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Review

Neutrophils and extracellular traps in crystal-associated diseases

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Crystalline material can cause a multitude of acute and chronic inflammatory diseases, such as gouty arthritis, silicosis, kidney disease, and atherosclerosis. Crystals of various types are thought to cause similar inflammatory responses, including the release of proinflammatory mediators and formation of neutrophil extracellular traps (NETs), processes that further promote necroinflammation and tissue damage. It has become apparent that the intensity of inflammation and the related mechanisms of NET formation and neutrophil death in crystal-associated diseases can vary depending on the crystal type, amount, and site of deposition. This review details new mechanistic insights into crystal biology, highlights the differential effects of various crystals on neutrophils and extracellular trap (ET) formation, and discusses treatment strategies and potential future approaches for crystal-associated disorders.

Highlights

Deposits of crystals within the body, or exposure to external crystalline material, can cause diverse metabolic and inflammatory acute as well as chronic medical disorders.

Neutrophils are crucial in the immune response to these pathogenic crystals, from contributing to necroinflammation and driving further tissue damage through the release of proinflammatory mediators and neutrophil extracellular trap (NET) formation to limiting inflammation.

The mechanisms of how neutrophils respond to these pathogenic crystals, specifically their mode of cell death (e.g., NETosis, necrosis, necroptosis, and/or pyroptosis), depends on the crystal type, site of crystal deposition, and amount of crystals, all of which influence the intensity of inflammation.

Targeting NET components and certain forms of neutrophil death as well as crystallization are novel treatment targets that may improve outcomes in crystal-associated disorders.

Crystals as contributors to inflammation and organ injury

Crystals can either form through aberrant crystallization of intrinsic organic material inside the human body or enter the body (usually via the lung) as extrinsic crystals and microparticles. Intrinsic crystals from inorganic minerals are physiologically important and tolerated by the immune system to provide structural stiffness and durability. However, abnormal crystallization and deposition of organic material (Box 1) cause diverse medical disorders that can manifest as either acute or chronic organ injuries, including **gouty arthritis** (see Glossary), gallstones, **Alzheimer's disease**, and **atherosclerosis**. Extrinsic crystals (Box 1), such as occupational dust, can lead to pneumoconiosis, silicosis, and cancer [1].

Organ injuries and diseases caused by these crystals are commonly associated with inflammation, cell death, fibrosis, thrombosis, and/or obstruction of vessels and ducts. During these processes, neutrophils are crucial. Several forms of crystal-related neutrophil death have been identified, including **neutrophil extracellular traps (NETs)**, **apoptosis**, **NETosis** and **necroptosis**. NETs are important not only for pathogen clearance [2] during host defense, but are also involved in driving inflammation and further tissue damage (Box 2) [3]. Recent evidence indicates that aggregated NETs can even limit inflammation [4]. Thus, the identification of NETs has helped us to refine our view of the role of neutrophils in numerous crystal-related diseases (Figure 1). It has become apparent that the type of neutrophil death may vary depending on the crystalline material, the site of crystal deposition, and the amount of crystals in the body [5]. This suggests that the immune response and severity of inflammation might differ in the tissue depending on the crystal type. In this review we outline new mechanistic insights into crystal biology and immunity, and address the differential effects of numerous crystals on neutrophils and ET formation as well as on NETs in crystal-related disease manifestations. This will inform our discussion on potential treatment strategies and provide future research opportunities for crystal-associated diseases. Our purpose is to provide a succinct, yet comprehensive, overview of the latest updates in this field.

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Box 1. Crystal formation and types of crystals

Crystallization

In general, crystallization inside the human body occurs through reduced solubility (leading to supersaturation), nucleation, and crystal growth [100]. Nucleation begins in the liquid phase, in which small molecules (e.g., minerals) cluster and agglomerate together. After crystals nucleate, they start growing immediately and can eventually form larger crystalline particles, known as aggregates. The crystallization process involves many factors such as pH, ionic strength, body temperature, low solubility, connective tissue factors and proteins, reduced urine volume, or a combination of these factors, which determine crystal type, shape, size, and distribution [101]. Additionally, fibers (probably collagen) in the synovial fluid of patients with gout might also affect MSU crystal formation by templated nucleation [102].

Under healthy condition, crystallization is tightly regulated: for example, the formation of life-essential structures such as teeth. At the same time, the body has different strategies in place to avoid harmful crystallization: for example, (i) a strong blood flow can limit crystal nucleation and growth [103], (ii) plasma and urine proteins (e.g., apolipoproteins, uromodulin, Tamm–Horsfall protein) and small molecules (e.g., citrate) [104,105], (iii) excretion of minerals via the urinary tract, and (iv) dietary or drug metabolites [106] can reduce supersaturation. However, when crystals aggregate and continue to form in the human body, the overloaded particles will induce necroinflammation, obstruction, fibrosis, and sometimes even organ failure [1].

Intrinsic pathological crystals

In the case of an imbalance of proteins, lipids, and mineral precipitation, formation and deposition of endogenous crystals will occur in the body, which applies to various diseases (see Figure 1 in the main text). Many factors contribute to the crystallization process and influence the size, shape, and distribution of crystals. Such pathological crystals can cause not only kidney stones and crystal nephropathy (UA, CaOx crystals) but also gallstones and atherosclerotic plaques, gouty arthritis, neurodegenerative diseases, and the propagation of the malaria parasite within infected patients [1].

Extrinsic pathological crystals

Apart from intrinsic crystals, various extrinsic crystals can enter and deposit in the human body (see Figure 1 in the main text). Airborne environmental pollutants and occupational dusts such as silica and asbestos can be easily inhaled and accumulate in the lung. Other sources of extrinsic crystalline material include cosmetics or implants. In recent years, the exposure of humans to nanoparticles is rising due to their wide applications (e.g., vaccine adjuvants and nanomedicines) [16]. Several drugs can also precipitate within kidney tubules and cause crystal nephropathy due to their insolubility in the human urine (e.g., sevelamer, acyclovir, methotrexate, indinavir, and sulfadiazine).

Box 2. Mechanisms of ET formation in neutrophils

Neutrophils are crucial for host defense [3], but also in non-infectious diseases. Once recruited to the site of tissue injury, neutrophils form NETs and subsequently promote necroinflammation (see Figure 2 and Table 1 in the main text). Recent studies indicate that NETosis can be classified into two forms: suicidal and vital [107]. Suicidal NETosis occurs in a NOX-independent manner through Ca^{2+} influx and mitochondrial ROS production [108]; it involves chromatin decondensation, nuclear swelling, and membrane rupture. By contrast, vital NETosis seems to be oxidant-independent without membrane rupture, where neutrophils release mitochondrial DNA and/or nuclear DNA in vesicles but retain their ability to migrate, phagocytose, and kill pathogens [108,109]. Although it seems that NETs are decorated with a conserved set of proteins (e.g., complement, PAD4, cytokines) and enzymes (e.g., NE, MPO, histones) [110], the composition of NETs varies depending on the type and concentration of crystals and the time of exposure. Not only the composition but also post-translational modifications of proteins are heterogeneous in NETs [111]. Whether different NET compositions can exhibit different biological functions, and whether crystals can induce vital NETs, are currently unknown. Similarly, it is unclear how neutrophils exactly decide between phagocytosis and NET formation when combating crystals. Studies suggest that neutrophils selectively release NETs in response to larger crystals, while smaller particles are phagocytosed [112].

