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REPORT

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Administration of fingolimod before endovascular therapy arrests local leukocyte responses in acute ischemic stroke

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Features of lymphocytes exerted at the luminal side of the brain microvasculature remain unknown in acute human ischemic stroke. Here, we used an endovascular technique to aspirate local blood with a microcatheter placed distally to the occlusive clot before recanalization in patients undergoing endovascular therapy (EVT). By comparing patients treated with fingolimod (a lymphocyte inhibitor) or not prior to EVT, we provide direct evidence for lymphocyte influx to the ipsilateral brain microvasculature. First, we demonstrated that several types of lymphocytes reached the target territory via retrograde collateral flow, and that their counts locally were higher than in the peripheral circulation. Second, although stroke-induced immunodepression was apparent in the peripheral circulation, we found a time-dependent accumulation of lymphocytes in the occluded vascular compartment. Third, lymphocytes' invasion from the periphery into the local territory's cerebral circulation was clearly blocked by a 6-hour fingolimod intervention prior to recanalization treatment. This study, akin to fingolimod-modulated vascular-inflammation, paves a potential way for ultra-early treatment strategies before recanalization.

INTRODUCTION

During hyperacute ischemic stroke (AIS), lymphocytes (especially T cells) have been identified as key orchestrators of subsequent thrombotic activity in the local microcirculation of occluded vascular fields, leading to progressive penumbral tissue loss in animal stroke models[1]. A first human observational study using microcatheter aspiration from occluded cerebral vessels demonstrated lymphocyte accumulation in the ischemic vasculature prior to recanalization [2]. However, whether this process can be therapeutically modulated before recanalization remains unknown. We are currently conducting a phase 2 trial of adjunctive antilymphocyte therapy with fingolimod prior to endovascular therapy (EVT) to reduce thrombotic activity and enhance collateral perfusion (**Fingolimod Intervention in Endovascular Treatment of Ischemic Stroke, NCT04629872**). In this prospective mechanistic observational substudy, we retrieved blood samples via distal microcatheter aspiration from trial participants to characterize local lymphocyte responses and to determine whether fingolimod administered before EVT attenuates lymphocyte accumulation at the occluded site. We hypothesize that ultra-early fingolimod administration reduces local lymphocyte infiltration through inhibition of lymphocyte egress from lymphoid organs, independent of its systemic lymphopenic effect.

MATERIALS AND METHODS

Study design and participants

This is a prospective mechanism-observational study in patients with large-vessel occlusion (LVO) of the anterior circulation undergoing EVT. Ethical approval was obtained from the ethics committee of the First Affiliated Hospital of Fujian Medical University (approval number: MRCTA, ECFAH of FMU [2020]296, approval date: 23 July 2020), and written informed consent was obtained from legal representatives.

Clinical and imaging inclusion criteria were defined as follows: (1) first-ever ischemic stroke with neurological baseline deficit equivalent to the National Institute of Health Stroke Scale (NIHSS) ≥ 6; (2) multimodal imaging prior to endovascular therapy comprising cranial noncontrast computed tomography (CT; Toshiba Aquilion 320-slice CT scanner, Japan), multiple CT-angiography and CT-perfusion scan in order (a) to exclude hemorrhage or extensive infarction, (b) to noninvasively determine the occlusion site, (c) to confirm patient eligibility for endovascular therapy according to current guidelines in the extended therapeutic time window ≤ 24 hours, and (d) to exclude individuals with poor collateral status for avoiding empty aspiration; and (3) periprocedural (invasive angiographic) confirmation of LVO of the following sites: distal internal cerebral artery (ICA), middle cerebral artery (MCA) M1 segment, or proximal M2 segment, respectively.

Samples collecting

Microcatheter aspiration was performed from the M2 segment of the middle cerebral artery (MCA) during persisting occlusion after clot penetration[2,3]. The following two arterial blood sample triplicates were drawn by microcatheter aspiration: (1) distal to the embolus under occlusive condition (labeled as brain B) and (2) the external iliac artery (labeled as peri B). Control samples were obtained from patients who underwent routine catheter-based angiography.

Lymphocyte analyses

Lymphocyte analyses followed samples collecting within 3 hours. Isolated mononuclear cells were immunostained with antibodies to CD3-Percp, CD4-PE, CD8-APC, CD19-APC, CD56-PE (Biolegend, USA) (Figure 1A). Data were acquired using a Beckman Cytoflex S flow cytometer and analyzed with Flow Jo software (Tree Star, USA).

