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Abstract

During atherosclerosis, lipid-rich plaques are formed in large- and medium-sized arteries, which can reduce blood flow to tissues. This situation becomes particularly precarious when a plaque develops an unstable phenotype and becomes prone to rupture. Despite advances in identifying and treating vulnerable plaques, the mortality rate and disability caused by such lesions remains the number one health threat in developed countries. Vulnerable, unstable plaques are characterized by a large necrotic core, implying a prominent role for necrotic cell death in atherosclerosis and plaque destabilization. Necrosis can occur accidentally or can be induced by tightly regulated pathways. Over the past decades, different forms of regulated necrosis including necroptosis, ferroptosis, pyroptosis and secondary necrosis have been identified and these may play an important role during atherogenesis. In this review, we describe several forms of necrosis that may occur in atherosclerosis and how pharmacological modulation of these pathways can stabilize vulnerable plaques. Moreover, some challenges of targeting necrosis in atherosclerosis such as the presence of multiple death-inducing stimuli in plaques and extensive cross-talk between necrosis pathways are discussed. A better understanding of the role of (regulated) necrosis in atherosclerosis and the mechanisms contributing to plaque destabilization may open doors to novel pharmacological strategies and will enable clinicians to tackle the residual cardiovascular risk that remains in many atherosclerosis patients.

Nonstandard abbreviations and acronyms

4HNE	4-hydroxynonenal
ACSL4	Acyl-CoA synthetase long chain family member 4
ApoE	Apolipoprotein E
ASC	Apoptosis inhibitor speck-like protein
BiKE	Biobank of Karolinska Endarterectomies
CARD	Caspase recruitment domain
DAMP	Damage associated molecular pattern
FasL	First apoptotic signal ligand
FDA	Food and drug administration
Fer-1	Ferrostatin-1
GPX4	Glutathione peroxidase 4
GSDMD	Gasdermin D
GSDME	Gasdermin E
GSH	Glutathione
HeCES	Helsinki Carotid Endarterectomy Study
HMGB1	High mobility group box 1
HMOX1	Heme oxygenase 1
IL-1β	Interleukin-1β
IL-18	Interleukin-18
iNOS	Inducible nitroc oxide synthase
LDL	Low density lipoprotein
Lip-1	Liproxstatin-1
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MLKL	Mixed lineage kinase domain-like protein
Nec-1s	Necrostatin-1s

NF-κB	Nuclear factor-κB
NLRP3	Nucleotide-binding oligomerization domain-like, leucine-rich repeat and pyrin domain containing receptor 3
Nrf2	Nuclear factor erythroid 2-related factor 2
NT-GSDMD	N-terminal cleavage product of gasdermin D
oxLDL	Oxidized low density lipoprotein
PCSK9	Proprotein convertase subtilisin/kexin type 9
PRR	Pattern-recognition receptor
PYD	Pyrin domain
RAGE	Receptor for advanced glycation end-products
RHIM	RIP homotypic interaction motif
RIPK	Receptor-interacting protein kinase
ROS	Reactive oxygen species
RSL3	Ras selective lethal 3
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRADD	TNF receptor type 1-associated death domain protein
TRAIL	TNF-related apoptosis inducing ligand
VSMC	Vascular smooth muscle cell

Atherosclerosis is a progressive inflammatory disease of large and medium-sized muscular arteries and typically leads to the formation of plaques, which can reduce blood flow to tissues. When a plaque develops an unstable phenotype, it is prone to rupture, which can lead to myocardial infarction, stroke and sudden death. The global aim in the treatment of atherosclerosis is the prevention of cardiovascular complications.[1] Lifestyle changes including dietary lipid lowering, regular physical activity, smoke cessation, and blood pressure control are necessary measures in the prevention of the disease. If lifestyle changes are not sufficient, treatment with medications, such as those that lower circulating lipids, is recommended. However, despite tremendous advances in identifying and treating vulnerable plaques, the mortality rate and disability caused by such lesions still remains the number one health threat in developed countries. Statins and Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) inhibitors reduce LDL-cholesterol to low levels but do not

eliminate residual cardiovascular risk as a result of other atherogenic lipoproteins or pathways for atherosclerotic cardiovascular disease, including inflammation, that are independent of LDL-cholesterol.[2] This highlights the need for additional therapeutic strategies to prevent atherosclerotic plaque formation and rupture. Because cell death is a prominent feature of advanced atherosclerotic plaques, with a major impact on atherogenesis and plaque destabilization[3], pharmacological modulation of cell death in atherosclerosis represents a promising therapeutic approach. Indeed, plaque cells may undergo diverse types of death of which apoptosis is the best-characterized. However, electron microscopic examination of human plaques showed that the vast majority of disintegrating macrophages and vascular smooth muscle cells (VSMCs) have an ultrastructure typical of necrosis (30±18% necrotic versus 1±2% apoptotic cells).[4,5] This finding suggests that, although cell death by apoptosis clearly occurs in advanced human plaques, cells that accumulate in vulnerable plaques die by necrosis.

Necrotic cell death is characterized by an increased cell volume (oncosis), organelle swelling and chromatin condensation, which eventually culminates in plasma membrane rupture and the release of intracellular compounds.[6] Accumulation of necrotic cells and their contents triggers the formation and enlargement of a central necrotic core (**Figure 1**), which is a hypocellular region containing lipids and cellular debris.[7] The majority (80%) of necrotic cores in advanced human atherosclerotic plaques are larger than 1 mm^2, which compromises >10% of the lesion area.[8] However, in 65% of plaque ruptures, the necrotic core occupies >25% of the plaque area,[8] suggesting that it plays a pivotal role in unstable atherosclerotic plaques. Virtually all advanced human plaques have areas of necrosis.[9,10] Noteworthy is that both 'early' and 'late' stages of necrotic cores are recognized. Areas of 'early' necrotic core formation typically show free cholesterol with mostly intact macrophages and extracellular matrix made up of proteoglycans. In contrast, 'late' stage necrotic cores show numerous cholesterol clefts, cellular debris and absence of extracellular matrix.[10] It should be noted that the core region is not only critical for plaque stability but also for thrombogenicity. The necrotic core contains high concentrations of tissue factor,[11] suggesting that plaque cells undergoing necrosis mediate thrombus formation after plaque rupture.

Apart from the formation and enlargement of a central necrotic core, necrotic macrophages in advanced plaques are a source of pro-inflammatory cytokines and damage associated

molecular patterns (DAMPs).[12] The release of DAMPs promotes inflammation in the plaque, thereby contributing to plaque instability. High mobility group box 1 protein (HMGB1) is one of the best studied DAMPs in atherosclerosis, and is abundantly produced by plaque macrophages.[13] Once released in the extracellular space, HMGB1 interacts with different receptors including receptor for advanced glycation end-products (RAGE). Binding of HMGB1 triggers the transcription of pro-inflammatory cytokines in an NF-κB dependent manner, thereby promoting further plaque development.[14] Experimental evidence has shown that HMGB1 expression increases during atherogenesis.[13] Neutralization of HMGB1 in $ApoE^{-/-}$ mice reduces plaque area through inhibition of immune cell accumulation and macrophage migration.[15] Interestingly, increasing experimental evidence suggests that statins attenuate plaque formation partly by reducing the expression of HMGB1 and RAGE.[16-18] The release of HMGB1 and other DAMPs or pro-inflammatory mediators is not only characteristic of necrosis but also occurs in senescent cells. Indeed, senescence is a dynamic process resulting in cell cycle arrest and accompanied by a pro-inflammatory senescence-associated secretory phenotype (SASP), which is pro-atherogenic.[19,20] Consequently, senescent cells are pro-inflammatory and undergo metabolic changes, but they remain viable. This contrasts with apoptotic and necrotic cells which lose viability irreversibly and are destined to disappear, either silently (apoptosis) or leaving a pro-inflammatory footprint behind (necrosis). Different intraplaque cell types such as endothelial cells, VSMCs, macrophages and T cells can undergo senescence or cell death but the total number of senescent versus dying cells in atherosclerotic plaques remains elusive and is complicated by their shared characteristics (e.g. increased cell volume, DNA breaks, release of pro-inflammatory cytokines and DAMPs) and interplay.[20,21]

Necrosis in atherosclerotic plaques can occur accidentally (e.g. when cholesterol crystals puncture the plasma membrane) or can be induced following activation of tightly regulated pathways (**Figure 1**). In this review, we describe different types of regulated necrosis that may occur in atherosclerosis and how pharmacological targeting of these types of death can stabilize vulnerable plaques and contribute to the beneficial effects of currently applied plaque stabilizing therapies.

