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Importance of Receptor-targeted Systems in the Battle Against Atherosclerosis

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Abstract

Atherosclerosis is the leading cause of death in the Western World and has been for decades a field of intense research. Yet, while there is a rich and diverse literature describing in detail the players and mechanisms involved in this complex disease in cell and animal models, we remain today with virtually no reliable markers for early diagnosis and targeted treatments options. This review is centered upon the latter. We summarize the latest studies focused on detecting endothelial dysfunction during the early stages of atherosclerosis, when the disease is asymptomatic and describe strategies recently proposed to image and target advanced plaque.

Keywords

Atherosclerosis; biomarkers; endothelial cells; receptors; early diagnosis; direct targeting

INTRODUCTION

The challenges associated with the treatment of atherosclerosis span the poles of disease stages, from difficulty in detection of asymptomatic early phases or subclinical events to the identification and determination of targets for treatment once plaque shows high risk of rupture and thrombosis. Each of these poles presents unique challenges and opportunities. Identification of biomarkers and detection of molecules secreted by endothelial cells (EC) lining blood vessels lumen have helped define the early aspect of atherogenesis, EC activation and significantly advanced mechanistic insight into vascular disease. Yet, the

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CONFLICT OF INTEREST

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numerous biomarkers displayed in the preliminary stages of atheroma are common to many other pathologies, limiting unequivocal correlation with atherosclerosis and selective targeting. The advanced stages of atheromatous plaque formation are the polar opposite in many different respects. Not only are the lesions far advanced, but realistic *in vitro* models that distinctively recapitulate plaque cellular and extracellular composition and their intricate interplay are extremely difficult to obtain. As a result, the complex environment can only be accurately studied in human subjects. Advanced *in vivo* animal models offer great insight, but must be applied with caution, given the difficult extrapolation of animal model results to human trials. Nevertheless, significant progress has been achieved with the formulation of receptor-targeted systems that enable imaging, detection and even treatment of certain components of the advanced plaque.

The Challenge of Detecting Endothelial Activation in Subclinical Human Atherosclerosis

Decades of research have come to define vascular disease as a chronic inflammatory disease of blood vessels. Much attention has been given to the endothelium, and its critical role in the early stages of atherogenesis has become widely recognized. This organ consisting of a single monolayer of endothelial cells lining blood vessels is more than a mere physical barrier between flowing blood and neighboring cells and tissues. The intact endothelium functions as a marvelous factory of molecules, big (proteins) and small (nitrous oxide), that ensure blood flow and vessel tone. It has also been described as a dynamic gate-keeper for numerous proteins, lipids, and cells present in blood.

Sustained oxidative and fluid shear stresses alter the biochemical profile of the endothelium. With endothelial cell dysfunction or injury the balance at the fluid-solid tissue interface is lost and with the vital anti-proliferative, anti-coagulant, and anti-adherent properties of the endothelial barrier. The switch from a quiescent endothelium to a dysfunctional one provides a unique opportunity to detect atherosclerosis at its infancy.

With *in vitro* cell culture available in the late 1970s, the number of studies focused on the endothelial cell machinery has grown exponentially. The search for cellular products or by-products of any kind, the so-called biomarkers, directly linked to endothelial dysfunction has become the Holy Grail of early diagnosis of cardiovascular disease. In this context, the ideal biomarker is chemically stable and found in sufficient concentration to be easily and quickly detected in a safe and little or non-invasive fashion. Direct *in situ* imaging or quantification as a solute in blood or urine of such biomarker should be correlated to extent of disease in years to come. Despite the overwhelming effort of the scientific community in academia and industry alike, to date a marker of early endothelial dysfunction directly linked and univocally correlated to disease progression remains elusive. From the myriad molecules produced by endothelial cells, only a few have shown promise as candidate biomarkers.

