

# Atherosclerosis is a major human killer and non-resolving inflammation is a prime suspect

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## Abstract

The resolution of inflammation (or inflammation-resolution) is an active and highly coordinated process. Inflammation-resolution is governed by several endogenous factors, and specialized pro-resolving mediators (SPMs) are one such class of molecules that have robust biological function. Non-resolving inflammation is associated with a variety of human diseases, including atherosclerosis. Moreover, non-resolving inflammation is a hallmark of ageing, an inevitable process associated with increased risk for cardiovascular disease. Uncovering mechanisms as to why inflammation-resolution is impaired in ageing and in disease and identifying useful biomarkers for non-resolving inflammation are unmet needs. Recent work has pointed to a critical role for balanced ratios of SPMs and pro-inflammatory lipids (i.e. leucotrienes and/or specific prostaglandins) as a key determinant of timely inflammation resolution. This review will focus on the accumulating findings that support the role of non-resolving inflammation and imbalanced pro-resolving and pro-inflammatory mediators in atherosclerosis. We aim to provide insight as to why these imbalances occur, the importance of ageing in disease progression, and how haematopoietic function impacts inflammation-resolution and atherosclerosis. We highlight open questions regarding therapeutic strategies and mechanisms of disease to provide a framework for future studies that aim to tackle this important human disease.

## Keywords

Atherosclerosis • Ageing • Inflammation • Immunology • hematopoiesis

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## 1. Introduction

Atherosclerosis is a major killer worldwide and is characterized by the accumulation of immune cells and lipids in medium and large arteries.<sup>1</sup> Life-threatening clinical manifestations of atherosclerosis include (but are not limited to) myocardial infarctions and ischaemic strokes and are collectively known as major adverse cardiovascular events (MACEs). These life-threatening events typically occur due to plaque rupture or erosion which results in acute atherothrombosis and vascular occlusion. The pathogenesis of atherosclerosis is linked to persistent inflammation and dyslipidaemia including high levels of low-density lipoprotein (LDL). Current therapeutic strategies to limit LDL such as statins and PCSK9 inhibitors are effective at reducing LDL levels in the blood; however, MACEs are only reduced by ~50%,<sup>2</sup> which is likely due to the pro-inflammatory nature of the disease. Indeed, chronic inflammation (or the residual inflammatory risk, RIR) is thought to be a culprit in the progressive and non-resolving nature of atherosclerotic cardiovascular disease.<sup>3</sup> Interestingly, significant LDL lowering with statins and PCSK9 inhibitors

as seen in the SPIRE trials, still demonstrated evidence of RIR in individuals, which suggests that dramatic cholesterol lowering may not be enough to fully terminate the progressive nature of this disease.<sup>4</sup> Moreover, this finding suggests that inflammation must be mitigated to potentially control the progression of this disease. CANTOS was the first demonstration in humans that blockade of interleukin-1 $\beta$  (IL-1 $\beta$ ) reduced MACEs.<sup>5</sup> COLCOT was another trial in which anti-inflammatory colchicine also reduced MACEs.<sup>6</sup> Collectively, results from these trials provide direct evidence that blockade of pro-inflammatory factors can reduce MACEs. Moreover, they offer much needed momentum towards understanding key mediators and mechanisms associated with failed non-resolving in the arterial wall.

While inflammation is a clear culprit in atherosclerosis, directly targeting/blocking inflammation poses some risks, including immune suppression and an increased likelihood for infection, which was noted in the CANTOS trial. Therefore, identifying factors and cellular programmes that can control inflammation without compromising host defense may be ideal treatments for atherosclerosis that can potentially be used with

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therapies that already exist. This review will focus on a particular family of ligands called 'specialized pro-resolving mediators' or SPMs that function to control inflammation and promote tissue repair in a manner that does not compromise host defense.<sup>7</sup> This review will also pinpoint some mechanisms as to why SPMs may not be readily made within atherosclerotic plaques and how restoration of the key defective mediators may help limit the progression of atherosclerosis.

## 2. SPMs activate inflammation-resolution programmes

While it was previously thought that passive disappearance of pro-inflammatory factors was sufficient for the cessation of inflammation, it is now known that the resolution of acute inflammation (or inflammation-resolution) is an active and highly coordinated process.<sup>7</sup> Inflammation-resolution is governed by a panoply of endogenous factors that include SPMs,<sup>7</sup> protein/peptide mediators such as annexin A1<sup>8</sup> and interleukin 10, gases such as carbon monoxide and hydrogen sulfide,<sup>9,10</sup> and nucleotides such as adenosine and inosine.<sup>11</sup> Here, we specifically highlight how SPMs impact inflammation-resolution in atherosclerosis and discuss key gaps in our understanding of non-resolving inflammation in this context.

SPMs are actively biosynthesized at the onset of acute inflammation to counterbalance numerous pro-inflammatory signals. This 'push-and-pull' of pro-inflammatory and pro-resolving ligands ultimately controls the magnitude and duration of inflammation.<sup>7</sup> Recent work has pointed to the balance between SPMs and pro-inflammatory ligands like leukotrienes (LTs) or some of the prostaglandins (PGs) as a key determinant to whether inflammation resolves in a timely manner or persists.<sup>12,13</sup> SPMs are biosynthesized from arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or n-3 docosapentaenoic acid via the actions of lipoxygenases (LOXs) and cyclooxygenases (COXs)<sup>7,14</sup> (Figure 1). This superfamily of mediators includes lipoxins, resolvins, protectins, their aspirin-triggered isomers, maresins, cysteinyl-conjugated SPMs (CTRs), and 13-series resolvins (RvTs).<sup>14-20</sup> SPMs are not immunosuppressive in animal models<sup>13,21</sup> and are protective in several disease models,<sup>7</sup> including atherosclerosis (Table 1), which will be the focus of this review.

