



# Inflammation and thrombosis – testing the hypothesis with anti-inflammatory drug trials

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3 **INFLAMMATION AND THROMBOSIS – TESTING THE HYPOTHESIS**  
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5 **WITH ANTI-INFLAMMATORY DRUG TRIALS**  
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10 **Running title: Inflammation and Thrombosis**  
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**ABSTRACT**

The hypothesis of atherosclerosis as an inflammatory process has been a *Leitmotiv* in cardiology for the past 20 years, and has now led to the launch of clinical trials aimed at testing whether drugs that primarily target inflammation can reduce cardiovascular events. Inflammation indeed drives all phases of atherosclerosis, from inception, through progression, and ultimately acute thrombotic complications (plaque rupture and probably plaque erosion). Since plaque rupture and erosion cause most acute coronary syndromes, **appropriately tuned** anti-inflammatory treatments should limit myocardial infarction and cardiovascular death. Beyond interrupting inflammation-related plaque disruption, such treatments might however also ameliorate the propensity to thrombosis once the trigger (plaque rupture or erosion) has occurred.

Several lines of evidence support this view: experimental data document the role of inflammation in platelet activation, tissue factor-mediated coagulation, hyperfibrinogenemia, impaired activity of natural anticoagulants (including those expressed by endothelial cells), and reduced fibrinolytic activity. Supporting evidence also derives from the involvement of inflammation in venous thrombosis, a process that commonly occurs in the absence of traditional risk factors for atherosclerosis but associates with several inflammatory diseases including obesity. Ongoing trials, in addition to evaluating effects on primary outcomes, will afford the opportunity to probe the possibility that anti-inflammatory interventions that yield salutary changes in biomarkers of the thrombotic/fibrinolytic balance also translate into reduction of clinical events.

**Keywords:** inflammation; thrombosis; methotrexate; colchicine; interleukin-1 receptor antagonist.

## INTRODUCTION

The hypothesis that inflammation drives atherosclerosis has gained currency in cardiovascular medicine. Inflammation contributes pivotally to all stages of atherosclerosis, from its inception (endothelial dysfunction, recruitment of immune cells, LDL modifications, foam cell formation, and apoptosis), and ultimately acute thrombotic complications triggered by plaque rupture or by plaque erosion (1). Inflammatory activation, involving multiple components of innate or acquired immunity (2), predisposes plaques to such acute complications (3). As corroborating histopathological evidence, circulating and easily measurable indices of inflammation, such as high-sensitivity (hs) C-reactive protein (CRP), myeloperoxidase, and leukocyte count, correlate with cardiovascular risk for events such as myocardial infarction, stroke and cardiovascular death (4, 5). Emerging biomarkers of inflammation in cardiovascular diseases include microparticles (MPs), which are small vesicles released from activated or apoptotic cells and containing signal transduction proteins, messenger (m)RNAs, and micro (mi)RNAs: inflammatory stimuli promote production of these particles that themselves can regulate inflammation (6). The links between inflammation and cardiovascular disease have inspired clinical trials in progress to test whether drugs that target inflammation primarily can reduce cardiovascular events (7-9).

Thrombosis contributes critically to the natural history of atherosclerosis, often being the final actor by precipitating its acute clinical manifestations: rupture or erosion of atherosclerotic lesions expose the highly thrombogenic subendothelial layers to the flowing blood, and initiate platelet adhesion and aggregation and fibrin formation, precipitating clot formation (10). Inflammation can therefore cause thrombosis indirectly, by fostering atherosclerosis and its complications. Inflammation might, however, also promote clot accumulation directly, increasing the thrombotic risk as a consequence of increased blood thrombogenicity or impaired fibrinolysis, as first hypothesized over a decade ago (11). Support for this hypothesis derives from evidence of a role for inflammation in venous thromboembolism (VTE), a process that can occur in the absence of traditional risk factors for atherosclerosis in a vascular bed usually spared by this disease. Proof of this concept may now emerge from secondary analyses of ongoing clinical trials with anti-inflammatory agents.

This review therefore aims to analyze the links between inflammation and thrombosis, to help set the stage for interpreting the results of clinical trials of anti-inflammatory agents in patients at risk for atherosclerotic events. **Other reviews have recently covered the topic (12-16), but this paper focusses primarily on the clinical translation of these concepts.**

## ARTERIAL THROMBOSIS VERSUS VENOUS THROMBOEMBOLISM

Traditional classifications have viewed arterial thrombosis and VTE as distinct pathological entities, with clear differentiating features. According to this classical schema, *white thrombus* characterizes the nascent arterial thrombosis, composed of an aggregate of platelets enmeshed in fibrin strands at the site of plaque rupture or erosion; while clot propagation (*red thrombus*) consists mostly of red blood cells entrapped in a growing fibrin network. Such propagation of the initial thrombus often occurs in cases of total arterial occlusion, such as in ST segment-elevation myocardial infarction (STEMI). Conversely, according to the classical view, venous thrombosis typically starts with the formation of a *red thrombus*, less dependent on platelets. Of the trio of precipitating factors postulated by Virchow, in VTE vessel wall injury traditionally lags in pathogenic relevance after hypercoagulability and stasis. Inherited thrombophilia, due to factor (F) V variants leading to resistance to activated protein C (APC) or the prothrombin variant G20210A, or the rarer natural anticoagulant antithrombin (AT) or protein C or protein S deficiencies, but also acquired thrombophilia in the setting of lupus anticoagulant or the anti-phospholipid antibody syndrome, increase the risk of VTE. Their link with arterial thrombosis (except in the case of the lupus anticoagulant) remains, however, unclear (17-19).

