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The European Guidelines on Diagnosis and Management of Neutropenia in Adults and Children: A Consensus Between the European Hematology Association and the EuNet-INNOCHRON COST Action

Francesca Fioredda[1], Julia Skokowa[2], Hannah Tamary[3,4], Michail Spanoudakis[5], Piero Farruggia[6], Antonio Almeida[7,8], Daniela Guardo[1], Petter Höglund[9,10,11], Peter E. Newburger[12], Jan Palmblad[10,11], Ivo P. Touw[13], Cornelia Zeidler[14], Alan J. Warren[15,16,17], David C. Dale[18], Karl Welte[19], Carlo Dufour[1], Helen A. Papadaki[20,21]

Correspondence: Francesca Fioredda (francescafioredda@gaslini.org); Helen A. Papadaki (e.papadaki@uoc.gr).

ABSTRACT

Neutropenia, as an isolated blood cell deficiency, is a feature of a wide spectrum of acquired or congenital, benign or premalignant disorders with a predisposition to develop myelodysplastic neoplasms/acute myeloid leukemia that may arise at any age. In recent years, advances in diagnostic methodologies, particularly in the field of genomics, have revealed novel genes and mechanisms responsible for etiology and disease evolution and opened new perspectives for tailored treatment. Despite the research and diagnostic advances in the field, real world evidence, arising from international neutropenia patient registries and scientific networks, has shown that the diagnosis and management of neutropenic patients is mostly based on the physicians' experience and local practices. Therefore, experts participating in the European Network for the Innovative Diagnosis and Treatment of Chronic Neutropenias have collaborated under the auspices of the European Hematology Association to produce recommendations for the diagnosis and management of patients across the whole spectrum of chronic neutropenias. In the present article, we describe evidence- and consensus-based guidelines for the definition and classification, diagnosis, and follow-up of patients with chronic neutropenias including special entities such as pregnancy and the neonatal period. We particularly emphasize the importance of combining the clinical findings with classical and novel laboratory testing, and advanced germline and/or somatic mutational analyses, for the characterization, risk stratification, and monitoring of the entire spectrum of neutropenia patients. We believe that the wide clinical use of these practical recommendations will be particularly beneficial for patients, families, and treating physicians.

[1]Unit of Hematology, IRCCS Istituto Giannina Gaslini, Genova, Italy
[2]Department of Oncology, Hematology, Immunology, Rheumatology, and Clinical Immunology, University Hospital Tübingen, Germany
[3]The Rina Zaizov Hematology/Oncology Division, Schneider Children's Medical Center of Israel, Petah Tikva, Israel
[4]Sackler School of Medicine, Tel Aviv University, Israel
[5]Department of Hematology, Warrington and Halton Teaching Hospitals NHS foundation Trust, Warrington, United Kingdom
[6]Pediatric Onco-Hematology, ARNAS Civico Di Cristina Benfratelli Hospital, Palermo, Italy
[7]Department of Hematology, Hospital da Luz Lisboa, Portugal
[8]Faculdade de Medicina, Universidade Católica Portuguesa, Lisbon, Portugal
[9]Clinical Immunology and Transfusion Medicine Clinic, Karolinska University Hospital, Stockholm, Sweden
[10]Center for Hematology and Regenerative Medicine (HERM), Department of Medicine Huddinge, Karolinska Institutet, Stockholm, Sweden
[11]Department of Hematology, Karolinska University Hospital, Stockholm, Sweden
[12]Department of Pediatrics, UMass Chan Medical School, Worcester, MA, USA
[13]Department of Hematology and Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, Netherlands
[14]Department of Oncology, Hematology, Immunology and Bone Marrow Transplantation, Hannover Medical School, Hannover, Germany
[15]Department of Hematology, University of Cambridge, United Kingdom
[16]Cambridge Institute for Medical Research, University of Cambridge, United Kingdom
[17]Wellcome Trust–Medical Research Council Stem Cell Institute, University of Cambridge, United Kingdom
[18]University of Washington, Seattle, WA, USA
[19]University Children's Hospital Tübingen, Germany
[20]Hemopoiesis Research Laboratory, School of Medicine, University of Crete, Heraklion, Greece
[21]Department of Hematology, University Hospital of Heraklion, Crete, Greece
JS and HT have contributed equally to this work.
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INTRODUCTION

