigma-Aldrich Chemistries GmbH, Taufkirchen) for 30 minutes at 850 rpm. After incubation, the 96-well plate was washed three times with 100 µL wash buffer for each well and washing step.

The next step involves the preparation of the 100x concentrated Streptavidin Phycoerytin solution (Bio-Plex- Streptavidin-PE (100x)). A shortly shaken volume of 60 µL of the Streptavidin Phycoerytin was diluted with a 5940 µL assay buffer and transferred to a tube, protecting it from light in the process. A volume of 50 µL from the vortexed Streptavidin Phycoerytin dilution was added to each well. After that, the plate was covered with an aluminum sealing tape, and was incubated on the shaker (IKA® MTS 2/4 multi-well plate shaker, Sigma-Aldrich Chemistries GmbH, Taufkirchen) for ten minutes at 850 rpm at room temperature. After incubation, the 96-well plate was washed three times with 100 µL wash buffer for each well and washing step.

The last step before analysis involved a re-suspension of the wells with 125 µL assay buffer, which were covered with an aluminum sealing tape to protect from light, and shaken at 850 rpm for 30 seconds. The sealing tape was removed, and the plate was analyzed using the Bio-Plex 200 multiplex system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at Standard PMT Settings, CAL2 Low RPI Target.

The analysis of the measured cytokine concentration was performed using the Bio-Plex Manager™ Software (Bio-Rad). At each timepoint and for each patient, the value for each cytokine was collected. The median of the observed concentration (pg/mL) of each duplicate sample was calculated based on the intensity of fluorescence of the detected molecules using the standard curves of all cytokines (Figure 9). Outliers and out of range values were marked with an asterisk.



■ Standard ☐ Partial Outlier ☒ Outlier
Regression Type: Logistic - 5PL
Std. Curve: FI = 5.4135 + (28441.2 - 5.4135) / ((1 + (Conc / 1831.56)^-0.610806))^1.70132
FitProb. = 0.6392, ResVar. = 0.5633

Figure 9: Standard curve of IL-6

Exemplarily shown standard curve for IL-6, which is represented by the median concentration in pg/mL on the X-axis and the intensity of fluorescence (FI) on the Y-axis. The curve of IL-6 shows no outliers. The Bio-Plex manager™ Software from the manufacturer Bio-Rad was used.

4.6. Statistical analysis

Median values and the interquartile range (IQR) were calculated for CRP (mg/mL) and cytokines (pg/mL). The values for CRP and cytokines in the VO group and control group, respectively, were compared at the pre-operation (pre-OP) timepoint (day -20 to 0) and at the post-operation (post-OP) timepoints (days 3-5, 6-11, 40-56, 63-142) using box and whisker plots. To ensure a heterogeneity regarding age, body mass index (BMI), and sex, between the VO group and the control group, a two-sample Wilcoxon test was performed. The significance of the effects was identified by using p-values. P-values lower than 0.05 were considered statistically significant. Receiver operating characteristic (ROC) curves were plotted to investigate the predictive potential of CRP and the cytokines for diagnosis of VO. The ROC curve shows a relationship between the clinical sensitivity and the clinical specificity. Sensitivity is defined as the probability of a model to correctly predict a VO case (positive predictive values), whereas specificity is defined as the probability of a model to correctly predict the control group (negative predictive values). Larger areas below the ROC curves (AUC) signify higher specificity and sensitivity of the model and, therefore, also an increasingly accurate

differentiation between cases and non-cases. Also, the Youden's Index and their 95% confidence intervals were calculated showing the diagnostic potential. Using the ROC curve analysis and Youden's Index, cut-off values were identified to be able to statistically separate the two conditions of the two study groups by their concentration levels. A statistical power analysis for CRP and IL-6, as a representative of the candidate cytokines, was made, and it showed sufficient statistical power with even clearer results for IL-6 compared to CRP. Furthermore, an odds ratio above 1.2 and a sufficient number of patients (above 20) guarantees sufficient statistical relevance. To present the data with regard to bacterial characteristics, the difference between high-virulent VO group, low-virulent VO group, and control group was assessed by using a two-sample Wilcoxon test. All data processing and statistical analyses were performed using Microsoft Excel 2013 and SAS/STAT software 9.4 (SAS Institute Inc.: SAS/STAT User's Guide, Cary NC: SAS Institute Inc., 2014).

5. Results

5.1. Study participants

For this study, a group of patients with VO was recruited to determine the potential of cytokine profiling for diagnosing VO. This group was compared to a control group with *osteochondrosis intervertebralis*. Over a period of 22 months from October 2015 to July 2017, 98 patients were diagnosed with VO and underwent spondylodesis. However, 82 patients did not meet the inclusion criteria and were thus excluded from the CyProSpon study. Three patients were excluded from the VO group due to missing tissue samples, two patients were excluded due to late diagnosis of MODIC type 1, and one was excluded due to a withdrawal of consent. In addition, three patients were excluded from the control group. At this time, the study participants included 16 patients in the VO group and 20 patients in the control group with *osteochondrosis intervertebralis*. Serum samples of the remaining 36 participants were used for cytokine and CRP measurement.

At the timepoint of inclusion (n=36 patients), the median age of the patients was 71 (IQR: 61-76) years in the VO group and 65 (IQR: 59-73) years in the control group. The median BMI in the VO group was 29 (IQR: 24-31) kg/m^2. In the control group, this value was 29 (IQR: 27-32) kg/m^2. In the VO group, ten males and six females were included. In the control group, nine males and 11 females were included. Consequently, there was no significant difference in sex, age, and BMI of the VO group compared to the characteristics of the control group. Comorbidities, nicotine use, and alcohol consumption were equally common in both study groups.

5.2. CRP profile

In the pre-operative phase (-20-0d pre-OP), the median CRP concentration in the VO group was significantly (p=0.0255) higher than that in the control group (40.70 mg/L; IQR: 14.75-95.30 vs. 3.55 mg/L; IQR: 0.00-6.10 mg/L). In the early post-operative phase, the median CRP concentration increased in both groups (3-5d post-OP). This value is noticeably higher in the control group with a 34.65-fold increase from the pre-operative value (3.55 mg/L vs. 123.00 mg/L). In the VO group, the CRP levels increased 2.5-fold compared to the pre-operative timepoint (40.70 mg/L vs. 101.20 mg/L). At this timepoint, the concentration was also at its peak in both groups. A significant difference of values in the groups presented at 6-11d post-OP. At this point, CRP levels were 2.5-fold higher in the VO group than those in the control group. During the follow-up period, the CRP values decreased further and reach the baseline level in the control group. There is a 1.4-fold higher level in the VO group compared to controls at the last timepoint (6.20 mg/L; IQR: 0.00-13.35 vs. 4.30 mg/L; IQR: 0.00-5.80 mg/L). The CRP value for each group and timepoint is shown in Figure 10.

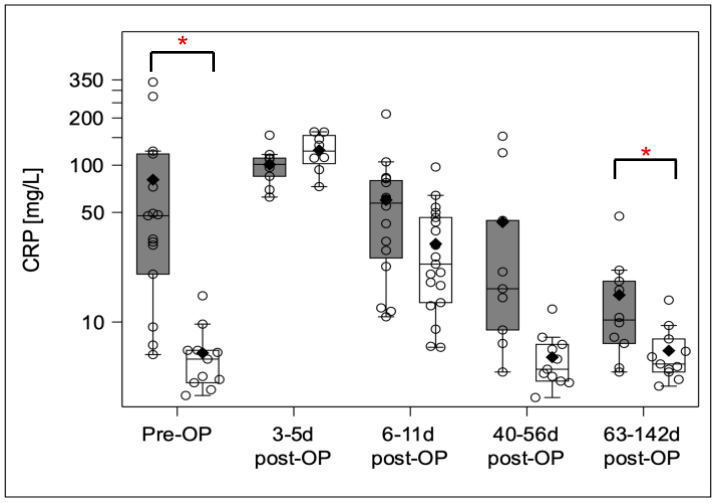


Figure 10: CRP plasma levels for VO patients (grey bars) and control patients (white bars)

Box-and-whiskers plot of CRP concentrations in mg/L (Y-axis) for all timepoints (X-axis). The absolute data points (open circles), minimum (endpoint of lower whisker), maximum (endpoint of upper whisker), 75th percentile (upper end of the box), 50th percentile (line in the box), 25th percentile (lower end of the box), and mean value (♦) are shown in the figure. Significant differences ($p < 0.05$) between groups are marked (*).

Figure from Brinkmann et al. [166].

5.3. Cytokine profiles

In total, 27 cytokines were measured in each serum sample. The cytokines included in the assay were IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, FGF basic, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-bb, RANTES, TNF-α, and VEGF. However, IL-2, IL-10, IL-15, and GM-CSF showed a high number of out-of-range and invalid values across all measured timepoints. Therefore, these cytokines were excluded from the statistical analysis. The remaining 23 cytokines were quantified and integrated into our statistical analysis. Out of these 23 remaining cytokines, ROC curves were drawn for the pre-operative timepoint and overall timepoints. The analyses of cytokine profiles were performed by using the median values of the cytokine level and the standard error of the median, comparing different timepoints and groups. Out of the total of 23 cytokines, five cytokines, IL-6, IL-8, IL-12(p70), MIP-1ß and VEGF showed significant differences for the pre-operative timepoint compared to the cytokine values of the control group (significance level $p < 0.05$). Furthermore, significantly higher values in the VO group were recorded at timepoint 6-11d post-OP for cytokines IL-8, IL12 (p70), FGF basic, and IL-17. Significantly higher IL-8 and FGF basic concentrations were also observed for the 40-56d post-OP timepoint. The last timepoint 63-142d post-OP showed no significant difference between

the two groups. In the following section, the cytokine profiles of the five cytokines with significant differences for the pre-operative timepoint are presented in detail (5.3.1.-5.3.5.).

5.3.1. Profile of IL-6

The median values (pg/mL) of IL-6 showed a significant (p=0.0429) 8.0-fold higher concentration in the VO group compared to the control group (25.16 pg/mL; IQR: 13.74-38.74 vs. 3.13 pg/mL; IQR: 2.81-5.40 pg/mL) in the pre-operative phase (Figure 11). However, the timepoint 63-142d was excluded for IL-6 due to values being under the lower level of quantification (LLOQ=2.2 pg/mL). Therefore, this timepoint is not shown in Figure 11.

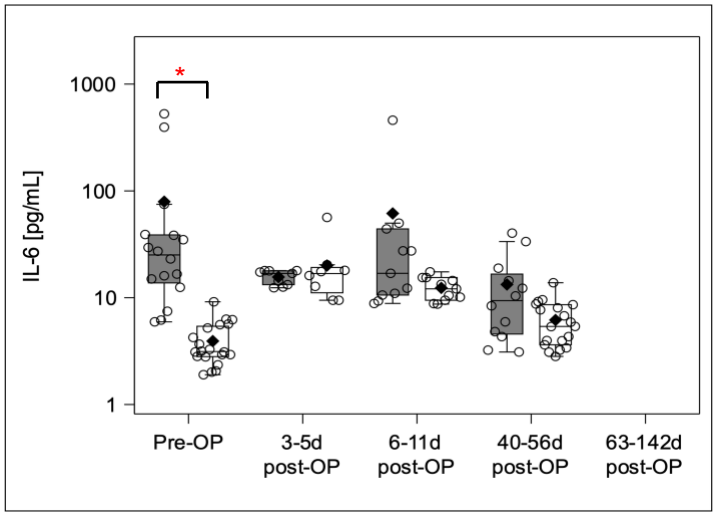


Figure 11: IL-6 serum levels for VO patients (grey bars) and control patients (white bars)
Box-and-whiskers plot of IL-6 concentrations in pg/mL (Y-axis) for the timepoints (X-axis). The absolute data points (open circles), minimum (endpoint of lower whisker), maximum (endpoint of upper whisker), 75th percentile (upper end of the box), 50th percentile (line in the box), 25th percentile (lower end of the box), and mean value (♦) are shown in the figure. Significant differences ($p < 0.05$) between groups are marked (*).
Figure from Brinkmann et al. [166].

5.3.2. Profile of IL-8

Values of the cytokine IL-8 showed a significant (p=0.0087) 1.8-fold higher level in the VO group at the pre-operative timepoint (-20-0d pre-OP) compared to the control group (17.49 pg/mL; IQR: 14.05-22.48 vs. 9.69 pg/mL; IQR: 7.70-10.99 pg/mL). At the timepoint 6-11d post-OP, a higher median value in the VO group was observed (22.04 pg/mL; IQR: 16.75-29.02) vs. 16.23 pg/mL; IQR: 13.72-19.21 pg/mL). In addition, the timepoint 40-56d post-OP also presented a significantly higher median value for the VO group (20.78 pg/mL; IQR: 18.19-

22.23 pg/mL vs. 13.83; IQR: 10.50-17.16 pg/mL). Both groups presented an increase in cytokine levels in the post-operative period (3-5d post-OP and 6-11d post-OP) (Figure 12).

