Disturbed Blood Flow Acutely Induces Activation and Apoptosis of the Human Vascular Endothelium

Nathan T. Jenkins, Jaume Padilla, Lerny J. Boyle, Daniel P. Credeur, M. Harold Laughlin, Paul J. Fadel

Abstract—There is strong and consistent evidence from in vitro studies that disturbed blood flow produces a proatherogenic vascular endothelial phenotype. However, data from human studies are lacking. To address this, a 220 mm Hg occlusion cuff was placed on the distal forearm of 10 young, healthy men to induce a localized region of disturbed blood flow in the proximal vasculature for 20 minutes. We hypothesized that disturbed blood flow would induce endothelial activation and apoptosis as indicated by increases in local concentrations of CD62E+ and CD31+/CD42b− endothelial microparticles, respectively. Distal cuff occlusion induced reductions in mean blood flow, mean shear, and antegrade shear, and increases in retrograde flow, retrograde shear, and oscillatory shear stress, confirming that our protocol produced a disturbed blood flow stimulus in the experimental arm. Relative to baseline (0 minutes), CD62E+ endothelial microparticles increased by ≈3-fold at 10 minutes and ≈4-fold at 20 minutes in the experimental arm (P<0.05). CD31+/CD42b− endothelial microparticles were elevated by ≈9-fold at the 20 minutes time point (P<0.05). There were no changes in the concentrations of either endothelial microparticle population throughout the experiment in the contralateral arm, exposed to normal resting blood flow (no cuffs). These findings indicate that disturbed blood flow acutely induces endothelial activation and apoptosis in humans, as reflected by release of microparticles from activated (CD62E+) and apoptotic (CD31+/CD42b−) endothelial cells. These data provide the first in vivo experimental evidence of disturbed blood flow-induced endothelial injury in humans. (Hypertension. 2013;61:615-621.)

Key Words: atherosclerosis ■ microparticles ■ shear stress

It is well established that geometrically irregular arterial regions, such as curvatures, branches, and bifurcations, are characterized by overall low shear stress combined with high retrograde flow and oscillatory shear stress.1 Collectively, such flow patterns represent a state of disturbed blood flow. Importantly, these arterial regions also display marked susceptibility to the development of atherosclerosis compared with segments exposed to laminar flow and moderate-to-high shear.2 Insights into the mechanisms underlying this association between disturbed blood flow and disease susceptibility have come primarily from in vitro studies. For example, early studies with cultured endothelial cells indicated that disturbed flow induces endothelial activation and cell turnover,4 whereas laminar shear stress mitigates proatherogenic processes, such as leukocyte adhesion.5 Similarly, recent studies indicated that proatherogenic genes are upregulated in cultured endothelial cells exposed to shear patterns of in vivo atheroprone regions relative to cells exposed to patterns observed in protected regions.6–8 These cell culture data are consistent with data indicating that oscillatory shear stress induces pathological remodeling and impairments in endothelium-dependent vasodilation in isolated arteries.9 Thus, the evidence from in vitro preparations that disturbed flow patterns produce an unfavorable vascular endothelial cell phenotype is strong and consistent.

Although the data are still limited, recently the association between disturbed blood flow and vascular dysfunction has begun to receive support from experimental in vivo studies. For example, in mice, induction of disturbed blood flow via partial carotid ligation produces rapid and profound endothelial dysfunction and atherosclerosis.10 In humans, our group and others have recently shown that there is evidence of increased proatherogenic blood flow and shear patterns in peripheral conduit arteries of subjects at increased risk for cardiovascular diseases and atherosclerosis.11–14 Surprisingly, only one study has tested whether disturbed blood flow can acutely influence the human vasculature in vivo. In an elegant experiment, Thijssen et al15 used 25, 50, and 75 mm Hg distal forearm cuff occlusion pressures to elicit progressive increases in brachial artery retrograde flow, and shear and concomitant reductions in mean flow and shear. This maneuver induced a dose-dependent reduction in brachial artery flow-mediated dilation (FMD), providing the first indication that disturbances in blood flow result in functional impairment of
the human endothelium in vivo. However, although FMD is a well-established index of vascular function, it does not allow for insights into the cellular/molecular underpinnings of the functional impairment.

