Neutrophils as regulators of cardiovascular inflammation

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Abstract | Neutrophils have traditionally been viewed as bystanders or biomarkers of cardiovascular disease. However, studies in the past decade have demonstrated the important functions of neutrophils during cardiovascular inflammation and repair. In this Review, we discuss the influence of traditional and novel cardiovascular risk factors on neutrophil production and function. We then appraise the current knowledge of the contribution of neutrophils to the different stages of atherosclerosis, including atherogenesis, plaque destabilization and plaque erosion. In the context of cardiovascular complications of atherosclerosis, we highlight the dichotomous role of neutrophils in pathogenic and repair processes in stroke, heart failure, myocardial infarction and neointima formation. Finally, we emphasize how detailed knowledge of neutrophil functions in cardiovascular homeostasis and disease can be used to generate therapeutic strategies to target neutrophil numbers, functional status and effector mechanisms.

The survival of organisms crucially depends on their capacity to protect themselves from exogenous pathogens and to repair the tissue damage resulting from trauma or infection. However, in a large number of conditions, the inflammatory reaction causes damage to host tissues and leads to organ dysfunction[1]. Inflammatory processes crucially regulate the onset, progression and outcomes of cardiovascular disease. The important role of monocytes and macrophages in cardiovascular inflammation has historically been appreciated[2]. By contrast, although neutrophils are the most abundant type of white blood cell in the human circulation and the principal cell type during acute inflammatory reactions, neutrophils have, until 10 years ago, received only limited attention in the context of cardiovascular inflammation[3]. In this Review, we discuss basic principles of neutrophil biology that are relevant to cardiovascular pathophysiology and further elaborate on how traditional and novel risk factors for cardiovascular inflammation influence neutrophil production and function. We then discuss the emerging knowledge of the contribution of neutrophils to the various stages of atherosclerosis and the clinical manifestations of atherosclerosis, including myocardial infarction, heart failure and stroke. Finally, we highlight the evolving strategies aimed at therapeutically interfering with neutrophil numbers, functional status and effector mechanisms.

Basic principles of neutrophil biology

Neutrophils are traditionally viewed as terminally differentiated, short-lived phagocytes with a rather uncontrolled mode of action. With their vast turnover, 10^{11} neutrophils need to be produced every day in the adult human body. In the steady state, neutrophil production predominantly occurs in the bone marrow, where in a cascade of proliferation and differentiation steps, neutrophils develop, mature and reside as a ready-to-go pool. Chemokine axes, adhesion molecules and growth factors control the release of neutrophils into the bloodstream. In the circulation, neutrophils undergo an ageing programme that leads to the downregulation of CXC-chemokine receptor 2 (CXCR2) and CD62L expression and the upregulation of CXCR4 expression, thereby rendering the neutrophils prone to clearance in the bone marrow[4]. Although neutrophils were traditionally considered to be a homogeneous cell population, diverse neutrophil subpopulations have been described (BOX 1). The ageing process is one of several mechanisms that contribute to neutrophil heterogeneity. Neutrophil ageing occurs in a circadian fashion with cycles of about 12 h (REF.[4]), which fits well with the described short lifespan of circulating neutrophils. Nevertheless, the exact half-life of circulating neutrophils is still under debate. Early studies estimated that human neutrophils circulate for about 4.3–17.5 h before infiltrating the peripheral tissues or homing back to the bone marrow[5,6]. However, a study that combined in vivo neutrophil labelling with the stable isotope deuterium and mathematical modelling showed a neutrophil lifespan of 5.4 days in humans and 18 h in mice[7]. This paradigm-shifting finding was rapidly challenged by several studies arguing that the disparities in modelling the relationship between the bone-marrow and blood neutrophil pools to calculate

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Key points

- Hypercholesterolaemia and hyperglycaemia heighten neutrophil production in the bone marrow and at extramedullary sites, thereby accelerating cardiovascular inflammation.
- Lifestyle factors, including stress, disturbed sleep and nutrition, influence cardiovascular inflammation in part by altering neutrophil production in the bone marrow.
- Neutrophils accelerate all stages of atherosclerosis by fostering monocyte recruitment and macrophage activation and through cytotoxicity.
- During complications of cardiovascular inflammation, such as neointima formation and myocardial infarction, neutrophils have reparative functions primarily by promoting endothelial regrowth and angiogenesis.
- In cardiac hypertrophy and stroke, neutrophil-driven macrophage activation and stimulation of coagulation have a negative effect.
- Time-specific and site-specific interference with neutrophil recruitment to large arteries and inhibition of neutrophil extracellular trap discharge or neutralization of active components of neutrophil extracellular traps are important targets to reduce cardiovascular inflammation.

the lifespan might account for the differences between studies and concluding that the half-life of circulating neutrophils is <1 day[8]. Therefore, further investigation is needed to resolve these controversies, to define the exact neutrophil lifespan in the circulation under steady-state conditions and to understand how the lifespan of circulating neutrophils is modulated during inflammation[9]. Similarly, the lifespan of neutrophils in tissues is unclear. A 2019 study suggested that mouse neutrophils migrate to and reside in the lymph nodes for up to 7 days[10]. In addition, upon inflammation, several factors can extend the neutrophil lifespan in tissues, including hypoxia[11], growth factors, such as granulocyte colony-stimulating factor[12] and granulocyte–macrophage colony-stimulating factor[13], and pathogens[14]. Whether the same mechanisms control neutrophil lifespan in the atherosclerotic lesion or the infarcted myocardium remains unknown.

Neutrophils can infiltrate naive tissues in mice, such as the liver and the intestine, in a mechanism reminiscent of that described for non-classical monocytes[15]. Whether this steady-state neutrophil infiltration also occurs in the heart or in large arteries is currently unknown. In acute inflammation, neutrophils are rapidly recruited to the affected tissues in a multistep recruitment cascade, which has been well characterized over the past 3 decades[16]. Of note, the molecular cues differ between tissues, therefore offering opportunities for tissue-specific inhibition strategies[16]. Although neutrophil recruitment was traditionally considered to be unidirectional (that is, from blood to tissue), evidence from mouse models suggests that neutrophils can migrate from the tissues to the blood and possibly home to other tissues, where neutrophils might be reprogrammed to facilitate their clearance or, alternatively, to generate secondary tissue damage[17,18].

When at the site of inflammation, neutrophils have a large inbuilt toolset ready for immediate use. This toolset includes the production and release of reactive oxygen species and bioactive lipid mediators. In addition, neutrophils carry a large number of preformed granule proteins, including several alarmins (such as cathelicidins) and proteases (such as cathepsin G, neutrophil elastase and myeloperoxidase) for rapid discharge and communication within their vicinity. Owing to the abundance of readily available armoury, neutrophils were long considered to be transcriptionally silent, a notion supported by the low amounts of mRNA carried by mature neutrophils. However, several studies have challenged this dogma. For example, in humans, the variability of the transcriptome during steady-state conditions is higher in neutrophils than in mononuclear cells[19]. The transcriptional plasticity of neutrophils becomes evident when neutrophils are stimulated with sterile or microbial insults[20–23]. Treatment of neutrophils with these agents alters the expression of many genes, some of which are regulated with differential kinetics, indicating that diverse chromatin-based elements regulate neutrophil transcriptional activity. In addition, evidence shows that PU.1, a transcription factor of critical importance during granulopoiesis, shapes the epigenome in active neutrophils[24]. PU.1 restrains the transcriptional activation of genes related to inflammation in neutrophils, which in turn reduces the tissue damage. Of note, the mRNA content and de novo synthesis of cytokines in neutrophils is much lower than in mononuclear cells on a per cell basis[25]. However, neutrophil recruitment exceeds the recruitment of monocytes and lymphocytes at sites of acute inflammation, thereby possibly compensating for the transcriptional differences by an overall greater neutrophil contribution to the cytokine pool. Nevertheless, the exact contribution of neutrophils to the cytokine milieu in large arteries or the heart is unknown, but given the supposedly continuous recruitment, fast turnover and transcriptional plasticity of neutrophils, one can anticipate that the neutrophil contribution in these tissues is currently underestimated.

Risk factors fuel granulopoiesis

Functional disturbance of haematopoietic stem and progenitor cells (HSPCs) is fundamental in inflammation-driven myelopoiesis, which sustains neutrophil numbers and promotes chronic inflammation. Cellular lipid and glucose metabolism and ageing are essential regulators of granulopoiesis and neutrophil function. In addition, altered metabolism and ageing are undoubted risk factors for cardiovascular disease. In this section, we discuss novel mechanistic implications of disturbed metabolism and ageing in neutrophil biology.

