Published in final edited form as:

Histochem Cell Biol. 2011 February ; 135(2): 141–152. doi:10.1007/s00418-011-0783-5.

The endothelial microenvironment in the venous valvular sinus: thromboresistance trends and inter-individual variation

W. E. Trotman

Department of Pathology, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405, USA

D. J. Taatjes

Department of Pathology, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405, USA

P. W. Callas

Biostatistics Unit, University of Vermont College of Medicine, 25 Carrigan Avenue, Burlington, VT 05405, USA

E. G. Bovill

Department of Pathology, University of Vermont College of Medicine, 89 Beaumont Avenue, Burlington, VT 05405, USA

Abstract

The valve sinuses of the deep venous system are frequent sites of venous thrombus initiation. We previously reported that, in comparison with the non-valvular luminal endothelium, the valve sinus endothelium had decreased expression of von Willebrand factor (vWF) and increased expression of endothelial protein C receptor (EPCR) and thrombomodulin (TM), suggesting alteration in the procoagulant/anticoagulant balance. We hypothesized that increased stasis in the deeper recesses of the venous valves would be associated with a gradient of increased thromboresistance. Expression of EPCR, TM, and vWF was analyzed via quantitative confocal immunofluorescence in residual saphenous veins collected following coronary artery bypass procedures. In agreement with our hypothesis, endothelial expression of vWF in the valve sinus decreased from the uppermost to the deepest region of the valve sinus. In contrast to our hypothesis, EPCR expression decreased from the uppermost to the deepest region of the valve sinus ($p < 0.001$) and TM expression remained unchanged throughout the valve sinus. Comparison of the non-valvular luminal endothelium with the valve sinus endothelium demonstrated significantly decreased vWF expression ($p < 0.001$) in the valvular sinus consistent with our previous report; however, we did not observe statistically significant differences in EPCR or TM expression in this comparison. In addition, remarkable inter-individual variation in expression of these three proteins was also observed. These findings suggest that the genesis of these observations is more complex than predicted by our initial hypothesis, likely due, at least in part, to the complex rheology of the valvular sinus microenvironment.

Keywords

Endothelial protein C receptor; Thrombomodulin; Venous thrombosis; Venous valve sinus; von Willebrand factor; Confocal microscopic image analysis

Introduction

The valvular sinuses of the deep venous system are generally accepted as frequent sites of venous thrombus initiation. The evidence for this association consists of autopsy studies (Paterson and McLachlin 1954; Sevitt 1974) and the association connecting the frequency of venous thrombosis to the number of valves in individuals (Liu et al. 1986). In the older literature, histologic evidence showed intact endothelium in non-trauma related deep venous thrombosis (DVT) with overlying red, fibrin-rich “venous thrombus” favoring tissue factor (TF) driven fibrin deposition over platelet adhesion as the initiating event (Sevitt 1974). Thrombus initiation at these sites is commonly attributed, at least in part, to stasis which has been objectively observed in venography studies, where contrast media lingered in the valvular sinuses for up to an hour following the study, particularly in the elderly (McLachlin et al. 1960). Valvular sinus stasis has been associated with hypoxia with a gradient of increasing hypoxia from the top to the bottom of the valve sinus (Hamer et al. 1981). Furthermore, blood flow within the valvular sinus has been characterized by the formation of vortices as observed by ultrasound (Lurie et al. 2003). In the valve sinus, the endothelium is exposed to two counter-rotating vortices (Karino and Motomiya 1984) with the deepest region of the valve exposed to the smaller vortex with the lowest velocity and thus, the greatest degree of stasis. Red cell clumping was observed in the smaller secondary vortex at the base of the valve. In addition, stasis prevents the efflux of activated clotting factors and increases the likelihood of leukocyte-endothelial interaction. These phenomena together create a potentially hypercoagulable micro-environment particularly in the deepest part of the sinus which is thought to be the anatomic site of thrombus initiation (Sevitt 1974).