Microparticles are small (≤1 µm diameter) vesicles formed from plasma membranes and released into the circulation on cellular activation, apoptosis, or injury of many cell types. Elevated circulating levels of endothelial microparticles (EMPs), first detected in the in vivo human circulation in 1999, are now well established as systemic markers of dysfunctional and diseased vascular endothelium. For example, EMPs are elevated in patients with atherosclerosis, coronary artery disease, hypertension, cerebrovascular disease, and the metabolic syndrome, among other cardiovascular and metabolic abnormalities. Interestingly, surface protein marker expression analysis of EMPs can reveal insights into the insult that triggered their release from the vascular wall. Constitutive markers such as CD31 are expressed on EMPs shed from apoptotic endothelial cells, whereas expression of inducible markers such as CD62E (E-selectin) indicates endothelial activation induced by a proinflammatory event. Thus, the examination of multiple EMP populations may provide novel information about the in vivo nature of disturbed flow-induced endothelial injury.

Therefore, the purpose of this study was to determine the acute effect of disturbed blood flow on the release of EMPs from the human vascular endothelium. We adapted the distal forearm cuff occlusion model introduced by Thijssen et al to create a localized region of disturbed blood flow, with the contralateral arm serving as a control. We hypothesized that disturbed blood flow would induce endothelial activation and apoptosis, as indicated by increases in local concentrations of CD62E+ and CD31+/CD42b− EMPs, respectively.

**Methods**

**Subjects**
Ten healthy men with a mean age of 29±1 year, height of 176±1, and weight of 83±2 kg (means±SEM) participated. All subjects were free of known cardiovascular, metabolic, and neurological diseases, and were nonsmokers, normotensive (resting blood pressure: 115±64), and were nonobese (body mass index: 26.7±0.5 kg/m²). The investigators provided verbal and written explanations of the study procedures, and the risks and benefits associated with participation, and subjects provided their written informed consent. All procedures adhered to the principles of the Declaration of Helsinki, and the study was approved by the University of Missouri-Columbia Health Sciences Institutional Review Board.

**Experimental Design and Protocols**
Subjects reported to the laboratory after an overnight fast, and abstained from caffeine and strenuous exercise for a minimum of 12 hours. Indwelling venous catheters were placed in a superficial antecubital vein of both arms, followed by 20 minutes of quiet rest period in the supine position in a dimly lit, temperature-controlled (20–22°C) room. The experimental setup and design are depicted in Figure 1A and Figure 1B, respectively. Two pneumatic cuffs (Hokanson) were placed on the experimental arm for 20 minutes: (a) a distal cuff, inflated to 220 mm Hg to produce a localized environment of disturbed blood flow (low mean flow and shear, low antegrade flow and shear, high retrograde flow and shear, and high oscillatory shear stress) in the proximal vasculature and (b) a proximal cuff positioned ≈3 cm distal from the axilla, inflated to 40 mm Hg to partially

![Figure 1. Experimental setup (A) and design (B). Small arrows in B indicate blood draws. Refer to the Methods section for details.](http://hyper.ahajournals.org/)}
occlude venous flow from the arm, thereby facilitating the trapping of EMPs that would be released during the experiment (Figure 1). The distal occlusion pressure of 220 mm Hg was selected to maximize a disturbed blood flow state and also to occlude backflow from the ischemic downstream region, which could contribute to increases in EMPs being measured upstream. The absence of contamination of blood from the distal ischemic region was assessed by measuring plasma lactate concentrations (YSI 2700, Yellow Springs, OH) in 0 and 20 minutes samples from the experimental arm (mean±SEM: 0.63±0.05 versus 0.61±0.04 mmol/L; P=0.69). Two separate control experiments were carried out in the contralateral arm. First, simultaneous blood flow (n=3) and EMP (n=7) measures were performed in the absence of any cuffs (see Figure 1A). Second, blood flow and EMP (n=3) measures were obtained with the application of a proximal 40 mm Hg occlusion cuff.

