Severe congenital neutropenia
Milestones of the history of congenital neutropenias

- „Agranulocytosis-Schultz-syndrome“
- „Preleukemic Syndrome“
- G-CSF (Phase 1-3 clinical trials)
- CSF3R mutations*
- SBDS mutations
- P14 mutations
- RUNX1 mutations*
- „Infantile genetic agranulocytosis – Kostmann-syndrome“
- Recombinant G-CSF
- Establishment of the SCNIR
- ELANE mutations
- HAX1 mutations
- G6PC3 mutations

* = acquired mutations
Severe congenital neutropenia

G-CSF Treatment

Patient 8716/01: 20 µg/kg/d

Patient 8716/27: 5 µg/kg/d

Patient 8716/27: 5 µg/kg/d
Identification of Neutropenia causing gene defects

Establishment of the SCNIR

1994

- ELANE
  - Horwitz M, Benson KF, Person RE et. al. (1999)

1999

- SBDS
  - Boocock GR, Morrison JA, Popovic M et. al. (2003)

2003

- CXCR4
  - Hernandez PA, Gorlin RJ, Lukens JN et. al. (2003)

- P14

2007

- HAX1

2009

- G6PC3
  - Boztug K, Appaswamy G, Ashikov A et. al. (2009)
### ELANE Mutations in Cyclic and Congenital Neutropenia

#### Linear Localization

**Congenital Neutropenia (CN)**
- 189 patients, 29 AML/MDS

**Cyclic Neutropenia (CyN)**
- 118 patients, 0 MDS/AML

#### 29 patients

<table>
<thead>
<tr>
<th>Exon 1</th>
<th>Exon 2</th>
<th>Exon 3</th>
<th>Intron III</th>
<th>Exon 4</th>
<th>Intron IV</th>
<th>Exon 5</th>
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<td>F43L</td>
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**3 patients**

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<tr>
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<td>c. -9 A&gt;G</td>
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**1 patient**

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#### 63 patients

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#### 49 patients

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<td>A233fs</td>
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**11 patients**

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#### 49 patients

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<th>Intron III</th>
<th>Exon 4</th>
<th>Intron IV</th>
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<td>G214ter</td>
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<td>IVS4 +6 3bp ins</td>
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</tbody>
</table>

### References

Unfolded protein response (UPR)

**Transcription of new BiP**

**Signal to nucleus**

**Apoptosis**

**Translational arrest**

**Protein degradation**

Adapted from Dudek, J. et al., Cell. Mol. Life. Sci. 2008
ATF6 is upregulated in myeloid cells of CN, but not CyN patients

CD33^+ bone marrow cells

Nustede R., et al., BJH 2016
Mutations affecting both isoforms are associated with neutropenia and a neurological phenotype:
Isoform 2 is critical for neuronal functions,
Mutations affecting isoform 1 only (e.g. Trp44X) are associated with neutropenia only.

Klein, C., et al., Nat Gen 2007
HCLS1 is phosphorylated by Lyn and Syk upon G-CSF stimulation.

HCLS1 is a Hematopoietic Cell specific Lyn Substrate 1.

HAX1 is a HCLS1 Associated protein X 1.
G-CSF failed to phosphorylate HCLS1 in hematopoietic cells of CN patients harboring HAX1 mutations.
HCLS1 is essential for myeloid differentiation

HCLS1 is involved in the nuclear transport of LEF-1 protein

(a) LEF-1 WT and LEF-1 HCLS1 binding MUT

(b) LEF-1 with HCLS1 WT, HCLS1 Y397F, and HCLS1 NLS in - G-CSF and + G-CSF conditions.

LEF-1

HCLS1

WT

Y397F

NLS

- G-CSF

+ G-CSF
LEF-1 and its target gene C/EBPα expression are downregulated in ELA2 – and HAX1 mutated CN patients

CN: congenital neutropenia; CyN: cyclic neutropenia;

Restoration of defective LEF-1 expression promotes granulocytic differentiation of CD34+ progenitors of CN patients

HCLS1 interacts with LEF-1 transcription factor inducing its nuclear translocation and activation upon G-CSF treatment

