Endothelial dysfunction in cerebral aneurysms

*Dallas L. Sheinberg, BS, David J. McCarthy, MSc, Omar Elwardany, MD, Jean-Paul Bryant, MSc, Evan Luther, MD, Stephanie H. Chen, MD, John W. Thompson, PhD, and Robert M. Starke, MD, MSc

Department of Neurosurgery, University of Miami, Florida

Endothelial cell (EC) dysfunction is known to contribute to cerebral aneurysm (CA) pathogenesis. Evidence shows that damage or injury to the EC layer is the first event in CA formation. The mechanisms behind EC dysfunction in CA disease are interrelated and include hemodynamic stress, hazardous nitric oxide synthase (NOS) activity, oxidative stress, estrogen imbalance, and endothelial cell-to-cell junction compromise. Abnormal variations in hemodynamic stress incite pathological EC transformation and inflammatory zone formation, ultimately leading to destruction of the vascular wall and aneurysm dilation. Hemodynamic stress activates key molecular pathways that result in the upregulation of chemotactic cytokines and adhesion molecules, leading to inflammatory cell recruitment and infiltration. Concurrently, oxidative stress damages EC-to-EC junction proteins, resulting in interendothelial gap formation. This further promotes leukocyte traffic into the vessel wall and the release of matrix metalloproteinases, which propagates vascular remodeling and breakdown. Abnormal hemodynamic stress and inflammation also trigger adverse changes in NOS activity, altering proper EC mediation of vascular tone and the local inflammatory environment. Additionally, the vasoprotective hormone estrogen modulates gene expression that often suppresses these harmful processes. Crosstalk between these sophisticated pathways contributes to CA initiation, progression, and rupture. This review aims to outline the complex mechanisms of EC dysfunction in CA pathogenesis.

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Cerebral aneurysms (CAs) have an estimated prevalence of 1%–3% and are responsible for 80%–85% of all spontaneous subarachnoid hemorrhages. CA formation and progression constitute a multifactorial disease process with dynamic contributions from genetic drivers, inflammatory reactions, and hemodynamic stress. There is substantial evidence showing that endothelial cell (EC) dysfunction plays a large role in CA pathogenesis, with damage or injury to the EC layer cited as the first event in CA formation. Through molecular crosstalk to vascular smooth muscle cells (VSMCs) and the intravascular lumen, ECs regulate vascular tone and local inflammatory reactions. Proper EC function is essential to vascular integrity through the regulation of VSMC proliferation, prevention of unnecessary platelet to vessel adhesion, and management of leukocyte recruitment. EC expression and functionality is affected by their interactions with various lumen-based molecular and mechanical stimuli.

EC dysfunction and damage is commonly reported in both in vivo and in vitro CA laboratory investigations. Histopathological and clinical observations of human CA pathogenesis have validated these laboratory findings. This review explores the literature in order to clearly delineate the contribution of EC dysfunction in CA disease.

Endothelial Cells and Hemodynamic Stress

Under physiologically normal conditions, there is a balance between the integrity of the vascular wall and the...
hemodynamic forces acting upon it. Current literature suggests that abnormal hemodynamic stress can incite pathogenic EC transformation that contributes to CA disease. Intravascular hemodynamic stress is composed of two separate entities, fluid force vectors and blood pressure, which fundamentally translate into perpendicular transmural pressure, circumferential cyclic stretch, and wall shear stress (WSS). Both transmural pressure and cyclic stretch are derived from blood pressure, whereas WSS is a tangential force exerted by flow vectors.

When examining hemodynamic force in CA disease, most of the literature focuses on the effect of WSS. Mathematically, WSS is the force that a fluid in motion exerts on the walls that surround it: \( WSS = \mu (dx/dy) \), where \( \mu \) is the dynamic viscosity of fluid, \( x \) is the flow velocity parallel to the wall, and \( y \) is the distance to the wall. Variations in the magnitude and duration of WSS have a significant effect on EC morphology and gene expression.