Brachial Artery Blood Flow Measures
Brachial artery blood velocities and diameters were measured using duplex Doppler ultrasound, as described in detail previously. Briefly, images of the brachial artery diameter and blood velocity profiles were acquired with a 5 cm proximal to the antecubital fossa in the anterior–medial plane at 30 Hz using a custom Labview program interfaced to the video output of the Doppler ultrasound. Offline analyses of brachial artery diameters and Doppler blood velocity profiles were performed using custom-designed edge-detection and wall-tracking software (Labview, National Instruments). Blood flow was calculated as $V_r = D/4 	imes 60$, where $V_r$ is time-average mean blood velocity (cm/s), $D$ is arterial diameter (cm), and mean shear rate ($\gamma$) was defined as $4V_r/D$. Antegrade and retrograde time-averaged blood velocities were used to calculate their respective shear rates. The oscillatory shear index, an indicator of the magnitude of shear oscillations, was calculated as follows: [retrograde shear/antegrade shear]+[retrograde shear]. The oscillatory shear index values range from 0 to 0.5, where a value of 0 corresponds to pure unidirectional shear and 0.5 indicates pure oscillation with a time-average shear of 0.

Plasma EMP Measures
Blood samples were obtained from both the experimental and control arm at 0, 10, and 20 minutes into 8 mL vials containing acid citrate dextrose. Microparticles were isolated from plasma samples (500 μL aliquots) were thawed at room temperature and centrifuged at 1500g for 20 minutes at room temperature to obtain platelet poor plasma. The top two thirds (ie, 335 μL) of platelet-poor plasma was diluted with 500 μL of sterile PBS before flow cytometric and were diluted with 500 μL of sterile PBS before flow cytometric analysis.

Samples were analyzed using a Beckman Coulter CyAn ADP flow cytometer in the University of Missouri Cell and Immunobiology Core Facility. EMPs were defined as CD31+/CD42b- or CD62E- events smaller than 1.0 μm. Size calibration was performed with 0.9 μm standard precision NIST Traceable polystyrene particle beads (Polyscience, Inc). Fluorescence minus one controls and unstained samples were used to discriminate true events from noise. The flow rate was set on medium and all samples were run for 3 minutes. Using calibrator beads (BD Truecount), we calculated that, on medium flow rate, the average rate of microparticle event analysis was 140 μL/min. EMP counts per microliter plasma were determined using the formula: 

\[
(\text{number of EMP events/sample size}) \times (\text{final sample volume}) / (\text{sample volume})
\]

Statistics
Data were analyzed using a 2-factor (arm×time) repeated measures ANOVA with SPSS software (version 18). In the event of significant interaction, Fisher’s least significant differences post hoc analysis was used to examine differences within arms (ie, baseline versus 10 minutes and baseline versus 20 minutes) and between arms at 10 and 20 minutes. Data are presented as mean±SE. P≤0.05 was considered statistically significant.

Results
CD62E- EMPs increased substantially at 10 and 20 minutes compared with baseline in the experimental arm (P<0.05; Figure 2A), indicating endothelial activation. Likewise, CD31+/CD42b- EMP concentrations were elevated at the 20 minutes time point (P<0.05; Figure 2B), suggestive of endothelial apoptosis. There were no changes in the concentrations of either EMP population over the same time period in the contralateral arm, when no cuffs were applied (n=7; Figure 2A and 2B). Additionally, in 3 subjects, EMPs were analyzed in samples obtained with a proximal 40 mm Hg venous occlusion cuff inflated on the contralateral arm. However, samples from one of these subjects appeared hemolyzed and were therefore not used for EMP analysis. The proximal 40 mm Hg cuff did not produce major changes in CD62E- or CD31+/CD42b- EMP concentrations in either subject from which acceptable samples were obtained (mean±SE: 1.7±0.1, 1.7±0.2, and 1.9±0.1 CD62E- EMPs/μL plasma at 0, 10, and 20 minutes, respectively; 0.9±0.2, 1.0±0.1, and 1.6±0.2 CD31+/CD42b- EMPs/μL plasma at 0, 10, and 20 minutes, respectively). These data suggest that the increases in EMPs observed in the experimental arm were driven by the disturbed blood flow patterns induced by the distal 220 mm Hg cuff.