Necroptosis

Research in the field of necrotic cell death was drastically changed by the discovery of small molecules, termed necrostatins, which inhibit receptor-interacting protein kinase (RIPK) 1-induced necroptosis in TNFα-treated cells.[22,23] This discovery led to the characterization of downstream necroptosis mediators, namely RIPK3 and mixed lineage kinase domain-like protein (MLKL) (**Figure 2**).[24-27] In addition to TNFα, other necroptosis triggers have been identified including TNF-related apoptosis inducing ligand (TRAIL), first apoptotic signal ligand (FasL), interferons, toll-like receptor (TLR) ligands, and virus-activated pathways.[28]

Activation of the RIPK1/RIPK3/MLKL-axis after TNFα stimulation

The response of cells to TNFα is complex and primarily depends on the ubiquitination and phosphorylation profile of RIPK1 (**Figure 2**).[29] Upon TNFα binding, TRADD and RIPK1 are recruited to form a membrane-bound complex I. Subsequently, the default response to TNFα signaling is ubiquitination of RIPK1 by cellular inhibitors of apoptosis (cIAP1/2) and linear ubiquitin chain assembly complexes (LUBAC) followed by the activation of pro-survival pathways including NF-κB and MAPK signaling. In this case, ubiquitinated RIPK1 merely serves as a scaffold for binding and activation of mediators of NF-κB and MAPK pathways. In contrast, when protein synthesis of endogenous apoptosis inhibitors is blocked, for example by cycloheximide, or when ubiquitination and NF-κB activation are reduced, RIPK1 switches from a pro-survival scaffold to a cytosolic pro-death protein.[30,31] In that case, necroptosis and apoptosis pathways compete and the presence and catalytic activity of caspase-8 plays a decisive role. Indeed, necroptosis proteins RIPK1 and RIPK3 are activated when caspase-8 is inhibited, combined with reduced RIPK1 ubiquitination and NF-κB signaling.[30] Inhibition of caspase-8 can be obtained pharmacologically with the pan-caspase inhibitor zVAD-fmk. However, the molecular mechanisms underlying caspase-8 inhibition and RIPK1/RIPK3 activation in atherosclerotic plaques are unclear. Most likely, oxidation or inducible nitric oxide synthase (iNOS)-driven S-nitrosylation (i.e. inactivation) of a critical thiol-residue in the active site of caspase-8, in combination with pro-apoptotic signaling, triggers necroptosis induction. Macrophages overexpressing iNOS frequently surround the necrotic core of human plaques, which favors this hypothesis.[32] Interestingly, genetic deletion or deficiency of caspase-8 (or its adaptor TRADD) is associated with embryonic lethality in mice, which is

attributed to necroptosis because deletion of RIPK3 or MLKL rescues these mice. Lethality is mainly caused by defects in vascular development, underlining the importance of these pathways in cardiovascular disease.[33] Deletion of MLKL in caspase-8 deficient mice still causes perinatal lethality meaning that necroptosis-independent cell death is induced upon caspase-8 inhibition in later stages of embryonic development. Of note, this phenotype is rescued by deletion of apoptosis inhibitor speck-like protein (ASC) or caspase-1, both part of the pyroptosis machinery (vide infra), demonstrating that pyroptosis is induced when apoptosis and necroptosis are inhibited. Indeed, caspase-8 represents a molecular switch between apoptosis, necroptosis and pyroptosis.[33]

Once activated, RIPK1 undergoes autophosphorylation and interacts with RIPK3 through a RIP homotypic interaction motif (RHIM) which results in RIPK3 oligomerization and the formation of a necroptotic complex called 'the necrosome'.[34,35] In the necrosome, RIPK3 is activated resulting in a series of autophosphorylations and recruitment and phosphorylation of MLKL via its pseudokinase domain. As the name implies, the pseudokinase domain of MLKL topologically resembles a protein kinase domain but has no catalytic activity whatsoever and serves merely as an interaction domain with RIPK3.[34] Finally, RIPK3-induced phosphorylation of MLKL triggers its oligomerization. Phosphorylated MLKL oligomers associate with phospholipids in the plasma membrane, which causes plasma membrane permeabilization, release of DAMPs and eventually necroptotic cell death.[27,36]

Necroptosis in atherosclerosis

The expression of necroptosis mediators RIPK3 and MLKL is elevated in human atherosclerotic plaques, both at the mRNA and protein level.[37,38] RIPK3 and MLKL mRNA are specifically upregulated in subjects with unstable compared to stable atherosclerotic plaques.[37] RIPK3 expression is also elevated in advanced plaques of $LDLr^{-/-}$ mice, predominantly in macrophages. Loss of RIPK3 reduces advanced atherosclerotic lesions in $ApoE^{-/-}$ or $LDLr^{-/-}$ mice, but has no effect on earlier stages of plaque development,[39] suggesting that macrophage necroptosis plays a major role in advanced plaques. Bone-marrow transplantation showed that loss of RIPK3 expression from bone-marrow derived cells is responsible for this atheroprotective effect.[39] Likewise, deletion of MLKL with antisense oligonucleotides or genetic deletion of MLKL reduces the necrotic area in advanced plaques but not in early atherosclerotic plaques of $ApoE^{-/-}$ mice.[40,41] These findings

clearly illustrate a role for RIPK3- and MLKL-mediated macrophage necroptosis in atherosclerosis.

Analogous with RIPK3, RIPK1 is mainly found in macrophages of human carotid lesions.[42] However, the role of RIPK1 in atherogenesis is not straightforward and is complicated by two intrinsic, albeit conflicting activities, namely a scaffolding function that promotes cell survival versus a kinase activity that triggers cell death. A full RIPK1 knock-out affects both kinase-independent scaffolding functions and kinase-dependent cell death and inflammation.[42] It is worth mentioning that $RIPK1^{-/-}$ mice die perinatally due to the loss of pro-survival, RIPK1-kinase independent signaling such as the NF-κB pathway.[43] In contrast, mice with a RIPK1 kinase dead (e.g. K45A) or inactivating (e.g. S25D) mutation are viable and display no obvious abnormalities. Similarly, mice with a deficiency of RIPK3 and MLKL, which are key executioners of necroptosis and regulated by RIPK1 kinase activity, are also viable and healthy. Therefore, inhibition of pathology-associated necroptosis may serve as a therapeutic target. However, in a comparative study using mice with inactive RIPK1 kinase ($RIPK1^{D138N/D138N}$), RIPK3 deficiency ($RIPK3^{-/-}$) and MLKL deficiency ($MLKL^{-/-}$) in several models of necroptosis-related inflammatory diseases, MLKL deficiency offered little protection in a kidney ischemia-reperfusion model and no protection at all in a model of systemic inflammation, as opposed to $RIPK1^{D138N/D138N}$ and $RIPK3^{-/-}$ mice.[44] In general, this favors targeting upstream RIPK1 and RIPK3 over MLKL. RIPK3 inhibitors have been developed and have been shown to block necroptosis *in vitro* and *in vivo*. However, they induce concentration-dependent apoptosis and therefore none of them moved into clinical trials.[45] In contrast, several specific RIPK1 kinase inhibitors were safe and well tolerated in phase I (NCT02302404,[46] NCT03590613 and NCT03305419,[47] NCT03757325) and phase II clinical trials (NCT02776033,[48] NCT02903966[49]). Because the classical RIPK1 kinase inhibitor Necrostatin-1s (Nec-1s) suffers from selectivity and potency problems *in vivo*, several research groups have focused on the development of alternative RIPK1 inhibitors over the past decade. Consequently, a new generation of alternative RIPK1 kinase inhibitors have recently been developed with increased potency and improved pharmacokinetic profiles as compared to Nec-1s, such as GSK'547, GSK'772, DNL747, DNL758 and GFH312 which are currently being tested in animal models and humans.[50-52] After passing phase I safety and tolerability trials, phase II clinical studies were completed to evaluate the effects of GSK'772 on psoriasis, ulcerative colitis and rheumatoid arthritis (NCT02776033,[48] NCT02903966,[49]

and NCT02858492[53]). Importantly, promising results were reported for GSK'772 in patients with active plaque psoriasis.[48] DNL758/SAR443122 also passed phase I and is currently included in a proof-of-concept study in patients with cutaneous lupus erythematosus (NCT04781816). Furthermore, GFH312 is currently included in a first-in-human trial (NCT04676711). Altogether, RIPK1 kinase inhibitors are readily moving into clinical stages and are reported to be safe and well tolerated so far, making the kinase activity of RIPK1 an attractive therapeutic target for necroptosis in atherosclerosis.

Pharmacological inhibition of RIPK1 by Nec-1s reduces plaque size and promotes plaque stability in $ApoE^{-/-}$ mice.[37] Similar observations apply after administration of RIPK1 antisense oligonucleotides that reduce but do not completely abrogate Ripk1 expression.[54] Additional *in vitro* experiments have unraveled the underlying mechanism by which necroptosis is induced in atherosclerosis. During plaque development, oxLDL increases ROS-mediated RIPK3 and MLKL gene expression in macrophages, which leads to necroptosis.[37] These findings demonstrate that inhibition of macrophage necroptosis could be a promising therapeutic strategy to prevent the development of a vulnerable plaque. However, $ApoE^{-/-}$ $RIPK1^{S25D/S25D}$ mice lacking active RIPK1 kinase develop larger plaques compared to $ApoE^{-/-}$ $RIPK1^{+/+}$ controls.[55] Moreover, pharmacological inhibition of RIPK1 with GSK'547 does not limit atherogenesis in $ApoE^{-/-}$ $Fbn1^{C1039G+/-}$ mice, a model of advanced atherosclerosis.[55] Accordingly, GSK'547 does not limit plaque formation in more advanced stages of atherogenesis while it decreases the plaque area in earlier stages.[56] Additionally, when RIPK1 expression is reduced by antisense oligonucletodies[54], there is a reduction in the macrophage ability to activate pro-inflammatory NF-κB while necroptotic cell death remains functional, suggesting RIPK1 expression may be tightly regulated to balance its pro-inflammatory vs. pro-death functions. Together, these results stress the complex and stage-dependent involvement of RIPK1 kinase activity in atherosclerosis.

