



Lipids, hyperreflective crystalline deposits and diabetic retinopathy: potential systemic and retinal-specific effect of lipid-lowering therapies

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Abstract

The metabolically active retina obtains essential lipids by endogenous biosynthesis and from the systemic circulation. Clinical studies provide limited and sometimes conflicting evidence as to the relationships between circulating lipid levels and the development and progression of diabetic retinopathy in people with diabetes. Cardiovascular-system-focused clinical trials that also evaluated some retinal outcomes demonstrate the potential protective power of lipid-lowering therapies in diabetic retinopathy and some trials with ocular primary endpoints are in progress. Although triacylglycerol-lowering therapies with fibrates afforded some protection against diabetic retinopathy, the effect was independent of changes in traditional blood lipid classes. While systemic LDL-cholesterol lowering with statins did not afford protection against diabetic retinopathy in most clinical trials, and none of the trials focused on retinopathy as the main outcome, data from very large database studies suggest the possible effectiveness of statins. Potential challenges in these studies are discussed, including lipid-independent effects of fibrates and statins, modified lipoproteins and retinal-specific effects of lipid-lowering drugs. Dysregulation of retinal-specific cholesterol metabolism leading to retinal cholesterol accumulation and potential formation of cholesterol crystals are also addressed.

Keywords Cholesterol · Cholesterol crystals · Diabetic retinopathy · Dyslipidaemia · Fibrate · Hyperreflective crystalline deposits · Lipids · Review · Statin

Abbreviations

ABCA1	ATP-binding cassette subfamily A member 1
ABCG1	ATP-binding cassette subfamily G member 1
ACCORD	Action to Control Cardiovascular Risk in Diabetes
BRB	Blood–retina barrier
CC	Cholesterol crystal
C5a	Complement factor 5a
C5aR	Complement factor 5a receptor
CR3	Complement receptor 3
DMHCA	<i>N,N</i> -dimethyl-3- β -hydroxy-cholenamide

DMO	Diabetic macular oedema
EPA	Eicosapentaenoic acid
ETDRS	Early Treatment Diabetic Retinopathy Study
FIELD	Fenofibrate Intervention and Event Lowering in Diabetes
GM-CSF	Granulocyte-macrophage colony stimulating factor
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HSC	Haematopoietic stem cell
IRF3	Interferon regulatory factor 3
LDLR	LDL receptor
LENS	Lowering Events in Non-Proliferative Retinopathy in Scotland
LXR	Liver X receptor
NETosis	Neutrophil extracellular trap formation
NLRP3	NOD-like receptor family pyrin domain containing 3
OCT	Ocular coherence tomography
PDR	Proliferative diabetic retinopathy

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PPAR α	Peroxisome proliferator-activated receptor alpha
REC	Retinal endothelial cell
RPE	Retinal pigment endothelium
SR-B	Scavenger receptor class b
STDR	Sight-threatening diabetic retinopathy
TLR	Toll-like receptors
TG	Triacylglycerol
VEGF	Vascular endothelial growth factor

Introduction

Hyperglycaemia is a major risk factor for diabetic retinopathy and improving blood glucose control substantially protects against diabetic retinopathy onset and progression in people with type 1 or type 2 diabetes [1, 2]. Furthermore, metabolic memory exists for blood glucose levels and diabetic retinopathy [3–5]. While not as strong or consistent as the links between glucose and diabetic retinopathy, and between lipids and CVD, evidence from clinical studies suggests associations between traditional lipid profiles and diabetic retinopathy [6]. There is also evidence of metabolic memory of some vascular beds for lipids and lipid-lowering drugs [7], likely modulated by epigenetics [8].

In this review we discuss associations between traditional blood lipid levels and diabetic retinopathy, challenges in this research area, and the effects of lipid-lowering drugs on diabetic retinopathy, including a meta-analysis of relevant trials. We then discuss the potential retinal-specific mechanisms of lipid-lowering and of immune cells in diabetic retinopathy, and the role of the bone marrow in vascular repair, before providing a summary and suggesting future directions.

Associations between circulating lipid levels and diabetic retinopathy

Since recognition of the association between circulating lipid levels and retinal hard exudates in the 1950s [9, 10], cross-sectional and longitudinal studies have evaluated associations between traditional lipid classes, total cholesterol, triacylglycerols (TGs), HDL-cholesterol and (usually calculated) LDL-cholesterol, and the novel prothrombotic proatherogenic lipoprotein(a). Some illustrative studies are summarised in Table 1 [11–28]. Associations are not always consistent, possibly relating to differences in study size, diabetes type and duration, definitions of diabetic retinopathy, lipid assays and other factors (discussed next).

Challenges in evaluating the roles of lipids and lipid-lowering drugs in retinopathy

Many factors make it difficult to discern the relative importance of lipids and lipid-lowering drugs in diabetic retinopathy [6]. Likely confounding factors include interactions between lipids, blood glucose levels and other retinopathy risk factors such as obesity, renal dysfunction and smoking. Other challenges are the long duration over which retinopathy develops and the potential for different lipid effects on the different stages (e.g. initiation or progression) of diabetic retinopathy. Differences between studies in the type of diabetes, diabetes duration, retinopathy status at initial visit and duration of follow-up and in the definitions of diabetic retinopathy and methods used to assess it may also contribute. Furthermore, most clinical studies use relatively simple measures of circulating lipoproteins, such as total, LDL-cholesterol and HDL-cholesterol and TG levels for what are complex quantitative and qualitative changes in lipoproteins. Additionally, circulating lipid levels do not best reflect extravasated and modified lipids, such as in the retina, nor intra-retinal lipid metabolism, which may be more pathogenic than unmodified lipoproteins in plasma. Often only a single measure of blood lipid levels is evaluated in relationship to diabetic retinopathy status (or only a few measures) and there may be little consideration of confounding effects such as blood glucose levels, metabolic memory for glucose, lipids and some drugs [7, 8], unmeasured genetics, epigenetic effects and other yet to be determined factors.

