Mutations in the ELANE Gene are Associated with Development of Periodontitis in Patients with Severe Congenital Neutropenia

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Received: 26 January 2011 / Accepted: 11 July 2011 / Published online: 29 July 2011 © The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract

Background Patients with severe congenital neutropenia (SCN) often develop periodontitis despite standard medical and dental care. In light of previous findings that mutations in the neutrophil elastase gene, ELANE, are associated with more severe neutropenic phenotypes, we hypothesized an association between the genotype of SCN and development of periodontitis.

Methods Fourteen Swedish patients with SCN or cyclic neutropenia harboring different genetic backgrounds were recruited for periodontal examination. Peripheral blood, gingival crevicular fluid (GCF), and subgingival bacterial samples were collected. The levels of cytokines and antibacterial peptides were determined in GCF and plasma by multiplex immunoassay and immunoblotting, respectively. Subgingival bacterial samples were analyzed using 16S rDNA pyrosequencing.

Results ELANE mutations correlated with more severe periodontal status than the HAX1 or unknown mutations in patients with SCN. The subjects with mutant ELANE had higher levels of IL-1β in GCF. Using principal coordinate analysis of the subgingival microbiota, patients with ELANE mutations and reference subjects with periodontitis tended to cluster differently from patients with HAX1 or unknown mutations and non-periodontitis reference subjects.

Conclusion This study demonstrates an association between ELANE mutations in SCN and the development of periodontitis with skewed subgingival microbiota, indicating a potential role of ELANE mutations in the pathogenesis of periodontitis.

Keywords Kostmann neutropenia · neutrophil elastase 2 · periodontal diseases · subgingival microflora

Introduction

Severe congenital neutropenia (SCN, also known as Kostmann disease) includes a heterogeneous group of disorders characterized by chronic low absolute neutrophil counts (ANC) (below 0.5×10⁹/l) in the peripheral blood, early onset of bacterial infections, and mostly a maturation arrest of the myelopoiesis in the bone marrow at the level of promyelocyte/myelocyte stage [1–3]. Recent studies have revealed that a number of inherited gene mutations may cause SCN [4]. Heterozygous mutations in the ELANE gene
demonstrated that periodontal health is still unclear. Previous studies have and phenotype in terms of gene mutations in SCN and ELANE and that patients with periodontitis [15]–[19], the correlation between genotype and phenotype in terms of gene mutations in SCN and periodontal health is still unclear. Previous studies have demonstrated that ELANE mutations correlate with more severe disease manifestation in patients with SCN [20], and that patients with ELANE mutations require higher doses of G-CSF compared to patients with HAX1 mutations [3]. In light of these findings, we hereby address the hypothesis that ELANE gene mutations are associated with the occurrence of periodontitis in subjects with SCN. The underlying parameters that are believed to contribute to periodontal disease were studied, including sub-gingival microbiota composition, proinflammatory cytokines, as well as innate immune components HNP1–3 and pro-LL-37/LL-37.

Materials and Methods

Participants

From 2006 to 2008, patients with SCN (n=13) or CyN (n=1) were recruited from Karolinska University Hospital, Sweden and numbered periodontitis–neutropenia (PN) 1-to-14 according to recruitment date. The subjects ranged in age from 6 to 50 years with various forms of SCN or CyN. Ethical permission was granted by the local ethical committee at Karolinska University Hospital (2006/176-31/4). All subjects or their parents provided informed consent before participating in this study.

Clinical Examination

The clinical examination involved recording visible plaque index (%), bleeding on probing (BOP, %), probing depth (mm), and radiographs which were taken in order to determine the occurrence of alveolar bone loss. The distance between enamel cement junction and marginal bone (mm) was measured on the radiographs and alveolar bone loss was diagnosed when the distance exceeded 3.0 mm. Based on the clinical examination, the patients were categorized as either being healthy, suffering from gingivitis or periodontitis, or edentulous, respectively. Gingivitis was diagnosed when BOP exceeded 25%, while periodontitis was diagnosed when the patient exhibited both alveolar bone loss for more than three teeth and periodontal pockets exceeding 4 mm for the same teeth.

