CORRESPONDENCE

REVISED Lupeol and pristimerin do not inhibit activation of the human sperm CatSper Ca(2+)-channel [version 2; peer review: 2 approved, 1 approved with reservations]

Anders Rehfeld [1-3], Christian Marcus Pedersen [4]

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v2 **First published:** 24 Feb 2022, **11**:222
https://doi.org/10.12688/f1000research.109279.1
Latest published: 02 Aug 2022, **11**:222
https://doi.org/10.12688/f1000research.109279.2

Abstract

Opposing findings have been published on the regulation of the sperm-specific Ca^{2+} channel CatSper (cation channel of sperm) in human sperm cells by the plant triterpenoids lupeol and pristimerin. While the original study on this topic found these triterpenoids to act as potent inhibitors of human CatSper, subsequent studies have failed to replicate such an inhibitory effect. It has been suggested that these issues could in part be due to purity issues and/or batch variation between the plant-derived extracts of lupeol and pristimerin obtained for the studies. The aim of this study was to elucidate this controversy by investigating the batches of lupeol and pristimerin used in our previous study with state-of-the-art ^{1}H-, ^{13}C- and 2D-nuclear magnetic resonance (NMR) methods to reveal potential purity and/or batch variation issues. When comparing the NMR-spectra obtained from ^{1}H-NMR and ^{13}C-NMR with previously published NMR-spectra for lupeol and pristimerin, we could confirm that both the lupeol and pristimerin batch were ≥95 % pure. These results confirm the validity of the findings in our previous study for lupeol and pristimerin, showing that lupeol and pristimerin do not inhibit activation of CatSper in human sperm. In conclusion, using ^{1}H-, ^{13}C- and 2D-NMR methods, we confirm that the lupeol and pristimerin batches used in our previous study were ≥95 % pure and thereby fail to identify any purity issues and/or batch variation that could explain the observed inability of lupeol and pristimerin to inhibit activation of CatSper in human sperm.

Keywords

Fertility, CatSper, Male reproduction, Lupeol, Pristimerin, Sperm function

Open Peer Review

Approval Status ✓ ✓ ?

	1	2	3
version 2 (revision) 02 Aug 2022	✓ view	✓ view	
version 1 24 Feb 2022	? view	? view	? view

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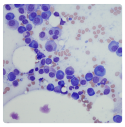
Any reports and responses or comments on the article can be found at the end of the article.



This article is included in the Cell & Molecular Biology gateway.

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Author roles: Rehfeld A: Conceptualization, Funding Acquisition, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing; **Marcus Pedersen C**: Data Curation, Methodology, Visualization, Writing – Review & Editing

Competing interests: Anders Rehfeld holds a patent P2493DK00 "ZAFIRLUKAST DERIVATIVES FOR USE AS CONTRACEPTIVE AGENTS".

Grant information: AR is funded by a BRIDGE - Translational Excellence Programme grant funded by the Novo Nordisk Foundation, grant agreement number: NNF18SA0034956.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Rehfeld A and Marcus Pedersen C. **Lupeol and pristimerin do not inhibit activation of the human sperm CatSper Ca(2+)-channel [version 2; peer review: 2 approved, 1 approved with reservations]** F1000Research 2022, **11**:222 https://doi.org/10.12688/f1000research.109279.2

First published: 24 Feb 2022, **11**:222 https://doi.org/10.12688/f1000research.109279.1

REVISED **Amendments from Version 1**

The manuscript has been updated to address the suggestions made by the three reviewers. Specifically, the discussion has been revised to address the specific issues raised by Mannowetz *et al.*, 2018 to explain the irreproducibility of their results. Furthermore, we now state that the implicated groups have been contacted to offer them to conduct NMR-analyses of the lupeol and pristimerin batches used in their experiments.

Any further responses from the reviewers can be found at the end of the article

The putative inhibitory action of the two plant triterpenoids lupeol and pristimerin on the activation of the human sperm CatSper Ca(2+)-channel has recently been debated in the scientific literature. The original study on this subject (Mannowetz *et al.*, 2017) indicated that these triterpenoids act as very potent and efficacious inhibitors of progesterone-activated CatSper-currents in human sperm cells with IC_{50}-values in the lower nM range, and a follow-up study by the same research group confirmed the inhibitory action of pristimerin on progesterone-induced Ca(2+)-influxes via CatSper through measurements in the principal piece of the flagellum in single human sperm cells (Mannowetz *et al.*, 2018).

