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Commentary

The role of cholesterol crystals and ocular crystal emboli in retinal pathology

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A B S T R A C T

Cholesterol crystals (CC) can be responsible for a range of clinical syndromes in the retina from asymptomatic plaques to retinal artery occlusion with clinical trials providing evidence for the efficacy in lipid lowering therapies in preventing ocular pathology. Much of the literature has focused on CC in retinal circulation as a marker of poor systemic health and have attempted to use them to categorize risk of mortality and stroke. More recently cholesterol accumulation and CC formation have been linked to development of diabetic retinopathy with CC formation in the retina due to aberrant retinal cholesterol homeostasis and not simply systemic dyslipidemia.

1. Introduction

The metabolically active retina obtains essential lipids by endogenous biosynthesis and from systemic circulation [1]. Cholesterol crystals (CC) can cause a wide range of retinal pathology including diabetic retinopathy, retinal artery occlusion (RAO) or can also be asymptomatic [2–4]. Retinal CC emboli can be visualized in the vasculature on retinal fundus exam and associated with pathology in the eye ranging from asymptomatic plaques to RAO [3,4]. CCs can present within the retina as hyper-reflective crystalline deposits and have been identified in the retina of murine models of type 1 and type 2 diabetes and in a high-cholesterol diet-fed pig model [2,5]. Cell culture studies demonstrated that treatment of retinal cells with CCs can recapitulate key aspects of diabetic retinopathy including inflammation, cell apoptosis and breakdown of the blood retinal barrier (BRB) integrity [2]. Strategies to remove CCs, including fenofibrate, statins and α-cyclodextrin, resulted in correction of diabetic retinopathy emphasizing their critical role in retinal pathology [2,6].

While many randomized controlled trials (RCTs) have shown the efficacy of lipid lowering drugs in preventing diabetic retinopathy, this is not due to reducing serum levels of cholesterol as RCTs have not shown any association between drug treatment results and reduction of serum cholesterol levels [5,7]. Recent studies concerning ocular CCs suggest they are the result of abnormal local retinal cholesterol homeostasis [2]. Animal and in vitro studies support a role for CCs formation due to the presence of retinal cholesterol pools [5]. Cholesterol in the retina is maintained by two methods: internal synthesis and transport primarily across the outer BRB by retinal pigment epithelial cells (RPE) [8]. The RPE utilizes ATP-binding cassette transporters and apolipoproteins to transport cholesterol to the choroidal circulation, a process called "reverse cholesterol transport" [8,9]. Furthermore, the RPE and neural retina convert cholesterol into oxysterols that function as protective ligands for liver X receptors (LXR) [10]. LXR receptors agonists function as regulators of reverse cholesterol transport [10]. The function of the RPE and oxysterol conversion are reduced in diabetic retinopathy, leading to an accumulation of cholesterol within the retina due to a reduction of LXR activation and less elimination of cholesterol into choroidal circulation [7,11].

We recently demonstrated that CCs contribute to inflammation, apoptosis, BRB breakdown, and loss of vision in rodent models of diabetic retinopathy [2]. CCs are known to activate the complement cascade, NLR3P inflammasome, and inflammatory cytokines [12,13]. Therefore, prevention of CC formation or their dissolution after formation may significantly reduce the symptoms and inflammation seen in diabetic retinopathy.

This review covers key aspects of CCs formation and how they impact the retina. Four main topics are considered: diabetic retinopathy, asymptomatic Hollenhorst Plaques, retinal artery occlusion, and age-related macular degeneration. For each topic, the role of CCs in the generation of the clinical findings and the basic research studies that

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support the relevance of CCs to ocular disease in humans is discussed.

