Antioxidants & Redox Signaling

Antioxidants & Redox Signaling: http://mc.manuscriptcentral.com/liebert/ARS

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Journal:	*Antioxidants and Redox Signaling*
Manuscript ID	Draft
Manuscript Type:	Forum Article
Date Submitted by the Author:	n/a
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Keyword:	Aging, Lipids, Inflammation
Manuscript Keywords (Search Terms):	anti-inflammatory, oxidised phospholipids, oxysterols, metabolism, eicosanoids, reactive oxygen species

ARS Forum Review

Inflammation, lipid (per)oxidation and redox regulation

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Running Head - Lipid (per)oxidation in inflammation and ageing

Abstract

Significance: Inflammation increases during the ageing process. It is linked to mitochondrial dysfunction and increased ROS production. Mitochondrial macromolecules are critical targets of oxidative damage; they contribute to respiratory uncoupling with increased ROS production, redox stress, and a cycle of senescence, cytokine production and impaired oxidative phosphorylation. Targeting the formation or accumulation of oxidised biomolecules, particularly oxidised lipids, in immune cells and mitochondria could be beneficial for age-related inflammation and comorbidities.

Recent Advances: Inflammation is central to age-related decline in health and exhibits a complex relationship with mitochondrial redox state and metabolic function. Improvements in mass spectrometric methods have led to the identification of families of oxidised phospholipids, cholesterols and fatty acids that increase during inflammation and which modulate Nrf2, PPARγ, AP1 and NFkB redox sensitive transcription factor activity. The kinetic and spatial resolution of the modified lipidome has profound and sometimes opposing effects on inflammation, promoting initiation at high concentration and resolution at low concentration of oxidised phospholipid.

Future Directions: There is an emerging opportunity to prevent or delay age-related inflammation and vascular co-morbidity through a resolving (oxy)lipidome that is dependent on improving mitochondrial quality control and restoring redox homeostasis.

Innovation: (Per)oxidised lipids are central mediators in the initiation and resolution of inflammation. Generalised targeting of lipid peroxidation with antioxidants such as tocopherols as a primary prevention in age-related, inflammatory vascular disease has not proven successful. A more nuanced view is emerging where ROS/NO production is desirable during early phases of inflammation and for resolution. Indeed, the cell-targeted amplification of ROS in neutrophils may be required during inflammation to promote resolution. Phytochemicals that promote resolving oxylipid mediators and improve mitochondrial quality control merit further investigation as inhibitors of underlying sterile inflammation and to mitigate age-related vascular disease.

Key words: anti-inflammatory, oxidised phospholipids, oxysterols, metabolism, eicosanoids, reactive oxygen species

1. Introduction

Inflammation is an important physiological process that ensures homeostasis and survival. But as we age, inflammation is less well-controlled and may come at a cost. Usually, physiological inflammation is triggered by non-self materials that activate surveillance systems (complement and resident immune cells). The chemotactic mediators produced by activated complement and resident cells recruit nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase (NOX)-dependent reactive oxygen species (ROS)-producing monocytes, macrophages and neutrophils to kill the invaders. This is followed by lymphocyte recruitment and finally the switch-off of inflammation, in a process known as resolution. Figure 1 illustrates the key effector molecules and different cells involved during the phases of physiological inflammation. Central to the control of physiological inflammation is the metabolic inter-dependence of activation and resolution, and their redox sensitivities (72). Mitochondria are central to inflammation and during ageing, impaired mitophagic quality control may promote low-level chronic inflammation with associated co-morbid diseases.

This review considers the mechanistic relationship between modified lipids, produced enzymatically and by free radical reactions during inflammation, and mitochondrial metabolism, the inflammasome and the resolution phase of inflammation. We consider the impact of ageing and age-related vascular diseases on lipid oxidation and mechanisms by which the phytochemical classes, flavonoids and oxo-carotenoids, may mitigate inflammation by preserving mitochondrial function and enhancing nitric oxide ($^{\bullet}$NO) availability.

2. Physiological inflammation and redox regulation

The initial lines of defence towards invading pathogens are non-specific circulating proteins of the complement cascade combined with tissue resident cells of the innate immune system such as macrophages and dendritic cells. The complement cascade proteins will release chemotactic fragments following contact with unexpected damage-associated molecular pattern molecules (DAMPs) on pathogens (155). A well-known DAMP is bacterial lipopolysaccharide, LPS, that is used widely in studies of inflammation. LPS binds to Toll-like receptor (TLR)4 triggering endocytosis and NOX2 activation with associated production of superoxide anion radicals by neutrophils and macrophages (90). The tissue-resident macrophages are present in low numbers throughout the body, ready to perform a random surveillance role and initiate physiological inflammation. To achieve rapid deployment, innate immune cell activity is tightly coupled to differential use of substrates for energy and the metabolic phenotype (72). While inflammatory macrophages are glycolytic, rapidly producing ATP, alternatively activated anti-inflammatory macrophages are polarised toward mitochondrial biogenesis, oxidative phosphorylation and fatty acid oxidation (167). The high degree of metabolic plasticity of these cells enables them to mount a rapid response to infection and damage (159) and then switch to resolution.

Intertwined metabolic pathways play a major role in immune function and inflammation. To enable switching between different substrate sources according to inflammatory environmental triggers, glucose, amino acid and fatty acid metabolism are coordinated by the activity of redox sensitive transcription factors, nuclear factor erythroid 2-related factor 2 (Nrf2), peroxisome proliferator-activated receptor gamma (PPARγ) and hypoxia-inducible factor 1-alpha (HIF1α) (72,159,191). It is lipid peroxidation products that are electrophilic activators of these transcription factors.

Oxidative damage to lipids, proteins and DNA is frequently observed during inflammation. Extracellular oxidised lipids are ligands for TLR4, either directly activating receptor or acting as a competitor for LPS and inhibiting receptor activation. Mitochondrial DNA is also recognised as an endogenous DAMP by the intracellular receptor, TLR9 (129). DAMPs also activate local resident innate macrophages, which possess a series of pattern recognition receptors (PRRs) including TLR4 and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) that are highly conserved (46), to produce chemotactic cytokines and activate the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome (78). In addition to being activated by oxidised lipids, the NLRP3 inflammasome can also be activated by endogenous metabolic DAMPs including many different native lipids; fatty acids, ceramides and free cholesterol (210,226,231).

The cells that respond most rapidly to infection and the emerging chemotactic gradients are neutrophils which phagocytose extracellular pathogens, killing them via NADPH oxidase-dependent ROS production within the phagosome (61). To increase their efficiency in pathogen capture, neutrophils also produce neutrophil extracellular traps (NETs) from mitochondrial DNA. NADPH oxidase -dependent ROS production and formation of NETS are also essential for the resolution of necrosis-induced inflammation (18) (Figure 2). Neutrophil nets, together with proteins within the activated complement and the clotting cascades immobilise pathogens and provide a physical barrier, preventing infection from spreading around the body.

While NADPH oxidase activation is essential for adequate NET production (61), ROS production causes non-specific bystander oxidative damage. Neutrophils themselves will die rapidly due to low antioxidant enzyme levels within 24 hours of recruitment; they are not equipped to survive under conditions of oxidative stress and this is exacerbated with age (92).

Monocytes and lymphocytes appear later than neutrophils and within 24 hours. The monocytes display a range of phenotypes and functions that are dependent on the local environment; for example, if concentrations of DAMPS and pro-inflammatory cytokines are high, monocytes differentiate to macrophages and adopt an M1-type of pro-inflammatory highly migratory phenotype to target infection to distant sites. This phenotype favours further NADPH oxidase activation with superoxide anion radical production, expression and activity of nitric oxide synthases (NOS) with associated nitic oxide production (although uncoupled NOS which is more common during ageing increases peroxynitrite $ONOO^-$ production) as reactive nitrogen species (RNS) and the production of inflammatory mediators. M1 type macrophages depend on glycolysis to meet the metabolic requirement for rapid energy production (Figure 3). The pentose phosphate pathway is also upregulated under oxidative stress and the presence of lipid peroxides in M1 macrophages via the Nrf2 pathway (128). This increases availability of NADPH as an essential reducing agent to restore the antioxidant glutathione and redoxin cycles. In an Nrf2 dependent process, the antioxidant enzymes mitochondrial superoxide dismutase-2 (SOD2), glutathione reductase, thioredoxin reductase and peroxiredoxin 1 but not catalase are upregulated in macrophages, enhancing their chance of survival during a high ROS environment (151). Neutrophils do not have this extent of adaptation and antioxidant activity. Instead, after killing pathogens, neutrophils die, newly recruited and resident macrophages sense the presence of apoptotic neutrophils via surface receptors such as CD36 and intercellular adhesion molecule 3, and then clear them in a non-inflammatory phagocytic process

(203). Local tissue macrophages may also "cloak" the damaged site to prevent further neutrophil recruitment (207).

Resolution of inflammation was once thought to be a passive process that simply 'burns out' with time, allowing for tissues to return to homeostasis. However, resolution of inflammation is now recognised as an active programme that involves sequential synthesis of lipid mediators of inflammation (**described in Section 4**)(178,180). To meet the demands for degradation of invaginated material and remodelling the extracellular environment to promote healing, macrophages adopt mitochondrial-dependent oxidative phosphorylation and secrete anti-inflammatory lipids formed by an enzyme-catalysed oxidation reaction from the n-3 fatty acids and transforming growth factor beta (TGFβ), enabling the resolution of normal physiological inflammation (72). The enzyme-catalysed oxidation of n-3 fatty acids leads to the formation of protectins, maresins and resolvins (D- and E-series) which normally resolve acute inflammation and prevent chronic inflammation from developing. They affect macrophage differentiation (explained in more detail in section 4 and illustrated in Figure 5). Interestingly, administration of resolvin D3 was also able to reduce joint inflammation in an arthritis model, suggesting that resolvins may also reverse and resolve chronic phase of inflammation. D- and E-series resolving effects are not limited to the innate immune system; they may also target recruited T and B cells at an inflammatory site. In parallel, the recruited lymphocytes (T cells normally) will recognise ROS-regulated, enzymatically-trimmed foreign molecules that have been produced from a pathogen (141). Processed antigens are carried on the surface of dendritic antigen presenting cells within a specialised protein, within the major histocompatibility class, that enables the antigen to be recognised as foreign by T cells. T cells in turn are activated, switch to glycolysis and promote the adaptive immune system to produce foreign-antigen specific antibodies on future contact with antigen and enable a specific targeted immune response to be elicited (204).

Dysregulation at each phase of inflammation may lead to a process that has been termed sterile inflammation which becomes more common with age when there is no pathogenic stimulus; non-resolution of inflammation is common during age co-morbidities.