Little data are available with respect to the mechanism(s) underlying the local events at the vessel wall during venous thrombogenesis; however, the venous valvular sinus appears to represent a unique microenvironment. In recent years, the endothelium has been demonstrated to have a remarkable ability to adapt to a wide range of microenvironments, both with respect to morphology (Aird 2007a, b) and the expression of critical cell surface and cytoplasmic proteins (Aird 2005; Chi et al. 2003). As an example, we recently demonstrated, by quantitative confocal fluorescence analysis, that the valve sinus has a thromboresistant phenotype when compared with the adjacent luminal endothelium, characterized by higher endothelial expression of endothelial protein C receptor (EPCR) and thrombomodulin (TM) and lower expression of von Willebrand factor (vWF) (Brooks et al. 2009). In that study, we analyzed the valve sinus endothelium in only the deepest region of the valve sinus with the greatest degree of stasis. To better understand the genesis of this thromboresistant micro-environment, we have hypothesized that TM and EPCR expression would be highest in the deepest region of the valve sinus, and conversely vWF expression would be lowest in the deepest recess of the sinuses, characterized in each instance by a progressive expression gradient as a function of the degree of stasis and resulting hypoxia. In pursuit of this hypothesis, we measured the endothelial expression patterns of the anticoagulant proteins TM, EPCR, and the procoagulant vWF longitudinally at several locations along the midline of the valve sinus and adjacent non-valvular luminal endothelium.

Materials and methods

Tissue processing

Nine greater saphenous veins were collected from nine patients undergoing Coronary Artery Bypass Grafting at Fletcher Allen Health Care, Burlington, VT. These saphenous vein specimens were from a different set of patients than those analyzed in our previous publication (Brooks et al. 2009). The mean age of the patients was 67 years (standard

deviation = 8; range = 59–75). This study was reviewed and approved by the Human Experimentation Committee of the University of Vermont and informed consent was obtained in accordance with the Declaration of Helsinki.

Immediately following surgical removal, the specimens were gently perfused in an antegrade fashion with the donor's heparinized blood and then immersed in heparinized blood in a bowl on the surgical tray. All harvested veins were received for processing within 2–3 h of harvest. Each specimen was then perfused in antegrade fashion with 10% neutral buffered formalin to flush out the blood and subsequently in a retrograde direction to fix the valve shut. Following perfusion, specimens were further fixed by immersion in 10% neutral buffered formalin for 24 h. The fixed specimens were opened longitudinally, and longitudinal segments were taken at the midline of the valve cusp (Fig. 1a), and embedded on edge in paraffin with the deepest region of the valve facing the block face.

Tissue sectioning and immunofluorescence

Several 5-micron serial sections were taken starting at the deepest region of the valve sinus (midline) (Fig. 1a). One of these sections was stained with hematoxylin and eosin to check for intact endothelium. Adjacent sections were used for subsequent immunofluorescence staining. Tissue sections were subjected to routine processing for immunofluorescence including deparaffinization, rehydration, and antigen retrieval with 10 mM sodium citrate. Slides were then blocked in pre-immune donkey serum for 1 h at room temperature. The following primary antibodies were used in cocktail form for an overnight incubation at 4°C: monoclonal mouse anti-Thrombomodulin (anti-TM; clone #1009, Lab Vision, Fremont, CA), polyclonal goat anti-Endothelial Protein C Receptor (anti-EPCR; R&D Systems, Minneapolis, MN), and polyclonal rabbit anti-von Willebrand Factor (anti-vWF; DAKO, Carpinteria, CA). After several rinses with phosphate buffered saline, secondary antibodies were applied in cocktail form at 4 µg/mL for an 1-h incubation at room temperature: donkey-anti-rabbit Alexa488, donkey-anti-goat Alexa647, and donkey-anti-mouse Alexa555 (Invitrogen, Carlsbad, CA). Nuclei were visualized with 4,6-diamino-2-phenylindole hydrochloride (DAPI). A secondary antibody negative control was used to check for non-specific binding of the secondary antibodies and to set minimum threshold values for quantitative fluorescence analysis (see below). For control slides, pre-immune donkey serum was left on the slide during primary antibody incubation. A cocktail of species-appropriate fluorescence conjugated secondary antibodies listed earlier was then added to the slides as a negative control. No immunofluorescence labeling of those slide was observed. These slides were used to set minimum threshold intensity valves.