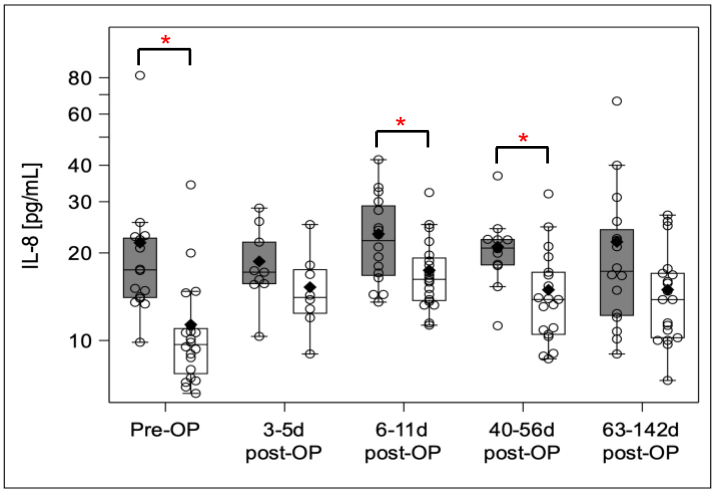


Figure 12: IL-8 serum levels for VO patients (grey bars) and control patients (white bars)
Box-and-whiskers plot of IL-8 concentrations in pg/mL (Y-axis) for all timepoints (X-axis). The absolute data points (open circles), minimum (endpoint of lower whisker), maximum (endpoint of upper whisker), 75th percentile (upper end of the box), 50th percentile (line in the box), 25th percentile (lower end of the box), and mean value (♦) are shown in the figure. Significant differences ($p < 0.05$) between groups are marked (*).
Figure from Brinkmann et al. [166].

5.3.3. Profile of IL-12 (p70)

The values of the cytokine IL-12 (p70) were at a 2.7-fold higher level (p=0.0137) in the VO group compared to the control group at the pre-operative timepoint (43.82 pg/mL; IQR: 30.43-64.59 pg/mL vs. 16.19 pg/mL; IQR: 12.10-19.62 pg/mL). A significant (p=0.0360) 1.7-fold elevation was shown at the timepoint 6-11d post-OP (69.07 pg/mL; IQR: 42.63-94.48 pg/mL vs. 38.79 pg/mL; IQR: 29.57-57.02 pg/mL). In both groups, an increase in the cytokine levels was shown in the post-operative period, with a peak at 6-11d post-OP. A decrease until the end of follow-up was observed but the concentration was higher than the baseline cytokine level, respectively, compared to pre-operative level (Figure 13).

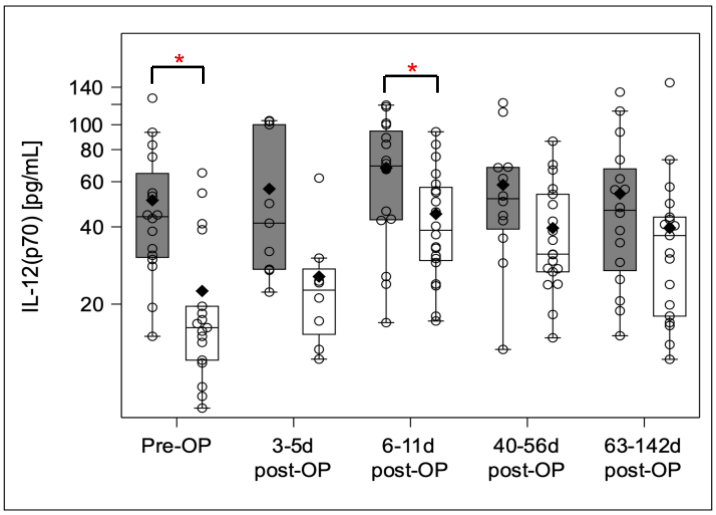


Figure 13: IL-12 (p70) serum levels for VO patients (grey bars) and control patients (white bars)

Box-and-whiskers plot of IL-12 concentrations in pg/mL (Y-axis) for all timepoints (X-axis). The absolute data points (open circles), minimum (endpoint of lower whisker), maximum (endpoint of upper whisker), 75th percentile (upper end of the box), 50th percentile (line in the box), 25th percentile (lower end of the box), and mean value (♦) are shown in the figure. Significant differences ($p < 0.05$) between groups are marked (*).

Figure from Brinkmann et al. [166].

5.3.4. Profile of MIP-1ß

There is a significant (p=0.0284) 1.2-fold difference between the median values of the cytokine MIP-1ß between the VO group and the control group at the pre-operative timepoint (156.73 pg/mL; IQR: 128.10-230.91 pg/mL vs. 131.12 pg/mL; IQR: 103.66-170.05 pg/mL). In both groups, an increase in cytokine concentration was noticed after surgery, with a peak in both groups at the 3-5d post-operative timepoint. The median values of the cytokine MIP-1ß showed no significant difference or increase at any other timepoint (Figure 14).

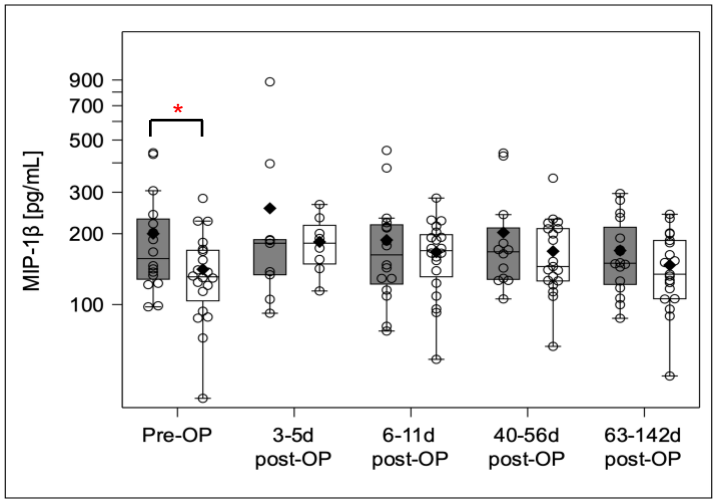


Figure 14: MIP-1ß serum levels for VO patients (grey bars) and control patients (white bars)

Box-and-whiskers plot of MIP-1ß concentrations in pg/mL (Y-axis) for all timepoints (X-axis). The absolute data points (open circles), minimum (endpoint of lower whisker), maximum (endpoint of upper whisker), 75th percentile (upper end of the box), 50th percentile (line in the box), 25th percentile (lower end of the box), and mean value (♦) are shown in the figure. Significant differences ($p < 0.05$) between groups are marked (*).

Figure from Brinkmann et al. [166].

5.3.5. Profile of VEGF

The median values of VEGF show a significant (p=0.0053) 3.1-fold difference between the VO and the control groups at the pre-operative timepoint (99.25 pg/mL; IQR: 46.62-164.41 pg/mL vs. 32.41 pg/mL; IQR: 24.49-48.52 pg/mL). The cytokine level increases up to a peak at the 6-11d post-operative timepoint. There was a 1.4-fold elevation in the VO group (140.15 vs. 99.25 pg/mL) and 2.5-fold elevation in the control group compared to the pre-operative values (82.01 vs. 32.41 pg/mL) (Figure 15).

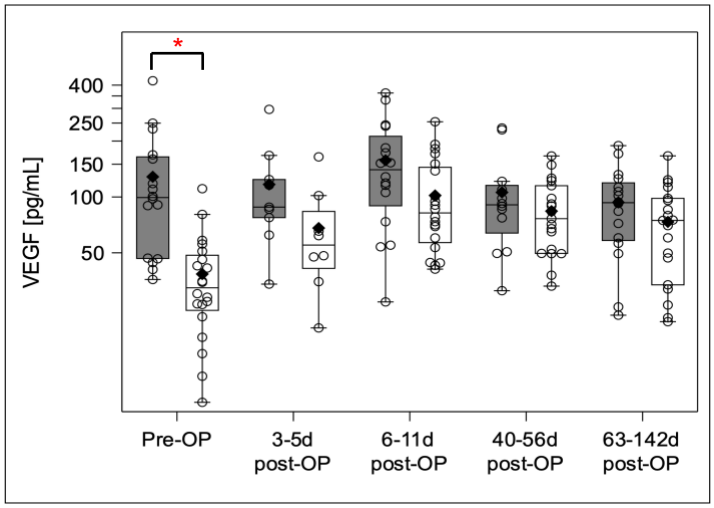


Figure 15: VEGF serum levels for VO patients (grey bars) and control patients (white bars)

Box-and-whiskers plot of VEGF concentrations in pg/mL (Y-axis) for all timepoints (X-axis). The absolute data points (open circles), minimum (endpoint of lower whisker), maximum (endpoint of upper whisker), 75th percentile (upper end of the box), 50th percentile (line in the box), 25th percentile (lower end of the box), and mean value (♦) are shown in the figure. Significant differences ($p < 0.05$) between groups are marked (*).

Figure from Brinkmann et al. [166].

5.4. ROC curves and diagnostic potential for VO

In Figure 16, the ROC curve and the area under the curve (AUC) are shown for CRP and the four cytokines at the pre-operative timepoint (-20-0d pre-OP). The AUC classifies the accuracy of a diagnostic test with 0.91-1.00 = excellent; 0.81-0.90 = good; 0.71-0.80 = fair; 0.61-0.70 = poor; and 0.51-0.60 = fail. CRP, IL-6, IL-8, IL-12 (p70), and VEGF show a large area below the curve in the ROC curves. The AUC for the CRP ROC curve is 0.9219 (95% CI: 0.812-1.000). For IL-6, the AUC is 0.9781 (95% CI: 0.942-1.000); for IL-8, the AUC is 0.8500 (95% CI: 0.713-0.987); for IL-12 (p70), the AUC is 0.8493 (95% CI: 0.718-0.981) and for VEGF, the AUC is 0.8750 (95% CI: 0.751-1.000). To summarize, IL-8, IL-12 (p70) and VEGF seem to be good markers for VO. CRP and IL-6 are excellent markers for detecting VO, with a slightly higher accuracy in the values of IL-6 (AUC: 0.9219 vs. 0.9781). Notably, the combination of all four cytokines scored the highest value (AUC: 0.9926).

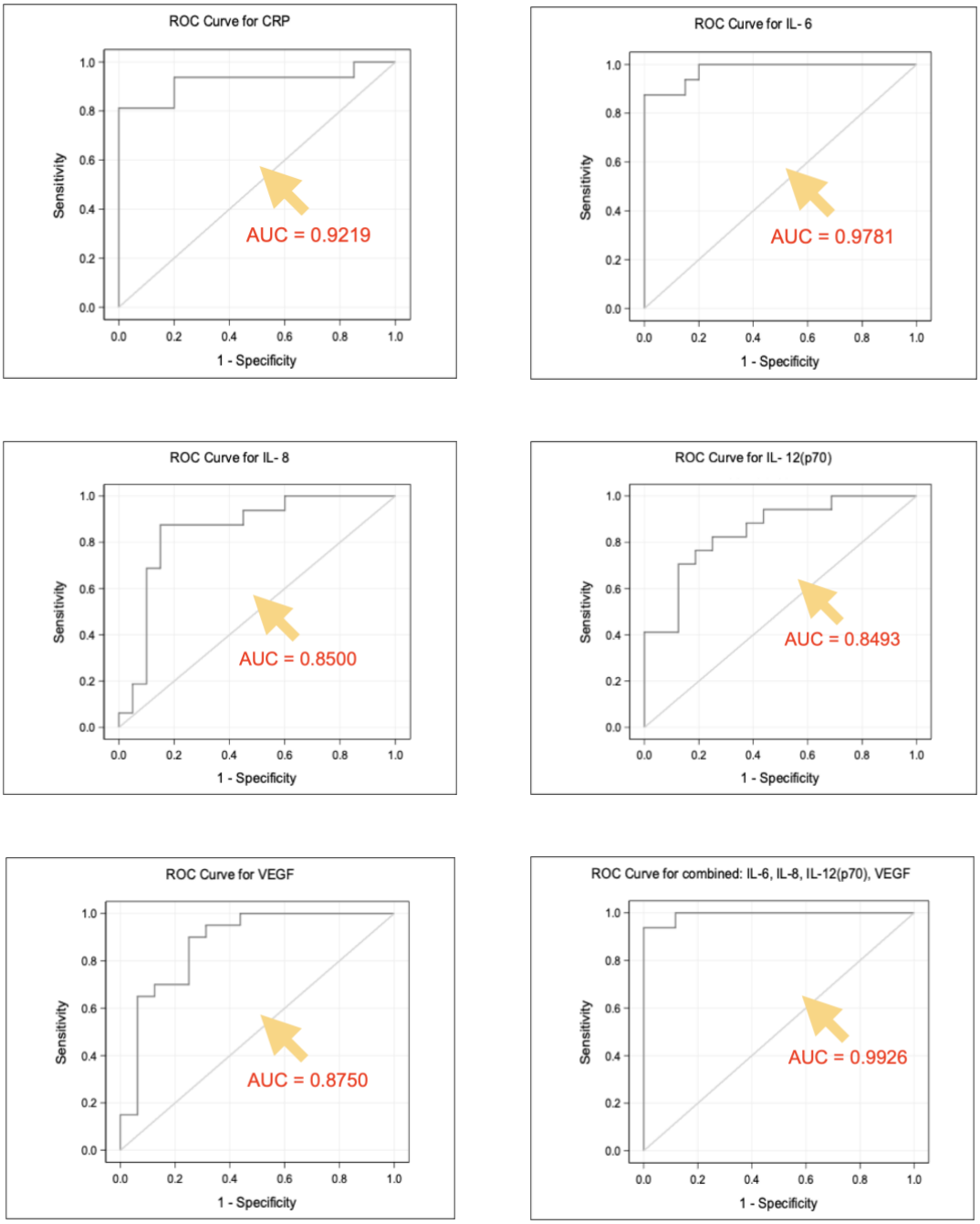


Figure 16: ROC curves and AUCs for CRP, IL-6, IL-8, IL-12 (p70), VEGF, and all candidate cytokines combined at pre-OP timepoint (-20-0d pre-OP)

AUC is the area under the curve, which was plotted on sensitivity (Y-axis) against 1 - specificity (X-axis).

