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REVIEW

Clinical update

Cholesterol crystal induced arterial inflammation and destabilization of atherosclerotic plaque

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Evolution of plaque that is prone to rupture is characterized by inflammation and physical changes. Accumulation of low-density lipoprotein in the sub-intima provides esterified cholesterol (ESC) to macrophages and smooth muscle cells that convert it into free cholesterol (FRC) by cholesteryl ester hydrolases (CEHs). Membrane-bound cholesterol carriers transport FRC to high-density lipoprotein (HDL). Impaired HDL transport function and altered composition can lead to extracellular accumulation of FRC, whereas impaired membrane carrier activity can lead to intracellular FRC accumulation. Saturation of FRC can result in cholesterol crystallization with cell death and intimal injury. Disequilibrium between ESC and FRC can impact foam cell and cholesterol crystal (CC) formation. Cholesterol crystals initiate inflammation via NLRP3 inflammasome leading to interleukin-1β (IL-1β) production inducing C-reactive protein. Eventually, crystals growing from within the plaque and associated inflammation destabilize the plaque. Thus, inhibition of inflammation by antagonists to IL-1β or agents that dissolve or prevent CC formation may stabilize vulnerable plaques.

Keywords Atherosclerosis • Cholesterol crystals • IL-1β • Vascular inflammation • Vulnerable plaque • Vulnerable patient

Introduction

Understanding the mechanism of plaque rupture and thrombosis can greatly impact therapeutic approaches for both prevention and treatment of acute cardiovascular events, the leading global medical cause of morbidity and mortality.[1] The histological description of plaque rupture is a snapshot of events at a single point in time. Thus, morphological descriptions of the vulnerable plaque such as thin-cap fibroatheroma (TCFA) do not take into account the preceding dynamic events that had led to rupture.[2] However, recent *in vitro* studies have demonstrated that cholesterol expands in volume when crystallizing from a liquid to a solid state and larger amounts of cholesterol cause greater volume expansion.[3,4] Thus, sharp-tipped crystals growing within the plaque could eventually perforate the fibrous cap. Both post mortem and *ex vivo* human studies as well as animal studies support the concept of cholesterol crystals (CCs) perforating the fibrous cap.[4–8] Moreover, CCs have been noted to form early in plaque development and can trigger local and systemic inflammation.[9] This review will focus on the process of cholesterol metabolism within the arterial wall leading to cholesterol crystallization that triggers inflammation and vascular injury.

Initiation and progression of atherosclerosis

Plaque formation is a highly dynamic process that begins with entry of cholesterol into the arterial wall via low-density lipoprotein (LDL) particles. Once LDL is oxidized, it is assimilated by macrophages to become foam cells.[10] Initially, however, the monocyte-derived macrophages in atherosclerotic plaques have anti-atherogenic properties that facilitate reverse cholesterol transport by producing nascent high density lipoprotein (HDL).[11] Cholesterol in LDL particles exists in an esterified form but it is the process leading to free cholesterol (FRC) accumulation within the arterial wall that predisposes to CC formation. Factors that lead to FRC accumulation include imbalances between esterified cholesterol (ESC) and FRC and changes in HDL functionality.[12] Thus, disruption in cholesterol homeostasis can lead to crystal build-up

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within the plaque's necrotic core causing mechanical injury and plaque rupture. Although plaque rupture occurs in the majority of cases,[3–8] the mechanism of plaque erosion causing thrombosis requires further elucidation.[13]

Equilibrium between esterified and free cholesterol

Cholesterol ester hydrolase (CEH) enzymes convert ESC to FRC, whereas acyl-coenzyme A cholesterol acyltransferase 1 (ACAT-1) converts FRC back to the esterified state. Therefore, a disruption in the equilibrium between esterification and de-esterification can result in the accumulation of ESC or FRC within foam cells. Kellner-Weibel demonstrated that inhibition of ACAT resulted in liquid crystalline and cholesterol monohydrate crystals formation in the plasma membrane bilayer of macrophages.[14] These membrane cholesterol domains act as nucleation sites for formation of crystals that are released into the extracellular space.[14,15] Studies have evaluated the effects of enhancing CEH and blocking ACAT-1 on atherosclerosis. Zhao *et al.*[16] studied the effect of enhancing neutral CEH activity in macrophages using a transgenic atherosclerotic mouse that overexpresses CEH. They demonstrated a reduction in the necrotic core size and an increase in the number of viable foam cells in the presence of normal HDL function. The increased CEH activity did not result in accumulation of FRC due to increased reverse cholesterol transport out of tissues via HDL. Other studies found that oestrogens enhanced CEH activity whereas testosterone had no effect (*Figure 1*).[17] Accumulation of FRC in cells leads to cholesterol crystallization and cell death.[18]