This classical – totally dichotomous – view has recently come under scrutiny. Current concepts link the pathogenesis of arterial and venous thrombosis **more tightly**. Clinical evidence supports this reassessment: patients with arterial thrombosis have an increased risk of venous thrombotic events; a history of VTE associates with the risk of future arterial events; and the two conditions share several risk factors, including age, obesity, hypercholesterolemia, hypertension, lupus anticoagulant, hyperhomocysteinemia, microalbuminuria and non-alcoholic hepatosteatosis. Moreover, evidence has emerged that platelets do indeed participate in venous thrombosis to a degree previously underestimated (20, 21). This commonality has therapeutic implications: current studies support roles for aspirin, low-molecular weight heparin and warfarin in the treatment of both venous and arterial thrombosis (22-27). As a consequence, the *silo thinking* of VTE and arterial thrombosis as completely separate entities has given place to a new concept that includes VTE in a panvascular syndrome that comprises thrombotic complications affecting the veins as well as the coronary, peripheral, and cerebrovascular arterial beds (28). The prevalence of carotid plaques in patients with unexplained venous thrombosis **that exceeds that** in those with events attributable to a well-identified cause (47.1% **vs** 27.4%), has stimulated consideration of a systemic thrombophilic state as an underlying feature (22). More recent evidence supports inflammation as a common

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3 pathogenic contributor to both arterial and venous thrombosis, giving rise to the concept of  
4 *inflammation-induced thrombosis* (26).  
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## 9 **RECIPROCAL RELATIONS BETWEEN INFLAMMATION AND THROMBOSIS**

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12 Inflammation and hemostasis can participate in a pathogenic positive feedback loop: inflammation  
13 unleashes hemostatic mechanisms, which in turn can amplify inflammation (29-31). Both  
14 **mechanisms participate** in host defenses that help eliminate or contain pathogens, limit tissue  
15 damage, and – in order to resolve – evoke counter-regulatory mechanisms that tend to restore  
16 homeostasis. In particular, fibrin deposition can serve to wall-off invading agents and confine the  
17 consequent inflammatory response to a limited area, e.g., abscess formation in coagulase-positive *S.*  
18 *aureus* infections (32). An exaggerated or insufficiently controlled thrombotic response may  
19 however contribute to disease, such as in the case of the systemic activation occurring with severe  
20 infection or sepsis. This **scenario** results from a spatially uncontrolled systemic inflammatory  
21 response, and leads to multiple organ dysfunction and possibly failure in the setting of disseminated  
22 intravascular coagulation (32, 33).  
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33 The interrelationship between inflammation and hemostasis reaches back to the early stages of  
34 evolution: invertebrates have a single cell type, the hemocyte, which can perform both anti-  
35 inflammatory/immune and hemostatic functions. This overlap between innate immunity and  
36 hemostasis has endured over millions of years of evolution (34). Cross talk between these the two  
37 functions involves cell receptor-mediated signaling, cell-cell interactions, and the production of  
38 cell-derived microvesicles, with dual functions, by endothelial cells, leukocytes and platelets (35).  
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45 Inflammation affects the hemostatic system leading to a procoagulant state by affecting all cellular  
46 and humoral components of hemostasis, including vascular endothelial cells, platelets, the plasma  
47 coagulation cascade, the physiologic anticoagulant pathways, and fibrinolytic activity (31).  
48 Likewise, many elements of the hemostatic system, such as platelets, thrombin, FXa, the tissue  
49 factor (TF)-FVIIa complex, fibrinogen, and fibrin, can augment inflammation. Thrombin activation  
50 not only promotes fibrin generation, but **also** – by cleaving protease-activated receptors (PARs) –  
51 promotes the production of pro-inflammatory mediators, such as cytokines, chemokines, growth  
52 factors, and leukocyte adhesion molecules. Indeed, the crosstalk between inflammation and  
53 thrombosis generates a vicious circle that can amplify pathological processes and tissue damage.  
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3 Thus, therapeutic targeting of either coagulation or inflammation may influence the onset and the  
4 progression of multiple diseases the pathogenesis of which involves both processes (31, 32, 36).  
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## 9 10 **THROMBOSIS AS AN INFLAMMATORY PROCESS**

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12 Inflammation can perturb the hemostatic system, promoting a pro-thrombotic state, through several  
13 mechanisms (summarized in **Table 1 and Figure 1**): in particular, inflammation affects endothelial  
14 function, increases platelet reactivity, and activates plasma coagulation by activating procoagulant  
15 mechanisms and muting anticoagulant mediators (37).  
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### 20 21 **Endothelial cell dysfunction**

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24 Vascular endothelial cells furnish an antithrombotic surface that, in normal conditions, prevents the  
25 inappropriate activation of hemostasis through a balanced production of pro- and anti-inflammatory,  
26 pro- and anti-coagulant, anti- and pro-fibrinolytic molecules. Inflammation leads to an imbalance  
27 favoring procoagulant and antifibrinolytic components, including von Willebrand factor (VWF),  
28 thromboxane (TX)<sub>A2</sub>, plasminogen activator inhibitor (PAI)-1, TF, and cell adhesion molecules,  
29 causing a shift from an anticoagulant, anti-inflammatory and vasodilatory endothelial suite of  
30 functions to a proinflammatory and prothrombotic state. Endothelial cells contribute to  
31 *inflammation-induced thrombosis* acting at all levels of hemostasis, including the activation of  
32 platelets and other inflammatory cells, TF-mediated thrombin generation, reduction of physiologic  
33 anticoagulant functions, and the reduction/suppression of fibrinolytic activity. A number of small  
34 molecules also serve as pro- or anti-inflammatory mediators that may have impacts on thrombosis  
35 and hemostasis, including prostaglandins, **reactive oxygen species**, nitric oxide, and regulators of  
36 tissue remodeling enzymes (31) (**Figure 1**).  
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### 48 49 **Platelet activation**

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51 Platelets not only participate in primary hemostasis, but also contribute to *inflammation-induced*  
52 *thrombosis*. Platelet activators include not only thrombin and ADP, but also molecules implicated in  
53 mediating inflammation, such as bacterial endotoxins (38) and the lipid mediator platelet-activating  
54 factor (PAF) (38). Activated platelets express on their surface or secrete pro-inflammatory and pro-  
55 coagulant substances, such as adhesion molecules, growth factors, cytokines (including  
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3 CD154/CD40 ligand) and the inhibitor of fibrinolysis PAI-1, and they allow the surface assembly of  
4 coagulation factors (39). Moreover, platelets contribute to a systemic prothrombotic state through  
5 the generation of TF-expressing MPs and the expression of P-selectin, which, through the activation  
6 of the transcription factor nuclear factor (NF)- $\kappa$ B, in turn promotes the production of  
7 proinflammatory molecules by endothelial cells and leukocytes, as well as the expression of TF in  
8 monocytes, endothelial and also smooth muscle cells (31, 32, 36, 40).  
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### 14 **Modulation of plasma coagulation**

