Expanding the genetic landscape of congenital neutropenia: *CXCR2* mutations in three families revealed through whole exome sequencing

The list of genes associated with severe congenital neutropenia (CN), a pathogenic condition characterized by a profound decrease in neutrophil numbers and high susceptibility to bacterial infections, is constantly growing.[1] While most patients with CN receive a genetic diagnosis based on the known causative genes, 22% of CN patients, according to the Severe Congenital Neutropenia International Registry database, do not have an identified genetic mutation.[2] In this study, we performed whole exome sequencing (WES) and prioritization of variants in three non-consanguineous families with genetically unclassified neutropenia after negative Sanger sequencing of *ELANE, HAX1*, and *JAGN1*, genes most frequently associated with CN. All subjects studied provided written informed consent according to the Institutional Review Board of the University Hospital of Heraklion.

The index case (II:1) in family A (Figure 1) was identified at the age of 30 years with long-standing severe neutropenia with an average absolute neutrophil count (ANC) 0.3×10^9/L and frequent upper respiratory tract and skin infections. At that time, the neutropenia was characterized as autoimmune, based on the positive granulocyte immunofluorescence and granulocyte agglutination tests, associated with the presence of an IgG autoantibody with strong positivity against Fcγ-RIIIb in the monoclonal antibody immobilization of granulocyte antigen (MAIGA) assay. The bone marrow (BM) aspiration and biopsy revealed an increased number of myeloid cells at all stages of differentiation with particularly increased BM neutrophils and with a myeloid:erythroid cell ratio of approximately 7:1. The patient commenced on recombinant human granulocyte colony stimulating factor (rhG-CSF) and responded well to low doses of lenograstim, i.e., 131.5 μg/day (2 μg/kg/day) every 3 days, with normal ANC and resolution of infections. A detailed family investigation during the follow-up showed that the patient's mother and two siblings had long-standing mild neutropenia (average ANC 1.5×10^9/L) with absence of anti-neutrophil antibodies and no history of infections.

The index case (II:4) in family B (Figure 1) was diagnosed with long-standing severe neutropenia (average ANC 0.5×10^9/L) at the age of 14 years. She had a long history of infectious episodes of pharyngitis and bronchitis (1-3 times per year) and recurrent oral ulcerations (more than 12 times per year). The BM aspiration and biopsy showed an increased number of myeloid cells with normal maturation and a myeloid: erythroid cell ratio of 6:1.