3. Sterile inflammation and ageing

During ageing, the carefully orchestrated acute inflammatory response that enables effective recognition of tissue damage and removal of pathogens is less well regulated. A process described as "sterile" inflammation is frequently seen and is manifested in specific organs and tissue e.g. in the vasculature during atherosclerosis. Atherosclerosis is a maladaptive phenotype that promotes immune cell recruitment activation and impairs resolution, can accelerate ageing and age-related co-morbidities, and will be considered further.

In addition to the production of ROS by NADPH oxidase, a significant increase in mitochondrial ROS production has been observed during ageing possibly due to ineffective mitophagy (196). Some have reported that overproduction of mitochondrial ROS may increase inflammation e.g. in atherosclerosis (223). Mitochondrial ROS generated by myeloid cells within atherosclerotic plaques increase the concentration of the free-radical mediated cholesterol oxidation product, 7-ketocholesterol (7-KC), which is the most abundant oxysterol in low density lipoprotein (LDL), and enhance the formation of atherogenic neutrophil NETs (224). An exaggerated "sterile" inflammatory response is generated to cholesterol-rich lipoproteins that are retained in the arterial wall in the absence of any infection or tissue damage (186). Shirai et al have identified that circulating monocytes from atherosclerosis

patients are primed to produce more inflammatory cytokines via mitochondrial ROS-mediated oxidation of the glycolytic enzyme pyruvate kinase M2 (188). ROS play a role in regulating pro-inflammatory priming of monocytes in response to oxidised lipids through epigenetic and metabolic re-programming e.g. by protein acetylation (191). For effective priming and memory of an inflammatory event, the acetylation of macrophage histone proteins is rate limiting according to the availability of one metabolite, acetyl CoA (formed in abundance by during lipid metabolism), that is consumed by mitochondria. These observations highlight that lipids are essential sources of energy and are increasingly recognised as important regulators of inflammatory signalling.

Histone deacetylation is necessary to switch macrophage activity towards resolution of inflammation. The associated modulation of gene expression is closely regulated by the activity of sirtuin (SIRT) proteins and availability of the reducing agent nicotinamide adenine dinucleotide (NADH); this again provides another link between inflammation and redox state, as the availability of NADH is controlled by mitochondrial activity. Macrophage SIRT3 plays an essential anti-inflammatory role through regulating mitochondrial bioenergetics and redox homeostasis as well as controlling activation of the inflammatory protein complex, known as the inflammasome (97). The NLRP3 inflammasome has emerged as an immune sensor that causally links systemic inflammation to ageing (230). It is responsible for the proteolytic activation of the inflammatory cytokines IL-1β and IL-18 and mitochondria are involved; the NLRP3 inflammasome is organised following activation at the mitochondria and this is achieved through its binding to cardiolipin, a highly abundant phospholipid lipid in mitochondrial membranes (50) (84).

An increase of ROS/RNS production by immune cells is observed with age due to mitochondrial uncoupling and sterile inflammation and causes lipid peroxidation. There are many classes of lipids that may be oxidised or nitrated. These include phospholipids and cardiolipin, sterols and fatty acids. Oxidised cardiolipins are found circulating at higher concentrations during ageing and are proinflammatory (220). It remains to be seen whether oxidised cardiolipin accumulates in mitochondria with age and whether this influences inflammasome activity. Mass spectrometry methods are now becoming sufficiently sensitive to measure oxidised lipids in subcellular organelles to address this question.

Over the following sections we review three principle classes of oxidised lipids; 1) oxidised phospholipids, such as those formed by free radical oxidation of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (PAPC), that have been shown to modulate signalling induced by bacterial lipopeptide or (LPS, (51,52,117); 2) oxysterols produced by free radical reactions or enzymatically-produced cholesterol oxidation, which amongst other inflammatory effects, can regulate the activation of NLRP3 inflammasome by LPS (37); and 3) the products of 12/15 lipoxygenase (12/15 LOX); specialised pro-resolving mediators e.g. lipoxins and resolvins, acting on G protein coupled receptors (GPCRs). These lipid per/oxidation products are stable and can exert distant sites from their site of production. This raises the potential for oxidised lipids to exert distant effects of ROS on inflammation in a time and concentration dependent manner.

We have previously described increased levels of lipid peroxidation products in the age-related diseases rheumatoid arthritis, Alzheimer's disease and cardiovascular disease (38-40,71) and atherosclerosis is a common comorbidity in all conditions. Since mitochondria have an important role in proinflammatory signalling role during ageing (69) we consider in the following sections how a vicious cycle of lipid (per)oxidation may perpetuate mitochondrial dysfunction and inflammation.

3.1 Peroxidised phospholipids, nitrated fatty acids, inflammation and age-related vascular disease

A. Oxidised phospholipids (OxPLs)

Phospholipids have structural roles in cell membranes and while maintaining membrane integrity they may also function as second messengers (3,23). Phospholipids are substrates for synthesis of lipid mediators including phosphoinositides, diacylglycerides, platelet-activating factor, sphingosine-derived phospholipids, phosphatidic acids, and eicosanoids (135).

Phospholipid bound polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA), docosahexaenoic acid (DHA) present in the second position of the glycerol backbone of phospholipids are also major targets for non-enzymatic oxidation by ROS/RNS to produce the primary oxidation products, peroxyl radicals and hydroperoxides (23). Subsequent molecular rearrangement, cyclisation or fragmentation and oxidation yield a diverse pool of oxidised phospholipids (OxPLs). This includes full-length or shortened carbon chains containing oxygen functional groups such as hydroxy-, keto-, epoxy, hydroperoxyl- and prostane groups as well as carboxylic and aldehydic terminal groups (23). In addition to being esterified within phospholipids, esterified oxidised PUFAs can also be detected as cholesterol esters and triglycerides. All exert distinct biological effects compared to their reduced parent molecules in part due to increased polarity but also due to their chemical reactivity (22). The source of oxidants predicts the likely subcellular targets e.g. mitochondrial DNA and cardiolipin are readily oxidised as mitochondria become more uncoupled, and the nature of ROS/RNS informs the chemistry of oxidation and the products that are formed.

Several OxPL species may result from one individual precursor (48). For example, PAPC (the major phospholipid) oxidation results in oxygenated full-length products e.g. epoxyisoprostane (PEIPC) and epoxycyclopentenone (PECPC) as well as fragmented or truncated products. Truncated PAPC products include 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-phosphatidylcholine [POVPC] and 1-palmitoyl-2-glutaroyl-sn-glycero-phosphatidylcholine [PGPC] and have carbonyl groups that readily form adducts with other biomolecules especially proteins (23,153). OxPLs can bind covalently to lysine, histidine, arginine and cysteines via Schiff bases or as Michael adducts as well as to the amine groups of other phospholipids. Improvements in MS methodologies have led to the identification of membrane and cytosolic targets of proteins in cultured mouse macrophages that may be modified by OxPLs; these included proteins involved with apoptosis, stress response, lipid metabolism and transport (194).

Using model membranes, OxPLs with an aldehyde or a carboxyl group at their truncated sn-2-chain end e.g. POVPC, PGPC were shown to be released rapidly, to be inflammatory and to increase local membrane permeability whereas oxygenated full-length products PEIPC and PECPC were released more slowly and stabilised membranes (80). This led the authors to suggest that early truncated oxidation products may promote vascular leakiness associated with inflammation whereas the release of full length oxygenated products, perhaps as ROS levels reduce during later phases of inflammation, may support resolution. In support of this hypothesis, the phospholipase catalysed release of 5,6-epoxyisoprostane that is present in PEIPC has been shown to mimic the effects of pro-resolving prostanoids, through Michael addition to essential Kelch Like ECH Associated Protein 1 (KEAP-1) thiols, facilitating Nrf2 activation. Together these studies illustrate how OxPL are involved in induction and resolution of inflammation (24,234).

OxPLs present on oxidised lipoproteins, senescent and apoptotic cells are pattern associate molecular patterns (PAMPs) that are recognised and removed by soluble and cell-associated PRR, including scavenger receptors such as CD36, natural (germ line-encoded) antibodies, vascular endothelial growth factor receptors on endothelial cells and C-reactive protein (23). Downstream signalling pathways in endothelial and mononuclear cells include receptor tyrosine kinase and MAP kinases, that signal through intermediates to promote inflammation by regulating Activator Protein 1 (AP1), NF-κB, PPARγ and Nrf2 pathways leading to the increased expression of cytokines and chemokines including TNFα, IL-1β, IL-6, IL-8, MCP-1, MIP2, the lipid oxidation enzyme cyclooxygenase (COX)-2, and the homing receptors CCR1, CCR2 and CCR5 (22). In endothelial cells, a high concentration of oxidised PAPC was shown to disrupt barrier function due to increases in intracellular ROS and downstream activation of Src (193) that catalysed the phosphorylation of the adherens junction protein vascular endothelial cadherin (VE-cadherin) at Tyr-731 and Tyr-658. This was not observed in endothelial cells treated with low OxPAPC concentrations, which conversely showed potent barrier protective effects (193) (19). Additionally, OxPCs with different chain lengths also show opposite effects on barrier function where short chain OxPCs are reported to have disruptive barrier function whilst long-chain OxPC induce protective barrier function (19,60,117).

Elevated concentrations of fragmented OxPLs exert proinflammatory effects in monocytes and promote foam cell formation but, in contrast to LPS, do not activate granulocytes. Similar to the effects of OxPL on endothelial cells, it has been suggested that proinflammatory effects of OxPLs on monocyte/macrophages may be observed more frequently in sites of local to lipid accumulation sites (136). In contrast, at distant sites and with lower concentrations, OxPL may be anti-inflammatory, possibly by competing with PAMPs for TLR4 binding. Consistent with this, it has also been shown that when mouse macrophages are treated with OxPLs, they undergo differentiation resulting in a phenotype that is less phagocytic and with higher Nrf2 activation. However, these authors also fractionated the OxPLs and observed that truncated species of OxPAPC induced reprogramming of macrophage metabolism (increased *Glut1* expression, the major glucose transporter) to support antioxidant gene expression (e.g. haemoxygenase 1, *Ho1* thioredoxin reductase, *Txnrd1*; and *Gclm,* glutamate cysteine ligase*)*, while the full-length OxPAPC treated macrophages showed increased expression of cytokines and chemokines e.g. *Il-1β*, *Il-6*, and *Cxcl1* genes encoding interleukin-1β, interleukin-6 and the chemokine (C-X-C motif) ligand 1 (174,175).

