Epigenetic and Molecular Biomarkers in Chronic Low Back Pain and Modic Changes

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Study protocol: Elaboration of the final categorization of bacterial growth and of the PCR methods used.

For the full protocol, se Bacterial growth in patients with low back pain and Modic changes: protocol of a multicentre, case-control biopsy study - PubMed

Background

Bacterial infection and Modic changes (MCs) as a cause of low back pain (LBP) is debated. Results diverged between two randomized controlled trials examining the effect of amoxicillin vs. placebo in patients with chronic LBP (cLBP) and MCs. Previous biopsy studies have been criticized with regards to methods, limited number of patients and controls, and measures to reduce the risk of perioperative contamination. In this study, we minimize contamination risk, include a control group, and optimize statistical power. The main aim of the present study is to compare bacterial growth between patients with and without MCs.

Methods and analysis:

Study design
This multicenter, case-control study examines disc material and vertebral body biopsies of patients with cLBP undergoing lumbar fusion or lumbar disc herniation surgery. Cases have MCs at the level of tissue sampling, and controls do not. Previously operated patients will be included as a subgroup. Tissue will be sampled before routine antibiotic prophylaxis with separate instruments and operating tables, and after all personnel have changed gloves. We will apply microbiological methods and histology on all tissue samples and pre-specify criteria for significant bacterial growth, possible contamination, and no growth.

In the present study, we have designed methods specifically to reduce the risk of preoperative bacterial contamination of the tissue samples, we have included a control group, and we will assess bacterial growth in subgroups.

Categorization of bacterial growth and of the PCR methods

The following tissues are collected: dermis, sub-fascial tissue, nucleus pulposus, annulus fibrosus, and endplates. Endplate and annular biopsies are only performed in patients undergoing fusion surgery. In addition, air samples from the operating theatre during surgery are collected as negative controls.

For each tissue sample, bacterial growth is recorded and the bacteria are identified at species level. Initially, the microbiologist grades the plates as "no growth", "possible contamination", and "significant growth".

"Possible contamination" means that the bacteria may be derived from the environment and could have been introduced at any step from the sample is taken to the analyses in the laboratory. Therefore, we have included negative controls in each step.

We will perform direct 16S rDNA amplicon nanopore sequencing on all frozen tissue samples and air samples. Nanopore sequence reads are searched against a 16S rDNA database and the read counts per bacterium are evaluated for each sequencing batch containing 96 samples with regard to the included negative and positive controls. Other broad metagenomic methods may be considered, e.g. Illumina sequencing.

Since *Cutibacterium acnes* is considered the main pathogen in this setting, the investigators will also use a specific PCR on all samples. To validate any positive findings, negative DNA extraction control and negative PCR control will be included in each run. Since *C. acnes* DNA is known to be highly prevalent within the air and environment generally, the negative controls may give a late positive signal due to environmental contamination. To reach a conclusion on either positive or negative finding for the *C. acnes* PCR, we will use a cutoff of 37 cycle threshold (Ct) values which is the lowest Ct value for any negative controls.

In addition, we will use whole genome sequencing on *C. acnes* isolates for phylogenetic analyses to compare isolates found in different samples from the same patient.

In cases of a culture-negative but 16S rDNA nanopore-positive biopsy, the sample is classified as "no growth" when we find the same bacterial species in the air control sample as in the biopsy.

After the study was designed and the method article was prepared, nanopore sequencing technology became available and was incorporated into the present analysis. Although not part of the original protocol, 16S rDNA nanopore sequencing was applied to all samples to complement the diagnostic approach.

The results derived from 16S rDNA nanopore sequencing will be included as part of prespecified sensitivity analyses to evaluate the robustness of the main finding. These analyses allow assessment of whether the inclusion of sequencing-based detection influences the overall estimates and conclusions, while maintaining the original study design.

Based on cultivation alone, samples will be graded as "significant growth", "possible contamination" or "no growth". Before unblinding, in preparation for the sensitivity analyses, all samples of nucleus and annulus will be classified into a final categorization of “significant growth”, "possible significant growth", “nanopore positive” or "no growth" based on cultivation and PCR (Table 1).

