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Increased Lysosomal Membrane Permeabilization in Oxidant-exposed Macrophages of Human Fibrotic Lungs

Hans L. Persson[1] and Linda K. Vainikka[2]

[1]Division of Pulmonary Medicine, Department of Medical and Health Sciences, Faculty of Health Sciences, Linköping University, Department of Respiratory Medicine UHL, Centre for Surgery and Oncology, County Council of Östergötland, Linköping, Sweden.
[2]Division of Experimental Pathology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden.

ABSTRACT: A disrupted balance of reduced glutathione (GSH) and iron (Fe) and subsequent enhanced susceptibility of lysosomes of lung macrophages (LMs) to oxidants may play a role in lung fibrogenesis. We assessed cellular Fe/GSH, lysosomal membrane permeabilization (LMP), and cell death in cultures of oxidant exposed LMs. LMs from 7 lung fibrosis patients and healthy subjects were exposed to a physiologic concentration of H_2O_2 for 1 h. LMP was assessed with acridine orange green fluorescence, apoptosis/necrosis were estimated by apoptotic DNA and typical morphology, Fe was assessed with Prussian blue staining/atomic absorption spectrophotometry, and GSH was evaluated using a GSH assay kit. Oxidant-induced LMP and cell death were more pronounced in cultures of LMs from patients with lung fibrosis, and these cells contained less GSH and more cytochemically stained Fe. These observations indicate that LMP may be involved in fibrosis development, possibly through activation of the inflammasome complex. Further studies are warranted for a detailed understanding.

KEYWORDS: apoptosis, fibrosis, inflammation, iron, macrophage

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CORRESPONDENCE: lennart.persson@liu.se

Introduction

Lung fibrosis is an end-stage process involving the alveoli and alveolar ducts. Although the etiology of inflammatory lung disorder is different or even unknown (idiopathic), the pathophysiological mechanisms behind the fibrotic process display striking similarities, regardless of the initial trigger of inflammation. A common feature is oxidative lung cell injury and death due to an excess of reactive oxygen species (ROS).[1] Iron (Fe) exacerbates lung tissue injury by promoting hydroxyl radical ($HO^{\bullet}$) formation through a Fenton-type chemistry ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet}$).[2,3] Often, lysosomal membrane permeabilization (LMP) and leakage of cathepsins in to the cytosol of oxidant-exposed lung macrophages (LMs) precedes inflammasome activation, inflammation, and fibrogenesis.[4,5]

Fe is normally deposited in the interior of lysosomes of phagocytic cells, primarily LMs, in an un-reactive state bound to different proteins.[6] Potentially hazardous Fe becomes separated from ROS by a protective shield of intracellular antioxidative enzyme systems, for which the major antioxidant reduced glutathione (GSH) is particularly important.[2,7] This defense system is overwhelmed if cells and lysosomes are exposed to excessive amounts of ROS, as is the case in the inflamed lung.[8–10] Free or loosely bound lysosomal Fe^{2+} promotes peroxidation of lysosomal membranes, which in turn results in LMP, leakage of cathepsins, and ensuing cell death.[11] Moderate LMP is thought to activate programmed cell death, known as apoptosis, while extensive and more complete lysosome rupture will result in unregulated necrosis.[12,13]

Based on this information, we hypothesized that lysosomes of LMs harvested from human fibrotic lungs would be more vulnerable to oxidant challenge in vitro, which in turn would be associated with a disturbed balance of protective GSH and harmful Fe. More detailed studies are warranted to determine the exact role of the lysosome-inflammasome pathway in LMs on fibrotic tissue remodeling.

Methods

Ethical considerations. According to the guidelines of the Declaration of Helsinki, the study protocol was approved by the local Ethical Committee (Linköping, Sweden).

Study population. Bronchoscopies with bronchoalveolar lavage (BAL) were carried out after obtaining informed and written consent. In all control cases, including 7 healthy and non-smoking subjects, pulmonary disease was thoroughly ruled out by bronchoscopy, chest X-ray, and lung function tests. Patients included 7 subjects with lung fibrosis confirmed by high resolution computer tomography and lung biopsy. Four subjects had stage IV lung sarcoidosis, 2 patients had idiopathic lung fibrosis, and 1 patient had fibrosis due to rheumatoid arthritis. Table 1 presents the demographic details of the subjects and the BALF characteristics.