Following the properties of fluid dynamics, blood flows within the vessels in either a laminar or turbulent manner. Laminar flow is unidirectional, consisting of parallel flow vectors that do not mix during fluid transport. Pulsatile laminar blood flow occurs within the vasculature under normal conditions. Conversely, turbulent flow consists of nonuniform, chaotic, and volatile flow vectors. When blood encounters arterial branch points, the change in vessel angle results in an increased WSS at the bifurcation apex, with larger branching angles resulting in higher WSS magnitudes. In comparison, flow within an aneurysm dome is turbulent. Normal laminar flow exerts medium WSS, whereas turbulent flow exerts low WSS because of less parallel fluid vectors. ECs respond differently when subjected to abnormal WSS magnitudes.

Throughout the pulsatile cardiac cycle, cerebral ECs are subjected to an estimated WSS of 9.5–15 dyne/cm², with higher levels localized at the arterial bifurcation apex. Because of the variation in WSS magnitude and duration, it has proven difficult to properly organize a cohesive EC WSS response profile. In addition to the deviations from normal WSS magnitude, there are acute and prolonged changes in WSS that affect ECs differently. Thus, in their review of hemodynamic stress in cerebral vascular disease, Nixon et al. organized the EC WSS response profiles according to prolonged and acute WSS. Because of the variation in WSS magnitude and duration, it has proven difficult to properly organize a cohesive EC WSS response profile. In addition to the deviations from normal WSS magnitude, there are acute and prolonged changes in WSS that affect ECs differently. Thus, in their review of hemodynamic stress in cerebral vascular disease, Nixon et al. organized the EC WSS response profiles according to prolonged and acute WSS.

When subjected to normal WSS, ECs demonstrate healthy maintenance and proliferation through the suppression of cyclin-dependent kinase activity. Furthermore, they effectively balance the mTOR signaling cascade through dual activation of antimitotic AMPK and proliferative Akt. ECs exposed to prolonged normal WSS typically promote a low inflammatory environment through the downregulation of nuclear factor kappa B (NF-κB) and epigenetic gene repression. With respect to cellular morphology, normal WSS ECs organize themselves into stretched, elongated rows that are oriented in the direction of flow, containing long parallel actin cytoskeletons.

In recent decades many investigators have shown that deviations from normal WSS contribute to various aspects of EC dysfunction in CA pathogenesis. Initially in CA models in rodents, Kondo et al. found that normally high WSS leads to CA formation. Building on this, Fukuda et al. observed that the initial stages of CA pathogenesis occurred at the vessel bifurcation intimal pad and the region distal to it, which they defined as the juxta-apical groove (JAG). They noted that these JAG regions were subjected to the highest magnitude of WSS and were associated with EC degenerative changes that ultimately led to CA formation. Given these morphological observations, they classified the early EC degenerative changes into five consecutive grades (Table 1). In later studies, Jamous et al. similarly observed that CA formation began with high WSS and EC injury in the “area just distal to the apical intimal pad,” the same region Fukuda et al. defined as the JAG. Jamous et al. further studied CA formation at bifurcation sites, creating a 3-stage classification system that delineated aneurysm formation and progression (Fig. 1). Stage I was characterized by abnormal EC morphological changes and loss of eNOS expression. Stage II was marked by inflammatory zone formation, which catalyzed proteolytic destruction of the vascular wall. Stage III embodied the clinical representation of CA disease, with saccular dilation.

More recently, Aoki et al. uncovered a molecular pathway for high WSS–induced endothelial degeneration. They demonstrated that initial high WSS (approximately 15 dyne/cm²) induces an EC self-amplified loop of COX-2–PGE₂–EP₂–NF-κB (cyclooxygenase 2, prostaglandin E2, prostanooid receptor EP2, nuclear factor kappa B) that eventually leads to CA pathogenesis. In the middle of this pathway, the transcription factor NF-κB has a decisive role in aneurysm formation and progression. Specifically, it leads to increased expression of vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1), which are responsible for inflammatory cell adhesion and recruitment. Subsequent macrophage infiltration increases inflammatory cytokines (tumor necrosis factor alpha [TNF-α], interleukin 1 beta [IL-1β]) and matrix metalloproteinases (MMP-2/9), which propagate inflammatory response and the degradation of matrix proteins and apoptosis of smooth muscle cells (Jamous stage II). Furthermore, inhibition/knockout of COX-2 or EP₂ resulted in decreased NF-κB expression with a reduced incidence of CA formation.

