Inherited disorders of granulopoiesis: understanding pathogenesis to advance new therapies

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The central importance of neutrophils for human health is clearly demonstrated by the clinical presentation of patients with severe congenital neutropenia (CN) and cyclic neutropenia (CyN). The majority of patients with CN with neutropenia as their only symptom do not survive the first year of life unless they are treated with recombinant human cytokine granulocyte colony-stimulating factor (rhG-CSF) or undergo allogeneic bone marrow transplantation. Understanding the processes of defective granulopoiesis downstream of CN-associated gene mutations sheds light on the mechanisms that regulate granulopoiesis and granulocyte functions. Furthermore, given that CN is a preleukemia syndrome, studying CN can help to define the role of defective granulopoiesis and deregulated neutrophil functions in leukemogenesis. The development of advanced experimental models of CN and CyN overcomes the limitations of insufficient patient material for research, leading to breakthroughs in the understanding of their pathogenesis and the development of new potential therapies.

BIOLOGY OF GRANULOPOIESIS

Granulopoiesis is the tightly regulated process by which hematopoietic stem cells (HSCs) produce neutrophils and other granulocytes, enabling rapid frontline defense while preventing damaging inflammation. Based on the classical schema, HSCs are committed to common myeloid progenitors (CMPs) under myelopoietic stimuli. CMPs can then differentiate into granulocyte−monocyte progenitors (GMPs), which have the capacity to develop into monocytes or granulocytes. Mature neutrophils are generated from GMPs through several stages, during which myeloblasts differentiate into promyelocytes, the cells in which the first granules appear. These azurophilic granules contain myeloperoxidase, defensins, bactericidal/permeability-increasing protein (BPI), azuracidin, and elastase. Promyelocytes mature into myelocytes, which, in addition to azurophilic granules, form specific granules containing lactoferrin, metalloproteinases, cathelicidin, lipocalin 2, and olfactomedin 4. At the next stage of granulocytic differentiation, cells lose proliferative capacity and become committed to the neutrophil lineage, transitioning into metamyelocytes that form gelatinase granules containing gelatinase, arginase 1, and lysozyme. These cells mature into neutrophils, which contain additional secretory granules with plasma proteins, membrane receptors, and alkaline phosphatase [1]. Neutrophils are divided into band and segmented cells, based on the shape of the nucleus and maturation stage; segmented neutrophils are terminally differentiated cells.

The process of granulopoiesis is regulated by transcription factors, cytokines, the bone marrow niche, as well as metabolic and circadian cues. Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) play a central role in activating intracellular signal transduction that ultimately regulates transcription factors including C/EBPα, C/EBPβ, C/EBPε, PU.1, and Id1/2. These transcription factors govern granulopoiesis differently and sometimes selectively at various stages of maturation and under different physiological and pathological conditions. Steady-state granulopoiesis, for example, is mainly regulated by C/EBPα, whereas emergency granulopoiesis is triggered by C/EBPβ.

NEUTROPHIL FUNCTIONS

The most important function of neutrophils is to eliminate invading pathogens, but they also play a central role in initiating and resolving inflammation [2]. The extravasation and migration of neutrophils to infected sites occurs in multiple steps. Chemokines such as CXCL2 and leukotriene B4 (LTB4) that are released by activated perivascular leukocytes induce the adhesion of neutrophils to endothelial cells and their rolling along the vessel wall. Subsequently, neutrophils are primed by substances including cytokines such as CXCL8 and/or bacterial peptides. This leads to firm adhesion to the endothelial cells and transendothelial migration (diapedesis) through the pericyte layer and the basement membrane. Extravasated neutrophils migrate to

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the site of infection along a gradient of chemokines or formylated peptides secreted by tissue resident cells or bacteria [3,4].

Mechanisms that neutrophils use to combat infections include phagocytosis, the release of various granule components into the extracellular space or into the phagosome and the formation of neutrophil extracellular traps (NETs). Phagocytosis occurs within a few minutes and is enhanced by cell priming and by opsonization with complement as well as IgG antibodies [5,6]. Following phagocytosis, primary and secondary granules fuse with the phagosome and release their antimicrobial contents. Through the fusion of specific granules with the phagosome, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is assembled from the membrane-bound and cytoplasmic components [7]. NET formation based on the extrusion of decondensed chromatin decorated with granule proteins to trap and kill microbes [8]. Depending on the activating compound, NET formation occurs either in a NADPH oxidase-dependent or -independent manner. Activation of the NADPH oxidase-dependent reactive oxygen species (ROS) production occurs via protein kinase C activation and induces the release of neutrophil elastase (NE). Thus, the activation of the glycoprotein-specific dexamethasone dehydrogenase and breakdown of the actin cytoskeleton is induced. Subsequently, NE migrates to the nucleus, where it mediates chromatin relaxation by cleaving histones [9]. Following cleavage of the nuclear membrane, chromatin fills the cell and is released as NETs after cell lysis [9,10]. In NADPH oxidase-independent NET formation, neutrophil activation triggers an influx of Ca^{2+}, leading to the rupture of the granules and the release of NE. Ca^{2+} activates protein arginine deiminase 4 (PAD4), leading to the citrullination of histones, the decondensation of chromatin, and the release of NETs [3–6]. Other studies demonstrate that even viable neutrophils can form NETs [11–13], consisting of mitochondrial or nuclear DNA, in a rapid and nonlytic manner [14,15].

The weapons of neutrophils include BPI protein, as well as AMPs, including α-defensins and cathelicidin, and are stored in granules. Most AMPs in neutrophil granules exert their microbicidal effect by destroying bacterial membranes. Neutrophil serine proteases are not only involved in NET formation but can also directly kill microbes or inactivate bacterial toxins [16,17]. Furthermore, the granules also contain lysozyme, which cleaves cell wall peptidoglycans, as well as lipocalin and lactoferrin, which limit the availability of iron to bacteria.

Neutrophil recruitment and activation are key events for acute inflammation, such as acute lung injury, and are necessary to prevent acute infection by bacteria or fungi. Physiologically, acute inflammation is followed by a recovery phase, which is important for tissue homeostasis. Neutrophils are not only targets of specific proresolving mediators (SPMs), but they are also producers of bioactive lipids such as arachidonic acid, docosahexaenoic acid, and other anti-inflammatory mediators, such as Annexin A1 [18,19]. Thus, they regulate the resolution of inflammation, which is essential for maintaining the balance between protective inflammatory response and overwhelming inflammation.

SEVERE CONGENITAL NEUTROPENIA SYNDROMES

Severe congenital neutropenias (CNs) include syndromes with isolated severe neutropenia or additional abnormalities in other blood lineages, organs, or tissues. In most of the genetic subtypes of CN, peripheral blood neutrophil counts are <500/mL. There are more than 30 autosomal dominant and recessive genes mutated in CN. The largest group of patients with isolated CN carries autosomal dominant mutations in the *ELANE* gene, which encodes the protein neutrophil elastase (NE) [20,21]. Variable disease penetrance is associated with, for example, autosomal dominant mutations in *SRP54*, a critical component of the signal recognition particle (SRP) complex [22]; autosomal dominant *CLPB* mutations encoding caseinolytic peptidase B, an adenosine triphosphatase implicated in protein folding and mitochondrial function [23,24]; autosomal recessive mutations in *HAX1* (HCLS1 associated protein X1) [25]; or autosomal recessive *SBDS* (Shwachman–Bodian–Diamond Syndrome) mutations [26]. Not all patients with Shwachman-Diamond syndrome (SDS) have neutropenia. CN-associated gene mutations have recently been summarized in several review articles [20,27]. New mutated genes associated with CN are continuously being identified, such as autosomal recessive *COPZ1* mutations, a heterozygous *SRPRA* mutation, and a homozygous *SRP19* mutation [28,29]. All patients with CN are at risk of developing severe bacterial infections shortly after birth due to very low neutrophil counts and require chronic treatment (daily or every other day) with recombinant human granulocyte colony-stimulating factor (rhG-CSF) [20]. Depending on the mutated gene or the position of the mutation within the same gene, patients with CN respond differently to rhG-CSF, and some patients do not respond at all. Some patients with CN may experience adverse effects to rhG-CSF therapy, such as bone pain and splenomegaly. CN is a preleukemia bone marrow failure syndrome with a high incidence of myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), or, in rare cases, biphenotypic leukemia or chronic myelomonocytic leukemia [20]. Patients who do not respond to rhG-CSF or who develop MDS or leukemia require allogeneic s transplantation with relatively good outcomes [20]. Established in 1994, the Severe Chronic Neutropenia International Registry (SCNIR) continuously monitors and analyses the clinical and genetic status of patients, their treatment requirements, and survival outcome [30,31]. The recently published European guidelines on the diagnosis, management, and therapy of patients with neutropenia provide a valuable framework for the standardized care of these patients [32,33].