As expected, the distal forearm cuff reduced brachial artery blood flow, mean shear, and antegrade shear, and increased retrograde shear and the oscillatory shear index (all P<0.05; Figure 3). These results confirm that the cuff successfully induced a localized disturbance in blood flow in the experimental arm. Representative Doppler velocity profiles are provided in Figure 3D. Brachial artery diameter...
did not change throughout the protocol in the experimental arm (0.40±0.01, 0.41±0.01, and 0.41±0.01 cm at 0, 10, and 20 minutes, respectively; P>0.05). There were no statistically significant changes in blood flow or shear patterns in the contralateral arm, when no cuff was applied (Table, part A). When the proximal 40 mm Hg cuff was applied (Table, part B), there were slight but significant increases in retrograde shear and the oscillatory shear index (P<0.001), although the magnitude of these increases were not as robust as those observed in the experimental arm (Figure 2).

**Discussion**

The major finding of this study is that disturbed blood flow acutely induces endothelial activation and apoptosis in humans, as reflected by release of microparticles from activated (CD62E+) and apoptotic (CD31+/CD42b−) endothelial cells. These data provide the first in vivo experimental evidence of disturbed blood flow-induced endothelial injury in humans.

The 2 EMP populations we examined are well-established markers of endothelial activation (CD62E+) and apoptosis (CD31+/CD42b−). These cellular processes are important components of a damaged endothelium. Our findings are consistent with previous in vitro data indicating that disturbed flow upregulates the expression of E-selectin, as well as other adhesion molecules and markers of inflammation on the vascular endothelium.

Additionally, in vivo experimental conditions that chronically produce disturbed blood flow, such as that seen in atheroprotective regions, have been shown to induce substantial vascular dysfunction, injury, remodeling, and atherosclerosis in rodents. It has also been reported that low in vivo mean shear rates are associated with elevated circulating EMP levels in humans. Our data extend these previous findings by providing experimental evidence that disturbed blood flow confers an injurious stimulus to the endothelium in humans. In addition, from our data, it seems reasonable to expect that circulating EMPs could preferentially originate from classically atheroprotective segments of the vasculature (eg, branch points, curvatures, bifurcations), as these regions are characterized by disturbances in flow. It will be interesting for future studies to examine the extent to which elevations in proatherogenic shear patterns are responsible for the consistently observed phenomenon of elevations in basal circulating EMP levels in patients with cardiovascular diseases.

**Table. Blood Flow and Shear Patterns in the Contralateral Arm**

<table>
<thead>
<tr>
<th>Variable</th>
<th>0 min</th>
<th>10 min</th>
<th>20 min</th>
<th>P for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. No cuffs, n=3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter (cm)</td>
<td>0.40±0.01</td>
<td>0.41±0.02</td>
<td>0.41±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Mean flow (mL/min)</td>
<td>60.4±15.4</td>
<td>69.7±20.4</td>
<td>73.2±23.1</td>
<td>NS</td>
</tr>
<tr>
<td>Mean shear (s−1)</td>
<td>82.6±23.6</td>
<td>89.2±49.8</td>
<td>90.1±29.5</td>
<td>NS</td>
</tr>
<tr>
<td>Antegrade shear (s−1)</td>
<td>86.5±21.0</td>
<td>94.0±25.4</td>
<td>91.3±26.7</td>
<td>NS</td>
</tr>
<tr>
<td>Retrograde shear (s−1)</td>
<td>−3.5±2.3</td>
<td>−4.3±3.6</td>
<td>−2.3±1.6</td>
<td>NS</td>
</tr>
<tr>
<td>Oscillatory shear index</td>
<td>0.05±0.04</td>
<td>0.07±0.06</td>
<td>0.04±0.03</td>
<td>NS</td>
</tr>
<tr>
<td><strong>B. Proximal cuff only (40 mm Hg), n=3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter (cm)</td>
<td>0.42±0.02</td>
<td>0.45±0.02</td>
<td>0.45±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Mean flow (mL/min)</td>
<td>106.5±20.5</td>
<td>57.2±7.5</td>
<td>51.2±5.3</td>
<td>NS</td>
</tr>
<tr>
<td>Mean shear (s−1)</td>
<td>131.9±40.8</td>
<td>49.8±8.2</td>
<td>42.9±5.6</td>
<td>NS</td>
</tr>
<tr>
<td>Antegrade shear (s−1)</td>
<td>138.5±38.6</td>
<td>70.2±8.0</td>
<td>67.5±4.8</td>
<td>NS</td>
</tr>
<tr>
<td>Retrograde shear (s−1)</td>
<td>−8.0±1.1</td>
<td>−19.9±0.6</td>
<td>−24.1±1.3</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Oscillatory shear index</td>
<td>0.07±0.03</td>
<td>0.23±0.03</td>
<td>0.27±0.02</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