Neutropenia, defined as a reduction in the absolute neutrophil count (ANC) below the lower limit of the normal range for the age and ethnic origin of the affected subject, is a common cause of referral to both adult and pediatric hematologists. The disorder, as an isolated blood cell deficiency, may be acute/transient or chronic/persistent; acquired, likely acquired, or congenital; primary or secondary to a number of disease entities; benign or premalignant, predisposing to myelodysplastic neoplasms (MDS), or acute myeloid leukemia (AML).[1] In recent years, advances in genomics have identified novel genes implicated in the pathogenesis and/or evolution of neutropenia, unraveled the underlying pathogenic mechanisms, and opened the way for novel tailored therapies.[2–7] The role of well-organized patient registries, by studying individual cases and families, has been particularly important for the recognition of novel entities and accumulating and sharing experience on diseases natural history.[8]

A number of comprehensive reviews have been produced by experts in the field aiming to disseminate this knowledge and guide clinicians for the accurate diagnosis, follow-up, and treatment of neutropenia patients, particularly those with chronic disease.[1–7,9–12] Real world data, however, arising from a survey within the Cooperation in Science and Technology European Network for the Innovative Diagnosis and Treatment of Chronic Neutropenias (EuNet-INNOCHRON; https://eunet-innochron.eu/) involving physicians with special interest in neutropenias from 28 European countries, Armenia, Israel, Turkey and the United States of America have shown that the work-up of patients with chronic neutropenia is mostly based on the physicians' experience and local practices rather than on the guided clinical and laboratory evidence.[13] Thus, the diagnosis and monitoring of neutropenic patients remains varied and challenging. It is also challenging to perform a smooth transition of neutropenic patients from pediatric to adult medical care, and the treating physicians need to be aware of the complications related to neutropenia and the risk of progression to MDS/AML, and finally they need to be familiar with specific situations like pregnancy and genetic counseling. Overall, continuous education of hematologists on known and arising neutropenia entities and guided diagnostic, follow-up, and treatment strategies are particularly important.

Taking into account the research advances in the field of neutropenias and their translation to clinical practice, progress in genomics and the availability of genetic testing in routine practice and the accumulated experience from long-standing patient registries, the European Hematology Association (EHA) has collaborated with the EuNet-INNOCHRON to develop guidelines for the classification, diagnosis, monitoring, and management of patients across the whole spectrum of chronic neutropenias. Our hope is that these recommendations are widely used clinically to benefit patients, families, and treating physicians.

METHODS

The guidelines working group (GL-WG) consisted of the chairs, steering committee, and expert panel including the chairs of the European and North-American Branches of the Severe Chronic Neutropenia International registry. All 17 members of the GL-WG are active members of EuNet-INNOCHRON and were selected for their recognized expertise in research and clinical practice on neutropenias. A memorandum of understanding was signed between the EHA and the GL-WG chairs representing also the EuNet-INNOCHRON, to verify the absence of conflict of interests of the GL-WG members and their compliance with the rules adopted by the EHA Guidelines Committee. EHA provided organizational assistance and support as well as funding for the systemic literature review.

Five main topics were identified: (1) definition and classification of neutropenias, (2) diagnostic approach, (3) natural history and follow-up, (4) treatment, and (5) special situations, and a number of key questions were formulated for each topic. The GL-WG members were further divided into subgroups and based on the literature review and their own experience, responses to the topic questions were generated. Literature review on all topics except treatment, comprised case control and cohort studies. The treatment topic, having much higher evidence based on randomized trials, was reviewed by Cochrane Hematology (https://haematology.cochrane.org/de) and was decided to be presented as a separate document.

Expert recommendations were developed and discussed by the entire panel in multiple rounds of communication with subsequent adjustments based on the replies. To achieve the greatest possible agreement, a voting procedure was followed according to the Delphi approach.[14] Consensus was reached if agreement was obtained from >75% of the panelists.

DEFINITION AND CLASSIFICATION OF NEUTROPENIA

Definition of neutropenia

The definition of neutropenia varies according to the patient's ethnic origin and age (Table 1). The widely accepted cutoff level of ANC for the definition of neutropenia in Caucasian newborns and infants up to the age of 1 year is 1.0×10^9/L;[15] however, neutropenia is defined as an ANC level of 2.5×10^9/L for term/near-term neonates 72–240 hours following delivery, and 1.0×10^9/L for preterm neonates.[4,16,17] A more detailed characterization of neutropenia in newborns is given in the "Specific situations" section of this article. From the age of 1 year to adulthood the cutoff level for neutropenia is 1.5×10^9/L.[1,14,18,19] For Caucasian adults, the ANC threshold of 1.8×10^9/L is adopted for the definition of neutropenia according to the World Health Organization and the MDS/AML expert groups.[20–22]