Plasma, GCF, and Subgingival Bacteria Sampling

Peripheral blood was collected and coagulation was inhibited using EDTA. Following centrifugation, plasma was gathered from the top layer and subsequently stored at −80°C in aliquots. For each subject, GCF was collected from the mesial surface of an incisor or for PN2 from a deciduous molar by inserting a paper strip (PerioPaper, Oralflow Inc.) into the gingival sulcus for 15 s. The strip was then analyzed using a Periotron Model 8000 (Oralflow Inc.), and the volume was calculated by interpolation from a standard curve. The two edentulous patients (PN4 and PN9) did not provide GCF samples. Individual strips were then placed into a sterile tube containing 120 μl PBS buffer (pH=6.8), 0.01 M EDTA, 0.3% bovine globulin, 0.005%
Plasma and GCF samples were analyzed for IL-1
Luminex Cytokine Immunoassay

Plasma and GCF samples were analyzed for IL-1β, IL-4, IL-6, IL-17, IFN-γ, and TNF-α concentrations using fluorescent bead-based Luminex cytokine immunoassays, which were performed using the Bio-Plex system (Bio-Rad laboratories). Samples were thawed on ice and homogenized in a vortex mixer for 1 min before analysis. The cytokine concentrations were determined using a human cytokine LINCOplex kit (Millipore) according to the manufacturer’s instructions and were expressed as ng/ml in GCF and pg/ml in plasma.

Gel Electrophoresis and Immunoblotting

Plasma and GCF samples were analyzed for pro-LL-37 and mature LL-37 peptide content using Western blotting. GCF samples were further tested for HNP1–3 using the same method. The GCF samples were treated with 60% acetonitrile containing 1% trifluoroacetic acid for 2 h on a shaker at 4°C to extract small peptides from the periopaper. Following centrifugation, the extraction supernatant was then transferred into a sterile tube, kept at −80°C, and lyophilized until dry. The GCF extract and plasma were dissolved in NuPAGE SDS sample buffer (Invitrogen) and lyophilized until dry. The GCF extract and plasma were electrophoresed in 1.0 mm 4–12% NuPAGE Bis–Tris gels (Invitrogen) under reducing conditions. Immunoblotting was performed as previously described [21] using the following antibodies: rabbit anti-LL-37 (Innovagen, Sweden), mouse anti-alpha defensin 1+2+3 antibody (Abcam), goat anti-rabbit, and goat anti-mouse immunoglobulins (Dako, Denmark). Detection was carried out using chemiluminescence (SuperSignal West Pico, Pierce).

454 Pyrosequencing

The microbiota of subgingival bacterial samples from the patients and reference individuals was analyzed using a 454 FLX pyrosequencing facility according to previously described methods with minor modifications [22, 23]. Briefly, DNA extraction was performed using DNeasy Blood and Tissue kit (Qiagen) with proteinase K treatment at 56°C for 16 h. For each extracted DNA sample, three 50 μl PCR mixes were prepared containing 1× PCR buffer, 200 μM dNTP PurePeak DNA Polymerization Mix (Pierce Nucleic Acid Technologies), 0.5 μM of each primer, 0.5 U Phusion F-530L enzyme (Finzyme), and 2 μl template-DNA. The primer pairs, amplifying the hypervariable 16s rRNA gene V3-V4 regions, were: 341f (5’ CCTACGGGNGGCWGCAG) with adaptor B and 805r (5’ GACTACHVGGGTATCTAATCC) with adaptor A and a sample-specific sequence barcode. The PCR conditions were 95°C for 5 min, 26 cycles of 95°C for 40 s, 58°C for 40 s, and 72°C for 1 min, followed by 72°C for 7 min. A PCR reaction without template was also used as a control for each primer pair. After analyses in agarose gel (1% w/v in TBE buffer), the samples with the same barcode were pooled and PCR reactions were purified using an Agencourt AMPure system (Beckman Coulter Genomics). The DNA concentrations were measured using Qubit (Invitrogen), and the quality control was performed with a Bioanalyzer 2100 using the DNA 1000 chip (Agilent Technologies). The samples were diluted to 3 ng/μl, and 5 μl of each sample was pooled. Region V4 was sequenced using 454 pyrosequencing with a standard amplicon kit and run in the 454-FLX (Roche, Switzerland) [24].