Statistical analysis

Data distribution was tested using the Kolmogorov-Smirnov or the Shapiro-Wilk test. As data were not normally distributed, quantitative variables are reported as median (IQR) and qualitative variables as number (%). Quantitative variables were analysed using Wilcoxon matched-pairs signed rank test or Mann-Whitney tests. Qualitative variables were analysed with chi-squared test. Spearman's analysis was used for correlation.

Data availability

Data are available upon request to the corresponding author.

RESULTS

Between November 2020 and April 2021, we assessed 70 consecutive patients with LVO stroke for study eligibility. According to the applied prospective protocol, 22 EVT patients were included: 12 patients received only EVT; 4 patients were treated with tissue-type plasminogen activator (tPA) prior to EVT (tPA + EVT), and 6 patients had fingolimod prior to EVT (Fin + EVT). As Table 1 showed, median patient age was 76 years in the EVT group, 71 years in the tPA+ EVT group and 59 years in the Fin+ EVT group ($P > 0.05$). Sex was similarly distributed (40-50% men) in the different groups ($P > 0.05$). Median NIHSS was 14 and median ASPECTS was 8 in the different groups, all along with a good collateral circulation at admission for EVT ($P > 0.05$).

The median time from symptom onset to initial blood sampling was 790 min (522-996) in the EVT group, 382 min (286-561) in the tPA+ EVT group and 525 min (404-834) in the Fin+ EVT group. Compared to the peripheral circulation in AIS patients under occlusion condition, cerebral vascular territory showed obvious stroke-induced lymphocytes increased during the

