Diagnosis of infected and aseptic non-union correlating local gene expression and systemic proteomics, miRNA, and immune cells profiles.

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INTRODUCTION: A critical diagnosis in patients presenting with fracture non-union is the differentiation between infected (INF) and aseptic (AS) non-union. A preoperative diagnosis, without requiring culture of invasive biopsies, would be preferable as intraoperative decisions largely differ between both scenarios. This can be challenging, moreover, in case of low-grade infection lacking clear clinical or radiological signs of infection, or in cases where standard blood markers such as white blood cell count or C-reactive protein do not show robust and reproducible results. The aim of this study was to profile preoperative blood samples from patients with non-union and submitted for proteomics, miRNA analyses and peripheral blood mononuclear cells (PBMCs) immunophenotyping and cross-referenced with gene expression data from non-union tissue biopsies to identify potential biomarkers.

METHODS: This prospective multicenter study enrolled patients undergoing revision surgery of femur or tibia non-union. One hundred thirty-seven patients were recruited in the eight level I trauma centers in Germany between January 2019 and April 2022. Patients with implant removal after regular fracture healing (HEAL) were included as a control-group. Preoperative blood samples, intraoperative tissue samples, sonication of osteosynthesis material and 1-year-follow-up questionnaire were taken. Non-union patients were grouped into INF or AS after assessing bacterial culture and histopathology of intraoperative biopsies. Diagnosis of infection followed the fracture related infection consensus group criteria, with additional consideration of healing one year after revision surgery. Intraoperative tissue samples were stored immediately in RNAlater® and stored at –80 °C until RNA isolation using standard TRI Reagent® extraction method. Several genes, including *matrix metalloproteinase-1* (*MMP-1*) and *osteocalcin* (*OCN*) were analyzed using qPCR (AS n=62, INF n=43 and HEAL n=32). Targeted proteomics was used to investigate a predefined panel of 45 cytokines in preoperative blood samples (AS n=62, INF n=43 and HEAL n=32). The 45 cytokines were chosen according to the results from a previous analysis using Olink proximity extension assay with the 96-inflammation-panel. Statistical differences were calculated with Kruskal Wallis and Dunn´s post hoc test. Cytokines with less than 80% of samples being above the lower limit of detection range (LLDR) were excluded for this study. Isolation of miRNA from patients' plasma and miRNA sequencing was performed by Qiagen according to standard protocols (AS, INF and HEAL n=8 per group). Peripheral blood mononuclear cells (PBMCs) were immunophenotyped using high-dimensional mass cytometry (AS and INF n=11 per group; HEAL n=8). This included simultaneous determination of immune cells like T cells, B cells and dendritic cells. The study was approved by the Ethics Committee of the Institutional and National Medical Board (Bavarian State Chamber of Physicians, ID 2016-16041). In addition, each study center received further approval from their local ethics committees.

RESULTS SECTION: In total 62 AS, 43 INF, and 32 HEAL patients were recruited. Patients in the two non-union groups (INF and AS) did not differ concerning smoking, diabetes or initial open or closed fracture. Microbiological analyses of intraoperative samples and sonication fluid from the osteosynthesis material revealed a higher occurrence of *Cutibacterium acnes* (*C. acnes*) and Coagulase-negative staphylococci (CoNS) compared to other pathogens.
Proteomics analyses revealed significant increased expression of Macrophage Colony Stimulating Factor 1 (MCSF-1), Hepatocyte Growth Factor (HGF), Interleukin (IL)-6, and Matrix Metalloproteinase 1 (MMP-1) (Figure 1 A) in INF patients compared to AS and HEAL patients. Additionally, *MMP1* was also found to be significantly more expressed in the local tissues of INF patients compared to AS (p=0.0096) and HEAL patients (p<0.0001) (Figure 1 B). MiRNA analyses showed marked differences between HEAL *vs* AS and HEAL *vs* INF patients. Two miRNAs were identified as differentially expressed between AS and INF: hsa-miR-16-5p and has-miR-486-5p. The PBMCs immune profiling showed that AS patients have a significant increase in Th2 CD4+ T cells (p=0.015) and a significant decrease in dendritic cells (p=0.049) compared to HEAL patients. Furthermore, AS patients showed a significant higher amount of B cells (p=0.014) compared to INF patients. Moreover, INF patients showed a significant reduction in Th1 cells compared to HEAL patients (p=0.007).

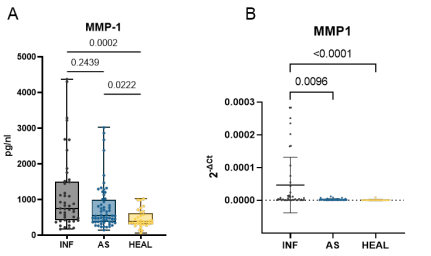

Figure 1. Expression of MMP1. A. Proteomic analyses of blood samples; B. Gene expression analyses of local tissue.

DISCUSSION: Conventional clinical diagnostic tools for infected non-union such as microbiology and histopathology can sometimes result in low sensitivity, low infection detection rate and false negative results. The wrong diagnosis of infected or aseptic non-union causes delays in the correct treatments and prolongs medical care needed for the patient. This study shows that preoperative blood samples have potential for an earlier detection of infection. Proteomics data revealed several differences in plasma cytokines between INF, AS and HEAL patients. More interesting, one of the cytokines highly expressed in the preoperative blood samples of INF patients, MMP-1, was also found to be locally expressed in the intraoperative samples. Increased expression of MMP1 was also found in the study of Ormsby *et al.*, in periprosthetic joint infection (PJI) patients [1]. Our results confirm the important role of MMP-1 in infected non-union as for PJI. The miRNAs identified in AS and INF patients in the first set of analyzed samples have been reported to be involved in osteogenesis suppression [2] and chondrocytes proliferation [3]. The immune profiling suggests that T-helper cell responses are biased in favor of humoral Th2 cell immune responses in non-union patients. Cohort expansion is needed to confirm these results, and specific subsets require a closer evaluation (ongoing experiments). Although no single biomarker is sufficient to differentiate these patients preoperatively in isolation, future multivariant analysis of the cytokine data, miRNAs, gene expression and immune cells in combination with clinical characteristics may provide valuable diagnostic insights.

SIGNIFICANCE/CLINICAL RELEVANCE: Correct diagnosis of infection is crucial in surgical treatment of non-union, but it is often challenging and can easily be missed. This study provides a set of biomarkers that could potentially be addressed preoperatively to aid in the diagnosis of infected non-union and therefore help to choose the correct treatment earlier.

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