RESEARCH NOTE

Effect of LXR/RXR agonism on brain and CSF Aβ40 levels in rats [version 2; referees: 1 approved, 2 approved with reservations]

Songli Wang¹, Paul Wen², Stephen Wood²

¹Genome Analysis Unit, Amgen Inc., San Francisco, CA, USA
²Department of Neuroscience, Amgen Inc., Thousand Oaks, CA, USA

Abstract
Alzheimer’s disease (AD) is characterized pathologically by the presence of amyloid plaques and neurofibrillary tangles. The amyloid hypothesis contends that the abnormal accumulation of Aβ, the principal component of amyloid plaques, plays an essential role in initiating the disease. Impaired clearance of soluble Aβ from the brain, a process facilitated by apolipoprotein E (APOE), is believed to be a contributing factor in plaque formation. APOE expression is transcriptionally regulated through the action of a family of nuclear receptors including the peroxisome proliferator-activated receptor gamma and liver X receptors (LXRs) in coordination with retinoid X receptors (RXRs). It has been previously reported that various agonists of this receptor family can influence brain Aβ levels in rodents. In this study we investigated the effects of LXR/RXR agonism on brain and cerebrospinal fluid (CSF) levels of Aβ40 in naïve rats. Treatment of rats for 3 days or 7 days with the LXR agonist, T0901317 or the RXR agonist, bexarotene did not result in significant changes in brain or CSF Aβ40 levels.
The E4 allele of apolipoprotein E (APOE) is the largest genetic risk factor for sporadic, late-onset AD. The presence of a single copy of E4 increases the risk for Alzheimer’s disease 3-fold and individuals with 2 copies are 15 times more likely to develop AD. Data showing that APOE4 carriers begin to accumulate amyloid deposits earlier in life relative to non-carriers has led to the hypothesis that increased risk associated with an E4 genotype may be the result of the effects of APOE on Aβ production, turnover and/or clearance from the central nervous system (CNS).

The expression of genes encoding lipid-transport proteins, including APOE is transcriptionally regulated by the ligand-activated nuclear receptors, peroxisome proliferator-activated receptor gamma (PPARγ) and liver X receptors (LXRs) which form obligate heterodimers with retinoid X receptors (RXRs). Activation of these receptors has been shown to affect the activation state of macrophage and microglia. Based on the processes influenced by this nuclear receptor family it is a reasonable hypothesis that agonism of one or more members of the family could have beneficial effects on Aβ homeostasis in the CNS. In fact, several groups have demonstrated that LXR agonism with either GW3965 or T0901317 results in reduced amyloid plaque burden and/or soluble Aβ levels in amyloid precursor protein (APP) transgenic mouse models. Using non-transgenic rats, Suon et al. demonstrated a statistically significant increase in CSF Aβ and a decrease in soluble brain Aβ following T0901317 treatment.

In addition, it was reported that a highly selective, blood-brain-barrier-permeant, RXR agonist, bexarotene (Targretin), enhanced clearance of soluble Aβ in an APP transgenic mouse model in an APOE-dependent manner. In the same study, Aβ plaque burden was reduced by more than 50% within 72 hours. Further, bexarotene treatment also resulted in a similar reduction (~25%) in brain interstitial fluid (ISF) levels of Aβ in non-transgenic, C57Bl/6 mice 7–12 hours following a single administration. Attempts to replicate the bexarotene findings resulted in mixed results (see reviews by Tesseur & De Strooper and Tousi). This study aims to examine the robustness of the hypothesis that RXR or LXR agonism affects soluble Aβ homeostasis in the CNS.

