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Clinical Research

# Effects of Statins on Endothelial Progenitor Cell Proliferation from Peripheral Blood of Stable Coronary Artery Disease Patient

Feranti Meuthia, Yudi Her Oktaviono, Djoko Soemantri

**Background:** Atherosclerotic lesions develop as the result of an inflammatory process initiated by endothelial damage. Endothelial progrenitor cells (EPCs), which is derived from bone marrow, participate in endothelial repair and new vessel growth. Cardiovascular pharmacotherapies have been shown to improve overall numbers and function of EPCs in patients with cardiovascular risk and cardiovascular disease. Studies have reported that statins exert beneficial effects on EPCs by enhancing EPC proliferation and differentiation. Thus, we require a research to analyze the effects of three different statins on EPC proliferation. The objective of this study is to analyze the effect of statins on EPC proliferation from peripheral blood of stable coronary artery disease patient.
**Methods:** This is an *in vitro* true experimental post-test only control group design. The mononuclear cells (MNCs) were isolated from peripheral blood of stable coronary artery disease patient and were cultured in CFU-Hill medium for three days. Then samples were put into four groups, simvastatin experiment group, atorvastatin experiment group, rosuvastatin experiment group and control group. Each experiment group was divided into three subgroups with different doses, 0.1 $\mu$mol/L, 0.5 $\mu$mol/L, and 2.5 $\mu$mol/L then incubated for 48 hours. EPC proliferation was evaluated afterwards with MTT cell proliferation assay. Immunocytochemistry method was performed for EPC identification to evaluate expression of $CD34^+$. CFU-Hills were observed to confirm functional characteristics of EPC. Data were analyzed by independent T-test and ANOVA.
**Results:** MTT cell proliferation assay showed a significant increase of EPC proliferation in simvastatin, atorvastatin, and rosuvastatin groups compared with control group ($0.237\pm0.007$, $0.248\pm0.01$, $0.231\pm0.008$ vs $0.17\pm0.008$, $p<0.05$). It also revealed significant difference in EPC proliferation between each experiment groups, which atorvastatin showed the highest effect. EPC proliferation in atorvastatin is higher than simvastatin group ($0.248\pm0.01$ vs. $0.237\pm0.007$, $p<0.05$), and simvastatin is also higher than rosuvastatin group ($0.237\pm0.007$ vs. $0.231\pm0.008$, $p<0.05$). CFU-Hill counts demonstrated highest number in rosuvastatin group, followed by atorvastatin, and simvastatin. Immunocytochemistry showed positive expression of CD34.
**Conclusion:** Statins increase EPC proliferation from peripheral blood of stable coronary artery disease patient. Atorvastatin showed the highest EPC proliferation, followed by simvastatin, and rosuvastatin. Each statins increased EPC proliferation dose-dependently.

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**Keywords:** EPC proliferation, simvastatin, atorvastatin, rosuvastatin

Penelitian Klinis

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# Efek Pemberian Statin terhadap Proliferasi *Endothelial Progenitor Cell* pada Darah Tepi Penderita Penyakit Jantung Koroner Stabil