The index case (II:6) in family C (Figure 1) was diagnosed at

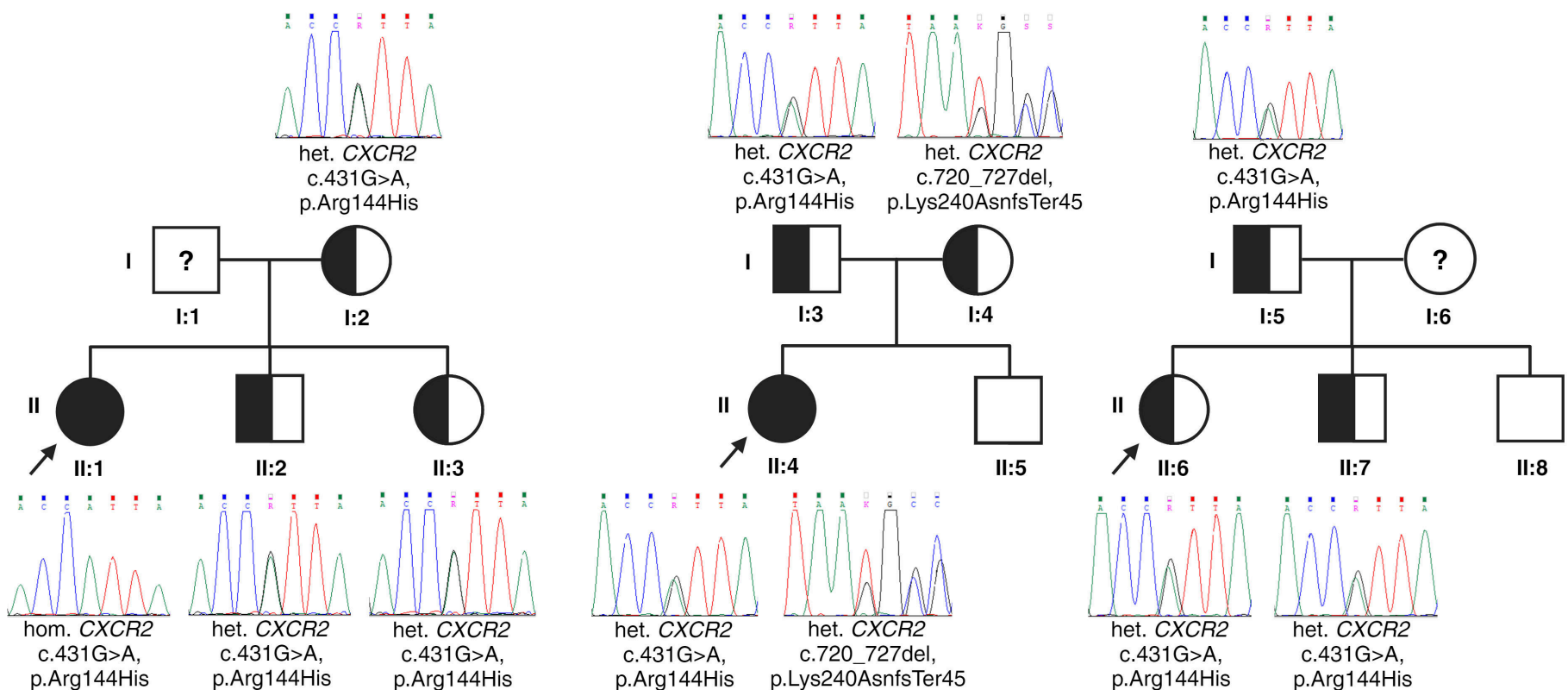

Figure 1. *CXCR2* variants identified in three unrelated families. Family pedigrees with identified homozygous (patient II:1), compound heterozygous (patient II:4), and heterozygous (subjects I:2, I:3, I:4, I:5, II:2, II:3, II:6, II:7) *CXCR2* variants. The results of whole exome sequencing were further confirmed by Sanger sequencing. The arrows show the index cases initially identified as neutropenia cases.

the age of 32 years with long-standing mild neutropenia (average ANC 1.2x10^9/L) without a history of recurrent infections. A BM aspiration and biopsy have not been performed in this patient. The family history revealed that one of the patient's brothers (II:7) had chronic mild neutropenia, too. The clinical and laboratory findings of the neutropenic members of the three families are summarized in Table 1.

WES analysis of DNA samples isolated from peripheral blood (PB) of members of family A revealed a *CXCR2* homozygous variant ENST00000318507.7:c.431G>A, p.Arg144His in the index case (II:1). The mother (I:2) and two siblings (II:2 and II:3) were heterozygous carriers of the *CXCR2* p.Arg144His variant (Figure 1). WES analysis in family B showed that the index case (II:4) carried compound heterozygous variants of *CXCR2*, i.e., ENST00000318507.7:c.431G>A, p.Arg144His and ENST00000318507.7:c.720_727del, p.Lys240AsnfsTer45. Sanger sequencing of the first generation of family B revealed that the parents (I:3 and I:4) were heterozygous carriers of missense *CXCR2* p.Arg144His and frameshift *CXCR2* p.Lys240AsnfsTer45 variants. The shift of the reading frame due to 8 bp deletion leads to the loss of one-third of the CXCR2 protein sequence. In family C, the index case (II:6), her brother (II:7) and father (I:5) were heterozygous carriers of the *CXCR2* p.Arg144His variant. The results of WES were further confirmed by Sanger sequencing.

The *CXCR2* p.Lys240AsnfsTer45 variant identified in family B (cases II:4 and I:4) has not been reported in the gnomAD and ClinVar databases. A homozygous missense variant in *CXCR2* that affects Arg144 but leads to another amino acid exchange (Cys) has been recently reported in a CN patient with CXCR2 deficiency.[3] The pathogenicity of missense *CXCR2* p.Arg144His variant was estimated using combined annotation-dependent depletion (CADD), sorting intolerant from tolerant (SIFT), polymorphism phenotyping v2 (PolyPhen-2), and REVEL, and CADD tools. All algorithms predicted the damaging effect of p.Arg144His. An allele frequency of *CXCR2* p.Arg144His in the genome aggregation database (gnomAD) comprises 0,002%, with no homozygous carriers recorded. According to ACMG/AMP guidelines for the interpretation of sequence variants,[4] *CXCR2* p.Arg144His variant was classified as "likely pathogenic" supported by the following ACMG/AMP criteria: PP3, PM2, and PM5. The classification of *CXCR2* p.Lys240AsnfsTer45 as "likely pathogenic" was supported by the fulfillment of ACMG/AMP criteria PVS1 and PM2.

