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Elastase-dependent congenital neutropenia

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Abstract

Congenital neutropenia, which refers to an inherited deficiency in neutrophils, is a rare pathologic condition that affects approximately 0.0001-0.0009% of the general population. While congenital neutropenia can result from mutations in approximately 30 genes, its leading cause is gain-of-function mutations in the *ELANE* gene, which encodes the neutrophil granule serine protease, neutrophil elastase. This review focuses on established and novel concepts in the genetic, molecular and cellular mechanisms underlying neutrophil elastase-dependent neutropenia, and discusses possible new avenues for neutropenia research as well as potential novel treatment options that target pathogenic elastase variants.

Keywords: Neutropenia, neutrophil elastase, *ELANE* gene editing, neutrophil elastase inhibitor, secretory leukocyte protease inhibitor

INTRODUCTION

Congenital neutropenia is a genetic disorder in which there is a lower-than-normal number of neutrophils. In cases of severe neutropenia, circulating neutrophil counts, which are normally around 1500-8500/μl blood in healthy human individuals, drop below 500/μl, and in very severe cases, they can be as low as < 200/μl to close to zero[1,2]. Since neutrophils play a key role in immune surveillance, mainly against bacteria and fungi, an inherited deficiency of them predisposes the sufferer to recurrent bacterial, or fungal

infections, which occur early in life and can be life-threatening[1-3]. Mutations of *ELANE*, the gene that encodes neutrophil elastase (NE), are the most frequent cause of the inherited forms of neutropenia. Here we review the current knowledge and emerging questions regarding how *ELANE* mutations contribute to a shortage of, and/or dysfunction in neutrophils, and discuss possible strategies that target NE in the context of neutropenia, focusing on *ELANE* gene editing and NE inhibitors. An overview of the full spectrum of congenital neutropenic disorders is available elsewhere[3-5].

TYPES AND MAIN CAUSES OF NEUTROPENIA

Chronic neutropenia is a result of hereditary bone marrow-failure syndrome or an acquired deficit of neutrophils[6]. Whereas acquired neutropenia can occur secondary to autoimmune diseases, chemotherapy or drug reactions, congenital (hereditary) neutropenia is a genetically heterogeneous group of primary hematologic conditions that are mainly characterized by an anomalous neutrophil developmental program (granulopoiesis) leading to insufficient production of mature neutrophils in the bone marrow and, subsequently, low neutrophil counts in the bloodstream[3]. In rare cases, such as WHIM syndrome, mature neutrophils fail to exit the bone marrow, which also leads to peripheral neutropenia[7]. Mutations in more than 30 genes, such as *ELANE, HAX1, G6PC3, SBDS, G6PT, CXCR4, TAZ, VPS45* or *CXCR4* have been identified so far as causative factors in inherited neutropenia[3,8]. Neutrophil differentiation block observed in congenital neutropenia can be associated with changes in the bone marrow, such as bone marrow fibrosis; thus, it is not necessarily always an autonomous neutrophil cell phenomenon[9]. However, cell-intrinsic neutrophil maturation arrest, as a primary cause of neutropenia, has been reported for at least some neutropenia genes[3]. Despite the diverse underlying genetic defects that likely affect the function of various subcellular compartments in neutrophil precursors, such as ER or mitochondria, the proteins encoded by these genes may share (a) signaling pathway(s). Disruption of these pathways at different stages, by defective products of neutropenia-causative genes, would then have similar hematopoietic manifestations[3].

Mutations of the *ELANE* gene are the most common genetic aberration, accounting for about half of the cases of severe congenital neutropenia (SCN). Whereas a hallmark of untreated SCN is either chronic or severe quantitative and qualitative deficit in neutrophils[6], *ELANE* mutations are also the main cause of milder cyclic neutropenia (CyN), characterized by episodes of neutrophil scarcity recurring in a cyclic fashion, usually every 3 weeks. CyN-related *ELANE* mutations, which are either different from or overlap with those occurring in SCN, are found in > 80% of CyN patients[10]. The majority of SCN and CyN cases arise due to germline mutations in the *ELANE* gene and are of a dominant nature, typically affecting only one *ELANE* allele, and leaving the other *ELANE* allele intact[1]. In addition to heterozygous, autosomal dominant *ELANE* mutations, other models of inheritance, including single gene autosomal recessive, X-linked and sporadic patterns, have also been described in congenital neutropenia in association with different genes. For example, the *HAX1* gene that causes SCN, also known as Kostmann disease, is an autosomal recessive genetic mutation[3].

