Comment inserted in the website of Retraction of Arnica paper.

June 21, 2019

Authors' reply to the retraction note

The 20 of June 2019 the journal PlosOne has retracted our article "*Arnica montana Stimulates Extracellular Matrix Gene Expression in a Macrophage Cell Line Differentiated to Wound-Healing Phenotype*" (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0166340), that was published in November 2016 [1]. The results of this work provide new insights into the action mechanism of Arnica montana (Arnica m.) in homeopathic dilutions in tissue healing and repair, and identify extracellular matrix regulation by macrophages as a possible therapeutic target. The retraction note appears in the journal website at https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0219007. We and the other co-authors are completely opposed to a decision that seems unjustified and seriously damaging to the way a scientific community proceeds.

The manuscript was sent on 5 February 2016, accepted on 26 August after a long and thorough review process and published on 10 November 2016. The retraction of the paper more than 30 months after the publication is incredible and, in hindsight, not motivated by any finding of errors or methodological defects. Our technical response to the editorial note of retraction is explained in a detailed manner here below (the Editorial statements are followed point-by-point by the author's responses)

1. The retraction note states that "*concerns were raised about the concentration of Arnica montana (Arnica m.) used in the experiments, and that the reported gene expression changes are within the range of what would be expected for standard noise within an RNA-seq dataset*."

RESPONSE: Readers must know that when a sort of "inquiry" was opened on our work, we asked about the source of the "concerns", but no further information was provided. Such a lack of clarity on the origin of the criticisms does not seem to correspond to a normal debate in a crucial scientific discussion. In any case, following Editor request, we provided all the original data, in addition to those that we had already deposited at the time of the first publication. We sent to PLOS ONE as many as 17 files containing the original data. Subsequently, we received a list of queries and we responded to all the questions raised. On the light of this correspondence, the retraction of the paper appears as unjustifiable. The concentration of Arnica m. used in our study was addmittedly low, as we clearly noted, where we used solutions diluted over 10.000 times from starting crude extract. However, these are the Arnica m. dilutions normally used by other investigators in laboratory settings [2–8] and also in several clinical trials [9–17]. The interest and novelty of our research is actually the discovery that extremely low doses and high dilutions of a medicinal plant are active in a precise in vitro model used with macrophages. As an expected consequence of the very low doses used, also the cell responses were low, but strongly statistically significant. Just because the expected effects were small, we programmed an accurate protocol with five independent replications, each one of which done in triplicate wells. The RNA-seq analyses were done by independent researchers, not aware of the solutions used in different samples.

2. "*Specifically, the authors reported results of RNA-seq experiments and follow-up cell culture-based studies to examine effects of Arnica m. on macrophages, using dilutions of Arnica m. ranging from 2c to 15c (i.e. 10−4 to 10−30). The authors reported that sesquiterpene lactones were present in 2c experiments at a final concentration of 1.05 x 10−8; the exact concentration of other components was not reported. In Figs 1 and 2, the article reports an absorption spectrum and nanoparticle*

spectrum analysis for the Arnica m. 1c starting material, but not for the 2c or other solutions used in the study, or for a control solution. This raises concerns on whether there is sufficient evidence to demonstrate that biochemically active ingredients remain in the diluted solutions used in the experiments."

RESPONSE: To get a more complete overview of the doses / dilutions of *Arnica m.* normally used in medicine, we decided to test a series of dilutions from 2c (very low dose) to 15c (very high dilution). We considered the characterization of the starting material by dosing the sesquiterpene lactone compounds to be adequate, since it corresponds to the standard requirement of official pharmacopoeia and is sufficient to guarantee qualitative and quantitative information about the product used. UV-VIS spectrum and NPs-spectrum represent fingerprints of the starting solution that can be compared in future with solutions from different sources. These analyses were done on the *Arnica m.* 1c dilution because at this dilution we have the highest dose and best precision. The 2c dilution and the following dilutions (i.e 3c, 5c, 9c and 15c) are obtained by serial 100 times dilution/succussion, as clearly described in the Methods, starting from the same solution 1c, and using the same hydroalchoolic solvent. We recorded the spectra of 2c dilution but we have not reported in the paper because, as expected from a 100 x diluted solution, the absorption peak was around the detection power of the instruments used. The spectrum of the control was not reported just because the control solution was included in the reference cuvette of the double-beam spectrometer. Since this point appears as one of the reasons of retraction, in a forthcoming paper we will show also this result and the baseline of control as the (obvious) baseline (zero absorbance, background noise). As a matter of facts, our major experimental result that sesquiterpene lactones of Arnica 2c (at a precise final concentration of 1.05×10^{-8} mol/l) have a statistically significant capacity to modulate gene expression of human macrophages is undisputable, even if the exact concentration of (supposed) other components was not reported. Other types of analysis were not performed since the scope of the investigation was the study of cellular and molecular action mechanisms of a popular plant medicine. The description of nature of the information remaining in the water structure after reiterated dilutions/succussion and the determination of the suitable tool to investigate it is an actual and complex topic, but was beyond of the scope of the study. According to current views in literature these changes are related to nano-heterogeneities of water solutions (see response to point 3 here below).