Cell cultures and treatments. BAL was performed during a fibreoptic bronchoscopy with sterile 0.9% (w/v) saline solution and the BALF retrieved (without blood stain) were prepared as previously described.[9,10] Briefly, BALF samples kept on ice were centrifuged at 200 × g for 10 min at + 4°C, leukocytes were counted and seeded in 35-mm Petri dishes (± cover slips). Following rinsing, the dishes (containing approximately 0.4 × 10^6 attached LMs) were then returned to standard culture conditions for 48 h. LMs were cultured in Dulbecco's Modified Eagle's Medium supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B (all from GIBCO, Grand Island, NY, USA), and 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria). Following rinsing, LMs were oxidatively stressed using a stable concentration of approximately 120 µM H_2O_2 for 60 min under standard culture conditions, which was achieved by adding glucose oxidase (GO; Sigma-Aldrich Inc., St Louis, MO, USA) directly to the culture medium. Oxidant challenge was ended by rinsing and adding culture medium, after which LMP and cell death were analyzed.

Assessment of lysosomal membrane permeabilization. Early and minor LMP was monitored using the AO-relocation test, which was thoroughly standardized for human LMs as previously described.[8–10] Briefly, LMs were stained with AO (2.5 µg/mL) for 15 min at 37°C, rinsed with complete culture medium, oxidatively stressed for 1 h as described above, rinsed again, and then prepared for analysis. To initiate controlled LMP in J774 murine macrophages the synthetic lysosomotropic detergent O-methyl-serine dodecylamide hydrochloride (kindly provided by Gene M. Dubowchik, Bristol-Myers Squibb, Wallingford, CT, USA) was used. The AO-green (FL1 = 530 nm) fluorescence was recorded on log scale using a BD LSR Flow Cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) that was equipped with a 488-nm exciting argon laser. CellQuest software was used for data acquisition/analyses and data were expressed as arbitrary units (AU).

Cell death assays. Ten hours after the 1-h oxidant challenge, the percentage of cells displaying apoptotic or necrotic morphology of the initial numbers of cells was determined (detached cells were all necrotic). Cells with an apoptotic or necrotic morphology (ie, cytoplasmic budding/pycnotic fragmented nuclei/apoptotic bodies and membranous rupture/nuclear swelling, respectively) were counted using phase contrast microscopy in a blinded fashion as previously described.[8–10] Fragmented apoptotic DNA, stained with propidium iodide (Sigma), was assessed using cytofluorometry.[14]

Prussian blue staining of ferric Fe. LMs on cover slips were fixed in paraformaldehyde and stained for ferric Fe (Fe^{3+}) by the Prussian blue staining procedure.[8–10] The cellular amount of Fe^{3+} was scored independently by two examiners according to the Golde method.[15] Thus, each LM was graded on a scale of 0–4 (0 = no blue color, 1 = faint blue staining in cytoplasm, 2 = dense blue color in minor portion of cytoplasm or medium color intensity throughout cell, 3 = deep blue staining in most of cytoplasm, 4 = dark blue throughout cytoplasm) and a Golde Index for 100 LMs was calculated with zero being the minimum and 400 the maximum score.

Determination of total cellular Fe and GSH. The total amount of Fe in LMs was assessed using atomic absorption spectrophotometry, equipped with an iron lamp (243.3 nm),

Table 1. Patient and BALF characteristics of the population studied. Data are presented as the means ± 1 SD. P-value vs healthy control patients is indicated. BAL(F): bronchoalveolar lavage (fluid).

