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# Upregulation of Ecto-5'-Nucleotidase by Rosuvastatin Increases the Vasodilator Response to Ischemia

Patrick Meijer, Constantijn W. Wouters, Petra H.H. van den Broek, Maarten de Rooij, Gert Jan Scheffer, Paul Smits, Gerard A. Rongen

**Abstract**—3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins) are effective in the primary and secondary prevention of cardiovascular events. Although originally developed to improve lipid profile, statins have demonstrated a surplus of beneficial pleiotropic effects, including improved endothelial function, reduced inflammation, and increased tolerance to ischemia-reperfusion injury. In preclinical studies, increased ecto-5'-nucleotidase activity, the key enzyme in extracellular adenosine formation, plays an important role in these effects. Because human data are absent, we explored the effects of rosuvastatin on ecto-5'-nucleotidase activity and the clinical relevance of increased extracellular adenosine during ischemia in humans in vivo. The forearm vasodilator responses to 3 increasing periods of forearm ischemia (2, 5, and 13 minutes) were determined during placebo and caffeine (an adenosine receptor antagonist) infusion into the brachial artery. At the end of an 8-day treatment period with rosuvastatin (20 mg per day), this whole procedure was repeated. During both experiments, ecto-5'-nucleotidase activity was determined. Vasodilator responses are expressed as the percentage increase in forearm blood flow ratio from baseline. Rosuvastatin increased ecto-5'-nucleotidase activity by  $49 \pm 17\%$  and enhanced the vasodilator response after 2, 5, and 13 minutes of ischemia in the absence ( $146 \pm 19$ ,  $330 \pm 26$ , and  $987 \pm 133$  to  $312 \pm 77$ ,  $566 \pm 107$ , and  $1533 \pm 267$ ) but not in the presence of caffeine ( $98 \pm 25$ ,  $264 \pm 54$ , and  $727 \pm 111$  versus  $95 \pm 19$ ,  $205 \pm 34$ , and  $530 \pm 62$ ). Rosuvastatin increases extracellular formation of adenosine in humans in vivo probably by enhancing ecto-5'-nucleotidase activity. This action results in the improvement of reactive hyperemia and may further enhance the clinical benefit of statins, in particular in conditions of ischemia. (*Hypertension*. 2010;56:722-727.)

**Key Words:** adenosine ■ ecto-5'-nucleotidase ■ caffeine ■ ischemia ■ reactive hyperemia

3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors (statins) are effective in the primary and secondary prevention of cardiovascular events.<sup>1,2</sup> Although originally developed to improve lipid profile, statins have demonstrated a surplus of beneficial pleiotropic effects, including improved endothelial function, reduced inflammation, and increased tolerance to ischemia-reperfusion injury.<sup>3-7</sup> These effects are independent of plasma cholesterol lowering and improve outcome after cardiovascular events.<sup>8,9</sup>

Increased extracellular adenosine formation has been implicated as one of the underlying mechanisms. This is supported by several preclinical studies demonstrating the activation of ecto-5'-nucleotidase (CD73) activity, increased stimulation of adenosine receptors, and adenosine-mediated protection against ischemia-reperfusion injury by statins.<sup>10-12</sup> The enzyme CD73 dephosphorylates extracellular AMP, which forms the rate-limiting step in the formation of extracellular adenosine.<sup>13</sup> We recently demonstrated increased extracellular adenosine formation in healthy human

volunteers after a 1-week treatment with rosuvastatin using a pharmacological approach. In addition, we demonstrated rosuvastatin-induced augmentation of postocclusive reactive hyperemia (PORH).<sup>5</sup> However, we did not assess CD73 activity as a potential source of extracellular adenosine, nor did we investigate the role of adenosine in rosuvastatin-induced augmentation of PORH. CD73 activity plays a pivotal role in the formation of atherosclerosis and increased cellular resilience against ischemia-reperfusion injury observed after ischemic preconditioning.<sup>14-16</sup> An increase in CD73 activity may therefore reduce atherosclerosis formation and improve cellular tolerance against ischemia. The extent of PORH in the human forearm correlates well with the vasodilator impairment in the coronary circulation.<sup>17</sup> Indeed, it reflects vascular health status, because increased forearm PORH is associated with a reduced incidence of stroke and cardiovascular disease in patients with essential hypertension.<sup>18</sup> Compared with normotensive subjects patients with essential hypertension have a reduced vasodilator response to