Figures adapted from Brinkmann et al. [166].

The diagnostic potential of MIP-1ß was not sufficient (AUC: 0.7594), data not shown. The AUCs for the other cytokines are shown in Table 6.

Table 6: Median cytokine concentrations (pg/ml) with interquartile range (IQR), the area under the curve (AUCs), and 95% confidence interval (CI) at the pre-OP timepoint (-20-0d pre-OP)

		IL-1ß	IL-1ra	IL-4	IL-5	IL-7	IL-9	IL-13	IL-17	Eotaxin	FGF basic	G-CSF	IFN-γ	IP-10	MCP-1	MIP-1α	MIP-1ß	PDGF-bb	RANTES	TNF-α
VO	Median	2.0	32.8	3.5	6.1	7.1	86.5	2.6	33.2	63.0	41.2	18.8	27.6	546.9	146.2	4.0	156.7	1.203	10.191	33.1
	IQR*	1.6 2.8	24.8 85.1	3.0 4.1	3.8 9.0	3.6 9.7	68.7 112.9	1.9 3.8	19.3 60.1	43.9 104.3	31.3 65.7	13.8 43.6	23.5 39.7	262.7 1.139	87.0 205.9	3.7 5.2	128.1 230.9	818.3 1.437	9.077 10.894	27.0 41.6
Control	Median	1.5	19.6	3.3	6.5	3.7	75.9	3.1	28.9	58.2	33.0	13.0	19.9	446.0	102.8	3.4	131.1	891.9	10.133	27.5
	IQR*	1.2 1.8	13.3 28.8	3.1 3.5	5.4 13.7	3.5 7.2	70.1 87.4	2.1 4.7	20.2 43.2	39.4 71.9	28.5 44.9	11.2 18.5	16.5 27.2	284.2 523.8	73.5 137.8	3.0 5.0	103.7 170.1	631.7 1.286	9.884 10.901	23.1 32.3
	P-value	0.238	0.152	0.262	0.928	0.466	0.096	0.771	0.443	0.349	0.160	0.163	0.450	0.122	0.058	0.589	0.028	0.110	0.653	0.272
	AUC	0.746	0.750	0.734	0.703	0.700	0.728	0.740	0.659	0.694	0.706	0.763	0.719	0.684	0.747	0.656	0.759	0.722	0.817	0.725
	CI 95% LL	0.571	0.574	0.563	0.447	0.480	0.557	0.502	0.468	0.503	0.519	0.593	0.535	0.493	0.562	0.461	0.586	0.549	0.629	0.542
	CI 95% UL	0.921	0.927	0.905	0.955	0.921	0.899	0.977	0.851	0.884	0.893	0.932	0.902	0.876	0.931	0.851	0.933	0.895	1.000	0.908

VO: vertebral osteomyelitis, *upper value: quartile 1, lower value: quartile 3, LL: lower limit, UL: upper limit.

Table from Brinkmann et al. [166].

Additionally, values for a better understanding of the diagnostic potential for VO are presented in Table 7. The sensitivity, specificity, positive predictive value, negative predictive value for CRP and the four cytokines was as follows; sensitivity (CRP 0.88, IL-6 1.00, IL-8 0.94, IL-12 (p70) 0.94, VEGF 0.69), specificity (CRP 0.90, IL-6 0.85, IL-8 0.80, IL-12 (p70) 0.71, VEGF 0.95), positive predictive value (CRP 0.88, IL-6 0.84, IL-8 0.79, IL-12 (p70) 0.75, VEGF 0.92), and negative predictive value (CRP 0.90, IL-6 1.00, IL-8 0.94, IL-12 (p70) 0.92, VEGF 0.79). A ROC curve analysis and Youden's index were used to define the optimal cut-off values of CRP and cytokines (CRP 7.1 mg/L, IL-6 5.9 pg/mL, IL-8 13.3 pg/mL, IL-12 (p70) 19.4 pg/mL, VEGF 89.7 pg/mL). The number of detected VO cases is also shown in Table 7. For CRP, IL-6, IL-8, IL-12 (p70), and VEGF there were 14 cases, all 16 cases, 15 cases, 15 cases, and 11 cases detected, respectively.

Table 7: The diagnostic potential of CRP and selected cytokines for differentiating VO from *osteochondrosis intervertebralis*

Diagnostic marker	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Youden's index	Cut-Off value [mg/L] for CRP, [pg/mL] for cytokines	No. of VO patients detected (m/f)
CRP	0.88	0.90	0.88	0.90	0.78	7.1	14 (9m/5f)
IL-6	1.00	0.85	0.84	1.00	0.85	5.9	16 (10m/6f)
IL-8	0.94	0.80	0.79	0.94	0.74	13.3	15 (10m/5f)
IL-12 (p70)	0.94	0.71	0.75	0.92	0.64	19.4	15 (10m/5f)
VEGF	0.69	0.95	0.92	0.79	0.64	89.7	11 (7m/4f)

The diagnostic potential of CRP, IL-6, IL-8, IL-12 (p70), and VEGF represented by the sensitivity, specificity, positive predictive value, negative predictive value, the Youden's index, Cut-off values, and number of VO patients detected.
Table from Brinkmann et al. [166].

5.5. Differences in cytokine and CRP concentrations between high-virulent VO group, low-virulent VO group, and control group

According to their infectious bacterial agents, the VO group was divided into a high-virulent group (n=10) with the species *S. aureus* (MRSA), *Streptococcus dysgalactiae*, *E. coli,* and *S. lugdunensis* and a low-virulent group (n=6) with the species *S. epidermidis*, *Parvimonas micra*, and *P. acnes*. The distribution of pathogens and their virulence are shown in Figure 17.

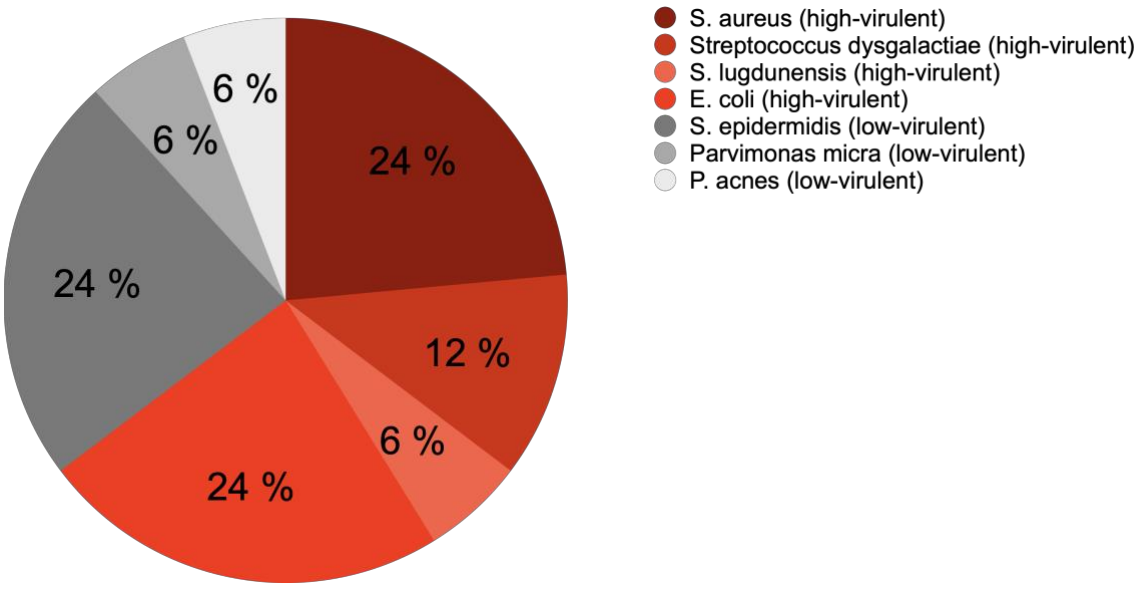


Figure 17: Distribution of pathogens in the VO group and division by virulence (high- or low-virulent)

S.: Staphylococcus, *E. coli*: *Escherichia coli, P. acnes*: *Propionibacterium acnes*

As shown in Table 8, comparisons were made between 1) high- vs. low-virulent, 2) high-virulent vs. control group, and 3) low-virulent vs. control group. These comparisons were only made for the timepoint before operation. Significantly higher median values were found in the high-virulent VO group than in the control group. In detail, the results were CRP (31.65 mg/l; IQR: 7.13-72.60 mg/L vs. 3.55 mg/L; IQR: 0.00-6.10 mg/L; p=0.0390), IL-6 (19.03 pg/mL; IQR: 7.46-39.07 vs. 3.13 pg/mL; IQR: 2.81-5.40; p=0.0496), IL-8 (21.23 pg/mL; IQR: 15.10-22.75 pg/mL vs. 9.69 pg/mL; IQR: 7.70-10.99 pg/mL; p=0.0310), IL-12 (p70) (44.53 pg/mL; IQR: 31.00-54.27 pg/mL vs. 16.19 pg/mL; IQR: 12.10-19.62 pg/mL; p=0.0286), and VEGF (94.19 pg/mL; IQR: 44.30-160.12 pg/mL vs. 32.41 pg/mL; IQR: 24.49-48.52 pg/mL; p=0.0388). Compared to the control group, the cytokine concentration in the high-virulent VO group showed a 6.1-fold IL-6 elevation, a 2.1-fold IL-8 elevation, a 2.8-fold IL-12 (p70) elevation and a 2.9-fold VEGF elevation. Conversely, there were no significant differences between the high- and the low-virulent VO group as well as for the comparison between the low-virulent VO group and the control group.

Table 8: Concentrations of significant diagnostic markers for high-virulent VO group, low-virulent VO group and control group at pre-OP timepoint (-20-0d pre-OP)

			Significant diagnostic markers				
Group	**Virulence**		**IL-6**	**IL-8**	**IL-12 (p70)**	**VEGF**	**CRP**
VO	**High**	Mean ± SEM	115.0 ± 66.4[a]	18.5 ± 1.6[a]	42.6 ± 6.8[a]	95.4 ± 22.0[a]	87.7 ± 42.4[a]
		Median	19.0	21.2	44.5	94.2	31.7
		IQR	7.5-39.1	15.1-22.8	31.0-54.3	44.3-160.1	7.1-72.6
	Low	Mean ± SEM	34.4 ± 10.8[a,b]	14.7 ± 0.7[a,b]	45.5 ± 12.3[a,b]	131.1 ± 35.6 [a,b]	68.9 ± 21.6
		Median	32.2	14.5	38.0	109.4	48.5
		IQR	16.6-38.4	14.0-17.4	29.9-74.9	89.8-168.7	33.8-118.0
Control	**n.a.**	Mean ± SEM	3.9 ± 0.4[b]	11.3 ± 1.4[b]	22.5 ± 4.1[b]	38.4 ± 5.5[b]	3.5 ± 0.9[b]
		Median	3.1	9.7	16.2	32.4	3.6
		IQR	2.8-5.4	7.7-11.0	12.1-19.6	24.5-48.5	0.0-6.1

Values with different superscripts within the same column are statistically different ($p < 0.05$).

VO: vertebral osteomyelitis, n.a.: not applicable, SEM: standard error of mean, IQR: interquartile range.

Table from Brinkmann et al. [166].

In addition, the cut-off values for CRP (7.1 mg/L) and cytokines (IL-6 5.9 pg/mL, IL-8 13.3 pg/mL, IL-12 (p70) 19.4 pg/mL, VEGF 89.7 pg/mL) were used to identify the number of detected cases for each group and diagnostic marker as shown in Table 9. In the high-virulent VO group, 8 cases, 10 cases, 9 cases, 9 cases, and 6 cases were detected for CRP, IL-6, IL-8, IL-12 (p70), and VEGF, respectively. In the low-virulent VO group, mostly all cases were detected, except for one patient of this group, which had a VEGF concentration below the cut-off value. There was a low number of detected cases in the control group, namely 2 cases, 3 cases, 4 cases, 5 cases, and 1 case were detected for CRP, IL-6, IL-8, IL-12 (p70), and VEGF, respectively.

