9

Markers of Vascular Damage and Repair

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1. Introduction

Damage to endothelial cells is a crucial event during the pathogenesis of vasculitis. The vasculitides cause different clinical manifestations, depending on the extent and acuity of endothelial damage as well as their preponderance to affect some organ-specific endothelial cells and spare others. About 40 years ago circulating endothelial cells (CEC) were first observed in peripheral blood. Since then CEC have been established as a reliable indicator of vascular injury and damage and more sophisticated detection techniques, such as immunomagnetic isolation and fluorescence-activated cell sorting (FACS), have become available to detect and enumerate them. Based on current concepts of pathogenesis, detached endothelial cells, and/or their soluble and cellular debris, must be detectable in peripheral blood of vasculitis patients. In hindsight, it is therefore surprising that for many years few, if any, attempts were made to evaluate their use as clinically relevant markers of endothelial damage. Endothelial Microparticles (eMP) have been described as another potential marker of endothelial damage. eMP are markers of activation, cell injury or apoptosis. They are the product of exocytic budding and consist of cytoplasmic components and phospholipids. Fluorescence-activated cell sorting (FACS) is the preferred technology for isolating MP and different surface markers of the parent cells have been used. eMP can reflect endothelial activation and damage, although differences between eMP and CEC remain ill-defined. Another approach to measuring endothelial damage is to assay soluble markers, such as thrombomodulin or von Willebrand factor. However, these markers also have their limitations. It is also worthwhile to remember that all approaches struggle with the fact that many endothelial markers are also expressed on non-endothelial cells (Table 1). More recently, interest has focused on endothelial repair and damage and endothelial progenitor cells have been studied, again with different methodologies. Recent evidence has also revealed interesting interactions between CEC and healthy endothelium in vitro although the relevance of these findings for human vascular disease in vivo remains unclear. Here, we review markers of endothelial damage and repair in vasculitis. We discuss the implications of these findings for the pathogenesis, their potential clinical utility, and also review the limitations of each approach. Finally, we review the phenotype of CEC, mechanisms of detachment and interactions with other cell subsets.

2. Soluble endothelial markers

Endothelial cells express a broad variety of proteins [1] but only few of these have been studied in serum or plasma in vascular disease. Currently, von Willebrand factor (vWF), thrombomodulin [2], soluble E-Selectin and circulating angiopoietin [3] are best described [4-7]. It must be noted that several factors may influence the levels of these circulating proteins. For example, thrombomodulin undergoes renal excretion. Hence, serum levels are influenced by renal function. Other confounding factors, such as liver function, clotting or fibrinolysis may also influence these proteins. In addition, these soluble markers do not distinguish between endothelial activation and damage. Some investigators compared levels of these markers with numbers of CECs. A recent study found a correlation between CECs, von Willebrand factor ($p=0.002$) and plasma tissue factor ($p=0.02$) [8]. It is also clear that necrotic endothelial cells will release, either in situ or after their detachment from the basement membrane, a variety of other, nonspecific, soluble factors. In this regard, Bruchfeld and colleagues recently reported elevated levels of High-mobility group box-1 protein (HMGB1), a nuclear and cytosolic protein that is released from necrotic cells [9]. However HMGB1 is also actively secreted from monocytes and macrophages. Angiopoietin-2 (Ang-2) is another new soluble marker investigated in small vessel vasculitis. Ang-2 is bound to the endothelial specific angiopoietin Tie Ligand–receptor, which is a regulator of endothelial cell detachment. Circulating Ang-2 is elevated in small vessel vasculitis and closely correlates with vasculitis activity score [3]. Ang-2 therefore reflects a potential new mediator of endothelial cell detachment in vasculitis although theses findings need to be validated by analyzing a larger cohort.

