**Loss-of-function mutations in *CSF3R* cause moderate neutropenia with fully mature neutrophils: two novel pedigrees**

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*To the Editor:*

The growth factor granulocyte colony-stimulating factor (G-CSF) has been described as a key regulator of neutrophil development by inducing proliferation, differentiation, and survival of myeloid progenitors. In case of infection, G-CSF stimulates emergency granulopoiesis (Panopoulos and Watowich 2008). The receptor for G-CSF (G-CSF-R), encoded by *CSF3R*, is a member of the type I cytokine receptor superfamily. One of the major pathways activated upon G-CSF-R stimulation is the Janus kinase (JAK)-signal transducer and activators of transcription (STAT) pathway, leading to phosphorylation of STAT1, 3 and 5, which subsequently can induce transcription of their target genes (Dong *et al.,* 1998).

To date, 8 families with 11 (inherited) loss-of-function mutations in *CSF3R* in 10 patients have been reported (supplementary Table SI) (Dror *et al.,* 2000; Druhan *et al.,* 2005; Klimiankou *et al.,* 2015; Sinha *et al.,* 2003; Triot *et al.,* 2014; Ward *et al.,* 1999; Yilmaz Karapinar *et al.,* 2019; Yilmaz Karapinar *et al.,* 2018). These patients suffer from congenital neutropenia and cannot be treated with G-CSF due to refractoriness. The consequence of G-CSF-R deficiency for cellular differentiation, effector functions and signal transduction in human primary neutrophils have not been studied in detail.

Genetic testing on the first patient with moderate-to-mild neutropenia was performed at the age of 40 (Fig 1A-B; panel I). She had been admitted with several infectious episodes for which intravenous antibiotics were prescribed. A bone marrow aspirate showed a left shift in granulopoiesis. Upon treatment with G-CSF (before genetic analysis was performed) no improvement in neutrophil count was repeatedly noticed (for patient history, see Supporting Information). Genetic analysis identified compound heterozygous mutations in *CSF3R* (NM_000760.3) with a novel conserved missense variant (c.359G>A, p.(G120D); CADD score 29) and a nonsense variant with a population frequency of approximately 1:5000 (gnomAD AF = 0.0003; BRIDGE/GEL AF = 0.0007 at c.1640G>A, p.(W547*)).

A second case, from a consanguineous family, had mild neutropenia and suffered from unexplained febrile episodes for about 5 years (Fig 1A-B; panel II). Genetic analysis identified a homozygous missense mutation in *CSF3R* (c.1318C>T, p.(R440*)). Out of 4 other siblings, one was also homozygous (patient 3), one a heterozygous carrier, and one did not have the genetic variant. Whilst being mildly neutropenic, patients 2 and 3 were clinically not severely affected (Fig 1B; Supporting Information).

All reported inherited loss-of-function mutations in *CSF3R* are present in the extracellular domain of *CSF3R*, of which 4 are localized near or in the conserved WSXWS motif (Fig 1C). The mutations in

patient 1 were localized in the so-called CRH region and third FNIII domain, respectively (Fig 1C). The mutation in the FNIII domain leading to a stop-codon (p.W547*) has been previously described (Klimiankou *et al.,* 2015). The homozygous mutations in patients 2 and 3 are localized in the second FNIII domain and lead to a premature stop.

Circulating neutrophils demonstrated a normal morphology and segmented nuclei (Fig 1D). Neutrophils from the UK samples showed some apoptotic cells, which can be explained by the shipment of blood samples to our laboratory. Flow cytometric analysis did not reveal differences in expression of the neutrophil maturation marker EGF module-containing mucin-like hormone receptor 3 (EMR3) (Matmati *et al.,* 2007) between control and patient neutrophils (Figure S1).

Label-free quantitative mass spectrometry-based proteomics was performed on neutrophils of patients, patients' family members, and controls. No large differences between granule proteins and components of the NADPH-oxidase complex were found, indicating intact neutrophil development at all stages (Hoogendijk *et al.,* 2019) (Figure S2, A-B). Of the 2,849 proteins which were identified and quantified, 10 proteins showed altered expression between patients and controls (Figure S3). Altered expressed proteins include proteoglycan 3 (PRG3), eosinophil cationic protein (RNASE3) and eosinophil peroxidase (EPX), indicative of some eosinophil contamination which was also observed by cytospin (i.e. 5.3 ± 3.3% in controls vs. 10.3 ± 2.6% in patients).