Inhibitors of ACAT-1 have been developed and evaluated in both animal models and in humans. The rationale was the potential prevention of macrophages conversion into foam cells in order to suppress plaque growth. Although animal studies on ACAT inhibition were found to be promising in reducing atherosclerosis, this was not the case in humans.[19] This raises concern about the limitations of animal studies regarding translation to humans. In fact, several clinical trials, in particular the Carotid Atherosclerosis Progression Trial Investigating Vascular ACAT Inhibition Treatment Effects (CAPTIVATE) randomized trial using pactimibe, demonstrated acceleration of atherosclerosis and was associated with a significant increase in major cardiovascular events.[20] Also, ageing may reduce ACAT-1 activity, as indicated by a study demonstrating that older rabbits had less ACAT-1 activity.[21]

Not all FRC is derived via the esterification/de-esterification pathway. Other major sources include dying foam cells and red blood cells with rich cholesterol membranes released from injured vasa vasorum contribute to the lipid pool.[22] The release of cellular contents from dying cells can also alter the local pH milieu. Many of these local physical changes can enhance cholesterol crystallization.[23] Eventually, extracellular lipid deposits evolve to form a lipid pool encased between the fibrous cap and the arterial wall giving the plaque its characteristic shape of an inflated ellipse.

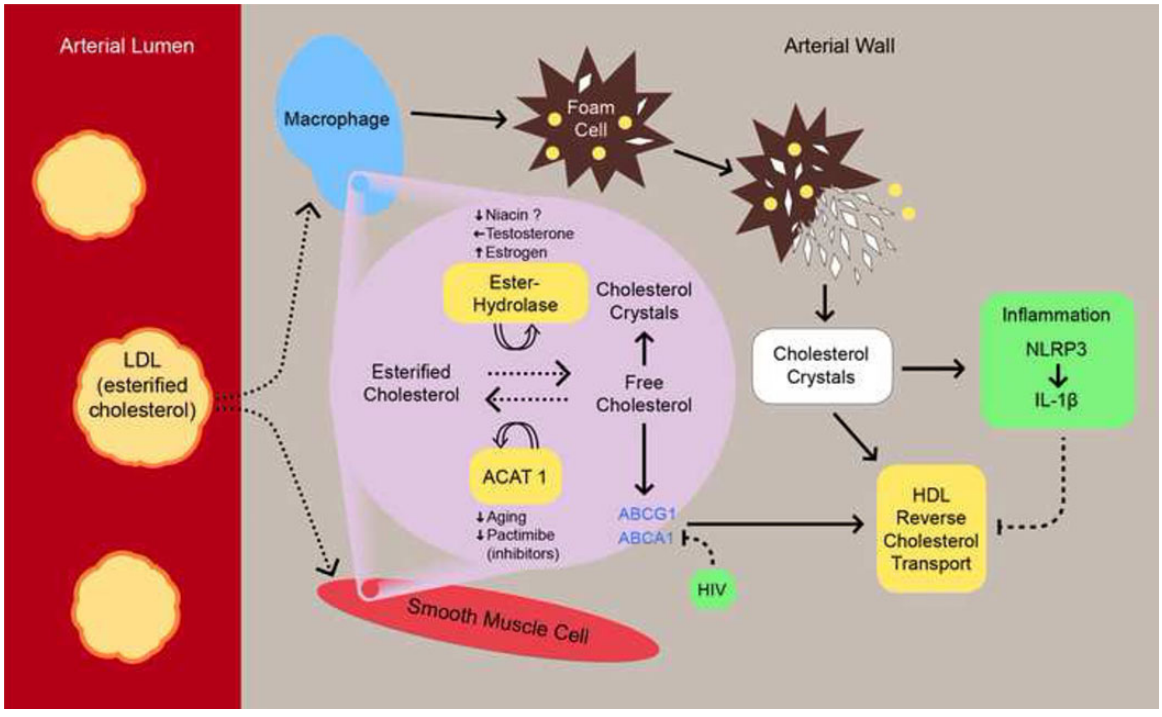

Figure 1 Cholesterol transport within cells and extracellular space. Equilibrium between esterified and free cholesterol is noted with membrane transporters driving free cholesterol into the extracellular space where it is taken up by high-density lipoprotein. Dying foam cells overloaded with esterified cholesterol and crystals release their content into the extracellular space. Free cholesterol build-up in the extracellular space leads to crystallization. ABCA1, ABCG1, ATP-binding cassette A-1, G-1; ACAT 1, acyl-coenzyme A cholesterol acyltransferase 1; HDL, high-density lipoprotein; HIV, human immunodeficiency virus; IL-1β, interleukin-1β; LDL, low-density lipoprotein; NLRP3, NLRP3 inflammasome.