Metabolic alterations promote myeloid cell production. Hypercholesterolaemia and hyperglycaemia are two of the most important modifiable risk factors for cardiovascular disease. Apart from their profound effect on metabolism, these conditions alter inflammatory responses by reprograming HSPC function and subsequent myelopoiesis (FIG. 1). Both metabolic alterations influence HSPC biology through diverse mechanisms, including direct effects on cell function or indirect effects through alterations of the HSPC–niche interaction. Increased accumulation of cholesterol in the cell membrane as a consequence of defective cholesterol efflux induces HSPC proliferation and mobilization and a differentiation bias towards the myeloid cell lineage

Granulopoiesis
Production of granulocytes, including eosinophils, basophils and neutrophils; comprises the differentiation process from the haematopoietic stem cell into the mature cell.

Epigenome
The profile of chemical changes in the DNA and associated histones of an organism.

Myelopoiesis
Production of myeloid cells, including eosinophils, basophils, neutrophils and monocytes.

Box 1 | **Neutrophil heterogeneity**

The traditional view of neutrophils as a homogeneous population of terminally differentiated and transcriptionally silent leukocytes has been challenged over the past 10 years[138]. Multiple studies have demonstrated the existence of neutrophil subpopulations with phenotypic and functional differences, and with causal implications in both homeostatic and pathogenic functions. This neutrophil diversity is influenced by their maturation status, their site of production, their capacity to proliferate and by inflammatory signals[139,140]. Although scarce, evidence of neutrophil heterogeneity is also found in cardiovascular inflammation. For instance, as neutrophils age in the circulation, they undergo full transcriptomic and proteomic reprogramming, with important functional implications. Aged neutrophils have a highly active phenotype with an increased capacity to produce neutrophil extracellular traps[65] and to induce vascular and myocardial damage[64] compared with freshly produced neutrophils. Given that both young and aged neutrophils coexist in time and space, identification and modulation of their relative proportions might be important when considering their influence on cardiovascular diseases. Parallel to the M1–M2 macrophage paradigm, a similar classification has been adopted for neutrophils in the context of stroke. Administration of a peroxisome proliferator-activated receptor-γ agonist[141] or recombinant IL-27 (REF.[142]) after stroke in rodent models promotes neutrophil polarization towards an anti-inflammatory phenotype with beneficial effects on tissue and neurological recovery. Low-density granulocytes (LDGs) are neutrophils that, owing to their density, are isolated in vitro from the low-density fraction after centrifugation in a Ficoll density gradient[138]. LDGs are a subset of pro-inflammatory neutrophils, the level of which is increased in patients with autoimmune diseases compared with healthy individuals. The number of circulating LDGs is strongly associated with atherosclerotic plaque burden in patients with lupus erythematosus[143] or psoriasis[144]. However, evidence supporting the implication of LDGs in cardiovascular disease without autoimmune causes is lacking. In the context of cardiovascular inflammation, studies deciphering neutrophil heterogeneity to target therapeutically the pathogenic neutrophil subpopulations without altering host defence are warranted. To this end, we recommend a comprehensive analysis of neutrophil heterogeneity with the use of combined and complementary novel techniques to measure molecular diversity at the cell surface (for example, with multicolour flow cytometry) as well as proteomic and transcriptomic (for example, with small conditional RNAs) levels (BOX 3).

in mice[26,27]. Similar effects contributing to dysregulated myelopoiesis were shown when cholesterol efflux was affected in osteoblastic and endothelial niche cells[27,28]. Neutrophil production in mice can also be regulated from distal haematopoietic niches, mediated by macrophage production of IL-23 that in turn leads to systemic release of granulocyte colony-stimulating factor[15], a process that is fuelled by hypercholesterolaemia[27]. Similarly to the influence of cholesterol metabolism on stem cell function, regulation of glycolysis is important in balancing HSPC quiescence and proliferation[29], and hyperglycaemia has been shown to increase granulopoiesis in mice[30]. Moreover, disruption of glucose uptake in HSPCs prevents neutrophilia in hypercholesterolaemic *Apoe*$^{-/-}$ mice, which in turn attenuates the development of atherosclerosis compared with control mice[31]. Hyperglycaemia promotes the release of the alarmins S100A8 and S100A9 from neutrophils. The interaction of S100A8 and S100A9 with myeloid progenitor cells and Kupffer cells drives myelopoiesis[30] and IL-6-mediated thrombocytosis[32], respectively, and contributes to accelerated atherogenesis and atherothrombosis. Despite the immunometabolic role of cholesterol and glucose in controlling HSPC activity in response to haematopoietic needs (such as during infection), the dysregulation of cholesterol and glucose metabolism in cardiometabolic diseases sustains the chronicity of the inflammation.

Trained immunity
Immune memory of the innate immune system, involving epigenetic programming of myeloid cells enabling a stronger immune response to secondary stimuli.

Clonal haematopoiesis of indeterminate potential
(CHIP). Clonal expansion of blood cells as a result of somatic mutations in genes that confer a growth advantage to haematopoietic stem cells.

Inflammasome pathways activate neutrophils. The inflammasome complex is a potential master regulator of haematopoiesis by integrating cholesterol and glucose metabolism with myeloid lineage differentiation. In the context of granulopoiesis during chronic inflammation, the inflammasome has a central role during myeloid lineage specification because caspase 1-dependent cleavage of the transcription factor GATA1 boosts neutrophil production at the expense of erythroid differentiation[33], thereby providing an explanation for the prevalent combination of neutrophilia and anaemia in patients with chronic inflammation. In addition, IL-1β directly stimulates precocious granulocyte lineage differentiation through activation of a PU.1-dependent gene programme[34]. IL-1β signalling-dependent control of HSPC proliferation occurs through regulation of glucose and cholesterol metabolism in these cells, which is a critical process during trained immunity[35] Conversely, hypercholesterolaemia mediates long-term increased proliferation and increased immune responses of myeloid progenitor cells in an NLRP3 inflammasome-dependent fashion[36]. This connection between inflammasome activation and cholesterol and glucose metabolism in the context of granulopoiesis and cardiovascular inflammation requires further investigation. In addition, disrupted cholesterol efflux in neutrophils can also increase NLRP3 inflammasome activation, promote neutrophil infiltration and release of neutrophil extracellular traps (NETs) in the atherosclerotic lesion and consequently accelerate atherosclerotic lesion formation[37]. Therefore, these results provide evidence for the role of the inflammasome and cholesterol metabolism in regulating neutrophil activation beyond controlling myelopoiesis.

Clonal expansion of myeloid cells during ageing promotes cardiovascular inflammation. As we age, HSPCs accumulate somatic mutations. Certain somatic mutations in HSPCs lead to the expansion of blood-cell clones without the development of haematological malignancies, a process known as clonal haematopoiesis of indeterminate potential (CHIP), and have been associated with increased risk of cardiovascular diseases[38,39]. CHIP is primarily restricted to cells of the myeloid lineage and has been linked to loss-of-function mutations in *DNMT3A*, *TET2* and *ASXL1* and the gain-of-function mutation *JAK2*^{V617F} (REF.[39]). Studies using competitive, partial bone-marrow transplantation approaches in mice showed that HSPC-derived clones deficient in the epigenetic regulator TET2 expanded rapidly compared with TET2^{+} cells and promoted atherosclerosis[40] and heart failure[41] through increased NLRP3 inflammasome activity in macrophages. By contrast, transplantation of bone-marrow cells expressing JAK2-V617F — which is a constitutively active form of the tyrosine-protein kinase JAK2 — into hypercholesterolaemic *Ldlr*$^{-/-}$ mice led to the activation of JAK2–signal transducer and activator of transcription signalling and induced marked erythrophagocytosis and neutrophil infiltration, leading to accelerated atherogenesis and increased vulnerability at advanced stages of atherosclerosis compared with mice receiving wild-type bone-marrow cells[42]. *JAK2*^{V617F} is associated with increased spontaneous NET release[42,43]