Of further importance to understanding the potential role of lipid-lowering drugs in ameliorating diabetic retinopathy is the direct and indirect effects of these drugs. Their pleiotropic effects are common to most lipid-lowering drug classes, in spite of different mechanisms of action and predominant lipid targets. A novel approach to help elucidate the role of lipids in diabetic retinopathy are Mendelian randomisation studies, in which the impact of genes involved in high or low lipid levels are related to the condition of interest [29], and a recent study is described herein.

Effects of lipid-lowering drugs

Lipid-lowering drugs are broadly classified by their predominant lipid target. Unlike CVD, there is a relative paucity of evidence and sometimes inconsistent evidence regarding the effect of lipid-lowering drugs on diabetic retinopathy. Basic science studies can be very helpful in preclinical drug testing and in elucidating mechanisms [30–39] but results do not always translate to the human setting. We now summarise key lipid-lowering drug trials that have evaluated diabetic retinopathy endpoints and highlight evidence gaps.

Table 1 Clinical studies demonstrating the diversity of associations between circulating traditional lipid profiles and diabetic retinopathy

Study	Diabetes type	Type of DR	<i>n</i>	Blood lipids evaluated	Results
Cross-sectional studies					
Brown et al [12]	T1D+T2D	Exudative DR	31	TC, TG	Increased serum TG in participants with vs without DR
Sacks et al [23]	T1D+T2D	DR (PDR, moderate or severe DMO) or ETDRS scale ≥ 20	2535	TC, TG, LDL-C, HDL-C	Higher TG and lower HDL with vs without DR, but not once adjusted for hypertension and HbA _{1c}
Raman et al [22] (SN-DREAMS)	T2D	CSMO, non-CSMO	1414	TC, TG, HDL-C, LDL-C	High serum LDL-C, non-HDL-C and HDL-C ratio related to non-CSMO High serum TC related to CSMO
Benarous et al [11]	T1D+T2D	NPDR (mild, moderate, severe), PDR, DMO (mild, moderate, CSMO)	500	TC, TG, LDL-C, HDL-C, non-HDL-C	Serum lipids independently associated with CSMO only No associations with DR, mild or moderate DMO, or macular thickness
Wong et al [26] (MESA)	T1D+T2D	DR, DMO, CSMO, STDR	778	TC, TG, LDL-C, HDL-C	No associations with any DR, DMO or CSMO
Cetin et al [13]	Not specified	NPDR, PDR, DME	199	TC, HDL-C, LDL-C, VLDL-C, TG	Serum lipid levels not associated with severity of DR or DMO
Tan et al [25] (SEED study)	T2D	DR, severe non-PDR, STDR, DMO, CSMO,	2877	TC, LDL-C	Higher TC and LDL-C associated with lower risk of any type of DR
Guerci et al [16]	T1D	DR, NPDR, PDR	341	Lp(a)	Higher Lp(a) levels were associated with more severe DR, and Lp(a) >300 mg/l (30 mg/dl) was associated with higher PDR
Longitudinal studies					
Dodson and Gibson [15] (7 years)	T2D Hypertension	DR with exudative maculopathy	52	TC, HDL-C, HDL ₂ , LDL-C, VLDL-C, TG	Higher HDL ₂ subfraction with exudative maculopathy
Klein et al [18] (WESDR; 30 years)	T1D	PDR, DMO	903	TC, HDL-C	Serum lipids not associated with incidence of PDR or macular oedema, nor was statin use
Chew et al [14] (ETDRS; 5 years)	T2D+T1D	Hard exudates	2709	TC, TG, HDL-C, LDL-C, VLDL-C	High TC, TG and LDL-C associated with higher risk of hard exudates
Miljanovic et al [20] (DCCT; 6.5 years)	T1D	CSMO, hard exudate, DR progression, PDR	1441	TC, LDL-C, HDL-C, TG, TC / HDL	Higher serum lipids associated with higher risk of CSMO and retinal hard exudates No lipids associated with DR progression or development of PDR after adjustment for HbA _{1c}
Klein et al [17] (WESDR substudy; 5 years)	T1D	DR severity, PDR, hard exudate incidence and progression, DMO	251	TC/HDL	Univariate analyses: TC/HDL-C was associated with all incident retinal lesions Multivariate analyses: no association was significant
Morton et al [21] (ADVANCE trial 5 years)	T2D	New or worsening DR	11,400	Baseline HDL-C	HDL-C levels not related to DR
Lloyd et al [19] (EDC; 2 years)	T1D	DR (Airlie House Classification) Progression, PDR	657	TC, TG, LDL-C, HDL-C	TG and LDL-C predictive DR progression and of development of PDR
Singh et al [24] (DiaGene; 6.97 years)	T2D	NPDR, PDR	1886	Plasma Lp(a) levels Two SNPs modulating Lp(a) levels	No association between Lp(a) levels or SNPs with incident or prevalent DR

Table 1 (continued)

Study	Diabetes type	Type of DR	<i>n</i>	Blood lipids evaluated	Results
Meta-analyses					
Yau et al [27] (META-EYE)	T1D+T2D	DR, PDR, STDR, DMO, CSMO	22,896	TC	Higher TC associated with higher prevalence of DMO
Zhou et al [28]	T1D+T2D	DR	4366	TC, TG, LDL-C, HDL-C	TC, TG, HDL-C: no difference between DR vs no DR LDL-C: higher in DR vs no DR

CSMO, clinically significant macular oedema; DR, diabetic retinopathy; EDC (Pittsburgh) Epidemiology of Diabetes Complications; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; Lp(a), lipoprotein(a); MESA, Multi-ethnic Study of Atherosclerosis; META-EYE, Meta-Analysis for Eye Disease; NPDR, non-proliferative diabetic retinopathy; SEED, Singapore Epidemiology of Eye Diseases; SN-DREAMS, Sankara Nethralaya Diabetic Retinopathy Epidemiology and Molecular Genetic Study; T1D, type 1 diabetes; T2D, type 2 diabetes; TC, total cholesterol; WESDR, Wisconsin Epidemiologic Study of Diabetic Retinopathy

TG-lowering and LDL-lowering drugs

As yet there are no large placebo-controlled diabetic retinopathy primary endpoint trial results related to the (TG-lowering) fibrates, fish oils or eicosapentaenoic acid (EPA). Apart from some early small intervention studies (1–50 participants) with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) [40–43], there are no large, randomised controlled trials for (LDL-lowering) drugs with a diabetic retinopathy primary endpoint. The LDL-lowering drug classes of interest are as follows: statins; ezetimibe; resins; proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors; and bempedoic acid (the first in the LDL-lowering drug class of ATP citrate lysase inhibitors, which acts upstream of HMG-CoA reductase) [44].

