
June 2009

HemoShear Case Report

Assessing the Pleiotropic Effects of Statin Therapy on Human Vascular Cells using HemoShear's Arterial Surrogate Model

Authors:

Nicole Hastings, PhD

Director of Scientific Studies

Brett R. Blackman, PhD

Chief Scientific Officer

Brian R. Wamhoff, PhD

Vice President for Research & Development



Transforming Drug Discovery

and safety through innovative surrogate models

Case Study

Our Technology:

HemoShear has developed a cell-based human surrogate model of an artery to screen drug compounds for vascular safety, toxicity, and efficacy (**Figure 1**). The model recreates the vascular anatomy by plating primary human endothelial and smooth muscle cells on opposing sides of transwell membrane and stimulates the endothelial layer with fluid shear forces that mimic the exact hemodynamics measured from the human circulation. The application of regional “Healthy” or “Disease-prone” arterial hemodynamics induces the endothelial and smooth muscle cell layers to acquire phenotypes similar to the comparative regions in the in vivo circulation. As a result, the HemoShear technology is far more predictive of human response to new drug therapies.

Background and Study Objectives:

Statins, HMG-CoA reductase inhibitors, are the most widely prescribed class of drug for lipid-based cardiovascular diseases (e.g., atherosclerosis). Statins are widely prescribed to reduce systemic levels of LDL cholesterol and triglycerides, and thus have shown tremendous clinical benefit. Over the past two decades, considerable basic and clinical research has been conducted to evaluate positive off-target, or pleiotropic effects of statins. Statin therapy in humans has been shown to improve endothelial function, reduce inflammation,

stabilize atherosclerotic plaques, reduce vessel remodeling, and prevent thrombus formation. While positive attributes of statins have been reported on from clinical trial data, the specific mechanism of action on human vascular cells is unknown. This is because studies of this nature cannot be performed in living humans and until now, there were no human surrogate models of the vasculature to test this. The objectives of this case study are to:

- Demonstrate that the HemoShear human vascular technology can predict the anti-inflammatory, anti-remodeling and anti-thrombotic pleiotropic attributes of statin therapy
- Uncover new and meaningful mechanisms of action for promoting these statin-induced phenotypes
- Uncover the regional responses of human endothelial and smooth muscle cells in healthy and disease-prone areas of the arterial vasculature in response to statin therapy
- Determine if statin therapy could reduce atherosclerotic burden through reducing permeability (i.e., enhancing barrier function)
- Demonstrate that HemoShear’s technology is far more predictive of a human response than traditional cell culture methods

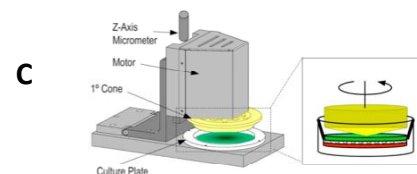
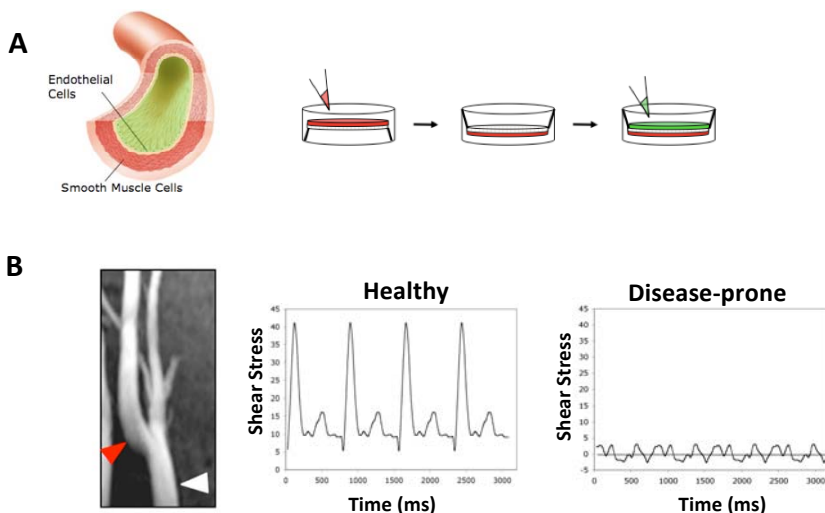


Figure 1. Human hemodynamic endothelial – smooth muscle cell co-culture model. (A) Plating conditions are optimized for endothelial (green) and smooth muscle cell (red) confluence on opposing sides of a transwell membrane. (B) Human hemodynamic shear stress flow patterns were acquired from MRI in the Healthy (common carotid; white arrow) and Disease-prone (internal carotid sinus; red arrow) regions. (C) Human healthy or disease-prone hemodynamics are mimicked on the endothelial layer of the in vitro flow device to produce in vivo regional vascular cell phenotypes.

