Pathophysiology and targeted treatment of cholesterol crystal embolism and the related thrombotic angiopathy

Chongxu Shi1 | Elmina Mammadova-Bach2,3 | Cong Li2 | Dong Liu1 | Hans-Joachim Anders2

Abstract

Cholesterol crystal (CC) embolism is a complication of advanced atherosclerotic plaques located in the major arteries. This pathological condition is primarily induced by interventional and surgical procedures or occurs spontaneously. CC can induce a wide range of tissue injuries including CC embolism syndrome, a spontaneous or intervention-induced complication of advanced atherosclerosis, while treatment of CC embolism has remained empiric. Vascular occlusions caused by CC embolism may exceed the ischemia tolerance of many tissues, particularly when small arteries are affected. The main approach to CC embolism is primary prophylaxis in patients at risk by stabilizing atherosclerotic plaques and avoiding unnecessary catheter interventions. During CC embolism, the use of platelet inhibitors to avoid abnormal activation and aggregation and anticoagulants may reduce the risk of vascular occlusions and tissue ischemia. This probably explains the relatively low prevalence of clinical manifestations of CC embolism, which are frequently found in autopsy studies. In this review, we summarized the current knowledge on the pathophysiology of CC embolism syndrome deriving from clinical observations and experimental mouse models. Furthermore, we described the risk factors of CC embolism in humans as well as the experimental studies based on empiric treatments. We also discuss potential therapeutic interventions based on recent experimental data and emerging drug options evolving from other research domains. Given the substantial unmet medical need to improve the outcomes of CC embolism, the identification of effective treatment strategies is urgently needed.

Keywords
atherosclerosis, cholesterol crystal embolism, injury, ischemia, vascular occlusion

Abbreviations: ADP, Adenosine diphosphate; AKI, Acute kidney injury; ATP, Adenosine triphosphate; CC, Cholesterol crystal; CRP, C reactive protein; eNOS, Endothelial NO synthase; FGN, Fibrinogen; GFR, Glomerular filtration rate; GP, Glycoprotein; HUS, Hemolytic uremic syndrome; KLF2, Krüppel-like Factor 2; LDL, Low-density lipoproteins; NO, Nitric oxide; PAR-Gq, Protease-activated receptor; PS, Phosphatidylserine; SMC, Smooth muscle cell; TF, Tissue factor; TMA, Thrombotic microangiopathies; TNFR, Tumor necrosis factor receptor; tPA, Tissue-type plasminogen activator; TTP, Thrombotic thrombocytopenic purpura; vWF, von-Willebrand-Factor.

This manuscript is part of Special Collection Emerging Mechanisms and Therapeutic Targets in Cardiovascular Immunology.
1 | INTRODUCTION

Cholesterol crystal (CC) embolism is a complication of advanced atherosclerosis and can affect almost every organ through microvascular obstructions and ischemic tissue necrosis. CC embolism originating from the atheromatous plaques of the abdominal aorta affects lower limbs (“blue toe syndrome”) as well as solid organs such as the kidney (AKI), the pancreas (ischemic pancreatitis), and the small intestines (intestinal ischemia). When the source of CC is plaques in the ascending aorta, ocular or cerebral embolism can occur as well as ischemic lesions in the upper limbs. The pathological consequences of CC embolism are frequently fatal when ischemic necrosis of the small intestines leads to bacterial peritonitis or when bilateral kidney embolism leads to uremia. Milder episodes of CC embolism to non-vital organs are frequently undetected. Indeed, autopsy studies report a high prevalence of CC embolisms which are not recognized during a lifetime.

So far, treatment of CC embolism has remained empirical due to a limited number of randomized controlled trials. Hence, only three trials are available (www.clinicaltrials.gov) in this pathological context: a study testing prednisolone therapy (NCT01452100), a study testing the efficacy of blood purification (NCT01726868), and a diagnostic study of magnetic resonance imaging following angiographies and angioplasty-stenting of the renal artery (NCT00027469).

In this review, we describe the risk factors and pathophysiological nature of CC embolism syndrome. We present new experimental and conceptual progress about the different steps of disease pathogenesis, including the molecular mechanisms of CC embolism initiation, CC embolism-induced thrombosis and thromboinflammation, ischemic injury, and cell death. In addition, we analyze the effects of anti-inflammatory and anti-platelet agents at each stage of CC embolism progression, potentially providing novel preventive and therapeutic approaches for patients diagnosed with CC embolism syndrome.

2 | RISK FACTORS FOR CC EMBOLISM

The incidence of clinically evident CC embolism syndrome has been reported to vary between 0.09% and 2.9%. Notably, the true incidence is probably much higher since many cases of CC embolism syndrome are often overlooked as reported by autopsy studies. Several risk factors contribute to the development of CC embolism syndrome, including interventional vascular procedures, cardiovascular surgery, hypertension, diabetes mellitus, gender, age, and the use of anticoagulants and thrombolytic treatments (Figure 1).