Immunostaining of G-CSF-R showed greatly reduced expression on patient neutrophils. In the second pedigree, flow cytometry showed intermediate expression on the heterozygous family members, while the unaffected sibling had G-CSF-R surface staining comparable to unrelated healthy controls (Fig 2A and Figure S4). Intermediate G-CSF-R expression did not result in reduced neutrophil counts (data not shown).

G-CSF is important for neutrophil survival. Although granulocyte-macrophage colony-stimulating factor (GM-CSF) has overlapping functions with G-CSF, it signals via a different receptor (GM-CSF-R) (Mehta *et al.,* 2015). When comparing the response to both growth factors, G-CSF failed to induce survival of patient neutrophils (Fig 2B and Figure S5). Additionally, we tested the priming capacity of G-CSF by formyl-Met-Leu-Phe-induced reactive oxygen species (ROS) production in neutrophils. Again and in contrast to control cells, patient neutrophils did not show an enhanced ROS production upon pre-incubation with G-CSF while being responsive to GM-CSF (Fig 2C).

To confirm the absence of G-CSF-R signalling, STAT3 phosphorylation was evaluated upon incubation with GM-CSF or G-CSF. Both control and patient neutrophils showed STAT3 phosphorylation upon GM-CSF stimulation, whereas only control neutrophils demonstrated STAT3 phosphorylation after G-CSF stimulation (Figure 2D and Figure S6). These data show that the mutations in *CSF3R* in these patients resulted in lack of G-CSF-R expression and function.

Although the missense variant (c.359G>A, p.(G120D)) in patient 1 might result in residual expression of G-CSF-R, functional analysis on primary neutrophils and *in-vitro* and *in-vivo* refractoriness to G-CSF proves that this missense variant has a deleterious impact on receptor signalling.

Despite lack of G-CSF-R expression and signalling in neutrophils, the neutropenia in these patients is moderate-to-mild. This finding implies that the myeloid progenitors are able to generate a pool of normal differentiated and functionally mature neutrophils. This is reminiscent of G-CSF-R knockout models in mice, in which neutrophil counts were decreased for 50% at most (Liu *et al.,* 1996). Bone marrow samples of patients were not available for functional analysis. Still, there is a lack of immediate response to G-CSF-induced release of neutrophils from the bone marrow upon infection or exogenous administration, as exemplified in the first patient. In healthy controls, there is low expression of G-CSF-R on bone marrow neutrophils compared to circulating neutrophils as assessed by flow cytometry (Figure S7). These results suggest that G-CSF-R is present on committed progenitors for accelerating neutrophil production required for 'emergency neutrophilia' at the final stage of segmented neutrophils and immediate release of the bone marrow reserve pool. G-CSF-induced mobilization of hematopoietic progenitor cells coincides with cleavage of C-X-C Motif Chemokine Receptor 4 (CXCR4) and its ligand (stromal cell-derived factor 1, SDF-1) by neutrophil proteases in the bone marrow (Levesque *et al.,* 2003). As the CXCR4/SDF-1 axis is also implicated in neutrophil retention (Kawai and Malech 2009), it is expected that the same mechanism applies for the egress of mature neutrophils. The increase of G-CSF-R expression at the final stage of neutrophil development would also be compatible with the strong pro-survival effect of G-CSF on circulating and bone marrow neutrophils (data not shown).

In the absence of G-CSF-R, neutrophil differentiation in the bone marrow can still proceed under the influence of other factors like GM-CSF, interleukin-6 and as yet unidentified factors (Mehta *et al.,* 2015). However, these factors combined do not seem to fully compensate for G-CSF deficiency because neutrophil production remains quantitatively impaired in individuals which lack GCSF-R expression.

In sum, our data support the notion that the major role of G-CSF in neutrophil expansion and release may be during inflammatory responses when a larger bone marrow output and prolonged survival is required to cope with serious infections but not for neutrophil differentiation per se.

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## Authorship contributions

Contribution: E.G.G.S., S.O.B. and T.W.K. wrote the manuscript; E.G.G.S., A.T.J.T., and T.W.K. designed the experiments. E.G.G.S., and A.T.J.T. performed experiments and analyzed the data; S.O.B. and T.W.K. analyzed clinical data; NIHR BioResource, J.M. and K.v.L. performed DNA analysis; I.C.K. and F.P.J.v.A. performed mass spectrometry analysis; and all authors read, revised and approved the manuscript.