Methods

Drug Treatment

Atorvastatin (generously provided by Pfizer via Compound Transfer Agreement) was resuspended in DMSO to a working concentration of 1mM. Cells were exposed to a final concentration of 1uM representing a dosage of 20mg/day based on 12% bioavailability.

Cell Culture and Flow Experiments

Human primary smooth muscle and endothelial cells were plated on opposing sides of four transwell membranes in parallel, as previously described (Hastings, et al., 2007). Upon confluency, dishes were pre-conditioned to healthy or diseased blood flow patterns for 24 hours in order to recalibrate vascular wall *in vivo* phenotypes. Atorvastatin or vehicle control was then added to fresh media, which was subsequently perfused in to and out of the system at a constant exchange rate, where each flow condition received drug and control treatments for an additional 24 hours. For each flow experiment, two additional transwell plates were seeded with cells and received atorvastatin and control treatments for 24 hours, but were not exposed to hemodynamic conditions (referred to as Traditional Condition). See **Figure 2** for schematic of experimental conditions.

Gene Array Analysis

At the termination of the experiment, cells were gently scraped from either side of the dish, maintaining separate cell populations. Total mRNA was isolated from each cell type (Invitrogen, #12183018A). Gene analysis was performed using StellarArray technology (Lonza), where 96 custom-selected genes were examined for fold change in drug treatment, relative to control using StellarArray GPR software and other statistical analyses. All genes were placed into functional categories and plotted, including Apoptosis, Cholesterol Transport, Coagulation, Contractility, Oxidative/ER Stress, Anti-inflammatory, Pro-inflammatory, Migration--Proliferation--Remodeling, and Transcriptional Regulation.

Secreted Protein Analysis

During flow treatment, media was collected for analysis of secreted proteins at distinct four-hour time intervals: (1) prior to drug treatment, (2) immediately following drug treatment, (3) prior to flow cessation. Media was collected on ice, aliquoted, and stored at -80°C until analysis. Examination of 20 potential secreted proteins was performed using BioPlex analysis (Bio-Rad).

Endothelial Barrier Function

Proprietary label-free technology was developed to measure real-time changes in endothelial barrier function under hemodynamic flow conditions in the presence of atorvastatin or vehicle.

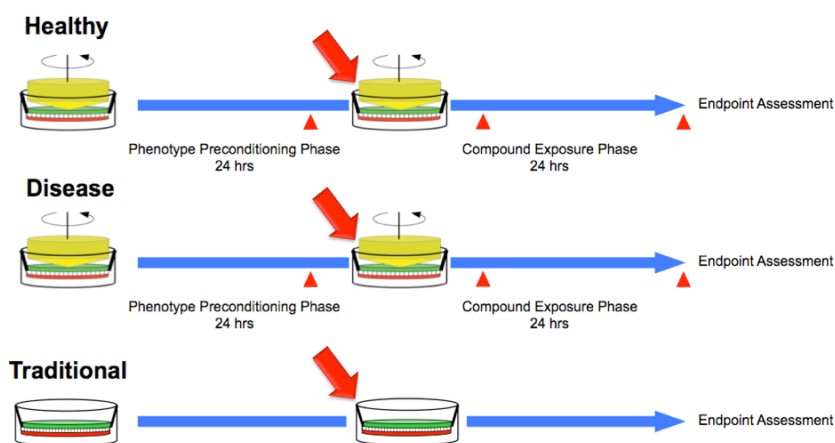


Figure 2. Schematic of experimental protocol for testing effects of Atorvastatin using HemoShear's Technology vs. Traditional culture. Large red arrow indicates the administration of Atorvastatin to the cultures. Red arrowheads indicate time points in which media effluent was sampled for post-analysis of biomarker secretion.



