HHS Public Access

Author manuscript

Nat Rev Dis Primers. Author manuscript; available in PMC 2018 February 21.

Published in final edited form as:

Nat Rev Dis Primers. ; 3: 17032. doi:10.1038/nrdp.2017.32.

Severe congenital neutropenias

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Abstract

Severe congenital neutropenias are a heterogeneous group of rare haematological diseases that are characterized by impaired maturation of neutrophil granulocytes. Patients with severe congenital neutropenia are prone to recurrent, often life-threatening infections beginning in their first months of life. The most frequent pathogenetic defects are autosomal dominant mutations in *ELANE*, which encodes neutrophil elastase, and autosomal recessive mutations in *HAX1*, whose product contributes to the activation of the granulocyte-colony stimulating factor (G-CSF) signalling pathway. The pathophysiological mechanisms of these conditions are the object of extensive research and are not fully understood. Furthermore, severe congenital neutropenias may predispose to myelodysplastic syndromes or acute myeloid leukaemia. Molecular events in the malignant progression include acquired mutations in *CSF3R* (encoding G-CSF receptor) and subsequently in other leukaemia-associated genes (such as *RUNX1*) in a majority of patients. Diagnosis is based on clinical manifestations, blood neutrophil count, bone marrow examination, and genetic and immunological analyses. Daily subcutaneous G-CSF administration is the treatment of choice and leads to a substantial increase in blood neutrophils count, reduction of infections and drastic improvement of quality of life. Haematopoietic stem cell transplantation is the alternative treatment. Regular clinical assessments (including yearly bone marrow examinations) to monitor treatment course and detect chromosomal abnormalities (e.g. trisomy 21, monosomy 7) as well as somatic pre-leukaemic mutations are recommended.

Graphical abstract

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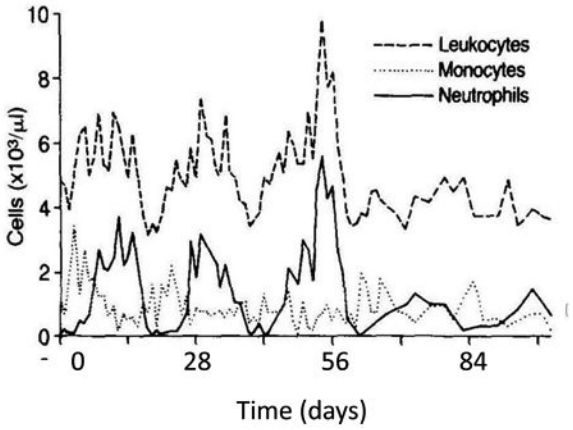
Competing interests: D.C.D. is a consultant and receives research support from Amgen; Inc., a manufacturer of granulocyte colony-stimulating factor (G-CSF) used to treat neutropenia. All other authors declare no competing interests.

Author contributions: Introduction (K.W. and D.C.D.); Epidemiology (D.C.D., J.S.); Mechanisms/pathophysiology (J.S., I.P.T. and K.W.); Diagnosis, screening and prevention (D.C.D., K.W. and J.S.); Management (C.Z., K.W. and J.S.); Quality of life (C.Z. and K.W.); Outlook (K.W.); Overview of the Primer (K.W.)



Introduction

The severe congenital neutropenias comprise a group of inherited disorders of haematopoiesis characterized by impaired differentiation of neutrophilic granulocytes and, as a result, severe chronic neutropenia, defined as blood absolute neutrophil counts (ANCs) $< 0.5 \times 10^9$/L. Bone marrow examination in the majority of patients reveals a maturation arrest of myelopoiesis at the level of promyelocytes[1], which generally leads to reduced neutrophil counts but elevated numbers of atypical promyelocytes (Figure 1). In these patients, the risk of infections such as otitis, gingivitis, skin infections, pneumonia, deep abscesses and septicaemia begins in the neonatal period,[1,2] and, without proper treatment, continues throughout life. Furthermore, patients with severe congenital neutropenia have an increased risk of developing leukemia. Mutations in numerous genes have been linked to congenital neutropenia.

Although neutropenia is a hallmark shared by all of the severe congenital neutropenias, the severity of neutropenia varies, and might vary even in the same patient over time. The increasing knowledge of the mutations and the intracellular signalling pathways implicated in severe congenital neutropenias has helped us to understand the underlying disease mechanisms of aberrant molecular haematopoiesis and leukemogenesis.

In this Primer, we focus on the genetic mutations that lead to severe forms of congenital neutropenia and discuss recent advances in understanding severe congenital neutropenias, from epidemiology, genetics and pathophysiology to screening, diagnosis and management.

Epidemiology

Prevalence

Severe congenital neutropenia is a rare condition: its prevalence is estimated to be 3-8.5 cases per million individuals[3,4]. The establishment of registries for patients with severe chronic neutropenia has helped to identify cases and the prevalence and relative frequency of each specific condition. Prevalence depends on several factors: the success of the mother's pregnancy, the frequency of neonatal deaths from infections or other causes, the pattern of inheritance for the specific genetic disorder and the ascertainment of the diagnosis through

measurement of the blood ANC early in life. The physician's awareness and availability of basic diagnostic tools are also contributing factors. Most infants worldwide do not have a complete blood cell count done at birth, and most cases of neutropenia early in life are attributable to prematurity or immunological mechanisms. For this reason, severe congenital neutropenia is usually an inferred diagnosis based on fever and recurrent infections early in life and measurement of the blood ANC associated with these events.

Inheritance

Demographics also play an important part in epidemiology. Worldwide autosomal dominant disorders appear to be more common, and, and the recessive disorders are usually diagnosed in consanguineous populations. Differences in the rate of consanguinity are probably the main reason that could explain the observed differences in the prevalence of specific neutropenia-associated mutations between Europe and North America. For example, the prevalence of severe congenital neutropenia due to *HAX1* mutations in Europe is high (11%) probably owing to the large number of consanguineous families of Turkic or Arabic origin. By contrast, in the United States no patients with *HAX1* mutations have been detected so far. In Israel, a unique pattern of mutations with a high prevalence of *G6PC3* mutations (25%) was found[5]. These informations are very useful in clinical practice. When using genetic sequencing to confirm a diagnosis of severe congenital neutropenia, sequencing *ELANE* should be considered first, as mutations in *ELANE* are are most common, unless the patient's family history and clinical clues from the physical examination or general laboratory testing suggest otherwise. As targeted-next generation sequencing and exome sequencing become more widely used as diagnostic tools, we can expect that new variants associated with severe congenital neutropenia will be identified, adding to our knowledge and perhaps making diagnosis somewhat more-confusing.

Other hereditary factors are involved in determining the prevalence of congenital neutropenia. Individuals of African heritage have lower blood neutrophil counts compared with persons of white European or Asian origin[6]. Within the African heritage group, the presence of a specific polymorphism in *DARC* (encoding the Duffy antigen) has been linked with neutropenia[6,7]. This condition is now often referred to as “benign ethnic neutropenia”, as it is not associated with infections or evolution to myeloid malignancies. There are other forms of benign ethnic neutropenia and probably others to be discovered[7]. Interestingly, individuals carrying the neutropenia-associated Duffy antigen are not predisposed to develop other forms of neutropenia, as reflected by the scarcity of persons of African heritage in the Severe Chronic Neutropenia International Registry (SCNIR) or in the French Neutropenia Registry.

Mortality

The mortality rate for severe congenital neutropenia prior to the antibiotic era (1950s) was as high as 90%[8], and even with antibiotics, >80% of patients would die from severe bacterial infections[9]. A breakthrough in the treatment of severe congenital neutropenias occurred with the availability of recombinant granulocyte-colony-stimulating factor (G-CSF) for clinical use[10] (Figure 2). Treatment with G-CSF resulted in an increase of neutrophils to more than 1×10^9/L [11,12,13], and a statistically significant decrease in the number and severity of

infectious episodes, leading to a dramatic improvement of the quality of life[14]. The clinical benefits of G-CSF therapy were clearly demonstrated in a Phase III clinical trial[14]. Intriguingly, granulocyte-macrophage colony-stimulating factor (GM-CSF) did not lead to an increase in ANC in the majority of patients[13]

Today, G-CSF is the treatment of choice for severe congenital neutropenias. Overall survival is now estimated to exceed 80%, including patients developing malignancies[15,16], although approximately 10% of patients (mainly G-CSF non-responders) still die from sepsis or severe bacterial infections[3,16,17]. In patients without non-haematologic involvement, survival mainly depends on their response to G-CSF treatment, whereas in the subtypes of severe congenital neutropenias that also present with extra-haematopoietic manifestations, survival depends on both the severity of neutropenia and of organ defects. For example, in Barth syndrome, a severe congenital neutropenia associated with congestive heart failure (Table 1), survival is approximately 51% at five years, and largely depends on the severity of heart failure and availability of a heart transplant.[18] However, because genetic analyses of severe congenital neutropenias are relatively recent and the majority of patients are children, adolescents and young adults, there is limited data correlating age, risk factors and life expectancy for each specific aetiology of severe congenital neutropenia. Evolution to myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML) are other major possible complications; data from the SCNIR indicates that the cumulative incidence of leukemia was estimated to be 22% after 10 years in a population of 374 patients with severe congenital neutropenia, regardless of their genetic diagnosis[16,17]. Of note, in this study the risk of leukaemia increased with the dose of G-CSF, as less responsive patients, who required higher doses of G-CSF, had a cumulative incidence of leukemia of 40% after 15 years, compared with 11% in more-responsive patients[16,17]. The proposed interpretation was that a poor response to G-CSF defines an ‘at risk’ population of patients with severe congenital neutropenia and predicts an adverse outcome. In the French Neutropenia registry at the time of the analysis, 13 cases of leukemia were recorded among 231 patients observed for a total of 3166 person years[19]. The cumulative incidence of MDS or AML was 2.7% at 10 years and 8.1% at 20 years[19]. In the SCNIR, 44 cases of leukemia were recorded among 374 patients observed for a total of 2043 person years.[16,17] The main difference in the methodologies of these two studies was the inclusion of patients who did not receive G-CSF in the French registry, whereas all patients in the SCNIR analysis had received G-CSF. Of note, patients wits *SBDS* mutations (which cause Shwachman-Diamond syndrome (Table 1) the risk was comparable to that of patients with *ELANE* or *HAX1* mutations[17].

Mechanisms/pathophysiology

The establishment of the SCNIR and several national registries has enabled the performance of genetic and molecular studies in these rare disorders and prompted the identification of mutations in >20 genes that impair the neutrophil differentiation program (Figure 3).