Gender-specific risk factors not only contribute to different development of atherosclerosis but also indirectly promote CC embolism, associated with other risk factors, such as smoking, dyslipidemia, hypertension, and diabetes mellitus. Males tend to develop atherosclerotic plaques earlier and have a higher plaque burden compared to females. Males also develop more non-culprit lesions, coronary artery lesions, frequent plaque ruptures, larger total necrotic core volume, and more comorbidities.

Age is another important risk factor in CC embolism syndrome. Spontaneous plaque ruptures are predominantly observed in patients over 60 years old, with an incidence of 0.79%–3.4%.

Smoking is another risk factor for CC embolism syndrome. Various chemical components, such as nicotine, present in tobacco smoke directly cause endothelial damage and enhance inflammatory responses in the vascular wall due to the oxidant and proinflammatory properties of nicotine molecules. The size of calcified atherosclerotic plaques is directly proportional to the number of cigarettes consumed per day. Moreover, smoking cessation is one of the most important health interventions for reducing the risks of cardiovascular diseases, cancer, and mortality at any age.

Surgical interventions involving the aorta or its major branches are associated with the highest risk of plaque rupture and CC embolism. Abnormalities consistent with CC embolism were observed in 21.7% of the autopsy series.
from patients who underwent myocardial revascularization or heart valve surgery. Coronary revascularization surgery-related CC embolism (26.1%) was three times more common than valve surgery (8.9%). In a prospective study of 1786 patients undergoing cardiac catheterization in Japan, the incidence of CC embolism syndrome was reported as 1.4%. Abdominal aortic aneurysm (AAA) is also a causative factor for the development of CC embolism, 2.9% of AAA patients with CC embolism was detected. However, coronary angioplasty is a lower risk factor, only 0.09% of patients developed CC embolism. Interventional procedures or cardiovascular surgery are the most common risk factors, it may also trigger spontaneous plaque rupture, and the outcome is in 70% of cases generally.

Thrombolytic therapy is also associated with CC embolism syndrome. Anticoagulants and fibrinolytics may promote CC embolism syndrome by causing plaque hemorrhage and plaque rupture. However, the exact pathomechanisms, linking anticoagulants/fibrinolytics and CC embolism syndrome remain unclear.

Increased inflammation is also a critical risk factor for CC embolism syndrome. Patients with CC embolism syndrome have often significantly higher levels of C reactive protein (CRP) in their plasma compared to those without CC embolism syndrome (0.7 vs 2.4 mg/dL). Therefore, increased CRP levels are considered an independent predictor of CC embolism syndrome (OR 4.6).

3 | CLINICAL MANIFESTATIONS OF CC EMBOLISM

The kidney is a commonly affected organ by CC emboli, but only around 50% of patients experience noticeable clinical symptoms. The onset of symptoms can vary, i.e. some patients exhibit symptoms shortly after CC embolism, while others may experience a delay of several weeks to months. Kidney CC embolism primarily manifests in the following conditions: (i) AKI accompanied by symptoms originating from multiple sites or organs, typically resulting from either larger arteries or the emboli, (ii) subacute kidney injury attributed to either the inflammatory response caused by CC emboli or the consecutive lodging of new emboli, (iii) chronic kidney disease, characterized by kidney ischemia or vascular sclerosis, is often asymptomatic because CC emboli are mainly detected in kidney biopsies or autopsies. CC embolism can affect the cerebral and retinal arteries, deriving from the ascending aorta and proximal aortic arch. Symptoms include psychological disturbances, headaches, neurological deficits, temporary vision loss and mild paralysis of the lower extremities. Hollenhorst plaques are known as diagnostic features of CC embolism occurring in retinal arteries. Embolism affecting the arteries of the kidneys and arteries below the abdomen, including the legs and feet, typically originates from the descending thoracic and abdominal aorta. Patients with CC embolism can also experience gastrointestinal complications, characterized by mucosal ulceration, bleeding, diarrhea, and stomach pain. Skin lesions, such as livedo reticularis, cyanosis, and ulcers, are observed in 35–96% of patients. Other symptoms associated with CC embolism are hemoptysis, femoral head necrosis, dyspnea and rhabdomyolysis.