Sequences were excluded if there was no perfect match with the primer or barcode, ambiguous nucleotides, or the sequence was shorter than 200 nucleotides excluding the primer/barcode. Non-redundant reads with the primer/barcode removed were aligned and sorted into operational taxonomic units (OTU) using complete linkage clustering and a 3% distance threshold, which was performed using the Pyrosequencing Pipeline at Ribosomal Database Project (RDP) [25]. 16S rRNA gene sequences from RDP 10.22 were converted into a local BLAST database. The OTUs were BLAST searched against the database with a 95% identity threshold over at least 180 nucleotides. Different OTU hits were sorted to the taxonomic level for further analysis.

The different sequence identification levels were analyzed and visualized with regards to relative abundance as a heat map using MultiExperiment Viewer v4.6 software [26]. Principal coordinate analysis (PCoA) was performed and visualized in Fast UniFrac (http://bmf.colorado.edu/fastunifrac/) [27] using normalized weighted abundance. The Shannon diversity index was calculated using the R package vegan (http://CRAN.R-project.org/package=vegan) for each sample, and the significance was tested using the Wilcoxon rank sum test.
Results

Clinical Findings

The medical history of the patients (n=14) is presented in Table I. All patients except PN14 were diagnosed before 1 1/2 years of age. Of all the patients with SCN, six exhibited ELANE mutations, four HAX1 mutations, and three unknown mutation(s). Two patients (PN8 and PN9) had received HSCT before they were recruited into the study, and three patients (PN7, PN8, and PN9) had not received G-CSF treatment by the time the clinical examinations were performed.

The periodontal conditions of the patients are described in Table II. Except for PN4 and PN9 who were edentulous and PN11 who underwent professional dental care 1 year previously, the other patients had had a last dental visit between 2-to-6 months prior to the clinical examination. Their toothbrushing habits were at least once per day. Of all the patients with SCN, four were classified as being periodontally healthy, two as having gingivitis, five as having periodontitis, and two subjects as edentulous due to periodontitis at an early age. Of the six patients with ELANE mutations, five were diagnosed with periodontitis or edentulous. Conversely, within SCN cases, the subjects with HAX1 or unknown mutations were mostly (six out of seven) classified as being healthy or having gingivitis. ELANE mutations are significantly correlated with the occurrence of periodontitis/edentulism compared to HAX1 or unknown mutations (P=0.025; Table III). The single patient with CyN displayed a healthy periodontium.

Antimicrobial Peptide and Cytokine Levels in GCF and Plasma

Subjects who had received HSCT and the edentulous patients (n=3) were excluded from the GCF analysis. HSCT-transplanted patients (n=2) were excluded from the plasma analysis.

| Subjects | Age | Sex | Diagnosis | Gene mutation | Age at diagnosis | ANCc (×10^9/L) | G-CSF/Durationd/ | Other important clinical findings |
|----------|-----|-----|-----------|---------------|-----------------|---------------|----------------|---------------------------------|
| PN1      | 14  | M   | SCN       | D89H          | 4 months        | 2.1           | Yes/Since diagnosis/7.8 | Bronchial asthma                |
| PN2      | 6   | M   | SCN       | C122S         | 1 week          | 0.9           | Yes/Since diagnosis/25 | G-CSF resistant; T-S prophylaxis |
| PN3      | 17  | M   | SCN       | C26S          | 1 week          | 0.5           | Yes/Since 1991/5.0    | T-S prophylaxis                  |
| PN4      | 50  | F   | SCN       | C26S          | 3 weeks         | 0.5           | Yes/Since 1991/2.5    |                                  |
| PN5      | 19  | F   | SCN       | C26S          | 3 months        | 0.2           | Yes/Since 1991/4.5    | Atopic dermatitis               |
| PN6      | 17  | M   | SCN       | W44X          | 1 1/2 years     | 1.5           | Yes/Since diagnosis/4.3 | Ureter reflux and kidney infection |
| PN7      | 14  | M   | SCN       | wt            | 2 months        | 1.0           | No               |                                  |
| PN8      | 23  | M   | SCN (HSCTp) | wt            | 2 months        | 1.7           | No               | HSCT at 7 months of age; Epilepsy; ADHD |
| PN9      | 31  | F   | SCN (HSCTp) | L92H          | 2 weeks         | 3.1           | No               | Developed G-CSFR mutations; HSCT 2004 |
| PN10     | 21  | F   | CyN       | wt            | 11 months       | 9.2           | Sporadically       | Developed G-CSFR mutations; Epilepsy |
| PN11     | 19  | F   | SCN       | Q190X         | 13 months       | 7.0           | Yes/Since 1991/3.0   | Motor proficiency difficulties |
| PN12     | 8   | M   | SCN       | wt            | 14 months       | 1.6           | Yes/Since diagnosis/9.0 | Special school; Splenomegaly |
| PN13     | 17  | M   | SCN       | wt            | 1 year          | 5.7           | Yes/Since diagnosis/4.2 |                                  |
| PN14     | 27  | F   | SCN       | wt            | 5 years         | 1.2           | Yes/Since 1991/1.5   |                                  |