Table 9: Number of detected cases for each diagnostic marker and each group (high-virulent VO group. low-virulent VO group, and control group)

Diagnostic marker	Cut-Off value [mg/L] for CRP, [pg/mL] for cytokines	No. of patients detected in high-virulent VO group	No. of patients detected in low-virulent VO group	No. of patients detected in control group
CRP	≥ 7.1	8 /10	6 /6	2 /20
IL-6	≥ 5.9	10 /10	6 /6	3 /20
IL-8	≥ 13.3	9 /10	6 /6	4 /20
IL-12 (p70)	≥ 19.4	9 /10	6 /6	5 /20
VEGF	≥ 89.7	6 /10	5 /6	1 /20

6. Discussion

6.1. Epidemiological data of the study population

In this study, the goal was to determine whether cytokine levels can discriminate between VO and degenerative diseases of the spine. Therefore, the inclusion criteria for this study was the indication for an operation of the spine due to either the diagnosis of VO or *osteochondrosis intervertebralis*. The patients with *osteochondrosis intervertebralis* served as an ideal control group because of their spinal condition with a non-infectious spinal presentation treated with the same surgical approach as the VO patients. A comparable situation between both study groups needed to be guaranteed by the following inclusion criteria to reduce bias between groups, which were age between 40 and 85 years and both genders. At the timepoint of inclusion, a similar mean age could be calculated for both groups. In fact, the ages were 69 years in the VO group versus 66 years in the control group. In the VO group, 37.5% were women and 62.5% were men. The control group included a higher percentage of women - 55% women and 45% men. Comparing our VO group to other VO study groups, this distribution was slightly deviant. In other VO study groups, a lower mean age of 64 years was described [2,4,6]. Also, comparatively more men were affected with 56-68% of men being included in the studies. The percentage of men in the current study lies approximately in the middle of the range [1,4,6,23,29,31].

In addition to these epidemiological criteria, the study groups were also similar in terms of other physical conditions of the patients such as the amount of comorbidities and unhealthy lifestyle habits, for example, high alcohol consumption, nicotine-abuse, and obesity. For instance, the mean BMI in the VO group of 28 kg/m^2 was similar to the mean BMI of 29 kg/m^2 in the control group. Comorbidities in the control group were distributed as follows; 20% of patients had diabetes mellitus, 75% had arterial hypertension, and 15% had hypothyroidism. This was very similar to the comorbidities in the VO group, which included 19% of patients with diabetes mellitus, 75% of patients with arterial hypertension, and 19% of patients with hypothyroidism. In several other VO studies, diabetes mellitus was an even more common comorbidity with 24-35% of patients being affected [11-15].

Cytokine levels might be influenced by many factors such as age, gender, BMI, alcohol abuse, and diseases (e.g. diabetes mellitus type 2) [167-173]. In fact, the cytokine levels in this study were analyzed using regression models adjusted for sex, age and BMI. With regard to age, patients under 52 years and patients above 85 years were not included. Furthermore, different types of bacteria and therapeutical use of antibiotics may also have an influence on cytokine concentrations [174,175]. In the current study, only the VO group was tested for bacterial pathogens, and a wide variety of bacteria was verified. In the control group, possible bacterial pathogens remain unidentified. The control group received peri-operative antibiotic therapy

(Cefazolin, 2g), and no other antibiotic treatment was necessary in this group. The patients with VO received antibiotic treatment dependent on the microbiological findings and bacterial resistance. This treatment was discontinued for at least three days prior to surgery with the exception of three patients. Moreover, the time of day and the presence of severe pain can alter cytokine concentration [176,177]. However, the presence of pain was not documented in this study. The blood samples in this study were collected in the mornings.

The exclusion criteria for this study were specifically strict concerning the infectious point of view; all patients with present infectious foci other than from the spine were excluded. In addition, malignancies or autoimmune diseases were excluded. This was not done in other studies [12]. Moreover, an exclusion of the *osteochondrosis intervertebralis* MODIC type 1 took place in the process of the study in order to have a clear diagnosis of VO within the VO group and in order to have an entirely non-infectious presentation of the spine within the control group.

6.2. CRP

CRP belongs to the important pillars on which a diagnosis of a VO case rests and is, together with leucocyte count and ESR, the most frequently used parameter for laboratory examination [4,5,47,67,68]. CRP is generally increased in infectious conditions of the spine [4,6,18,31,178,179]. In the current study, the VO group presented with significantly higher median values of CRP (40.70 mg/L; IQR: 14.75-95.30). In contrast, the control group presented with low values (3.55 mg/L; IQR: 0.00-6.10 mg/L), which verifies the group as a non-infectious control group. Other VO studies observed even higher levels of CRP, which might be due to less accurate exclusion criteria. For instance, infections other than the spine were not excluded [4,18,178,180]. Our results show an accuracy of 92%, a sensitivity of 88%, and a specificity of 90% when using CRP for diagnosing VO. This contrasts with other VO studies, where the sensitivity, described with 84-100%, was higher than the specificity [18,47,179]. CRP often shows pathological values in acute cases, but in subacute or chronic cases with, for instance, low-virulent bacteria or previous antibiotic therapy, there might be none or only a slight alteration [3,178]. Interestingly, higher CRP levels shorten the time to reaching a definitive diagnosis [6].

Comparing the pre-operative timepoint and the first post-operative timepoint, it is clear that CRP changed in line with the spinal surgery. Especially the control group reacted with a 35-fold elevation, whereas the VO group only reacted with a 2.5-fold elevation. This timepoint (3-5d post-OP) represented the peak of CRP levels for both study groups. Due to this effect, a significant discrimination between VO and the control group is not possible for the following timepoints (3-5d post-OP and 6-11d post-OP). A study by Larsson et al. demonstrated that CRP is relatively directly affected by operations. The higher CRP levels typically vanish within three weeks after the operation [181]. In accordance with these findings, the effect of the

operation vanished between the timepoints 6-11d post-OP and 40-56d post-OP in our study groups.

In the follow-up period, CRP values mostly decreased. CRP values below 5 mg/L are considered normal. At the last timepoint of the study (63-142d post-OP), the CRP values of the control group reached the pre-operative levels, whereas in the VO group, the CRP values remained slightly positive above 5 mg/L (4.30 mg/L; IQR: 0.00-5.80 mg/L vs. 6.20 mg/L; IQR: 0.00-13.35). In either case, CRP is frequently used in clinical settings for monitoring therapy success and is explored by other studies, which state a beginning of CRP decrease already two weeks after the start of treatment for VO [179,182,183].

6.3. Cytokines

The findings of this study demonstrate the potential use of cytokine measurement to discriminate between a non-infectious degenerative spinal disease and VO. A wide-ranging test of 27 cytokines using a multiplex assay was done for both study groups at the pre-operative and post-operative timepoints. In the following, mainly results of the pre-operative timepoint are presented as it is the most interesting when aiming to answer the question whether cytokines can be used for the diagnosis of VO. Significantly higher values in the VO group were shown for the cytokines IL-6, IL-8, IL-12, MIP-1ß, and VEGF at the pre-operative timepoint. The most compelling evidence for diagnosing VO and significant values were shown for the candidates IL-6, IL-8, IL-12 and VEGF. These cytokines showed the highest significant accuracy when taking into account the logistic regression and the AUCs. Good markers for diagnosing VO according to the AUCs are IL-8, IL-12 and VEGF. IL-6 and CRP are classified as excellent markers for detecting VO, notably, with a slightly higher accuracy calculated for IL-6 (AUC: 0.9781 vs. 0.9219). Moreover, the combination of all four cytokines scored the highest value (AUC: 0.9926). The results from the Youden's index were in line with these findings. A combination of an ROC curve analysis and Youden's index were used to determine the ideal cut-off values of CRP and cytokines (CRP 7.1 mg/L, IL-6 5.9 pg/mL, IL-8 13.3 pg/mL, IL-12 19.4 pg/mL, VEGF 89.7 pg/mL). With the use of these cut-off values, the number of detected VO cases can be shown in our study. In total, for CRP, IL-6, IL-8, IL-12, and VEGF, the number of detected cases were 14 cases, 16 cases, 15 cases, 15 cases and 11 cases, respectively. Furthermore, to understand the clinical use of CRP and the four cytokines, other important values were presented in this study. IL-6 showed the highest value for sensitivity (1.00) and the highest negative predictive value (1.00). VEGF had the highest specificity (0.95) and positive predictive value (0.92).

Other than the present study, there are no available studies that I am aware of, which address the possibility to diagnose VO using a wide range of cytokines through a minimally-invasive manner by simply drawing blood. A new study by Loft et al. examined the immune

response of 49 infectious spondylodiscitis patients by measuring 8 cytokines. Some common cytokines measured in our study were tested, namely IL-6, IL-8 and IL-12. Loft et al. created study groups that were tested with three substances by either stimulating with lipopolysaccharide from *E. coli* or two other substances from viruses. Furthermore, an unstimulated control group was also included in the study. Thus, the current study is not entirely comparable to the aforementioned study design of Loft et al.. However, the unstimulated control group is comparable to our VO group, and interestingly, the concentration of IL-6 also showed a significantly higher concentration compared to healthy individuals at the timepoint before treatment [184]. Another study by Lang et al. noted an increased IL-8 concentration via biopsy at infectious intervertebral disc and adjacent vertebral bodies of VO patients [185]. It must be highlighted that our results were solely based on blood samples, and it remains unclear if cytokine concentrations at the site of the infected tissue are actually equally increased. For the sake of comparability, the results of our study were mainly compared to those of other studies that used peripheral blood tests and mostly neglect studies where biopsies or cavity fluids were evaluated for cytokine concentrations. Local and systemic cytokine concentrations may be different. The systemic responses to maximum local concentrations at the site of infection may be minimal or non-existent [186,187]. In our study, it was crucial to use systemic cytokine levels in order to achieve a quick and minimally-invasive method of diagnosis.

In recent years, the exploration of cytokines for clinical use is subject of an increasing number of studies. For many infectious diseases, cytokine levels may be a valuable diagnostic tool. Importantly, the cut-off values are not well established, but the use of ROC curve analysis, which was done in the current study, is the best way to provide diagnostic accuracy [138]. Cytokines can be used for diagnosis but also as a monitoring tool or as a target for therapy. Studies found that cytokines, especially IL-6, can act as a diagnostic marker in some infectious conditions such as neonatal infections or severe sepsis [188,189]. For the latter, cytokines can act as a good predictor for patient outcome and mortality [190-192]. For rheumatoid arthritis and psoriasis, TNF-alpha is used as a therapeutic target although it is discussed controversially [193,194]. Less well established is the cytokine use for other conditions such as endometriosis or for identifying complicated Covid-19 cases [195,196]. A study by Zarghooni et al. tested the cytokines of patients and found that IL-6, IL-1ra, IL-12, and MCP-1 are candidates for diagnosing patients with implant-associated bacterial infections [197]. With regard to IL-6, these findings are supported by Glehr et al., who tested for infections of knee and hip arthroplasties [198]. One other study found a high diagnostic value also for IL-6 when testing for post-surgery infections of the spine [199]. A review by van den Berg et al. reported that there is a lack of evidence for the use of pro-inflammatory cytokines to determine the severity of non-specific low back pain [200]. However, there are more studies testing local cytokine concentrations in

spinal biopsy samples rather than testing cytokine concentrations of peripheral blood samples. Several studies about different spinal diseases like degenerative disc disease or herniated discs have been exploring local concentrations [201-203]. The use of cytokines has been explored for all of these conditions, so our study contributes to this field by determining whether cytokines can be used as diagnostic markers for VO.

Of the four candidate cytokines, three had major similarities; IL-6, IL-8, and IL-12 are pro-inflammatory cytokines and are all produced by macrophages. All three cytokines intensely perform as immunomodulators. Notably, a study by Takada et al. found a correlation between macrophage concentration and IL-6 concentration in intervertebral disc material of rats [204]. IL-6, as a mediator in the acute-phase reaction, plays an important role in the fever reaction and regulates the CRP secretion in the liver [132,140,147,148]. In the current study, a correlation of CRP and IL-6 was seen as both concentrations decreased over the course of the study. Additionally, the functions of IL-6 include the induction of antibody-forming plasma cell maturation and haematopoiesis [132,139,140,142]. It is noteworthy that IL-6 regulates osteoblasts and provokes bone resorption [144-146]. IL-8 plays an essential role in angiogenesis [154-156]. It acts chemotactically and stimulates an upregulation of neutrophil granulocytes with the result of increased adhesion on the endothelium and extracellular matrix. At the site of tissue inflammation, IL-8 induces immune cell recruitment, release of myeloperoxidase, and free oxygen radicals formation [132,143,153]. Via an interaction with pathogens like bacteria, an increased secretion and activation of IL-12 occurs. The active form IL-12 (p70) reinforces the functioning of natural killer cells [132,157]. In the early immune response, activities attributed to IL-12 include secretion of other cytokines and the differentiation of naive T-cells to T helper cells. It contributes to an increased local immune response [132,157,158]. VEGF shares a similarity to IL-8 as VEGF also has a role of regulating angiogenesis and lymphangiogenesis [159-161]. In contrast to the other cytokines, the key stimulator of VEGF for secretion is hypoxic stress [160,162]. Under this circumstance, VEGF, which is secreted by many different cells, stimulates macrophages and monocytes [160,163]. To emphasize, the essential similarity of all of these cytokines may be the functional connection to macrophages. In short, all four cytokines seem to play a prominent role as a network locally, at the site of infection, which contributes directly to inflammation and regeneration.