It should be noted that some individuals of African and Middle Eastern descent display normal ANCs in the range from 0.5 to 1.5×10^9/L and less frequently even lower.[23–25] This variation, previously termed ethnic neutropenia, is usually inherited as an autosomal recessive trait associated with a polymorphism (rs2814778, –46T>C) in the GATA box in the promoter region of the atypical chemokine receptor-1 (*ACKR1*) gene, also known as the duffy antigen receptor for chemokines (*DARC*). In homozygosity (C/C), the polymorphism results in the absence of Duffy antigen expression specifically on red blood cells, a phenotype known as Duffy-null.[26] The GL-WG suggests the introduction of the term *ACKR1/DARC*-associated neutropenia (ADAN), instead of ethnic neutropenia, to emphasize the genetic rather than the ethnic basis of this entity.[27]

Classification of neutropenia

It has long been recognized that the risk and outcome of bacterial infections resulting from neutropenia depends on the individual's capacity to recruit and deliver neutrophils to tissues rather than only on the ANC in the peripheral blood (PB).[28,29] However, validated methods to estimate total body neutrophil

Table 1

ANC Cutoff Level for Definition of Neutropenia According to Age

Age	ANC[a]
From 14 d to 1 yr	$<1.0 \times 10^9$/L
Children >1 y to adulthood*	$<1.5 \times 10^9$/L
Adults[a]	$<1.8 \times 10^9$/L

[a]ANC $<1.5 \times 10^9$/L are considered normal in individuals from African and the Middle Eastern ancestry.

ANC = absolute neutrophil count.

counts are not clinically available; thus, we still extrapolate the quantitative correlation between circulating ANC and infections in patients undergoing chemotherapy to classify neutropenias as mild when ANC is between 1.0 and 1.5 (or 1.8 for adults) × 10^9/L, moderate when ANC is 0.5 to 1.0 × 10^9/L, and severe when ANC is <0.5 × 10^9/L.[19,30] The term agranulocytosis is used for severe neutropenias with ANC <0.2 × 10^9/L and is usually associated with a high risk of severe, life-threatening infections.[19] Neutropenia is also characterized as acute or chronic depending on whether the duration is <3 or >3 months, respectively.[19]

Further to the above traditional classifications, the GL-WG agreed on an extended, pathogenesis-based classification that categorizes neutropenias as congenital (Table 2) versus acquired and likely acquired (Table 3) with respective subcategories. Congenital neutropenia (CN) comprises a group of genetic diseases characterized by impaired production, differentiation and survival of neutrophils in the bone marrow (BM), susceptibility to infections, and increased propensity to MDS/AML transformation.[3–5,15,31–33] CN can be further subclassified into disorders where neutropenia is the only abnormality and those where neutropenia is associated with extrahematological manifestations, immunodeficiency/immune dysregulation, metabolic disorders and nutritional deficiencies, or as part of more complex BM failure syndromes. The classification also takes into consideration the genes that have been identified as responsible for each CN subtype.

Consensus was reached to classify acquired neutropenia as primary or idiopathic, associated with the presence of antineutrophil antibodies or other unknown mechanisms; and secondary due to infections, autoimmune diseases, exposure to drugs, nutritional deficiencies, hypersplenism, or hematologic diseases.[9–12,35–39] Likely acquired neutropenia includes mostly idiopathic or autoimmune neutropenia (AIN) of childhood, which usually seems to have a benign and uncomplicated course but does not resolve after 24–36 months (long lasting) or neutropenia, with and without antibodies, which arises after the age of 3 years (late onset).[18,40–42] A proportion of these patients display lymphopenia due to low B cells and natural killer (NK)-cells subsets, and mutation analysis of genes related to immunodeficiency/immune-dysregulated disorders has identified pathogenic variants in individual cases.[42] The GL-WG agreed that this group of pediatric or adolescent patients, provisionally classified as acquired idiopathic, need a thorough work-up to exclude an underlying genetic cause.

DIAGNOSIS

What is important to know from patient/family history?

The initial step for the investigation of patients with neutropenia is a detailed past medical and family history. A summary of recommendations on the content of patient/family history is presented in Box 1.

General inquires

The age of first detection of neutropenia is an important piece of information; in fact, although CN can rarely be diagnosed in adulthood,[43–47] it is usually diagnosed in early childhood. Results of previous cell blood counts (CBC) could clarify the duration of neutropenia and differentiate between acute and chronic neutropenia.[1,10,11,19] It is also important to know whether the neutropenia was an incidental finding or part of an acute illness. The clinical history should investigate the frequency, type, severity of infections, and need for hospitalization. Inquiries about clinical events such as fever, mouth ulcers, sore throat/odynophagia, gingivitis, sinusitis, otitis, skin ulcers and cellulitis, deep tissue infections, episodes of pneumonia, gastrointestinal symptoms, and perianal infections are particularly important. Periodic patterns of recurrent infections could indicate cyclic neutropenia (CyN).[1,10,11,19] It is also important to know whether previous febrile or inflammatory episodes were associated with a spontaneous increase in ANC and how they were treated.[1]