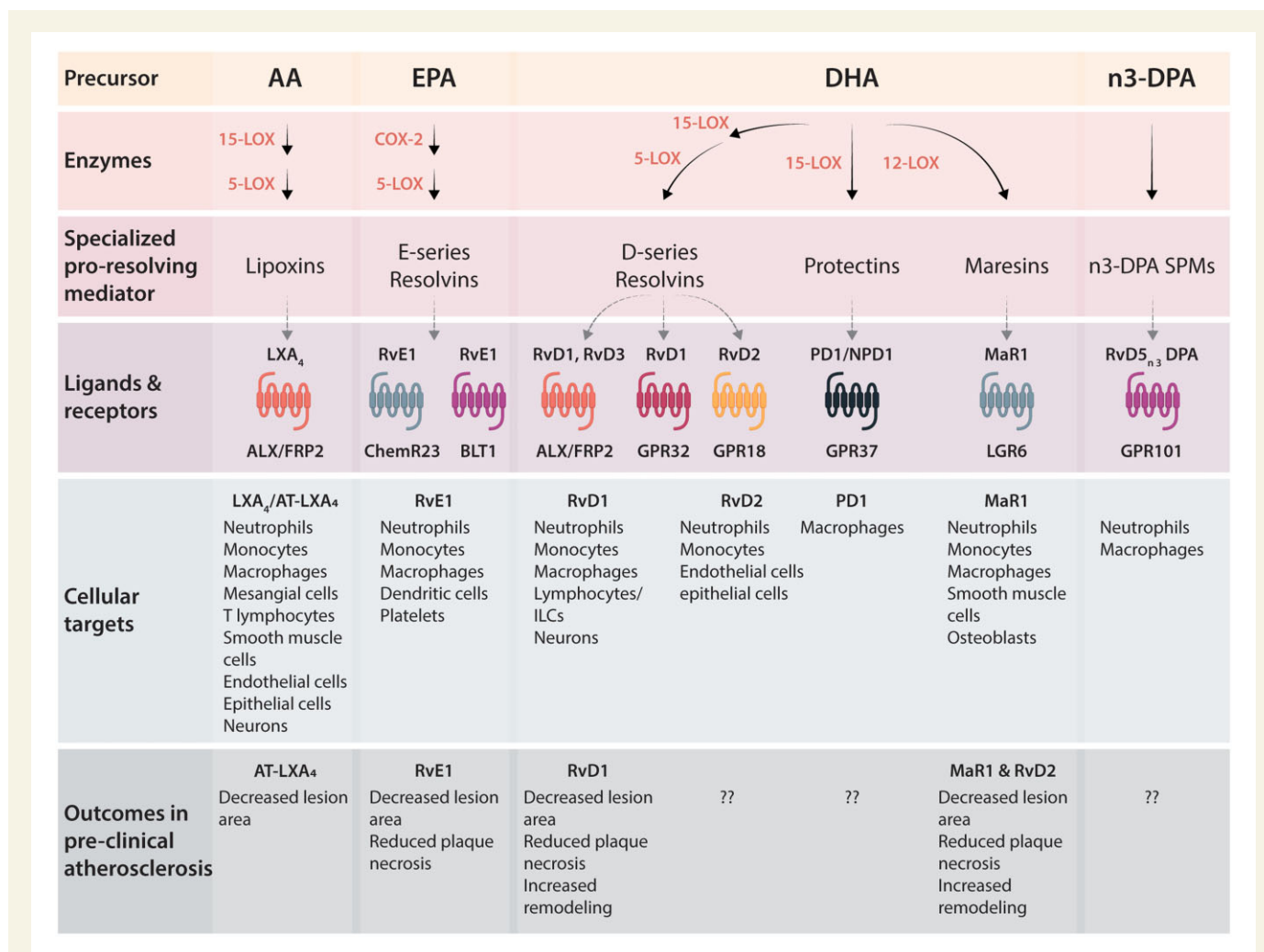
SPMs have distinct chemical structures and are potent ligands that bind and activate specific G-protein coupled receptors<sup>7,32-36</sup> (Figure 1). As a testament to their potency, the concentration of Resolvin D1 (RvD1) in local, resolving blister exudates from humans is ~85pM.<sup>37</sup> RvD1 enhances the clearance of apoptotic cells (i.e. efferocytosis) in this dose range,<sup>38</sup> supporting the idea that low concentrations of SPMs can evoke significant biological activity on human cells. Deletion of key SPM receptors, such as ALX/FPR2, GPR18, or LGR6, leads to exacerbated inflammation and defective resolution.<sup>39-42</sup> In the context of atherosclerosis, removal of RvE1's receptor, ChemR23, resulted in increased lesion size and plaque necrosis, demonstrating a critical role for endogenous RvE1 in thwarting atherosclerosis progression.<sup>28</sup> These findings provide compelling evidence that SPMs are essential endogenous mediators of the inflammation-resolution response and therefore critical for limiting disease associated with non-resolving inflammation. Indeed, they are protective in several disease models including injury-induced neointimal hyperplasia,<sup>43,44</sup> myocardial infarction,<sup>45,46</sup> and atherosclerosis.<sup>26,29,30,47</sup> A consequence of ongoing inflammation in atherosclerosis is damage to the arterial wall. In addition to limiting pro-inflammatory lipid mediators and cytokines, SPMs stimulate the host to repair and regenerate tissue.

Therefore, SPM therapies may be an attractive new approach towards treatment of atherosclerosis, with the dual properties of both safely limiting inflammation and enhancing repair.