Germline mutations

***ELANE* mutations—**The majority of patients with autosomal dominant severe congenital neutropenia harbor heterozygous *ELANE* mutations[20,21]. *ELANE* encodes neutrophil

elastase (NE), a cytotoxic serine protease that stored in azurophil granules and is released after neutrophil activation. NE hydrolizes multiple protein substrates, including cell-surface proteins (G-CSF-Receptor, VCAM, c-kit, and CXCR4). It is also involved in the function of neutrophil extracellular traps (networks of fibers that bind pathogens) [20-22]. More than 200 different *ELANE* mutations have been identified, randomly distributed over all exons as well as in introns 3 and 4 [22,23,24]. There is a substantial overlap in the mutations causing severe congenital neutropenia and those causing cyclic neutropenia, revealing that genotyping alone is not sufficient to establish a clinical diagnosis[23-26]. Some *ELANE* mutations, such as p.C151Y or p.G214R, cause a more-severe phenotype in terms of increased risk of leukemogenesis, poor G-CSF response, and risk of severe infections[16]. In addition, there are cases of phenotypically healthy parents with mosaicism of *ELANE* mutations (that is, who harbor two populations of cells, one with *ELANE* mutations the other with wild type *ELANE*) whose children have inherited neutropenia in an autosomal dominant way[27,28].

***HAX1* mutations—**Homozygous mutations in *HAX1*, which encodes HCLS1-associated protein X-1 (HAX-1), can be found in patients with autosomal recessive congenital neutropenia especially in consanguineous pedigrees, such as the families in which severe congenital neutropenia was originally described [4,29,30]. There are two HAX-1 splice isoforms and genotype-phenotype studies have shown that *HAX1* mutations affecting both isoforms (mainly p.Q190X in the original patients and p.R86X in Japanese patients) cause severe congenital neutropenia with neurological symptoms[31,32] (for example, developmental delay and epileptic seizures). Mutations affecting only one isoform (mainly p.W44X, found in consanguineous families of Turkic origin) lead to severe congenital neutropenia without neurological involvement[30]. HAX-1 is a ubiquitously expressed protein, but the binding partner of HAX-1, hematopoietic lineage cell-specific protein (HCLS1), is an essential adaptor protein of the G-CSF receptor (G-CSFR) signalling[33,34]. Indeed, mice with deletions of *Hcls1* are neutropenic[33]. In patients with *HAX1* mutations, neutropenia probably results from the abrogated activation of HCLS1 and its downstream signaling, which is essential for granulopoiesis.

Mutations causing extra-haematopoietic abnormalities—Severe congenital neutropenia could also be caused by rare mutations (Figure 3) that lead to severe neutropenia in combination with non-haematopoietic organ failures (Table 1). Examples of other affected tissues or organs include heart (*G6PC3*[35], *TAZ*[36]), urogenital system (*G6PC3*)[35], bone (*SBDS*[37]), exocrine pancreas (*SBDS*), skin (*LAMTOR2*[38], *RAB27A*[39]) and liver (glycogen storage; *SLC37A4*[40,41]) (Tables 1, 2). These mutations affect various functions of haematopoietic stem cells and myeloid cells (Figure 4). In patients harboring one of these mutations, the bone marrow morphology shows a great maturation clearly arrests at the level of promyelocytes[21,30].

Multigenic mutations—Intriguingly, some patients with severe congenital neutropenia harbour inherited mutations in more than one neutropenia-associated gene. Combinations of *G6PC3* and *ELANE* or *HAX1* and *ELANE* mutations have been reported[42]. In the latter combination, the influence of each mutation is hard to analyze, as both mutations lead to severe neutropenia. In combinations of *G6PC3* with *ELANE* or *HAX1*, patients may show

symptoms of both inherited mutations[42]. The additional mutation besides the driving monoallelic autosomal or biallelic recessive mutations may have an influence on the disease severity. Next generation sequencing-based evaluation of all known neutropenia-causing mutations will probably reveal more cases of digenic or even multigenic mutations and lead to a better understanding of the influence of each mutation.

Germline *CSF3R* mutations—Of note, germline bi-allelic *CSF3R* mutations affecting the extracellular part of G-CSFR and leading to reduced or completely abrogated G-CSF signal transduction have been reported in rare cases of severe congenital neutropenia[43-45]. The pattern of inheritance of these mutations is: one mutated allele from the mother, the other mutated allele from the father (Table 1) Interestingly, some patients with germline bi-allelic *CSF3R* mutations respond to GM-CSF[43].

Pathological mechanisms

The dysregulated molecular pathways that underlie the arrest of granulopoiesis at the promyelocyte stage can vary based on the different genetic aetiologies. However, some pathological mechanisms are shared by different mutations. We will focus our discussion on the mechanisms that are better characterized, owing to the larger numbers of patients with a common aetiology.

Endoplasmic reticulum stress and apoptosis of myeloid cells—Mutant neutrophil elastase could not be properly folded, processed, secreted or degraded in myeloid cells of patients with severe congenital neutropenia associated with *ELANE* mutations, depending on which elastase domain was affected by the mutation[30,46,47]. Also, a neutropenia-associated *ELANE* mutation that disrupts the translational start generates a shorter form of neutrophil elastase, which leads to aberrant localization of the mutated protein[48]. Intracellular accumulation and mislocalization of the mutant neutrophil elastase induces endoplasmic reticulum (ER) stress and activates the unfolded protein response (UPR)[49-52], leading to increased apoptosis associated with up-regulation of the master ER chaperone 78 kDa glucose-regulated protein (GRP-78), *XBP1* mRNA splicing and activation of *ATF6*[49-52]. The magnitude of UPR activation varies with different *ELANE* mutations[49-52]. Interestingly, drastically diminished levels of *ELANE* mRNA in promyelocytes and neutrophil elastase in plasma of patients with severe congenital neutropenia have been reported[53,54], a finding that questions how these low levels of mutated protein could induce the UPR. It is also not clear why the same *ELANE* mutations activate the UPR in patients with severe congenital neutropenia but not in patients with cyclic neutropenia. Patients with severe congenital neutropenia have diminished levels of anti-leukoproteinase (SLPI), the natural inhibitor of neutrophil elastase, in promyelocytes and plasma[55]. SLPI might regulate the extent of UPR triggered by mutant neutrophil elastase and, therefore, a deficiency in SLPI might explain how even low levels of neutrophil elastase can induce UPR. Patients with cyclic neutropenia have normal levels of SLPI, and this could protect cells from UPR. Expression of the ER-stress related proteins GRP-78 and phosphorylated eukaryotic translation initiation factor 2A (eIF-2A) are also increased in neutrophils from patients with *G6PC3* mutations[35].

Elevated apoptosis was observed in the bone marrow myeloid progenitor cells of patients with severe congenital neutropenia with *ELANE* or *HAX1* mutations[47,56,57]. Diminished expression of anti-apoptotic proteins apoptosis regulator Bcl-2 (Bcl-2), Bcl2-associated agonist of cell death (Bcl-xL) and baculoviral IAP repeat-containing protein 5 (survivin), but elevated expression of Bcl-2-related protein A1 (BFL-1) and induced myeloid leukaemia cell differentiation protein Mcl-1 (mcl1/EAT) was detected[56,57]. HAX-1 is also expressed in mitochondria, functioning as an anti-apoptotic protein; mutated *HAX1* causes apoptosis of myeloid progenitors by loss of mitochondrial function[30]. Furthermore, mitochondrial release of cytochrome c was also increased in myeloid progenitors from patients with *HAX1* mutations but not in controls[35,47]. Interestingly, these patients had elevated survivin levels in myeloid cells[58]. Morphologic studies and laboratory evidence suggest that apoptosis and removal of the weakened cells by marrow macrophages is the final step in this pathway. Neutrophils from patients with *JAGN1* mutations also revealed increased apoptosis[59]. An aberrant glycosylation of neutrophil proteins (for example, NADPH oxidase) was reported in patients with *G6PC3* and *SLC37A4* mutations, which led to increased ER stress and apoptosis[35,60].

Deregulated transcription factors expression—In addition to neutropenia, the majority of patients with severe congenital neutropenia have increased levels of monocytes and eosinophils (2- to 4-fold above normal)[61]. Monocytosis could be explained as a compensatory mechanism of innate immunological defense in the absence of neutrophils. It might also be a consequence of the deregulated signalling of the transcription factors that are responsible for the commitment of the myeloid progenitors toward neutrophils or monocytes [62,63]. In patients with severe congenital neutropenia, expression of the granulopoiesis-activating protein CCAAT/enhancer binding protein alpha (C/EBPα) is severely diminished and expression of monopoiesis-activating transcription factor PU.1 (PU.1) is unaffected or slightly elevated[64,65]. Thus, disturbed C/EBPα:PU.1 expression ratio with a strong shift toward PU.1 expression might have a decisive role in the dysregulation of myelopoiesis in severe congenital neutropenia. An increased blood and bone marrow eosinophil counts is also a common finding in severe congenital neutropenia patients[61]. Constitutive expression of the transcription factor, Inhibitor of DNA binding 1, (Id1) in human hematopoietic cells modestly enhanced neutrophilic differentiation and inhibited eosinophilic development[66]. G-CSF upregulates Id1 in myeloid progenitor cells of healthy individuals, but not in CN patients. Therefore, defective Id1 expression may cause eosinophilia and may contribute to the lack of neutrophilic granulocytes in CN patients[65].

G-CSFR signal transduction

The G-CSF signalling pathway is altered in patients with severe congenital neutropenia due to the mutations in the genes involved in the G-CSF signalling. This explains why therapy with a very high therapeutical dose of G-CSF is effective in most cases. Severe congenital neutropenia patients have no defects in G-CSF production or G-CSFR expression (their serum contains increased G-CSF levels[67] and myeloid cells express increased numbers of G-CSFR[68]). The dysregulation in the G-CSFR signal transduction occurs at the level of effector proteins downstream in the pathway, leading to the severe deregulation of the transcription of genes that promote myeloid cell proliferation or differentiation (Figure

5)[65,69-73]. The consequence of this altered protein expression profile is diminished or absent neutrophil differentiation.

Mechanisms of G-CSF therapy—By increasing the stimulation of G-CSFR signalling, G-CSF therapy induces compensatory mechanisms of granulopoiesis in patients with severe congenital neutropenia. The enzyme nicotinamide phosphoribosyltransferase (NAMPT) is necessary for the activation of NAD-dependent protein deacetylase sirtuin-1 (SIRT1), which in turn activates transcription factors C/EBPα and C/EBPβ in healthy individuals. C/EBPα is responsible for steady-state granulopoiesis, whereas C/EBPβ is crucial for "emergency" granulopoiesis[74], which is normally required in situations such as infections. In patients with severe congenital neutropenia, G-CSF treatment activates the NAMPT- and SIRT1-induced C/EBPβ-dependent granulopoiesis (Figure 5)[75] The levels of NAMPT and SIRT1 were markedly elevated in myeloid cells and in plasma from G-CSF treated patients, as compared to healthy individuals [75].

Interestingly, Nicotinamid (Vitamin B3), the substrate of NAMPT, is capable of increasing the ANC in healthy individuals[75] and is considered for clinical use in severe congenital and cyclic neutropenia patients.

