Aus dem Zentrum für Molekulare Medizin
der Universität zu Köln

Leitung Vergleichende Medizin
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Cytokine-producing peripheral blood mononuclear cells (PBMCs) for discrimination of vertebral osteomyelitis and degenerative spinal diseases

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Medizinischen Fakultät
der Universität zu Köln

vorgelegt von
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aus Dormagen

promoviert am: 28. Februar 2024

Gedruckt mit Genehmigung der Medizinischen Fakultät der Universität zu Köln

Druckjahr 2024

Dekan: Universitätsprofessor Dr. med. G. R. Fink

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Die in dieser Arbeit angegebenen Experimente mit zugehörigen Ergebnissen sind Teil der CyProSpon-Studie. Die Patientenrekrutierung, Probengewinnung und Aufbereitung wurde von mir selbst und zwei weiteren Promovierenden dieser Studie Frau Julia Brinkmann, ehemals Klein, und Herr Jan Scharrenberg durchgeführt:

Die dieser Arbeit zugrunde liegenden Experimente, inklusive Analyse und Auswertung der Zytokin-produzierenden PBMCs, sind von mir mit Unterstützung von Herrn Martin Thelen und der veterinärmedizinisch-technischen Assistentin Frau Antje Beckmann durchgeführt worden. Die bildliche Aufarbeitung und Auswertung der Ergebnisse wurden von mir mit der Unterstützung von Mohamed Majjouti durchgeführt.

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Köln, den 27.11.2023

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Acknowledgements

First of all, I would like to thank Prof. Dr. Esther Mahabir-Brenner for her time, effort, and dedication to make this project successful. I am grateful for the opportunity to carry out this research project in her working group and the associated comprehensive scientific support.

I am deeply grateful to all contributors of this study – Prof. Dr. Norma Jung, Prof. Dr. Jan Siewe, and Dr. Ayla Yagdiran for their clinical advice and discussion input.

My gratitude also goes to Malte Heykants and Antje Beckmann for their assistance with laboratory work, revision of numerous data, and motivational support. I would especially like to thank Mohamed Majjouti for his contribution to the revision of numerous tables and graphs and his always supportive and encouraging words.

I would like to thank Priv. Doz. Dr. Hans Schlößer's research group, especially Martin Thelen for his great support with the laboratory work and valuable advice.

Furthermore, I would like to thank my fellow students Julia Brinkmann, Dr. Jan Scharrenberg and Eva-Carina Zeißler for their great support and especially for all the work we managed to do for the whole study.

Lastly, I would like to thank my wonderful family and friends who have always been there for me and encouraged me.

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Glossary

ALIF	Anterior lumbar interbody fusion
APCs	Antigen-presenting cells
C	Control
CMMC	Center for Molecular Medicine Cologne
CO_2	Carbon dioxide
CoNS	Coagulase-negative staphylococci
CRP	C-reactive protein
CT	Computer tomography
CyProSpon	Cytokine Profiles in Spondylodiscitis
d	Days
DCs	Dendritic cells
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
F-18 FDG	Fluorine-18 fluorodeoxyglucose
g	Gram
GCP-V	Good Clinical Practice-Regulation
h	Hours
HIV	Human immunodeficiency virus
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
MC	Modic changes
MCP-1	Monocyte chemotactic protein-1
ml	Milliliter
µl	Microliter
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
M. tuberculosis	*Mycobacterium tuberculosis*
NK-cells	Natural killer cells
P. acnes	*Proprionibacterium acnes*
PBMC	Peripheral blood mononuclear cell
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction

PET	Positron emission tomography
PLIF	Posterior lumbar interbody fusion
Post-OP	Postoperative
Pre-OP	Preoperative
PRRs	Pattern recognition receptors
rpm	Rounds per minute
RT	Room temperature
S. aureus	*Staphylococcus aureus*
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor alpha
TLIF	Transforaminal lumbar interbody fusion
VEGF	Vascular endothelial growth factor
VO	Vertebral osteomyelitis
VPF	Vascular permeability factor

1. Summary

Vertebral osteomyelitis (VO) is a rare but dreaded disease of the spine. A major challenge lies in its earliest possible diagnosis and treatment. The average time between the first symptoms and a final diagnosis ranges between 2 and 6 months. These difficulties can lead to a highly acute course with neurological deficits, sepsis, organ failure and may result in death. Previous work in our group showed that IL-6, IL-8, IL-12, and VEGF were candidate cytokines for the discrimination of vertebral osteomyelitis and degenerative spinal diseases. The aim of the present study was to gain an understanding of the immune cells responsible for the production of these candidate cytokines along with the associated changes in immune cells during vertebral osteomyelitis. This approach may enable a clear differentiation between vertebral osteomyelitis and degenerative spinal diseases. Therefore, the levels of cytokine-producing peripheral mononuclear blood cells (PBMCs) were measured via fluorescence activated cell sorting (FACS).

The study was a single-center-control study performed at the Orthopedic Department of the University of Cologne in co-operation with the Department for Internal Medicine I and the Center for Molecular Medicine Cologne (CMMC) between 2015 and 2019. Inclusion criteria were medical need for operative stabilization due to VO or erosive osteochondrosis and exclusion criteria were autoimmune diseases, chronic infections or active cancer. A total number of 36 patients were included in the study: 16 patients being part of the VO group and 20 patients with erosive osteochondrosis in the control group. The average age was 68.8 years and 62.5 % of the VO group were male. There were two time points of blood drawing, one before surgery (pre-OP) and one 40-56 days after surgery (post-OP). For both groups at both timepoints, FACS analysis was conducted, and seven cell populations were screened, namely lymphocytes, B-cells, T-cells, which were then divided into CD4 T-cells and CD8 T-cells as well as NK-cells, and monocytes. The results were statistically analyzed via paired t-test within the groups and a two-sample-t-test (method of Satterthwaite) to detect the differences between the VO group and the control group. Furthermore, similarity matrices were created for B-cells, T-cells, and monocytes for both study groups at both time points to determine co-regulatory processes.

IL-6-positive cells were produced by all cell populations analyzed but there were no significant differences between both study groups at the pre-operative time point. The similarity matrices showed positive correlations of IL-6 with VEGF and/or IL-12 pre-OP. Monocytes showed by far the highest number of IL-8-positive cells compared to other cell populations analyzed and the proportion of $CD8^+$ T-cells in the VO patients was significantly lower than in the controls pre-OP. There was a significant higher number of IL-12-positive lymphocytes in the VO group than in the controls pre-OP. IL-12 showed positive correlations with IL-6 and VEGF in the similarity matrices. VEGF-positive cells showed the most significant differences

between both study groups pre-OP, whereby the proportion of positive cells was higher in the VO group than in the controls (lymphocytes, B-cells, T-cells, and $CD8^+$ T-cells). In addition, the number of VEGF-positive cells was the highest in comparison to the other cytokine-positive cell populations and VEGF showed positive correlations with IL-6, IL-8, and IL-12 in the similarity matrices pre-OP.

Based on the present results, a clear differentiation between vertebral osteomyelitis and degenerative spinal disease is possible pre-operatively, especially with the IL-8-positive $CD8^+$ T-cells, IL-12-positive lymphocytes, and VEGF-positive cells (lymphocytes, B-cells, T-cells, and $CD8^+$ T-cells).

2. Zusammenfassung

Die vertebrale Osteomyelitis (VO) ist eine seltene, aber gefürchtete Erkrankung der Wirbelsäule. Eine große Herausforderung besteht in ihrer möglichst frühzeitigen Diagnose und Behandlung. Die durchschnittliche Zeitspanne zwischen den ersten Symptomen und einer endgültigen Diagnose beträgt zwischen 2 und 6 Monaten. Diese Schwierigkeiten können zu einem hochakuten Verlauf mit neurologischen Ausfällen, Sepsis, Organversagen und unter Umständen zum Tod führen. Frühere Arbeiten unserer Studiengruppe haben gezeigt, dass anhand von IL-6, IL-8, IL-12 und VEGF eine Unterscheidung zwischen VO und degenerativen Wirbelsäulenerkrankungen möglich ist. Das Ziel der vorliegenden Studie war es, ein Verständnis der Immunzellen zu erlangen, die für die Produktion dieser Zytokine verantwortlich sind, sowie der damit verbunden Veränderungen in den Immunzellen während VO. Dafür wurde die Menge der Zytokin-sekretierenden peripheren mononukleären Blutzellen (PBMCs) mittels fluoreszenzaktivierte Zellsortierung (FACS) gemessen.

Die Single-Center-Control-Studie wurde von 2015 bis 2019 an der Orthopädischen Klinik der Universität zu Köln in Kooperation mit der Klinik für Innere Medizin I und dem Zentrum für Molekulare Medizin Köln (ZMMK) durchgeführt. Einschlusskriterien waren die medizinische Notwendigkeit einer operativen Stabilisierung aufgrund von VO oder erosiver Osteochondrose und Ausschlusskriterien waren Autoimmunerkrankungen, chronische Infektionen oder eine aktive Krebserkrankung. Insgesamt wurden 36 Patienten in die Studie eingeschlossen: 16 Patienten gehörten zur VO-Gruppe und 20 Patienten mit erosiver Osteochondrose zur Kontrollgruppe. Das Durchschnittsalter betrug 68,8 Jahre und 62,5 % der VO-Gruppe waren männlich. Es gab zwei Zeitpunkte der Blutentnahme, einen vor der Operation (prä-OP) und einen 40-56 Tage nach der Operation (post-OP). Für beide Gruppen wurde zu beiden Zeitpunkten eine FACS-Analyse durchgeführt und dabei sieben Zellpopulationen untersucht: Lymphozyten, B-Zellen, T-Zellen, unterteilt in $CD4^+$-T-Zellen und $CD8^+$-T-Zellen, Monozyten und NK-Zellen. Die Ergebnisse wurden mittels eines gepaarten t-Test innerhalb der Gruppen und eines Zwei-Stichproben-t-Test (Methode von Satterthwaite) statistisch ausgewertet, um Unterschiede zwischen der VO-Gruppe und der Kontrollgruppe zu ermitteln. Außerdem wurden Ähnlichkeitsmatrizen für B-Zellen, T-Zellen und Monozyten für beide Studiengruppen zu beiden Zeitpunkten erstellt.

IL-6-positive Zellen wurden von allen analysierten Zellpopulationen produziert, aber es gab keine signifikanten Unterschiede zwischen beiden Studiengruppen zum präoperativen Zeitpunkt. Die Ähnlichkeitsmatrizen zeigten positive Korrelationen von IL-6 mit VEGF und/oder IL-12 vor der OP. Monozyten wiesen im Vergleich zu den anderen untersuchten Zellpopulationen die mit Abstand höchste Anzahl IL-8-positiver Zellen auf, und der Anteil der $CD8^+$ T-Zellen war bei den VO-Patienten signifikant niedriger als bei den Kontrollen vor der OP. Die Zahl der IL-12-positiven Lymphozyten war in der VO-Gruppe signifikant höher als in

den Kontrollen vor der OP. IL-12 zeigte positive Korrelationen mit IL-6 und VEGF in den Ähnlichkeitsmatrizen. VEGF-positive Zellen wiesen die meisten signifikanten Unterschiede zwischen den beiden Studiengruppen vor der OP auf, wobei der Anteil der positiven Zellen in der VO-Gruppe höher war als in den Kontrollen (Lymphozyten, B-Zellen, T-Zellen und CD8+ T-Zellen). Darüber hinaus war die Zahl der VEGF-positiven Zellen im Vergleich zu den anderen Zytokin-positiven Zellpopulationen am höchsten und VEGF zeigte positive Korrelationen mit IL-6, IL-8 und IL-12 in den Ähnlichkeitsmatrizen vor der OP.

Basierend auf den vorliegenden Ergebnissen ist präoperativ eine Differenzierung zwischen vertebraler Osteomyelitis und degenerativer Wirbelsäulenerkrankung möglich, insbesondere anhand der IL-8-positiven $CD8^+$ T-Zellen, IL-12-positiven Lymphozyten und den VEGF-positiven Zellen (Lymphozyten, B-Zellen, T-Zellen und $CD8^+$-T-Zellen).

3. Introduction

3.1 Vertebral osteomyelitis

Vertebral osteomyelitis (VO) is a rare but serious infection of the spine, also known as spondylodiscitis, pyogenic spinal infection or spondylitis, whereas the latter terminology also includes chronic infectious cases such as spondylitis ankylosans (Bechterew's disease) [1-3]. The disease is primarily caused by bacterial infection and typically begins in the intervertebral discs before spreading to the adjacent vertebral bodies. VO accounts for 2 to 7% of all osteomyelitis cases [4], making it the third most common site for osteomyelitis after the femur and tibia [5]. In most cases, the disease is chronic with non-specific symptoms such as nightly back pain or B-symptoms (night sweat, fever, weight loss) [6]. These non-specific symptoms often lead to delayed diagnosis and, consequently late start of the proper antimicrobial therapy. The average delay in accurate diagnosis ranges between 2 and 6 months after the onset of symptoms [1]. Delayed diagnosis can lead to a highly acute course of the disease, causing severe complications such as sepsis, neurological deficits or organ failure and may even result in death. Patients suffering from acute VO have been reported to be hospitalized for 30 to 57 days with a mortality rate of 2 to 20% [1,4,7-9]. Hence, the development of laboratory tools for early diagnosis is important to improve the management of VO.

3.1.1. Epidemiology and Pathogenesis

The incidence of VO in Europe ranges from 0.4 to 2.4 per 100,000 individuals and is positively correlated with advanced age. There are two distinct peaks regarding the incidence, the first peak occurring in patients younger than 20 years (0.3/100,000) and the second peak in patients over the age of 70 (up to 6.5/100,000), with the latter being more prevalent. Among patients over 70 years of age, male patients exhibit a higher incidence rate than females, with a ratio of 1.5-2:1. This gender disparity may be due to a greater prevalence of comorbidities in older male patients [7,8].

According to a 14-year retrospective study conducted in Denmark, the incidence of VO has increased in recent years [10]. This increase may be attributed to improved diagnostic methods, increased surgical interventions in older patients or other risk factors identified in previous studies [8,9,11]. The incidence of post-operative VO ranges from 0.5% to 18.8% and varies depending on the surgical techniques employed [7].

VO has various etiologies, with the three primary mechanisms being: 1) hematogenous dissemination from an existing infection in the body, 2) extension of an infection from surrounding structures, and 3) iatrogenic infection, particularly in cases involving surgery and the implantation of foreign objects [8,9,11]. Hematogenous spread is the most frequent route of infection, which depends on the individual patient's comorbidities and risk factors, including

conditions such as diabetes mellitus, advanced age, multimorbidity, obesity, intravenous drug addiction, immunodeficiency, cancer, HIV, chronic kidney disease or liver cirrhosis [1]. Bacterial endocarditis is also a significant trigger of hematogenous spread since infected patients have an increased risk of up to 20% for developing VO and 33.3% of VO patients may develop bacterial endocarditis [7,11,12]. Mylona et al. [13] conducted a study involving 1,008 patients and found that urogenital infections were the most common trigger, accounting for 17% of cases, followed by endocarditis with 12%, which corresponds to the previous findings [8,14]. Skin/soft tissue infections were also identified as a significant factor, accounting for 11% of cases [14]. In complicated cases, VO may lead to inflammation of the surrounding tissue and the development of abscesses. A retrospective study by McHenry et al. involving 253 VO patients showed that 17% of patients developed epidural abscesses and 26% had a paravertebral abscess. The study further revealed that 15% of patients developed VO after undergoing spinal surgery [15].

3.1.2. Microbiology

VO is classified into two categories: specific and non-specific. The former is caused by an endogenous *Mycobacterium tuberculosis* infection and accounts for the majority of cases worldwide, the spine being the most commonly affected site [1,8]. VO caused by *Mycobacterium tuberculosis* is more frequently diagnosed in younger patients and in patients from developing countries, particularly Russia [4]. In contrast, non-specific VO is more prevalent in Europe and is predominantly associated with a monomicrobial infection with less than 10% of cases resulting from polymicrobial infections [4]. The main causative pathogen of non-specific VO is *Staphylococcus (S.) aureus*, responsible for 30 to 80% of infections [1,4,7,8,11].

Gram-negative bacteria including *Escherichia (E.) coli* or *Pseudomonas (P.) aeruginosa* have also been reported as causative pathogens of VO, accounting for 7 to 33% of infections [7,8]. Especially, *P. aeruginosa* is often found in patients with intravenous drug addiction [4].

Up to 20% of the infections are caused by gram-positive bacteria such as *Streptococci* and *Enterococci.* These infectious agents are often associated with patients with diabetes mellitus, bacterial endocarditis or after dental treatment [4,7] . Gram-positive, coagulase-negative staphylococci (CoNS) bacteria are responsible for 5-16% of these infections, with *S. epidermidis* being the most identified pathogen. Furthermore, *S. epidermidis* is frequently associated with infections that occur following surgical procedures and the implantation of foreign objects [4,7,8].

