To the editor:

Two cases of cyclic neutropenia with acquired *CSF3R* mutations, with 1 developing AML

Maksim Klimiankou,[1,*] Sabine Mellor-Heineke,[2,*] Olga Klimenkova,[1] Elisa Reinel,[1] Murat Uenalan,[3] Siarhei Kandabarau,[2] Julia Skokowa,[1] Karl Welte,[4,†] and Cornelia Zeidler[2,†]

[1]Department of Hematology, Oncology, Immunology, Rheumatology, and Pulmonology, University Hospital Tuebingen, Tuebingen, Germany; [2]Department of Molecular Hematopoiesis and Severe Chronic Neutropenia International Registry, and [3]Department of Experimental Hematology, Hannover Medical School, Hannover, Germany; and [4]Department of Pediatrics, University Hospital Tuebingen, Tuebingen, Germany

Congenital neutropenia (CN) and cyclic neutropenia (CyN) are rare genetic disorders of hematopoiesis predominantly caused by *ELANE* mutations.[1-3] Due to overlaps in their genetic profiles, CyN can be distinguished from CN by cycling neutrophil counts, usually at 21-day intervals, in the former. In contrast to CN, CyN is also characterized by cycling of platelets, monocytes, and reticulocytes.[4,5] Infectious episodes are usually less severe in patients with CyN vs CN. Although *CSF3R* mutations are frequent in patients with CN and these patients are at increased risk of leukemic transformation, *CSF3R* mutations and transformation to acute myeloid leukemia (AML) have not been reported to date in patients with CyN.[6-11]

This report describes a 17-year-old female with CyN who developed AML (French-American-British classification M2). She was first diagnosed with severe neutropenia at age 4 weeks while experiencing *Pseudomonas* septicemia; at that time, serial blood counts were not collected. Treatment with granulocyte colony-stimulating factor (G-CSF), at a dose of 5.7 μg/kg per day, was initiated at age 2 years. The amount of G-CSF administered was not adjusted for increasing body weight, resulting in a dose of 1.7 μg/kg per day at age 13 years. After a liver abscess at age 14 years, she was referred to our center. She presented with large variations in absolute neutrophil count (ANC), suggesting a cyclic pattern of ANC. Because her median ANC was deemed insufficient, her G-CSF dose was increased to 7.5 μg/kg per day. This relatively high dosage is still in the range of G-CSF dosages required for CyN patients (0.5-10.5 μg/kg per day); however, it is higher than the median dose (2.6 μg/kg per day) in CyN patients within our European branch of the Severe Chronic Neutropenia International Registry (SCNIR) and might be associated with the risk of malignant development. Sequential neutrophil counts, determined at least 3 times per week for 6 weeks, over 2 time periods 4 months apart, confirmed a diagnosis of CyN (Figure 1A-B). Cycling platelet counts have been reported previously in patients with CyN, but not CN.[4,5] No cytogenetic aberrations were detected prior to a diagnosis of AML. BM at the time of AML showed 33% blasts, monosomy 7, and trisomy 21. The patient recently received a BM transplant from an HLA-matched unrelated donor and was released from the hospital after successful engraftment.

Interestingly, this CyN-AML patient was found to harbor 2 *ELANE* mutations, p.Ala233Pro and p.Val235TrpfsX (NP_001963.1), both located on 1 allele (Figure 1C). The *ELANE* fragment spanning c.697G<C and c.703delG was cloned from genomic DNA isolated from this patient's peripheral blood MNCs. Sequencing of 20 individual bacterial clones showed that all clones carried both *ELANE* mutations (Figure 1C). Sequencing of DNA isolated from peripheral blood MNCs of both parents revealed no *ELANE* mutations (Figure 1C). Moreover, neither parent has shown signs of neutropenia or episodes of bacterial infections. Interestingly, the levels of expression of ELANE protein were much lower in BM polymorphonuclear cells of this CyN-AML patient as compared with healthy controls (Figure 1D).