Study Results

HemoShear Technology Demonstrates Different Global Responses Relative to Traditional Culture Models

Over thirty years of literature demonstrates that endothelial cells exposed to flow in cell culture models are fundamentally different than cells under static, no-flow conditions. HemoShear's technology can re-create the in vivo biology/phenotype of endothelial and smooth muscle cells to match the regional "healthy" or disease-prone segment of a human artery wall. In **Figure 3**, we show that co-cultures treated with Atorvastatin have a different genetic response when exposed to hemodynamic flow conditions compared to traditional (no flow) experiments. Scatter plots demonstrate no correlation between Atorvastatin under hemodynamic conditions vs. Atorvastatin in Traditional conditions for both endothelial and smooth muscle cells.

HemoShear's Technology Differentiates Atorvastatin's Response in Healthy and Disease-prone Regions of the Artery

Concordance plots were generated to determine the similarity (and differences) that exists between hemodynamic and traditional conditions. Data was stratified based on each cell type and gene "functional" category. **Figure 4** corroborates data in **Figure 3** and also highlights the differences that exist depending on whether the co-culture was pre-conditioned to emulate a healthy or disease-prone phenotype. Interestingly, anti-inflammation, which has been the most widely promoted off-target benefit of statin's, shows the greatest differences between hemodynamic and traditional conditions.

HemoShear Technology Confirms Anti-inflammatory Properties of Atorvastatin

HemoShear's technology has confirmed one of the most promising off-target, pleiotropic benefits of statins that has been reported on from human clinical trials; the anti-inflammatory properties of statins. Statin's off-target effects reduce

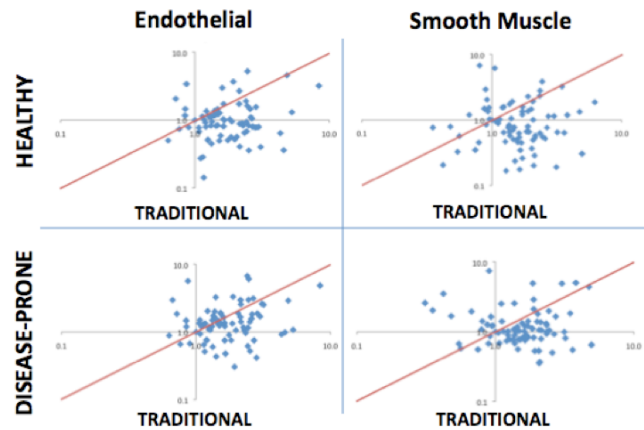


Figure 3. Scatter plots assessing the correlation between HemoShear flow conditions vs. traditional culture methods. Data represents the correlation of gene responses between Healthy or Disease-prone conditions vs. traditional culture. Data points are Atorvastatin treatment normalized to its respective vehicle control.

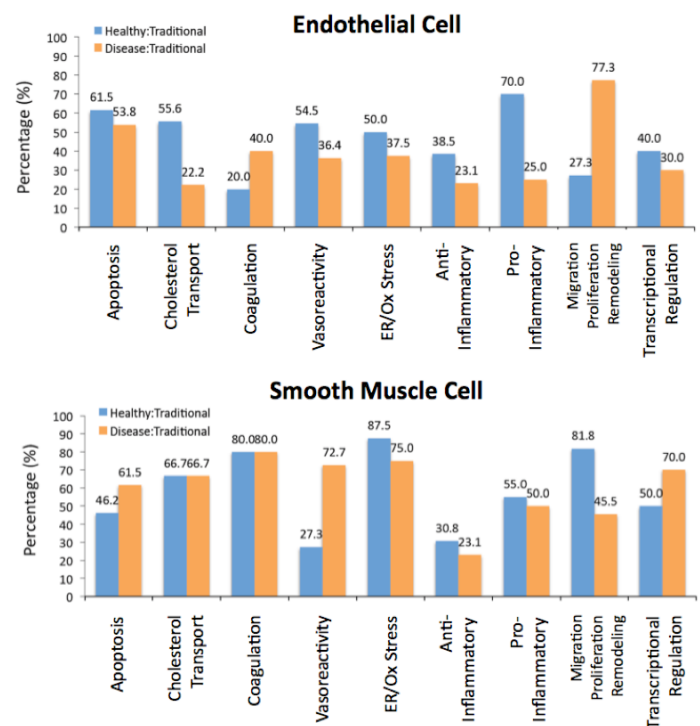


Figure 4. Concordance plots of genetic analysis stratified by vascular cell type and biological category. Data are the percentage of similar gene responses with Atorvastatin treatment normalized to vehicle control for hemodynamic relative to traditional culture conditions.