Materials and methods

In vivo pharmacodynamic studies: All procedures were approved by the Amgen Institutional Animal Care and Use Committee. Young male Sprague-Dawley rats (175–200 g) were purchased from Harlan (Indianapolis, IN) and were maintained on a 12h light/dark cycle with unrestricted access to food and water until use. Rats were dosed orally for 3 and 7 consecutive days with AMG8155, a proprietary small molecule BACE1 inhibitor, at 3 mg/kg in 2% HPMC and 1% Tween 80, pH 2, bexarotene (Alfa Aesar, Ward Hill, MA) at 100 mg/kg in 30% Labrasol, 1% Tween 20, 2% Provodone and 0.05% BHA, pH7.0 (Vehicle 3), and T0901317, a LXR agonist (Fisher Scientific, Pittsburgh, PA), at 30 mg/kg in 0.5% NaCl, 2% Tween 80 (Vehicle 4). 4 hours post dose on the last day of study, rats were euthanized with CO2 inhalation for 2 minutes and the cisterna magna was quickly exposed by removing the skin and muscle above it. Cerebrospinal fluid (CSF) was collected with a 30 gauge needle inserted through the dura membrane.
covering the cisterna magna. CSF samples with visible blood contamination were discarded. Blood was withdrawn by cardiac puncture and plasma was obtained by centrifugation at 15,000 rpm for 10 min at 4°C for drug exposure. Brains were removed and, along with the CSF, immediately frozen on dry ice and stored at -80°C until use. The frozen brains were subsequently homogenized in 10 volumes (w/v) of 0.5% Triton X-100 in TBS with protease inhibitors cocktails. The homogenates were centrifuged at 355,000 rpm for 30 min at 4°C.

Quantification of Aβ40 and APOE in brain and CSF: Samples are analyzed for Aβ levels by immunoassay with a MSD imager. Briefly, 96-well avidin plates (MesoScale Discovery, Inc., Gaithersburg, MD) were coated with biotinylated-anti-Aβ antibody 4G8 (mouse monoclonal, Cat# Sig 39240-1000, Covance Research Products, Princeton, NJ) at 10 μg/ml in PBS. Samples were co-incubated in the plate overnight at 4°C along with a ruthenium-labeled anti-Aβ antibody specific for the C-terminal region of Aβ40 (ConFab40; Amgen, Thousand Oaks, CA). Plates were then washed, 150 μl/well read buffer T (MesoScale Discovery, Inc.) was added, and plates were read immediately on a Sector 6000 imager according to the manufacturer’s recommended protocol (MesoScale Discovery, Inc.). All samples were assayed in triplicate and analyzed by using Prism version 5.04 (GraphPad Software Inc., San Diego, CA). Data was analyzed by one-way analysis of variance and Dunnett’s multiple comparison test.

APOE levels in brain (50 μg homogenates) and CSF (10 μl) were analyzed by Western blot following PAGE using 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Blots were probed with primary antibodies to APOE (goat polyclonal, EMD Millipore; 1:1000) and the loading control, actin (ThermoFisher Scientific; 1:200) for 60 min at 4°C and then washed with TBST (Tris-buffered saline, 0.1% Tween 20) three times at room temperature, followed by (Goat-anti-mouse) secondary antibody (ThermoFisher Scientific; 1:1000) for 30 min at 4°C. Densitometric analysis of ApoE was performed (exposure time of 4 minutes with a relative intensity of 2.0, Odyssey imaging system, with application software Version 3.0) followed by an unpaired t-test using GraphPad Prism 5.04 software.

Measurement of Plasma, CSF, and Brain Drug Concentration: Aliquots of plasma (50 μl) were combined with 300 μl of acetonitrile containing 125 μl structurally related internal standard (IS), vortexed, and centrifuged. Supernatant was transferred into a plain polypropylene 96-well plate for sample analysis. Brain tissue samples were homogenized by using a Covaris (Woburn, MA) acoustic homogenizer. Aliquots of 50 μl homogenate were combined with acetonitrile containing a structurally related IS, vortexed, and centrifuged at 1,900 g for 5 minutes. Supernatant was transferred into a 96-well plate for sample analysis. Analytical standards and tissues were measured by liquid chromatography mass spectrometry (Shimadzu Pumps Autosampler Prominence for HPLC and PE Sciex API 4000 for MS, with Analyst 1.6.1 software) using atmospheric-pressure chemical ionization and multiple reaction monitoring in the positive ion mode.

Results

Our aim in this study was to investigate the effects of RXR/LXR agonism on Aβ homeostasis in the CNS of non-transgenic rats using the RXR agonist, bexarotene and the LXR agonist, T0901317. As a positive control, we included a β-secretase inhibitor (AMG8155). Compounds and appropriate vehicle controls were administered to naïve Sprague Dawley rats at doses indicated in Table 1 for either 3 or 7 consecutive days.