The increased production of IL-1 β by OxPAPC-treated monocyte macrophages and endothelial cells indicates activation of the inflammasome by OxPAPC. Again pleiotropic effects of OxPAPC on inflammasome activation are described; it has been shown by some authors that OxPAPC binds directly to caspase-4 and caspase-11, competes with LPS binding, and consequently inhibits LPS-induced pyroptosis, IL-1β release and septic shock (32), whereas others have shown that POVPC injection resulted in the production of caspase-1, IL-1β, and IL-18 in wild-type, but not in NLRP3-deficient, mice. Furthermore, POVPC-induced inflammasome activation was dependent on an increase in mitochondrial ROS; ROS were increased following POVPC-mediated increases in intracellular Ca^{2+} signaling and mitochondrial destabilisation (229), after transient POVPC stimulation of transient receptor potential channels (7). Collectively, these studies illustrate that OxPL are effectors of bioenergetic switch, mitochondrial ROS production and altered redox state, potentially at distant sites, and their local concentrations may influence whether a pro- or anti-inflammatory phenotype predominates.

B. Isoprostanes

Prostaglandin D_2 and prostaglandin E_2 GPCR receptors are important targets for prostanoid-like products formed from OxPAPC and PEIPC, but not POVPC (70). Common stable products of non-enzymatic free radical attack on phospholipids are isoprostane-like structures and chain-shortened products containing carboxylic acid groups, carbonyl and hydroxyl groups. These include arachidonic acid-derived F2α-isoprostanes and other isoprostanes from α-linolenic, eicosapentaenoic and docosahexaenoic such as F1-phytoprostanes, F3-isoprostanes and F4-neuroprostanes respectively (212). F2α-isoprostanes regulate vasoactive, mitogenic and inflammatory properties but also may have inhibitory action via cAMP signalling from the thromboxane A2 receptor (TBXA2R) on platelets (89). Formation of F2α-isoprostanes is significantly increased in age-associated diseases such as obesity, diabetes and atherosclerosis (16,195). F2α-isoprostanes may also compete with receptors to inhibit drug action – it has been suggested that aspirin-insensitivity in cardiovascular diseases may be due to TBXA2 prostanoid receptor activation by F2α-isoprostanes (16). The rearrangement of F2α endoperoxide intermediates results in the formation of D_2/E_2-isoprostanes which can undergo further rearrangements generating the cyclopentenone A/J-isoprostanes (77). In contrast to the inflammatory effects of isoprostanes, the cyclopentenone deoxy-A2/J2-isoprostane-phosphocholines 15d-PGJ2 and 15d-PGJ2-PC, formed from 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, prevent foam cell formation, induce anti-inflammatory and anti-oxidant responses in macrophages through targeting redox sensitive thiols within the NF-κB, PPARγ and Nrf2 pathways (77,175). Some suggest that it is Nrf2 that is largely responsible for the anti-inflammatory actions of oxidised lipids (59).

Table 1 summarises the biological effects of OxPL and isoprostanes on inflammation and atherosclerosis, illustrating both pro- and anti-inflammatory effects (110).

C. Nitrated fatty acids

RNS derived from •NO, including ONOO-, nitrogen dioxide ($^{\bullet}NO_{2)}$ and nitrous oxide can also oxidise and nitrate unsaturated fatty acids via the homolytic addition of ($^{\bullet}NO_{2)}$ to a double bond, to form nitrated lipids (21,132). In addition, •NO terminates peroxyl radical-induced chain propagation reactions of lipid peroxidation at a faster rate than tocopherol. Nitration specific products from arachidonic, linoleic and oleic acids include nitroalkenes, nitro-nitrile esters, nitrohydroxy species and β-nitroalkyl radicals. In addition, hydrogen abstraction may also occur, producing similar fragmented peroxidation products to oxygen radical species attack (21,108). Nitrated fatty acids were first described in 2003 and are considered to be protective in the vasculature; they are the subject of several reviews (91,206). They have potent electrophilic activity, forming adducts with proteins though Michael addition reactions (8,170). Their formation has been reported in the plasma membrane, mitochondria, lipoproteins and triglycerides and they may be transported to distant sites within lipoproteins (54). Free nitro-fatty acids are found at very low concentrations in the circulation, probably released by phospholipases (162). They can target extracellular receptors through nucleophilic attack and may be taken up in the esterified form by scavenger receptors. Intracellular nitrated fatty acids contribute to reversible and exchangeable nitrated fatty acid-thiol adducts occur under biological conditions (162) and are effective inhibitors of NF-kB; alkylation of macrophage p65 by nitroalkene fatty acids inhibits NF-kB DNA binding activity and represses downstream inflammatory target gene expression (36). Nitrolipids are also potent agonists of PPARγ and Nrf2, binding to thiol on

KEAP1 and releasing Nrf2 which directs antioxidant and anti-inflammatory and metabolic gene expression in monocytes/macrophages and endothelial cells. There are intrinsically different potencies of regioisomers of nitro fatty acids (8). In contrast to ROS-inducing activity of OxPLs, nitrolipids have been reported to inhibit activation of the NADPH oxidase via nucleophilic attack on thiol residues in p47 Phox (109), possibly involving the formation of a covalent Michael adduct between NO_2-AA and critical nucleophilic residues (*e.g.* histidines or cysteines) preventing the formation of the active complex. Within mitochondria, mild uncoupling by nitrolipids has been described which in turn promoted adaptation during ischemia and reperfusion, a sterile inflammatory response involving production of ROS (126,127), and cardioprotection.

To date there are no reports describing any change of nitrolipids with age, however, it is known that endothelial NOS efficiency decreases with age due to uncoupling (44), potentially reducing the rate of anti-inflammatory nitro-lipid formation and increasing $ONOO^-$-mediated lipid peroxidation. Furthermore, our previous study has shown that the median concentration of plasma linoleic acid is reduced by 50% in healthy adults over 50 years, reducing availability of a major substrate for nitration by $^{\bullet}NO$ (145). The in situ generation of nitrolipids within mitochondria highlights their significant role for coordinating the regulation of metabolism and inflammation, driving an anti-inflammatory phenotype (213), and efforts to increase eNOS coupling may contribute to anti-inflammatory activity via nitrolipid formation to mitigate risk and severity of inflammageing and atherosclerosis.

3.2 Oxysterols, inflammation and age-related vascular disease

ROS oxidise the unsaturated rings of cholesterol yielding several oxysterols that differ in the position of hydroxyl group addition. In addition to free radical mediated oxidation, some oxysterols are formed by specific enzymatic oxidation reactions to produce short-lived intermediates that are involved in cholesterol excretion pathways; they may also be taken up in the diet (Figure 4). Compared to highly abundant cholesterol that circulates in blood at millimolar levels, oxysterols are reported to found in 1,000-10,000 times lower concentrations around micromolar to submicromolar levels in human plasma (40). Regardless of the source or the concentration, oxysterols are taken up by a range of receptors (LDL receptors, EBI1; Epstein-Barr-virus-induced G-protein coupled receptor 2, also known as GPR183) , scavenger receptors, G-protein coupled receptor 17 GPRI7, C-X-C Motif Chemokine Receptor 2 CXCR2, glucocorticoid receptor, purinergic P2X7 receptor, Smoothened, Frizzled Class Receptor SMO, Glutamate Ionotropic Receptor NMDA Type NMDA, NPC Intracellular Cholesterol Transporter 1 NPC1; see Figure 4) and exert a range of biological effects. At high concentrations, oxysterols are cytotoxic and pro-atherogenic compared to cholesterol, especially in vascular cells.

Similar to other oxidised lipids, addition of a hydroxyl, epoxide, hydroperoxyl, carboxyl or ketone moieties to the steroid nucleus or to the side chain, increases the polarity of oxysterols (20,73). To date, the subcellular distribution of oxysterols has not been characterised, although increased concentrations are predicted to occur in mitochondria during ageing, which may influence cellular metabolism. In membranes, a change in behaviour in micro environments by oxysterols has been shown to affect membrane fluidity (55), cell signalling and metabolism (27,68). Oxysterols are stable species that are found within lipoproteins or free in serum; they can diffuse within LDL to distant sites,

may cross the blood brain barrier (42,111,200) and are taken up by discrete receptors to influence cell cholesterol and mitochondrial metabolism (Figure 4).

Enzymatically produced oxysterols have been studied in relation to health and vascular diseases. The cytochrome P450 family gene, *CYP27A1*, encodes a mitochondrial enzyme which catalyses the formation of 27-hydroxycholesterol (27-OHC) and modulates the acidic biosynthetic pathway for bile acids. 27-OHC is the most prevalent enzymatically produced oxysterol in human circulation ranging from 150- 730 nM but increases more than two fold in atherosclerotic lesions (25). As competitors for binding estrogen receptor α, atherogenic properties of 27-OHC have been attributed to the attenuation of estrogen-related atheroprotection (208). Additionally, 27-OHC induces adverse effects in the brain by passing through the blood-brain-barrier and interrupts local cholesterol homeostasis (120).

We have shown previously that 27-OHC alters the distribution of membrane microdomains in neuron-like cells to increase the activity of beta secretase to produce amyloid beta (41) in vitro. In neurones, cholesterol is converted into 24S-hydroxycholesterol (24-OHC). Excess 24-OHC crosses the blood-brain-barrier to reach the blood and is metabolised by the liver, converted into biliary acids and eliminated into the bile. Both 24-OHC and 27-OHC increase proportionally to the number of e4 alleles in individuals with cognitive decline and a positive correlation has been reported for APOE genotype and 24-OHC levels in cerebrospinal fluid from patients with Alzheimer's disease and mild cognitive impairment (143). Another prospective ageing study reported that lower cholesterol present in ApoE epsilon 4 carriers was related to a higher rate of decline of information processing speed and a higher ratio of 27-OHC to cholesterol was related to a lower level of general performance and memory functioning. The authors concluded that lower total cholesterol and high oxysterol levels may be considered as a frailty marker, predictive of lower cognitive functioning in the elderly (209).

Both 27-OHC and 25-hydroxycholesterol (25-OHC) regulate immune cell function. 25-OHC is synthesised by the enzyme cholesterol 25-hydroxylase. Initial studies by Park and Scott revealed that 25-hydroxylase gene expression is low in resting macrophages under standard cell culture conditions but is rapidly induced by two orders of magnitude when cells are activated with TLR ligands (146). Other oxysterols, 7α-OHC and 7α, 25-dihydroxycholesterol (7α,25-OHC) are reported as a high-affinity ligands for the inflammatory receptor EBI2/ GPR183 that is induced in B cells upon viral infection (149).

In addition to a direct antiviral role, 25-OHC also regulates transcription of inflammatory genes. 25-OHC amplified the TLR-induced gene expression through a positive feedback response mediated, at least in part, via the transcription factor, AP1 (67). The induction of pro-inflammatory cytokines by 25-OHC is also dependent on increased NFκB activation, most likely following the activation of p38 MAPK and JNK (185). Umetani et al reported the activation of endothelial and macrophage NFκB pathway by 27-OHC (208). Peritoneal macrophages from 27-OHC treated mice upregulated mRNA level of TNF-α, IL-1β and IL-6 by 3 to 6 fold and promoted monocyte-endothelial cell adhesion through estrogen receptor (ER)-α driven pathway (208). Several closely related sterol regulatory transcription factors are regulated by oxysterols including LXR, RXR, RORα, RORβ, RORγ, ERα. Some oxysterols are able to induce metabolic reprogramming upon uptake; they are involved in polarising M1 to M2 macrophages through the LXR transcription factor (76). In this process, the oxysterol activated LXR pathway drives macrophages to recognise dead cells, promotes cholesterol efflux and down regulates inflammatory

gene expression, further illustrating the relationship between oxidised lipids, metabolism and inflammation.