## Conflict of interest

The authors declare that they have no relevant conflicts of interest.

## Declaration of funding sources

## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
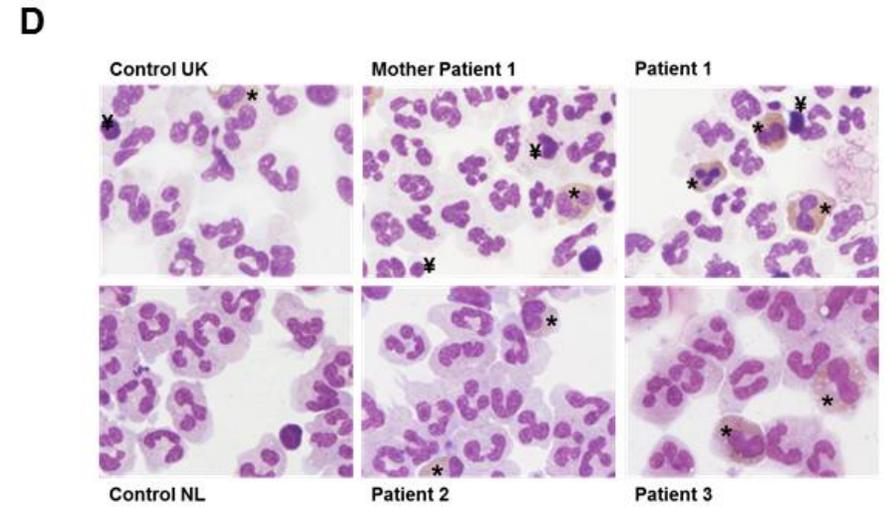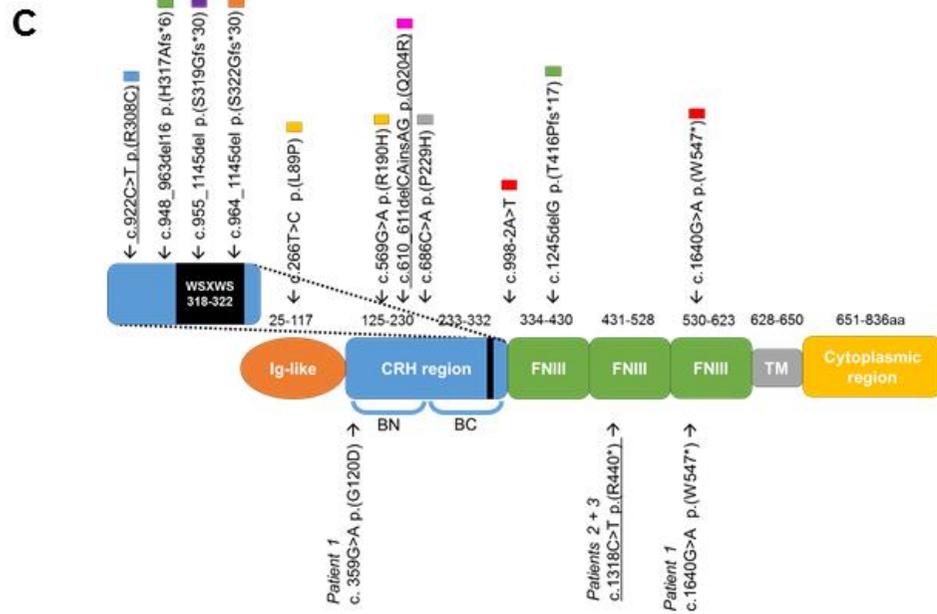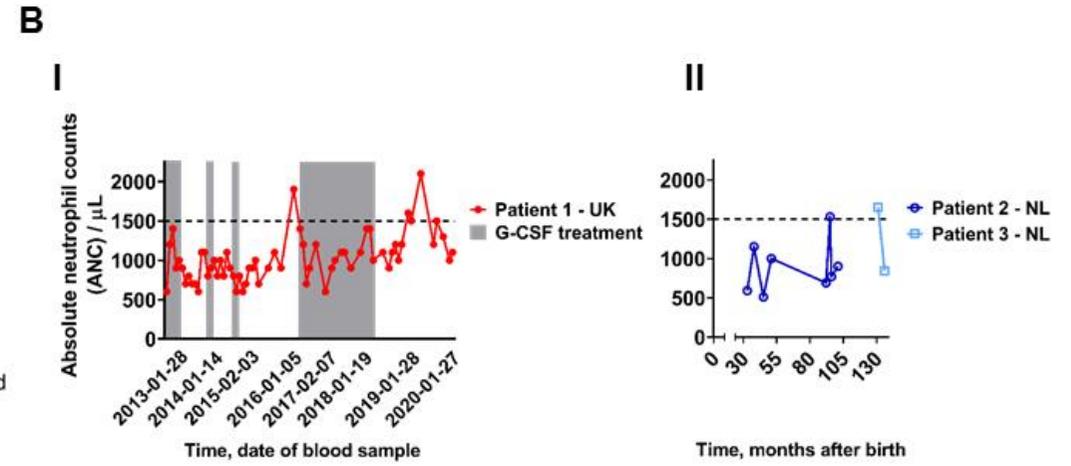
## References