PATHOGENESIS OF CN

Several studies have described the pathomechanism underlying the maturation arrest of granulopoiesis downstream of CN-associated mutations. The pathogenesis of SDS, as a ribosomopathy, has been also comprehensively investigated [34–36]. This review focuses on the pathogenesis of CN. Some of the dysregulated neutropenia-causing signaling pathways downstream of mutated proteins are shared across different forms of the disease, and some are unique to the specific mutated genes (Figure 1). The most investigated subgroup of CN is *ELANE*-associated CN. *ELANE* mutations are gain-of-function mutations with >200 different amino acid substitutions described to date [37–40]. Depending on the position of the mutation, mutant NE cannot be properly folded, intracellularly processed and/or secreted, with varying degrees of retained enzymatic activity reported. The most common hypothesis links accumulated mutant NE to increased unfolded protein response (UPR) and endoplasmic reticulum (ER) stress, leading to maturation arrest and apoptosis of myeloid progenitor cells [41–46]. Different *ELANE* mutations activate different UPR pathways, and the ultimate link between the UPR and dysregulated signaling remains unclear [47]. It has been proposed that dysregulation of transcription factors, including downregulation of C/EBPα and LEF-1 and upregulation of PU.1, C/EBPß, and

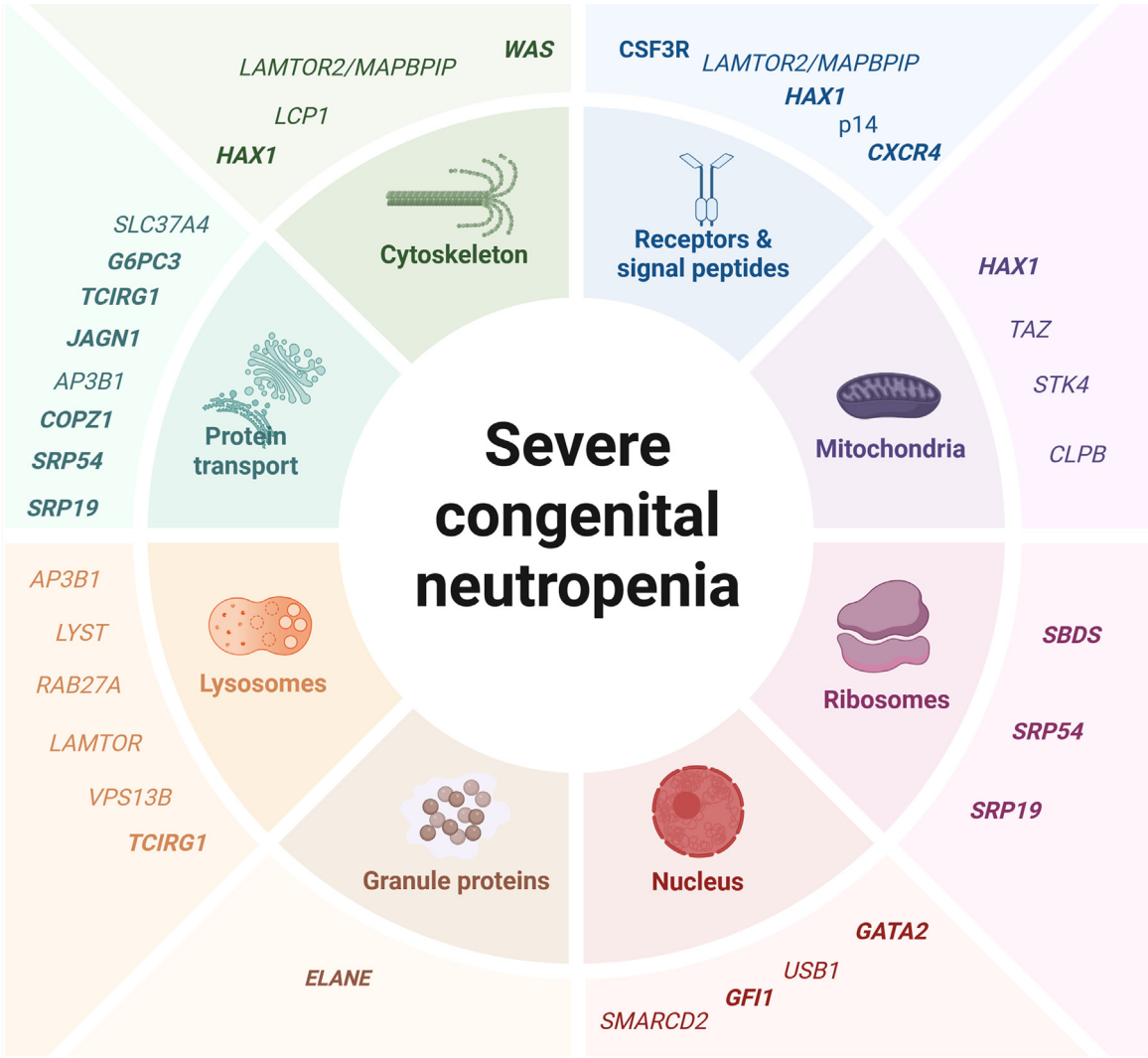

Figure 1 Graphic summary of mutated genes in CN grouped by biological function. Genes covered in this review are highlighted in bold.

phospho-STAT5, as well as diminished expression of the adaptor protein HCLS1 in myeloid progenitors can cause defective granulocytic differentiation [48–52]. Interestingly, these processes are shared between *ELANE* and *HAX1* mutant cells. Due to this transcriptional dysregulation, physiological blood concentrations of G-CSF fail to induce C/EBPα-dependent steady-state granulopoiesis, and chronic high-dose G-CSF treatment is required to induce C/EBPß-dependent emergency granulopoiesis in CN. This process of emergency granulopoiesis requires stimulation of the NAD^+-producing enzyme, nicotinamide phosphoribosyltransferase (NAMPT), and subsequent activation of protein deacetylation by the NAD^+-dependent protein deacetylase SIRT1 [53]. The crucial role of reduced C/EBPα expression in the pathogenesis of CN is further supported by the fact that restoring C/EBPα in CN $CD34^+$ cells rescued in vitro granulocytic maturation [41]. Furthermore, treatment of CN $CD34^+$ cells with the pan-CDK inhibitor flavopiridol restores defective granulopoiesis by mimicking the inhibitory effects of C/EBPα on CDKs, which is essential to initiate granulocytic differentiation of hematopoietic progenitors [54]. Interestingly, dominant negative mutations in the transcription factor *GFI1* cause CN by inducing aberrant overexpression and accumulation of NE, and some patients with *GATA2* mutations have neutropenia [55,56]. These findings clearly demonstrate that disrupted transcriptional regulation of the granulopoietic differentiation in hematopoietic stem and progenitor cells (HSPCs) may cause CN. *G6PC3*, which encodes the ubiquitously expressed glucose-6-phosphatase-ß located in the neutrophil ER, facilitates the storage of glucose and its mobilization to the cytosol upon cell activation. Loss-of-function mutations in *G6PC3* blocks early glycolysis and promotes apoptosis as well as increased ER stress and UPR responses [57,58].