Anti-inflammatory properties of oxysterols have also been reported; Vurusaner et al have shown that low micromolar concentrations of 27-OHC can activate the redox sensitive transcription factor, Nrf2 and subsequently the target genes, HO-1 and NQO-1 in monocyte-like cell line, U937 (217). Early and transient generation of ROS levels by 27-OHC enhanced MEK-ERK/PI3K-Akt phosphorylation, which in turn reduced the subsequent ROS production suggesting the ability of oxysterols as pro-survival inducers at low concentration (217,218). In the same cell line, 27-OHC activated an autophagic response by expressing upregulated microtubule-associated protein 1A/1B-light chain 3 (LC3) II/LC3 I ratio and Beclin 1 levels in a MEK-ERK/PI3K-Akt dependent manner (219).

To study the short term effect of free radical mediated cholesterol oxidation products acquired from the diet, Vine et al analysed non-enzymatically produced 7β-hydroxycholesterol (7β-OHC), 7- KC and 5α,6α-epoxycholesterol after two weeks of feeding rabbits with chow supplemented with oxidised cholesterol (containing 6% oxysterols) (214). The authors did not observe any changes to 7β-OHC levels but five times higher levels of 5α,6α-epoxycholesterol and double the levels of 7-KC were found in triglyceride-rich lipoproteins from oxidised cholesterol-fed animals compared to the purified cholesterol-fed animals. The oxidised cholesterol-fed animals also had a 64% increase in total aortic cholesterol. This is consistent with the observation that 7α-OHC, 7β-OHC and 7-KC are found in relatively high concentration in foam cells and fatty streaks (26). Foam cells are formed after oxidised lipid loading into recruited macrophages; and it is estimated that oxysterols comprise up to 50% of total sterol content of OxLDL-loaded cells (26). The underpinning mechanism of plaque formation is likely to be via 7-KC enhanced leukocyte-endothelial interactions (199). In an attempt to prevent foam cell formation, macrophage lysosomal lipase hydrolyses the ingested cholesteryl esters. In concert, 25-OHC is synthesised, probably in mitochondria where it maintains membrane integrity, and prevents mitochondrial ROS production and NLRP3 inflammasome activation. Lysosomal lipase activity is also required for LXR-mediated activation of cholesterol efflux (Figure 4) (211); free intracellular cholesterol is transported to the mitochondria via steroidogenic acute regulatory protein where CYP27H catalyses oxysterol synthesis. 27-OHC is a regulatory oxysterol that not only activates LXR and mediates lipid efflux, but also modulates PPARγ activity and suppresses IL-6, TNFα and IL-1β production by macrophages (133). This evidence supports an important role for oxysterols in regulation of inflammation via the mitochondrion.

In summary there is accumulating evidence for a role of oxysterols in regulating inflammation. The biological effects may be pro- or anti-inflammatory according to oxysterol concentration. However, whether they undergo covalent interactions or act as partial agonists/antagonists remains to be determined.

3.3 Eicosanoids, inflammation and age-related vascular disease

The eicosanoids are another group of oxidised lipids that integrate with metabolism, regulating both the initiation and resolution of inflammation (**Figure 5**)(178,180). The immune system sentinels, neutrophils and monocytes/macrophages, require a directional signal both to migrate to the site of tissue injury, during the acute inflammation phase, and back out, removing cellular debris and clearing out invading pathogens without tissue injury. The cardinal 'signals' come in the form of

chemoattractant bioactive lipids that are produced by the coordinated action of receptors, phospholipases and oxygenases. Thus, they are considered to play key roles driving both pro-inflammatory and pro-resolving responses(179). While they are also electrophilic in nature and are formed through enzyme-controlled free radical reactions, the biological effects are not mediated through covalent modification of targets but rather as conventional ligands for receptors and transcription factors.

Pro-inflammatory lipid mediators are produced and released by the host cells with an aim to create chemotactic gradient that will support transendothelial migration of professional phagocytes to the site of injury. They include eicosanoids, namely leukotrienes (Lts)(147,166), prostaglandins (PGs)(154) and thromboxanes (TXs)(202), Figure 6. These lipid mediators are synthesised from membrane-derived AA within minutes of an acute challenge. Phospholipase A2 (PLA2) hydrolyses phospholipids at the sn2 position to generate fatty acid substrates for oxidation by cyclooxygenases (COX) and lipoxygenases (LOX).

Production of **leukotrienes** from free AA is initiated by the enzyme 5-lipoxygenase (5-LOX). The most potent of all, leukotriene B_4 (LtB_4) is produced mainly by neutrophils and pro-inflammatory macrophages. By ligating to G-protein coupled receptor BLT1/2, LtB_4 supports further recruitment and activation of circulating leukocytes to the site of cellular injury(198).

Metabolism of AA by cyclooxygenase (COX) and thromboxane synthase leads to the formation of **thromboxanes**. Thromboxane A_2 (TXA_2), mainly produced by monocytes and macrophages, exerts its pro-inflammatory actions through binding to the thromboxane receptors TP that belong to the family of G-protein coupled receptors(201). Thromboxane is best known for being a potent platelet aggregant and vasoconstrictor(130).

The **prostaglandin** profile in tissues depends on the activity of COX-1 and COX-2, enzymes with both cyclooxygenase and peroxidase activity(189). During homeostasis COX-1 is the dominant catalyst in formation of prostaglandins. Upon receiving an inflammatory stimulus, the expression of COX-2 becomes upregulated, leading to the synthesis of pro-inflammatory prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)(202). Hence, the prostaglandin profile is dependent on both the levels of expression of enzymes responsible for their synthesis and the cell activation state. Indeed, TXA_2 is produce by the resting macrophages, while the synthesis of PGE_2 is favoured upon cellular activation.

The actions of $PGF_{2\alpha}$, are mediated by its individual receptors FP, PGD_2 and PGE_2 activate multiple receptors that are differentially expressed in immune cells, thereby triggering immune responses(202). In the context of this review, the most important cellular response mediated by PGE_2 and PGD_2 is the lipid mediator "class switch". While both known for their pro-inflammatory activities(58), PGE_2 and PGD_2 each promote a switch in the upregulation of key enzymes to promote synthesis of dual-acting specialised pro-resolving lipid mediators of inflammation (SPM)(100).

The dual-acting nature of SPM is reflected in their ability to selectively stop further infiltration of neutrophils to the site of acute challenge while supporting pro-resolving futures, such as recruitment and activation of monocytes without pro-inflammatory stimuli (non-phlogistic stimulation), phagocytosis of apoptotic cells and microorganisms, exit of phagocytes via lymphatics and upregulation of antimicrobial agents(177,178).

Lipoxins, namely lipoxin A_4 (LxA_4) and lipoxin B_4 (LxB_4), are the first SPM to be recognised for their dual-acting nature(176). They are derived from AAin a form of trihydroxy derivatives via two different routes. For example, in mucosal tissue, lipoxins are released due to the interactions between leukocytes and epithelial cells. Here, synthesis of lipoxins is initiated in epithelial cells by the 15-lipoxygenase (15-LOX), leading to the formation of 15-hydroxy-eicosatetraenoic acid (15-HETE) intermediate. This substrate is provided to neutrophils for the final synthesis of lipoxins by 5-LOX. In blood vessels lipoxin synthesis is ensured by an interplay between leukocytes and platelets. Leukocyte-derived 5-LOX metabolises AA to form epoxide intermediate LtA_4. Studies have shown that more than 50% of LtA_4 is further metabolised by platelet 12-lipoxygenase (12-LOX) to produce lipoxins(47,56,160). In macrophages, a unique two-step activation mechanism ensures controlled production of lipoxins; first TLR4 activation results in accumulation of an esterified form of the COX-2-derived lipoxin precursor 15-HETE which is stored within membrane phospholipids. Subsequent activation of P2X7 by extracellular ATP leads to phospholipase A2 activation, hydrolysis of 15-HETE from membranes and its conversion to lipoxins by 5-LOX, linking inflammasome activation to resolution (134). LxA_4 binds to lipoxin A4 receptor (ALX) on leukocytes thereby triggering cell-specific responses. In neutrophils, this interaction will stop neutrophil chemotaxis, adherence(57,101,144) and transmigration into the inflamed sites(86,184), while in monocytes it stimulates chemotaxis(112) and non-phlogistic responses(66,123). LxA_4-ALX interaction in T cells blocks secretion of TNFα providing the link between the innate and adaptive immune systems (94).

E-series resolvins, namely resolvin E1 (ResE1) and resolvin E2 (ResE2), are derived from 18-hydroxy-eicosapentaenoic acid (18-HEPE) via two pathways. EPA can be converted into its hydroxy derivative in the presence of aspirin or by the cytochrome P450 enzymes, which can be transformed by 5-LOX in leukocytes (mainly neutrophils) to ResE1 and ResE2. ResE1 exerts its dual-action nature by interacting with at least two G protein coupled receptors. Interaction with BLT1 on neutrophils prevents transendothelial migration(10,181) while interaction with chemokine-like receptor 1 on monocytes supports non-phlogistic phagocytosis of apoptotic leukocytes(173). In peripheral blood leukocytes, ResE1 prevents the decrease in mitochondrial respiration, membrane potential, and the imbalance of mitochondrial fission and fusion that is induced by TNFα; since mitochondrial fission alone can induced proinflammatory cytokines, a novel anti-inflammatory mechanism of ResE1 may be through maintenance of mitochondrial integrity (79).

Docosahexaenoic acid (DHA) is a PUFA that is highly enriched in brain, retina and synapses(17,165). Hence, **D-series resolvins** (ResD1-D4) derived from DHA via metabolism by 15-LOX/5-LOX(183) are of a great importance as 'guards' of neural tissues(114). It is important to note that synthesis of D-series resolvins can be also triggered with aspirin. In microglia, both ResD1 and its aspirin analogue indirectly block synthesis of pro-inflammatory interleukin-1β, which is rapidly released following brain injury(83). They also protect macrophages from efferocytosis-induced death via ResD1 mediated activation of cAMP-PKA signaling, which in turn inhibits p47 phox phosphorylation, suppressing NOX activation and limiting further oxidative damage (99). In the liver during ischemia reperfusion, ResD1 regulates mitophagy, mitochondrial biogenesis and mitochondrial fission via thioredoxin 2, and reduces mitochondrial oxidative stress (88). In another elegant study in macrophages, the binding of ResD2 to the GPR18 receptor was shown not only to inhibit the priming but also to expedite the deactivation of the NLRP3 inflammasome during the resolution process, probably by preventing apoptosis-associated speck-like protein oligomerisation at the mitochondria and inflammasome assembly (107).

