
June 2010

HemoShear Case Report

Examination of the Vascular Response to Pioglitazone in a Human Surrogate Arterial Model

Authors:

Nicole E. Hastings, PhD, Director of Scientific Studies
Brett R. Blackman, PhD, Chief Scientific Officer
Brian R. Wamhoff, PhD, Vice President of R&D



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. 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:

Pioglitazone hydrochloride (trade name ACTOS) is a drug of the family thiazolidinedione (TZD), commonly prescribed to non-insulin dependent diabetes (Type II) patients in order to decrease sensitivity to insulin resistance. TZDs act through a mechanism of peroxisome proliferator-activated receptor gamma (PPAR- γ) activation, thus influencing glucose regulated genes.

TZDs are compounds not recommended for patients with heart failure symptoms due to fluid retention and build-up which can cause or exacerbate heart problems. However, evidence supporting positive pleiotropic effects of pioglitazone on the cardiovascular system has recently been reported. Studies have highlighted beneficial effects of pioglitazone, including a reduction in blood pressure. In addition, pioglitazone can attenuate production of inflammatory and pro-atherogenic molecules in the vascular system (Schernthaner, Int J Clin Pract, 2009). Among these proteins are potent secreted factors known to exacerbate a localized inflammatory or unstabilizing response, such as Interleukin-6 (IL-6), Monocyte Chemoattractant

Protein-1 (MCP-1), and Metalloproteinase-9. Data from the Schernthaner study further supported the hypothesis that pioglitazone reduces the thickness of the carotid intimal region. While the mechanisms of pioglitazone and other TZDs on the vasculature are beginning to be elucidated, much information regarding pleiotropic effects is unknown.

HemoShear’s human surrogate model of the vasculature was used in the current study to understand the effects of pioglitazone at a molecular level.

The objectives of this case study were to:

- Examine the human vascular response to pioglitazone using HemoShear technology and high throughput PCR array analysis, focusing on inflammation, apoptosis, cell stress, remodeling, proliferation, and migration-related gene changes.
- Uncover the regional responses of human endothelial and smooth muscle cells in Healthy and Disease-prone areas of the arterial vasculature and highlight the importance of flow-exposure systems compared to traditional cell culture models.
- Determine if pioglitazone influences endothelial cell permeability (i.e., enhances barrier function)

Methods

Drug Treatment

Pioglitazone (Toronto Research Chemicals, Inc.) was resuspended in methanol to a working concentration of 16.1mM. Cells were exposed to a final concentration of 16.1 μ M representing a dosage of 30 mg/day.

Cell Culture and Flow Experiments

Human primary smooth muscle and endothelial cells were plated on opposing sides of four transwell membranes simultaneously, 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. Pioglitazone 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 the “traditional condition”, two additional transwell plates were seeded with cells and received pioglitazone and control treatments for 24 hours, but were not exposed to hemodynamic conditions. See **Figure 1** 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 StellARray technology (Lonza), where 96 custom-selected genes were examined for fold change in drug treatment, relative to control using StellARray 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.

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 pioglitazone or vehicle.

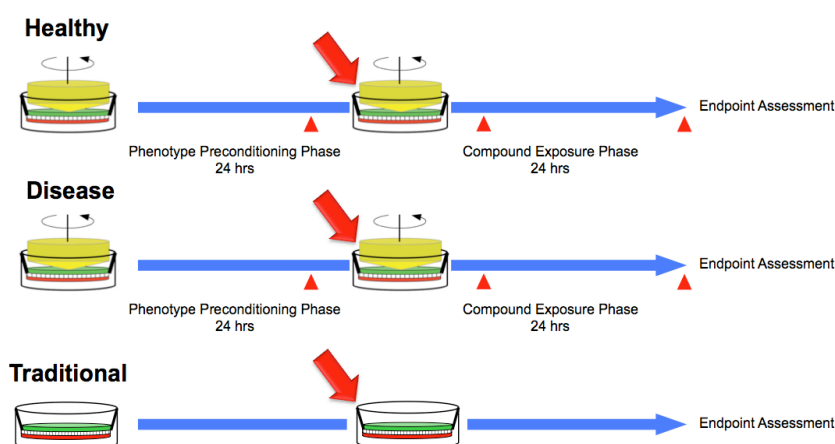


Figure 1. Schematic of experimental protocol for testing effects of pioglitazone using HemoShear's Technology vs. Traditional culture. Large red arrow indicates the administration of pioglitazone to the cultures.