CD/antigen name	Other names	Expression by non-endothelial cells
CD31	PECAM-1	Platelets, monocytes, neutrophils, T cell subsets
CD62e	E-selectin	Activated endothelial cells
CD54	ICAM-1	Endothelial cells, activated B and T lymphocytes, monocytes
CD105	Endoglin	Endothelial cells, activated monocytes, tissue macrophages, erythroid marrow precursors
CD106	VCAM-1	Activated endothelial cells, stromal cells
CD141	Thrombomodulin	Endothelial cells, keratinocytes, platelets, monocytes, neutrophils
CD146	P1H12, S-endo-1	Endothelial cells, activated T-Lymphocytes, melanoma cells, trophoblast
Tissue factor		Endothelial cells, monocytes/macrophages

Table 1. Antigens of endothelial cells, which are also present on non-endothelial cells

3. Circulating endothelial cells in vasculitis

Circulating endothelial cells (CEC) are detectable in peripheral blood after they have been detached from the damaged endothelial monolayer, probably leaving behind a denuded basement membrane. Those cells were first described almost 40 years ago [10] although methods of their identification were rather primitive. ANCA-associated small-vessel

vasculitis serves as a paradigm of an endothelial disorder. Therefore it is not surprising that high numbers of CEC are detected in ANCA vasculitis and correlate with disease activity [11]. Phenotypic analysis, however, proved more difficult than anticipated. It is quite clear from the concept of small vessel vasculitis that CEC cannot be specific to ANCA vasculitis. Dang and colleagues reported elevated CEC numbers in aortoarteritis [12] while Nakatani et al. demonstrated CECs in patients with Kawasaki disease [13]. CEC are also elevated in systemic lupus erythematosus [14] and Behcet's [15]. In a broader sense, CEC are also markedly elevated in other, non-vasculitic, forms of widespread acute vasculopathy, such as thrombotic microangiopathy [16]. In addition, CEC can be useful to monitor treatment and to distinguish between relapse and infection in difficult cases [17]. Patients with relapse of vasculitis had markedly elevated numbers of circulating endothelial cells and indeed similar cell numbers when compared to patients at their initial vasculitic presentation[18]. Patients with limited disease due to granulomatous ANCA-associated vasculitis had only slightly elevated cell numbers, which were similar to those seen in remission. Patients with infection had no elevated CEC numbers [18]. These findings gave us confidence in the clinical use of CEC in vasculitis [17, 19] although prospective data on the clinical use of CEC are lacking.

	CEC	**EPC**
Cell type	Mature endothelium	Endothelial progenitor cell
Origin	Vessel wall	Bone marrow
Morphology	Cells, a-nuclear carcasses or sheets of multiple cells 10-100µm	Diameter less than 20µm
Characteristic properties	VWF CD 31 Thrombomodulin CD 146 UEA-1	CD 133 CD 34 TIE-2 KDR Uptake of acetylated LDL UEA-1 (unclear)
Colony-forming potential	None (controversial)	Yes
Laboratory methods	Immunomagnetic isolation, FACS	FACS, culture assays

Table 2. Characteristic properties of CEC and EPC

3.1 CEC and vasculitis: Immunomagnetic isolation and FACS in competition

The mainstay of immunomagnetic isolation is the use of paramagnetic particles (Dynabeads™), which have been coated with anti-endothelial antibodies as reviewed in great detail elsewhere [10]. Briefly, whole blood is incubated with antibody-labeled magnetic Dynabeads™. Next, target cells with bound anti-endothelial antibody and Dynabeads™ are recovered with a magnet. CEC can then be enumerated after acridine staining. Immunomagnetic capturing is mostly performed using the cell surface marker CD 146 [20].

A variety of factors has been considered to influence CEC counts [21]. To avoid false positive results caused by traumatic venepuncture (resulting in dislodgement of endothelial cells from the vessel wall) it is recommended to discard the first tube of blood [20]. Adding albumin

or EDTA and Fc-blocking agents is employed to reduce non-specific binding of anti-CD 146-coupled beads to leukocytes although this remains a concern even in experienced hands [22]. Moreover, activated T-lymphocytes and other cell subsets may under some circumstances also harbor CD 146 and lead to artifacts [23]. We therefore developed a secondary stain with Ulex Europaeus lectin 1 (UEA-1). [24] Even so and despite the proposal of a consensus for definition of CEC the approach remains time consuming and require considerable experience. Automated systems have been described but these are costly [25].

Flow cytometry is an alternative technique to isolate and enumerate CEC [26-31]. The technique holds considerable promise, as several surface markers can be used concurrently. For example, CD 146 expression on activated T cells can be distinguished from CD 146 on endothelial cells by co-staining with CD45 or CD3 (or both). CD 133 may help to identify EPC because it is not present on CEC or any mature endothelial cells. The addition of viability stains, such as propidium iodide or 7-AAD, may also help to identify EPC. Markers of endothelial activation can be studied as well. Most groups define CEC using flow cytometry as CD146+, CD34+ and CD45- [32]. Others have defined CEC as CD31bright, CD34+CD45-, CD133- [33 34]. However, CD31 bright could also include platelets, resulting in falsely elevated numbers of CEC [35].