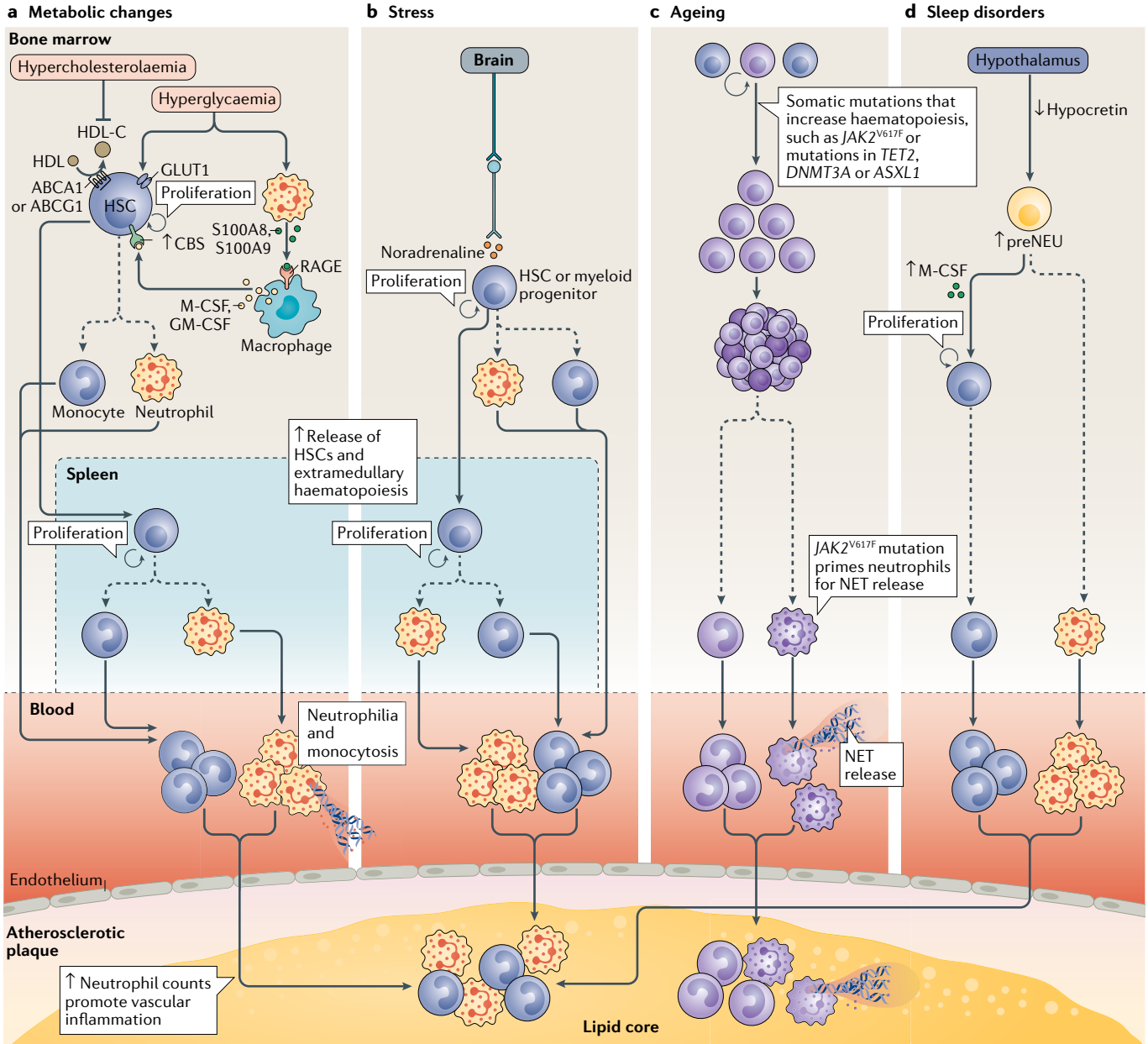

Fig. 1 | **Risk factor–neutrophil interaction network.** Under normal conditions, neutrophils and monocytes are produced in the bone marrow in a process referred to as 'myelopoiesis'. During myelopoiesis, haematopoietic stem cells (HSCs) differentiate into granulocyte–macrophage progenitors and subsequently into either monocytes or preneutrophils (preNEU) and then mature neutrophils. Many risk factors for cardiometabolic disorders accelerate leukocyte production, with a bias towards myelopoiesis. This increased myelopoiesis in turn sustains high myeloid cell counts in blood, thereby fuelling chronic inflammation. **a** | Metabolic disturbances, such as hypercholesterolaemia and hyperglycaemia, directly promote HSC proliferation. Under hypercholesterolaemic conditions, increased cholesterol content in the plasma membrane of HSCs and myeloid progenitor cells as a result of defective cholesterol efflux promotes an increase in the cell surface levels of the common β-subunit (CBS) of the IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF) receptors, which in turn leads to increased proliferation in response to these growth factors. During hyperglycaemia, influx of glucose in the cell through glucose transporter 1 (GLUT1) directly increases HSC proliferation. Indirectly, hyperglycaemia increases the release of S100A8 and S100A9 from neutrophils. Binding of S100A8 or S100A9 to the receptor for advanced glycation end products (RAGE) in bone marrow-derived macrophages induces the production of macrophage colony-stimulating factor (M-CSF) and GM-CSF, which in turn increase myeloid cell production. **b** | Stress induces the release of noradrenaline from sympathetic nerve fibres, which drives HSC proliferation and increased myeloid cell production. Hypercholesterolaemia, hyperglycaemia and stress can result in increased release of HSCs from the bone marrow to the blood and subsequent extramedullary haematopoiesis in organs, such as the spleen, further fuelling the increased myeloid cell levels. **c** | Ageing of the host can result in the acquisition of HSCs with somatic mutations in certain genes, such as *TET2*, *DMMT3A*, *ASXL1* or *JAK2*, that lead to the clonal expansion of functionally altered, proatherogenic myeloid cells. **d** | Disturbed or fragmented sleep patterns result in reduced expression of hypocretin, a negative regulator of haematopoiesis. The low hypocretin level leads to increased production of M-CSF by preNEU and increased myelopoiesis, which in turn results in neutrophilia and monocytosis. High neutrophil counts promote vascular inflammation and are a predictor of future cardiovascular events. HDL-C, HDL cholesterol; NET, neutrophil extracellular trap.

Box 2 | **Neutrophil-based cardiovascular risk assessment**

As accumulating evidence supports a central role of neutrophils and their mediators in the development of cardiovascular disease, further investigation is needed to demonstrate their potential value as diagnostic tools. Stratification of patients with cardiovascular disease is usually on the basis of the assessment of core Framingham risk factors (age, blood pressure, total-cholesterol or LDL-cholesterol level, ratio of total cholesterol to HDL cholesterol, and smoking), in combination with other factors, such as circulating C-reactive protein (CRP) levels, family history of cardiovascular disease or presence of diabetes mellitus[145]. However, in an effort to adapt to the rapid evolution of technological advances and big-data integration techniques, new factors are being analysed to improve the assessment of the risk of cardiovascular disease[146]. Subclinical analyses of disease with the use of imaging techniques (such as CT and MRI), 'omic' phenotyping (genetic, transcriptomic, proteomic and metabolomic) or microbiota analysis, together with novel data integration and mining techniques, are examples of these improvements. In the context of inflammatory factors, the CANTOS trial[147] has been an important step to introduce immunotherapy as a potential treatment for cardiovascular disease; however, immunotherapy is also associated with important secondary effects. The immunotherapy assessed in the CANTOS study, canakinumab (a human monoclonal antibody that neutralizes IL-1β signalling), has increased protective capacity in patients with elevated CRP levels compared with those with low CRP levels[147], and also in patients carrying *TET2* somatic mutations compared with non-carriers[148]. These findings, which are currently being analysed, correlate with augmented inflammasome activation in *Tet2*-mutant myeloid cells in mice[40]. Therefore, efficient stratification of patients on the basis of inflammatory factors might be necessary for greater efficacy of cardiovascular immunotherapy. In the context of neutrophils, different studies have associated elevated neutrophil numbers[81] or levels of myeloperoxidase[149,150] and circulating DNA[151] (as a surrogate marker of neutrophil extracellular traps) with the risk of cardiovascular disease. However, whether these factors increase or add predictive value to the core Framingham factors needs to be evaluated. Beyond soluble biomarkers, neutrophil migration capacity[152] and the levels of specific neutrophil subsets[143,144] have been shown to be strong predictive factors for disease. Therefore, complementary evaluation of neutrophil activity (for example, neutrophil extracellular trap release, degranulation and migration) and heterogeneity is of interest when assessing the individual risk of cardiovascular disease, for patient stratification and for developing personalized treatments. However, more standardized and cost-effective methods are needed to analyse cell function or heterogeneity at a single-cell level before these techniques can be implemented in the clinic.

and coagulation[43], both processes with great relevance to atherothrombosis. Taken together, the discovery of CHIP as a central mediator of the risk of cardiovascular inflammation increases our understanding of cardiovascular pathology and provides novel therapeutic and diagnostic opportunities (BOX 2).

Lifestyle regulates granulopoiesis

Lifestyle factors profoundly influence the risk of cardiovascular disease and are gaining increasing attention in clinical disease prevention guidelines[44]. In this section, we describe the latest studies on how stress, disrupted sleep and the gut microbiota, which is a sensor and an effector of nutrition, influence neutrophil biology and, thereby, alter cardiovascular inflammation.