Thus, systemic conditions that lead to cholesterol crystallization may result in multiple plaque ruptures in various arterial beds resulting in a 'vulnerable patient' rather than just a single 'vulnerable plaque'.[24]

High-density lipoprotein functionality and free cholesterol accumulation

High-density lipoprotein reverse transport of cholesterol out of arterial wall cells requires the conversion of ESC to FRC so that it can be mobilized via membrane-bound transporters, ATP-binding cassette A-1 and G1 (ABCA-1, ABCG-1).[25,26] A failure of this pathway will lead to both ESC and FRC build-up. Thus, with dysfunctional HDL, FRC will accumulate in both the cell membrane and the extracellular space promoting the formation of CC. Of note, one of the conditions leading to HDL dysfunction is inflammation.[12] As atherosclerosis builds in the arterial wall, there is a rise in both local and systemic inflammation that can induce HDL dysfunction leading to a vicious positive feedback cycle that favours atherosclerosis build-up with further worsening of HDL function. Moreover, HDL has been shown to dissolve CC directly.[27] This is an area of active research. Various cholesterol ester transfer protein inhibitors that increase HDL levels continue to be evaluated. Initial trials with torcetrapib and dalcetrapib were not successful. However, a current trial with anacetrapib is still ongoing.[28]

Inflammation and plaque destabilization

Inflammation plays a critical role at various stages of atherogenesis, triggering both local and systemic responses.[29] Locally, oxidized LDL causes endothelial dysfunction and induces the expression of various proinflammatory cytokines, chemokines and adhesion molecules via TRA-F3IP2 protein which is an upstream regulator of the NF-κB and AP-1 pathways.[30] Moreover, macrophages are activated by oxidized LDL and release cytokines, such as monocyte chemoattractant protein-1 (MCP-1) which signals other macrophages to the site and metalloproteinases that have autolytic properties.[31] Also, oxidized LDL induces CD14 and toll-like receptor 4 (TLR-4) which in turn induces NLRP3 inflammasome and pro-IL-β protein expression.[32] Other inflammatory biomarkers increase during atherogenesis including TNF-α which promotes foam cell formation and apoptosis, impairs endothelial function, and enhances smooth muscle cell migration and proliferation.[33] As reported in earlier studies, we have demonstrated that CC form very early in atherogenesis and activate NLRP3 leading to the secretion of IL-1β.[9] Similar findings have been reported for monosodium urate

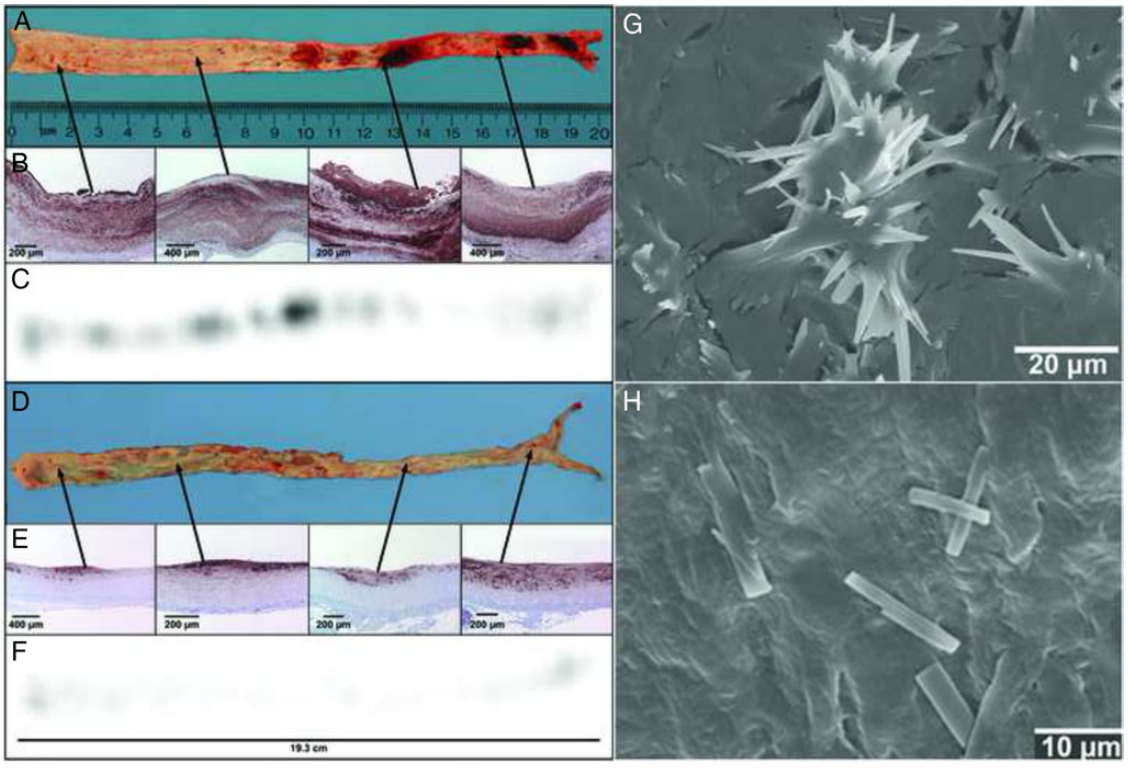

Figure 2 Macrophage and cholesterol crystal density in atherosclerotic rabbit aortas. (*A*) Aorta of rabbit on an atherogenic diet demonstrating extensive plaque and thrombosis. (*B*) RAM 11 staining demonstrates dense macrophage accumulation (brown stain). (*C*) Positron emission tomography scan demonstrates high fluorodeoxyglucose uptake of corresponding artery. (*D*) Aorta of rabbit on atherogenic diet and ezetimibe demonstrating minimal plaque and no thrombosis. (*E*) RAM 11 staining demonstrates minimal macrophage accumulation. (*F*) Positron emission tomography scan demonstrates low flurodeoxyglucose uptake. (*G*) Scanning electron microscopy of atherosclerotic rabbit aorta with extensive cholesterol crystals perforating the intima. (*H*) Scanning electron microscopy demonstrates few cholesterol crystals on intimal surface with ezetimibe treatment. Modified and reproduced with permission.[7]