In contrast to these findings, two studies from independent research groups failed entirely to replicate any inhibitory action for neither lupeol nor pristimerin on progesterone-induced Ca(2+)-influxes through CatSper in populations of human sperm cells (Brenker *et al.*, 2018b; Rehfeld, 2020) and progesterone-activated CatSper-currents in single human sperm cells (Brenker *et al.*, 2018b), even when exposing the sperm cells to lupeol and pristimerin at much higher µM concentrations.

The complete failure of these studies to replicate the findings from (Mannowetz *et al.*, 2017; Mannowetz *et al.*, 2018) is highly concerning since a patent has been filed (Lishko & Mannowetz, 2018) and a company (YourChoice Therapeutics, CA, US) has been formed based on the original discovery by (Mannowetz *et al.*, 2017; Mannowetz *et al.*, 2018) that lupeol and pristimerin act as potent inhibitors of human CatSper and could thus potentially be used as novel male and female contraceptives.

Since the publication of the most recent study on this matter (Rehfeld, 2020), the corresponding author was contacted by researchers who questioned the validity of the results presented in the study for lupeol and pristimerin, i.e., the inability to reproduce the inhibitory action of these triterpenoids on human CatSper, and suggested that the failure to identify such an inhibitory effect on human CatSper could be due to purity issues and/or batch variation between the plant-derived extracts of lupeol and pristimerin obtained for the study from Cayman Chemicals (MI, USA).

Although Cayman Chemicals stated that the lupeol and pristimerin batches were delivered with a purity of ≥98 %, we fully agreed with these researchers that it would be good scientific conduct and of general interest of the field of human sperm physiology to examine the two stocks solutions used in (Rehfeld, 2020), i.e., a 5 mM pristimerin dimethylsulfoxid (DMSO) stock and a 1 mM lupeol ethanol stock, using state-of-the-art ^{1}H-, ^{13}C- and 2D-nuclear magnetic resonance (NMR) methods (Bruker 500 MHz Ultrashield Plus equipped with a CryoProbe, Bruker, Germany) to reveal potential purity and/or batch variation issues in these stocks.

To prepare the stocks for the NMR-measurements, we first evaporated the ethanol from the lupeol stock, removed the DMSO from the pristimerin stock using an evaporation system (V-10 evaporator, Biotage, Sweden), and exchanged the solvent for both triterpenoids to deuterated chloroform ($CDCl_3$). The raw NMR data can be found as *Underling* data (Rehfeld, 2022a).When comparing the NMR-spectra obtained on the two stocks from ^{1}H-NMR and especially ^{13}C-NMR (see *Extended data* (Rehfeld, 2022b)) with previously published NMR-spectra for lupeol and pristimerin (Espindola *et al.*, 2018; Shwe *et al.*, 2019), we could confirm that Cayman Chemicals had indeed provided us with batches containing lupeol and pristimerin, respectively. Furthermore, the NMR-data showed that both lupeol and pristimerin were ≥95 % pure (*Extended data* (Rehfeld, 2022b)), despite the prolonged storage at -20 °C since conducting the experiments for (Rehfeld, 2020).

Taken together, the results provided here confirms the validity of the findings in our previous study for lupeol and pristimerin (Rehfeld, 2020), i.e., that the two plant triterpenoids lupeol and pristimerin do not inhibit activation of CatSper in human sperm. The findings in (Rehfeld, 2020) are therefore still in line with the observations by (Brenker *et al.*, 2018b) and still contradicting the putative inhibitory action of lupeol and pristimerin on human CatSper described in (Mannowetz *et al.*, 2017; Mannowetz *et al.*, 2018).