Stress induces neutrophilia. Psychosocial factors such as stress and depression are strong predictors of future cardiovascular events independently of age and sex[45,46]. Individuals who have experienced stressful events have a higher risk of myocardial infarction, stroke, arrhythmias and arterial thrombosis[47,48], with a prevalent increase in the number of circulating neutrophils[49]. In preclinical studies, atheroprone mice exposed to chronic stress have increased HSPC proliferation, mobilization and subsequent myelopoiesis compared with non-stressed atheroprone mice[49] (FIG. 1). These effects are caused by the release of noradrenaline from sympathetic nerve fibres, which triggers HSPC proliferation, and can be blocked by administration of β_3-adrenergic receptor blockers[49]. Consequent stress-associated monocytosis and neutrophilia are associated with higher infiltration of these cells into atherosclerotic lesions, leading to exacerbated inflammation and accelerated atheroprogression[49]. In humans, a prospective study supported these observations, showing that the activity in the amygdala, a brain region linked to emotional stress, is an independent predictor of cardiovascular disease and is strongly associated with bone-marrow activity and arterial inflammation[50]. Social stress also induces extramedullary haematopoiesis in mice by promoting HSPC homing to the spleen, where HSPCs proliferate and differentiate into myeloid cells[51]. In contrast to the bone marrow, production of monocytes and neutrophils in the spleen persists after stress cessation[51], suggesting that the spleen sustains the inflammation and promotes its chronicity.

Disturbed sleep accelerates myelopoiesis. Alterations in sleep patterns are significantly associated with the risk of cardiovascular disease[52]. A study published in 2019 shed light on the mechanisms linking disturbed sleep and atherosclerosis[53]. In this study, mice subjected to sleep fragmentation had increased myelopoiesis, elevated levels of circulating neutrophils and Ly6C^{high} monocytes and augmented atherosclerosis compared with mice with unfragmented sleep. Heightened myelopoiesis in mice subjected to sleep fragmentation was a consequence of reduced expression of the sleep-related protein hypocretin, which inhibits haematopoiesis, resulting in elevated production of macrophage colony-stimulating factor 1 by immature neutrophils within the bone marrow and increased myelopoiesis[53]. As observed with chronic stress, this study exemplifies the importance of neural regulation of myelopoiesis and indicates that perturbation of this regulation leads to systemic cardiovascular inflammation.

The gut microbiota links nutrition with inflammation. The gut microbiota emerges as a central component to integrate nutrient signalling, metabolism and immune system function[54]. Metabolic alterations such as hypercholesterolaemia[55], hyperlipidaemia[56] and high salt consumption[57] modify the composition of the gut microbiota, which in turn produces metabolites with a profound effect on the risk of cardiometabolic diseases[58–60]. Over the past 5 years, several studies have suggested that alterations in the gut microbiota composition in cardiovascular disease are associated with dysregulation of neutrophil production and mobilization. For instance, transplantation of pro-inflammatory faecal microbiota into atheroprone mice accelerates atherosclerosis development and induces increased blood neutrophil counts and influx to the atherosclerotic lesion compared with atheroprone mice receiving autologous transplantation[61]. Similarly, gut microbiota alterations after an obesogenic diet in aged mice induce neutrophilia and are associated with sustained inflammation after myocardial

Extramedullary haematopoiesis
Production of blood cells outside the bone marrow; for example, in the spleen.

infarction[62]. In a mouse model of ischaemic stroke, gut microbiota depletion with antibiotic treatment restricted intestinal γδ T cell migration towards the meninges after stroke and reduced IL-17 production by γδ T cells and neutrophil infiltration into the stroke region, thereby improving outcomes after ischaemic stroke[63]. Together, accumulating studies demonstrate the effects of gut microbiota dysbiosis in systemic inflammation and neutrophilia; however, causal links between gut microbiota-dependent regulation of immune function and chronic cardiovascular inflammation are scarce. Finally, studies suggest that microbiota derivatives also regulate neutrophil ageing, a process linked to a pro-inflammatory neutrophil phenotype driving vascular damage and myocardial infarction[64,65].

Neutrophils accelerate atherosclerosis

Atherosclerosis is the pathology underlying myocardial infarction and stroke and, therefore, accounts for the majority of deaths worldwide[66]. The onset of atherosclerosis is characterized by arterial wall damage driven by unbalanced blood lipid profiles, oscillating shear forces and pro-inflammatory cytokines. Arterial endothelial cell activation leads to myeloid cell adhesion to the endothelium and infiltration into the arterial intima. Accumulation of immune cells, lipoproteins and cell debris in the arterial intima contributes to atherosclerotic plaque engrossment and instability, the breeding ground for plaque rupture and cardiovascular complications.

Time and space define neutrophil recruitment. Accumulating evidence indicates that neutrophil trafficking is defined by space and time. Strategic localization of chemotactic molecules guides the recruitment of neutrophils to areas of interest. In atheroprone mice, cathepsin G — an antimicrobial polypeptide with proteolytic and chemotactic properties that is released from neutrophils — is specifically deposited on arterial endothelial cells after high-fat diet feeding but not on the endothelium of postcapillary venules after treatment with tumour necrosis factor (TNF)[67]. In arterial endothelial cells, cathepsin G promotes neutrophil and monocyte firm adhesion and extravasation (FIG. 2), as shown by intravital microscopy[67] (BOX 3). This distribution of cathepsin G in the vasculature reveals a possible mechanism to avoid indistinct infiltration of myeloid cells during acute stimulation (as induced by