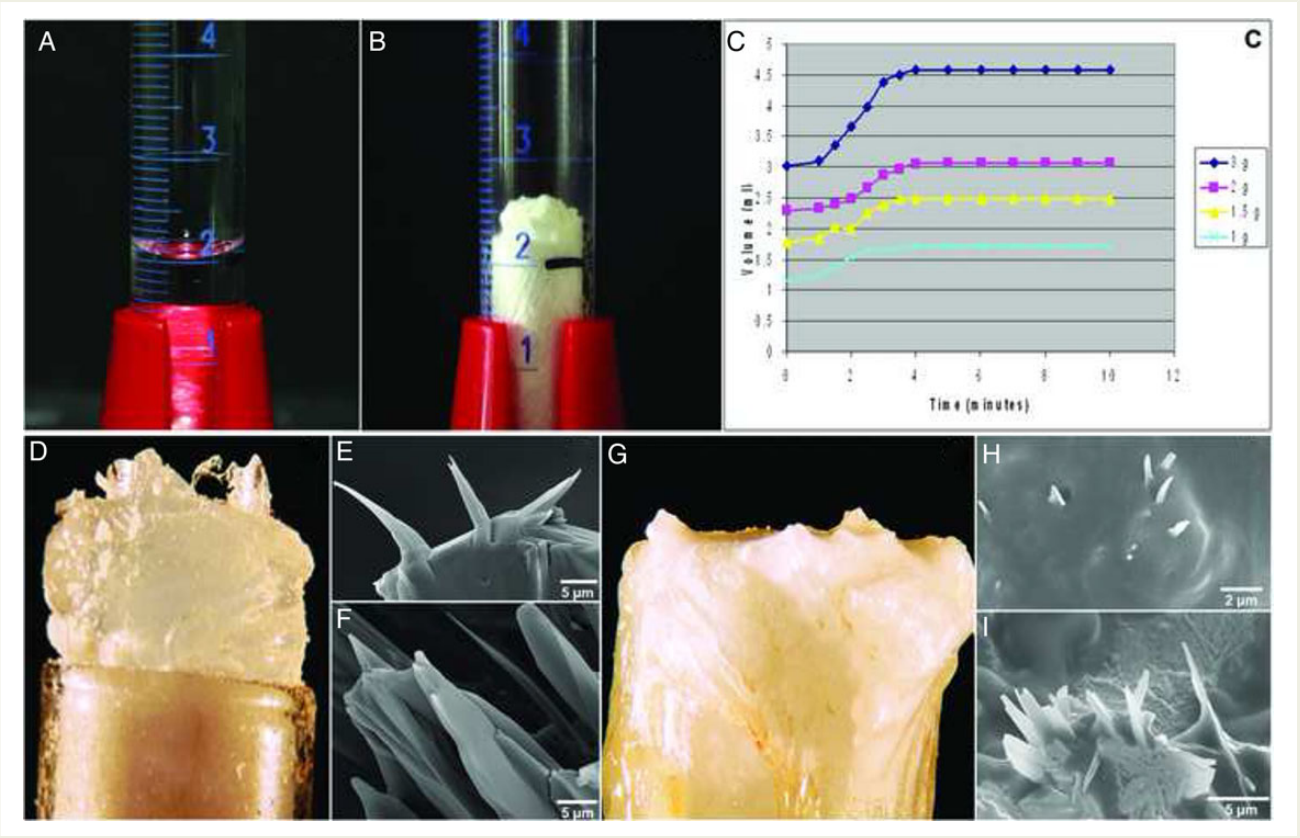

Figure 3 Volume expansion during cholesterol crystallization. (*A*, *B*) Upon crystallization, liquid cholesterol in graduated cylinder expands in volume above the meniscus line. (*C*) Graph of volume expansion is greater with increasing amounts of cholesterol (1–3 *g*). (*D*) Cholesterol crystals are seen above the edge of the test tube after crystallization. (*E*, *F*) Scanning electron micrographs demonstrate sharp-tipped crystal geometries. (*G*) When a fibrous membrane is placed over the mouth of the test tube, crystals perforate the membrane (*H*, *I*). Reproduced with permission.[3,56,57]

crystals in gout.[34] Systemically, IL-1β then induces inflammation via IL-6[35] which elicits an acute-phase response in the liver.[36] Several clinical studies have demonstrated that C-reactive protein produced during an acute-phase response is a marker for risk of developing acute cardiovascular events.[37] The inflammatory process results in positive remodelling of the arterial wall and destabilizes the plaque.