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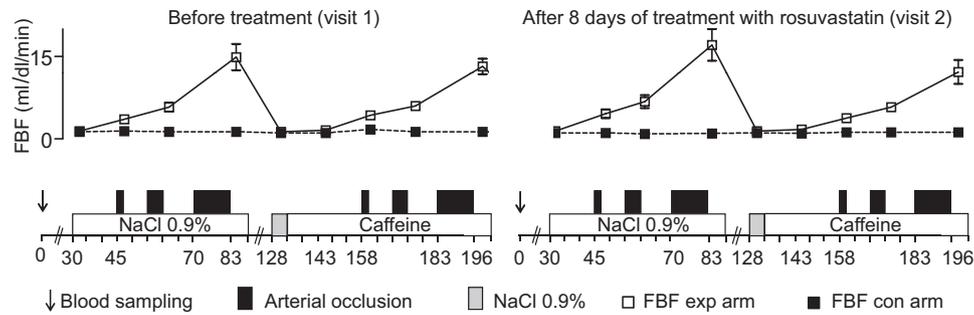
P.M. and C.W.W. contributed equally to this article.

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**Figure 1.** Schematic overview of the experimental protocol, indicating arterial occlusion, placebo and caffeine infusion, and FBF measurements. In addition, the graph above the protocol represents the absolute FBFs, at baseline and reperfusion, in the experimental and control arms ( $n=8$ ).

reactive hyperemia and may therefore benefit from therapies aimed at or associated with an improvement of PORH.<sup>19</sup> Insight into the underlying mechanism of rosuvastatin-induced augmentation of reactive hyperemia may help us in optimizing the beneficial effects of statins and improve treatment of patients at risk for cardiovascular events. We, therefore, assessed the involvement of adenosine in rosuvastatin-induced augmentation of PORH by testing the following hypotheses: a 1-week treatment with rosuvastatin enhances CD73 activity of circulating mononuclear blood cells, and caffeine, an adenosine receptor antagonist, inhibits the effect of rosuvastatin on forearm PORH.

## Methods

### Subjects

The study was performed in accordance with the Declaration of Helsinki of the World Medical Association and approved by the institutional review board of the Radboud University Nijmegen Medical Centre. Ten healthy volunteers (5 women; aged 19 to 24 years) with a normal medical history, physical examination, blood pressure, body mass index, fasting plasma lipid profile, and glucose concentration gave written informed consent before entering the study. Experiments were performed in a temperature-controlled room ( $23 \pm 1^\circ\text{C}$ ) in the morning after an overnight fast and  $\geq 24$  hours of caffeine abstinence. All of the experiments were performed according to institutional guidelines.

### Procedures

At the start of each experiment, a 27-gauge needle (B. Braun Medical B.V.) was inserted into the brachial artery of the nondominant arm for intra-arterial drug administration. In both arms, forearm blood flow (FBF) was measured simultaneously with venous occlusion plethysmography using mercury-in-silastic-strain gauges and occluded hand circulation as described previously.<sup>20</sup>

### Experimental Design

Figure 1 shows the design of the study. All volunteers received an 8-day treatment with rosuvastatin (20 mg per day) and entered our research facility twice, the first time before the start of treatment with rosuvastatin and the second time on the final day of treatment with rosuvastatin. On both visits forearm vasodilator responses to 3 increasing periods of forearm ischemia (2, 5, and 13 minutes) were determined, once during the infusion of placebo (saline 0.9%, 50  $\mu\text{L}/\text{min}$  per 100 mL of forearm volume) and once during the administration of caffeine (90  $\mu\text{g}/\text{min}$  per 100 mL of forearm volume) into the brachial artery.

