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**Critical Care**

**α7 Nicotinic acetylcholine receptor agonist GTS-21 attenuates ventilator-induced tumour necrosis factor-α production and lung injury**

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**Editor’s key points**

- Mechanical ventilation causes inflammation and lung damage.
- In this mouse study, stimulation of the cholinergic anti-inflammatory pathways reduced cytokine release after 4 h ventilation.
- The agonist used may be useful in patients in the future.

**Background.** Mechanical ventilation (MV) induces an inflammatory response that can lead to lung injury. The vagus nerve can limit the inflammatory response through the cholinergic anti-inflammatory pathway. We evaluated the effects of stimulation of the cholinergic anti-inflammatory pathway with the selective partial α7 nicotinic acetylcholine receptor (α7nAChR) agonist GTS-21 on inflammation and lung injury induced by MV using clinically relevant ventilator settings. Furthermore, we investigated whether altering endogenous cholinergic signalling, by administration of the non-specific nAChR antagonist mecamylamine and the peripherally acting acetylcholinesterase inhibitor neostigmine, modulates the MV-induced inflammatory response.

**Methods.** C57BL6 mice were injected i.p. with either the selective α7nAChR agonist GTS-21 (8 mg kg⁻¹), the acetylcholinesterase inhibitor neostigmine (80 μg kg⁻¹), the nAChR antagonist mecamylamine (1 mg kg⁻¹), or a placebo; followed by 4 h of MV (8 ml kg⁻¹, 1.5 cm H₂O PEEP).

**Results.** MV resulted in release of cytokines in plasma and lungs compared with unventilated mice. Lung and plasma levels of tumour necrosis factor (TNF)-α, but not of interleukin-10, were lower in GTS-21-treated animals compared with placebo (P<0.05). In addition, GTS-21 lowered the alveolar–arterial gradient, indicating improved lung function (P=0.04). Neither neostigmine nor mecamylamine had an effect on MV-induced inflammation or lung function.

**Conclusions.** MV with clinically relevant ventilator settings results in pulmonary and systemic inflammation. Stimulation of the cholinergic anti-inflammatory pathway with GTS-21 attenuates MV-induced release of TNF-α, which was associated with reduced lung injury. Modulation of endogenous cholinergic signalling did not affect the MV-induced inflammatory response. Selective stimulation of the cholinergic anti-inflammatory pathway may represent new treatment options for MV-induced lung injury.

**Keywords:** GTS-21; mecamylamine; mechanical ventilation; neostigmine; ventilator-induced lung injury

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Recently, it was discovered that stimulation of the efferent vagus nerve attenuates the innate immune response. This so-called ‘cholinergic anti-inflammatory pathway’ is based on binding of the vagal neurotransmitter acetylcholine (ACh) to the $\alpha_7$ nicotinic ACh receptor ($\alpha_7nAChR$) present on resident macrophages and other immune cells resulting in inhibition of pro-inflammatory cytokine release. In several animal studies, 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21), a selective partial $\alpha_7nAChR$ agonist, has proven to be effective in attenuating the immune response and improving outcome. GTS-21, which has been primarily developed for the treatment of Alzheimer’s disease and schizophrenia, has been administered to human volunteers and patients and is well tolerated with no clinically significantly safety findings. Therefore, GTS-21 might represent a new clinically relevant treatment option for inflammatory conditions.

The cholinergic anti-inflammatory pathway is regarded as a regulatory system, a reflex-type response to limit excessive inflammation. Endogenous activation of the cholinergic anti-inflammatory pathway results in increased ACh release by the vagus nerve. Recently, it was shown that cholinesterase inhibition attenuates the cytokine response and lowers mortality in experimental sepsis, suggesting that cholinesterase inhibition reinforces the cholinergic anti-inflammatory response by increasing ACh availability. Therefore, cholinesterase inhibition might represent another clinically relevant means of limiting excessive inflammation.

In the present study, we investigated the effects of direct stimulation of the cholinergic anti-inflammatory pathway by GTS-21 on inflammation and lung injury induced by MV using clinically relevant ventilator settings in mice. To determine whether alterations of endogenous cholinergic signalling modulate the MV-induced inflammatory response, we studied the effects of the acetylcholinesterase inhibitor neostigmine and the non-specific nAChR antagonist mecamylamine.