Anaerobic bacteria are responsible for a relatively small proportion of infections, comprising less than 4% of cases. Specifically, *Propionibacterium acnes* is associated with post-operative inflammation resulting from the implantation of foreign objects [4]. In contrast,

fungal infections account for only 1 to 3% of all cases of VO and are typically observed in immunocompromised patients due to malignant diseases, infectious diseases or pharmacologically induced immunosuppression [4].

3.1.3. Clinical Presentation

At the beginning of VO, patients present with non-specific symptoms, which is typical of this disease. Mylona et al. reported that 85% of patients experience back pain as the most common initial symptom [13], with back pain peaks occurring at night [7]. Depending on which segmental section of the vertebral column is affected, the pain can be detected at different locations. The most common location of infection is the lumbar section with 58% of all cases, followed by the thoracic section with 30%. The cervical spine is rarely involved, accounting for only 10% of cases [4,8]. In most cases, the back muscles are more rigid and tense. Therefore, twisting of the upper body is painful and restricted [11,16]. Physical examination findings such as pain on percussion or pain on compression may suggest the presence of a VO [14].

Fever is a common symptom of VO, with approximately 50% of patients reporting its onset. The use of painkillers may influence the presence of fever in affected patients [8,16]. One-third of cases also exhibit neurological symptoms, resulting from epidural abscesses, which compress the spinal cord and lead to motor weakness, sensitivity disorder, paralysis, and sphincter dysfunction. The severity and location of these symptoms vary depending on the location of the abscess. Factors influencing abscess formation include diabetes mellitus and higher age [8,11,15]. In some cases, patients may experience a poor disease progression, leading to the subsequent development of B-symptoms [6]. In highly acute cases, VO can lead to significant pain and fever peaks, thereby causing severe suffering [7,14]. Conversely, up to 15% of patients do not report pain [17], demonstrating the non-specific and atypical nature of this disease.

3.1.4. Diagnosis

Since symptoms are non-specific and atypical, many differential diagnoses such as erosive osteochondrosis, degenerative spinal disease and disc herniation should be considered. Additionally, VO may present similarly to other diseases such as osteoporotic fractures, destruction caused by tumors or metastatic seeding and Bechterew's disease [1,7].

Accurate diagnosis of VO requires a comprehensive evaluation that includes detailed anamnesis, physical examination, body temperature measurement and laboratory tests [4]. C-reactive protein (CRP) is a commonly used diagnostic marker for infection, with a sensitivity of 98 to 100% [8,18] but it is important to pay attention to specificity since CRP levels are also elevated in non-infectious diseases such as cancer [8].

Medical imaging can determine the extent of VO. Radiographs are easy to perform and useful for monitoring disease progression and detecting other differential diagnoses such as osteoporotic fractures or tumorous processes [14]. However, this method is not appropriate for early detection of VO. Magnetic resonance imaging (MRI) is the method of choice for early diagnosis due to its high sensitivity and accuracy of approximately 90% [8,19]. MRI can reveal bone oedema of the adjacent vertebral bodies and hyperintensity of the disc in a T2-weighted image in the early stages of the disease. Furthermore, it can detect potential abscesses and determine the degree of infection in the entire spine [20]. Contrast media such as Gadolinium can enhance the specificity of MRI and enable better differentiation between infection, degenerative process, and tumors [9,21,22]. In cases, where MRI is contraindicated, such as in patients with pacemakers or other magnetic implants, computer tomography (CT) imaging is necessary. CT imaging can provide detailed assessment of bone structure regarding stability and destruction of the spine. It is also useful via CT-guided biopsy to detect the pathogen and release the abscess [1,19].

Positron emission tomography (PET) with fluorine-18 fluorodeoxyglucose (F-18 FDG) is a promising method for detecting VO with a lower radiation dose than other imaging techniques [1,23]. Stumpe et al. suggested in a study with 38 patients that F-18 FDG PET could be advantageous over MRI in differentiating between VO and degenerative changes in the spine [24]. Another study involving 57 patients with suspected spinal infections after surgery showed an overall accuracy of 68% and a negative predictive value of 100% for F-18 FDG PET [25].

In case of suspicion of VO, it is crucial to obtain a minimum amount of 2 to 3 blood cultures within 24h to rapidly detect the causative pathogen [1]. Positive blood culture results were observed in 30 to 78% of cases according to a review by Mylona et al. [13]. Blood cultures should be obtained in periods without fever. It is important to note that preceding antibiotic therapy can negatively influence the results of blood cultures. Therefore, antibiotic therapy should be initiated after pathogen detection tests [8] except for unstable patients with sepsis or severe neurological symptoms who require immediate antibiotic therapy [22].

If blood culture fails to detect the pathogen, an invasive biopsy should be performed [1,8]. The most common one is the CT-controlled biopsy, which is superior to blood cultures, with a diagnostic accuracy of 47 to 100% [7]. Due to the small amount of tissue sample, only 50% of CT-controlled biopsies result in pathogen detection [1]. The greatest amount of tissue and therefore, the best pathogen detection can be performed with an intra-operative tissue sample since 68 to 92% of pathogens can be detected with this method [26,27].

In cases of negative biopsy results due to early antibiotic treatment, the polymerase chain reaction (PCR) can be used to detect the pathogen although it is more vulnerable to

contamination [8,28]. Rapid diagnosis using these methods allows for early targeted antibiotic therapy.

Scharrenberg et al. recently found that the biomarker suPAR (soluble urokinase-type plasminogen activator receptor) is more specific than CRP in distinguishing VO from degenerative spinal disease. The combination of suPAR with CRP further increases the diagnostic potential [29]. In the latest research, Brinkmann et al. identified four cytokines (IL-6, IL-8, IL- 12 (p70) and VEGF) as potential biomarkers for differentiating VO from degenerative spinal disease [30].

3.1.5. Therapy

The most important goals of therapy are to cure the infection, relieve pain and restore stability of the spine. Currently, there are two main therapeutic approaches available: conservative treatment and surgical intervention [9]. So far, there is no prospective randomized controlled study concerning which therapy is superior [31]. A conservative approach requires an uncomplicated course and contraindications for surgery. The common opinion is that cases with a serious course involving severe neurological or hemodynamic symptoms, instability of the spine and failure of the conservative treatment indicate surgical intervention [1,11,32,33].

Initial therapy involves hospitalization, immobilization of the spine, specific calculated antibiotic treatment and adequate analgesia. Intravenous administration of antibiotics should last at least six weeks [14]. Michiels et al. recommended Cefepim, Flucloxacillin with Ceftriaxon or Acylamminopenicillin with a beta-lactamase-inhibitor for the initial intravenous therapy [14]. However, the choice of the appropriate antibiotic depends on the results of the detection of the pathogen and individual antibiogram. Later, oral antibiotics with good bioavailability such as Quinolones or Lincosamides can be used [8,14,22]. Successful conservative therapy can be monitored by periodic laboratory checks and medical imaging. The erythrocyte sedimentation rate (ESR) is the most suitable laboratory marker because normal ESR indicates complete recovery [14,34].

Surgical intervention focusses on removal of the infected areas, drainage of abscesses, decompression of neural structures and stabilization and reconstruction of the spine. The affected spinal area determines the anatomical approach; for example, an anterior approach is used for the thoracic part, while a retroperitoneal approach is often chosen for the lumbar part. Spine fusions such as posterior lumbar interbody fusion (PLIF), transforaminal lumbar interbody fusion (TLIF) or anterior lumbar interbody fusion (ALIF) are commonly performed, and titanium cages are often used for vertebral replacement [14,16]. This intervertebral fusion method allows for faster post-operative mobilization and shorter hospitalization times compared to the conservative treatment [1,14]. The surgical intervention can be performed as a

one-stage or two-stage procedure, the latter being performed in patients in poor physical condition who require a recovery period between operations [1,34,35].

3.2 Osteochondrosis intervertebralis

Osteochondrosis intervertebralis describes a progressive form of changes in the vertebral bodies, where erosive osteochondrosis describes a far advanced stage. These changes result from a degenerative loss of function of the adjacent intervertebral disc, which promotes pathological load distribution of the spine. As a result, erosion and structural changes of the surrounding tissue occur, leading to reduced disc space and compression of the nerve canal, which can cause severe back pain [36-38]. When conservative methods fail to relieve pain, surgical spine fusion of the affected spinal area is the last option [39]. Notably, osteochondrosis shows similar symptoms and requires the same surgical therapy as VO.

Osteochondrosis can be classified into three groups according to radiological parameters as conceived by Modic and Ross [36]. This concept describes the different changes in the adjacent vertebral bone marrow, excluding the presence of tumors, other malignancies, or seropositive rheumatic diseases. These groups can be distinguished by their different signal intensity in T1-weighted and T2-weighted images. Modic changes type 1 (MC1) represent the acute form of osteochondrosis and are associated with inflammation of the bone marrow with surrounding oedema. MC1 changes appear hypointense in T1-weighted images and hyperintense in T2-weighted images. Modic changes type 2 (MC2) are hyperintense in T1-weighted sequence and hyper- or isointense in T2-weighted sequence, indicating a fatty degeneration. Modic changes type 3 (MC3) are hypointense in both sequences and present sclerotic realignment [36,40,41]. It is important to note that MC1 changes in the early stages can resemble VO, making it a very important differential diagnosis [42]. Furthermore, MC1 changes are associated with inflammation of the bone marrow and may lead to an increase of inflammatory characteristics, similar to those seen in VO. Therefore, it can be difficult to determine the exact cause of the inflammation [40].

3.3 The human immune system

The human immune system is divided into two classes: the non-specific innate immunity and the specific acquired immunity. Both types co-operate to protect the body against pathogenic infections. The innate immunity consists of various systems that collaborate to combat pathogens via both physical barriers such as the skin and mucosa and functional barriers such as soluble factors like cytokines and acute phase proteins. In addition, components of the innate immune system include the complement system as well as immune cells such as granulocytes, monocytes, macrophages, natural killer cells (NK-cells), and

dendritic cells (DCs) [43]. The second important part of the immune system is the specific acquired immunity, which consists of secretory antibodies, cytokines and especially, lymphocytes (T- and B-cells). This type of immunity functions through two phases: the induction phase and the effector phase [43,44].

Cytokines are a class of proteins that play a crucial role in regulating cell proliferation and differentiation and are integral components of the immune system. They contribute to immunity and inflammation in various tissues and promote the differentiation of immune cells such as lymphocytes and antigen-presenting cells (APCs) including DCs, monocytes and macrophages [43]. Moreover, cytokines co-ordinate the communication between immune cells and non-immune cells. The biological function of each cytokine depends on the target cells and their corresponding receptors. Cytokines can be classified into different groups based on their specific actions such as interleukins (IL), growth factors, chemokines, interferons and tumor necrosis factors [45,46].

When a pathogen enters the body, the innate immune system provides the first line of defense, primarily through the mononuclear phagocyte system consisting of mobile monocytes and macrophages. These cells recognize the presence of the pathogen via pattern recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs) on the surface of the pathogen. PRRs can be found soluble, membrane-bound or in the cytoplasm. Soluble PRRs bind to the pathogen surfaces, facilitating phagocyte recognition, a process known as opsonization. This process is aided by various proteins including surfactant-proteins, antibodies, and complement factors. Membrane-bound PRRs, on the other hand, detect pathogens directly without previous opsonization. Once the pathogen is recognized, phagocytes initiate destruction by phagocytosis, whereby the pathogen is engulfed and trapped in a phagosome. Lysozyme and proteases are produced, creating a bacteriostatic environment inside the phagosome, leading to pathogen destruction. During this phase, regulatory molecules are released, intensifying the inflammatory response. Prostaglandins, leukotrienes and complement factors produced by macrophages increase fluid influx into the vessels, thereby raising the concentration of leukocytes. Furthermore, macrophages produce various cytokines such as tumor necrosis factor (TNF), which promote inflammation by increasing vascular permeability fluid influx, thrombocyte stimulation, and activation of immune cells such as NK-cells and dendritic cells. In addition, IL-8 is produced, attracting neutrophil granulocytes, and IL-12 stimulates NK-cells [43,44,47].

Acquired immunity is an immune response that develops over time due to exposure to various pathogens or foreign substances. Its main function is to protect the body against specific pathogens by producing a targeted response. This immune response occurs in two stages. During the induction phase, antigen-presenting cells (APCs) such as dendritic cells (DCs) inform lymphocytes about an infection. Upon interaction with the corresponding antigen

receptor, lymphocytes are activated and develop into effector cells. In the effector phase, the cell-mediated component of the immune response contributes to pathogen elimination by triggering programmed cell death of infected cells. Simultaneously, the humoral component produces specific antibodies directed against the pathogen [44].

After recovery from an infection, specific antibodies as well as memory B- and T-cells work together to provide a faster and more effective immune response when the same pathogen reappears. Memory T-cells can recognize and destroy infected cells more quickly than during the primary immune response. Memory B cells can quickly produce the specific antibodies that successfully neutralized the pathogen during the initial infection. [47].

3.3.1. Peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) include several types of leukocytes such as T- and B-cells, NK-cells, macrophages, monocytes and DCs [48,49]. Lymphocytes represent 25 to 40% of the leukocytes in the peripheral blood and are classified into three main groups: T-cells, B-cells, and NK-cells. T- and B-cells belong to the acquired immune system and specifically recognize antigens [43]. Each cell type possesses various distinguishing characteristics such as cell-specific membrane-bound glycoproteins known as cluster of differentiation molecules (CD molecules). The expression of CD molecules on cell surfaces allows for the identification and differentiation of cell populations [43,50]. Immunofluorescence methods can be used to analyze these cells, and targeted therapies via mononuclear antibodies can be developed based on these findings. All leukocytes express CD45 on their surface but individual cell types exhibit additional specific surface markers. For instance, T-cells express CD3 molecules involved in signaling pathways activation, while B-cells express CD19 molecules that function as bridge molecules to transport cytoplasmatic signaling proteins to them [50].

T-cells form the cell-mediated part of the acquired immune system. Their main function is to recognize and respond to specific foreign antigens presented by antigen-presenting cells (APCs) such as dendritic cells and macrophages. There are two main types of T-cells: $CD8^+$ cytotoxic T-cells and $CD4^+$ T-helper-cells. T-cells are activated and differentiate into effector cells depending on the type of cell they communicate with such as macrophages, B-cells, neutrophil or eosinophil granulocytes [44,51].

Naïve $CD8^+$ T-cells are activated when their T cell receptors (TCRs) recognize specific antigens presented by major histocompatibility complex (MHC) class I molecules on the surface of infected cells. These antigens are fragments of proteins that are produced by the infecting pathogen. This process known as antigen presentation leads to the development into effector cytotoxic T-cells, which can destroy infected cells and differentiate into T-memory cells that strengthen the immune response in case of a future infection with the same antigen.

Furthermore, naïve $CD4^+$ T-cells are activated through antigen presentation and stimulation by cytokines such as IL-2, IL-12, and IL-18, leading to the development into active T-helper cells that produce IL-2 and IFN-γ. The latter acts as a growth and differentiating factor for T-helper cells and promotes the development of $CD8^+$ T-cells [44].

B-cells form the humoral immune response by recognizing foreign structures and producing antibodies against them, thereby receiving a co-stimulatory signal from T-helper cells. These processes are regulated by various cytokines such as IL-4, IL-5, IL-13, and IFN-γ. IL-6 works as a growth factor for mature plasma cells. Upon activation, B-cells differentiate into plasma cells that produce antibodies that bind to the pathogens and mark them for destruction by cell-mediated defense mechanisms. Additionally, a subset of B-cells develop into memory B-cells that provide rapid and specific antibody-mediated protection upon a subsequent exposure to the same antigen [43,44,52].

Natural killer (NK) cells account for 10 to 15% of the lymphocytes in the peripheral blood [43]. They are larger in size than other lymphocytes and express the surface molecule CD56. NK-cells produce granules and cytokines, and their primary role is to eliminate altered cells such as those infected with viruses, intracellular bacteria or cancer cells. Similar to cytotoxic T-cells, NK-cells can directly kill infected cells by releasing cytotoxic molecules such as perforin and granzymes. NK-cells are activated by various cytokines such as IL-2, IL-15, TNF, and IL-12. The specific cytokines produced by NK-cells can vary depending on the type of infection or stimulus. NK-cells can produce pro-inflammatory cytokines such as IFN-γ, TNF and chemokines, including CXCL8/IL-8 and CCL3, as well as cytokines that limit inflammation such as IL-10. NK cell-mediated cytotoxicity is particularly important in the early stages of viral infections before the acquired immune response has time to develop. Additionally, NK-cells play a crucial role in controlling cancer by recognizing and eliminating tumor cells [43,44,53].