Leukaemic progression

Severe congenital neutropenia should be regarded as a preleukaemic condition whereby a substantial proportion of patients develop leukaemia[9,16,17,19,76-80], predominantly AML (undifferentiated (M0), myeloblastic (M1) or myelomonocytic (M4), but acute lymphoblastic leukaemia (ALL)[81] and chronic myelomonocytic leukaemia (CMML)[82] have also been reported. Leukaemic progression is not restricted to patients with *ELANE*-associated severe congenital neutropenia. In the European branch of the SCNIR, 17 of the 118 patients with *ELANE* mutations (11%) and 6 of the 41 patients harbouring *HAX1* mutations have developed leukaemia (12,7%). Of the 88 patients with Shwachman-Diamond syndrome, 7 developed leukaemia (9%). However, the number of patients in most other genetic subgroups of congenital neutropenias is too small to make any reliable statement about leukaemia risk. Studies on individual patients, regardless of their genotype, including a patient with cyclic neutropenia[83], have reported leukaemic progression.

***CSF3R* mutations—**A major risk factor for leukaemogenesis in patients with severe congenital neutropenia is the presence of somatic (acquired) *CSF3R* mutations (mostly C-to-T nonsense mutations that convert glutamine codons into premature stop codons) that result in the truncation of approximately 100 amino acids of the cytoplasmic domain of G-CSFR (figure 6). Functional studies in murine myeloid cell lines showed that these truncated G-CSFRs blocked G-CSF-induced neutrophil differentiation and maturation in these cell line models[84,85]. This observation fueled the concept that the membrane-distal C-terminal region of G-CSFRs harbored a "differentiation domain" from which specific differentiation signals emanated[84-86]. Extended mutation analysis has shed further light on the crucial functions of the G-CSFR C-terminus, which have been reviewed in detail elsewhere[87,88]. G-CSFR truncation mutants show prolonged half-life at the plasma membrane and, therefore, act dominantly over the wild type receptors[89], resulting in an elevated G-CSF-induced

proliferative response and sustained activation of STAT5 in myeloid progenitors[90,91], which could promote the clonal expansion of hematopoietic stem cells and progenitor cells[92].

The presence of acquired *CSF3R* nonsense truncating mutations has been shown in the leukaemic haematopoietic clones of patients with severe congenital neutropenia who progress to MDS or AML[89,93,94,95]. The frequency of *CSF3R* mutations in patients with severe congenital neutropenia was estimated at around 30-35% in the non-leukaemic patients, compared with approximately 80% in those who also had AML or MDS, supporting the association between the acquisition of *CSF3R* mutations and leukaemic transformation[78]. Importantly, multiple haematopoietic clones with distinct *CSF3R* mutations frequenlty coexist in the same patient, indicating a strong selective pressure favouring the expansion of such clones[78]. The time between the first detection of clones with *CSF3R* mutations and signs of malignant transformation is highly variable: some patients progressed to MDS or AML within a few months[78,95], whereas others did not develop leukaemia for many years, even if they had high numbers of mutated haematopoietic clones, indicating that additional cooperating events are involved in malignant transformation[78,96-99].

A crucial question that emerged from the discovery of hematopoietic clones harboring somatic *CSF3R* mutations was whether, and if so how, G-CSF treatment has an effect on the proliferation of such clones. Data from long-term follow-up studies of patients with severe congenital neutropenia (in whom *CSF3R* mutant clones were detected receiving G-CSF indicated that the percentage of *CSF3R* mutant clones did not systematically increase, and sometimes even temporarily declined during G-CSF treatment[78,97]. However, this decline does not necessarily mean that these clones have disappeared entirely, as it might simply be a matter of the sensitivity of detection method. Recently, next-generation deep sequencing has been established as a highly sensitive analysis technology that enables detection of haematopoietic stem cells clones harbouring *CSF3R* mutations that occur with a frequency <1%[97,98]. We have hypothesized that if one of these clones is hit by a second leukemia-associated mutations, it will acquire a proliferative advantage that would ultimately lead to overt leukaemia[97,98].

Acquisition of a second leukaemia-associated mutation—In a collaborative study involving patients from different registries, the patterns of acquisition of leukaemia-associated mutations in haematopoietic stem cell clones were investigated using next-generation sequencing[97,98]. Of the 31 patients who had progressed to MDS or AML, 20 (64.5%) had clones with mutations in *RUNX1* and these mutations mostly occurred in clones which had already acquired *CSF3R* mutations. This pattern was seen in severe congenital neutropenia associated with mutations in *ELANE*, *HAX1*, *G6PT, GFI1* and *WAS*[97].

The time course between acquisition of one or more leukemia-associated mutations and development of leukaemia varies considerably[96,97]. Thus, *CSF3R* and *RUNX1* mutation analyses cannot be used for diagnostic purposes, but can be helpful to screen for the risk of malignant transformation. The presence of these mutations is a strong predictor of later leukemic development and, therefore, should result in a more intensive diagnostic

monitoring to evaluate the growth of pre-leukaemic cell clones. Screening for *CSF3R*, *RUNX1* and other (less frequent) mutations associated with secondary malignancies, such as mutations in *ASXL1*, *SUZ12, EP300*, *FIZ1* internal tandem duplication and *NPM1*, is advised[96,97]. The high frequency of cooperating *RUNX1* and *CSF3R* mutations in patients with severe congenital neutropenia suggests a novel and unique molecular pathway of leukaemogenesis, not seen thus far in any other type of adult or paediatric malignancy[97] (Figure 7). Independent from the genetic subtypes, leukaemic progression in patients with severe congenital neutropenia is often preceded by one or more chromosomal abnormalities, such as monosomy 7 and trisomy 21[97].

Diagnosis, screening and prevention

Diagnosis

Presenting signs and symptoms—Severe congenital neutropenia is usually diagnosed by early childhood. In neonates, a diagnostic clue could be an acute and severe umbilical infection, which can occur within the first days of life. During the first weeks of life, the child might begin to have fevers associated with respiratory symptoms, including signs of pneumonia[13]. A few weeks or months later cellulitis or deep tissue abscesses might present. Severe gingivitis and periodontitis (Figure 8) can develop within the first two years[100].

There are often long delays between the first symptoms and the confirmation of the diagnosis. Children presenting with fever and symptoms of a respiratory or skin infection do not routinely receive a white blood cell count with differential count to discover neutropenia, in most cases these common problems are not caused by severe congenital neutropenia. Furthermore, neutropenia in early childhood is commonly an autoimmune disorder [101,102], which usually remits spontaneously over the first 3 or 4 years of life, so low ANCs is often dismissed as unconcerning. Early diagnosis of severe congenital neutropenia depends upon a skillful clinician recognizing that recurrent fevers and infections suggest an underlying haematological or immunological problem. The most relevant clue often is that the patient is more severely ill than it would be expected. Recurrent and painful mouth sores or teeth or gums problems are other important clues to prompt blood cell counts (Figure 8).

When neutropenia is one of the manifestations of a syndrome (Tables 1 and 2), the other features, such as organ failures, diarrhoea, malabsorption, failure to thrive etc., are often recognized first and the diagnosis of severe congenital neutropenia follows. It cannot be over emphasized that a careful physical examination is the critical starting point for the diagnosis of severe congenital neutropenia. In addition, the family history is extremely important to determine whether there are multiple persons in the same family with the same or similar medical problems and if the patient's parents are consanguineous.

Blood neutrophil counts—Patients with severe congenital neutropenia have chronically reduced ANCs, but the blood counts are first measured when the patient has a fever and infections, and, therefore, in this occasion the ANC might be a little higher or even lower than it would be usual for this specific patient. The stress of inflammation might mobilize all of the available neutrophils from the bone marrow reserves, thereby raising the ANC, but the stress of infection could also exhaust the supply, thereby leading to a more-extreme degree

of neutropenia. For instance, ANCs > 0.5×10^9/L without G-CSF treatment are quite rarely seen in patients with severe congenital neutropenia not even during infection due to mutations in *ELANE, HAXI* or *G6PC3* [13,14,35], whereas patients with autoimmune neutropenia can have ANCs > 1.0×10^9/L during bacterial infections[101]. Patients with *SBDS* mutations might initially have normal ANCs that would drop to second over time[103]. For these reasons, it is very helpful to obtain a series of blood counts once or twice a week for 2-3 weeks in patients suspected of having severe congenital neutropenia.

Bone marrow examination—If severe congenital neutropenia is suspected, bone marrow examination is useful as an early step in diagnosis. At the initial examination, biopsies as well as aspirates are helpful to rule out (or confirm) leukaemia, aplastic anemia or myelodysplasia. Typical findings in the bone marrow of patients with severe congenital neutropenia are elevated numbers of promyelocytes and myelocytes and a paucity of metamyelocytes, band cells and mature neutrophils[104], indicating maturation arrest of granulopoiesis. Quantitatively, the bone marrow can be described with the myeloid to erythroid cell ratio and the immature to mature myeloid cell ratio. To obtain results that are representative of the baseline conditions, it is important to do the bone marrow examination when the patient is in a reasonably stable condition (that is, after treatment is given for an acute infection). In most patients, the morphology of the myeloid cells is not indicative of the cause of neutropenia[104]. Cytogenetics tests to exclude chromosomal aberrations are done on the initial bone marrow sample, although the result is almost always normal at the time of diagnosis. It is very useful to save some unstained slides and a frozen sample of the patient's bone marrow cells for future testing, whenever possible.

Genetic analyses—The pattern of inheritance can assist in confirming the diagnosis. For example, autosomal dominant mutations in *ELANE*[20-22] are the most common cause of cyclic neutropenia and severe congenital neutropenia. (Table 1, Box 1) Mutations in *ELANE* should be suspected and might be recognized in the first months of life if a child is ill and a family member already has the diagnosis of severe congenital neutropenia associated with *ELANE* mutations. On the other hand, the diseases inherited in a recessive manner, for example, severe congenital neutropenia due to mutations in *HAX1*[30], *G6PC3*[35], *JAGN1*[59] or *SBDS*[37] among others, are usually recognized as the first case in a family. Severe congenital neutropenias associated with mutations in *TAZ*[36] (which cause Barth syndrome) or *WAS*[105] have X-linked inheritance.

Genetic testing is playing an increasingly central role in diagnosing severe congenital neutropenia. Germline mutations can be identified by DNA sequencing of blood, saliva or another tissue samples. Clinical clues pointing to a syndrome matching the patient's presentation can help to determine which gene should be tested first (Table 1). For instance, in an infant with neutropenia who also presents gastrointestinal symptoms, short stature and failure to thrive (common manifestations of Shwachman-Diamond syndrome) *SBDS* should be sequenced. Similarly, a young boy with neutropenia, shortness of breath and symptoms of heart failure (indications of Barth syndrome) should first have *TAZ* sequenced first or a patient with severe neutropenia, urogenital or heart deformities should be sequenced for *G6PC3*.