Recently it was shown that cell-surface CXCR2 expression in neutrophils is reduced in patients with *CXCR2* deletion or p.Arg144Cys missense variant but not p.Arg212Trp variant.[3] We thus evaluated surface CXCR2 expression in PB neutrophils from members of all three families as well as healthy individuals by flow cytometry. We observed a mutant-dosage effect in the surface expression of CXCR2. The subjects carrying biallelic variants (homozygous index case from family A (II:1) and compound heterozygous index case from family B (II:4) demonstrated decreased expression of surface CXCR2 in comparison to healthy individuals (Figure 2A). The heterozygous parents from family B

Table 1. Clinical profile and laboratory data of the neutropenic family members.

Clinical and laboratory parameters	Family A				Family B	Family C	
	Subject I:2, heterozygous *CXCR2* p.Arg144His (mother)	Patient II:1, homozygous *CXCR2* p.Arg144His (daughter)#	Subject II:2, heterozygous *CXCR2* p.Arg144His (son)	Subject II:3, heterozygous *CXCR2* p.Arg144His (daughter)	Patient II:4, heterozygous *CXCR2* p.Arg144His, heterozygous *CXCR2* p.Lys240AsnfsTer45 (daughter)	Patient II:6, heterozygous *CXCR2* p.Arg144His, (daughter)	Subject II:7, heterozygous *CXCR2* p.Arg144His, (son)
Clinical data							
Age in years at diagnosis	56	30	43	28	14	27	30
Infections	No	Yes	No	No	Yes	No	No
rhG-CSF treatment	No	Yes	No	No	No	No	No
Hematological values							
WBC x10^9/L	3.8	2.0	2.7	3.5	3.5	3.9	3.4
Neutrophils x10^9/L	1.7	0.3	1.0	1.7	0.5	1.2	1.3
Lymphocytes x10^9/L	1.5	1.3	1.0	1.3	2.4	2.2	1.6
Monocytes x10^9/L	0.5	0.3	0.5	0.4	0.3	0.3	0.3
Hemoglobin g/dL	12.9	12.6	14.4	13.6	12.6	12.4	13.3
Hematocrit %	38.7	37.8	43.3	40.8	36.9	37.3	39.5
Platelets x10^9/L	228	294	191	202	254	202	158
Immunoglobulin levels mg/dL							
IgG (701-1,600)*	960	2,240	1,310	1,150	2,300	1,040	1,390
IgM (25-170)*	77	242	64.9	148	121	127	83.4
IgA (48-368)*	321	644	253	172	431	181	249

*The parenthesis shows the normal range values. #This patient displayed positive autoantibodies against Fcγ-RIIIb in addition to the homozygous *CXCR2* variant. WBC: white blood cells; MCV: mean corpuscular volume; rhG-CSF: recombinant human granulocyte colony-stimulating factor.