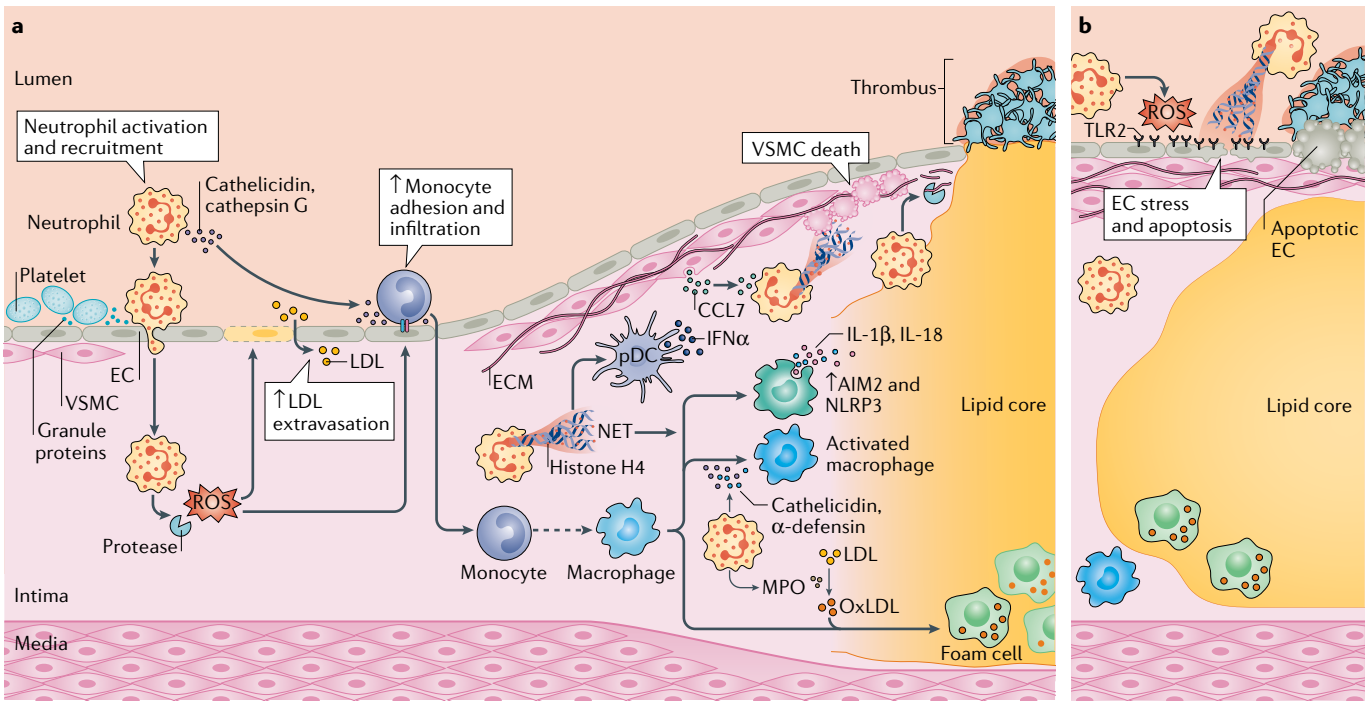

Fig. 2 | **Stage-dependent contribution of neutrophils to atherosclerosis.** **a** | During atherogenesis, platelet-derived chemokines, such as CC-chemokine ligand 5, promote neutrophil activation and recruitment. At the luminal side, activated neutrophils secrete granule proteins, including cathelicidin and cathepsin G, which directly or indirectly promote myeloid cell recruitment. Neutrophil secretion of reactive oxygen species (ROS) and proteases at the luminal and intimal sides of the atherosclerotic plaque results in activation and dysregulation of the endothelial cell (EC) layer and the underlying extracellular matrix (ECM), enabling leukocyte infiltration and LDL extravasation. During atherosclerosis progression, the neutrophil-derived granule proteins cathelicidin and α-defensin stimulate macrophage activation towards a pro-inflammatory state. Neutrophils secrete myeloperoxidase (MPO), which mediates oxidation of LDL (oxLDL), promoting foam cell formation. Neutrophil extracellular traps (NETs) stimulate plasmacytoid dendritic cells (pDCs) to produce proatherogenic interferon-α (IFNα) and macrophages to produce IL-1β and IL-18 on NET recognition via the NLRP3 and absent in melanoma 2 (AIM2) inflammasomes. During the late stages of atherosclerosis, neutrophils can destabilize the plaque by secreting NETs containing cytotoxic histone H4, which perforates and eventually lyses vascular smooth muscle cells (VSMCs). VSMC death is also induced by neutrophil-derived metalloproteinases via degradation of the ECM. VSMC death and ECM degradation result in thinning of the fibrous cap and the formation of rupture-prone vulnerable plaques. **b** | Neutrophils can trigger luminal EC desquamation, a process known as plaque erosion. Neutrophils stimulate EC stress and apoptosis, resulting in EC detachment. This process is regulated by Toll-like receptor 2 (TLR2) signalling in ECs and by NETs. CCL7, CC-chemokine ligand 7.

Box 3 | **Methods to study neutrophil involvement in cardiovascular inflammation**

Reliable detection of neutrophils in atherosclerotic lesions has been a shortcoming in the definition of the role of neutrophils in atherosclerosis. The lack of consistency between studies in the use of neutrophil markers to detect both mouse and human neutrophils, together with the use of markers shared by neutrophils and other (activated) myeloid cells (including neutrophil elastase, myeloperoxidase, Ly6B.2 (detected with anti-Ly6B.2 antibody, clone 7/4) and CD66b) has led to confusing results. In mice, the use of Ly6G (detected with anti-Ly6G antibody, clone 1A8) as a specific marker for neutrophils is widely recognized and has permitted a robust analysis of the number of neutrophils within atherosclerotic lesions[68,95]. By contrast, for human specimens, the options are less clear, and only the antigen CD177 is a specific marker for human neutrophils and can be detected within the atherosclerotic lesion[95]. However, CD177 is expressed only in some neutrophils[153], which can result in an underestimation of the actual number of neutrophils present in the atherosclerotic tissue. In addition to traditional 2D analysis of tissue-infiltrated neutrophils in atherosclerotic lesions, the newly developed techniques for tissue clearing enable 3D imaging of atherosclerotic lesions with increased depth and resolution (Supplementary Figure 1; Supplementary Video 1). Tissue clearing is of critical relevance to understand the function of neutrophils in their microenvironment. These imaging modalities can be combined with a mouse model expressing the fluorescent protein tdTomato under the control of the *Ly6g* promoter[154], thereby allowing reliable neutrophil detection in intact tissues.

Circulating blood cell counts can be manipulated both pharmacologically and genetically. Antibody-based neutrophil depletion by repeated administration of anti-Ly6G antibody (clone 1A8) is widely used to study neutrophil involvement in disease, including atherosclerosis and myocardial infarction, in mouse models[68,80,95]. An example of a genetic approach is the ablation of the prosurvival factor MCL1 in mice with the use of a *loxP*-flanked *Mcl1* gene construct and expression of Cre recombinase under the regulation of the *Ly6g* promoter[95] or the *S100a8* promoter[15], which results in persistent neutropenia. Importantly, the *S100a8* promoter is active earlier during neutrophil development than the *Ly6g* promoter; therefore, use of *S100a8* promoter-regulated *Cre* expression results in the elimination of both immature and mature neutrophils, whereas only mature neutrophils are affected when the $Ly6g^{Cre}$ mouse model is used. Models to generate neutrophilia include the pharmacological inhibition or genetic disruption of myeloid *Cxcr4* (REF.[95]), which encodes the chemokine receptor CXCR4, which controls the retention of neutrophils within the bone marrow and the lungs[155]. CXCR4 in neutrophils not only controls the release to and the retention in the blood but also prevents the molecular ageing of neutrophils[64]. Therefore, mice with neutrophil-specific deletion of *Cxcr4* can be used as a model to study aged neutrophils.

Infiltration of myeloid cells into tissues is a crucial process during stroke, myocardial infarction and atherosclerosis. In mice, real-time myeloid cell infiltration into brain, heart and large arteries can be visualized with the use of intravital microscopy. Protocols for each tissue are available. Neutrophils can be labelled with anti-Ly6G antibodies (1A8 clone) or with the use of Ly6G-reporter mice. For tissues with imaging artefacts derived from respiratory and pulsatile movement, mechanical stabilization or triggered recording can be used to visualize luminal adhesive and interstitial neutrophil movement in four dimensions[75,156].

In the single-cell era, the rapid evolution of single-cell omic techniques has contributed to the identification of neutrophil heterogeneity at the transcriptomic and surface protein levels[157,158]. Although these approaches are applied to study cell heterogeneity within the atherosclerotic lesion[159–162], the low number of neutrophils in atherosclerotic lesions and the low amount of RNA per neutrophil has hampered the identification of intimal neutrophil subpopulations at a transcriptomic or proteomic level. Therefore, more neutrophil-focused, single-cell analyses are needed to determine neutrophil heterogeneity in cardiovascular inflammation. Importantly, these omic studies require subsequent mechanistic studies to confirm the causal implication in disease of the identified subpopulations.

TNF treatment) and chronic stimulation (as induced by high-fat diet feeding) settings. In addition to cathepsin G, the chemokine network also contributes to recruit neutrophils differently in arteries and peripheral venules. Whereas CC-chemokine receptor 1 (CCR1), CCR2, CCR5 and CXCR2 govern infiltration in large arteries, only CCR2 and CXCR2 do likewise in small vessels of mice[68]. In mice, platelets activated by oscillatory shear stress deliver CC-chemokine ligand 5 (CCL5), the ligand of CCR1 and CCR5, on the endothelium of arteries, but not veins, thereby promoting neutrophil accumulation towards atherosclerosis-prone vessels[68]. Of note, immobilized CCL5 also induces cathepsin G secretion from neutrophils, therefore providing a mechanism by which platelets and neutrophils sequentially stimulate myeloid cell recruitment to the arterial intima[67]. Not only is the site of neutrophil infiltration important, but so is the timing. Circadian rhythms are a ubiquitous feature of living organisms, as exemplified by the observation in baboons that 82% of all genes are under circadian control[69]. In this context, adhesion of neutrophils and monocytes to the arterial endothelium and infiltration into atherosclerotic lesions has been shown to have a circadian pattern in mice, with a peak during the transition from the active phase to the resting phase of the mice[70,71]. This 24-h pattern was shifted by 12 h in the microcirculation[70], thereby offering a recruitment pattern controlled by time and space. Mechanistically, rhythmic release of myeloid cell-derived CCL2 determined the circadian recruitment pattern of neutrophils and monocytes into atherosclerotic lesions[70]. These findings are in agreement with the identification in mice of an internal timer in neutrophils, controlled by the circadian clock component aryl hydrocarbon receptor nuclear translocator-like protein 1 (also known as BMAL1), which regulates CXCR2-dependent diurnal changes in neutrophil ageing and related functions and cell counts, leading to diurnal neutrophil infiltration into tissues[64]. Another study in mice provides an additional explanation for circadian arterial recruitment of neutrophils: rhythmic sympathetic innervation of large arteries, but not of veins, activates the arterial endothelium and increases the levels of endothelial adhesion molecules[71].