Confocal microscopy

Immunostained slides were imaged with a Zeiss 510 META confocal laser scanning microscope (Zeiss MicroImaging, Thornwood, NY). A Plan-Neofluar 25×/0.8 NA multi-immersion objective lens was used for quantitative analysis. All images were acquired in 12-bit multi-track mode, with appropriate laser excitation and emission filters as previously described (Brooks et al. 2009). To capture confocal images for quantitative analysis, the detector gain was adjusted between specimens so that each of three fluorophore channels representing each antibody, respectively, only had one pixel of saturation. The detector gain adjustment was necessary to avoid unquantifiable pixel saturation resulting from the variable staining intensities observed between patient samples. However, within the individual specimens, the detector gain was held constant for each antibody allowing for intra-specimen comparisons of staining intensity. A percentage conversion for changes in detector gain settings was used to allow for direct comparisons of pixel intensities amongst the different patient samples (Sedgwick 2008). Amplifier gain was held constant for all antibodies.

Quantitative analysis

Images were analyzed using MetaMorph Imaging Series 7.1 image analysis software (Molecular Devices, Sunnyvale, CA). Images were opened as a set of four planes representing separate fluorophores. To merge all four fluorophores, an overlay image was created, with careful assigning of different colors for each antibody. Blue, white, red, and green were chosen to represent nuclear staining, vWF, TM, and EPCR immunostaining, respectively. This newly created reference image allowed for accurate drawing of a region of interest to be transferred to individual fluorophore images for each integrated intensity measurement. Graphic outlines highlighting three 400 μ m longitudinal segments of cusp, valve wall, and non-valvular luminal endothelium were traced on each image for quantitation (Fig. 1b).

The fluorescence intensity of antibody binding in each representative area was assessed by assigning a minimal pixel intensity threshold value (600 for anti-EPCR and 500 for anti-vWF and anti-TM), determined by secondary antibody control incubations. The integrated intensity measurement was determined automatically in each 400- μ m long region of interest by measuring all pixels with intensity values greater than the minimum set threshold. The intensity values were logged onto an excel spreadsheet from which charts were created. For each protein, arbitrary intensity units in each representative area (nine) were compared after numbers were corrected for adjustments in gain settings.

Areas of analysis

The two areas of analysis in the midline of each valve included overall location and region (Fig. 1b). Overall locations included the valve wall, cusp, and non-valvular luminal endothelium, respectively. Regional analysis included proximal (P), middle (M), and distal (D) regions within each overall location. In total, nine representative areas were analyzed to quantify EPCR, TM, and vWF expression, respectively, including three regions of the valve cusp (PC, MC, DC), the valve wall (PW, MW, DW), and non-valvular luminal endothelium (PL, ML, DL). Figure 2 is a representative example of the composite images used for the analysis of the confocal immunofluorescence data. Figure 3 is a representative section of a venous valve and vein wall with split and merged confocal images, which was merged into the composite image shown in Fig. 2.

Statistical analysis

The primary analysis compared trends in intensities of each factor across regions within each vein. For statistical testing, the null hypothesis was that there was no difference in intensities for the regions within a vein, and the alternative hypothesis was that intensities increased or decreased across regions. Although we had a priori scientific hypotheses about the expected direction of those trends, we did not use directional hypotheses for statistical testing so that we would not miss differences that were in the opposite direction of what we expected (i.e., we used two-sided rather than one-sided hypothesis testing). Repeated measures analysis of variance was used to test for trends in intensities across regions within veins. This analytic method determined if the trends in intensities across the three regions within each vein occurred more often than would be expected by chance for the nine veins (Winer et al. 1971).

We also compared non-valvular luminal endothelial intensities with intensities in each region in the valve wall and valve cusp using paired *t* tests. Comparing DL to DW for vWF, for example, the difference in vWF intensity between DL and DW was computed for each of the nine veins. The paired *t* test then analyzed the mean of these differences to determine if the differences in intensities were on average significantly different than zero or not.