Table 1:

Final categorization of bacterial growth and PCR-based methods for each tissue sample

Culture of primary plate and enrichment broth	**PCR-based methods**			
	Positive *C. acnes* specific PCR[1]	**Negative** *C. acnes* specific PCR[3]		
		16S rDNA nanopore sequencing		
		Possible pathogen[4], same as culture[1]	**Possible pathogen[4], different from culture[2]**	**Negative[3] or non-pathogen[4]**
No growth, single colonies outside the streak, or clinically irrelevant species (i.e., *Micrococcus* spp).	No growth	NA	Nanopore positive only	No growth
<5 single colonies on primary plate and growth of <5 colonies from enrichment broth **or** **No growth on primary plate, but growth of ≥5 single colonies from enrichment broth**	Possible significant growth	Possible significant growth	Nanopore positive only	No growth
Growth on primary plate and from enrichment broth, or growth ≥5 single colonies on primary plate regardless of growth or not in enrichment broth	Significant growth	Significant growth	Significant growth	Significant growth

NA; not applicable

[1] positive for the same species as the cultivated microbe.

[2] positive for a different species than the cultivated microbe.

[3] negative for the cultivated microbe

[4] Which bacteria that were regarded as possible pathogens were based on the literature of reviews and case-reports.

Planned analyses

The analysis are detailed under Study Description at ClinicalTrials.gov

Statistics and power:

These are also detailed under Study Description.

Ethics and dissemination:

The Regional Committees for Medical and Health Research Ethics in Norway (REC South East, reference number 2015/697) approved the study. Study participation requires written informed consent. The study is registered at ClinicalTrials.gov (NCT03406624).

Discussion

This protocol describes a detailed and pre-specified strategy for conducting a biopsy study in patients with or without MCs undergoing spinal fusion or lumbar disc herniation surgeries.

The infection theory postulates that disc degeneration and/or herniation may lead to neovascularization, allowing bacteria into the intervertebral disc. Cytokine and propionic acid production may cause local inflammation and, consequently, MCs. The main aim is to determine if there is an association between MCs and bacterial growth.

The methodology of previous biopsy studies has been questioned. Potential contamination of surgical samples is a common argument against the theory that bacteria lead to MCs. Some studies have used percutaneous techniques, with a high risk of contamination. Other studies did not specify if separate instruments were used for the surgical approach and for the biopsy sampling. Furthermore, routine administration of prophylactic antibiotics and/or steroids were administered before biopsy sampling in some studies, or not specified in others. In our study, the ethical board gave permission to delay routine antibiotic prophylaxis by one hour after the incision. This will allow us to obtain biopsies unaffected by preoperative antibiotics. We have argued that delaying antibiotics by one hour from skin incision carries a minimal risk of increased surgical site infection. Cefazolin (negatively charged) and clindamycin (positively charged) can penetrate cancellous bone and synovial fluids. However, the amount of antibiotics that reach the avascular disc within this short time is probably negligible, especially for negatively charged antibiotics such as cefazolin. It has been shown that intervertebral disc tissues receiving positively charged clindamycin contained a significantly greater percentage of the antibacterial dose than tissue from patients receiving negatively charged cefazolin. Our target population of elective immune competent patients (with few comorbidities) are expected to have a low risk of postoperative infection.

Furthermore, anterior (retroperitoneal) surgical approaches are associated with fewer positive bacterial samples compared to traditional posterior approaches. In our study, only posterior approaches are used.

The pre-specification of microbiological endpoints of “significant growth”, “no growth”, and “probable contamination” are crucial for this study. The definition of infection or no infection may appear straightforward. However, *Cutibacterium acnes* is present in normal skin flora and is a well-known contaminant in microbiological laboratories. In our study, we expect different grades of bacterial growth, as outlined in table 1.

The interpretation of findings in the different tissue layers should also be discussed. The dermis is included as a “positive control sample” since we expect to find bacterial growth from the normal flora, especially *Cutibacterium acnes*. In contrast, the sub-fascial tissue is considered a "negative control" as the tissue is well beneath the dermis, where no normal flora is expected, and is not part of the affected MC region.