Author’s response to reviews

Title: Cellular pharmacodynamic effects of Pycnogenol® in patients with severe osteoarthritis: a randomized controlled pilot study

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Reviewer reports:

Sonja Maria Walzer (Reviewer 1): In this study the authors describe the cellular pharmacodynamic effect of pycnogenol in serum, synovialfluid and chondrocytes in OA patients on gene expression and changes of specific markers like MMPs. Pycnogenol was given over 3 weeks to patients suffering from OA prior to the surgical intervention of joint replacement. The used methods are well described and the clinical application of pycnogenol and the relevance of the pharmacodynamic effect is of interest for the musculoskeletal research community.

We would like to thank the reviewer for appreciating our methods and her interest on our study. Thank you for taking the time to evaluate our manuscript and for the helpful comments.

Do the authors think that a three weeks intake is representing the effect of pycnogenol on OA? Are there any short time effects shown in the literature and will this effect improve the patients outcome according to the progression of the OA? Which benefit is expected for the patients?

Before we initiated this current investigation three randomized placebo-controlled double-blind studies with osteoarthritis (OA) patients taking Pycnogenol have been published (see references 6-8 in the reference list). All studies revealed time-dependent effects on symptoms of OA. For example, in the study of Cisar et al. (reference # 8) statistically significant differences between Pycnogenol and placebo regarding the overall WOMAC score (summarizing pain, stiffness and daily activities) were observed after six weeks. In other studies (e.g. reference # 7) measurements were made only after three months.
In a clinical context, an improvement of OA symptoms is typically a “late” event and is preceded by changes on a cellular level. Based on the clinical studies we hypothesized that changes on a cellular would occur earlier than 4-6 weeks. The three weeks of intake were finally chosen because this is the time period that the clinicians routinely schedule between pre-surgery meeting with the patients and the elective surgery. During this meeting a basic examination was performed, the patients were supplied with the pine bark extract and pre-intervention blood samples were obtained.

Clearly, the benefit for the patients is not the change of inflammatory or chondrometabolic markers, but the reduction of OA clinical symptoms. Since the latter had already been demonstrated in clinical trials our study aimed at providing a rational basis for the reported clinical effects. Besides the pharmacokinetic aspect (analysis of Pycnogenol constituents and metabolites in serum, blood cells and synovial fluid; reported in a separate manuscript) we addressed the pharmacodynamic aspect on a cellular level in the present manuscript.

We included these additional explanations into the manuscript.

- Which effect would the authors expect given this product to OA patients over 1 year? Compliance? When will be the right point of time to start? Are there any studies about the safety of the substance?

Since there are no clinical studies yet that investigated the effects of Pycnogenol over one year we can only make educated guesses. We would expect that the cartilage degradation would be slowed down and symptoms of knee OA would be attenuated. We would think that the best time to start would be as early as the first mild symptoms occur. Clearly, severe OA cannot be healed, but it might be possible to extend the time period until a knee replacement surgery becomes a medical requirement.

As for the compliance: we do not yet have data over one year. However, from our experience with the current investigation we can conclude that there is a cohort of patients is very open towards a regular intake of plant extracts. They rather prefer herbal remedies over regular use of analgesics and typically their motivation is very high thus the compliance excellent. We can report that after the end of the present study some patients initially refused to return the residual Pycnogenol capsules that we needed for counting the number of used capsules. Those patients argued that they wanted to continue the intake of Pycnogenol because they hoped to postpone a surgery of the other knee.

Regarding the safety: currently there are 76 completed clinical studies with Pycnogenol including 8325 patients. A recent review (Med Res Arch 2015; 3: 1-11) reported a rate of 2% mild unwanted effects in clinical studies. The safety is therefore well documented and the current study was immediately approved by the ethics committee of the Medical Faculty.

- Are there any effects/findings beside inflammation?
There are various review articles summarizing the effects of Pycnogenol, e.g. there is an independent scientific and clinical monograph of Pycnogenol by the American Botanical Council (www.herbalgram.org) and many other reviews. We did not detail all reported effects in the manuscript, but focused on those that might be relevant in the context of OA. Anti-inflammatory effects have been described before, the present study adds new insights on chondrometabolic markers that play a role in OA.