Unlike immunomagnetic isolation, FACS does not permit visualization of the cell. Furthermore the cell numbers obtained with FACS differ markedly from those obtained with immunomagnetic isolation, whereby higher numbers are usually observed with FACS. In addition, there is considerable discrepancy in these numbers between different groups that employ FACS. It is remarkable that most if not all investigators using immunomagnetic isolation enumerate in the range of 10 CEC/ml blood in healthy individuals while those using FACS report cell numbers in the thousands per ml with a fairly broad range [10]: Holmen and colleagues measured a mean of 50 CEC/ml in healthy controls [26], Mancuso et al. counted 1,200 CEC/ml of rested cells [36] and Jacques N et al. 6.5 CEC/ml [37]. Two groups compared CEC counts measured with both methods in the same populations. Goon et al. measured 8 CEC/ml in healthy controls comparable to CEC detection by IB (4.5CEC/ml) [32]. In contrast, Clarke et al. detected lower numbers of CEC by FC compared to IB [38] suggesting limited sensitivity for the detection of CECs. Further validation studies are required to determine the influence of gating, CEC phenotype, and "lysing", which could reduce recovery of CEC.

In comparison to immuno-magnetic isolation, FACS holds considerable promise and technical advantages. In addition, FACS is less time consuming and easily amenable to standardization. Cost is difficult to compare with immunomagnetic isolation, given the expenditure for the FACS counter and the fact that the cost of experienced staff is difficult to gauge. Very recently alternative approaches have combined the two techniques [39]. This novel tool has to be validated in other clinical settings and populations.

3.2 Phenotype and mechanisms of detachment of CEC in vasculitis

Endothelial cells can be activated by various stimuli, such as pro-inflammatory cytokines, growth factors, infectious agents, lipoproteins, or oxidative stress. Loss of integrity of the endothelial layer eventually leads to cell detachment of cells [40]. Such detachment can be caused by defective adhesive properties of the endothelial cells, by action of proteases and/or cytokines or, by mechanical injury. Endothelial adhesive molecules of the integrin and cadherin family, such as vitronectin and fibronectin and VE cadherin, respectively,

promote adhesion of endothelial cells to matrix [40-41]. Loss of these survival signals triggers detachment and apoptosis of endothelial cells [42]. Protective factors have been described as well: In sickle cell disease endothelial apoptosis is impaired by VEGF. This has also been shown in vitro, where VEGF inhibits apoptosis of unanchored culture cells [43]. Release of proteases by granulocytes is another well-documented cause of endothelial cell detachment [44-45 46 47]. Finally, mechanical force can detach endothelial cells from the basement membrane as shown in patients undergoing percutaneous catheter interventions [48].

Not much is known regarding the phenotype of CEC. This is caused mainly by the paucity of CEC even in active disease and in the difficulty of characterizing these cells further. Moreover, it is difficult to say with certainty whether or not phenotypic changes were induced by the isolation procedure itself. The viability of CEC remains particularly controversial. Our own data suggest that CEC in ANCA-associated small-vessel vasculitis are mainly necrotic [11] and we were unable to culture these cells. Others, however, describe culture of CEC that were isolated by FACS. In contrast, two-thirds of CEC in normal subjects are believed to be apoptotic [49]. Lin et al could also demonstrate that vessel-wall derived CD146+ CEC can be viable, although they have limited growth capability [50]. Another group was recently able to grow CEC for about 10 days, but no significant proliferative capacity was observed [26].