The discussion above sets a focus on the measurements at the pre-operative timepoint in order to find benefits for a better and faster diagnosis of VO. Not only are these findings interesting but also the measurements of the other post-operative timepoints. These values also give valuable insights into the specificities of cytokines in relation to this disease. The results show that the concentrations of IL-6 and IL-12 decreased over the course of the follow-up. Therefore, monitoring these two cytokines can be relevant in understanding the course of the disease and the therapeutical success. The cytokines IL-8 and VEGF might also be

candidates for monitoring therapy success. However, for these two cytokines to become relevant, a longer follow-up period would have to be observed.

In this study, the control group consisted of patients who presented with *osteochondrosis intervertebralis*, which involves degenerative changes of the vertebral bodies and the intervertebral discs. Back pain, radicular pain, and herniated discs may be linked to this condition. Studies support the idea that the degenerated disc diseases include an involvement of a local invasion of macrophages, which leads to an increased secretion of cytokines, namely IFN-y, IL-1a, IL-1ß, IL-6, IL-17, and TNF-a [205-207]. The literature states - although partially contradicting - that an increased concentration of IL-1ß and TNF-a could be linked to these spinal conditions [206,208,209]. This, however, is not supported by the results of our group since no elevation of these cytokines was found in the control group. It must be noted that these studies only measured local cytokine concentrations in tissue biopsies and that the concentration in the blood stream remained unexplored.

6.4. Microbiological findings and levels of CRP and cytokines

Notably, due to the small cohort of 16 VO patients, the significance of the usage of percentage has to be made with caution. However, in the present study, the pathogen *S. aureus* was found in 24% of the patients (n=4), *S. epidermidis* in 24% of the patients (n=4), *E. coli* in 24% of the patients (n=4), and *Streptococcus dysgalactiae* in 12% of the patients (n=2). Each of the other pathogens (*Parvimonas micra*, *P. acnes*, and *S. lugdunensis*) was only found in one of the patients, which equals 6% (n=1) for each pathogen. This can be seen as an illustration to notice the large spectrum of pathogens throughout our VO study cohort and the fact that there is no leading causative pathogen. In contrast to these results, in many other studies, *S. aureus* was the most frequently isolated pathogen being responsible for all VO cases [8,12,14,17]. The distribution of the other infectious pathogens was slightly less common in other VO cohorts compared to our results reporting for example, *E. coli* in 9-12% of the patients and *S. epidermidis* in 3-14% of the patients [5,14,17,18].

Within our VO study group, 62,5% of patients (n=10) presented high-virulent pathogens, including the species *S. aureus* (MRSA), *Streptococcus dysgalactiae*, *E. coli,* and *S. lugdunensis*, and 37,5% of patients (n=6) presented low-virulent pathogens, including the species *S. epidermidis*, *Parvimonas micra*, and *P. acnes*. It is important to state that the control group was not tested by blood culture or biopsy so that possible infectious pathogens remained unidentified. There was only one study reporting low- and high-virulent VO pathogens in humans. Lopez et al. present a comparative study of spondylodiscitis patients caused by either *S. aureus* or *S. epidermidis*, but only a small cohort was included. Difficulties in the diagnostic process were reported for patients with low-virulent bacteria due to an indolent presentation of the patient. Moreover, uncertainty about the causative pathogen was reported because of

higher rates of sample contamination [51]. In general, low-virulent candidates can cause chronic, slow-growing infections of the spine with missing symptoms and low levels of the usual biomarkers for infection such as CRP, which makes early diagnosis difficult [51,56,57]. In the present study, the low-virulent patients showed slightly lower mean values in CRP than the mean values in the high-virulent group. For this reason, it could be useful for the clinical practice to examine other effective biomarkers like cytokines when there are low concentrations of infection markers and a low-virulent microbial status. However, for the candidate cytokines of this study, there were no significant differences between the high- and the low-virulent group.

The present study compared CRP and candidate cytokine concentrations between the high-virulent VO group, the low-virulent VO group, and the control group. The results showed significantly higher median values in the high-virulent VO group compared to those in the control group. Specifically, CRP and IL-6 showed the highest elevations with an 8.9-fold elevation for CRP and a 6.1-fold elevation for IL-6 in the high-virulent group. The comparison between the high- and the low-virulent VO group as well as between the low-virulent and the control group showed no significant differences. In general, the lowest values were counted in the control group, which confirms the non-infectious presentation of this group. When considering cut-off values and detected cases for each group, it is clear that the least detected cases were present in the control group. In fact, the most detected cases were present in the low-virulent group. Especially for IL-6, all cases were detected from the low- and high-virulent group, which shows the high clinical relevance for this diagnostic marker.

The results of the present study show that the discrimination between the high-virulent group and the control group is more accurate than the discrimination between the low-virulent group and the control group. It is reported that low-virulent bacteria can also play a role in various spinal conditions such as degenerative disc disease or disc herniations [52-55,112,114,117,210]. This might signal that the overlap of these conditions are fluent when considering the infection status. It shows that these seemingly non-infectious conditions can develop into spinal infections. In addition, antibiotic treatment showed a significant effect for these spinal diseases with mostly *P. acnes* being the causative pathogen [211]. In patients with MODIC type 1, low-virulent bacteria seem to occur [112,113,116]. MODIC changes are classified with the help of an MRI, and this classification categorizes *osteochondrosis intervertebralis* into type 1 - 3 according to the alterations in the vertebral end-plate and bone marrow. Type 1 changes show an inflammation and edema of the bone marrow. It also involves similar characteristics as the MRI findings in the beginning of a VO as well as elevated inflammatory biomarkers. These findings contrast with the characteristics of type 2 and type 3, which do not present inflammatory characteristics [122-124]. A study by Rannou et al. investigated CRP levels in the different MODIC type patients and found the highest values for MODIC type 1 patients [212]. The

osteochondrosis intervertebralis patients of the present study acted as the control group. Most of these patients were classified according to MODIC by an MRI and the results showed only MODIC type 2 and type 3 cases. However, not all control patients received a recent MRI, so some patients who had started to develop MODIC type 1 were not yet diagnosed. In addition to that, there were no blood cultures or biopsies taken of the control group so that possible hidden local infections with low-virulent pathogens were not discovered. However, the control group of the present study showed the lowest concentrations of the candidate biomarkers, which suggests that local infections might not have been present.

It would be a clinically meaningful benefit if clinicians could discriminate between high- and low-virulent causative pathogens for VO via a simple, minimally-invasive blood test. In summary, the discrimination of VO patients with high-virulent bacteria and patients with *osteochondrosis intervertebralis* is reliable, but cytokines can be of limited diagnostic value when patients with low-virulent bacteria are involved.

6.5. Clinical relevance, limitations of this study, and further research

Until now, this is the first study investigating the suitability of cytokine levels for diagnosing VO. The findings of the present study are clinically relevant because early diagnosis of VO is of practical importance in order to provide early treatment with antibiotics. Early treatment is important for a good patient outcome and for reducing medical costs. Provided that specific and sensitive biomarkers are non-existent and MRI-findings are often negative in early stages, a delay in diagnosis and treatment of VO often occurs [2,4,6,12,33,39,59]. The use of MRI is the gold standard for diagnosing VO and provides an accuracy of 94% [39,47,60,74,77,78]. In the current study, the measurement of all potential diagnostic markers (IL-6, IL-8, IL-12 and VEGF) together scored an accuracy of 99%. Given the existing findings, there is considerable potential in using circulating cytokine levels as a diagnostic method for VO. Many cytokines seem to be adequate markers for potentially indicating different infectious diseases [138]. Furthermore, it would be useful to know what bacterial species might be involved in the early stages of VO, and it is ideal to be able to measure this by solely using the minimally-invasive method of drawing blood from a potential patient. Cytokines might help to identify a more specific and prompt antibiotic treatment for a VO patient. However, our current findings provide no evidence that one can discriminate between low- and high-virulent pathogens via cytokine levels in VO patients.

Some weaknesses and limitations of this study should be mentioned. Firstly, with a total of 36 patients, the groups of this study are quite limited. However, there are many VO studies with even smaller cohorts [9,18,30,31,55,72,82,92,107,185]. The current study reviewed 98 VO patients as possible study participants and 82 patients did not meet the inclusion criteria. Thus, the exclusion and inclusion criteria were narrow in order to achieve the goals of the research

question. Nevertheless, the patient groups could be larger in order to receive more reliable results. Secondly, there were no blood cultures or biopsies taken from the control patients. As mentioned above, not only in inflammatory diseases of the spine but also in non-inflammatory diseases of the spine, studies reported that bacterial culture can be present [52-55,112,114,117,210]. Thirdly, in order to have a clearer discrimination between an infectious and a non-infectious group, all patients of the control group should have undergone an MRI, like the patients in the VO group did. This would have enabled us to completely exclude the more inflamed condition MODIC change type 1 within the control group. Fourthly, the follow-up times were 63-142 days, and the timeframe could have been extended further in order to receive more reliable information about the effect of treatment results, the course of the disease, and the risk for complications, and thus, the monitoring potential of cytokines. Finally, one has to keep in mind that cytokines may also be elevated due to other factors, as mentioned above. In this study, the analysis of cytokine levels were adjusted to gender, age and BMI, which can be confounders.

In conclusion, this study can offer a basis for further research as IL-6, IL-8, IL-12 (p70) and VEGF showed to be significantly reliable to discriminate between VO and degenerative spinal diseases - although, low-inflammatory conditions with low-virulent pathogens may interfere with the diagnostic potential. Also, an exclusion within the control group of patients with MODIC type 1 should be considered. A reasonable setup of a future study would be to compare cytokine levels of VO patients to those of *osteochondrosis intervertebralis* patients without positive culture results and MODIC type 1. In addition, future research would benefit from larger patient groups and longer follow-up schedules.

7. References

1. Cramer J, Haase N, Behre I, Ostermann PAW. Spondylitis und Spondylodiszitis. *Trauma und Berufskrankheit* 2003; **5**(3): 336-41.
2. Sobottke R, Seifert H, Fatkenheuer G, Schmidt M, Gossmann A, Eysel P. Current diagnosis and treatment of spondylodiscitis. *Deutsches Arzteblatt international* 2008; **105**(10): 181-7.
3. Michiels I, Jager M. [Spondylodiscitis : Current strategies for diagnosis and treatment]. *Der Orthopade* 2017; **46**(9): 785-804.
4. Frangen TM, Kalicke T, Gottwald M, et al. [Surgical management of spondylodiscitis. An analysis of 78 cases]. *Unfallchirurg* 2006; **109**(9): 743-53.
5. Lehner B, Akbar M, Rehnitz C, Omlor GW, Dapunt U, Burckhardt I. [Standards of microbiological diagnostics of spondylodiscitis]. *Der Orthopade* 2012; **41**(9): 702-10.
6. Jean M, Irisson JO, Gras G, et al. Diagnostic delay of pyogenic vertebral osteomyelitis and its associated factors. *Scand J Rheumatol* 2017; **46**(1): 64-8.
7. Grammatico L, Baron S, Rusch E, et al. Epidemiology of vertebral osteomyelitis (VO) in France: analysis of hospital-discharge data 2002-2003. *Epidemiol Infect* 2008; **136**(5): 653-60.
8. Fantoni M, Trecarichi EM, Rossi B, et al. Epidemiological and clinical features of pyogenic spondylodiscitis. *Eur Rev Med Pharmacol Sci* 2012; **16 Suppl 2**: 2-7.
9. Hopkinson N, Patel K. Clinical features of septic discitis in the UK: a retrospective case ascertainment study and review of management recommendations. *Rheumatol Int* 2016; **36**(9): 1319-26.
10. Zadran S, Pedersen PH, Eiskjaer S. Vertebral Osteomyelitis: A Mortality Analysis Comparing Surgical and Conservative Management. *Global Spine J* 2020; **10**(4): 456-63.
11. Priest DH, Peacock JE, Jr. Hematogenous vertebral osteomyelitis due to Staphylococcus aureus in the adult: clinical features and therapeutic outcomes. *South Med J* 2005; **98**(9): 854-62.
12. Mylona E, Samarkos M, Kakalou E, Fanourgiakis P, Skoutelis A. Pyogenic vertebral osteomyelitis: a systematic review of clinical characteristics. *Semin Arthritis Rheum* 2009; **39**(1): 10-7.
13. Doutchi M, Seng P, Menard A, et al. Changing trends in the epidemiology of vertebral osteomyelitis in Marseille, France. *New Microbes New Infect* 2015; **7**: 1-7.
14. Park KH, Cho OH, Lee JH, et al. Optimal Duration of Antibiotic Therapy in Patients With Hematogenous Vertebral Osteomyelitis at Low Risk and High Risk of Recurrence. *Clin Infect Dis* 2016; **62**(10): 1262-9.
15. Kim DY, Kim UJ, Yu Y, et al. Microbial Etiology of Pyogenic Vertebral Osteomyelitis According to Patient Characteristics. *Open Forum Infect Dis* 2020; **7**(6): ofaa176.
16. Gouliouris T, Aliyu SH, Brown NM. Spondylodiscitis: update on diagnosis and management. *J Antimicrob Chemother* 2010; **65 Suppl 3**: iii11-24.
17. Kehrer M, Pedersen C, Jensen TG, Lassen AT. Increasing incidence of pyogenic spondylodiscitis: a 14-year population-based study. *J Infect* 2014; **68**(4): 313-20.
18. Jeong DK, Lee HW, Kwon YM. Clinical Value of Procalcitonin in Patients with Spinal Infection. *J Korean Neurosurg Soc* 2015; **58**(3): 271-5.
19. Hadjipavlou AG, Mader JT, Necessary JT, Muffoletto AJ. Hematogenous pyogenic spinal infections and their surgical management. *Spine (Phila Pa 1976)* 2000; **25**(13): 1668-79.
20. Loibl M, Stoyanov L, Doenitz C, et al. Outcome-related co-factors in 105 cases of vertebral osteomyelitis in a tertiary care hospital. *Infection* 2014; **42**(3): 503-10.
21. Gupta A, Kowalski TJ, Osmon DR, et al. Long-term outcome of pyogenic vertebral osteomyelitis: a cohort study of 260 patients. *Open Forum Infect Dis* 2014; **1**(3): ofu107.
22. Breuninger M, Yagdiran A, Willinger A, et al. Vertebral Osteomyelitis After Spine Surgery: A Disease With Distinct Characteristics. *Spine (Phila Pa 1976)* 2020; **45**(20): 1426-34.
23. McHenry MC, Easley KA, Locker GA. Vertebral osteomyelitis: long-term outcome for 253 patients from 7 Cleveland-area hospitals. *Clin Infect Dis* 2002; **34**(10): 1342-50.
24. Wiley AM, Trueta J. The vascular anatomy of the spine and its relationship to pyogenic vertebral osteomyelitis. *J Bone Joint Surg Br* 1959; **41-B**: 796-809.