Cell Adhesion Molecules—Atherosclerosis is a complex and multivariable disease, but it is widely accepted that one of the first steps in the process of plaque formation involves the endothelium surface becoming sticky to circulating monocytes. Activated endothelial cells express a family of transmembrane cell adhesion receptors, commonly referred as cell adhesion molecules (CAMs), which enable intercellular interaction. In the early stages of

atherosclerosis, endothelial cells first express E-selectin which promotes monocyte rolling, followed by vascular cell adhesion molecule 1 (VCAM-1) which allows monocyte firm adhesion on the endothelium surface and intercellular adhesion molecule 1 (ICAM-1), responsible for monocyte transendothelial migration. Soluble forms of these receptors (sCAMs) are found in plasma as a result of shedding from the surface of activated endothelial cells. With inflammation, the plasma levels of soluble CAMs increase. Attempts to correlate CAMs concentration with coronary artery calcification and carotid artery stenosis and intimal-media thickness have shown mixed results. sICAM-1, however, has been directly correlated with cardiovascular disease in a number of epidemiologic studies, and recent studies link this marker of endothelial dysfunction with early changes in the arterial intima decades before the development of clinical cardiovascular disease. The soluble form of another adhesion molecule expressed by endothelial cells and responsible for lymphocyte infiltration, sVAP-1, has been directly correlated with thickening of the intima/media and carotid plaque development [1].

Inflammatory Cytokines—Members of the interleukin family and their receptors, traditionally associated with leucocytes, are also expressed by endothelial and smooth muscle cells. Tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-18 (IL-18), and interferon gamma (IFN γ) belong to pro-inflammatory cytokines and plasma concentrations are increased in patients with ischemic heart disease. While abundant literature exists that defines *in vitro* and in animal models the role of these molecules, studies in humans are focused in cases of advanced disease state. To our knowledge, only an inverse correlation has been shown between IL-6 levels and flow-mediated vasodilation in health individuals. Also, recent studies show that IL-18 [2] and CXCL5 [3] can be used to predict subclinical atherosclerosis in patients with type 2 diabetes.

Clotting Factors—Quiescent endothelial cells confer blood vessels' anticoagulant properties. On one hand endothelial cells shield smooth muscle cells, which constitutively express tissue factor (TF), from circulating blood. On the other, endothelial cells are responsible for controlling coagulation by synthesizing antithrombotic substances such as thrombomodulin, heparan sulfate, tissue-type plasminogen activator (t-PA), tissue factor pathway inhibitor, protein C and protein S. Activation of endothelial cells alters their phenotype, which becomes pro-thrombotic. Elevated levels of tissue factor, plasminogen activator inhibitor-1 (PAI-1) and fibrinogen have been found in early-stage and advanced-stage atherosclerotic plaques. Direct correlation in healthy human studies, however, between soluble prothrombotic or anti-thrombotic factors in blood has not been established nor has early endothelial dysfunction been associated with the localized nature of atheromatous plaque.

Matrix Metalloproteinases (MMPs)—MMPs have been shown to play a role in the formation of atherosclerotic lesions, mostly in the later stages of the disease, facilitating vessel remodeling and smooth muscle cell infiltration. Indeed, MMP-9 serum levels are elevated in patients with stable coronary artery disease and double or triple during acute coronary syndrome [4]. MMP-2 and MMP-9 activity are significantly elevated in plaques of

human specimens obtained at autopsy. MMPs can disrupt basement membrane enabling monocyte migration into the tunica media. Yet, early clinical studies investigating circulating MMPs in premature atherosclerosis did not have appropriate healthy controls [5]. Earlier promise has been hampered by more recent studies that show no difference in MMP-9 plasma levels between patients and controls [6].

C-reactive Protein (CRP)—CRP is an acute phase protein associated with increased risk for cardiovascular events. Its role in atherogenesis has been established *in vitro* and in animal models [7]. While the Jupiter trial distinguished statin effect based on CRP levels, the Dallas Heart study [8] showed CRP as a poor predictor of atherosclerotic burden, and CRP status as a risk factor remains controversial [9]. Some have postulated that CRP is a marker of inflammation at a specific point in time and not predictive of future events, and others have shown that the protein may itself be a causal agent for thrombosis rather than hyperplastic disease. Indeed under oxidative stress endothelial cells release CRP [10]. Bisioendial *et al* [11] showed that infusion of CRP in healthy volunteers upregulated markers of inflammation and coagulation.