Methods

Reagents

3-[(2,4-Dimethoxybenzylidene)-anabaseine (GTS-21) was a kind gift from Comentis Inc. (San Francisco, CA, USA). Neostigmine methylsulphate was purchased from Centrafarm (Etten-Leur, The Netherlands), lipopolysaccharide (LPS, derived from *Escherichia coli* serotype O55:B5) and mecamylamine hydrochloride from Sigma-Aldrich (St Louis, MO, USA), ketamine from Eurovet (Bladel, The Netherlands), dexmedetomidine from Pfizer (Berlin, Germany), and atropine from Pharmachemie (Haarlem, The Netherlands). LPS was further purified as described previously. RPMI culture medium (RPMI 1640 Dutch modification, ICN Biomedicals; Costa Mesa, CA, USA) was supplemented with 10% fetal calf serum, 10 $\mu$g ml$^{-1}$ gentamicin, 10 mM L-glutamine, and 10 mM pyruvate.

Animals

All procedures described were in accordance with the requirements of the Dutch Experiments on Animals Act, the EC Directive 86/609, and approved by the Animal Ethics Committee of the Radboud University Nijmegen Medical Centre. Male C57BL6 mice (Charles River, Sutzfeld, Germany) aged 10–12 weeks and weighing 23–29 g were used in all experiments. The in vivo experimental protocols are listed below. The protocol for isolation and ex vivo stimulation of alveolar macrophages is given in the Supplementary material.

Experimental protocols

Non-ventilated control group

A group of eight mice that were immediately killed after anaesthesia induction were used as non-ventilated controls in all following protocols (NV group).

GTS-21 protocol

Mice received either an i.p. injection of phosphate-buffered saline (PBS, 200 $\mu$l) 30 min before the start of ventilation (placebo–MV group, $n=18$) or an i.p. injection of GTS-21 (8 $mg$ kg$^{-1}$ in 200 $\mu$l PBS) 30 min before the start of ventilation (GTS21–MV group, $n=16$). Earlier reports have demonstrated that the anti-inflammatory effects of GTS-21 in mice are dose-dependent and that 4 $mg$ kg$^{-1}$ significantly attenuates cytokine production. To maximize its effects, we used 8 $mg$ kg$^{-1}$, which is still a non-toxic dose according to the manufacturer. One mouse in the GTS21–MV group died shortly after the start of MV.

Neostigmine/mecamylamine protocol

Mice received either an i.p. injection of 200 $\mu$l PBS 30 min before the start of ventilation (placebo–MV group, $n=15$) or of neostigmine (80 $\mu$g kg$^{-1}$ i.p. in 200 $\mu$l PBS) 30 min before the start of ventilation (neostigmine–MV group, $n=14$), or of mecamylamine (Sigma-Aldrich, 1 mg kg$^{-1}$ dissolved in 200 $\mu$l PBS) 30 min before the start of ventilation (mecamylamine–MV group, $n=15$).

Neostigmine and mecamylamine doses were based on previous studies. Three mice died before the start of MV (two in the neostigmine–MV group and one in the placebo–MV group).

Instrumentation and MV

With the exception of the NV group, all mice were mechanically ventilated for 4 h in an identical fashion. Before intubation, mice were anaesthetized with an i.p. injection of 126 $mg$ kg$^{-1}$ ketamine, 0.4 $mg$ kg$^{-1}$ dexmedetomidine, and 0.5 $mg$ kg$^{-1}$ atropine. Atropine is a vagolytic drug, but it predominantly antagonizes muscarinic ACh receptors (mAChRs); it is therefore not expected to interfere with the anti-inflammatory effects of the vagus nerve to a great extent. Mice were intubated with a tracheal tube and the carotid artery was cannulated to administer Ringer’s lactate with heparin (4 IU ml$^{-1}$) and to monitor arterial pressure. Mice were ventilated (MiniVent $^R$ type 845; Hugo
Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) with clinically relevant settings: a tidal volume (V) of 8 ml kg⁻¹, a breathing frequency of 160–170 min⁻¹, a PEEP of 1.5 cm H₂O, and an inspired oxygen fraction (FIO₂) of 45%. Anaesthesia was maintained by administration of 18 mg kg⁻¹ ketamine, 20 μg kg⁻¹ dexmedetomidine, and 37.5 μg kg⁻¹ atropine as a bolus every 30 min via an i.p. placed catheter. Furthermore, depth of anaesthesia was checked regularly by elicitation of the pedal withdrawal reflex. When mice reacted to the stimulus by pedal withdrawal, an increase in heart rate or arterial pressure (a sign of discomfort), or both, an additional dose of maintenance anaesthesia was administered. Rectal temperature was monitored throughout the experiment and was kept between 36.5°C and 37.5°C using a heating pad and blankets.