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**Latar Belakang:** Lesi atherosklerotik merupakan akibat dari proses inflamasi yang diawali oleh kerusakan endotel. *Endothelial progenitor cell* (EPC), yang berasal dari sumsum tulang, berpartisipasi dalam perbaikan endotel dan pertumbuhan pembuluh darah baru. Farmakoterapi kardiovaskular telah dibuktikan dapat memperbaiki jumlah dan fungsi EPC pada penderita dengan risiko kardiovaskular dan penyakit kardiovaskular. Banyak studi melaporkan bahwa statin memiliki efek yang menguntungkan terhadap EPC yaitu dengan meningkatkan proliferasi dan diferensiasi. Oleh karena itu, kami melakukan penelitian untuk menganalisis efek tiga statin yang berbeda terhadap proliferasi EPC. Tujuan penelitian ini adalah untuk menganalisis efek pemberian statin terhadap proliferasi EPC pada darah tepi penderita penyakit jantung koroner stabil.
**Metode:** Penelitian ini kami lakukan secara *in vitro true experimental post-test only control group design*. Sel mononuklear diisolasi dari darah tepi penderita penyakit jantung koroner stabil dan kultur dilakukan dalam medium CFU-Hill selama tiga hari. Sampel kemudian dibagi menjadi empat kelompok, yaitu kelompok simvastatin, atorvastatin, rosuvastatin dan kelompok kontrol. Tiap kelompok yang diberi perlakuan tersebut dibagi lagi menjadi tiga subkelompok dengan dosis yang berbeda, yaitu 0,1 µmol/L, 0,5 µmol/L, dan 2,5 µmol/L kemudian diinkubasi selama 48 jam. Proliferasi EPC dievaluasi setelahnya dengan *MTT cell proliferation assay*. Metode imunositokimia dilakukan untuk identifikasi EPC dengan mengevaluasi ekspresi $CD34^+$. Pemeriksaan dan penghitungan CFU-Hill dilakukan untuk mengonfirmasi karakteristik fungsional EPC. Data dianalisis dengan uji T independen dan ANOVA.
**Hasil:** *MTT cell proliferation assay* menunjukkan peningkatan bermakna terhadap proliferasi EPC pada kelompok simvastatin, atorvastatin, dan rosuvastatin dibandingkan dengn kelompok kontrol (0,237±0,007, 0,248±0,01, 0,231±0,008 vs. 0,17±0,008, p<0,05). Proliferasi EPC juga berbeda antar-kelompok statin, dengan efek tertinggi didapatkan pada kelompok atorvastatin. Proliferasi EPC pada kelompok atorvastatin lebih tinggi daripada kelompok simvastatin (0,248±0,01 vs. 0,237±0,007, p<0,05), dan simvastatin lebih tinggi daripada kelompok rosuvastatin (0,237±0,007 vs. 0,231±0,008, p<0,05). Penghitungan CFU-Hill memperlihatkan jumlah tertinggi pada kelompok rosuvastatin, diikuti atorvastatin, dan simvastatin. Pemeriksaan imunositokimia menunjukkan ekspresi positif terhadap CD34.
**Kesimpulan:** Statin meningkatkan proliferasi EPC pada darah tepi penderita penyakit jantung koroner stabil. Efek tertinggi tampak pada kelompok atorvastatin, diikuti kelompok simvastatin, dan rosuvastatin. Tiap statin meningkatkan proliferasi dengan bergantung dosis (*dose-dependent).*

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**Kata kunci:** proliferasi EPC, simvastatin, atorvastatin, rosuvastatin

## Introduction

Cardiovascular disease (CVD) remains the biggest cause of deaths worldwide. It is responsible for over 17 million deaths per year.[1] The underlying disease process in

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the blood vessels that results in coronary heart disease is known as atherosclerosis.[2] Atherosclerotic lesions are initiated by endothelial damage which develop as the result of an inflammatory process over many years then become coronary artery disease.[3] Stable coronary artery disease is generally characterized by a disease causing exercise- and stress-related chest symptoms due to narrowing of ≥50% in the left main coronary artery and ≥70% in one or several of the major coronary arteries.[4] Endothelium has the ability to repair itself. It is not only driven by local cells, but also with the contribution of circulating cells, which have been termed endothelial progenitor cells (EPCs). EPCs derive from bone marrow and can be mobilized to the peripheral circulation upon a variety of stimuli including tissue ischemia through the release of growth factors.[3] Experimental and clinical studies have shown that atherosclerosis is associated with reduced numbers and dysfunction of EPCs, and there are various ways to increase circulating EPCs and improve their function.[5] Cardiovascular pharmacotherapies have been shown to improve overall numbers and function of EPCs, such as statins, angiotensin converting enzyme (ACE) inhibitors, calcium channel blockers (CCBs).

Hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, have already led to important improvements in primary and secondary prevention of coronary artery disease (CAD).[6] Besides lipid lowering, statins can reduce vascular inflammation, decrease platelet aggregability and thrombus deposition, and increase endothelium-derived nitric oxide production.[7,8] Moreover, different doses of different statins have been reported to be useful in increasing EPC numbers and function.[9] Here, we need a research to analyze the effects of three different statins on EPC proliferation.