PN periodontitis–neutropenia, wt wildtype, T-S trimethoprim–sulfamethoxazole, G-CSFR G-CSF receptor, ADHD attention deficit hyperactivity disorder

p HSCT before participating in this study. PN8 had mixed chimerism and had been treated with low dose (0.5 μg/kg/day) of G-CSF until adult age; PN9 had 100% donor and normal ANC

Compound heterozygous HAX1 (cHAX1) mutations [61]

Reference range is 2.0–8.0

G-CSF treatment started in Sweden in 1991
Neither ANC ($P=0.116$), plasma pro-LL-37 ($P=0.106$), GCF $\alpha$-defensin ($P=0.703$), or GCF pro-LL-37/LL-37 ($P=0.450$) levels were significantly different in patients with ELANE mutations compared to HAX1 or unknown mutations. However, an interesting observation was that patients with unknown gene etiology exhibited low levels of both HNP1–3 and LL-37, maybe reflecting a similar gene defect. The LL-37 levels in all but one patient with SCN were 10- to 1,000-fold lower than in those two patients (PN8 and PN10) that exhibited normal ANC levels without G-CSF treatment at the time of sampling (Tables I and IV).

In addition, the level of IL-1$\beta$ ($P=0.038$) in GCF was significantly higher in the ELANE mutation group compared to HAX1 or unknown mutation group (Table V).

Table II: Periodontal status of the patients with congenital neutropenia

| Subjects | Gene mutation | Full mouth | Periodontal status | Tested site |
|----------|---------------|------------|-------------------|-------------|
|          | ELANE         | HAXI       | VPI (%) | BOP (%) | PD>4 mm | Massive Plaque Index | BOP (%) | PD (mm) | ECJ-MB (mm) |
| PN1      | D89H          | wt         | >50     | >50     | No      | Gingivitis | 41, 31 | 3     | 2    |
| PN2      | C122S         | wt         | >50     | >50     | Yes     | Periodontitis | 55, 85 | 4, 3  | 4, 3 |
| PN3      | C26S          | wt         | >50     | >50     | Yes     | Periodontitis | 41, 31 | 6     | 9    |
| PN4      | C26S          | wt         | –       | –       | –       | Edentulous   | –       | –     | –    |
| PN5      | C26S          | wt         | >50     | >50     | Yes     | Periodontitis | 41, 31 | 6     | 5    |
| PN6      | wt            | W44X       | >50     | >50     | Yes     | Periodontitis | 41, 31 | 4     | 3.5  |
| PN7      | wt            | Q190X and E31KfsX54 | <15 | 0 | No | Healthy | 41, 31 | 2 | 2 |
| PN8      | wt            | Q190X      | <25     | <15     | No      | Healthy     | 41, 31 | 2 | 1.5 |
| PN9      | L92H          | wt         | –       | –       | –       | Edentulous   | –       | –     | –    |
| PN10     | wt            | Q190X      | <15     | <15     | No      | Healthy     | 41, 31 | 2 | 1.2 |
| PN11     | wt            | Q190X      | 0       | 0       | No      | Healthy     | 41, 31 | 2 | 2    |
| PN12     | wt            | Q190X      | <15     | <15     | No      | Healthy     | 41, 31 | 2 | 2    |
| PN13     | wt            | wt         | >50     | >50     | Yes     | Periodontitis | 41, 31 | 4 | 3.5 |
| PN14     | wt            | wt         | <25     | >25     | Yes     | Gingivitis   | 41, 31 | 3 | 2.5 |