Following 3 and 7 days of dosing, animals were evaluated for both compound levels and pharmacodynamic endpoints. APOE levels were quantitated in brain homogenate and CSF by Western blot. Aβ40 levels were quantitated in the same compartments using immunoassay as described in the Materials and methods section. Following 3 and 7 days of dosing, APOE levels were increased in brain and CSF in the T0901317 treated animals compared to vehicle treated animals (Figure 1). Changes in CSF were statistically significant at both 3 (p = 0.0002) and 7 days (p = 0.0007) whereas changes in brain were statistically significant at day 3 (p = 0.030) but did not reach significance at day 7 (p = 0.056). Bexarotene treatment also resulted in a statistically significant increase in CSF APOE levels compared to vehicle treated animals following both 3 (p = 0.019) and 7 days (p = 0.002) of dosing (Figure 2). APOE levels in brain following bexarotene treatment trended towards an increase however these changes were not statistically significant. Soluble Aβ40 levels were unchanged in brain and CSF following 3-day (Figure 3) and 7-day (Figure 4) treatment with either bexarotene or T0901317. The positive control BACE inhibitor, AMG8155 effectively reduced Aβ40 levels by 70% and 71% in CSF and by 67% and 69% in brain in the 3-day and 7-day studies respectively (Figure 3 and Figure 4).

Drug levels of bexarotene and T0901317 were measured in plasma and brain homogenate following 3 and 7 days of dosing (Table 2). Total levels of both compounds achieved single-digit to low double-digit μM levels in brain and showed good uptake in brain relative to plasma in both dosing paradigms.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bexarotene</td>
<td>100</td>
</tr>
<tr>
<td>AMG8155</td>
<td>3</td>
</tr>
<tr>
<td>T0901317</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 1 lists the 3 compounds tested in this study along with the respective doses (mg/kg).
Figure 1. LXR agonist, T0901317 significantly increased APOE levels in rat CSF following 3 and 7 days of dosing at 30 mg/kg. APOE was also increased in brain however the changes only reached statistical significance at day 3. A) Western blot analysis of APOE in brain and CSF. B) Densitometric analysis of the bands was performed as described in the Materials and Methods section; data are presented as the mean plus standard deviation; Vehicle 4 (black bars) and T0901317 (gray bars).
Figure 2. RXR agonist, bexarotene significantly increased APOE in rat CSF following 3 and 7 days of dosing at 100 mg/kg. APOE changes in brain were not statistically significant. A) Western blot analysis of APOE in brain and CSF. B) Densitometric analysis of the bands was performed as described in the Materials and Methods section; data are presented as the mean plus standard deviation; Vehicle 4 (black bars) and bexarotene (gray bars).
Figure 3. Aβ40 levels in (A) CSF and (B) brain were unchanged following 3 days of treatment with bexarotene (triangles) or T0901317 (diamonds). Positive control BACE inhibitor AMG8155 (squares) reduced Aβ40 levels 70 and 67% in CSF and brain respectively following a single administration.

Figure 4. Aβ40 levels in (A) CSF and (B) brain were unchanged following 7 days of treatment with bexarotene (triangles) or T0901317 (diamonds). Positive control BACE inhibitor AMG8155 (squares) reduced Aβ40 levels 71 and 69% in CSF and brain respectively following a single administration.
Conclusion
In this study we demonstrate that 3-day or 7-day treatment of naïve rats with the LXR agonist, T0901317 or the RXR agonist, bexarotene treatment results in an increase in APOE levels in CSF without observable effects on CSF or brain Aβ levels.

Although this study sought to examine the robustness of the hypothesis that RXR/LXR agonism affects soluble Aβ homeostasis, it was not designed to explicitly replicate any one prior study. Differences in findings between labs could very well be a result of a variety of factors related to the methodologies employed, something that has been nicely reviewed for bexarotene previously.