Pyroptosis

Besides necroptosis, other types of regulated necrosis have been observed in atherosclerotic plaques, even though their significance is not always clear-cut. Among the most well-defined is pyroptosis, a pro-inflammatory form of regulated cell death that is characterized by the formation of plasma membrane pores via caspase-1-dependent cleavage of gasdermin D

(GSDMD), which is highly expressed in different tissues and cell types and well conserved in mammals.[57,58]

Canonical inflammasome-mediated pyroptosis

Canonical induction of pyroptosis involves cleavage (activation) of caspase-1 through a large supramolecular complex, known as an inflammasome (**Figure 3**). The NLRP3 (nucleotide-binding oligomerization domain (NOD)-like, leucine-rich repeat (LRR) and pyrin domain containing receptor 3) inflammasome is currently the best characterized inflammasome and consists of NLRP3, ASC and procaspase-1. First, a priming step, is required to increase the transcription of pro-IL-1β, NLRP3 and ASC. The priming step is induced by recognition of extracellular molecules such as LPS, TNFα or IL-1β by pattern-recognition receptors (PRRs). Subsequently, NF-κB will be activated and the C-terminal LRR on NLRP3 will be deubiquitinated, allowing NLRP3 activation. Assembly of the inflammasome occurs through ASC which contains a pyrin domain (PYD), that interacts with the N-terminal PYD in NLRP3, and a caspase recruitment domain (CARD) for binding of procaspase-1.[59]

Once assembled, the NLRP3 inflammasome can be activated by low intracellular potassium concentrations. Indeed, potassium efflux induced by ionophores such as nigericin or by cation channels such as the P2X7 receptor and TWIK2 channel induced by ATP, are known inducers of NLRP3-dependent pyroptosis.[60,61] Furthermore, cathepsin B is required for NLRP3 activation.[62] Mounting evidence suggests that oxLDL, crystals of cholesterol and calcium phosphate, and fibrillar ligands in atherosclerotic plaques activate NLRP3 inflammasomes through lysosomal rupture and subsequent cathepsin release, which in turn leads to cleavage and activation of procaspase-1.[63-66] Active caspase-1 exerts proinflammatory effects by converting pro-IL-1β and pro-IL-18 into their bioactive forms. Furthermore, GSDMD is N-terminally cleaved by caspase-1. Subsequently, NT-GSDMDs oligomerize and bind to phospholipids in the cell membrane where they induce pore formation. NT-GSDMD-induced pores are approximately 10-15 nm in diameter, in contrast to the much smaller MLKL-induced channels, and a large number of these NT-GSDMD pores disrupt the plasma membrane and physiological ionic gradients.[67,68] The damaged membrane starts blebbing, a phenomenon that is also observed in apoptotic cells although in a much slower fashion.[67,69] Pyroptotic cells flatten while releasing cellular content through the NT-GSDMD-pores, such as IL-1β, IL-18, ATP, HMGB1 and cleaved GSDMs, which amplify

inflammation and pyroptosis induction.[68,69] Interestingly, secretion of IL-1β and IL-18 does not require cell lysis and is temporally associated with GSDMD-dependent pore formation, suggesting that these pores are sufficient to mediate cytokine release.[70]

Non-canonical pyroptosis induction

Caspase-11 can directly sense cytoplasmic LPS without the need for TLR4 or a canonical inflammasome, because it contains an LPS-binding site located on its CARD domain, forming a procaspase-11-LPS complex, also called the non-canonical inflammasome (**Figure 3**).[71] Active caspase-11 cleaves GSDMD which induces pore formation and pyroptosis, similar to caspase-1. Next to direct induction of pyroptosis, caspase-11-induced formation of NT-GSDMD also serves as an activator of the NLRP3 inflammasome. In this way, caspase-11 indirectly triggers canonical caspase-1-dependent pyroptosis and, subsequently, the maturation and release of IL-1β and IL-18.[72]

Pyroptosis in atherosclerosis

Recent studies have shown that components of the NLRP3 inflammasome are present in human atherosclerotic plaques and are expressed in macrophages and foam cells around the necrotic core.[73,74] Both mRNA and protein levels of NLRP3, caspase-1, ASC, IL-1β and IL-18 are increased in human plaques compared to normal arteries.[74-77] In patients with coronary atherosclerosis, the aortic expression of the NLRP3 inflammasome is correlated with disease severity and clinical risk factors for cardiovascular disease (e.g., hypertension, diabetes, smoking, LDL cholesterol).[75] Moreover, the highest NLRP3 expression levels were observed in unstable lesions as compared to stable lesions and non-atherosclerotic arteries.[73] All these epidemiological studies highlight a possible role of the NLRP3 inflammasome in atherogenesis and plaque destabilization. Interestingly, it has been demonstrated that ATP and cholesterol crystals induce pyroptosis both in the presence and absence of LPS priming in cultured plaques.[76] Both inducers are relevant in the context of atherosclerosis as cholesterol is involved in every stage of plaque development, while extracellular ATP is expected to be more abundant in advanced stages of plaque development and destabilization due to excessive cell death.[66,76,78] Another possible activator of the NLRP3 inflammasome in atherosclerosis is nicotine. Indeed, smoking is a major risk factor for

atherosclerosis which is at least partly attributable to activation of the ROS/NLRP3-axis and pyroptosis induction.[79,80]

Many studies have targeted proteins involved in canonical (NLRP3, ASC, caspase-1, IL-1β and IL-18) and non-canonical (caspase-11) pyroptosis signaling in atherosclerotic models and reported beneficial effects on atherogenesis (reviewed elsewhere: [57,81]). However, often these targets are not limited to pyroptosis signaling but are also involved in other pro-inflammatory and cell death pathways. For example, caspase-1 is reported to be pro-apoptotic, besides its pyroptotic properties, and deletion of caspase-1 activity is linked to lytic, non-pyroptotic cell death.[82] NLRP3 is also linked to RIPK1 and RIPK3 signaling and subsequently, apoptosis and necroptosis.[83-85] In addition, targeting one single player might not suffice as both canonical and non-canonical pathways play a role in atherosclerosis. This is supported by the considerable risk for recurrent cardiovascular events in participants of the CANTOS trial treated with the IL-1β antibody canakinumab.[86]

GSDMD is the common executor of both canonical and non-canonical pyroptosis. Interestingly, GSDMD mRNA is upregulated in peripheral blood monocytes of patients with coronary artery disease. Moreover, expression of GSDMD and NT-GSDMD is increased in ApoE$^{-/-}$ and WT mice fed a high fat diet as compared to chow-fed controls.[87] Expression of NT-GSDMD was also observed in human endarterectomy specimens.[88] Thus, GSDMD is actively involved in pyroptosis during atherogenesis in both humans and mice, making GSDMD an attractive pharmacological target for pyroptosis in atherosclerosis. Disulfiram is an FDA-approved drug used for the treatment of alcohol abuse and was recently identified as a potent GSDMD inhibitor. It covalently modifies cys191 (in human, cys192 in mouse) in GSDMD to block pore formation at nanomolar concentrations,[89] but is metabolized to carbon disulphide, which promotes atherosclerosis, thus not recommended for long-term anti-atherosclerosis therapy. Dimethyl fumarate, used as an immunomodulator in multiple sclerosis, has been identified as an alternative GSDMD inhibitor,[90] and reduces aortic plaque area in hyperglycemic ApoE$^{-/-}$ mice.[91] More recently, genetic deletion of GSDMD as well as pharmacological inhibition with necrosulfonamide reduce infarct size and heart failure in a mouse model of acute myocardial infarction,[92,93] underlining the involvement of GSDMD in cardiovascular disease and the possibility to use it as a pharmacological target in atherosclerosis. This is supported by the observation that GSDMD deficiency decreases the formation of inflammatory plaques in ApoE$^{-/-}$ mice.[88] However, no effect on the initiation

and formation of stable aortic plaques was observed. Thus, GSDMD is mainly involved in the transition to an inflammatory, vulnerable plaque phenotype in advanced stages of atherosclerosis and appears to be a promising target for limiting plaque destabilization.

Gasdermin E-mediated secondary necrosis in atherosclerosis

Besides the canonical caspase-1/inflammasome pathway that activates GSDMD, gasdermin E (GSDME) was recently identified as an alternative effector of programmed necrosis under pro-apoptotic conditions.[94] GSDME leads to membrane pores and programmed necrosis of apoptotic cells, known as secondary necrosis, after cleavage by caspase-3. Efficient clearance of apoptotic cells – a process called efferocytosis – is essential for preventing secondary necrosis. Unfortunately, efferocytosis is strongly impaired in advanced atherosclerosis,[95] making secondary necrosis a main feature of advanced plaques. Stimulation of efferocytosis is challenging as most phagocytes are lipid-filled foam cells with limited phagocytosis potential. Nevertheless, ongoing therapeutic efforts aimed at boosting efferocytosis have shown promising results.[96] It should be noted that GSDME, after cleavage by caspase-3, can also form pores in the mitochondrial membrane resulting in the release of pro-apoptotic molecules such as cytochrome c. This event creates a positive feedback loop that promotes caspase-3 activation and further GSDME cleavage, ultimately augmenting apoptotic cell death and secondary necrosis. Surprisingly, there are no studies describing (cleaved) GSDME expression in atherosclerosis. It should be noted that GSDME is transcriptionally controlled by p53 and is essential in the p53-mediated response to DNA damage. Because DNA damage, phosphorylated (active) p53 and cleaved caspase-3 are abundantly present in advanced plaques,[97] this alternative, GSDME-mediated pathway of programmed necrosis, besides GSDMD, definitely needs further attention in the context of atherosclerotic plaque destabilization.