4 | MOLECULAR MECHANISMS AND PATHOGENESIS OF CC EMBOLISM

4.1 | Rupture of vulnerable plaques

The relationship between CC embolism and aortic atherosclerosis was first suggested in 1945 by Flory. This concept was later studied by Kealy who demonstrated the presence of advanced plaques in several cases of peripheral embolism, often accompanied by the formation of aneurysms. CC embolism occurs when small atheromas found in major arteries, including the aorta, break and release CC into smaller arteries. The central pathology underlying atherosclerosis, a chronic condition, involves the formation of atheromatous or fibrofatty plaques. These plaques are the primary pathological feature in atherosclerosis, and their instability can lead to the release of CC emboli, contributing to CC embolism. Lipids, inflammatory infiltrates, smooth muscle cells (SMCs) and connective tissues are present within the plaques. Two simultaneous mechanisms contribute to plaque rupture: the gradual loss of SMCs from the fibrous cap and the degradation of the collagen-rich cap matrix, leading to thinning of the fibrous cap. At the rupture site, SMCs are usually absent and ruptured caps contain fewer SMCs than intact caps. Activated macrophages and foam cells can degrade the collagen matrix components of the fibrous cap in the lesion. Once the atherosclerotic plaque ruptures, it becomes exposed to the vascular endothelium bloodstream, leading to partial or complete occlusion of the blood vessel lumen. In addition, macrophages and SMCs at the rupture site can produce tissue factors (TF), contributing to the procoagulant phenotype and the development of acute coronary syndrome, which is the main clinical manifestation of atherosclerotic progression (Figure 2). The onset of acute coronary diseases can be triggered by simple daily activities or the circadian rhythm, which has a higher incidence in the morning. Increased heart rate and blood pressure can lead to plaque rupture, with enhanced
coagulability and platelet reactivity further amplifying the thrombotic response to the ruptured plaques. Variuos factors influence the vulnerability of plaques to rupture, including the expression of adhesion molecules, local cytokine release, endothelial cell dysfunction, activation of monocytes and macrophages, complement activation and the presence of proteolytic enzymes. Collectively, these factors contribute to the destabilization of plaques, thereby increasing the risk of rupture.

4.2 CC-induced vascular occlusion

During CC embolism, the hemostatic balance is disturbed by the rupture of the endothelial layer, leading to the exposure of the vascular extracellular matrix. Following vessel injury, subendothelial matrix proteins, including collagen, become exposed to the flowing blood. Exposed collagen anchors von-Willebrand-Factor (vWF) and initiates interactions between platelet glycoprotein (GP) Ibα and vWF, as well as GPVI and collagen, which are crucial steps in platelet activation. Platelets also express various integrins on their surface, which, upon outside-in activation by their extracellular ligands, promote platelet adhesion to the injured vessel wall. Additionally, activated platelets release numerous bioactive molecules and secondary mediators, such as fibrinogen (FGN), vWF, adenosine diphosphate/adenosine triphosphate (ADP/ATP), and serotonin, from their alpha (α) and dense delta (δ) granules. These molecules further enhance the prothrombotic process, promoting the recruitment of circulating platelets to the developing thrombi. The extrinsic and intrinsic blood coagulation pathways trigger the second wave of hemostasis, leading to the generation of thrombin. Thrombin, in turn, converts soluble fibrinogen into fibrin, thereby reinforcing platelet activation and clot formation. Activated platelets also expose phosphatidylserine (PS) on their surface, facilitating the binding of coagulation factors that
stimulate thrombin generation and activate the coagulation cascade. Recently, we provided compelling evidence regarding the significant impact of CC on platelet adhesion and activation, thereby promoting prothrombotic functions. While CC alone does not induce platelet activation or adhesion, upon stimulation with thrombin, CC exerts a potent effect on platelets, leading to their activation and degranulation. This dynamic process results in the release of mediators such as FGN and ATP, thereby fostering a prothrombotic microenvironment. CC stimulates platelet activation through the GPVI-ITAM and PAR-Gq signaling pathways, ultimately leading to the inside-out activation of integrin αIIbβ3 and the exposure of P-selectin. Collectively, these intricate mechanisms contribute to enhanced platelet reactivity and the formation of fibrin clots.

Thromboinflammation, a term used to describe the simultaneous activation of thrombotic and inflammatory responses, is observed in various diseases, including CC embolism. The presence of CC emboli within arterioles triggers a series of inflammatory reactions, including endothelial dysfunction, cytokine release, immune response and the formation of intravascular thrombi. In the initial 24 h, neutrophils are the first inflammatory cells to infiltrate the affected arterioles, followed by the infiltration of monocytes that differentiate into macrophages and form foreign body giant cells responsible for engulfing large CCs. Platelet–neutrophil interactions contribute to the activation and release of proinflammatory cytokines and chemokines, which further promote thrombus formation and exacerbate the inflammatory response. Using the kidney CC embolism model, we recently showed that extracellular traps play a critical role in the formation of emboli. Indeed, the observed immunothrombotic complication was associated with the presence of platelets, red blood cells, leukocytes, fibrin mesh and extracellular DNAs that are released by damaged endothelial cells, neutrophils and activated platelets. Thus, the reciprocal interaction between platelets, innate immune cells and activated vascular endothelium influences both the thrombotic potential and immune response, thereby linking thrombosis to the inflammatory site.

Although thrombosis is a characteristic feature of thrombotic microangiopathies (TMAs) observed in CC embolism syndrome, not all cases of thrombosis are classified as TMAs. TMAs were introduced to specifically refer to a group of disorders that share the common feature of microvascular thrombosis and associated organ dysfunction. Other forms of TMAs can be regulated by different mechanisms depending on underlying conditions (platelet consumption, erythrocyte fragmentation, ischemic injury, thrombotic thrombocytopenic purpura (TTP), antiphospholipid syndrome, hemolytic uremic syndrome (HUS)).