To assess inter-individual variation, we used one-way analysis of variance to compare intensities of vWF, EPCR, and TM within the valvular sinus across the nine veins in the study. This analysis was conducted separately for the valve wall and valve cusp endothelium. Looking at vWF in the valve wall endothelium for example, one-way analysis of variance tested to determine if the mean intensity of vWF at this location was the same or different across the nine individual veins.

We conducted each statistical test using a type I error rate of 0.05, which means that if there were truly no differences, we would incorrectly conclude that there is a difference 5% of the time for each test. Although there are methods for reducing this error rate when making a number of statistical comparisons, we did not adjust for multiple comparisons because as shown in an earlier study in the field, it is preferable to minimize type II error (Brooks et al. 2009). By describing all comparisons that were made, the overall likelihood of a type I error can be assessed in context (Perneger 1998; Rothman 1990).

Results

Comparison of the non-valvular endothelium with the valvular sinus endothelium

In a recent paper, we observed that the expression of the procoagulant protein vWF was lower at the base of the valvular sinus compared with the non-valvular luminal endothelium just distal to the valve cusp. Conversely, we observed that expression of the anticoagulant proteins EPCR and TM were higher at the base of the valvular sinus compared with the non-valvular luminal endothelium (Brooks et al. 2009). Comparison of the same endothelial loci in the present study confirmed this observation (Fig. 4). However, the present study extended the evaluation of the non-valvular endothelium to two additional loci of non-valvular luminal endothelium extending distally from the vein wall attachment of the valve cusp, including middle and distal endothelium (Fig. 5a). These three loci (proximal, middle, and distal) demonstrated statistical significance for a linear trend toward increased expression of vWF (1.7-fold), EPCR (2.5-fold) and TM (1.5-fold) (see Fig. 5a). These trends were unexpected and likely due to the microenvironment in the region of non-valvular luminal endothelium chosen for observation in the previous study which was next to the distal attachment site of the valve cusp.

In response to this observation, expression of each of the three proteins at the distal-most, non-valvular luminal endothelial mid-line loci for each specimen was compared with alternative non-valvular luminal endothelial loci between ~0.75 and ~1.25 cm proximal or distal to the valve. This was done to determine whether the distal most non-valvular endothelial loci were representative of nonvalvular endothelium. This analysis was performed on freshly cut sections from the original blocks. No statistical differences in expression levels were observed for each of the three proteins at each pair of loci (Fig. 5b), demonstrating that the distal-most of the three original measures of non-valvular luminal endothelium is representative of the non-valvular venous endothelium.

Thus, to assess the relative thromboresistance of the sinus endothelium compared with the non-valvular luminal endothelium, the expression of all three proteins at the distal-most non-valvular luminal endothelial loci were compared with the proximal, middle, and distal regions within the valve sinus. The vWF expression in the nonvalvular luminal endothelium was found to be significantly higher than expression in the proximal, middle, and distal regions for both valve sinus wall ($p < 0.001$) and valve cusp ($p < 0.001$). The higher vWF expression in the nonvalvular luminal wall endothelium compared with the sinus endothelium ranged from 3.0- to 6.7-fold increased (Fig. 6a) in individual cases. EPCR expression in the valve sinus was shown to be significantly different from the nonvalvular luminal endothelium in only the proximal region ($p < 0.05$) of the valve cusp (Fig. 6b).

Although EPCR expression at the proximal loci of the valve wall endothelium was higher than the non-valvular luminal endothelium ranging from 1.25- to 1.7-fold in individual cases, it did not reach statistical significance. Conversely, EPCR expression at the distal loci in the valve sinus ranged from no change to 20% lower than the non-valvular luminal endothelium within any single individual. TM expression in the valve sinus did not show any significant differences across all regions in all sections when compared with the distal-most non-valvular luminal endothelium (Fig. 6c).