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18 TF acts as the pivotal initiator of inflammation-induced thrombin generation, thus bridging  
19 inflammation and thrombosis (32). Various cell types constitutively express this transmembrane  
20 molecule. Most TF-expressing cells localize in tissues that do not contact blood directly, such as in  
21 the adventitial layer of blood vessels. TF comes in contact with blood in case of breaches in  
22 vascular integrity or when induced in vascular endothelial cells or circulating blood cells (31, 32,  
23 36). The source of TF may differ in various inflammatory conditions: in atherosclerotic plaques,  
24 macrophage-derived foam cells account for much TF expression, likely as a result of stimulation by  
25 pro-inflammatory cytokines such as CD154/CD40 ligand (41). Rupture of atheromatous plaques  
26 permits contact of blood coagulation factors with TF residing within the lesion (32, 42). In sepsis,  
27 TF expressed on the endothelium and on circulating mononuclear cells in response to endotoxins  
28 and/or proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1, or IL-6  
29 (43), leads to systemic activation of coagulation (31, 32). Cells that do not ordinarily express TF  
30 can acquire it from MPs (34, 36). MPs act as biological shuttles, enabling otherwise unusual cell  
31 interactions, and may represent one important intersection between thrombosis and inflammation by  
32 furnishing a reservoir of TF released from cells with either inducible (monocytes, macrophages,  
33 endothelial cells and platelets) or constitutive expression (cells in the adventitia or in certain  
34 tumors). Stimuli for TF production and its release in MPs include endotoxin, cytokines,  
35 microorganisms, thrombin, low shear stress, oxidative stress, hyperglycemia, smoking,  
36 dyslipidemia, complement and immune reaction. Beside the circulating pool of MPs, a pool of MPs  
37 sequestered in the plaque provides another TF reservoir in atherothrombosis. Apoptotic  
38 macrophages and smooth muscle cells may contribute to accumulation of TF-bearing MPs in the  
39 lipid core (6). TF exposed to blood binds to FVIIa, which then catalyzes the conversion of FX into  
40 FXa directly or through the activation of FIX. FXa, FVa, prothrombin (FII) and calcium constitute  
41 key components of the prothrombinase complex on the surface of activated cells, able to generate  
42 thrombin, which then converts fibrinogen into fibrin (31, 32, 39, 44).  
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3 The main physiological anticoagulant mechanisms include antithrombin (AT), the protein C system  
4 and TF pathway inhibitor (TFPI). These mediators function to prevent blood clotting in  
5 physiological conditions and to limit coagulation activation to the site of vascular injury.  
6 Impairment in their function represents an important mechanism of the procoagulant state  
7 associated with inflammation (31). Antithrombin (AT, formerly AT III), a serine protease inhibitor,  
8 targets thrombin and FXa. AT levels decrease markedly in inflammation because of consumption  
9 (related to ongoing thrombin generation), impaired synthesis (as a result of the acute phase  
10 response), and degradation by neutrophil elastase (32). Additionally, cytokines can reduce the  
11 synthesis of glycosaminoglycans on the endothelial surface, which otherwise normally promote the  
12 anticoagulant activity of AT (31).

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14 Protein C undergoes activation by thrombin bound to the endothelial cell membrane molecule  
15 thrombomodulin. Activated protein C (APC) cleaves the essential cofactors of coagulation FVa and  
16 FVIIIa. In addition, by binding to the endothelial protein C receptor (EPCR), it increases its own  
17 activation mediated by the thrombomodulin–thrombin complex, and inhibits induced TF expression  
18 on mononuclear cells. Inflammation limits the protein C system due to reduction of  
19 thrombomodulin, produced by TNF and IL-1 on the endothelial surface (36).

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21 The third physiological anticoagulant mechanism is TFPI, a serine protease inhibitor of the TF-  
22 FVIIa complex that attaches to the endothelium via glycosaminoglycans. The impact of  
23 inflammation on TFPI function has received little attention. Proinflammatory cytokines can reduce  
24 glycosaminoglycan synthesis in endothelial cells, affecting TFPI function (31, 32).

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26 The fibrinolytic system also modulates thrombosis induced by inflammation. Plasmin lyses clots by  
27 degrading fibrin. Generation of plasmin from plasminogen depends on the action of tissue-type  
28 plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Type-1  
29 plasminogen activator inhibitor (PAI-1) can inhibit both of these plasminogen activators, reducing  
30 their fibrinolytic potential. Inflammatory states transiently increase fibrinolytic activity, due to the  
31 release of tPA stored in vascular endothelial cells, followed by a more sustained increase of  
32 endothelial PAI-1 production induced by TNF and IL-1. Moreover, platelets also contain preformed  
33 PAI-1 stored in alpha-granules, and release it upon their activation. Thus, inflammatory signaling  
34 yields a net inhibition of fibrinolysis, contributing to *inflammation-induced thrombus accumulation*  
35 (31, 32).