- According to the design: There is no placebo group included.

The current study did not aim at measuring clinical effects, e.g. symptoms reported by the patients. As explained above, the aim was to investigate pharmacokinetic aspects (analysis of Pycnogenol constituents and metabolites in serum, blood cells and synovial fluid; reported in a separate manuscript) as well as pharmacodynamic aspects on a cellular level. To the best of our knowledge it is not possible to deliberately influence the concentration or expression of inflammatory or chondrometabolic markers, just as it is not possible to deliberately influence the concentration of constituents of metabolites in serum, blood cells or synovial fluid. Pharmacokinetic trials are typically open-label studies without placebo control. Therefore, we think that it was scientifically justified to compare the data with data of an untreated group in the present pilot study.

We now discussed this point more detailed in the manuscript.

- The authors are choosing patients with severe OA. Which long time benefit do the authors expect for patients with severe OA? (outcome measures,.....)

We chose patients with severe OA because it is the most ethical option to simultaneously obtain synovial fluid and cartilage samples. The patients were already scheduled for surgery, so the sampling of those specimen did not require additional invasive procedures for the patients. In knee replacement surgery the synovial fluid and knee fragments are usually discarded.

We do not think that patients with severe OA would have vast benefits from taking Pycnogenol in the 3 weeks between inclusion and the knee replacement surgery, which was already scheduled. However, as discussed above, we have good reason from results of the clinical studies (cited in references 6-8 in the reference list) that the patients could have less pain and less joint stiffness.

We now discussed this point more detailed in the manuscript.

Stefan Toegel (Reviewer 2): This study investigates the effect of a nutritional extract (Pycnogenol) on molecular marker levels in articular chondrocytes, synovial fluid and serum obtained from arthroplasty patients. The authors argue that this setting allows studying the activity of a dietary supplement (including its bioactive metabolites!) in an in vivo setting that also takes bioavailability into account. This is a good point and makes the manuscript interesting.
However, there are a number of limitations and weaknesses of the manuscript which should be addressed before publication can be considered.

We would like to thank the reviewer for his understanding of our intention and taking the time to evaluate our manuscript and for the helpful comments.

*) OA patients - who received neither Pycnogenol nor placebo - served as control. Although the authors indicate this limitation in their work, the scientific value is compromised by this limitation.

The current study did not aim at measuring clinical effects, e.g. symptoms reported by the patients. The aim was to investigate pharmacokinetic aspects (analysis of Pycnogenol constituents and metabolites in serum, blood cells and synovial fluid; reported in a separate manuscript) as well as pharmacodynamic aspects on a cellular level. To the best of our knowledge it is not possible to deliberately influence the concentration or expression of inflammatory or chondrometabolic markers, just as it is not possible to deliberately influence the concentration of constituents of metabolites in serum, blood cells or synovial fluid. Pharmacokinetic trials are typically open-label studies without placebo control. Therefore, we think that it was scientifically justified to have an untreated control group in the present study. We would like to emphasize that this is a pilot study. When we started the study it was not clear at all whether we would observe any changes in inflammatory or chondrometabolic markers after only three weeks of intake of Pycnogenol. We would like to point out that this is – to the best of our knowledge – the first human study that indicates cellular effects of a plant extract in various specimen of OA patients.

We now discussed this point more detailed in the manuscript.

*) The impact of the study is largely limited by the fact that only one statistically significant result (that is relevant for the study) is presented (Figure 3). Due to the lack of other significant results, large parts of the discussion are rather speculative.

We reported not only the statistically significant decrease of ADAMTS-5 concentration in serum, but also the statistically significant decrease of MMP-3, MMP-13 and IL-1beta expression in chondrocytes. Those cartilage degradation markers have been discussed as relevant in other OA studies, e.g. in numerous animal studies.