$PN$ periodontitis–neutropenia, $wt$ wildtype, $VPI$ visible plaque index, $BOP$ bleeding on probing, $PD$ probing depth, $ECJ-MB$ enamel cement junction–marginal bone

Subgingival microbiota analysis from tooth 41, or from tooth 55 in PN2; cytokine and AMP analysis from tooth 31, or from tooth 85 in PN2

Although the levels of IL-17, IL-6, and TNF-$\alpha$ were not statistically different in the GCF, it may be noted that mean and median values in ELANE mutation group are higher than in HAX1 or unknown mutation group. The GCF cytokine levels of IFN-$\gamma$ and IL-4 were both below the detection limit in all samples. Moreover, there was no significant difference in terms of the cytokine levels in plasma between the two subgroups of patients.

Subgingival Microflora

We generated 8,810 high-quality 16S rRNA gene sequences from 12 subgingival bacterial samples (PN4 and PN10 were excluded due to edentulism). The sequences represented seven major phyla: Actinobacteria (19.5%), Bacteroidetes (19.5%), Firmicutes (22.2%), Fusobacteria (16.6%), Proteobacteria (15.9%), Spirochaetes (2.0%), and candidate domain TM7 (1.3%). The predominant genera were Fusobacterium, Streptococcus, Rothia, Prevotella, Corynebacterium, Neisseria, Veillonella, Capnocytophaga, and Leptotrichia, which in total accounted for 66% of all sequences. The Shannon diversity index was similar in patients with ELANE mutations and HAX1 or unknown mutations according to Wilcoxon rank sum test ($P=0.788$). To illustrate subgingival microbiota composition of individuals, a heat map was generated to reveal the abundance of different taxa in samples from both patients with neutropenia and references (Fig. 1). A sample tree was also created using hierarchical clustering using
Pearson correlation of absolute distance and complete linkage clustering (Fig. 1). In both hierarchical clustering and UniFrac PCoA (Fig. 2), three out of four samples from patients with \textit{ELANE} mutations were clustered together with all reference samples of periodontitis.

**Discussion**

Although the prognosis and quality of life of patients with congenital neutropenia were improved dramatically following the introduction of G-CSF therapy 20 years ago [28], some patients still suffer from frequent periodontal infections despite efficient and adequate oral hygiene. In the current study, we demonstrate for the first time a link between mutations affecting \textit{ELANE} encoding neutrophil elastase and the occurrence of periodontitis. The periodontal condition of the patients in the present study varied from healthy to severe periodontitis and two patients were edentulous due to periodontitis, indicating great variation of the chronic inflammatory response in the periodontium among patients with congenital neutropenia. Recent genetic studies have identified multiple gene mutations in SCN, with the most common mutations affecting the \textit{ELANE} (\textit{ELA2}) gene. To date, more than 45 distinct \textit{ELANE} mutations have been described in SCN and CyN [29]. Our current findings demonstrate a correlation between \textit{ELANE} mutations and periodontitis in patients with SCN, concurring with the view that \textit{ELANE} mutations are correlated with more severe disease manifestations and having a relatively poorer response to G-CSF treatment [3, 20]. However, the number of patients in our study is limited since SCN is a rare disease. Further investigation involving a larger cohort will be needed to confirm our findings.

Many of the patients with \textit{ELANE} mutations had low ANC and therefore may be expected to have more severe periodontal disease. However, it is both neutrophil functionality and neutrophil counts over time that can affect the outcome of periodontal disease. Moreover, factors such as oral hygiene and diet may influence the outcome of oral health, which we have attempted to account for by using questionnaires for patients and their dentists.

The antimicrobial peptides HNP1–3 and LL-37 are produced during neutrophil maturation in the bone marrow and are stored as pro-peptides in neutrophil granules. The pro-LL-37 (hCAP18) is also detectable in plasma from healthy individuals [30]. We previously demonstrated that plasma pro-LL-37 levels were low in patients with SCN in spite of G-CSF elevated neutrophil levels, thus reflecting impaired neutrophil development [21, 31]. In the current study, pro-LL-37 levels in plasma were low in all patients with SCN and did not differ between the \textit{ELANE} mutation group compared to \textit{HAX1} or unknown mutation group.