Fibrates In an early (1968) trial, a 3 year study of clofibrate in 23 individuals with exudative diabetic retinopathy and 25 control individuals, clofibrate significantly reduced exudate severity but did not improve other retinal vascular lesions or visual acuity; there was no association between exudate severity or improvement and serum lipid levels [45]. Subsequently, there have been much larger RCTs of fenofibrate, though retinopathy was not the main focus of these trials. The peroxisome proliferator-activated receptor alpha (PPAR α)-agonist fenofibrate has positive diabetic retinopathy-related findings from two major placebo-controlled RCTs in type 2 diabetes, the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial [46] and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) Lipid Eye Trial [47].

The FIELD trial was a cardiovascular trial wherein microvascular complications were not the primary endpoint, thus not all participants had retinal imaging, ocular coherence tomography (OCT) was not used, and the then-standard therapy for sight-threatening diabetic retinopathy (STDR) was retinal laser photocoagulation. In the FIELD trial ($n = 9795$), co-micronised fenofibrate 200 mg once daily for a median of 5 years significantly reduced STDR incidence requiring retinal

laser therapy by 31%, with all STDR events being verified by adjudicating ophthalmologists. Similar benefits were seen for proliferative diabetic retinopathy (PDR) and diabetic macular oedema (DMO). In the FIELD ophthalmology substudy ($n = 1012$), which included serial retinal images, the composite exploratory endpoint (two-step progression of Early Treatment Diabetic Retinopathy Study (ETDRS) severity scale grade, DMO or laser photocoagulation) was significantly reduced by 34% by fenofibrate allocation, with results driven by the pre-existence of diabetic retinopathy [46].

In the ACCORD Eye Lipid trial ($n = 1593$), simvastatin therapy was combined with fenofibrate 160 mg daily or placebo for a mean of 4 years. Simvastatin with fenofibrate group had significantly reduced diabetic retinopathy progression (by ≥ 3 ETDRS severity scale steps based on grading of seven-field stereoscopic fundus photo of all participants or need for laser or vitrectomy therapy) by 40% compared with simvastatin with placebo group [47]. Participants were also examined by an ophthalmologist or optometrist at baseline and at 4 years.

In both the FIELD and the ACCORD Eye Lipid trials, results were consistent regarding fenofibrate showing similar magnitude of benefit in diabetic retinopathy which was independent of both baseline lipids and changes in traditional lipid classes and without benefit to visual acuity [46, 47].

Cultured retinal cell and diabetic animal models support potentially protective mechanisms of fibrates being due to a combination of anti-inflammatory effects, suppression of vascular endothelial growth factor (VEGF) via modulation of the PPAR α -Wnt- β -catenin signalling pathway, outer blood-retina barrier (BRB) breakdown, neuroprotection and anti-apoptotic effects on retinal microvascular cells [30, 31, 33, 35–39, 48]. Fenofibrate has been shown to reverse the adverse effects of hyperglycaemia-induced metabolic memory in endothelial cells [7, 8] through a sirtuin (silent mating type information regulation 2 homolog 1 (SIRT1)-dependent mechanism [49]. Benefits of the intraocular administration of fibrate have been demonstrated [31, 50] but as yet there are no human studies using this route. Ocular delivery may increase

retinal drug levels, reduce toxic systemic drug levels and side effects and enable treatment in those for whom the systemic drug is contraindicated (e.g. individuals with end stage renal disease). Based on non-primary endpoint retinal outcome data, fenofibrate is approved in 19 (mainly low and low-middle income) countries in which intraocular anti-VEGF agents are less-widely available for use. However, it has not been approved by major regulators (European Medicines Agency [EMA], Food and Drug Administration [FDA]) for type 2 diabetes with diabetic retinopathy.

As yet, there are no completed fibrate trials specifically addressing diabetic retinopathy in type 1 or type 2 diabetes, although several trials with primary endpoints related to diabetic retinopathy are in progress: the Fenofibrate and Microvascular End-points (FAME-1) Eye trial in adults with type 1 diabetes and existent diabetic retinopathy (ETDRS severity scale 35–53) (results anticipated in 3 years) [51]; the Lowering Events in Non-Proliferative Retinopathy in Scotland (LENS) trial in people with type 1 and type 2 diabetes [52, 53]; and the Fenofibrate for Prevention of Diabetic Retinopathy Worsening trial in the USA [54]. As well as fenofibrate studies, retinopathy-related data related to other more recently developed fibrates, such as fenofibric acid and pemafibrate, are merited.

Omega-3 fatty acids Omega-3 fatty acids, found in fish, nuts, fish oils or purified EPA supplements, also lower TGs. There are very limited data relating the effects of fish oils or EPA supplements to diabetic retinopathy. Most studies have focused on CVD, as did the *Prevención con Dieta Mediterránea* (PREDIMED) study testing Mediterranean diets supplemented with extra virgin olive oil or nuts vs a control diet [55]. In this type 2 diabetes trial ($n = 3482$), 75% of the participants met the dietary long-chain ω -3 polyunsaturated fatty acid (LC ω 3PUFA) recommendation (≥ 500 mg/day) and over 6 years of follow-up there were 69 new-onset STDR cases, with (on adjusted analyses) a 48% reduced risk of incident STDR in those meeting vs. not meeting the dietary target. However, these data are observational and robust clinical trials using omega-3 fatty acid and EPA supplements with detailed primary diabetic retinopathy endpoints are merited.

Statins HMG-CoA reductase inhibitors (statins) have major primary and secondary cardioprotective effects in diabetes [56] but a meta-analysis of 13 trials ($n = 91,140$; mean follow-up of 4 years) revealed that statins can significantly increase the risk of new-onset type 2 diabetes (OR 1.09; 95% CI 1.02, 1.17). People with statin-induced new-onset diabetes should be screened for diabetic retinopathy, and the overall cardiovascular and death risk/benefit ratio for most people offered statin therapy remains favourable [57].