2. Cholesterol crystals and diabetic retinopathy

The utility of lipid lowering agents such as statins and fibrates in diabetic retinopathy has been explored by many RCTs. Some studies and RCTs suggest circulating cholesterol levels have no impact on diabetic retinopathy [5]. Recent meta-analysis of 13,454 patients over 8 randomized controlled trials demonstrated that lipid lowering agents (combined fenofibrate and statins) significantly reduce the progression of diabetic retinopathy [OR = 0.77 (95%CI: 0.62, 0.96), $P = 0.02$] with potential effects on preventing diabetic macular edema [OR = 0.60 (95%CI: 0.34, 1.08), $P = 0.09$] [14]. This finding agrees with other systematic reviews and meta-analysis data on clinical trials and cohort studies examining statin use in diabetic retinopathy which significantly reduced prevalence of diabetic retinopathy (HR: 0.68 (0.55, 0.84), $p < 0.001$; I^2 95%), non-proliferative diabetic retinopathy (HR: 0.80 (0.66, 0.96), $p = 0.02$; I^2 93%), advancement to proliferative diabetic retinopathy (HR: 0.69 (0.51, 0.93), $p = 0.01$; I^2 90%), prevalence of diabetic macular edema (HR: 0.56 (0.39, 0.80), $p = 0.002$; I^2 82%), and reduction in procedural techniques like pan-retinal laser photocoagulation, vitrectomy, and intravitreal injection with anti-vascular endothelial growth factor [15,16]. However, not all studies and meta-analyses have demonstrated positive effects of statins on diabetic retinopathy progression leaving their clinical role unclear [5,17,18]. Furthermore, many of these analyses on lipid lowering agents were conducted on macrovascular studies examining cardiovascular outcomes with diabetic retinopathy being secondary or tertiary outcomes [5,19,20]. The LENS trial, a recent RCT studying the effect of fenofibrate on diabetic retinopathy progression, demonstrated a reduction in progression to referable or treatable retinopathy (HR 0.73; 95 % CI, 0.58 to 0.91; $P = 0.006$) [21]. However, more, large RCTs need to be performed to clarify fenofibrates role in diabetic retinopathy.

Hammer et al. identified hyper-reflective crystalline deposits found in diabetic retinopathy on ocular coherence tomography (OCT) as CCs utilizing scanning electron microscopy (SEM) and immunohistochemistry in human donor tissue [2]. Importantly, traditional use of organic solvents in preparation of the SEM was avoided to prevent dissolving the CC [2,22]. This is not a standard procedure of SEM analysis and explains why they have not been reported previously (Fig. 1). When human and bovine retinal endothelial cells (BREC) were exposed to CCs, the cells expressed inflammatory markers including IL-6 and IL-8 and reduction of tight junction markers [2]. Diabetic mice exhibit activated microglia that engulf CCs, resulting in elevated C5AR1 mRNA expression, complement MAC activation, and breakdown of the BRB [2]. BRB breakdown led to increases in systemic absorption of cholesterol and reduction of synthesis within the retina as a compensatory mechanism [2]. Treatment with α-cyclodextrin and fenofibrate in BREC dissolved CCs and prevented release of inflammatory cytokines by the cells [2]. In vivo treatment of diabetic mice with α-cyclodextrin corrected breakdown of the BRB and diabetic retinopathy [2].

3. Asymptomatic Hollenhorst plaques

In the 1960s, Dr. Robert Hollenhorst described specific emboli in the retina as yellow, orange refractile plaques at retinal artery bifurcations [4]. He originally postulated that these crystals were CCs and investigated his hypothesis by planting atherosclerotic human specimens and CCs into the carotid arteries of rhesus monkeys and dogs [23]. The refractile yellow and orange crystals were reproduced strongly suggesting that CCs explained these emboli [23]. Further case reports of autopsies demonstrated that these refractile crystals were histologically CCs [23]. Hollenhorst continued his work demonstrating that retinal CC embolism is associated with atherosclerotic risk factors such as diabetes, hypertension, and smoking [23]. He is also credited for establishing the connection between retinal embolism and decreased survival of patients