When there are no congenital organ anomalies or other clinical clues, the standard approach for children who present with severe neutropenia and whose bone marrow examination shows maturation arrest is sequencing candidate genes that have been associated with severe congenital neutropenia. It is reasonable to sequence *ELANE* first, as it is the most common mutation. Because *ELANE* mutations can cause severe congenital neutropenia or cyclic neutropenia[22,23,24] sequential blood counts at regular intervals, best every day or ever other day, are needed to make the diagnosis of cyclic neutropenia with greatest confidence. If a heterozygous mutation in *ELANE* is found, most clinicians do no further genetic testing. If *ELANE* sequencing is negative, other individual genes could be analysed, based on family history and available resources. In consanguineous families, search for autosomal recessive mutations would be recommended (starting with *HAX1*). If these tests are also negative, sequencing of a panel of neutropenia-associated genes or whole exome sequencing would be recommended. The panel sequencing approach is being used increasingly as first choice and could be ultimately less expensive and more-informative, as it can also uncover multi-gene mutations.[104]

Genetic testing is becoming increasingly precise, but the mechanisms whereby the mutated protein causes the patient's specific disease features are still poorly understood. Different neutropenia-associated *ELANE* mutations have consequences of different severity[16,17]. Longitudinal records of the SCNIR show that no cases of MDS or AML have occurred in patients with some specific *ELANE* mutations in many patient-years of observation[23], and careful statistical analysis shows highly significant genotype-phenotype correlations for these mutations.[23] It should not be surprising that mutations associated with more-benign phenotypes are prevalent. Longitudinal follow-up of patients to determine the clinical significance and prognostic importance of specific neutropenia-associated mutations is a vital function of patient registries.

A common problem with genetic diagnosis is the finding of novel variants of unknown clinical significance. An initial step to determine the significance of the genetic variant is to map it through an extensive family study, to rule out family segregation. Whole exome sequencing would be a reasonable next step, if available, using global databases as control to identify common non-pathogenic variants; this approach can lead to the discovery of new causes for neutropenia.[106] It is usually best to review genetic findings in light of a registry and repository focused on the neutropenia-causing genes. In the European branch of the SCNIR, in 22 % (118/537) of the registered patients a genetic diagnosis could not be obtained.

Immunological diagnostics—Young children with neutropenia are commonly tested for anti-neutrophil auto-antibodies[101]. These tests are useful to support a suspected diagnosis of autoimmune neutropenia, but they are not definitive. In a substantial proportion, probably at least one-third, of children with presumed autoimmune neutropenia, test results are negative even with repeated testing. Anti-neutrophil antibodies have also been reported in patients with severe congenital neutropenia. [107, 108].

When evaluating patients presenting with fever, infections and neutropenia, it is good practice to take an initially broad approach to assess the immunological status: lymphocyte

counts, lymphocytes subsets numbers and immunoglobulin levels should be measured. Most patients with severe congenital neutropenia have normal or even elevated lymphocyte counts[61], increased blood monocyte counts[61] and normal or increased immunoglobulin levels, whereas patients with immunodeficiency syndromes usually also have lymphocytopenia and immunoglobulin deficiencies, and they might have severe neutropenia. Warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome is a unique condition that should be considered in patients with warts, severe lymphocytopenia and severe neutropenia, who often have a total white cell count < 1.0×10^9/L.

Screening

It is not routine practice to perform blood cell counts in infants at birth or in the first week of life. The incidence of severe congenital neutropenias is considered too low to justify this practice. Blood counts are not routinely done when children present with febrile illnesses either, again because these illnesses are common in the general population.

Prevention

Prevention of these genetic disorders is challenging. Genetic counseling that clearly presents all the options for diagnosis and management is the mainstays for excellent care, and the body of evidence on which couselling is based is rapidly evolving. Prenatal genetic diagnosis (through amniocentesis) is possible for the autosomal dominant disorders, whereas, for the recessive disorders, DNA sequencing of the potential parents in addition to genetic counselling might help parents understand the risk of having affected offspring. Genetic testing should also be offered to relatives of patients with recessive disorders, especially if they are eligible as a HSC donor for the affected family member.

Another important aspect is prevention of the consequences of neutropenia. In addition to early G-CSF treatment, maintaining excellent oral hygiene, good nutritional status and healthy living patterns can help reduce the risk of infections, whereas there is no substantial evidence supporting the benefit of prophylactic antibiotics. The use of myeloid growth factors is a preventive strategy (see below). As discussed, the risk of leukaemic transformation can be managed with regular screening for somatic mutations or karyotype anomalies.

Management

Initiation of G-CSF therapy at diagnosis

G-CSF is the first choice treatment in almost all patients with severe congenital neutropenia or cyclic neutropenia (Figure 9), who require G-CSF treatment to achieve and maintain blood ANCs > 1.0×10^9/L[11-14]. In general, at the beginning of treatment, G-CSF is given daily subcutaneously; by starting with a low (1–3 μg/kg per day) or moderate dose (5 μg/kg per day) in patients with cyclic neutropenia or severe congenital neutropenia, respectively, the adverse effects (bone pain, headache and myalgias) can be generally avoided. The dose is gradually increased in some patients at about 10-14 day intervals until the patient consistently maintains an ANC >1×10^9/L [100], a threshold that is usually sufficient to ameliorate the symptoms. The genetic subtype does not correlate with the G-CSF dose

needed to raise the ANC to this level[109]. G-CSF treated patients should receive blood counts at least several times per year to assure proper treatment. The patient is considered a "non-responder" at G-CSF dosages >50 μg/kg per day and ANCs < 0.5×10^9/L, but some experts use a lower threshold (25 μg/kg per day) for defining a non-responder or poor responder www.depts.washington.edu/registry[110] and www.scnir.org[111] These patients should be at least considered as candidates for haematopoietic stem cell transplantation, based on data of the SCNIR[111]. G-CSF is most effective as a preventative treatment, but patients with severe congenital neutropenia presenting with infections should start receiving a combination of G-CSF and antibiotics as soon as possible.

Long-term management

Because of the variety of genetic subtypes of severe congenital neutropenia, most with additional clinical features (Tables 1 and 2), the long-term management of patients usually requires the cooperation of a team of specialists who see the patient on a regular basis. G-CSF therapy can correct the neutropenia in Shwachman-Diamond syndrome and Barth syndrome but it does not alter most of the other manifestations of these conditions.

Most patients on G-CSF treatment can maintain a normal or near normal ANC, but they may still experience some infections and develop gingivitis and periodontitis. Failure to comply with the need for continuous treatment is the most common reason for treatment failures. Consistent dental care is particularly important because once the integrity of the dental-gingival barrier to prevent bacterial penetration below the border of the enamel is destroyed it usually does not recover. Periodontal disease then develops and can last for a lifetime. G-CSF treatment can reverse the quantitative deficiency of neutrophils, but the full functions of these cells in patients with severe congenital neutropenia may not be completely restored. Neutrophils from patients have shown defective generation of oxygen radicals and reduced *in vitro* chemotaxis.[112] *CYBB*, a gene coding for a key component of NADPH-oxidase, is downregulated in patients with severe congenital neutropenia[113]. Neutrophil dysfunction could also be due to specific defects in the neutrophils bactericidal proteins (for example, deficient antibacterial peptide LL-37)[114-117]. However, G-CSF treatment has many effects to enhance the functions of neutrophils. It primes the metabolic burst associated with phagocytosis and stimulates production of a number of neutrophil antibacterial proteins, and thereby mediate a net increase in the functional activity of neutrophils.[118]

The risk of long-term haematological complications, such as the risk of developing AML or MDS, varies considerably across the spectrum of genetic aetiologies of severe congenital neutropenia. The specific risk for each gene or mutation is difficult to determine, because the number of known cases worldwide is too small and the duration of follow-up is too short to draw strong conclusions. In patients (both children and adults) with subtypes with a known high risk, (for example, mutations in *ELANE*, *HAX1* or SBDS), monitoring with annual bone marrow evaluations including cytogenetic analyses and *CSF3R* mutational screenings is recommended[110,111]. As karyotype aberrations or leukemia associated mutations such as *Runx1* occur prior to leukaemia[97], their detection might suggest to initiate bone marrow transplantation before overt leukaemia develops.

The development of early-onset osteopenia and osteoporosis has been reported in approximately 40% of patients with severe congenital neutropenia, based on bone density measurements[119]. The underlying pathological mechanisms are still unclear, and fortunately fractures are very infrequent events. The development of osteopenia and osteoporosis appears independent from the underlying genetic defect, but further genotype-phenotype correlation analyses are required.

Stem cell transplantation

Owing to the efficacy of G-CSF therapy, haematopoietic stem cell transplantation (HSCT) is no longer essential for saving the lives of patients with severe congenital neutropenia. However, there are two conditions in which HSCT is the only currently available treatment option: patients who do not respond to G-CSF treatment[120,121-123] and patients who develop AML or MDS[120]. In the former case, following successful transplantation, patients normalize their peripheral blood counts and do not require further G-CSF treatment. Deciding when the optimal time point for HSCT is in patients who do not respond to G-CSF is very difficult, as HSCT could still be too toxic to be recommend in some patients. In the case of patients who develop MDS or AML, the chances of survival are dramatically reduced without HSCT. Before 2000, all patients who received standard chemotherapy for AML without HSCT died, and those treated with chemotherapy followed by HSCT had very poor outcomes: most succumbed from transplant-related infectious complications, for example fungal infections and graft versus host disease[120]. Since 2001, only mild chemotherapy instead of standard chemotherapy are used to lower the leukemic blast load followed by conditioning for HSCT. Therefore, prognosis for these patients has improved substantially, primarily owing to the practice of anticipating HSCT when possible (for example, as soon as the patient develops AML or MDS, without waiting for progression to AML)[124], avoiding anti-leukaemic chemotherapy prior to HSCT and modifications in the preparative regimens. Low-dose ara-C and thioguanine can be used to reduce peripheral blast counts and treat organomegaly. Chemotherapy regimens similar to those used to treat AML are only warranted in patients who do not respond to this milder treatment and have peripheral blast counts that cannot be maintained below $5 \times 109/L$ or who have a rapidly progressing disease or organ involvements – especially pulmonary involvement. The improved evaluation of the HSC donor might also play a part.

Regardless of the reason for HSCT, 5-year survival is approximately 80% for patient enrolled in the European branch of the SCNIR[124]. However, HSCT-related morbidity owing to acute or chronic graft versus host disease, bacterial or viral infections and the increased risk for AML and secondary malignancies after chemotherapy or irradiation are still major risks to be considered. With current transplant regimens, the AML relapse rate is low: in an analysis of 136 patients by the European Bone Marrow Transplant group, 3-year event free survival was 71% overall[121]. Of note, the authors of this study state that “in a precancerous disease like congenital neutropenia full donor chimerism (all hematopoietic cells are donor derived) is very important to achieve in order to prevent the re-emergence of predisposed clones[121].”