CN is a preleukemic condition, with >20% of patients developing leukemia after 20 years.[6-11] Approximately 80% of CN patients who develop AML are heterozygous for *CSF3R* mutations, suggesting the involvement of these mutations in leukemogenesis.[6-12] To date, *CSF3R* mutations have never been reported in patients with CyN.[7] Deep sequencing of DNA from this patient's BM MNCs obtained 3 years before the diagnosis of AML revealed a clone with the acquired *CSF3R* mutation p.Gln741X at a 3% allele frequency (Figure 2A). Two years later, or 1 year before AML diagnosis, the *CSF3R* mutant allele frequency in this patient's BM MNCs was 8%. At the time of AML diagnosis, all *CSF3R*-expressing BM MNCs (predominantly leukemic blasts) were positive for the p.Gln741X mutation (Figure 2A). Because cooperative *RUNX1* and *CSF3R* mutations are present in >65% of CN-AML/MDS patients,[12] we tested the patient's BM MNCs, obtained at the time of leukemia development, for *RUNX1* mutations, finding that the *RUNX1* mutation p.Asp171Asn was present at an allele frequency of 10%.

A CFU assay was performed to determine the stage of myeloid differentiation at which *CSF3R* mutations occurred in this CyN-AML patient. Of the BM MNCs isolated at the time of overt AML, 80% were abnormal CFU-blasts, 16% were CFU-G colonies, and 4% were CFU-GM colonies (Figure 2B). All 22 CFU-blast colonies sequenced were positive for the *CSF3R* p.Gln741X and *RUNX1* p.Asp171Asn mutations (Figure 2C).

To determine whether other CyN patients harbor *CSF3R* mutations, we performed deep sequencing of *CSF3R* in 18 additional CyN patients, finding that cell clones from 1 patient aged 15.4 years had an acquired *CSF3R* mutation. This patient and her sister had inherited CyN from their father, with all 3 harboring an *ELANE* p.Val190_Phe199del (NP_001963.1) mutation and presenting with cycling hematopoiesis (Figure 2D). Time-course analysis of the acquisition of *CSF3R* mutations in this patient showed that 2.6% of the *CSF3R* alleles in BM MNCs obtained at age 13 years possessed the p.Gln749X mutation, with the mutant allele frequency increasing to 9% and 8% after an additional 1.5 and 2.4 years, respectively. At the last time point, an additional minor mutant clone was identified, with the *CSF3R* mutation p.Gln739X and an allele frequency of 0.34% (Figure 2E). This CyN patient has no signs of AML or myelodysplastic syndrome (MDS). Taken together, these findings suggest that CyN patients with familial type inheritance and typical CyN-associated *ELANE* mutations also may acquire *CSF3R* mutations.