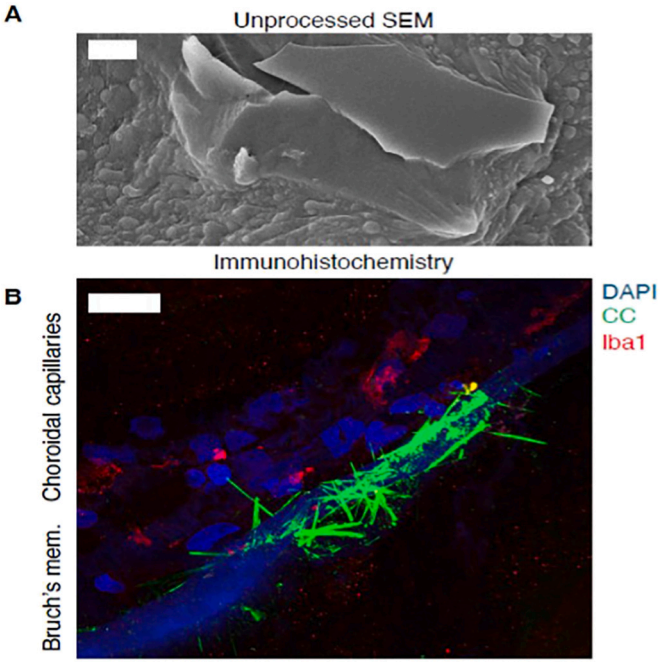

Fig. 1. Cholesterol crystals in human retina. Cholesterol crystals in a donor retina with proliferative diabetic retinopathy were visualized via unprocessed SEM (A) and immunohistochemistry methodology (B). 10 μm thick sections of a human eye have been stained for nuclei (DAPI), cholesterol crystals (CC), and microglia (Iba1). Scale bars = 2.5 μm (A) and 10 μm (B).

by 13 % at one year and 40 % at eight years [23]. These associations have provided the framework for subsequent studies that quantified the risk of retinal cholesterol emboli for stroke, vision loss, and other serious complications including death. Given Dr. Hollenhorst's large contribution to this area of research, CC emboli in the eye are referred to as Hollenhorst plaques [23].

Since Dr. Hollenhorst's original work demonstrated how CCs were closely related to carotid artery disease and stroke, studies have focused on determining the risk of stroke/TIAs, mortality, and proper clinical tests to perform when asymptomatic Hollenhorst plaques (HP) are found on routine exam. A systematic review examining asymptomatic HPs found 9 studies that examined 780 patients and calculated a total of a 12 % incidence of stroke in a follow up range of 0.5 to 7 years [24]. However, the mortality rate from stroke in the same population was 0.15 % [24]. This number was much lower than the number reported originally by Dr. Hollenhorst which found a decreased survival rate of 13 % after 1 year and 40 % after 8 years [23]. Ghoneim et al. suggested that older studies, performed 25 to 40 years prior, had higher incidences of stroke in the follow up period, but newer studies report a decreased rate of stroke in their populations [24–28]. The reduction of strokes of asymptomatic retinal CCs in newer studies was attributed to the improvement in management of atherosclerotic risk factors [24]. However, there are fewer recent studies examining this relationship, and no study has been conducted that examines this relationship with a population of patients in the past 15 to 20 years [24]. Therefore, the relationship between HPs, modern management of risk factors, and stroke rate remains unclear.

Although the rate of strokes may be decreasing in asymptomatic patients, HPs remain a significant indicator of poor overall health with significantly increased risk of stroke and all-cause mortality [28]. Current studies have not been able to elucidate the role of carotid artery endarterectomy (CEA) or carotid artery stenting (CAS), and it remains unclear whether there is true benefit in surgically treating asymptomatic patients with retinal emboli [24,29]. Few studies have directly studied the efficacy of CEA in patients with asymptomatic HPs although the