### Ecto-5'-Nucleotidase Activity

We determined the activity of CD73 exposed on the surface of intact mononuclear cells by quantifying the conversion of  $1,N^6$ -

ethenoadenosine 5'-monophosphate to  $1,N^6$ -ethenoadenosine<sup>21</sup> in the presence and absence of the CD73-specific inhibitor  $\alpha,\beta$ -methyleneadenosine 5'-diphosphate. The difference in these 2 activities reflects CD73 activity. To eliminate the influence of nonspecific dephosphorylation of  $1,N^6$ -ethenoadenosine 5'-monophosphate to  $1,N^6$ -ethenoadenosine and  $1,N^6$ -ethenoadenosine transport, CD73 activity was measured in the presence of  $\beta$ -glycerophosphate and dipyridamole.  $\beta$ -Glycerophosphate forms a competitive substrate for nonspecific phosphatases eliminating the contribution of nonspecific dephosphorylation of AMP. Dipyridamole is an equilibrative nucleoside transport inhibitor that, in this concentration, prevents adenosine transport across the cell membrane.

At the start of each experiment 8 mL of venous blood was sampled and collected in cell preparation tubes (BD Vacutainer CPT), which were centrifuged at 1600g for 20 minutes to isolate mononuclear cells. After harvesting they were washed twice in PBS (5.39 mmol/L of  $\text{Na}_2\text{HPO}_4$ , 1.29 mmol/L of  $\text{KH}_2\text{PO}_4$ , and 0.15 mol/L of NaCl [pH 7.4]), followed by centrifugation for 15 minutes at 319g. The obtained pellets were resuspended in 1 mL of Hanks' balanced salt solution (Invitrogen).

Pilot experiments were performed to confirm optimal ( $V_{\text{max}}$ ) (maximum CD73 velocity) substrate concentrations (data not shown). For analysis, 30  $\mu\text{L}$  of homogenate were buffered in Hanks' balanced salt solution containing 10.0 mmol/L of  $\text{MgCl}_2$ , 10.8 mmol/L of  $\beta$ -glycerophosphate, 20  $\mu\text{mol}/\text{L}$  of dipyridamole, and 0.2 mmol/L of  $1,N^6$ -ethenoadenosine 5'-monophosphate with or without 400  $\mu\text{mol}/\text{L}$  of  $\alpha,\beta$ -methyleneadenosine 5'-diphosphate. All of the assays were performed for 5 minutes at  $37^\circ\text{C}$  in a total volume of 100  $\mu\text{L}$ . The reaction was terminated by adding 50  $\mu\text{L}$  of perchloric acid 70% and the obtained lysate centrifuged for 3 minutes. The supernatant was used for measurement of  $1,N^6$ -ethenoadenosine concentration by ion-paired reverse-phase high-performance liquid chromatography with fluorescence detection set at 280/420 nm. For separation, an altima HP C18 AQ column was used with a mobile phase containing 50 mmol/L of  $\text{NH}_4\text{H}_2\text{PO}_4$ , 5 mmol/L of hexane sulfonic acid (pH 3.0), and acetonitrile as the organic modifier.

### Other Analytic Procedures

In all of the volunteers, blood was collected before and at the end of oral treatment to determine fasting serum lipid profile with a commercially available kit (Aeroset, Abbott). Compliance to caffeine abstinence was monitored by determination of plasma caffeine concentration before each experiment. Plasma caffeine concentrations were determined by use of reversed-phase high-performance liquid chromatography with UV detection set at 273 nm according to Schreiber-Deturmeny and Bruguerolle.<sup>22</sup>

### Solutions

The caffeine (Genfarma) solution was freshly prepared to reach a final syringe concentration of 90  $\mu\text{g}$  per 50  $\mu\text{L}$ . Rosuvastatin tablets contained 20 mg per tablet and were obtained from Astra Zeneca SA.

**Table. Effect of Treatment With Rosuvastatin on Lipid Profile**

Biochemistry	Day 1	Day 8
Fasting total cholesterol, mmol/L	4.2±0.1	3.3±0.1*
Fasting low-density lipoprotein cholesterol, mmol/L	2.3±0.1	1.5±0.1*
Fasting triglycerides, mmol/L	1.0±0.1	0.8±0.1
Fasting high-density lipoprotein cholesterol, mmol/L	1.4±0.1	1.4±0.1

Data are expressed as mean±SE.

\* $P<0.05$  day 1 vs day 8, paired  $t$  test ( $n=8$ ).