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57 Fibrinogen also participates in the inflammatory response. The liver synthesizes fibrinogen, an  
58 acute phase reactant the blood concentration of which increases in conditions characterized by  
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3 infection or inflammation (44). Hyperfibrinogenemia not only serves as a biomarker of thrombotic  
4 risk, but can contribute causally to both venous and arterial thrombosis (45). An elevated plasma  
5 fibrinogen level (>4 mg/mL) associates **significantly** with increased risk of VTE **in a** concentration-  
6 **dependent manner** (45). Elevated fibrinogen levels increase blood viscosity and red blood cell  
7 agglutination in the setting of a hyperviscosity syndrome that increases blood flow shear stress,  
8 leading itself to endothelial cell and platelet activation (44). Moreover, several clinical studies have  
9 suggested that abnormal fibrin accumulation modulates thrombosis: clots with increased fibrin fiber  
10 density and resistance to fibrinolysis associate with both arterial and venous thrombosis (46-52).  
11 The structure of the fibrin network reflects fibrinogen and thrombin concentration: indeed, elevated  
12 levels of fibrinogen and thrombin increase fibrin network density, clot stiffness, and the resistance  
13 of thrombi to fibrinolysis, contributing to clot accumulation and persistence (45).  
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### 22 **Neutrophil extracellular traps: a novel link between innate immunity and thrombosis**

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24 Polymorphonuclear leukocytes participate pivotally in host defenses against infection or injury.  
25 These neutrophilic leukocytes, attracted to sites of microbial invasion or tissue damage, release their  
26 granular contents, produce reactive oxygen species, generate lipid mediators, and then die, a process  
27 probably involving apoptosis. In the throes of death, neutrophils release strands of their nuclear  
28 DNA that become decorated with various proteins, among them the pro-oxidant enzyme  
29 myeloperoxidase, and the pro-coagulant tissue factor. These strands of extruded DNA provide a  
30 “solid state” reactor at sites of acute inflammation that can locally amplify oxidant stress and trigger  
31 thrombosis. Moreover, in combination with a fibrin network, these structures, known as neutrophil  
32 extracellular traps or NETs, participate in the propagation thrombosis, entrap platelets, and thus  
33 promote local thrombosis.  
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42 When NETs form on the intimal surface of veins or arteries, they can amplify and extend local  
43 thrombosis. Recent experimental work has documented that NETs provide a scaffold for thrombus  
44 formation, and participate in platelet activation (20). Further observations implicated NETs in  
45 experimental (53) and in human venous thrombosis (54). Markers of NET formation correlate with  
46 the severity of human coronary atherosclerosis and evidence for a prothrombotic state (13). In  
47 addition, NETs themselves can amplify inflammation by activating the NLRP3 inflammasome, a  
48 multimeric intracellular assembly of proteins that generates the active forms of the pro-  
49 inflammatory cytokines IL-1 beta and IL-18 (55). Thus, NETs mediate a newly recognized  
50 mechanism that links innate immunity and inflammation. This pathway likely promotes the  
51 prolongation and propagation of local inflammatory and thrombotic responses in both veins and  
52 arteries (56).  
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## CLINICAL IMPLICATIONS

The increased thrombotic risk encountered in patients with conditions characterized by a proinflammatory state, such as autoimmune diseases, infections and sepsis, underscores the links between inflammation and thrombosis (**Table 2 and Figure 2**) (57). Patients with rheumatoid arthritis have a 1.5 to 6 times increased risk of VTE, a reflection in part of their active systemic inflammation. Inflammatory cytokines implicated in rheumatologic diseases, such as IL-6, IL-8, or TNF- $\alpha$ , can activate hemostatic pathways promoting a thrombotic tendency, and also inhibit fibrinolysis (58-61). Thromboembolic complications, both venous and arterial, also commonly cause serious extra-intestinal complications in patients with inflammatory bowel diseases inflammation likely contributes to their elevated risk of thrombosis (62-64).

The acute phase of two prototypic autoimmune skin diseases, chronic autoimmune urticaria and bullous pemphigoid, also associate with hypercoagulability, although only the latter condition has clearly increased thrombotic risk. The crosstalk between inflammation and thrombosis likely participates in the pathophysiology of both autoimmune diseases. The stronger activation of systemic coagulation in patients with bullous pemphigoid may contribute to the increased thrombotic risk. In these patients, the reduction in coagulation activation observed after immunosuppressive treatments may contribute to the healing of cutaneous manifestations, but also to the reduction of the associated thrombotic risk (65).

Systemic inflammation can participate in the pathogenesis of the increased rates of thrombosis documented in other autoimmune diseases, such as autoimmune hemolytic anemia, immune thrombocytopenic purpura, systemic lupus erythematosus, systemic vasculitides, the antiphospholipid antibody syndrome, Sjögren's syndrome, and systemic sclerosis (66-70). Similarly, increased microvascular thrombosis is found in chronic and acute infections and in sepsis, where it contributes to multiple organ dysfunction. Indeed, strategies to manage microvascular thrombosis appear to reduce morbidity and mortality outcome in clinical studies (71, 72), illustrating the antithrombotic potential of aggressive anti-inflammatory treatment during the active phases of several types of diseases.

## ANTI-INFLAMMATORY THERAPIES IN CARDIOVASCULAR DISEASE

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The inflammatory aspects of atherosclerosis and its complications have already inspired several clinical trials aimed at testing the possibility that anti-inflammatory therapies will reduce cardiovascular events in various settings of risk, including trials with COX-2 inhibitors (coxibs), anti-TNF agents, etc. (5, 8, 73). Most such trials have either used agents not selective for targeting inflammation, or have lacked sufficient power to address cardiovascular endpoints.

Statin treatment reduces the incidence of all-cause mortality and major coronary events in appropriately selected individuals in the setting of both secondary and primary prevention (74). Statin therapy, beyond its effect on lipids, reduces the concentration of CRP (75), a biomarker that adds to prediction of risk of a first myocardial infarction and ischemic stroke in healthy men and women (76, 77). Patients who achieve lower CRP levels on statin therapy have better clinical outcomes regardless of low-density lipoprotein (LDL) cholesterol levels (78). The *Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin* (JUPITER) tested the proposition that inflammatory status could identify a group of individuals in primary prevention that would benefit from statin treatment. This study demonstrated that rosuvastatin (20 mg daily) reduced cardiovascular risk in individuals with above median levels of CRP (>2 mg/L) and below median LDL-cholesterol values (LDL <130 mg/dL) (79). Yet, because statins lower both cholesterol LDL and CRP, JUPITER did not aim to test whether rosuvastatin reduced events by an anti-inflammatory mechanism independent of reduced LDL. Highly relevant to the thesis of this review, JUPITER also documented a reduction of both provoked and unprovoked VTE, likely related to the anti-inflammatory effect of the statin or a conceivably direct antithrombotic effect, as VTE risk depends little if at all on LDL (80).