## Methods

Blood samples were taken from patients with stable coronary artery disease which were men, 40-59 years old, had narrowing of ≥50% in the left main coronary artery and ≥70% in one or several of the major coronary arteries from angiography.

Patients with history of acute myocardial infarction, acute limb ischemia, stent placement, coronary artery bypass grafting, diabetes mellitus, smoking, and anemia were excluded.

Written informed consent was obtained from patients before peripheral blood sampling. The protocol was approved by local ethics committee (320/Panke. KKE/IV/2016).

### *Isolation and culture of EPCs*

Forty ml of blood was diluted with phosphate buffered saline (PBS) containing 2% of fetal bovine serum (FBS). It was then centrifuged on Ficoll-Histopaque density gradients and interface mononuclear cells (MNCs) were collected. After two washes in PBS containing 2% of FBS, the pellet were diluted with CFU-Hill Liquid medium kit, and these cells were counted using hemocytometry. Total mononuclear cells (cell density $5x10^6$ cells/ml) were plated on fibronectin coated well plates. After two days, nonadherent cells were transferred into new fibronectin coated well plates for colony counts and MTT cell proliferation assay.

### *Statin*

After 24 hours of another incubation, three doses of statins, which were 0.1 µmol/L, 0.5 µmol/L, dan 2.5 µmol/L of simvastatin (Abcam®), atorvastatin (Tocris®), and rosuvastatin (Tocris®) were added to three different plates. Colony counts and MTT cell proliferation assay were determined after 48 hours of incubation.

### *EPC proliferation assay*

Effects of statins on EPC proliferation were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each well was supplemented with MTT and incubated for a further span of 4 hours. Optical density (OD) was measured at 595 nm.

### *Colony counting*

EPC colony was combination of 15 or more EPC, which can be round, spindle-shape, or cobblestone-like shape. All colonies from each fibronectin coated well plate were analysed with an inverted light microscope.

### *Immunofluorescence assay*

Cells were washed with PBS and fixed with 3% formaldehyde for 15 minutes. Cells then added with

1% serum and washed again with PBS. Cells were stained with a FITC anti-CD34 antibody. Expression of the cells was documented with fluorescence microscope.

### *Statistical analysis*

Data are expressed as mean±SD. To analyse differences, one-way ANOVA was used. Differences were considered significant if p-values were <0.05. All statistical analyses were performed with SPSS for Windows (IBM Corp., Armonk, NY).

## Results

### *Statin increases EPC proliferation*

To investigate the effect of statins on EPC proliferation, we added three different types of statins on EPC cultures. MTT cell proliferation assay of the lowest dose of each statins, which was 0.1 µmol/L, showed a significant increase of EPC proliferation in simvastatin, atorvastatin, and rosuvastatin groups compared with control group (0.237±0.007, 0.248±0.01, 0.231±0.008 vs. 0.17±0.008, p<0.05). It also revealed significant difference in EPC proliferation between each experiment groups, which atorvastatin showed the highest effect. EPC proliferation in atorvastatin was higher than simvastatin group (0.248±0.01 vs. 0.237±0.007, p<0.05), and simvastatin was also higher than rosuvastatin group (0.237±0.007 vs. 0.231±0.008, p<0.05) (**Figure 1**).

EPC proliferation induced by statins was dose dependent. Three different doses of simvastatin, atorvastatin and rosuvastatin, which were 0.1 µmol/L, 0.5 µmol/L, 2.5 µmol/L, respectively, increase EPC proliferation according to the dosage. 2.5 µmol/L rosuvastatin expressed the highest EPC proliferation (0.243±0.005 vs. 0.232±0.003, p<0.05). 0.5 µmol/L rosuvastatin showed a non-significant increase of EPC proliferation than 0.1 µmol/L rosuvastatin (0.232±0.003 vs. 0.231±0.008). 2.5 µmol/L simvastatin showed significantly higher EPC proliferation than 0.5 µmol/L simvastatin (0.26±0.006 vs. 0.244±0.003, p<0.05), and 0.1 µmol/L simvastatin also demonstrated lower effect than 0.5 µmol/L simvastatin (0.237±0.007 vs. 0.244±0.003, p<0.05). Atorvastatin group also showed similar results. 2.5 µmol/L atorvastatin significantly increased EPC proliferation than 0.5 µmol/L atorvastatin (0.2740±0.005 vs. 0.263±0.004, p<0.05) and 0.1 µmol/L atorvastatin had the lowest effect (0.248±0.01 vs. 0.263±0.004, p<0.05) (**Figure 2**).