**Material harvesting**

After 4 h of ventilation, mice were killed by exsanguination. We chose this specific time point based on previous work of our group, where a transient increase in inflammatory mediators was demonstrated with nearly all mediators peaking after 4 h of MV. Blood gas analysis was performed using an i-STAT Blood Gas Analyzer (Abbot, Hoofddorp, The Netherlands) and plasma was stored at −80°C until analysis. Immediately after exsanguination, the lungs were carefully removed. The middle lobe of the right lung was fixed for light microscopic histological examination (see Supplementary material). The left lung (cytokine and myeloperoxidase determination, see Supplementary material) and one lobe of the right lung (mRNA analysis, see Supplementary material) were snap-frozen in nitrogen and stored at −80°C until analysis.

**Statistical analysis**

Data were normally distributed (calculated by the Kolmogorov-Smirnov test). Since there was a considerable variation in the LPS response between alveolar macrophage cultures obtained from three different mice, the effect of GTS-21 was expressed as per cent of the LPS-induced tumour necrosis factor (TNF-α) production in the absence of GTS-21. Alveolar–arterial gradient was calculated using the formula: FIO₂ × (Atmospheric pressure – H₂O pressure) – (PO₂/0.8) – PO₂. The Grubbs test (extreme studentized deviate method) was performed and significant outliers were excluded from analysis (maximum of one exclusion per data set). Statistical differences between the groups were detected by one-way analysis of variance (ANOVA, with the Bonferroni post hoc test to check for significant differences between ventilated groups) or unpaired two-sided Student’s t-tests as appropriate. The Pearson correlation analysis was used to investigate the relation between TNF-α, IL-6, and keratinocyte-derived chemokine (KC) concentrations. A P-value of <0.05 was considered statistically significant. All tests were performed with Graphpad Prism 5 (Graphpad Software, La Jolla, CA, USA).

**Results**

**Treatment with the selective partial α7nAChR agonist GTS-21 attenuates MV-induced TNF-α production at the transcriptional level**

In ventilated placebo-treated mice, both pro-inflammatory (TNF-α, IL-6, and KC) and anti-inflammatory [interleukin (IL)-10] mediators were increased in plasma compared with non-ventilated controls (Fig. 1a). Similarly, in lung homogenates, TNF-α, IL-6, and KC levels were elevated in ventilated animals compared with the NV group (Fig. 1a). Pre-treatment with GTS-21 significantly attenuated MV-induced TNF-α levels in plasma and lung homogenates. Although plasma and lung concentrations of TNF-α correlated with levels of the other pro-inflammatory cytokines IL-6 and IL-10 in our model (Supplementary Figure 1), GTS-21 pre-treatment did not affect these cytokines or the anti-inflammatory mediator IL-10. Four hours of MV up-regulated TNF-α and IL-10, but not IL-6 mRNA expression in lung tissue, measured by quantitative polymerase chain reaction analysis (Fig. 2). GTS-21 pretreatment abrogated the MV-induced TNF-α up-regulation while not significantly affecting IL-10 expression, indicating that GTS-21 inhibits TNF-α production at the transcriptional level. Since alveolar macrophages are important cytokine-producing cells in the lung, we wanted to confirm whether GTS-21 inhibited TNF-α transcription and production in these cells. Therefore,
we isolated murine alveolar macrophages and stimulated them with LPS in the presence and absence of GTS-21 and found that GTS-21 inhibited LPS-induced TNF-α production (Fig. 3A) and TNF-α gene expression (Fig. 3B).