Elevated apoptosis of CN hematopoietic cells may be a cause of dysregulated signal transduction or even the primary cause of neutropenia. For instance, HAX1 is an antiapoptotic protein, maintaining the inner mitochondrial membrane potential, and its loss of function in *HAX1*-CN leads to increased apoptosis [25,59]. In contrast, G-CSF treatment in CN demonstrates an antiapoptotic effect through

the restoration of antiapoptotic proteins. Cario et al. [46] showed a diminished expression of Bcl-2 and Bcl-xL, but increased levels of BCL2 family members Bfl-1/A1 and Mcl-1 in progenitor cells of G-CSF-treated patients with CN. Also located in the mitochondrial compartment, CLPB functions as a potent protein disaggregase and ATP-dependent chaperone essential for mitochondrial proteostasis [60]. Heterozygous missense variants clustering in the ATP-binding pocket act dominant negatively and impair mitochondrial function without prominent ER stress [23,61]. A recent study by Fan et al. [62] has defined a shared CLPB–HAX1 axis in CN, showing the critical importance of mitochondrial proteostasis in differentiation of neutrophil granulocytes.

Protein transport machinery is also affected in CN hematopoietic cells, exemplarily in patients with *SRP54, SRPRA, SRP19, COPZ1*, or *JAGN1* mutations. This disruption ultimately leads to cellular stress through the activation of ER stress and UPR [22,63]. *SRP54, SRPRA*, and *SRP19* mutations disrupt protein targeting to the ER leading to defective protein synthesis and cellular stress [22,28]. *JAGN1* mutations impair ER–Golgi vesicle-mediated protein trafficking, which can also lead to accumulation of unprocessed proteins in the ER and induce ER stress [64]. A most recently identified homozygous truncating mutation in *COPZ1* also showed a disruption in retrograde COPI-dependent Golgi-to-ER transport most likely due to the destabilization of the COPI complex [29].

The interplay among UPR activation, increased apoptosis, and oxidative stress seems to contribute to the proinflammatory environment present in CN [29,44,65–67]. For instance, truncating mutations in *COPZ1* led to increased expression levels of the interferon-stimulated genes and the IFN-α response gene *MX1* [29]. Moreover, recent studies have indicated the possible contribution of a sustained proinflammation state in the progression of CN toward leukemia [67–69]. These findings demonstrate that not only the failure of the innate immune response, but also systemic inflammation, may contribute to the acquisition of secondary somatic mutations and accelerate the leukemogenic transformation.

Patients with the most common forms of CN exhibit maturation arrest of granulopoiesis at the promyelocyte stage in bone marrow, suggesting that different cellular processes converge at a common point that is crucial for the granulocytic differentiation. The high level of granule protein synthesis in myeloid progenitors and the massive production of neutrophils make these processes especially vulnerable to any defects in proteins involved in neutrophil maturation. Therefore, it is expected that many mutated proteins (e.g., NE, G6PC3, JAGN1, SRP54, SRPRA, SRP19, COPZ1, and SBDS) are involved in regulating protein maturation, trafficking, and post-translational processing, particularly in the ER and the Golgi apparatus, as well as in UPR and ER stress homeostasis. Other mutated proteins are involved in mitochondrial integrity and proteostasis (HAX1 and CLPB), metabolism (G6PC3), or transcriptional regulation of granulopoiesis (GFI1, HAX1 through HCLS1 deregulation). This can also affect the maturation of highly active promyelocytes. Disruption of these essential cellular mechanisms at early stages of granulopoiesis can result in ER stress, UPR, and cell death or directly impaired transcriptional regulation of granulopoiesis, including diminished levels of the transcription factor C/EBPα. Severely reduced C/EBPα levels indicate that diverse upstream genetic lesions converge on a common downstream defect in C/EBPα-dependent transcriptional control of granulopoiesis.

PATHOGENESIS OF CYCLIC NEUTROPENIA

Cyclic neutropenia (CyN) is characterized by regular oscillations of peripheral blood neutrophils from nearly normal or even higher than normal to severely low counts, generally within 21-day cycles. Absolute neutrophil counts at nadir are often <200/mm^3 and are associated with fever, mouth ulcers, pharyngitis, sinusitis, and, in some cases, more severe infections with gram-negative bacteria [20]. In patients with CyN, rhG-CSF treatment does not abrogate the cycling of neutrophil counts but increases the amplitude of neutrophil counts during the cycle, leading to a shorter period of severe neutropenia and a reduction in the cycle length from 21 days to approximately 14 days [70]. There are a few cases of AML reported [71].

The pathomechanism responsible for the neutrophil cycling in CyN is still not fully identified. Intriguingly, the same *ELANE* mutation may cause both types of disease: CN and CyN [20]. However, the UPR-induced inhibition of proliferation and differentiation of HSCs reported in CN is incomplete in CyN [72]. Some early undifferentiated CD49f$^+$CD34$^+$ escaper cells in patients with CyN are not affected by UPR because they do not express *ELANE* [41]. The hypothesis that escaper HSCs evade the effects of the UPR is further supported by findings from Avellino et al. [73], showing that *ELANE* is not expressed in long-term HSCs.

Unlike CD34$^+$ cells from patients with CN, CD34$^+$ cells from CyN patients showed higher expression levels of C/EBPα and C/EBPα target transcription factors, such as MLL1, MEIS1, HOXA9, and HLF, at the peak of the neutrophil cycle, exceeding the levels observed in healthy controls [41]. It is known that C/EBPα can induce expression of *ELANE* [74,75]. At the peak of the neutrophil cycle, large amounts of NE protein are released from neutrophils into the patients' serum. Indeed, NE concentration in serum from CyN correlates with neutrophil numbers [41]. The high serum concentration of NE at the neutrophil peak of the cycle leads to direct inhibition of CD34$^+$ cell proliferation [76] and proteolytic cleavage of G-CSF and its receptor proteins [76,77]. NE indirectly regulates granulopoiesis through its capacity to cleave G-CSF protein [76,77]. We also demonstrated that NE treatment digests human recombinant G-CSF [78]. Therefore, NE, which is released from neutrophils at the peak of the cycle, is a strong candidate for mediating the negative feedback loop. This is in agreement with Horwitz et al. [79], who favored a feedback model in which mature neutrophils secrete an inhibitor of myelopoiesis, the concentration of which depends on the number of neutrophils present. Due to its low release at the nadir, which is attributable to low neutrophil counts and low expression of C/EBPα [41], NE is unable to affect G-CSF levels, thereby lifting the inhibition. Consequently, both endogenous and pharmacologic G-CSF become available to induce the proliferation and differentiation of the remaining CD49f$^+$CD34$^+$ cells during the ascending phase of the cycle, and this, together with the increasing expression of C/EBPα in CD34$^+$ cells, leads to an increase in neutrophil numbers toward the peak of the cycle to replenish them. It remains to be investigated how mutated NE contributes to the feedback regulation of granulopoiesis in patients with CyN.

LEUKEMOGENESIS

Patients with CN have a significantly elevated risk of developing MDS or AML [80,81]. Long-term studies indicate an annual transformation risk

of 2.3% per year after the first decade of rhG-CSF therapy, cumulating to ~20% to 30% after 10–15 years on treatment [80,82]. Leukemogenesis in CN is a multistep process. Inherited mutations impairing granulopoiesis-related signaling pathways provide a permissive environment for the acquisition of somatic genetic alterations that lead to leukemogenic transformation. Independent of the CN-causative gene mutation, the most frequent early somatic lesions in CN are stop codon mutations in *CSF3R*, the gene encoding the G-CSF receptor [20]. These *CSF3R* mutations lead to a truncation of the cytoplasmic C-terminal domain of the G-CSF receptor [83–85], resulting in exaggerated proliferation but impaired terminal neutrophil maturation [83,86]. The truncated receptor also fails to recruit SHP1, eliminating a negative feedback regulation [87]; abrogates SOCS3-mediated inhibition of STAT5 phosphorylation [88,89]; causes prolonged AKT/ERK signaling [89]; and cannot recruit SHIP and CIS adaptors [90,91].