While high WSS contributes to CA formation, turbulent flow within the aneurysm dome exerts low WSS and contributes to growth and rupture. ECs subjected to low WSS appear round with short disorganized actin filaments and have high turnover rates with increased apoptotic activity via the protein kinase C zeta (PKCζ) and p53 pathway. Furthermore, ECs exposed to low WSS have an increased expression of NF-κB, potentiating the already present macrophage infiltration. The combina-
TABLE 1. Summary of CA formation stages and associated EC signaling pathways

| Stage I | Pathological Changes | Signaling Pathways |
|---------|----------------------|--------------------|
| Grade 1 | No damage            | ↑ WSS → ↑ NADPH oxidase → ↑ ROS |
| Grade 2 | Mild damage: wavy rippling of EC plasma membrane | ↑ COX-2–PGE₂–EP₃–NF-κB |
| Grade 3 | Moderate damage: some vacuoles in EC cytoplasm | ↑ MAP kinase → ↓ claudin, occludin |
| Grade 4 | Severe damage: EC deformation w/ or w/o many vacuoles in cytoplasm & nucleus | ↑ Ca²⁺-calmodulin binding + PKB → ↑ eNOS |

| Stage II | Grade 5 | Severe damage, macrophage infiltration, inflammatory zone formation, intimal pad elevation |
|----------|---------|------------------------------------------|
|          | ↑ TNF-α, IL-1 → ↑ COX-2 → ↑ ROS |
|          | ↑ TNF-α → ↑ YAP + TAZ → ↑ VCAM-1 |
|          | ↑ TNF-α, IL-1β → ↑ PKCζ + MAP kinase → ↑ NF-κB |
|          | ↑ NF-κB → ↑ VCAM-1, ICAM-1, MCP-1 |
|          | ↑ Macrophages → ↑ MMP-2/9 |
|          | ↑ ROS + IFN-γ, TNF-α, IL-1β → ↑ iNOS |
|          | ↑ iNOS → ↓ eNOS, nNOS; ↑ ROS |
|          | ↑ PKCζ & p63 |
|          | ↑ Macrophages → ↑ MMP-2/9 |

| Stage III | Destruction & protrusion of vessel wall, saccular dilatation |
|-----------|-------------------|
|           | ↑ PKCζ & p63 |
|           | ↑ Macrophages → ↑ MMP-2/9 |

↑ = increase; ↓ = decrease; → = leads to; COX-2 = cyclooxygenase 2; eNOS = endothelial nitric oxide synthase; EP₃ = prostaglandin receptor EP₂; ICAM-1 = intracellular adhesion molecule-1; IFN-γ = interferon gamma; IL = interleukin; iNOS = inducible NOS; MAP kinase = mitogen-activated protein kinase; MCP-1 = monocyte chemotactic protein-1; MMP-2/9 = matrix metalloproteinases 2/9; NADPH oxidase = nicotinamide adenine dinucleotide phosphate oxidase; NF-κB = nuclear factor kappa B; nNOS = neuronal NOS; PGE₂ = prostaglandin E₂; PKB = protein kinase B; PKCζ = protein kinase C zeta; ROS = reactive oxygen species; TAZ = transcriptional co-activator with a PDZ-binding motif (also known as WWTR1); TNF-α = tumor necrosis factor alpha; VCAM-1 = vascular cell adhesion molecule-1; YAP = Yes-associated protein.

These molecular pathways are not necessarily restricted to the above stages or grades. There is carryover between stages or grades throughout CA pathogenesis. Additionally, there is significant crosstalk between these pathways. For further information, refer to Fig. 1.