SCN and CyN remain incurable diseases, although in the majority of patients, these conditions can be kept stable by regular, subcutaneous infusions of granulocyte colony-stimulating factor (G-CSF). Neutropenia can evolve into myeloid malignances, such as myelodysplastic syndrome or acute myeloid leukemia (MDS/AML). For effective therapy, patients with SCN typically require high doses of G-CSF. Those patients who receive the largest doses of G-CSF appear to be at the greatest risk of progressing to myeloid malignances[11,12]. In patients unresponsive to G-CSF or with an increased risk of MDS/AML, hematopoietic stem cell transplantation might be a potential therapeutic option[3].

ELANE MUTATIONS LEADING TO NEUTROPENIA

During the promyelocytic stage of granulopoiesis in the bone marrow, NE is synthesized as zymogen and post-translationally activated in the neutrophil precursors [Figure 1A]. The NE activation steps include N-terminal trimming by cathepsin C, which is required for NE protease activity. In addition, NE may be modified at C-terminus by an as yet unidentified protease[1]. The proteolytically-processed C-terminal is thought to serve as a binding site for adaptor protein complex 3 (AP3), implicated in the transport of NE to lysosome-like granules[1,13].

NE mainly localizes to the primary granules of the neutrophil precursors, where it remains stored in a catalytically active, ready-to-use form. Neutrophils that are released from the bone marrow into the circulation, followed by recruitment to peripheral tissues, rely on catalytically active NE for their full spectrum of antimicrobial, pro-inflammatory and tissue-remodeling functions[14]. In addition to the localization of NE to the primary granules in the neutrophil precursors and mature neutrophils, the presence of this protease out of the granules, including in cytosol, at the cell surface and in the nucleus, has also been documented[15-17]. In mature, circulating neutrophils, transient release of NE from the granules is likely to be required for the physiological functions of these cells, such as generating and releasing neutrophil extracellular traps, or regulating neutrophil migration[15,17]. However, NE misplaced from the granules in the neutrophil precursors in the bone marrow is associated with neutrophil pathology, such as SCN (see below).

ELANE-based neutropenia results from the gain-of-function of mutant NE rather than diminished levels of native protein which might also be a consequence of monoallelic mutations[1]. This is supported by several lines of research. For example, a blockade of NE expression leads to the restoration of normal granulopoiesis and neutrophil differentiation, regardless of the type of underlying genetic mutation[18].

The *ELANE* gene consists of 5 exons and 6 introns, and > 200 mutations, distributed over all exons as well as some noncoding regions, have been described in association with congenital neutropenia[3,19]. However, to link genetic *ELANE* variants with pathologic outcomes, mechanistic studies are required. Mouse models of *ELANE*-targeted neutropenia have failed to accurately phenocopy human neutropenia because basal granulopoiesis was not disrupted in mice upon expression of abnormal murine NE, targeted at a position orthologous to human pathogenic NE mutation. A lack of suitable experimental neutropenia models has hampered the testing of *ELANE* mutants *in vivo* in the context of this pathology[20]. Human, murine or rat promyelocytic cell lines transduced with mutant *ELANE,* or patient neutrophil or patient-derived induced pluripotent stem cell (iPSC) lines, are typically used to unravel the biochemical and biological consequences of different *ELANE* mutations in myeloid progenitors. On the basis of these studies, several *ELANE* mutations have been identified to interfere with neutrophil maturation potential [Table 1].

According to recent studies, a comprehensive gene editing screen in the human hematopoietic stem and progenitor cells (HSPCs) dissects the genetic bases of *ELANE* neutropenia pathogenicity. This model, in which HSPCs were edited with sgRNAs that targeted a broad range of *ELANE* mutants *in vitro*, faithfully recapitulated the known genetic features of *ELANE*-based neutropenia[21]. In addition, human-edited HSPCs have also been shown to produce an *ELANE* pathogenic variant-dependent abnormal hematopoietic engraftment function, following infusion into specific immunodeficient mice. Thus, these new xenograft models of gene-edited human HSPCs also enable the outcome of *ELANE* mutations to be tested in *in vivo* settings, which was not possible before with *ELANE* transgenic mice[21].