Protectins represent another class of bioactive lipid mediators produced from DHA via lipoxygenase pathway, with protectin D1 (PD1) being the most potent(12). They are produced by neutrophils, macrophages, T cells, glial cells and retinal pigment epithelium(45). Although they it is known that they can bind leukocytes and retinal pigment epithelium cells, the exact binding partner is yet to be determined(115). PD1 upregulates CCR5 expression in neutrophils(9) and stimulates non-phlogistic uptake of apoptotic leukocytes by macrophages(173). **Maresins** represent the third class of SPM derived from DHA via metabolism by 12-LOX/5-LOX. The exact receptors for maresins remain unknown. However there is an evidence that maresins limit neutrophil transendothelial migration(182), promote uptake of apoptotic leukocytes by macrophages(102), and shorten the resolution phase(197).

During ageing, inflammageing is associated with lower SPM concentration (11). Lower maresin, ResD and ResE concentrations during ageing associate with poor cardiovascular outcomes (75). Lower SPM concentration effects on heart function relate to a combination of altered metabolism and increased dietary intake of n-6 fatty acids that correlate with inflammation and oxidative stress (87). In support, delivery of resolvins in nanoparticles can reverse a decline in cardiac function in animal models by promoting efferocytosis and phenotype switching [145]. In a balloon model of vascular injury, both ResD1 and D2 inhibit vascular smooth muscle activation, monocyte recruitment and ROS production (124) and ResD2 promotes revascularisation post-ischemia via GPR18 receptor dependent mechanisms (233). In the brain, histological post-mortem brain studies from people with cognitive impairment but not healthy people have also shown increased levels of the cyclooxygenase, COX-2, greater oxidative damage and more oxidised lipid deposition (28,62,164). Furthermore, the eicosanoid precursors DHA and AA, are significantly dysregulated in the brains of patients with varying degrees of Alzheimer pathology (2,190). Therapeutic activation the resolution pathways has great potential for the treatment of inflammatory diseases. Could appropriate nutrition be a link between innate and adaptive immune system? Studies with n-3 PUFAs supplementation for inflammatory resolution and neuroinflammation have not shown consistent outcomes (1,85,164). It is known that older adults lack delta-6-desaturase (D6D) activity when compared to younger adults (145) which is required for conversion of fatty acids to n-3 PUFAs. In the absence of D6D, AA may predominate as a proinflammatory substrate for COX-2. Furthermore, before tissue-specific treatments become available, it is important to address whether dietary supplementation with PUFA or SPM intermediates can be beneficial for the local production of SPM, or if their supplementation in excess may have deleterious effects due to the increased risk for auto-oxidation. Instead, novel approaches that address mitochondrial quality may prevent the activation of the inflammasome (31,98), generation of high concentrations of oxidised lipids and enable macrophage differentiation to a resolving M2 phenotype and yielding anti-inflammatory outcomes.

This review is focussed on involvement of oxylipins in inflammation and its resolution with relation to age-related vascular disease. The vasculature itself is exposed to a range of oxygen tension, but the variance is less than in other tissues. It is worthy of note that some studies that have explored oxylipins under physiological conditions of extreme oxygen tension, for example in the lung (higher oxygen tension) or exercising muscle (hypoxia). The free-radical dependent formation of oxidised phospholipids and fatty acids is increased during hyperoxia and during reperfusion post-ischemia, however, their biological effects in lung and muscle are similar to those reported in the vasculature with both pro- and anti-inflammatory effects being described (119,161,232). Nevertheless, there is a

consistent view that the enzyme catalysed eicosanoids play an important role in the regulation of inflammation and may be targeted to improve muscle regeneration (30,65,233).

3.4 Analytical challenges of measuring products of lipid oxidation

The foregoing discussion has highlighted that oxidised lipids generated from PUFA, cholesterol and phospholipids through enzymatic or free radical mediated reactions are structurally similar, with similar chemical and physical properties. These properties make it difficult to simultaneously separate, and accurately identify and quantify oxidised lipids in biological samples (103,104). Since the regulation of different lipid oxidation species may shift the balance between inflammatory and anti-inflammatory status, and because these processes are fine-tuned, accurate and precise quantitation are important. It is also noteworthy that most of the oxidised lipids are unstable compounds that can degrade or further oxidise during sample collection, handling and storage. Therefore, special care and attention is needed starting from the point of experimental design. These challenges have been reviewed by others (152,168), which emphasise the need of standardised approaches to sample preparation lipid extraction and storing lipid oxidation products. For example, removal of highly abundant parent lipids (cholesterol, phospholipids) and concentration of oxidised species are required to avoid interference and to optimise detection limits. Techniques such as sample evaporation, liquid-liquid extraction and solid phase extraction have been reported in literature. Each of these techniques has their limitations on sample recovery and extraction efficiencies (104). Advances in analytical techniques, availability of high sensitive instruments and column chemistries are playing a major role in overcoming these challenges (43). However, still the complex nature of lipid species and their interaction with solid phase matrix in different strengths may pose challenges to extract all species with a same efficiency (116).

In recent years, much effort has been invested to accurately quantify lipid oxidation products from minimum sample volumes (4,5,192). The development of ultra-high performance liquid chromatography methods with short runtimes using targeted and sensitive quantification using quadrapole ion trap mass spectrometers has been successfully applied to different types of biological samples (6,163,221) and is likely to revolutionise our understanding of the spatial and temporal distribution of these modified lipids.

4. Flavonoids mitigating lipid oxidation, inflammation and age-associated vascular disease

Two classes of phytochemicals that are less-well studied but have positive age-related health outcomes are the oxocarotenoids and flavonoids. Oxocarotenoids are metabolised by mitochondria, promote membrane integrity and vascular risk factors. On the other hand, in the PREDIMED study the increase in circulating flavonoids were associated with preventing negative cardiovascular outcomes in older adults following dietary supplementation with olive oil and with or without nuts, and for type diabetes, catechins, proanthocyanins and hydroxybenzoic acid afforded the greater protection (205). The next two sections focus on the mechanisms and effects of these phytochemicals on oxidised lipids; despite being under-investigated in clinical studies, they show promise to exert anti-inflammatory effects and protect against age-related vascular disease.

Diet is a major determinant of healthy aging and the flavonoid phytonutrients may extend healthy lifespan and prevent age-related vascular mortality (53,227). This hypothesis is based on human epidemiological and clinical intervention studies and lifespan studies in model species. The mechanisms are not fully understood and include modulation of nitric oxide-dependent arterial function (106,171) regulation of NADPH oxidase, (158) immune and inflammatory function modulation via Nrf2 activity modulation of the inflammasome, (49) CNS/neuronal cell function

modulation (13). Pre-clinical studies in mice have shown that flavonoids may attenuate inflammation through inhibition of the inflammasome and promoting mitochondrial biogenesis is also enhanced in many tissues (125,225). Mitochondrial biogenesis increases the efficiency of mitochondrial metabolism and promotes differentiation to anti-inflammatory phenotype.

Flavonoids are the biggest group of polyphenolic secondary plant metabolites found in human diets (216). Major classes of flavonoids are flavan-3-ol monomers, theaflavins, proanthocyanidins, anthocyanidins, flavones, flavonols, and flavanones - they differ in their abundance in foods, absorption, metabolism, distribution, and excretion (156). An important reason for the lack of clarity of molecular targets and mechanisms is the likely importance of considering the absorption, distribution, metabolism, and excretion (ADME) of flavonoids when investigating their mechanisms; this was previously underappreciated (138). Most flavonoids undergo significant metabolism during absorption and therefore, the circulating metabolites significantly differ from the flavonoids in food. For instance, flavanols in cocoa consist of monomers, mainly (-)-epicatechin (20%) and procyanidins (80%) which are oligomers with a degree of polymerisation of up to 10 monomers. However, recent research has shown that (-)-epicatechin is methylated, sulphated and glucuronidated during absorption in the small intestine while procyanidins are not absorbed as such and are broken down by the gut microflora, reaching the circulation much later e.g. as valerolactones (139). While studies in the past have shown that many polyphenols including (-)-epicatechin and procyanidins can mediate a number of effects in vitro including inhibition of NADPH oxidase with the half maximal inhibitory concentration (IC50) values in the micromolar range, these are seldomly reached in vivo limiting the understanding of how flavanols mediate health benefits in humans in vivo (169). More recent work with structurally related (-)-epicatechin metabolites at physiologically relevant concentrations in human endothelial cells using systems biology-based network analysis suggests that epicatechin metabolites trigger complex nutri(epi)genomic changes that subsequently modulate endothelial cell adhesion function and permeability. This study also identified key molecular and cellular targets of epicatechin associated with their vascular protective effects *in vivo* (121). Flavanol metabolites reduce monocyte adhesion to endothelial cells through modulation of expression of genes via p38-MAPK and p65-NF-kB pathways (33). Whether there is any effect on concentration of (per)oxidised lipids is unknown. Comparison of the 253 differentially expressed genes with the 4787 TNFα-modulated genes revealed an overlap of 66 genes. Interestingly, among these 66 common genes, 44 presented opposing gene expression profiles, suggesting that epicatechin metabolites could partially counteract the genomic effect induced by TNFα in endothelial cells. Another interesting class of flavonoids are anthocyanins which give blueberries their blue colour. While the parent compounds are not likely to be absorbed in relevant amounts, there are many smaller phenolic degradation products that reach micromolar plasma concentrations and may lead to significant health effects (157). Ex vivo experiments showed that anthocyanins and their gut metabolites can affect adenosine diphosphate-induced platelet activation and their aggregation with monocytes and neutrophils (96) and attenuate monocyte adhesion and transendothelial migration through nutrigenomic mechanisms regulating endothelial cell permeability (95).

Human flavonoid intake varies considerably, and epidemiological data rely on food frequency questionnaires. Based on food consumption data from the European Food Safety Authority and the FLAVIOLA Food Composition Database, it was estimated that in Europe the mean daily intake is 430 mg/d and the median id 160 mg/d with tea and fruit being the main source (216). Data from

anthropological research (82) and observational studies (81) suggested that flavonoids can reduce the risk of ageing-related pathologies such as cardiovascular disease although results from more recent studies were less clear (118,122,215) and the main beneficial effect emerges when comparing very low intake with low intake. Overall, a systematic review suggests that the dietary intakes of six classes of flavonoids, namely flavonols, anthocyanidins, proanthocyanidins, flavones, flavanones and flavan-3-ols, significantly decrease the risk of CVD (222). Unfortunately, randomised controlled or prospective trials studying human morbidity or mortality as an endpoint for flavonoid supplementation are scarce with the exception of the ongoing COSMOS trial (35).