Ferroptosis

Ferroptosis was discovered when the small molecules erastin and Ras Selective Lethal 3 (RSL3) were designed to induce cytotoxicity in cells expressing oncogenic mutant RAS proteins.[98-100] When characterizing the cytotoxicity induced by erastin and (1S,3R)-RSL3, a unique necrotic morphology featuring smaller mitochondria and increased membrane

density was observed. Moreover, ferroptosis is characterized by excessive iron-dependent lipid peroxidation. This can occur by enzymatic peroxidation of poly-unsaturated fatty acids (PUFAs) in phospholipid (PL) bilayers through the Acyl-CoA Synthetase Long Chain family member 4 (ACSL4)/Lysophosphatidyl Acyltransferase 3 (LPCAT3)/15-Lipoxygenase (LOX)-axis (**Figure 4**). Lipid peroxidation can also be induced non-enzymatically through Fenton chemistry, which forms hydroxyl radicals, and free radical chain reactions. Both enzymatic and non-enzymatic lipid peroxidation require free ferrous iron (Fe^{2+}), which resides in a cytosolic labile iron pool. Under physiological conditions, accumulation of ferrous iron in the cytosol and growth of the labile iron pool is limited by ferritin as it can store up to 4500 iron molecules.[101] Another ferroptosis limiting factor is ferroptosis suppressor protein 1 (FSP1), which reduces Coenzyme Q10 (CoQ_{10}) to ubuiqinol. The latter traps lipid peroxyl radicals, accompanied by the formation of CoQ_{10}, thereby preventing the formation of lipid peroxides.[102,103]

When growth of the labile iron pool exceeds the buffering capacity of ferritin (non-canonical pathway) or when formed lipid peroxides are not cleared properly (canonical pathway), excessive lipid peroxidation of PUFAs in PL bilayers occurs. This affects the chemical and geometric properties of the lipid bilayer, leads to membrane pore formation and destroys the barrier function of the plasma membrane. Together, these events lead to cell lysis and eventually cell death.[104] Additionally, lipid peroxides are degraded to toxic lipid aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4HNE), which adds an extra layer of cytotoxicity. Indeed, MDA and 4HNE easily bind covalently to proteins and DNA, thereby impairing several signaling processes. Furthermore, MDA binding on epitopes generates oxidative self-epitopes which can be recognized by scavenger receptors on phagocytes or as DAMPs by PRRs and initiate innate immune responses. MDA-modified proteins and lipoproteins also trigger adaptive immune responses.[105] Once lipid peroxides are formed, MDA and 4HNE may further amplify ROS signaling.

Canonical induction of ferroptosis

Canonical ferroptosis induction involves impaired glutathione peroxidase 4 (GPX4), either through direct inhibition of GPX4 or through depletion of its substrate glutathione (GSH) (**Figure 4**).[106] GPX4 catalyzes the reduction of lipid peroxides in cellular membrane environments and simultaneously oxidizes and consumes GSH. Full-body depletion of GPX4

in mice results in embryonic lethality and shRNA mediated GPX4 knockdown sensitizes cells to undergo ferroptosis, underlining the importance of GPX4 in normal physiology by preventing excessive lipid peroxidation.[107] (1S,3R)-RSL3 and erastin are both inducers of canonical ferroptosis. (1S,3R)-RSL3 (and not the other diastereomers of RSL3) directly targets and inhibits GPX4.[107] Erastin inhibits the X_c-antiporter system which transports cystine, a key precursor in the synthesis of GSH, into the cell in exchange for glutamate. By inhibiting this system, erastin decreases the entry of cystine into the cell and induces downstream depletion of the cellular antioxidant GSH, the substrate of GPX4, thereby impairing GPX4 activity and triggering accumulation of ROS.[107,108]

Non-canonical induction of ferroptosis

Non-canonical ferroptosis is induced when ferrous iron accumulates in the labile iron pool (**Figure 4**).[109] This occurs when the finely regulated iron balances are disturbed due to overloading of cells with iron (e.g. overload with hemoglobin, hemin, ferrous ammonium sulfate or iron chloride), or due to excessive activation of heme oxygenase 1 (HMOX1), decreased ferroportin expression or enhanced transferrin expression.[109-111] Ferroportin and transferrin regulate iron export to and transport in the circulation, respectively. HMOX1 is responsible for the catabolism of hemoglobin to heme and Fe^{2+}. This is highly relevant in the context of phagocytosis of erythrocytes, a process called erythrophagocytosis, which is responsible for the clearance of aged or damaged erythrocytes by macrophages.[112]

Ferroptosis in atherosclerosis

Several epidemiological studies have reported a relationship between iron levels and atherogenesis.[113,114] Indeed, restriction of dietary iron intake or iron chelation with deferoxamine leads to a significant decrease in experimental plaque formation.[115-117] Moreover, iron depletion through frequent blood donation is associated with a decreased cardiovascular risk.[118-120] Given that lipid peroxidation, intraplaque hemorrhage and iron deposition are key features of advanced human plaques, ferroptosis is suggested to play a role in plaque destabilization, however, to date no direct evidence exists. Erythrocytes are released in the plaque during intraplaque hemorrhages, thereby increasing the cholesterol and iron content of the plaque. Macrophages surrounding intraplaque hemorrhages phagocytose erythrocytes leading to HMOX1 activation and high intracellular levels of heme

and iron.[32,121,122] *In vitro* experiments demonstrated that erythrophagocytosis by macrophages induces ferroptosis (Puylaert P., unpublished data). The latter results in the release of intracellular content and DAMPs into the plaque, which may contribute to exponential growth of the necrotic core, amplification of the inflammatory cycle and eventually plaque destabilization. This hypothesis is supported by the observation that atherosclerotic lesions contain MDA, a toxic lipid peroxidation product, and adaptive IgG antibodies with specificity for MDA.[105] MDA can modify epitopes and is therefore capable of inducing undesired pro-inflammatory responses in atherosclerosis. MDA can also bind to LDL, leading to the formation of proatherogenic and immunogenic modified LDL. Interestingly, immunization studies have shown atheroprotective effects of neutralizing endogenous MDA.[105] Similarly, 4-HNE can bind to apolipoprotein B on LDL, which leads to uptake of LDL by macrophages and contributes to foam cell formation. Next to the presence of lipid peroxidation products in plaques, iron-positive foam cells are present in human plaques, HMOX1 expression is increased in human aortic endothelial cells and hemoglobin, HMOX1 and ferritin accumulate in advanced human plaques.[32,122,123] These observations are suggestive of growth of the labile iron pool in plaque cells, and thus, combined with lipid peroxidation, of the occurrence of ferroptosis in plaques.

The first specific ferroptosis inhibitors that were identified by high-throughput screening of small molecule libraries are ferrostatin-1 (Fer-1) and liproxstatin-1 (Lip-1).[106,124] These are potent inhibitors of (1S,3R)-RSL3- and erastin-induced ferroptosis showing EC_{50} values in the nanomolar range. The anti-ferroptotic acitivity of both Fer-1 and Lip-1 can be attributed to their potent radical trapping effects, especially in lipid bilayers.[125,126] Another radical trapping agent that inhibits ferroptosis is α-tocopherol, albeit with a lower potency in lipid bilayers as compared to Fer-1 and Lip-1. Pharmacological inhibition of ferroptosis with Fer-1 was recently reported to reduce plaque burden in $ApoE^{-/-}$ mice as well as in diabetic $ApoE^{-/-}$ mice.[123,127] Both studies also observed decreased iron levels in serum and in the aorta and increased GPX4 and SLC7A11 (subunit of the X_c-antiporter) expression which, at least partly, explains the observed atheroprotective effects. Accordingly, GPX4 overexpression in $ApoE^{-/-}$ mice inhibited plaque progression.[128] Furthermore, inhibition of enzymatic lipid peroxidation with a pharmacological 15-LOX blocker decreased plaque progression in atherosclerotic rabbits with pre-established plaques and genetic deletion of 15-LOX resulted in decreased plaque burden in $ApoE^{-/-}$ mice.[129,130] Together, these studies are highly suggestive of

involvement of iron-dependent lipid peroxidation in atherogenesis. Combined with the availability of novel Fer-1 analogs with improved potency and ADME properties such as UAMC-3203 and UAMC-3206,[131-133] this makes ferroptosis a promising target to explore in atherosclerosis.

Regulated necrosis in human atherosclerotic plaques

As described above, several studies have reported the expression of proteins related to necroptosis, pyroptosis or ferroptosis in plaques from both humans and animals. Observational studies in human atherosclerotic plaques are summarized in Table 1. Moreover, many compounds targeting regulated necrosis have been developed and some are already moving into clinical trials (Table 2). This makes targeting necroptosis, pyroptosis and ferroptosis an interesting approach to explore in atherosclerosis.

Concluding remarks

Research over the last two decades has demonstrated that necrotic cell death is critically involved in the formation and destabilization of atherosclerotic plaques. Different forms of regulated necrosis can be distinguished by analyzing a combination of mechanistic characteristics (e.g., presence of phosphorylated MLKL during necroptosis and GSDMD-pore formation during pyroptosis) and morphological features (e.g. mitochondrial abnormalities during ferroptosis, cytoplasm flattening and membrane blebbing during pyroptosis). The most prominent characteristics of necroptosis, pyroptosis and ferroptosis are described in Table 3 (and more extensively reviewed elsewhere).[134,135] Hitherto, many qualitative analyses of morphological and mechanistic markers of regulated necrosis in atherosclerotic plaques have been performed. However, a quantitative analysis to estimate the true percentage of intraplaque cells undergoing necroptosis, pyroptosis and ferroptosis (and hence their contribution to atherogenesis) is currently lacking.