Skokowa J. et al., Nature Medicine, 2012
### Glucose-6-Phosphatase Komplex

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene</th>
<th>Expression</th>
<th>Phenotype</th>
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</thead>
<tbody>
<tr>
<td>GSD1a</td>
<td>G6PC1</td>
<td>Liver, kidney, intestine</td>
<td>GSD</td>
</tr>
<tr>
<td>GSD1b</td>
<td>G6PT</td>
<td>ubiquitous</td>
<td>GSD + CN</td>
</tr>
<tr>
<td>G6PC3-deficiency</td>
<td>G6PC3</td>
<td>ubiquitous</td>
<td>CN</td>
</tr>
</tbody>
</table>

**Diagram:**

- **Cytosol**
  - G6PT
  - G6PC1
  - G6PC3
  - ER Lumen

**Chemical Reaction:**

\[
\text{glucose-6-phosphate} + \text{Mg}^{++} + \text{H}_2\text{O} \rightarrow \text{glucose} + \text{Pi}
\]
JAGN1 deficiency causes aberrant myeloid cell homeostasis and congenital neutropenia

JAGN1-mutant granulocytes are characterized by ultrastructural defects, absence of secretory vesicles and aberrant N-glycosylation of multiple proteins, and increased apoptosis.

---

**Family A**

I-1 □  I-2 ○  I-3 □  I-4 □

II-1 ○  II-2 (P1) ○  II-3 (P2)

II-4 (P3) □  II-5 □  II-6 (P4) □  II-7 (P5)
c.998-2A>T

p.W547*

Healthy individual

CN patient

Ig-like domain

Cytokine receptor homology region

Fibronectin III-like domains

Transmembrane region

Intracytoplasmic region

Neutrophils Monocytes

G-CSFR

isotype ctrl healthy individual

isotype ctrl CN patient

anti-G-CSFR healthy individual

anti-G-CSFR CN patient

G-CSF (up to 110μg/kg/day)

GM-CSF 6 μg/kg/day twice a week

GM-CSF 3 μg/kg/day twice a week

ANC, x1000/ul

Time, months after birth

Figure 1
Distribution of gene mutations in 226 European congenital neutropenia patients
Neutropenia causing mutations

- Most of cases of SCN are attributable to ELANE mutations, but there are
- mutations in genes affecting G-CSF signaling (CSF3R, HAX1)
- genes affecting glucose homeostasis (SLC37A4, G6PC3),
- lysosomal function (LYST, RAB27A, ROBLD3/p14, AP3B1, VPS13B, TCIRG1),
- ribosomal proteins (SBDS, RMRP), mitochondrial proteins (HAX1, TAZ),
- immune functions (STK4, GFI1, CXCR4), and X-linked (WAS)
- ultrastructural defects, absence of secretory vesicles and
How does G-CSF induce granulopoiesis (overcome senescence) in CN, if both LEF-1 and HCLS1 are severely downregulated?
How does G-CSF induce granulopoiesis in CN?

LEF-1 dependent **steady-state** granulopoiesis

LEF-1 independent **emergency** granulopoiesis

G-CSF induces C/EBPβ in CN!
Nampt triggers myeloid differentiation of CD34+ cells

**Western Blot Analysis:**
- **Nampt:** 55 kDa
- **SIRT1:** 110 kDa
- **C/EBPβ:** 37.5 kDa
- **β-actin:** 45 kDa

**Flow Cytometry:**
- **Control:** 20%
- **Nampt:** 84%

**G-CSF Production:**
- **Ctrl:** 0 ng/ml medium
- **Nampt:** 0.6 ng/ml medium

**Significance:**
- **** Signifies statistical significance.
G-CSF induces Nampt/PBEF and NAD$^+$ in myeloid progenitors from CN patients

G-CSF → STAT3/Nampt → NA → NAD$^+$ → SIRT1

Regulation of transcription

G-CSF induces Nampt/PBEF and NAD$^+$ in myeloid progenitors from CN patients

Nampt triggers myeloid differentiation of CD34+ cells

- ctrl
- Nampt
- G-CSF
- Nampt + G-CSF

% of CD16+ cells

Time (d)

0 7 10 14

% of CD15+ cells

Time (d)

0 7 10 14

- NAMPT

+ NAMPT

10 μm
Vitamin B3 treatment of patient with cyclic neutropenia

- Treatment with G-CSF
- Treatment with Vitamin B3 without G-CSF

Neutrophil granulocytes in peripheral blood (x 10^3 µl⁻¹)

Graphs showing neutrophil granulocytes levels with and without G-CSF treatment.
G-CSF signaling pathways

G-CSF

G-CSFR

Vitamin B3
(Nicotinamide)