Putative mechanisms that may underlie flavonoid benefit as identified in worms include energy-restriction-like effects, inhibition of insulin-like-growth factor signalling, induction of antioxidant defence mechanisms, lowered inflammation, hormesis, and antimicrobial properties. Lifespan studies show that flavonoids can promote longevity in a range of species (142). In most studies, worms and flies experienced lifespan extension when supplemented with flavonoids either as extracts or single compounds. Studies with mutant worms and flies give hints as to which gene products may be regulated by flavonoids and consequently enhance longevity. Although flavonoids can work as radical scavengers because of their chemical structure; their in vivo antioxidative action is more likely caused by them stabilising and up-regulating the antioxidant transcription factor Nrf2. Interestingly, in flies, cocoa powder was tested in SOD-deficient mutants and it prolonged life-span in flies that did not have the cytosolic SOD1 but was detrimental for flies without the mitochondrial SOD2. These findings suggest that when oxidative stress levels are very high, flavonoids could further increase oxidative damage (14). The metabolome of flavonoids differs widely between mice and men making comparative conclusions difficult (137), and studies in mice are not discussed further here. Readers are directed to a few studies in mice (140,157,172).

Taken together, while flavonoids are promising phytonutrients that may extend a healthy lifespan and limit vascular co-morbidities, this remains to be demonstrated clinically. The underlying mechanisms of individual flavonoids and their circulating putatively bioactive metabolites and their interactions with inflammatory pathways remain to be elucidated taking their ADME into account.

5. Carotenoids, inflammation and age-related dementia and vascular disease

PUFA are highly susceptible to peroxidation. Carotenoids can quench singlet oxygen and scavenge lipid peroxides [9–11]. They are defined into two groups according to their polarity: xanthophylls (polar carotenoids such as astaxanthin, β-cryptoxanthin, lutein, and zeaxanthin) and carotenes (nonpolar carotenoids such as α-carotene, β-carotene, and lycopene). In contrast to carotenes which are metabolised by BCO1 and retained in the cytoplasm, the enzyme BCO2 is associated with the inner mitochondrial membrane and metabolises xanthophylls (which accumulates in the inner mitochondrial membrane) into long-chain apo-carotenoids. BCO2 functions as a key regulatory enzyme that prevents toxicity caused by carotenoid accumulation and loss of BCO2 function is associated with the development of mitochondrial oxidative stress and metabolic diseases [16]. The uptake, distribution and metabolism of carotenoids have been attributed to the activities of different protein transporters (SR-BI, CD36, NPC1L1), digestion enzymes (PNLIP, CES), cleavage enzymes (BCO1/2), intracellular transporters (FABP2) and receptors (LPL, APOC/E, LDLR). It is predicted that carotenoids are widely bioavailable [37]. At a molecular level zeaxanthin not only attenuates lipid oxidation by scavenging mitochondrial ROS but also promotes mitochondrial biogenesis (105). The

oxocarotenoids are effective modulators of the physical properties of both natural and model membranes, increasing their rigidity and thermostability (93). Oxo-carotenoids stabilise membranes to a greater extent than β-carotene; they are incorporated into bilayers and span the membrane with their lipophilic core, being further anchored in the aqueous area with –OH groups, thus functioning like a molecular rivet (74,93).

Several intervention studies have investigated whether carotenoids improve vascular outcomes. Despite the lack of benefit of astaxanthin on vascular risk factors in renal transplant patients (34), a systematic review and meta-analysis has confirmed that increasing tomato and lycopene intake has positive effects on blood lipids, blood pressure and endothelial function (29). Smaller studies have also identified positive effects of lutein in early atherosclerosis (228,235). Previously, we have shown that a decrease of circulating carotenoids and tocopherols was associated in correlated with increased protein and lipid oxidation (148) in dementia and vascular disease patients that was correlated with the degree of cognitive impairment (39). In the MARK-Age study, lower levels of β-cryptoxanthin and zeaxanthin were found among 2220 randomly recruited age-stratified persons, in those who were physically, cognitively or psychologically frail [34]. Notably, carotenoid concentrations were inversely associated with the risk of being cognitively frail after adjusting for confounders (131).

Vascular disease is a common co-morbidity with dementia(15). Evidence from cross-sectional and observational studies has grown for an association between elevated serum cholesterol in mid-life and later development of dementia(187). Furthermore, chronic low-grade inflammation relates to age-related cognitive impairment (150). The epigenetic oxidative redox shift theory of ageing proposes that sedentary behavior associated with age triggers an oxidised redox shift and impaired mitochondrial function inducing an epigenetic vicious circle that promotes inflammation (64).

Conversely, optimal nutrition may negatively influence dementia risk through epigenetic mechanisms. The “LEARn” (latent early life-associated regulation) model suggests that environmental stress – likely and often oxidative stress and/or nutritional imbalance - marks a gene which later during life and in the presence of a secondary trigger, e.g. inflammation, it will be expressed aberrantly causing overt pathology (113). Preliminary studies on the role of vascular- and lifestyle-related preventive strategies show that vascular risk control and lifestyle improvement are indeed able to slow down the progression of cognitive impairment (63). Plasma levels of several lipophilic antioxidant micronutrients are significantly associated, independently of fruit and vegetable intake, with validated, accurate measures of both cognitive and physical performance in persons with mild cognitive impairment (MCI). This represents a further step in the field of nutritional cognitive neuroscience.

Taken together, while carotenoids are promising phytonutrients that may limit vascular co-morbidities, this remains to be demonstrated clinically. The underlying mechanisms of individual oxocarotenoids and their interactions with mitochondria and inflammatory pathways remain to determined. In light of the increasing attention towards the nutritional cognitive neuroscience of carotenoids [43, 44], the use of computerised measures of cognitive performance might be further implemented in future studies investigating their effects on cognitive and physical impairment.

Overall conclusion

Inflammation associates with ROS/RNS production and with changes to the (oxy)lipidome. (Per)oxidised lipids are central mediators in the initiation and resolution of inflammation. Generalised targeting with antioxidants such as tocopherols as a primary prevention in age-related, inflammatory vascular disease has not proven successful. A more nuanced view is emerging where ROS/NO production is desirable during early phases of inflammation and for resolution. Indeed, the cell-targeted amplification of ROS in neutrophils may be required in some circumstances (Reshetnikov et al. 2018) to promote resolution. Phytochemicals that promote resolving oxylipid mediators and improve mitochondrial quality control merit further investigation as inhibitors of underlying sterile inflammation and to mitigate age-related vascular disease.

Table 1

Model	Target	Outcome	OxPL	Reference	Year
HEK, vascular smooth muscle cells	TRPC5 calcium channel	Increase in calcium permeability	PGPC PCPC	(7)	2010
Pulmonary endothelial cells	Cytoskeletal remodelling, Src Kinase mediated VE-cadherin phosphorylation	Reduced permeability with low concentration and full length oxPL. High concentration and fragmented oxPL increase permeability	PEIPC PGPC POVPC	(19,193)	2012, 2013
Myeloid cells	Chemokine and cytokine production via Nrf2	Decreased inflammatory response, Reduced sepsis	OxPAPC with epoxy-cyclopentenone	(24)	2015
Macrophages not DCs	Non-canonical inflammasome pathway, TLR4	Decrease in IL-1b and decrease n sepsis	OxPAPC	(32)	2018
Endothelial cells	Cell adherens junction	Fragment disrupts barrier Complex mix enhances barrier	LysoPC OxPAPC	(80)	2013
Macrophages, smooth muscle	TLR2, TLR4, MD2	Decrease in LPS-mediated inflammatory signalling	Ox PAPC, POVPC, PGPC	(51)	2008
Bone-marrow derived macrophages	Mitochondrial ROS, inflammasome	Increased IL-1b production due to mito-ROS and calcium influx activating NLRP3	POVPC	(229)	2017

HEK = hamster embryonic kidney cells

Figure Legends

Figure 1. Key mediators and cells involved in physiological and sterile inflammation responses

Figure 2. Innate immune recruitment to inflammatory sites; oxidised phospholipids contribute to the chemotactic gradient for recruitment of inflammatory cells. Their local production in the mitochondria results in impaired mitochondrial activity and failure to remove damaged mitochondria exacerbates ROS production. MtDNA and OxPL enhance inflammasome activation and pro-inflammatory cytokine release.

Figure 3. Monocyte and macrophage phenotype and metabolism

Figure 4: Cellular distribution of oxysterols. Cellular cholesterol is maintained by regulatory mechanisms that control synthesis, uptake, metabolism and efflux. Cells uptake cholesterol and oxysterols packed lipoproteins aided by many membrane receptors, such as LDL receptor (LDLR) family, EBI2 , G-protein coupled receptor, GPR17, C-X-C motif chemokine receptor 2 (CXCR2) and scavenger receptors. Inside the cell, endosomes containing cholesterol/oxysterol are transported to Endoplasmic reticulum (ER) mitochondria or peroxisomes. Within ER, sterols are further metabolised with help of many protein complexes including, oxysterol binding protein (OSBP), OSBP-related proteins (ORPs), the cellular nucleic acid binding protein, the sterol regulatory element binding protein (SREBP), insulin induced gene protein (INSIG) and Niemann-Pick protein C1 (NPC1). These metabolic activities within ER and golgi transfer signals to nucleus either to up- or down regulate endogenous cholesterol synthesis via 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) pathway. Cellular cholesterol and oxysterol levels are also sensed a family of nuclear receptors family; Liver X receptors (LXR), Retinoid X receptor (RXR), Retinoic acid receptor (RAR)-related orphan proteins (RORα, RORβ and RORγ) and Estrogen receptor α (ERα). Activation of these transcription factors upregulate multiple genes involved in cellular cholesterol homeostasis, including ATP-binding cassette transporter A1 (ABCA1), ATP binding cassette transporter G1 (ABCG1), and APOE. Mitochondrial translocator protein (TSPO) has high affinity for cholesterol and uptake cholesterol from ER or from lipid droplets. Steroidogenic acute regulatory protein (StAR) transport cholesterol from the outer to inner membrane in mitochondria. Peroxisome proteins ATP-binding cassette transporter D1 (ABCD1) and acyl-CoA oxidase 1 (ACOX1) are also involve in transport of cholesterol and oxysterols.

Figure 5. Lipid-derived mediators of inflammation are key players that drive both pro-inflammatory and anti-inflammatory and pro-resolving responses. Infiltration of neutrophils from blood vessels into the site of injury, and later pro-inflammatory macrophages (M1 macrophages), is supported by leukotriene B_4 and prostaglandin $F_{2\alpha}$. Resolution phase is initiated by the synthesis of prostaglandins that induce "class switch" from pro-inflammatory (LtB_4, $PGF_{2\alpha}$) to the specialised pro-resolving mediators (SPM, such as lipoxins, resolvins, protectins and maresins). Their dual acting nature stops neutrophils from further infiltrating to the sites of injury, while supporting chemotaxis of pro-repair macrophages (M2 macrophages), phagocytosis of apoptotic material, and their exit via lymphatics.