Many patients with CN carry preleukemic clones with stop codon *CSF3R* mutations for many years before a "second hit" drives progression to overt MDS/AML [81,92,93]. HSPCs clones with *CSF3R* mutations can exhibit a dynamic behavior, with fluctuations in their size, including disappearance and reappearance during life [92–94]. This dynamic nature suggests ongoing clonal competition and selection of mutated cells even in the preleukemic phase. It underscores the complexity of monitoring preleukemia clones in patients with CN and the challenge of defining precise intervention points. Early detection of clonal progression in patients with CN is recommended because prompt intervention can be lifesaving. Recent European Guidelines on Diagnosis and Management of Neutropenia advise annual bone marrow biopsies with cytogenetics and targeted gene panel sequencing to detect leukemogenic events at an early stage [32].

The most frequent secondary somatic mutations identified in patients with CN-MDS/AML, in addition to *CSF3R* mutations, are genetic lesions in *RUNX1*. In a study conducted by Skokowa et al. [81], *RUNX1* mutations were reported in 64.5% CN-MDS/AML cases (N = 31). Notably, 80.5% of these *RUNX1*-mutated cases also harbored *CSF3R* mutations. *RUNX1* mutations in CN-MDS/AML typically affect the Runt homology domain (RHD), which is crucial for DNA binding on-target genes. Later events in CN-MDS/AML can include mutations in epigenetic regulators such as *ASXL1*, *SUZ12*, and *EP300* [81,93]. *NRAS* mutations have also been reported, although in a small number of patients [81,95].

Patients with SDS also face a high risk of developing leukemia (approximately 19% by age 20 years and 36% by age 30 years) [96]. Unlike other genetic types of CN (e.g., *ELANE, HAX1*, and *JAGN1*), SDS is frequently associated with somatic mutations in the *TP53* gene, whereas mutations in *CSF3R* are notably absent. Clonal hematopoiesis driven by *TP53* mutations is observed in nearly one half of patients with SDS, often emerging years before the onset of overt leukemia [97]. These genetic lesions in the *TP53* allele represent the predominant genetic drivers of leukemia transformation in SDS [35,36]. *SBDS* mutations cause impaired ribosome biogenesis, leading to p53-mediated growth arrest in HSPCs [98]. This disruption of late 60S ribosome biogenesis induces translational stress, which in turn exerts strong selective pressure for somatic adaptations that favor cells with disabled p53 surveillance. Acquired *TP53* mutations allow HSPCs to bypass the growth arrest, thereby conferring a selective advantage [36]. Interestingly, SDS mutant cells show two routes of clonal escape: a "maladaptive" pathway, driven by heterozygous missense or nonsense mutations in *TP53*, and a "compensatory" pathway that provides a genetic rescue via heterozygous loss-of-function mutations or microdeletions in *EIF6* [36]. The complete inactivation of p53 checkpoint usually occurs by loss of the remaining wildtype *TP53* allele either via copy neutral loss of heterozygosity (LOH), deletion of 17p, or acquisition of a second *TP53* mutation [35]. In addition to biallelic inactivation of *TP53*, leukemic transformation in SDS is accompanied by further genomic abnormalities, such as monosomy 7, isochromosome 7q, or complex karyotype [99,100], as well as acquired mutations in *PRPF8, CSNK1A1*, and RAS pathway genes or chromatin modifiers [36]. Isochromosome 7q, one of the most recurrent cytogenetic abnormalities in patients with SDS, seems not to promote malignant transformation [35]. Similarly to other bone marrow failure syndromes, early surveillance can increase overall survival in patients with SDS, especially when AML/MDS has been detected on routine bone marrow examination [100].

DEFECTIVE NEUTROPHIL FUNCTIONS IN CN

Although treatment of patients with CN with rhG-CSF increases neutrophil counts and significantly reduces the frequency of infections, neutrophils from rhG-CSF-treated patients with CN still exhibit functional defects. Myeloid progenitor cells from patients with CN have abnormal neutrophil primary granules, as assessed using electron microscopy [101]. Additionally, neutrophils or plasma from patients with CN exhibit severely reduced or even absent levels of certain antimicrobial peptides, including α-defensins HNP1-3, pro-LL-37 and cathelicidin LL-37, *ELANE*/NE and SLPI, as well as several, although not all, granule components [102–106]. Functionally, neutrophils from patients with CN exhibit defects in F-actin polymerization; calcium flux upon stimulation with fMLP and phorbol-myristate-acetate (PMA); chemotaxis in response to fMLP, C5a, IL-8, LTB4, and PAF; and cytoskeletal regulation. In contrast, their ability to produce ROS following stimulation with PMA remains intact. Furthermore, neutrophils retain the capacity to phagocytose zymosan A particles and to produce ROS simultaneously as well as to kill *Staphylococcus aureus* [107]. It has also been demonstrated that neutrophils from patients with CN express increased levels of fMLP receptor, but reduced expression of C5a receptors. After incubation with PMA, the expression level of fMLP receptors remains normal, whereas the re-expression of fMLP receptors is reduced [108]. The expression of the adhesion glycoproteins CR3 (CD11b/CD18) and LFA-1 (CD11a/CD18) remains unaffected; however, upon preactivation, the cells express high levels of Fcγ-RI (CD64), Fcγ-RII (CD32), and CD14, as well as low levels of Fcγ-RIII (CD16) and HLA-DR. These abnormalities may affect neutrophil functionality, potentially impairing both antibacterial and antifungal immune responses in CN [107–111]. Interestingly, patients with CN suffer from severe periodontitis and gingivitis, which might be explained by these functional defects. However, it should be noted that these investigations were performed on cells isolated from patients with rhG-CSF-treated CN or CyN; therefore, some of the observed differences in neutrophil function may be therapy induced.

EXPERIMENTAL MODELS OF CN AND CN/AML

In recent years, significant progress has been made in the development of experimental models to study CN. These models have contributed to overcoming the challenge of limited primary patient material, leading to significant advances in our understanding of the genetic and molecular mechanisms underlying the disease and the design of potential therapies. Here, we summarize currently available

in vitro and *in vivo* models and their contribution to delineating the pathogenesis of CN.

Engineered primary human hematopoietic stem and progenitor cells

The genetic manipulation of healthy donor HSPCs using vector-mediated transgene expression or genome editing techniques has served as an excellent model for exploring the role CN-associated mutations in disease biology. The overexpression of mutants *SRP54* [112] or *CLPB* [23] in primary $CD34^+$ HSPCs recapitulated the impairment of granulocytic maturation observed in patients. CRISPR/Cas9 gene editing of *COPZ1* in primary $CD34^+$ HSPCs confirmed the induction of neutropenia by mutated *COPZ1*, also showing dysregulated JAK/STAT, G-CSFR, interferon, and STING signaling [29]. Following this line of research, gene editing of healthy donor $CD34^+$ HSPCs has been used to investigate the impact of *ELANE* mutations on granulocytic differentiation, showing that frameshift mutations in the late, but not the early, *ELANE* exons led to neutrophil maturation arrest. In contrast, missense mutations are pathogenic irrespective of their location [113].

Induced Pluripotent Stem Cells

The generation of patient-derived induced pluripotent stem cell (iPSCs) is a promising in vitro technology for modeling CN. iPSCs are generated from somatic cells, such as fibroblasts or peripheral blood mononuclear cells obtained from patients with CN, by reprogramming them via the expression of four specific genes, known as Yamanaka factors (*Oct4, Sox2, c-Myc*, and *Klf-4)*. This process preserves the original CN-associated mutation while producing an unlimited source of patient-derived cells [114]. iPSCs are then differentiated into HSPCs and neutrophils using well-established protocols, providing a robust platform to study the effect of CN mutations in early hematopoiesis as well as in granulopoiesis [41,115,116].