Dong, F., Liu, X., de Koning, J.P., Touw, I.P., Hennighausen, L., Larner, A. & Grimley, P.M. (1998) Stimulation of Stat5 by granulocyte colony-stimulating factor (G-CSF) is modulated by two distinct cytoplasmic regions of the G-CSF receptor. *J Immunol,* **161,** 6503-6509.

Dror, Y., Ward, A.C., Touw, I.P. & Freedman, M.H. (2000) Combined corticosteroid/granulocyte colony-stimulating factor (G-CSF) therapy in the treatment of severe congenital neutropenia unresponsive to G-CSF: Activated glucocorticoid receptors synergize with G-CSF signals. *Exp Hematol,* **28,** 1381-1389.

Druhan, L.J., Ai, J., Massullo, P., Kindwall-Keller, T., Ranalli, M.A. & Avalos, B.R. (2005) Novel mechanism of G-CSF refractoriness in patients with severe congenital neutropenia. *Blood,* **105,** 584-591.

Hoogendijk, A.J., Pourfarzad, F., Aarts, C.E.M., Tool, A.T.J., Hiemstra, I.H., Grassi, L., Frontini, M., Meijer, A.B., van den Biggelaar, M. & Kuijpers, T.W. (2019) Dynamic Transcriptome-Proteome Correlation Networks Reveal Human Myeloid Differentiation and Neutrophil-Specific Programming. *Cell Rep,* **29,** 2505-2519 e2504.

Kawai, T. & Malech, H.L. (2009) WHIM syndrome: congenital immune deficiency disease. *Curr Opin Hematol,* **16,** 20-26.