Monocytes are part of the mononuclear-phagocytic system, which functions in the breakdown of infected cells and cellular waste, antigen presentation, and T-helper cell activation via cytokine production. Monocytes account for 1-6% of leukocytes in peripheral blood [43]. When monocytes encounter an infection or injury, they migrate to the affected area and differentiate into macrophages, specialized cells that engulf and digest foreign substances. Furthermore, monocytes secrete various substances involved in the immune response and inflammation, including lysosomal enzymes for phagocytosis, complement factors, reactive oxygen metabolites and cytokines such as IL-1, IL-6, IL-10, IL-12, chemokines, TNF, colony-stimulating factors, TGF-β, and IFN [44,52]. Among these cytokines, IL-1 is an endogenous pyrogen that contributes to the fever response and activates T-helper cells as well as stimulates the production of acute phase proteins, fibrinogen, collagenase, and prostaglandins, acting as an inflammation mediator. Recognition of apoptotic cells by macrophages triggers an anti-inflammatory response, which is mediated by the release of IL-10. IL-10 production by

monocytes is often associated with modulation of the immune response and the suppression of inflammation [54]. In addition to their function in the immune response, monocytes also contribute to tissue repair and regeneration by secreting growth factors and cytokines that stimulate new blood vessel growth and tissue formation [55].

Dendritic cells (DCs) are a type of phagocytic cell that can differentiate from monocytes or the precursors of T- and B-cells. DCs account for less than 0.1% of leukocytes in peripheral blood and resemble monocytes in size and appearance [43]. Their most important function is antigen recognition and presentation, which triggers the acquired immune response. Therefore, DCs express various surface receptors that enable them to detect, ingest, and present pathogens to other immune cells. DCs produce several cytokines such as IFN α/γ, IL-2 and IL-6 [43,52]. Unlike other antigen-presenting cells, DCs can produce IL-12 at an early stage of the immune response and hence, initiate the differentiation of T-cells. This property makes DCs a crucial mediator of the immune response, bridging innate and acquired immunity [43].

3.3.2. Cytokines

The present study focuses on four different cytokines: interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12 (p70)) and vascular endothelial growth factor (VEGF) as well as the PBMCs responsible for their production. These cytokines have been identified by our research group as potential biomarkers for differentiating between VO and *osteochondrosis intervertebralis* [30].

The main function of IL-6 is to promote inflammation and immune response. The pleiotropic cytokine is produced by various immune and non-immune cells such as B- and T-lymphocytes, fibroblasts, endothelial cells, mononuclear phagocytic cells, mast cells and mesenchymal stromal cells. IL-6 has various effects on different cells and processes including the activation of the production of acute phase proteins such as CRP, fever progression, and the differentiation of B-cells into plasma cells. The latter activates the production of immunoglobulins and helps to intensify the immune response. Furthermore, IL-6 can also lower the amount of anti-acute phase protein such as transferrin and albumin [43,49,56]. Due to its strong impact on pro-inflammatory processes, IL-6 is often measured in laboratory tests to assess the extent of inflammation and immune response [57]. Elevated levels of IL-6 are associated with a variety of acute and chronic inflammatory diseases such as rheumatoid arthritis, sepsis, and inflammatory bowel diseases. IL-6 plays an increasing role in the development of autoimmune diseases and chronic inflammation and offers approaches for therapeutic options such as IL-6 receptor blockades. Antibodies against the IL-6 receptor such as Tocilizumab are approved for the treatment of rheumatoid arthritis and show excellent efficacy and tolerability. These antibodies bind to the IL-6 receptor and inhibit IL-6 signaling [56,58].

IL-8, also known as CXCL8, belongs to the group of chemotactic cytokines that is produced by various cells such as macrophages, T-cells, monocytes, and fibroblasts. IL-8 plays a significant role in inflammation as a major mediator, responsible for the chemotactic recruitment and migration of neutrophils to the site of infection. Additionally, IL-8 is involved in angiogenesis, the process of forming new blood vessels in the vascular system, by promoting the proliferation and migration of endothelial cells. IL-8 has been implicated in various pathological conditions including chronic inflammatory diseases and cancer [43,46,48,49].

IL-12 is responsible for the immunological defense against bacterial, viral, and parasitic infections caused by intracellular pathogens. It is mainly produced by APCs such as monocytes, DCs, macrophages as well as by B and T-cells. The primary function of IL-12 is to activate NK-cells and T-cells as well as to promote the differentiation of naïve T-cells into T-helper type-1 cells and facilitate their development into cytotoxic cells. Upon activation, these cells produce cytokines such as IFN-γ and TNF that trigger the activation of macrophages and NK-cells, resulting in a robust cell-mediated immune response. IL-12 also increases antibody production by B-cells and promotes the development of memory T-cells, which provide long-term protection against recurrent infections [43,49,59]. IL-12 (p70) is the biologically active form of IL-12 and is therefore measured in multiplex cytokine assays [60].

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a signaling protein that mediates the development of the vascular system. It plays a significant role in embryonic vasculogenesis and regulates angiogenesis in adults by promoting the growth and proliferation of endothelial cells that line the blood vessels. This function allows the formation of new blood vessels, which is important for normal development, wound healing and tissue repair [61-63]. VEGF reacts to hypoxia by inducing neovascularization, a mechanism that has also been observed in tumor cells [64]. While VEGF primarily activates mainly endothelial cells, it also stimulates the migration of monocytes and macrophages [65,66]. Several therapeutic approaches such as monoclonal antibodies (e.g., Bevacizumab) have been developed to target VEGF and block its activity. These therapies are commonly used to treat cancer, eye diseases or even in cardiovascular medicine [63,67].

3.4 Flow cytometry

Flow cytometry is a high throughput method for analyzing cell sub-populations. In this technique, individual cells are rapidly passed through a light beam, which generates scattered light patterns reflecting cell properties such as size and morphology. These patterns enable the identification and characterization of specific cell sub-populations based on their distinct features. Furthermore, the relative proportion of each sub-population can be quantified [68,69].

A specialized form of flow cytometry, known as fluorescence activated cell sorting (FACS) was invented in the late 1960s and later commercialized by Becton Dickson. FACS

uses fluorescently labelled markers such as antibodies, which link specifically to their cell surface antigen. The fluorescence intensity of the labelled cells is proportional to the quantity of the targeted cell type [70-72].

3.5 Aim of this study

The term 'CyProSpon' refers to the study entitled 'Cytokine Profiles in Spondylodiscitis', which aims to develop a more accurate diagnostic method for patients with spondylodiscitis, referred to in this thesis as vertebral osteomyelitis (VO). Diagnosis of VO is often delayed, leading to an elevated risk of severe complications, and the need for surgical intervention is high. In a previous publication of CyProSpon data, soluble urokinase plasminogen activating receptor (suPAR) was found to be superior to CRP in diagnosing VO [29]. In another publication of data from the CyProSpon study, the cytokine profiles of patients with VO were analyzed via multiplex cytokine assays and compared to a control group of patients without an infection [30]. For the interpretation of the results and to extend the knowledge about this disease, it is important to obtain an overview of the cytokine-producing cells, namely the PBMCs. We wanted to gain an understanding of the immune cell profile responsible for cytokine production, along with the associated changes in immune cells during vertebral osteomyelitis, thereby enabling a clear differentiation between vertebral osteomyelitis and degenerative spinal diseases. To date, there are no reports about cytokine-producing PBMCs in patients with VO. Based on cytokine profile analyses, four cytokines (IL-6, IL-8, IL-12 (p70); further referred to as IL-12 to simplify readability, and VEGF) were selected for PBMC measurement. These cytokines showed significant differences between both study groups and were identified as potential markers for discriminating between VO and *osteochondrosis intervertebralis* [30]. In this study, we employed flow cytometry to identify the cell populations responsible for producing the four chosen cytokines. To this end, the cells were labelled with fluorescent cytokine markers and analyzed via FACS to quantify the cytokine-producing cell sub-populations. The proportion of cells producing each cytokine was compared between each patient group to determine if it is possible to differentiate between VO and degenerative spinal diseases.

4. Material and Methods

4.1 Study design

The single-center case-control study was conducted by members of the Center for Molecular Medicine Cologne, University of Cologne (CMMC) in collaboration with the Orthopedic Department and the Department for Internal Medicine I of the University Hospital of Cologne. The study consisted of two parts: a clinical part including recruitment, patient information and blood draws and was conducted at the University Hospital of Cologne as well as an experimental part performed at the laboratories of the CMMC. The active recruitment phase took place from 2015 to 2017 while the experimental phase continued until 2019.

The Declaration of Helsinki (seventh revision, 2013) for ethical performance in studies including human patients was fulfilled. Furthermore, the study was performed according to the German national guidelines of “good clinical practice” and “good laboratory practice” (GCP-V, Federal Ministry of Justice, Berlin, Germany, last revision 2012). Permission was granted by the Ethics Committee of the University of Cologne (Trial protocol: Uni-Köln 9-2014) and the study was registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT02554227). The ethical agreement was extended two times during the clinical part of the study. The first time, the age range of the participants was increased from 80 up to 85 years and the thoracic part of the spine was added to the defined affected areas. The second adjustment allowed the measurement of suPAR (doctoral thesis of Jan Scharrenberg). Both modifications were permitted by the Ethics Committee of the University of Cologne. All patient data and samples were anonymized.

4.2 Study population

There were various criteria for the inclusion of a patient in the CyProSpon study. At first, all patients needed a medical indication for an operative stabilization of the spine due to vertebral osteomyelitis or erosive osteochondrosis (Modic changes type 2/3). Based on these two diseases, the study population was divided into two groups: the VO group and the erosive osteochondrosis group as the control group. The affected area was defined as a thoracic or lumbar vertebral part and should involve removal of the intervertebral disc. The groups consisted of male and female patients, and their ages ranged from 40-85 years. Additionally, all the patients gave a signed declaration of consent, indicating their voluntary participation in the study.

The exclusion criteria were patients with osteochondrosis having Modic changes type 1 and patients with autoimmune diseases, chronic infections such as HIV, Hepatitis B and C or other acute infections besides the spine or active cancer.

All patients underwent surgical treatment of the spine in the form of an interbody fusion such as PLIF, TLIF, ALIF or a decompression. Additionally, the removed intervertebral disc was replaced by a cage. The surgical interventions were fulfilled under standard general anesthesia and all patients were intubated. All control patients received an antibiotic prophylaxis pre-operative in form of a cephalosporin of the first or second generation (Cefazolin or Cefuroxime). The antibiotic treatment of the VO group varied widely.

Out of 98 patients screened, 36 patients were included in the study. In total, 16 patients were allocated to the VO group (Patient ID 1-16), and the control group (C = control group, Patient ID 21-40) consisted of 20 patients. The mean age in both groups was comparably high with an average age of 67.1 years (68.8 years in the VO group vs. 65.8 years in the control group). The proportion of male patients in the VO group was higher than that in the control group (62.5% vs. 45.0%) and the proportion of female patients was higher in the control group (55.0% vs. 37.5%). All patient data including age and gender are shown in Table 1.

Table 1: Epidemiological data of the study population including age and sex

	VO	**Control**
No. of patients	16	20
Male	10 (62.5%)	9 (45.0%)
Female	6 (37.5%)	11 (55.0%)
Average age (range) in years	68.8 (53-85)	65.8 (52-80)
Age median (IQR)	71 (61-76)	65 (59-73)

Tables 2 and 3 provide a detailed overview of the study group including relevant comorbidities and lifestyle factors at the time point of inclusion.

Table 2: Demographic and clinical features of the vertebral osteomyelitis patients

Patient			Blood draw		Relevant comorbidities and lifestyle factors
No.	Age (years)	Gender (m/f)	pre-OP (days)	post-OP (days)	
1	76	m	0	n.a.	None
2	79	m	0	n.a.	HT, type 2 DM, CHD, ischemic cardiomyopathy with low left-ventricular function, nicotine abuse (20 PY)
3	58	f	-1	48	HT, type 2 DM, paroxysmal atrial fibrillation, obstructive sleep apnea syndrome, hypothyreosis, hepatic steatosis, chronic renal insufficiency, M. Meulengracht, obesity (BMI 35)
4	66	m	-1	50	HT, type 2 DM, diabetic nephropathy, obstructive sleep apnea syndrome, obesity (BMI 31), Ex-nicotine abuse (45 PY)
5	71	m	-1	43	HT, paroxysmal atrial fibrillation, middle grade aortic stenosis and low-grade insufficiency, hypothyroidism
6	53	f	-1	50	Nicotine abuse (15 PY)
7	68	f	-14	43	HT
8	75	f	0	56	HT, CHD, obesity (BMI 31)
9	63	m	0	42	Regular consumption of alcohol
10	71	m	0	47	HT, regular consumption of alcohol
11	54	m	0	41	Obesity (BMI 31), nicotine abuse (30 PY)
12	72	f	0	47	HT, obesity (BMI 32)
13	59	m	-8	40	HT, type 2 DM, regular consumption of alcohol
14	77	m	-9	41	HT
15	85	m	-20	n.a.	HT, atrial fibrillation, hypothyroidism
16	73	f	-1	n.a.	HT, type 2 DM

m: male, f: female, BMI: body mass index, CHD: coronary heart disease, DM: diabetes mellitus, HT: hypertension, PY: pack year, pre-OP: before surgery, post-OP: after surgery, day of surgery = day 0, n.a. = complete blood draw missing

Distribution of comorbidities and lifestyle factors:
HT: 12 (7m/5f); type 2 DM: 5 (3m/2f); nicotine abuse: 4 (3m/1f); alcohol: 3 (3m/0f); obesity: 5 (2m/3f)

Table 3: Demographic and clinical features of the control patients

Patient			Blood draw		Relevant comorbidities and lifestyle factors
No.	Age (years)	Gender (m/f)	pre-OP (days)	post-OP (days)	
21	80	f	0	42	HT, type 2 DM, hypothyroidism, CHD
22	75	f	-1	42	HT
23	70	f	-1	40	HT, CHD, obesity (BMI 36)
24	74	f	0	48	HT, obstructive sleep apnea syndrome, obesity (BMI 31)
25	78	f	-1	42	HT
26	54	m	0	41	CHD, PAD, nicotine abuse (20 PY)
27	57	f	0	40	HT, nicotine abuse (40 PY)
28	58	m	0	41	HT, atrial fibrillation, obesity (BMI 34)
29	66	m	0	48	HT, gastro-esophageal reflux disease, regular consumption of alcohol
30	63	f	0	43	HT, PAD
31	52	m	0	43	Nicotine abuse (40 PY)
32	63	f	0	44	HT, hypothyroidism, obesity (BMI 33)
33	72	m	-1	40	HT
34	59	f	-1	48	Hypothyroidism, liver cirrhosis, type 2 DM, nicotine abuse (40 PY)
35	77	m	0	44	HT, type 2 DM, CHD, PAD
36	72	f	0	41	Osteoporosis, regular consumption of alcohol
37	60	m	0	43	Obesity (BMI 31)
38	72	f	0	42	HT, type 2 DM, obesity (BMI 31)
39	53	m	-1	41	HT, obesity (BMI 35)
40	61	m	0	n.a.	HT, aortic valve stenosis, hypothyroidism, CHD

m: male, f: female, BMI: body mass index, CHD: coronary heart disease, DM: diabetes mellitus, HT: hypertension, PAD: peripheral artery disease, PY: pack year, pre-OP: before surgery, post-OP: after surgery, day of surgery = day 0, n.a. = complete blood draw missing

Distribution of comorbidities and lifestyle factors:
HT: 15 (6m/9f); type 2 DM: 4 (1m/3f); nicotine abuse: 4 (2m/2f); alcohol: 2 (1m/1f); obesity: 7 (3m/4f)

The VO diagnosis was confirmed by a completion of microbiological results, clinical presentation, and diagnostic imaging (MRI or, if contraindicated, CT). Special interest was directed to the identification of the causative pathogen. Therefore, blood cultures and tissue biopsies were taken. In 25% of the patient cases, an infection with *Staphylococcus aureus* was detected. Furthermore, *S. epidermidis* and *E. coli* were identified as the causative pathogen in 25% of the cases. Two cases (12.5%) showed an infection with *Streptococcus dysgalactiae* and in one patient case (6.3%), a co-infection with *S. aureus* and *E. coli* was verified. All patients were treated with an appropriate antibiotic therapy in co-operation with the Department of Infectiology of the University Hospital of Cologne. Table 4 shows the individual microbiological results for each VO patient.