As CC grow and accumulate in the extracellular space within the necrotic core, they can eventually reach and perforate the overlying intima.[3,4] This will then trigger a systemic response by activating intimal surface cytokines that attract monocytes.[38] This process has been demonstrated in an atherosclerotic rabbit model. By lowering serum cholesterol levels with ezetimibe, arterial wall CC content, plaque rupture and thrombosis were significantly reduced (*Figure 2*).[7] These findings are consistent with the reduced cardiovascular events reported in The Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT).[39] Thus, atherogenesis is a highly dynamic process that responds well to lipid-lowering interventions.[4]

Local conditions that enhance plaque rupture

In vitro and *in vivo* studies have shown that as FRC accumulates in the sub-intimal space it is subjected to local physico-chemical conditions that can enhance crystallization.[4,23] As mentioned earlier, when liquid FRC crystallizes, it occupies a significantly greater volume in the solid state compared with the liquid state (*Figure 3*). Cholesterol has been found in the liquid state in the arterial wall making the process of CC expansion leading to plaque rupture a realistic possibility.[40] Evidence that supports this hypothesis has been derived from several sources:

(1) Autopsy findings demonstrated that only patients who died from acute myocardial infarction had CC perforating the intimal surface and fibrous cap while those who died of causes other than myocardial infarction had arterial plaques without CC perforating the surface.[5]
(2) Autopsy findings of patients who died from myocardial infarction revealed CC perforating the intima overlying ruptured plaques not only of the culprit artery but also in other coronary arteries of the same heart (*Figure 4*).[5]
(3) Greater plaque burden observed in atherosclerotic rabbits was associated with higher arterial wall cholesterol content, CC density, serum inflammation, macrophage infiltration in plaque and significantly greater events of intimal disruption with thrombosis.[7] Moreover, lowering total serum cholesterol reduced the crystal content and associated thrombosis (*Figure 2*).