Analysis of expression gradients within the valve sinus

We hypothesized that increasing stasis of blood flow, characteristic of the deeper recesses of the venous valves, would be associated with a gradient of increased thromboresistance from the uppermost to the deepest regions of the valve sinus. To address this hypothesis we evaluated the endothelial expression of vWF, EPCR, and TM from the uppermost to the deepest regions of the valvular sinus (Fig. 6a–c). In support of our hypothesis, vWF expression in the valve sinus wall demonstrated a statistically significant gradient ($p < 0.001$) of decreasing endothelial expression (Fig. 6a). The decreased endothelial expression of vWF along the sinus wall at the distal most point ranged from 30 to 57% of the proximal most point within any single individual. Contrary to our hypothesis, EPCR expression demonstrated a statistically significant trend toward decreasing endothelial expression across the valve cusp ($p < 0.001$) (Fig. 6b). The decreased expression of EPCR at the distal loci of the sinus ranged from 44 to 60% of the proximal loci within any single individual. Although EPCR expression in the regions of the valve sinus wall did not reach statistical significance for a linear trend, there was a similar proximal to distal trend toward decreasing expression (Fig. 6b). Contrary to our hypothesis, TM expression in the valve wall and the valve cusp did not show statistically significant trends across any of the valvular sinus regions (Fig. 6c).

Inter-individual variation in expression of vWF, EPCR and TM in the valve sinus

Considerable inter-individual variation in endothelial expression was observed for all three proteins, both in the valvular wall and the valve cusp endothelium. It is also apparent that there are some individuals who have consistently high or low levels of expression for all these proteins. Figure 7 plots the vein wall endothelial expression of vWF (Fig. 7a), EPCR (Fig. 7b), and TM (Fig. 7c), respectively. Within the valve wall endothelium as displayed in figure Fig. 7, the ANOVA test for inter-individual variability demonstrated p values of 0.0003 for vWF, 0.03 for EPCR, and <0.0001 for TM. Similar p values were observed for the valve cusp endothelium (data not shown) for vWF ($p < 0.0001$), EPCR (0.54), and TM (<0.0001). So, all three proteins show significant inter-individual variability within the valve sinus at all locations except EPCR in the valve cusp. Figure 8a, b show examples of an individual with high expression compared with one with low expression of all three proteins.

Discussion

In this study we hypothesized that the endothelium of the valve sinus would express greatest anticoagulant and least procoagulant activity at the deepest recess of the valve sinus as a result of stasis and accompanying hypoxia. In agreement with our hypothesis, vWF expression was found to be lowest in the deepest region of the valve sinus wall and valve cusp endothelium and increased in a graded fashion to the uppermost region of the valve sinus wall. Contrary to our hypothesis, EPCR expression was found to be lowest at the deepest region and highest at the uppermost region of the valve sinus on both valve wall and valve cusp endothelium. TM expression was similar across all regions and sections in the valve wall and valve cusp.

We also reproduced the results of our recent study which demonstrated decreased expression of vWF and increased expression of EPCR and TM in the deepest portion of the valve sinus compared with the non-valvular luminal endothelium when comparing the same anatomic locations examined in that study (Brooks et al. 2009). However, in the present study we evaluated additional non-valvular endothelial loci more distal to the valve cusp attachment and demonstrated a gradient of increasing expression of these three proteins from proximal to distal loci. We chose to compare the valve endothelial expression of the three proteins with the distal-most non-valvular luminal endothelial loci as most representative of the non-valvular luminal endothelium (see Fig. 1b). A comparison of the distal-most non-valvular luminal endothelial loci, with the valvular sinus endothelium, revealed that vWF in the depth of the sinus was lower than the non-valvular luminal endothelium as previously observed. However, although EPCR tended to be higher at the uppermost loci in both the valve sinus wall and the valve cusp compared with the non-valvular endothelium, these changes did not reach statistical significance, and EPCR expression at the deepest loci in the sinus tended to be equal to or moderately lower than the non-valvular vein wall. TM showed little change across all loci for the same comparison.