### Statistical Analysis

All of the CD73 activity measurements were performed in duplicate, averaged for each subject and visit, and differences between the 2 visits were analyzed by paired  $t$  tests. To correct for random changes in FBF unrelated to the intervention, the ratio of simultaneously measured FBF in intervention and control arms was calculated (FBF ratio). The FBF ratios of the last 4 minutes of reference measurements (during intra-arterial saline or caffeine, as appropriate) and first 3 minutes of each reperfusion period were averaged to 1 value. To reduce the influence of intra-individual changes between visits and interindividual variations in the baseline FBF ratio, results are expressed as the percentage increase in FBF ratio from baseline measurements. An ANOVA for repeated measures was performed on FBF ratio with rosuvastatin, caffeine, and arterial occlusion time as within-subject factors to determine the effect of caffeine on rosuvastatin-enhanced PORH. Results are expressed as mean±SE.

### Results

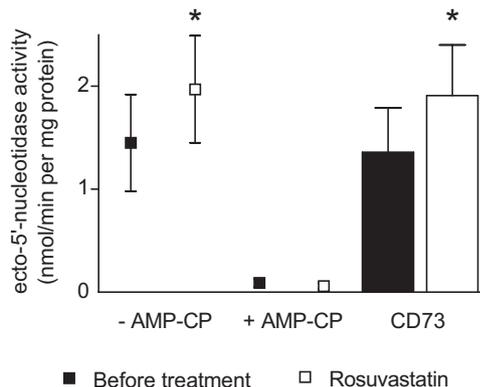
The experiments were successful in 8 volunteers. Baseline serum caffeine concentrations were  $<1.5$  mg/L during both experimental days, indicating adequate compliance to the caffeine-free diet. Treatment with rosuvastatin significantly reduced fasting total cholesterol and low-density lipoprotein cholesterol (Table).

### Ecto-5'-Nucleotidase Activity

Rosuvastatin significantly increased CD73 activity with  $49\pm17\%$  compared with baseline (Figure 2).

### Forearm Circulation

The absolute changes in FBF are presented in Figure 1. FBF ratio returned to baseline before the start of the second set of



**Figure 2.** Ecto-5'-nucleotidase (CD73) activity of intact mononuclear cells. Experiments were performed in the presence and absence of the specific CD73 inhibitor  $\alpha,\beta$ -methyleneadenosine 5'-diphosphate (AMP-CP). The difference between these two measurements reflects CD73 activity (bars). \* $P<0.05$ , data are mean±SE ( $n=8$ ).

ischemic challenges on each study day. Rosuvastatin treatment did not influence baseline FBF ratios nor the course of FBF in the control arm between study days. Rosuvastatin treatment significantly increased the vasodilator response to reactive hyperemia (Figure 3). In the presence of caffeine, this augmentation of the vasodilator response was abolished. When analyzing both experimental days separately, caffeine significantly altered the vasodilator response to reactive hyperemia while on rosuvastatin ( $P=0.03$ ) but not during baseline conditions (before rosuvastatin treatment,  $P=0.2$ ).

### Discussion

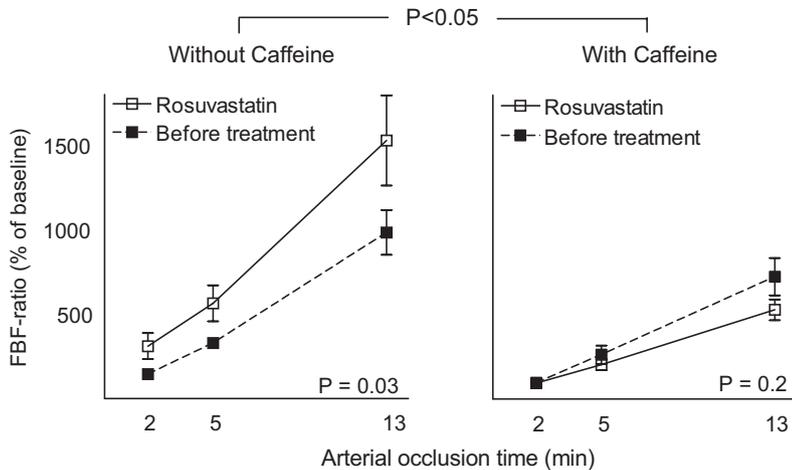
We recently demonstrated by using a pharmacological approach that rosuvastatin increases extracellular adenosine formation. We now demonstrate that this is at least partly mediated by increased CD73 activity. In addition, we underline the clinical relevance of the increased adenosine formation as it augments PORH after rosuvastatin treatment.