3.3 Circulating endothelial cells as potential mediators of disease

It has been speculated that CEC themselves could be pro-inflammatory [40]. In general, damaged eukaryotic cells have been shown to release a variety of pro-inflammatory factors, to initiate pro-inflammatory pathways in other cell subsets, such as a Toll-like-receptor-2/NFκB-dependent reaction in monocytes [51]. In highly active vasculitis, the healthy endothelium must surely encounter a vast array of apoptotic and/or necrotic endothelial cells and their debris. Disturbed clearance of apoptotic cells may play a role in systemic lupus erythematosus [52]. Interestingly, apoptotic and necrotic endothelial cells and their fragments are rapidly internalized by healthy endothelium [53]. Support for these findings came from other studies demonstrating the phagocytic capability of endothelial cells [54]. We could also show that endothelial cells exposed to apoptotic and necrotic cells exhibit enhanced adhesion properties for leukocytes and that isolated CEC from patients with vasculitis aggravated these effects further [53]. These effects on binding properties could be explained in part by release of IL-8 and MCP1, which serve as chemo-attractants. Interestingly, apoptotic and necrotic cells induced different patterns of effects in healthy endothelium. Enhanced IL-8 and MCP1 levels in serum have been detected in patients with active vasculitis and ANCA induce the synthesis of these chemokines in various cell subsets [55]. Endothelial synthesis of these mediators triggered by ANCA [56] and circulating endothelial cells [53] may contribute to the pro-inflammatory state associated with vasculitis.

We have investigated this topic further and became interested in thrombospondin (TSP-1) as a possible mediator. This multi-functional glycoprotein is a known endogenous inhibitor of angiogenesis [57] and modulates cell adhesion and proliferation [58]. We were able to show that apoptotic cells induce expression of TSP-1 in endothelial cells [59] and that TSP-1 facilitates engulfment of apoptotic cells by phagocytes [59]. We speculate that under pathological conditions with high numbers of un-cleared dying cells in the circulation endothelial-derived elevated TSP-1 level may serve as an attraction signal for phagocytes promoting enhanced recognition and clearance of apoptotic cells.

It is probably fair to say that at present we do not understand the interactions between CEC and healthy endothelium and other cell subsets. Further studies, for example in animal models, are surely warranted. Figure 1 summarizes proven and proposed interactions of circulating endothelial cells with healthy endothelium.

4. Microparticles in vasculitis: Just smaller than CEC or different, too?

Microparticles (MP) are sub-micrometric fragments derived from plasma membranes in response to a variety of events, such as activation, injury, or apoptosis. Loss of phospholipid asymmetry and increased surface expression of phosphatidylserine are crucial events in this process [60-61]. On their surface these particles express antigens that reflect their cellular origin, which permits their enumeration and characterisation by flow cytometry. In addition eMP have a functional role as mediators of inflammation or coagulation. In general, microparticles have attracted considerable interest in vascular disease although a consensus definition of these particles and a uniformly accepted protocol for their enumeration is still lacking [62]. To make matters even more complicated, endothelial microparticles represent a small subgroup of all MP found in plasma[63]. Specific endothelial microparticles were first described in 1990 by Hamilton and colleagues [64]. On balance, it is probably fair to say that the field of microparticles is fraught with similar technical issues as that of CEC and that further standardisation is eagerly awaited.

We studied endothelial microparticles (EMP) by FACS analysis and found elevated EMP in active vasculitis [65]. Similar results had previously been obtained in a paediatric cohort of vasculitis patients [60 66]. Particle counts also correlated with disease activity [65]. The difficult bit is that CECs and microparticles may not reflect the same disease process. Incidentally, the same holds true for soluble endothelial markers, such as soluble von Willebrand factor or thrombomodulin: CEC, EMP and soluble markers may represent different mechanisms of endothelial activation and damage. For example, soluble markers and EMP may already be elevated in endothelial activation whereas presence of CEC probably reflects true damage. Interestingly CEC and eMP also follow different kinetics in ANCA-associated vasculitis: CEC decline slowly during successful immunosuppressive therapy while activated eMP probably represent an early marker that normalises quickly [65]. To make matters even more complicated, each of these markers may underlie different confounding factors: eMP are elevated in patients with renal diseases including those on hemodialysis [67] and could reflect vascular damage in these patients whereas CEC are not increased in renal failure [11]. Nevertheless, ESRD patients with and without a history of cardiovascular disease causing possible endothelial damage had similar levels of EMP [68]. This illustrates that phenotyping of microparticles and characterization of subgroups of microparticles for each different disease process will be crucial as each disease process will release different microparticles.

Finally, EMP may also have pathogenetic importance in vasculitis. Microparticles are now regarded as crucial players at the interface of atherosclerosis and inflammation [69]. MP are generally capable of inducing cytokine release [70] and leukocyte MP induce endothelial IL-6 and MCP-1 production [71]. It has been demonstrated that endothelial microparticles convert plasminogen into plasmin [72] and are tissue-factor positive [73] . Burkhart et al. demonstrated recently that microparticle tissue factor activity is increased in PR3-ANCA vasculitis patients with active disease [74].