Study Results

Pioglitazone Induces Primarily Anti-Inflammatory Properties on Smooth Muscle Cells

PPARs, expressed in both endothelial and smooth muscle cells, are known to have protective and anti-inflammatory effects; thus, we hypothesized that activation of PPAR- γ via pioglitazone would yield anti-inflammatory responses. PCR gene array heat plot analysis demonstrated a strong inhibition of inflammatory genes predominantly in smooth muscle cells under both flow conditions (**Figure 2**). Smooth muscle cells exposed to healthy flow also exhibited upregulation of some anti-inflammatory genes, which indicates beneficial responses due to PPAR- γ agonism. Further, endothelial cells exhibited more modest regulation than smooth muscle cells of inflammatory pathway related markers due to Pioglitazone exposed to both Healthy and Disease flow conditions. Together these results suggest pleiotropic effects of pioglitazone that appear to convey a more protective effect on the arterial vasculature.

A close examination of the genes associated with pioglitazone-induced protective effects in smooth muscle cells revealed that the genes important to the development of atherosclerosis and inflammation were reduced due to pioglitazone treatment when cells were preconditioned by the HemoShear technology. Importantly, the smooth muscle cell inflammatory response to pioglitazone is opposite for traditional (no flow) cultures compared to both healthy and disease flow conditions, where pioglitazone in traditional cultures shows an induction of inflammatory genes (**Figure 3**). The data demonstrating a reduction in pro-inflammatory genes in smooth muscle correlate with data generated from human studies, where pioglitazone has effects of reducing soluble levels of CRP, IL6, ICAM1 and VCAM1 (Takase et al., *Metabolism*, 2007). Reduction of ICAM1 is a positive feature of pioglitazone because elevated soluble levels of ICAM are known to contribute to carotid intimal-medial thickness (IMT), a clinical metric

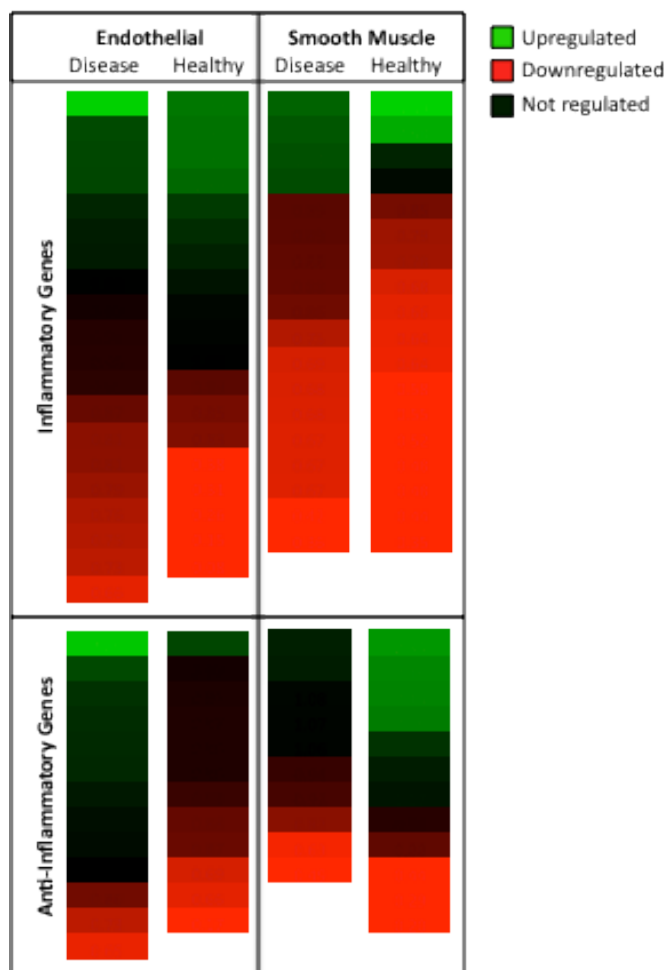


Figure 2. Heat plot analysis demonstrating inflammatory response due to pioglitazone. Fold changes from pioglitazone treated condition relative to control were determined and each gene having Inflammatory and Anti-Inflammatory characteristics were assigned a colormetric value based on level of induction (green) or reduction (red) of gene expression. Data is sorted in order from high to low expression. n=3 biological replicates.