inflammatory genes/proteins and promote anti-inflammatory genes/proteins in the cells of the

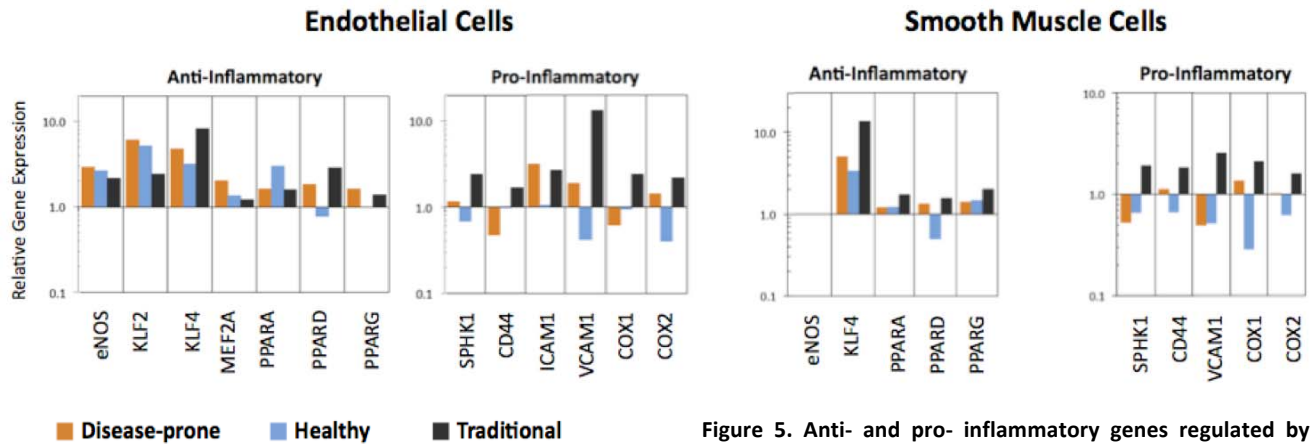


Figure 5. Anti- and pro- inflammatory genes regulated by Atorvastatin. Gene expression data per condition (Disease-prone, orange; Healthy, blue; Traditional, black) represent the response to Atorvastatin relative to vehicle control.

vascular wall. **Figure 5** highlights a cohort of genes that are anti- or pro- inflammatory and compares the effect of a low dose of Atorvastatin on the regulation of these genes in response to healthy or disease-prone hemodynamics or traditional cultures. Overall across all genes observed, Atorvastatin **reduced pro-inflammatory** genes (Healthy < Disease-prone < Traditional) and **promoted anti-inflammatory** genes (Disease-prone > Healthy > Traditional) in endothelial and the underlying smooth muscle cells. The HemoShear Technology resulted in a physiologically relevant response, whereby anti- and pro- inflammatory genes demonstrated opposing responses. This is in sharp contrast to the Traditional cell co-cultures where Atorvastatin elicited both an up-regulation of both anti- and pro-inflammatory genes. These results highlight the advantages of HemoShear’s technology and the concerns with interpreting results from traditional culture methods.

Biomarker Assessment:

HemoShear’s technology can also examine proteins (i.e., biomarkers) secreted by the endothelial and smooth muscle cell layers as a function of hemodynamic condition and compound treatment. Media effluent collected after statin or vehicle control treatment was assessed for several pro-inflammatory cytokines/chemokines secreted from the endothelial or smooth muscle layer. **Figure 6** shows the results for four of these factors; IL-6, MCP-1, IL-15, IL-9. In general, a low dose of

Atorvastatin treatment reduced secretion of these pro-inflammatory cytokines. While this was true for most conditions, the technology demonstrated that Atorvastatin did not have a significant effect on IL-6, MCP-1, and IL-9 secretion in the smooth muscle cells exposed to the Disease-prone condition. Further, it is important to note that