### 4.3 Programmed cell death pathways in CC embolism

The subsequent inflammatory response can be influenced by the programmed cell death pathways, detected in the parenchymal and tubular cells during kidney ischemia. The molecular and cellular mechanisms of ischemic necrosis are well-studied in many thromboinflammatory diseases, including myocardial infarction, ischemic stroke, and acute kidney injury. The measurement of infarct size is the primary endpoint of most experiments predicting long-term organ functionality in the heart, brain, or kidney. Tissue hypoxia in the kidney first impairs the functional capacity of parenchymal cells by depleting ATP but prolonged ischemia leads to ischemic cell necrosis. Different tissues have a different sensitivity to ischemia time but without therapeutic revascularization, CC embolism-related vascular occlusions may exceed ischemia tolerance of most tissues, especially when small ends are affected, e.g., in the brain, the skin, the small intestine, and the kidney. Ischemic necrosis is not a passive process but involves numerous signaling pathways of regulated necrosis discovered and characterized during the last decade (Figure 4). This chapter describes the various types of reprogrammed cell deaths that arise in response to proinflammatory and prothrombotic stimuli during CC embolism.

#### 4.3.1 Necroptosis

Necroptosis is a programmed form of necrosis, or inflammatory cell death, triggered by outside-in signaling pathways downstream effectors of pro-inflammatory death receptors such as tumor necrosis factor receptor 1 (TNFR1). When caspase-8-mediated apoptosis is deactivated, receptor-interacting protein (RIP)-kinase 1 and RIP-kinase 3 dissociate from the receptor complex. Consistently, Mlkl-deficient mice are protected from CC embolism-related ischemic necrosis of the kidney, indicating the central role of necroptosis in this process. Mlkl-deficient mice are also protected from ischemic necrosis of the kidney induced by transient kidney pedicle clamping as well as from ischemic necrosis in the brain and the heart induced by other means, e.g., arterial ligation. This suggests a general contribution of the necroptosis signaling pathway to ischemic tissue necrosis.

#### 4.3.2 Apoptosis

The first known form of programmed or regulated cell death was apoptosis, which for a long was defined by...
TUNEL positivity, an analytical method detecting breaks in double-stranded DNA. However, the discovery of other forms of regulated necrosis clarified the unspecific nature of TUNEL positivity, which is now considered more a general marker of cell death. Currently, each form of regulated cell is defined by a unique signaling pathway. For example, apoptosis is characterized by cell death that occurs in the presence of caspase-3 cleavage and can be inhibited by caspase-3 inhibitors. With the use of these analytical tools, it appears that apoptosis does not contribute significantly to ischemic cell necrosis. Indeed, apoptosis is a non-immunogenic form of homeostatic cell death, e.g., with major importance in the silent deletion of autoreactive immune cells or of cells with significant DNA damage to prevent malignant transformation.

4.3.3 | Netosis

In 2004, the group of Arturo Zychlinsky first described an alternative form of neutrophil bacterial killing, i.e., the release of NETs. NETosis has two distinct forms, suicidal and vital NETosis. NET release is frequently associated with neutrophil necrosis, driving necroinflammation in many pathological conditions, including ischemic tissue necrosis NETs mostly form inside the microvasculature, and primarily cause direct endothelial cell injury and intravascular thrombosis. Activated platelets can directly induce NETosis, thereby amplifying necroinflammatory and thrombotic events.

NETosis does not only imply the release of nuclear DNA and histones but also the release of toxic proteases from neutrophil granules. DNase I treatment has been proposed as an
effective tool to neutralize NETs but this treatment does not influence lytic enzymatic activities. Systemic administration of DNase I is potent to prevent ischemic tissue necrosis in CC embolism to dissolve the chromatin mesh contributing to intravascular thrombosis at the site of the crystal clot. It has been proposed that perilesional neutrophil infiltrates in the kidney may be involved in NET formation. Therefore, we speculate that inhibiting NETosis may help to limit the size of tissue infarction in CC embolism.

Neutrophil infiltration and consequent tissue necrosis are regulated by the circadian rhythm, which was explained by the differential expression of adhesion molecules and cytokine on vascular endothelium and immune cells during CC embolism. Indeed, the expression of adhesion molecules (Icam-1, Vcam-1) on endothelial cells is lower during the day than at night, the release of chemokine (CXCL1) from neutrophils cells is less than at night, the expression of CXCR2 is lower, while CXCR4 is high, which leads to more tissue necrosis during the night.

4.3.4 | Necroinflammation

Ischemic necrosis is accompanied by rapid neutrophil recruitment followed by pro-inflammatory macrophages, which both contribute to perilesional inflammation. The auto-amplification loop between ischemic necrosis and perilesional inflammation also referred to as necroinflammation, involves damage-associated molecular patterns (DAMPs) and proinflammatory cytokines (interleukins), as well as cytotoxic DAMPs such as extracellular histones that trigger both cell death and inflammation.

CC-induced TMAs are a complex pathological process, involving thrombosis, tissue necrosis and inflammation. Thrombosis within the microvasculature leads to the release of cytokines and chemokines from activated platelets. These mediators activate endothelial cells and attract leukocytes to the site of injury.