Unquestionably, we would have liked to present more statistically significant results. However, as explained before, this investigation was designed as a pilot study. There was no pre-study data on the effect size of Pycnogenol on inflammatory or chondrometabolic markers in OA patients. The current investigation provides a basis for a rational calculation of the sample size of subsequent studies that would thus not be underpowered.

In the present study the limited number of participants and the high inter-individual variability of data hindered confirming results with statistical methods. Despite of that fact we still do report
important statistically significant results. Moreover, all tendencies that were not statistically significant are consistent with the clinical study results.

We now discussed this more detailed in the manuscript.

*) line 46: "…investigated in serum…"

Thank you for the indicating the missing word, we changed the manuscript accordingly.

*) Official gene symbols should be used throughout the manuscript to indicate mRNA levels and avoid confusion with protein levels (e.g. IL1B instead of IL-1ß which would indicate the protein).

Thank you for the indicating this, we changed the manuscript accordingly.

*) The conclusion of the abstract should be revised to be more specific and related to the study.

We changed the abstract according to the suggestion of the reviewer.

*) The manuscript should unambiguously state why endstage OA patients were used in this study. Is there any benefit expected from clinical application of the extract in endstage patients?

We chose patients with end-stage OA because it is the most ethical option to simultaneously obtain synovial fluid and cartilage samples (along with serum and blood cell samples). The patients were already scheduled for surgery, so the sampling of synovial fluid and cartilage did not require additional invasive procedures for the patients. In knee replacement surgery the synovial fluid and knee fragments are usually discarded. In fact, the surgery team is so used to dispose of synovial fluid and knee fragments that they occasionally forgot to collect those samples for us during surgery (reported in the manuscript under “Results” lines 247 / 248 (page 11, line 13 and 15).

We do not think that patients with end-stage OA with a knee replacement surgery being already scheduled would have vast benefits from the intake of Pycnogenol. We would think that the disease progression might be slowed down and that a knee replacement surgery might be postponed for a while if Pycnogenol is taken earlier, e.g. after diagnosis of OA. As reported in previous clinical trials which evaluated symptoms (references 6-8 in the reference list; those patients had mild to moderate OA) we would think that the patients have less pain, joint stiffness, more daily activities and thus a higher quality of life.

We now discussed this point more detailed in the manuscript.

*) The first phrase of the introduction (line 61) should be revised to improve wording.

We changed this according to the suggestion of the reviewer.
*) The statement in line 82 ("Subsequent investigations…") should indicate the biological background of the previous studies. Which cells, which disease context…?

We changed this according to the suggestion of the reviewer.

*) Methods: Regarding synovial fluid, please provide details on how samples were collected and processed (centrifugation, pre-treatment,…)

The synovial fluid samples were centrifuged at 1000 g for 10 min using the Megafuge 1.0 R Thermo Scientific (Waltham, MA, USA). No other particular pre-treatment was performed except for the samples used in DMMB assay (which is described there, see lines 221 ff), since for this test the samples had to be less viscous to allow for homogeneous distribution of the colorant.

We added this according to the manuscript.

*) line 157 ("All samples were shock-frozen.."). This statement is misleading as it obviously does not include "knee fragments" (preceding sentence).

We added "serum and synovial fluid samples" to the sentence for clarification. "All serum and synovial fluid samples were shock-frozen and stored at -80 °C." The knee fragments were not shock-frozen to guarantee the survival of chondrocytes.

We changed this accordingly.

*) Methods, line 174 ("Residual articular cartilage…"). Which anatomical tissue regions were selected for cell isolation (loaded/unloaded, un/affected)?

The complete available cartilage was removed from the joint pieces to obtain an average expression of the marker genes, since it is not possible to definitely distinguish between affected and unaffected areas macroscopically. The cartilage is known as tissue with low cell number, so digesting the whole cartilage was necessary to obtain a sufficient chondrocyte cell number for RNA isolation and cell culture experiments.

We added this information to the manuscript.

*) The authors cite the MIQE guidelines. Did they check for RNA integrity of the preparations using qualified methods as outlined in these guidelines?