NETs are proinflammatory and cytotoxic. Under conditions where NETs are inappropriately released or not rapidly degraded and cleared, they can be pathogenic and cause tissue inflammation and damage (e.g., in Alzheimer's disease, chronic obstructive pulmonary disease, and cystic fibrosis) [113]. In non-crystalline autoimmune or autoinflammatory diseases such as rheumatoid arthritis, NETs can even act as autoantigens. In turn, immune complexes containing NET autoantigens – such as double-stranded DNA, histones, and MPO coupled with autoantibodies – can further promote innate and adaptive immune responses [114], suggesting that NETs, if not removed from tissues or the circulation, might exacerbate inflammation and tissue damage. Thus, the immune system has certain mechanisms in place to prevent or diminish inadvertent immunological responses, including clearance of apoptotic neutrophils [115,116], degradation of NETs by DNase I and complement factor C1q [117], as well as removal of NETs [118]. Evidence even suggests now that NETs have anti-inflammatory effects and contribute to the resolution of inflammation. For example, in gouty arthritis, neutrophils when present in high numbers form aggregated NETs in response to MSU crystals and subsequently release proteases that can trap, cleave, and inactivate proinflammatory cytokines [119].

Glossary

Alzheimer's disease: a progressive brain disorder beginning with mild memory loss and eventually leading to a gradual decline in memory, thinking, behavior, and social skills.
Apoptosis: the orchestrated collapse of a cell characterized by membrane blebbing, cell shrinkage, condensation of chromatin, and fragmentation of DNA, followed by rapid engulfment of the corpse by neighboring cells. Unlike necrosis, apoptosis is a silent process without inducing an inflammatory response.
Atherosclerosis: a build-up of fats, cholesterol, calcium, and other substances in arteries (known as plaque) in the heart, legs, brain, and kidneys. It can lead to heart attack, stroke, or heart failure, among other conditions.
Crystallopathy: a disease caused by crystalline material associated with tissue injury, acute or chronic inflammation and cell death (known as necroinflammation), remodeling of tissues leading to atrophy and scarring, and obstruction of ducts or vessels by larger crystal masses.
Damage-associated molecular patterns (DAMPs): intracellular mediators released into the extracellular space during cell necrosis upon plasma membrane rupture; DAMPs act as danger signals by eliciting proinflammatory responses.
EETosis: a regulated active form of NADPH-oxidase-dependent eosinophil extracellular trap cell death, involving membrane rupture, release of granule proteins, and filamentous chromatin structures, but not accidental necrosis or apoptosis.
Ferroptosis: an intracellular iron-dependent form of cell death that is distinct from apoptosis, necrosis, and autophagy. It involves mainly genetic changes in iron homeostasis and lipid peroxidation metabolism during cell death.
Gouty arthritis: a form of inflammatory arthritis associated with acute onset of severe pain, swelling, redness, and tenderness due to the deposition of monosodium urate crystals.
Necroinflammation: the inflammatory response to necrotic cell death. It is associated with the release of DAMPs from dying cells, which further promotes inflammation and tissue injury.
Necroptosis: a form of programmed lytic NETosis requiring receptor-

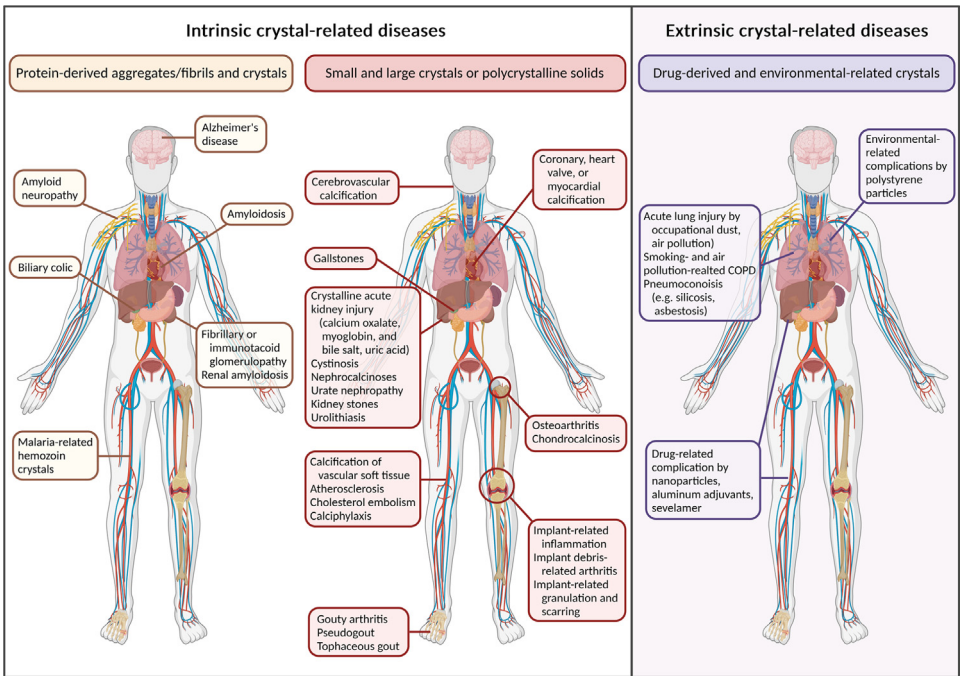

Figure 1. Crystal-associated diseases grouped according to the crystal type. Intrinsic or extrinsic crystal-related disease manifestations can be broadly grouped in three categories, according to the origin of crystals: protein-derived crystals, small and large crystals or polycrystalline solids, and drug-derived and environment-related crystals. The predominant pathological mechanisms of these crystal-related disorders include necroinflammation with neutrophil extracellular trap (NET) formation by smaller crystals and chronic tissue remodeling leading to tissue atrophy and scarring, and obstruction of ducts, cavities, or vessels by larger crystal masses, calculi, or stones. Abbreviation: COPD, chronic obstructive pulmonary disease. Figure created with BioRender.

Mechanisms for crystal interaction with the immune system

The crystallization process is complex and involves many factors which affect the size, shape, rigidity, stiffness, and electrical surface charge of crystals (Box 1), thus dramatically influencing the outcomes of their interaction with tissues and cells in the human body [5]. This includes how immune cells sense and select crystals, what factors influence crystallization and immune cell function, the mode of crystal-related cell death, and the associated inflammatory processes.