The data presented here do not explain the discrepancy between the results by (Mannowetz *et al.*, 2017; Mannowetz *et al.*, 2018) and (Brenker *et al.*, 2018b; Rehfeld, 2020). In their follow-up study (Mannowetz *et al.*, 2018) suggested that the irreproducibility of their findings was due to two issues: 1) Differences between electrophysiological protocols. It was claimed that (Brenker *et al.*, 2018b) used an electrical driving force of only 20 mV (generated by stepping from a holding potential of −80 mV to −100 mV) to activate CatSper currents. This was argued to be too small to reliably assess inward CatSper currents under conditions in which the bath and pipette solutions contain equal concentrations of the major permeant ion, i.e., in the absence of a chemical driving force. 2) Differences in the Ca(2+)-imaging assays used to measure Ca(2+)-influxes in human sperm cells. (Rehfeld, 2020) and (Brenker *et al.*, 2018b) measured Ca(2+)-influxes in populations of human sperm, whereas (Mannowetz *et al.*, 2018) measured Ca(2+)-influxes in the principal piece of the flagellum using single-cell imaging. (Mannowetz *et al.*, 2018) claimed that CatSper-mediated Ca(2+)-influxes must be recorded specifically in the principal piece of the flagellum in order to avoid

interference from strong fluorescent signals from the head, which could mask the fluorescence changes in the flagellum. These two arguments put forward by (Mannowetz *et al.*, 2018) are discussed below.

First, the experimental protocol used by (Brenker *et al.*, 2018b) was also used by this group in two recent publications (Brenker *et al.*, 2018a; Schiffer *et al.*, 2020) to record CatSper-currents from human sperm. Recordings from CatSper-deficient sperm by (Schiffer *et al.*, 2020) demonstrate that the currents recorded under these conditions are indeed carried by CatSper. In contrast to the cesium-based bath and pipette solutions used by (Mannowetz *et al.*, 2017), (Brenker *et al.*, 2018b) used a sodium-based divalent-free (NaDVF) bath solution together with a cesium-based pipette solution. This means that inward CatSper currents are carried by Na^+ and outward currents by Cs^+. Under these specific conditions the reversal potential (V_{rev}) for monovalent CatSper currents is ~30 mV (Brenker *et al.*, 2018a; Schiffer *et al.*, 2020). The driving force (V_{DF}) for monovalent CatSper currents at a certain membrane potential (V_m) is given by $V_{DF} = (V_m - V_{rev})$. Accordingly, at a membrane potential of −100 mV, the driving force is ~130 mV rather than 20 mV as suggested by (Mannowetz *et al.*, 2018), why sizeable inward currents via CatSper can be recorded at this membrane potential using the experimental protocol in (Brenker *et al.*, 2018b). Thus, the argument put forward by (Mannowetz *et al.*, 2018) is based on a biophysical misconception. Moreover, Figure 2D in (Brenker *et al.*, 2018b) demonstrates the failure of pristimerin and lupeol to inhibit resting and progesterone-activated CatSper currents across the entire range of membrane potentials tested and not just at −100 mV as shown in Figure 2E in (Brenker *et al.*, 2018b). Taken together, the claim by (Mannowetz*et al.*, 2018) that the irreproducibility of their results is due to differences in the electrophysiological protocols seems invalid.

Secondly, concerning the differences in Ca(2+)-imaging protocols. Already in 1996, Ca(2+)-imaging in populations of human sperm was used to identify potent antagonists of the progesterone-induced Ca(2+)-influxes in human sperm cells (Blackmore *et al.*, 1996). In (Rehfeld, 2020) the sperm population based Ca(2+)-imaging assay confirmed the inhibitory action of the specific CatSper inhibitor RU1968 (Rennhack *et al.*, 2018), but completely failed to identify any inhibition of progesterone-induced Ca(2+)-influxes by lupeol and pristimerin in experiments testing these compounds side-by-side, see Figure 6 in (Rehfeld, 2020). This fails to support the claim by (Mannowetz *et al.*, 2018) that the irreproducibility of their results is due to differences in Ca(2+)-imaging protocols.