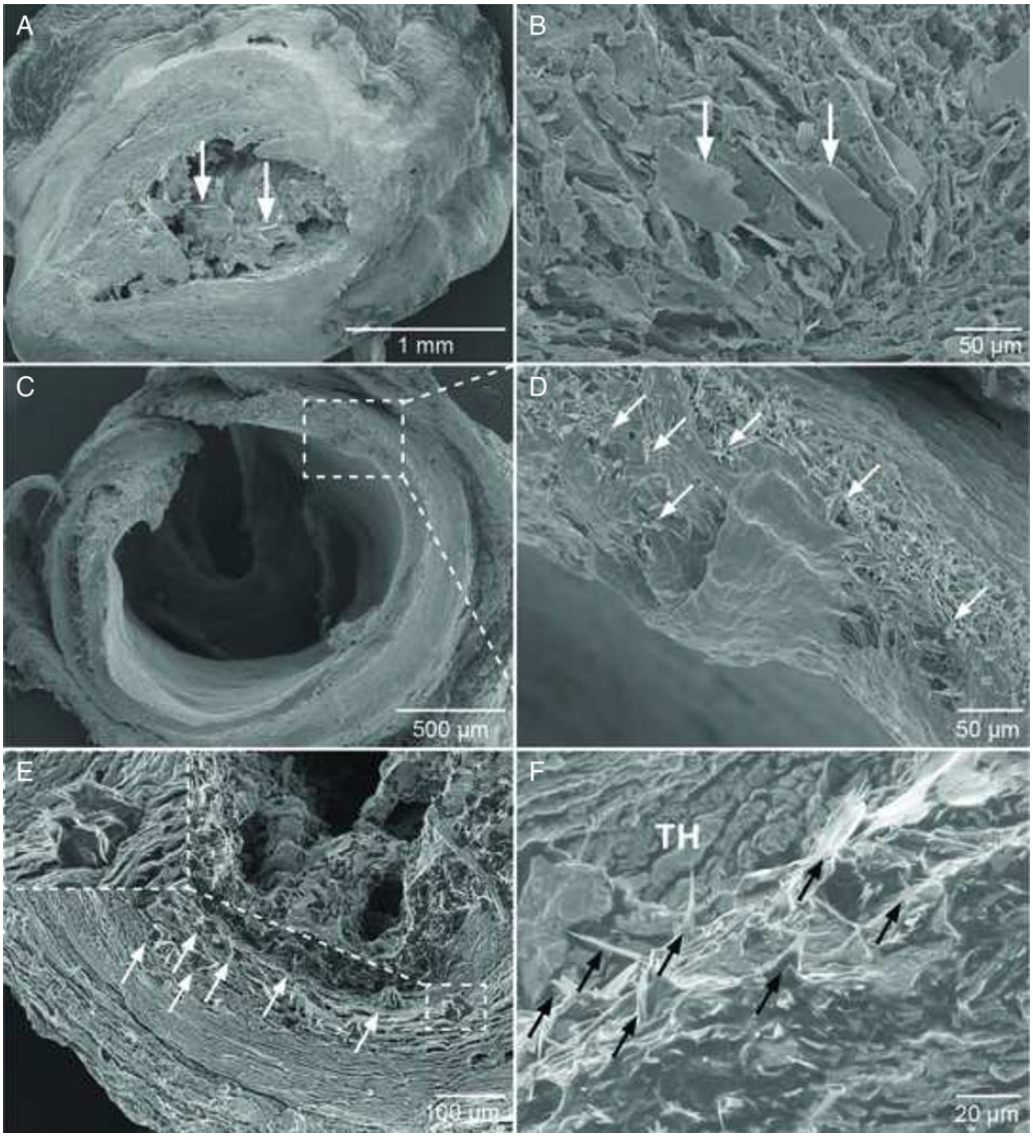

Figure 4 Scanning electron micrographs of coronary arteries of patients who died during acute myocardial infarction. (*A*, *B*) Circumflex artery totally occluded with thrombus that is loaded with cholesterol crystals (arrows). (*C*, *D*) Right coronary artery that was not the culprit but had evidence of crystals perforating the intima (arrows). (*E*, *F*) Left anterior descending culprit artery with crystals perforating the intima (insert; arrows) and thrombus (TH) formation.[5] Modified and reproduced with permission. Courtesy of Abela GS.

(4) Optical coherence tomography (OCT) has confirmed the presence of CC in human coronary arteries during percutaneous procedures and suggested an association between the presence of CC with potentially vulnerable plaques (*Figure 5*).[41,42]

Several physical conditions such as a rise in cholesterol saturation, a minor drop in local temperature (1−2°C), an alkaline pH, and hydration of the cholesterol molecule to form cholesterol monohydrate are recognized to enhance cholesterol crystallization.[23] Moreover, combinations of these conditions (i.e. drop in temperature and cholesterol saturation) had a synergistic effect on crystallization. Although there is no known causal relationship between CC and plaque rupture in humans, the current studies suggest that the same process could be occurring *in vivo*. Volume expansion does occur with sharp-tipped crystals perforating fibrous tissue during *in vitro* experiments.[3,5] Similar findings of crystals perforating atherosclerotic plaque caps have been demonstrated in post mortem human coronary arteries and *ex vivo* human carotid plaques obtained at surgery.[5] Also, findings of crystals perforating the intima similar to the human data were present in an atherosclerotic rabbit model.[7] Therefore, the sum total of these findings suggests that the process of plaque rupture involves penetration of sharp-tipped CC into the fibrous cap. Additionally, one can speculate that the growth of crystals within the necrotic core can stretch the fibrous cap to create TCFA. The thinned cap is then perforated by sharp-tipped CC emerging from the necrotic core, further weakening it and

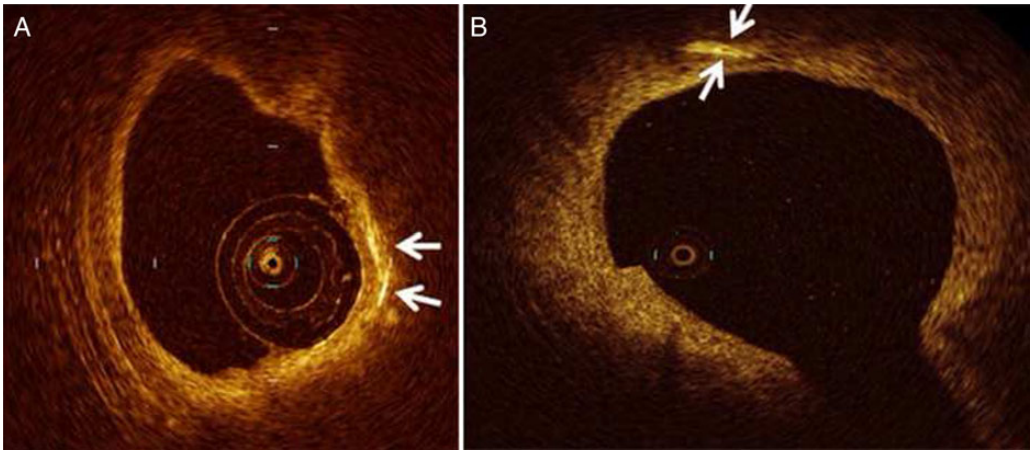

Figure 5 Optical coherence tomographic images of human coronary arteries during percutaneous intervention. (*A*[41], *B*[42]) Cholesterol crystals (arrows) are defined as thin, linear regions of high intensity frequently associated with a fibrous cap or necrotic core within the plaque. Reproduced with permission.

causing rupture (see Supplementary material online, *Video*). This concept is consistent with an earlier observation by Loree *et al.*[43] who demonstrated a strong correlation between a thin fibrous cap and a large necrotic core in vulnerable plaques[2] that also contain a large amount of CC.[5] The basic process is similar to eruptive skin xanthomas rich in CC or gouty tophi filled with monosodium urate crystals over the skin. These are shared features of crystalloid diseases infiltrating soft tissues.