Several iPSC-based models have been established to investigate CN-causing gene mutations, such as *ELANE* [41,66,116–118], *SBDS* [119], *HAX1* [65,120,121], *TCIRG1* [122], and *G6PC3* [123]. These models successfully reproduced several key cellular defects observed in patients' HSPCs, including NE mislocalization, UPR induction, and increased ROS levels in *ELANE*-mutant iPSCs, as well as mitochondrial dysfunction along with elevated apoptosis in *HAX1*-mutant iPSCs [42,44,66,121]. Patient-derived iPSCs are also a valuable model for drug discovery and are frequently used to evaluate potential therapies targeting defective granulopoiesis, as will be addressed later in the review.

An important feature of iPSC model is the possibility to generate patient-derived isogenic iPSCs lines, in which CN-causing inherited mutations are corrected [65] or mutant genes are knockout [124] using genome editing. In this setting, we can estimate isolated effects of specific mutations on granulocytic differentiation, avoiding the impact of interindividual genetic and epigenetic backgrounds. Isogenic iPSC lines can also be generated by inserting CN-causing mutations in iPSCs derived from healthy donors. This approach has been used to investigate the effects of CN-causing mutations in *COPZ1, ELANE, SRPRA*, and *SRP19* on granulopoiesis and deregulated intracellular signaling [28,29,42]. The importance of iPSC-based disease modeling is particularly evident when assessing the pathogenicity of genetic variants identified through whole-exome or whole-genome sequencing in patients with CN, where patient numbers are often limited—sometimes unique—and access to primary material is scarce, especially in cases in which, due to disease severity, patients have already undergone bone marrow transplantation.

Disease modeling of the preleukemia and leukemia stages in CN allows investigations of stepwise leukemogenesis. The introduction of a *CSF3R* truncating mutation into CN patient-derived iPSCs to mimic a preleukemia stage showed that this early genetic event did not induce clonal expansion, but instead triggered a sustained proinflammatory state [68]. In a study by Dannenmann et al. [116], the authors recreated stage-specific CN leukemogenesis by introducing *CSF3R* and *RUNX1* mutations in patient-derived iPSCs. The combination of both mutations induced a block in neutrophil differentiation. Moreover, this iPSC model of leukemia stage in CN identified the overexpression of *BAALC* as a key oncogenic event during CN/AML transformation [116]. Similarly, iPSC-based models have been used to model the phenotype of bone marrow failure and subsequent leukemic progression in SDS. Patient-derived iPSCs from individuals with SDS were engineered to carry a del(7q), revealing hyperactivation of the TGFβ signaling. Inhibition of TGFβ using the small-molecule SD208 rescued erythroid and myeloid differentiation of SDS iPSCs, but not SDS del(7q) or healthy donor iPSCs [125]. iPSCs and primary HSPCs are also valuable tools for testing potential gene therapy strategies for CN. For instance, targeted inactivation of genes implicated in CN, such as *ELANE*, has been shown to restore granulocytic differentiation in vitro [113,124,126]. Similarly, correcting disease-causing mutations, such as *ELANE* and *COPZ1*, in patient-derived HSPCs or iPSCs, using either lentiviral transduction or CRISPR-mediated gene correction, successfully reversed defective myeloid differentiation [29,65,127]. Of note, CRISPR/Cas9-based gene editing for disease modeling has some pitfalls. Gene editing may introduce off-target genome modifications, which can generate unintended phenotypes and confound the interpretation of the results [128]. Additionally, genetically engineered in vitro systems are maintained in an artificial environment that lacks the complexity of the bone marrow niche and its interaction with other cells. In the future, improving differentiation protocols, establishing coculture methods, and advancing gene editing technologies will be essential for increasing the predictive capabilities of in vitro experimental models for CN.

Zebrafish as an *In Vivo* Model System to Study CN

The zebrafish (*Danio rerio*) has emerged as a powerful vertebrate model system for studying human diseases, including CN, due to the conserved molecular and cellular mechanisms underlying hematopoiesis when compared with humans [129]. The availability of transgenic lines, combined with the optical clarity and external development of zebrafish embryos, allows real-time visualization of hematopoietic cell development. Furthermore, genome editing tools facilitate the functional analysis of disease-associated genes. During the last years, several zebrafish models of CN have been established mimicking the CN-causing mutations in the orthologous zebrafish genes (Table 1) [9–10,88,105–121].

Unfortunately, no direct zebrafish ortholog exists for *ELANE*, the most mutated gene in CN patients. Zebrafish possess several genes encoding serine proteases with similar functions, such as *ela2l* and other elastase-like genes. Other CN-associated mutations such as *HAX1* and *CSF3R* have zebrafish orthologs, *hax1* and *csf3r*, respectively. Knockdown studies with antisense oligonucleotides (morpholinos) and knockout studies with CRISPR/Cas9 showed that the impaired function of Hax1 [130] and Csf3r [131,132] had a negative

Table 1 Zebrafish models of CN, SDS, and WAS

Human gene	Zebrafish orthologs	Phenotype	Method	References
		CN		
COPZ1	*copz1*	Decrease in neutrophils and macrophages, without affecting stem cells, myeloid progenitors or erythrocytes Bone deformities (scoliosis)	CRISPR/Cas9-induced truncation	[10]
CSF3R	*csf3r*	Decrease in neutrophils without affecting macrophages No response to G-CSF and increased susceptibility to bacterial infection	Morpholino-induced knockdown CRISPR/Cas9-induced knockout	[105,107,108]
CXCR4 (WHIM syndrome)	*cxcr4a* *cxcr4b*	Neutrophil retention in hematopoietic tissue Impaired neutrophil motility and wound recruitment No differences in apoptosis of neutrophils Peripheral neutropenia	Stable transgenic line expressing truncated Cxcr4b tagged with GFP under the control of the neutrophil-specific promoter mpo	[118]
G6PC3	*g6pc3*	Decrease of HSCs, lymphocytes, erythrocytes, and neutrophils	Morpholino-induced knockdown	[110]
GFI1	*gfi1aa* *gfi1ab* *gfi1b*	Broad impairment in hematopoiesis, including a decrease in *mpx*-expressing neutrophils	Morpholino-based knockdown of *gfi1aa*	[111]
		Expansion of neutrophil progenitors and MDS-like phenotype	Truncated *gfi1aa* lacking DNA binding domain	[112]
		No effect on hematopoiesis	*gfi1aa* lost-of-expression	[113]
HAX1	*hax1*	Neutropenia without affecting stem and progenitor cells, normal monopoiesis, and erythropoiesis Increase in apoptosis Impaired G-CSF signaling	Morpholino-induced knockdown CRISPR/Cas9-induced knockout	[106]
JAGN1	*jagn1a*	Decrease in HSCs, myeloid progenitors, macrophages, and neutrophils	Morpholino-induced knockdown CRISPR/Cas9-induced knockout Overexpression of the mutated Jagn1b variant	[109]
	jagn1b	Mimicked human *JAGN1*-associated CN leading to neutropenia without affecting other hematopoietic cell types Increased UPR, apoptosis, and impaired G-CSF signaling		
SRP19	*srp19*	Reduction in neutrophil numbers	Morpholino-induced knockdown	[9]
SRPRA	*srpra*	Reduction in neutrophil numbers	Neutrophil-specific Cas9 gene editing	[9]
VPS45	*vps45*	Reduction in neutrophil numbers	Morpholino-induced knockdown	[117]
		Reticular dysgenesis		
AK2	*ak2*	Defects in HSPC development and profound impairment of lymphoid and myeloid development	Morpholino-based knockdown Deficient mutant	[121]

(continued)

Table 1 (*Continued*)