	LUNG FIBROSIS PATIENTS (n = 7)	HEALTHY SUBJECTS (n = 7)
Mean age	64 ± 17	61 ± 14
Gender	2 females/5 males	2 females/5 males
BAL recovery (ml)	143 ± 14	137 ± 14
Cell count (10^6 cells/L)	271 ± 143	240 ± 144
% macrophages	68 ± 22 (0.031)	90 ± 6
% lymphocytes	22 ± 19	5 ± 3
% neutrophils	9 ± 16	5 ± 5
% eosinophils	1 ± 3	0 ± 1
% basophils	0 ± 0	0 ± 0

which has a lower detection limit of 0.65 μg Fe/L.[8–10] GSH was estimated using the Glutathione Assay Kit (BioCat GmbH, Heidelberg, Germany) and a Wallace 1420 Victor Plate Reader (PerkinElmer, Waltham, MA, USA). Both cellular Fe and GSH were normalized to the protein concentration of each sample.[8–10]

Statistical analysis. The results were reported as the means ± 1 SD. Student's t-test was used for statistical comparisons [$P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)].

Results

Lung fibrosis macrophages and their lysosomes are very susceptible to oxidants. The baseline green AO fluorescence values of non-oxidatively stressed LMs from fibrotic lungs and healthy controls did not differ significantly. Following oxidative stress, LMs from fibrotic lungs exhibited a significantly greater increase in green AO fluorescence corresponding to a more pronounced leakage of AO from AO-loaded lysosomes than LMs from healthy subjects (Fig. 1A–C).

Cell death in oxidatively stressed cultures of LMs from fibrotic lungs were pronounced and these cultures displayed significantly more dead cells, both apoptotic ($P < 0.01$) and necrotic cells ($P < 0.001$), than oxidant challenged cultures of LMs from healthy subjects did (Fig. 2A–G).

GSH-iron balance is pro-oxidative in lung fibrosis macrophages. Cell characteristics decisive for lysosomal