association with ipsilateral stroke and carotid artery stenosis exists. One study had 28 patients (24 were asymptomatic and 4 were symptomatic) with HPs that underwent ipsilateral CEA and 39 patients with HPs that did not have CEA performed [24,29,30]. The average annual rate of stroke was 3 % in patients who did not have CEA and 2.4 % in patients who underwent CEA [24,29,30]. Due to the lack of sufficient studies, the asymptomatic HP patient could be compared to the asymptomatic patient with significant carotid stenosis to better clarify the risk and benefit assessment of undergoing CEA on asymptomatic HP patients [29]. A network meta-analysis of RCTs found that major strokes (OR 0.59, 95 % CI 0.32–1.1) and major strokes and mortality (OR 0.56, 95 % CI 0.27–1.2) were not significantly reduced by CEA and best modern medical therapy (BMT) when compared to BMT alone, but CEA significantly reduced minor strokes when compared to BMT alone (OR 0.35, 95 % CI 0.21–0.58) [31]. In this study, modern best medical therapy was defined to occur past the year 2000 [31]. Furthermore, the annual stroke rate of asymptomatic patients with >50 % stenosis in the internal carotid is far less than that of symptomatic patients, and perioperative risk will always be present in CEA [32]. Therefore, new clinical trials are underway to determine if BMT is sufficient in treating asymptomatic CAS [32,33]. These new studies may provide key insights for asymptomatic severe CAS and Hollenhorst plaques. However, it is recommended that patients with asymptomatic HPs receive a carotid artery doppler ultrasound (CDUS) to assess for the degree of stenosis which may influence clinical judgement toward CEA [34]. Given the risk for cerebrovascular accidents, when an asymptomatic HP is found, aggressive management of atherosclerotic risk factors is recommended [34].

4. The release of CCs from atherosclerotic plaques

The mechanism of plaque rupture with release of CCs from atherosclerotic plaques was described by Abela et al. [35,36]. The basic principle is that when cholesterol undergoes a phase transition from a liquid to a solid state it occupies a greater volume causing rapid expansion of the atherosclerotic plaque core and sharp tipped crystals (that can be stained with Bodipy, Fig. 2 left panel) perforate the fibrous cap leading to thrombosis and arterial occlusion. Crystals are then released into the circulation embolizing distally to end organs such as the retina (Fig. 2 right panel) triggering inflammation and ischemia. This was demonstrated by angioscopic studies in live humans by Komatsu et al. [37]. As shown in Fig. 3, atherosclerotic lesions are composed of plaque hemorrhage and a thick fibrous cap. Following endarterectomy, light and scanning electron microscopy demonstrate extensive cholesterol crystals with intraplaque hemorrhage and the fibrous cap. Furthermore, CCs in the plaque can cause hemorrhage into the plaque core by cutting the vasa vasorum [38,39]. This enhances plaque growth by providing a