Other Biomarker Candidates—Circulating endothelial cells (CECs), mature and progenitor, and endothelial microparticles (EMPs) have been proposed as candidates to track endothelial dysfunction (Davì & Patrono, 2007; Burnier, Fontana, Kwak, & Angelillo-Scherrer, 2009; Smadja *et al.*, 2011; Burger & Touyz, 2012; Devaraj, Kumaresan, & Jialal, 2011). Flow cytometric detection and distinction among CECs and EMPs has become possible recently [17] overcoming the technical challenges imposed by sample centrifugation that may provoke shedding of CEC and subcellular size of EMPs (less than 1 μ m). Contradictory studies, however, have also been recently published, where circulating angiogenic cell phenotypes were not associated with vascular function after adjusting for traditional risk factors [18].

Asymmetric dimethylarginine (ADMA), an inhibitor of NO synthase, is elevated in young hypercholesterolemic adults, and linked to impaired vasodilation due to endothelium dysfunction [19]. This small endogenous molecule has also been reported higher in patients suffering from early-stage rheumatoid arthritis [20]. Moreover, elevated concentrations of ADMA have been associated with greater carotid intima media thickness in young children [21].

A proteomic pattern approach of urine polypeptides has been correlated using capillary electrophoresis-mass spectrometry analysis with the same molecules found in human plaque at different time points during its progression [22]. Another interesting high-through put study of circulating lipids, lipoprotein subclasses and small molecules has been published from The Cardiovascular Risk in Young Finns study and shows promise in better risk stratification for subclinical atherosclerosis [23].

Receptor-Targeted Imaging and Treatment of Advanced Atherosclerotic Plaque

In atherogenesis, the formation of a mature plaque follows the early cellular events of endothelial cells activation and recruitment of inflammatory cells from the blood stream. The atheromatous plaque is characterized by a growing necrotic core, mainly due to

activated macrophage accumulation and tissue breakdown, and atherosclerotic neovascularization. This complex environment cannot be reliably recapitulated using *in vitro* models. Animal models and clinical trials with human subjects in contrast have enabled the successful development of receptor-targeted systems for imaging and treatment of biomarkers and bioprocesses that could lead to ameliorate conditions or prevent the development of a vulnerable plaque susceptible to fatal rupture.

One of the most important of such atheroprone bioprocesses is angiogenesis of the *vasa vasorum* in the adventitia and its penetration into the atheroma [24][25]. Initial neovascularization in atherosclerosis, which is triggered by hypoxia and Toll-like receptors, may help remove LDL when its concentration is lower in the neovessel circulation than in the intima [24], Linggen 2008 J Clin Pathol). The contribution of angiogenesis to the development of the disease, however, is more detrimental than beneficial. Evidence suggests that it promotes plaque growth, intraplaque hemorrhage and plaque destabilization and rupture. The same leaky nature of the neovessels that leads to hemorrhage and the 2- to 3-fold higher expression of CAMs by neovascular endothelium with respect to arterial luminal endothelium (Moreno *et al.*, 2006; Brien *et al.*, 1993), all lead to the recruitment of activated macrophages into the atheromatous plaque (Moreno *et al.*, 2006; [27]). Intra-plaque macrophages secrete metalloproteinases and serine proteases that degrade the extracellular matrix, causing thinning of the fibrous cap and leading to plaque rupture and thrombosis. In fact, many studies have revealed that ruptured plaques present the highest degree of neovascularization (Moreno *et al.*, 2006). The tremendous implications of angiogenesis in the development of atherosclerotic plaque and its complications have led it to emerge as one of the key biological process for targeted imaging and therapy for atherosclerosis ([28], [29]).

IMAGING OF ANGIOGENESIS IN ATHEROSCLEROSIS MRI

Contrast Agents

Various contrast agents have been studied in attempting to achieve non-invasive examination of angiogenesis in atherosclerosis ([25], [30]). Gadolinium-based (Gd) contrast agents have been thoroughly explored to detect permeability in vessel structures, and thus identify angiogenic tissue in atherosclerotic plaques [30]. The change of signal intensity with time shows the concentration of the contrast agent, and permeability is calculated by comparing the concentration of retained contrast agent in the vessel to concentration of the agent that has leaked from the vessel and entered the surrounding tissue. Several studies focused on using such Gd-based contrasts for *in vivo* imaging of atherosclerotic angiogenesis in humans have confirmed a strong correlation between the extravasation rate of Gd-based contrast agent and macrophage and neovasculature burden [30].