Recognition of crystals by immune cells

Cells of the immune system can distinguish and differentially respond to small variations in crystal morphology and size. Immune cells, including macrophages and neutrophils, ingest particles within the nanometer to micrometer size range [1]. Crystals can be directly recognized by neutrophils and other immune cells via receptors: for example, the inhibitory C-type lectin receptor Clec12a, CD14 and signal inhibitory receptor on leukocytes-1 (SIRL-1) for monosodium urate (MSU) crystals [6,7], macrophage receptor with collagenous structure (MARCO) and low-density lipoprotein receptor (LDLR) for MSU, calcium pyrophosphate dehydrate (CPPD), and calcium oxalate (CaOx) crystals [8], and Clec4e (known as Mincle) and MACRO for cholesterol crystals [8,9]. However, further studies are needed to identify receptors for the recognition of other crystalline material by neutrophils. Although receptors are important for phagocytosis of larger crystalline material, other physical characteristics of crystals also play an important role in cell activation without involving receptors, including ion influx and efflux [10]. It is currently

interacting protein kinases 1 and 3 (RIPK1 and 3) and mixed lineage kinase domain-like (MLKL) protein.

NETosis: a cell death program of neutrophils releasing neutrophil extracellular traps (NETs) and undergoing necrosis (suicidal NETosis), whereby NET-like structures containing chromatin decondensed nuclear DNA coated with neutrophil cytosolic and granule proteins – such as neutrophil elastase, citrullinated histone H3, PAD4, ROS, and myeloperoxidase – are released upon activation and membrane rupture.

Neutrophil extracellular traps (NETs): NETs are generated by viable neutrophils releasing mitochondrial DNA and granule proteins. This process requires an active NADPH oxidase, rearrangements in the cytoskeleton, and glycolytic ATP production without membrane rupture.

NOD-like receptor protein 3 (NLRP3) inflammasome: a multimeric protein complex activated by diverse danger signals/stimuli to initiate an inflammatory response through the release of the proinflammatory cytokines IL-1β and IL-18.

unknown whether neutrophils require receptors also for the endocytosis of small crystals, or whether this occurs in an indirect way without receptors.

Crystallization and the influence on immune cells

The formation of intrinsic crystals occurs in a multistep process involving many factors such as pH, body temperature, concentration, low solubility, proteins, reduced urine volume, or a combination of these factors, which affect crystal properties and the associated immune response (Box 1) [1]. For example, temperatures lower than 37°C promote MSU crystal formation *in chemico* and increase MSU-crystal-mediated **NOD-like receptor protein 3 (NLRP3) inflammasome** activation and interleukin (IL)-1β release in macrophages [11], while a higher temperature of 39°C seems to influence the characteristics of MSU crystals and hence promotes respiratory burst in neutrophils [12]. Differential effects of microcrystals have also been reported in human neutrophils *in vitro*. While both MSU and CPPD crystals induced IL-8 release in tumor necrosis factor (TNF)α and granulocyte-macrophage colony-stimulating factor (GM-CSF)-primed neutrophils, macrophage inflammatory protein 1 (MIP-1) secretion was reduced in TNFα primed neutrophils upon MSU or CPPD crystal stimulation, suggesting that inflammatory mediators can affect the response of immune cells to crystals [13].

Crystal-induced cell death

The interaction between crystals and cells can induce a broad range of immune responses. Crystals are taken up by cells into phagolysosomes where this material is broken down and digested, whereas an overload of indigested crystals will induce cell stress, actin depolymerization, reactive oxygen species (ROS) production, and NLRP3 inflammasome activation [14]. Many crystals are cytotoxic to different cell types by inducing lysosomal rupture, tyrosine phosphorylation, activation of cathepsins, and mitochondrial dysfunction, as well as ET formation in neutrophils and other immune cells (Boxes 2 and 3 and Figure 2) [15]. The mode of crystal-induced cell death varies depending on the crystal type and size (Table 1). While bigger crystals induce NETs (NETosis), single small crystals do not, but rather are taken up into the phagolysosome or endosomes without being degraded where they may influence cell activation, differentiation, and function [16,17]. For example, CaOx or MSU crystals can induce receptor-interacting serine/threonine-protein kinase 3 (RIPK3)–mixed lineage kinase domain like pseudokinase (MLKL)-dependent necroptosis in neutrophils and NET formation [15]. MSU crystals induce size-dependent pyroptotic cell death in neutrophils and macrophages via NLRP3 inflammasome

Box 3. Mechanisms of ET formation in other immune cells

Except for neutrophils, recent studies have revealed that other immune cells – such as macrophages, mast cells, basophils, and eosinophils – can release ETs (Figure 2). Macrophages and monocytes were the first cells reported to release ETs when stimulated with statins [120]. In a mouse model of rhabdomyolysis-induced acute kidney injury, macrophages release ETs (METs) containing DNA fibers, histones, and granule proteins [121]. In response to calcium phosphate crystals, macrophages are capable of releasing METs [122] as observed with NETs [123]. However, unlike granulocytes (neutrophils, eosinophils, and basophils), monocytes cannot form METs in response to MSU crystals [124]. In addition, eosinophil cytolysis represents a regulated active mode of cell death of activated eosinophils (EETosis) [26] similar to that observed in neutrophils. Like neutrophils, eosinophils actively select their death program, promoting inflammation and/or efficient elimination of CLCs [28], thrombosed coronary atherosclerotic plaques [125], and MSU crystals [124]. Interestingly, silica crystals induce NETosis but not EETosis, suggesting that the size and shape of crystals may play a role in EET formation. However, a recent study has demonstrated that the absence of elastase results in the failure of plasma membrane breakdown, leading to a lack of EET formation [126]. Unlike neutrophils and eosinophils, basophil ET formation does not require phagocytosis of MSU crystals for the release of extracellular DNA, but the cell contact with MSU crystals triggers the upregulation of the activation marker CD203c [124], suggesting a role for basophil ETs in crystal-related diseases. Mast cells are known for their role in allergic diseases, as well as many other infectious conditions. In response to pathogens, both human and murine mast cells can release NET-like ETs with antimicrobial activities [127]. However, in contrast to NETs, DNase I alone was not sufficient to dismantle mast cell ETs only on addition of tryptase-degrading MPO. Whether crystalline material can induce mast-cell ETs is currently unknown.