In a recent review article on natural products with a potential for nonhormonal male contraception (Shunnarah *et al.*, 2021) the authors discuss the controversy regarding the action of lupeol and pristimerin on human CatSper and state the following: "*These contradictory findings suggest a need for further studies to confirm the action or lack of action of the plant triterpenoids on the CatSper channel. The conflicting results also demonstrate the difficulties in reproducibility of results, which is often a barrier to studies of natural compounds in general.*". We fully agree with this statement and believe that our study follows up on this suggestion. We encourage other researchers to test lupeol and pristimerin for actions on human CatSper in their own lab and offer them the opportunity to send us their batches of lupeol and pristimerin for state-of-the-art ^{1}H-, ^{13}C- and 2D-NMR analyses. Also, we have contacted the research groups of behind the implicated studies on this controversy (Brenker *et al.*, 2018b) and (Mannowetz *et al.*, 2017; Mannowetz *et al.*, 2018) with this opportunity.

In conclusion, using state-of-the-art ^{1}H-, ^{13}C- and 2D-NMR methods, we confirm here that the lupeol and pristimerin stocks used in (Rehfeld, 2020) were ≥95 % pure and thereby fail to identify any purity issues and/or batch variation that could explain the observed inability of these triterpenoids to inhibit activation of CatSper in human sperm.

Data availability

Underlying data

Figshare. Raw ^{1}H-, ^{13}C- and 2D-NMR data for lupeol and pristimerin in MestReNova (Mnova) format. https://doi.org/10.6084/m9.figshare.19181087.v1 (Rehfeld, 2022a).

Raw ^{1}H-, ^{13}C- and 2D-NMR data for lupeol and pristimerin in MestReNova (Mnova) format are also available at the BMRbig repository, part of the Biological Magnetic Resonance Data Bank (BMRB), with ID: BMRbig35, https://bmrbig.org/released/bmrbig35.

Extended data

Figshare: Supplementary file 1. https://doi.org/10.6084/m9.figshare.19134488.v1 (Rehfeld, 2022b).

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Open Peer Review

Current Peer Review Status: ✔ ✔ ?

Version 2

Reviewer Report 22 August 2022

https://doi.org/10.5256/f1000research.135864.r146345

✔ **Tao Luo**
Institute of Life Science, School of Life Sciences, Nanchang University, Nanchang, China

The authors have made a comprehensive revision according to my issue and addressed all my issue. Now, I have no additional comments. I think the manuscript is suitable for indexing at this version.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Male infertility

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 09 August 2022

https://doi.org/10.5256/f1000research.135864.r146343

✔ **João Ramalho-Santos**
[1] CNC-Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
[2] DCV-Department of Life Sciences, University of Coimbra, Coimbra, Portugal
[3] CIBB-Center for Innovative Biomedicine and Biotechnology, University of Coimbra, Coimbra, Portugal

I understand the authors' response in terms of the context it provided to my initial comments/reservations. I have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Sperm physiology, male and female infertility, reproductive and stem cell biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 May 2022

https://doi.org/10.5256/f1000research.120762.r129827

?

Huafeng Wang
Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT, USA

The effects of lupeol and pristimerin on human CatSper have aroused a controversy since two PNAS papers were published from different groups (Mannowetz et al., 2017 and Brenker et al., 2018). This manuscript was aiming to uncover a potential possibility for a better explanation by examining the purity of lupeol and pristimerin, together with their previous work (Molecular Human Reproduction, 2020). It is believed the data in this manuscript was solid and convincing. However, is the lupeol and pristimerin tested in this manuscript from same batch with the other two groups? If not, it would be great to test those from the two groups individually, even though the purity was not mentioned in this controversy (Mannowetz et al., 2018 and Brenker et al., 2018).

Is the rationale for commenting on the previous publication clearly described?
Yes

Are any opinions stated well-argued, clear and cogent?
Partly

Are arguments sufficiently supported by evidence from the published literature or by new data and results?
Partly

Is the conclusion balanced and justified on the basis of the presented arguments?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: sperm, CatSper, ion channel, electrophysiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Jul 2022

Anders Rehfeld, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

We have now contacted these groups to offer them NMR-analyses of their lupeol and pristimerin batches.

Competing Interests: No competing interests were disclosed.

Reviewer Report 13 April 2022

https://doi.org/10.5256/f1000research.120762.r129824

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Tao Luo
Institute of Life Science, School of Life Sciences, Nanchang University, Nanchang, China

Different research groups showed contradictory results on whether plant triterpenoids, lupeol and pristimerin, inhibit the activation of CatSper. It has been suggested that these issues could in part be due to purity issues and/or batch variation between the plant-derived extracts of lupeol and pristimerin obtained for the studies.