The most prominent and consistent findings in this study are the observed differences in venous sinus endothelial vWF expression both in comparison with non-valvular luminal endothelium and across the observed expression gradient present in the valve sinuses. This finding is consistent with our previous study and suggests that the endothelial microenvironment within the valvular sinuses may be conditioned to have less platelet reactivity, especially in the deepest recesses of the sinus, the initiation point of most venous thrombi. In a recent study, vWF expression was shown to vary considerably across various vascular beds (Pusztaszeri et al. 2006). The mechanism underlying the differential expression of vWF in the venous sinus is unclear but likely relates to the peculiar microenvironment of the sinus, characterized by cyclical vortical flow, stasis, and hypoxia (Hamer et al. 1981; Lurie et al. 2003). Persistence of these conditions, associated with many of the clinical risk factors for venous thrombosis, is likely associated with activation of the endothelium and greater frequency of interactions with the cellular elements of the blood. Leukocytes have been observed at the endothelial surface shortly after the initiation of experimental thrombosis in animal models (Zhou et al. 2009), an event likely associated with activation of the endothelium and the expression of TF. However, platelets do not become prominent until after the initiation event, which is morphologically characterized as a red cell-rich fibrin thrombus directly adjacent to intact endothelium (Sevitt 1974). Platelets appear to be important in the subsequent propagation of the thrombus as evidenced by the presence of linear layers of platelets alternating with red cell-rich fibrin thrombi referred to as the Lines of Zahn (Zahn 1874) and irregular “lakes” of platelets scattered throughout the red cell-rich venous thrombus (Sevitt 1974). Our findings with respect to vWF may partially explain the paucity of platelets at the thrombus initiation site and the relative ineffectiveness of prophylactic anti-platelet therapy for venous thrombosis; however, the underlying mechanism remains to be determined.

Abnormalities of the protein C (PC) system are the most frequent biochemical abnormality associated with venous thrombosis, pointing out the important role of this system for endothelial thromboresistance (Rosendaal 1999; Vossen et al. 2005). Our results suggest that endothelial expression levels of TM, similar to those observed in the non-valvular luminal endothelium, are adequate to resist thrombus formation in the venous sinus. On the other hand, we observed modest differences in endothelial expression of EPCR in the valvular venous sinus compared with the non-valvular luminal endothelium, although for the most part these differences were small, especially in the deepest part of the venous sinus. The greatest differences in endothelial EPCR expression were at the most proximal points measured on the valve cusps which may be due to the physical movement and contact of the

cusps with each other and the vein wall. Turbulent flow and physical contact likely affect orientation and activation state of the valvular cusp and adjacent valve wall endothelium (Lurie et al. 2003) where the cytoprotective effects of APC may be important to resist thrombus formation in these locations (Mosnier et al. 2007).

Finally, the significant inter-individual variation in endothelial expression of all three of these proteins suggests that thromboresistance of the venous vascular bed may vary as a function of both the relative and absolute levels of endothelial expression of these procoagulant and anticoagulant proteins. Given the growing recognition of the importance of vascular bed heterogeneity (Aird 2007a, b) in the genesis of thrombosis, these findings suggest that the investigation of endothelial expression profiles, reflecting inter-individual variation in vascular endothelial phenotypes, may help to improve our understanding of venous thrombogenesis.

There are limitations to this model. Saphenous veins are superficial and are at best only a surrogate model for deep veins. However, the causal pathway of superficial venous thrombosis shares risk factors with DVT and is itself a risk factor for DVT (Decousus and Leizorovicz 2005). The commonality in the causal pathway of superficial and venous thrombosis supports usage of the saphenous vein as a means to model the deep veins. At present there are no other reliable, non-invasive, and readily accessible means of studying fresh specimens of the deep veins. This may change in the near future with the advent of modern in vivo imaging technologies. A second limitation is the surgical source of the saphenous veins. After the veins are harvested, they are kept in a container on the sterile surgical tray immersed in the patient's heparinized blood. The presence of heparin abrogates our ability to look at some coagulation proteins of interest like endothelial cell TF pathway inhibitor. Finally, since the patients in this study have advanced atherosclerosis, they may have endothelial dysfunction which is associated with abnormal endothelial regulation including impaired vasomotor control and a pro-thrombotic/inflammatory state (Bonetti et al. 2003; Widlansky et al. 2003). Although impaired endothelial regulation could affect our observations, recent data suggest that endothelial dysfunction is associated with increased risk of venous thrombosis (Migliacci et al. 2007). Thus, changes associated with endothelial dysfunction may be relevant to understanding the microenvironment of the venous valvular sinus.