Table 4: Microbiological results for all VO patients and antibiotic treatment

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

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

4.3 Blood sampling and PBMC isolation

4.3.1. Blood draws and CRP measurement

All blood samples were taken at the Orthopedic Department of the University Hospital of Cologne. Blood was drawn from each patient before surgery (pre-OP) and 40-56 days after surgery (post-OP). The first time point was during the stationary period of the patients and time point two was during routine check-ups at the Department of Orthopedics, University Hospital of Cologne. A standard version of blood drawing was used. A peripheral vein of each fasting patient was punctured with a maximum duration of the stasis of 2 minutes. Blood was collected in monovettes filled with lithium heparin (Sarstedt, Nümbrecht, Germany). In some cases, an existing central venous catheter was used. Blood was analyzed in the Central Laboratory of the University Hospital of Cologne for standard parameters including C-reactive protein (CRP). Therefore, an immunoturbidimetric assay (CRP-Latex-Test Gen 3, Roche Diagnostics, Rotkreuz Switzerland) in combination with an automated analysis system (Cobas C 702, Roche Diagnostics, Rotkreuz Switzerland) was used. A CRP concentration above 5 mg/L was classified as pathological.

4.3.2. PBMC Isolation

Blood samples from the patients in the VO group and the control group for PBMC isolation were collected in two EDTA monovettes (S-Monovette®, EDTA KE/9 mL, Sarstedt, Nümbrecht, Germany) and taken to the laboratory at the Center for Molecular Medicine, University of Cologne. Both samples were transferred to a 50 mL Falcon™ centrifuge tube (Corning, New York, USA) and filled up with Dulbeccos Phosphate Buffered Saline (PBS, ThermoFisher Scientific, Waltham, USA) until a volume of 50 mL was obtained. Two 50 mL Falcon™ centrifuge tubes were filled with 15 mL Biocoll Separating Solution (Sigma Aldrich, St. Louis, USA) each. The 50 mL blood/PBS-mix was divided in two and carefully overlayed the Biocoll fluid. Both tubes with Biocoll and an upper layer of blood/PBS-mix were immediately centrifuged (EBA 20 Centrifuge, Hettich Lab Technology, Tuttlingen, Germany) for 20 minutes at 2,000 rpm with minimal brakes at 21°C. The interphase from both tubes was removed and transferred to a new 50 mL Falcon™ tube. Afterwards, it was filled up to 45 mL with PBS and centrifuged for 10 minutes at 1,400 rpm and 21°C. The supernatant was removed, and the pellet was re-suspended in 10 mL PBS.

If red blood cells were visible in the cell pellet, the pellets were treated with BD FACS™ Lysing Solution (Becton Dickinson, Franklin Lakes, USA). Therefore, 200 µL BD FACS™ Lysing Solution and 1800 µL deionized H_2O were mixed and added to the cell pellet and re-suspended. Tubes were filled up with 10 mL PBS and again centrifuged for 10 minutes at 1,400 rpm and 21°C. The supernatant was removed, and the pellet was re-suspended in 10 mL PBS.

A volume of 10 µL was removed and mixed with 90 µL 0.4% Trypan Blue Solution (ThermoFisher Scientific) to count the number of cells with the use of a BLAUBRAND® Neubauer counting chamber (OptikLabor, Lancing, England). The aim was to freeze $5x10^6$ cells per mL. Therefore, the 10 mL PBS-liquor was divided to produce the corresponding number of aliquots and centrifuged for 10 minutes at 1,400 rpm and 21°C. Subsequently, the supernatant was removed, and the pellet was re-suspended in 1 mL freezing medium consisting of 20% Dimethylsulfoxide (DMSO, Sigma Aldrich, St. Louis, USA) and 80% fetal calf serum (FCS, ThermoFisher Scientific). The labelled aliquots were frozen slowly at -80°C in storage tubes (NuncTM 1.8 mL, ThermoFisher Scientific) and stored at -150°C until analysis.

4.4 PBMC profiling

4.4.1. Establishing intracellular cytokine staining

To establish a suitable protocol for intracellular cytokine staining, a preliminary experiment was first performed with blood from healthy donors obtained from the blood bank of the University Hospital of Cologne. The procedure established from this preliminary experiment was then used for PBMC profiling of the study cohort. PBMCs were either stained untreated, treated with Monensin/Brefeldin A for 2h (both BioLegend, San Diego, USA) [73,74] or stimulated with lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, USA) for 6h or 24h and then treated with Monensin/Brefeldin A 2h before staining. Afterwards, cells were washed, stained and analyzed by flow cytometry. Monensin and Brefeldin A act as inhibitors of protein transport and thus prevent the secretion of cytokines leading to a brighter signal in flow cytometry [73]. LPS is part of the cell membrane of gram-negative bacteria and should stimulate the PBMCs to produce cytokines [75].

The PBMCs were thawed and counted to attain a density of $3x10^6$ cells/mL. Therefore, the cell suspension was transferred into a 15 mL Falcon™ tube (Corning, New York, USA), 10 mL PBS were added, and cells were centrifuged (EBA 20 Centrifuge, Hettich Lab Technology, Tuttlingen, Germany) for 4 minutes at 1,400 rpm (317 x g) and 21°C. The supernatant was removed, and the cell pellet was re-suspended in 5 mL serum-free Gibco™ AIM V™ Medium (ThermoFisher Scientifc). A volume of 10 µL cell suspension was mixed with 10 µL Trypan Blue Solution to count the number of living cells with the use of an automatic cell counter (Countess II, ThermoFisher Scientific). The individual samples were filled up with medium until a cell density of $3x10^6$ cells/mL was obtained.

A volume of 100 µL containing $0.3x10^6$ PBMCs was added to a 96-well flat bottom plate (Sarstedt, Nümbrecht, Germany). Lyophilized Lipopolysaccharides *E. coli* (LPS 055: B5, Sigma) was dissolved in 1 mL PBS and diluted with medium to a final concentration of 200 ng/mL. When dissolved, 100 µL of the LPS pre-dilution was added to the wells resulting in a

final concentration of 100 ng/mL and the cells were incubated for 6h or 24h at 37°C with 5% CO_2 (Inkubator ED53, Binder, Germany). The wells for PBMCs that were untreated or treated with Monensin/Brefeldin A were filled up with 100 µL AIMV medium before incubation. Monensin/Brefeldin A was added at a ratio of 1:1000 to the appropriate wells for the last 2h of the incubation.

After incubation, cells were harvested and transferred to a new 96-well U-bottom plate (Sarstedt) and centrifuged (Heraeus Megafuge 16R, ThermoFisher Scientific) for 4 min at 1,400 rpm and 4°C. The supernatant was discarded, and cells were washed with 180 µL PBS followed by centrifugation for 4 min at 1,400 rpm and 4°C. Subsequently, cells were resuspended in 50 µL PBS containing a FcR block (Fc receptor binding inhibitor polyclonal antibody, eBioscience™, ThermoFisher Scientific, dilution 1:100 and a live/dead marker (Zombie UV™, BioLegend, 1:200) to block unspecific binding and discriminate live and dead cells. FcR block and live/dead staining was performed for 15 min at 4°C in the dark. Subsequently cells were centrifuged for 4 min at 1,400 rpm and 4°C. Cells were washed with 150 µL cell wash (Becton Dickinson, Franklin Lakes, USA) and centrifuged for 4 min at 1,400 rpm and 4°C and the supernatant was discarded.

We next stained the surface markers using a master mix that contained the antibodies of interest (detailed list of antibodies in Table 5). A volume of 50 µL surface master mix was added to the cells and incubated for 20 min at 4°C. Afterwards, cells were washed twice with 150 µL cell wash and centrifuged twice for 4 min with 1,400 rpm at 4°C.

For fixation, 180 µL fixation buffer (Cyto-Fast Fix/Perm Buffer Set by BioLegend) were added to the cells, which were incubated for 20 min in the dark and RT. This was followed by centrifugation for 4 min at 1,400 rpm and RT after which the supernatant was discarded. In preparation for the intracellular stain, 180 µL permeabilization buffer (BioLegend) were added to the cells, which were subsequently centrifuged for 4 min at 1,400 rpm and RT and the supernatant was removed.

For intracellular staining, two new master mixes containing antibodies against intracellular targets were prepared. The master mix for the first stain included permeabilization buffer and antibodies against IL-8 and IL-12. The mix for the second stain included permeabilization buffer and antibodies against IL-6 and VEGF. A volume of 50 µL of the appropriate master mix was added to the cells and cells were incubated for 20 min in the dark at RT. Afterwards, the cells were washed with 150 µL cell wash and centrifuged for 4 min at 1,400 rpm and RT. As a next step, the supernatant was removed. The cell pellets were re-suspended with 70 µL cell wash and measured on a Cytoflex Lx flow cytometer (Beckman Coulter, Brea, USA).

Table 5: Antibodies for flow cytometric analysis

Article-No.	Fluorochrome	Epitope	Dilution	Clone
Live/Dead staining, FcR-Block				
eBioscience 14-9161-73	FcR block		1:100	
BioLegend #423108	BUV395	Zombie	1:200	
Surface master mix				
BioLegend	PerCp Cy5.5	CD8	1:50	SK1
BioLegend	PECy7	CD56	1:75	5.1H11
BioLegend	AF700	CD3	1:50	SK7
BioLegend	BV785	CD45	1:150	HI30
BioLegend	APC-Fire 750	CD4	1:75	SK3
BioLegend	BV421	CD14	1:50	M5E2
Becton Dickinson	BUV737	CD19	1:50	SJ5C1
Intracellular staining mix 1				
BioLegend #514604	FITC	IL-8	1:25	BH0814
Becton Dickinson #559325	PE	IL-12	1:25	20C2
Intracellular staining mix 2				
BioLegend #501107	PE	IL-6	1:25	MQ2-13A5
R&D System #IC2931A	AF647	VEGF	1:25	23410

4.4.2. Results of the preliminary test

The preliminary experiments implicated use of Monensin/Brefeldin for 2h before intracellular cytokine staining to increase sensitivity and detection while maintaining the natural cytokine production of the cells. An artificial stimulus in the form of bacterial LPS was not performed. In addition, initial experiments of the same cohort by multiplex cytokine analysis indicated secretion of cytokines using untreated serum [30].

Furthermore, DCs were excluded from analysis as various surface markers are needed to define different DC subsets [76]. Also, DCs contribute to less than 0.1% of PBMCs.

4.4.3. Preparation for PBMC staining

After evaluation of the results of the preliminary test, it was decided to treat the PBMCs of the study group with Monensin/Brefeldin A only (1:1000 dilution) and incubate them for 2h before staining. The cells were thawed, counted, adjusted to a density of 1.5×10^6 and plated on a 96-well plate. They were allowed to rest for 30 min before adding Monensin/Brefeldin A. Cells were incubated for an additional 2h. Afterwards, staining as described for the preliminary experiments was performed, including FcR block, live/dead stain, surface stain, fixation, and intracellular staining (see 4.4.1).

4.5 Flow cytometric analysis

The flow cytometry analysis was conducted in the laboratories of the CMMC, University of Cologne with the assistance of the research group of Dr. Hans Schlößer and, especially with Martin Thelen. Flow cytometry data were analyzed with Kaluza Analysis Version 2.1 (Beckman Coulter). The flow cytometry gating strategy of cytokine-producing cells is shown in Figure 1.

Samples containing at least 100 living cells of interest (T-cells, B-cells, NK cells or monocytes were included in the analysis. As such, some patient samples were excluded for either all cell populations or only for one specific cell population as shown in Table 6. Stain 1 and Stain 2 stand for two separate measuring rounds; in Stain 1, IL-8 and IL-12 were measured and IL-6 and VEGF were measured in Stain 2.

Table 6: Excluded patient samples for FACS analysis having less than 100 viable cells of the target population (T-, B-, NK-cells or monocytes)

	Cell type	Control		VO	
		pre-OP	post-OP	pre-OP	post-OP
IL-8, IL-12	All populations		22.4, 37.4		
	$CD8^+$ T-cells			7.1	
	Monocytes	40.1	39.4	9.1, 13.1, 14.1, 15.1, 16.1	12.4, 13.4, 14.4
IL-6, VEGF	All populations		22.4		
	B-cells		33.4		
	$CD8^+$ T-cells		33.4		
	Monocytes	33.1, 40.1	33.4, 39.4	5.1, 7.1, 13.1 14.1, 15.1, 16.1	12.4, 13.4, 14.4
	NK-cells		33.4		12.4

4.6 Gating Strategy

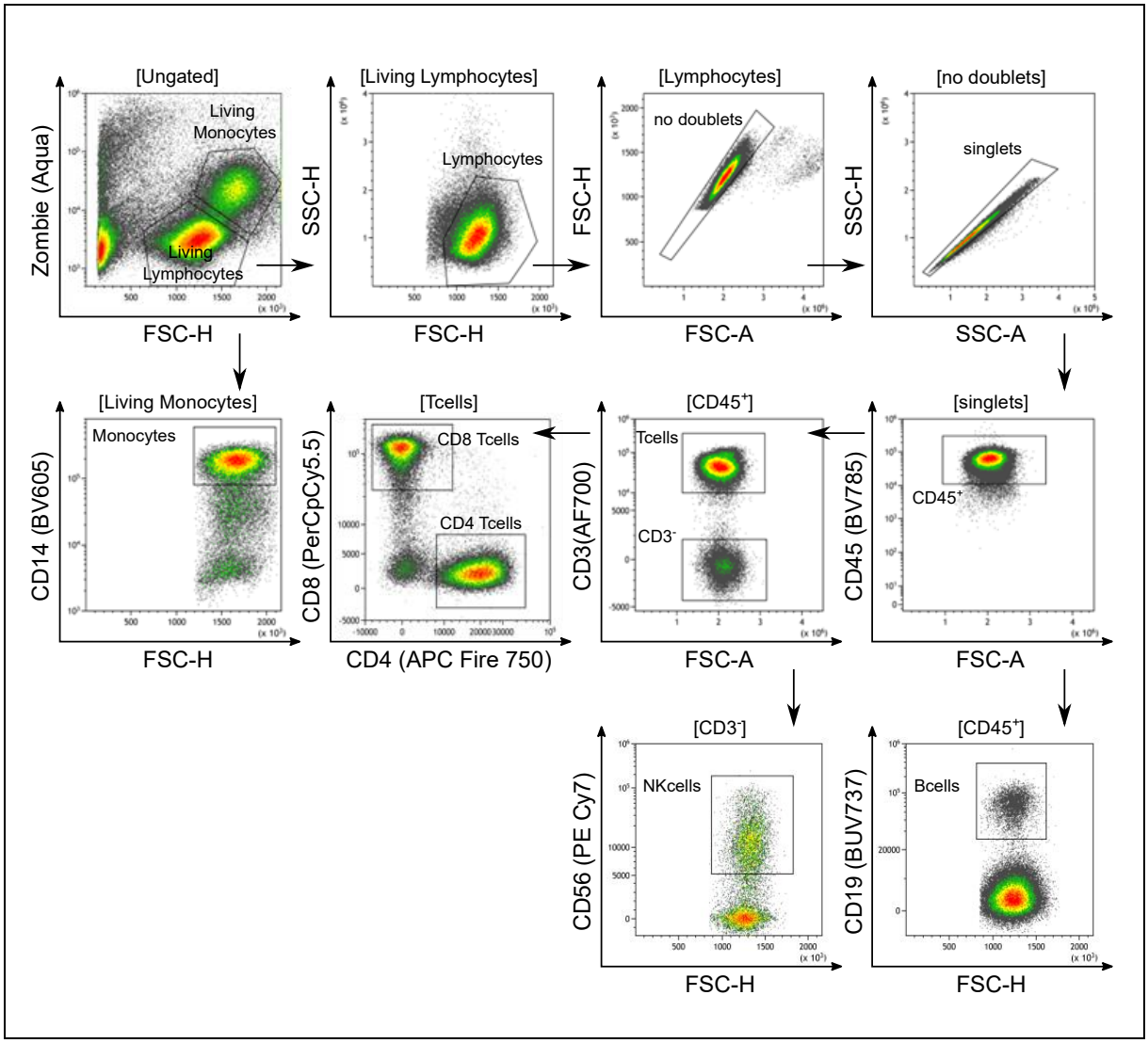

Figure 1: Flow cytometry gating strategy of cytokine-producing cells

Dead cells were excluded. Monocytes were defined by gating on living cells and CD14 expression. Lymphocytes were selected by gating for size and granularity of the event (Forward Scatter-height (FCS-H) versus Side Scatter-height (SSC-H) and SSC-H versus SSC-A for excluding doublets and gate single cells. $CD45^+$ lymphocytes were gated from the single cell gate (singlets) by CD45 expression ($CD45^+$). B-cells were defined by gating on $CD45^+$ lymphocytes singlets and the expression of CD19. T-cells were defined by gating on $CD45^+$ lymphocytes singlets and the expression of CD3, whereby $CD8^+$ T-cells were defined by gating on T-cells expressing CD8 and no CD4 (defined as $CD4^+$ T-cells). NK-cells were defined by gating on $CD3^-$-cells expressing CD56.