Table 1. Necroptosis-, pyroptosis- and ferroptosis-related proteins in human atherosclerotic plaques

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Ref
RIPK3	Gene expression upregulated in unstable carotid plaques	• Gene expression analysis on carotid endarterectomy specimens (n=127 plaques from BiKE) vs. disease-free artery (n=10 organ donors without cardiovascular history) • Gene expression analysis on unstable (n=87 symptomatic patients) vs. stable (n=40 asymptomatic patients) carotid plaques from BiKE	[37,136]
	Protein levels increased in carotid lesions with necrotic core	• Western blot analysis of plaques with vs. without necrotic core (n≥6 per group from 12 autopsy samples and 6 carotid endarterectomies)	[38]
RIPK1	Protein level increased in carotid lesions with necrotic core	• Western blot analysis of plaques with vs. without necrotic core (n≥6 per group from 12 autopsy samples and 6 carotid endarterectomies)	[38]
	Expressed in macrophages (but not smooth muscle cells) in carotid lesions	• Double immunofluorescence staining of advanced carotid plaque selected from a library of endarterectomy specimens from patients with carotid stenosis >70%	[42]
	Expressed in macrophages and endothelial cells in coronary plaques	• Immunohistochemical analysis of early plaques vs. arteries with pathological intimal thickening obtained from CVPath Institute Sudden Cardiac Death registry	[54,137]
	Gene expression upregulated in early carotid plaques	• Gene expression analysis on carotid endarterectomy specimens from 34 patients: plaque tissue (mostly stage IV and/or V lesions) vs. adjacent macroscopically 'intact tissue' (almost exclusively stage I and II lesions)	[54,138]
MLKL	Gene expression upregulated in unstable carotid plaques	• Gene expression analysis on carotid endarterectomy specimens (n=127 plaques from BiKE) vs. disease-free artery (n=10 organ donors without cardiovascular history) • Gene expression analysis on unstable (n=87 symptomatic patients) vs. stable (n=40 asymptomatic patients) carotid plaques from BiKE	[37,136]
	P-MLKL levels increased in advanced coronary plaques	• Immunohistochemical analysis of advanced lesions (n=11) vs. arteries with pathological intimal thickening (n=5) obtained from CVPath Institute Sudden Cardiac Death registry	[37,137]

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Ref
NLRP3	Gene and protein levels upregulated in unstable carotid plaques	• Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology • Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs. mesenteric arteries from 10 patients with early intestinal tumors • Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology	[73]
	Gene and protein levels upregulated in advanced coronary plaques	• Gene expression analysis and immunohistochemistry of coronary artery specimens from 10 explanted hearts (4 patients undergoing heart transplantation, 6 donor hearts not fulfilling transplantation criteria, all male): advanced (stage IV-VI) vs. early (stage I-III) lesions from the same coronary tree (n=10 per group)	[74]
	Expressed in aortic plaques and expression level correlated with coronary atherosclerosis severity and risk factors	• Immunohistochemical analysis of ascending aorta specimens from 36 patients undergoing coronary artery bypass graft surgery (severity determined using Gensini scoring) vs. 10 healthy renal arteries from kidney donors	[75]
	Gene levels upregulated in carotid plaques and expressed in intraplaque macrophages and smooth muscle cells	• Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs. disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history) • Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE	[76]
	Expression positively correlated with degree of stenosis and plaque severity stage	• Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction and diabetes mellitus)	[139]
ASC	Gene and protein levels upregulated in unstable carotid plaques	• Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology • Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs. mesenteric arteries from 10 patients with early intestinal tumors • Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on	[73]

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Ref
		plaque morphology	
	Gene and protein levels upregulated in advanced coronary plaques	• Gene expression analysis and immunohistochemistry of coronary artery specimens from 10 explanted hearts (4 patients undergoing heart transplantation, 6 donor hearts not fulfilling transplantation criteria, all male): advanced (stage IV-VI) vs. early (stage I-III) lesions from the same coronary tree (n=10 per group)	[74]
	Gene levels upregulated in carotid plaques and expressed in intraplaque macrophages and smooth muscle cells	• Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs. disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history) • Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE	[76]
Caspase-1	Gene and protein levels upregulated in unstable carotid plaques	• Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology • Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs. mesenteric arteries from 10 patients with early intestinal tumors • Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology	[73]
	Gene and protein levels upregulated in advanced coronary plaques	• Gene expression analysis and immunohistochemistry of coronary artery specimens from 10 explanted hearts (4 patients undergoing heart transplantation, 6 donor hearts not fulfilling transplantation criteria, all male): advanced (stage IV-VI) vs. early (stage I-III) lesions from the same coronary tree (n=10 per group)	[74]
	Gene levels upregulated in carotid plaques	• Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs. disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history) • Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE	[76]
	Cleaved caspase-1 upregulated in symptomatic plaques in vascular smooth muscle cells that are transdifferentiating to	• Double immunofluorescence staining of carotid endarterectomy specimens: symptomatic vs. asymptomatic patients (n=12 per group) with >70% stenosis	[140,141]

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Ref
	macrophages		
	Expression positively correlated with degree of stenosis and plaque severity stage	• Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction and diabetes mellitus)	[139]
IL-1β	Gene and protein levels upregulated in unstable carotid plaques	• Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology • Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs. mesenteric arteries from 10 patients with early intestinal tumors • Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology	[73]
	Gene levels upregulated in carotid plaques	• Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs. disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history) • Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE	[76]
	Expression increased in symptomatic plaques in vascular smooth muscle cells that are transdifferentiating to macrophages	• Double immunofluorescence staining of carotid endarterectomy specimens: symptomatic vs. asymptomatic patients (n=12 per group) with >70% stenosis	[140,141]
IL-18	Gene and protein levels upregulated in unstable carotid plaques	• Gene expression analysis on carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology • Immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis vs. mesenteric arteries from 10 patients with early intestinal tumors • Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 30 patients with >70% stenosis: unstable vs. stable plaques (n=15 per group) based on plaque morphology	[73]

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Ref
	Gene levels upregulated in carotid plaques	• Gene expression analysis on carotid endarterectomy specimens (n=106 plaques from BiKE) vs. disease-free arteries (n=9 iliac arteries, n=1 aorta intima from organ donors without cardiovascular history) • Immunohistochemical analysis of 3 carotid endarterectomy specimens from BiKE	[76]
	Gene and protein levels upregulated in carotid plaques and correlated with plaque instability	• Western blot analysis of 12 endarterectomy specimens (from 40 carotids collected from 35 patients) vs. 5 control arteries (2 carotids and 3 mammary arteries collected at autopsy or during coronary artery bypass graft surgery) • Immunohistochemical analysis of 6 endarterectomy specimens (from 40 carotids collected from 35 patients) • Gene expression analysis on 22 endarterectomy specimens (from 40 carotids collected from 35 patients): symptomatic (n=13) vs. asymptomatic (n=9) patients; unstable (n=14) vs. stable (n=8) plaques (based on ulceration)	[77]
GSDMD	Cleaved GSDMD expressed in macrophage- and smooth muscle cell-rich areas of advanced carotid plaque	• Double immunohistochemical staining of advanced carotid plaque selected from a library of endarterectomy specimens from patients with carotid stenosis >70%	[88]
PTGS2	Expression positively correlated with degree of stenosis and plaque severity stage	• Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction and diabetes mellitus)	[139]
GPX4	Expression negatively correlated with degree of stenosis and plaque severity stage	• Immunohistochemical analysis of 40 coronary artery samples obtained from 4 autopsy cases (causes of death: occupying lesions, cerebral hemorrhage, myocardial infarction and diabetes mellitus)	[139]
Ferritin	Expressed in carotid plaques and upregulated in unstable plaques and symptomatic patients	• Immunohistochemical analysis of 52 endarterectomy specimens from patients with >50% stenosis (Linköping Carotid Study): ruptured (n=19) vs. vulnerable (n=9) vs. stable (n=8) plaques (based on morphology and collagen staining); symptomatic (n=44) vs. asymptomatic (n=8) patients	[142]

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Ref
	Expressed in early plaques and accumulation in advanced plaques	• Immunohistochemical analysis of 35 carotid endarterectomy specimens from patients with varying degree of carotid atherosclerosis (n=18 advanced and ruptured plaques, n=8 early plaques) vs. healthy arteries (non-diseased parts of carotid arteries and n=4 mammary artery specimens)	[122]
	Expression in the intima of fatty streaks in areas rich in macrophages and TUNEL-positivity	• Immunohistochemical analysis of coronary artery and thoracic aorta specimens: fatty streak (n=12 autopsy cases with general atherosclerosis) vs. normal arteries (n=19: normal areas of arteries from 12 autopsy cases with general atherosclerosis and 7 young autopsy cases without atherosclerosis)	[143]
Transferrin receptor	Expressed in carotid plaques and upregulated in unstable plaques and symptomatic patients	• Immunohistochemical analysis of 52 endarterectomy specimens from patients with >50% stenosis (Linköping Carotid Study): ruptured (n=19) vs. vulnerable (n=9) vs. stable (n=8) plaques (based on morphology and collagen staining); symptomatic (n=44) vs. asymptomatic (n=8) patients	[142]
HMOX1	Gene and protein levels upregulated in symptomatic carotid plaques and correlated with intraplaque iron deposits and hemorrhage	• Gene expression analysis on carotid endarterectomy specimens from 4 HeCES patients with bilateral stenosis: symptomatic vs. asymptomatic side/plaques • Gene expression analysis on carotid endarterectomy specimens from 40 HeCES patients with unilateral stenosis: symptomatic (n=22 patients with ipsilateral stroke symptoms) vs. asymptomatic (n=18 patients without cerebrovascular symptoms) • Western blot and immunohistochemical analysis of carotid endarterectomy specimens from 22 patients: symptomatic (n=13 patients with confirmed ipsilateral stroke) vs. asymptomatic (n=9 asymptomatic patients with normal brain imaging)	[144]
	Gene expression upregulated in advanced carotid plaques	• Gene expression analysis on carotid endarterectomy specimens from 34 patients: plaque tissue (mostly stage IV and/or V lesions) vs. adjacent macroscopically 'intact tissue' (almost exclusively stage I and II lesions)	[138]
	Gene expression upregulated in unstable carotid plaques	• Gene expression analysis on unstable (n=40 asymptomatic patients) vs. stable (n=87 symptomatic patients) carotid plaques from BiKE	[136]
	Expressed in intraplaque macrophages together with	• Immunohistochemical analysis of carotid endarterectomy specimens from 15 patients with >70% stenosis	[32]