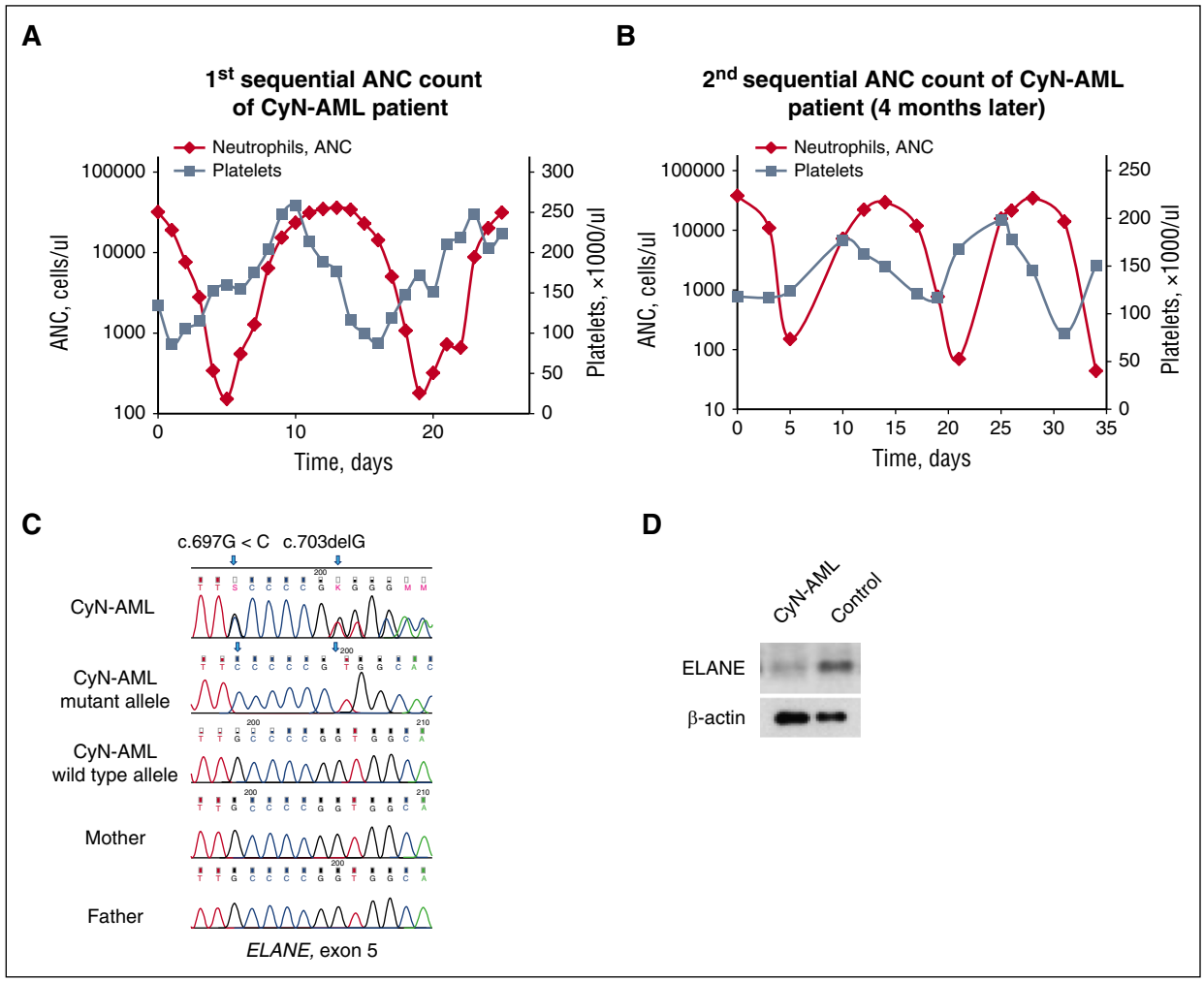

Figure 1. Patient characteristics associated with neutropenia. (A-B) Representative patterns of neutrophil (blue line) and platelet (orange line) cycling in the CyN-AML patient during G-CSF treatment. ANCs and platelet counts were plotted for 25 days (left) and for 35 days (right), accordingly. (C) Genetic characterization of double de novo *ELANE* mutations in the CyN-AML patient. The blue arrows above the Sanger sequencing traces indicate the positions of the mutations. Cloning of a genomic DNA fragment spanning *ELANE* c.697G<C and c.703delG showed that both mutations were present on the same allele. Two representative examples of sequenced clones are shown. Sequencing of *ELANE* exon 5 revealed no *ELANE* mutations in parental DNA isolated from peripheral blood MNCs. (D) Western blot analysis of ELANE protein expression in BM polymorphonuclear cells of the CyN-AML patient and healthy donors. β-actin was used as a loading control. The level of ELANE protein expression was lower in BM polymorphonuclear cells of the CyN-AML patient as compared with healthy donors. The blot shown is representative of 3 independent experiments. BM, bone marrow; MNC, mononuclear cell.