Acknowledgments

The authors would like to acknowledge the expert support of Jennifer Díaz in preparing the manuscript for submission. The work from the laboratory of EGB was supported by P01-HL046703 from the National Institutes of Health and a Transatlantic Network for Excellence in Cardiovascular Research grant from Fondation Leducq, Paris. Purchase of the confocal microscope used in this project was supported by S10 RR019246-01 from the National Center for Research Resources (DJT). The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources or the National Institutes of Health.

References

- Aird WC. Spatial and temporal dynamics of the endothelium. *J Thromb Haemost.* 2005; 3:1392–1406. [PubMed: 15892866]
- Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res.* 2007a; 100:158–173. [PubMed: 17272818]
- Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circ Res.* 2007b; 100:174–190. [PubMed: 17272819]
- Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol.* 2003; 23:168–175. [PubMed: 12588755]

- Brooks EG, Trotman W, Wadsworth MP, Taatjes DJ, Evans MF, Ittleman FP, Callas PW, Esmon CT, Bovill EG. Valves of the deep venous system: an overlooked risk factor. *Blood*. 2009; 114:1276–1279. [PubMed: 19436051]
- Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, Brown PO. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci USA*. 2003; 100:10623–10628. [PubMed: 12963823]
- Decousus H, Leizorovicz A. Superficial thrombophlebitis of the legs: still a lot to learn. *J Thromb Haemost*. 2005; 3:1149–1151. [PubMed: 15946201]
- Hamer JD, Malone PC, Silver IA. The PO₂ in venous valve pockets: its possible bearing on thrombogenesis. *Br J Surg*. 1981; 68:166–170. [PubMed: 7470818]
- Karino T, Motomiya M. Flow through a venous valve and its implication for thrombus formation. *Thromb Res*. 1984; 36:245–257. [PubMed: 6515603]
- Liu GC, Ferris EJ, Reifsteck JR, Baker ME. Effect of anatomic variations on deep venous thrombosis of the lower extremity. *Am J Roentgenol*. 1986; 146:845–848. [PubMed: 3485360]
- Lurie F, Kistner RL, Eklof B, Kessler D. Mechanism of venous valve closure and role of the valve in circulation: a new concept. *J Vasc Surg*. 2003; 38:955–961. [PubMed: 14603200]
- McLachlin AD, McLachlin JA, Jory TA, Rawling EG. Venous stasis in the lower extremities. *Ann Surg*. 1960; 152:678–685. [PubMed: 13774059]
- Migliacci R, Becattini C, Pesavento R, Davi G, Vedovati MC, Guglielmini G, Falcinelli E, Ciabattini G, Dalla Valle F, Prandoni P, Agnelli G, Gresele P. Endothelial dysfunction in patients with spontaneous venous thromboembolism. *Haematologica*. 2007; 92:812–818. [PubMed: 17550854]
- Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. *Blood*. 2007; 109:3161–3172. [PubMed: 17110453]
- Paterson JC, McLachlin J. Precipitating factors in venous thrombosis. *Surg Gynecol Obstet*. 1954; 98:96–102. [PubMed: 13122392]
- Perneger TV. What's wrong with Bonferroni adjustments. *Br Med J*. 1998; 316:1236–1238. [PubMed: 9553006]
- Pusztaszeri MP, Seelentag W, Bosman FT. Immunohisto-chemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *J Histochem Cytochem*. 2006; 54:385–395. [PubMed: 16234507]
- Rosendaal FR. Risk factors for venous thrombotic disease. *Thromb Haemost*. 1999; 82:610–619. [PubMed: 10605758]
- Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology*. 1990; 1:43–46. [PubMed: 2081237]
- Sedgwick, J. Scientific imaging with photoshop: methods, measurement and output. New Riders; Berkeley: 2008. Guidelines for specific types of images; p. 44-74.
- Sevitt S. The structure and growth of valve-pocket thrombi in femoral veins. *J Clin Pathol*. 1974; 27:517–528. [PubMed: 4138834]
- Vossen CY, Conard J, Fontcuberta J, Makris M, VDM FJ, Pabinger I, Palareti G, Preston FE, Scharrer I, Souto JC, Svensson P, Walker ID, Rosendaal FR. Risk of a first venous thrombotic event in carriers of a familial thrombophilic defect. The European prospective cohort on thrombophilia (EPCOT). *J Thromb Haemost*. 2005; 3:459–464. [PubMed: 15748234]
- Widlansky ME, Gokce N, Keaney JF Jr, Vita JA. The clinical implications of endothelial dysfunction. *J Am Coll Cardiol*. 2003; 42:1149–1160. [PubMed: 14522472]
- Winer, BJ.; Brown, DR.; Michels, KM. Statistical principles in experimental design. McGraw-Hill; New York: 1971.
- Zahn FW. Untersuchungen über thrombose. *Virchows Arch*. 1874; 62:81–124.
- Zhou J, May L, Liao P, Gross PL, Weitz JI. Inferior vena cava ligation rapidly induces tissue factor expression and venous thrombosis in rats. *Arterioscler Thromb Vasc Biol*. 2009; 29:863–869. [PubMed: 19265029]