## 3. Prime suspect: impaired inflammation-resolution in atherosclerosis

The balance of lipid mediators in the vasculature has been studied and appreciated for decades.<sup>48</sup> For example, the timely and controlled synthesis of thromboxane activates platelets to prevent blood loss and initiate the recruitment of inflammatory cells required for repair. Prostacyclin limits platelet activation while swiftly restoring flow in the vasculature. Thromboxane and prostacyclin are synthesized through COX and balance between these lipid mediators is critical for maintaining vascular homeostasis. Disruption in this balance (as we have learned from selective COX-2 inhibitors) can lead to catastrophic outcomes such as acute thrombotic events.<sup>49</sup> Consequently, it is not surprising that disproportions of other lipid mediators may have maladaptive outcomes in vascular diseases, and, in fact, imbalances in SPMs and LTs or PGs are associated with atherosclerosis.<sup>29,30,50</sup> Specifically, we found that SPMs were imbalanced with LTs in highly necrotic regions of human plaques compared with less necrotic regions.<sup>29</sup> In an effort to demonstrate causation, we investigated *Ldlr*<sup>-/-</sup> mice and found that highly necrotic, advanced lesions had a decreased SPM:LT ratio compared with lesions with minimal necrosis, and RvD1 treatment during advanced atherosclerosis reduced necrosis and increased plaque remodelling in mice.<sup>29</sup> The low dose of RvD1 given also increased several other species of intraplaque SPMs and improved the SPM:LT ratio in lesions,<sup>29</sup> suggesting that RvD1 stimulates a feed-forward pro-resolution mechanism that can repair damage within the arterial wall. At the same time, a separate group found a decrease in the SPM:LT and SPM:PG ratio in advancing murine plaques. Viola *et al.*<sup>30</sup> found that the SPMs that were the most reduced in advanced plaques were RvD2 and Maresin 1 (MaR1). Exogenous administration of MaR1 and RvD2 to *ApoE*<sup>-/-</sup> mice with established atherosclerotic disease limited necrosis and atheroprotection. Several SPMs (and other pro-resolving ligands) limit atherosclerosis in different animal models (summarized in Table 1). Together, these results suggest that the imbalance in SPMs in plaques may drive plaque necrosis and atheroprotection.

Based on the work of several groups around the world the SPM:LT/PG ratio may also be a potential biomarker for atherosclerosis and susceptibility for MACEs in humans. For example, a decreased RvD1:leucotriene B<sub>4</sub> (LTB<sub>4</sub>) in saliva was associated with increased intimal-medial thickness,<sup>51</sup> a metric associated with advancing disease. Analysis of human plasma revealed significantly lower RvD1 levels in highly symptomatic stroke patients compared with asymptomatic patients,<sup>52</sup> therefore suggesting that low levels of RvD1 may be associated with specific outcomes or disease trajectories. The RvE1:LTB<sub>4</sub> ratio was also lower in the context of aortic valve disease in humans,<sup>53</sup> again suggesting that this ratio is critical in vascular disease. The ability to predict responses to treatments and/or potential outcomes is clinically relevant and currently lacking. Collectively, the examples used here were determined by different assays, including ELISA and LC-MS/MS suggesting that several different types of analysis can generate meaningful data, which may be leveraged to develop more routine screens for improved patient care.



**Figure 1** Schematic of SPM generation and their actions via specific GPCRs on select cellular targets. Precursor fatty acids, including arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or n-3 docosapentaenoic acid ( $n_3$ -DPA), are biosynthesized into several families of SPMs by the action of enzymes including 5-LOX, 15-LOX, and COX-2. The receptors for many SPMs have been identified, as shown, but many remain to be elucidated. SPMs also have distinct cellular targets, though more work is needed to examine cell-specific expression of receptors under different conditions and in different tissues.

The SPM:LT ratio in the aforementioned studies were largely determined by decreased levels of SPMs, suggesting that there may be a defect either in the synthesis of key SPMs during atheroprogession or an exuberance of their breakdown. While catabolism of SPMs may be a viable hypothesis, some metabolites of RvD1 and RvD2, as examples, have equipotent biologic actions, at least with regard to limiting monocyte adhesion to adipocytes.<sup>54</sup> Therefore, we postulate that reduced or defective synthesis, plays a critical role in SPM:LT imbalances in atherosclerosis. There are several possible mechanisms that may underly this imbalance on a cellular level including factors that affect key enzymes, oxidative stress, and SPM production.<sup>55</sup> We previously uncovered a new pathway that explains, in part, why endogenous SPMs could be defective in atherosclerosis.<sup>29,55</sup> Under conditions of oxidative stress as seen in inflammation, 5-LOX is phosphorylated and translocates to the nuclear membrane in macrophages, which favours the biosynthesis of the pro-inflammatory mediator, LTB<sub>4</sub><sup>56,57</sup> (Figure 2). In this context, RvD1 promotes nuclear exclusion of 5-LOX and thereby suppresses LTB<sub>4</sub> and enhances production of the pro-resolving molecule, lipoxin A<sub>4</sub> (LXA<sub>4</sub>) in

macrophages.<sup>55</sup> Newer work suggests that the SPM:LT ratio may also involve the interaction between the DAMP high-mobility group box 1 (HMGB1) and with Complement 1q (C1q). Liu *et al.*<sup>58</sup> found that HMGB1 drives LTB<sub>4</sub> production via phosphorylation and nuclear translocation of 5-LOX in macrophages. However, when HMGB1 is present with C1q, 5-LOX is cytoplasmic a subsequent increase in SPMs is observed.<sup>58</sup> Interestingly, C1q deficiency is associated with systemic lupus erythematosus, a patient population with a well-known predisposition for accelerated atherosclerosis.<sup>59</sup> There are likely numerous permutations as to how SPM:LT ratios are regulated at the signalling level and via enzymatic activity and uncovering these pathways may lead to new strategies that can restore the SPM:LT/PG balance.

Other processes that can impact SPM production include transcellular biosynthesis between interacting cells<sup>7</sup> and the release of precursors from cellular phospholipids.<sup>60</sup> Therefore, the cell composition and the phenotype of cells within plaques may be critical determinants as to whether SPM can be formed. For example, human 'M2-like' macrophages are more robust generators of SPMs than 'M1-like' macrophages<sup>61,62</sup>.