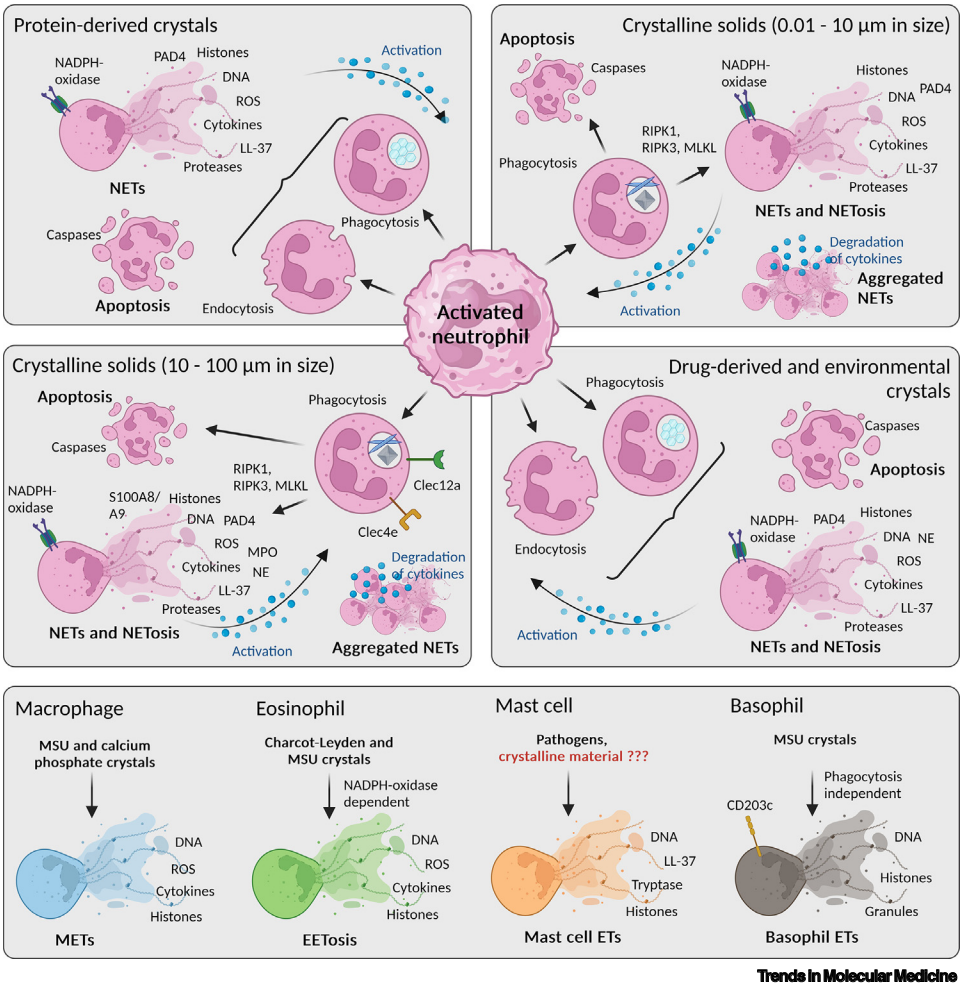

Figure 2. Molecular mechanisms of crystal-induced neutrophil extracellular trap (NET) formation. Upon activation, neutrophils take up crystalline material by phagocytosis or endocytosis, which triggers a cascade of inflammatory processes including NET formation, cell death, and release of proinflammatory mediators that further promote necroinflammation and tissue injury. The inflammatory processes related to neutrophils share certain pathomechanisms, such as apoptosis, NET formation, release of proinflammatory mediators – for example, histones, reactive oxygen species (ROS), proteases, neutrophil elastase, cytokines – and neutrophil activation, but can also vary depending on the crystalline material. The crystalline-material-related neutrophil responses can be categorized in four groups: protein-derived crystals, crystalline solids sized 0.01–10 µm and 10–100 µm, and drug-derived and environmental crystals. While smaller crystals – such as protein-derived, drug- and environment-derived crystals – are taken up via phagocytosis or endocytosis, larger crystals are mainly internalized by phagocytosis via receptor-interacting serine/threonine-protein kinase 1 (RIPK1)–RIPK3–mixed lineage kinase domain like pseudokinase (MLKL)-dependent neutrophil necroptosis and/or NETs/NETosis. NETs are not only considered proinflammatory but also anti-inflammatory under conditions where neutrophils are present in high numbers and, therefore, contribute to the resolution of inflammation (e.g., in acute gouty arthritis). Additionally, other cells, such as macrophages, mast cells, basophils, and eosinophils, are also able to release extracellular traps depending on the stimuli. Abbreviations: Clec, C-type lectin receptor; ET, extracellular trap; MPO, myeloperoxidase; MSU, monosodium urate; NE, neutrophil elastase; PAD4, peptidyl arginine deiminase 4. Figure created with BioRender.

activation and gasdermin D, while larger MSU crystals trigger NET formation [18]. Small silica crystals can induce caspase 9-dependent apoptosis in lung epithelial cells, causing airway inflammation and epithelial barrier damage [19] as well as NETosis [20]. Various nanomaterials used for cancer therapy were reported to induce iron-dependent **ferroptosis** [21]. Crystal masses that

Table 1. Mechanisms of crystal- and microparticle-related NET formation in human diseases[a]

Crystal type	Crystal-associated disease	Tissue location	NET formation	Mode of neutrophil death	Signaling pathway	Refs
Amyloid fibrils and amorphous aggregates	Degeneration disease	Brain, peripheral tissues	Yes	Apoptosis, necrosis	NADPH-oxidase/ROS-Ca^{2+}, mitochondrial damage, apoptosis	[23,128]
Charcot–Leyden crystals	Allergy	Lung	?	Necrosis	NADPH oxidase	[28,129]
Heme/hemozoin crystals	Malaria	Vascular	No/yes	?	Heme: neutrophil proteases, CDK6, PKC, and ROS; Hemozoin: TLR9/NF-κB, NLRP3/IL-1	[30–32,130]
CaOx crystals	Oxalosis	Kidney	Yes	Necroptosis, NETosis	RIPK1–RIPK3–MLKL-dependent pathway, ROS, NLRP3	[10,14,15]
Calcium carbonate	Gallstone/kidney stone	Pancreas, kidney	Yes	NETosis	PAD4-dependent pathway	[38]
Brushite crystals	Kidney stone	Kidney, bone	?	?	CD11b, CD18, CD55, and CD66a	[34,131]
Hydroxyapatite crystals	Osteoarthritis	Joints, tendons, vessels	?	Apoptosis	NF-κB pathway/NLRP3	[39,132,133]
MSU crystals	Gout/UA nephrolithiasis	Joints, kidney	Yes	NETosis, necroptosis, apoptosis, autophagy, pyroptosis	Purinergic receptors P2Y and P2Y6 ROS, RIPK1–RIPK3–MLKL, PI3K pathway, syk and src Endosomal acidification, lysosomal NADPH-oxidase-dependent and -independent	[14,18,45,134]
CPPD	Pseudogout	Joint	Yes	NETosis, apoptosis, necroptosis	NLRP3, ERK/MEK and PI3K/Akt pathway, HSP90, RIPK1–RIPK3–MLKL	[15,49,135]
Cholesterol crystals	Atherosclerosis, kidney C	Vascular, kidney	Yes	NETosis, necrosis, pyroptosis	ROS, NE, PR3, NLRP3/IL-1b RIPK1–RIPK3–MLKL	[14,51,53]
Pigment calculi/ mixed calculi/ cholesterol calculi	Gallstone	Gallbladder	Yes	NETosis	ROS	[81]
Nanoparticles (e.g., quartz, titanium, silver, gold, cationic lipid)	Oxidative stress and systemic inflammation	Blood, lung, heart, brain	Yes	NETosis, apoptosis	ROS, NADPH oxidase, JNK, NF-kb	[56]
Alum	Adjuvants	Muscle	Yes	NETosis, necrosis	Syk and PI3K	[136]
Asbestos	Asbestosis/mesothelioma	Lung	Yes	NETosis, necroptosis, apoptosis	DNA release, ROS independent, IL-1, RIPK1–RIPK3–MLKL	[14,70]
Silica	Silicosis	Lung	Yes	NETosis, apoptosis	ROS, IL-1, RIPK1–RIPK3–MLKL	[14,70]
Sevelamer crystals	Gastrointestinal complications	Gastrointestinal tract	Yes	NETosis, necrosis	?	[65–67]
Polystyrene	Multiorgan	Multiorgan	Yes	NETosis, necrosis	ROS-NLRP3, PAD4, NE	[74]