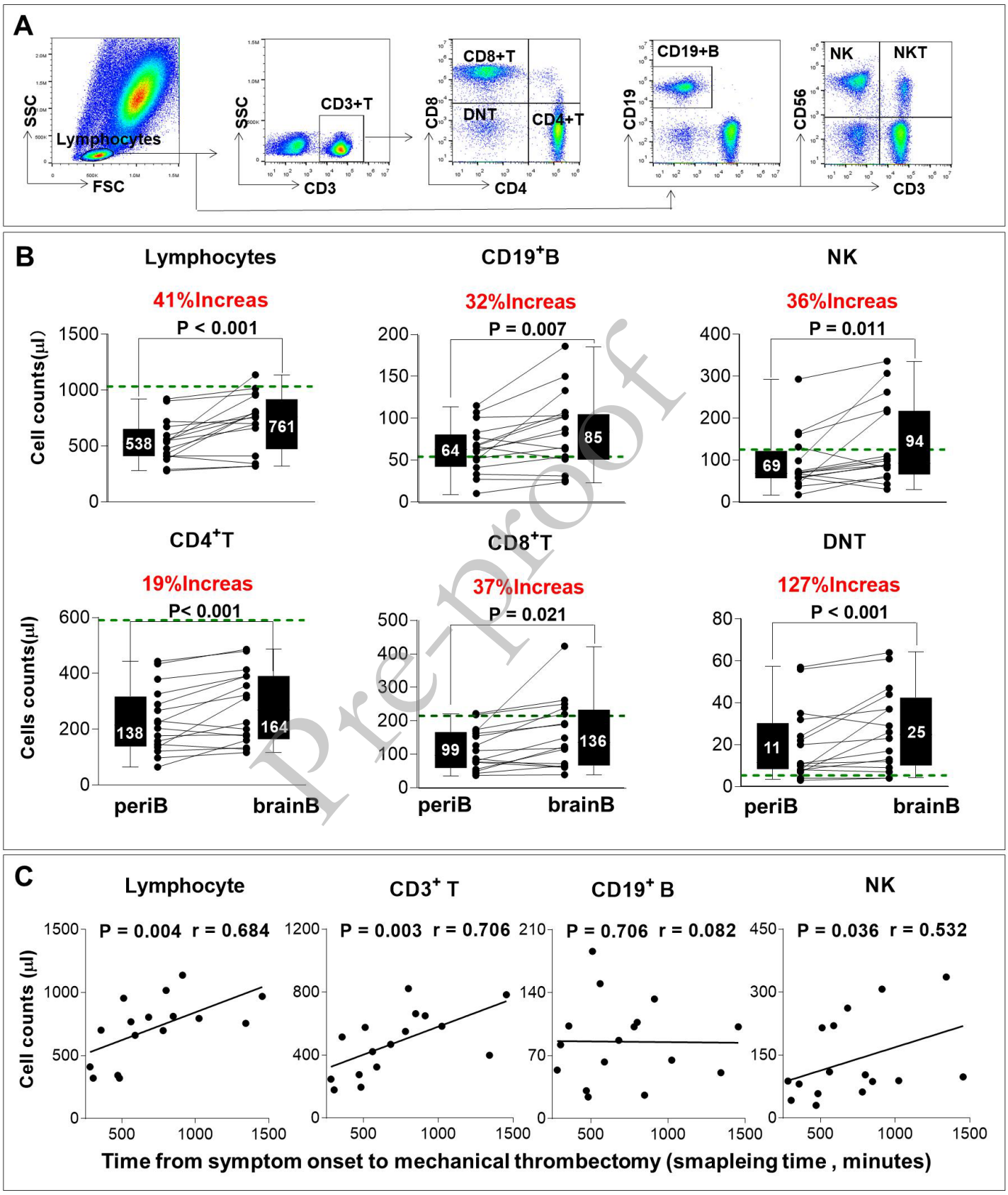


Figure 1. Stroke-induced lymphocytes persistently influx to the local circulation of occluded vascular territory via retrograde collateral flow A, Gating strategy of flow cytometry; double negative T cells (DNT). B, Lymphocyte subtype comparison; data are given as median (top and bottom of the box indicate the interquartile; the error bar indicates the Minimum and Maximum); Wilcoxon matched-pairs signed rank test was used; Green dashed lines represent the lowest number of peripheral bloods from the controls without stroke (N = 5). C, Correlation analysis, Spearman's correlation coefficients were used.

median of 12 hours after symptom onset (Median of increase counts: lymphocytes, 223/ul; CD3$^+$T, 140/ul; CD19$^+$ B, 21/ul; NK, 25/ul). This increasing count of the lymphocytes was primarily attributable to a significant and strong increase in the T cell subpopulation (Median of increase counts: CD4$^+$

Table 1. Baseline characteristics of patients

Characteristic	EVT (N = 12)	tPA + EVT (N = 4)	Fin + EVT (N = 6)
Age, year, median (IQR)	74.0 (61.3-81.8)	71.0 (53.8-80.0)	59.0 (50.3-61.5)
Male, No (%)	6 (50)	2 (50)	2 (33.3)
Medical history, No (%)			
Hypertension	10 (83.3)	1 (25.0)	3 (50.0)
Atrial fibrillation	7 (58.3)	2 (50.0)	2 (33.3)
Diabetes	1 (8.3)	0 (0.0)	2 (33.3)
Hyperlipidemia	2 (16.7)	0 (0.0)	1 (16.7)
Smoking	4 (33.3)	0 (0.0)	2 (33.3)
Previous stroke	4 (25.0)	0 (0.0)	1 (16.7)
Etiology, No (%)			
Large artery atherosclerosis	3 (25.0)	0 (0.00)	2 (33.3)
Cardioembolic	7 (58.3)	3 (75.0)	3 (50.0)
Undetermined	2 (16.7)	1 (25.0)	1 (16.7)
NIHSS at admission, median (IQR)	13.0 (5.0-16.5)	14.5 (12.5-20.3)	14.0 (6.5-16.5)
ASPECTS admission, median (IQR)	7.0 (4.5-8.8)	5.0 (2.8-7.3)	8.0 (4.3-9.3)
Occlusion location, No (%)			
ICA	2 (16.7)	1 (25.0)	3 (50.0)
M1	10 (83.3)	2 (50.0)	2 (33.3)
M2	0 (0.0)	1 (25.0)	1 (16.7)
Onset-to-EVT time, min, median (IQR)	790 (522-996)	382 (286-561)	525 (404-834)

Abbreviation: EVT, endovascular therapy; Fin, Fingolimod; tPA, tissue-type plasminogen activator; NIHSS, National Institutes of Health Stroke Scale; ASPECTS, Alberta Stroke Program Early CT Score; ICA, internal carotid artery; M1/M2, M1/M2 segment of middle cerebral artery; IQR, interquartile range.