**Table 1** Pro-resolving ligands are protective in atherosclerosis

Treatment	Animal model	Diet	Context	Outcome
Proteins/peptides				
Ac2-26 <sup>22</sup> (i.v.)	Ldlr <sup>-/-</sup>	Western diet	Advanced athero (progression)	↓ Lesional necrosis, MMPs, ROS ↑ Lesional fibrous caps and IL-10 mRNA
Ac2-26 <sup>23</sup> (i.v.)	ApoE <sup>-/-</sup>	Western diet	Early athero (atherogenesis)	↓ Leucocyte migration into arterial wall
IL-10 <sup>24</sup> (i.v.)	Ldlr <sup>-/-</sup>	Western diet	Advanced athero (progression)	↓ Lesional necrosis, MMPs, ROS ↑ Lesion fibrous caps
Human recombinant annexin 1 <sup>25</sup> (i.p.)	Ldlr <sup>-/-</sup>	Western diet	Mid-advanced athero (progression)	↓ Lesion area, lesional necrosis
SPMs				
RvE1 <sup>26</sup> (oral-topical)	Rabbit	High cholesterol	Early athero (atherogenesis)	↓ Lesion area
RvE1 <sup>27</sup> (oral)	ApoE*3 Leiden	Western diet	Mid-advanced athero (progression)	↓ Lesion area, lesional mRNA of Adam17, CD44, CCR5, CCL2, CD74
RvE1 signalling ChemR23 <sup>28</sup>	Erv1/Chemr23 <sup>-/-</sup> x ApoE <sup>-/-</sup> Erv1/Chemr23 <sup>-/-</sup> → Ldlr <sup>-/-</sup>	Western diet	Advanced athero (progression)	Removal of ChemR23 lead to ↓ Lesion and necrotic area
RvD1 <sup>29</sup>	Ldlr <sup>-/-</sup>	Western diet	Advanced athero (progression)	↓ Lesional necrosis, MMPs, ROS ↑ Lesion fibrous caps, efferocytosis
RvD2 and Mar1 <sup>30</sup>	ApoE <sup>-/-</sup>	Western diet	Advanced athero (progression)	↓ Lesional necrosis ↑ Lesion fibrous caps
ATL <sup>31</sup>	ApoE <sup>-/-</sup>	Western diet	Advanced athero (progression)	↓ Lesional area ↑ Lesion collagen

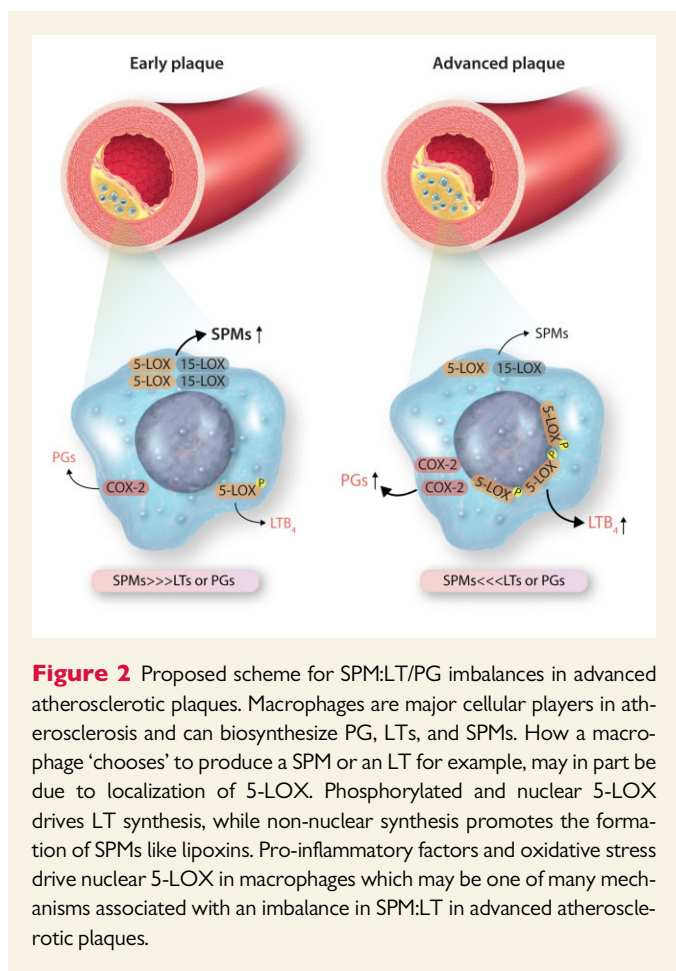
M2-macrophages in atherosclerosis are critical for regression, in part through their ability to synthesize SPMs.<sup>63,64</sup> Other reports demonstrate that five-lipoxygenase activating enzyme, a protein that facilitates the synthesis of LTB<sub>4</sub>, also plays an important role in the production of SPMs.<sup>65,66</sup> Leucotriene A<sub>4</sub> hydrolase is yet another intriguing target for atherosclerosis and potentially other vascular diseases, because inhibition of this enzyme has been linked to a decrease in LTB<sub>4</sub> and an increase in lipoxins.<sup>65,67</sup>

Lastly, macrophage function, and in particular the ability to carry out efferocytosis, facilitates the synthesis of SPMs over LTs.<sup>61,68,69</sup> This feed-forward circuit was first demonstrated in murine systems<sup>68</sup> and was further recapitulated in human macrophages that had ingested apoptotic PMN.<sup>61</sup> A potential mechanism for the increase in SPMs during efferocytosis is transcellular biosynthesis.<sup>61</sup> How apoptotic cell corpses are handled once ingested and whether the enzymes within those cells are recycled, donated, or broken down are not known and would be of immense interest. For example, do dead cells become organelle or enzyme 'donors' and if so, does that facilitate the synthesis of SPMs over LTs? Another mechanism that promotes production of SPMs during efferocytosis is through MerTK signalling.<sup>69</sup> Cai *et al.*<sup>69</sup> demonstrated that MerTK signalling leads to increased SPMs and that silencing of MerTK resulted in reduced SPMs. Ga6-stimulated MerTK signalling leads to decreased phosphorylation of 5-LOX, increased SPMs and decreased LTs.<sup>55,69</sup> MerTK signalling in human macrophages decreased activity of the

mitogen-activated protein kinase p38 and the kinase MK2, resulting in increased non-phosphorylated, cytoplasmic 5-LOX, and enhanced SPM biosynthesis.<sup>70</sup> The ability to bind target cells for efferocytic clearance is also highly regulated, in part via integrin-dependent adhesion. Endothelial cells and macrophages can secrete the molecule developmental endothelial locus 1 which regulates binding to neutrophils, efficient efferocytosis, and production of SPMs.<sup>71</sup> This work suggests that specific signalling pathways and cell-cell interactions associated with dead cell clearance can impact SPM generation.