Risk of leukemia in CN patients

First reports on leukemias in CN:
Gilman PA, et al., Blood 1970
### G-CSF Treatment by Neutropenia-Genotype

<table>
<thead>
<tr>
<th>Neutropenia Code</th>
<th>No Leukemia (n)</th>
<th>Median G-CSF dose (µg/kg/d)</th>
<th>Leukemia (n)</th>
<th>Median G-CSF dose (µg/kg/d)</th>
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<tr>
<td>ELANE-CN</td>
<td>72</td>
<td>4,9</td>
<td>11</td>
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<td>HAX1-CN</td>
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* Median G-CSF Dose for all Congenital Patients **4,85 µg/kg/d**
and for all Cyclic Patients **1,6 µg/kg/d**
Congenital Neutropenia
Incidence of Leukemia
CI at 30 Years by Genetic Subtype

Log Rank p=0.649
VAFs of CSF3R mutant clones in CN and CN/AML patients
CSF3R mutations
VAFs of CSF3R mutant clones in CN and CN/AML patients.
Leukemia-associated mutations in 31 CN/AML patients

Targeted deep sequencing

23 (74 %) CSF3R
20 (64,5 %) RUNX1

2 FLT3-ITD
4 EP300
2 SUZ12
1 CREBB
1 CBL
1 NRAS

!!! Neg. for: CEBPA, DNMT3A, IDH1, IDH2, NPM1, TET2
High frequency of cooperating \textit{RUNX1} and \textit{CSF3R} mutations in 31 CN/AML patients
Segregation of \textit{RUNX1} and \textit{CSF3R} mutations in blasts of CN/AML patient

\begin{itemize}
    \item N=48
    \item \textit{RUNX1} MUT + \textit{CSF3R} MUT: 43
    \item \textit{RUNX1} MUT only: 4
    \item \textit{CSF3R} MUT only: 1
\end{itemize}
First detection of *CSF3R*- and *Runx1* mutations in months prior to AML

<table>
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<th>Runx1 mut</th>
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<tr>
<td># 6</td>
<td>-192</td>
<td>-36</td>
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<tr>
<td># 7</td>
<td>-36</td>
<td>-12</td>
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<td># 14</td>
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<td># 16</td>
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<td># 19</td>
<td>-36</td>
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</table>
G-CSF treatment in combination with mutations in \textit{CSF3R} and \textit{RUNX1} are leukemogenic

Skokowa et al., EHA 2014 Presidential Symposium
Figure 1

A 1st sequential ANC count of CyN-AML patient

B 2nd sequential ANC count of CyN-AML patient (4 months later)

C

- CyN-AML
- CyN-AML mutant allele
- CyN-AML wild type allele
- Mother
- Father

ELANE, exon 5

c.697G>C c.703delG

D

ELANE
β-actin

Blood 2016
Mutated RUNX1 enhanced clonogenic capacity of lin- cells from d715 Csf3r mice

BM lin- cells from d715 Csf3r mice

48 h expansion

72 h transduction with lentiviral constructs with WT or MUT RUNX1

FACS sorting BFP+ cells

1st replating

2nd replating

number of colonies

<table>
<thead>
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<th>WT RUNX1</th>
<th>R139G RUNX1</th>
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number of colonies

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<th>ctrl BFP</th>
<th>WT RUNX1</th>
<th>R139G RUNX1</th>
<th>R174X RUNX1</th>
</tr>
</thead>
<tbody>
<tr>
<td>no colonies</td>
<td></td>
<td>**</td>
<td></td>
</tr>
</tbody>
</table>

number of cells 2 weeks after plating, x 10^4

<table>
<thead>
<tr>
<th>ctrl BFP</th>
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</tbody>
</table>
The two-hit hypothesis of leukemogenesis in CN

ELANE-, HAX1-mutations, Genotoxic stress
HSC

1st hit

pre-leukemia stem cell

CSF3R mutation

2nd hit

RUNX1 mutation

leukemia stem cell

Monosomy 7
Trisomy 21

leukemic blasts

diminished LEF-1, C/EBPa, HCLS1
hyperactivated NAMPT/SIRTs, Akt, STAT5a
deacetylated p53, FOXO3a, LEF-1
Improvement of maturation arrest after genetic correction

Correction of ELANE mutations in iPSCs from a patient with congenital neutropenia by CRISP/Cas9 technology

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