representing progression of atherosclerosis plaque formation. Furthermore, pioglitazone can slow progression of IMT in type II diabetic patients, which may be due to the reduction of these soluble adhesion molecules and inflammatory markers. Data from the current study suggest that pioglitazone effects on smooth muscle are contributing to the overall inhibition of plaque formation.

The response to pioglitazone in endothelial cells in the current study is much more complex,

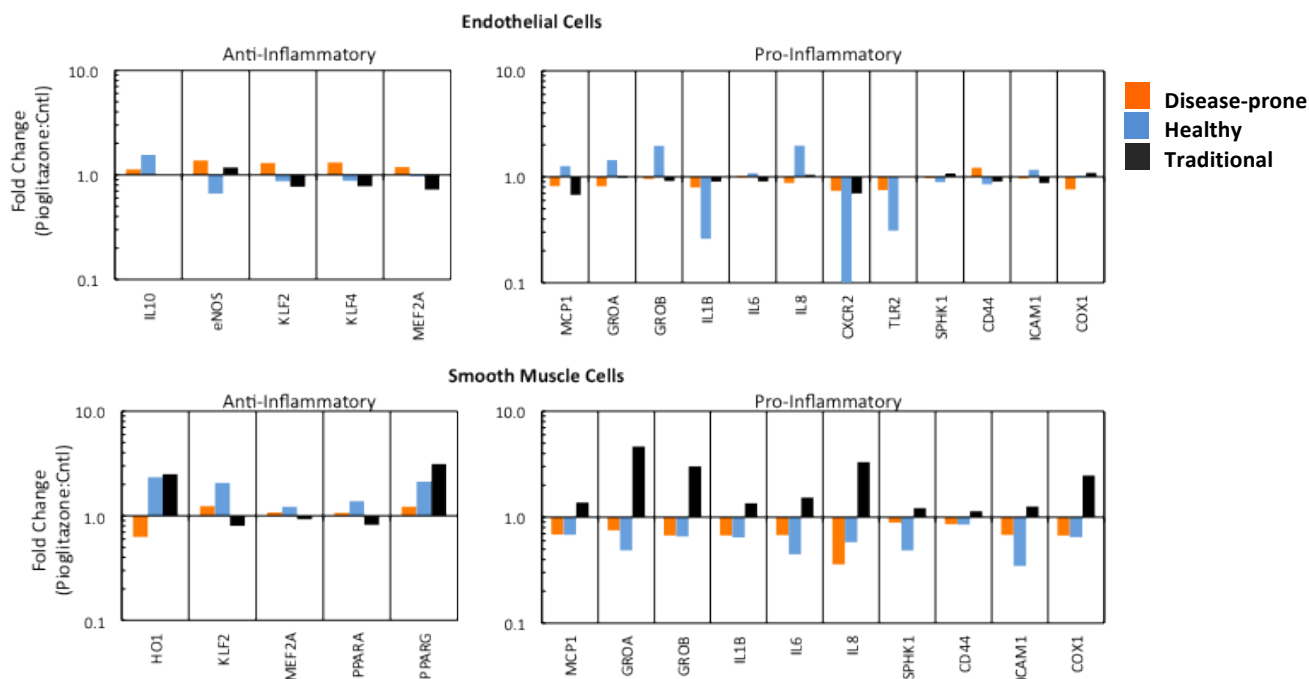


Figure 3. Anti- and pro- inflammatory genes regulated by pioglitazone in endothelial and smooth muscle cells. Gene expression data per condition (Disease-prone, orange; Healthy, blue; Traditional, black) represent the fold change response to Pioglitazone relative to vehicle control for endothelial (top) and smooth muscle cells (bottom) for select inflammatory related genes. n=3 biological replicates.