Mechanism of plaque rupture and clinical implications

Not all plaque ruptures lead to a heart attack or stroke. Studies have demonstrated that during a heart attack, several arteries are often involved with plaque rupture, yet not all develop occlusive thrombosis.[42] Therefore, other factors are involved in determining the event severity and outcome. Based on the current proposed role of CC in plaque rupture, it would be expected that plaques with large necrotic cores will release greater amounts of CC into the circulation causing more injury and arterial thrombosis.[5] Thus, plaque burden is of critical importance and this has been recognized in clinical studies.[42,44]

Imaging of vulnerable plaques

Using scanning electron microscopy (SEM), CC could be observed perforating the intima over plaques visible only when tissue samples are processed by air or vacuum dehydration but not when using the standard approach with ethanol (*Figure 4*).[5,45] This was also confirmed by confocal microscopy of fresh human plaque samples obtained from the operating theatre during carotid endarterectomy and kept at 37°C. When crystals were stained with Bodipy, a fluorescence dye, and the intima counterstained with acyl-LDL, CC were found to be protruding through the intimal surface (*Figure 6*).[5] This was performed at 37°C without any tissue fixation or dehydration. Despite limitations due to dissolving cholesterol during standard tissue preparation methods, light microscopy also reveals CC penetrating the fibrous cap and communicating as empty channels or clefts with the intimal surface (*Figure 6*). Recently, Liu *et al.*[6] made a similar observation of CC perforating the plaque cap using micro-OCT of human carotid plaques. Using coherent anti-Stokes Raman scattering (CARS), Lim *et al.*[46] were also able to identify CC at the arterial intimal surface. Recently, we used β-cyclodextrin conjugated with super paramagnetic iron oxide nanoparticles to successfully image CC in atherosclerotic rabbit aortas with magnetic resonance imaging.[47] Thus, molecular imaging techniques may provide additional means to detect CC *in vivo*.

Therapeutic implications

Based on studies regarding the role of CC, pharmacological compounds that have the potential of dissolving CC or inhibiting their formation may provide an effective therapeutic approach to reduce the incidence of acute cardiovascular events. In our studies, the observations by SEM were made possible by avoiding tissue processing with ethanol, because ethanol dissolves CC rendering them and their role in atherosclerosis invisible.[3,5,45] Also, recent studies have demonstrated that statins can dissolve CC and interfere with crystal formation.[4] Thus, maximizing inhibition of CC formation and their dissolution could prove to be an effective therapy in stabilizing vulnerable plaques. Also, the recent introduction of proprotein convertase subtilisin/kexin type 9 inhibitors that are very effective in lowering LDL may greatly impact CC formation.[48,49]

Another therapeutic target is reduction of the inflammation induced by CC. Recently, a clinical trial, Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), was designed to evaluate the efficacy of an antibody that selectively inhibits IL-1β, canakinumab, in reducing plaque inflammation and cardiovascular events.[50] Canakinumab is used in the treatment of gout and other inflammatory conditions. However, the results of