To our knowledge, this is the first report of acquired *CSF3R* mutations in CyN patients, with 1 patient subsequently developing AML. The diagnosis of CyN in the first patient was verified through 2 independent courses of neutrophil counts, with this patient also showing cycling of platelets and monocytes. The second patient, with inherited CyN, was positive for an acquired *CSF3R* mutation, but has no signs of AML or MDS. Long-term data of the SCNIR have shown no risks of acquisition of *CSF3R* mutations and of myeloid transformation in patients with CyN to date.[7,8] The risk of malignant transformation in patients who acquired *CSF3R* mutations, however, has been documented in patients with CN. In our recent analyses using the same deep-sequencing technology as has been used for the 2 CyN patients, we searched for the presence of *CSF3R* mutations in 45 patients with *ELANE*-positive CN. Sixteen of the 45 patients (35.5%) harbored *CSF3R* mutations; 8 of them (18%) had developed AML. The incidence of *CSF3R* mutations in CN suggests that clonal populations with *CSF3R* mutations can expand in CN patients and never evolve into malignant hematopoiesis. This finding may have parallels with recent studies showing that clonal populations with mutations in AML-associated genes are common in older adults and much more common than the frequency of AML.[13]

Because we have identified only 2 CyN patients with *CSF3R* mutations, with 1 developing AML and the other without malignant transformation to date, the clinical importance of annual *CSF3R* mutational analysis to identify CyN patients at high risk of developing leukemia remains unanswered. The CyN-AML patient we identified differs from other patients with CyN in that she required doses of G-CSF that were high (but still within the treatment range of CyN patients in our SCNIR). Genotype–phenotype correlations indicate that the same *ELANE* mutation can result in either CN or CyN, depending on the genetic background.[10,14] The second CyN patient described here had typical familial CyN, inheriting from her father the *ELANE* p.Val190_Phe199del mutation, which is common in both CyN and CN. She responded to a very low dose (1.5 μg/kg per day) G-CSF therapy. Although CN and CyN are closely related disorders with overlapping molecular and clinical phenotypes,[2] platelet cycling was not reported in CN patients in the SCNIR,[7] suggesting that both