It has several important roles in vascular physiology including tone modulation, inhibition of smooth muscle cell proliferation, suppression of proinflammatory mediators, and maintenance of proper EC function and integrity. Nitric oxide (NO) is a hydrophobic molecule with a short half-life, allowing for easy passage through neighboring cell membranes and a short-lived localized effect. While eNOS is primarily regulated through Ca²⁺-calmodulin binding, it can alternatively be activated by laminar WSS through protein kinase B phosphorylation. In addition to upregulating eNOS activity, laminar WSS induces transient and prolonged eNOS mRNA transcription through c-Src–dependent pathways. When subjected to pathogenic low WSS, ECs fail to express eNOS. In contrast, acute or prolonged high WSS results in increased eNOS transcription and activation, which helps to prevent initial vessel injury (stage I of CA formation) through vasodilation. This vasodilation decreases WSS by increasing the distance to the vessel wall (see WSS equation above). Fukuda et al. supported this theory in CA models in the rat after observing that eNOS is upregulated at arterial bifurcation JAG points. This high WSS–induced eNOS expression is modulated by EC environments; for example, eNOS is decreased in hypoxia or hyperglycemia and increased by homocysteine. In rat CA tissue, however, eNOS expression was pathogenically decreased, suggesting that sometime during CA formation the endothelium loses the ability to effectively produce eNOS. Despite this theory, Aoki et al. observed that eNOS knockout mice had the same CA prevalence as wild-type mice. Surprisingly, the eNOS knockout mice effectively protected against CA formation through a compensatory increase in nNOS expression, which sufficiently replaced prior eNOS NO production. However, nNOS compensation is not observed in clinical CA disease. This laboratory to clinical discrepancy is likely attributable to the inability of a genetic knockout model to replicate the inflammatory environment observed in CA pathogenesis.
In CA formation, Jamous stage II is characterized by an increased inflammatory environment. While eNOS and nNOS exhibit CA protective effects, iNOS is expressed by macrophages and VSMC in abnormal conditions such as mechanical injury and inflammation. Increased iNOS expression has been observed in human CA tissue. iNOS activation and expression is calcium independent and is increased by reactive oxygen species (ROS) and inflammatory mediators such as IFN-γ, TNF-α, and IL-1β. Typically, the quantity of NO produced by eNOS is much less than that synthesized by iNOS, which generates large cytotoxic quantities of NO. In a porcine cerebral artery model, iNOS induction was shown to impair eNOS and nNOS activity, partly through a negative NO feedback loop. Other than the iNOS inhibition, abnormal genetic or environmental eNOS activity has been shown to contribute to CA pathogenesis. Originally discovered to have a significant association with coronary vasospasm, the T-786C eNOS single nucleotide polymorphism (SNP) is a thymine (T) to cytosine (C) substitution at a locus 786 that may affect CA pathogenesis. Human ECs containing this eNOS SNP have significantly reduced eNOS activity. A meta-analysis suggested that the polymorphism was associated with an increased risk of CA incidence in Asian populations. In patients with ruptured aneurysms, Khurana et al. found that homozygous C/C eNOS patients seemed to clinically present similar to T/T patients, whereas a heterogeneous T/C eNOS genotype was independently associated with a larger (> 10 mm) aneurysm size (p = 0.03). However, a more rigorous investigation by Akagawa et al., which balanced the enrollment of Korean and Japanese CA
patients, failed to find any association between CA size and the heterozygous genotype.\textsuperscript{1} To date, there remains no clear molecular explanation for why a homozygous C/C mutant genotype might behave similar to a homozygous wild-type genotype, with T/C heterozygotes demonstrating worse CA pathogenesis. In addition to genetically mutated eNOS, abnormal inflammatory environments or estrogen levels can dramatically alter eNOS functionality.

Estrogen and Endothelial Dysfunction

The steroid hormone estrogen has been shown to have antiinflammatory, antioxidiant, and vasoprotective effects.\textsuperscript{14,70} In ECs and VSMCs, estrogen binds to cytosolic estrogen receptors alpha (ER\textsubscript{a}) and beta (ER\textsubscript{b}). Upon binding to estrogen, the ER complex translocates into the nucleus to modify gene expression. Estrogen’s vasoprotective function is primarily attributable to its modulation of eNOS, elimination of ROS, and regulation of prostanooid metabolism.

Estrogen has multiple different pathways that upregulate both eNOS and nNOS. Strione et al. demonstrated that estrogen-ER\textsubscript{a} binding in cerebral ECs leads to both a rapid and a prolonged increase in eNOS activity via PI3-kinase/Akt and MAPK signaling pathways.\textsuperscript{14,63} In eNOS-deficient states, estrogen can initiate nNOS compensation, which helps to prevent CA formation.\textsuperscript{3,40} Furthermore, estrogen-deficient ECs downregulate eNOS expression, leading to increased CA pathogenesis.\textsuperscript{57} Estrogen-ER\textsubscript{a} binding also promotes vasoprotection through prostanoid (PGI\textsubscript{2})-mediated vasodilatation. In rodent models, the administration of estradiol enhances PGI\textsubscript{2} production via pathways involving COX-1 and PGI\textsubscript{2} synthase.\textsuperscript{57}