Table 1. Selected *ELANE* variants and their biologic consequences in experimental cell models

Mutation variant	Protein change	Experimental model	Molecular and biological consequences [Ref]
NM_001972.4(ELANE):c.170C > T	A57V	SCN iPSCs	Diminished granulocytic differentiation[18]
NC_000019.10:g.852986A.T	I60F	SCN iPSCs	Elevated ELANE &NE expression, ROS levels & PML-NBs numbers; impaired neutrophil differentiation[26]
NM_001972.4(ELANE):c.211T > C	C71R	U937 cells	Protein processing changed, mistargeting, loss or reduction of proteolytic activity of NE[28]
		HL60, NB4 cells; BM CD34 + cells	Increased ATF-6, ATF-4 mRNA & expression of ATF-6 targeted genes[25]
NC_000019.10:g.853345G.T	R103L	SCN iPSCs	Elevated ROS levels[26]
NM_001972.4(ELANE):c.353T > A (p.Ile118Asn)*	I118N	SCN iPSCs	Increased BIP and ATF-6 m RNA; decreased CEBPA and CEBPB mRNA, mislocalization of a proteolytically active NE, upregulated apoptosis, impaired granulocytic differentiation[30]
NM_001972.4(ELANE):c.416C > T	P139L	U937 cells	Loss or reduction in proteolytic activity of NE, increased ER stress and apoptosis[28]
		SCN iPSCs	Elevated number of PML-NBs[26]
NM_001972.4(ELANE):c.452G > A	C151Y	SCN iPSCs	Diminished granulocytic differentiation[18]
		SCN iPSCs	Increased ATF-6 mRNA, reduced granulocytic differentiation[38]
NM_001972.4(ELANE):c.640G > A	G214R (G185R)	murine 32D cells	increased GRP78 mRNA expression, unperturbed NE traffic, elevated apoptosis, diminished granulocytic differentiation[39]
		SCN iPSCs	Increased ATF-4 mRNA, reduced granulocytic differentiation[38]
		murine 32D cells, NB4 cells	unchanged ATF-4, ATF-6 mRNA level; reduced TF expression, mislocalization & loss or reduction in enzymatic activity of NE, viability not changed, impaired granulocytic differentiation[22]

Mutation variants (cDNA or protein) based on ClinVar (nih.gov). Alternative name is given in brackets. Clinical significance of the NE variants: likely pathogenic or pathogenic. *uncertain significance. TF: Transcription factors.

Most of these *ELANE* mutations are missense mutations, or in-frame insertion or deletions (indels), that lead to the expression of a mutant NE. Frameshift mutations that result in out-of-frame stop codons and nonsense mutations have also been described in association with *ELANE* neutropenia, but only involving distal exon 4 and exon 5 [Figure 1B]. These findings suggest that, when the premature stop codons are confined to the early *ELANE* exons, the NE protein is not translated[19]. A pooled CRISPR gene editing of the mutated early *ELANE* exons 1-4 and the mutated terminal *ELANE* exons (distal part of exon 4) and exon 5 in HSPCs have confirmed these predictions and revealed two different strategies that bypass neutrophil maturation arrest. The first mechanism was attributed to the elimination of erroneous mRNA that was transcribed from the mutated early exons (1-4), by a nonsense-mediated decay [Figure 1B]. This strategy prevented the translation of a mutant *ELANE* allele that contained premature stop codons in early exons, and was found to be a key determinant of *ELANE* variant pathogenicity. The other mechanism, which involved *ELANE* variants that escape a nonsense-mediated decay, resulted from a blockade of translation by a mutation (late exon -2 frame indels) that shortened the 3'-nontranslated region (3'-UTR) of *ELANE*[19]. Thus, a nonsense-mediated decay and repression of the mutated *ELANE* translation can rescue neutrophil precursors from maturation arrest [Figure 1B]. *ELANE* variants that bypass these rescue routes are translated, leading to deficiency in mature neutrophils. These findings are in line with clinical data that show only a -1, but not -2 frameshift mutation in terminal *ELANE* exons in patients with congenital neutropenia[19].

A knowledge of these two general escaping neutropenia rules suggests potential universal strategies for correcting pathogenic mutations, independent of *ELANE* neutropenia variants[8,19]. For example, to take advantage of nonsense-mediated decay, the corrective *ELANE* edits need to involve early exons. Another strategy may be based on placing corrective indels in late exons, and/or directly targeting the length and/or

