Title: Cutibacterium acnes is present in non-herniated human discs; its positivity rate correlates with the patient's age.

Introduction:

The presence of bacteria in the intervertebral discs (IVDs) and their role in disc degeneration is an area of controversy. Numerous studies have detected *Cutibacterium acnes* and other microbes with 16S DNA Sequencing and microbial cultures. However, those studies fail to determine whether the bacteria are *in-vivo* disc bacteria or perioperative contamination. Capoor *et al.* [1] performed confocal scanning laser microscopy for a limited number of herniated IVDs and detected *C. acnes* biofilms within the human specimens. Our study investigated the presence of Gram-positive bacteria *C. acnes* and *Staphylococcus aureus* in non-herniated human IVDs. Furthermore, expression of cellular recognition receptors Toll-like receptor (TLR) 2, TLR4 and NLR family pyrin domain containing 3 (NLRP3) and the pyroptosis marker Gasdermin D were investigated.

Methods:

Immunohistochemical staining for Gram-positive bacteria, *S. aureus, C.acnes* TLR2, TLR4, NLRP3 and Gasdermin D was performed on 75 non-herniated human IVD samples. Cell detection and classification was performed using QuPath. Fluorescently labelled *S. aureus* cells were co-cultured with human NP cells in monolayer across multiplicity of Infection (MOI) range (1:10- 1:100), and analysed by confocal imaging. Furthermore, human nucleus pulposus (NP) cells in monolayer were treated with Lipopolysaccharide (LPS) (5-50µg/ml) and Peptidoglycan (PGN) (5-50 µg/ml) for 48h, and cells in 3D alginate with PGN for up to 72h. Secretome analysis was performed using Luminex for cytokines, chemokines, matrix degrading enzymes and other secreted factors. Statistical analysis was performed using Kruskal-Wallis, Dunn's multiple comparison test and Pearson correlation.

Results

Co-culture of S. aureus with NP cells showed internalisation of bacteria. Immunohistochemical staining demonstrated gram positive bacteria was solely detected within cells and not as biofilm within the tissue. The positivity rate of *C. acnes* ranged between 5-99%. The number of *C.acnes* positive cells showed a correlation with the age of the patients ($r=0.41$, $p= 0.007$). However, it did not correlate with grade of degeneration. The positivity rate of TLR2 ranged between 5-99% and TLR4 from 3-72%. TLR2 and TLR4 showed a strong correlation ($r= 0.62$, $p= 1.5e-006$). A significant decrease in TLR2 was observed in females showing a mid-degenerative grade compared to females showing no signs of degeneration. Investigation of the presence and the correlation between NLRP3, GasderminD, *S. aure*us and the above-mentioned factors is undergoing. Treatment of NP cells with LPS and PGN resulted in an increase of several catabolic cytokines such as IL-1, TNF, IL-6 and IFN-γ alongside increased production of chemokines, neurotrophic and angiogenic factors associated with IVD degeneration.

Conclusion

This study demonstrated the presence of Gram-positive bacteria such as *C. acnes* in non-herniated and cadaveric human disc samples. The internalisation of bacteria by human NP cells was demonstrated and aligns with previous publications. Furthermore, this shows a correlation between age and the presence of *C. acnes* as well as a strong correlation between the two TLRs. Moreover, bacterial cell membrane components triggered a catabolic response in human disc cells. Ongoing interaction studies between bacteria and NP cells will give us insight it to the potential role of bacteria in disc degeneration.