Fig. 1. Normal venous valvular anatomy. **a** The *small inset* shows the line of longitudinal opening of the vein at the valvular commissure. The main figure demonstrates the anatomy of the venous valve with the *dashed line* representing the plane of sections taken for histology. The *arrow* denotes the direction of blood flow. **b** This diagram demonstrates the size and locations chosen for immunofluorescence evaluation of the endothelium both inside and outside of the valvular sinus

Fig. 2. Immunofluorescence image of venous valve from saphenous vein specimen. Pseudocolors for vWF, EPCR and TM are *white*, *green*, and *red*, respectively. *Arrows* denote locations of immunofluorescence evaluation. Abbreviations are defined in Fig. 1b

Fig. 3. Representative section of a venous valve and vein wall with split and merged confocal images. **a** DAPI binds to DNA (*blue*), **b** vWF (*white*), **c** EPCR (*red*), **d** TM (*green*) and **e** composite image with DAPI, vWF, EPCR, and TM

Fig. 4.

Comparison of the nonvalvular endothelium with the valvular sinus endothelium from the same locations as described in our recent publication (Brooks et al. 2009) reproduces our previous findings. The expression of the procoagulant protein vWF was lower at the base of the valvular sinus compared with the non-valvular luminal endothelium just distal to the valve cusp, whereas the expressions of the anticoagulant proteins EPCR and TM were higher at the base of the valvular sinus compared with the non-valvular luminal endothelium (paired *t* test = superscript “a”)

Fig. 5.

a Three loci of nonvalvular vein wall endothelium extending distally from the vein wall attachment of the valve cusp, including proximal, middle, and distal endothelium demonstrate a statistically significant gradient of increased expression for vWF, EPCR, and TM (ANOVA contrast for linear trend = *superscript "b"*). **b** Comparison of expression for vWF, EPCR, and TM at the distal-most, non-valvular endothelial loci for all specimens, with alternative nonvalvular endothelial loci between ~0.75 and ~1.25 cm proximal or distal to the valve. No statistical differences in expression levels were observed for any of the three proteins at each pair of loci. AL alternative non-valvular vein wall luminal endothelium (paired *t* test = *superscript "a"*; NS non-significant)

Fig. 6. Graphic representation of comparison between nonvalvular luminal endothelium and valvular sinus cusp and wall endothelium for **a** vWF, **b** EPCR, and **c** TM expression, respectively (paired *t* test compared to “DL” = superscript “a”; NS non-significant. ANOVA contrast for linear trend = superscript “b”; NS non-significant)

Fig. 7. Graphic representation of inter-individual variability of **a** vWF, **b** EPCR, and **c** TM, respectively, for all individual subjects' results including the non-valvular sinus endothelium and the valve sinus luminal wall endothelium. Inter-individual differences were tested only for the leaflet and vein wall valvular sinus endothelium for each protein (ANOVA test between subject variability = "c")

Fig. 8. Two cases, representative of the wide inter-individual variation in expression of vWF, EPCR, and TM. In case 1 (a), all three proteins are expressed at a markedly higher level than in case 7 (b)