This manuscript aimed to elucidate this controversy by investigating the batches of lupeol and pristimerin used in their previous study using state-of-the-art 1H-, 13C- and 2D-nuclear magnetic resonance (NMR) methods. The authors confirm that the lupeol and pristimerin batches used in their previous study were ≥95 % pure. Therefore, they concluded that the purity issues and/or batch variation could not explain the inability of lupeol and pristimerin to inhibit activation of CatSper in human sperm found in their previous study. The opinions stated are well-argued, clear and cogent. The arguments were sufficiently supported by evidence from the new data.

However, the manuscript did not discuss or respond to the other two important issues argued by Mannowetz et al. 2018:

1. An electrical driving force of 20 mV generated from a holding potential of −80 to −100 mV, as shown by Brenker et al. 2018, is neither enough to reliably assess inward CatSper currents

nor to estimate “fold current increase”.

2. If it is of interest to study CatSper-mediated calcium influx into spermatozoa, individual principle pieces (PPs) as regions of interest must be analyzed.

If these issues are addressed, the controversy may be better elucidated.

Is the rationale for commenting on the previous publication clearly described?
Yes

Are any opinions stated well-argued, clear and cogent?
Yes

Are arguments sufficiently supported by evidence from the published literature or by new data and results?
Yes

Is the conclusion balanced and justified on the basis of the presented arguments?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Male infertility

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Jul 2022

Anders Rehfeld, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

We have now addressed these two issues in the discussion of the updated manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Report 02 March 2022

https://doi.org/10.5256/f1000research.120762.r125363

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João Ramalho-Santos

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My comments have to do with the fact that, while the topic is very worthwhile, this is not exactly (in my view) a straightforward Correspondence. The steps are as follows:

1. Originally a paper is published in Proceedings of the National Academy of Sciences of the United States of America (PNAS) in 2017 showing that Lupeol and pristimerin strongly inhibits the CatSper channel, the implication being that they could be used in male contraception, and apparently a company was formed with that in mind.

2. In 2018 (also in PNAS) other authors question this data suggesting that there are, in fact, no effects.

3. The original 2017 authors contest this, stand by their results and suggest that their data is valid and that the negative results are based on monitoring different things. Sample purity is not mentioned in this exchange.

4. It should be noted that none of the authors of this correspondence were involved in this controversy, the first author did work with the team that published the 2018 paper, and confirmed the absence of results with CatSper not being inhibited by Lupeol and pristimerin in a 2020 Molecular Human Reproduction (MHR) paper.

In this correspondence the authors perform NMR spectra to show that Lupeol and pristimerin are as pure as possible, and that the absence of inhibition of CatSper is therefore a true result. One authors is an expert on sperm (and CatSper in particular), the other on chemical structures, so I have no issues with the data here at all. However, I doubt that this will in any meaningful way solve the contradiction. Using other batches of Lupeol and pristimerin might be a possibility, or at least framing the discussion a bit more thoroughly and discussing/updating the issues raised in the 2018 exchange, that, as stated, never mention sample purity. But I do respect the authors not wanting to do this, as they were not part of those papers.

Is the rationale for commenting on the previous publication clearly described?
Partly

Are any opinions stated well-argued, clear and cogent?
Yes

Are arguments sufficiently supported by evidence from the published literature or by new data and results?
Partly

Is the conclusion balanced and justified on the basis of the presented arguments?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Sperm physiology, male and female infertility, reproductive and stem cell biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 03 Mar 2022

Anders Rehfeld, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

I fully agree with the reviewer in his comment that this is not a straightforward Correspondence. I would have preferred to be able to publish all my data on this issue as a Letter to the original PNAS paper, but unfortunately, there was a 6-month limit to submit such Letters to articles in PNAS and I did not have any data ready within that timeframe. My way into writing this Correspondence was this:

1. I read the original 2017 PNAS paper and was very excited about the novel CatSper-inhibitor compounds Lupeol and Pristimerin, which seemed to be highly potent and efficacious compared to other known CatSper-inhibitors. I therefore ordered these compounds to use them in my own studies as CatSper-inhibitors.