4.7 Statistical Analysis

For each cytokine, the median value and the interquartile range (IQR) were calculated by group and time point. For each cell population, the mean value and the standard error of the mean (SEM) were calculated by group, time point, and produced cytokine. A paired t-test was used to compare the mean values within the VO group or the control group by time points

(VO pre-OP vs. VO post-OP and control pre-OP vs. control post-OP). Furthermore, a two-sample t-test (method of Satterthwaite) was used to detect the differences between the VO group and the control group at both study intervals (VO pre-OP vs. control pre-OP and VO post-OP vs. control post-OP). Differences with a p-value <0.05 were defined as statistically significant. The statistical analysis was performed by SAS/STAT software UE (SAS Institute Inc: SAS/STAT User's Guide, Cary NC: SAS Institute Inc, 2014).

A similarity matrix is a probabilistic representation of similarities among data points. It enables the demonstration of correlation between two measured values. In our study, we used the MORPHEUS software for versatile matrix visualization and analysis (Morpheus, hhtps:// software.broadinstitute.org/Morpheus). Hierarchical clustering was performed as the initial step to group individual cytokines produced by PBMCs and a Pearson correlation was used as metric. The results were visualized using a heat map.

5. Results

The study population was divided into two groups: the vertebral osteomyelitis (VO) group and the erosive osteochondrosis group as the control group. Both groups needed an operative stabilization of the spine due to their diseases. Blood was drawn at two different time points: before surgery (pre-OP) and 40-56 days after surgery (post-OP).

5.1 CRP concentrations

To assess the level of inflammation in each patient group, C-reactive protein (CRP) was measured. A statistically significant difference was found between the control group and VO group both pre-OP ($p = 0.0255$) and post-OP ($p = 0.0366$). All CRP concentrations are presented in Table 7 and were previously reported [30].

Table 7: CRP concentration in mg/L per group and time point

		Control			VO			
		mean	median	IQR	mean	median	IQR	x-fold (*)
CRP (mg/L)	pre-OP	3.5	3.6	0.0-6.1	75.7	40.7	14.8-95.3	21.6
	post-OP	3.5	4.1	0.0-5.8	32.5	11.6	2.4-32.7	9.3

*based on mean values

5.2 Serum cytokine levels and cytokine production by PBMCs

The serum cytokine levels were obtained within the framework of the CyProSpon study and reported in a previous publication “The diagnostic value of cytokines for the discrimination of vertebral osteomyelitis and degenerative diseases of the spine” [30].

For cytokine measurement, samples from some patients could not be used due to measurement errors and high outliers, leading to exclusion of patient no. 11 and 16. Due to other medical treatments, it was not possible to perform the post-operative blood collection from 4 VO patients (1, 2, 15, and 16) and from 1 control patient (40). The serum samples of all other patients of this study were used for cytokine measurements and PBMC characterization. Interleukin-6 (IL-6), interleukin-8 (IL-8, interleukin 12 (IL-12), and vascular epithelial growth factor (VEGF) were selected for further analysis in the serum of the patients included. The values for the chosen cytokines are presented in Table 8 and were previously published [30].

Table 8: Serum cytokine levels in pg/mL per group and time point

	Cytokine	Control (pg/mL)			VO (pg/mL)			x-fold (*)
		mean	median	IQR	mean	median	IQR	
pre-OP	IL-6	3.9	3.1	2.8-5.4	79.3	25.2	13.7-38.7	20.3[a]
	IL-8	11.3	9.7	7.7-11.0	21.7	17.5	14.1-22.5	1.9[b]
	IL-12	22.5	16.2	12.1-19.6	50.8	43.8	30.4-64.6	2.3[a]
	VEGF	38.4	32.4	24.5-48.5	128.4	99.3	46.6-164.4	3.3[b]
post-OP	IL-6	6.2	5.4	3.6-8.6	13.3	9.4	4.6-16.6	2.1
	IL-8	14.9	13.8	10.5-17.2	21.0	20.8	18.2-22.2	1.4[a]
	IL-12	39.6	31.3	26.7-53.6	58.3	51.5	39.2-68.2	1.5
	VEGF	83.9	76.4	49.6-115.0	105.9	90.7	63.9-115.4	1.3

[a]: $p<0.05$; [b]: $p<0.01$; *: based on mean values

We revealed that VO was associated with increased levels of IL-6, IL-8, IL-12, and VEGF. The levels of these cytokines showed a significant difference between both study groups, implicating their use for the discrimination of VO and *osteochondrosis intervertebralis*. Therefore, we aimed to assess the production of these cytokines by different immune cell populations isolated from the VO and control groups of patients. For this, we treated the isolated PBMCs with Monensin/Brefeldin A then analyzed the cytokine production by flow cytometry. To study different populations, we classified the cells as follows: Living cells were gated for 1) All lymphocytes ($CD45^+$), 2) B-cells ($CD45^+$ $CD19^+$), 3) T-cells ($CD45^+$ $CD3^+$), 4) $CD4^+$ T-cells ($CD3^+$ $CD4^+$), 5) cytotoxic T-cells ($CD3^+$ $CD8^+$), 6) Monocytes (CD14+) and 7) NK-cells ($CD3^+$ $CD56^+$).

5.2.1. IL-6 in serum and IL-6-producing PBMCs

The cytokine IL-6 is a potent pro-inflammatory mediator that plays a crucial role in activating the production of acute-phase proteins (such as CRP) and mediating the development of T-cells. Laboratory tests often utilize IL-6 to assess the extent of inflammation and immune response due to its strong impact on pro-inflammatory processes [43,57]. The mean serum level of IL-6 was found to be 20.3-fold higher in the VO group compared to the control group pre-OP (79.3 pg/mL vs. 3.9 pg/mL; p=0.0429). Post-OP, the mean serum level of IL-6 showed a 2.1-fold higher level in the VO group than in the control group (13.3 pg/mL vs. 6.2 pg/mL; p=0.0759).

Figure 2 presents the percentage of the indicated PBMC subsets that produced IL-6. The VO group demonstrated two significant differences between the pre-OP and the post-OP time points. Firstly, there was a significant increase in the proportion of IL-6-producing lymphocytes at the post-OP time point compared to the pre-OP time point (2.63% vs. 2.04%,

p=0.0478). Secondly, also the $CD4^+$ T-cells in the VO group showed a statistically significant increase in the proportion of IL-6-producing cells at the post-OP time point compared to the pre-OP time point (3.03% vs. 2.18%, p=0.0405). At the pre-operative time point, the proportion of several PBMC sub-populations producing IL-6 were non-significantly higher in the VO group compared to the control group, namely B-cells (1.49% vs. 1.06%), $CD8^+$ T-cells (1.02% vs. 0.69%), and NK-cells (1.96% vs. 1.9%). Taken together, there were no statistical differences in the proportion of PBMCs producing IL-6 between the VO and the control groups. All other differences in the proportion of PBMCs were not statistically significant.

Notably, the highest proportion of IL-6-positive cells was found in the monocytes of the control group pre-OP (approx. 6%) while the lowest proportion was seen in the B-cells (approx. 1%) and $CD8^+$ T-cells (approx. 0.6%) pre-OP. It is important to note here that lymphocytes incorporate other sub-populations such as B-cells, T-cells and NK-cells. The same is true for T-cells, which include $CD4^+$ T-cells and $CD8^+$ T-cells, respectively. All statistical analyses are presented in Table 9.

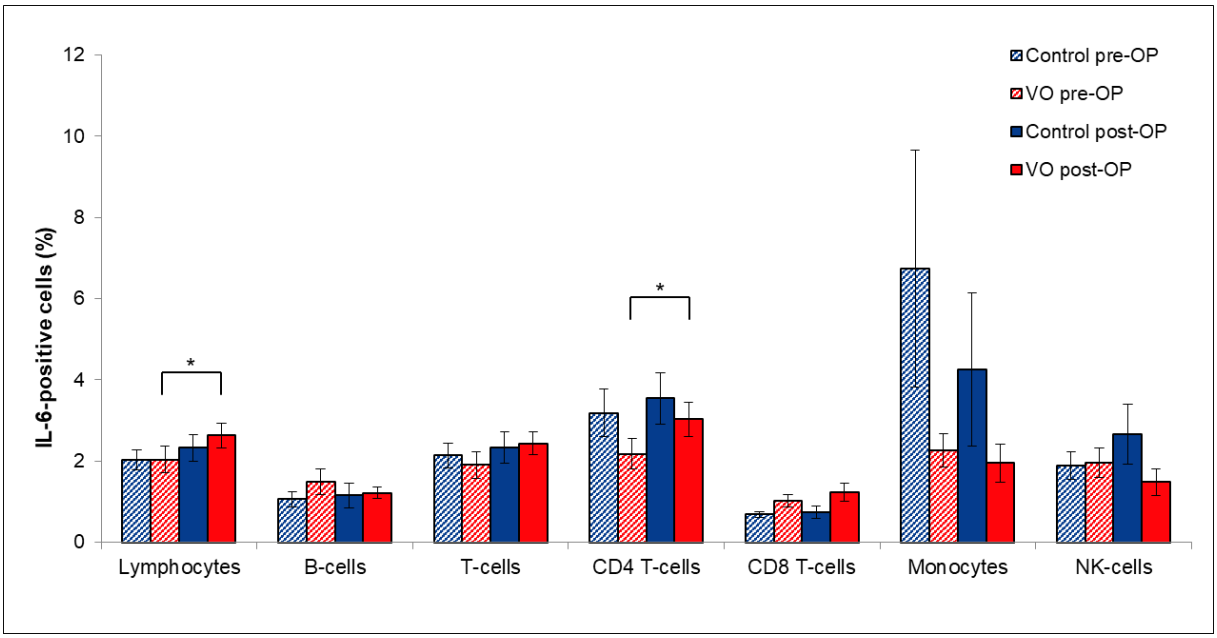

Figure 2: IL-6-positive cells per group and time point

Table 9: P-values of the two-sample t-test and paired t-test for IL-6 production

PBMCs	Control vs. VO		pre-OP vs. post-OP	
	pre-OP	post-OP	Control	VO
Lymphocytes	0.9695	0.5127	0.5270	0.0478*
B-cells	0.2606	0.8493	0.6961	0.7514
T-cells	0.6053	0.8350	0.8398	0.0811
$CD4^+$ T-cells	0.1602	0.5055	0.8404	0.0405*
$CD8^+$ T-cells	0.0674	0.0800	0.8481	0.1223
Monocytes	0.1468	0.2509	0.3958	0.4499
NK-cells	0.9066	0.1588	0.3582	0.1747

*: $p<0.05$

5.2.2. IL-8 in serum and IL-8-producing PBMCs

IL-8 plays a significant role in inflammation as a major mediator and is responsible for the chemotactic recruitment and movement of neutrophils to the site of infection. Additionally, IL-8 is involved in the regeneration and formation of new blood vessels in the vascular system [43,46,49]. The mean serum level of IL-8 pre-OP was 1.9-fold higher in the VO group compared to the control group (21.7 pg/mL vs. 11.3 pg/mL; p=0.0087). Post-OP, the mean serum level of IL-8 showed a 1.4-fold higher level in the VO group than in the control group (21.0 pg/mL vs. 14.9 pg/mL; p=0.0323).

Figures 3 and 4 demonstrate the percentage of the PBMC populations that produced IL-8, indicating four significant differences. The control group had a higher proportion of IL-8-positive cells than the VO group at the post-operative timepoint, with respect to lymphocytes (1.31 % vs. 0.7%, p=0.032), T-cells (0.45% vs. 0.2%, p=0.0116%) and $CD8^+$ T-cells (0.35% vs. 0.14%, p=0.0271). The same pattern was observed in B-cells, $CD4^+$ T-cells and NK-cells with a non-significantly higher proportion of IL-8-positive cells in the control group than in the VO group at the post-OP time point. The $CD8^+$ T-cells at the pre-OP time point also showed significantly more IL-8-positive cells in the control group than in the VO group (0.3% vs. 0.15%, p= 0.0141). Although B-cells and $CD4^+$ T-cells showed similar distribution patterns at both time points between both study groups as the T-cells, the differences were not statistically significant.

Monocytes had the highest proportion of IL-8-positive cells, with 16%, and is the only sub-population that showed an increase in the proportion of IL-8-positive cells in the VO group post-OP compared to pre-OP (17.53% vs.14.67%). All other IL-8-positive sub-populations in

the VO group decreased non-significantly from the pre-OP to the post-OP stage. All statistical results are presented in Table 10.

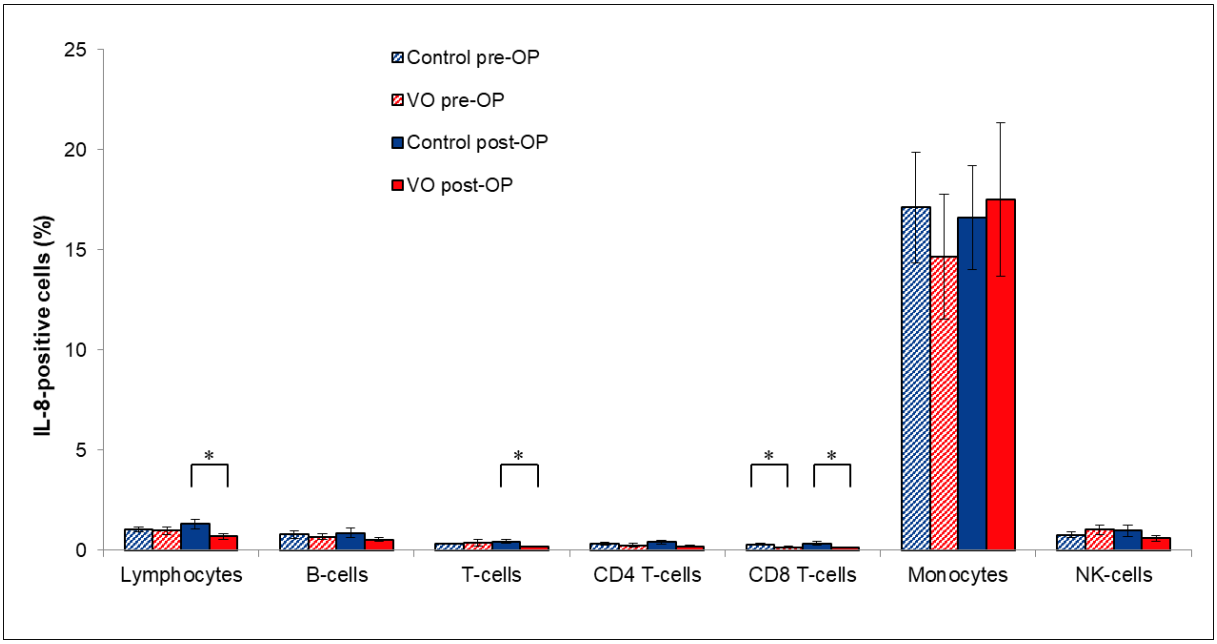

Figure 3: IL-8-positive cells per group and time point

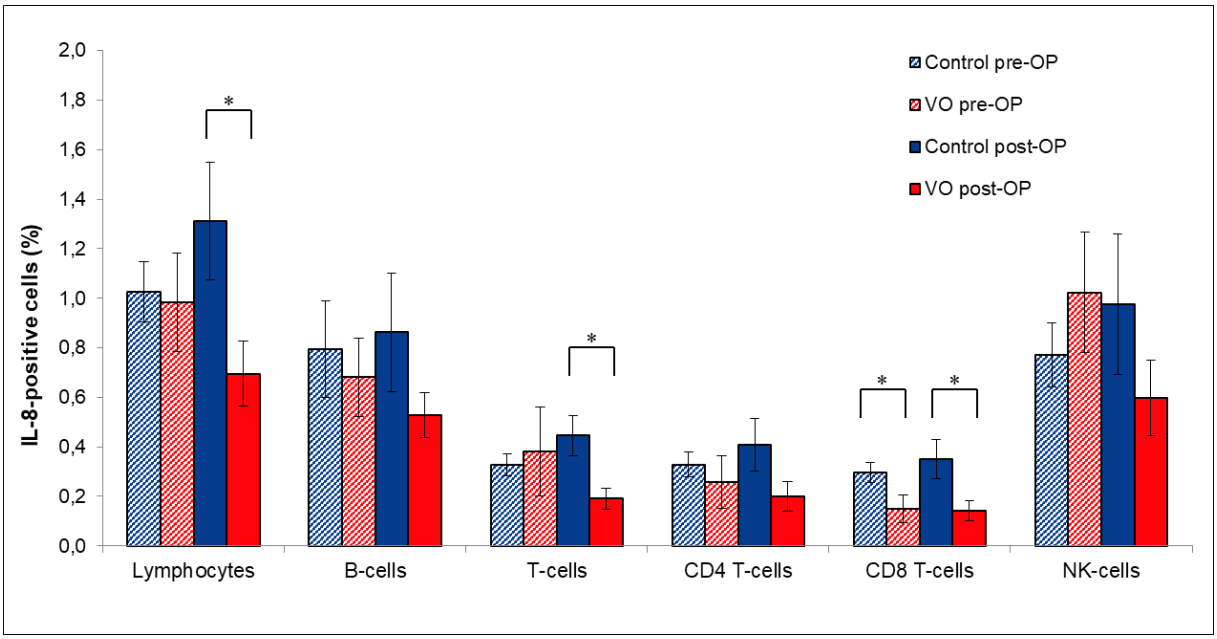

Figure 4: IL-8-positive cells per group and time point except monocytes

Table 10: P-values of the two-sample t-test and paired t-test for IL-8 production

PBMCs	Control vs. VO		pre-OP vs. post-OP	
	pre-OP	post-OP	Control	VO
Lymphocytes	0.8582	0.0320*	0.3077	0.2235
B-cells	0.6560	0.2038	0.5159	0.5108
T-cells	0.7782	0.0116*	0.0764	0.3020
$CD4^+$ T-cells	0.5443	0.0994	0.2857	0.5225
$CD8^+$ T-cells	0.0414*	0.0271*	0.3192	0.9724
Monocytes	0.5601	0.8470	0.7249	0.3112
NK-cells	0.3717	0.2516	0.3971	0.0603

*: $p<0.05$

5.2.3. IL-12 in serum and IL-12-producing PBMCs

IL-12 is responsible for the immunological defense against bacterial, viral, and parasitic infections. The most important function of IL-12 is the activation of the T-helper cell-mediated immune response. By affecting these defense mechanisms, IL-12 influences the severity and duration of intracellular infections [49]. The mean serum level of IL-12 pre-OP was found to be 2.3-fold higher in the VO group compared to the control group (50.8 pg/mL vs. 22.5 pg/mL, p=0.0137). Post-OP, the mean serum level of IL-12 showed a 1.5-fold higher level in the VO group than in the control group (58.3 pg/mL vs. 39.6 pg/mL, p=0.0735). The serum level in both groups increased when comparing pre-OP to post-OP time points.