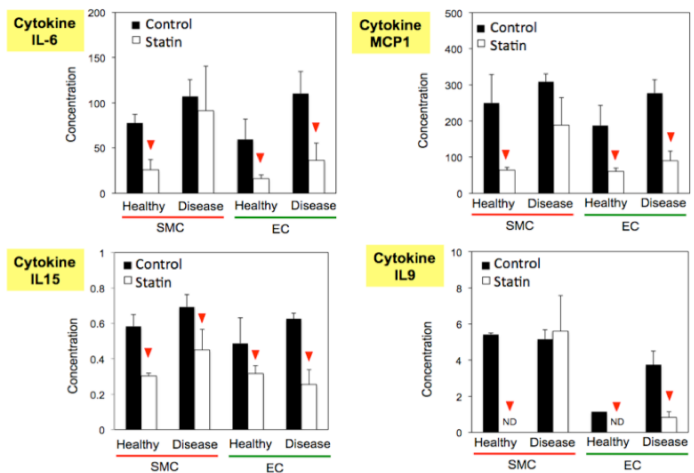


Figure 6. Cytokine secretion profile is reduced upon Atorvastatin treatment. Inflammatory cytokines secreted from the endothelial and smooth muscle cell layers were measured in the media following Atorvastatin or vehicle control conditions in response to hemodynamic flow. Red arrowheads indicate reduction following treatment.

both IL-15 and IL-9 are not well described in the literature in the context of atherosclerosis, and we

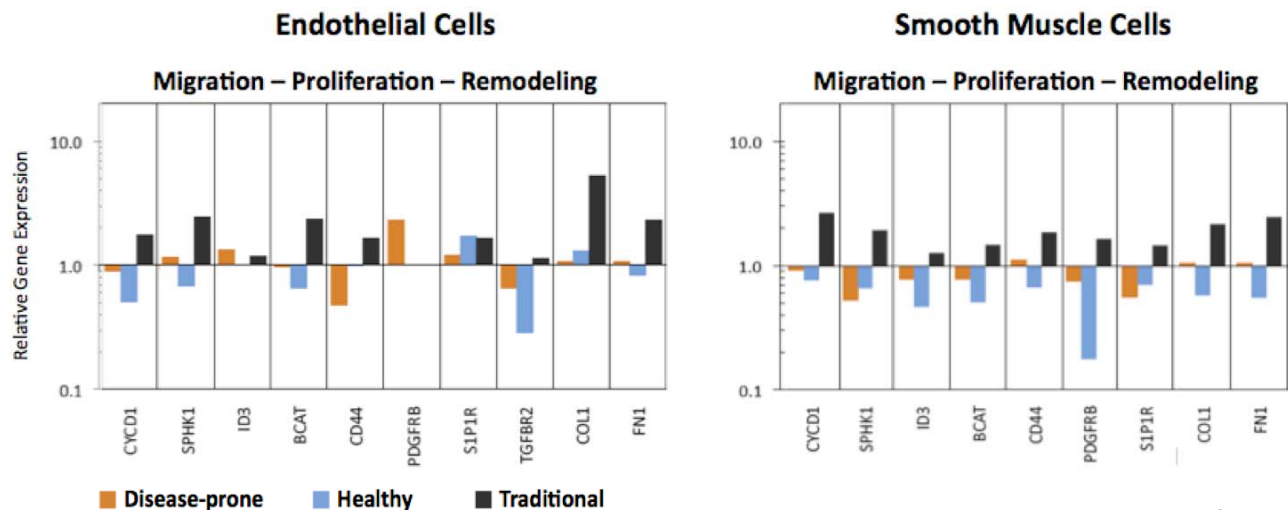


Figure 7. Atorvastatin attenuates the expression of vascular migratory, remodeling and proliferation genes. Gene expression data per condition (Disease-prone, orange; Healthy, blue; Traditional, dark grey) represent the response to Atorvastatin relative to vehicle control.

were able to uncover significant effects of the statin on their secretion profile; thus highlighting the potential for biodiscovery with the technology.

HemoShear's Technology Indicates that Atorvastatin Promotes a Quiescent Vascular Cell Phenotype: A divergent response compared to the traditional co-culture model

Additional evidence points to multiple pleiotropic effects of statin therapy, including attenuation of vascular remodeling and even atherosclerotic plaque stabilization. Through gene analysis, HemoShear has identified multiple pathways in which statin therapy may reduce vascular cell remodeling and enhance a non-proliferative quiescent state. **Figure 7** highlights up to 10 genes involved in vascular migration, proliferation, and remodeling. In general, HemoShear's technology demonstrates that a low dose of Atorvastatin reduced the expression of genes that are well known to promote the "remodeling" phenotype in both endothelial and smooth muscle cells. In sharp contrast to this finding, statin treatment in the traditional culture model caused the majority of these remodeling genes to increase in expression.