NS indicates not statistically significant (P>0.05).
Our data complement and extend the important study of Thijssen et al., which demonstrated that increased retrograde flow and shear can acutely impair arterial function in humans. One potential caveat is that we used a complete (220 mm Hg) distal occlusion rather than partial (eg, 75 mm Hg) because we were concerned with a combination of occlusion, venous pressure downstream of the occlusion cuff (ie, in the hand) might eventually exceed that of the cuff, resulting in contamination of sampling region with blood that had been exposed to a partially ischemic and high-pressure environment. These stimuli could theoretically promote the release of EMPs independent of any changes in blood flow patterns. Nevertheless, despite our slight deviation from the protocol employed by Thijssen et al., our findings concur with theirs by demonstrating that disturbed blood flow confers a deleterious signal at the cellular level resulting in endothelial activation and apoptosis in humans. Furthermore, we also observed no changes in EMPs in the contralateral arm exposed to normal resting blood flow, further supporting that our observations in the experimental arm were because of local disturbances in blood flow.

Although we have provided evidence that disturbed blood flow can induce the release of distinct EMP populations, the heterogeneity of circulating EMPs should be kept in mind. Peterson et al. demonstrated that EMPs induced by in vitro exposure to tumor necrosis factor-α were dramatically different from both control and plasminogen activator inhibitor-1–induced EMPs, using a comparative proteomics approach. It seems likely that a similar comparative proteomics experiment might also reveal a distinct phenotype of EMPs evoked by disturbed blood flow. Additionally, although our experimental protocol was successful in inducing a localized region of disturbed blood flow, we were not able to attribute our observations to specific component(s) of the disturbed flow profile. This will be an important issue for future studies to address, as recent data indicate that low time average-shear stress and fluid flow reversal produce somewhat distinct proatherogenic responses at the molecular level. Furthermore, our control experiments with a proximal 40 mm Hg cuff on the contralateral arm induced a moderately disturbed blood flow stimulus. However, this condition was not sufficient to induce an increase in CD62E+ or CD31+/CD42b− EMPs in the contralateral limb. It was somewhat surprising that (a) the proximal low-pressure cuff altered arterial flow and shear patterns, and (b) we did not detect any changes in EMP concentrations in spite of this moderate disturbance in flow. These data might suggest the existence of a disturbed flow threshold for the acute induction of endothelial injury.

A few limitations of our study warrant discussion. First, although the flow and shear data using the proximal cuff in the contralateral arm indicated minimal changes in EMPs, these data should be interpreted with caution, given the small sample size. Overall, future studies designed to determine a dose–response relationship between magnitude of flow disturbance and release of EMPs from the vascular wall are needed. In addition, it might have been advantageous to incorporate measurements of brachial artery FMD into our study, as this would have allowed a direct comparison with the study of Thijssen et al., and it would have been interesting to correlate our changes in EMPs with changes in FMD. However, an unavoidable caveat to an assessment of FMD with our protocol was that there would have been substantial reactive hyperemia on release of the 220 mm Hg occlusion cuff before a postintervention FMD assessment. Thus, we would not have been able to link our EMP findings with FMD data because the reactive hyperemia (ie, increases in flow and shear), and not disturbed blood flow, would have been the final stimulus to which the brachial artery endothelium was exposed. Exposing the limb to a reactive hyperemia would confound our experimental design, making any interpretation difficult.