25. Batson OV. The vertebral system of veins as a means for cancer dissemination. *Prog Clin Cancer* 1967; **3**: 1-18.
26. Le Moal G, Roblot F, Paccalin M, et al. Clinical and laboratory characteristics of infective endocarditis when associated with spondylodiscitis. *Eur J Clin Microbiol Infect Dis* 2002; **21**(9): 671-5.
27. Pigrau C, Almirante B, Flores X, et al. Spontaneous pyogenic vertebral osteomyelitis and endocarditis: incidence, risk factors, and outcome. *Am J Med* 2005; **118**(11): 1287.
28. Quast MB, Carr CM, Hooten WM. Multilevel lumbar spine infection due to poor dentition in an immunocompetent adult: a case report. *J Med Case Rep* 2017; **11**(1): 328.
29. Nolla JM, Ariza J, Gomez-Vaquero C, et al. Spontaneous pyogenic vertebral osteomyelitis in nondrug users. *Semin Arthritis Rheum* 2002; **31**(4): 271-8.
30. Schinkel C, Gottwald M, Andress HJ. Surgical treatment of spondylodiscitis. *Surg Infect (Larchmt)* 2003; **4**(4): 387-91.
31. Zarrouk V, Feydy A, Salles F, et al. Imaging does not predict the clinical outcome of bacterial vertebral osteomyelitis. *Rheumatology (Oxford)* 2007; **46**(2): 292-5.
32. Ratcliffe JF. An evaluation of the intra-osseous arterial anastomoses in the human vertebral body at different ages. A microarteriographic study. *J Anat* 1982; **134**(Pt 2): 373-82.
33. Lerner T, Hackenberg L, Rosler S, Joosten U, Halm H, Liljenqvist U. [Surgical therapy of unspecific and specific Spondylodiscitis]. *Zeitschrift fur Orthopadie und ihre Grenzgebiete* 2005; **143**(2): 204-12.
34. Roy MS, J.; Kerr, K. G.; Conroy, J. L. Pathophysiology and Pathogenesis of Osteomyelitis: InTech; 2012.
35. Bhavan KP, Marschall J, Olsen MA, Fraser VJ, Wright NM, Warren DK. The epidemiology of hematogenous vertebral osteomyelitis: a cohort study in a tertiary care hospital. *BMC Infect Dis* 2010; **10**: 158.
36. D'Agostino C, Scorzolini L, Massetti AP, et al. A seven-year prospective study on spondylodiscitis: epidemiological and microbiological features. *Infection* 2010; **38**(2): 102-7.
37. Cornett CA, Vincent SA, Crow J, Hewlett A. Bacterial Spine Infections in Adults: Evaluation and Management. *J Am Acad Orthop Surg* 2016; **24**(1): 11-8.
38. Inoue S, Moriyama T, Horinouchi Y, et al. Comparison of clinical features and outcomes of staphylococcus aureus vertebral osteomyelitis caused by methicillin-resistant and methicillin-sensitive strains. *Springerplus* 2013; **2**(1): 283.
39. Nickerson EK, Sinha R. Vertebral osteomyelitis in adults: an update. *Br Med Bull* 2016; **117**(1): 121-38.
40. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* 1997; **10**(3): 505-20.
41. Gill SR, McIntyre LM, Nelson CL, et al. Potential associations between severity of infection and the presence of virulence-associated genes in clinical strains of Staphylococcus aureus. *PLoS One* 2011; **6**(4): e18673.
42. Wright JA, Nair SP. Interaction of staphylococci with bone. *Int J Med Microbiol* 2010; **300**(2-3): 193-204.
43. Jenkins A, Diep BA, Mai TT, et al. Differential expression and roles of Staphylococcus aureus virulence determinants during colonization and disease. *mBio* 2015; **6**(1): e02272-14.
44. Tuchscherr L, Pollath C, Siegmund A, et al. Clinical S. aureus Isolates Vary in Their Virulence to Promote Adaptation to the Host. *Toxins (Basel)* 2019; **11**(3).
45. Jin T, Zhu YL, Li J, et al. Staphylococcal protein A, Panton-Valentine leukocidin and coagulase aggravate the bone loss and bone destruction in osteomyelitis. *Cell Physiol Biochem* 2013; **32**(2): 322-33.
46. Moon MS. Tuberculosis of the spine. Controversies and a new challenge. *Spine (Phila Pa 1976)* 1997; **22**(15): 1791-7.
47. Jung N, Seifert H, Siewe J, Fätkenheuer G. Spondylodiszitis. *Der Internist* 2013; **54**(8): 945-53.
48. Renker EK, Mohring K, Abel R, et al. [Urogenic spondylodiscitis]. *Der Orthopade* 2009; **38**(4): 355-6, 8-60, 62-4.

49. Jeon I, Kong E, Yu D, Hong CP. Clinical and Radiological Analysis of Pyogenic Vertebral Osteomyelitis Immediately after Successful Antimicrobial Therapy: Considerations for Assessing Therapeutic Response. *Diagnostics (Basel)* 2020; **10**(11).
50. Carragee EJ. Pyogenic vertebral osteomyelitis. *J Bone Joint Surg Am* 1997; **79**(6): 874-80.
51. Lopez J, Tatar Z, Tournadre A, et al. Characteristics of spontaneous coagulase-negative staphylococcal spondylodiscitis: a retrospective comparative study versus Staphylococcus aureus spondylodiscitis. *BMC Infect Dis* 2017; **17**(1): 683.
52. Stirling A, Worthington T, Rafiq M, Lambert PA, Elliott TS. Association between sciatica and Propionibacterium acnes. *Lancet* 2001; **357**(9273): 2024-5.
53. Yuan Y, Zhou Z, Jiao Y, et al. Histological Identification of Propionibacterium acnes in Nonpyogenic Degenerated Intervertebral Discs. *Biomed Res Int* 2017; **2017**: 6192935.
54. Tang G, Wang Z, Chen J, Zhang Z, Qian H, Chen Y. Latent infection of low-virulence anaerobic bacteria in degenerated lumbar intervertebral discs. *BMC Musculoskelet Disord* 2018; **19**(1): 445.
55. Chen Y, Wang X, Zhang X, et al. Low virulence bacterial infections in cervical intervertebral discs: a prospective case series. *Eur Spine J* 2018; **27**(10): 2496-505.
56. Foreman SC, Schwaiger BJ, Meyer B, et al. Computed Tomography and Magnetic Resonance Imaging Parameters Associated with Poor Clinical Outcome in Spondylodiscitis. *World Neurosurg* 2017; **104**: 919-26 e2.
57. Akgun D, Burger J, Pumberger M, Putzier M. C-reactive protein misdiagnoses delayed postoperative spinal implant infections in patients with low-virulent microorganisms. *Eur Spine J* 2019; **28**(12): 2990-5.
58. Schiedo RM, Lavelle W, Sun MH. Lumbar Spinal Candida Glabrata Treated Without Surgical Intervention: A Case Report. *Cureus* 2017; **9**(6): e1371.
59. Butler JS, Shelly MJ, Timlin M, Powderly WG, O'Byrne JM. Nontuberculous pyogenic spinal infection in adults: a 12-year experience from a tertiary referral center. *Spine (Phila Pa 1976)* 2006; **31**(23): 2695-700.
60. Raghavan M, Lazzeri E, Palestro CJ. Imaging of Spondylodiscitis. *Semin Nucl Med* 2018; **48**(2): 131-47.
61. Russo A, Graziano E, Carnelutti A, et al. Management of vertebral osteomyelitis over an eight-year period: The UDIPROVE (UDIne PROtocol on VErtebral osteomyelitis). *Int J Infect Dis* 2019; **89**: 116-21.
62. Akbar M, Lehner B, Doustdar S, et al. [Pyogenic spondylodiscitis of the thoracic and lumbar spine : a new classification and guide for surgical decision-making]. *Der Orthopade* 2011; **40**(7): 614-23.
63. Herren C, Jung N, Pishnamaz M, Breuninger M, Siewe J, Sobottke R. Spondylodiscitis: Diagnosis and Treatment Options. *Deutsches Arzteblatt international* 2017; **114**(51-52): 875-82.
64. Srinivasan D, Terman SW, Himedan M, Dugo D, La Marca F, Park P. Risk factors for the development of deformity in patients with spinal infection. *Neurosurg Focus* 2014; **37**(2): E2.
65. Osenbach RK, Hitchon PW, Menezes AH. Diagnosis and management of pyogenic vertebral osteomyelitis in adults. *Surg Neurol* 1990; **33**(4): 266-75.
66. Nagashima H, Tanishima S, Tanida A. Diagnosis and management of spinal infections. *J Orthop Sci* 2018; **23**(1): 8-13.
67. Zimmerli W. Clinical practice. Vertebral osteomyelitis. *N Engl J Med* 2010; **362**(11): 1022-9.
68. Cheung WY, Luk KD. Pyogenic spondylitis. *Int Orthop* 2012; **36**(2): 397-404.
69. Dubost JJ, Lopez J, Pereira B, Couderc M, Tournadre A, Soubrier M. Serum procalcitonin measurement is not a useful biomarker in the detection of primary infectious spondylodiscitis. *Joint Bone Spine* 2017; **84**(4): 503-4.
70. Berbari EF, Kanj SS, Kowalski TJ, et al. 2015 Infectious Diseases Society of America (IDSA) Clinical Practice Guidelines for the Diagnosis and Treatment of Native Vertebral Osteomyelitis in Adults. *Clin Infect Dis* 2015; **61**(6): e26-46.