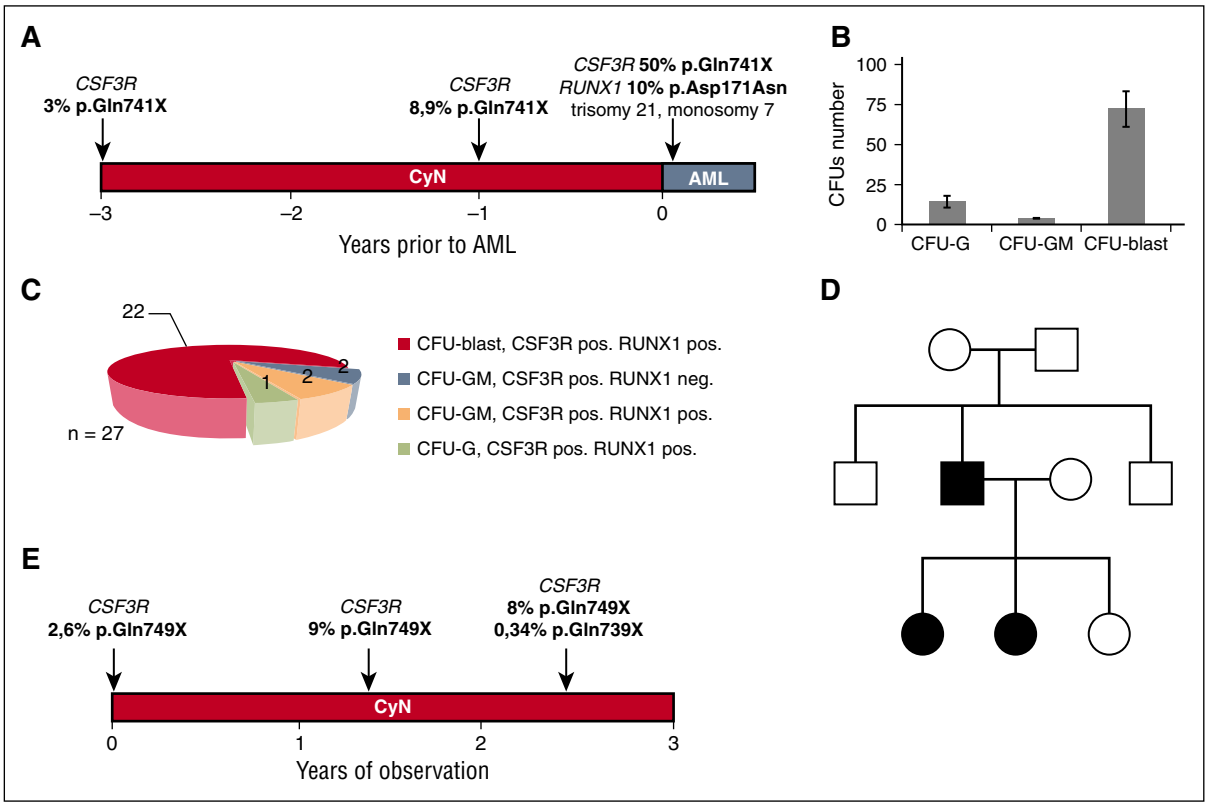

Figure 2. Acquired *CSF3R* mutations in the CyN-AML and CyN patients described in this study. (A) The time course and frequency of *CSF3R* mutations in the CyN-AML patient, as determined by deep sequencing, for 3 years prior to the development of overt AML. Mutant allele frequency and mutation position are indicated above each time point. All mutations were considered heterozygous, and the number of cells with a *CSF3R* mutation was estimated to be twice the frequency of the mutant allele. The *RUNX1* mutation p.Asp171Asn was present only after development of MDS. (B) CFU-blast colonies were predominant after incubation for 14 days with a cytokine cocktail, consisting of 10 ng/mL rhG-CSF, 10 ng/mL rhGM-CSF, 10 ng/mL rhIL-3, 10 ng/mL rhSCF, and 1 U/mL rhEPO. (C) Diagram of the distribution of *CSF3R* and *RUNX1* mutations in different CFU colonies isolated from a sample from the CyN-AML patient taken after the development of AML. (D) Autosomal dominant inheritance of the *ELANE* mutation p.Val190_Phe199del in a CyN patient with an acquired *CSF3R* mutation. The *ELANE* mutation was inherited by 2 daughters from their affected father, who also showed cyclic hematopoiesis. (E) Time course and frequency of *CSF3R* mutations in a patient with familial CyN, as determined by deep sequencing, starting from the date of the first mutation analysis. Mutant allele frequency and mutation position are indicated above each time point. All mutations were considered heterozygous, and the number of cells with *CSF3R* mutations was estimated to be twice the frequency of the mutant allele. CFU, colony-forming unit; EPO, erythropoietin; G, granulocyte; GM, granulocyte-macrophage; IL, interleukin; rh, recombinant human.

patients described in this report had classical CyN, not masked CN. Haurie et al reported that in CyN, the available evidence indicates a broad involvement of the entire hematopoietic system, because cycling is typically observed in more than 1 of the mature hematopoietic cell types.[15] This characterization is in agreement with our definition of CyN. In our opinion, the clinician has to decide on the clinical and molecular data available to classify a patient as CyN or CN, and prognostic counseling is based on this clinical classification.

*M.K. and S.M.-H. contributed equally to this study.

†K.W. and C.Z. contributed equally to this study.

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Contribution: M.K. performed the main experiments; C.Z. and S.M.-H. provided the patient material and clinical data; E.R. performed the Sanger sequencing and cloning; O.K. performed the CFU assays; M.U., S.K., and M.K. analyzed the deep-sequencing data of *CSF3R*; and J.S., K.W., C.Z., S.M.-H., and M.K. analyzed the data and wrote the manuscript.

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Correspondence: Karl Welte, University Children's Hospital, Hoppe-Seyler-Strasse 1, 72076 Tübingen, Germany; e-mail: karl.welte@med.uni-tuebingen.de.