Interestingly, Tada et al. observed that ER\textsubscript{b} agonists, but not ER\textsubscript{a} agonists or estradiol, significantly reduced the CA incidence in ovariecotomized mice.\textsuperscript{64} This protective effect was negated after NO inhibition and ER\textsubscript{b} knockout, suggesting that the protective effect of estrogen-ER\textsubscript{b} occurred through NO generation. These authors’ findings challenge the role of estrogen-ER\textsubscript{a}, a known regulator of eNOS, in CA disease. Further studies have suggested that estrogen-ER\textsubscript{b} binding may help to prevent aneurysm rupture.\textsuperscript{65} In ovariecotomized mice, Xing et al. demonstrated that estrogen-ER\textsubscript{b} activation decreased the expression of NF-kB, a known CA inflammatory transcription factor.\textsuperscript{70} In a similar rodent model, Maekawa et al. demonstrated that treatment with the ER modulator bazedoxifene significantly decreased aneurysm rupture incidence from 52\% to 17\%.\textsuperscript{46} Furthermore, bazedoxifene decreased levels of IL-1\textbeta and MMP-9 and restored basal mRNA levels of ER\textbeta and ER\textalpha.\textsuperscript{48}

Other studies have examined the effect of estrogen on oxidative stress, showing that each ER has a distinct role in the expression of superoxide dismutase 2 (SOD-2), an enzyme that aids in ROS elimination. ER\textalpha is responsible for increased SOD-2 activation, while ER\textbeta regulates its basal expression.\textsuperscript{53} Estrogen also mediates the expression of NADPH oxidase, an enzyme that produces ROS. Estrogen-deficient states lead to a downregulation of SOD-2 and upregulation of NADPH oxidase, which results in oxidative stress and subsequent vascular injury.

The role of estrogen in CA pathogenesis has been clinically validated. In a meta-analysis of 68 studies, Vlak et al. reported an overall female/male unruptured aneurysm prevalence ratio of 1.57, which rose to 2.2 among adults older than 50 years.\textsuperscript{68} This suggests an increased CA risk in postmenopausal estrogen-deficient women. Other investigations have significantly linked female sex to aneurysm formation (OR 4.73), one of which even cited it as the sole independent risk factor in elderly women (ages 70–84 years).\textsuperscript{3,39}

Endothelial Cell-to-Cell Adhesions

EC-to-EC (EC-EC) junctions are composed of tight junctions, adherens junctions, and PECAM-1 (CD31), which maintain vessel wall integrity and regulate paracellular transport. As previously discussed, abnormal WSS and iNOS activity lead to increased quantities of inflammatory cells (macrophages, B lymphocytes, T lymphocytes, and natural killer cells) in CA tissue. There is mounting evidence that EC-EC junction dysfunction significantly contributes to CA pathogenesis.

Tight junctions are constructed from occludins, claudins, and junctional adhesion molecules. Using scanning and transmission electron microscopes in rats, Tada et al. observed EC-EC junction morphological changes at arterial bifurcations in early aneurysm stages.\textsuperscript{66} Early endothelial damage consisted of interendothelial gap formation without obvious arterial wall depressions, coinciding with Jamous stage I. Gap formation was followed by leukocyte adhesion and migration into the paracellular space, coinciding with Jamous stage II. Further progression led to expansion of this defect resulting in outward bulging of the vessel wall, coinciding with Jamous stage III. Initial EC gap formation was associated with a reduction of the tight junction proteins occludin and ZO-1, with persistent normal expression of adherens junction proteins and PECAM-1. Thus, the EC degeneration observed in early CA disease is likely accompanied by interendothelial gap formation due to a pathological reduction in tight junction proteins.\textsuperscript{53} Furthermore, the unaltered expression of PECAM-1 likely promotes leukocyte traffic through these interendothelial gaps, promoting a CA inflammatory environment.

ROS generation also contributes to interendothelial gap formation, cellular morphological change, and actin filament reorganization.\textsuperscript{44} Low WSS decreases the expression of occludins via a combination of ROS and MAPK signaling pathways.\textsuperscript{73} Ultimately, these cellular changes result in increased paracellular permeability and enhanced inflammatory cell infiltration.