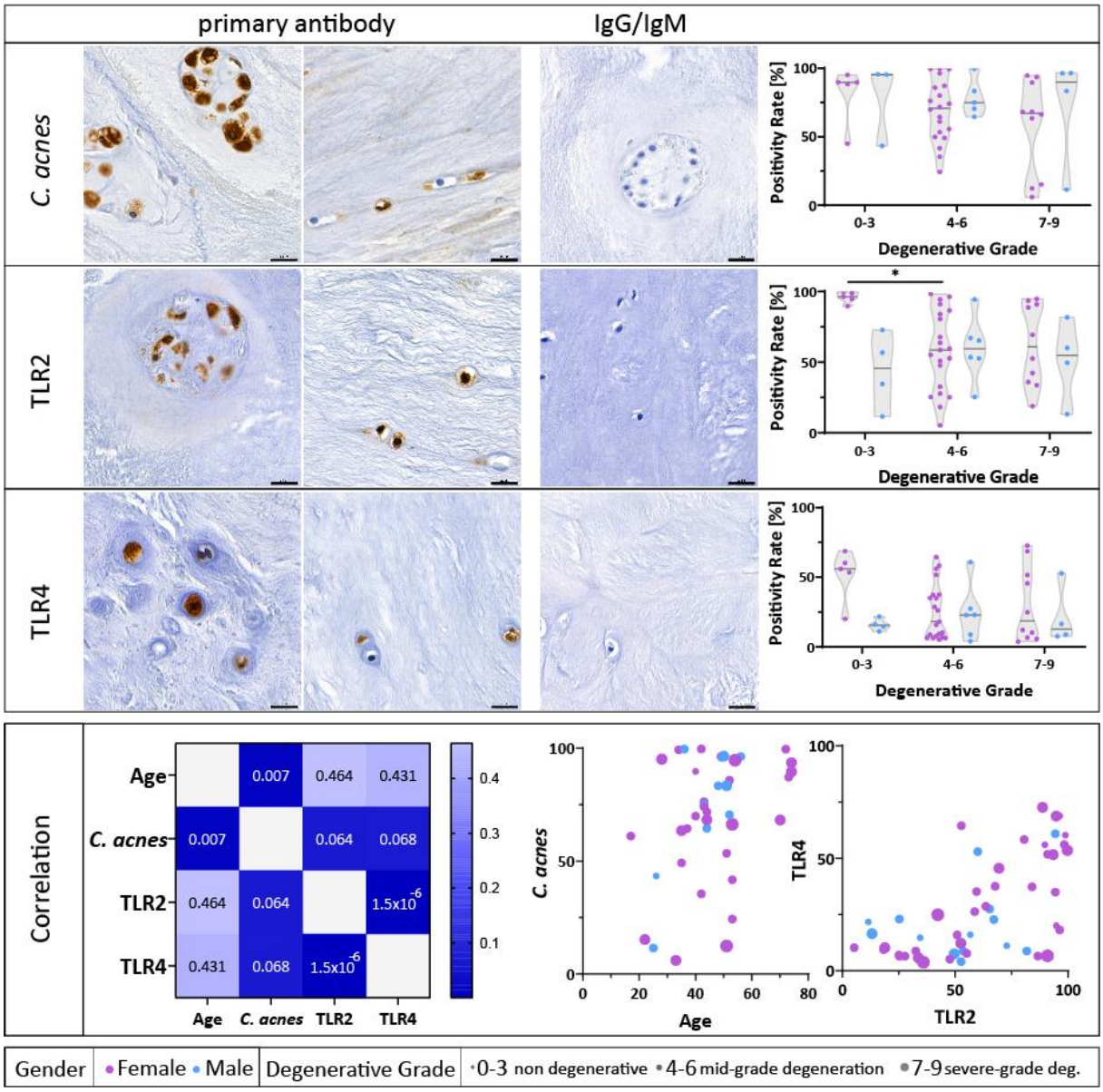

Figure Legend: Will be included in Illustrator:

(Top) Immunohistochemical staining for C. acnes, TLR2 and TLR4 demonstrated their presence in the human disc tissue. The IgG/IgM isotope control were negative. Positivity rate of TLR2 is significantly lower in females showing mid-grade degeneration compared to non-degenerative phenotype females. (Bottom) A matrix showing the p-values of a Pearson-correlation study. C. acnes immunopositivity was correlated with age. A strong correlation was detected between TLR2 and TLR4.

1: Coenye T, Spittaels KJ, Achermann Y. The role of biofilm formation in the pathogenesis and antimicrobial susceptibility of *Cutibacteriumacnes*. Biofilm. 2021 Dec 9;4:100063. doi: 10.1016/j.bioflm.2021.100063. PMID: 34950868; PMCID: PMC8671523.