[a]Abbreviations: CaOx, calcium oxalate; CCE, cholesterol crystal embolism; CPPD, calcium pyrophosphate dihydrate; IL-1, interleukin-1; JNK, c-Jun N-terminal kinases; MLKL, mixed lineage kinase domain like pseudokinase; MSU, monosodium urate; NE, neutrophil elastase; NLRP3, NLR family pyrin domain containing 3; PAD4, peptidyl arginine deiminase 4; PKC, protein kinase C; RIPK1, receptor-interacting serine/threonine-protein kinase 1; ROS, reactive oxygen species; ?, not known.

are too large to be internalized are segregated from the parenchymal tissue by macrophages, known as granulomas, without inducing cell death *per se* [22].

Crystal-induced necroinflammation

Crystal-induced cell death is accompanied by the release of **damage-associated molecular patterns (DAMPs)**, such as high mobility group box 1 (HMGB1), histones, adenosine triphosphate (ATP), uric acid (UA), and double-stranded DNA. These molecules further trigger inflammation by activating Toll-like receptors, the complement system, and inflammasomes, and promote the release of cytokines, chemokines, kinins, and lipids [5]. In addition, most crystals act directly as danger signals upon phagocytosis and trigger the activation of the NLRP3 inflammasome and IL-1β release in immune and non-immune cells, depending on the tissue, which further promotes inflammation and tissue injury in a vicious cycle known as **necroinflammation**.

NETs in crystal-related disease manifestations

The incidence of inflammatory diseases is increasing, both in developed and developing countries, concomitant with a rise in living standards, the adoption of a western lifestyle, and economic development. This also applies to **crystallopathies** (some of which are described in more detail later) according to the crystal composition, morphology, and their ability to induce NETs and NETosis (Figure 1, Box 2, and Table 1).

Intrinsic crystal-associated diseases

Intrinsic crystals can be classified into protein-derived aggregates/fibrils and crystals, crystalline solids, and polycrystalline solids that cause a multitude of crystal-associated diseases (Figure 1).

Protein-derived crystals – such as amyloid fibrils and protein aggregates (e.g., amyloid, transthyretin, immunoglobulin light and heavy chains) – are associated with protein misfolding diseases, including Alzheimer's and Parkinson's disease [1]. For example, aggregated amyloid fibrils can trigger NAPDH-oxidase-dependent NET formation in human neutrophils [23], which induces a metabolic shift in the pentose phosphate pathway [24], but can also be digested by neutrophil elastase (NE) into fibril fragments that are toxic to other cells [23]. This may explain why NE and histones were found in amyloid deposits in patients with systemic amyloidosis [23]. NETs have been observed in the cerebral vasculature and parenchyma of mice and in individuals with Alzheimer's disease, suggesting that NETosis may contribute to central nervous system inflammation and damage in humans [25]. Of note, at high concentrations, such protein aggregates and amyloid fibrils can form birefringent microcrystals due to fragmentation or monomer dissociation of the fibrils and an increase in intermolecular pleated β-sheet and hydrogen bonding. It remains to be investigated whether amyloid fibrils and microcrystals exhibit similar or differential immunological effects. In addition, Charcot–Leyden crystals (CLCs) are eosinophilic, refractile, non-birefringent, thin, hexagonal bipyramidal structures, commonly found in humans, caused by allergic diseases such as asthma, parasitic infections, or idiopathic eosinophilic inflammation [5]. In humans, eosinophils and basophils are the primary sources of CLCs. CLC formation is an active process associated with the release of **EETosis** via NADPH oxidase [26]. Other eosinophilic crystalline structures – including hemoglobin crystals, skeinoid and rosenthal fibers, and reinke crystals – have also been observed in certain forms of cancer and sickle cell hemoglobin C disease [27]. Although current studies indicate that CLC formation is associated mainly with eosinophil EETosis [28], further investigation is required to identify the inflammatory potential of CLCs and other eosinophilic crystals in neutrophils and NET formation. Hemozoin is an insoluble crystalline pigment (protein-derived) produced by the malaria parasite *Plasmodium* during infection. Following red blood cell rupture, hemozoin crystals (0.1–1.0 μm) are released into the circulation and cleared by phagocytes [29]. Experimental studies have shown that extracellular heme,

but not hemozoin crystals, induces NETosis and NET formation in TNFα-primed neutrophils independently of peptidyl arginine deiminase 4 (PAD4) and NADPH oxidase 2 (NOX2) [30]. Unlike heme, hemozoin crystals can be internalized in phagosomes and vesicles/vacuoles by human neutrophils, which leads to morphological abnormalities without cell membrane rupture and NET release [31]. Although hemozoin crystals do not directly induce NETs, they contribute to immune activation and inflammation [32]. Effects of parasite-induced NETs during malaria are reviewed elsewhere.