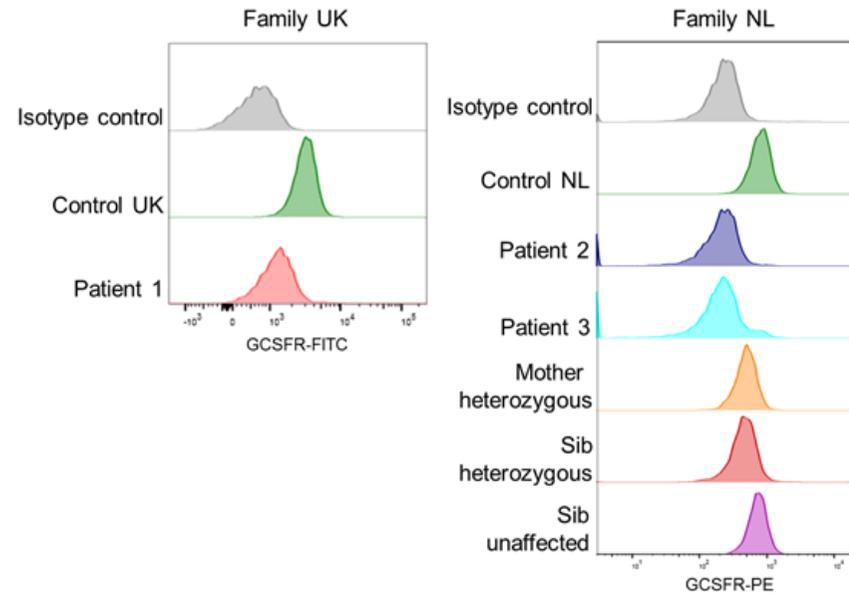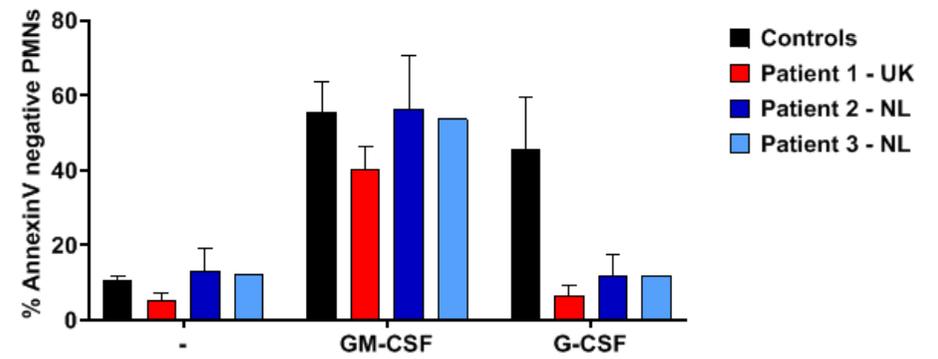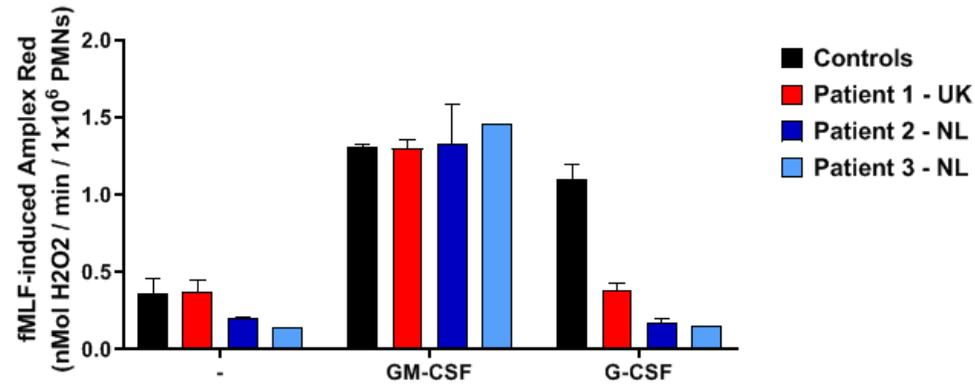Klimiankou, M., Klimenkova, O., Uenalan, M., Zeidler, A., Mellor-Heineke, S., Kandabarau, S., Skokowa, J., Zeidler, C. & Welte, K. (2015) GM-CSF stimulates granulopoiesis in a congenital neutropenia patient with loss-of-function biallelic heterozygous CSF3R mutations. *Blood,* **126,** 1865-1867.

Levesque, J.P., Hendy, J., Takamatsu, Y., Simmons, P.J. & Bendall, L.J. (2003) Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *J Clin Invest,* **111,** 187-196.

Liu, F., Wu, H.Y., Wesselschmidt, R., Kornaga, T. & Link, D.C. (1996) Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice. *Immunity,* **5,** 491-501.

Matmati, M., Pouwels, W., van Bruggen, R., Jansen, M., Hoek, R.M., Verhoeven, A.J. & Hamann, J. (2007) The human EGF-TM7 receptor EMR3 is a marker for mature granulocytes. *J Leukoc Biol,* **81,** 440-448.

Mehta, H.M., Malandra, M. & Corey, S.J. (2015) G-CSF and GM-CSF in Neutropenia. *J Immunol,* **195,** 1341-1349.

Panopoulos, A.D. & Watowich, S.S. (2008) Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and 'emergency' hematopoiesis. *Cytokine,* **42,** 277-288.

Sinha, S., Zhu, Q.S., Romero, G. & Corey, S.J. (2003) Deletional mutation of the external domain of the human granulocyte colony-stimulating factor receptor in a patient with severe chronic neutropenia refractory to granulocyte colony-stimulating factor. *J Pediatr Hematol Oncol,* **25,** 791-796.

Triot, A., Jarvinen, P.M., Arostegui, J.I., Murugan, D., Kohistani, N., Dapena Diaz, J.L., Racek, T., Puchalka, J., Gertz, E.M., Schaffer, A.A., Kotlarz, D., Pfeifer, D., Diaz de Heredia Rubio, C., Ozdemir, M.A., Patiroglu, T., Karakukcu, M., Sanchez de Toledo Codina, J., Yague, J., Touw, I.P., Unal, E. & Klein, C. (2014) Inherited biallelic CSF3R mutations in severe congenital neutropenia. *Blood,* **123,** 3811-3817.