Patients with metabolic syndromes often have a pro-inflammatory state, due to the accumulation of several disease conditions, such as atherosclerotic cardiovascular disease, insulin resistance and diabetes mellitus. Recently, we showed that hyperglycemia aggravated TMA and glomerular filtration rate by increasing neutrophil recruitment, necroinflammation and infarct size during CC embolism. In line with this, CC enhanced the prothrombotic response of hyperglycemic platelets. Altogether, these studies indicate that a CC-induced prothrombotic environment can exacerbate necroinflammation. Of note, the long-term outcomes of renal TMA depend on the regenerative capacity of different cell types through intrinsic or extrinsic progenitor potential. In general, the suppression of necroinflammation can be achieved by blocking proinflammatory and prothrombotic mediators or pathways that regulate necrosis. However, the efficacy of these treatments may vary depending on the degree of injury and the stage of the disease.
5 | TARGETED TREATMENTS OF CC EMBOLISM

The adhesion and activation of immune cells and platelets are tightly regulated at different steps of CC embolism, thereby influencing the inflammatory response, thrombus formation and ischemia in patients with CC embolism. Several signaling receptors and bioactive mediators from immune cells, platelets and vascular endothelium are involved in this process (Table 1). In this chapter, we discuss the potential of anti-inflammatory, anti-coagulant and fibrinolytic therapies at different steps of CC embolism.

5.1 | The site of CC emission

Statins are commonly used to reduce atherosclerotic lesions by effectively decreasing the levels of low-density lipoproteins (LDL) and stabilizing or regressing the established atherosclerotic plaque. Furthermore, statins have pleiotropic effects, such as reducing inflammation and oxidative stress in the vascular system. Statins also block cholesterol synthesis in the liver by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, thereby reducing the production of mevalonate, which is an essential metabolic product for cholesterol synthesis. Other studies suggested that statins not only limit hyperlipidemia but also decrease oxidative stress, activating anti-inflammatory and anti-proliferative mechanisms on vascular endothelial and smooth muscle cells. It was shown that atorvastatin and simvastatin exert a positive effect on nitric oxide (NO) levels by increasing the synthesis and activity of endothelial nitric oxide synthase 3 (eNOS) via the inhibition of miRNA-221 and miR-222. Increased levels of NO are associated with a low concentration of ROS, reduced vascular endothelium permeability and inflammatory response (Figure 5).

### Table 1  Possible therapeutic interventions in CC embolism syndrome.

<table>
<thead>
<tr>
<th>Targeted Pathomechanism</th>
<th>Specific pathway</th>
<th>Pharmacological inhibitor/device</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stabilization of vulnerable plaque</td>
<td></td>
<td></td>
<td>[72–78]</td>
</tr>
<tr>
<td>Arterial occlusion by crystal clots</td>
<td></td>
<td></td>
<td>[54]</td>
</tr>
<tr>
<td>Ischemic necrosis</td>
<td>MPT-driven necrosis</td>
<td>Cyclosporin A</td>
<td></td>
</tr>
<tr>
<td>Necroinflammation</td>
<td>NIM811</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necroptosis</td>
<td>Necrostatins, phenytoin, ...</td>
<td></td>
<td>[60,61]</td>
</tr>
<tr>
<td>Autophagy</td>
<td>3-methyladenine</td>
<td></td>
<td>[79]</td>
</tr>
</tbody>
</table>

**Figure 5**  Targeted treatments at the site of CC emission. The mechanism of statin therapy on endothelial cells. On one hand, statin therapy can reduce inflammation and oxidative stress in the vascular system, because it regulates endothelial proinflammatory factors such as ROS release by inhibiting miR-222, and miR-221, and decreasing the levels of LDL. On the other hand, statin can also enhance the activity of KLF2 decreasing the thrombomodulin and fibrinolytic activity. ROS: Reactive oxygen species, KLF2: Kruppel-like transcription factor 2, LDL: low-density lipoprotein, NO: nitrogen oxide, eNOS: Endothelial nitric oxide synthase.
effects by inhibiting the release and expression of thromboxane A2, ox-LDL and CD36 receptors and enhancing the production of eNOS, which improves the production of platelet NO. In its turn, the NO inhibits platelet activation and aggregation, thereby suppressing blood clotting and coagulation by downregulating the tissue factor expression and activity. In addition, statins can inhibit thrombosis by upregulating Krüppel-like Factor 2 (KLF2) levels, which promotes the expression of thrombomodulin and fibrinolytic activity. Whether statins may have anti-inflammatory and anti-thrombotic effects in CC embolism awaits future investigation.