Crystalline solids can vary in morphology and size. Small microparticles – including CaOx, calcium carbonate, brushite crystals, and hydroxyapatite – range from 0.01 to 10 μm in size. CaOx can crystallize in the tubules of the kidney and cause acute and chronic CaOx nephropathy as well as kidney stones. Two types of CaOx crystals have been identified in the urine: CaOx monohydrate (dumbbell shaped) and CaOx dehydrate (bipyramid shaped); the former are predominantly found in patients with kidney stones [33]. In CaOx crystal-induced acute kidney injury, neutrophils undergo RIPK1–RIPK3–MLKL-dependent neutrophil necroptosis and/or form NETs [14] upon phagocytosis, and subsequently contribute to necroinflammation and tissue damage. Brushite crystals are frequently found in patients with kidney and urinary stones. In patients with brushite stones, neutrophils get activated and release NETs–NETosis like that observed with CaOx crystals [34]. However, further studies are needed to unravel the pathomechanisms of brushite crystal-related inflammation. Additionally, calcium carbonate is present in an amorphous form but can also exist as polymorphous calcium carbonate, as hydrated crystals, and as calcium carbonate hemihydrate [35]. The morphology (layered, rhombohedral, irregular, needlelike, spherulitic, or cube-like in shape), size (from nanoscale to micron-scale), and aggregation of calcium carbonate crystals can be influenced by the concentration of acidic amino acids [36]. These crystals can activate neutrophils *in vitro* [37], and induce NETs and aggregated NETs in the pancreas, leading to ducal occlusion and pancreatitis. Aggregated NETs require PAD4 activity and may contribute to pancreatic stone formation [38]. Hydroxyapatite is an essential ingredient of normal bones and teeth. However, hydroxyapatite crystals can deposit periarticularly and intra-articularly (around and in joints and tendons), causing osteoarthritis [39]. Phagocytosis of hydroxyapatite crystals by macrophages promotes calcification of vessel walls and inflammation, depending on the crystal size [40]. Synthetic hydroxyapatite crystals are used as biomaterial, although it has been shown that these crystals can trigger local inflammation upon being released from implanted prosthetics [41], inducing human neutrophil cell membrane rupture and lysozyme release. Whether these crystals trigger NET formation and NETosis is currently unknown.

MSU, UA, CPPD, and cholesterol are classified as large crystalline solids (10–100 μm). The crystallization of MSU and UA within joints, periarticular tissues, and the kidney depends on physiological factors such as temperature, pH, the articular hydration state, and cation concentrations, and the presence of extracellular matrix proteins influence MSU and UA crystal formation [42]. These needle-like crystals have been implicated in gouty arthritis [43], tumor lysis syndrome, and chronic UA nephropathy [44]. MSU crystals can trigger NET formation, NETosis, and autophagy [45], but not EETs. Although NET release is often associated with exacerbated inflammation, aberrant accumulation of aggregated NETs – due to a high density of neutrophils, and DNA coating of crystals, as well as the release of neutrophil microvesicles – can be anti-inflammatory and favors the resolution of inflammation [4,46]. Interestingly, MSU crystals can also be found in the synovial fluid of patients even after a gout attack has resolved without evidence for clinical inflammation [43], possibly due to crystal-coating proteins and the anti-inflammatory effects of soluble UA on neutrophils [47]. CPPD crystals (rhomboid or elongated in shape) tend to deposit in joints and cause pseudogout characterized by periodic acute joint flares [48]. They activate resident macrophages to release IL-1β,

which recruits neutrophils into the inflamed joint. After being phagocytosed by neutrophils, CPPD crystals induce NADPH-oxidase-independent NETs [49] that are associated with the activation of the heat shock protein 90 (HSP90), PI3K, and CXCR2 (in contrast to MSU crystals), suggesting the involvement of different signaling pathways in NETosis. Compared with MSU crystals, CPPD crystals show a stronger NET-inducing signal in neutrophils.

Cholesterol is a lipid of endogenous or environmental origin. The consumption of high-fat, high-cholesterol diets can trigger the formation of monohydrate cholesterol crystals leading to hypercholesterolemia and atherosclerosis. Recently, cholesterol crystal-induced NETs have been identified as major drivers of atherosclerotic plaque formation due to the prothrombotic properties of NETs that stimulate fibrin deposition [50]. For cholesterol crystals to induce NETosis, the translocation of ROS, NE, and PR3 into the nucleus is required [51]. Experimental studies have shown that inhibiting PAD enzymes does not affect cholesterol crystal-induced NET formation, and that NE- and PR3-deficient mice do not form NETs in a model of atherosclerosis [51]. In addition, cholesterol-crystal-induced NETs can also activate T helper type 17 (Th17) cells and macrophages to sustain chronic sterile inflammation. Thus, NETs modulate immunothrombosis. In a mouse model of cholesterol crystal embolism, Shi *et al.* showed that not the crystals themselves, but the clots formed around the crystals, cause arterial obstruction and kidney failure [52], and that cholesterol crystals clots contain small amounts of NETs. This indicates that the contribution of cholesterol-crystal-induced NETosis to immunothrombosis may depend on different factors, and this requires further investigation [52]. Evidence now suggests that gasdermin D, a pore-forming protein facilitating the secretion of IL-1β and IL-18, regulates cholesterol-crystal-induced neutrophil activation and maturation, as well as pyroptosis [53]. In addition, gasdermin D has also been implicated in the release of NETs [54].

Polycrystalline solids are composed of many crystallites of varying size and orientation. They form when different crystal materials continuously deposit. These large crystal masses – but also implants (plastic or silicone) that are too big to be internalized – contribute to granuloma formation and persistent inflammation with NET formation and tissue fibrosis, for example, in progressive lung fibrosis, chronic tophaceous gout, chronic UA nephropathy, thrombosis, aseptic osteolysis, kidney stones, and gallstones [1,44,52]. In mice and humans with gallstones, NETs drive crystal aggregation and gallstone formation [55]. Whether crystal formation and growth depend on NETs other than gallstones is still unknown.

Extrinsic crystal- and nanomaterial-related diseases

Like intrinsic crystals, extrinsic crystals can be broadly classified into drug-derived crystals and environmental pollutant-related crystals that trigger differential effects on NETs.

Drug-derived crystals, including nanoparticles (NPs, 0.01–1 μm), are a class of amorphous materials which tend to aggregate irreversibly. Nanomedical NPs are designed for drug delivery due to their controllable properties and small size for tissue penetration [16]. The use of NPs in experimental and clinical settings has increased exponentially due to the wide range of biomedical applications, but this also carries the risk of unpredictable and adverse effects. Neutrophils can take up smaller NPs via endocytosis and larger NPs via phagocytosis, which can lead to neutrophil ROS and myeloperoxidase (MPO), degranulation, apoptosis, and NET formation, depending on the NP type, concentration, size, and exposure time, and subsequent sterile inflammation and tissue damage [56]. As antimicrobial materials, silver NPs rapidly induce atypical human neutrophil cell death by a process involving caspases, ROS, and NETs release [57]. Interestingly, small silver NPs (5 nm), but not large silver NPs (100 nm), induce NET formation via activation of PAD4 and NE [58]. Large gold NPs (40 and 100 nm) have been shown to be more effective