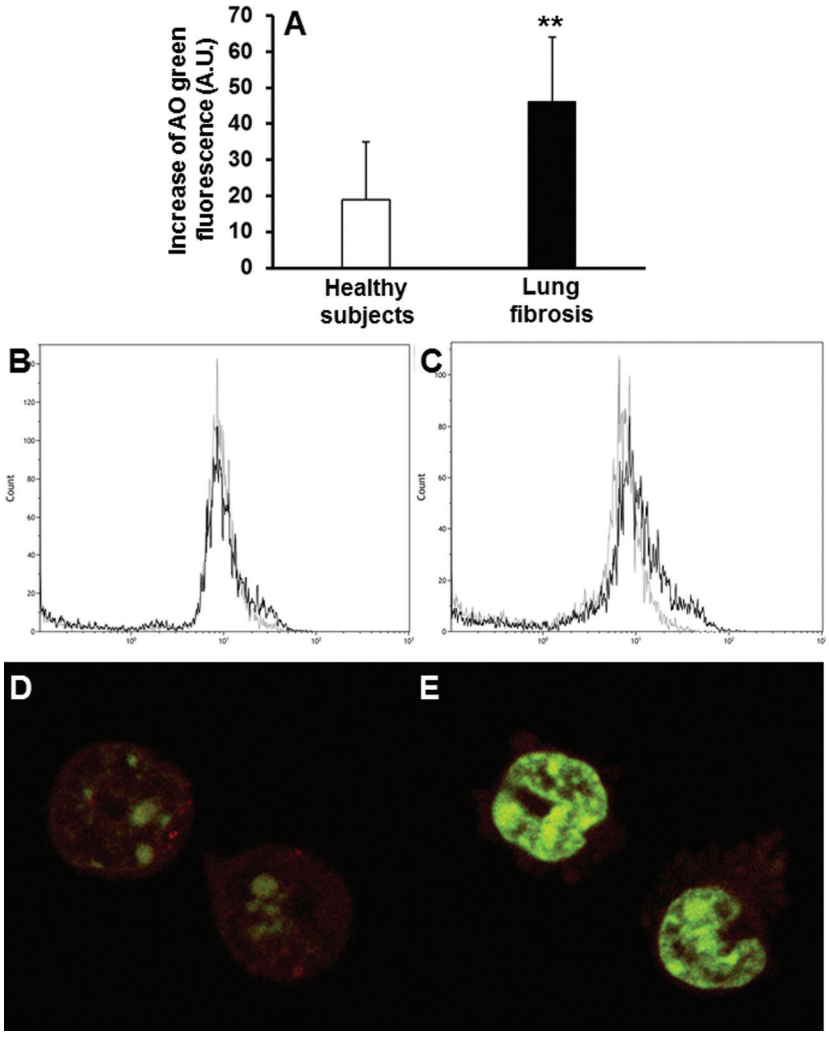

Figure 1. (**A**) Lysosomal membrane permeabilization (LMP) in cultures of oxidatively stressed human lung macrophages that were retrieved from healthy subjects (n = 7) and patients with lung fibrosis (n = 7). LMP was assessed as the increase of cytosolic/nuclear green fluorescence generated by acridine orange leakage into the cytosol in lung macrophages immediately after a 1-h oxidant exposure. Post-oxidant increase expressed as arbitrary units (AU) is indicated. Representative curves of acridine orange-green fluorescence from 10,000 LMs pre- (gray) and post- (black) oxidant challenge retrieved from (**B**) healthy subjects and (**C**) patients with lung fibrosis. Detailed micrographs of acridine orange relocation from lysosomes to the cytosol and nucleus appearing in the same murine J774 macrophages before (**D**) and after (**E**) treatment with the lysosomotropic detergent O-methyl-serine dodecylamide hydrochloride (200 μM) for 5 min. Note the nearly complete loss of intact red-fluorescent lysosomes and massive leakage of acridine orange into the cytosol, resulting in a strong green fluorescence. For details, see the Methods section. Values are the means ± 1 SD. Significant differences are indicated as follows: **P < 0.01 (vs healthy subjects).