Pregnancy

The number of patients with severe congenital neutropenia who reach adulthood and wish to have children is increasing. Based on two observational studies, administration of G-CSF throughout pregnancy is safe and well tolerated[125,126]. No noticeable adverse effects have been reported. No major differences in pregnancy outcome, complications in the newborn baby and infections in the mother were seen between treated and untreated women[125]. Thus, continuation of G-CSF treatment throughout pregnancy in women with severe congenital neutropenia is recommended.

Quality of life

The availability of G-CSF therapy drastically changed the quality of life for patients with severe chronic neutropenia and their families[127]. Both life expectancy and the ability to participate in normal life activities improved substantially, owing to decreased risk and fear of dying from infections. The majority of children with severe congenital neutropenia and stable ANCs have no physical limitations that prevent them from participating in all aspects of daily life, such as attending kindergarten or school, practicing sports, meeting with friends, etc [110,111]. (Bolyard AA et al, http://www.depts.washington.edu/registry, Understanding severe chronic neutropenia: A handbook for patients and their families. February 2010.)[110] Regardless of their genetic subtype, the majority of patients with severe congenital neutropenia now reach adulthood[110.111]. Many patients desire family planning, and are prepared to accept the substantial risk of transmitting the neutropenia-causing mutation to their offspring. Most adults are in good conditions with long-term G-CSF treatment, but data is limited so far to about 25 years of G-CSF injections. The risk of developing a haematological malignancy at any time can negatively influence quality of life. However, with improving long-term survival and decreasing transplant related morbidity, outcomes for stem cell transplantation are steadily improving[120,121,124].

Outlook

Since the late 1980s[11], substantial progress has been made in the diagnosis, understanding of the molecular and genetic heterogeneity, pathophysiology and treatment. Early detection of severe congenital neutropenia already in the first months of life, G-CSF therapy and HSCT have contributed to survival rates of >80%. However, regular genetic screening is needed to detect and monitor the molecular events associated with leukaemogenesis[128].

To reduce the risk of leukemogenesis, treatment strategies using low dose or no G-CSF need to be investigated. In inducible pluripotent stem cells (iPSCs) obtained from patients harboring *ELANE* mutations, the neutrophil elastase inhibitor sivelestat promotes the differentiation of promyelocytes into mature neutrophils[129] (although the mechanism is still unclear). Thus, sivelestat could be an alternative or adjuvant treatment. Furthermore, the use of nicotinamide (Vitamin B3) in addition to G-CSF treatment to stimulate emergency granulopoiesis by activating the NAMPT/SIRT pathway[75] might enable to reduce G-CSF dosages and, therefore, reduce the risk of acquisition of C*SF3R* mutations and leukemogenesis.

iPSCs have been generated from somatic cells of patients with either *ELANE* or *HAX1* mutations[129-131]. With the establishment of efficient protocols for *in vitro* haematopoietic and myeloid differentiation of iPSCs cells[132,133], these cells could be used as an experimental model to study the pathophysiology of disease. Successful restoration of defective granulopoiesis *in vitro* has been achieved by lentivirus- or CRISPR/Cas9-based correction of mutated *ELANE* or *HAX1* in iPSCs[129-131]. Thus, correction of the underlying gene mutation *ex vivo* with the CRISPR/Cas9 technology followed by autologous stem cell transplantation is a promising future therapeutic option.

The discovery of > 20 mutated neutropenia-causing genes responsible for severe congenital neutropenia within the last 20 years was only possible owing to the improvement of next-generation sequencing technologies. However, in a substantial number of patients with severe congenital neutropenia no mutations could be detected so far. Careful anamnestic inquiries, genetic counselling and tailored next-generation sequencing and bioinformatic analysis might help to detect mutations in these patients, especially in those who acquired *CSF3R* mutation, as this event is exclusive of severe congenital neutropenia and, therefore, a further confirmation of the diagnosis.

Acknowledgments

This manuscript is dedicated to the late Dr. Laurence Boxer, a pioneer of neutropenia research.

The work was supported by grants from National Institute of Health (NIH), Deutsche Forschungsgemeinschaft (DFG), Bundesministerium für Bildung und Forschung (BMBF), Deutsche Jose-Carreras Leukämie-Stiftung e.V., Amgen Foundation.

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Box 1

Cyclic neutropenia

Cyclic neutropenia (a type of congenital neutropenia, is an autosomal dominant disorder that is characterized by oscillating levels of blood neutrophils, monocytes, platelets, reticulocytes and lymphocytes, usually with a 21-day periodicity[12,134,135,136,137]. Family studies later confirmed the inheritance pattern and the absence of associated hematological and non-hematological malignancies[138]. Mutations in *ELANE* can cause both cyclic neutropenia and other types of severe congenital neutropenia[21,23] and, although there is some overlap, genotype-phenotype correlations indicate that there is a cluster of mutations that are more likely to be associated with cyclic neutropenia[22,23,24]. Cyclic neutropenia has a lower risk of evolution to myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) and milder morbidity[23]. However, when neutrophil counts are at the lowest point, patients regularly have painful mouth ulcers, respiratory symptoms, cellulitis, abscesses and severe, even fatal, infections[12].

The central pathophysiological mechanism of cyclic neutropenia is failure of the bone marrow to maintain a consistent production of mature neutrophils[20,21,139]. When granulocytopoiesis stops, early myeloid progenitors accumulate, then leave the bone marrow and can be observed in the blood. Severe neutropenia recurs when the bone marrow supply is exhausted. This process then repeats regularly. Researchers have puzzled over the mechanisms of the cycling of neutrophils; several mathematicians have proposed explanations[139-145]. The first model, by Michael Mackey, postulates that the production of neutrophils is governed by long-range stimulatory factors in a long feedback loop that has a built-in time delay in the maturation of promyelocytes to fully differentiated neutrophils. Cell loss early in this process (at the promyelocyte stage) would be associated with oscillations in blood neutrophil counts[143,144,145]. Furthermore, if such cell loss were very severe, there might be no apparent oscillations, because neutrophil counts would remain continuously extremely low. Intriguingly, G-CSF treatment in patients with cyclic neutropenia does not abrogate cycling, but increases ANC, shortens the cycle periodicity from the usual 21 days to about 14 days and prevents serious infections[12].

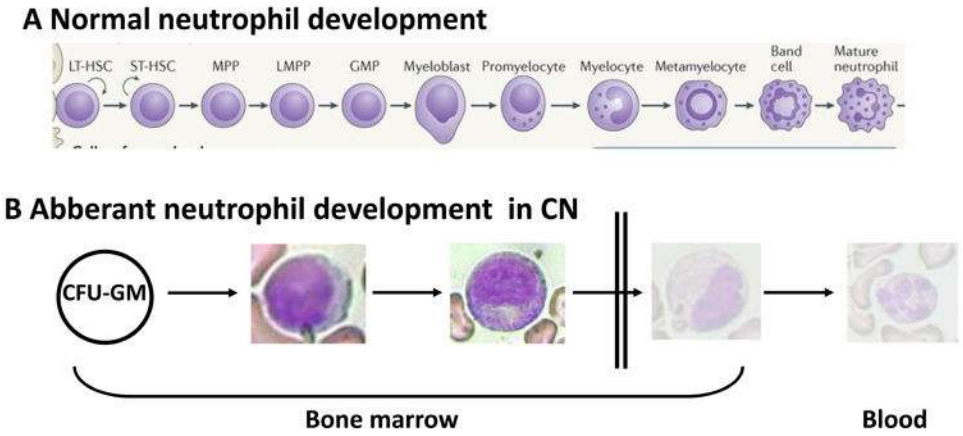

Figure 1. Maturation arrest of granulopoiesis in patients with severe congenital neutropenia

A | Normal differentiation of myeloid progenitor cells into neutrophils in the bone marrow and peripheral blood. **Right:** In a May Grunewald Giemsa stained bone marrow smear of a healthy individual promyelocyte (black arrow) and neutrophil granulocyte (white arrow) are indicated.

B | In patients with severe congenital neutropenia, the maturation of granulocytic precursor cells arrests at the stage of promyelocytes and myelocytes, which accumulate in the bone marrow. **Right:** In a hematoxylin-eosin stained bone marrow smear of a patient with severe congenital neutropenia there are no mature granulocytes and elevated numbers of promyelocytes (arrow). The morphology of promyelocytes is also different: in patients with severe congenital neutropenia, promyelocytes have multiple vacuoles (white arrow), a bulked nucleus and are much bigger than promyelocytes of healthy individuals.

ST-HSC, short-term haematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-primed multipotent progenitor; GMP, granulocyte-macrophage progenitor.

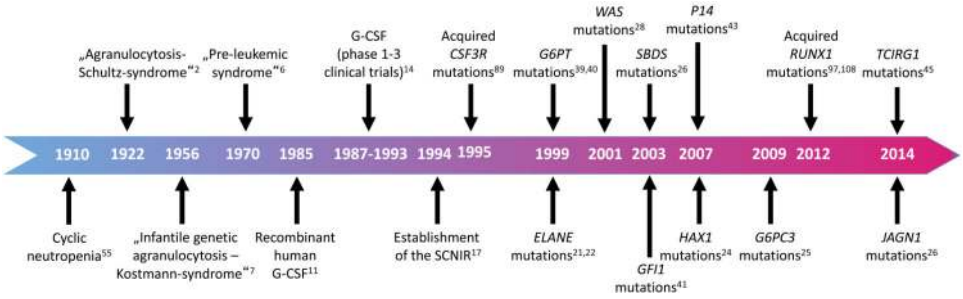

Figure 2. Milestones of the history of severe congenital neutropenia

Severe congenital neutropenia was first described in 1922 (Ref[146]), but referred to as ”agranulocytosis“ or Schultz syndrome [8,147,148]. In 1956, Rolf Kostmann described for the first time the autosomal recessive inheritance of familial agranulocytosis and named it as ”infantile genetic agranulocytosis“[29] Subsequently, the term ”Kostmann syndrome” was used for many years for patients with severe congenital neutropenia. In 1959, a family with dominant inheritance of neutropenia was reported for the first time[149] and in 1970 severe congenital neutropenia was first recognized as a preleukemic syndrome[9]. Recombinant human granulocyte-colony stimulating factor (G-CSF) (for example filgrastim and lenograstim became available for clinical research in 1987[10] and was approved by the FDA for the treatment of congenital neutropenia in 1993 (Ref[14]). First description of the: GFI1 gene mutation[150] the CXCR4[151] TCIRG1[152]. First description of the acquired CSF3R mutations in severe congenital neutropenia patients[153]