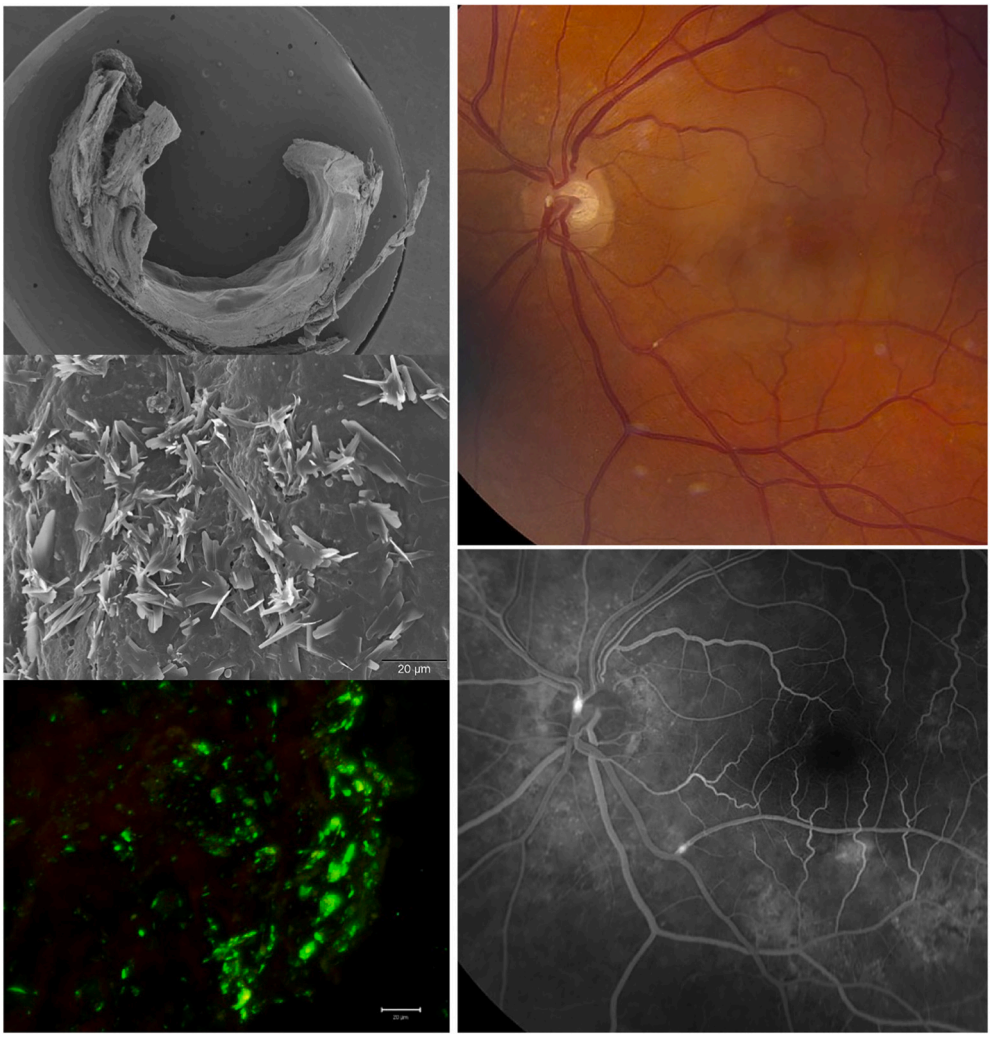

Fig. 2. (Left panel), (top) Low power scanning electron micrograph of carotid artery plaque. (Middle) Surface scanning of the plaque demonstrates extensive cholesterol crystals. (Bottom) Fluorescence image of cholesterol crystals on the intimal surface of the artery using Bodipy stain for cholesterol crystals. (Right panel) Hollenhorst plaques in retinal arteries of embolized cholesterol crystals (arrows, Modified from Elizabeth Gauger, MD and Toni Venckus, CRA University of Iowa). Reproduced with permission [68].