in inducing ROS via NOX2 and promote NET release in human neutrophils compared with small gold NPs (10 nm) [59]. Cationic lipid NPs, but not their neutral counterparts, also trigger NETs [60]. Furthermore, high doses of polystyrene NPs and nanodiamonds trigger a self-limiting inflammation with NETs in mice [17], suggesting that NET formation may depend largely on the dose of NPs and the activation status. The agglomerates of superparamagnetic iron oxide NPs were shown to elicit NETs, while human serum albumin can reduce agglomeration and NET formation [61]. In addition, aluminum adjuvants – including aluminum phosphate, aluminum hydroxide, and aluminum potassium sulfate – are widely used in vaccines for both humans and animals [62]. These aluminum adjuvants form insoluble crystals nanometers to micrometers in size, triggering innate and adaptive immune responses [62]. Experimental studies showed that alum crystals induce DNA release in human neutrophils, independently of ROS production [63], and that neutrophils release fibrin-like ETs in the presence of aluminum adjuvants in mice [64]. Several drugs can also precipitate within kidney tubules and the gastrointestinal tract. An example is sevelamer, a phosphate-binding drug used for the treatment of hyperphosphatemia in patients with chronic kidney disease [65]. It is a non-absorbable resin able to bind the phosphate in the gastrointestinal tract and kidney, where sevelamer crystals can form and injure the mucosa and kidney tubules, leading to inflammation and crystal-induced nephropathy [66]. In general, sevelamer crystals display broad, curved, and irregularly spaced 'fish scales' with a variable eosinophilic to rusty brown color on hematoxylin and eosin staining and a violet color on periodic acid–Schiff staining with diastase [66]. Sevelamer crystals can induce intestinal epithelial cell barrier dysfunction and trigger the formation of both NETs and monocyte extracellular traps (METs) [67].

Environmental pollutant-related crystals – such as asbestos, silica, and polystyrene particles – enter the human body primarily by inhalation. Asbestos is a naturally occurring fibrous silicate mineral (μm to cm). The structural properties of the different asbestos fibers play an important role in their pathogenicity. Prolonged inhalation of large amounts of asbestos can cause various diseases, including pulmonary fibrosis, pleural disease, or mesothelioma and lung cancer [68]. Amphibole fibers are considered more toxic than chrysotile fibers, and as these fibers accumulate in more distal parts of the lung, they are not cleared as effectively and have a longer half-life [69]. Asbestos can be sensed by the NLRP3 inflammasome leading to IL-1β secretion [70]. *Ex vivo* experiments showed that asbestos can trigger RIPK1–RIPK3–MLKL-dependent neutrophil necroptosis and NET formation in neutrophils [14]. Silica crystals, however, are considered harmless and non-toxic, although inhalation of silica can cause pulmonary inflammation (silicosis) [20]. *In vitro* studies show that silica crystals can induce NETosis via both oxidative and autophagic pathway activation [71]. Silica crystal-stimulated DNA release from neutrophils along with numerous proinflammatory cytokines and cytotoxic contents is somewhat comparable with that induced by MSU crystals [72]. Along with the disease progression, aggregated NETs might be formed that have anti-inflammatory effects on the local environment, as in gouty arthritis. Persistent exposure to silica crystals, especially for coal-miners and smokers, confers a great risk of developing pneumoconiosis silicosis, a chronic, progressive, irreversible, and incurable disease characterized by pulmonary fibrosis [20]. Finally, polystyrene microplastics or nanoplastics derived from the widespread use of plastics exist in various environmental media. Due to their small size and specific surface area, polystyrene particles can easily be inhaled, consumed orally, or absorbed through the skin, causing toxicity and inflammation. Compared with microplastic, nanoplastics are more easily swallowed by organisms and accumulate in biological tissues, resulting in extensive toxic effects [73]. Nanoplastics induce neutrophil infiltration and NET formation in the liver via mechanisms involving the ROS–NLRP3 inflammasome axis, PAD4, and NE. Moreover, overexpression of PAD4 induced by nanoplastics further mediates histone citrullination and chromatin depolymerization in mice [74].

Proposed treatment options for crystal-related diseases

Crystalline materials can cause a variety of diseases, as already outlined. The mechanisms by which crystals mediate disease progression are gradually becoming apparent. As discussed, certain crystals (mainly large crystals) can induce NETs or NETosis in neutrophils but also in other immune cells, while small crystals do not (Boxes 2 and 3 and Table 1). NETs in turn display bidirectional effects in crystal-related diseases. On the one hand, they cause inflammation, drive tissue damage, and promote further crystal growth, but on the other hand they contribute to the resolution of inflammation. Common therapeutic approaches for crystal-associated diseases involve the use of steroids and colchicine to inhibit inflammation. However, other treatment targets should also be considered: for example, crystallization, inflammation, NETosis, and NET-derived products. Currently, various therapeutic drugs are used in the clinic that target NETs in other diseases, and these are expected to effect an improved outcome also in crystal-associated pathologies (Table S1 in the supplemental information online).

Crystallization and crystal-induced inflammation as therapeutic targets

Dissolving existing crystals and inhibiting crystal deposition in the human body can be an effective approach. Some misfolded proteins can be refolded or stabilized by pharmacological chaperones or aromatic small molecules [75]. Recombinant uricase, an enzyme that breaks down UA, can dissolve MSU crystals. Cholesterol crystals can be dissolved with cyclodextrin, which reduces atherosclerotic plaque size and cholesterol load in a murine model of atherosclerosis [76]. Urinary alkalizing agents, such as citrate, bicarbonate, and hydroxycitrate, have been shown to be beneficial in preventing CaOx monohydrate and UA crystallization in the kidney [77]. Gallbladder and kidney stones are usually big (large crystal masses); thus, no efficient drugs can currently dissolve them, and they require surgical removal or shockwave interventions [78]. Besides targeting crystallization, crystal-induced inflammation is associated mainly with NLRP3 inflammasome activation and IL-1β release. The IL-1 receptor blocker anakinra and the IL-1 blocking-related agents rilonacept and canakinumab are clinically used to suppress IL-1β-mediated inflammation (e.g., in gouty arthritis). Besides blocking IL-1, several NLRP3 antagonists have been tested in preclinical studies, including arglabin in atherosclerosis and β-hydroxybutyrate precursor 1,3-butanediol in hyperoxaluria and nephrocalcinosis [79–81]. Whether these drugs exhibit similar effects and also improve outcomes in other crystal-induced acute and chronic inflammatory diseases requires further investigations. It is important to mention here that not all crystal types trigger NLRP3 activation and IL-1β release, including aluminum salts and NPs [82]. Therefore, it is important to understand the pathomechanisms of each crystallopathy to develop specific drugs.