3. "*The RNA-seq results obtained using the most concentrated Arnica m. solution (2c) are reported in Table 1, wherein mean Log2 Fold Change values are reported in the range from -0.36 to 0.3, i.e. the authors reported changes in gene expression in the range of 0.75–1.25-fold following Arnica m. treatment as compared to cells in the control group. A number of concerns have been raised about these results: a)Questions have been raised as to whether RNA-seq data within this fold range reflect biologically significant or reproducible changes in gene expression; b)The statistical strength of the results reported in Table 1 and Fig 5 have been called into question, and specifically concerns have been raised as to whether the p values reported in Table 1 provide a valid statistical assessment or clear representation of the relationship between the gene expression mean and standard error values in columns 4–7; c) Follow-up experiments using pooled samples of cells treated with more dilute solutions (3c, 5c, 9c, 15c) yielded results in approximately the same range of fold change, as reported in Fig 5, calling into question the specificity of the reported results. The PLOS ONE Editors have discussed the study design and results reported in this article with experts in RNA-seq analysis, statistical analysis and members of our Editorial Board. Based on our assessment and the advice received, and in light of the above concerns, we have determined that*

the results presented in this article do not provide sufficient support for claims about effects of Arnica m. on gene expression. Hence, we are retracting this article due to concerns about the study design and about the validity and reliability of the reported conclusions. We regret that these issues were not fully addressed during the article's pre-publication peer review."

RESPONSE: This question in its entirety reveals that the Editor and the experts he consulted do not "believe" our results, despite the experimental and statistical evidence we provide. As discussed above, it must be considered that a low effect of the 2c solution was expected, since it contains nanomolar doses of the supposed active ingredients. The effects are small but statistically significant.
a) The question of the biological significance of the small canges recorded has been widely discussed in the paper including this paragraph: "The slight effect in this *in vitro* model does not mean that the modulating effect will also be small *in vivo,* in whole organisms. Whereas conventional anti-inflammatory drugs are designed to suppress the underlying enzymatic mechanism of inflammation (e.g. prostaglandins, cytokines) and act at considerably high doses, homeopathic treatment is designed to regulate only the pathological aspects and malfunctioning tissues, because the inflammatory process in itself is seen as an expression of natural healing dynamics. In these conditions, even a 20-30% increase of macrophage activity in production of key-proteins such as fibronectin may have a decisive positive outcome of tissue healing and repair. Moreover, given the variety of *Arnica m.* effects and the multiplicity of its alkaloids, flavonoids, and sesquiterpene lactones[18], it is conceivable that the picture of its action is much more complex and could involve modulation of different cells and further pathways. The field of pharmacologic regulation of connective tissue and cell matrix by natural and chemical compounds is open to further studies and developments[19]." It is therefore quite astonishing that the same journal that reported the discussion requires the same, already given and published, explanation. Furthermore, changes in FBN1 gene expression are validated by changes in the production of fibronectin protein as shown by ELISA analysis. That this change is biologically significant is suggested by the fact that Fibronectin is the main protein produced by macrophages during wound healing and even a small increase could play an important role in the healing process, which has been amply illustrated in the discussion.