5.2 The sites of peripheral occlusion and ischemic necrosis

The presence of the crystal component, which occupies only a small fraction of the vascular lumen, results in arterial thrombosis and vascular occlusion, thereby inducing acute kidney injury, which is followed by a sudden drop in glomerular filtration rate (GFR), and ischemic kidney infarction (Figure 6). This thrombo-inflammatory process involves the complex interplay between platelets, neutrophils and vascular endothelium. During CC embolism, the hemostatic balance is disturbed by the rupture of the endothelial layer, leading to the exposure of the vascular ECM. CC can enhance platelet attachment to the exposed collagen, further amplifying platelet reactivity by promoting the exposure of P selectin, the release of fibrinogen and ATP and the increasing the adhesion of circulating platelets to the CC-induced thrombi. Consequently, the prothrombotic environment becomes abundant in FGN and thrombin, leading to the generation of a fibrin clot, which can further induce the recruitment of circulating platelets to the growing thrombus sites. Accordingly, preemptive injection of fibrinolytic drugs, anticoagulants, DNase I and antiplatelet agents was shown to completely prevent crystal clot formation, acute kidney injury and kidney

![Figure 6](https://faseb.onlinelibrary.wiley.com/doi/10.1096/fj.202301316R) Targeted treatments at the peripheral vascular occlusion. Damaged endothelium release tissue factor (TF) and ecDNA activate platelets, meanwhile, the role of ecDNA as a DAMP can also trigger an inflammatory response. Activated platelets release granule contents, and cause blood coagulation. DNase I can degrade ecDNA released from damaged endothelial cells to prevent platelet activation and inflammation. DNase I prevents ecDNA mesh formation, platelet activation, and aggregation. Clopidogrel can be combined with P2Y12 to prevent platelet activation and aggregation, urokinase prevents coagulation via balancing the plasmin in the system, and heparin involves in various steps of coagulation.
infarction without affecting the crystal component inside the arteries.\textsuperscript{54}

Ischemic necrosis is a common form of tissue injury, e.g., in stroke, myocardial infarction or limb ischemia but despite a myriad of experimental studies demonstrating tissue-protective effects from targeted interventions, the medical practice remains focused on revascularization or fibrinolytic and antithrombotic drugs. One of the reasons is that whenever ischemic necrosis is a consequence of vascular occlusion, tissue-protective interventions have limited potential as long as vascular occlusion and ischemia persist. For example, genetic or pharmacological inhibition of CC embolism-related ischemic necrosis of the kidney did not improve the early decline of kidney function as further proof that vascular obstruction is an upstream mechanism of CC embolism.\textsuperscript{54} Therefore, the drug pipeline of necrosis inhibitors at best may help to increase the window of opportunity for revascularization strategies, e.g., with fibrinolytic or antithrombotic drugs, in established CC embolism.\textsuperscript{54}

5.2.1 | Fibrinolysis

Fibrin-rich clot formation plays an important role in the process of hemostasis, serving as the main product of the coagulation cascade and also acting as the ultimate substrate for fibrinolysis. The process of fibrinolysis involves the conversion of plasminogen into plasmin by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator and the breakdown of fibrin degradation through the hydrolytic action of plasmin.\textsuperscript{85} However, the intrinsic fibrinolytic system cannot effectively reduce thrombus growth and intravascular coagulation during CC embolism to restore blood flow and revascularization. Platelet-rich thrombi are resistant to tPA-induced thrombolysis, compared to red blood cell-rich clots.\textsuperscript{86} This resistance is linked to the presence of the platelet-derived alpha subunit of factor XIII and protease inhibitors.\textsuperscript{86} Therefore, fibrinolytic therapies are used in the treatment of thrombotic disease conditions, such as stroke, pulmonary embolism and deep vein thrombosis, thereby helping to dissolve blood clots and restoring blood flow and promoting tissue healing and revascularization. We showed that after 24 hours, urokinase significantly reduced the number of occlusions in the arteries, while the crystal component persisted. Urokinase treatment could improve GFR loss and significantly reduce the size of kidney infarcts, kidney injury, neutrophil infiltration, vascular injury, and kidney tubular cell death.

Although fibrinolytic drugs could completely prevent crystal clot formation, acute kidney injury and kidney infarction without affecting the crystal component inside the arteries.\textsuperscript{54} However, these interventions can be associated with bleeding complications, thereby limiting their effectiveness in clinical settings. Recently, we showed that Glu-plasminogen extracts from human plasma injected intravenously 4 h after CC injection into the left kidney artery of mice attenuated thrombotic angioopathy, AKI, and cortical necrosis in a dose-dependent manner.\textsuperscript{87} An intermediate dose had a transient effect, which renders Glu-plasminogen a well-controllable intervention although no bleeding complications occurred during operative or post-operative periods.\textsuperscript{87} Therefore, Glu-plasminogen substitution could be a potential therapeutic approach in patients with CC embolism syndrome. Although Glu-plasminogen treatment can restore balance to local fibrinolysis in the injured kidney arteries, inhibiting fibrin-rich blood clot formation, it may probably require continuous anticoagulant therapy to prevent recurrent crystal clot formation.