Early relatively small statin studies (1–50 participants with diabetes) suggested statin benefit for retinopathy, including

protection against late-stage diabetic retinopathy complications and visual acuity loss [23, 41, 58]. In early statin CVD trials, such as the primary CVD prevention Collaborative Atorvastatin Diabetes Study (CARDS) trial in type 2 diabetes [59], no diabetic retinopathy benefit was evident [59]. These relatively small trials of short duration designed for cardiovascular primary endpoints are not conclusive for a condition such as diabetic retinopathy that develops very slowly. Large observational studies, usually with more potent statins, do support some statin benefit for diabetic retinopathy but no consistent improvement in visual acuity [60–65]. Indeed, several studies support different aspects of statin benefit in the development or progression of diabetic retinopathy: the Longitudinal Health Insurance Database in Taiwan adults with type 2 diabetes ($n = 37,894$) [64]; a Taiwanese population-based cohort ($n = 219,359$) with type 2 diabetes and dyslipidaemia [65]; a large USA health insurance claims database study of adults with type 2 diabetes ($n = 269,782$) [61]; and a Japanese prospective clinical practice study of 40- to 75-year-old individuals with type 1 and type 2 diabetes ($n = 363$ and $n = 5489$, respectively) [63]. However, not all observational studies evaluating statins and risk of diabetic retinopathy result in positive findings [60].

Potential mechanisms by which statins may reduce retinopathy risk, other than their lipid benefits, include anti-inflammatory, antioxidant, anti-platelet, anti-clotting, vasodilatory, immunomodulatory, cell-signalling, epigenetic and stem-cell effects [66]. Other lipid-lowering drugs, such as the fibrates, have similar pleiotropic effects [37].

Meta-analysis of statin and/or fibrate trials for diabetic retinopathy endpoints

A recent systematic review and meta-analysis evaluated statin and/or fibrate RCTs for prevention and progression of diabetic retinopathy using Cochrane guidelines, the PRISMA Statement and GRADE approach regarding evidence certainty [67]. Included were four fibrate trials, three statin trials and one fibrate plus statin trial, with eight for diabetic retinopathy therapy and four for diabetic retinopathy prevention. Without increased adverse events in 1309 participants, fibrates were associated with a 45% risk reduction of DMO incidence. There were no other positive outcomes. Trial quality was not ranked highly in this meta-analysis [68], nor were 13 trials in a separate similar meta-analysis [67].

Potential retinal-specific mechanisms of lipid lowering in diabetic retinopathy

As described above, lipid-modifying therapies (in particular fenofibrate) may have some protective effects against diabetic retinopathy but existent trials are limited by retinopathy not

being the main endpoint. Moreover, the connection between the benefits of lipid-lowering drugs in the retina and the effect of these drugs on traditional blood lipid classes is not established, suggesting that non-traditional lipoprotein-related measures, such as lipoprotein subclasses, modified lipoproteins and/or retinal-specific effects could be at play.

Cholesterol input into retina

The retina is a highly specialised organ in which lipid levels are tightly regulated independently of their systemic levels. Under normal conditions, retinal cholesterol levels are controlled by a balance of input and output mechanisms [69]. Cholesterol input in the retina includes local biosynthesis [69–75] and uptake from the circulation (Fig. 1). Local cholesterol biosynthesis is the primary source of cholesterol in the retina. Most retinal cells express the enzymes involved in cholesterol biosynthesis, with the highest levels found in Muller cells and photoreceptor inner segments (Fig. 1). Unique to the retina are the BRBs separating the neuroretina from the systemic circulation, requiring specialised cholesterol transport mechanisms [70, 71]. The inner BRB is comprised of retinal endothelial cells (RECs) connected by tight junctions and is virtually impermeable to cholesterol under normal conditions (Fig. 1). Retinal pigment epithelium (RPE) cells, also connected by tight junctions, make up the outer BRB (Fig. 1). LDL receptors (LDLRs) and scavenger receptors class B (SR-B) type 1 and type 3 (CD36) [70, 72, 76–81] expressed on the basal membrane of the RPE provide the mechanism for controlled cholesterol-rich lipoprotein particle uptake from the circulation [74, 82] through the outer BRB (Fig. 1). Once in the RPE, cholesterol is transported through the apical membrane by ATP-binding cassette subfamily A member 1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1) transporters and delivered by lipoproteins to the neural retina [75, 76, 83–86].