Cell death-related genes and proteins	Expression in human atherosclerotic plaques	Methods	Ref
	hemoglobin and iron deposits		
	Expressed in atherosclerotic tissue and accumulation in advanced carotid plaques	Combined analysis of • Gene expression in carotid endarterectomy specimens from 34 patients: plaque tissue (mostly stage IV and/or V lesions) vs. adjacent macroscopically 'intact tissue' (almost exclusively stage I and II lesions) • Gene expression in carotid endarterectomy specimens from 30 patients with >70% stenosis: advanced (n=8) vs. early (n=9) plaques	138,145,146
	Gene expression in coronary plaque areas with hemorrhage	• Immunohistochemical analysis of autopsy cases from sudden coronary deaths (CVPath Institute Sudden Coronary Death registry): plaque areas with prior hemorrhage vs. areas without hemorrhage	147

BiKE= Biobank of Karolinska Endarterectomies, HeCES: Helsinki Carotid Endarterectomy Study

Table 2. Human intervention studies targeting necroptosis, pyroptosis or ferroptosis

Target	Drug	Study	Main findings	Ref
RIPK1	GSK'772	Phase I single center, randomized, placebo-controlled, double-blind study in healthy, male, adult volunteers (identifier: NCT02302404)	• Single and repeated doses were safe and well tolerated • Pharmacokinetic profiles showed dose linearity over the range tested (up to 120 mg bid) • High level of RIPK1 target engagement	46
		Phase IIa multicenter, randomized, placebo-controlled, double-blind, repeat-dose study in patients with mild-to-moderate active plaque-type psoriasis (identifier: NCT02776033)	• 60 mg bid or tid for 84 days was generally safe and well tolerated • Improved clinical efficacy measures and biomarkers in patients with mild-to-moderated active plaque psoriasis	48
		Phase IIa multicenter, randomized, placebo-controlled, double-blind study in patients with active ulcerative colitis (identifier: NCT02903966)	• 60mg tid for 84 days was generally safe and well tolerated • No clinical improvement in patients with active ulcerative colitis	49
		Phase IIa, multicenter, randomized, placebo-controlled, double-blind study in patients with moderate to severe rheumatoid arthritis (identifier: NCT02858492)	• 60mg bid or tid for 84 days was generally safe and well tolerated • No clinical improvement in patients with moderate to severe rheumatoid arthritis	53
		Phase I single center, randomized, placebo-controlled, double-blind study in healthy volunteers in the UK (NCT03305419) and in Japan (identifier: NCT03590613)	• Similar pharmacokinetics and tolerability between Western and Japanese subjects	47
		Non-randomized, open-label study to assess the pharmacokinetic profile of a modified-release prototype coated tablet formulation (identifier: NCT03649412)	• The GSK DiffCORE^{TM} technology overcame food effects and can be used in once daily dosing regimen	148
	GSK3145095	Phase I/II open-label study in patients with solid tumors (identifier: NCT03681951)	*Study terminated*	149
	DNL747/ SAR443060	Randomized, double-blind, placebo-controlled first-in-human study	• Single and multiple ascending dosing 100-400 mg bid for 14 days was generally well tolerated and safe • High level of RIPK1 target engagement • Simultaneous preclinical studies revealed long-	150

Target	Drug	Study	Main findings	Ref
			term toxicity *Production terminated*	
		Phase Ib multicenter, randomized, double-blind, placebo-controlled crossover study in patients with Alzheimer's disease (identifier: NCT03757325)	• 50 mg bid for 28 days was safe and well tolerated • High level of RIPK1 target engagement (but lower than 200 mg bid) • Simultaneous preclinical studies revealed long-term toxicity *Production terminated*	[150]
		Phase Ib multicenter, randomized, double-blind, placebo-controlled crossover study in patients with amyotrophic lateral sclerosis (identifier: NCT03757351)	• 50 mg bid for 28 days was safe and well tolerated • High level of RIPK1 target engagement (but lower than 200 mg bid) • Simultaneous preclinical studies revealed long-term toxicity *Study terminated*	[150]
	DNL758/ SAR443122	Phase IB, randomized, double blind, placebo-controlled study in hospitalized patients with severe COVID-19 (Identifier NCT04469621)	• 300mg bid for 14 days was considered well tolerated and safe • Preliminary results showed trends towards clinical improvement but a larger confirmatory trial to assess clinically significant effects is required	[151]
		Phase II, multicenter, randomized, double-blind, placebo-controlled proof of concept study in patients with moderate to severe subacute or chronic cutaneous lupus erythematosus (identifier: NCT04781816)	*Ongoing* (estimated study completion: March 2023)	
	DNL788/ SAR443820	Phase I, open-label, crossover study in healthy volunteers (identifier: NCT04982991)	• *"Robust target engagement was demonstrated at doses that were generally well tolerated"* Published on Denali Therapeutics Inc website on 6/10/2021	[152]
		Phase II multicenter, randomized, double-blind, placebo-controlled study in patients with amyotrophic lateral sclerosis (HIMALAYA study, identifier: NCT05237284)	*Ongoing* (estimated study completion: September 2023)	
	GFH312	A first-in-human, randomized, double-blind, placebo-controlled study in healthy subject (identifier: NCT04676711)	*Ongoing* (estimated study completion: August 2022)	

Target	Drug	Study	Main findings	Ref
IL-1β	Canakinumab	Randomized, double-blind, placebo-controlled, event-driven trial in the prevention of recurrent cardiovascular events among stable post-myocardial infarction patients with hsCRP ≥ 2 mg/L (Canakinumab Anti-inflammatory Thrombosis Outcome Study, CANTOS trial, identifier: NCT01327846)	• Quarterly subcutaneous administration of 150 mg canakinumab reduced the risk for recurrent cardiovascular events independent of lipid lowering • Quarterly subcutaneous administration of canakinumab decreased hsCRP levels after 48 months • A residual cardiovascular risk remains in patients despite treatment with high-intensity statins and canakinumab which was associated to IL-18 and IL-6	86,153
NLRP3 inflammasome	Colchicine (237 clinical trials on clinicaltrials.gov)	Phase III colchicine cardiovascular outcomes trial (COLCOT, identifier: NCT02551094)	• Low dose colchicine (0.5 mg once daily) reduced the risk of ischemic cardiovascular events in patients recruited within 30 days after myocardial infarction • Patients benefit from early initiation of colchicine treatment (time-to-treatment initiation effect)	154,155
		Phase III open-label, randomized, controlled trial to study the effect of low dose colchicine on the natural history of patients with stable coronary artery disease (LoDoCo, identifier: ACTRN12610000293066)	• Addition of 0.5 mg/day colchicine to standard therapy in patients with stable coronary artery disease reduced risk of cardiovascular events	156
		Phase III double-blind, randomized, controlled, investigator-initiated, event-driven trial to study the effect of low dose colchicine for secondary prevention of cardiovascular disease in patients with established, stable coronary artery disease (LoDoCo2, identifier: ACTRN12614000093684)	• Addition of 0.5 mg/day colchicine to standard therapy in patients with stable coronary artery disease decreased the occurrence of cardiovascular events	157
		Phase IV trial of anti-inflammatory therapy during percutaneous coronary intervention (identifiers: NCT02594111, NCT01709981)	• No effect on the risk for post-percutaneous coronary intervention-related myocardial infarction • Colchicine attenuated the increase in inflammatory-markers after percutaneous coronary intervention	158

Target	Drug	Study	Main findings	Ref
		Phase IV efficacy and safety study of colchicine in improving the stability of coronary plaques in patients with acute coronary syndrome (COLOCT, identifier: NCT04848857)	*Ongoing* (estimated study completion: July 2023)	
	OLT-1177 (dapansutrile)	Phase I single center, randomized, dose escalation study in healthy volunteers (NCT02134964)	• Daily oral administration of up to 1000 mg for 8 days was generally safe and well tolerated	[159]
		Phase IIa open-label, dose adaptive, proof-of-concept study in patients with monoarticular gout flare	• Orally administered 100-2000 mg/day was generally safe and well tolerated • Reduced target joint pain and joint and systemic inflammation were reported after 7 days treatment	[160]
		Phase Ib single-center, randomized, double-blind study in patients with NYHA II-III systolic heart failure (identifier: NCT03534297)	• Orally administered 500-2000 mg/day was generally safe and well tolerated in patients with stable HFrEF	[161]
		Phase 2 pilot, single-center, open-label, proof-of-concept study in patients with Schnitzler's syndrome (identifier: NCT03595371)	*Ongoing* (estimated study completion: February 2023)	
		Phase 2 multi-center, randomized, double-blind, placebo-controlled study in patients with moderate COVID-19 symptoms and evidence of early cytokine release syndrome (identifier: NCT04540120)	*Ongoing* (estimated study completion: July 2023)	
Caspase-1	VX765 (belnacasan)	Phase II randomized, double-blind, placebo-controlled study in patients with treatment-resistant partial epilepsy (identifier: NCT01048255)	• *"The primary endpoint of the study was safety and tolerability, and results from the study showed a similar safety profile for VX-765 as compared to placebo. Secondary endpoints and additional analyses evaluated the clinical activity of VX-765, and results support the initiation of a larger and longer-duration."* Published on Vertex Pharmaceuticals Inc website on 10/3/2011	[162]
		Phase IIb randomized, double-blind, placebo-controlled, parallel-group, dose-ranging study in patients with	*Terminated*	

Target	Drug	Study	Main findings	Ref
		treatment-resistant partial epilepsy (Identifier: NCT01501383)		
		Phase II randomized, double-blind, placebo-controlled study in patients with chronic plaque psoriasis requiring systemic therapy (identifier: NCT00205465)	*Completed: results to be reported*	

Table 3. Overview of the most prominent morphological and mechanistic characteristics of necroptosis, pyroptosis and ferroptosis.