NETs as a therapeutic target

Growing evidence suggests that NETs promote crystal growth and that an impaired clearance of NETs mediates further tissue damage; therefore, targeting NETosis and NETs may provide new therapeutic approaches for certain crystal-associated diseases. Drugs that specifically target NETosis are designed to degrade NET structures and deactivate NET components. NET structures containing chromatin can be dismantled by recombinant DNase I, a drug that showed beneficial effects in preclinical studies of atherosclerosis and acute kidney injury [52,83,84]. In the clinic, DNase I is safely used for the treatment of cystic fibrosis [85]. Due to the DNA-targeting nature of DNase I, it is inefficient for other components of NETs, such as histones and NE. Instead, targeting histones can be achieved by using histone antibodies or PAD4 inhibitors; the latter can prevent histone citrullination [86,87]. Targeting NETs and NETosis by inhibiting PAD4 can effectively block gallstone formation in mice [55]. In addition, the anticoagulant heparin has been shown to deactivate histones [88], and to reduce cholesterol crystal aggregation and infarct size in cholesterol crystal-induced embolism in mice [52]. Inhibition of NE and MPO might also prevent NETosis by blocking chromatin decondensation. Although targeting NADPH-oxidase-

Clinician's corner

The formation and accumulation of crystalline material in tissues is a hallmark of many metabolic and inflammatory conditions. Although the human body has different strategies in place to avoid unnecessary or harmful crystallization of soluble substances, an overload of crystals in the human body can cause necroinflammation, obstruction, fibrosis, and sometimes even organ failure.

Over the past decade it has become apparent that the size, shape, rigidity, stiffness, and electrical surface charge of crystals dramatically influence the pathology (see Boxes 1–3 in the main text). Several lines of evidence support the involvement of NETs and NETosis in the pathogenesis of crystal-associated diseases (see Figure 1 in the main text). Even though the proinflammatory effects of crystal-induced NETs are similar, there are differences in the pathomechanisms depending on the crystal type (see Table 1 in the main text). Recent data indicate that aggregated NETs can even limit inflammation. Thus, the immune response and severity of inflammation seem to differ in tissues depending on the crystalline material; a better understanding of the pathomechanisms of each crystallopathy is therefore essential for the development of specific drugs.

In clinical practice, common drugs to inhibit acute inflammation in patients with crystal-associated diseases are steroids and colchicine, but also other treatment targets – such as the IL-1β signaling pathway with anakinra or urinary alkalizing agents – have become available (see Table S1 in the supplemental information online). Given the importance of NETs and NETosis in crystallopathies, new therapeutic agents (such as PAD4 and cell death inhibitors) have shown promising results in preclinical models, some of which will hopefully become available for patients suffering from crystal-associated disorders.

mediated ROS production reduced NET release *in vitro*, *in vivo* experiments showed opposite effects [89,90]. Colchicine approved for the treatment of acute gouty arthritis [43,91] can reduce neutrophil rolling [92] and degrade NETs by destabilizing the actin cytoskeleton and reducing NOX2/ROS production, tyrosine phosphorylation, and Ca^{2+} influx [93,94].

An additional approach could be to inhibit neutrophil recruitment or cell death [95]. For example, metoprolol, a β1-adrenergic-receptor antagonist, can reduce neutrophil migration and inhibit the growth of gallstones in mice [55], and decrease infarct size in animal or human coronary obstruction [95]. Of note, the β2 integrin inhibitors efalizumab and enlimomab used in patients were withdrawn from the market because of substantial side effects, while blocking selectin reduced vaso-occlusive events in preclinical and clinical studies [95]. Thus, further research is needed to overcome issues associated with targeting neutrophil recruitment: for example, selectivity and window of therapy during the disease course. Given that certain crystals not only induce NETosis but also necroptosis, pyroptosis, and ferroptosis, targeting crystal-induced cell death represents another interesting approach [96]. For example the RIPK1 inhibitors (necrostatin1s, PN10, Cpd27, ponatinib, pazopanib, GSK'481 and GSK'963) and the MLKL inhibitor necrosulfonamide have been shown to block necroptosis, while deferoxamine and ferrostatins are used to inhibit ferroptosis in preclinical studies [1]. Other strategies may include blocking intracellular signals that are essential for initiating NET formation (Table S1).

Concluding remarks

Clinically diverse crystal-associated and microparticle-related disorders are now known to share common molecular pathological mechanisms, including immune-cell activation, release of proinflammatory mediators, and cell death. However, the intensity of inflammation and certain pathological mechanisms – such as NET formation, NETosis, necroptosis, autophagy, and pyroptosis – can vary depending on the size, type, and shape of the crystalline material, as well as the site of crystal deposition in the human body. A better understanding of crystal and neutrophil biology is required to assess whether results obtained from *in vitro* and preclinical studies can be translated to humans, and whether targeting several forms of neutrophil death could be useful to improve disease outcomes (see Outstanding questions).

It will also be important to assess whether age and sex hormones may influence crystal formation and NET formation (NETosis) in crystallopathies. Many diseases disproportionately affect one sex: autoimmune diseases are more common in premenopausal women, while the incidence of other disorders – such as gouty arthritis, malignant cancers, atherosclerosis-related complications, kidney stone disease and certain infections – is higher in men [97–99]. Women experience more gout attacks following menopause, suggesting immune modulatory properties of sex hormones. Whether this applies also to other crystal-related diseases will require further investigation.

Finally, given the importance and multitude of NET-related proteins and enzymes that further promote necroinflammation and tissue damage in crystal-associated disorders, several therapeutic approaches seem to be promising, such as PAD4 and cell death inhibitors (Table S1), but also preventing crystallization. There is increasing hope that some of these evolving drugs will eventually become innovative cures for patients suffering from crystal-associated disorders (see Clinician's corner).

Outstanding questions

What neutrophil subpopulations (immature or mature) form NETs in crystal-related diseases? Do circadian rhythm and neutrophil aging play a role in NET formation in this context?

How does a neutrophil decide to undergo apoptosis, NETosis, necroptosis, or NET formation (viable NETs) following activation, phagocytosis, or endocytosis of small versus large crystals? Via surface receptors? Is it tissue-dependent?

Do smaller crystals induce viable NETs and larger crystals NETosis and necroptosis? Do viable NETs contribute to the pathogenesis of crystallopathies similar to NETosis?

Do neutrophil- and NET-associated biomarkers have the potential to monitor disease activity, guide clinical treatment decisions, and improve patient care in crystal-associated disorders? Measuring NET components such as MPO and NE in sera could be an intriguing addition but will require further validation.

Can NET and NETosis inhibitors be utilized synergistically with other common drugs, such as alkalizing agents, steroids, and anakinra, to improve efficacy? Are there potential side effects?

What role do sex hormones and the age of a person play in crystal formation and NET release in the pathogenesis of crystallopathies?

Why do MSU crystals form in some hyperuricemic individuals but not others? It is possible that other factors present within the serum or joints of patients with gouty arthritis promote MSU crystallization in the presence of elevated tissue urate concentration. Are there convenient ways to inhibit MSU crystallization despite local supersaturation to prevent future gout attacks?

Author contributions

S.S. conceptualized the manuscript. S.S. and Q.M. contributed to the draft and its revisions, and have approved the submitted version. S.S. and Q.M. generated the figures with BioRender software.

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Declaration of interests

The authors declare that there are no conflicts of interest related to this article.

Supplemental information

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