T, 54/ul; $CD8^+$ T, 37/ul; DNT, 14/ul) (Figure 1B). Correlation analysis revealed that lymphocyte brain-vascular invasion was time dependent, the counts of which were positively associated with the time from symptom onset to initial blood sampling (Figure 1C).

As Figure 2 shows, after 6 hours of fingolimod treatment prior to EVT, the number of lymphocytes (especially T cells) decreased significantly in the blood of occluded local vascular fields relative to that in patients treated with EVT alone. Specifically, fingolimod recipients manifested 71%, 81% and 59% decreases, respectively, in lymphocytes, $CD4^+$ T cells and $CD8^+$ T cells.

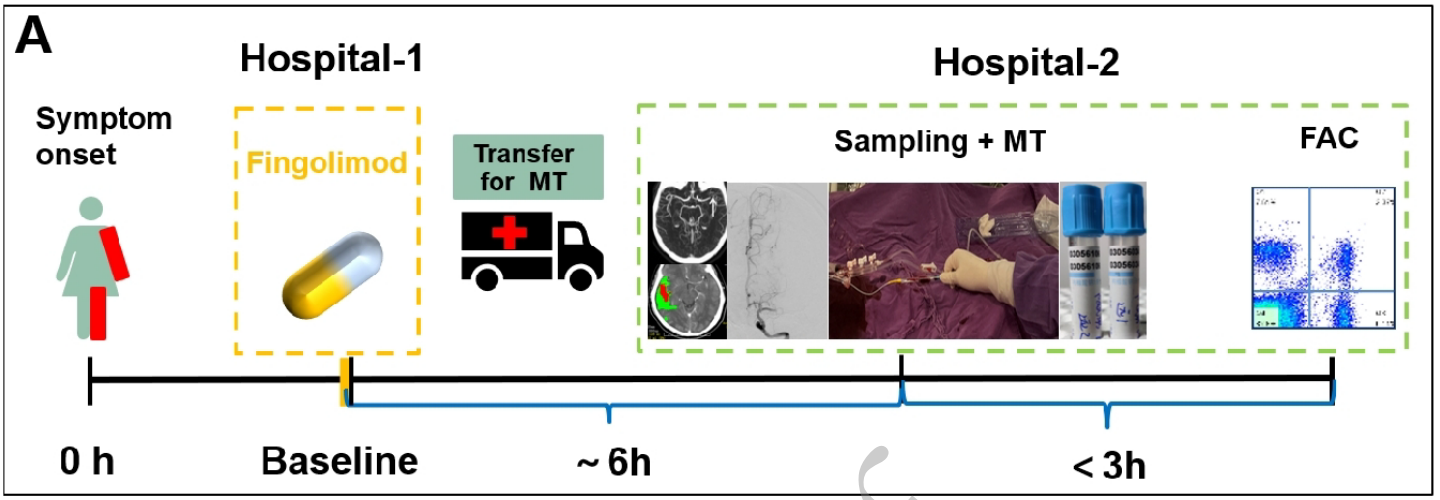
DISCUSSION

The specific features of lymphocytic invasion into occluded circulation fields during acute human ischemic stroke are unknown. However, use of an endovascular technique by which patients' blood was aspirated with a micro-catheter placed distally to the occlusive clot before recanalization proved useful. With higher selection criteria for enhancing the homogeneity, we demonstrated the main findings. First, several distinct lymphocyte subsets reach the occluded vascular territory despite complete embolic occlusion. Their counts in the local cerebral circulation were higher than those in the systemic circulation. Furthermore, although stroke-induced immunodepression was clear in patients' peripheral circulations, lymphocytes time-dependently accumulated in the stroke-afflicted cerebral circulation. Importantly, the lymphocytes' invasion from the periphery to the stroke-afflicted cerebral circulation was clearly blocked by 6-hours of fingolimod intervention prior to recanalization treatment.

Our measurements suggest that lymphocytes may enter the ischemic vasculature via retrograde collateral flow as anterograde occlusion time extends. Additionally, those counts increased to about 35% peripheral levels during 12 hours after stroke onset (Figure 1), which is consistent with results in a previous, similar study (24% lymphocyte increase during about 7 hours after stroke onset)[2]. This finding supports early applying vessel-lymphocyte inhibitors, not tissue-lymphocyte inhibitors, prior to endovascular treatment for orchestrating the focal luminal side of the vasculature inflammatory response. Given our finding that the largest increase of a lymphocyte subset was T cells (specifically $CD4^+$ T cells), the value of T cell inhibitors is probably optimized.