Figure 6. Pathways of formation and molecular structures of eicosanoids

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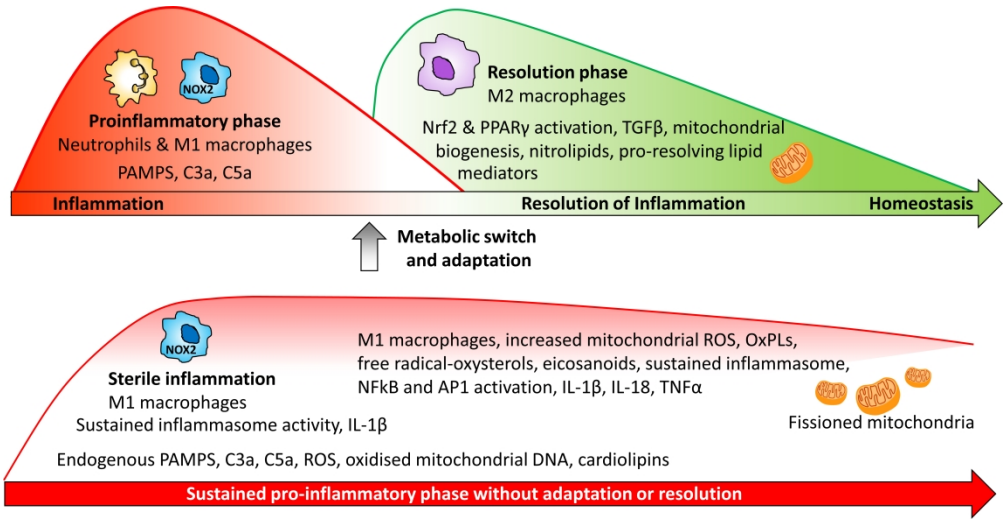
List of Abbreviations

5-LOX	5-lipoxygenase
7α,25-OHC	7α, 25-dihydroxycholesterol
7β-OHC	7β-hydroxycholesterol
7-KC	7-ketocholesterol
12-LOX	12-lipoxygenase
12/15 LOX	12/15 lipoxygenase
15-HETE	15-hydroxy-eicosatetraenoic acid
15-LOX	15-lipoxygenase
18-HEPE	18-hydroxy-eicosapentacnoic acid
24-OHC	24S-hydroxycholesterol
25-OHC	25-hydroxycholesterol
27-OHC	27-hydroxycholesterol
AA	arachidonic acid
ABCD1	ATP-binding cassette transporter D1
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP binding cassette transporter G1
ACOX1	acyl-CoA oxidase 1
ADME	absorption, distribution, metabolism, and excretion
ALX	lipoxin A4 receptor
AP1	Activator Protein 1
COX	cyclooxygenases
CXCR2	C-X-C motif chemokine receptor 2
D6D	Delta 6 desaturase
DAMPS	damage-associated molecular patterns
DHA	Docosahexaenoic acid
EBI2	Epstein-Barr-virus-induced G-protein coupled receptor 2
ER	Endoplasmic reticulum
Gclm	glutamate cysteine ligase
GPCR	G protein coupled receptors
HIF1α	Hypoxia-inducible factor 1-alpha
HMGR	3-hydroxy-3-methylglutaryl CoA reductase
Ho-1	haemoxygenase 1
IC50	inhibitory concentration
INSIG	insulin induced gene protein
KEAP-1	Kelch Like ECH Associated Protein 1
LEARn	latent early life-associated regulation
LC3	light chain 3
LDL	low density lipoprotein
LDLR	LDL receptor
LOX	lipoxygenases
LPS	lipopolysaccharide
LtB_4	leukotriene B_4
Lts	leukotrienes
LxA_4	lipoxin A_4
LxB_4	lipoxin B_4
LXR	Liver X receptors

lyso-PC	lysophosphatidyl choline
MCI	mild cognitive impairment
NADH	Nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate oxidase
NET	neutrophil extracellular traps
NLR	NOD-like receptors
NLRP	nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing
$^{\bullet}NO$	nitric oxide
$^{\bullet}NO_2$	nitrogen dioxide
NOD	nucleotide-binding oligomerization domain
NOS	nitric oxide synthases
NOX	(NADPH) oxidase
NPC1	Niemann-Pick protein C1
Nrf2	nuclear factor erythroid 2-related factor 2
OSBP	oxysterol binding protein
ORP	OSBP-related proteins
OxPLs	Oxidised phospholipids
PAPC	1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine
PEIPC	epoxyisoprostane
PECPC	epoxycyclopentenone
PD1	protectin D1
PGs	prostaglandins
PGE_2	prostaglandin E_2
PGD_2	prostaglandin D_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGPC	1-palmitoyl-2-glutaroyl-sn-glycero-phosphatidylcholine
PLA2	Phospholipase A2
POVPC	1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-phosphatidylcholine
PPARγ	peroxisome proliferator-activated receptor gamma
PUFA	polyunsaturated fatty acids
PRR	pattern recognition receptors
ResE1	resolvin E1
ResE2	resolvin E2
RNS	reactive nitrogen species
RXR	Retinoid X receptor
RAR	Retinoic acid receptor
ROS	reactive oxygen species
SOD2	superoxide dismutase-2
SIRT	sirtuin
SPM	specialised pro-resolving lipid mediators of inflammation
SREBP	sterol regulatory element binding protein
StAR	Steroidogenic acute regulatory protein
TBXA2R	thromboxane A2 receptor

TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TSPO	Mitochondrial translocator protein
TXs	thromboxanes
TXA_2	Thromboxane A_2
Txnrd1	thioredoxin reductase
VE-cadherin	vascular endothelial cadherin



Key mediators and cells involved in physiological and sterile inflammation responses

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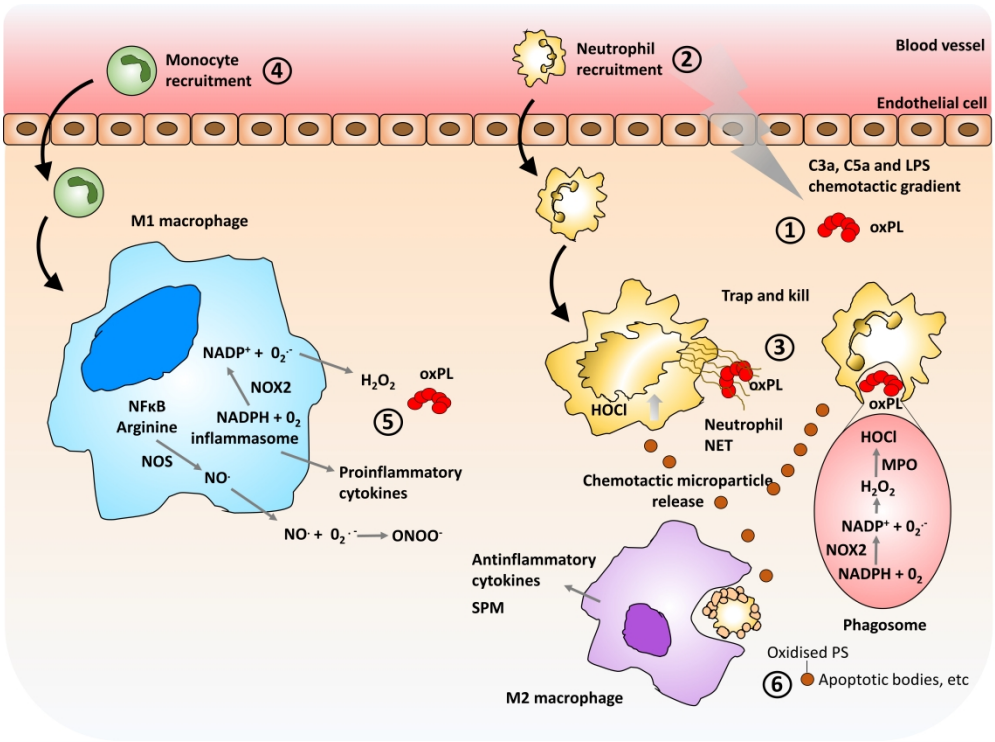

Innate immune recruitment to inflammatory sites; oxidised phospholipids contribute to the chemotactic gradient for recruitment of inflammatory cells. Their local production in the mitochondria results in impaired mitochondrial activity and failure to remove damaged mitochondria exacerbates ROS production. MtDNA and OxPL enhance inflammasome activation and pro-inflammatory cytokine release.

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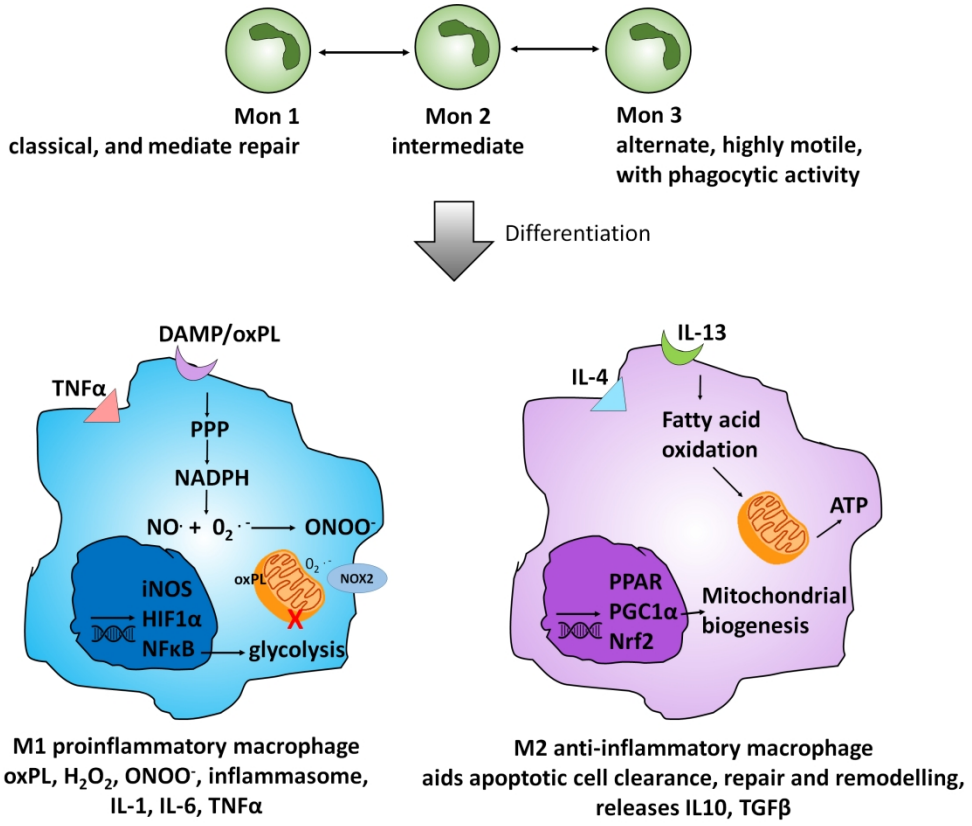