Enhanced results can be achieved when using more complexly-engineered contrast agents. Sirol *et al.* synthesized the Gd-based chelate Gadofluorine, and made use of its hydrophobic fluorinated side chains to form nano-micelles in hydrophilic environments. Testing Gd-chelate micelles in hypercholesterolaemic rabbits confirmed their accumulation in lipid rich areas of plaque [30].

In 2010, Morishige *et al.* designed enhanced superparamagnetic nanoparticles targeted to macrophages to detect plaque vulnerability by quantifying macrophage burden [31]. When testing these macrophage-targeted monocrystalline iron oxide nanoparticles (MION-47) in cholesterol-fed New Zealand White rabbits, results proved this method with potentially clinically translatable utility in assessing plaque condition [31]. In 2011, Woodward *et al.* patented a multifunctional MRI or PET scanning tracer capable of targeting angiogenesis in cancer or atherosclerosis, for imaging and monitoring [32]. The structure of this tracer is an amphiphilic comb-like nanostructure conjugated first with an oligopeptide comprising a fragment of a natriuretic peptide, and second, with a signaling moiety (which may vary depending on the imaging technique used). The great variety of embodiments that the tracer can comprise allows it to be useful in imaging and monitoring of human atherosclerotic plaque or angiogenesis, stroke and heart attack [32].

Non-invasive MRI imaging of angiogenesis has been further advanced by conjugating contrast agents to antibodies or peptides to specifically target certain antigens known to be biomarkers of angiogenesis. To this end, several more recent studies have focused on assessing the efficacy of $\alpha_v\beta_3$ integrin-targeted contrast agents ([25]; [33]). The $\alpha_v\beta_3$ integrin, a cell surface glycoprotein receptor, is highly upregulated in both inflamed angiogenic endothelial cells and activated macrophages that have filtered into the plaque ([28]; [25], [34]). Winter *et al.* formulated a $\alpha_v\beta_3$ integrin-targeted paramagnetic Gd-based contrast agent by conjugating perfluorocarbon emulsion-encapsulating nanoparticles with multiple Gd paramagnetic chelates to Arg-Gly-Asp (RGD), a short amino acid sequence binder of the $\alpha_v\beta_3$ integrin. When tested in cholesterol-fed New Zealand White rabbits, the study revealed MRI enhancement in at sights of neovasculature, indicating the successful targeting of the contrast to the $\alpha_v\beta_3$ integrin [25].

In 2011, Kitagawa *et al.* conjugated ferritin nanoparticles to RGD, to similarly target $\alpha_v\beta_3$ integrin for neoangiogenesis imaging in abdominal aortic aneurysm (AAA) lesions [28]. A heavy-chain ferritin protein cage nanoparticle was coated with RGD peptides, and later conjugated to a Cy5.5 fluorophore. Successful specific targeting of the $\alpha_v\beta_3$ integrin was assessed by near-infrared fluorescence, revealing the potential of enhancing molecular imaging of macrophages and angiogenic endothelial cells in a mouse AAA model [28].

Imaging and Detection by Targeted Fluorescence

Detection of plaques at high risk of rupturing is critical to predicting and potentially preventing acute events and reducing associated patient mortality. An ideal diagnostic tool would be a non-invasive indicator that allows plaque vulnerability assessment. Both the high concentration of macrophages accumulated along the plaque neovasculature and the activated nature of neovascular endothelium are attractive targets for targeted imaging of angiogenesis within the plaque. Lam MK *et al.* developed a VEGF receptor-targeted indicator that enables near infrared imaging of VEGF receptors. The indicator, a Cy5.5-conjugated single-chain VEGF (scVEGF) that mimics the anti-VEGF antibody, allows for mapping of VEGF expression throughout the atherosclerotic plaque [35]. VEGF, a highly specific mitogen of endothelial cells, is induced by hypoxia and various cytokines, and promotes EC proliferation, vessel permeabilization and angiogenesis [36].