71. Sehn JK, Gilula LA. Percutaneous needle biopsy in diagnosis and identification of causative organisms in cases of suspected vertebral osteomyelitis. *Eur J Radiol* 2012; **81**(5): 940-6.
72. Shibayama M, Nagahara M, Kawase G, Fujiwara K, Kawaguchi Y, Mizutani J. New needle biopsy technique for lumbar pyogenic spondylodiscitis. *Spine (Phila Pa 1976)* 2010; **35**(23): E1347-9.
73. Fenollar F, Levy PY, Raoult D. Usefulness of broad-range PCR for the diagnosis of osteoarticular infections. *Curr Opin Rheumatol* 2008; **20**(4): 463-70.
74. Palestro CJ, Love C, Miller TT. Infection and musculoskeletal conditions: Imaging of musculoskeletal infections. *Best Pract Res Clin Rheumatol* 2006; **20**(6): 1197-218.
75. Militz MH, D. Diagnosepfad und Behandlung der Spondylodiszitis. *Trauma und Berufskrankheit* 2012; **14 (2)**: 171-6.
76. Jevtic V. Vertebral infection. *Eur Radiol* 2004; **14 Suppl 3**: E43-52.
77. Modic MT, Feiglin DH, Piraino DW, et al. Vertebral osteomyelitis: assessment using MR. *Radiology* 1985; **157**(1): 157-66.
78. Maiuri F, Iaconetta G, Gallicchio B, Manto A, Briganti F. Spondylodiscitis. Clinical and magnetic resonance diagnosis. *Spine (Phila Pa 1976)* 1997; **22**(15): 1741-6.
79. Pschyrembel W, Arnold U. Pschyrembel Klinisches Wörterbuch 2013. 264th ed. Berlin: De Gruyter; 2012.
80. Weber M, Heller KD, Wirtz D, Zimmermann-Picht S, Keulers P, Zilkens KW. [Percutaneous CT-controlled puncture and drainage of spondylodiscitis--a minimally invasive method]. *Z Orthop Ihre Grenzgeb* 1998; **136**(4): 375-9.
81. Morales H. Infectious Spondylitis Mimics: Mechanisms of Disease and Imaging Findings. *Semin Ultrasound CT MR* 2018; **39**(6): 587-604.
82. Schmitz A, Risse JH, Grunwald F, Gassel F, Biersack HJ, Schmitt O. Fluorine-18 fluorodeoxyglucose positron emission tomography findings in spondylodiscitis: preliminary results. *Eur Spine J* 2001; **10**(6): 534-9.
83. Afzelius P, Heegaard PMH, Jensen SB, et al. [(99m)Tc]-labelled interleukin-8 as a diagnostic tool compared to [(18)F]FDG and CT in an experimental porcine osteomyelitis model. *Am J Nucl Med Mol Imaging* 2020; **10**(1): 32-46.
84. Love C, Palestro CJ. Nuclear medicine imaging of bone infections. *Clin Radiol* 2016; **71**(7): 632-46.
85. Stumpe KD, Zanetti M, Weishaupt D, Hodler J, Boos N, Von Schulthess GK. FDG positron emission tomography for differentiation of degenerative and infectious endplate abnormalities in the lumbar spine detected on MR imaging. *AJR Am J Roentgenol* 2002; **179**(5): 1151-7.
86. Guerado E, Cervan AM. Surgical treatment of spondylodiscitis. An update. *Int Orthop* 2012; **36**(2): 413-20.
87. Zarghooni K, Rollinghoff M, Sobottke R, Eysel P. Treatment of spondylodiscitis. *Int Orthop* 2012; **36**(2): 405-11.
88. Homagk N, Jarmuzek T, Hofmann GO, Homagk L. [Therapy of Spondylodiscitis by Severity]. *Zeitschrift fur Orthopadie und Unfallchirurgie* 2017; **155**(6): 697-704.
89. Karadimas EJ, Bunger C, Lindblad BE, et al. Spondylodiscitis. A retrospective study of 163 patients. *Acta Orthop* 2008; **79**(5): 650-9.
90. Weissman S, Parker RD, Siddiqui W, Dykema S, Horvath J. Vertebral osteomyelitis: retrospective review of 11 years of experience. *Scand J Infect Dis* 2014; **46**(3): 193-9.
91. Petkova AS, Zhelyazkov CB, Kitov BD. Spontaneous Spondylodiscitis - Epidemiology, Clinical Features, Diagnosis and Treatment. *Folia Med (Plovdiv)* 2017; **59**(3): 254-60.
92. Sobottke R, Rollinghoff M, Zarghooni K, et al. Spondylodiscitis in the elderly patient: clinical mid-term results and quality of life. *Archives of orthopaedic and trauma surgery* 2010; **130**(9): 1083-91.
93. Bornemann R, Muller-Broich JD, Deml M, Sander K, Wirtz DC, Pflugmacher R. [Diagnosis and Treatment of Spondylodiscitis/Spondylitis in Clinical Practice]. *Zeitschrift fur Orthopadie und Unfallchirurgie* 2015; **153**(5): 540-5.
94. Huttner B, Opravil M. [Infectious spondylitis]. *Z Rheumatol* 2006; **65**(1): 7-11.

95. Fleege C, Wichelhaus TA, Rauschmann M. [Systemic and local antibiotic therapy of conservative and operative treatment of spondylodiscitis]. *Der Orthopade* 2012; **41**(9): 727-35.
96. Li HK, Rombach I, Zambellas R, et al. Oral versus Intravenous Antibiotics for Bone and Joint Infection. *N Engl J Med* 2019; **380**(5): 425-36.
97. Bernard L, Dinh A, Ghout I, et al. Antibiotic treatment for 6 weeks versus 12 weeks in patients with pyogenic vertebral osteomyelitis: an open-label, non-inferiority, randomised, controlled trial. *Lancet* 2015; **385**(9971): 875-82.
98. Rutges JP, Kempen DH, van Dijk M, Oner FC. Outcome of conservative and surgical treatment of pyogenic spondylodiscitis: a systematic literature review. *Eur Spine J* 2016; **25**(4): 983-99.
99. van Ooij A, Beckers JM, Herpers MJ, Walenkamp GH. Surgical treatment of aspergillus spondylodiscitis. *Eur Spine J* 2000; **9**(1): 75-9.
100. Blumberg HM, Burman WJ, Chaisson RE, et al. American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. *Am J Respir Crit Care Med* 2003; **167**(4): 603-62.
101. Pappas PG, Kauffman CA, Andes D, et al. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2009; **48**(5): 503-35.
102. Klockner C, Valencia R, Weber U. [Alignment of the sagittal profile after surgical therapy of nonspecific destructive spondylodiscitis: ventral or ventrodorsal method--a comparison of outcomes]. *Der Orthopade* 2001; **30**(12): 965-76.
103. Segreto FA, Beyer GA, Grieco P, et al. Vertebral Osteomyelitis: A Comparison of Associated Outcomes in Early Versus Delayed Surgical Treatment. *Int J Spine Surg* 2018; **12**(6): 703-12.
104. Hadjipavlou AG, Katonis PK, Gaitanis IN, Muffoletto AJ, Tzermiadianos MN, Crow W. Percutaneous transpedicular discectomy and drainage in pyogenic spondylodiscitis. *Eur Spine J* 2004; **13**(8): 707-13.
105. Dimar JR, Carreon LY, Glassman SD, Campbell MJ, Hartman MJ, Johnson JR. Treatment of pyogenic vertebral osteomyelitis with anterior debridement and fusion followed by delayed posterior spinal fusion. *Spine (Phila Pa 1976)* 2004; **29**(3): 326-32; discussion 32.
106. Shiban E, Janssen I, Wostrack M, et al. A retrospective study of 113 consecutive cases of surgically treated spondylodiscitis patients. A single-center experience. *Acta Neurochir (Wien)* 2014; **156**(6): 1189-96.
107. Hee HT, Majd ME, Holt RT, Pienkowski D. Better treatment of vertebral osteomyelitis using posterior stabilization and titanium mesh cages. *J Spinal Disord Tech* 2002; **15**(2): 149-56; discussion 56.
108. Kowalski TJ, Berbari EF, Huddleston PM, Steckelberg JM, Mandrekar JN, Osmon DR. The management and outcome of spinal implant infections: contemporary retrospective cohort study. *Clin Infect Dis* 2007; **44**(7): 913-20.
109. Hendrickx L, Van Wijngaerden E, Samson I, Peetermans WE. Candidal vertebral osteomyelitis: report of 6 patients, and a review. *Clin Infect Dis* 2001; **32**(4): 527-33.
110. Pennekamp W. [Degenerative alterations of the spinal column: rational imaging]. *Internist (Berl)* 2013; **54**(7): 818-26.
111. Ohtori S, Koshi T, Yamashita M, et al. Existence of pyogenic spondylitis in Modic type 1 change without other signs of infection: 2-year follow-up. *Eur Spine J* 2010; **19**(7): 1200-5.
112. Albert HB, Lambert P, Rollason J, et al. Does nuclear tissue infected with bacteria following disc herniations lead to Modic changes in the adjacent vertebrae? *Eur Spine J* 2013; **22**(4): 690-6.
113. Dudli S, Liebenberg E, Magnitsky S, Miller S, Demir-Deviren S, Lotz JC. Propionibacterium acnes infected intervertebral discs cause vertebral bone marrow lesions consistent with Modic changes. *J Orthop Res* 2016; **34**(8): 1447-55.
114. Lin Y, Tang G, Jiao Y, et al. Propionibacterium acnes Induces Intervertebral Disc Degeneration by Promoting iNOS/NO and COX-2/PGE(2) Activation via the ROS-Dependent NF-kappaB Pathway. *Oxid Med Cell Longev* 2018; **2018**: 3692752.

115. Slaby O, McDowell A, Bruggemann H, et al. Is IL-1beta Further Evidence for the Role of Propionibacterium acnes in Degenerative Disc Disease? Lessons From the Study of the Inflammatory Skin Condition Acne Vulgaris. *Front Cell Infect Microbiol* 2018; **8**: 272.
116. Singh S, Siddhlingeswara GI, Rai A, Iyer RD, Sharma D, Surana R. Correlation Between Modic Changes and Bacterial Infection: A Causative Study. *Int J Spine Surg* 2020; **14**(5): 832-7.
117. Rajasekaran S, Soundararajan DCR, Tangavel C, et al. Human intervertebral discs harbour a unique microbiome and dysbiosis determines health and disease. *Eur Spine J* 2020; **29**(7): 1621-40.
118. Butler D, Trafimow JH, Andersson GB, McNeill TW, Huckman MS. Discs degenerate before facets. *Spine (Phila Pa 1976)* 1990; **15**(2): 111-3.
119. Ahlhelm F, Naumann N, Maher A, Shariat K, Ulmer S. [Degenerative intervertebral disc processes : Current aspects of diagnosis]. *Radiologe* 2019; **59**(10): 925-38.
120. Jensen MC, Brant-Zawadzki MN, Obuchowski N, Modic MT, Malkasian D, Ross JS. Magnetic resonance imaging of the lumbar spine in people without back pain. *N Engl J Med* 1994; **331**(2): 69-73.
121. Peterson CK, Bolton JE, Wood AR. A cross-sectional study correlating lumbar spine degeneration with disability and pain. *Spine (Phila Pa 1976)* 2000; **25**(2): 218-23.
122. de Roos A, Kressel H, Spritzer C, Dalinka M. MR imaging of marrow changes adjacent to end plates in degenerative lumbar disk disease. *AJR Am J Roentgenol* 1987; **149**(3): 531-4.
123. Modic MT, Steinberg PM, Ross JS, Masaryk TJ, Carter JR. Degenerative disk disease: assessment of changes in vertebral body marrow with MR imaging. *Radiology* 1988; **166**(1 Pt 1): 193-9.
124. Crockett MT, Kelly BS, van Baarsel S, Kavanagh EC. Modic Type 1 Vertebral Endplate Changes: Injury, Inflammation, or Infection? *AJR Am J Roentgenol* 2017; **209**(1): 167-70.
125. Albert HB, Manniche C. Modic changes following lumbar disc herniation. *Eur Spine J* 2007; **16**(7): 977-82.
126. Yoshihara H, Yoneoka D. National trends in the surgical treatment for lumbar degenerative disc disease: United States, 2000 to 2009. *Spine J* 2015; **15**(2): 265-71.
127. Schol J, Sakai D. Cell therapy for intervertebral disc herniation and degenerative disc disease: clinical trials. *Int Orthop* 2019; **43**(4): 1011-25.
128. Kelso A. Cytokines: structure, function and synthesis. *Curr Opin Immunol* 1989; **2**(2): 215-25.
129. Rose-John S, Heinrich PC. Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem J* 1994; **300 (Pt 2)**(Pt 2): 281-90.
130. Cohen MC, Cohen S. Cytokine function: a study in biologic diversity. *Am J Clin Pathol* 1996; **105**(5): 589-98.
131. Loppnow H. [Cytokines: classification, receptors, mechanisms of action]. *Internist (Berl)* 2001; **42**(1): 13-4, 7-27.
132. Holländer GA. Immunologie: Grundladen für Klinik und Praxis: Urban & Fischer; 2006.
133. Keustermans GC, Hoeks SB, Meerding JM, Prakken BJ, de Jager W. Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples. *Methods* 2013; **61**(1): 10-7.
134. Dinarello CA. Proinflammatory cytokines. *Chest* 2000; **118**(2): 503-8.
135. Gogos CA, Drosou E, Bassaris HP, Skoutelis A. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis* 2000; **181**(1): 176-80.
136. van Deventer SJ. Cytokine and cytokine receptor polymorphisms in infectious disease. *Intensive Care Med* 2000; **26 Suppl 1**: S98-102.
137. Su DL, Lu ZM, Shen MN, Li X, Sun LY. Roles of pro- and anti-inflammatory cytokines in the pathogenesis of SLE. *J Biomed Biotechnol* 2012; **2012**: 347141.
138. Monastero RN, Pentyala S. Cytokines as Biomarkers and Their Respective Clinical Cutoff Levels. *Int J Inflam* 2017; **2017**: 4309485.
139. Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB J* 1990; **4**(11): 2860-7.