Whereas the current report was focused solely on soluble Aβ, we recognize that RXR/LXR agonism may affect AD pathology in other ways (e.g. increased phagocytic clearance of amyloid deposits). LXR or RXR agonism may also affect cognitive decline in AD patients via non-Aβ-dependent mechanisms not yet fully understood. Moreover, this study exclusively assessed Aβ40 and it remains possible that LXR/RXR agonism may result in Aβ42-specific changes. In most prior studies that have examined changes in soluble Aβ40 and Aβ42 homeostasis, both Aβ species are affected in a similar manner. However, one published report showed that LXR agonism with T0901317 resulted in a selective reduction in Aβ42 in the hippocampus only. Such region-specific changes would not have been detected under our current experimental protocol as whole brain homogenates were analyzed.

Following 3 and 7 days of dosing, compound levels were measured in brain homogenate and plasma. Total (t) compound concentrations (μM) are reported in each case. The brain to plasma ratio is also shown (far right-hand column).

Table 2. Compound Exposure Table.

<table>
<thead>
<tr>
<th>Treatment Duration</th>
<th>Compound</th>
<th>[brain] t, μM</th>
<th>[plasma] t, μM</th>
<th>[brain]t/[plasma] t</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>Bexarotene (100 mg/kg)</td>
<td>6.26</td>
<td>5.74</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td>T0901317 (30 mg/kg)</td>
<td>14.44</td>
<td>5.30</td>
<td>2.72</td>
</tr>
<tr>
<td>7 days</td>
<td>Bexarotene (100 mg/kg)</td>
<td>3.41</td>
<td>4.76</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>T0901317 (30 mg/kg)</td>
<td>11.68</td>
<td>4.30</td>
<td>2.72</td>
</tr>
</tbody>
</table>

Following 3 and 7 days of dosing, compound levels were measured in brain homogenate and plasma. Total (t) compound concentrations (μM) are reported in each case. The brain to plasma ratio is also shown (far right-hand column).

Finally, others have shown that the effects of LXR/RXR agonism vary depending on APOE isoform. Treatment of EFAD mice (mice expressing 5XFAD mutations and h-APOE3 or h-APOE4) with bexarotene or bexarotene analog, LG100268, resulted in an increase in APOE4 lipidation and subsequent decrease in soluble, oligomeric Aβ levels. Likewise, in naïve human APOE3 or APOE4 targeted replacement mice, bexarotene treatment increased APOE4 lipidation and decreased E4-associated Aβ42 and hyperphosphorylated tau accumulation in the hippocampus.

The current study was performed in naïve rats expressing endogenous APOE, therefore, human APOE isoform-specific effects would have been beyond the scope of this study.

It remains to be seen how well any of these preclinical findings translate to human clinical trials. Recently, the effect of bexarotene on amyloid burden was assessed in patients with Alzheimer’s disease (AD) in a small, proof-of-concept trial. Although the primary outcome of the trial was negative, data suggest that bexarotene resulted in lowering of amyloid burden in APOE4 non-carriers.

We hope that these findings will stimulate future discussion in the Alzheimer’s research community on the impact of LXR/RXR agonism on central Aβ homeostasis.

Data availability
Open Science Framework: Dataset: Effect of LXR/RXR agonism on brain and CSF Aβ40 levels in rats, doi: 10.17605/OSF.IO/3NS64

Author contributions
S. Wang: Participated in research design; wrote or contributed to the writing of the manuscript.

P. Wen: Participated in research design; conducted experiments; performed data analysis; wrote or contributed to the writing of the manuscript.

S. Wood: Participated in research design; performed data analysis; wrote or contributed to the writing of the manuscript.

Competing interests
All authors are employees and stockholders of Amgen, Inc.

Grant information
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References


Open Peer Review

Current Referee Status: ☑️ ️ ️

Version 2

Referee Report 11 May 2016
doi:10.5256/f1000research.9090.r12557

Daniel M. Michaelson
Department of Neurobiology, Sagol School of Neurosciences, Myriam Lebach Chair in Molecular Neurodegeneration, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

This communication revealed that treatment of rats with the LXR/RXR agonists bexarotene and T0901317 increased the brain levels of apoE but had no effect on the levels of Ab40.