Macrovasculature versus microvasculature. One of the hallmarks of established atherosclerotic lesions is the formation of neovasculature, which has been extensively shown in human plaques, in which vasa vasorum are a predominant access point for leukocyte infiltration into the lesion[72,73]. In mice, rolling and adhesion of neutrophils and monocytes occur in the arterial lumen in early atherosclerotic lesions[68,74], and in vivo extravasation has been observed at the bifurcation of the carotid artery[75]. However, observations in advanced stages of atherosclerosis in mice indicate a prevailing myeloid cell adhesion in venules (but not arterioles or capillaries) in the atherosclerotic lesion compared with the arterial lumen[76]. Whether the main infiltration route of neutrophils into the atherosclerotic plaque is through the arterial lumen or the adventitial vessels needs further analysis. Moreover, neutrophil exit routes also need to be taken into consideration in the context of neutrophil dynamics in inflammation. Abluminal to luminal transmigration of neutrophils has been observed in venules after ischaemia–reperfusion injury in mice[17]. Neutrophils re-enter the luminal space through reverse transendothelial migration, a process not observed with monocytes in this inflammatory model. Mechanistically, neutrophils underwent reverse transendothelial migration in vessels that expressed low levels of junctional adhesion molecule C, suggesting that this endothelial junction protein has a critical role in the process. In a mouse model of sterile hepatic injury, neutrophils accumulated in the injured area and then surrounded the network of damaged vasculature, driving the dismantling of injured vessels and inducing revascularization[18]. After promoting tissue repair, these neutrophils re-entered the healthy vasculature or migrated into healthy tissue through the interstitium within 24 h after the injury. Neutrophils that had emigrated were found in the lung, where they showed a prolonged retention time and higher CXCR4 expression compared with neutrophils that had not emigrated, and in the bone marrow, where neutrophils had high levels of CXCR4 and annexin V (REF.[18]). This study implies that, after performing crucial repair functions, neutrophils exit sterile inflammation sites, move to the lung and are then redirected to the bone marrow for their withdrawal. In other inflammatory settings, neutrophils are mediators of angiogenesis and tissue regeneration either directly[77,78] or indirectly by attuning macrophage responses[79,80]. Therefore, neutrophils might drive atherosclerotic plaque angiogenesis to promote their own recruitment or possibly their exit from the plaque.

Neutrophils initiate atherosclerosis. In humans, circulating neutrophil counts are predictors of future adverse cardiovascular events[81,82]. In mice, the number of circulating neutrophils positively correlates with the size of developing lesions[68]. Inducing neutropenia in mice during the initial stages of atherosclerosis diminishes lesion sizes and alters lesion cellular composition, with reduced macrophage accumulation[68]. Neutrophils trigger various mechanisms that promote monocyte recruitment[83] (FIG. 2). Among these mechanisms, secretion of chemotactic proteins is an important mechanism for arterial monocyte recruitment. Cathepsin G, cathelicidin or complexes formed of neutrophil-derived α-defensin and platelet-borne CCL5, when immobilized on endothelial cells, induce firm monocyte adhesion in mouse models of vascular inflammation[67,84,85]. During the initial stages of atherogenesis, the dysregulation and activation of the endothelial layer and disintegration of the underlying extracellular matrix enables the increased adhesion and transmigration of immune cells and the transfer of LDL from the luminal side of the artery to its basal aspect. Neutrophil granule proteins, such as neutrophil-derived azurocidin, proteinase 3 and α-defensin, increase the surface expression of adhesion molecules in endothelial cells and regulate endothelial cell permeability in mice[86].

In addition to regulating monocyte entry into atherosclerotic lesions, neutrophils and their secretory products also shape macrophage fate and function. For example, hydrochlorous acid produced by myeloperoxidase leads to the oxidation of LDL and, thereby, accelerates foam cell formation[87]. In addition, granule proteins directly activate human and mouse macrophages, inducing a pro-inflammatory signature in macrophages that promotes the secretion of cytokines that have been shown to foster atherosclerosis[88]. Beyond degranulation, neutrophils can also communicate with the environment by displaying proteins in NETs. Over the past 5 years, much research has been dedicated to understanding the role of NETs during early atherosclerosis. In mice, pharmacological inhibition of NET release or deficiency in myeloid cells of protein-arginine deiminase type 4 (PAD4), an enzyme essential for NET formation, resulted in reduced atherosclerosis development compared with control mice[89,90]. Mechanistically, NETs containing DNA–cathelicidin-related antimicrobial peptide (CRAMP) complexes induce interferon-α production in plasmacytoid dendritic cells, contributing to atherosclerosis formation in atheroprone mice[91]. CRAMP deficiency or plasmacytoid dendritic cell depletion reduces atherosclerosis burden in these mice[91]. In addition, NETs can drive atherosclerosis through the activation of macrophages. In atherosclerotic mice, NETs can prime macrophages to produce the proatherogenic cytokine IL-1β through stimulation of the NLRP3 inflammasome[92]. Direct cleavage of pro-IL-1β into its mature form by neutrophil serine proteases might be an alternative mechanism to generate IL-1β[93]. Taken together, these findings indicate that neutrophils fuel central processes known to promote atherosclerosis development.

Neutrophils promote atherosclerotic plaque instability. Rupture-prone atherosclerotic lesions are typically lipid-rich plaques, histomorphologically dominated by macrophages, with large necrotic cores and with fibrous caps composed of vascular smooth muscle cells (VSMCs) and collagen[94]. Thinning of the fibrous cap as a result of collagen degradation and VSMC death is thought to be pivotal for plaque rupture. Analyses of human thin fibrous cap atheroma specimens showing higher neutrophil counts in rupture-prone lesions than in stable lesions indicate a contribution of neutrophils to plaque destabilization[72]. In agreement with this finding, neutrophil levels in the arterial intima have been shown

Angiogenesis
Formation of blood vessels from pre-existing vessels.

to positively correlate with signs of plaque instability in humans and mice[95]. Moreover, neutropenia and neutrophilia were shown to increase or decrease, respectively, atherosclerotic plaque VSMC content and stability in mice[95]. Mechanistically, activated VSMCs stimulate neutrophils to release NETs through the secretion of CCL7. These NETs are rich in histone H4, which has cytotoxic effects in human and mouse VSMCs by inducing the formation of pores in the plasma membrane that lead to cell death[95]. Reoccurring histone H4-mediated VSMC lysis in atherosclerotic lesions leads to the thinning of the fibrous cap (FIG. 2). Additionally, NETs and double-stranded DNA released from necrotic cells can be sensed by macrophages through the absent in melanoma 2 (AIM2) inflammasome[96]. Activation of the AIM2 inflammasome resulted in strong production of the proatherogenic cytokines IL-1β and IL-18 and led to atherosclerotic plaques with traits of instability in a mouse model of advanced atherosclerosis[96]. In addition, endotoxaemia increases the risk of plaque rupture[97], and evidence from studies in mice indicates that neutrophils might contribute to this process[98]. Induction of endotoxaemia in atherosclerotic mice increases the production of leukotriene B4 in atherosclerotic plaques, and this lipid is a strong chemoattractant for neutrophils. The heightened infiltration of neutrophils into advanced lesions was shown to induce collagen degradation and necrosis in the lesions[98].

Neutrophils promote desquamation of endothelium in atherosclerotic plaques. Given the changes in the cardiovascular risk factor profile in the past 20 years[99], with smoking being largely banned in public places in many Western societies and hyperlipidaemia and hypertension being better controlled pharmacologically, the morphological presentation of atherosclerotic lesions is shifting. The frequency of thin fibrous cap atheroma, albeit still the greatest, is declining, whereas matrix-rich, lipid-poor plaques with low macrophage numbers are becoming more frequent[94]. Atherosclerotic plaques with these characteristics do not tend to rupture but instead undergo superficial erosion. However, similarly to vulnerable plaques, neutrophils are present in human eroded plaques[100]. In human specimens, neutrophils colocalize with patches of Toll-like receptor 2 (TLR2) on endothelial cells[100]. Stimulation of TLR2 on endothelial cells results in endothelial cell stress and apoptosis, an effect that is increased in the presence of neutrophils[100]. Not surprisingly, the number of luminal apoptotic endothelial cells correlates with the amount of neutrophils, NETs and TLR2 staining in human atherosclerotic lesions[100]. Similar results were found in a newly established mouse model of endothelial erosion[101]. In this model, the presence of neutrophils adhered to the endothelium negatively correlated with endothelial continuity, a process that was regulated by TLR2-dependent activation of endothelial cells. These effects were abolished with TLR2 deficiency, neutropenia or blockade of neutrophil adhesion, overall suggesting the involvement of neutrophils in endothelial erosion. A study in atherosclerotic mice provided genetic evidence for the involvement of NETs during endothelial erosion[102] (FIG. 2). Inhibition of NETosis (induced by PAD4 deficiency) or treatment with DNase I (which degrades NET DNA) reduced endothelial discontinuity and endothelial cell apoptosis in these mice.

Neutrophils in cardiovascular diseases

Beyond their role as pro-inflammatory cells, evidence shows that neutrophils also contribute to tissue repair. Such reparative functions engage various mechanisms, including clearance of debris, release of proresolving mediators, discharge of microvesicles containing anti-inflammatory cargo and scavenging of cytokines[103,104].