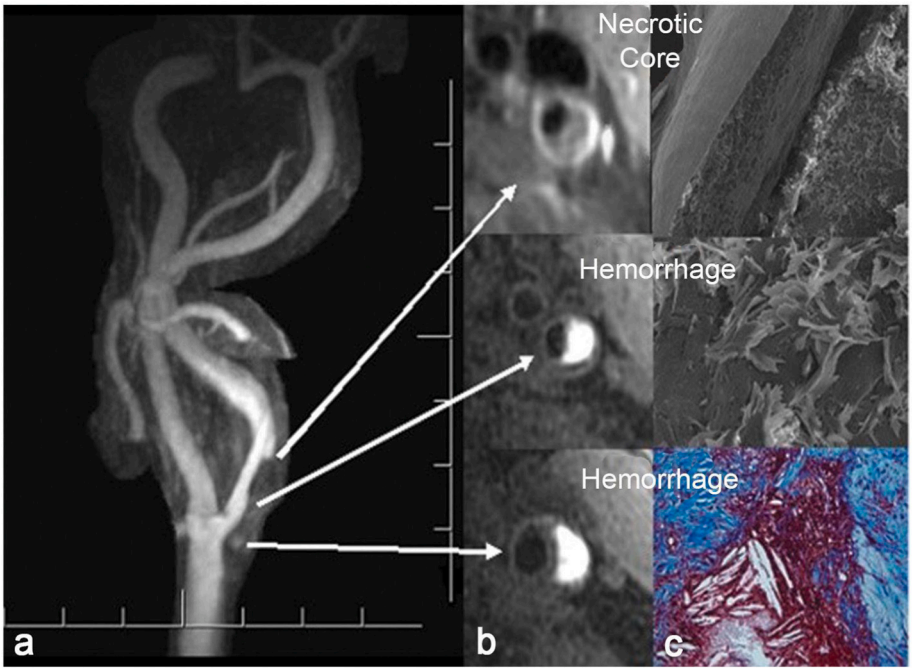

Fig. 3. Left carotid artery confirming intraplaque hemorrhage by black-blood T1-weighted cross sectional images using 3D magnetization-prepared rapid acquisition gradient echo sequence, where the intraplaque hemorrhage is bright. (a,b) Along the inferior aspect of the intraplaque hemorrhage there is a 1 mm thick fibrous cap between the dark lumen and the bright deep intraplaque hemorrhage. Superiorly there is a well-defined fibrous cap (<500 μm) between the lumen and lipid core. (c) Light and scanning electron microscopy of endarterectomy specimen demonstrates extensive cholesterol crystals with intraplaque hemorrhage and thin fibrous cap. Modified and reproduced with permission [38].

great amount of cholesterol from the red blood cell membranes [40]. Also, release of CCs into the circulation can trigger arterial spasm that can worsen the ischemia in the distal circulation [41].

5. Retinal artery occlusion from CC

Retinal artery occlusions have many potential causes, but the non-arteritic form accounts for 90 % of cases [42]. Retinal emboli are the most common cause of nonartertic RAO and CC are responsible for 46 to 80 % of all emboli [42–44]. RAO is highly associated with cerebral stroke [45]. One meta-analysis of incidence of acute cerebral ischemia after RAO detected by magnetic resonance imaging (MRI) found stroke rates occurring at 30 % for central RAO (CRAO) and 25 % for branch RAO within 7 days of initial RAO [45]. Therefore, management typically involves being admitted to the emergency department for an acute stroke although some experts debate the utility of this examination with no neurological symptoms [45,46]. Once the acute stroke workup is complete, patients with RAO will undergo evaluation of sources of emboli [46]. This is in stark contrast to asymptomatic HPs which undergo outpatient assessment of risk factors.

Few studies exist that directly examine the effects of CC emboli on retinal vasculature in animal models [47,48]. Although animal models exist that recapitulate RAO, few of these animal models utilize embolization and even fewer use CCs or atheroembolic material [47,48]. Most of these studies aim to examine the effect of ischemia on the retina, and embolism models are not specific to CRAO, but instead, create an ocular ischemic syndrome model [47]. Therefore, more specific models are preferred for CRAO, but CCs in the retinal vasculature and the pathogenic mechanisms that they specifically induce in the retina are understudied. In kidney CC emboli animal models, CCs induce vascular occlusion by initiating endothelial damage, thrombin activation, platelet recruitment, and aggregation of neutrophils and proinflammatory cytokines [49,50]. Therefore, the CCs themselves constitute a small volume of the clot size [50]. CCs remain after clot thrombolysis, but reperfusion does occur [50]. Since CC emboli to the retina are so common, it may be beneficial to study their effects on retinal vasculature and compare retinal CC emboli to similar emboli in the brain and kidney. Clinical trials have provided the available data on management options for ocular crystal emboli.