More recently, however, trials with drugs that target inflammation more specifically have attempted to avoid confounding due to effects on other determinants of cardiovascular risk. The *Low-Dose Colchicine* (LoDoCo) trial used a prospective randomized open blinded endpoint (PROBE) design to test the effects of colchicine on cardiovascular events in a relatively small but intriguing study. The results provided strong preliminary evidence in support of the concept that an anti-inflammatory drug could reduce cardiovascular events (7). This study points to the urgent need for a larger double-blind placebo-controlled trial of colchicine in the context of cardiovascular prevention. The *LosmApimod To Inhibit p38 MAP kinase as a Therapeutic target and modify outcomes after an acute coronary syndromE* (LATITUDE)-TIMI-60 study investigated the effects an inhibitor of p38 MAP kinase, losmapimod, on current events in patients with acute coronary

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3 syndromes. The part A of the study followed over 3,500 patients, and the results did not support  
4 expanding of this preliminary part into a larger-scale outcome trial (81).  
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7 Two phase III studies (**Table 3**) currently underway provide further opportunities to test anti-  
8 inflammatory interventions of distinct mechanisms on recurrent cardiovascular events in at-risk  
9 populations. The *Canakinumab Anti-inflammatory Thrombosis Outcomes Study* (CANTOS) has  
10 enrolled patients stable post-myocardial infarction with hsCRP >2 mg/L despite secondary  
11 prevention strategies. CANTOS examines whether canakinumab, a human monoclonal antibody  
12 that selectively neutralizes IL-1 $\beta$ , will reduce rates of recurrent myocardial infarction, stroke, and  
13 cardiovascular death (82). **A specific modulation of the IL-1 pathway as a therapeutic strategy in the  
14 atherosclerosis finds support in the recent appreciation of the role of the “inflammasome” in  
15 atherogenesis, as outlined above (55), since caspase-1, an end-product of the inflammasome,  
16 cleaves the pro-inflammatory cytokines IL-1 $\beta$  and IL-18 into their active forms, which when  
17 released from cells, promote local inflammation (83-85). The discovery that cholesterol crystals  
18 activate the inflammasome in human macrophages, resulting in the release of active IL-1 $\beta$   
19 underscores the interrelation between atherogenic risk factors and vascular inflammation (86, 87).**  
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30 The *Cardiovascular Inflammation Reduction Trial* (CIRT) **is enrolling** patients with elements of the  
31 metabolic syndrome and post-MI or with stable multivessel coronary artery disease. **Distinct** from  
32 CANTOS, that studies a strategy interfering with one specific anti-inflammatory **mediator**, CIRT  
33 will evaluate the efficacy in reducing cardiovascular events of very low-dose (10-25 mg weekly)  
34 methotrexate, a broadly acting anti-inflammatory drug commonly used for the treatment of  
35 rheumatoid arthritis (88, 89). Since plaque rupture and erosion trigger most arterial thromboses,  
36 these treatments may limit reduce the hard endpoints by reducing inflammatory drivers of  
37 thrombosis and impaired fibrinolysis. The hypothesis of *inflammation-induced thrombosis* predicts  
38 a reduction of VTE events, as prespecified in the trial designs. Indeed these ongoing trials could  
39 provide a clinical confirmation of the direct role played by inflammation in the pathogenesis of  
40 thrombosis.  
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49 **A third ongoing phase IV trial of the effects of anti-inflammatory therapy on the incidence of  
50 cardiovascular events is ENTRACTE (*A Clinical Outcomes Study to Evaluate the Effects of IL-6  
51 Receptor Blockade With Tocilizumab in Comparison With Etanercept on the Rate of  
52 Cardiovascular Events in Patients With Moderate to Severe Rheumatoid Arthritis*), which compares  
53 tocilizumab, a humanized monoclonal antibody against the IL-6 receptor, to etanercept, a soluble  
54 TNF receptor, in patients with rheumatoid arthritis (**Table 3**) (90)**  
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## CONCLUSIONS

Anti-inflammatory therapy to prevent atherothrombotic events, as in other therapeutic areas, may entail unwanted effects. The inflammatory and immune responses contribute importantly to host defenses. These pathways participate in combatting infections, in tumor surveillance, as well as in wound healing and tissue repair. Hence, the quest to identify anti-inflammatory therapies that will selectively inhibit effector arms of innate and adaptive immunity involved in atherothrombosis without causing undue interruption of host defense mechanisms may require multiple attempts. While rodent and other experiments can prove highly informative from the mechanistic viewpoint, they seldom predict accurately the full spectrum of efficacy or of adverse effects encountered in clinical trials (91). Moreover, the high degree of redundancy in inflammatory signaling pathways presents a challenge, but also a potential advantage. A myriad of mediators (e.g., cytokines, chemokines, and lipid mediators) may promote the pathogenesis of the thrombotic complications of atherosclerosis. This menu provides a plethora of potential targets for intervention. From one perspective, this redundancy raises the hope that narrow blockade of a single mediator may provide Paul Ehrlich's hypothesized "magic bullet" to interrupt a pathogenic pathway, while the complementary mechanisms will limit undesired actions. Yet, the very multiplicity of mediators in host defenses indicates that the mere interruption of one of dozens of possible targets may not suffice to reduce events, as parallel pathways provide detours and bypasses should only one path be blocked. The survival value of host defenses has doubtless fostered this ability to compensate.