Cholesterol output from retina

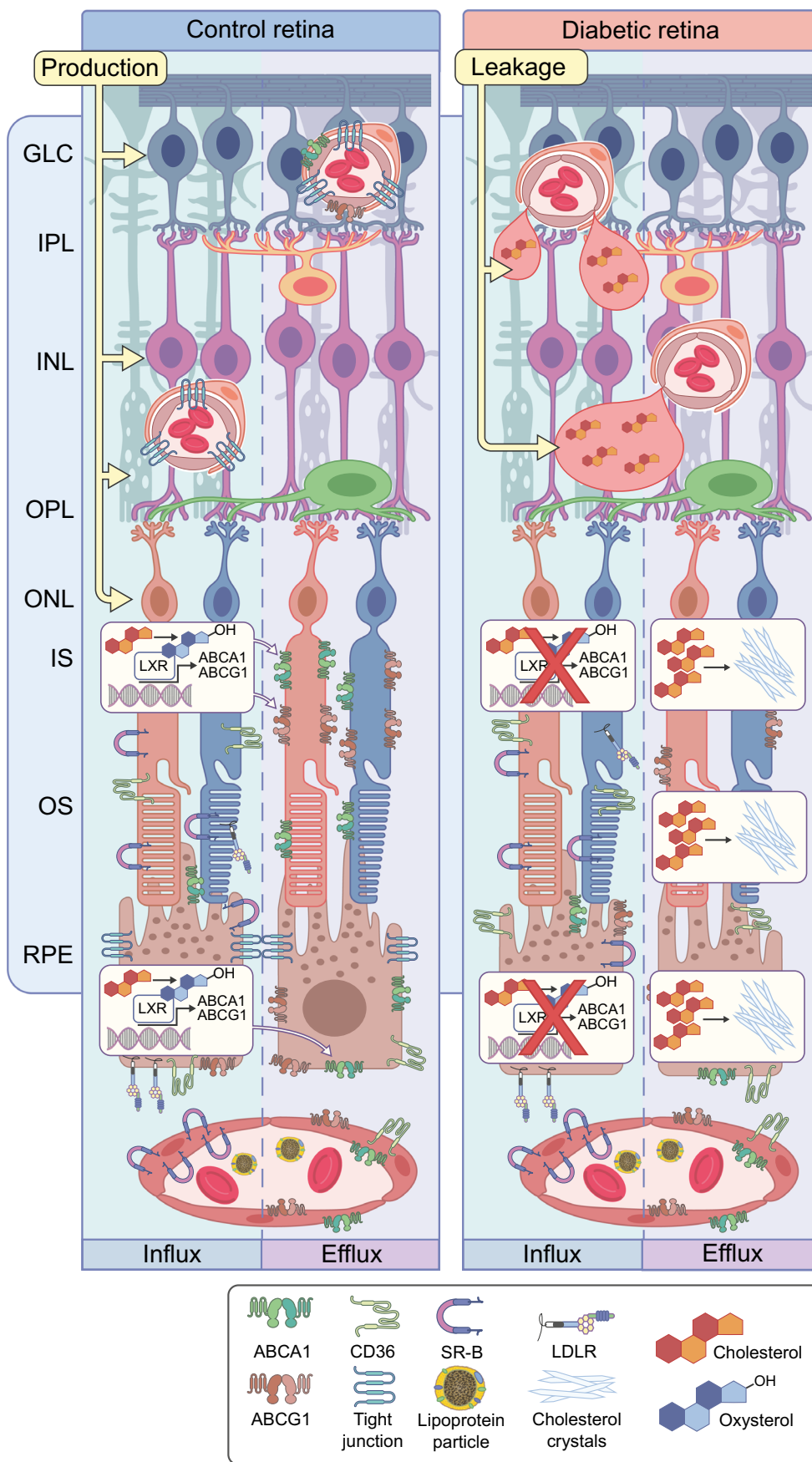
In a healthy retina, cholesterol input is balanced by cholesterol removal. Cholesterol efflux is aided by the ABCA1 and ABCG1 transporters on the RPE cells and endothelial cells, which deliver cholesterol to lipoprotein particles in the choroidal or retinal circulation through a process called reverse cholesterol transport [75, 76, 83–86]. In addition to reverse cholesterol transport, cholesterol is removed from the retina through metabolism to more soluble oxysterols by cytochrome P450s, 27A1 and 46A1 [75, 76, 83–87]. Oxysterols are rapidly diffused into the systemic circulation and are delivered to the liver for conversion to bile acids. Oxysterols are the activating ligands for liver X receptor (LXR).

Dysregulation of retinal cholesterol in diabetes

LXR downregulation In diabetes, LXR is downregulated leading to dysregulation of retinal cholesterol metabolism and increased proinflammatory signalling. Moreover, there is reduced oxysterol production due to a decrease in cytochrome P450 27A1 and cytochrome P450 46A1 in the diabetic retina, further reducing LXR activity. Diabetes-induced disruption of the LXR signalling and decrease of oxysterol production, result in diminished cholesterol removal and increased cholesterol accumulation in the retina [88–90] (Fig. 1). In addition to cholesterol dyshomeostasis in the diabetic retina, the breakdown of the outer and inner BRBs (Fig. 1) in diabetes further promotes cholesterol accumulation in the retina. Activation of LXR in animal models of diabetes restores cholesterol transport, leads to normalisation of cholesterol homeostasis and repression of inflammatory genes, such as those coding for inducible nitric oxide synthase (iNOS), IL-1 β , intercellular adhesion molecule-1 (ICAM-1), and C-C motif chemokine ligand 2 (CCL2) in the retina [89], and prevents the formation of diabetes-induced acellular capillaries.

Cholesterol crystal formation Disruption in cholesterol homeostasis and metabolism leads to a high cholesterol concentration in the diabetic retina, comparable with the levels that have been previously shown to lead to spontaneous formation of cholesterol crystals (CCs) in atherosclerotic plaques [91–93]. Accumulation of cholesterol in the diabetic retina thus creates a favourable environment for the formation of CCs [94–101] (Fig. 1). Indeed, the crystalline structures consistent with CCs (hyperreflective crystalline deposits) were recently identified by spectral-domain OCT in the retina and choroid of individuals with diabetes [102], age-related macular degeneration [103–105] and Coats disease [106]

Fig. 1 Dysregulation of cholesterol metabolism leading to cholesterol accumulation and formation of hyperreflective crystalline deposits (CCs) in diabetic retina. Cholesterol plays an important role in normal retinal function, and the balance of retinal cholesterol production, uptake by RPE cells and elimination is tightly controlled. At the outer BRB, cholesterol uptake from circulation by RPE cells through CD36, SR-B and LDLR is normally balanced by oxidation to oxysterols, LXR activation and production of ABCA1 and ABCG1, which control cholesterol efflux through RPE cells. Reduced production of oxysterols, decreased LXR expression and activity and thus decreased expression of ABC cassette cholesterol efflux transporters, collectively lead to cholesterol accumulation in the neuroretina. At high concentrations, cholesterol has the propensity to crystallise, forming proinflammatory and pro-atherogenic CCs. The endothelial cells of the inner BRB are not permeable to cholesterol uptake; however, endothelial cells express ABC cassette cholesterol efflux transporters. Inner BRB breakdown in diabetes leads to leaky blood vessels. Coupled with decreased expression of ABC transporters, this can lead to accumulation of cholesterol in the inner retina. GLC, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, photoreceptor outer segment. This figure is available as part of a [downloadable slideset](#)



and are associated with diabetic retinopathy progression. The presence of hyperreflective deposits has been proposed as a novel prognostic biomarker in diabetic retinopathy [102, 106–111]. Although cholesterol is likely to be the main component of crystalline structures in hyperreflective deposits, a positive identification of these deposits cannot be made based on OCT alone. Moreover, CCs are often overlooked in traditional scanning electron microscopy and immunohistochemistry as the ethanol used as a dehydrating agent in standard tissue preparations can dissolve the CCs, masking their presence and their potential involvement in pathogenic mechanisms.