RAOs do not have clear treatment guidelines [42]. Given the variety of etiologies for RAO, numerous treatment strategies have been established to treat RAOs including intraocular pressure drugs, anterior chamber paracentesis, carbogen, ocular massage, intravenous thrombolytics, intraarterial thrombolytics, intravenous methylprednisolone, neodymium: yttrium-aluminum-garnet laser embolectomy (nd:YAG), surgical embolectomy and others [42,46]. Most therapies have not demonstrated improvements of visual acuity [46]. Recent studies have examined nd:YAG embolectomy as a potential therapeutic option. In a recent RCT, nd:YAG improved visual acuity (VA) by Snellen chart in 5 of 7 patients compared to medical management that showed 2 of 7 patients with improvement [51]. Furthermore, a meta-analysis of case reports, case studies, and retrospective reviews demonstrated that improvement of VA for patients with starting VA of worse than 20/200 was on average 12 lines ($p < 0.001$) [52]. However, in patients with a VA of better than 20/200 the average improvement was 2.5 lines ($p < 0.001$) [52]. This study demonstrated a vitreous hemorrhage rate of 57 % with 15 % of all cases requiring additional vitrectomy [52]. In the nd:YAG arm of one study, 5 of 7 patients' clinical course was complicated by vitreous hemorrhage [51]. The rate of vitreous hemorrhage requiring vitrectomy may be related to power of the laser being above 2.4 mJ [52]. However, the rate of vitreous hemorrhage not requiring surgery remained the same regardless of the laser's power [52]. Overall, it has been suggested that nd:YAG may be a treatment option for RAO in patients that have significantly poor vision with VA over 20/200 [52].

Although nd:YAG embolectomy has demonstrated improvement in VA in patients, it is not standard of care for RAO [46]. This is due to the lack of RCTs available with large study sizes that assess rates of complication with vitreous hemorrhage and subsequent need for vitrectomy [46]. However, it may offer a potential treatment option in the right candidate with significant VA loss exceeding 20/200 [52]. A recent

meta-analysis examining the use of intravenous and intraarterial thrombolysis given within 4.5 h of onset of symptoms found that 80.9 % of patients improved by one Snellen chart line, 74.3 % improved 3 Snellen chart lines, and 39.0 % had VA better than 20/100 [53]. In their analysis, 0.5 % of patients had intra-ocular hemorrhage, 0.6 % had ischemic stroke, 1.0 % had cerebral hemorrhage, and 0.1 % mortality rate from cerebral hemorrhage [53]. Therefore, due to the improvement in vision and serious but rare complications, thrombolysis demonstrates promise as a treatment for nonarteritic RAO. More studies are needed to further delineate clear guidelines for treatment in nonarteritic RAO.

6. Age-related macular degeneration and cholesterol pathology

Age-related macular degeneration (AMD) has also been associated with dysfunction of cholesterol homeostasis in the retina. Mutations in genes associated with cholesterol and lipid homeostasis are associated with AMD [54]. Systemically, AMD has been associated with elevated high-density lipoprotein (HDL) and increased oxysterols, but it has had varied associations with lipid lowering therapy, like statins, leaving the impact of systemic cholesterol on AMD unclear [54–56]. In the retina, the pathogenesis of AMD is complex but has been associated with cholesterol homeostasis abnormalities such as reduced functioning of ATP binding cassette protein A1 (ABCA1) which is normally used for reverse cholesterol transport [55,57]. It is classically associated with lipid deposition in the form of drusen and subretinal drusenoid deposits in the macula [58]. However, distinct from the drusen deposits, OCT has imaged HCDs, referred to as the "onion sign," that are theorized to be CCs in neovascular AMD. This was further supported by ex vivo histologic findings of donor eyes corresponding to HCD on OCT imaging [59]. The onion sign is only found by OCT in 5 to 7 % of the population of AMD patients [59]. Furthermore, in avascular fibrosis, CC have also been correlated to reflective lines in OCT imaging at the basal lamina of the RPE [60]. These two studies suggest CCs form in the retina as AMD progresses from soft drusen and lipids to hemorrhage and fibrosis [59,60]. Although CCs are present in stages of AMD, their role in the pathogenesis and inflammation in AMD has not been directly examined. AMD has been described to have NLRP3 inflammasome activation and other pro-inflammatory cytokines that CCs have been previously associated with, but this has been attributed to drusen deposition, RPE stress and mitochondrial damage, and other inflammatory conditions [12,13,61–63]. However, there is no study in AMD examining how CCs contribute to inflammation, endothelial cell tight junction degradation, BRB breakdown, and apoptosis as there is in diabetic retinopathy [2]. Therefore, more studies examining the exact role of CCs in AMD could provide further insight into the pathogenic mechanisms of AMD progression.