In GCF, elevated levels of both HNP1–3 and LL-37 are produced during neutrophil maturation in the bone marrow and are stored as pro-peptides in neutrophil granules. The pro-LL-37 (hCAP18) is also detectable in plasma from healthy individuals [30]. We previously demonstrated that plasma pro-LL-37 levels were low in patients with SCN in spite of G-CSF elevated neutrophil levels, thus reflecting impaired neutrophil development [21, 31]. In the current study, pro-LL-37 levels in plasma were low in all patients with SCN and did not differ between the \textit{ELANE} mutation group compared to \textit{HAX1} or unknown mutation group.

In GCF, elevated levels of both HNP1–3 and LL-37 have been reported in subjects with chronic periodontitis, most likely due to enhanced neutrophil influx [32]. In our study, the GCF levels of HNP1–3 and LL-37 did not appear to be statistically different between patients harboring different mutations, possibly due to the wide range of values in both

| Diagnosis | Gene mutation | Subjects Periodontal status | GCF HNP1–3 (μg/ml) | GCF pro-LL-37 | Plasma pro-LL-37 <d>
|-----------|--------------|-----------------------------|--------------------|---------------|----------------|
| SCN       | ELANE        | PN1 Gingivitis              | 192                | 0.25          | 0.01          |
|           |              | PN2 Periodontitis           | 428                | 0.82          | <0.01         |
|           |              | PN3 Periodontitis           | <4                 | 10.56         | <0.01         |
|           |              | PN5 Periodontitis           | <4                 | 0.05          | <0.01         |
|           | HAX1         | PN6 Periodontitis           | 40                 | 6.21          | 0.03          |
|           |              | PN7 Healthy                 | <4                 | 6.93          | <0.01         |
|           |              | PN11 Healthy                | 247                | 78.18         | 0.17          |
|           |              | PN12 Healthy                | 5                  | 0.08          | 0.06          |
|           |              | PN13 Periodontitis          | 4                  | 0.38          | 0.10          |
|           |              | PN14 Gingivitis             | 4                  | 0.13          | 0.09          |
|           |              | SCN (HSCTb)                | HAX1               | PN8 Healthy   | 840           | 324.0         | 0.31          |
|           |              | CyN unknown                 | PN10 Healthy       | 227           | 132.8         | 0.54          |

Table IV: Antimicrobial peptides in GCF and plasma from patients<sup>a</sup>

| Cytokines (ng/ml) | ELANE mutations (n=5) | HAX1 or unknown mutations (n=6) | P value<sup>a</sup> |
|------------------|-----------------------|---------------------------------|---------------------|
|                  | Median                | Mean±SD                         | Median              | Mean±SD              |               |
| IL-1β            | 9.3                   | 13.1±9.3                        | 2.9                 | 5.1±6.8              | 0.038          |
| IL-17            | 0.5                   | 0.5±0.4                         | 0.1                 | 0.2±0.1              | 0.131          |
| IL-6             | 3.0                   | 3.8±3.8                         | 0.8                 | 0.8±0.5              | 0.186          |
| TNF-α            | 0.5                   | 0.5±0.4                         | 0.01                | 0.03±0.05            | 0.089          |

Table V: Cytokines in GCF from patients with SCN harboring different genetic mutations

<sup>a</sup>Mann–Whitney test
groups. The α-defensins HNP1–3 are stored in primary granules in neutrophils, and the GCF levels of HNP1–3 have been reported to vary widely in healthy subjects [33, 34]. The considerable difference of GCF HNP1–3 in the present study is compatible with our previous observation that HNP1–3 levels vary from deficiency to normal levels in neutrophils from patients with SCN [14]. To date, gingival LL-37 has been demonstrated to have two main sites of origin, neutrophils and epithelial cells [35]. In the absence of neutrophil-derived LL-37 in the GCF of patients with SCN, it was possible to determine the epithelial contribution to LL-37 levels, which was found to be noticeably low. LL-37 levels below bactericidal concentrations have been demonstrated to serve as a chemo-attractant or modulator of host inflammatory responses in concert with other epithelial-derived cytokines [36–38]. Thus, in the absence of efficient neutrophil antibacterial clearance due to deficiency of neutrophil granule peptides, epithelial-derived peptides might even augment periodontal inflammation.