	Necroptosis	Pyroptosis	Ferroptosis
Morphological characteristics	• Cell swelling • Plasma membrane rupture • Moderate chromatin condensation	• Lack of cell swelling • Plasma membrane rupture • Membrane blebbing and formation of pyroptotic bodies • Moderate chromatin condensation	• Mitochondrial abnormalities
Mechanistic markers	• P-MLKL-mediated membrane disruption	• Cleaved GSDMD-pores	• Lipid peroxidation • Iron accumulation

Pharmacological inhibition of regulated necrosis improves several features of plaque stability, such as lowering plaque inflammation, reducing oxidative stress, and increasing collagen content and fibrous cap thickness. However, different forms of regulated necrosis can occur simultaneously, particularly in advanced atherosclerotic plaques, because of the coordinated action of multiple death-inducing stimuli. Indeed, severity stages in human coronary atherosclerosis positively associate with the expression of both ACSL4 (ferroptosis), caspase-1 and NLRP3 (pyroptosis).[139] Furthermore, the expression of several ferroptosis- and necroptosis-related proteins was upregulated in atherosclerotic rabbits and did not respond to atorvastatin or PCSK9 antibody.[163] This finding highlights the possible contribution of these different forms of regulated necrosis to the residual cardiovascular risk that remains in atherosclerosis patients despite efficient lipid-lowering therapy. Accordingly, it may be necessary to target multiple death pathways. Dimethyl fumarate is a GSDMD inhibitor (pyroptosis) that reduces atherogenesis in hyperglycemic $ApoE^{-/-}$ mice by increasing Nrf2 expression and decreasing ROS production.[91] Nrf2, once activated, binds to the antioxidant responsive element (ARE) in the nucleus, thereby inducing the expression of antioxidant enzymes such as HMOX1.[164] Overexpression of HMOX1 can trigger non-canonical ferroptosis induction, thus dimethyl fumarate indirectly targets ferroptosis. Moreover, dimethyl fumarate also inhibits NF-κB and promotes apoptosis. Therefore, the atheroprotective effects of dimethyl fumarate treatment are possibly attributable to combined targeting of Nrf2, ferroptosis, pyroptosis and apoptosis. Similarly, tanshinone IIA, a flavonoid used in

traditional Chinese medicine, has atheroprotective effects due to targeting of either apoptosis,[165] ferroptosis,[166] or NLRP3-mediated pyroptosis.[167]

Another aspect that complicates therapeutic inhibition of regulated necrosis is the crosstalk between cell death mechanisms.[168] Apoptosis, autophagy, and (regulated) necrosis were initially considered mutually exclusive states. However, recent findings reveal a balanced interplay between these types of death so that blocking one type of death may sensitize cells to initiate another death pathway. For example, inhibition of caspases by the pan-caspase-inhibitor zVAD-fmk is sufficient for preventing apoptosis in many experimental models, but it may facilitate the necroptosis program downstream of TNFR. Conversely, active caspase-8 promotes apoptosis and simultaneously cleaves RIPK1 and RIPK3, thereby preventing activation of the RIPK1/RIPK3/MLKL-axis and necroptosis induction.[169,170] These observations underline the fine interplay between apoptosis and necroptosis pathways, with caspase-8 representing the molecular switch.[33] Importantly, caspase-8 can also be activated on inflammasomes when pro-pyroptotic caspase-1 is absent or inhibited.[82] Moreover, necroptosis induction via TLR activation (e.g. by poly(I:C) on TLR3 or by LPS on TLR4) upregulates the transcription of NLRP3 while RIPK1/RIPK3 signaling can induce NLRP3 inflammasome activation. During the execution phase of necroptosis, MLKL pores are formed which induce potassium efflux, a known activator of the NLRP3 inflammasome.[83] Similarly, pannexin-1 channels are formed during apoptosis, which also induce potassium efflux and NLRP3 inflammasome activation.[171] Altogether, there is a clear overlap and crosstalk between pyroptosis, apoptosis and necroptosis pathways, which was recently termed PANoptosis.[172] It should be noted, however, that crosstalk with ferroptosis is also described. For example, NLRP3-dependent pyroptosis regulates downstream ferroptosis in a mouse model of type 2 diabetes-induced cardiac remodeling and contractile dysfunction. Moreover, the pyroptosis inhibitors MCC950 and necrosulfonamide inhibit high glucose/high fat-induced ferroptosis *in vitro*.[173]

In conclusion, the simultaneous occurrence of different types of regulated necrosis and intense cross-talk between cell death pathways provide a strong scientific rationale for recommending combination therapy to prevent necrotic core formation in advanced plaques. Simultaneous inhibition of different types of cell death by combination therapy could be an important emerging concept in the field of atherosclerosis, but so far there is little experimental evidence to support this approach.

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Highlights

- The majority of dying cells in advanced human atherosclerotic plaques undergo necrosis.
- Necrotic death stimulates atherogenesis and plaque instability through induction of inflammation and enlargement of a central necrotic core.
- Apart from accidental necrosis, regulated (programmed) necrosis is a major contributor to necrotic core formation and plaque destabilization.
- Cells in atherosclerotic plaques may undergo different types of regulated necrosis including necroptosis, pyroptosis and ferroptosis.
- Regulated necrosis can be efficiently blocked with potent and selective inhibitors targeting key regulators in the necrosis pathway.

Figures legends

Figure 1. Overview of generally known regulated necrosis triggers in the atherosclerotic plaque and their impact on plaque progression and destabilization. In atherosclerosis, necrosis can occur accidentally or be induced by tightly regulated pathways, eventually leading to the development of a large hypocellular region containing remnants of dead cells known as the necrotic core. During plaque progression, the hypoxic environment inside the plaque can induce neovascularization. Since intraplaque neovessels are commonly leaky, inflammatory blood cells are recruited, and intraplaque hemorrhage occurs. Macrophages surrounding intraplaque hemorrhage phagocytose erythrocytes (erythrophagocytosis), leading to HMOX1 activation, high intracellular levels of heme and iron, and consequently ferroptosis. Moreover, oxLDL increases ROS-mediated RIPK3 and MLKL gene expression in macrophages, triggering necroptosis. As apoptotic bodies accumulate in advanced plaques, efferocytosis falls short and gets impaired, resulting in secondary necrosis. Cholesterol crystals in atherosclerotic plaques activate NLRP3 inflammasomes through lysosomal rupture leading to the induction of pyroptosis. Different forms of regulated necrosis contribute to expansion of the necrotic core which induces inflammation, plaque destabilization, fibrous cap rupture and thrombus formation. Image created with Biorender.

Figure 2. Overview of TNFα-induced apoptosis and necroptosis pathways and potential targets for pharmacological inhibition. As tumor necrosis factor α (TNFα) binds to trimeric tumor necrosis factor receptor 1 (TNFR1), recruitment of tumor TNFR1-associated death domain (TRADD) and receptor-interacting protein kinase 1 (RIPK1) is initiated. Consequently, TNFR-associated factor 2 (TRAF2), TRAF5, cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 are recruited to TRADD, thereby forming complex I. CIAP1/2 subsequently ubiquitinates RIPK1 with K63-linked ubiquitin chains, allowing the recruitment of linear ubiquitin chain assembly complexes (LUBAC). Thereupon, LUBAC generates M1-linked ubiquitin chains, which then are added to RIPK1. Subsequently, M1- and K63-ubiquitin chains act as a scaffold for the recruitment of IKKα/IKKβ/NEMO and TAB2/TAB3/TAK1, respectively. Next, TAK1 phosphorylates IKKβ and the downstream MAPKs JNK, p38, and ERK, which activate transcription factor AP-1. Phosphorylated IKKβ activates IκBα resulting in the release of nuclear factor κB (NF-κB). Subsequently, NF-κB translocates to the nucleus, resulting in the transcriptional upregulation of pro-survival genes. When cIAP1/2 is depleted by smac mimetic-induced degradation or genetic ablation, RIPK1 ubiquitination and NF-κB signaling are decreased. Simultaneous RIPK1 de-ubiquitination by cylindromatosis protein (CYLD) will result in the release of RIPK1 from complex I. Subsequently, RIPK1 engages with FADD, leading to the recruitment of pro-caspase-8 and FLICE-like protein long isoform (c-FLIP) heterodimer and pro-caspase-8 homodimer, together forming complex IIa. Pro-caspase-8 and c-FLIP heterodimer inhibit the activation of caspase-8, thereby stimulating cell survival. In contrast, pro-caspase-8 homodimer generates active caspase-8, which in turn activates caspases-3 and -7 to induce apoptotic cell death. Whenever RIPK1 is not ubiquitinated and transcription of apoptosis inhibitors is disrupted (for example by cycloheximide), complex IIb, consisting of RIPK1, RIPK3, pro-caspase-8 and c-FLIP, will be formed. In contrast to complex IIa, the kinase activity of RIPK1 is crucial for the induction of apoptosis via complex IIb. Pharmacological inhibition of apoptotic cell death can be achieved by pan-caspase inhibitors such as zVAD-fmk. When caspase-8 is inhibited, activation of RIPK1 will not result in RIPK1-kinase dependent apoptosis, but will initiate the necroptosis pathway. In the cytosol, RIPK1 binds to RIPK3, resulting in a series of auto- and trans-phosphorylations of RIPK1 and RIPK3. Phosphorylated RIPK3 consequently recruits and phosphorylates mixed lineage kinase domain-like protein (MLKL) leading to MLKL oligomerization. MLKL oligomers migrate to the plasma membrane where they induce

necroptotic cell death by membrane permeabilization and deregulation of calcium and sodium channels. The activation state (green = active, yellow = inactive) of RIPK1, caspase-8 and c-FLIP plays a decisive role in the cell's fate. Pharmacological inhibition of necroptosis is achieved with inhibitors of RIPK1 kinase activity, RIPK3, or MLKL. Image created with Biorender.