where changes in gene expression are variable across conditions and gene categories. In general, compared to smooth muscle cells, modulation of the endothelial cell inflammatory phenotype was more subtle. For the Healthy and Disease-prone Flow conditions, gene modulation in endothelial cells due to Pioglitazone were much more modest. Though changes were small, some of the divergent responses in anti- and pro-inflammatory related genes could be explained by different basal levels of gene expression prior to pioglitazone exposure. For example, eNOS and KLF2 are endothelial cell anti-inflammatory genes that are high after Healthy Flow exposure compared to Disease-prone Flow (Hastings, et al., AJP, 2007). Exposure to pioglitazone yielded divergent responses likely due to signaling pathways dependent on starting levels of these genes. The IL1 β response in endothelial cells due to pioglitazone was one of the strongest from the pro-inflammatory category. IL1 β is a potent inflammatory mediator, known to stimulate production of TNF α . While pioglitazone has also been shown to reduce blood-circulating levels of

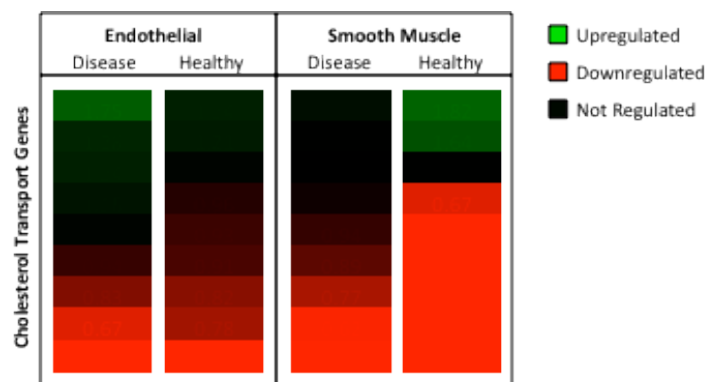
TNF α in diabetic patients (Ceriello, *Diabetes*, 2008), reduction in IL1 β suggests local suppression of these inflammatory pathways.

Together, data demonstrate that pioglitazone has a primarily anti-inflammatory effect on smooth muscle cells and a modest anti-inflammatory effect on endothelial cells under hemodynamic flow conditions, thus promoting vascular health.

Pioglitazone Alters Cholesterol Transport Related Genes in Endothelial and Smooth Muscle Cells

Cholesterol transport-associated genes are critical to regulating flux of these important sterol molecules. In the vasculature, cholesterol build-up in cells leads to development of plaque formation and atherosclerosis. Examination of a subset of genes related to cholesterol metabolism demonstrated effects on both endothelial and smooth muscle cells under Healthy and Disease-prone Flow conditions. Pioglitazone has been shown to alter the balance of the atherogenic lipid profile, such that HDL levels are increased

Figure 4. Heat plot analysis demonstrating cholesterol metabolism regulation due to pioglitazone. Fold changes from pioglitazone treated condition relative to control were determined and each gene having Cholesterol Transport characteristics were assigned a colorimetric value based on level of induction (green) or reduction (red) of gene expression. Data is sorted in order from high to low expression. n=3 biological replicates.



and free-fatty acid levels reduced (Erdmann and Wilcox, *Q J Med.*, 2009). Changes in circulating HDL and FFA levels will undoubtedly impact the blood vessel wall health (ie., driving down inflammation and NF-kB activation), but in the current study, direct effects of pioglitazone on the vasculature were uncovered. Overall, the most robust response was a reduction in cholesterol transport related genes in smooth muscle cells exposed to Healthy Flow (**Figure 4**). Closer study of these genes identified the downregulated genes such as ABCG1 (mediator of cholesterol efflux across membranes), SREBP1 and SREBP2 (complex regulators of cholesterol biosynthesis), as well as LXRB (a nuclear receptor