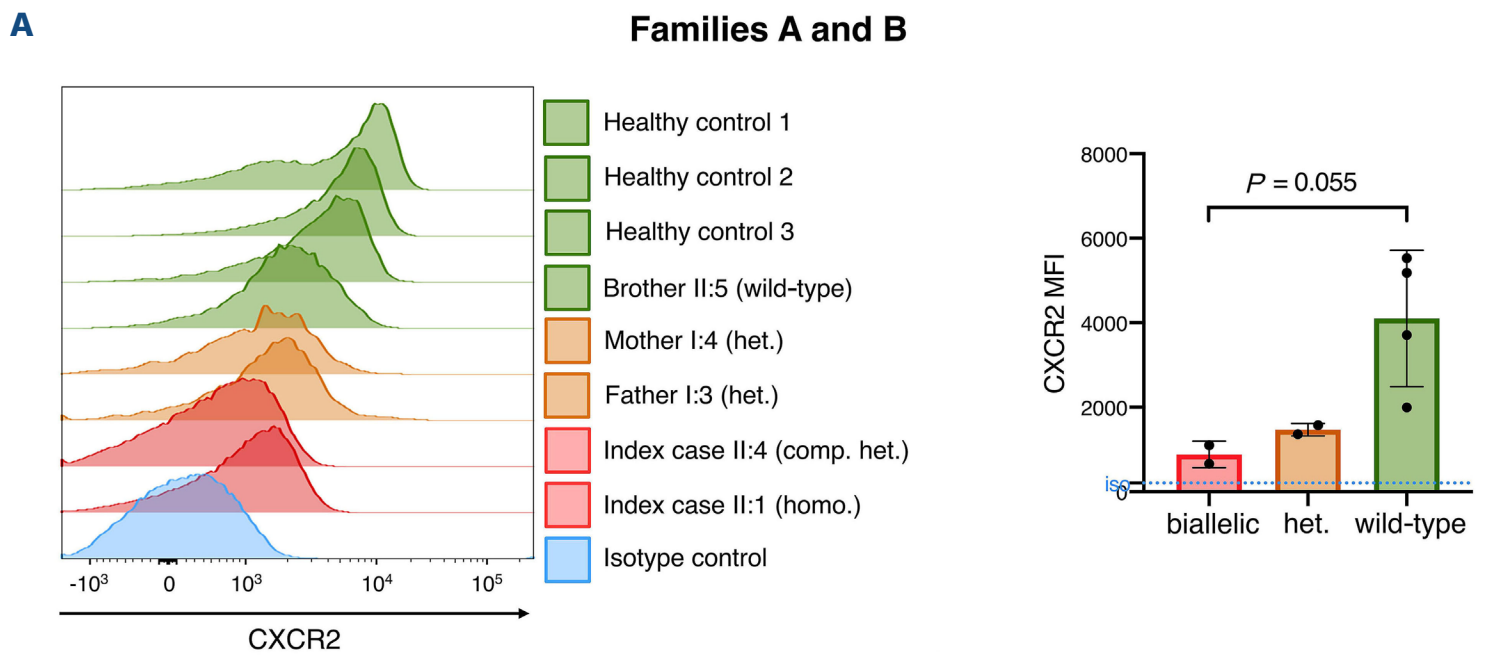

Figure 2. Reduced neutrophil surface expression of CXCR2 and neutrophil migration capacity in subjects carrying *CXCR2* variants. (A, B) Flow cytometry analyses (FACS) (left) and mean fluorescence intensity (MFI) quantification (right) of CXCR2 expression on peripheral blood neutrophils in members of families A and B (A), family C (B), and healthy individuals. For families A and B, neutrophils were isolated using density gradient centrifugation or EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies, Catalog # 19666) according to manufacturer's protocol. The multi-color flow cytometry antibody panel compromised CD45 BV510, CD15 PE, CD16 APC, and CXCR2/IL-8RB FITC. 7-aminoactinomycin D was used to assess viability. Neutrophils were defined as $CD45^{+}CD15^{+}CD16^{+}$ cells. Multi-color FACS analysis was performed using BD FACSCanto™ II flow cytometer. For family

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C, neutrophils were isolated using EasySep™ Direct Human Neutrophil Isolation Kit. Surface expression of CXCR2 was evaluated using human CXCR2/IL-8RB FITC conjugated antibody, and the analysis was performed using BD LSRFortessa™ cell analyzer. Red represents biallelic (homozygous and compound heterozygous) subjects, orange represents heterozygotes, and green represents wild-type individuals. Isotype control is indicated in blue. Graphs display individual experiments. In MFI plot, dots represent individual subjects, bars represent median values for each group, whiskers represent standard deviation. Statistical analysis was performed using Kruskal-Wallis with Dunn's multiple comparisons test. (C) Impaired CXCL8-induced neutrophil chemotaxis in patients II:1 (homozygote) and II:4 (compound heterozygote). Chemotaxis assays were performed in triplicate, with neutrophils purified from whole blood with EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies, Catalog # 19666). Blood samples from all the subjects were collected simultaneously and processed equally. The chemotaxis experiments were performed using the IncuCyte® Chemotaxis Assay (Essen BioScience); samples were added in the upper wells, and CXCL8 (5 nM or 20 nM) or vehicle were added in the bottom wells. Cells in the upper wells (responding cells) were tracked and counted every 30 minutes for 15 hours in the IncuCyte Live-Cell Analysis System. Results are expressed as a percentage of responding neutrophils.

(I:3 and I:4) had intermediate CXCR2 expression between the compound heterozygous index case (II:4) and healthy controls (Figure 2A). In family C, all heterozygous subjects (I:5, II:6, II:7) also displayed decreased surface expression of CXCR2 compared to the healthy individuals (Figure 2B). However, the mechanisms underlying the turnover of CXCR2 protein harboring distinct types of mutations remain to be investigated.