Since CCs are very stable physiologically and are not easily amenable to dissolving *in vivo*, they become a source of chronic inflammation. CCs are recognised by the innate immune system as foreign bodies because of their shape, firmness and inability to be dissolved [97, 112–123]. CCs can activate all three complement pathways (classical [121], lectin [124] and alternative [115, 123]) in the extracellular space and can activate intracellular complement system [125–128]. Emerging experimental and clinical evidence supports the link between the complement system and diabetic vascular complications, including diabetic retinopathy and atherosclerosis [129–154].

Membrane attack complex is the final step in the activation of complement. Increased membrane attack complex deposition is found in eyes with diabetic retinopathy but not in those without [148] and is believed to be the result of reduced levels of complement regulatory proteins and sustained activation of the alternative complement pathway [147]. Circulating extracellular vesicles, such as exosomes, activate the complement system via the classical pathway and contribute to microvascular damage in diabetic retinopathy [155]. Moreover, genetic association studies have linked complement genes to diabetic retinopathy, as exemplified by the finding that intronic SNP rs2269067 in the *C5* gene is a risk factor for PDR in the Chinese Han population [139]. CCs were shown to activate the complement factor 5a (C5a) and C5a receptor (C5aR) pathways leading to the upregulation of complement receptor 3 (CR3) [121]. CR3 (CD11b/CD18), a β_2 integrin, is a highly versatile pattern-recognition receptor that activates leucocytes, mediates phagocytosis and promotes leucocyte transmigration [156]. CR3 and C5aR link CC to leucocyte activation [157]. CC-induced cytokine/chemokine production occurs through interaction of C5a with C5aR [157].

Importantly, CCs also induce inflammation via the NOD-like receptor family pyrin domain containing 3 (NLRP3)

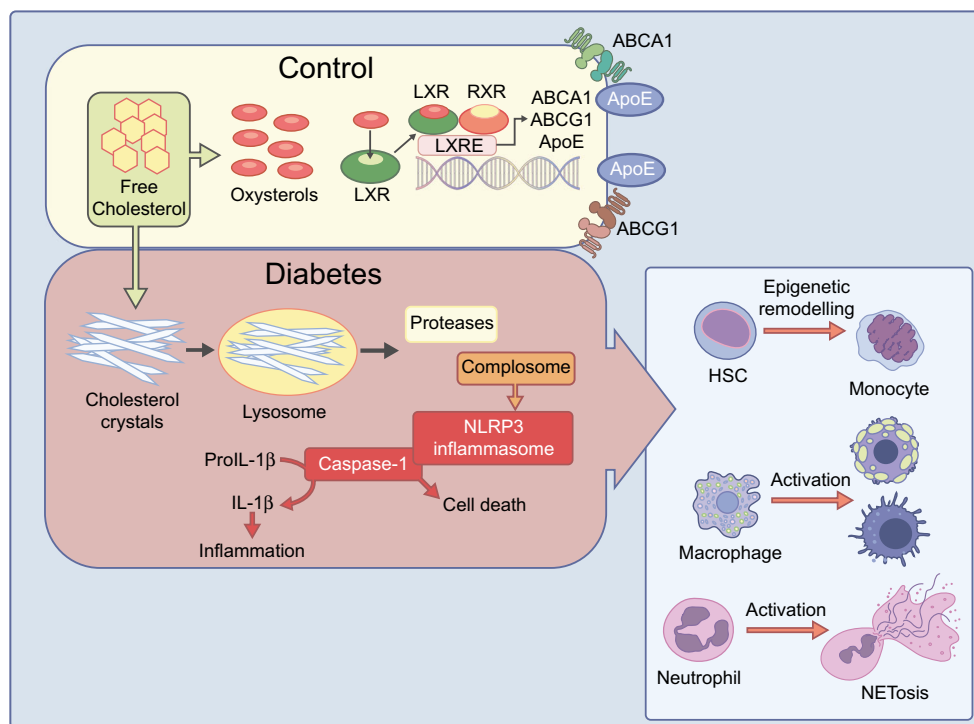


Fig. 2 Dysregulation of cholesterol metabolism in diabetes and immune system. In a healthy retina (control), oxidation of cholesterol to oxysterols, LXR activation and production of ABCA1 and ABCG1 maintains cholesterol homeostasis. However, in diabetes, the disruption of these processes can lead to hypercholesterolaemia and CC formation, which can activate the innate immune system and trigger the NLRP3 inflammasome. CCs, when interacting with innate immune receptors, can enhance

tissue damage and initiate inflammatory responses through secretion of IL-1 β and inflammation. This can lead to HSC epigenetic reprogramming resulting in the differentiation of HSC towards the monocyte lineage, neutrophil activation and NETosis, and macrophage activation and foam cell formation. ApoE, apolipoprotein E. This figure is available as part of a [downloadable slideset](#)

inflammasome, which mediates the formation of activated IL-1 β [97, 158], a key inflammatory mediator in diabetic retinopathy. HDL, the only known element in the body that can dissolve CCs [96] is often reduced and/or dysfunctional in diabetes and has poor accessibility to deep tissue sites such as the retina. Thus, diabetic individuals are particularly at risk for developing CCs.

Potential immune cell-specific mechanisms of lipid lowering in diabetic retinopathy

In addition to retinal-specific effects described above, lipid-lowering drugs can affect the retina through specific effects on immune cells. Lipid dysregulation can contribute to low-grade chronic inflammation [159, 160] and VEGF receptor 2 (VEGFR2) activation, resulting in increased retinal endothelial permeability and cell injury [161].