involved in lipid homeostasis control) (**Figure 5**). The significant downregulation of ABCG1 is surprising, since these proteins aid in cholesterol and oxysterol efflux; however, a recent report showed that in smooth muscle LXR-dependent reverse cholesterol transport is mediated by ABCA1 (Delvecchio, *et al.*, *AJP*, 2008). Although further investigation is required, perhaps this shift in ratio of ABCG1 to ABCA1 allows for more efficient cholesterol transport in smooth muscle. SREBP regulation may be helping to maintain this balance of cholesterol efflux proteins. Together these data suggest that pioglitazone can lead to a region-specific reduction in the efficiency of cholesterol transport in smooth muscle cells. Interestingly, this response was not consistent in endothelial cells, which are directly exposed to blood contents, and the first line of defense for molecules crossing the vasculature. Endothelial cells exposed to pioglitazone showed a strong decrease in oxidized LDL receptor (LOX-1) expression regardless of flow condition (**Figure 5**). This inhibition of LOX-1 reduces binding potential of oxidized LDL, thus reducing potential inflammatory responses (ICAM-1, IL-6, IL-8, COX-2), redox balance (HO-1) and lipid metabolism (LDLR, ABCA1). Other cholesterol transport related genes yielded less dramatic and divergent

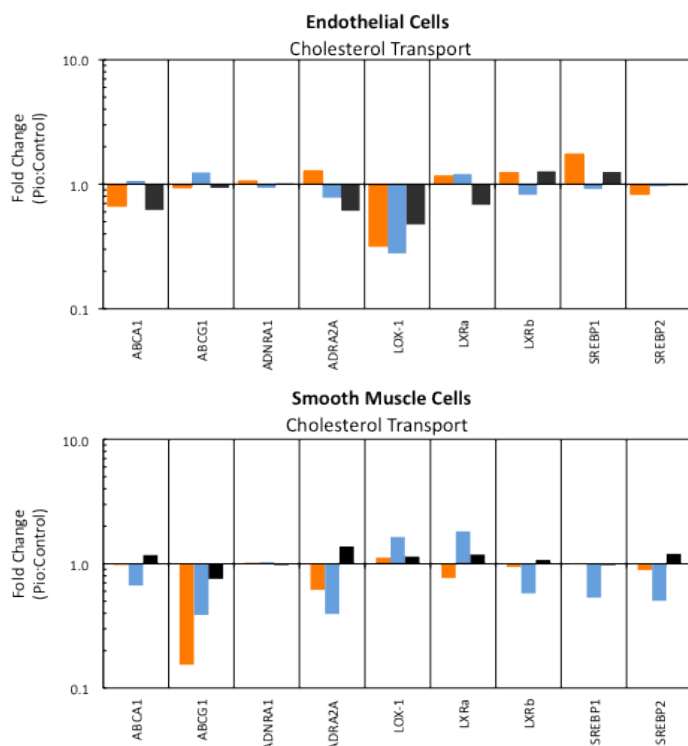


Figure 5. Cholesterol transport related genes regulated by pioglitazone. Gene expression data per condition (Disease-prone, orange; Healthy, blue; Traditional, black) represent the fold change response to pioglitazone relative to vehicle control for endothelial (top) and smooth muscle cells (bottom) for select cholesterol transport/metabolism related genes. n=3 biological replicates.



responses based on flow condition in endothelial cells, suggesting that LOX-1 is more directly regulated by activation of the PPAR- γ pathway. Interestingly, negative regulation of PPAR- γ in the system was observed in endothelial cells exposed to the Healthy Flow condition at this 24 hour time point, but positive regulation for the Disease-prone Flow condition. Increased PPAR- γ was noted in smooth muscle cells for both flow conditions. (*Data not shown*) This may provide mechanistic insight into the chronic exposure of pioglitazone on vascular cell responsiveness. In endothelial cells, PPAR- γ was increased in the Disease condition by pioglitazone, but decreased in the Healthy and Traditional condition. This may suggest different basal levels of PPAR- γ due to flow preconditioning and the ability of pioglitazone to further induce gene expression under specific flow conditions. Further investigation of the influence of pioglitazone on endothelial and smooth muscle cells is warranted to identify mechanisms by which the drug acts to alter both direct and off target effects related to cholesterol metabolism.