5.2.2 | Anticoagulant therapies

Heparin is an anti-coagulant to prevent blood clotting during surgical interventions, kidney dialysis, and for the management of various diseases such as VTE, heart attacks, and angina. Heparin affects the blood coagulation pathway by inactivating thrombin.\textsuperscript{88} Heparin induces a conformational change in antithrombin III, thereby facilitating a complex formation with thrombin. Although heparin is considered an anti-coagulant, it can also have anti-inflammatory and anti-proliferative and profibrinolytic effects.\textsuperscript{88} Heparin has also been shown to protect against organ and tissue damage caused by histones, as well as decrease the levels of NETs in a mouse model of sepsis.\textsuperscript{89} Similar to urokinase, heparin could also prevent crystal clot formation, improving GFR loss and reducing acute kidney injury and infarction during crystal clot embolism.\textsuperscript{54} However, heparin treatment may cause adverse effects such as hemorrhagic diathesis, intra-organ bleeding, osteoporosis and thrombocytopenia. Heparin-induced thrombocytopenia is frequently caused by the production of antibodies against heparin-platelet factor 4 (PF4) complexes, which can also activate neutrophils and promote NET formation, thereby enhancing thrombosis.\textsuperscript{90} Low-molecular-weight heparin (LMWH) offers several benefits compared to heparin, including decreased risks of HIT and osteoporosis, longer bioavailability and half-life, and predictable anticoagulant response. However, both LMWH and unfractionated heparin (UFH) can increase the risk of bleeding in patients with kidney disease, due to their accumulation in kidney tissue.\textsuperscript{91}

In order to minimize the bleeding risk, various clinical therapies have been recommended, including reducing...
the dosage, adjusting treatment based on anti-Xa heparin levels, and using lower doses of LMWH.\textsuperscript{92} Nonetheless, the risk of bleeding may differ depending on the particular clinical circumstance. For example, patients with venous disease may experience lower bleeding risks with LMWH compared to UFH, whereas those with acute coronary syndrome may be at higher risk for bleeding with LMWH.\textsuperscript{92}

### 5.2.3 | Anti-P2Y\textsubscript{12}-based antiplatelet therapies

The platelet P2Y\textsubscript{12} receptor is a G\textsubscript{i}-coupled ADP binding receptor that regulates thrombus stability by amplifying platelet aggregation and activation, granule secretion and procoagulant activity.\textsuperscript{93} The P2Y\textsubscript{12} receptor is considered a prime candidate for anti-thrombotic medication, due to its central role in the purinergic signaling pathway.\textsuperscript{94} Clopidogrel is a selective P2Y\textsubscript{12} receptor antagonist, which is used in the treatment and prevention of heart attacks, ischemic stroke and peripheral artery diseases.\textsuperscript{94} In the CC embolism model, preventive injection of clopidogrel completely protected mice from intravascular obstructions, GFR loss, acute kidney injury and kidney infarction without affecting the crystal component inside the arteries. However, clopidogrel treatment could be associated with bleeding complications, which complicates their usage in clinical settings. In line with this, genetic or functional deficiency of the P2Y\textsubscript{12} receptor in mice or humans induces longer bleeding times and severe hemorrhage.\textsuperscript{94,95} Mice treated with a high dose of clopidogrel displayed bleeding times that can last up to 30 minutes and even result in death from bleeding.\textsuperscript{95} Hence, bleeding risks represent the major drawbacks when clopidogrel is administered, either alone or in combination with aspirin.\textsuperscript{94,96} These challenges led to the development of new anti-P2Y\textsubscript{12} drugs, such as prasugrel, ticagrelor and cangrelor. More recently, in patients with myocardial infarction, selatogrel emerged as a new potent, selective and reversible P2Y\textsubscript{12} blocker, inducing a rapid and strong platelet inhibition without major bleeding complications. Further studies are needed to test the effect of selatogrel on CC-induced AKI and thromboinflammation.

#### 5.2.4 | DNase I-based anti-thrombotic therapy

DNase I is an enzyme that degrades DNA, leading to the dissolution of extracellular DNA and NET formation. Although DNase I treatment has a strong impact on NETosis during immunothrombosis, it also prevents thrombus growth without the involvement of neutrophils.\textsuperscript{54,97} Interestingly, platelet activation, P-selectin exposure, aggregation in response to collagen, collagen-related peptide, or thrombin were suppressed when washed platelets were preincubated with DNase I.\textsuperscript{54} In addition, DNase I treatment strongly inhibited CC-induced fibrin production and ATP release from activated platelets.\textsuperscript{54} In line with this, in vivo neutrophil depletion in peripheral blood did not affect the severity of disease in the mouse model of CC embolism, whereas DNase I treatment dramatically reduced the number of occluded arteries, ischemic organ failure and kidney infarction in vivo.\textsuperscript{54}

Tissue-factor-expressing neutrophils contribute to thrombosis in the laser-induced arterial injury model.\textsuperscript{98} DNase I treatment could also prevent thrombosis in this in vivo model.\textsuperscript{97} The electron microscopy studies could not detect neutrophil-associated extracellular trap structures. DNase I treatment also affects platelet functions, probably reducing fibrin formation and increasing ATP/ADP hydrolysis.\textsuperscript{97} DNase I treatment also limits the venous thrombus growth in a mouse model of HIT. DNase I administrated systematically could potentially serve as an effective treatment for preventing ischemic tissue necrosis in CC embolism. However, its inhibitory effect is limited to the early CC embolism (i.e., the first 3h).\textsuperscript{54} Extracellular DNA also plays a significant role in the hemostatic system, as it activates factor XI and factor XII.\textsuperscript{99} Furthermore, extracellular DNA can be incorporated into the fibrin clot thereby inhibiting the anticoagulant activities of drugs such as unfractionated heparin and enoxaparin.\textsuperscript{100} Conversely, RE31 DNA aptamers have been shown to inhibit the generation of thrombin formation, thereby accelerating fibrinolysis and suppressing thrombosis.\textsuperscript{101,102} Damaged endothelial cells can also release potent procoagulant molecules, TF, extracellular DNA and the endogenous fibrinolysis inhibitor (plasminogen activator inhibitor 1).\textsuperscript{103} Extracellular DNA can activate platelet aggregation and contribute to thrombus formation.\textsuperscript{54} Future studies are required to test the effects of DNA aptamers in CC-induced thrombosis and thromboinflammation.