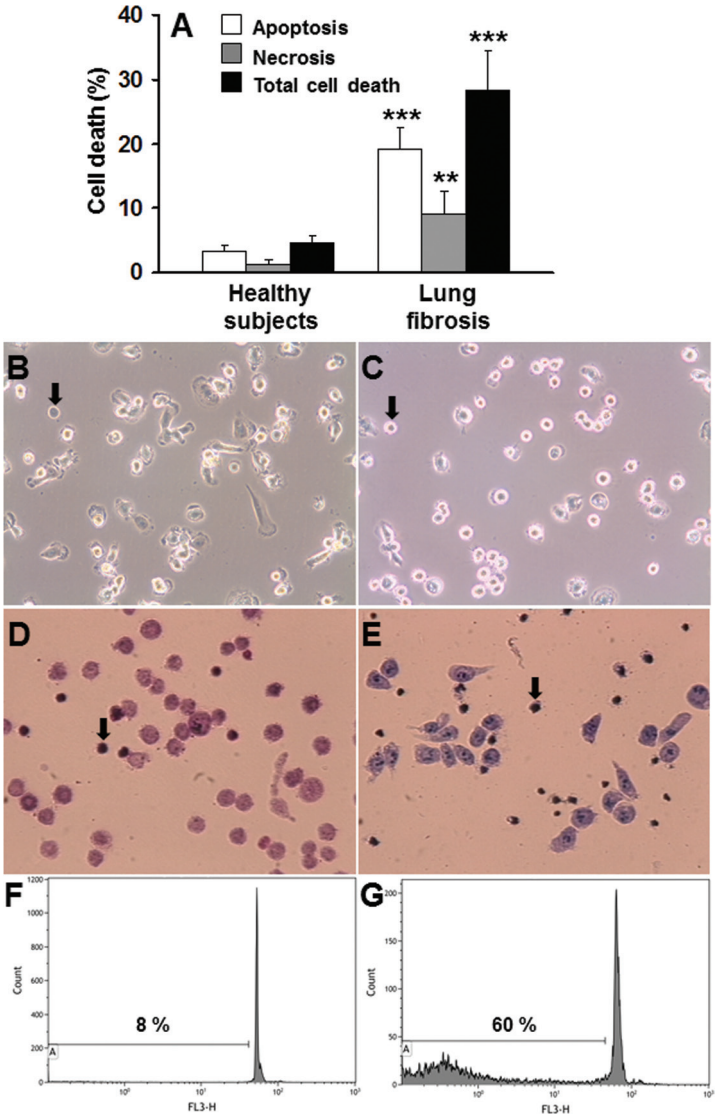

Figure 2. (**A**) Apoptosis, necrosis, and total cell death in cultures of oxidatively stressed human lung macrophages retrieved from healthy subjects (n = 7) and patients with lung fibrosis (n = 7). Cell death (apoptosis and necrosis), which appeared 10 h after the end of oxidant challenge in cultures of lung macrophages from healthy subjects (**B**) and patients with lung fibrosis (**C**) was assessed by typical morphology using a phase contrast microscope. The fraction of apoptotic cells at 10 h post-oxidative stress in cultures of lung macrophages from healthy subjects (**D** and **F**) and patients with lung fibrosis (**E** and **G**) was also assessed using Giemsa staining (**D** and **E**) and by the fraction of apoptotic sub-G_1 DNA (% of total is indicated; **F** and **G**). All methods gave similar results for apoptosis. Representative micrographs and stack bars of oxidant-challenged cultures of lung macrophages are shown. Representative apoptotic cells are indicated with black arrows. Detached cells were all necrotic. In the non-oxidatively stressed cultures, cell death was <3%. For details, see the Methods section. Values are the means ± 1 SD. Significant differences are indicated as follows: **P < 0.01 and ***P < 0.001 (vs healthy subjects).

Fe reactivity were evaluated in the LMs (Table 2). LMs of fibrotic lungs contained significantly less GSH ($P < 0.05$), while total cellular Fe did not differ significantly. In contrast, the Golde index mean value of LMs from fibrotic lungs, corresponding to cytochemically stained ferric Fe, was significantly higher than that of LMs from healthy control subjects (Fig. 3A–B); 31 ± 11 ($P < 0.01$) and 10 ± 16, respectively (normal range: 4–25).[16]

Discussion

Fibrosis, which is characterized by increased deposition of extracellular matrix proteins in tissues, is a terminal stage of many inflammatory lung disorders. A growing body of evidence supports that the development of fibrotic diseases in humans is initiated by increased oxidative stress.[1] Thus, decreased GSH is observed in human fibrotic diseases and in experimental fibrosis models.[1] GSH is a highly concentrated antioxidant in the cell and a major determinant for cellular redox status. Consequently, maintenance of intracellular GSH homeostasis is of vital importance for normal cell functions. Many inflammatory lung disorders and lung fibrosis are also associated with increased amounts of Fe in LMs and lung tissues,[2,3] which is a transition metal that promotes oxidative tissue injury due to its participation in Fenton-type chemistry.

Table 2. GSH and iron in human LMs. Data are presented as the means ± 1 SD. P-value vs healthy control patients is indicated.

ASSAYS	LUNG FIBROSIS PATIENTS (n = 7)	HEALTHY SUBJECTS (n = 7)
GSH (ng/mg protein)	30 ± 13 (0.035)	40 ± 8
Cellular Fe (ng/mg protein)	4 ± 2 (0.374)	5 ± 3
Golde Index	31 ± 11 (0.008)	10 ± 16

Although the present study included relatively few participants, the two populations, patients with fibrotic lung disease and healthy subjects, were well matched regarding both age and gender (Table 1). None of the subjects included was on any medication that would interfere with lysosomal integrity and redox status. BALF from the fibrotic lungs contained significantly less macrophages, while the number of lymphocytes and neutrophils were increased (Table 1). In agreement with previous studies,[1] LMs of human fibrotic lungs demonstrated significantly less GSH (Table 2). This depletion of GSH was likely important for the response of lysosomes and cells to oxidant challenge. Unlike previous studies,[2,3] we found no convincing difference between the two groups of LMs regarding their content of Fe (Table 2). Although cytochemical staining of LMs demonstrated significantly more ferric Fe in cells retrieved from the fibrotic lungs, assessment of total cellular Fe did not differ.