Ample data show that a decreased SPM:LT or PG ratio is associated with worsened disease, but what about the opposite? Does an increased SPM:LT or PG ratio correlate with plaque resolution/regression in humans? Welty *et al.*<sup>72</sup> began to tackle this question in a recent study in which they monitored coronary artery disease patients given Lovaza (which is an omega-3 EPA and DHA ethyl ester mixture), for 30 months. First, they found that that some individuals had a low plasma omega-3 index and other had a high plasma omega-3 index.<sup>72</sup> Those with a high omega-3 fatty acid (FA) index were more likely to be women and to have lower white blood cell and neutrophil counts compared with those with a low omega-3 FA index.<sup>72</sup> Most individuals with a high omega-3 index exhibited an increased SPM:LT ratio and coronary plaque regression, which was independent of LDL-C and hsCRP levels. This work suggests that the SPM:LT ratio may be a more precise measure of residual inflammation in the setting of already low hsCRP. Results from the REDUCE-





**Figure 2** Proposed scheme for SPM:LT/PG imbalances in advanced atherosclerotic plaques. Macrophages are major cellular players in atherosclerosis and can biosynthesize PG, LTs, and SPMs. How a macrophage 'chooses' to produce a SPM or an LT for example, may in part be due to localization of 5-LOX. Phosphorylated and nuclear 5-LOX drives LT synthesis, while non-nuclear synthesis promotes the formation of SPMs like lipoxins. Pro-inflammatory factors and oxidative stress drive nuclear 5-LOX in macrophages which may be one of many mechanisms associated with an imbalance in SPM:LT in advanced atherosclerotic plaques.

IT and EVAPORATE trials demonstrated that Vascepa (which is an EPA ethyl ester) reduced the incidence of cardiovascular events and reduced plaque volume<sup>73,74</sup> again suggesting that omega-3 FAs may aid in plaque regression. Whether Vascepa's protective actions are via the synthesis of SPMs is not known and a deeper exploration of these patient samples may yield important mechanistic clues as to why this omega-3 regimen showed a benefit. Conversely, the STRENGTH trial, which tested the use of free acid EPA and DHA in cardiovascular patients with hypertriglyceridaemia was stopped early due to no reductions in MACE between the control (corn oil) and the EPA and DHA treatment group.<sup>75</sup> There are key differences in REDUCE-IT versus STRENGTH trials and the obvious is EPA ethyl ester vs. free acid EPA+DHA, respectively. On the surface, one would postulate that the addition of DHA may be a reason for these discrepant findings, but until there is a trial with DHA alone, jumping to that conclusion may be premature. A glaring difference in the trials are the oils used in the control groups. REDUCE-IT used mineral oil whereas the STRENGTH trial used corn oil as a control. Corn oil is rich in omega-6 FAs and has small but appreciable amounts of omega-3 alpha-linolenic acid (ALA). ALA can be converted to EPA in humans<sup>76</sup> and so the differences between control and experimental groups may be muddled.

Moreover, an intriguing component of the Welty *et al.*<sup>72</sup> study was that there was a small subset of individuals who had a high omega-3 index but still exhibited atherosclerosis progression. These individuals did not have an increased SPM:LT ratio, which suggests that not all individuals respond to omega-3 supplementation and specifically not all individuals can

make SPMs in the presence of omega-3's. Why some humans can synthesize SPMs over LTs in the presence of omega-3's and why other cannot is not known. One possibility is that confounding factors, like diabetes status, smoking, sedentary lifestyle or unhealthy diet can increase inflammation or oxidative stress may drive localization of biosynthetic enzymes in a manner that is not suitable to synthesize SPMs.<sup>29</sup> Another explanation may be hidden in subtle diet-gene interactions. For example, individuals with a specific 5-LOX variant who also ingested a diet rich in AA had increased carotid atherosclerosis compared to individuals with non-variant 5-LOX, while those with the same mutation but who ingested a diet rich in omega-3 FAs had less carotid atherosclerosis compared with the control population.<sup>77</sup> These results suggest that certain single-nucleotide polymorphisms (SNPs) may not associate with disease itself but may reveal pathologic or protective changes when in combination with lifestyle choices, such as diet. How other lifestyle choices such as exercise and stress interact with the genetic landscape of certain individuals may reveal specific clues as to who may benefit from specific treatments and who cannot. More experiments will be needed to further understand mechanisms regulating differences in SPM synthesis among individual patients.

## 4. What drives failed resolution in atherosclerosis?

Smoking, diabetes status, and dyslipidaemia are well-known risk factors for atherosclerosis and cardiovascular diseases, and inflammation-resolution is impaired in these contexts.<sup>78,79</sup> Other non-traditional risk factors such as poor diet, stress, and ageing itself all contribute to atherosclerosis, though whether inflammation-resolution programmes are defective in these conditions is vastly underexplored. Inflammation-resolution 'programmes' encompass a variety of mediators and cellular processes, which requires a coordinated effort between several cell types, factors, and organ systems. At the same time, cardiovascular disease is a result of the intersection of many cell types and organ systems. One such tissue, the blood, continuously bathes vessels, and plaques. Therefore, the blood and its source, the bone marrow, are critical players in chronic inflammatory diseases and will be a major focus of the remaining part of the review.

Immune cells are important responders to damage and can be recruited to areas of injury, and exuberant immune activity increases MACEs.<sup>80</sup> Cells of myeloid origin have been shown to contribute to plaque progression and instability in animal models,<sup>81,82</sup> and both increased neutrophils and monocytes have been linked with atheroprotection in humans.<sup>83,84</sup> Neutrophils are found in advanced human plaques,<sup>85,86</sup> a state also characterized by imbalances in SPM:LT/PG ratios.<sup>29</sup> Neutrophils can contribute to impaired inflammation-resolution if hyperactivated and release neutrophil extracellular traps or NETs that further promote inflammation and thrombosis.<sup>87</sup> Indeed, platelets have also been linked with atheroprotection and myocardial infarction,<sup>88,89</sup> and both platelet production and activity can be impacted by stress, disease status, infection, and ageing. While mature myeloid cells can be recruited into plaques and modulate the local microenvironment, increasing evidence supports the idea that altered blood production, or haematopoiesis, can impact atheroprotection.<sup>90</sup> Increases in haematopoietic stem cell (HSC) clones carrying somatic mutations correlates strongly with coronary artery disease, and emerging clinical evidence demonstrating a strong link between clonal haematopoiesis and cardiovascular disease has been extensively reviewed elsewhere.<sup>91-94</sup>