Anti- Coagulation and Thrombosis Properties of Atorvastatin are Similar to Recent Human Trial Data Using Another Statin

Recent human clinical data demonstrated that statin (i.e., Crestor®) therapy reduced thrombotic events. Using our genetic screen, HemoShear has identified potential mechanisms by which reduced

thrombosis/coagulation may occur at the vessel wall. In **Figure 8**, a low dose of Atorvastatin treatment reduced genes associated with thrombosis and coagulation in both the endothelial and smooth muscle cell layer. Platelet activator inhibitor-1 (PAI-1), for example, which is elevated in vascular disease and metabolic syndrome and promotes thrombosis formation, was down regulated in endothelial and smooth muscle cells conditioned to healthy or disease-prone conditions. This is in contrast to traditional cultures that demonstrated an opposing increase in PAI-1. Similarly, thrombospondin-1 (THBS1), which is active in platelet aggregation, was either not affected by statin treatment or its expression was significantly reduced under hemodynamic conditions. Once again, statin treatment caused an opposing response in traditional cultures that responded with an increase in THBS1. Lastly, thrombomodulin (THMD) binds thrombin and degrades clotting factors. Atorvastatin increased the expression of THMD in endothelial and smooth muscle cells in the disease-prone condition and only the endothelial cells in the healthy condition. It is well appreciated that thrombotic events in atherosclerosis may occur through plaque rupture or erosion. This may involve both the endothelium as well as exposed underlying smooth muscle cells in the neointima. HemoShear's technology, more

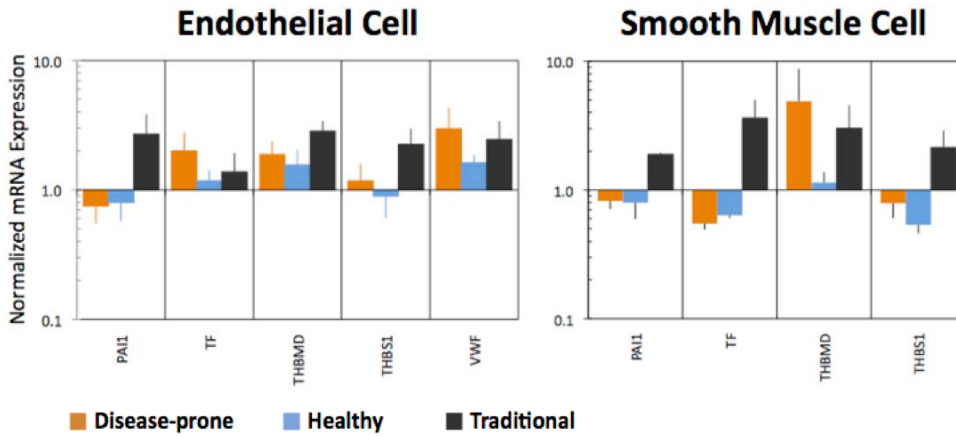


Figure 8. Atorvastatin attenuates pro- thrombotic and coagulation genes. Gene expression data per condition (Disease-prone, orange; Healthy, blue; Traditional, dark grey) represent the response to Atorvastatin relative to vehicle control.

so than traditional cultures, demonstrated that atorvastatin could reduce the propensity of thrombosis and/or coagulation at the vessel wall interface.

HemoShear’s Technology Uncovers a Potentially New Statin Indication: Reducing Vascular Permeability

HemoShear has developed a label-free method to measure real-time changes in endothelial barrier function (i.e., permeability) in the context of healthy and disease-prone human hemodynamic conditions. Using this technology, endothelium exposed to disease-prone hemodynamic conditions are ~25% more permeable compared to healthy hemodynamic conditions (*data not shown*). This result mimics differences observed in human and animal vasculature. We tested whether Atorvastatin could provide additional benefit to vascular wall biology and function by reducing endothelium permeability in regions prone to the development of atherosclerosis (i.e., disease-prone regions). **Figure 9** shows a time course of the effect of Atorvastatin treatment on endothelium barrier function from 12 to 24 hours following administration of the drug relative to the vehicle control. Atorvastatin treatment markedly reduced endothelium permeability (i.e., enhanced barrier function) from 15 to 80%. This is the first report of a statin reducing permeability in the context of a human surrogate model of the arterial vasculature and may provide additional insight into the functional benefit of statin therapy beyond reducing vessel wall inflammation.