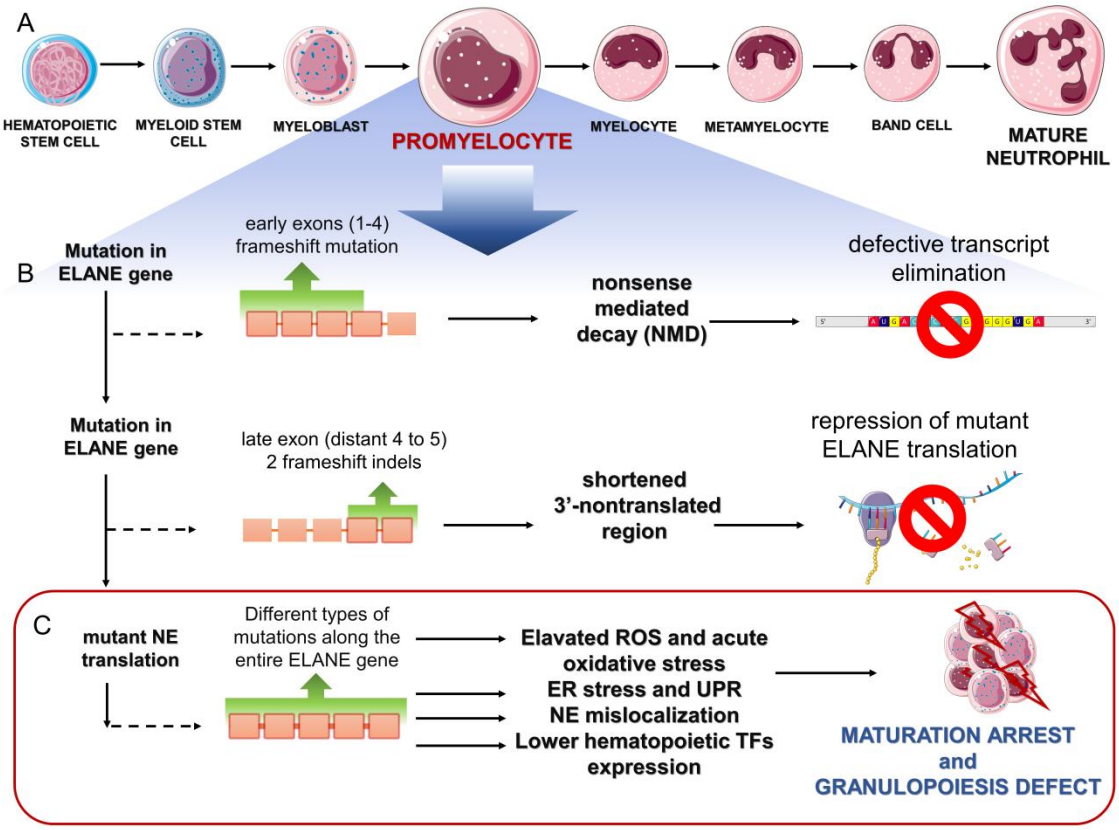

Figure 1. Outline of main steps leading to disruption of NE-mediated granulopoiesis. (A): Neutrophil development stages in the bone marrow, highlighting promyelocyte stage when differentiation blockade occurs. (B): *ELANE* frameshift mutations inducing cellular surveillance pathways that safeguard the quality of *ELANE* transcripts and prevent the production of mutant NE. (C): Mechanisms of mutant NE-induced neutropenia. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

structure of the 3'-UTR to inhibit the translation of pathogenic *ELANE* variants.

MECHANISMS OF NE-MEDIATED NEUTROPENIA

The above findings help to explain the genetic bases of *ELANE* neutropenia. What remains less clear is how the presence of NE mutants following their translation from *ELANE* variants that avoid quality control mechanisms leads to a neutrophil shortage. Because NE is a protease, it is likely that neutropenia-causative *ELANE* mutations affect the enzymatic activity of NE. However, while most diminish or abrogate NE proteolytic potential, some mutations appear not to alter the proteolytic function of this protein[22,23]. Given that uncommon genetic alterations or biochemical features underlie the toxicity of NE mutants, at the molecular level, the pathogenic effects of NE variants might potentially be attributed to disruption of the NE protein structure, changes to NE subcellular distribution and/or altered interaction of mutant NE with other proteins, which overall affect the lifespan of neutrophil precursors, or their maturation or proliferation ability.

Several, not necessarily mutually exclusive, cellular mechanisms have been proposed to explain the impact of diverse NE mutations on promoting neutropenia. These include (i) NE misfolding followed by activation of the unfolded protein response (UPR) and apoptosis of neutrophil precursors; (ii) acute oxidative stress followed by upregulation of mutant NE levels; (iii) mutation-driven NE mislocalization; and/or (iv) a

decreased expression of genes encoding key hematopoietic transcription factors as a result of the production of pathogenic NE variants [Figure 1C].