### Rosuvastatin Increases Ecto-5'-Nucleotidase

Previous studies already reported on the stimulatory effect of statins on CD73 activity. This was demonstrated for lovastatin in rat endothelial cells,<sup>10</sup> pitavastatin in canine in vivo,<sup>11</sup> and atorvastatin in human vascular endothelial cells.<sup>23</sup> Our results support these previous findings and are the first to demonstrate this effect in a human in vivo setting. The underlying mechanism still has to be unraveled. Inhibition of mevalonate is one of the potential mechanisms, because it not only reduces the synthesis of cholesterol but also isoprenoids, which are implicated in isoprenylation of small GTPases, including members of the Rho family. Inhibition of Rho may result in reduced endocytosis of CD73 and subsequently increased expression of CD73 on the cell membrane.<sup>10</sup> It has also been demonstrated that phosphatidylinositol 3-kinase and protein kinase C may influence CD73 activity by influencing its phosphorylation state and may thereby form an alternative route for statin-induced enhancement of CD73 activity.<sup>11,24</sup> Regardless of the underlying mechanism, activation of CD73 by statins is likely to contribute to the clinical efficacy of statins in a setting of cardiac infarction, because inhibition of CD73 attenuates the limiting effect of statins on infarct size in animals.<sup>11</sup> Furthermore, we have shown previously in a human forearm model of ischemia-reperfusion injury that endogenous adenosine is likely to contribute in rosuvastatin-induced protection against ischemia-reperfusion injury.<sup>5</sup>

### Rosuvastatin Enhances PORH in the Absence But Not in the Presence of Caffeine

The additional increase in PORH provided by rosuvastatin was abolished by the adenosine receptor antagonist caffeine indicating the involvement of enhanced adenosine receptor stimulation. Previous observations already showed that rosuvastatin did not affect the vasodilator response to adenosine, which excludes an effect of rosuvastatin on adenosine clearance, adenosine receptors, or postreceptor signaling.<sup>5</sup> Thus, the increased adenosine receptor stimulation observed during this study results from increased availability of extracellular adenosine. This conclusion is supported by data from the



**Figure 3.** Percentage increase in FBF ratio after arterial occlusion compared with baseline. *P* values indicate the level of significance for the effect of rosuvastatin on FBF ratio after arterial occlusion in the absence (left) and presence of caffeine (right) and the interaction between rosuvastatin and caffeine (left versus right, ANOVA for repeated measures). Data are mean  $\pm$  SE ( $n=8$ ).

current study indicating activation of CD73 on mononuclear cells as a result of treatment with rosuvastatin.

Potential limitations to our study design need to be addressed. First, because this study was neither randomized nor placebo controlled, a nonspecific time effect could have interfered. However, we have shown previously that repeated ischemia on a single day<sup>25</sup> or with a 1-week interval<sup>26</sup> results in similar reactive hyperemia. This observation makes it superfluous to explore potential carryover effects in this study. We opted for a limited study design to reduce the experimental load for our volunteers as much as possible. Second, we did not test the effect of caffeine on an adenosine-independent vasodilator, and, therefore, our findings may potentially be the result of adenosine-independent effects. A previous study from our institute demonstrated that intra-arterial caffeine (90  $\mu\text{g}/\text{min}$  per 100 mL of forearm volume) did not influence the vasodilator response to the endothelium-independent vasodilator sodium nitroprusside, which rules out a nonspecific effect of caffeine on PORH.<sup>27</sup>