Due to the very low amount of tissue obtained from all patients and therefore the very low amount of cells and RNA isolated from the tissue, we had decided to check the RNA quantity and purity (A260/A280 ratio) for further processing and not the RNA integrity via agarose gels stained with ethidium bromide (for which about 200 ng RNA is needed) referring to point 5 of
the MIQE guideline stating that “The only situation in which this requirement does not apply is when the quantity of total RNA extracted is too low to permit quality assessment.”

*) The citation and general adherence to the MIQE guidelines is appreciated. However, there is one issue regarding the use of reference genes. The authors state that SDHA was found to be unstable under the experimental conditions. So why was it still used for normalization to produce the data shown in figures, but then omitted to allow re-calculation which sometimes even improved significance? If omission of SDHA is justified, then the presentation of the data (that were generated using the 2 remaining reference genes) would be more straightforward and meaningful.

According to the MIQE guidelines a minimum of two validated reference genes should be used for normalization procedures, though ideally the use three reference genes was recommended (see e.g. PLoS One 201; 8(3): e59180). Thus, we followed that suggestion and aimed at using three reference genes. However, we then noticed that SDHA was less suitable and therefore we calculated results with and without inclusion of SDHA. We do not want to leave the impression that we suppress data or report incomplete data. Therefore, we prefer to report the results with and without inclusion of SDHA so that other researchers can get the full picture.

*) Line 275ff: Please indicate in which group the "tendency towards down-regulation" was observed.

…and a clear tendency to down-regulation in the Pycnogenol® group…

We added this information to the manuscript.

*) The result "Correlation analysis of biomarkers" (line 330ff) is rather off-topic and not related to the study aims. It should be shifted to the supplementary data.

In addition to our statistically significant results a correlation analysis strengthens the scientific value of the study. The results of our correlations show (patho-)physiologic coherences.

*) The relation of the results presented in the final paragraph of the results section (line 337ff) to the study is unclear. It appears that these data are obtained from a different study submitted elsewhere (line 338f). Please report full data and the respective methods or delete the entire section if reported elsewhere.

The reviewer is right, we now give a reference to access that study which contains a complete and detailed description of all data and methods used.

*) Discussion (line 349). In fact, this study does not investigate the "molecular effects of the…extract…on … markers". Please revise wording.

The sentence was revised: “…cellular effects of the maritime pine bark extract Pycnogenol® on various catabolic and inflammatory…”
We changed this accordingly.

*) The legend to Figure 2 should indicate what the data are referred to. What is "1"? Levels in control groups should be given in the figure.

The figure shows the relative gene expressions of the target genes in the Pycnogenol® group in relation to relative gene expressions of the target genes in the control group, generated using the REST®2009 software of Pfaffl et al.. Therefore, “1” is the relative gene expression of the particular targets (conc. of gene of interest/conc. of reference gene) in the control group. Since more than one reference gene was referred to, the relative gene expression of targets was calculated by dividing the concentration of the gene of interest by the geometric mean of the concentration of the several reference genes.

We added this information.

*) Figure 3: the legend should provide more details on data processing. The figure should indicate the statistical difference.

Each dot symbolizes the difference of serum concentrations regarding ADAMTS-5 in one patient (concentration of ADAMTS-5 in serum after intake subtracted by the concentration of ADAMTS-5 in serum before intake). A mean decline of 31.12 +/- 67.85 ng/mL in the Pycnogenol® group (n= 15) opposed a mean increase of 33.37 +/- 72.82 ng/mL in the controls (n= 15). The difference of those concentration differences between the groups was statistically significant (p= 0.02), which was even more pronounced after exclusion of one identified outlier in the control group (p= 0.001).

We added this information to the manuscript.

*) Figure 3 and 4 can be combined and presented as 2 panels of the same figure.

We would rather prefer to have two separate figures because the one shows a significant, the other a non-significant effect and both have very different numbers of patients with quantifiable concentrations of the investigated markers. However, if the editor wishes to have these figures combined we can certainly do so.