In another study, Nahrendorf *et al.* attached a VCAM-1-specific peptide, expressed on an M13 bacteriophage, to a magnetofluorescent nanoparticle. Because upregulated expression of VCAM-1 is characteristic of dysfunctional, activated endothelium, this peptide conjugate was internalized first by endothelial cells and then by smooth muscle cells (SMC) and macrophages in a mouse model of atherosclerosis [30].

As mentioned earlier, imaging of macrophage burden in atherosclerosis is closely associated with plaque vulnerability and even response to vascular intervention. Macrophage infiltration into the fibrous cap is abundant at sites of plaque rupture, and such cells are easily recruited and activated by dysfunctional endothelium expressing high levels of cell adhesion molecules ([37]; [27]). Macrophages are easily activated without antigenic specificity by many atherosclerotic risk factors, and cause indiscriminate tissue damage when secreting numerous pro-inflammatory, procoagulant, and proteolytic molecules, and effector molecules ([27]; [38]; [39]). Macrophages also produce proteases which cause degradation of the extracellular matrix, thinning of the fibrous cap and overall destabilization of the plaque (Boyle, 2005; Tabas, 2009; Dollery & Libby, 2006). Furthermore, macrophage apoptosis is known to contribute to necrotic core formation, mainly by promoting inflammation, plaque instability, leading to development of a vulnerable plaque and its eventual fatal rupture (Virmani *et al.*, 2005; Hu, Du, Chu, & Otsuka, 2010; Tabas, 2009).

Deguchi *et al.* took advantage of a special probe designed for imaging matrix metalloproteinase activity after myocardial infarction and applied it to monitoring metalloproteinase action in atheroma to assess plaque vulnerability (Chen *et al.* 2005 Circulation, [41]). This near-infrared fluorescence probe is activated by proteolytic cleavage by MMP2 and MMP9. Other studies used macrophage-targeted, near-infrared fluorescent magnetofluorescent nanoparticles to image *in vivo* macrophage activity in plaque with high-resolution laser scanning fluorescence microscopy ([42], [43]). In yet another more recent study, Saxena *et al.* used NIFR fluorescent dextrancoated nanosensors (CLIO-VT680) to map inflammatory-activated macrophages *in vivo* in endovascular injured murine carotid arteries [44]. This study confirmed the possibility of *in vivo* monitoring of macrophage response to arterial injury.

BIOMARKERS OF ANGIOGENESIS, ANGIOGENESIS INHIBITORS AND THE ART OF LOCAL DELIVERY

The profound implications of angiogenesis in promoting intra-plaque hemorrhage, plaque rupture and thrombosis have suggested that anti-angiogenetic therapy can bring plaque stabilization and possibly plaque regression. Anti-angiogenic substances, many of which have already met successes in hindering oncologic angiogenesis also have the potential to stabilize or reverse atherosclerotic disease progression [29].

The vitronectin receptor, $\alpha_v\beta_3$ integrin, described in the previous section as a target for imaging, may also be considered a target for antiangiogenic treatment. In one study, Maile *et al.* used a monoclonal antibody targeted against $\alpha_v\beta_3$ which successfully inhibited development of atherosclerotic lesions in diabetic pigs [45]. Some anti-angiogenic

substances have caused adverse effects to humans when administered as free drugs in clinical doses. By locally delivering these substances to the specific desired molecular receptors, such as the $\alpha_v\beta_3$ integrin, the dosage could be reduced significantly and thus prevent the adverse effects found for some of the substances in humans [29]. For instance, fumagillin is an effective angiogenesis inhibitor that disables endothelial proliferation [46], though its water soluble form (TNP-470) has proven to cause neurocognitive adverse effects in humans when used at high doses [47][29]. Furthermore, the concept of using targeted paramagnetic nanoparticles to deliver antiangiogenic drugs is extremely useful in non-invasive MRI or US imaging of real time effects of receptor-targeted drugs. With these ligand-directed, lipid-encapsulated nanoparticles [33], even quantification of ligand-bounded particles can be achieved using the high fluorine signal from the system's core [48]. Using this technique, Winter *et al.* conjugated fumagillin to targeted nanoparticles thereby significantly reducing the necessary fumagillin dose to obtain an antiangiogenic effect.