Human gene	Zebrafish orthologs	Phenotype	Method	References
		SDS		
SBDS	*sbds*	Neutropenia, besides atrophy in the pancreas, liver, and intestine	Morpholino-induced knockdown CRISPR/Cas-induced knockout	[114,115]
SRP54	*srp54*	Neutropenia and reduced chemotaxis Diminished exocrine pancreas	Morpholino-induced knockdown CRISPR/Cas-induced knockout	[88,116]
		WAS/XLN		
WAS	*was*	Normal neutrophil and macrophage numbers but reduced recruitment to wounding, delayed phagocytosis, and defective bacterial clearance hWASpI294T expression led to neutropenia, mimicking XLN	Morpholino-induced knockdown Null mutant generated through TILLING Overexpression of XLN-inducing variant hWASpI294T	[119,120]

CN=congenital neutropenia; *G-CSF*=granulocyte colony-stimulating factor; *HSCs*=hematopoietic stem cells; *HSPCs*=hematopoietic stem and progenitor cells; *MDS*=myelodysplastic syndrome; *GFP*=green fluorescent protein; *mpo*=myeloperoxidase; *XLN*=X-linked neutropenia; *UPR*=unfolded protein response; *CRISPR*=clustered regularly interspaced short palindromic repeats; *Cas9*=CRISPR-associated protein 9; *TILLING*=targeting induced local lesions in genomes.

effect on neutrophil development, leading to neutropenia without affecting other myeloid cells, such as macrophages. Moreover, *csf3r*-deficient zebrafish demonstrated impaired neutrophil function indicated by increased susceptibility to bacterial infections and reduced neutrophil migration to sites of injury [132]. These models have also been used to study the G-CSF pathway, showing impaired signaling in both cases. However, *hax1* morphants, unlike *csf3r* mutants [129,131,132], respond to *g-csfa* overexpression by activating an alternative Hax1-independent pathway of granulopoiesis [130]. Another gene involved in CN development, *JAGN1*, has two zebrafish paralogs, *jagn1a* and *jagn1b*. Morpholino-induced knockdown studies demonstrated that the impairment of Jagn1b mimicked the phenotype of patients with CN, leading to neutropenia without affecting other hematopoietic cells, such as stem cells, myeloid progenitors, macrophages, or erythrocytes. Additional knockout studies and the overexpression of a patient-specific mutation confirmed that interfering with the function of Jagn1b leads to neutropenia in zebrafish, allowing the establishment of a *JAGN1*-CN zebrafish model [133]. Resembling the *HAX1*-CN zebrafish model, this model showed impaired G-CSF signaling but responded to *g-csfa* overexpression. Further analysis of the underlying pathomechanism of *JAGN1*-associated neutropenia revealed activation of the UPR and increased apoptosis upon *jagn1b* knockdown [133]. A less well-characterized CN mutation in zebrafish is *G6PC3*, with its zebrafish ortholog, *g6pc3*. Morpholino-induced knockdown studies showed a general impairment in hematopoiesis, affecting stem cells, macrophages, lymphocytes, and neutrophils [134]. The study of *GFI1*-associated neutropenia in zebrafish is more complicated due to the existence of three orthologs, *gfi1aa, gfi1ab*, and *gfi1b,* playing different roles in hematopoiesis. Modeling Gfi1aa showed contradictory data depending on the used strategy. Morpholino-based knockdown showed a broad impairment in hematopoiesis, including a decrease in *mpx*-expressing neutrophils [135]. However, a Gfi1aa-mutant line expressing a truncated form of Gfi1aa lacking the DNA binding domain showed an expansion of neutrophil progenitors along with an MDS-like phenotype [136]. Another mutant established by a gene trap transposon resulted in an absent *gfi1aa* expression, without showing any effect on hematopoiesis [137].

Zebrafish has also been used to model *SBDS* and *SRP54*, mimicking the mutations in the zebrafish orthologs *sbds* and *srp54*. Morpholino-induced knockdown and CRISPR/Cas9-induced knockout studies demonstrated that interfering with *Sbds* [138,139] or *Srp54* [112,140] function leads to neutropenia and atrophy in the pancreas, mimicking the phenotype observed in patients with SDS. Noteworthy, knockdown or heterogenous knockout of *srp54* did not affect the development of other hematopoietic lineages, including HSPCs, erythrocytes, and lymphocytes, as indicated by a stable expression of markers such as *runx1, c-myb, globin,* and *rag1* [112,140]. However, homozygous *srp54* knockout embryos showed absence of peripheral erythrocytes due to impaired blood circulation as well as a lack of *rag1* expression due to early onset of lethality [112]. In addition to SRP-related genes, mutations in vacuolar protein sorting-associated protein 45 (VPS45), a key regulator of vesicular trafficking, have also been implicated in CN. The morpholino-induced knockdown of the zebrafish ortholog *vps45* also showed a significant reduction in neutrophil numbers [141].

A stable transgenic zebrafish line has also been established, in which *cxcr4b* with the WHIM-causing mutation is expressed under the control of the neutrophil promoter myeloperoxidase (mpo) and labeled with gfp. The authors reproduced the clinical phenotype of patients, showing neutrophil retention in hematopoietic tissue, impaired neutrophil motility, wound recruitment, and peripheral neutropenia. This model was also used to further investigate the underlying mechanism of neutrophil retention and offers a valuable platform for screening potential treatments to restore neutrophil functionality [142]. Similarly, zebrafish models have been established for Wiskott−Aldrich syndrome (WAS) and X-linked neutropenia (XLN). Both syndromes are caused by different mutations affecting the same protein, namely, WASp. Morpholino-induced knockdown studies and the generation of a null mutant showed no effect on neutrophil counts but an increased susceptibility to bacterial infection along with a deficit in neutrophil and macrophage responsiveness to wounding,

mimicking the phenotype of WAS patients [143,144]. However, the expression of the constitutively active human WAS p.I294T variant, which induces XLN, in the WASp null mutant zebrafish led to neutropenia, mimicking the phenotype observed in patients with XLN [144]. Another rare congenital syndrome characterized by severe immunodeficiency, including CN, is reticular dysgenesis, which is caused by biallelic mutations in the *AK2* gene. Modeling this syndrome in zebrafish using morpholino-based knockdown and the *ak2*-deficient mutant zebrafish line showed a broad range of hematopoietic defects, including defects in HSPC development and a profound impairment of lymphoid and myeloid development. These results were indicated by a lack of *ikaros* and *rag1* expression in the thymus arguing for an impaired lymphocyte development and a reduction in the expression of *mpx* and *hbae1*, indicating impaired granulopoiesis and erythropoiesis, respectively [145].

Furthermore, zebrafish is a valuable *in vivo* platform to functionally characterize CN-causing candidate gene mutations in unclassified CN. Recently, a novel truncating mutation in *COPZ1* has been identified in patients with CN [29]. To assess its role in granulopoiesis, zebrafish ortholog *Copz1* was truncated using CRISPR/Cas9, revealing a decrease in neutrophils and macrophages, without affecting stem cells, myeloid progenitors, or erythrocytes. Interestingly, this model showed bone deformities, reproducing the skeletal anomalies observed in patients [29]. A similar study was performed to confirm the pathogenicity of the newly identified CN-associated mutations in *SRPRA* and *SRP19*. The authors demonstrated that interfering with the expression of either of these two genes using morpholino or gene editing impaired granulopoiesis in zebrafish embryos [28].

Mouse Models of CN and Leukemia

The mouse is the primary mammalian model for investigating human physiology and disease due to the high degree of genomic sequence similarity between species [146]. Over the years, mouse models have been instrumental in the characterization of genes involved in CN through the modification of the mouse genome, the transplantation of edited murine HSPCs or the engraftment of human primary cells into "humanized" mice (Table 2) [24,45,74,89,102,123–124,126,128–143].