Monocyte and macrophage phenotype and metabolism

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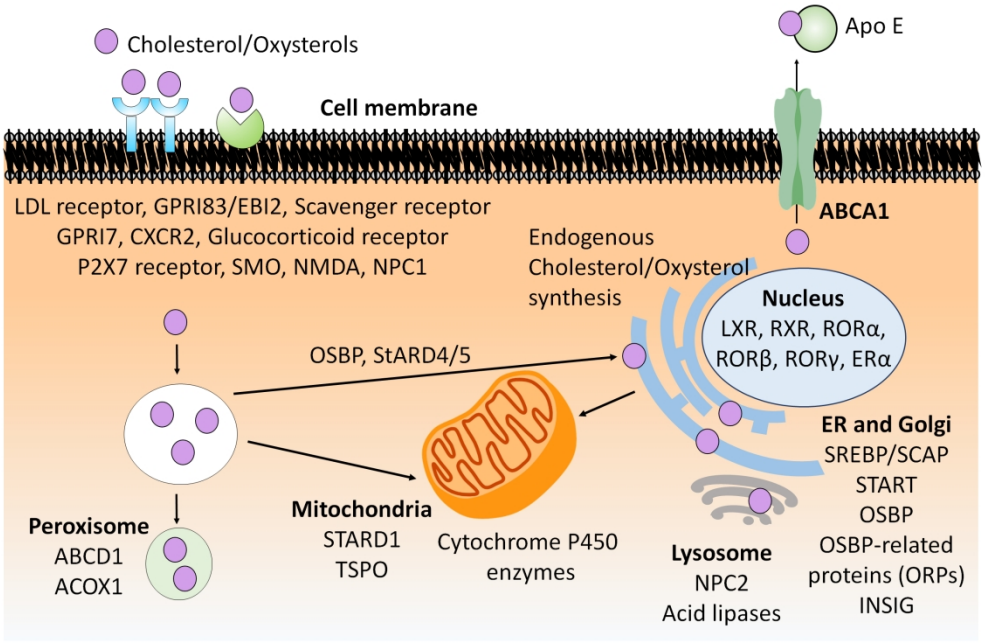

Cellular distribution of oxysterols. Cellular cholesterol is maintained by regulatory mechanisms that control synthesis, uptake, metabolism and efflux. Cells uptake cholesterol and oxysterols packed lipoproteins aided by many membrane receptors, such as LDL receptor (LDLR) family, EBI2 , G-protein coupled receptor, GPR17, C-X-C motif chemokine receptor 2 (CXCR2) and scavenger receptors. Inside the cell, endosomes containing cholesterol/oxysterol are transported to Endoplasmic reticulum (ER) mitochondria or peroxisomes. Within ER, sterols are further metabolised with help of many protein complexes including, oxysterol binding protein (OSBP), OSBP-related proteins (ORPs), the cellular nucleic acid binding protein, the sterol regulatory element binding protein (SREBP), insulin induced gene protein (INSIG) and Niemann-Pick protein C1 (NPC1). These metabolic activities within ER and golgi transfer signals to nucleus either to up- or down regulate endogenous cholesterol synthesis via 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) pathway. Cellular cholesterol and oxysterol levels are also sensed a family of nuclear receptors family; Liver X receptors (LXR), Retinoid X receptor (RXR), Retinoic acid receptor (RAR)-related orphan proteins (RORα, RORβ and RORγ) and Estrogen receptor α (ERα). Activation of these transcription factors upregulate multiple genes involved in cellular cholesterol homeostasis, including ATP-binding cassette transporter A1 (ABCA1), ATP binding cassette transporter G1 (ABCG1), and APOE. Mitochondrial translocator protein (TSPO) has high affinity for cholesterol and uptake cholesterol from ER or from lipid droplets. Steroidogenic acute regulatory protein (StAR) transport cholesterol from the outer to inner membrane in mitochondria. Peroxisome proteins ATP-binding cassette transporter D1 (ABCD1) and acyl-CoA oxidase 1 (ACOX1) are also involve in transport of cholesterol and oxysterols.

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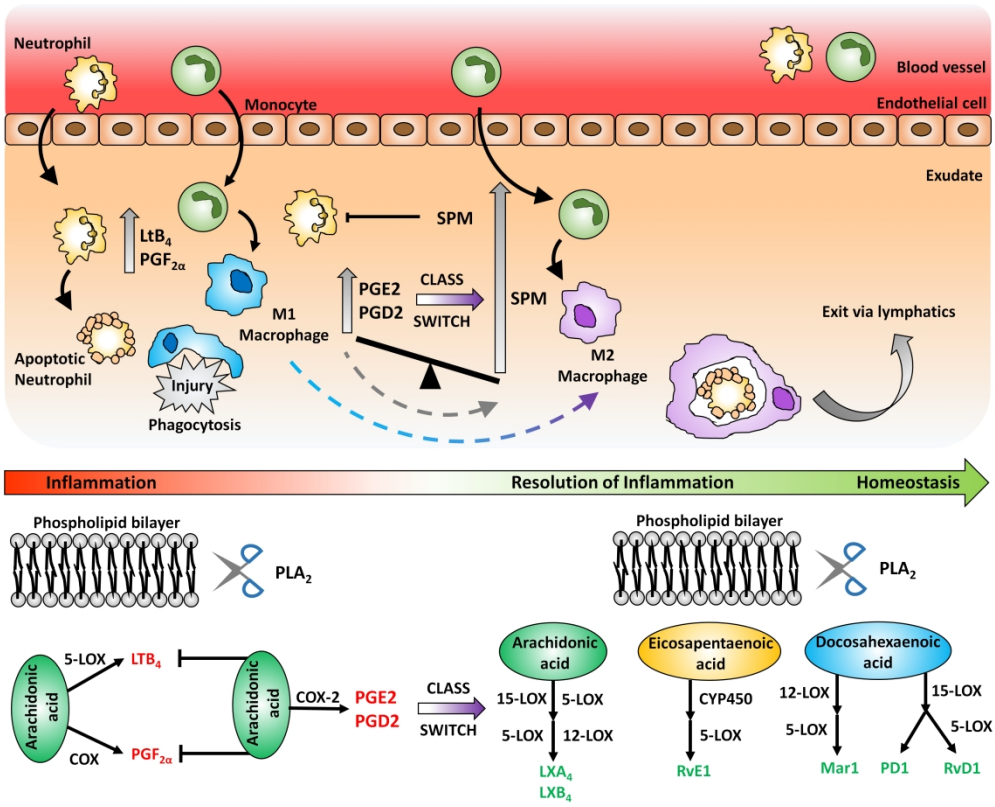

Lipid-derived mediators of inflammation are key players that drive both pro-inflammatory and anti-inflammatory and pro-resolving responses. Infiltration of neutrophils from blood vessels into the site of injury, and later pro-inflammatory macrophages (M1 macrophages), is supported by leukotriene B4 and prostaglandin F2α. Resolution phase is initiated by the synthesis of prostaglandins that induce "class switch" from pro-inflammatory (LtB4, PGF2α) to the specialised pro-resolving mediators (SPM, such as lipoxins, resolvins, protectins and maresins). Their dual acting nature stops neutrophils from further infiltrating to the sites of injury, while supporting chemotaxis of pro-repair macrophages (M2 macrophages), phagocytosis of apoptotic material, and their exit via lymphatics.

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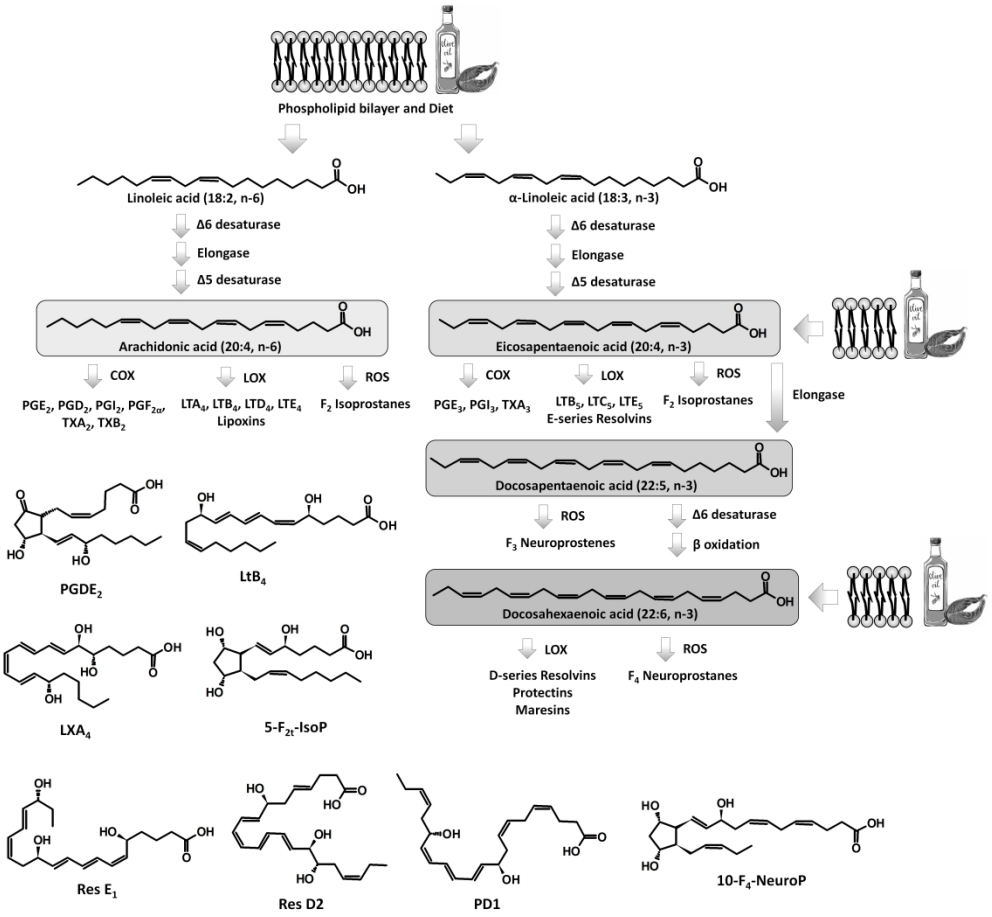

Pathways of formation and molecular structures of eicosanoids

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