References

1. Horwitz M, Benson KF, Person RE, Aprikyan AG, Dale DC. Mutations in ELA2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. *Nat Genet.* 1999;23(4):433-436.
2. Dale DC, Person RE, Bolyard AA, et al. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. *Blood.* 2000;96(7):2317-2322.
3. Horwitz MS, Corey SJ, Grimes HL, Tidwell T. ELANE mutations in cyclic and severe congenital neutropenia: genetics and pathophysiology. *Hematol Oncol Clin North Am.* 2013;27(1):19-41, vii. [vii.].
4. Hammond WP IV, Price TH, Souza LM, Dale DC. Treatment of cyclic neutropenia with granulocyte colony-stimulating factor. *N Engl J Med.* 1989;320(20):1306-1311.
5. Wright DG, Kenney RF, Oette DH, LaRussa VF, Boxer LA, Malech HL. Contrasting effects of recombinant human granulocyte-macrophage colony-stimulating factor (CSF) and granulocyte CSF treatment on the cycling of blood elements in childhood-onset cyclic neutropenia. *Blood.* 1994;84(4):1257-1267.
6. Touw IP. Game of clones: the genomic evolution of severe congenital neutropenia. *Hematology Am Soc Hematol Educ Program.* 2015;2015(1):1-7.
7. Zeidler C, Germeshausen M, Klein C, Welte K. Clinical implications of ELA2-, HAX1-, and G-CSF-receptor (CSF3R) mutations in severe congenital neutropenia. *Br J Haematol.* 2009;144(4):459-467.
8. Germeshausen M, Ballmaier M, Welte K. Incidence of CSF3R mutations in severe congenital neutropenia and relevance for leukemogenesis: Results of a long-term survey. *Blood.* 2007;109(1):93-99.

9. Donadieu J, Leblanc T, Bader Meunier B, et al; French Severe Chronic Neutropenia Study Group; Experience of the French Severe Chronic Neutropenia Study Group. Analysis of risk factors for myelodysplasias, leukemias and death from infection among patients with congenital neutropenia. *Haematologica.* 2005;90(1):45-53.
10. Makaryan V, Zeidler C, Bolyard AA, et al. The diversity of mutations and clinical outcomes for ELANE-associated neutropenia. *Curr Opin Hematol.* 2015;22(1): 3-11.
11. Rosenberg PS, Zeidler C, Bolyard AA, et al. Stable long-term risk of leukaemia in patients with severe congenital neutropenia maintained on G-CSF therapy. *Br J Haematol.* 2010;150(2):196-199.
12. Skokowa J, Steinemann D, Katsman-Kuipers JE, et al. Cooperativity of RUNX1 and CSF3R mutations in severe congenital neutropenia: a unique pathway in myeloid leukemogenesis. *Blood.* 2014;123(14):2229-2237.
13. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med.* 2014;371(26):2488-2498.
14. Newburger PE, Pindyck TN, Zhu Z, et al. Cyclic neutropenia and severe congenital neutropenia in patients with a shared ELANE mutation and paternal haplotype: evidence for phenotype determination by modifying genes. *Pediatr Blood Cancer.* 2010;55(2):314-317.
15. Haurie C, Dale DC, Mackey MC. Cyclical neutropenia and other periodic hematological disorders: a review of mechanisms and mathematical models. *Blood.* 1998;92(8):2629-2640.

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To the editor:

Mutations in succinate dehydrogenase B (*SDHB*) enhance neutrophil survival independent of HIF-1α expression

Robert Jones,[1,2,*] Kate E. McDonald,[1,2,*] Joseph A. Willson,[3] Bart Ghesquière,[4,5] David Sammut,[1] Eleni Daniel,[2] Alison J. Harris,[3] Amy Lewis,[1] A. A. Roger Thompson,[1] Rebecca S. Dickinson,[3] Tracie Plant,[3] Fiona Murphy,[3] Pranvera Sadiku,[3] Brian G. Keevil,[6] Peter Carmeliet,[4,5] Moira K. B. Whyte,[3] John Newell-Price,[2,†] and Sarah R. Walmsley[3,†]

[1]Department of Infection, Immunity, and Cardiovascular Disease and [2]Department of Oncology and Metabolism, University of Sheffield, Sheffield, United Kingdom; [3]Medical Research Council/University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom; [4]Laboratory of Angiogenesis and Vascular Metabolism, Vesalius Research Center, VIB, Leuven, Belgium; [5]Department of Oncology, KU Leuven, Leuven, Belgium; and [6]School of Medicine, University of Manchester, Manchester, United Kingdom