Neutrophils regulate endothelial cell function during cardiovascular healing. A central pathophysiological process underlying neutrophil-driven repair is angiogenesis, an essential process for delivery of nutrients and oxygen to healing tissue. Growing evidence shows that neutrophils promote angiogenesis, and a subset of proangiogenic neutrophils ($CXCR4^{high}VEGFR^{+}CD49d^{+}$) has been identified in humans and mice[77]. This neutrophil population might be important in settings in which neutrophils control the direct regrowth of blood vessels[18]. However, alternative mechanisms of neutrophil-driven endothelial recovery and angiogenesis have been identified in the context of arterial injury and myocardial infarction[79,85]. In experimental models of restenosis, neutrophils transiently infiltrate the affected artery[105]. Blockade of neutrophil recruitment increases neointima development and inhibits repair of the injured endothelial lining[105]. Mechanistic insights into how neutrophils directly contribute to endothelial repair derive from neutropenic mice and from mice lacking cathelicidin, a cationic antimicrobial polypeptide with chemotactic features[85]. Deposition of cathelicidin along the lumen of injured arteries induces the activation of circulating endothelial progenitor cells in an *N*-formyl peptide receptor 2 (FPR2)-dependent manner[85]. Endothelial progenitor cells recruited in this way contribute to re-endothelialization by directly covering the injured site but also in a paracrine fashion with the release of angiogenic growth factors[105] (FIG. 3).

Myocardial infarction is accompanied by rapid and pronounced neutrophil infiltration, and the prevailing view has been that neutrophils augment cardiac damage[106]. However, studies published in the past 3 years indicate that a well-titrated partnership between initially arriving neutrophils and macrophages infiltrating the heart at a later time shape the healing response after myocardial infarction[79,80,107]. Similarly, neutrophil depletion in mice led to impaired cardiac function, increased fibrosis and progressive heart failure after myocardial infarction[80]. Mechanistically, cardiac macrophages in neutropenic mice had altered polarization states characterized by a heightened inflammatory profile and a lower capacity to engulf apoptotic cells compared with macrophages from mice with normal neutrophil counts[80]. A previous study identified that the cytokine oncostatin M, which is produced by neutrophils and macrophages, induced dedifferentiating cardiomyocytes to release regenerating islet-derived protein 3β, which in turn modulated the degree of macrophage accumulation

Microvesicles
Also known as microparticles. Type of extracellular vesicles of approximately 50–1,000 nm in diameter that are released from the plasma membrane of cells.

Neointima
Type of scar tissue in blood vessels formed as a consequence of a surgical intervention, such as angioplasty or stent placement.

in the heart to fine-tune cardiac healing[108]. Annexin A1 is among the most abundant proteins stored in neutrophils, and, in myocardial infarction, neutrophils are the primary source of annexin A1 in the infarcted area[79]. Mice lacking annexin A1 had worsened healing after myocardial infarction, an observation that was linked to failed repolarization of macrophages to a proangiogenic phenotype and their reduced capacity to produce proangiogenic growth factors compared with macrophages from wild-type mice[79] (FIG. 3). Therefore, this study identified a regulatory circuit centred on neutrophil-borne annexin A1 that stimulates a proangiogenic state in macrophages characterized by release of vascular endothelial growth factor A. Treatment of mice with annexin A1 or overexpression of annexin A1 in pigs via activation of macrophage FPR2 strikingly improved angiogenesis in the failing heart and protected from the development of heart failure[79].

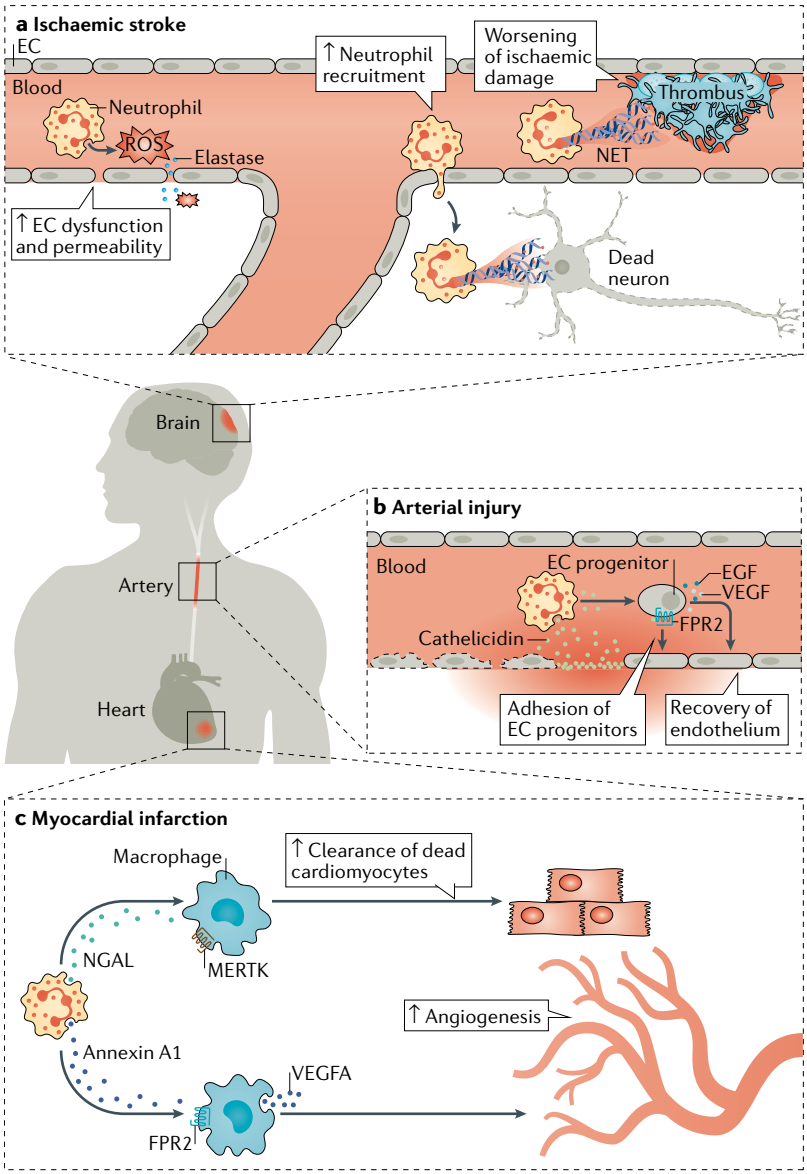

Fig. 3 | **Neutrophils in cardiovascular complications.** Neutrophils are crucially involved in cardiovascular complications, in which they can have inflammatory or resolving functions. **a** | After ischaemic stroke, dying neurons attract neutrophils to the stroke area. Neutrophils can aggravate the damage through secretion of reactive oxygen species (ROS) and neutrophil elastase, which increase endothelial cell (EC) dysfunction and permeability. In addition, neutrophil extracellular traps (NETs) promote thrombus growth, thereby increasing stroke volume. Finally, neutrophils increase neuronal cell death in a process that is likely to involve NETs. **b** | At sites of arterial injury, activated neutrophils deposit cathelicidin antimicrobial peptide (also known as LL37 in humans and CRAMP in mice), which promotes adhesion of circulating EC progenitors via *N*-formyl peptide receptor 2 (FPR2). In a paracrine fashion, cathelicidin stimulates the release of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) from EC progenitor cells. Both processes complement each other to promote EC recovery. **c** | Neutrophils influence cardiac healing after myocardial infarction in several ways. First, neutrophil gelatinase-associated lipocalin (NGAL) stimulates macrophages in a MERTK-dependent manner inducing a shift towards a reparative phenotype, exemplified by the removal of cell debris. Second, neutrophils recruited to the heart secrete annexin A1, which, via FPR2 ligation, promotes macrophage differentiation towards a proangiogenic phenotype, leading to VEGFA secretion, which in turn favours angiogenesis in the ischaemic heart.

Neutrophil–macrophage interaction in cardiac hypertrophy. Heart failure is a growing public health problem, with pressure overload-mediated cardiac hypertrophy being an important risk factor for the development of this condition[109]. A rapid influx of neutrophils into the heart occurs after induction of pressure overload in mice[110] (FIG. 3). In these mice, induction of neutropenia alleviated cardiac hypertrophy and dysfunction and reduced the influx of monocytes and the activation of macrophages in the myocardium[110]. These findings support the concept that neutrophils are important regulators of monocyte recruitment and macrophage activation[83,111]. Bone marrow-derived monocytes are required during cardiac remodelling[112], and therefore the protective effects of neutrophil depletion might be mediated, at least in part, by an indirect effect on monocyte accumulation in the injured myocardium. Neutrophils produce large amounts of CXC-chemokine ligand 1 (CXCL1), a chemokine involved in monocyte recruitment to the myocardium during hypertrophy in mice[113]. Therefore, reduced cardiac CXCL1 expression in neutropenic mice could mechanistically be linked to lower monocyte infiltration into remodelling hearts during hypertrophy.