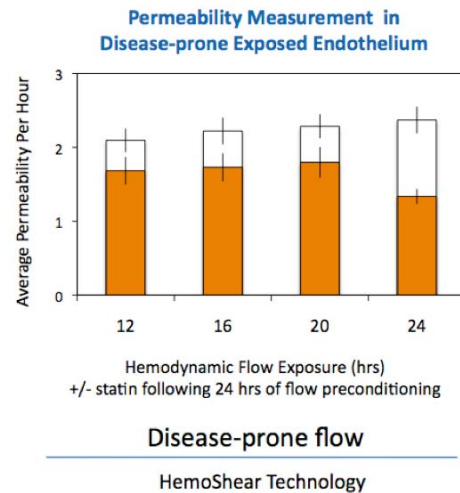
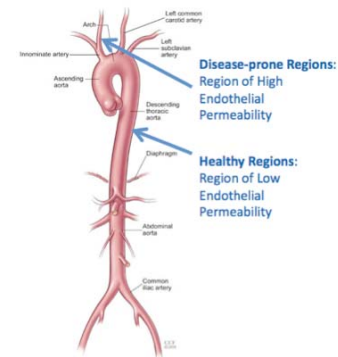


Figure 9. Atorvastatin treatment enhances endothelium barrier function.



Study Conclusions

The goal of this study was to demonstrate that HemoShear's human surrogate model of the arterial vasculature was more predictive of human clinically relevant benefits of atorvastatin relative to traditional cell culture methods. The experimental study was able to directly test the hypothesis that statins exert positive pleiotropic effects directly on the cells of the blood vessel wall.

HemoShear's technology demonstrated:

- Positive clinical correlation using the HemoShear method and opposite and confusing correlation using traditional culture methods,
- Anti-inflammatory effects of statins on endothelial and smooth muscle cells under healthy and disease-prone conditions,
- Attenuation of vascular remodeling and enhancement a non-proliferative, quiescent phenotype in endothelial and smooth muscle cells,
- Anti-thrombotic and anti-coagulation response in BOTH endothelial and smooth muscle cell gene pathways,
- Statins may enhance endothelial barrier function (i.e., reduce permeability),
- Additional mechanisms of action of a statin on endothelial and smooth muscle cell biology.



Study Team

Scientific Oversight and Data Interpretation

Brett Blackman, Ph.D., Chief Scientific Officer

Brian Wamhoff, Ph.D., Vice President, R&D

Nicole Hastings, Ph.D., Director of Scientific Studies

Study Planning, Conduct, Sample Analysis and Reporting

Nicole Hastings, Ph.D., Director of Scientific Studies

David Scott, M.S., Laboratory Specialist



Biographical Sketches

Brett R. Blackman, Ph.D., Chief Scientific Officer

Dr. Blackman is co-founder of HemoShear and co-inventor of the HemoShear technology. He is a tenured associate professor in the Department of Biomedical Engineering at the University of Virginia. Since August of 2002, Dr. Blackman has led an NIH-funded research program investigating the role of the hemodynamic environment in regulating vascular endothelial cell biology in atherosclerosis. Prior joining the University of Virginia, Dr. Blackman spent 3.5 years training as a postdoctoral research fellow in the Vascular Research Division of Brigham & Women's Hospital and Harvard Medical School. There he developed the first cell-culture-based model to simulate human hemodynamic flow patterns on isolated human endothelial cells, which laid the foundation for current research identifying the importance of precise hemodynamic conditions for regulating arterial vascular biology. Dr. Blackman has 19 peer-reviewed publications, serves on UVa's School of Engineering and Sciences Dean's Research Advisory Committee, is a member of the Robert M. Berne Cardiovascular Research Center, is a peer reviewer for more than 12 scientific journals, and is a standing member of the American Heart Association Bioengineering & Biotechnology study section. Dr. Blackman obtained a BS in mechanical engineering from Drexel University and a PhD in bioengineering from the University of Pennsylvania.