140. Borish LC, Steinke JW. 2. Cytokines and chemokines. *J Allergy Clin Immunol* 2003; **111**(2 Suppl): S460-75.
141. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J* 2003; **374**(Pt 1): 1-20.
142. Kishimoto T. IL-6: from its discovery to clinical applications. *Int Immunol* 2010; **22**(5): 347-52.
143. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta* 2014; **1843**(11): 2563-82.
144. Lowik CW, van der Pluijm G, Bloys H, et al. Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin-6 production by osteogenic cells: a possible role of interleukin-6 in osteoclastogenesis. *Biochem Biophys Res Commun* 1989; **162**(3): 1546-52.
145. Ishimi Y, Miyaura C, Jin CH, et al. IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol* 1990; **145**(10): 3297-303.
146. Littlewood AJ, Russell J, Harvey GR, Hughes DE, Russell RG, Gowen M. The modulation of the expression of IL-6 and its receptor in human osteoblasts in vitro. *Endocrinology* 1991; **129**(3): 1513-20.
147. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J* 1990; **265**(3): 621-36.
148. Oberhoffer M, Vogelsang H, Russwurm S, Hartung T, Reinhart K. Outcome prediction by traditional and new markers of inflammation in patients with sepsis. *Clin Chem Lab Med* 1999; **37**(3): 363-8.
149. Jekarl DW, Lee SY, Lee J, et al. Procalcitonin as a diagnostic marker and IL-6 as a prognostic marker for sepsis. *Diagn Microbiol Infect Dis* 2013; **75**(4): 342-7.
150. Barre M, Behnes M, Hamed S, et al. Revisiting the prognostic value of monocyte chemotactic protein 1 and interleukin-6 in the sepsis-3 era. *J Crit Care* 2018; **43**: 21-8.
151. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* 2010; **40**(7): 1830-5.
152. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol* 2014; **6**(10): a016295.
153. Hammond ME, Lapointe GR, Feucht PH, et al. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *J Immunol* 1995; **155**(3): 1428-33.
154. Koch AE, Polverini PJ, Kunkel SL, et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 1992; **258**(5089): 1798-801.
155. Heidemann J, Ogawa H, Dwinell MB, et al. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. *J Biol Chem* 2003; **278**(10): 8508-15.
156. Li A, Dubey S, Varney ML, Dave BJ, Singh RK. IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J Immunol* 2003; **170**(6): 3369-76.
157. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; **13**: 251-76.
158. Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* 1998; **16**: 495-521.
159. Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol Sci* 2001; **22**(4): 201-7.
160. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; **9**(6): 669-76.
161. Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes Cancer* 2011; **2**(12): 1097-105.

162. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992; **359**(6398): 843-5.
163. Clauss M, Gerlach M, Gerlach H, et al. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med* 1990; **172**(6): 1535-45.
164. Aydin S. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides* 2015; **72**: 4-15.
165. Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: From Clinical Significance to Quantification. *Adv Sci (Weinh)* 2021; **8**(15): e2004433.
166. Brinkmann J, Zeissler EC, Scharrenberg JS, et al. The diagnostic value of cytokines for the discrimination of vertebral osteomyelitis and degenerative diseases of the spine. *Cytokine* 2022; **150**: 155782.
167. Alexandraki K, Piperi C, Kalofoutis C, Singh J, Alaveras A, Kalofoutis A. Inflammatory process in type 2 diabetes: The role of cytokines. *Ann N Y Acad Sci* 2006; **1084**: 89-117.
168. Achur RN, Freeman WM, Vrana KE. Circulating cytokines as biomarkers of alcohol abuse and alcoholism. *J Neuroimmune Pharmacol* 2010; **5**(1): 83-91.
169. Pham MN, Hawa MI, Pfleger C, et al. Pro- and anti-inflammatory cytokines in latent autoimmune diabetes in adults, type 1 and type 2 diabetes patients: Action LADA 4. *Diabetologia* 2011; **54**(7): 1630-8.
170. Herder C, Carstensen M, Ouwens DM. Anti-inflammatory cytokines and risk of type 2 diabetes. *Diabetes Obes Metab* 2013; **15 Suppl 3**: 39-50.
171. Kleiner G, Marcuzzi A, Zanin V, Monasta L, Zauli G. Cytokine levels in the serum of healthy subjects. *Mediators Inflamm* 2013; **2013**: 434010.
172. Larsson A, Carlsson L, Gordh T, Lind AL, Thulin M, Kamali-Moghaddam M. The effects of age and gender on plasma levels of 63 cytokines. *J Immunol Methods* 2015; **425**: 58-61.
173. Velikova TV, Kabakchieva PP, Assyov YS, Georgiev Tcapital A C. Targeting Inflammatory Cytokines to Improve Type 2 Diabetes Control. *Biomed Res Int* 2021; **2021**: 7297419.
174. Henderson B, Wilson M. Cytokine induction by bacteria: beyond lipopolysaccharide. *Cytokine* 1996; **8**(4): 269-82.
175. Bode C, Diedrich B, Muenster S, et al. Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis in vitro. *Int Immunopharmacol* 2014; **18**(1): 27-34.
176. Koch A, Zacharowski K, Boehm O, et al. Nitric oxide and pro-inflammatory cytokines correlate with pain intensity in chronic pain patients. *Inflamm Res* 2007; **56**(1): 32-7.
177. Nilsonne G, Lekander M, Akerstedt T, Axelsson J, Ingre M. Diurnal Variation of Circulating Interleukin-6 in Humans: A Meta-Analysis. *PLoS One* 2016; **11**(11): e0165799.
178. Chelsom J, Solberg CO. Vertebral osteomyelitis at a Norwegian university hospital 1987-97: clinical features, laboratory findings and outcome. *Scand J Infect Dis* 1998; **30**(2): 147-51.
179. Homagk L, Marmelstein D, Homagk N, Hofmann GO. SponDT (Spondylodiscitis Diagnosis and Treatment): spondylodiscitis scoring system. *J Orthop Surg Res* 2019; **14**(1): 100.
180. Beronius M, Bergman B, Andersson R. Vertebral osteomyelitis in Goteborg, Sweden: a retrospective study of patients during 1990-95. *Scand J Infect Dis* 2001; **33**(7): 527-32.
181. Larsson S, Thelander U, Friberg S. C-reactive protein (CRP) levels after elective orthopedic surgery. *Clin Orthop Relat Res* 1992; (275): 237-42.
182. Khan MH, Smith PN, Rao N, Donaldson WF. Serum C-reactive protein levels correlate with clinical response in patients treated with antibiotics for wound infections after spinal surgery. *Spine J* 2006; **6**(3): 311-5.
183. Waheed G, Soliman MAR, Ali AM, Aly MH. Spontaneous spondylodiscitis: review, incidence, management, and clinical outcome in 44 patients. *Neurosurg Focus* 2019; **46**(1): E10.
184. Loft JA, Moller DL, Thudium RF, et al. The Induced Immune Response in Patients With Infectious Spondylodiscitis: A Prospective Observational Cohort Study. *Front Immunol* 2022; **13**: 858934.

185. Lang S, Loibl M, Glasner J, et al. Vertebral osteomyelitis is characterised by increased RANK/OPG and RANKL/OPG expression ratios in vertebral bodies and intervertebral discs. *Eur Cell Mater* 2021; **42**: 438-51.
186. Fullilove S, Jellis J, Hughes SP, Remick DG, Friedland JS. Local and systemic concentrations of tumour necrosis factor-alpha, interleukin-6 and interleukin-8 in bacterial osteomyelitis. *Trans R Soc Trop Med Hyg* 2000; **94**(2): 221-4.
187. Bastian D, Tamburstuen MV, Lyngstadaas SP, Reikeras O. Systemic and local cytokine kinetics after total hip replacement surgery. *Eur Surg Res* 2008; **41**(4): 334-40.
188. Laborada G, Rego M, Jain A, et al. Diagnostic value of cytokines and C-reactive protein in the first 24 hours of neonatal sepsis. *Am J Perinatol* 2003; **20**(8): 491-501.
189. Boskabadi H, Maamouri G, Tavakol Afshari J, et al. Evaluation of serum interleukins-6, 8 and 10 levels as diagnostic markers of neonatal infection and possibility of mortality. *Iran J Basic Med Sci* 2013; **16**(12): 1232-7.
190. Selberg O, Hecker H, Martin M, Klos A, Bautsch W, Kohl J. Discrimination of sepsis and systemic inflammatory response syndrome by determination of circulating plasma concentrations of procalcitonin, protein complement 3a, and interleukin-6. *Crit Care Med* 2000; **28**(8): 2793-8.
191. Bozza FA, Salluh JI, Japiassu AM, et al. Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. *Crit Care* 2007; **11**(2): R49.
192. Iwase S, Nakada TA, Hattori N, et al. Interleukin-6 as a diagnostic marker for infection in critically ill patients: A systematic review and meta-analysis. *Am J Emerg Med* 2019; **37**(2): 260-5.
193. Ronholt K, Iversen L. Old and New Biological Therapies for Psoriasis. *Int J Mol Sci* 2017; **18**(11).
194. Rubbert-Roth A, Szabo MZ, Kedves M, Nagy G, Atzeni F, Sarzi-Puttini P. Failure of anti-TNF treatment in patients with rheumatoid arthritis: The pros and cons of the early use of alternative biological agents. *Autoimmun Rev* 2019; **18**(12): 102398.
195. Bedaiwy MA, Falcone T, Sharma RK, et al. Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. *Hum Reprod* 2002; **17**(2): 426-31.
196. Coomes EA, Haghbayan H. Interleukin-6 in Covid-19: A systematic review and meta-analysis. *Rev Med Virol* 2020; **30**(6): 1-9.
197. Zarghooni K, Hackenberg RK, Sander G, Mahabir E. Suitability of serum cytokine profiling for early diagnosis of implant-associated infections after orthopaedic surgery: A preliminary prospective study. *Cytokine* 2019; **116**: 88-96.
198. Glehr M, Friesenbichler J, Hofmann G, et al. Novel biomarkers to detect infection in revision hip and knee arthroplasties. *Clin Orthop Relat Res* 2013; **471**(8): 2621-8.
199. Lenski M, Tonn JC, Siller S. Interleukin-6 as inflammatory marker of surgical site infection following spinal surgery. *Acta Neurochir (Wien)* 2021; **163**(6): 1583-92.
200. van den Berg R, Jongbloed EM, de Schepper EIT, Bierma-Zeinstra SMA, Koes BW, Luijsterburg PAJ. The association between pro-inflammatory biomarkers and nonspecific low back pain: a systematic review. *Spine J* 2018; **18**(11): 2140-51.
201. Lee S, Moon CS, Sul D, et al. Comparison of growth factor and cytokine expression in patients with degenerated disc disease and herniated nucleus pulposus. *Clin Biochem* 2009; **42**(15): 1504-11.
202. Sadowska A, Touli E, Hitzl W, et al. Inflammaging in cervical and lumbar degenerated intervertebral discs: analysis of proinflammatory cytokine and TRP channel expression. *Eur Spine J* 2018; **27**(3): 564-77.
203. Koerner JD, Markova DZ, Schroeder GD, et al. Correlation of Early Outcomes and Intradiscal Interleukin-6 Expression in Lumbar Fusion Patients. *Neurospine* 2020; **17**(1): 36-41.
204. Takada T, Nishida K, Doita M, Miyamoto H, Kurosaka M. Interleukin-6 production is upregulated by interaction between disc tissue and macrophages. *Spine (Phila Pa 1976)* 2004; **29**(10): 1089-92; discussion 93.
205. Shamji MF, Setton LA, Jarvis W, et al. Proinflammatory cytokine expression profile in degenerated and herniated human intervertebral disc tissues. *Arthritis Rheum* 2010; **62**(7): 1974-82.

206. Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. *Nat Rev Rheumatol* 2014; **10**(1): 44-56.
207. Koroth J, Buko EO, Abbott R, et al. Macrophages and Intervertebral Disc Degeneration. *Int J Mol Sci* 2023; **24**(2).
208. Le Maitre CL, Hoyland JA, Freemont AJ. Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1beta and TNFalpha expression profile. *Arthritis Res Ther* 2007; **9**(4): R77.
209. Wang Y, Che M, Xin J, Zheng Z, Li J, Zhang S. The role of IL-1beta and TNF-alpha in intervertebral disc degeneration. *Biomed Pharmacother* 2020; **131**: 110660.
210. Coscia MF, Denys GA, Wack MF. Propionibacterium acnes, Coagulase-Negative Staphylococcus, and the "Biofilm-like" Intervertebral Disc. *Spine (Phila Pa 1976)* 2016; **41**(24): 1860-5.
211. Albert HB, Sorensen JS, Christensen BS, Manniche C. Antibiotic treatment in patients with chronic low back pain and vertebral bone edema (Modic type 1 changes): a double-blind randomized clinical controlled trial of efficacy. *Eur Spine J* 2013; **22**(4): 697-707.
212. Rannou F, Ouanes W, Boutron I, et al. High-sensitivity C-reactive protein in chronic low back pain with vertebral end-plate Modic signal changes. *Arthritis Rheum* 2007; **57**(7): 1311-5.

8. Appendix

8.1. List of figures

8.2. List of tables

9. Publications

Abstract submitted for the Deutsche Wirbelsäulenkongress:

Suitability of cytokines for discrimination of vertebral osteomyelitis and degenerative diseases of the spine

Julia Brinkmann, Jan Simon Scharrenberg, **Eva-Carina Zeißler**, Mohamed Majjouti, Max Joseph Scheyerer, Ayla Yagdiran, Norma Jung, Jan Siewe, Esther Mahabir (9./11.12.2020, Berlin) 15th Deutscher Wirbelsäulenkongress (prize won)

Published article:

The diagnostic value of cytokines for the discrimination of vertebral osteomyelitis and degenerative diseases of the spine

Julia Brinkmann, **Eva-Carina Zeißler**, Jan Simon Scharrenberg, Julia Schenk, Mohamed Majjouti, Max Oberste, Ayla Yagdiran, Max Joseph Scheyerer, Norma Jung, Jan Siewe, Esther Mahabir (2021) https://doi.org/10.1016/j.cyto.2021.155782