Pioglitazone has No Effect on Endothelial Cell Permeability during Disease-prone Flow

Pioglitazone effects on endothelial cell permeability during Disease-prone flow were examined as part of this study. Barrier function becomes compromised when permeability is increased, thus the vessel wall is more susceptible to infiltration of inflammatory mediators from the blood stream. Vascular permeability due to pioglitazone is unexplored in the literature, and has never before been tested under hemodynamic conditions. Endothelial cells in monoculture were plated and preconditioned to Disease-prone flow using HemoShear's proprietary label-free real-time permeability technology. Cells were then exposed to a 16.1 μ M dose of pioglitazone for an additional 24 hours. Resistance measurements were converted to permeability (1/Resistance) and normalized to the initial resistance value. No significant differences compared to vehicle control were observed in response to the pioglitazone treatment (*data not shown*). In Disease-prone Flow regions of the vasculature for a standard daily dose, pioglitazone does not alter barrier function.

Study Conclusions

In this study, we examined the response of pioglitazone on human endothelial and smooth muscle cells using traditional (no flow) methods, as well as HemoShear's Healthy and Disease-prone flow patterns. Under hemodynamic flow conditions, pioglitazone reduced the smooth muscle cell inflammatory response, suggesting protective effects of PPAR- γ activation by this drug on the vasculature. This response, which was the most robust observation due to pioglitazone treatment, was completely opposite for smooth muscle cells exposed to the traditional, no-flow condition. Reduction of some endothelial cell inflammatory markers was also detected, supporting overall influence of pioglitazone on vascular arterial wall health.

We demonstrated pioglitazone's ability to reduce gene expression of the receptor for oxidized LDL in Endothelial Cells as well as alter regulation of other cholesterol transport regulated genes in smooth muscle cells. Downregulation of LOX-1 functionally may indicate a reduction in binding of monocytes/inflammatory cells to the endothelium and thus protective effects on the vasculature.

As with our previous case study with atorvastatin, this current study with pioglitazone highlights the differences between traditional and flow-based culture models. Ultimately, the hemodynamic environment is critical to studying physiologically relevant responses of the vasculature and emphasizes the need for more predictive human-based models such as HemoShear's technology.

Future work investigating TZDs in the HemoShear system involves a direct comparison of different diabetes compounds (e.g., rosiglitazone, pioglitazone, and troglitazone) to demonstrate the sensitivity of the technology to distinguish between drugs within a single class. Testing of pioglitazone and other TZD compounds in an elevated glucose model may yield a more relevant response in conditions under which patients would use such drugs.



Study Team

Scientific Oversight and Data Interpretation

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

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

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



Biographical Sketches

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

Dr. Blackman is co-inventor of the HemoShear technology and co-founder of HemoShear. He is a tenured associate professor in the Department of Biomedical Engineering at UVa. Since August 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 to joining UVa, Dr. Blackman spent more than three 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 published 17 peer-reviewed articles, 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 also co-inventor of the HemoShear technology and co-founder of HemoShear. He is a tenure-track associate professor at UVa and leads an NIH-funded laboratory that studies vascular disease at the Cardiovascular Division of UVa'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 in 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 with a minor in business administration from Rhodes College and obtained his PhD from the University of Missouri, where he developed swine models of diabetes and atherosclerosis. Dr. Wamhoff has 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. He has authored or coauthored 32 publications, two book chapters, and three commentaries. Dr. Wamhoff serves as peer reviewer for more than 10 major scientific journals and is 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, the 2008 Atherosclerosis, Thrombosis, and Vascular Biology (ATVB) Irvine Paige Award, and the 2010 American Physiological



Biographical Sketches

Society New Investigator Award for Cardiovascular Research. 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.

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 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 co-author 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 discovery and 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 work in strategic partnerships with 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, and inflammation. We are already developing a hemodynamic, co-culture liver model, and we plan to develop hemodynamic models of the blood-brain barrier, kidney, and other organ systems.

The present HemoShear artery model provides 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. Our partnerships will result in competitive advantages for our customers by developing innovative drugs with superior efficacy and safety characteristics.