Mouse models for *ELANE* deficiency or CN-causing *ELANE* mutations have failed to fully replicate the phenotype observed in patients. Targeted disruption of the murine *Elane* gene did not cause neutropenia but reduced the capacity of NE-deficient neutrophils to clear gram-negative bacterial infections [147]. A knock-in model reproducing in the mouse ortholog the *ELANE* p.V72M mutation, found in patients with CN, had normal granulopoiesis [148]. The same group generated a second transgenic mouse carrying the orthologous *ELANE* p.G192X mutation, which produced a truncated NE protein that degraded rapidly. In this model, *Elane* p.G193X mice also showed normal granulopoiesis but developed marked neutropenia when exposed to ER stress induction [43]. Another study used a mouse neutrophil differentiation model to characterize the role of the CN-causing *ELANE* p.G185R mutation in NE-deficient Hoxb8 progenitors. This model is based on conditional expression of Hoxb8 fused to the ER receptor in NE-deficient hematopoietic mouse cells, generating progenitor cells that differentiate into morphologically and functionally mature neutrophils upon estrogen withdrawal [149]. After transplantation into lethally irradiated mice, no difference was observed in the capacity to differentiate into mature neutrophils between NE-deficient cells and cells expressing wildtype or mutant NE [150]. *ELANE* mutations in mice may not result in a CN phenotype due to several reasons, such as species-specific differences in granulopoiesis as well as NE structure and function, or the inability of mutated murine NE to induce cellular stress, as it is observed in human cells [43,150]. In addition to genetically engineered mouse models, the use of immunodeficient "humanized" animals that engraft functional human cells and tissues also offers a robust platform for disease modeling and validation of potential therapies [151]. A recent study was able to reproduce the CN disease phenotype in immunodeficient mice. Gene edited human $CD34^+$ HSPCs carrying a truncated *ELANE* p.R220 mutation in exon 5 showed defective neutrophil maturation and increased UPR when transplanted into humanized mice [113]. Additionally, edited patient-derived HSPCs have been transplanted into immunodeficient mice to validate novel therapeutic strategies targeting *ELANE* [126,152]. Here, both studies used unedited HSPCs carrying *ELANE* mutations (p.V101E; p.L172P) as controls and successfully reproduced the neutrophil differentiation defect.

The role of other genes mutated in the pathogenesis of CN, such as *HAX1, JAGN1, GFI1*, or *G6PC3*, has also been investigated using mouse models. In the context of *HAX1*, homozygous mice with a targeted deletion of *Hax1* showed severe neurodegeneration with premature death at the age of 12 weeks. Deletion of *Hax1* resulted in pronounced effects on lymphopoiesis, a reduction in the HSCs pool, and increased apoptosis of granulocytes upon cytokine withdrawal; however, no impact on steady-state granulopoiesis has been reported [153,154]. Although *Jagn1* deficiency results in embryonic lethality, mice carrying a hematopoietic lineage-specific deletion are viable. Conditional *Jagn1* knockout mice showed normal numbers of neutrophils, but impaired response to *Candida albicans* infection. Loss of *Jagn1* resulted in defective neutrophil migration and cytotoxicity, which was rescued with GM-CSF treatment [155]. The targeted disruption of *Gfi1* transcription factor exhibited severe neutropenia accompanied by an accumulation of immature monocytic cells due to the failure of myeloid progenitors to respond to G-CSF stimulation [156,157]. Moreover, *Gfi1* deficient mice showed elevated levels of inflammatory cytokines leading to systemic inflammation and reduced bone mass [156,158]. Similarly, Muench et al. [159] were able to induce a neutropenic phenotype in knock-in mice expressing CN patient-derived mutations in *GFI1* (p.N382S, p.K403R and p.R412X). *Gfi1* mutants were susceptible to neutrophil-dependent pathogens and showed elevated G-CSF levels in serum. Unlike *Gfi1* deficient mice, mutants showed normal numbers of HSCs, and only the $Gfi1^{N382S}$ mutant showed variability in HSCs fitness in transplantation assays [159].

In the case of *G6PC3*, Cheung et al. [160] showed that knockout of the mouse ortholog led to neutropenia associated with numerous cellular dysfunctions and susceptibility to bacterial infection. Neutrophils and bone marrow cells deficient in G6pc3 exhibited elevated ER stress and apoptosis, accompanied by disrupted glucose metabolism [161].

Zambetti et al. [98] generated mice with targeted downregulation of *Sbds* in *Cebpa*-expressing cells. *Sbds* deficiency in early hematopoietic progenitors showed neutropenia with arrest in myeloid differentiation. In contrast, knock-in models carrying mutations in the *WAS* gene did not recapitulate the profound neutropenia seen in patients but displayed neutrophil hyperactivation [162,163].

Table 2 Mouse models of CN, SDS, WAS, and leukemia progression

Human gene	Mouse orthologs	Phenotype	Method	References
		CN		
ELANE	*Elane*	Protein-deficient: reduced capacity to clear bacterial infection	Knockout	[123]
		V72M mutation: normal granulopoiesis	Knock-in	[124]
		G193X mutation: neutropenia when exposed to ER stress	Knock-in	[24]
		G185G mutation: normal neutrophil differentiation	Transplanted NE-deficient Hoxb8 progenitors	[126]
		R220: defective neutrophil maturation and increased UPR	Xenotransplant of edited human HSPCs	[89]
		V101E, L172P: defective neutrophil maturation	Xenotransplant of CN patient-derived HSPCs	[102,128]
G6PC3	*G6pc3*	Protein deficient: neutropenia; defects in neutrophil respiratory burst, chemotaxis, and calcium flux; enhances apoptosis in neutrophils; and increased susceptibility to bacterial infection	Knockout	[136,137]
GFI1	*Gfi1*	Protein deficient: severe neutropenia, reduced HSC numbers and elevated levels of inflammatory cytokines	Knockout	[132–134]
		Patient-derived mutations (N382S, K403R, R412X): neutropenic phenotype, suspectable to pathogens and elevated G-CSF levels in serum	Knock-in	[135]
HAX1	*Hax1*	Protein-deficient: severe neurodegeneration, effects on lymphopoiesis	Knockout	[129,130]
JAGN1	*Jagn1*	Normal neutrophil counts but impaired activity. Restored with GM-CSF	Conditional knockout in *Vav1*-expressing cells	[131]
		SDS		
SBDS	*Sbds*	Protein deficient: neutropenia with arrest in myeloid differentiation	Conditional knockout in *Cebpa*-expressing cells	[74]
WAS/XLN				
WAS	*Was*	Neutrophil hyperactivation	Knock-in	[138,139]
		CN leukemogenic progression		
CSF3R	*Csf3r*	Protein deficient: defective granulopoiesis	Knockout	[140]
		Δ175 truncation: hypersensitivity to G-CSF	Knock-in	[141–143]
+ *RUNX1*	*Runx1*	$Csf3r^{\Delta175}$ + $RUNX1^{D171N}$: enhanced progenitor proliferation; progression to AML upon expression of CXXC4 mutation with elevated proinflammatory response	Transplanted $Csf3r^{\Delta175}$ progenitors transduced with $RUNX1^{D171N}$ vector	[45]

AML=acute myeloid leukemia; *CN*=congenital neutropenia; *ER*=endoplasmic reticulum; *G-CSF*=granulocyte colony-stimulating factor; *GM-CSF*=granulocyte-macrophage colony-stimulating factor; *HSCs*=hematopoietic stem cells; *HSPCs*=hematopoietic stem and progenitor cells; *UPR*=unfolded protein response; *RUNX1*=runt-related transcription factor 1; *CXXC4*=CXXC finger protein 4.