In addition to the generation of blood cells, the bone marrow is an important site of cell removal, and the homeostatic process of cell clearance regulates bone marrow function, including the niches that maintain HSCs.<sup>95</sup> While the regenerative capacity of bone marrow is well-established as evidenced by the success of HSC transplantation, mechanisms of inflammation-resolution at this site have not been clearly defined. Lipidomic analysis in mice revealed higher SPM levels in the bone marrow, relative to the spleen, and efferocytosis of bone forming cells is important for homeostatic bone turnover.<sup>96,97</sup> Thus, inflammation-resolution processes operate in a variety of tissues, and these programmes may contribute to atheroprotection both locally and from a distance. The plasticity among immune cell types, the intersection of various systems, and the relatively slow progression of disease highlights the complexity of understanding mechanisms driving of cardiovascular disease. Many questions remain regarding how SPMs and SPM:LT/PG balances impact immune cell generation and myeloid function, and in turn, how haematopoietic output may influence the balance of SPMs and LTs/PGs in different tissues.

#### 4.1 Poor diet and a sedentary lifestyle drive atherosclerosis: healthy diet and regular exercise boost endogenous inflammation-resolution

Beyond genetics, an important driver of atherosclerosis is poor diet. This is particularly important in terms of inflammation-resolution as SPMs are biosynthesized from essential FAs. Diets rich in omega-3 FAs have been well documented to thwart the progression atherosclerosis<sup>98</sup> and whether that is due to the synthesis and action of SPMs is still not known but is a topic of great interest. As mentioned above, atherosclerosis is a complex disease that involves lipids, inflammation, and genetic factors, and effective treatments in the future will likely be multi-pronged. It would be harmful to suggest that a single pill can act as a 'magic bullet' to quell atherosclerosis. However, maintaining a healthy lifestyle, which includes a diet rich in omega-3 FAs and regular exercise is beneficial. Indeed, leisurely exercise limits all cause and cardiovascular mortality in humans.<sup>99</sup> One possible mechanism is related to the bone marrow and the production of inflammatory cells. Exercise protects mice from chronic leucocytosis which ultimately resulted in reduced atherosclerosis in mice.<sup>100</sup> Whether SPMs or resolution mechanisms are involved in exercise-induced reduction in haematopoietic output of inflammatory cells is not known. In other contexts, exercise was shown to increase RvD1 and promote efferocytosis.<sup>101</sup> Therefore, it is possible that exercise-induced RvD1 could limit atherosclerosis, given the already known protective roles of RvD1 in murine models of atherosclerosis.<sup>29</sup>

Increased dietary omega-3 FAs are appreciated to reduce systemic inflammation, though clear mechanisms are poorly understood. Mice fed a fish oil diet where there was a higher omega-3:omega-6 ratio exhibited increased primitive myeloid progenitors in the bone marrow, but when cultured *ex vivo*, reduced myeloid cell production was observed.<sup>102,103</sup> Fish oil did not impact circulating white blood cells, but the findings suggested that increased dietary omega-3 FAs suppressed the differentiation of myeloid progenitors and support the notion that regulating myeloid cell production may be one mechanism whereby diet changes reduce atherosclerosis. In contrast, mice fed a diet laden with saturated fat and cholesterol (i.e. the Western diet) exhibited immune activation and enhanced myelopoiesis, similar responses seen after exposure to pathogens, and these responses were mediated by the intracellular Nod-like receptor NLRP3.<sup>104</sup> These NLRP3-dependent responses result in

epigenetic reprogramming in myeloid progenitors and long-lived immune dysfunction that may facilitate the chronic immune responses observed in patients with unhealthy diets. Recent evidence in a murine model of *JAK2*<sup>V617F</sup>-induced myeloproliferation and Western diet has linked DNA-damage and the DNA-sensing inflammasome, AIM2, as an essential driver of atheroprotection via the production of IL-1 $\beta$ .<sup>105</sup> Thus, in combination with underlying genetic perturbations, diet may be particularly important for disease progression.

Dietary FAs can be metabolized to generate LTs, PGs, and SPMs, and the precise mechanisms regulating the systemic and haematopoietic effects of dietary changes are important questions. Recent evidence from a human study demonstrated that enriched marine oil supplement could induce changes to blood SPM levels and impact the transcriptomic profile of circulating cells, as well as improve phagocytic capacity by neutrophils and monocytes.<sup>106</sup> This proof-of-concept study reveals the relatively rapid changes to immune system function and systemic levels of SPMs occurs upon supplementation of omega-3 FAs. One caveat of this study is that all volunteers were healthy and relatively young. In an entirely different scenario, where diet is restricted by fasting, blood production is also modulated and, notably, fasting limits the number and inflammatory function of monocytes.<sup>107</sup> In humans, obesity has been shown to correlate with increased circulating monocytes, again supporting the critical role of diet in regulating atheroprotection and linking atheroprotection to changes in haematopoiesis. The intersection of diet and overall caloric consumption likely plays an important role in the synthesis and balance of SPMs and PGs/LTs, and therefore atheroprotection.