**GTS-21 does not influence MV-induced increases in pulmonary neutrophil influx or lung MPO levels**

As depicted in Supplementary Figure 2, 4 h of MV resulted in an increase in pulmonary neutrophils compared with non-ventilated animals, but GTS-21 pretreatment did not have an effect on neutrophil influx.

**GTS-21 lowers the arterial–alveolar gradient**

Both placebo- and GTS-21-treated animals exhibited stable haemodynamic parameters throughout the experiment (Table 1). Mean arterial pressure (MAP) decreased from \(~100 \text{ mm Hg}\) at the beginning of the ventilation period to \(80 \text{ mm Hg}\) after 4 h in both groups. Lung function after 4 h of MV was better in GTS-21 pretreated mice compared with placebo, reflected by a trend towards a higher \(P_{\text{aO}_2}\), and a significant reduction in the alveolar–arterial (\(\Delta-a\)) gradient.

**Neither the acetylcholinesterase inhibitor neostigmine nor the nAChR antagonist mecamylamine affects the MV-induced inflammatory response or change in lung function**

To investigate whether alterations of endogenous cholinergic signalling modulate the MV-induced inflammatory response, we determined the effects of acetylcholinesterase inhibition by neostigmine and nAChR blockade by mecamylamine. As shown in Table 2 and Figure 4, pretreatment with neostigmine or mecamylamine had no effect on MV-induced changes in inflammatory mediators, haemodynamic parameters, or blood gas measures.

**Discussion**

In the present study, we demonstrate that MV with clinically relevant settings elicits a local and systemic inflammatory response. Pretreatment with the selective partial \(\alpha_7\)nAChR agonist GTS-21 attenuates MV-induced TNF-α production at the transcriptional level and improves lung function.

Recently, it was demonstrated that administration of a centrally acting vagus-mimetic drug attenuates VILI induced by high tidal volumes (20 ml kg\(^{-1}\)). In the present study, we demonstrate that selective pharmacological stimulation of the peripheral branch of the cholinergic anti-inflammatory pathway attenuates VILI elicited by MV with clinically relevant settings as well. This study confirms earlier findings from our group and others that MV using clinically relevant settings results in local and systemic cytokine release. In addition to these studies, we show up-regulation of cytokine mRNA (TNF-α and IL-10) in lung tissue, suggesting that the MV-induced elevation of pulmonary cytokine levels is the result of local production and not merely of systemic spillover. As we have shown earlier, using electron microscopy, alveolar integrity is preserved with a tidal volume of 8 ml kg\(^{-1}\) in this model, indicating...
that the observed inflammatory response is a result of biotrauma.\textsuperscript{1,3,23} We used the same protocol in the present study and demonstrate that GTS-21 attenuates the MV-induced pro-inflammatory response (TNF-\(\alpha\)) at the transcriptional level, but does not dampen anti-inflammation (IL-10). These effects are associated with an improved lung function. How TNF-\(\alpha\) affects lung function is largely unknown, but a critical role for this cytokine in the pathophysiology of VILI has been underlined by others, as in a rabbit VILI model, intratracheal administration of anti-TNF-\(\alpha\) antibody improved oxygenation and respiratory compliance.\textsuperscript{24}

In the absence of a reduction in alveolar inflammatory cell infiltration, the beneficial effects of GTS-21 on oxygenation in our model might be mediated by effects on alveolar capillary membrane integrity, which was previously shown to be compromised in our model.\textsuperscript{9} However, since we did not measure wet/dry weight ratios in this study, this remains speculative. Another possible explanation for the improvement in lung function by GTS-21 might rely on regulation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase JNK phosphorylation. These signalling molecules have been implicated in the pro-inflammatory response related to oxygenation in VILI,\textsuperscript{25-29} and cholinergic stimulation has been shown to mitigate hypoxia-induced MAPK and JNK phosphorylation.\textsuperscript{30}