Role of neutrophils in stroke. Activation and recruitment of neutrophils aggravates ischaemic stroke[114]. Conversely, preventing neutrophil infiltration into the brain, by either neutrophil depletion or inhibition of neutrophil entry, protects against focal cerebral ischaemia in different mouse models by increasing neuronal survival and improving neurological recovery[115,116]. In humans, a high neutrophil–lymphocyte ratio is associated with poor neurological recovery after ischaemic stroke[117], suggesting a detrimental role of neutrophils in this setting. Mechanistically, neutrophils can amplify thrombosis by mechanisms involving cleavage of coagulation factors and activation of platelets[118]. Soluble mediators, including cathelicidins or serine proteases, which can also be present in NETs, mediate many of these effects[119,120]. Indeed, NETs might compromise thrombolysis in patients with acute ischaemic stroke[121], whereas treatment with DNase I was protective in a mouse model of ischaemic stroke[122]. In addition, neutrophils induce the death of neurons, possibly via a process involving NETs[123] (FIG. 3).

Therapeutic targeting of neutrophils

Our refined understanding of the regulatory roles of neutrophils in cardiovascular inflammation enables the development of potential therapeutic strategies. For simplicity, we focus on neutrophil recruitment and NET-instructed inflammation. Please refer to a previous review for a broader overview[3].

Blocking neutrophil recruitment. Neutrophils infiltrate inflamed arteries during all stages of atherosclerosis, and therefore inhibition of neutrophil recruitment stands out as an important therapeutic target. Despite the multitude of preclinical studies revealing the importance of chemokines in cardiovascular inflammation, clinical translation has not yet been successful. Nevertheless, several clinical trials of strategies targeting chemokines in atherosclerosis are ongoing. For example, small-molecule antagonists of CCR5, a receptor involved in the trafficking of neutrophils, monocytes and T cells in atherosclerosis, has consistently resulted in beneficial effects in mouse models of cardiovascular inflammation[124]. Results from a clinical trial assessing the effects of maraviroc, a CCR5 antagonist inhibiting viral entry, on atherosclerosis in patients with HIV infection are pending[125]. On the basis of the importance of CXCR2 for neutrophil recruitment in atherosclerosis[68], the CICADA trial[126] is currently examining the effect of CXCR2 inhibition on cardiovascular surrogate parameters and coronary plaque inflammation in patients with atherosclerotic coronary artery disease.

Several factors make the translation of chemokine targeting from preclinical models to the clinic challenging, especially when targeting leukocyte trafficking long term. Reasons include the redundancy of chemokines during neutrophil recruitment, the insufficient definition of the pathophysiological role of the targeted structure and the importance of the target during host defence responses[127]. In addition, the mice used in experimental studies lack genetic and microbiome diversity, and circadian rhythms differ between species. Therefore, a better understanding is needed to overcome these hurdles. Identification of artery-specific recruitment patterns, possibly centred on mechanisms triggered by oscillatory shear stress, might allow specific blockade of neutrophil trafficking with limited adverse effects. In this context, cathepsin G is exclusively released at sites of disturbed shear rates, which is an environment not found naturally in the microcirculation. Accordingly, antibody-assisted neutralization of cathepsin G limited atherogenic leukocyte recruitment in atheroprone mice fed a high-fat diet but did not affect myeloid cell recruitment into inflamed lungs of mice receiving aerosolized lipopolysaccharide[67]. In addition, long-term administration of anti-cathepsin G antibodies in atherosclerotic mice reduced atherosclerotic lesion sizes compared with untreated controls[67]. Similarly, studies published during the past 2 years provide important insight into tissue-specific circadian neutrophil recruitment patterns. In mice, arterial neutrophil adhesion was shifted by a 12-h phase compared with microvascular neutrophil recruitment[70,71], thereby providing a therapeutic window for intervention with high efficacy in the carotid artery and a low effect on microvascular neutrophil adhesion. To overcome the redundancy of molecules involved in neutrophil recruitment, endothelial cell reprogramming with therapeutic neutralization of several endothelial guidance cues might be an innovative approach. As an example, simultaneous silencing of E-selectin, P-selectin and cell-adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion protein 1) reduced myeloid cell recruitment in mouse models of myocardial infarction and atherosclerosis[128]. Similarly, instructing endogenous pathways that override the activation of integrin induced by various chemokines might be an innovative way to supress myeloid cell adhesion sufficiently. For example, annexin A1 and growth differentiation factor 15 counteract the activation of β2 integrins induced by a variety of chemokines and consequently suppress neutrophil recruitment during chronic inflammation in mice[129,130].

Preventing NET-driven inflammation. Given the importance of NETs in atherogenesis, plaque destabilization, plaque erosion and atherothrombosis, pathways of NET formation, NET-resident components and NET-instructed inflammation evidently stand out as promising therapeutic targets in cardiovascular inflammation. The interaction of neutrophils and platelets along the vessel lumen can be decisive for NET release. Therefore, disruption of platelet-derived CCL5–CXCL4 heteromers or prevention of platelet–neutrophil communication via high mobility group protein B1 or P-selectin might be approaches to limit NET release[131–133]. In atherosclerotic lesions, NET release can be triggered by several factors, including cholesterol crystals and peptides derived from necrotic cells[92]. Inhibition of CCL7, a chemokine released from activated VSMCs, efficiently blocks NET release in the fibrous cap[95]. Citrullination of histones is catalysed by the enzyme PAD4 (REF.[134]). This process releases the electrostatic bonds that constrain nuclear chromatin to the nucleosomes, consequently allowing DNA decondensation and NET release[134]. Therapeutic administration of Cl-amidine, a pan-PAD inhibitor, prevents NET release and reduces vascular inflammation in mouse models of atherosclerosis[90,95]. NETs are rich in cationic proteins, some of which have cytotoxic activities. Continuous delivery of cyclical peptides designed to neutralize the cationic charge accumulating at the histone H4 amino terminus was shown to inhibit NET-evoked killing of VSMCs and consequent plaque destabilization in a mouse model of atherosclerosis[95]. Given the profound effects of NET chromatin degradation with DNase I treatment in experimental studies on cardiovascular inflammation or thrombosis[102,135], systemic treatment with DNase I merits consideration as a therapeutic strategy. Moreover, dornase alfa, a recombinant human DNase I, is approved for the treatment of patients with cystic fibrosis. However, we should bear in mind that the liberation of histones from the chromatin scaffold by DNase I treatment strikingly increases their cytotoxicity[95], and further analyses are required to balance the benefits and risks of DNase I treatment. Finally, NET chromatin has been suggested to activate AIM2 inflammasomes in macrophages, thereby contributing to IL-1β

and IL-18 production in atherosclerotic lesions in mice. Therapeutic inhibition of the AIM2 inflammasome in mice increased lesion stability[96] and, therefore, might be an important therapeutic target downstream of NET release.

Specific targeting of neutrophils. Despite the appealing therapeutic avenues outlined herein, concerns about safety and specificity need to be taken into consideration. Given the close relationship between neutrophils and other myeloid cell lineages, selective targeting of neutrophils is challenging. However, a small number of studies successfully used strategies to interfere primarily with neutrophils. Mouse neutrophils adherent to the endothelium engulf albumin nanoparticles by engaging Fcγ receptors expressed on the surface of activated neutrophils[136]. When these particles were loaded with a tyrosine-protein kinase SYK inhibitor, which blocks β2 integrin signalling, adherent neutrophils were dislodged into the circulation[136]. In a different approach, phage display technology has been used to identify peptides that bind to CD177, a neutrophil-specific surface marker. Conjugation of these peptides to lipid-based nanoparticles enables specific targeting of human neutrophils[137].

Conclusions

Despite neutrophils being neglected in the context of cardiovascular research for a long time, research during the past decade has revealed important regulatory functions of neutrophils during cardiovascular inflammation. We now know that these cells fuel essentially all stages of atherosclerosis and that neutrophil proteins or processes instructed by neutrophils are important novel targets for development of therapeutics. However, neutrophils also have reparative effects in the context of cardiovascular inflammation. Such dichotomy in the outcome of neutrophil activity is fascinating and intriguing. A remaining question is whether these differential outcomes of neutrophil action are a consequence of neutrophil subsets exerting opposite functions. An alternative explanation is that neutrophils act in a context-dependent manner with environmental cues dictating the consequences of neutrophil activity. Regardless of the explanation, we need to bear in mind the dichotomous functions of neutrophils when designing neutrophil-targeted therapeutic strategies for the treatment of cardiovascular inflammation.

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All authors contributed equally to all aspects of the article.

Competing interests
C.S.-R. holds a patent on targeting histones in cardiovascular inflammation. O.S. has consulted for Novo Nordisk and AstraZeneca, has received a grant from Novo Nordisk to study the effect of circadian rhythms on atherosclerosis and holds a patent on targeting histones in cardiovascular inflammation. Q.B. and A.O.-G declare no competing interests.

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