Oxidative Stress and Endothelial Cells

There are multiple sources of oxidative stress that contribute to CA formation, progression, and rupture. Known CA risk factors that contribute to ROS generation include cigarette smoke, alcohol, and hypertension. In a prior review, we discussed the major pathways of oxidative stress in CA pathogenesis including atherosclerosis, hemodynamic stress, endothelial dysfunction, VSMC phenotypic modulation, vessel wall remodeling, and apoptotic cell...
death. These pathways differentially contribute to the formation of ROS, such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and peroxynitrite (ONOO$^-$).

The main enzymatic sources of ROS in the cerebral vasculature include COX-2, lipoxigenase, and NADPH oxidase. The latter is utilized by cerebral ECs, VSMCs, and macrophages to produce O$_2^-$ and H$_2$O$_2$ in response to growth factors, cytokines, and hemodynamic stress. In a positive feedback loop, free radicals propagate NADPH oxidase production of ROS. Similarly, the COX-2 pathway is a major source of O$_2^-$ generation in response to IL-1 and TNF-$\alpha$. Other studies have demonstrated that cyclooxygenase and lipoxygenase pathways may be important sources of free radicals in CAs.

Another potential source of ROS in the cerebral vasculature is NOS. While this primarily occurs through iNOS production of cytotoxic levels of NO and subsequent ONOO$^-$ formation, ROS are also formed through eNOS uncoupling, where eNOS starts to produce O$_2^-$.

Though the mechanisms behind eNOS uncoupling are not entirely understood, popular theories include eNOS S-glutathionylation, L-arginine depletion, and eNOS cofactor BH$_4$ oxidation to BH$_2$. Reversal of the above mechanisms through BH$_4$ or L-arginine supplementation has been shown to restore EC function in common cardiovascular diseases such as hypercholesteremia, hypertension, and type 2 diabetes mellitus. However, the role of eNOS uncoupling in CA disease still needs elucidation.

Oxidative stress impairs the endothelial barrier by increasing EC permeability, enhancing leukocyte adhesion, and altering endothelial signal transduction and redox-regulated transcription factors. In response to oxidative stress, macrophage release of proinflammatory cytokines TNF-$\alpha$ and IL-1$\alpha$ increases endothelial NF-$\kappa$B via PKC and MAPK-dependent pathways. TNF-$\alpha$ also upregulates endothelial VCAM-1 expression through the transcriptional activators YAP/TAZ. As previously discussed, this leads to numerous CA potentiating effects, including increased leukocyte recruitment, adhesion, and infiltration. In an amplifying cycle, these newly recruited macrophages potentiate ROS production via NADPH oxidase, resulting in additional inflammatory cytokine production. This cycle is further fueled by ROS impairment of EC tight junction proteins, which increases leukocyte vessel infiltration. The free radical scavengeredaravone has been shown to inhibit CA formation in animal studies. The treatment of CA disease through ROS targeting offers a promising new opportunity for future bench to clinic translation.

Overall, these mechanisms of oxidative stress in CAs demonstrate a strong link among inflammation, hemodynamic stress, and aneurysm pathogenesis.

Conclusions

There is extensive laboratory and clinical evidence supporting the role of EC dysfunction in CA pathogenesis. The processes leading to this dysfunction are multifactorial and include hemodynamic stress, hazardous NOS activity, oxidative stress, estrogen imbalance, and EC-EC junction compromise. These interrelated pathways ultimately lead to EC dysfunction with ensuing CA initiation and progression. EC dysfunction is characterized by the loss of vasoprotective, antiinflammatory, antiatherogenic, and vasodilatory properties. Further investigation into the mechanisms of this complex process is essential to clearly elucidate CA pathogenesis and aid in the development of novel treatment modalities.

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Author Contributions
Conception and design: Sheinberg, McCarthy, Elwardany, Bryant, Luther, Thompson, Starke. Acquisition of data: Sheinberg, McCarthy, Elwardany, Bryant, Luther, Thompson, Starke. Analysis and interpretation of data: Sheinberg, McCarthy, Elwardany, Bryant, Luther, Thompson, Starke. Drafting the article: Sheinberg, McCarthy, Elwardany, Bryant, Luther, Thompson. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Sheinberg.

Correspondence
Dallas L. Sheinberg: University of Miami Miller School of Medicine, Miami, FL. dls226@med.miami.edu.