#### 5.2.5 | Other potential targets

CC embolism disrupts the hemostatic balance by rupturing vasculature, exposing the subendothelial collagen matrix. This results in platelet adhesion and aggregation, causing abnormal fibrin clot formation, kidney ischemia, and perilesional inflammation. Activated platelets initiate a procoagulant surface, thereby triggering the recruitment of neutrophils and initiating thromboinflammation.\textsuperscript{54} CC strongly increases platelet aggregation response to thrombin and GPVI-specific agonists (collagen,
collagen-related peptide). Additionally, a significant increase in P-selectin exposure and αIIbβ3 integrin activation was observed indicating that CC embolism activates PAR-Gq (protease-activated receptor) and GPVI-ITAM (GPVI-immunoreceptor tyrosine-based activation motif) signaling.54 These findings suggest that CC-induced procoagulant and thromboinflammatory phenotypes may be regulated by multiple platelet activation pathways. Further investigations are necessary to elucidate the specific roles of platelet-derived molecules, including key adhesion receptors and secondary mediators, and to determine their therapeutic potential in modulating renal hemostasis, thrombosis, and thromboinflammation.

6 | CONCLUSIONS

CC embolism-related vascular occlusions and tissue ischemia are the consequences of CC-induced TMA. Endothelial injury, complement-mediated intravascular clotting involving platelet activation, NET release and formation of a fibrin and chromatin mesh as in other forms of TMA or arterial thrombosis. The main approach to CC embolism is primary prophylaxis in patients at risk by stabilizing atherosclerotic plaques and avoiding unnecessary catheter interventions. The broad use of platelet aggregation inhibitors and anticoagulants may reduce the risk of vascular occlusions and tissue ischemia in case CC embolism occurs, which probably explains the relatively low prevalence of clinical manifestations of CC embolism, which are frequently found in autopsy studies. Clinical manifestations of CC embolism have crystal clots as the primary therapeutic target and may respond to platelet aggregation inhibitors, anticoagulants, and fibrinolytic drugs.

In analogy to other forms of TMA, complement inhibitors may have a future in this context. Animal studies suggest that a single prophylactic dose of necrosis inhibitors can increase the window of opportunity for vascular interventions but if this could implement into the clinical practice of high-risk patients remains unknown. Nevertheless, the availability of an animal model of CC embolism-induced ischemic necrosis, tissue infarction, and organ failure improves our knowledge about CC embolism.

Like CC embolism syndrome, severe SARS-CoV-2 infection involves neutrophils, platelets, and complement activation in coagulation, organ injury, and immunothrombosis.104 This process is triggered by the release of proinflammatory cytokines, and NET formation, thereby activating platelets and enhancing fibrin generation. Despite the viral etiology, the composition of the clot, including platelet and fibrin-rich thrombi, extracellular DNA, vascular obstruction, tissue damage, and organ injury, shows similarities with CC embolism.104,105 Consistently, administrating therapeutic doses of anticoagulants was shown to reduce the occurrence of thrombotic events and mortality rates in patients with moderate SARS-CoV-2 infection.79 Understanding these mechanisms and the impact of anti-inflammatory and anti-thrombotic drugs can help to interconnect treatment strategies and propose new modalities for preventing and treating immunothrombosis complications.

AUTHOR CONTRIBUTIONS

Hans-Joachim Anders drafted the outline of the manuscript and all authors wrote parts of it, edited the complete version, and approved the final version.

FUNDING INFORMATION

HJA received support from the Deutsche Forschungsgemeinschaft (AN372/20-2 and 30-1). CS and DL were supported by the National Natural Science Foundation of China (82200462, 81870359). CL received support from the Chinese Scholarship Council.

DISCLOSURES

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, the National Natural Science Foundation of China and Chinese Scholarship Council. Open Access funding enabled and organized by Projekt DEAL.

ORCID

Chongxu Shi https://orcid.org/0009-0006-2012-2322
Hans-Joachim Anders https://orcid.org/0000-0003-2434-2956

REFERENCES


45. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation*. 1994;89(1):36-44. doi:10.1161/01.cir.89.1.36


---

**How to cite this article:** Shi C, Mammadova-Bach E, Li C, Liu D, Anders H-J. Pathophysiology and targeted treatment of cholesterol crystal embolism and the related thrombotic angiopathy. *The FASEB Journal*. 2023;37:e23179. doi:10.1096/ffj.202301316R