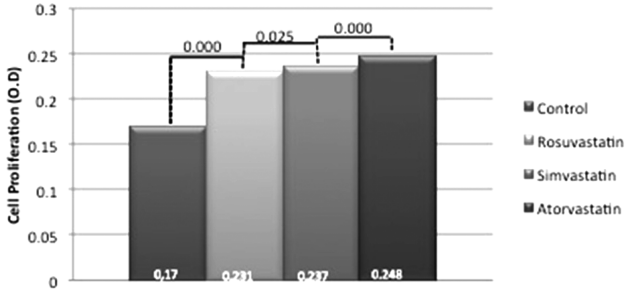


**Figure 1.** Statins increase EPC proliferation. EPC proliferation were expressed as optical density (O.D).

### *CFU counts*

The ability of EPCs to migrate to each other to form a colony represents their functional characteristics. We observed the EPC on the sixth day of culture to ensure they could form colonies (**Table 1** and **Figure 3**).

### *Immunofluorescence assay*

CD34 is one of the positive marker for EPC. After CFU counts, one of the well was washed with PBS and stained with a FITC anti-CD34 antibody. Expression of CD34 was green under fluorescence microscope (**Figure 4**).

## Discussions

The results of the present study demonstrate that statin significantly increases EPC proliferation in patients with stable CAD. There were different effects in EPC proliferation induced by each statins. Atorvastatin had the highest effect of EPC proliferation, and simvastatin could proliferate more EPC than rosuvastatin.

Many studies have reported the beneficial effects of statin on EPCs. Similar to this result, Llevadot, et al. reported that simvastatin increased EPC proliferative activity using EPC culture assay of peripheral blood from simvastatin-treated animals in vivo.[10] Vasa, et al. demonstrated that atorvastatin 40 mg treatment of patients with stable coronary artery disease was