Figure 5 presents the percentage of the PBMC population that produced IL-12, indicating six significant differences. At the pre-OP time point, lymphocytes showed a significantly higher proportion of IL-12-positive cells in the VO group compared to the control group (2.42% vs. 1.77%, p=0.0133). This trend was also observed in B-cells, T-cells, $CD8^+$ T-cells and monocytes, but was not statistically significant. At the post-OP timepoint, there were three significant differences between the control group and the VO group, with a higher proportion of IL-12-positive cells in the control group: $CD4^+$ T-cells (1.67% vs. 1.21%, p=0.0027), monocytes (2.62% vs. 0.64%, p=0.0003) and NK-cells (3.3% vs. 1.79%, p=0.00050). Monocytes also showed a significant increase in the proportion of IL-12-positive cells in the control group post-OP compared to pre-OP (2.62% vs. 1.61%, p=0.0400). Within the VO group, the proportion of IL-12-producing T-cells decreased significantly post-OP compared to pre-OP (1.79% vs. 1.52%, p=0.0157).

With approximately 1-3%, all cell populations showed similar levels of IL-12-positive cells except for monocytes in the VO group post-OP, which showed a lower percentage at 0.64%. All statistical results are presented in Table 11.

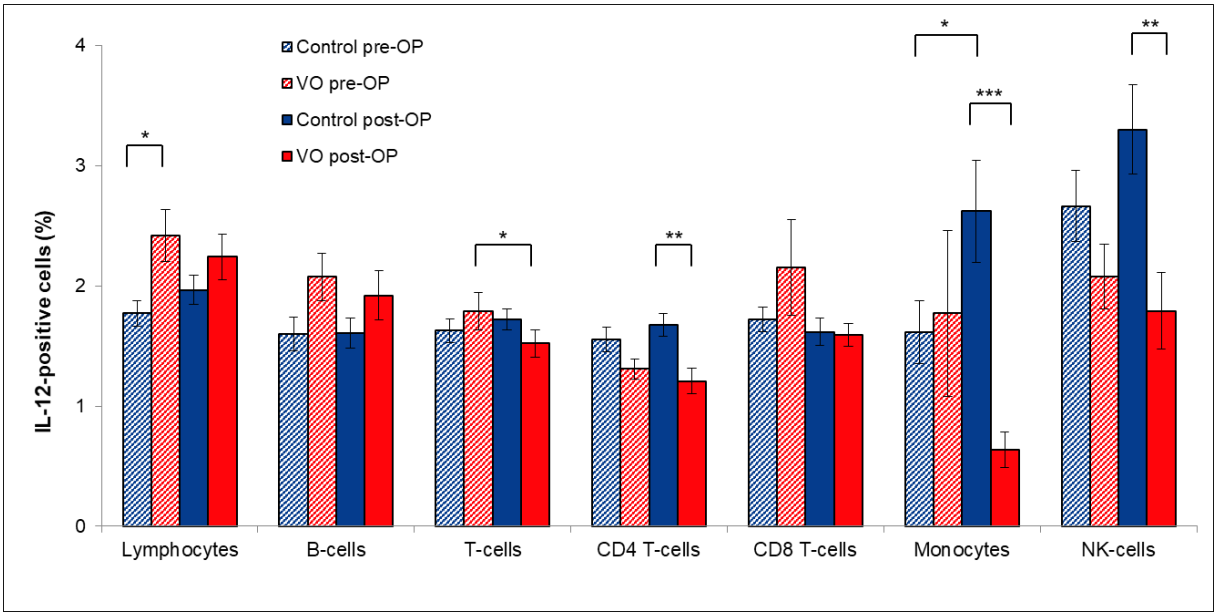

Figure 5: IL-12-positive cells per group and time point

Table 11: P-values of the two-sample t-test and paired t-test for IL-12 production

PBMCs	Control vs. VO		pre-OP vs. post-OP	
	pre-OP	post-OP	Control	VO
Lymphocytes	0.0133*	0.2391	0.0881	0.3892
B-cells	0.0581	0.2052	0.8475	0.4473
T-cells	0.3823	0.1818	0.3532	0.0157*
$CD4^+$ T-cells	0.0738	0.0027**	0.2484	0.2366
$CD8^+$ T-cells	0.3063	0.8583	0.2500	0.1801
Monocytes	0.8353	0.0003***	0.0400*	0.1631
NK-cells	0.1494	0.0050**	0.3072	0.2012

*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

5.2.4. VEGF in serum and VEGF-producing PBMCs

The cytokine VEGF is a potent mediator of the vascular system, induces embryonic vasculogenesis, and regulates angiogenesis in adults. VEGF activates mainly endothelial cells but also stimulates migration of monocytes and macrophages [63,65]. The mean serum level of VEGF pre-OP was found to be 3.3-fold higher in the VO group compared to the control group (128.4 pg/mL vs. 38.4 pg/mL, p=0.0053). Post-OP, the mean serum level of VEGF showed a 1.3-fold higher level in the VO group than in the control group (105.7 pg/mL vs. 83.9 pg/mL, p=0.0778).

Figure 6 shows the percentage of PBMCs producing VEGF with six statistically significant differences. Pre-OP, the proportion of VEGF-positive cells was higher in the VO group than in the control group: lymphocytes (19.7% vs. 11.88%, p=0.0033), B-cells (11.8% vs. 6.87%, p=0.0250), T-cells (21.94% vs.13.72%, p=0.0133) and $CD8^+$ T-cells (8.35% vs. 4.25%, p=0.0474). The same trend was observed in $CD4^+$ T-cells (31.93% vs. 19.4%), monocytes (1.94% vs. 1.84%), and NK-cells (5.9% vs. 4.1%) but the differences were not statistically significant. Overall, each PBMC sub-population had a higher proportion of VEGF-positive cells in the VO group than in the control group. B-cells and $CD4^+$ T-cells showed a significant increase in the proportion of VEGF-positive cells within the control group (10.18% vs. 6.87%, p=0.0459 and 29.48% vs. 19.4%, p=0.0392, respectively) post-OP. Lymphocytes, T-cells, $CD8^+$ T-cells and monocytes showed the same trend, but the differences were not statistically significant.

Notably, with up to 30%, $CD4^+$ T-cells showed the highest percentage of VEGF-positive cells, while less than 5% of $CD8^+$ T-cells, monocytes and NK-cells were VEGF-positive. All statistical results are presented in Table 12.

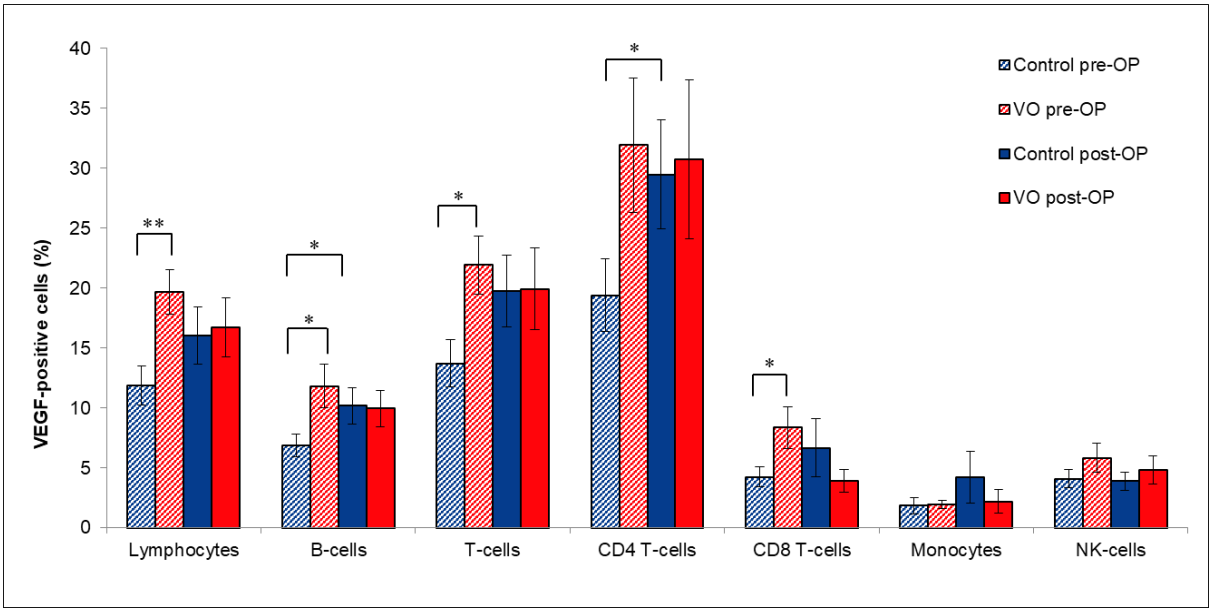

Figure 6: VEGF-positive cells per group and time point

Table 12: P-values of the two-sample t-test and paired t-test for VEGF production

PBMCs	Control vs. VO		pre-OP vs. post-OP	
	pre-OP	post-OP	Control	VO
Lymphocytes	0.0033**	0.8482	0.1493	0.0588
B-cells	0.0250*	0.9158	0.0459*	0.8465
T-cells	0.0133*	0.9655	0.0668	0.1669
$CD4^+$ T-cells	0.0608	0.8786	0.0392*	0.3863
$CD8^+$ T-cells	0.0474*	0.3038	0.3499	0.0580
Monocytes	0.8958	0.4016	0.1571	0.7726
NK-cells	0.2118	0.5088	0.7626	0.2073

*: $p<0.05$, **: $p<0.01$

5.2.5. All significant differences of cytokine-positive cells in the present study

The significant differences between the VO-group and the control group are summarized in Table 13. First, the differences at the pre-operative time point are shown followed by the differences at the post-operative time point. The significant differences within the study groups at both time points are presented in Table 14.

Table 13: Significant differences in cytokine-positive cells between both study groups

	Pre-OP vs. Pre-OP			
	IL-6	IL-8	IL-12	VEGF
VO vs. Control	-	CD8 T-cells (Control +)	Lymphocytes (VO +)	Lymphocytes (VO +) B-cells (VO +) T-cells (VO +) CD8 T-cells (VO+)
	Post-OP vs. Post-OP			
	IL-6	IL-8	IL-12	VEGF
VO vs. Control	-	Lymphocytes (Control +) T-cells (Control +) CD8 T-cells (Control +)	CD4 T-cells (Control +) Monocytes (Control +) NK-cells (Control +)	-

+: significantly higher proportion of cytokine-positive cells

Table 14: Significant differences in cytokine-positive cells within the study groups

	Pre-OP vs. Post-OP			
	IL-6	IL-8	IL-12	VEGF
VO	Lymphocytes (post-OP +) CD-4 T-cells (post-OP +)	-	T-cells (pre-OP +)	-
Control			Monocytes (post-OP +)	B-cells (post-OP +) CD4 T-cells (post-OP +)

+: significantly higher proportion of cytokine-positive cells

5.3 Similarity matrix

Intracellular flow cytometry revealed that B-cells, T-cells and monocytes were associated with the highest proportions of cytokine production. Therefore, we decided to generate a similarity matrix for these three sub-populations of PBMCs. A similarity matrix can demonstrate correlations between different cytokines and whether the cytokines are secreted by these subsets, B-cells, T-cells and monocytes. This approach could unmask possible co-regulatory processes.

5.3.1. B-cells

Figure 7 shows the similarity matrix for B-cells. In the control group, VEGF shows a positive correlation with IL-6 and a weaker positive correlation with IL-8 at the pre-operative time point. Moreover, VEGF shows a negative correlation with IL-12. No correlation was found between IL-6, IL-8 and IL-12. At the post-operative time point, two groups can be found; there is a positive correlation between IL-8 and IL-12 as well as between IL-6 and VEGF. Hierarchical clustering arranges the different cytokines together in groups. At the pre-operative time point, there are two main groups. IL-8, IL-6 and VEGF form one, the other is formed by IL-12. This constellation changes toward the post-operative time point where the two main groups are formed by IL-8 with IL-12 and by IL-6 with VEGF. In the VO group, a weak positive correlation is detected between IL-6 and VEGF. VEGF shows a negative correlation with IL-12, as in the control group pre-operative. Furthermore, a negative correlation is between VEGF and IL-8. At the post-operative time point, the correlations change, and the VO group shows a strong positive correlation between IL-8 and IL-12. Moreover, there is a stronger negative correlation of VEGF with IL-6, IL-8, and IL-12. Hierarchical clustering shows two main groups: IL-6 with VEGF as well as IL-8 with IL-12 in the VO group at the pre-operative time point. This is the same constellation as in the control group at the post-operative time point. In the VO group at the post-operative time point, there is a group with IL-6, IL-8 and IL-12 and VEGF stands alone.