The most prevalent theory of how altered NE produces a neutrophil deficit is that mutations in *ELANE* result in improper folding, triggering the stress response pathway (UPR) within the ER, which leads to apoptosis of neutrophil precursors. The expression of incorrectly folded NE variants can be expected to culminate in promielocytes stage of granulopoiesis, when NE becomes the most abundant protein. The bulk of misfolded NE is, therefore, likely to accumulate at high levels in the ER and induce the UPR response followed by cell death. This mechanism is supported by experimental evidence, showing typical markers of the canonical UPR pathways, such as activation of molecular sensors (ATF6, CHOP) and master regulator of ER stress (GRP78), in cell models expressing pathogenic NE mutants[24,25].

However, not all neutropenia-causative NE variants are capable of consistently evoking the classic UPR response in experimental cell models. Degradation of misfolded proteins, independent of the canonical UPR, can also be a consequence of other protective cell strategies[26]. Recent findings point to elevated levels of promyelocyte leukemia protein nuclear bodies (PML-NBs), an indicator of acute oxidative stress, which may provide an alternative protective strategy in cells that harbor misfolding NE mutations. PML-NBs can be formed to diminish excessive ROS levels in the affected cells, but the presence of PML-NBs in cells with NE misfolding variants also stimulates cell metabolism and boosts the expression of NE, including NE mutants[26]. Thus, the formation of PML-NBs, independent of canonical UPR responses, may further promote neutropenic state.

Many NE mutations are predicted not to lead to NE misfolding, since they do not substantially interfere with the structure of the core protein[26]. Instead, they may be responsible for the failure of NE to be properly distributed within the cells. The first clue regarding how mutant NE can contribute to neutropenia and support for mislocalization theory came from studies in dogs[1]. Dogs suffer from CyN, which is similar to the human version, but canine CyN is autosomal recessive and does not result from NE but from AP3 mutation. AP3 is a protein involved in the trafficking of cargo proteins from the trans-Golgi network to lysosomes, suggesting that AP3 may play a role in NE trafficking and accumulation in granules. If NE, in order to be distributed in granules, interacts with AP3, a mutation in either gene could cause disruption to the intracellular trafficking of NE following the accumulation of NE in inappropriate subcellular compartments. An altered NE distribution in AP3-deficient dogs with canine cyclic neutropenia is in line with NE, which serves as an AP3 cargo protein[1]. Therefore, chain-terminating C-terminal NE mutations that disrupt NE interaction with AP3 could be expected to be mislocalized within the cells. This is what is observed in SCN patients[1].

Among other mistrafficking, NE mutations are, for example, NE variants that lack the ER localizing signal sequence, which is associated with transcriptional start site mutations[27]. The mistrafficking of NE and its accumulation in the cytoplasm beneath the plasma membrane or in cytosol has been reported for several NE variants[28-30]. Furthermore, cells that express the pathogenic G185R NE mutant associated with clinically severe forms of neutropenia, or that express shorter NE isoforms, exhibit a different distribution pattern of NE with localization to the nuclear and plasma membrane or nuclei[27,31]. Either proteolytically active or inactive NE mutants appear to be associated with off-site intracellular accumulation[22,28,30]. However, the proteolytic activity of these mislocalized NE variants has yet to be imaged in intact cells. The atypical intracellular distribution of NE might result in increased immunoreactivity for NE, a lack of control by intracellular inhibitors, and a lower activation threshold in neutrophils[16]. Taken together, these data suggest that the mislocalization of NE either via mutation of NE itself or in NE adaptive proteins, such as AP3

complex that regulates its transport into granules, can result in neutropenia.

Although in most cases, *ELANE*-based neutropenia is attributed to apoptotic death of promyelocytes, especially in the context of the UPR, mutant *ELANE*-dependent impaired differentiation of neutrophil precursors has also been proposed as a mechanism of neutrophil shortage. When human and murine myeloid cell lines with an inducible expression of human *ELANE* were used, suppressed expression of transcription factors that contribute to granulocytic differentiation was observed[22]. Since the suppression of these factors coincided with an altered subcellular localization of NE within the cells, these findings suggest that the mislocalization of the pathogenic NE variant may perturb the transcriptional regulation of granulopoiesis without affecting cell viability[22].