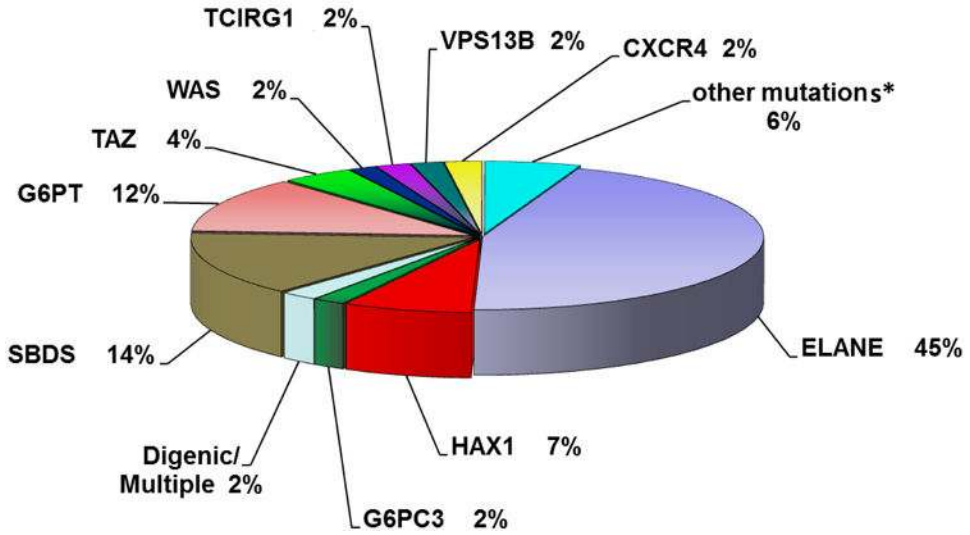

Figure 3. Genes with germline mutations associated with severe congenital neutropenia

Data based from 650 patients with severe congenital neutropenia registered in the European and North-American Branches of the Severe Chronic Neutropenia International Registry. *Mutations in *JAGN1*, *LAMTOR2*, *GFI1*, *LYST*, *USB1* or mitochondrial DNA.

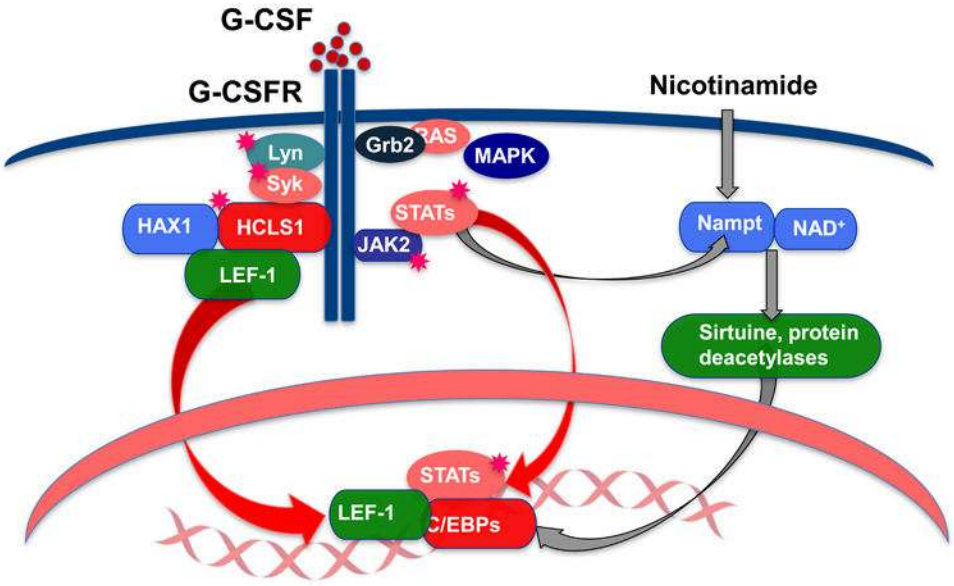

Figure 4. Main cellular localization of proteins mutated in patients with congenital neutropenia

Mutant proteins are distributed throughout different cellular compartments, including azurophil granules (neutrophil elastase (NE)), plasma membrane (granulocyte-colony stimulating factor receptor (G-CSFR) and C-X-C chemokine receptor type 4 (CXCR4)), endosomes and lysosomes (AP-3 complex subunit beta-1 (AP3B1), lysosomal-trafficking regulator (LYST), Ras-related protein Rab-27A (RAB27A) and ragulator complex protein LAMTOR2 (p14)), mitochondria (HCLS1-associated protein X-1 (HAX-1), tafazzin (TAZ), adenylate kinase 2 (AK2)), endoplasmic reticulum (glucose-6-phosphate exchanger SLC37A4 (G6PT1), glucose-6-phosphatase 3 (G6PC3), protein jagunal homolog 1 (JAGN1) and vacuolar protein sorting-associated protein 13B (VPS13B)), ribosomes (ribosome maturation protein SBDS (SBDS)), nucleus (zinc finger protein Gfi-1 (GFI1) and endothelial transcription factor GATA-2 (GATA2)) and cytoskeleton (Wiskott-Aldrich syndrome protein (WAS) and HCLS1-associated protein X-1 (HAX-1)). The functions of these proteins are described in Table 2.

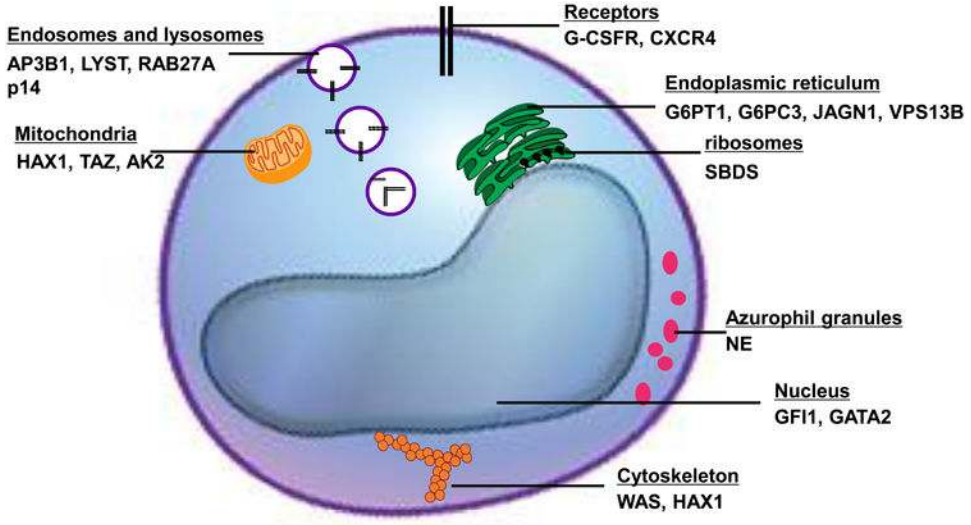

Figure 5. G-CSFR downstream signalling pathways

Main intracellular pathways involving key signalling molecules activated upon binding of granulocyte-colony stimulating factor (G-CSF) to G-CSF receptor (G-CSFR). Tyrosine-protein kinase JAK2 (JAK-2), which is hyperactivated in myeloid cells of patients with severe congenital neutropenia[69], leads to phosphorylation and activation of the signal transducer and activator of transcription (STAT) 3 and STAT5A, promoting proliferation of haematopoietic stem cells over granulocytic differentiation; sustained activation of STAT5A has been shown in patients with severe congenital neutropenia[70]. Tyrosine-protein phosphatase non-receptor type 11 (SHP-2) is another component of G-CSF signal transduction; SHP-2 induces dephosphorylation and, thereby, activation of tyrosine-protein kinase Lyn, which in turn in association with another tyrosine kinase Syk phosphorylates and activates haematopoietic lineage cell-specific protein (HCLS1), inducing myeloid differentiation of hematopoietic cells [33,34,71,72]. Substantially increased levels of SHP-2 have been observed in neutrophils from patients with severe congenital neutropenia[73]. In addition, lymphoid enhancer-binding factor 1 (LEF-1) a transcription factor member of the Wnt signalling pathway, is severely diminished in myeloid progenitors of patients with severe congenital neutropenia[64]. LEF-1 activates the granulocyte-specific transcription factor CCAAT/enhancer binding protein alpha (C/EBPα) and its target genes cyclin D1 and *c-MYC*, as well as anti-apoptotic factor baculoviral IAP repeat-containing protein 5 (survivin)[64]. Expression of all these proteins is severely diminished in myeloid cells of patients with severe congenital neutropenia, along with reduced activity of phosphatidylinositol 3-kinases (PI3K), serine/threonine-protein kinases (Akt), HCLS1-associated protein X-1 (HAX-1) and haematopoietic lineage cell-specific protein (HCLS1)[33,34]. As a consequence G-CSF therapy activates NAMPT and by this the compensatory emergency granulopoiesis pathway: NAMPT converts nicotinamide (NA) into nicotinamide adenine dinucleotide (NAD^+), activating NAD^+-dependent protein deacetylases, sirtuins (SIRTs), which in turn activate C/EBPβ[75]. Upregulated pathways are shown in red, downregulated in blue.

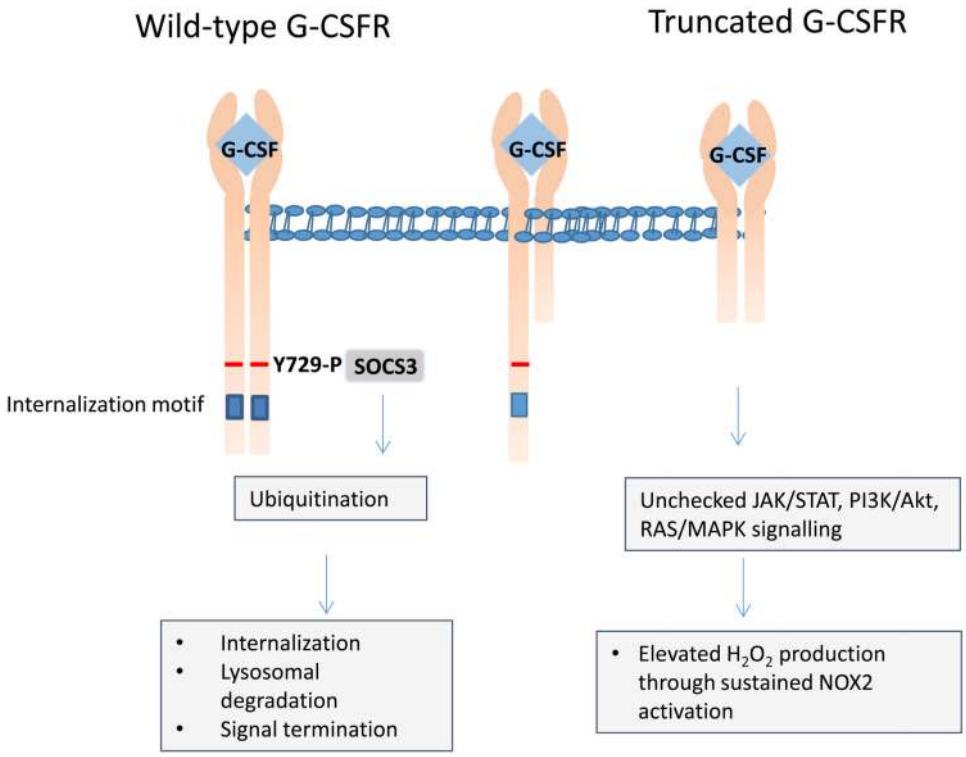

Figure 6. Dominant action of G-CSFR truncation mutants leads to sustained proliferation and survival signaling