7. Discussion

Clinical studies provide limited and sometimes conflicting evidence as to the relationships between circulating lipid levels and the development and progression of diabetic retinopathy [5]. Cardiovascular-system-focused clinical trials also evaluate some retinal outcomes and demonstrate the potential protective power of lipid-lowering therapies in diabetic retinopathy, but typically ocular endpoints were not the primary outcomes measured [5,19,20,64]. Systemic LDL-cholesterol lowering with statins did not afford protection against diabetic retinopathy in most clinical trials, but none of the trials focused on retinopathy as the main outcome [5,18,64,65]. Dysregulation of retinal-specific cholesterol metabolism leads to retinal cholesterol accumulation and formation of CCs [2,7]. However, systemic lipid levels do contribute to the development of atherosclerotic plaque that can break off into the circulation and result in retinal emboli that are typically asymptomatic but associated with risk of stroke and mortality [66]. Early in these studies, a significant risk of stroke and mortality was appreciated [24]. Newer studies suggest that this risk may be decreasing [24]. This improvement in the rate of stroke is attributed to improvement in medical management of risk factors associated with atherosclerosis [24]. Individuals with asymptomatic HPs often receive Carotid duplex ultrasound to evaluate the degree of stenosis and CEA or carotid artery stenting (CAS) candidacy [34]. In a comparable manner, the rates of major stroke and mortality were not significantly different in asymptomatic CAS whether treated with BMT alone or BMT and CEA or CAS [22]. New studies need to continue to explore the rate of strokes and mortality in patients with asymptomatic HPs with modern BMT.

Treatment with α-cyclodextrin (aCD) or 2-Hydroxypropyl-β-cyclodextrin (HPCD) has been recently proposed as treatment in disorders of retinal cholesterol homeostasis and atherosclerosis in systemic circulation [2,6,67]. Previously, cyclodextrins have been studied as a treatment option for hyperlipidemia and atherosclerotic plaques, and they have been shown to reduce the size of atherosclerotic plaques in rabbit models and circulating cholesterol levels in humans [67]. However, less studies exist examining the use of cyclodextrins in the diabetic retina. Hammer et al. demonstrated that introduction of aCD to diabetic mice models reduced inflammation, cell death, maintained retinal cell membrane integrity, and restored vascular barrier function [2]. It was also found that aCD could reduce the CCs in the retina layers, inhibit microglia activation, and decrease the values of unesterified and esterified cholesterol [2]. Similarly, HPCD reproduces many similar effects when given to CYP46a1$^{-/-}$ mice (a mice model for dysfunctional cholesterol homeostasis) including reductions in CC formation, total cholesterol content, and microglia activation [6]. These studies provide encouraging results, but also emphasize the need for additional research on the efficacy of cyclodextrins on CCs in diabetic retinopathy.

In conclusion, CCs are found in various retinal pathologies including asymptomatic plaques, RAO, diabetic retinopathy, and AMD. CC's contribution to the pathogenic mechanism of diabetic retinopathy help explain the intraretinal effects that fenofibrate and cyclodextrins exert on preventing progression of diabetic retinopathy [2]. Future studies need to examine cyclodextrins as a treatment option for diabetic retinopathy. When found on routine fundus exam, CCs have been classically associated with increased risk of stroke and mortality, but newer studies suggest that the risk is decreasing due to improvement in medical therapies and more aggressive pharmacological management [24]. New RCTs are examining the effects of BMT alone and CEA/CAS plus BMT and these studies may provide new clinical recommendations [32,33]. CCs have also been found in various stages of AMD, but their exact contribution to the pathophysiology of AMD remains unknown [59,60].

CRediT authorship contribution statement

Nicholas G. Medawar: Data curation, Writing – original draft. **Tim F. Dorweiler:** Data curation, Writing – original draft. **George S. Abela:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Julia V. Busik:** Conceptualization. **Maria B. Grant:** Conceptualization, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for [*Journal name*] and was not involved in the editorial review or the decision to publish this article.

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