NE INHIBITORS IN THE DIAGNOSIS AND TREATMENT OF NEUTROPENIA

Despite the fact that neutropenia-associated NE mutations may or may not severely reduce NE proteolytic activity, blocking NE enzymatic activity using small synthetic NE inhibitors can correct NE localization to primary granules, which suggests that NE inhibitors are required for the proper localization of NE and points to the possible therapeutic or diagnostic potential of NE inhibitors[32]. Some specific low molecular weight inhibitors of NE have been found to positively affect the differentiation of *ELANE*-mutated cells. Inhibitors such as MK0339 or Sivelestat could improve the ability of the promyelocytic cells to differentiate[32]. Neutropenia patient-derived iPSCs are normally able to differentiate into granulocytes only in the presence of high doses of G-CSF, but when additionally supplemented with Sivelestat, granulocyte differentiation was possible with a much lower dose of G-CSF; this might be potentially beneficial for future therapies - lowering the chance of the development of myeloid malignancies. In addition, the presence of a small NE inhibitor restored normal intracellular NE localization[18,30]. NE inhibitor MK0339 was also able to correct the defective granulocytic differentiation of iPSCs and HL60 cells expressing mutant NE. Moreover, the inhibitor promoted the differentiation of both iPSC cells derived either from healthy donors or SCN patients[32].

Since NE and other serine proteases stored in neutrophil primary granules can contribute to tissue damage and severe pathology such as SCN, they have to be tightly controlled. However, despite the fact that neutrophils are a rich source of different protease inhibitors (PI), the role of these proteins in neutrophil development and/or function remains obscure. For example, neutrophils have long been shown to produce various PI, such as α-1 PI (SerpinA1), SLPI or elafin (PI3)[33,34]. Although proteolytically-active NE mutants, like unmutated NE, are vulnerable to inhibition with SerpinA1[23], the ability of other endogenous NE inhibitors to control the activity of NE mutants remains largely unknown.

More recently, many more PI genes have been found to be expressed in neutrophils, including SERPINB1, SERPINB2, SERPINB8, SERPINB9, SERPINB10, SERPINE1, SPINK1, WFDC5, and WFDC12[35,36]. The expression of some of these genes increases in long-lived neutrophils exposed to inflammatory stimuli, with elafin, SERPINB9 and SLPI most highly upregulated[35].

Even though the expression levels of the majority of these PI in neutrophil precursors remain to be determined, the deficiency of one of them, SLPI, has been found to be associated with SCN. SLPI expression is strongly reduced in myeloid cells from SCN patients carrying the *ELANE* or *HAX1* mutations[37]. The treatment of myeloid cells with purified non-mutated NE highly increased the expression of SLPI. Likewise, silencing *ELANE* using specific shRNA has shown the dependence of SLPI expression on *ELANE* or *HAX1* levels. These results suggest that NE is needed to induce SLPI expression in myeloid progenitors. The knockdown of SLPI in bone marrow progenitors inhibited the G-CSF-driven formation of neutrophils by

decreasing the levels of genes responsible for proliferation and survival. These findings suggest that SLPI contributes to neutrophil production because it can regulate signal transduction downstream of G-CSFR in myeloid cells and improve their proliferation.

Another SLPI-dependent mechanism involved in neutropenia relates to the UPR response[25]. Correlative evidence links the severity of neutropenia, the expression of SLPI in myeloid cells from neutropenia patients, and the ability to trigger UPR stress pathways. These studies reported significantly lower levels of SLPI in patients with SCN compared to individuals with milder CyN[25]. Moreover, higher expression of SLPI in CyN has been suggested to provide protection against the UPR response. The combined suppression of SLPI and the transduction of *ELANE* mutant S126L in myeloid cells led to an elevation of the expression of several genes that are responsible for the induction of the UPR pathways. Taken together, these findings suggest that the magnitude of *ELANE*-triggered UPR response might be regulated by a natural inhibitor of NE - SLPI.

CONCLUSION

Gene editing technology has expanded our mechanistic understanding of how distinct *ELANE* mutations yield different outcomes, but has also potentially offered a universal approach for the treatment of *ELANE* neutropenia. In addition, previous experimental NE-based mouse models had limited clinical predictive power. The HSPCs models may offer a potential solution to this problem and provide a tool to further dissect the pathogenesis of congenital neutropenia. Finally, NE inhibitors need to be better explored in the context of neutropenia. A better understanding of the mechanisms underlying congenital neutropenia could help the development of alternative treatment options.

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Authors' contributions

Made substantial contributions to the conception and design of the study: Mazur A, Skrzeczynska-Moncznik J, Majewski P, Cichy J
Drafted the manuscript: Mazur A, Skrzeczynska-Moncznik J, Majewski P, Cichy J
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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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