Support for the modulation of immune cell behaviour in the prevention of atheromatous disease exists; however, for diabetic retinopathy evidence is lacking. Statins have a major primary and secondary cardioprotective effect in diabetes [56], yet diabetic individuals exhibit impaired plaque regression [162], possibly reflecting differences in statin effects on CCs by diabetes status. Clinical studies provide support for the inflammatory hypothesis of atherosclerosis [163], including the recent Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) showing that controlling vascular inflammation independent of lipid lowering could lower the rates of recurrent cardiovascular events [164]. However, none of these trials had diabetic retinopathy progression as an endpoint and thus assessment of the impact of controlling inflammation on diabetic retinopathy is lacking.

Hypercholesterolaemia increases the levels of circulating CCs [99, 165, 166]. As described above, CCs can activate the innate immune system [167] and can trigger the NLRP3 inflammasome [168]. CCs, when interacting with innate immune receptors, can enhance tissue damage and initiate inflammatory responses through secretion of IL-1 and IL-18 [169–171]. Hypercholesterolaemia is associated with increased proliferation and mobilisation of haematopoietic stem cells (HSCs) [172] and can directly regulate the function of HSCs through epigenetic reprogramming. This can occur through the upregulation or downregulation of different epigenetic enzymes (such as histone demethylases and methylases) or the upregulation of the granulocyte-macrophage colony stimulating factor (GM-CSF) receptor. GM-CSF receptor upregulation results in the differentiation of HSCs towards the monocyte lineage [173, 174] (Fig. 2). Inflammasome activation promotes neutrophil recruitment and neutrophil extracellular trap formation (NETosis) (Fig. 2) in atherosclerotic plaques, leading to the development of advanced atherosclerotic lesions [175]. Neutrophil expansion, activation and

mobilisation from the bone marrow is the result of hypercholesterolaemia [176, 177]. Yet only in preclinical studies has the impact of changes in the bone marrow been examined regarding diabetic retinopathy [178–180].

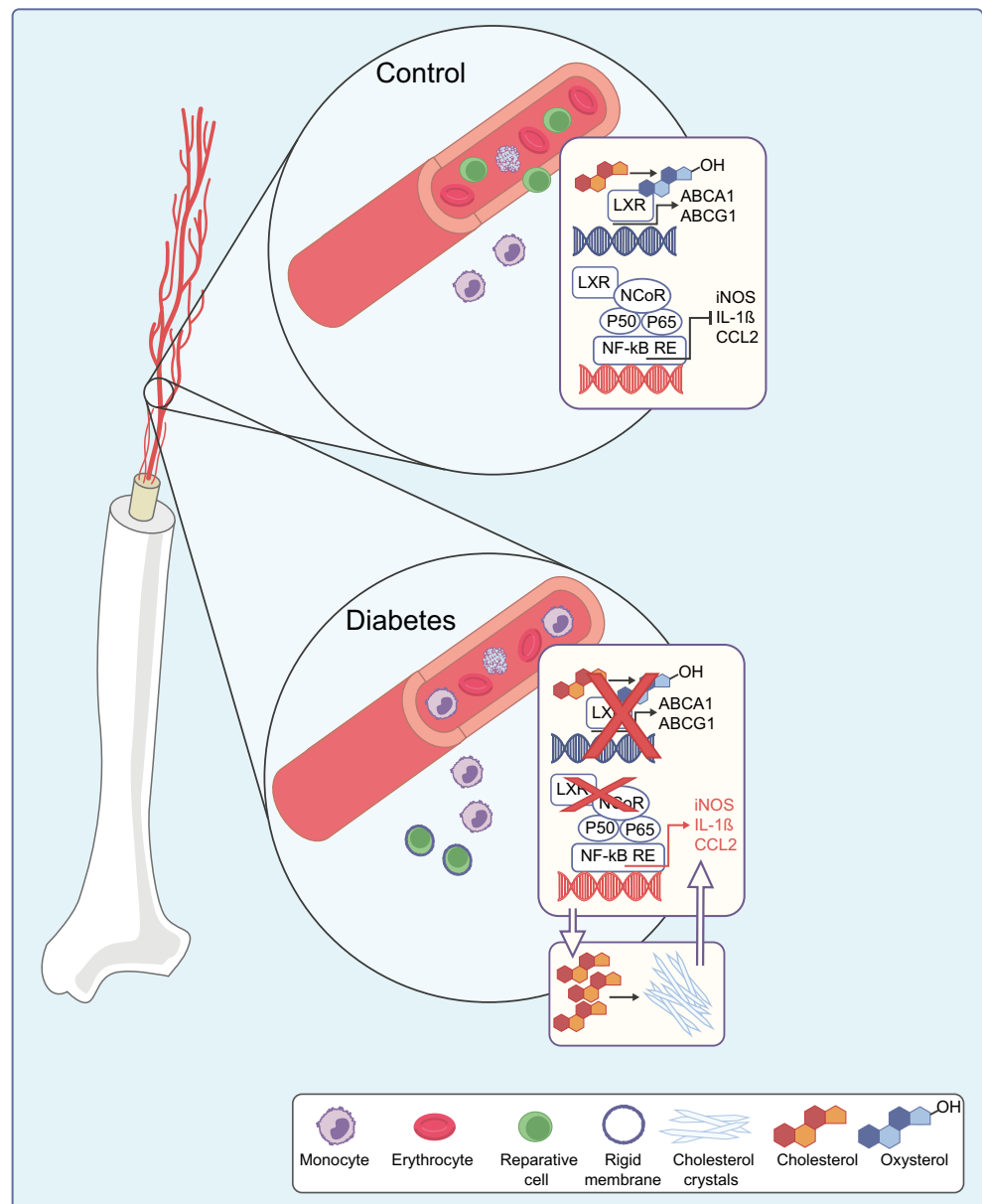
LXR is a critical modulator of cholesterol metabolism (Figs 2, 3). Its activation is a key cellular pathway regulating intracellular cholesterol and plays an important role in inflammation and disease pathogenesis [181]. LXR is a major regulator of ABCA1 and ABG1 transporter expression (Figs 2, 3). In addition to its role in cholesterol removal, LXR has pronounced anti-inflammatory properties through the inhibition of NF- κ B signalling (Fig. 3). Since diabetes is associated with chronic systemic inflammation, these clinical findings support the notion that activating Toll-like receptors (TLRs) can worsen cholesterol homeostasis. Thus, bacterial and viral particles that activate TLR3/4 profoundly inhibit both the expression of LXR target genes and the ability of macrophages to efflux excess cholesterol [182]. The activation of TLRs 3/4 suppresses the activity of LXR on corresponding genes in macrophages, thus reducing cholesterol efflux and contributing to hypercholesterolaemia-related inflammation [182]. The effect of TLRs occurs through the viral response transcription factor interferon regulatory factor 3 (IRF3). Expression and activation of IRF3 in macrophages is necessary and sufficient to block the transcriptional activity of LXR on the ABCA1 promoter and to inhibit cholesterol efflux from macrophages [182]. These findings provide an unexpected mechanism whereby microbial infection may contribute to cardiometabolic disease, such as type 2 diabetes, by interference with LXR-dependent cholesterol metabolism.