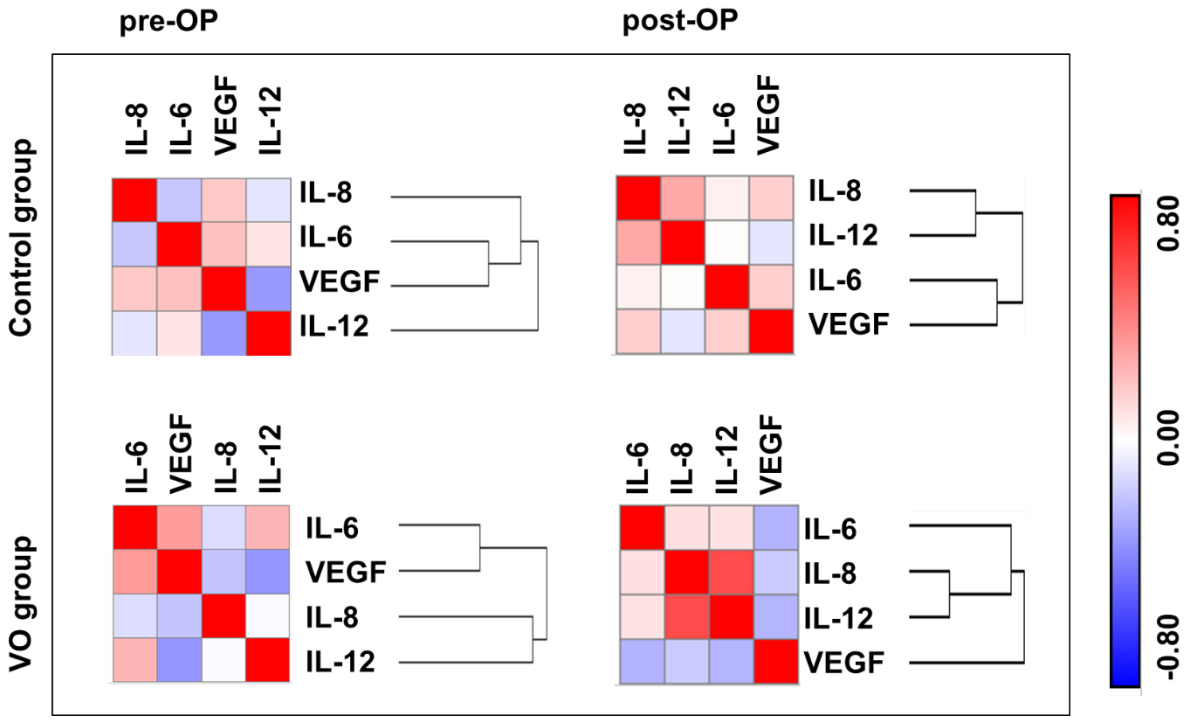

Figure 7: Similarity matrix for B-cells
Hierarchical clustering with a one-minus pearson-correlation with average-linkage, control group pre-OP (n=20), control group post-OP (n=18), VO group pre-OP (n=16), VO group post-OP (n=12)

5.3.2. T-cells

Figure 8 shows the similarity matrix for T-cells. The control group shows a positive correlation between IL-8 and IL-12 at the pre-operative time point. A negative correlation is detected between IL-8 and VEGF as well as between IL-12 and VEGF. At the post-operative time point, IL-6 and IL-8 show a positive correlation and IL-12 and VEGF a negative correlation. The dendrogram of the control group pre-operative, shows a large group with IL-6, IL-8, and IL-12 with IL-8 and IL-12 as a sub-group and VEGF stands alone. This distribution remains the same at the post-operative time point; only the sub-group distribution changes, instead of IL-8 and IL-12, IL-8 and IL-6 form a sub-group. The VO group shows a positive correlation between VEGF and IL-12 as well as between IL-6 and IL-12 at the pre-operative time point. At the post-operative time point, the VO cohort shows two separate correlation groups. There is a strong positive correlation between IL-8 and IL-12 and a weaker positive correlation between IL-8 and IL-6. The second group shows a weak positive correlation between IL-6 and VEGF. In the VO group, hierarchical clustering at the pre-operative time point reveals a group with IL-6, IL-12, and VEGF and IL-8 stands alone. At the post-operative time point, two main groups appear: IL-8 with IL-12 and IL-6 with VEGF.

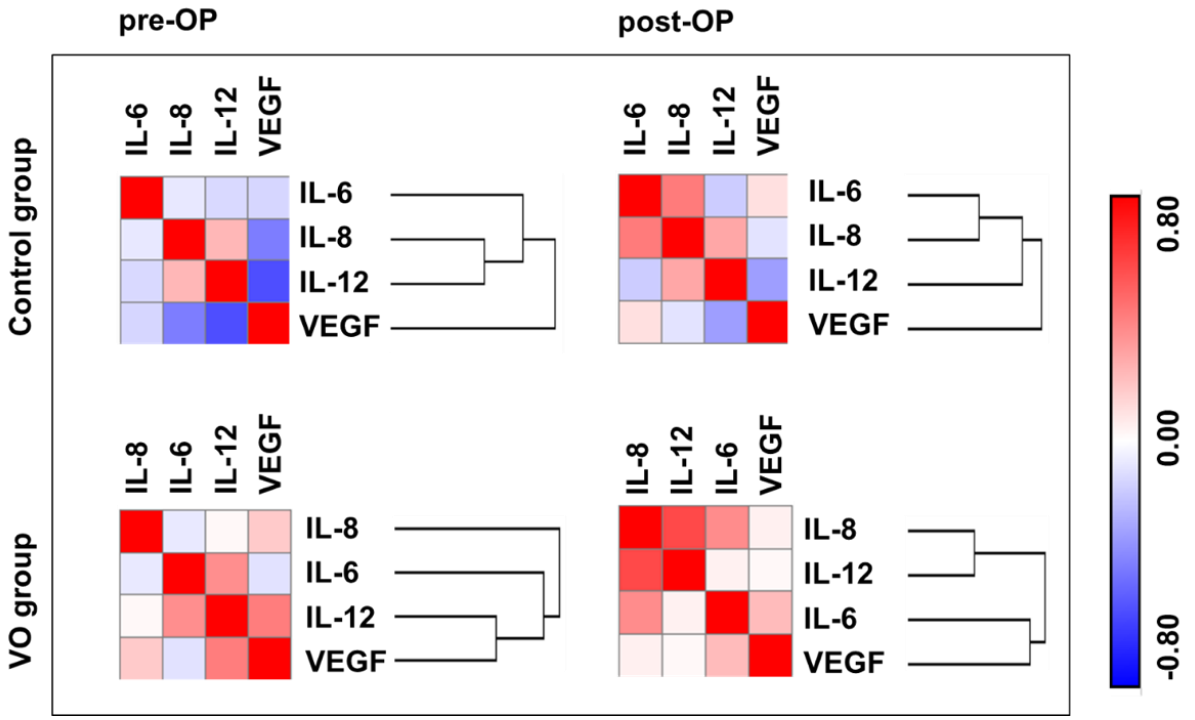

Figure 8: Similarity matrix for T-cells
Hierarchical clustering with a one-minus pearson-correlation with average-linkage, control group pre-OP (n=20), control group post-OP (n=18), VO group pre-OP (n=16), VO group post-OP (n=12)

5.3.3. Monocytes

Figure 9 shows the similarity matrix for monocytes. In the control group, there is a strong positive correlation between IL-6 and IL-12 and a weaker positive correlation between IL-12 and VEGF at the pre-operative time point. At the post-operative time point, the control group shows a more allocated cluster. A positive correlation was detected between IL-8 and IL-12 and a weaker positive correlation between IL-6 and VEGF. Hierarchical clustering of the control group at the pre-operative time point shows a major group with IL-6, IL-12 and VEGF and IL-8 stands alone. At the post-operative time point, the control group shows two main groups: IL-8 with IL-12 and IL-6 with VEGF.

The VO group shows an opposed cluster at the pre-operative time point. IL-8 shows a negative correlation with IL-12 and VEGF. There is a strong positive correlation between IL-12, VEGF, and IL-6. At the post-operative timepoint in the VO group, the strong positive correlation remains only between IL-6 and IL-12. VEGF shows a negative correlation with IL-12 and IL-8. The dendrogram of the VO group at the pre-operative time point shows a major group with IL-6, IL-12, and VEGF, with IL-12 and VEGF as a sub-group, and IL-8 stands alone. At the post-operative time point of the VO group, IL-8, IL-6, and IL-12 form a major group, whereby IL-6 and IL-12 form a sub-group, and VEGF stands alone.

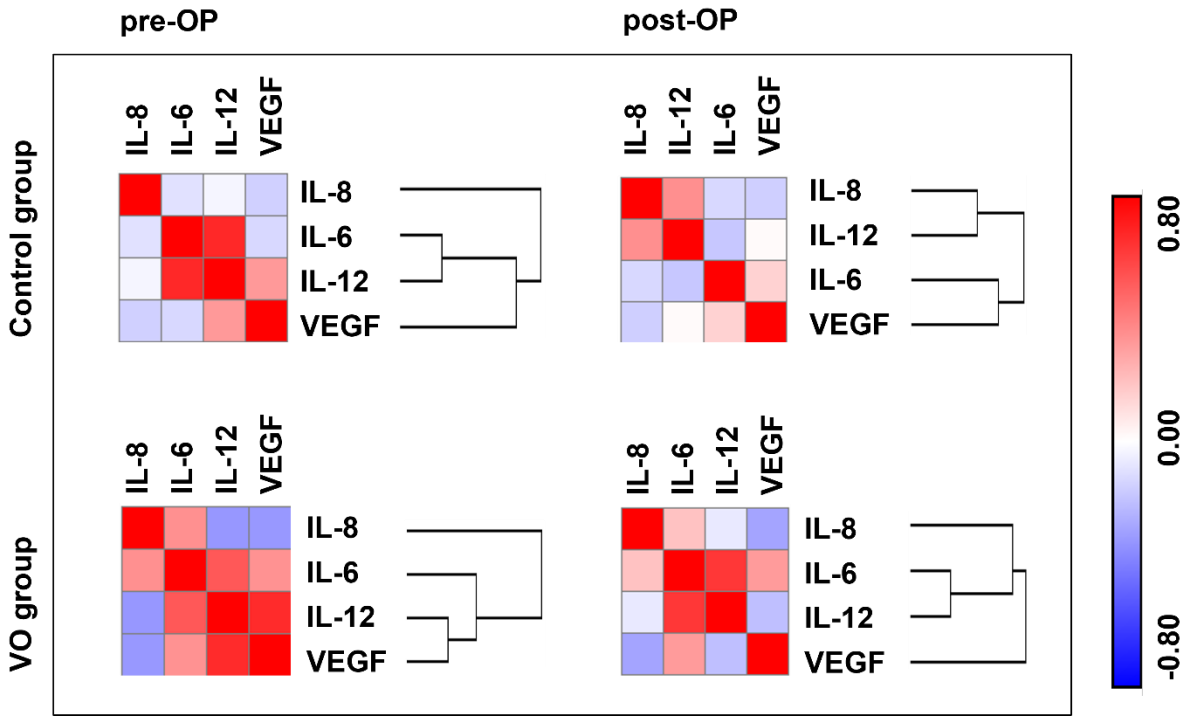

Figure 9: Similarity matrix for monocytes
Hierarchical clustering with a one-minus pearson-correlation with average-linkage, control group pre-OP (n=20), control group post-OP (n=18), VO group pre-OP (n=16), VO group post-OP (n=12)

5.3.4. Correlations of similarity matrices for the VO group pre-OP

In order to focus on the VO-group and highlight commonalities among all three similarity matrices created, the most interesting correlations of the VO group at the pre-operative time point are presented in Table 15.

Table 15: Correlations of similarity matrices for the VO group at the pre-operative time point

	Positive correlation	Negative correlation
B-cells	IL-6 and VEGF	VEGF and IL-12 VEGF and IL-8
T-cells	**VEGF** and **IL-12** VEGF and IL-8 IL-6 and IL-12	-
Monocytes	**VEGF** and **IL-12** VEGF and IL-6 IL-12 and IL-6	IL-8 and VEGF IL-8 and IL-12

Written in bold: strong correlation

6. Discussion

Vertebral osteomyelitis is a rare but dreaded disease of the spine. A major challenge lies in its earliest possible diagnosis and treatment. The average time between the first symptoms and a final diagnosis ranges between 2 and 6 months. These difficulties may lead to highly acute courses with sepsis, neurological deficits, organ failure and even death. Cytokine analysis has been proposed as a potential method for distinguishing vertebral osteomyelitis from other vertebral disorders. Our published work highlighted the value of serum cytokine profiling for diagnosis of vertebral osteomyelitis and showed that soluble urokinase plasminogen activating receptor (suPAR) was superior to CRP for diagnosing vertebral osteomyelitis [29,30].

During vertebral osteomyelitis, immune cells are typically recruited to the site of infection to help fight off the invading bacteria. In addition, other cells in the body such as fibroblasts and endothelial cells also contribute to the immune response. They help to create a supportive environment for the immune cells and aid in the repair of damaged tissue. Overall, the immune response to vertebral osteomyelitis is complex and involves the coordinated actions of many different types of cells. The objective of our study was to assess the potential involvement of different immune cell types in the production of several candidate cytokines, reported in a previous publication [30]. Furthermore, we wanted to gain an understanding of the immune cell profile responsible for cytokine production, along with the associated changes in immune cells during vertebral osteomyelitis, which may enable a clear differentiation between vertebral osteomyelitis and degenerative spinal diseases.

Even with the use of keywords such as “cytokines, PBMC, vertebral osteomyelitis”, currently, no relevant publication can be found in PubMed that specifically focuses on the characterization of cytokine-producing PBMCs. Therefore, this is the first study to investigate cytokine-producing PBMCs derived from patients with vertebral osteomyelitis, which were compared to those from control patients devoid of infections. Based on the results of the flow cytometry analysis of cytokine-producing PBMCs, a clear differentiation between vertebral osteomyelitis and degenerative spinal disease is possible pre-operatively, especially with the IL-8-positive $CD8^+$ T-cells and IL-12-positive lymphocytes. Compared to the control group, a significantly higher level of VEGF-positive cells was present in the VO group at the pre-operative time point: lymphocytes, B-cells, T-cells, $CD8^+$ T-cells.

Additionally, we obtained an epidemiological overview of the patient population affected by the disease as well as information about the pathogens involved.

6.1 Study population

In selecting the patients for the present study, the aim was to establish similar conditions for a control group in comparison to our vertebral osteomyelitis group. The disease

osteochondrosis intervertebralis shows similar symptoms and the same surgical therapy as vertebral osteomyelitis and serves as a good control group to the patients with vertebral osteomyelitis in this study. There are three groups of *osteochondrosis intervertebralis* classified based on radiological parameters, denoted as MC 1-3. MC1 represents the acute form of *osteochondrosis* and is associated with inflammation of the bone marrow accompanied by surrounding oedema, which can potentially result in an elevation of inflammatory markers akin to those observed in vertebral osteomyelitis. To ensure the study's accuracy and prevent any confounding factors, patients diagnosed with MC1 were deliberately excluded from the study to eliminate the possibility of simultaneous infection and the consequent alteration of the immune profile in the control group [36,40,42].

Our goal was to establish two study groups, each comprising 20 patients of equal size. However, the stringent exclusion criteria posed challenges in achieving this objective for the vertebral osteomyelitis group. Given the advanced age of patients with vertebral osteomyelitis, there were numerous concomitant comorbidities and pre-existing conditions such as cancer and immunological disorders. Due to these difficulties, we were only able to recruit 36 patients out of the initial 98 considered for the entire study, with just 16 being included in the vertebral osteomyelitis group during the intended recruitment phase.

6.1.1. Epidemiological data

The epidemiological data obtained in this study are consistent with those reported by other studies. Considering the gender distribution, the male to female ratio of 1.7:1 aligns with the findings reported by Fantoni et al. [7] and Grammatico et al. [77]. The mean age in our study population was 68.8 years, which is slightly above the range of 59 to 67 years described by other authors [32,77,78]. In relation to the high average age, our study population mirrors the demographic characteristics of the German population aged 65 and older. Out of the 36 patients, 27 (75%) had hypertension, which reflects the high prevalence of hypertension (60%) in this age group [79].

Numerous risk factors are associated with vertebral osteomyelitis, and some of these comorbidities were observed among the patients in our study group [80]. An important risk factor is excess weight, especially obesity. Over 50% of the total German population is overweight and as many as 19% are obese with a BMI over 30kg/m^2. Our study group had a higher incidence of obesity, with 12 out of 36 (33%) patients classified as obese, this is also particularly reflected in the VO group with 31% obese patients [81,82]. Another prominent factor is diabetes mellitus, whereby 5 out of 16 (31%) patients in the VO group were afflicted by this condition [83] [84]. Furthermore, in the control group, 4 out of 20 (20%) patients had diabetes mellitus. Another risk factor related to vertebral osteomyelitis is alcohol consumption [85]. In the VO group, 18% of patients reported regular alcohol consumption, which slightly exceeds the

German national average of 14% [81]. Other lifestyle factors such as nicotine consumption were equally common in both study groups. In our study, only 8 out of 36 patients reported using cigarettes, with an average of 22%, which is comparable to the average of the German population (25%) [86].

6.1.2. Microbiological findings

In the present study, 25% of the patients suffered from a *S. aureus* infection, which is with 30-80% the most frequently reported pathogen in Europe [1,4]. *S. epidermidis* was also detected in 25% of the patients and is often linked to post-operative infections and foreign objects implantation. The prevalence of *S. epidermidis* is described with up to 16% [4]. Also *E. coli* was the causative pathogen in 25% of the patients. Lehner et. al. reported gram-negative bacteria in up to 33% of pyogenic vertebral osteomyelitis [4]. Among others, these three causative pathogens represent the typical pathogen spectrum of vertebral osteomyelitis.

In two cases, *Streptococcus dysgalactiae* was found as the causative pathogen, whereby its severity can range from milder skin and soft tissue inflammation to severe sepsis [87]. Another patient suffered from a *Parvimonas micra* infection, which is normally a germ of the oral cavity and was also linked with spondylodiscitis as reported by Watanabe et al. in a review about bloodstream infections caused by *Parvimonas micra* [88]. *Propionibacterium acnes* belongs to the skin flora but caused vertebral osteomyelitis in one patient in our study group [89]. Another pathogen, *S. lugdunensis* is known for inducing a variety of infections such as abscesses of the soft and skin tissue and was the causative pathogen of one patient of our study group [90]. All VO patients received antibiotic therapy depending on the infectious agent and clinical history.