A key issue which is not yet resolved in this paper, and which I believe is important, is whether the present treatments with the LXR/RXR agonists in the rat result in partial/weak activation of the LXR/RXR system which affects the levels of apoE but not of the other related readout parameter (i.e. Ab40 in this case) or that the LXR/RXR system is robustly activated by the presently employed paradigm but that this does not affect the levels of Ab40.

Assessment of the "on target" effects of such pharmacological manipulations is important for determination of the significance and meaning of the Ab40 related negative finding and for addressing and reconciling difference between this study and previous reports (eg ref 12 of Suon et al 2010). Accordingly, we suggest that the expression of additional LXR\RXR driven genes be monitored (eg ABCA1 by either western blots or PCR) and if possible also Ab42.

The Suon study was performed utilizing a forebrain + hippocampus brain extract of 1 month old rat. The possible role of differences in these parameters in mediating the differences between the effects of the RXR\RXR treatment in the Suon study and the present manuscript should be discussed.

Competing Interests: No competing interests were disclosed.

I have read this submission. I 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.

Version 1

Referee Report 02 March 2016
doi:10.5256/f1000research.8469.r12696
Mary Jo LaDu, Conor Smith, Ana Valencia
Department of Anatomy and Cell Biology, University of Illinois at Chicago, Chicago, IL, USA

In this manuscript, Wang and colleagues report that treatment for 3 and 7 d bexarotene (Bex, an RXR agonist) or T0901317 (TO, an LXR agonist) induce an increase in apoE levels and but have no effect on Aβ40 in the CSF and brain of rats. While the subject matter is timely, the conclusions must be taken with reservations because of three major concerns:

1. Limited readouts and regions analyzed. It has been previously demonstrated that changes in the levels of Aβ40 and apoE in CSF and brain do not always correlate with efficacy in FAD-Tg mice. Furthermore, it has been shown that an increase of apoE levels by Bex can be beneficial or detrimental depending on the isofrom of apoE or the brain region analyzed.

2. The exclusion of Aβ42. It has been reported that CSF Aβ42 levels, and not Aβ40, are increased in AD patients compared to controls.

3. Comparison to previous reports. Suon and colleagues (2010) treated rats with LXR agonists TO (at the same dose) or GW3965 and reported an increase in apoE that correlated with an increase in Ab40/42 levels in CSF and a reduction of Aβ40 in brain. Wang and colleagues should reconcile their results with this work specifically and interpret their work in the context of the filed in general.

A minor observation is that in Figure 1A the vehicle is mislabeled as Vehicle 3 (should be Vehicle 4 for TO901317)

Within the AD field, there is an ongoing discussion of the effects of Bex on soluble and deposited Aβ levels in the brain and cognition, with contradictory findings. This is likely due to several confounding factors, including: variance within and across models and inconsistencies in the methods used to characterize and quantify the proteins of interest, particularly soluble Aβ. It is imperative to take these parameters into account when considering the efficacy of RXR or LXR agonists for AD therapeutics. As well, a phase I trial of Bex in AD patients recently concluded. While these results may be of limited significance and were not available for the submission of this manuscript, inclusion of their results in a revision of the Discussion would be helpful.

References

**Competing Interests:** No competing interests were disclosed.

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

Referee Report 29 February 2016
doi:10.5256/f1000research.8469.r12274

Sam Gandy Department of Neurology and Psychiatry, Mount Sinai School of Medicine, New York, NY, USA

Some controversy has surrounded the report in *Science* from Landreth and colleagues showing that bexarotene reduces the brain amyloid plaque burden from APP transgenic mice by 50% or more in a matter of days. Several groups of experts attempted to replicate the dramatic Landreth results but the dramatic results were not replicatable. The current *F1000Research* paper extends that replication attempt story by treating rats with bexarotene and then measuring APP metabolites and apoE in CSF. In agreement with the “second wave” of bexarotene studies, there was no effect of bexarotene on CSF levels of Aβ40 or Aβ42. The one point of agreement of all studies was that bexarotene does indeed modulation CSF levels of apoE. This argues against the development of bexarotene mimetics as Aβ lowering agents for the treatment or prevention of Alzheimer's disease. However, inasmuch as elevating apoE may be beneficial in clinical situations via a non-Aβ-dependent pathway (see http://www.alzforum.org/news/research-news/bexarotene-revisited-improves-mouse-memory-no-effect-plaques), bexarotene does reproducibly modulate CSF levels of apoE. The discovery of the entity of SNAP (for review, see Jack *et al.*, 2016) indicates that about one-third of clinically diagnosed Alzheimer's patients undergo cognitive decline via some as yet unknown non-Aβ-dependent pathway.