Evidence has also emerged to suggest that endothelial release of microparticles from adherent cells is actually protective and that inhibition of microparticle release leads to

endothelial detachment [75]. Moreover, pre-treatment of endothelial cells and monocytes with platelet derived MP modulates monocyte-endothelial cell interactions by increasing the expression of adhesion molecules on both cell types [76]. EMP have been shown to decrease nitric-oxide-dependent vasodilation and to be both pro-inflammatory and pro-coagulant [61]. MP have also been found to stimulate angiogenesis and differentiation of progenitor cells [77]. Finally, elegant studies in flow chambers have demonstrated that MP enhance leukocyte rolling [78]. Taken together, current data suggest that EMP may not only be a surrogate marker of vasculitis but that they may contribute to the pro-inflammatory and pro-coagulant status of the endothelium. It must be remembered, however, that findings in generic microparticles may not be applicable to EMP and vice versa.

5. CEC and EPC – an ongoing controversy

The role of endothelial progenitor cells (EPC) [79] in vascular disease and their potential role for therapy [80] have been reviewed recently [81]. Of note, the field of EPC is particularly hampered by lack of standardisation [82] [83]. Our knowledge about the kinetics of CEC detachment and EPC mobilisation as well as their interaction is equally limited. Very recently, the margins between endothelial progenitor cells and haematopoietic stem cells became somewhat blurred after proof that endothelial cells can be haematopoietic in mice [84].
We have previously studied numbers of circulating CD34+ progenitor cells and EPCs in vasculitis and demonstrated that these cells increased significantly after the institution of immunosuppressive therapy and with disease remission [85]. Others have previously described an increase in EPCs in inflammatory vascular diseases: Avouac and colleagues, for instance, described increased EPC numbers in scleroderma [86]. In contrast to de Groot and co-workers [85], other studies postulate an imbalance between CECs and EPCs in patients with vasculitis [26] [87]. Another study by Zavada and colleagues reports reduced EPC numbers as a risk factor for relapse in vasculitis [88]. Of note, the pattern of EPC in vasculitis may be different in children and one group reports increased numbers in active vasculitis [66]. EPCs were also measured in other subgroups of vasculitis. In Behcet's vasculitis EPC were decreased [89], in children with Kawasaki disease EPC were increased [90].
What make these studies so difficult to compare is, again, the lack of standardisation and the use of different assays and surface markers. Of note, the field of EPC is particularly hampered by lack of standardisation [82] [83]. The population of EPC may include a group of cells existing in a variety of stages ranging from immature hematopoietic stem cells to completely differentiated endothelial cells. Endothelial markers, such as CD-146 and UEA-1 are also present on EPC. However, the severely damaged morphology of cells obtained by CD-146-driven immunomagnetic isolation and our inability to growth these cells in culture [11] was regarded as indication that these cells are not EPC. Our own experience shows that CD 146 positive cells were CD 133 negative [91]. Very recently, however, Delorme and co-workers clearly demonstrated EPC among a population of cells isolated by CD-146-driven immunomagnetic isolation [92]. Although their findings need corroboration, new protocols of immunomagnetic isolation may be needed to exclude EPC. Table 2 summarizes characteristic properties of CEC and EPC.
Therefore, the studies mentioned above provide interesting food for thought but require independent confirmation. What stimulates EPCs in reaction to ischemia or other forms of insult? There is conclusive evidence that EPC are not stimulated by the non-specific acute

phase response [93] but by microvascular injury [94]. A variety of specific factors have been implicated in this mechanism: First, it is worthwhile to remember that erythropoietin (EPO) regulates EPCs [95]. Hence EPO treatment must always be corrected for when EPCs are measured in renal patients. Statins also influence EPC numbers [96]. Other factors that have been implicated as regulators of EPCs include vascular endothelial growth factor (VEGF), the angiopoetins, and platelet-derived growth factor CC (PDGF-CC). Haeme oxygenase 1 (HO1) has been implicated as well [97]. It is clear that EPCs are capable of homing in to sites of vascular damage. Mechanisms include CD18/ICAM-1 and sdf-1/CXCR4. Endothelial commitment requires histone deacylase (HDAC) activity and depends on the expression of the homoeobox transcription factor HoxA9 [98]. It is probably fair to say that EPCs will receive further scientific attention in vasculitis while a standard as to their definition and enumeration is eagerly awaited.