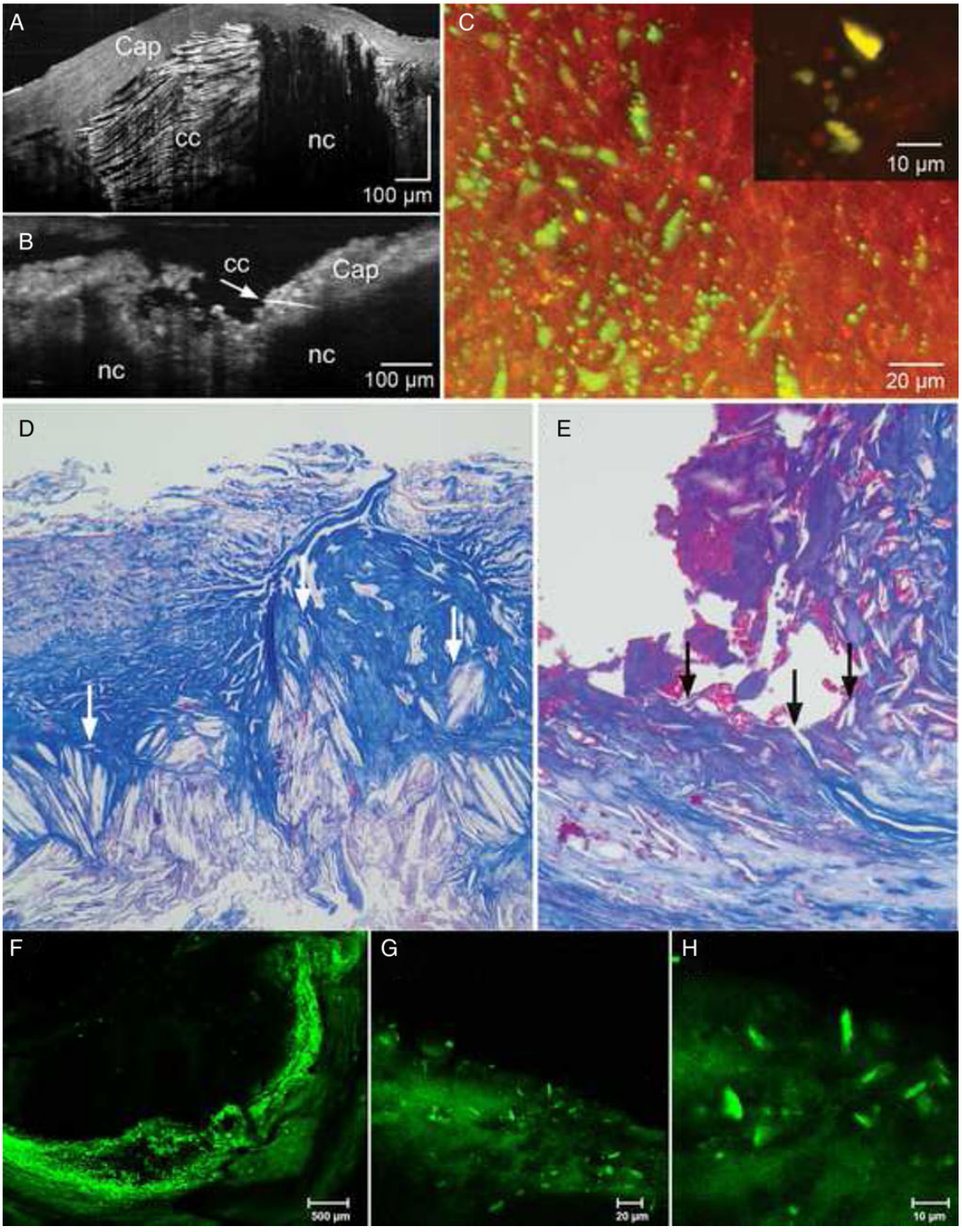

Figure 6 Evidence of cholesterol crystals perforating the fibrous cap and intima of human plaque. Micro-optical coherence tomography image of human carotid plaque with (*A*) dense concentration of cholesterol crystals beneath a bulging fibrous cap and (*B*) cholesterol crystals perforating the fibrous cap (arrow; nc = necrotic core). Courtesy of Tearney GJ and reproduced with permission.[6] (*C*) Fluorescence image of arterial wall surface demonstrating cholesterol crystals perforating the intima in fresh plaques at 37°C without fixation or ethanol dehydration. Insert is magnification of crystals.[5] (*D*) Light micrographs with clefts of cholesterol crystals penetrating the fibrous cap (arrows, magnification 12X) and (*E*) clefts connecting from the fibrous cap onto the lumen (arrows, magnification 16X). Courtesy of Abela GS. (*F*, *G*, *H*) Fluorescence image of fresh human carotid plaque demonstrating cholesterol crystals emerging from the plaque surface without processing with ethanol dehydration.[45] Modified and reproduced with permission.

the effects of canakinumab in cardiovascular disease are still pending. The use of other anti-inflammatory agents for atherosclerotic disease is being evaluated. Low-dose methotrexate is currently under investigation in patients with high cardiovascular risk in the Cardiovascular Inflammation Reduction Trial (CIRT).[51] Also, colchicine can interfere with macrophage and neutrophil activity and has been shown in a small trial to reduce cardiovascular events.[52] Anti-leukotrienes agents are currently being investigated for reducing cardiovascular inflammation, and lipoxins and resolvins are being proposed for their anti-inflammatory and pro-resolution properties.[53]

Future directions

The role of CC and their effect on clinical outcomes will require a broad approach that investigates the various stages of atherosclerotic plaque development, the evolution to vulnerable plaque and eventual rupture. Studies evaluating the effects of enhanced production of CEH in arterial wall cells, the role of various hormones and gene regulation on FRC accumulation may provide other therapeutic targets. Also, various pharmaceuticals (e.g. HIV treating agents) as well as certain viruses (i.e. HIV) enhance atherosclerosis by increasing FRC and promoting cholesterol crystallization.[54,55] Studies with other agents and viruses may need to be considered.

Summary

The role of CC in triggering inflammation as well as their mechanical and toxic effect on cells is critical to arterial wall injury. An imbalance in the intracellular equilibrium between ESC and FRC that favours FRC, especially when associated with low or dysfunctional HDL, will lead to accumulation of FRC and CC formation. Thus, developing agents that dissolve CC and/or that target inflammatory pathways may provide alternative approaches for stabilizing vulnerable plaques. The current data would support observations that a large plaque burden that contains more crystals is a critical feature of plaque ruptures with an associated inflammatory state that is reflective of a systemic condition with localized events.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Authors' contributions

G.A. handled funding and supervision. G.A. and A.J. drafted the manuscript. J.K.K. and F.E.S. made critical revision of the manuscript for key intellectual content.

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All authors have contributed to the design, analysis, development, critical revisions, and final approval of the manuscript. We would like to acknowledge Bruce H. Francis, M.D., F.A.C.E., for critically reviewing the manuscript and Vennila Dharman (Novartis Healthcare) for providing editorial support.

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