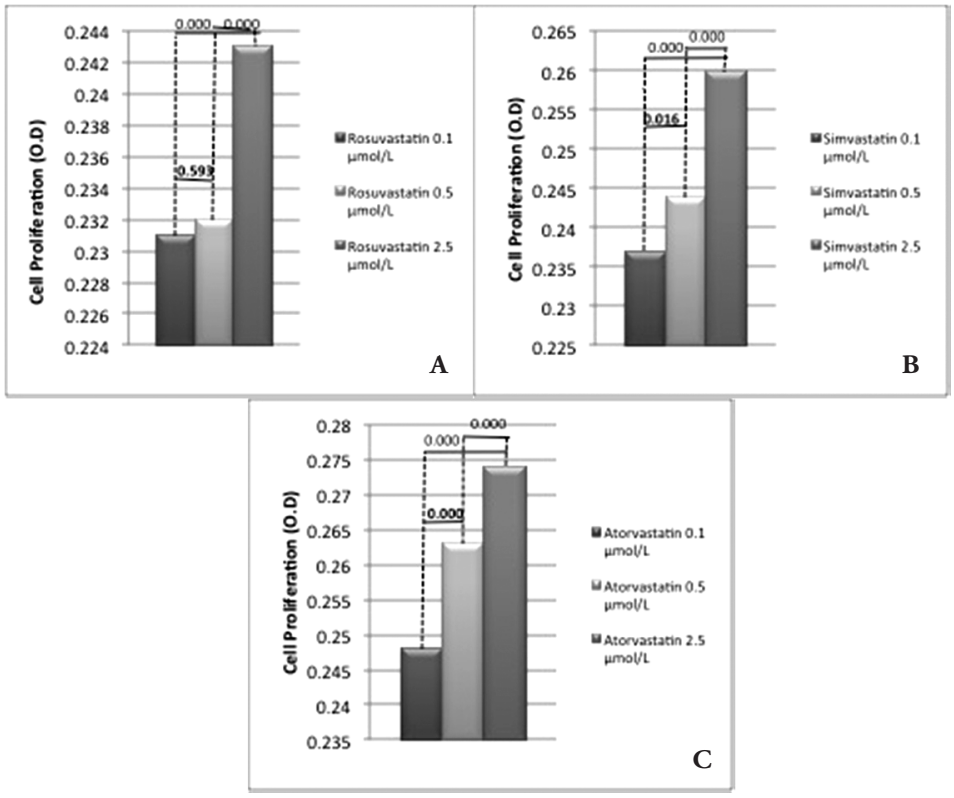


**Figure 2.** EPC proliferation in experiment groups. (A) Rosuvastatin group (B) Atorvastatin group (C) Atorvastatin group.

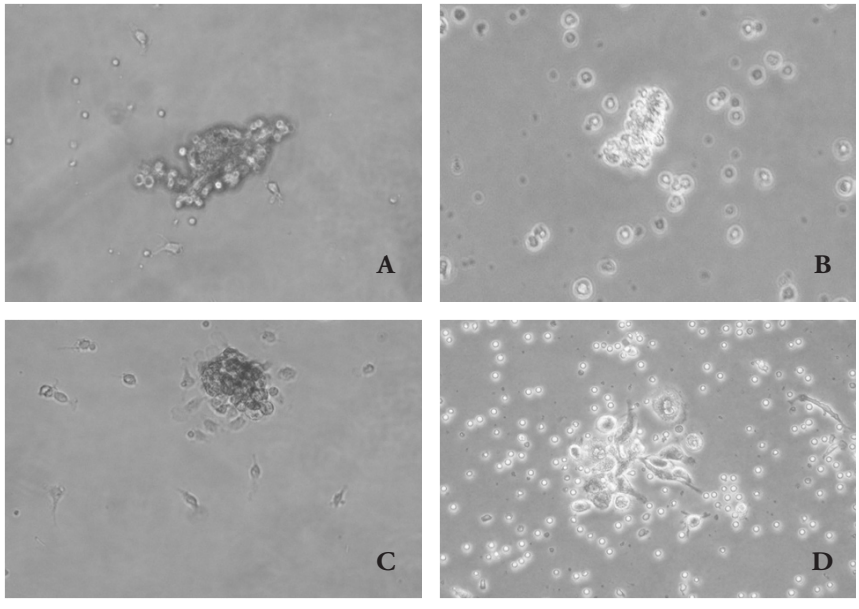


**Figure 3.** CFU in control and experiments group. (A) Control group (B) Simvastatin group (C) Atorvastatin group (D) Rosuvastatin group.

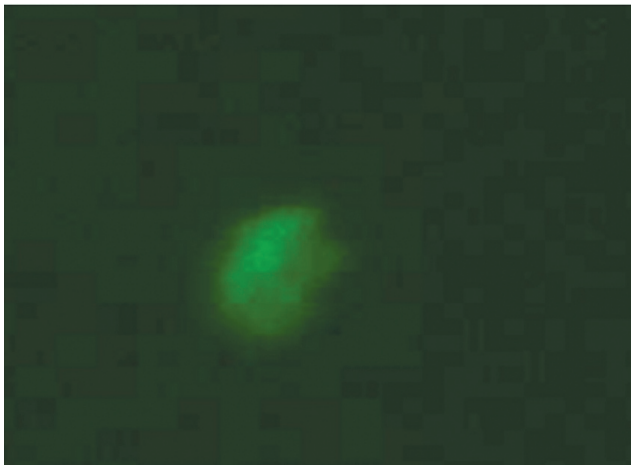

**Figure 4.** Immunofluorescence expression of CD34.

**Table 1.** CFU counts in control and experiments group.

| Group | CFU counts 0.1 µmol/L | CFU counts 2.5 µmol/L |
|---|---|---|
| Control | 34 | |
| Simvastatin | 76 | 172 |
| Atorvastatin | 142 | 175 |
| Rosuvastatin | 109 | 210 |