b) Concerning the statistics, since the expected differences in gene expression were small, the experimental design and statistics were defined consequently, choosing a high read depth in sequencing, using five independent experiments, appropriate batch-controlled model and Wald statistic test, corrected with false discovery rate (FDR) (0.05). We performed a total of 5 complete separate experiments; in each experiment, every treatment was performed in triplicate wells. Data were analyzed using a paired statistical approach (*Arnica m.* 2c versus control solvent) that properly minimized the inter-experimental variations. By this way we could unmask even small effects due to the treatments. The small changes of the DEGs were reproduced in all the experiments, as reported in the Supplementary Table 1(expression values of the 5 replicated experiments), supporting the significant p values obtained. How much this small change can be reproduced must be said by other researchers, as is customary in science. In fact, an analysis carried out by PLOS ONE on the data sent by us confirmed the significant increase in expression of Fibronectin, as documented by a previous correspondence with the Editor (17 November 2018). The other genes reported by us are poorly expressed and we cannot even determine the protein, as we correctly reported. However, even if their expression is low, they have a high biological meaning and internal coherence in the context of wound healing: besides FN-1, HSPG2, and FBN2 are proteins of the extracellular matrix, MACF1 directs cell movement through microtubule organization, LRP-1 has a role in restoring connective tissue integrity through the modulation of endocytosis and protein degradation. DESeq2 is sensitive to small, true differences as well as large true differences, all while controlling specificity/false positives, if you use the

FDR cutoff. The false discovery rate is a statistical method to reduce the risk of false positive calls when multiple repeated measures are done, typically in –omic assay. DEseq2 applies an algorithm to control the expected FDR below a specified level given a list of independent p-values. The outcome of the application of the FDR correction is that many genes with p not adjusted <0.05 actually are discarded because of the risk of being false positive. Such restriction on the number of the significant calls grants to get reliable results and is always used in RNA-seq dataset analysis. Recently we have confirmed the upregulation of FN-1 also with quantitative RT-PCR (manuscript in preparation).

c) Follow-up experiments comparing more diluted *Arnica m.* solutions with the control solution suggested that the gene-set is still modulated (Fig 5), giving a preliminary evidence of the existence of non-linear biological effects. The aspecificity of these results could be excluded by the following two considerations. The statistic values (Wilcoxon) express the low probability ($p<0.05$) that the maintenance of the direction the of change (Up-reg or-down-reg) is due to chance. Moreover, as proof of the absence of biases due to the control sample, we observed that analogues results were obtained calculating the fold changes using the data from the pooled control sample or from the average of the replicate control samples (data reported in the file "Data for Figure 5.xlsx" furnished to the Editor in the course of the inquiry). The effect of a 3c dilution is only slightly smaller than a 2c, although the expected concentration of active ingredients is 100 times lower. Significant *Arnica m.* activity on the set of genes considered is also found in the 5c and even higher dilutions. It can be understood that a referee not familiar with high-dilution pharmacology may have doubts about the "specificity" of these effects. Yet we have found and documented them, comparing with a "placebo" solution. An important methodological aspect, that PLOS ONE reviewers of original manuscript had appreciated, but was ignored by experts of this latter re-review, is that the tested samples were prepared by the special method of dilution followed by strong shaking (succussion), which is characteristic of homeopathic pharmacopoeia. Sequential dilution and succussion in the homeopathic production process changes the physico-chemical properties of the solutions, indicating that succussion may have an important influence on treatment effectiveness [20–22]. According to current views in literature these changes are related to nano-heterogeneities of water solutions (e.g. nanostructures, clusters or coherence domains) [22–29] and so highlight the need for further research. Science has always progressed when current theories were tested and even challenged by experimental evidence, not when experimental evidence was censored for not agreeing with dominant ideas.

> *4." In addition to the above, the PLOS ONE Editors hereby notify readers that the Competing Interests statement was incorrect for this article and should have explicitly stated that Boiron Laboratories, a company that provided funding support for this study, markets homeopathic products including various dilutions of Arnica m."*

RESPONSE: We totally disagree on the concern for which we omitted a possible conflict of interest. Actually we declared that this work was supported by Boiron Laboratoires Lyon, and that the funder had no role in data collection and analysis, interpretation, decision to publish, or writing the manuscript. We declared that this study was funded by Boiron Laboratoires with a research agreement in partnership with University of Verona. We declared that the tested medicine, at the 1c dilution, was provided by Boiron. *Arnica m.* is not a new product in development, nor is patented by Boiron Laboratories. It is a common medicine in the homeopathic repertoire, produced by all companies and it is marketed since decades worldwide. It is evident that the study was not finalized to improve the Boiron market but to obtain new knowledge in the field, to be shared with the scientific community.

The authors have no conflicts of interest

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