In the current study, we demonstrated that IL-1β levels were significantly higher in GCF samples from subjects with mutant ELANE. In addition, the mean and median levels of IL-17, IL-6, and TNF-α in patients with ELANE mutations were higher than in HAX1 or unknown mutations although these differences did not reach statistical significance, most likely due to the small size of the cohort and great variations within groups. It is known that elevated

![Fig. 1 Individual subgingival microbiota composition. The heat map depicts the abundance of the different taxa in individual subgingival samples. The color scale of relative abundance ranges from 0% (black) to 30% (red). The phylum level is shown on the left-hand side. The dominant taxa (genus level, except TM7) are displayed according to the relative abundance per sample. The sample tree was generated using hierarchical clustering with Pearson correlation of absolute distance and complete linkage clustering. PN8 is stem cell transplanted. R represents reference samples and patient PN10 (CyN).](image-url)
levels of IL-1β [39, 40], IL-6 [41], TNF-α [42], and IL-17 [43] in GCF are associated with severe periodontal disease, and that these cytokines may also be elevated in chronic periodontitis tissue [44–46]. Thus, the GCF from patients with ELANE mutations displays the presence of the strong proinflammatory cytokine IL-1β, which might be expected in the inflamed periodontium.

Oral microbiota in the healthy population has been determined using high-throughput 16S rDNA pyrosequencing in several studies [47–49], providing a rather comprehensive view of the oral commensal microbial community. To our knowledge, this is the first time that the 16S rDNA pyrosequencing technique has been employed to map subgingival microbiota in subjects with a congenital immunodeficiency. The predominant taxa from periodontal sites of individuals with SCN were similar to the microbiota previously reported from healthy subjects [49]. Although two patients with ELANE mutations were not included in microbiota analyses due to edentulism, hierarchical clustering and UniFrac PCoA analysis revealed that three out of four samples from the ELANE mutation group were clustered with the periodontitis reference individuals. The skewed periodontal microbiota in both periodontitis references and the ELANE mutation group of SCN cases indicates that periodontal pathogens of the genera Fusobacterium, Prevotella, Treponema, and TM7 domain are more likely to grow in the gingival crevices in patients with ELANE mutations compared to HAX1 or unknown mutations [50]. As an outlier, PN2 did not cluster with other individuals with ELANE mutations that may partly be explained by the fact that periodontal pathogens of young children may differ from those of adolescent or adults [51, 52].

The neutropenia of patients with SCN arises as a consequence of bone marrow neutrophil precursor accelerated apoptosis [53]. The two most frequently reported gene mutations of SCN are HAX1 and ELANE [54]. HAX-1 is a mitochondrial protein involved in maintaining the mitochondrial membrane potential, signal transduction, and cell survival [55, 56]. The HAX1 mutations in SCN result in HAX-1-deficient neutrophils and neutrophil precursors, which have been demonstrated to show enhanced apoptosis [57]. The mechanism by which ELANE mutations result in apoptosis of neutrophil precursors is less obvious, but it has been suggested that the accumulation of misfolded elastase proteins activates the unfolded protein response, leading to apoptosis [58, 59]. Although rescued by G-CSF treatment, the neutrophils with ELANE mutations still carry mutated elastase proteins that may be aberrant in their localization and functions such as proteolytic processing of other proenzymes or cytokines, which in turn may affect the local periodontal immune response [60]. Thus, it is possible that in addition to the antimicrobial peptide deficiency that is shared by all patients with SCN, the ELANE mutations may confer an additional neutrophil dysfunctionality, leading to more severe periodontal disease.

**Conclusion**

We herein report that patients with SCN that harbor mutations in the gene ELANE coding for neutrophil elastase present with more severe periodontal disease as compared to patients with HAX1 mutations or with unknown mutations. The periodontal pockets of the former group display a skewed microflora and elevated levels of proinflammatory cytokine IL-1β. The correlation between ELANE mutations and periodontal disease indicates that the serine protease elastase may have an important role in the local defense in the gingival pocket.
Acknowledgments The authors sincerely thank the patients and their families for their cooperation. The study was supported by grants from Swedish Children’s Cancer Foundation (G.C.), Söderbergs Foundation (L.E.), Swedish Research Council (K.P., M.A.), Åke Wiberg Foundation (K.P.), Swedish Society for Medicine (K.P.), and Swedish Patent Revenue Research Fund (T.M.).

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