associated with an 1.5-fold increase in the number of circulating EPCs by one week treatment and 3-fold throughout the 4-week period.[11] Another studies also used several types of statins but they did not directly compare the results. Spiel, et al. explored the effect of statin using simvastatin and rosuvastatin. The results showed that five days pre-treatment with 80 mg of simvastatin increased EPCs 2.1-fold, whereas pre-treatment with 40 mg of rosuvastatin led to a 1.9 fold increase as compared to baseline values.[12] Dimmeler, et al. also used three different types of statins, which were simvastatin, mevastatin, and atorvastatin. The results demonstrated that incubation of isolated human MNCs with statin increased the number of EPCs. Atorvastatin augmented the number of EPCs more than simvastatin, and mevastatin had higher number of EPCs than atorvastatin.[13]

Dimmeler, et al. demonstrated that statins enhanced the mobilization of EPCs from bone marrow to newly forming blood vessels through the PI3K/Akt pathway, which lead to an increase in endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) production, as NO is essential for EPC proliferation.[13,14] The mechanism underlying different effect of statins on EPC proliferation still needs to be determined. Although all statins share a common mechanism of action, they differ in terms of their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacy. Simvastatin are derived from fungal metabolites, but atorvastatin and rosuvastatin are fully synthetic compounds. Atorvastatin and simvastatin are relatively lipophilic compounds, while rosuvastatin are relatively hydrophilic.[15] These differences might influence the effect on EPC proliferation induced by different types of statin.

The result of this study also showed that simvastatin, atorvastatin, and rosuvastatin dose dependently increased EPC proliferation. Likewise, Dimmeler, et al. demonstrated that simvastatin, mevastatin, and atorvastatin dose dependently increased the number of differentiated EPCs.[13]

CFU counts represent the cumulative characteristics of EPC quantity and their functional characteristics, and cannot be reliably used for the estimation of EPC numbers in peripheral blood.[16] We only examined seven wells, which is control group, the low and high dose of each simvastatin, atorvastatin, and rosuvastatin. The results demonstrated that atorvastatin had the most CFU counts, while rosuvastatin had the most CFU counts in high dose group. Therefore, rosuvastatin might have another functional capacity better than atorvastatin and simvastatin. The amount of CFU also showed that it depends on the dose of statins.

EPCs appear to be a heterogenous group of cells originating from multiple precursors within the bone marrow and present in different stages of endothelial differentiation in peripheral blood. For this reason, the precise characterization of EPCs is difficult because many of the cell surface markers used in phenotyping are shared by hematopoietic stem cells and by adult endothelial cells. Currently, EPCs are defined as cells positive for both hematopoietic stem cell marker such as CD34 and an endothelial marker protein such as VEGFR2.[9] This study used CFU-Hill Liquid medium kit which has been developed specifically to support the culture from mononuclear cells of human peripheral blood. To ensure the colonies cultured were EPC, we used one of the EPC markers, which was CD34 and the result came out positive for CD34.

## Conclusion

The results of the present study define that statin increases EPC proliferation from peripheral blood of

stable coronary artery disease patient. Our data suggests that atorvastatin has the highest EPC proliferation, followed by simvastatin, and rosuvastatin. Each statin increases EPC proliferation dose-dependently.

## Acknowledgments

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## Abbreviations

ACE: angiotensin converting enzyme
CAD: coronary artery disease
CCB: calcium channel blocker
CFU: colony forming unit
CVD: cardiovascular disease
eNOS: endothelial nitric oxide synthase
EPC: endothelial progenitor cell
FBS: phosphate buffered saline
HMG-CoA: Hydroxymethyl glutaryl coenzyme A
MNC: mononuclear cell
NO: nitric oxide
PBS: phosphate buffered saline

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### Ethical Clearance

No. 320/Panke.KKE/IV/2016 from Research Ethics Committee, Dr. Soetomo Hospital, Surabaya.

### Publication Agreement

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### Conflict of Interest

The authors indicate no conflicts of interest.

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