Macrophage LXR signalling pathway is an important determinant of vascular damage including in the retina [89, 90, 180, 183] as LXR interferes with the action of NF- κ B on the promoters of inflammatory genes. LXR thus serves to integrate lipid metabolic and innate immune functions in macrophages (Figs 2, 3).

The diabetic bone marrow and vascular reparative dysfunction

Low-level chronic inflammation as seen in type 2 diabetes can alter the homeostatic balance of most tissues, including the bone marrow microenvironment [184]. Myeloidosis, defined as an increase in the proportion of myeloid-derived leucocytes, is a common feature in diabetic bone marrow and promotes the systemic inflammatory phenotype [185]. Bone marrow supernatant fractions from diabetic mice displayed significant increases in the levels of secreted proinflammatory proteins, including TNF- α , IL-3 and CCL-2, promoting differentiation towards myeloid cells. Bone marrow is also the source of vascular reparative cells, which are called myeloid angiogenic cells. In disease states such as

Fig. 3 Dysregulation of cholesterol metabolism in diabetes in bone marrow-derived cells. In control cells, LXR activation leads to increased expression of cholesterol efflux transporters ABCA1 and ABCG1, and inhibition of expression of NF- κ B-controlled inflammatory factors. Inhibition of LXR in diabetes leads to cholesterol accumulation, CC formation and increased inflammation. Increased membrane rigidity of reparative cells due to cholesterol accumulation further exacerbates diabetes-induced damage. NCoR, nuclear receptor corepressor; RE, response element. This figure is available as part of a [downloadable slideset](#)



diabetes and CVD myeloid angiogenic cells are reduced in number and function, thus leading to compromised endothelial recovery [186–188].

The molecular defect in these cells results in their compromised function [180, 189–194]. Migration of myeloid angiogenic cells is impacted by cell rigidity, which is markedly increased in the cells of diabetic individuals (Fig. 3). In diabetic dyslipidaemia, cholesterol accumulates in the plasma membrane, decreasing fluidity and thereby suppressing the ability of cells to transduce ligand-activated signalling pathways. *N,N*-dimethyl-3 β -hydroxy-cholenamide (DMHCA), a selective LXR agonist, specifically activates the cholesterol efflux arm of the LXR pathway with minimal stimulation of TG synthesis by the liver. DMHCA rejuvenates membrane

fluidity in myeloid angiogenic cells and reduces the pro-inflammatory microenvironment of the diabetic bone marrow. Using single-cell RNA sequencing on HSCs from untreated and DMHCA-treated diabetic mice, it was found that DMHCA increased the expression of several LXR target genes, confirming that DMHCA directly modulates these primitive cells. An increase in immediate early gene expression in HSCs was also observed, as the DMHCA-enhanced formation of membrane microdomains amplifies the transduction of intercellular signalling. Together, these findings suggest that DMHCA treatment normalises the bone marrow microenvironment. Thus, through modulation of LXR and other key signalling pathways, cholesterol homeostasis plays a critical role in immune function and in vascular repair. Loss

of the function of bone marrow-derived myeloid angiogenic cells can lead to inadequate repair of injured retinal capillaries, leading to vasodegeneration and development of diabetic retinopathy [195–199]. One area of ongoing research involves pharmacological strategies that normalise cholesterol synthesis and membrane fluidity of myeloid angiogenic cells to improve diabetic retinopathy outcomes.

Summary and future directions

Lipids are implicated in the pathogenesis of diabetic vascular complications, including retinopathy; lipid-lowering drugs may be protective, though large robust clinical trials and observational studies with detailed eye-related primary endpoints and lipid and lipoprotein-related measures are still merited. Studies with more recently available lipid-modulating drugs and of ocular drug delivery are also of interest. Usually, two or more trials with positive results will be required to change clinical practice and gain regulatory approval and, importantly, such treatments must then be translated into clinical practice. Recognition of effects specific to bone marrow, immune cells and the retina could help design more specific treatments. Further understanding of the accumulation of CCs and hyperreflective crystalline deposits generation in the diabetic retina as well as circulating cells could result in a novel prognostic biomarker and a novel therapeutic target in diabetic retinopathy.

Supplementary Information The online version contains a slideset of the figures for download, which is available to authorised users, available at <https://doi.org/10.1007/s00125-022-05655-z>.

Authors' relationships and activities AJJ is an investigator on the FIELD trial (post completion of its intervention stage), FAME-1 Eye and DCCT/EDIC trials and collaborates with Prof Emily Chew of the NEI on an unrelated study. The FAME-1 Eye trial, of which she is Co-Study Chairperson has been funded by the NHMRC (Australia), JDRF Australia and Abbott Europe for the investigator-initiated FAME-1 Eye trial. FIELD related biomarkers studies are funded by NHMRC Australia. Unrelated to this work AJJ is an Honorary Board Member of the International Diabetes Federation Western Pacific Region Executive Council, of the NGO Insulin For Life and an Honorary Member of the Diabetes Australia Research Advisory Council. Unrelated to this work she is an Advisory Board member for Abbott Diabetes, Medtronic, Sanofi-Aventis and Amgen. Unrelated to this work she has received peer-reviewed funding from the NHRMC, Medical Research Future Fund, JDRF International, JDRF Australia, Diabetes Australia Research Programme and investigator-initiated funding from Medtronic and Sanofi-Aventis. JVB is an Associate Editor of Diabetologia and consultant of Ceramedix Inc.

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