The molecular mechanisms of acute and chronic lung injury leading to inflammation and fibrosis are still not well-known. Recent studies indicate a role of the inflammasome in pulmonary fibrosis development and pathogenesis.[4,5] From this perspective, damage upon the lysosomes of lung cells may also be important. Indeed, agents known to promote the fibrotic process occurring in the asbestos- or silica-exposed lung, induce LMP as well as assembly of the inflammasome.[4,5,17] It is known that LMP leads to a leakage of cathepsins into the cytosol, activating the NLRP3 inflammasome, which cleaves IL-1β and IL-18 from their proisoforms.[4,5]

This is the first study to demonstrate that lysosomes of LMs of human fibrotic lungs are much more susceptible to oxidant challenge than LMs retrieved from healthy humans. In oxidatively stressed cultures, LMP was followed by pronounced cell death, mainly through apoptosis, but a considerable number of necrotic cells were also observed. These novel findings of the present study may represent an early event of great importance for the initiation of inflammation and fibrosis, considering the relatively recent discovery of a strong relationship between LMP and inflammasome activation.[4,5] In the inflamed lung, it is possible that lethal oxidant-induced injury on macrophageal lysosomes results in a release of ATP and uric acid, among a variety of other inflammation-triggering compounds.[4,5] ATP and uric acid, the latter as crystallized monosodium urate, will later work on viable LMs as potent danger-associated molecular patterns, which through LMP may activate the inflammasome complex.[4,5]

Conclusions

The present study indicates that oxidant-induced LMP may be of some importance for fibrosis development, possibly through the activation of the inflammasome complex in activated LMs. Further studies are warranted for a detailed understanding of the interaction between the lysosome and the inflammasome in this process.

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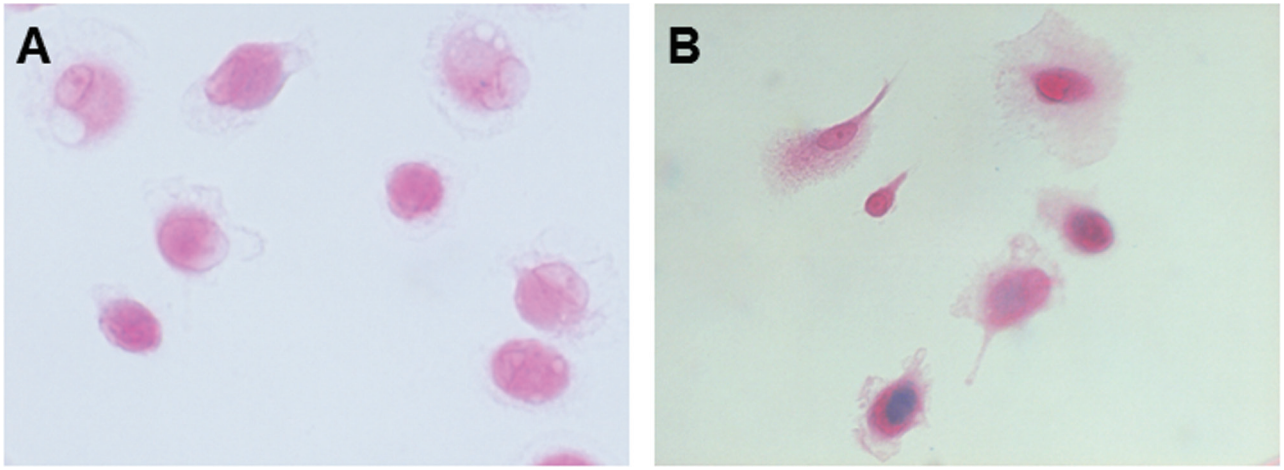

Figure 3. Prussian blue-stained ferric Fe in lung macrophages that were harvested from (**A**) a healthy subject and (**B**) a patient with lung fibrosis. Note the greater amount of Fe in the lung macrophages from the fibrotic lung.

Author Contributions

HLP designed the study, performed bronchoscopies, analyzed data, wrote the manuscript and obtained grant fundings. LKV performed experiments, analyzed data and wrote parts of the manuscript. Both authors have given their final approval of the version submitted. This study was performed at the Divisions of Pulmonary Medicine and Experimental Pathology, Linköping University, Sweden.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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