**Perspectives**

Our findings provide clear in vivo experimental evidence from human subjects to corroborate =30 years of in vitro work supporting the close association between disturbed flow patterns and a damaged/dysfunctional vascular endothelium. We also confirm and extend recent work indicating that disturbed blood flow can reduce arterial function in humans, as assessed by FMD. Our data suggest that the previously reported functional impairments may be related to proinflammatory and proapoptotic effects of disturbed blood flow. Additionally, the broader implications of our study relate to the growing understanding of the role of disturbed flow in the pathogenesis of vascular disease. Taken together with the accumulating evidence of proatherogenic shear patterns in conduit arteries of subjects with increased risk for cardiovascular diseases, our data stimulate the hypothesis that disturbed blood flow contributes to increased concentrations of circulating EMPs in these subjects. Notably, our study was conducted in the forearm, which is generally resistant to atherosclerosis. Thus, we speculate that disturbed blood flow-induced increases in EMP release might be even more pronounced in atheroprone arteries. Future studies of experimentally induced disturbed blood flow in atherosclerosis-susceptible vascular beds, for example, in conduit arteries of the leg, are clearly warranted.

Importantly, EMPs not only serve as biomarkers of damaged endothelium and diagnostic/prognostic markers of cardiovascular diseases, they also deliver inflammatory cytokines (eg, C-reactive protein), carry regulatory microRNAs, reduce endothelial nitric oxide synthesis, and promote thrombosis, inflammation, and the production of reactive oxygen species. Thus, our findings raise the idea that disturbed blood flow induces EMPs which may further promote endothelial dysfunction at distal sites, resulting in a feed-forward vicious cycle of vascular injury. Finally, it is important to bear in mind that our study volunteers were young healthy men. It is possible that the acutely altered blood flow patterns in the current study that resulted in endothelial activation and apoptosis could be matched by equal repair processes, such that it may not lead to long-term endothelial damage in these subjects. However, it seems reasonable to hypothesize that in subjects with diseases or risk factors associated with impaired endogenous endothelial repair mechanisms (eg, aging, obesity, type 2 diabetes mellitus, etc.), disturbances in blood flow could lead to irreparable endothelial damage. This hypothesis warrants future testing.

In summary, our study indicates that experimental induction of disturbed blood flow in the human forearm acutely increases local concentrations of CD62E+ and CDs1+/CD42b− EMPs, resulting in endothelial activation and apoptosis. However, these data should be interpreted with caution, given the small sample size. Overall, future studies designed to determine a dose–response relationship between magnitude of flow disturbance and release of EMPs from the vascular wall are needed. In addition, it might have been advantageous to incorporate measurements of brachial artery FMD into our study, as this would have allowed a direct comparison with the study of Thijssen et al., and it would have been interesting to correlate our changes in EMPs with changes in FMD. However, an unavoidable caveat to an assessment of FMD with our protocol was that there would have been substantial reactive hyperemia on release of the 220 mm Hg occlusion cuff before a postintervention FMD assessment. Thus, we would not have been able to link our EMP findings with FMD data because the reactive hyperemia (ie, increases in flow and shear), and not disturbed blood flow, would have been the final stimulus to which the brachial artery endothelium was exposed. Exposing the limb to a reactive hyperemia would confound our experimental design, making any interpretation difficult.
CD42b EMPs. As these EMP populations are well-validated markers of endothelial cell damage, our findings indicate that disturbed flow acutely induces activation and apoptosis of the human vascular endothelium in vivo.

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Disclosures
None.

References
What Is New?

• Disturbed blood flow has previously been shown to produce damage to vascular cells in cell culture and animal models.

• Here, we extend these findings to humans demonstrating that vascular wall endothelial cells release microparticles (markers of cellular injury) in response to an acute disturbed blood flow stimulus.

What Is Relevant?

• Disturbed blood flow is a hallmark characteristic of atherosclerosis-susceptible arterial regions. However, there are few previously published experimental studies providing solid evidence that disturbed flow actually causes endothelial injury at the cellular/molecular level in vivo. Ours is the first such study in humans.

Novelty and Significance

• Endothelial microparticles, which we find are released in response to a disturbed flow stimulus, can themselves promote the development of vascular disease.

• Recent studies also indicate that increased circulating levels of endothelial microparticles are strongly related to a number of cardiovascular diseases and risk factors, including hypertension.

Summary

We demonstrate that disturbed blood flow acutely induces endothelial injury in humans, as reflected by local release of endothelial cell microparticles in response to an experimental disturbed blood flow stimulus.
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