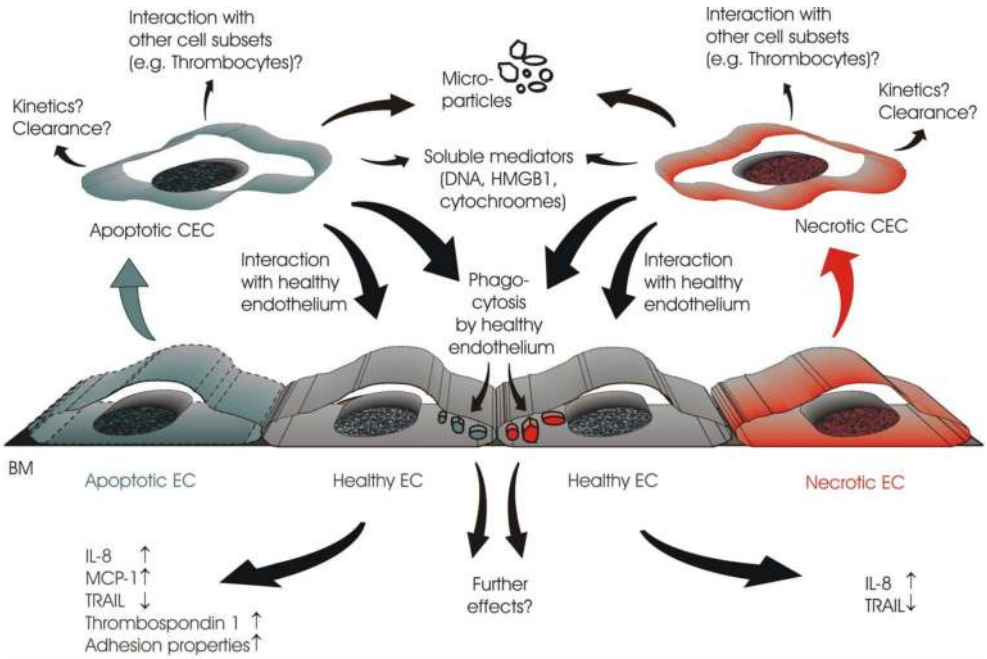

Fig. 1. Interactions of apoptotic and necrotic circulating endothelial cells with healthy endothelium; from [100], with permission

6. Conclusion

Endothelial activation and damage is a crucial event during the pathogenesis of vasculitis. Not surprisingly, markers of such damage are detectable in peripheral blood. Several markers have been studied. Circulating endothelial cells are an established and reliable marker of vascular damage. Cell numbers do correlate with the activity of vascular disease and their use in a clinical setting is on the horizon. In comparison, endothelial microparticles are smaller and their presence may reflect a different stage of the inflammatory process. For both approaches, the lack of standardization remains a matter of particular concern and

further multi-centre efforts should be encouraged. Interactions of CEC with the healthy endothelium and other cells deserve further attention, as does the phenotype of CEC. Endothelial repair is another facet of the inflammatory process although, again, progress is hampered by lack of standardization. Taken together, all of these markers may be useful to assess vascular inflammation and repair in a clinical setting.

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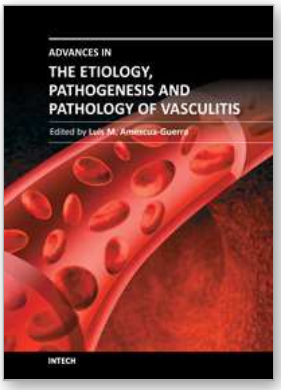

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This book represents the culmination of the efforts of a group of outstanding experts in vasculitis from all over the world, who have endeavored to devote their work to this book by keeping both the text and the accompanying figures and tables lucid and memorable. Here, you will find an amalgam between evidence-based medicine to one based on eminence, through an exciting combination of original contributions, structured reviews, overviews, state-of the-art articles, and even the proposal of novel pathogenetic models of disease. The book contains contributions on the etiology and pathology of vasculitis, the potential role of endothelial cells and cytokines in vascular damage and repair as well as summaries of the latest information on several primary and secondary vasculitis syndromes. It also covers selected topics such as organ-specific vasculitic involvement and quality of life issues in vasculitis. The editor and each of the authors invite you to share this journey through one of the most exciting fields of the medicine, the world of Vasculitis.

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