In practical terms, testing of a particular therapy in large-scale clinical trials requires not only a strong rationale, but also a degree of assurance regarding adverse actions. A number of biological approaches to neutralizing cytokine activities are making into the clinic, but for all considerations on the benefit-risk profile will be pivotal. For example, neutralizing IL-6 with a monoclonal antibody merits consideration as a therapeutic option for cardiovascular disease. Strong genetic evidence suggests causality of IL-6 in atherothrombotic events (92, 93). Yet, adverse perturbations and lipid profile might limit the utility of anti-IL-6 strategies in patients with established risk for atherosclerosis. Anti-TNF agents have transformed rheumatology, dermatology, and gastroenterology. Studies of TNF neutralization in patients with heart failure, however, not only failed to confer benefit, but raised notes of caution regarding adverse effects (94). On the other hand, lipid risk factors beyond LDL offer other potential targets for anti-inflammatory intervention. LDL itself associates weakly with indices of inflammation such as hsCRP. Yet, triglyceride-rich lipoproteins correlate strongly with this biomarker of inflammation (95). Thus, interventions that



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3 target triglyceride-rich lipoproteins or their associated pathogenic apolipoproteins might provide  
4 another avenue to quelling inflammation in atherothrombosis (96).  
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7 From the perspective of antithrombotic therapy, anti-inflammatory therapy does provide an avenue  
8 to reducing thrombotic risk without increasing bleeding. This happened in JUPITER, where  
9 rosuvastatin decreased the risk of deep venous thrombosis without producing an anticoagulant  
10 effect (97). Study of the anti-inflammatory treatments currently under scrutiny will not only  
11 evaluate efficacy, but also determine the safety of the interventions in individuals with heightened  
12 cardiovascular risk.  
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18 In sum, inflammation and thrombosis intertwine inextricably (30). Anti-inflammatory therapies may  
19 prevent thrombotic events not only by affecting the "solid-state" of the atherosclerotic plaque, but  
20 also by favorable effects on the "fluid phase" of the blood (11). The concerted effect of anti-  
21 inflammatory therapy on both compartments provides a potential new avenue for preventing  
22 thrombotic complications without augmenting bleeding risk, which is the Holy Grail of our field.  
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For Peer Review

## LEGEND TO FIGURES

**Figure 1.** Inflammation (top right) induces a pro-thrombotic state (bottom left) that influences plasma coagulation, endothelial cells, and platelets. Inflammation stimulates TF production triggering the coagulation cascade, inhibits the three main physiological anticoagulant mechanisms (AT III, APC and TFPI) and increases endothelial PAI-1 production inhibiting fibrinolysis. Inflammation favors the endothelial production of pro-coagulant and anti-fibrinolytic components, including VWF, TXA<sub>2</sub>, PAI-1, TF and cell adhesion molecules, causing a shift from an anticoagulant, anti-inflammatory and vasodilatory endothelial functions to a pro-inflammatory and pro-thrombotic state. Moreover, inflammation activates platelets, which express on their surface or secrete pro-inflammatory and pro-coagulant substances, such as adhesion molecules, growth factors, cytokines PAI-1, TF-expressing MPs and P-selectin.

Abbreviations: Ab: antibodies; APC: activated protein C; AT III: antithrombin III; MPs: microparticles; PAI-1: plasminogen activator inhibitor-1; TF: tissue factor; TFPI: tissue factor pathway inhibitor; t-PA: tissue-type plasminogen activator; TXA<sub>2</sub>: thromboxane A<sub>2</sub>; u-PA: urokinase-type plasminogen activator; VWF: von Willebrand factor; WBC: white blood cells.

**Figure 2.** Increased thrombotic risk in diseases with a proinflammatory state.

**Table 1: Direct mechanisms of inflammation-induced thrombosis (37)**

Endothelial cell dysfunction and activation

Platelet activation

Modulation of plasma coagulation

Augmented pro-coagulant functions Tissue Factor-mediated activation of coagulation

Reduction of endogenous anticoagulants: Antithrombin, Tissue Factor pathway inhibitor (TFPI); Protein C pathway

Inhibition of fibrinolytic activity

Hyperfibrinogenemia

Peer Review

**Table 2: Increased thrombotic risk in diseases with a pro-inflammatory state (57)**

<b>Autoimmune diseases</b>	Rheumatoid arthritis, inflammatory bowel diseases, chronic autoimmune urticaria, bullous pemphigoid, autoimmune hemolytic anemia, immune thrombocytopenic purpura, systemic lupus erythematosus, systemic vasculitides, the antiphospholipid antibody syndrome, Sjögren's syndrome and systemic sclerosis
<b>Infections and sepsis</b>	Chronic and acute

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Table 3. Ongoing phase III studies on drugs specifically targeting inflammation

TRIAL	DESIGN	AIM	DRUG TESTED	PRIMARY ENDPOINT	SECONDARY ENDPOINTS	REFERENCES
CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study)	A phase III, randomized, double-blind, placebo-controlled, multi-center, event-driven trial	Tests if reducing inflammation can reduce rates of recurrent cardiovascular events among stable post-myocardial infarction patients who remain at elevated vascular risk (hsCRP $\geq$ 2mg/L) despite usual care, including statin therapy	Low-dose methotrexate	Composite of non-fatal myocardial infarction, non-fatal stroke, or cardiovascular death	Total mortality, hospitalization for unstable angina requiring revascularization, new onset diabetes	82
CIRT (Cardiovascular Inflammation Reduction Trial)	A phase III, randomized, double-blind, placebo-controlled, multi-center, event-driven trial	Tests if reducing inflammation among stable coronary artery disease patients (prior myocardial infarction or angiographically demonstrated multivessel coronary artery disease) with type 2 diabetes or metabolic syndrome can reduce rates of cardiovascular events	Canakinumab, a human monoclonal antibody that neutralizes interleukin-1 $\beta$	Composite of cardiovascular death, non-fatal myocardial infarction and stroke	All-cause mortality, percutaneous or surgical coronary revascularization, hospitalization for congestive heart failure, incident venous thromboembolism, incident atrial fibrillation, incident diabetes, Incident peripheral artery disease, clinically worsening aortic stenosis	88, 89
ENTRACTE (A Clinical Outcomes Study to Evaluate the Effects of IL-6 Receptor Blockade With Tocilizumab in Comparison With Etanercept on the Rate of Cardiovascular Events in Patients With Moderate to Severe Rheumatoid Arthritis)	A phase IV, randomized, open-label, parallel-group, multicenter study	Evaluates the frequency of cardiovascular events on tocilizumab in comparison with etanercept in participants with rheumatoid arthritis.	Tocilizumab, a humanized monoclonal antibody against the interleukin-6 receptor and etanercept a soluble TNF receptor	Composite of cardiovascular death, non-fatal myocardial infarction and non-fatal stroke	A composite endpoint of major cardiovascular events plus non-fatal coronary revascularization procedures and hospitalization for unstable angina; as well as investigating the frequency of adverse events and serious adverse events	90