### Implications of Our Observations

Atherosclerosis is an inflammatory disease of large- and medium-sized arteries and the most important precursor of cardiovascular disease. Studies in atherosclerosis-susceptible mice, including CD73-deficient mice, demonstrate the correlation between reduced CD73 activity and enhanced vascular inflammation and subsequent atherosclerosis formation.<sup>14,16</sup> Therefore, increased CD73 activity may aid in the prevention of atherosclerosis. In addition, inhibition of CD73 or targeted gene deletion of CD73 intervenes with the infarct size-limiting effect of ischemic preconditioning.<sup>15,28</sup> This effect can be restored by administration of soluble CD73. Adenosine forms the likely mediator of CD73-dependent effects, because its activity is functionally associated with increased adenosine receptor stimulation.<sup>29</sup> This is also in line with the observation that administration of an adenosine receptor agonist restored the proinflammatory state of CD73-deficient mice to baseline wild-type values.<sup>16</sup> However, because we did not measure other adenosine forming enzymes, for example, alkaline phosphatase, we cannot rule out a significant contribution of this enzyme in rosuvastatin-induced augmentation of adenosine formation.

Reactive hyperemia in the human forearm depends on postocclusive dilation of arteriolar resistance vessels and correlates well with the degree of vasodilator impairment in the coronary circulation.<sup>17</sup> Indeed, PORH correlates with the incidence of cardiovascular events in patients with essential hypertension and decreases with an increase in cardiovascular risk factors.<sup>18,30</sup> In addition, the extent of PORH after coronary angioplasty reflects the clinical effectiveness of revascularization.<sup>31</sup> These findings support the use of PORH as a marker for cardiovascular disease and effectiveness of treatment. The improvement of reactive hyperemia by rosuvastatin, therefore, at least in part, represents its therapeutic effect. This effect is attenuated by caffeine, which supports the involvement of adenosine in rosuvastatin-induced effects on cardiovascular disease.

Intake of 2 cups of coffee results in a plasma caffeine concentration of  $\approx 9$  mg/L.<sup>32</sup> In our current design we aimed at optimal adenosine receptor blockade, which is obtained with 90  $\mu\text{g}/\text{min}$  per 100 mL of forearm volume.<sup>27</sup> During intra-arterial infusion of caffeine, the plasma concentration in the venous effluent depends on FBF and decreases with higher flows. Unfortunately, we did not collect blood during caffeine infusion and are, therefore, not able to provide caffeine concentrations in the venous effluent in the present study. However, in a previous trial with comparable design,<sup>20</sup> we measured plasma caffeine concentrations after 2 and 13 minutes of arterial occlusion and  $\approx 6$  minutes of reperfusion which were, respectively,  $15 \pm 2$  and  $8 \pm 1$  mg/L (mean  $\pm$  SE, unpublished data). This indicates that local caffeine concentrations in the present study approach values that occur during regular caffeine consumption. We studied the effects of acute caffeine administration. Previous studies have indicated the development of (partial) tolerance to the effects of caffeine on blood pressure. However, in chronic caffeine consumers the blood pressure response to adenosine continuously increased during a 2-week period of caffeine abstinence, suggesting that the interaction between caffeine and adenosine may be less prone to tolerance development. Therefore, we believe that our observations strongly support the advice that patients who are treated with statins should abstain from caffeine consumption to fully utilize its clinical benefit. Additional evidence for this advice would need a large clinical trial in

patients treated with statins randomized to long-term regular caffeine consumption or caffeine abstinence with mortality and morbidity as clinical end points. Such a trial is very expensive and, in our opinion, hardly feasible.

## Perspectives

In the last 20 years statins have been shown to improve outcome observed during acute coronary syndromes,<sup>8,9</sup> cardiac bypass surgery,<sup>33,34</sup> and percutaneous coronary interventions.<sup>35</sup> The underlying mechanism is extensively studied but nonetheless not fully understood, which may hamper the full potential of this class of drugs. Our results demonstrate for the first time the effect of rosuvastatin on CD73 in a human in vivo setting and confirm the clinical relevance of increased adenosine receptor stimulation in the beneficial effects exerted by statins. These findings are of particular interest, because pharmacological modulation, both beneficial and detrimental, of the transport and actions of adenosine is already in widespread use, for example, dipyridamole and caffeine. Indeed the combination of a statin with an inhibitor of adenosine uptake into the cell, dipyridamole and cilostazol, and subsequent increased extracellular adenosine concentrations potentiate the infarct size-limiting effect of statins in animals.<sup>36,37</sup> Our observations support caffeine abstinence in patients treated with a statin to fully utilize its clinical benefit.

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## Disclosures

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