Because *CSF3R* mutations are associated with CN progression to MDS/AML, transgenic mouse models have been generated harboring equivalent *CSF3R* mutations reported in patients. Unlike in *Csf3r* deficient mice, which showed reduced hematopoietic progenitors and impaired neutrophil production [164], no defects on granulopoiesis but a hypersensitivity to G-CSF were observed in *Csf3r*-Δ175 mice [165]. Interestingly, another study in mice carrying a similar *Csf3r* mutation showed reduced numbers of neutrophils in blood, but still hypersensitivity to G-CSF [166,167]. Although the underlying reasons for the observed differences in circulating neutrophil levels between these models remain uncertain, they may be attributed to variations in the targeting strategies or the mouse strains. These

models demonstrated that G-CSFR mutation alone is insufficient to develop leukemia but also serves as a valuable tool for investigating the coexpression of CN/AML associated mutations in vivo. Thus, Olofsen et al. [69] reported a *CSF3R/RUNX1* mutant mouse model of leukemic progression. Although coexpression of *Csf3r*-Δ175 and *RUNX1* p.D171N mutations enhanced progenitor proliferation, the sporadic expression of a CXXC4 mutation led to overt AML and was accompanied by an elevated proinflammatory response. Another study demonstrated that *Csf3r*-Δ175 truncation accelerated AML onset in PML-RARa transgenic mice, indicating its leukemogenic potential [168]. Overall, the progression from a CN phenotype toward leukemic transformation remains poorly understood, and the establishment of suitable CN/AML mouse models is a crucial step toward elucidating leukemogenesis.

Therapeutic Approaches for CN

An in-depth understanding of the pathogenesis of CN helps to identify druggable signaling pathways leading to neutropenia and thus new therapeutics. Recombinant human G-CSF (filgrastim, lenograstim) is still the treatment of choice for the majority of patients with CN and CyN. Intriguingly, GM-CSF treatment is not effective in the majority of patients [169]. Recently, several approaches have been proposed as alternatives or adjuncts to rhG-CSF therapy. *G6PC3*-mutated patients with CN can be successfully treated with the sodium-glucose cotransporter-2 (SGLT2) inhibitor empagliflozin [170–172]. The impaired activity of glucose-6-phosphatase in *G6PC3*-deficient patients with CN leads to the accumulation of 1,5-anhydroglucitol-phosphatase (1,5-AGP), a competitive inhibitor of hexokinase, resulting in reduced glycolysis and ATP depletion. Empagliflozin increases urinary excretion of 1,5-AG, leading to reduced levels of 1,5-AG6P and restoration of hexokinase activity and ATP production in neutrophils [170]. CXCR4 inhibitors, plerixafor or mavorixafor, are successfully used to treat patients with Warts, Hypogammaglobulinemia, Infections, and Myelokathexis (WHIM) syndrome caused by activating gain-of-function *CXCR4* mutations [173–175]. Treatment of *G6PC3*-CN patient-derived iPSCs or patients with CN with high-doses of nicotinamide (vitamin B3) in combination with rhG-CSF led to a reduction in the therapeutic G-CSF dose and a decrease in the frequency of infections [176].

NE inhibitors or UPR inhibitors restored defective granulopoiesis of *ELANE*-CN HSPCs, iPSCs, and cell lines expressing *ELANE* mutations in vitro, suggesting their therapeutic potential for patients with *ELANE*-CN [42,118,177]. In a drug repurposing study using isogenic iPSC lines from patients with *ELANE*-CN, a low dose of the pan-CDK inhibitor flavopiridol was shown to successfully restore defective granulocytic differentiation. This effect was observed in iPSCs-derived and primary $CD34^+$ cells from patients with CN with different genetic backgrounds (*ELANE, HAX1, SRP54*, and *JAGN1*), as well as zebrafish models of *HAX1* and *JAGN1* CN (54). Considering the anticancer properties of high-dose flavopiridol, further studies are needed to determine its potential to prevent or delay leukemogenic progression in CN.

De novo protein design enables the creation of new protein therapeutics with superior functions and properties. Recently reported *de novo* designed binders targeting G-CSFR demonstrated superior stability, protease resistance, binding to fewer G-CSFR domains compared with rhG-CSF and better specificity in inducing granulopoiesis [78,178]. These designed G-CSFR binders can potentially be explored for treatment of patients with CN for oral administration and for patients with mutations in the extracellular part of G-CSFR, which is not required for the binding of the designed proteins.

Gene Therapy Approaches for CN

As a group of genetic disorders, CN subtypes are obvious targets for which gene therapy would be highly beneficial in offering a permanent cure for the patients. However, despite the proposal of several promising strategies, no clinical trial has been conducted or registered to date [65,113,123,124,127,152,179]. The biggest challenge to gene therapy development is surely that CN is an ultrarare disease [180].

Gene therapy approaches for CN have been investigated preclinically only for *HAX1, ELANE*, and *G6PC3* genes (Table 3) [41,89,96,99–100,102–103,128,155,157]. For *HAX1* and *G6PC3*, lentiviral strategies have been proposed and tested successfully in patient-derived iPSCs [65,120,123]. Although Morishima et al. [120] successfully used a lentiviral expression construct to deliver the *HAX1* gene in patient-derived iPSCs, the system from Pittermann et al. [65] showed no successful restoration of granulopoiesis by lentiviral expression of *HAX1*, and the authors were only successful in

Table 3 Gene therapy strategies for CN

Gene	Inheritance pattern	Strategy	Status	References
ELANE	Autosomal dominant	CRISPR/Cas9 based knockout / knockdown	Preclinical in vivo proof of principle, primary patient cells	[89,100,102]
		Allele-specific knockout	Preclinical in vitro proof of principle, primary patient cells	[103,155]
		CRISPR/Cas9 and AAV6-based HDR	Preclinical in vivo proof of principle, primary patient cells	[103,128,157]
HAX1	Autosomal recessive	Lentiviral expression	Preclinical in vitro proof of principle in iPSC	[96]
		CRISPR/Cas9 and plasmid-based HDR	Preclinical in vitro proof of principle in iPSC	[41]
G6PC3	Autosomal recessive	Lentiviral expression	Preclinical in vitro proof of principle in iPSC	[99]

CRISPR=clustered regularly interspaced short palindromic repeats; *Cas9*=CRISPR-associated protein 9; *AAV6*=adeno-associated virus serotype 6; *iPSC*=induced pluripotent stem cell.

correcting the phenotype by CRISPR/Cas9 mediated homology-directed repair (HDR) of the mutation.

Significant efforts have been made to develop a CRISPR/Cas-based gene therapy approach targeting autosomal dominant mutations in the *ELANE* gene. To date, three approaches have been described to therapeutically target mutations in the *ELANE* gene using nucleases. The first is the complete knockout or knockdown of the *ELANE* gene [113,124,126]. The second is the correction of the mutation by HDR [127,152,181]. The third is the allele-specific knockout of the mutated allele [127,179]. The knockout approach is a universal strategy applicable to all patients with *ELANE*-CN. The only criticism of this approach is that, despite all the evidence that NE is a dispensable protein, only a clinical trial with long-term observation can ultimately prove its safety. HDR-mediated correction would not raise this question. However, this approach can only be applied to a limited number of mutations, as, in the best-case scenario, it would enable correction of all mutations situated in one exon. The allele-specific knockout would preserve NE expression and be applicable to 50%–75% of patients.

Because G-CSF is highly effective in preventing severe infections by increasing the absolute neutrophil counts [182], regulators and patients tend to have a much lower tolerance for risk compared with other genetic diseases that lack therapeutic alternatives [183–186]. This situation should not be mistaken as an absence of clinical need for a genetic cure from the patient's perspective. As evidenced by the cumulative 20% risk of leukemia, a 10% risk of sepsis [80], the significant impact on quality of life [187], and the risks and side effects of bone marrow transplantation [188], there is still an unmet clinical need for therapeutic alternatives among patients with CN.

The recent approval of the CRISPR/Cas gene therapy CASGEVY [189], the design of novel *in vivo* delivery methods [190], and the recent efforts by developers and regulators for innovative drug approval pathways [191,192] give us reasons for optimism that, despite many challenges in the development of gene therapies for CN, a solution is beginning to take shape.

Declaration of competing interest

The authors declare that they have no competing interests.

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