The native mature G-CSFR protein consists of 836 amino acids, whereas the truncated forms of the CSF3R protein usually vary between 715 and 790 amino acids in length. Truncated G-CSFRs lack the ability to undergo endocytosis, owing to the deletion of a di-leucine motif essential for internalization [154]. Furthermore, they are hampered in endosomal trafficking and lysosomal degradation, owing to the loss of (phospho-tyrosine Y729, the binding site of suppressor of cytokine signalling 3 (SOCS3), which mediates ubiquitination of a critical membrane-proximal lysine residue[87]. G-CSFR forms dimers upon activation by G-CSF. Both wild type G-CSFR homodimers and wild type-truncation mutant G-CSFR heterodimers are subject to normal internalization and lysosomal degradation, whereas the homodimeric truncation mutants accumulate at the plasma membrane. Owing to their prolonged half-life and residence time at the plasma membrane, the G-CSFR truncation mutants act dominantly over the wild type G-CSFR[89], resulting in an elevated G-CSF-induced proliferative response and sustained activation of signal transducer and activator of transcription 5A (STAT5A) and phosphatidylinositol 3-kinases (PI3K)- serine/threonine-protein kinases (Akt) in myeloid progenitors[90,91]. Sustained activation of STAT5 appeared essential for the clonal expansion of haematopoietic stem cells and progenitor cells in mice harbouring the CSF3R-d715 truncation mutation[92], whereas prolonged PI-3K-Akt activation resulted in the increased production of intracellular reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), in murine myeloid progenitors via activation of the NADPH oxidase complex 2 (Ref[91]). Increased H_2O_2 levels inactivate oxidation sensitive phosphatases (such as tyrosine-protein phosphatase non-receptor type 1 (PTPN1) and phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein

phosphatase PTEN (PTEN)) that might negatively control G-CSF signalling and cause oxidative damage to proteins, lipids and nucleotides.

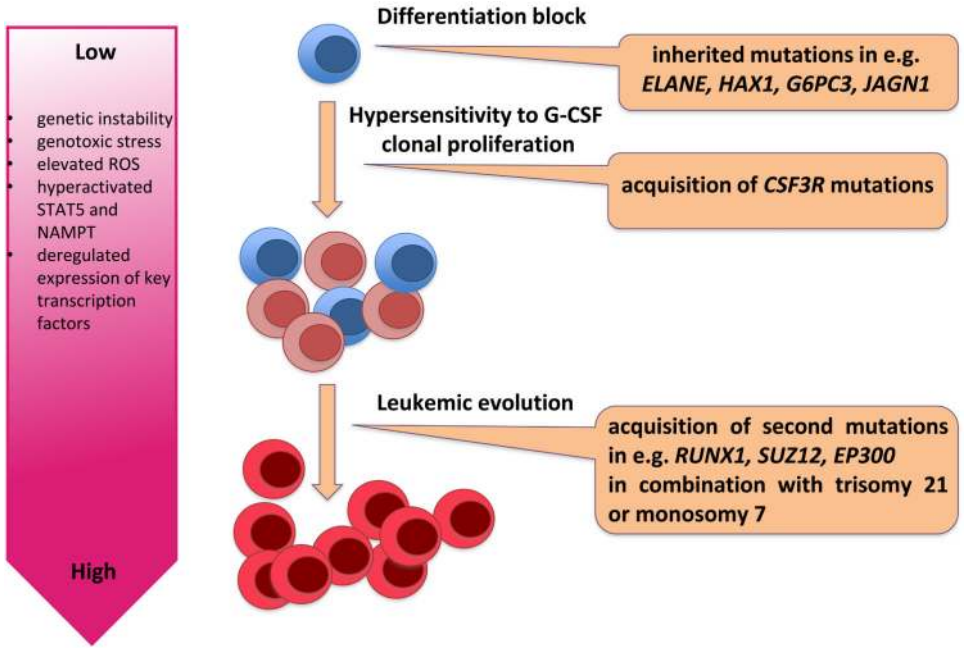

Figure 7. Model of leukaemogenesis in severe congenital neutropenia

The development of leukaemia is a multistep process characterized by a series of genetic changes that predispone haematopoietic cells with inherited severe congenital neutropenia-associated mutations to malignant transformation. Prolonged exposure of these cells to high dosages of G-CSF might result in the acquisition of *CSF3R* mutations that generate truncated G-CSFRs, thereby leading to hypersensitivity to G-CSF, defective G-CSFR signal transduction and clonal proliferation. Subsequently, the acquisition of additional leukemia-associated mutations (for example in *RUNX1*) or chromosomal aberrations, such as trisomy 21 and monosomy 7, ultimately lead to leukaemogenic transformation[97].

Figure 8. Severe gingivitis and periodontitis in patients with severe congenital neutropenia

Photographs of buccal cavities of two patients with severe congenital neutropenia caused by *ELANE* mutations show hypertrophic inflamed reddened gingiva and periodontitis. Peridontitis is common in severe congenital neutropenia patients[155].

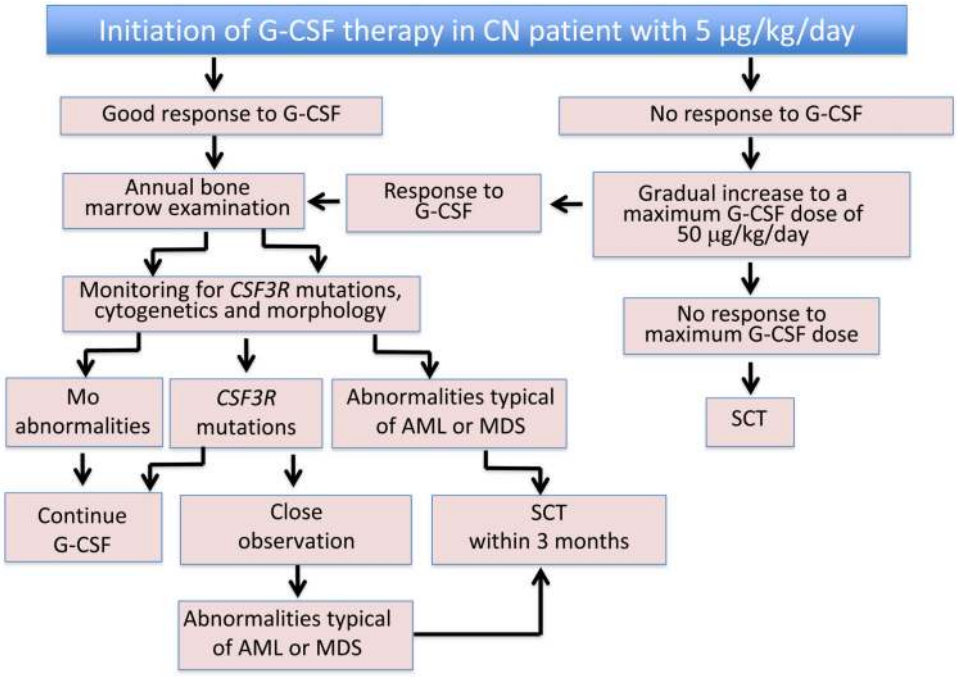

Figure 9. Algorithm for the management of patients with severe congenital neutropenia based on response to granulocyte-colony stimulating factor therapy

More than 90% of patients with severe congenital neutropenia respond well to the G-CSF treatment and reach a sustained absolute neutrophil count >1.0 × 10^9/L. G-CSF, granulocyte-colony stimulating factor; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome, abnormalities typical of AML or MDS: trisomy 21, monosomy 7, typical BM morphology.

Table 1

The congenital neutropenias*

Gene mutated	Disease	Other haematologic abnormalities	Non-haematologic abnormalities
Autosomal dominant inheritance			
ELANE	Severe congenital neutropenia	Monocytosis, eosinophilia and evolution to AML or MDS	Osteopenia
ELANE	Cyclic neutropenia	Cyclic hematopoiesis and evolution to AML orMDS	None
GFI1	Severe congenital neutropenia	Lymphopenia, increased numbers of immature myeloid cells in the peripheral blood	None
GATA2	Congenital neutropenia	Severe monocytopenia, dendritic cells and natural killer cells deficiencies, aplastic anaemia and evolution to AML or MDS	Mycobacteria, fungi or human papilloma virus infections; pulmonary dysfunction including pulmonary alveolar proteinosis ; warts and leg lymphedema
TCIRG1	Severe congenital neutropenia	None	In some patients, prominent hemangiomas that became more-prominent during G-CSF treatment
CXCR4	Congenital neutropenia, warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome	B cell defects and hypogammaglobulinemia	Warts
Autosomal recessive inheritance			
HAX1	Severe congenital neutropenia	Evolution to AML or MDS	Neurological phenotype in patients with two specific mutations
JAGN1	Severe congenital neutropenia	None	Short stature and bone and teeth defects
G6PC3	Severe congenital neutropenia	Thrombocytopenia and evolution to AML or MDS	Cardial defects, increased superficial veins visibility, urogenital malformations, endocrine abnormalities and skin hyper-elasticity
SLC37A4	Congenital neutropenia, glycogen storage disease, type Ib (GSDIb)	None	Hypoglycemia, fasting hyper-lactacidemia, glycogen overload of the liver, colitis, pancreatitis and osteoporosis
SBDS	Shwachman-Diamond syndrome	Thrombocytopenia, anemia, aplastic anemia, evolution to AML/MDS	Exocrine pancreatic insufficiency, cardiomyopathy, metaphyseal dysplasia, mental retardation and hepatic disease
STK4	Congenital neutropenia	Monocytopenia and T- and B-lymphopenia	Warts and atrial septal defects
CLPB	3-Methyglutaco-nic aciduria type VII	Evolution to AML or MDS	Psychomotor retardation, progressive brain atrophy, cataracts, 3-methylglutaconic aciduria, facial dysmorphism, cardiomyopathy or hypertrophy and hypothyroidism
AP3B1	Hermansky-Pud-lak syndrome 2	Impaired function of T cells and natural killer cells	Oculocutaneous albinism and haemorrhagic diathesis
LAMTOR2	p14 deficiency	Accumulation of neutrophils in the bone marrow, defective cytotoxicity and lymphoid immunodeficiency	Oculocutaneous albinism and stunted growth