2. I received Lupeol and Pristimerin, diluted them according to the recommendation of the vendor and started testing them in my own experimental setup. However, I could not identify any inhibitory effects of the compounds on CatSper in human sperm cells.

3. Puzzled, I contacted the authors of the original 2017 PNAS paper to discuss what I might have done wrong. The authors of the original 2017 PNAS paper suggested me to test Lupeol and Pristimerin prepared at different stock concentrations in different solvents, with or without sonication, as well as from different vendors. However, in the end I could still not observe any inhibition of human CatSper in my assay.

4. At this point I decided to re-examine both the two triterpenoids, Lupeol and Pristimerin, and the steroids investigated in the original 2017 PNAS paper using my own experimental setup. This collected work is what I published in the 2020 Molecular Human Reproduction paper.

5. After publishing this 2020 Molecular Human Reproduction paper, I was contacted per e-mail by researchers who questioned the validity of the results presented in the study for Lupeol and Pristimerin, and suggested that my failure to identify inhibitory effects on human CatSper could be due to purity issues and/or batch variation between the plant-derived extracts of Lupeol and Pristimerin obtained for the study. As the reviewer states, this purity issue was not mentioned in the 2018 PNAS exchange.

6. I had to agree with the researchers that this could of course be true and something that I should have investigated before submitting the 2020 Molecular Human Reproduction paper. I thus contacted, Christian Marcus Pedersen, a chemical scientist from the chemical department at the University of Copenhagen, and asked him to perform nuclear magnetic resonance analyses of the Lupeol and Pristimerin stocks I used for the 2020 Molecular Human Reproduction study.

7. Christian Marcus Pedersen performed nuclear magnetic resonance analyses of my Lupeol and Pristimerin batches and thankfully both compounds were present at high purity, even despite the prolonged storage at -20 °C since conducting the experiments for the 2020 Molecular Human Reproduction paper.

8. At this point I simply wanted to share these additional important data, which I thought were highly relevant for the discussion of the putative inhibitory effects of Lupeol and Pristimerin on human CatSper. Unfortunately, it was not possible for me to simply add these nuclear magnetic resonance data to the 2020 Molecular Human Reproduction paper, why I had to identify another journal to get the data published, accessible, and citable to other researchers of the human sperm physiology field. Luckily Correspondence articles at F1000 Research were suitable for exactly this.

Of note, I believe that I discussed several issues raised in the 2018 PNAS exchange quite thoroughly in the 2020 Molecular Human Reproduction paper, e.g., the claim that Ca^{2+}-signals must be recorded specifically from the principal piece of the flagellum only to be able to observe an inhibitory effect on CatSper in human sperm cells, which did not agree with my observations using the other CatSper-inhibitor RU1968. However, if other reviewers also suggest this, we can of course update our Correspondence to discuss the issues raised in the 2018 PNAS exchange more thoroughly.

I fully agree with the reviewer that this Correspondence may not solve this controversy. However, in a recent review article *"Natural Products with Potential for Nonhormonal Male Contraception"* (J. Nat. Prod. 2021, 84, 2762−2774), they also discuss the controversy regarding the action of Lupeol and Pristimerin on human CatSper, and state the following: *"These contradictory findings suggest a need for further studies to confirm the action or lack of action of the plant triterpenoids on the CatSper channel. The conflicting results also demonstrate the difficulties in reproducibility of results, which is often a barrier to studies of natural compounds in general."*.
I believe that we are following up on this exact suggestion with this Correspondence and I hope it will be helpful for solving the issue and reaching consensus in the human sperm physiology field at some point.

In the end, my hope is that other researchers reading our Correspondence will obtain Lupeol and Pristimerin themselves and test these compounds for effects on CatSper in human sperm cells in their own lab and hopefully at some point publish their findings like we have done here. This will be the only way to solve this controversy in my opinion.

Competing Interests: No competing interests were disclosed.

Author Response 05 Jul 2022

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As the other reviewers also suggest that we discuss the issues raised in the 2018 PNAS exchange, we have now update our Correspondence manuscript to address these issues more thoroughly.

Competing Interests: No competing interests were disclosed.