Our data are in accordance with recent in vitro studies of our group and others, demonstrating that GTS-21 limits TNF-\(\alpha\) mRNA expression and production in response to various Toll-like receptor agonists in isolated primary human monocytes.\textsuperscript{31,32} Similar to these in vitro studies, GTS-21 did not attenuate MV-induced IL-6 release in our model. I.P. GTS-21 also limits plasma TNF-\(\alpha\), but not IL-6, in a murine peritonitis model\textsuperscript{13} and GTS-21 administered locally in the airways inhibits TNF-\(\alpha\), but not IL-6 release, in the mouse lung after intratracheal LPS delivery.\textsuperscript{21} The anti-inflammatory effects of GTS-21 in our VILI model are probably attributable to its effect on alveolar macrophages since these are the most likely target for GTS-21,\textsuperscript{33,34} given that it inhibits LPS-induced TNF-\(\alpha\) transcription and production in these cells.

Despite limiting pulmonary TNF-\(\alpha\) production, lung neutrophil influx was not inhibited by GTS-21 in our study. This may be explained by the fact that although lower TNF-\(\alpha\) levels correlated with lower KC concentrations, GTS-21 did not significantly reduce KC in our model. KC (the murine homologue of human IL-8) is an important chemokine that plays a major role in attracting neutrophils.\textsuperscript{34,35} These findings are in accordance with those of the intratracheal LPS instillation model, where GTS-21 did not influence pulmonary neutrophil influx.\textsuperscript{21} In contrast, in the peritonitis study,\textsuperscript{13} GTS-21 did attenuate neutrophil influx into the pancreas, indicating that the effect of GTS-21 on neutrophil tissue infiltration may be dependent on other factors such as tissue type, dose, and timing of drug delivery.\textsuperscript{21} The lack of effects of TNF-\(\alpha\) reduction on neutrophil influx is of interest in view of the failure of anti-TNF-\(\alpha\) therapy in changing outcomes in clinical trials.

In our model, the peripheral acetylcholinesterase inhibitor neostigmine had no anti-inflammatory effects. We chose to administer the same dose (80 \(\mu\)g kg\(^{-1}\)) which was shown by Hofer and colleagues\textsuperscript{19} to have remarkable anti-inflammatory effects in a murine caecal ligation and puncture (CLP) sepsis model.\textsuperscript{19} In contrast, neostigmine in a comparable dose was not protective against endotoxin-induced

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### Table 1
Cardiorespiratory parameters after 4 h of MV in placebo- and GTS-21-treated ventilated animals. Mean (SD). P-values are \(t\)-test. Values in bold indicate significant P-value.

<table>
<thead>
<tr>
<th></th>
<th>Placebo–MV ((n=18))</th>
<th>GTS21–MV ((n=16))</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP 0 h (mm Hg)</td>
<td>104 (11)</td>
<td>104 (12)</td>
<td>0.95</td>
</tr>
<tr>
<td>MAP 4 h (mm Hg)</td>
<td>81 (11)</td>
<td>80 (10)</td>
<td>0.90</td>
</tr>
<tr>
<td>pH</td>
<td>7.36 (0.05)</td>
<td>7.38 (0.06)</td>
<td>0.26</td>
</tr>
<tr>
<td>(PaO_2) (mm Hg)</td>
<td>128 (21)</td>
<td>145 (28)</td>
<td>0.07</td>
</tr>
<tr>
<td>(PaCO_2) (mm Hg)</td>
<td>29 (5)</td>
<td>28 (5)</td>
<td>0.63</td>
</tr>
<tr>
<td>HCO(_3^-) (mmol litre(^{-1}))</td>
<td>16.3 (1.7)</td>
<td>16.4 (2.3)</td>
<td>0.88</td>
</tr>
<tr>
<td>Base excess</td>
<td>–9 (3)</td>
<td>–9 (2)</td>
<td>0.99</td>
</tr>
<tr>
<td>Lactate (mmol litre(^{-1}))</td>
<td>1.5 (0.4)</td>
<td>1.5 (0.8)</td>
<td>0.67</td>
</tr>
<tr>
<td>(A–a) gradient (mm Hg)</td>
<td>156 (18)</td>
<td>140 (23)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

### Table 2
Cardiorespiratory parameters after 4 h of MV in placebo-, neostigmine-, and mecamylamine-treated animals. Mean (SD). P-values are \(t\)-test.