Brian R. Wamhoff, Ph.D., Vice President of R&D

Dr. Wamhoff is a tenure track assistant professor and leads an NIH-funded laboratory that studies vascular disease at the Cardiovascular Division of the University of Virginia's Department of Medicine. Dr. Wamhoff began his fellowship at UVa to develop rodent models to investigate the molecular mechanisms of genes underlying vascular disease. During this time, he was also employed by Setagon, Inc., a Charlottesville start-up, as lead scientist to develop a novel drug-eluting stent for the treatment of blood vessel stenosis in humans. While at UVa, he has obtained more than \$3.3 million funding from Pfizer, the American Heart Association, NIH, and other organizations to study the fundamental mechanisms that regulate smooth muscle cell phenotypic switching in vascular disease.

Dr. Wamhoff obtained a BS in biology at Rhodes College and a minor in business administration, and obtained his PhD from the University of Missouri where he developed swine models of diabetes and atherosclerosis. Dr. Wamhoff filed more than 15 patents related to regulation of smooth muscle cell phenotypic switching in vascular disease, leading to licensing of the technology in 2007 to a major device company. Dr. Wamhoff has authored or coauthored 32 publications, two book chapters, and 3 commentaries. Dr. Wamhoff serves as peer reviewer for more than 10 major scientific journals and a grant reviewer for the American Heart Association. He has been the recipient of multiple awards, including the Robert M. Berne Trainee Achievement Award, the American Physiological Society Young Cardiovascular Investigator Award, and the 2008 Atherosclerosis, Thrombosis, and Vascular Biology (ATVB) Irvine Paige Award. Dr. Wamhoff devotes philanthropic time to the community by promoting health awareness as a local member of the board of directors for the American Heart Association.



Biographical Sketches

Nicole E. Hastings, Ph.D., Director of Scientific Studies

Dr. Hastings obtained her Ph.D. from the Department of Biomedical Engineering at the University of Virginia. Her research focused on elucidating mechanisms of cross-talk and phenotypic modulation of endothelial and smooth muscle cells during initiating stages of atherosclerosis. While developing HemoShear's technology, she has characterized and validated a novel *in vitro*-based human endothelial and smooth muscle co-culture model whereby hemodynamic shear stress patterns derived from atherosclerosis-prone or atherosclerosis-protective regions are applied to the endothelium. From this model, she identified that the endothelial cell secreted factor Interleukin-8 is released at higher levels during atheroprone flow, and examined its role in modulating the smooth muscle cell inflammatory phenotype, a critical and unexplored feature of the disease. Dr. Hastings has published two peer-reviewed manuscripts, is coauthor of four additional high-impact publications, and has presented at international meetings. Prior to obtaining her PhD at UVa, she obtained a bachelor's degree in Biomedical Engineering from North Carolina State University.



About HemoShear

HemoShear, LLC, located in Charlottesville, Virginia, was founded in early 2008 to guide drug development companies in the selection of innovative new drug candidates by leveraging the Company's unique proprietary, human surrogate technology, which closely mimics the human blood vessel system.

HemoShear can reduce risk and attrition in R&D programs, saving millions and even billions of dollars in misguided development decisions. We seek to establish strategic relationships with a limited number of pharmaceutical companies to identify targets and pathways of disease progression, assess compounds, and select optimal drug candidates.

HemoShear's innovative technology is far more predictive of human response to drugs in the vasculature, as compared with animal models and traditional static cell culture experiments. Our technology replicates human blood flow forces onto a culture of primary human endothelial and smooth muscle cells, stimulating the cells to respond as they do in the human blood vessel. HemoShear can test the effects of new drugs (mono-therapy) on the vasculature, in combination with other drugs (multi-therapy), and in specific physiological conditions such as inflammation and diabetes. HemoShear can target drugs intended to treat the cardiovascular system or drugs that may have off-target effects upon the vasculature. Examples include drugs intended to treat atherosclerosis, diabetes, inflammation and cancer. The HemoShear technology is highly adaptable and we plan to extend it to the liver, blood-brain barrier, kidney, and other organ systems, as well as to specific disease states such as cancer.

We believe HemoShear has most value to Pharmaceutical R&D during the discovery phase, providing rich data on how drugs affect the vasculature and their mechanisms of action, especially for new drug candidates that are intended to treat cardiovascular and metabolic diseases, vascular injury, and inflammation. HemoShear works in close, strategic, consultative partnerships with select customers to identify new targets and select optimal drug candidates. We anticipate such an engagement will result in competitive advantages by developing innovative drugs with superior efficacy and safety characteristics.