Ward, A.C., van Aesch, Y.M., Gits, J., Schelen, A.M., de Koning, J.P., van Leeuwen, D., Freedman, M.H. & Touw, I.P. (1999) Novel point mutation in the extracellular domain of the granulocyte colony-stimulating factor (G-CSF) receptor in a case of severe congenital neutropenia hyporesponsive to G-CSF treatment. *J Exp Med,* **190,** 497-507.

Yilmaz Karapinar, D., Akinci, B., Sahin Yasar, A., Hekimci Ozdemir, H., Onder Sivis, Z., Onay, H. & Ozkinay, F. (2019) Congenital Neutropenia Patient With Hypomorphic Biallelic CSF3R Mutation Responding to GCSF. *J Pediatr Hematol Oncol,* **41,** e190-e192.

Yilmaz Karapinar, D., Ozdemir, H.H., Akinci, B., Yasar, A.S., Sivis, Z.O., Onay, H. & Ozkinay, F. (2018) Management of a Patient With Congenital Biallelic CSF3R Mutation With GM-CSF. *J Pediatr Hematol Oncol*.

**Figure Legends**

**Fig 1.** *CSF3R* mutations and family pedigrees. (A) Pedigree of the UK patient (panel I) and NL patients (panel II). (B) Absolute neutrophil counts (ANC) of the UK patient during G-CSF treatment (panel I) and the NL patients (no treatment; panel II). Dashed horizontal line represents normal ANC counts. (C) Schematic representation of G-CSF-R showing the various structural domains and their amino acid positions. The zoomed panel shows the conserved WSXWS motif located in the CRH region. Mutations in the extracellular domain of G-CSF-R described in congenital neutropenia patients are displayed above the diagram, novel mutations below. Underlined mutations represent homozygous mutations. Boxes with identical color represent mutations in same patient/family. aa = amino acid (D) Cytospins of isolated neutrophils from controls and patients (original magnification x400; May-Grünwald/Giemsa stain). ¥ = apoptotic cell, * = eosinophil.

**Fig 2.** Functional characteristics of G-CSF-R-deficient primary neutrophils. (A) G-CSF-R expression on neutrophils was assessed by flow cytometry. (B) Viability upon overnight incubation with GM-CSF (20 ng/mL) or G-CSF (20 ng/mL) was assessed by flow cytometry (mean + range, n = 2 of patient 1 and 2, n = 1 of patient 3). (C) Priming capacity of neutrophils was assessed upon 30 minute pre-incubation with GM-CSF or G-CSF and subsequent stimulation with fMLF (1 µM) . Extracellular $H_2O_2$ was measured by Amplex Red assay (mean + range, n = 2 of patient 1 and 2, n = 1 of patient 3). (D) Phosphorylation of STAT-3 (p-STAT3) in neutrophils upon stimulation with GM-CSF and G-CSF (time of stimulation shown in minutes) was assessed by Western blot. GAPDH was used as loading control. Western blot of patient 3 is shown as representative blot.

**A**

I
Family UK

Pt1

II
Family NL

2003    Pt3      Pt2     2014    Not tested
        2008     2011            2017

**B**

I

Absolute neutrophil counts (ANC) / µL

2000
1500
1000
500
0

2013-01-28  2014-01-14  2015-02-03  2016-01-05  2017-02-07  2018-01-19  2019-01-28  2020-01-27

Time, date of blood sample

- Patient 1 - UK
- G-CSF treatment

II

2000
1500
1000
500
0

0   30   55   80   105   130

Time, months after birth

- Patient 2 - NL
- Patient 3 - NL

**C**

c.922C>T p.(R308C)
c.948_963del16 p.(H317Afs*6)
c.955_1145del p.(S319Gfs*30)
c.964_1145del p.(S322Gfs*30)

c.266T>C p.(L89P)
c.569G>A p.(R190H)
c.610_611delCAinsAG p.(Q204R)
c.686C>A p.(P229H)

c.998-2A>T
c.1245delG p.(T416Pfs*17)

c.1640G>A p.(W547*)

WSXWS 318-322

25-117   125-230   233-332   334-430   431-528   530-623   628-650   651-836aa

Ig-like    CRH region    FNIII    FNIII    FNIII    TM    Cytoplasmic region

BN    BC

Patient 1
c. 359G>A p.(G120D)

Patients 2 + 3
c.1318C>T p.(R440*)

Patient 1
c.1640G>A p.(W547*)

**D**

Control UK          Mother Patient 1          Patient 1

Control NL          Patient 2                 Patient 3

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