<table>
<thead>
<tr>
<th></th>
<th>Placebo–MV ((n=15))</th>
<th>Neostigmine–MV ((n=14))</th>
<th>Mecamylamine–MV ((n=15))</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP 0 h (mm Hg)</td>
<td>105 (10)</td>
<td>110 (12)</td>
<td>108 (9)</td>
<td>0.47</td>
</tr>
<tr>
<td>MAP 4 h (mm Hg)</td>
<td>81 (8)</td>
<td>84 (7)</td>
<td>83 (7)</td>
<td>0.43</td>
</tr>
<tr>
<td>pH</td>
<td>7.36 (0.05)</td>
<td>7.37 (0.05)</td>
<td>7.38 (0.05)</td>
<td>0.65</td>
</tr>
<tr>
<td>(PaO_2) (mm Hg)</td>
<td>143 (18)</td>
<td>152 (23)</td>
<td>143 (38)</td>
<td>0.57</td>
</tr>
<tr>
<td>(PaCO_2) (mm Hg)</td>
<td>30 (4)</td>
<td>31 (3)</td>
<td>32 (8)</td>
<td>0.64</td>
</tr>
<tr>
<td>HCO(_3^-) (mmol litre(^{-1}))</td>
<td>17.1 (2.1)</td>
<td>17.9 (1.7)</td>
<td>18.9 (3.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>BE</td>
<td>–8 (3)</td>
<td>–7 (2)</td>
<td>–6 (3)</td>
<td>0.12</td>
</tr>
<tr>
<td>Lactate (mmol litre(^{-1}))</td>
<td>2.0 (0.9)</td>
<td>2.1 (0.7)</td>
<td>2.2 (0.8)</td>
<td>0.89</td>
</tr>
<tr>
<td>(A–a) gradient (mm Hg)</td>
<td>140 (16)</td>
<td>129 (20)</td>
<td>137 (29)</td>
<td>0.42</td>
</tr>
</tbody>
</table>
Histopathological organ injury in mice. There are several possible explanations for the lack of an effect in our study. First, the cholinergic anti-inflammatory pathway is regarded as a reflex-type response to control excessive inflammation, and peripheral acetylcholinesterase inhibition can merely reinforce this endogenous pathway through increasing Ach availability at the acetylcholine receptors. The relatively mild MV-induced inflammatory response in our model (especially compared with the fulminant sepsis caused by CLP) may not be severe enough to activate this pathway. Along these lines, there would be no vagus-borne Ach release into the tissues, hence no pronounced anti-inflammatory effect of inhibition of Ach breakdown. The lack of endogenous activation of the cholinergic anti-inflammatory pathway might also explain why, in our study, nAChR blockade with mecamylamine did not worsen the MV-induced inflammatory response, while in murine pancreatitis, mecamylamine treatment led to an amplified inflammatory response and enhanced severity. However, mecamylamine is not selective for the α7nAChR and might therefore exert various other, yet unknown, effects. Future experiments with α7nAChR-deficient mice in our model could give a definitive answer whether the cholinergic anti-inflammatory pathway is activated in VILI. Another explanation for the ineffectiveness of neostigmine in our study might depend on the brain’s impermeability to neostigmine. Although Hofer and colleagues demonstrated that neostigmine was equally effective as physostigmine atropine. Therefore, further research regarding its suitability for human use, and therefore, further research regarding its effects on VILI is warranted.

In conclusion, our data show that stimulation of the cholinergic anti-inflammatory pathway by the selective partial α7nAChR agonist GTS-21 attenuates the inflammatory response induced by 4 h of MV using clinically relevant ventilator settings and improves lung function. Modulation of endogenous cholinergic signalling by either peripheral cholinesterase inhibition or nAChR blockade did not affect MV-induced inflammation or lung function. GTS-21 might be a promising compound because of its suitability for human use, and therefore, further research regarding its effects on VILI is warranted.

Supplementary material
Supplementary material is available at British Journal of Anaesthesia online.

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Conflict of interest
None declared.
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