Gene mutated	Disease	Other haematologic abnormalities	Non-haematologic abnormalities
USB1	Clericuzio type poikiloderma	None	Poikiloderma, generalized hyperkeratosis on palms and soles, short stature and recurrent pulmonary infections
VPS13B	Cohen syndrome	None	Psychomotor retardation, truncal obesity, microencephaly, skeletal dysplasia, hypotonia, myopia
VPS45	Congenital neutropenia[//]	Anisocytosis and poikilocytosis, hypergammaglobulinemia, renal extramedullary haematopoiesis, bone marrow fibrosis, progressive anaemia and thrombocytopenia	Nephromegaly, splenomegaly, osteosclerosis, neurologic abnormalities such as delayed development, cortical blindness, hearing loss and thin corpus callosum
CXCR2	Congenital neutropenia	Myelokathexis owing to impaired neutrophil release from the bone marrow to the peripheral blood	NA
EIF2AK3	Wolcott-Rallison syndrome	None	Early infancy-onset insulin-dependent diabetes mellitus, epiphyseal dysplasia, growth retardation, hepatic and renal dysfunction, developmental delay and exocrine pancreatic deficiency
LYST	Chédiak-Higashi syndrome	Defective natural killer cell function; lysosomal inclusion bodies in the myeloblasts, promyelocytes and granulocytes; macrophage activation and lymphoproliferative syndrome	Oculocutaneous albinism and neurodegeneration
RAB27A	Griscelli synd-rome, type 2	Defective cytotoxicity, hypogammaglobulinemia, thrombocytopenia, anaemia and haemophagocytosis	Oculocutaneous albinism
AK2	Adenylate kinase 2 deficiency	Severe lymphopenia	Inner ear hearing loss
RMRP	Cartilage-hair hypoplasia	Immunodeficiency and anaemia	Hypoplastic hair, skeletal dysplasia and cartilage hypoplasia
TCN2	Transcobalamin II deficiency	Megaloblastic anaemia and pancytopenia	Methylmalonic aciduria, failure to thrive, recurrent infections, mental retardation and neurologic abnormalities
Various autosomal inheritance[‡]			
CSF3R	Severe congenital neutropenia[§]	None	None
X-linked inheritance			
WAS	Congenital neutropenia	Monocytopenia, lymphopenia, reduced numbers of natural killer cells and abrogated phagocyte activity	None
TAZ	Barth syndrome	None	Cardiomyopathy, skeletal myopathy, stunted growth, cardiolipin abnormalities and 3-methylglutaconic aciduria
CD40LG	CD40 ligand deficiency, hyper-IgM syndrome type I (HIGM1)	Combined immunodeficiency; T cells, B cells and dendritic cells deficiencies; defective B cell class switch; markedly reduced levels of IgG, IgA and IgE but normal or elevated levels of IgM and reduced macrophage effector functions	Increased susceptibility to bacterial, viral and fungal infections and increased risk for developing autoimmune disorders and malignancies
Mitochondrial DNA inheritance			

Gene mutated	Disease	Other haematologic abnormalities	Non-haematologic abnormalities
Mitochondrial DNA deletion	Pearson syndrome	Refractory sideroblastic anaemia and vacuolization of bone marrow precursors and macrophages	Exocrine pancreas and renal insufficiency or fibrosis, endocrine abnormalities, neuromuscular degeneration and mitochondrial myopathy

[*] For completeness, all known genes that have been associated with congenital neutropenia are reported in this table, regardless of the severity of the phenotype.

[‡] dominant, homozygous recessive or compound heterozygous recessive

[§] no response to G-CSF, good response to GM-CSF with increased granulocyte counts

[//] poor or no response to G-CSF in some patients

AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome, G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage-colony stimulating factor; NA, not available

Table 2
Molecular pathological mechanisms in severe congenital neutropenias

Gene	Protein; functions	Pathological mechanisms	Knockout mouse model
ELANE	Neutrophil elastase; secondary granule protease	Activation of the UPR and apoptosis of myeloid progenitor cells	Normal granulopoiesis and high susceptibility to infections
HAX1	HCLS1-associated protein X-1; activation of HCLS1 adaptor protein in G-CSF signalling and anti-apoptotic functions	Reduced mitochondrial membrane potential, elevated apoptosis and abrogated G-CSFR signalling	Normal granulopoiesis and neurological defects
JAGN1	Protein jagunal homolog 1; early secretory pathway, cell adhesion and cytotoxicity	Aberrant N-glycosylation of multiple proteins, elevated apoptosis and poor to no G-CSF response	Normal granulopoiesis and severely impaired response to *Candida albicans*.
G6PC3	Glucose-6-phosphatase 3; Hydrolyzation of glucose-6-phosphate to glucose and phosphate in the ER	Impaired intracellular glucose homeostasis, UPR activation and elevated apoptosis of myeloid cells	Neutropenia but no cardiac or urogenital defects
SLC37A4	Glucose-6-Phosphate, exchanger; ransport of glucose-6-phosphate from the cytoplasm to the ER lumen, maintainment of glucose homeostasis and ATP-mediated calcium sequestration in the ER	Defective trans -ER transport, abnormal glycolysis and gluconeogenesis, elevated apoptosis of neutrophils and neutrophil dis-function	Lethal shortly after birth
WAS	Wiskott-Aldrich syndrome protein; regulation of actin rearrangement	Enhanced actin polymerization, altered cytoskeletal responses and genomic instability	NA
GFI1	Zinc finger protein Gfi-1; transcriptional repressor interacting with myeloid-specific transcription factors C/EBPα, C/EBPβ and PU.1	Diminished myeloid differentiation	Neutropenia and expansion of monocytic progenitors upon G-CSF treatment
STK4	Serine/threonine protein kinase 4; upstream component of the mitogen-activated protein kinase pathway	Enhanced loss of mitochondrial membrane potential and increased susceptibility to apoptosis	Normal granulopoiesis and progressive loss of T cells and B cells
GATA2	Endothelial transcription factor GATA-2; embryonic and definitive hematopoiesis and lymphatic angiogenesis	Deregulated proliferation and differentiation of HSCs and reduced numbers of HSCs pool	Embryonic lethality around day 13 owing to loss of vascular integrity
SBDS	Ribosome maturation protein SBDS; regulation of later steps of ribosome biogenesis and mitotic spindle stabilization	Mitotic spindle destabilization, genomic instability and enhanced apoptosis of HSCs	Embryonic lethality
TAZ	Tafazzin; acyltransferase in lipid metabolism and regulation of phospholipid membrane homeostasis	Destabilization of mitochondrial respiratory chain complexes and elevated apoptosis	NA
CXCR4	C-X-C chemokine receptor type 4; HSCs homing and myeloid cells retention to the bone marrow	Reduced egress of HSCs and mature neutrophils from the bone marrow	Severely reduced B-lymphopoiesis and lack of bone marrow myelopoiesis
AP3B1	AP-3 complex subunit beta-1; cargo protein in endosomal trafficking	Defective endosome formation and processing and endosomal or lysosomal defects in immune cells	Normal granulopoiesis
LAMTOR2	Ragulator complex protein LAMTOR2; regulation of endosomal trafficking and sorting, growth factor signalling and cell proliferation	Defective MAPK and ERK signaling, diminished phagocytosis and disturbed endosomal trafficking	Normal granulopoiesis
USB1	U6 snRNA Phosphodiesterase; exoribonuclease in RNA processing from pre-RNA	Diminished biogenesis of U6 small nuclear RNA and elevated apoptosis	NA
VPS13B	Vacuolar protein sorting-associated protein 13B; intracellular vesicle-	Glycosylation defects of newly synthesized proteins	NA

Gene	Protein; functions	Pathological mechanisms	Knockout mouse model
	mediated sorting and protein transport and Golgi complex integrity		
LYST	Lysosomal trafficking regulator; endosomal protein trafficking.	Defective sorting of endosomal resident proteins	Normal granulopoiesis
RAB27A	Ras-related protein Rab-27A; protein transport, small GTPase mediated signaling and lytic granule release	Defective vesicular trafficing, protein transport, endocytic and secretory pathway.	Normal granulopoiesis and reduction in coat color intensity
AK2	Adenylate kinase 2; transfer of the terminal phosphate group between ATP and AMP	Aberrant mitochondrial metabolism and regulation of apoptosis	Normal granulopoiesis
CD40LG	CD40 ligand; expressed on activated T cells, necessary for T cells to induce B cells to undergo Ig class-switching	Defective Ig class switch and defective *in vivo* clonal expansion of antigen-specific CD4+ T cells	Normal granulopoiesis, and decreased IgM response to thymus-dependent antigens
TCIRG1	V-type proton ATPase 116 kDa subunit a isoform 3; pH regulation of intracellular compartments and organelles of eukaryotic cells, including neutrophil phagocytic vacuoles and T-lymphocyte activation and immune response	Possible defective bone marrow nich regulation of granulopoiesis owing to osteopetrosis or osteoclasts activation or direct effects on promyelocyte differentiation	Osteopetrosis, granulopoiesis not studied yet
CSF3R	G-CSF receptor	no G-CSF response owing to the absent expression of G-CSFR on the cell surface	Mice with a neo cassette replacing exons 3-8 of *Csf3r* have a decreased number of circulating neutrophils and a 50% decrease of hematopoietic stem cells, myeloid progenitor cells and granulocytes in bone marrow
VPS45	Vacuolar protein sorting-associated protein 45; regulation of assembly of the SNARE (soluble N ethylma-leimide sensitive factor attachment protein receptor) complex, which plays an essential part in the trafficking and recycling of proteins through lysosomes, other endosomes and trans-Golgi complex	Degradation of key components of the SNARE complex and defective transport of proteins from the trans-Golgi network to endosomes, impaired cell motility, increased apoptosis, NADPH-oxidase dysfunction and diminished superoxide production by neutrophils and lack of surface expression of β1 integrin	NA
CXCR2	C-X-C hhemokine receptor type 2; chemotaxis of neutrophils to the site of inflammation and migration from the bone marrow to peripheral blood	Abolished IL8-induced Erk1/2 phosphorylation and chemotaxis	Neutrophils are preferentially retained in the bone marrowand have a severe defect in migration to sites of inflammation
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3;phosphorylation and inactivation of eIF2A; type I ER membrane protein induced by ER stress owing to misfolded proteins	Failure in translational initiation and repression of global protein synthesis and mitochondrial functions	Progressive loss of insulin-secreting beta cells and development of diabetes mellitus followed by loss of glucagon-secreting alpha cells, skeletal dysplasia at birth (including deficient mineralization, osteoporosis, and abnormal compact bone development) and postnatal growth retardation. Haematopoiesis was not studied
TCN2	Transcobalamin-2; transport of cobalamin (vitamin B12) from the blood stream to the cells	Defective plasma transport of Vitamin B12 resulting in B12 deficiency	NA

UPR, unfolded protein response; HCLS1, haematopoietic lineage cell-specific protein; G-CSFR, granulocyte-colony stimulating factor receptor; NA, not available; ER, endoplasmic reticulum, HSC, haematopoietic stem cell.