#### 4.2 Disrupted sleep and stress promote atherosclerosis: restoring circadian rhythms may boost endogenous resolution programmes

Natural sleep patterns that are also important to health and circadian rhythms are now well-appreciated to control inflammation and atheroprotection.<sup>108</sup> These daily rhythms that regulate our sleep cycle also regulate the bone marrow and its release of leucocytes and the ability of the kidney to filter blood and regulate blood pressure. Thus, disturbances in sleep caused by work or stress are poised to impact the very cells and organs that can contribute to progression of cardiovascular disease and incidence of MACEs. The release of HSCs and progenitors from the bone marrow into circulation is also regulated by internal clock genes<sup>109</sup> and this daily homeostatic fluctuation is thought to provide cells to distal tissues for repair and immunosurveillance.<sup>110</sup> Also, insufficient sleep is linked to increased myelopoiesis and atherosclerotic plaques in *Apoe*<sup>-/-</sup> mice.<sup>111</sup> Similar to the impact of sleep disturbances, exposure to stressful stimuli increased haematopoietic function in *Apoe*<sup>-/-</sup> mice and revealed enhanced development of unstable plaques.<sup>112</sup> Beyond systemic responses, evidence also suggests that circadian-dependent mechanisms are also at play within plaques themselves, wherein the timing of cell death and clearance regulates the growth of the necrotic core.<sup>113</sup> A prevailing biological function of sleep is one of restoration and repair and indeed, efferocytosis and phagocytosis in the bone marrow and other tissues, such as the brain, are dependent on circadian rhythms.<sup>110,114</sup> Collectively, sufficient sleep and reduced stress are beneficial to thwart atherosclerosis progression, but how these processes intersect with inflammation-resolution programmes is less understood. One clue is a recent paper that described diurnal synthesis of key SPMs.<sup>115</sup> These data support the notion that resolution programmes and/or resolution

therapy may be optimal at specific times of day, and disturbances in normal sleep patterns may impair inflammation-resolution.

### 4.3 Does the aged immune system set the stage for atherosclerosis?

In general, increasing age is a well-known and major risk factor for atherosclerosis. The impact of advanced age on the development and progression of atherosclerosis, as well as the treatment approaches for elderly patients with atherosclerosis, is a critically underexplored arena.<sup>116</sup> Chronological ageing involves, genetic, environmental, and metabolic changes that impact all organs systems.<sup>117</sup> Animal models suggest that ageing drives atherosclerosis in part through changes in the bone marrow.<sup>118</sup> Monocytosis was associated with increased atherosclerosis in ageing, and bone marrow transfers from aged mice into young *Ldlr*<sup>-/-</sup> mice produced larger plaques than *Ldlr*<sup>-/-</sup> recipients that received young marrow.<sup>118</sup> These findings suggest that the bone marrow from aged mice promoted atherosclerosis. Indeed, ageing is associated with profound changes to blood production, and a key feature of haematopoietic dysfunction in this context is the strong bias in myeloid cell production due to an expanded pool of myeloid-biased HSCs and progenitors.<sup>119,120</sup> At the same time, ageing is associated with reduced lymphoid cell generation that has been linked to a loss of lymphoid progenitor cells, in part, due to changes in the aged bone marrow microenvironment.<sup>121,122</sup> The function of mature cells is also compromised in ageing and linked with both poor immunity and more severe disease in response to infection.<sup>123–125</sup> Increased pro-inflammatory responses are observed in ageing and suggest that dysfunctional inflammation-resolution programmes are central to declining host defense and more pathological outcomes in response to infection in the elderly. Though no data exist to demonstrate that resolution impairments that arise during ageing cause atherosclerosis, evidence supports that the ageing landscape may set the scene for disease.

The aged immune system is vastly different than in younger individuals and new work in animal models suggests that haematopoietic cell senescence drives organ inflammation and damage.<sup>126</sup> Cellular senescence is a programme in which cells are halted in their proliferation while acquiring a secretory phenotype characterized by production of pro-inflammatory factors, referred to as the senescence-associated secretory phenotype or SASP.<sup>127</sup> Transient emergence of senescent cells upon injury is important for wound healing and can promote healing in arthritis.<sup>128</sup> However, in the context of ageing senescent cells are associated with ongoing inflammation or inflammaging.<sup>129</sup> Senescent cells exacerbate inflammation<sup>129</sup> and can also contribute to inefficient clearance of dead cells.<sup>130</sup> Briefly, we found that released factors from senescent cells decreased efferocytosis through enhanced MerTK cleavage.<sup>130</sup> RvD1 efferocytosis in this context rescued efferocytosis. Most of the senescence literature in the atherosclerosis arena has focused on smooth muscle and endothelial cells<sup>131,132</sup> and little attention has been given to haematopoietic-derived cells.<sup>133</sup> We recently found that haematopoietic cells promoted plaque necrosis and that removal of senescent cells during advanced atherosclerosis limited plaque necrosis and promoted the synthesis of key SPMs.<sup>134</sup> Moreover, we observed that senescent macrophages themselves were highly pro-inflammatory and were poor efferocytes,<sup>134</sup> therefore suggesting that senescent macrophages cannot carry out a key programme of inflammation-resolution. Moreover, these findings suggest that the accumulation of senescent macrophages or other haematopoietic cells in plaques may be a key driver of deranged resolution programmes in atherosclerosis.

Overall, we still do not fully understand why the aged immune system does not function as optimally as the young. SPMs,<sup>130,135</sup> drugs that target senescent cells,<sup>136</sup> and a restricted calorie diet<sup>137</sup> can limit inflammaging, which suggests that inflammaging is a 'modifiable' risk factor. As mentioned above, proper sleep cycles, a diet enriched in omega-3 FAs and regular exercise promote endogenous SPMs and resolution in mice.<sup>101,106,115</sup> Indeed, there is well documented literature that there is a lower cardiovascular disease risk for middle aged or elderly individuals who engage in regular exercise compared with their sedentary counterparts.<sup>138</sup> Whether SPMs are boosted in elderly humans who engage in routine exercise, or whether SPMs themselves can act in the bone marrow to correct age-related haematopoietic changes is not known and may provide important missing details for how to optimally treat elderly individuals.

Along these lines, ageing is associated with imbalances in the SPM:LT ratio in mice and humans in.<sup>135,139</sup> Mechanisms and consequences associated with these imbalances are underexplored. Efferocytosis, which is a major cellular programme of inflammation-resolution, is widely known to be impaired in ageing.<sup>135,140–145</sup> An efferocytosis receptor called TIM-4, was decreased on macrophages from elderly humans in the context of self-limited inflammation.<sup>146</sup> p38 signalling was attributed to decreased levels of TIM-4 and blockade of p38 restored efferocytosis in the elderly.<sup>146</sup> This work provides a rationale for therapeutic strategies that target p38 to promote efferocytosis in ageing.<sup>147</sup> Because p38 signalling is associated with several pro-inflammatory pathways, direct inhibition of the kinase over time may lead to immune suppression. Of note, RvD1 does not inhibit, but rather controls p38 activation and promotes a pro-resolution circuit in macrophages.<sup>55</sup> As mentioned above, MerTK promotes efferocytosis and increases SPMs.<sup>69</sup> Indeed, Gas6, which is a ligand for MerTK promotes SPM over LTs.<sup>70</sup> Interestingly, Frisch *et al.*<sup>143</sup> found that not only was efferocytosis impaired the bone marrow from aged mice but that levels of Gas6 were also significantly reduced the bone marrow from aged mice, compared with young controls. Whether SPMs were also decreased in aged bone marrow was not explored but would provide important mechanistic information. A deeper exploration of efferocytosis mechanisms in ageing may help inform the development of new tissue-reparative therapies for the elderly.