Neutrophils are unusual in their reliance on glycolysis to maintain their energy requirements[1] despite the presence of mitochondria and tricarboxylic acid (TCA) cycle intermediaries.[2] This metabolic adaptation is thought in part to underpin their survival and antimicrobial function in tissues that are typically hypoxic.[3-5] Despite their unique metabolism, little is known about the importance of flux between metabolic pathways in determining neutrophil survival responses. Recent work has demonstrated the importance of the hypoxia-inducible factor (HIF)/prolyl hydroxylase domain (PHD)–containing enzyme oxygen-sensing pathway in this regard, identifying both HIF-1α and PHD3 as critical regulators of neutrophil survival in hypoxia,[6,7] with the extended survival of neutrophils in hypoxia being dependent on HIF-1α expression. In parallel, an expanding body of work has addressed the role of HIF-1α in coordinating macrophage functional responses to proinflammatory mediators.[8-11] This work led to the observation that, in macrophages, lipopolysaccharide (LPS) causes an intracellular increase in succinate levels, resulting in HIF-1α stabilization and enhanced interleukin-1β signaling.[11] Subsequently, the metabolic rewiring of antimicrobial (M1) and tissue repair (M2) macrophages has been elucidated, with important consequences of TCA cycle activity and integrity for regulation of nitric oxide and N-glycosylation signaling, respectively.[12] Whether TCA cycle activity and succinate accumulation regulates HIF-1α and hypoxic survival in neutrophils is unknown.

Patients with rare germ line mutations in genes encoding the TCA cycle enzyme succinate dehydrogenase (SDH) allow us to directly question the role of the TCA cycle and mitochondrial respiratory chain in neutrophil survival responses. SDH oxidizes succinate to fumarate in the TCA cycle and is a ubiquinone oxidoreductase, also functioning in complex II of the respiratory chain.[13] SDH comprises four subunits (A-D), with inherited mutations of each of the subunits linked to the development of pheochromocytoma (PHEO) and paraganglioma (PGL) after somatic inactivation of the wild-type allele and loss of heterozygosity.[14-16] We questioned whether heterozygous germ line mutations in *SDHB (SDHBx)* would reduce SDH activity in the peripheral blood neutrophils of these patients, leading to accumulation of intracellular succinate, HIF-1α stabilization, and a pseudohypoxic survival phenotype, given the importance of the B subunit for SDH catalytic function and its high prevalence within PHEO/PGL patient populations.[13,17,18]

To determine whether succinate is implicated in regulating neutrophil survival responses, we isolated peripheral blood neutrophils from patients with heterozygous germ line *SDHBx* mutations in whom an increase in intracellular succinate would be predicted. In total, 20 individuals with frameshift, splice, missense, or nonsense mutations were studied (supplemental Table 1, available on the *Blood* Web site). Although all but 1 patient displayed plasma succinate levels within the normal range, a significantly higher plasma succinate level was observed in patients with *SDHBx* (Figure 1A). To confirm the consequence of *SDHB* mutations on intracellular succinate and to measure other TCA cycle and glycolytic intermediaries, peripheral blood neutrophils were isolated from 3 individuals with *SDHBx* and 3 healthy controls, and relative metabolite abundance was determined by gas chromatography–mass spectrometry (Figure 1B). Succinate was significantly more abundant in neutrophils isolated from patients with *SDHBx* than from controls. This finding was paralleled by increases in lactic acid and citric acid, but no changes in other TCA cycle intermediaries (α-ketoglutaric acid, fumaric acid, or malic acid) were observed. Thus, neutrophils heterozygous for mutant *SDHB* gene expression display the predicted elevation in intracellular succinate, but with no decrease in downstream TCA cycle intermediaries. Citric acid levels were increased, which may reflect an increase in biosynthetic requirements outside the TCA cycle. In keeping with the increased succinate in *SDHBx* neutrophils, a detectable increase in protein succinylation was also observed (Figure 1C).