An alteration in CXCL8-driven neutrophil chemotaxis was previously reported in patients harboring CXCR2 deletion. However, neutrophil migration towards chemoattractant varied in patients with distinct missense variants.[3] We evaluated the CXCL8-driven chemotactic responses of neutrophils derived from patients II:1 (homozygote) and II:4 (compound heterozygote) and healthy controls using a standard migration assay.[5] We found that neutrophils derived from healthy controls responded to CXCL8 at both 5 nM and 20 nM concentrations whereas neutrophils from both patients displayed CXCL8-response chemotaxis curves comparable to the vehicle control condition (Figure 2C). These observations confirm the loss-of-function phenotype of both *CXCR2* p.Arg144His and p.Lys240AsnfsTer45 variants. CXCR4 and its chemokine ligand CXCL12, in association with CXCR2 and its chemokine ligand CXCL8, contribute to neutrophil homeostasis by regulating neutrophil release from the BM to PB.[6] Specifically, the CXCR4/CXCL12 interaction results in neutrophil retention in the BM while CXCR2/CXCL8 promotes BM neutrophil release in the PB. Gain of function variants of *CXCR4* have been associated with neutropenia in the autosomal dominant inherited WHIM syndrome (warts, hypogammaglobulinemia, infections, myelokathexis).[6,7] Loss of function variants of *CXCR2* have also been described as a rare cause of neutropenia.[3,8] A population-based study showed rare and low-frequency of missense *CXCR2* variants (rs55799208, p.Arg153His; rs10201766, p.Arg236Cys; and rs61733609, p.Arg248Gln) associated with neutropenia and a homozygous truncating *CXCR2* variant (c.968delA) associated with severe neutropenia and myelokathexis in two siblings.[8] Recently, a study from the French Severe Chronic Neutropenia Registry described four patients from four family pedigrees with homozygous or compound heterozygous biallelic *CXCR2* variants (p.Arg144Cys, p.Arg212Trp, p.Arg289Cys, and *CXCR2* deletion).[3]

The CN patients presented in the current study displayed a missense p.Arg144His *CXCR2* variant in a homozygous state or as heterozygous in combination with heterozygous p.Lys240AsnfsTer45. To this date, there is only one report of *CXCR2* deficiency with clinical features involving severe neutropenia, transient episodes of lymphopenia and elevated IgG and IgA levels.[3] In this study, a *CXCR2* p.Arg144Cys variant was described as a cause of CN in homozygous or compound heterozygous state.[3] In contrast to our results, the majority of heterozygous carriers of *CXCR2* mutations tested in the French study displayed normal ANC despite the low neutrophil CXCR2 surface expression in flow cytometry analysis.

Interestingly, one of the patients with the homozygous p.Arg144His *CXCR2* variant (patient II:1, family A) displayed also positive autoantibodies against Fcγ-RIIIb which probably contribute to the more severe phenotype regarding the degree of neutropenia and frequency of infections in this patient. Positive anti-neutrophil antibodies can be frequently identified in patients with severe CN, delaying the proper diagnosis in some cases.[9] Notably, none of the patients described in our study displayed any of the key clinical features related to WHIM syndrome, including warts or other human papillomavirus-induced infections.[6,7] A BM aspiration/biopsy was performed only in patients with severe neutropenia carrying the homozygous (case II:1, family A) or compound heterozygous (case II:4, family B) *CXCR2* variants. Despite the hypercellular myeloid series with full maturation, also seen in WHIM syndrome, no dysmorphic neutrophils suggestive of myelokathexis were identified. Furthermore, hypogammaglobulinemia, typically seen in 70% of WHIM cases, was not observed in any of our patients (Table 1).

In summary, we report three unrelated non-consanguineous families from Crete, Greece, with CN associated with mutations in the *CXCR2* gene. Two variants have not been reported so far. Our results exemplify the importance of a detailed family history in the investigation of patients with unexplained, long-standing neutropenia and underline that CN may also be diagnosed in adulthood. Our findings also highlight the significance of advanced sequencing techniques for the identification of the genetic background in familial cases of neutropenia, even in patients with the presence of anti-neutrophil antibodies.

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Disclosures

No conflicts of interest to disclose.

Contributions

MK, JS and HAP designed the study. GT, EB, IM and HAP provided samples, collected biological and clinical data. MK, IT, MR and MS performed experiments and data analysis. MK, GT, IT, JS and HAP drafted the manuscript. All authors critically reviewed the collected biological and clinical data.

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Data-sharing statement

Technical information is available on request in order to assist other laboratories with the characterization of *CXCR2* variants.

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