**References**


**Competing Interests:** No competing interests were disclosed.

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

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**Discuss this Article**

**Version 1**
Gary Landreth, Case Western Reserve University, USA

Wang et al. have reported that they have failed to replicate the ability of bexarotene to clear soluble amyloid from the brain. The reason they failed is straightforward. This experiment has been repeated 6 times. This specific issue has been nicely summarized in a paper published by Tessier and De Strooper (2013), who were authors of one of the ‘Comments’ on our Science paper. I draw your attention to the summary table in that paper. The reason Amgen was unable to reproduce our results was that they used the wrong drug formulation. We used the clinically approved formulation of bexarotene (Targretin™), which is a microcrystalline form of the drug. Those laboratories who used this formulation found drug-induced ApoE expression and lowered Abeta levels. Those studies that solubilized the drug prior to administration failed to replicate the published results. The micronization of bexarotene results in very different pharmacokinetic compared to solubilized drug, which was clearly documented by Chen et al. (2014; Fig 8a) and in the FDA filings. The micronized form of the drug is slowly absorbed in the gut and results in sustained plasma (and brain) levels of the drug, whereas the solubilized drug is rapidly absorbed and cleared. The reason this is important is based on the well known mechanisms of nuclear receptor action. Nuclear receptors, like RXR, are ligand-activated transcription factors. Ligand binding to the receptor results in assembly of chromatin remodeling machinery on the enhancers and promoters of its target genes, resulting in epigenetic modifications that open chromatin structure, allowing induction of gene expression. This process takes time, thus there must be sufficiently high drug levels over an extended interval in order for this to be achieved.

The formulation issue was explicitly discussed in the literature and the FDA filing for bexarotene and was detailed on our ‘Response’ to the ‘Comments’ in Science (Landreth et al., 2013). The Amgen scientists (and others) clearly did not make an effort to understand and replicate the original study design, nor appreciate the importance of the formulation of the drug as it relates to nuclear receptor action. Although the Amgen study was narrowly focused on ApoE and soluble Abeta 40 and did not examine other aspects of our work, the same considerations apply to other contested outcomes of our study.

The Amgen study employed wild type Sprague-Dawley rats, whereas all other studies with bexarotene used murine models of AD. A study of the LXR agonist TO9001317 in rats that was similar to that of Amgen was published by Merck (Suon et al., 2010) and was only partially replicated in the Amgen study and was not cited.

Our work on bexarotene has led to clinical examination of its effects in Alzheimer’s disease. Cummings and colleagues published the outcome of a phase II study of bexarotene in mild to moderate AD patients last week (Cummings et al., 2016). While bexarotene treatment did not have a demonstrable effect in individuals possessing an ApoE4 allele, individuals that did not have an ApoE4 gene (representing about 80% of the population) exhibited a robust reduction in brain amyloid with a parallel appearance of Abeta42 in plasma. Thus, a brief 30 day treatment altered two canonical biomarkers of AD in patients with diagnosed disease. These findings are consonant with a recent case report reporting cognitive improvement with bexarotene treatment (Pierrot et al. 2015). I hasten to add that the small study size precludes making any hard conclusions from this trial and they should be interpreted cautiously. However, these preliminary clinical findings support and validate our original report using mouse models of the disease.

I don't think the Amgen study adds anything to what was previously known. The work has a logical flaw that
undermines the conclusion that they were unable to repeat our study outcomes, including the use of a different model. I think this study is emblematic of the problems associated with reporting ‘failure to replicate’ findings in studies that do not genuinely reproduce the published work.

**Competing Interests:** No competing interests were disclosed.