As mentioned above, chronological ageing is complex and includes cellular metabolic and epigenetic alterations that impact all organs and tissue systems,<sup>117</sup> and, from a practical standpoint, studying ageing is challenging and time-consuming. Therefore, the use of models that 'accelerate' or mimic ageing of the bone marrow compartment may be of use to home in on mechanisms that drive haematopoietic ageing. Sublethal radiation is a well-known model for accelerated ageing, particularly within the bone marrow.<sup>148–150</sup> This model leads to HSC senescence and mimics several features of ageing such as alterations in HSC functions like reduced clonogenicity and skewed differentiation towards myeloid lineages.<sup>149</sup> Senescence is associated with ageing and atherosclerosis and so the use of sub lethal radiation to understand mechanisms associated with haematopoietic and immune cell senescence may be reveal important mechanistic clues as to how senescence of immune cells impacts inflammation-resolution and/or diseases like atherosclerosis. Moreover, this model can accelerate the pace of research towards uncovering novel mechanistic based therapies and provide a more rapid platform for testing new strategies for age-related diseases. And so in addition to chronological ageing, more work should be focused on proof-of-concept models to augment our understanding of how ageing of the bone marrow and thus the birthplace of our immune cells can impact atherosclerosis.



## 5. Closing remarks: therapeutic opportunities and areas of future study

Pro-resolving mediators actively counteract inflammation without compromising host-defense.<sup>7</sup> In animal models, SPMs lower the threshold for antibiotic therapy and enhance host defense mechanisms regarding viral and bacterial infections and.<sup>13,151</sup> Because atherosclerosis is a long-term progressive disease, directly blocking inflammation for an extended time may lead to unintended host defense issues. A therapeutic strategy that activates the host to repair an injury (e.g. through boosting efferocytosis) may be a promising approach towards controlling inflammation and aiding regeneration of damaged tissue. It may be an approach that ultimately prevents atherosclerotic progression and/or can be utilized to improve current therapies aimed at driving plaque regression. Several important unanswered questions exist, however, and addressing the fundamental mechanisms balancing inflammation-resolution programmes in unique contexts and in distinct tissues may help refine strategies to improve health and limit cardiovascular disease.

First, the inflammation-resolution field needs more 'accessible' biomarkers. The discovery of SPMs paved a previously uncharted path towards our understanding of how the body resolves inflammation.<sup>7</sup> Identification of SPMs in tissue by LC-MS/MS is expensive due to the required equipment and expertise. SPM ELISAs may be helpful to profile inflammation-resolution patterns in individuals and if used in a multiplexing format, may yield a more high-throughput readout for patient care. Quantitative PCR (qPCR) and RNAseq have become standard laboratory techniques. And so we should be asking the question, as to whether there are inflammation-resolution RNA signatures. Data could be interrogated with RNA sequencing in archived databases and validated in newly generated data sets, and potentially reveal a 'resolution status'. Simple proof-of-concept experiments that directly compare self-limited vs. non-resolving inflammation can be profiled and used as a starting point to address this gap.

An additional open question is whether specific SNPs or epigenetic changes are associated with efficient inflammation resolution. Genetic and genomic patterns that may control inflammation-resolution are not known and if uncovered can provide strategies to (i) tailor current drugs for better use in humans or (ii) develop new personalized therapeutics to specifically enhance endogenous resolution. In humans, the innate inflammatory response can be assessed *in vivo* using models that include a systemic inflammatory reaction following endotoxin<sup>152,153</sup> or a contained, sterile inflammatory reaction following a cantharidin-induced skin blister.<sup>154,155</sup> Human skin blisters were used to stimulate self-limited acute inflammation in humans and this model revealed that there are individuals who resolve more swiftly than others in a manner that is associated with the presence of key SPMs and their receptor expression on cells within the exudate.<sup>156–158</sup> A deeper exploration of the DNA within the cells may reveal important genetic or epigenetic clues regarding inflammation-resolution mechanisms in humans.

Another hypothetical question is, if SPMs are a new drug, then which SPM should we choose to treat atherosclerosis? SPMs have common and distinct functions. One of the most widely studied common functions is the ability of nearly all SPMs to enhance efferocytosis. A distinct function, as an example, is in the context of platelets. EPA-derived RvE1 limits ADP-induced platelet aggregation whereas, DHA-derived PD1 does not.<sup>159</sup> However, DHA-derived RvD4 limits thrombosis in murine models,<sup>160</sup> and whether other SPMs carry out this function is not known.

Therefore, perhaps a combination of SPMs that can mitigate atherosclerotic plaque formation and limit platelet activation/thrombosis may be ideal.

Overall, atherosclerosis was previously thought to be an inevitable steady and degenerative accompaniment to ageing.<sup>161</sup> Instead, we now appreciate that atherosclerosis develops episodically, can regress, and that lifestyle and medical measures can dramatically modulate the course of disease.<sup>161</sup> Moreover, atherosclerosis is associated with defense mechanisms that have gone awry that ultimately lead to arterial damage.<sup>162,163</sup> The discovery of SPMs has shed light on an entirely new way to control inflammation, enhance host defense and promote repair and so this is an exciting time for inflammation-resolution and atherosclerosis research. Nevertheless, the continued identification of biomarkers, cellular processes, and factors associated with inflammation-resolution may: (i) reveal new ways to treat atherosclerosis and (ii) help inform treatment in a highly patient-specific manner.

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