Fingolimod is an immunomodulatory therapy that is clinically approved for treatment of multiple sclerosis, in which it's beneficial effects result from the rapid induction of vessel-lymphopenia. This advantageous effect was also seen in our clinical trials of fingolimod in stroke patients (no EVT)[4]. In stroke mice treated with fingolimod immediately before reperfusion, leukocyte numbers in the circulation decreased by 60% within 3 hours[5]. The data this study presents confirms the time window in which fingolimod treatment modulates the vascular lymphocyte response; that is, 6-hours preceding endovascular therapy.

Current opinions about immune intervention are mainly focused on brain parenchyma inflammation induced neural cell death[6,7]. However, the neurovascular unit is an organic system and ischemia induced injury comprises both the brain vascular system and parenchyma. Natalizumab inhibits leukocyte adhesion to endothelium but increases intravascular cell counts. In contrast, fingolimod inhibits lymphocyte egress from lymph nodes and reduces their counts in the cerebral microvasculature[5]. The discrepancy between the impact of natalizumab and fingolimod on imaging endpoints in early phase II stroke trials is striking[8-11]. Although both drugs have T lymphocytes as the presumed key target immune cell population, their effects on early infarct growth differ markedly. One possibility is that the detrimental effects of T cells in early infarct growth are mainly exerted at the luminal side of the microvasculature, outside the brain parenchyma. In conclusion, this exploratory mechanistic study provides preliminary evidence characterizing lymphocyte accumulation in the ipsilateral cerebral microvasculature during



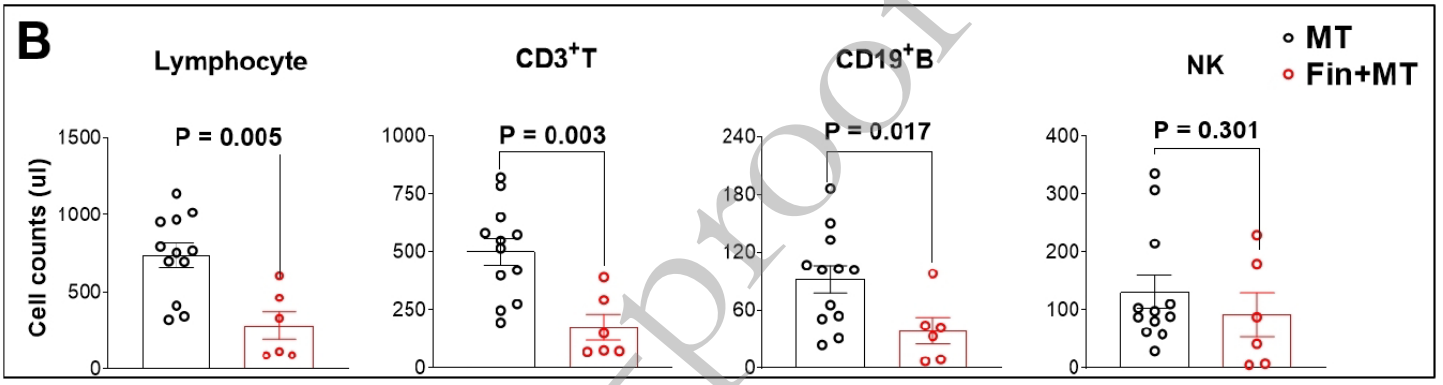


Figure 2. Receiving fingolimod at 6 hours prior to endovascular therapy adequately modulates lymphocytes-flux to the local circulation of occluded vascular territory A. Fingolimod interventional and sampling protocol. Patients who were transported by ambulance from hospital 1 to hospital 2 (thrombectomy-capable center), received oral fingolimod (0.5 mg Gilenya, Novartis) at hospital 1 about 6-hours preceding endovascular therapy. B. Receving fingolimod 6 hours before EVT arrests local leukocyte responses.

acute ischemic stroke. The observed reduction in local lymphocyte counts following fingolimod administration prior to EVT supports the biological plausibility of ultra-early immunomodulation[12]. However, whether this translates to improved clinical outcomes requires confirmation in adequately powered randomized controlled trials.

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DECLARATION OF INTERESTS

The authors declare no competing interests.