Figure 3. Overview of NLRP3-linked pyroptosis pathways and targets for pharmacological modulation. Canonical pyroptosis is characterized by assembly and activation of inflammasomes, which are large supramolecular complexes required for caspase-1 activation. The NLRP3 (nucleotide-binding oligomerization domain (NOD)-like, leucine-rich repeat (LRR) and pyrin domain containing receptor 3) inflammasome consists of NLRP3, ASC (apoptosis inhibitor speck-like protein) and procaspase-1. NLRP3 contains a C-terminal LRR, a central nucleotide domain called NACHT and an N-terminal pyrin domain (PYD). A priming step is required to increase the transcription of pro-IL-1β, NLRP3 and ASC, and is induced by recognition of extracellular molecules such as LPS, TNFα or IL-1β by pattern-recognition receptors (PRRs). Assembly of the inflammasome occurs through ASC which contains a PYD that interacts with the N-terminal PYD in NLRP3, and a caspase recruitment domain (CARD) for binding of procaspase-1. To allow proximity, and hence NLRP3 activation, between NLRP3 (mitochondria) and ASC (endoplasmic reticulum), activation of α-tubulin is required. The latter process can be pharmacologically inhibited by tubulin polymerization inhibitor colchicine. Activation of NLRP3 is induced by low intracellular potassium concentrations, for example by potassium efflux through ionophores or cation channels. Cathepsins are also required for NLRP3 activation and are released after lysosomal rupture induced by oxidized LDL (oxLDL), cholesterol and calcium crystals. Activation of NLRP3 results in cleavage of procaspase-1 to active caspase-1, which converts pro-IL-1β and pro-IL-18 to their bioactive forms and cleaves Gasdermin D (GSDMD) N-terminally (NT). NT-cleaved GSDMDs oligomerize and form NT-GSDMD pores, which allow the secretion of cytokines and eventually results in membrane lysis and cell death. Caspase-1 can be pharmacologically inhibited with VX-765. Pharmacological inhibition of GSDMD can be obtained with disulfiram, dimethyl fumarate or necrosulfonamide. Neutralization of IL-1β with canakinumab or anakinra provides another strategy to block pyroptosis-induced inflammation. Alternatively, pryoptosis can be induced through caspase-11, which can

directly sense cytoplasmic LPS without the need for an inflammasome, termed non-canonical pyroptosis. Caspase-11 contains an LPS binding site located on its CARD domain through which a procaspase-11-LPS complex is formed. Subsequently, caspase-11 is activated which results in NT-cleavage of GSDMD and pyroptotic death. Caspase-11-induced NT-GSDMDs also serves as activator of the NLRP3 inflammasome thus, caspase-11 indirectly triggers canonical pyroptosis. Image created with Biorender.

Figure 4. Overview of ferroptosis-inducing pathways and potential pharmacological targets. Ferroptosis is characterized by excessive iron-dependent lipid peroxidation of poly-unsaturated fatty acids (PUFAs) in phospholipid (PL) bilayers, which can be induced enzymatically or non-enzymatically and requires free ferrous iron (Fe2+). Enzymatic lipid peroxidation occurs through the Acyl-CoA Synthetase Long Chain family member 4 (ACSL4)/Lysophosphatidyl Acyltransferase 3(LPCAT3)/15-Lipoxygenase (LOX)-axis. Non-enzymatic lipid peroxidation includes Fenton chemistry, which forms hydroxyl radicals, and free radical chain reactions. Free ferrous iron resides in a cytosolic labile iron pool. Growth of the labile iron pool is limited by ferritin, which stores Fe2+. When the finely regulated iron balances are disturbed due to overloading of cells with iron, e.g., with hemoglobin, hemin, ferrous ammonium sulfate or iron chloride, or due to excessive activation of heme-oxygenase-1 (HMOX1), decreased ferroportin (iron transporter) expression or enhanced transferrin (iron receptor) expression, the labile iron pool grows beyond the buffering capacities of ferritin, which suffices to induce ferroptosis. Under physiological conditions, lipid peroxides formed in cellular membrane environments are reduced by glutathione peroxidase 4 (GPX4), thereby oxidizing glutathione (GSH). For the synthesis of GSH entry of cysteine (Cys) in exchange for glutamate (Glu) through the Xc-antiporter system is required. Inhibition of the Xc-antiporter with erastin, inhibition of glutathione synthesis or direct inhibition of GPX4 with 1S,3R-RSL3 induces canonical ferroptosis through accumulation of lipid peroxides. Excessive lipid peroxidation of PUFAs in PL bilayers affects chemical and geometric properties of the lipid bilayer which destroys the barrier function of the plasma membrane and leads to cell lysis and eventually cell death. Physiologically ferroptosis is limited by ferritin and by ferroptosis suppressor protein 1 (FSP1). FSP1 reduces Coenzyme Q10 (CoQ10) to ubiquinol, which traps lipid peroxyl radicals. Pharmacological ferroptosis inhibition is obtained with radical trapping agents such as α-tocopherol, Liproxstatin,

Ferrostatin-1 and derivates, with iron chelators (e.g., deferoxamine) or with blockers of the ACSL4/LPCAT3/15-LOX-axis (e.g., PRGL493, baicalcein, α-tocopherol and LOXblock-1). Image created with Biorender.

Figure 5. Cross talk between necroptosis, apoptosis, secondary necrosis, pyroptosis and ferroptosis. When TNFα binds to its receptor (TNFR) membrane-bound complex I is formed which contains receptor-interacting protein kinase 1 (RIPK1). In complex I RIPK1 is ubiquitinated (Ub) and phosphorylated (P) which keeps it in an inactive conformation. RIPK1 serves as a scaffold for pro-survival signaling such as MAPK and NF-κB pathways. Caspase 8 is inactivated by cellular FLICE-like protein (c-FLIP). When c-FLIP is absent, caspase 8 is released and engages with RIPK1 to form complex IIa. If RIPK1 is not ubiquitinated or de-ubiquinated it is released from complex I and engages with caspase 8 to form complex IIb. Both complex IIa and complex IIb activate caspase 8, which in turn activates apoptosis effector caspases 3 and 7. Caspase 8 also induces the mitochondrial release of pro-apoptotic proteins. Caspase 3 can cleave gasdermin E (GSDME), thus excessive caspase 3 activation leads to GSDME-mediated secondary necrosis. If RIPK1 is not ubiquitinated and caspase 8 is inactivated (e.g. by c-FLIP), RIPK1 is released from complex I, undergoes activating autophosphorylations and phosphorylates RIPK3. Phosphorylated RIPK1 and RIPK3 form a necrosome which recruits and phosphorylates mixed lineage kinase domain-like protein (MLKL). Subsequently, oligomerization of MLKL induces membrane permeabilization and eventually necroptosis. During necroptosis, apoptosis and secondary necrosis, potassium efflux occurs which is an activator of the NLRP3 (nucleotide-binding oligomerization domain (NOD)-like, leucine-rich repeat (LRR)- and pyrin domain-containing receptor 3) inflammasome. Cholesterol crystals indirectly also induce potassium efflux. Caspase 1 is activated on the NLRP3 inflammasome and in turn cleaves GSDMD N-terminally (NT) and activates IL-1β and IL-18. NT-GSDMDs oligomerize and form GSDMD-pores leading to osmotic lysis and cytokine release. In the absence of GSDMD, caspase 1 induces apoptosis through activation of caspase 3. Reciprocally, caspase 3 limits pyroptosis through inhibition of GSDMD. In the absence of caspase 1, caspase 8 can also become activated on the NLRP3 inflammasome and can cleave GSDMD. GSDMD and GSDME can also promote mitochondrial release of pro-apoptotic proteins, demonstrating intense cross talk between pyroptosis, apoptosis and secondary necrosis. Moreover, the necroptosis effector MLKL induces

assembly of the NLRP3 inflammasome, which can be activated by RIPK1 and RIPK3. Transcription of NLRP3 inflammasome components can be induced through NF-κB pathways, linking TNFα signaling and pyroptosis. Similarly, heme and oxidative stress can also activate the NLRP3 inflammasome, providing a link between ferroptosis and pyroptosis. Indeed, heme is formed together with ferrous iron (Fe^{2+}) when hemoglobin (Hb) is catabolized by heme-oxygenase 1 (HMOX1). This contributes to growth of the labile iron pool, which is limited by ferritin. Growth of the labile iron pool beyond the buffering capacity of ferritin, e.g., by excessive HMOX1 activation, increased expression of transferrin receptor (TfR) or decreased expression of ferroportin (Fpn), can induce ferroptosis through Fenton chemistry and non-enzymatic lipid peroxidation. Alternatively, enzymatic lipid peroxidation by 15-lipoxygenase (15-LOX) can also induce ferroptosis. Excessive lipid peroxidation can be limited by glutathione peroxidase 4 (GPX4) and ferroptosis suppressor protein 1 (FSP1). During lipid peroxidation toxic lipid aldehydes are formed such as malondialdehyde (MDA) and 4-hydroxynonenal (4HNE). The latter was recently reported to inhibit NLRP3 inflammasome activation. These examples illustrate that the fate of a cell depends on many factors (e.g., cell death stimulus, cellular environment, cell type and expression pattern of cell death executors) and is subjected to intense cross talk between cell death pathways. Image created with Biorender.