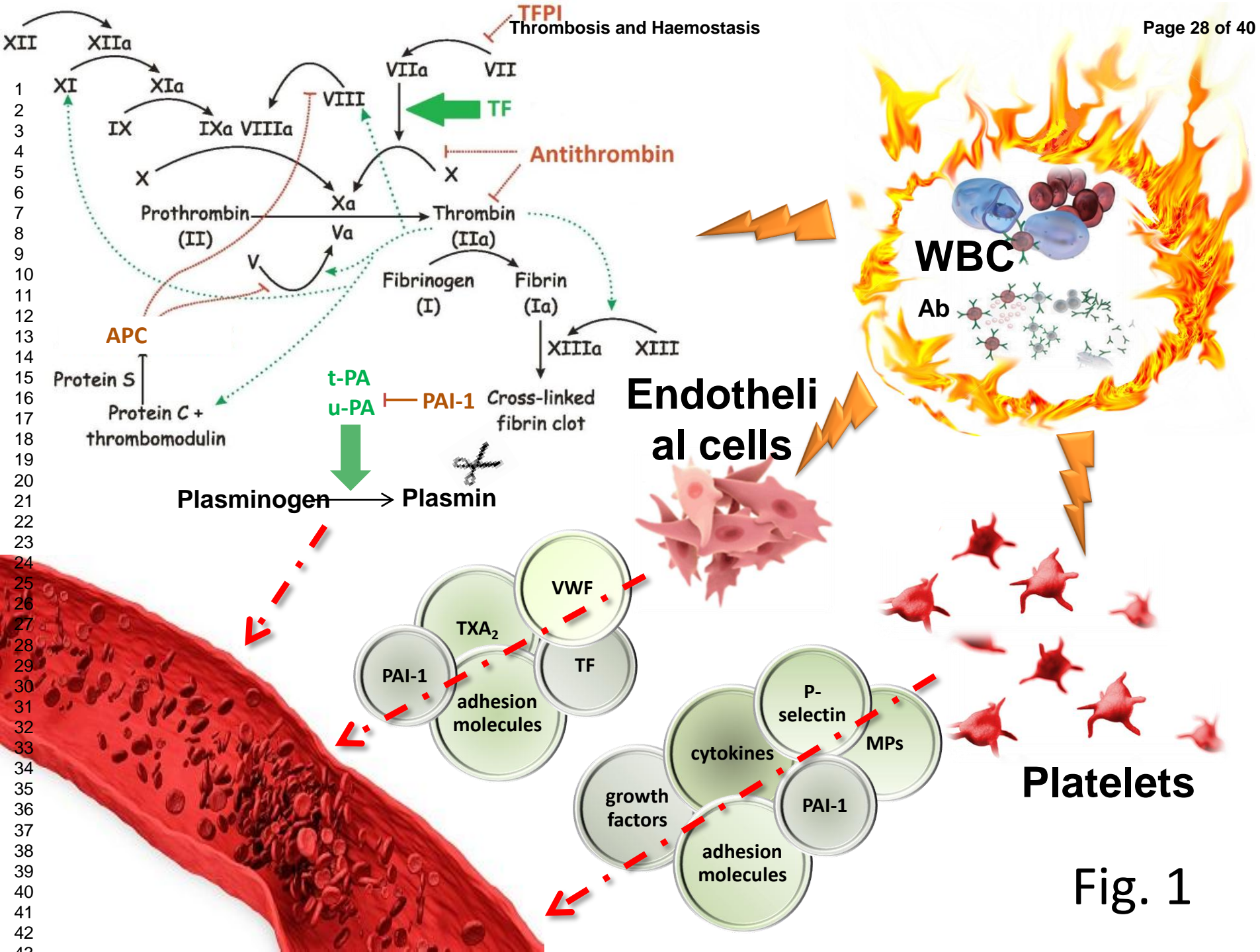


Fig. 1



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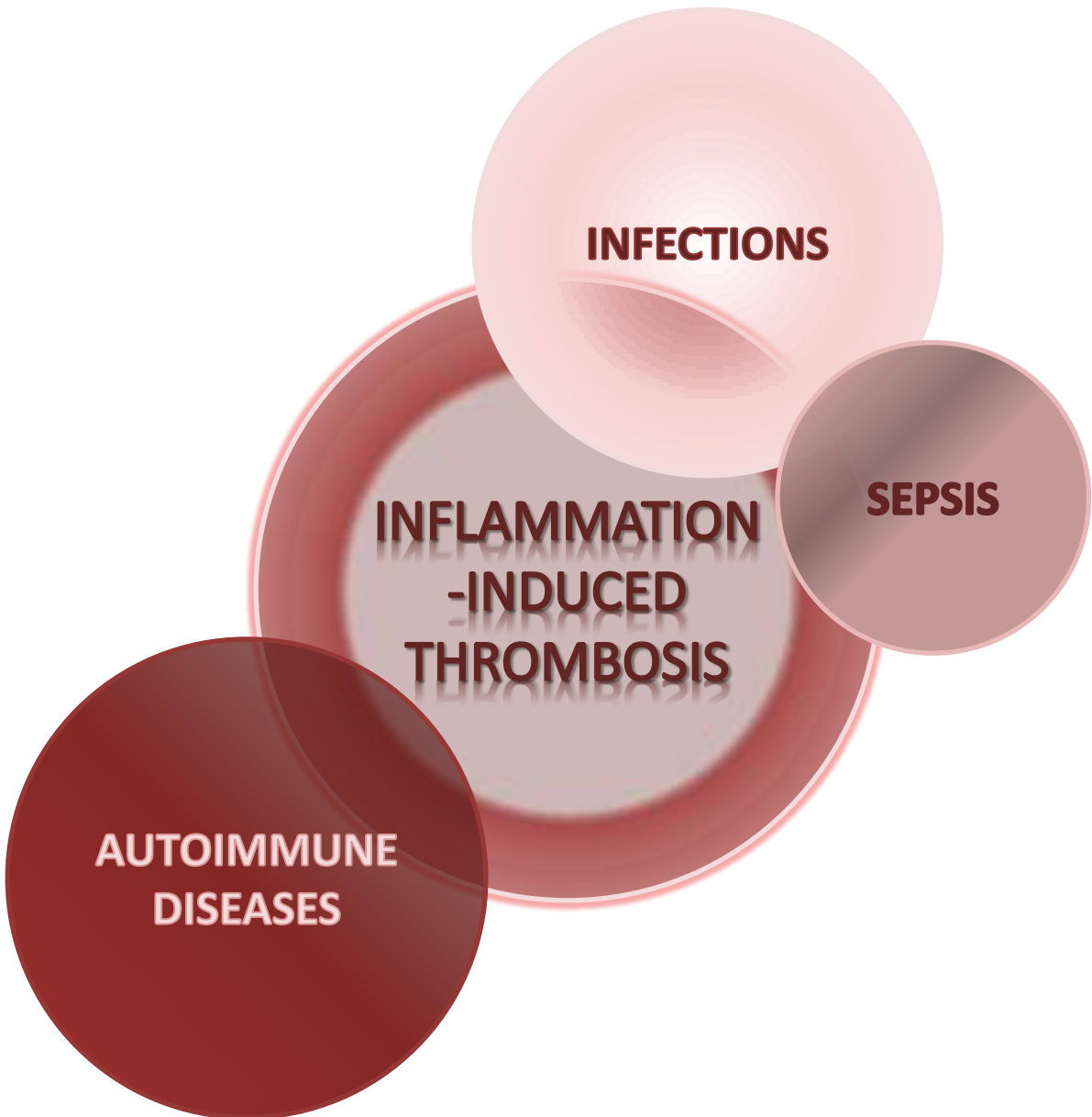