6.1.3. CRP level

The control group demonstrated consistently low CRP levels at both time points with a mean below 5 mg/L, confirming the inclusion criteria for this group [91]. Conversely, the VO group had higher CRP levels, with a mean of 75.746 mg/L pre-operative, indicating severe inflammation commonly associated with bacterial infections. Jung et al. reported a sensitivity ranging from 98 to 100% for CRP, making it as a valuable tool for the detection and monitoring of vertebral osteomyelitis. However, CRP has a lower specificity as it can also be increased in malignant or non-infectious diseases [8]. In our study, the combination of elevated CRP levels and the detection of typical pathogens served as strong inclusion criteria of patients in the vertebral osteomyelitis group. Jean et. al. reported a mean CRP concentration of 140 mg/L in a group of 88 patients with pyogenic vertebral osteomyelitis in France [92]. A Spanish study of 27 patients with abscess-associated spontaneous pyogenic vertebral osteomyelitis reported a

mean CRP level of 92 mg/L at admission [93]. In comparison, our study implemented stricter exclusion criteria, excluding patients with active infections other than those affecting the spine or the blood stream. This could explain the slightly lower CRP levels, with a mean of 75.75 mg/L pre-operative and potentially resulted in a less direct comparison with those earlier studies. It may indicate that the mean severity of vertebral osteomyelitis was lower in our study.

Elevated CRP concentrations related to surgery should vanish within about 21 days after surgical intervention [94]. The post-operative time point in this study was at least 40 days after surgery so this effect could be excluded. After surgery, CRP concentrations showed a decrease in the VO group. CRP levels correspond with the progression of the inflammation [91]. Therefore, this decline is a strong argument for a reversal of the inflammation process in the context of vertebral osteomyelitis and therefore, implicates the possibility of monitoring the therapeutic success of the antibiotic therapy via CRP [78].

In the literature, there is agreement on the importance of CRP as a parameter for diagnosing vertebral osteomyelitis [1,8,18]. However, there are cases in which patients have lower or normal C-reactive protein (CRP) levels despite vertebral osteomyelitis [14]. In 2019, Homagk et al. developed a scoring system for diagnosing vertebral osteomyelitis based on three main factors: 1) CRP level, 2) pain level based on NRS (Numeric rating scale), 3) medical imaging in the form of MRI [78], which underlines the importance of CRP as a diagnostic marker but points out the complexity of diagnostic analysis of vertebral osteomyelitis.

6.2 Cytokines and PBMCs

6.2.1. Cytokine levels

Research into the immunologic background of other diseases is more advanced than that of vertebral osteomyelitis. Many diseases can be diagnosed and monitored using different cytokine levels. Cytokine-directed therapy is also available for some diseases [95 96,97]. Bozza et al. analyzed the cytokine profiles of patients with severe sepsis to determine specific cytokine profiles that could predict disease progression, severity of complications, and mortality [96]. Rheumatoid arthritis is a good example of how cytokines, in this case TNF, are targeted as therapeutic agents [97]. Another interesting use for diagnosis via cytokine analysis is prosthetic infections. Infections of prosthetic joints of the knee and hip can be well diagnosed using the serum IL-6 level in combination with the level of IL-6/CRP in the synovial fluid [98].

A previous publication from our working group [30] showed that the cytokines: IL-6, IL-8, IL-12, and VEGF are candidate biomarkers for diagnosing vertebral osteomyelitis. The mean serum cytokine levels of the control group were lower than those in the VO group at both time points (pre-OP vs. post-OP), which underlines the difference between the two groups, thereby confirming the control group as the non-infectious reference and therefore, allows

discrimination between VO and degenerative spinal diseases. Another study of our research group emphasizes the importance of IL-6, IL-8, and IL-12, which showed higher serum cytokine levels in patients with implant-associated bacterial infections [95].

6.2.2. Cytokine-producing PBMCs and similarity matrix

The cytokines chosen for determination of the cytokine-producing PBMCs were previously reported as potential candidates for diagnosing VO [30]. Cytokines can be classified as being pro-inflammatory or anti-inflammatory. Notably, some cytokines such as IL-6 can show both pro-inflammatory and anti-inflammatory properties. This categorization of cytokines provides a broad perspective for understanding the signaling pathways triggered by the host immune response. A single cytokine can be secreted by various cells and exhibit both pro-inflammatory and anti-inflammatory effects depending on the particular context, leading to different immune responses [99]. Especially the pro-inflammatory cytokines IL-6, IL-8 and IL-12 were common in the detection of bacterial infections in previous studies of our research group [30,95]. Therefore, it is interesting to explore the potential contribution of different immune cells to cytokine production in VO patients, specifically examining which cell population primarily produces each cytokine. Furthermore, it is crucial to identify the cell population that produces the highest proportion of the selected cytokines and understand how the cytokine-producing cells differ between the two time points (pre-OP vs. post-OP) and/or the two groups (VO vs. Control). By analyzing PBMCs, we aim to uncover any differences between the VO group and the control group that could serve as a potential target for improving pre-operative diagnostic.

All VO patients received individual antibiotic therapy during the study. Antibiotics have different mechanisms of action, either fighting the bacterium by preventing its multiplication or leading to cell death of the bacterium [100] and thus have an immunomodulatory effect [101]. Some classes of antibiotics have anti-inflammatory effects that may lead to decreased cytokine release or reduced phagocytic activity of monocytes. Bode et al. demonstrated that antibiotics can modulate the immune response by regulating cytokine expression [101]. These findings are confirmed by the decreased cytokine levels of VO patients post-OP compared to pre-OP in the present study. The control group received a single-shot of antibiotic during surgery, which should have influenced the cytokine-producing PBMCs analyzed.

Commencing with IL-6, this cytokine has been consistently cited in various studies as a potential diagnostic biomarker for periprosthetic infection in patients with total knee or total hip arthroplasty [102], implant-associated infections [95], surgical spine infections after spinal surgery [103] or as a prognostic biomarker in patients afflicted with diverse bacterial infections [95,103,104]. Likewise, our study showed that IL-6 is produced by all cell populations analyzed with the highest contributors being $CD4^+$ T-cells and monocytes while the lowest contributors were B-cells and $CD8^+$ T-cells. In this study, flow cytometry showed no significant differences in IL-

6 levels between the VO and the control group at the pre-operative time point. These results were unexpected since the previous cytokine analyses had shown a significant difference between vertebral osteomyelitis and degenerative spinal diseases as well as the fact that IL-6 is a potent pro-inflammatory mediator [43,57]. This contradiction may imply that IL-6 in VO is not strongly produced by the cell series we analyzed. Notably, IL-6 is also produced by stromal cells, endothelial cells, fibroblasts, and hepatocytes, which may play a more crucial role than the set of cells we analyzed [105]. All three similarity matrices showed positive correlations of IL-6 with VEGF and/or IL-12 pre-OP, indicating a co-regulatory relationship. IL-6 may influence the expression of VEGF and IL-12. Previous studies showed that IL-6 has regulatory properties on VEGF expression [106], on the production of CRP [107] and on the development of T-cells [43].

In the present study, IL-8 was produced by all cell populations analyzed, and monocytes were by far the highest number of IL-8-positive cells (16%). Monocytes, which are stimulated by bacterial substances, produce a higher proportion of IL-8 [108]. There was a significantly lower proportion of IL-8-positive $CD8^+$ T-cells in the VO patients than in the controls at both the pre-operative and post-operative time points. These results could indicate that IL-8 is also secreted by other cells that were not analyzed such as endothelial cells, mesothelial cells or mast cells [109] [110] since the cytokine levels of IL-8 were significantly higher in the VO group. The lower proportion of IL-8-positive cells in the VO group at the post-operative time point may be due to successful antibiotic therapy and/or possible cure. The similarity matrix also confirms divergent cytokine correlations in the T cells, indicating a difference in the cytokine network of VO patients compared to patients with degenerative spinal disease. IL-8 also plays an important role in the regeneration and formation of new blood vessels and therefore contributes to wound healing and tissue repair after inflammation or surgery [43,46]. IL-8 showed a positive correlation with VEGF pre-OP in the similarity matrix of the T-cells. Both cytokines mediate angiogenesis and could be co-upregulated in VO during inflammation. Compared to the similarity matrix of T-cells post-OP, there is no specific correlation between both cytokines.

IL-12-positive cells were found in all cell populations at similarly high levels (1-3 %) in both study groups pre-OP. The VO group showed a significantly higher number of IL-12-positive lymphocytes than the controls at the pre-operative time point. This confirms the findings of the cytokine analysis, whereby the IL-12 level was significantly twice as high as that of the control group pre-OP. IL-12 is often increased within the framework of anti-bacterial defense [49]. This trend is also observed in the present study regarding B-cells, T-cells, and $CD8^+$ T-cells. Various studies pointed out the mediating effect of IL-12 on the innate and adaptive immunity and therefore, in preventing bone infections such as osteomyelitis. IL-12 is mostly responsible for the antigen-specific adaptive immunity by initiating the type 1 T helper cells immune response [111,112]. Comparing these results with the similarity matrices, IL-12

showed positive correlations with IL-6 and VEGF, showing a potential co-regulatory relationship among those pro-inflammatory cytokines at this stage of the VO disease. Within the VO group, the proportion of IL-12 producing T-cells decreased significantly post-OP compared to pre-OP, which could indicate a decline in anti-infective defenses possible due to successful antibiotic therapy and recovery. Similar to the results of the IL-8-positive cells, the IL-12-positive cells ($CD4^+$ T-cells, monocytes, NK-cells) in the VO group demonstrated significantly fewer cells compared to the control group post-OP.

The proportion of VEGF-positive cells was significantly higher in the VO group than in the control group in all cell populations analyzed (lymphocytes, B-cells, T-cells, and $CD8^+$ T-cells) at the pre-operative timepoint showing great potential for discriminating between VO and degenerative spinal disease. VEGF is primarily responsible for angiogenesis [113,114] and, thus, also is a mediator of inflammatory processes [114]. Inflammation always accompanies tissue injury and, thus, promotes the need for angiogenesis [115]. In the flow cytometric analyses of IL-6-, IL-8-, and IL-12-positive cells, monocytes usually had an equally high percentage of positive cells or even the highest. It is striking that monocytes and NK-cells have significantly fewer VEGF-positive cells than the other cell populations. The number of VEGF-positive cells was highest compared with the other cytokine-positive cell populations. VEGF was the only cytokine to show positive correlations with IL-6, IL-8, and IL-12 in the similarity matrices at the pre-OP stage. In B-cells and monocytes, VEGF was positively correlated with IL-6. This co-regulatory process may act pro-inflammatory and thus may explain the significant higher IL-6 serum levels at the pre-operative time point. In T-cells, VEGF showed a positive correlation with IL-8. Notably, these 2 cytokines play a major role in angiogenesis and wound healing [43,49,65]. Caine et al. previously described the up-regulating effect of IL-8 on VEGF [116]. VEGF showed strong positive correlations with IL-12 in T-cells and monocytes. In addition, the number of IL-12-positive/VEGF-positive lymphocytes was higher in the VO group than in the control group pre-OP. These findings lead to the assumption that they are both upregulated and belong to a tightly regulated network that may contribute to inflammation and therefore aid in differentiation of VO and degenerative spinal disease.

Taken together, IL-8-, IL-12-, and VEGF-positive cells can be investigated to aid in the diagnosis of VO.

6.2.3. PBMC profiling including PBMC isolation and flow cytometry

Numerous established procedures are available for isolating PBMCs. In our study, we employed the established Ficoll method to isolate cytokine-producing cells and analyze them via flow cytometry. Grievink et al. [117] compared three routine methods for isolation of PBMCs. These are the established Ficoll method, first described in 1968, then isolation by cell preparation tubes (CPTs) manufactured by Becton Dickinson, and third isolation by Sep-Mate

tubes with Lymphoprep by STEMCELL Technologies. The results showed that the Ficoll method showed the lowest percentage of spontaneously released cytokines. However, the Ficoll method requires more complex sample handling on the part of the laboratory technician and is therefore more dependent on the individual's level of experience in conducting the test. Nevertheless, it offers a more cost-effective alternative compared to the other two methods [117].

There are several approaches to analyze the isolated cytokine-producing PBMCs. An important point here is whether the cells should be stimulated prior to analysis. It is expected that this will lead to increased cytokine production by the individual cells and, thus, to better results. Untreated cells usually secrete cytokines below the detection level. There are several approaches to increase the intracellular production of cytokines to obtain better results in flow cytometry analysis [118]. The antibiotic Monensin interrupts transport processes, leading to cytokine accumulation in the Golgi complex [74]. Brefeldin A also inhibits protein production in a pre-Golgi compartment. Thus, both antibiotics inhibit intracellular protein transport [119], whereby Brefeldin A is more effective than Monensin [120].

Another common method is stimulation with lipopolysaccharide (LPS) from gram-negative bacteria [75]. LPS is a potent stimulator of immune cell activation, especially of proinflammatory processes. Interaction with LPS stimulates the cells of the innate immune response to produce cytokines and chemokines as an immune defense. Chaiwut and Kasinrerk [121] showed that monocytes are especially responsive to this type of stimulation, whereas T lymphocytes showed no response. In our study, we also analyzed other cell groups besides monocytes, which may not respond adequately to LPS [121]. Furthermore, this strong and artificial immune response triggered by LPS stimulation may not reflect the physiological status in VO patients, leading to a potentially biased result. In addition, LPS-induced activation may stress cells and potentially lead to cell death or changes in gene expression, which may confound experimental results [122,123].

In our preliminary experiments, we tested different conditions including stimulation with LPS and treatment with Monensin and Brefeldin A. Considering the results and potential drawbacks of LPS, we chose to use Monensin/Brefeldin for 2h before intracellular cytokine staining to increase sensitivity and detection while preserving the natural cytokine production of the cells. In addition, most of the vertebral osteomyelitis pathogens in our study were gram-positive rather than gram-negative. Moreover, the previous multiplex cytokine analysis was also performed without an artificial stimulus, so we wanted to maintain consistent experimental conditions [30]. We analyzed a total of seven cell populations and decided to exclude DCs from our analysis because they represent only less than 0.1% of leukocytes in peripheral blood [43].

Despite treatment of the cells with Monensin/Brefeldin A, the proportions of cytokine-positive PBMCs measured in this study were variable, with VEGF-positive cells showing the largest proportions, up to 30% positive cells. The IL-8-positive monocytes showed a similarly

high proportion with up to 15% positive cells, with the remaining cell populations ranging up to 1%. The IL-6- and IL-12-positive cells showed a comparable high proportion with up to 4% positive cells.

6.3 Strength, limitations and further research

There are some strengths of the present study. Notably, this is the first study that investigated cytokine-producing PBMCs derived from patients with vertebral osteomyelitis, which were compared to those from control patients devoid of infections to achieve a basis for discrimination between VO and degenerative spinal disease. Based on the results of the flow cytometry analysis of cytokine-producing PBMCs, a clear differentiation between vertebral osteomyelitis and degenerative spinal disease is possible pre-operatively, especially with the IL-8-positive $CD8^+$ T-cells and IL-12-positive lymphocytes. Compared to the control group, a significantly higher level of VEGF-positive cells (lymphocytes, B-cells, T-cells, and $CD8^+$ T-cells) was present in the VO group at the pre-operative time point. An advantage of the approach presented in this study is that access to PBMCs is simple and less invasive compared to biopsy harvesting. The epidemiological characteristics of the VO patients confirmed those previously reported in the literature.

However, the present study has some limitations. Regarding inclusion criteria, we excluded patients with Modic changes type 1 (MC1) in the VO group but did not screen patients in the control group for this type of osteochondrosis. We analyzed seven cell populations but must consider that cytokines are also produced by fibroblasts, hepatocytes, bone marrow, and mast cells [46]. Vertebral osteomyelitis is often caused by bacteria. Since neutrophils play an important role in the immune defense against bacterial infections, this cell subset would be interesting to analyze [124]. Furthermore, it is important to note that we analyzed the systemically active PBMCs from serum and not those from a local sample such as tissue.

Since our results show a clear difference between vertebral osteomyelitis and degenerative spinal disease, it would be helpful to confirm them with a larger cohort.

7. References

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8. Appendix

8.1 List of figures

8.2 List of tables

9. Publications

1) Published article of the CyProSpon-Study:

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 J Scheyerer, Norma Jung, Jan Siewe, Esther Mahabir. The diagnostic value of cytokines for the discrimination of vertebral osteomyelitis and degenerative diseases of the spine. Cytokine, 2022; 150, 155782. **IF = 3.8.**

2) Manuscript regarding cytokine-producing PBMCs in preparation