THE PATH AHEAD

In the light of recent biological advances, how can certain fundamental questions remain unresolved? Both suggested underlying mechanisms of atherosclerosis, such as the causality of inflammation in atherosclerosis, or the contribution of lipid oxidation to plaque development and growth, and the extrapolation of successes in animal models to humans, are yet to be unequivocally accepted or rejected [49]. As Dr. Libby *et al.* stated in Nature 2012, the insufficient number of clinical trials is becoming increasingly evident. Even the obvious differences between the animal models that attempt to reproduce a structurally different human vascular environment shine light on the growing necessity of human clinical trials [49].

Innovation in the health sector can only advance from scientific breakthroughs to therapeutic solutions in the clinic by intermingling disciplines in a directed manner. The pharmaceutical industry devotes in average more than \$6 billion per chemical entity subject to enter the market place and what is even more worrisome, 8–10 years to subject the candidate drug or diagnostic marker to the *in vitro*, preclinical and clinical validation needed for its regulatory approval. The same is true for biomedical devices. For example, the development of two dominant first generation drug eluting stents cost \$1 billion fifteen years ago and today this price of market entry has risen 6–10 fold. During the last decades, the pace of research and the number of publications and patent applications have continued to increase in an exponential manner and yet the number of products that reach the market remains stagnant. In the US the time from regulatory submission to regulatory approval along a PMA track has risen from 15 to 32 months. It is clear that an integrated approach to propulsion of technology from concept to clinic must be devised. It is increasingly further evident that critical clinical problems and complex cell-tissue-device interactions may be unraveled best by a pandisciplinary approach that brings engineers of all kinds and mathematicians together with biologists and physicians. Only integrated approaches-computational, *in vitro* and *in vivo*- embedded in a culture of open innovation will enable us to bridge the gap between scientific findings and clinical applications.

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Fig. 1. The role of endothelium in the pathway of atherosclerosis

In its quiescent, healthy state, the endothelium provides for essential regulatory processes that include vessel tone regulation, inhibition of SMC proliferation and transmigration and anticoagulant properties (A). Over several years of exposure to risk factors, the endothelium becomes activated, losing its functionality. This endothelial dysfunction causes the loss of alignment with flow, the upregulated expression of inflammatory and intercellular adhesion molecules which in turn causes recruitment of leukocytes and inflammatory cells. Endothelial dysfunction is significantly aggravated by flow disruptions, like those caused in recirculation areas of bifurcated regions (B). ECs in these areas express higher levels of CAMs (D, VCAM expression is determined by immunostaining with anti-VCAM-1) and are more prompted to monocyte attachment (E, monocytes are labeled with anti-CD14 and anti-tubulin) than those in regions of undisrupted flow (C). With time, a patient will eventually develop advanced atherosclerosis (F).

2.I

2.II

2.III

Fig. 2. The sequential development of atherosclerosis, with special attention set on atherogenesis of plaque

2.I) a. In the early stages of the disease, endothelial cells become activated, expressing several adhesion molecules that cause the recruitment of leukocytes, namely monocytes. These monocytes become activated macrophages and transmigrate into the intima, where their uptake of lipids causes them to morph into macrophage foam cells. b. As lesions progress, SMC migrate into the intima and begin to build a fibrous cap by secreting mainly collagen, proteoglycans and elastin. The apoptosis of both macrophage foam cells and SMC, and the accumulation of cholesterol lipids, begin the formation of a necrotic core. c. The formation of neovasculation from the adventitia vasa vasorum is mainly due to hypoxia in the atheromatous plaque. This leaky neovasculation (further described in Figure 2.II and 2.III) plays an important role in the progression of the vulnerable plaque, in which d. the fibrous cap is degraded by metalloproteases secreted by macrophages. When the plaque

finally ruptures, this may cause fatal thrombosis. 2.II) The neovasculature formed in atheroma is characterized by its leaky nature. The permeable junctions between the microvascular endothelial cells in these immature cells allow for extravasation of red blood cells (RBC) and intraplaque hemorrhage, which is responsible for further macrophage recruitment. In turn, the activated macrophages secrete metalloproteases that degrade the extracellular matrix and cause the plaque vulnerability that can lead to fibrous cap thinning and plaque rupture described in part 2.I of this figure.

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