Fig. 2

**Table 1: Di**

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Platelet acti  
Modulation

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1 **rect mechanisms of inflammation-induced thrombosis (37)**

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4 cell dysfunction and activation  
5 ivation  
6 of plasma coagulation  
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9 Augmented pro-coagulant functions Tissue Factor-mediated activation of coagulation

10 Reduction of endogenous anticoagulants: Antithrombin, Tissue Factor pathway  
11 inhibitor (TFPI); Protein C pathway

12 Inhibition of fibrinolytic activity

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14 Hyperfibrinogenemia  
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2 **Table 2: Increased thromb**  
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6 **Autoimmune diseases**  
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9 **Infections and sepsis**  
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**thrombotic risk in diseases with a pro-inflammatory state (57)**

Rheumatoid arthritis, inflammatory bowel diseases, chronic autoimmune urticaria, bullous pemphigoid, autoimmune hemolytic anemia, immune thrombocytopenic purpura, systemic lupus erythematosus, systemic vasculitides, the antiphospholipid antibody syndrome, Sjögren's syndrome and systemic sclerosis

Chronic and acute

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**Table 3. Ongoing phase III studies on drugs specificall**

<b>TRIAL</b>	<b>DESIGN</b>
CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study)	A phase III, randomized, double-blind, placebo-controlled, multi-center, event-driven trial
CIRT (Cardiovascular Inflammation Reduction Trial)	A phase III, randomized, double-blind, placebo-controlled, multi-center, event-driven trial
ENTRACTE (A Clinical Outcomes Study to Evaluate the Effects of IL-6 Receptor Blockade With Tocilizumab in Comparison With Etanercept on the Rate of Cardiovascular Events in Patients With Moderate to Severe Rheumatoid Arthritis)	A phase IV, randomized, open-label, parallel-group, multicenter study

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## y targeting inflammation

AIM	DRUG TESTED	PRIMARY ENDPOINT
Tests if reducing inflammation can reduce rates of recurrent cardiovascular events among stable post-myocardial infarction patients who remain at elevated vascular risk (hsCRP $\geq$ 2mg/L) despite usual care, including statin therapy	Low-dose methotrexate	Composite of non-fatal myocardial infarction, non-fatal stroke, or cardiovascular death
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**SECONDARY ENDPOINTS      REFERENCES**

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Total mortality, hospitalization for  
unstable angina requiring  
revascularization, new onset  
diabetes      82

All-cause mortality, percutaneous  
or surgical coronary  
revascularization, hospitalization  
for congestive heart failure, incident  
venous thromboembolism, incident  
atrial fibrillation, incident diabetes,  
Incident peripheral artery disease,  
clinically worsening aortic stenosis      88, 89

A composite endpoint of major  
cardiovascular events plus non-  
fatal coronary revascularization  
procedures and hospitalization for  
unstable angina; as well as  
investigating the frequency of  
adverse events and serious  
adverse events      90

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## Bulletpoint Tables

### What is known about this topic?

- Inflammation is an established mechanism for atherosclerosis inception, progression and complications, but its direct role in thrombosis, besides effects on the triggering substrate, is a much newer concept.
- Trials with statins have shown a previously unexpected reduction in venous thromboembolism, fueling the concept of “inflammation-triggered thrombosis”

### What does this paper add?

- Here we review the multiple pathogenetic links between inflammation and thrombosis, and
- Outline the ongoing trials with “pure” anti-inflammatory agents, now testing the hypothesis, all including venous thromboembolic episodes as endpoints.

**Thrombosis  
and  
Haemostasis**

My manuscript INFLAMMATION AND THROMBOSIS – TESTING THE HYPOTHESIS WITH ANTI-  
INFLAMMATORY DRUG TRIALS contains colour figures.

Yes, I would like to have the following coloured figures \_\_\_\_\_ published in colour. I agree to pay the charge of **EURO 450** for the first figure, as stated in our "Instructions to Authors". Any further figures in colour are free of charge. You are free to choose coloured or black and white. There is no charge for black and white figures.

No, I do not wish the figures to be published in colour.

MS Number: \_\_\_\_\_

Printed Name: Raffaele De Caterina, MD, PhD Date: 29 March 2016

Signature: 

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