Additional medical history

For suspected CN, the medical history should include any involvement of extrahematological sites (typical manifestations are described in Table 2). A detailed history should be taken of any symptoms denoting underlying autoimmune or other diseases that may result in secondary neutropenia (Table 3). Notably, many adult patients with AIN may present with various autoimmune manifestations (e.g., sicca symptoms, arthralgias, and Raynaud) without fulfilling the criteria for a definite diagnosis of an autoimmune disease.[10,11] Many patients also report chronic fatigue and malaise.[1,10,11,19] History of chronic viral infections such as viral hepatitis or HIV should also be obtained. Careful inquiry should be made concerning drug administration, including over the counter drugs, substances often denominated as natural supplements, and recreational drugs; neutropenia can be linked not only to drugs that the patient has recently started but also to drugs that have recently been discontinued.[1,10,11,19,36–39]

Family history

Detailed family history, including consanguinity and ethnic origin, is important and should be extended as far back as possible, especially if other members of the family have similar symptoms or laboratory findings. Also, it is important to know whether family members have died from infections or MDS/AML. Unexplained infant deaths or miscarriages can be also linked to CN.[1,19,48,49] Notably, the family history for neutropenia might be negative, because a mild or even moderate neutropenia might have been overlooked in previous blood tests; also, neutropenia may have varying severity within the same family. Therefore, CBCs from family members will definitely help to establish a diagnosis of familial neutropenia.

What should a detailed clinical examination comprise?

A summary of recommendations on the clinical examination of patients with neutropenias is presented in Box 2. Depending on the severity of neutropenia and the underlying pathogenetic mechanism, physical examination may reveal aphthous ulcers, tooth and gum abnormalities due to chronic periodontitis, evidence of acute or chronic sinusitis, pharyngitis or otitis; or skin abnormalities such as hyperpigmentation, partial albinism, rashes, ulcers or abscesses, and nail dystrophy. Abdominal examination in the setting of pain and fever may raise concerns of neutropenic colitis and abdominal sepsis. The perianal and perirectal area should also be carefully examined to exclude necrotizing cellulitis due to *Clostridium* species or other anaerobes, as neutropenic patients may have only subtle signs or symptoms of infection due to reduced inflammatory response.[19] The presence of lymphadenopathy, splenomegaly, and hepatomegaly may indicate infection or an associated underlying disease such as myeloid/lymphoid malignancy and metabolic disorders, among others.

In children, growth and development should be assessed in addition to examination of mucous membranes, gums, teeth, skin, nails, and tympanic membranes. Mutations in *ELANE* are very commonly associated with the development of periodontitis in patients with severe CN, whereas neurological problems may be present in up to 30% of patients with *HAX1* mutations.[50,51] Short stature, chondrodysplasia, skeletal, heart, urogenital abnormalities, increased visibility of superficial veins, myopathy, nail, hair, or skin abnormalities, signs of bronchiectasis due to recurrent chest infections, hepatosplenomegaly, as well as photophobia, nystagmus, and oculocutaneous albinism may be associated with specific CN syndromes (Table 2).[15,52]

In adults, it is important to assess signs related to possible underlying autoimmune or other neutropenia-associated

Table 2

Classification of Congenital Neutropenias

	Congenital Neutropenias		
	Genes Involved	**Type of Inheritance**	**Main Features/Notes**
Isolated			
Severe congenital neutropenia	*ELANE*	AD	Arrest of neutrophil maturation in bone marrow
	CSF3R	AD	Arrest of neutrophil maturation in bone marrow, unresponsive to G-CSF
	CXCR2	AR	No arrest of maturation and myelokathexis
	WAS gain of function	X-linked	(WASp-XLN) Maturation delay, monocytopenia
Cyclic neutropenia	*ELANE*	AD	Intermittent/cyclic impaired differentiation
Associated with various extrahematological manifestations			
Barth Syndrome (3-methylglutaconic aciduria type II)	*TAZ*	X-linked	No maturation arrest, hypertrophic cardiomyopathy, and myopathic syndrome
Charcot-Marie-Tooth neuropathy type B	*DNM2*	AD	Distal limb muscle weakness and atrophy due to peripheral neuropathy
Cohen syndrome	*VPS13B*	AR	No maturation arrest, psychomotor retardation, microcephaly, facial features, hypotonia, joint laxity, progressive, retino-choroidal dystrophy, and myopia
G6PC3 mutation	*G6PC3*	AR	Skin hyperelasticity and prominent superficial venous network, congenital heart disease, arrhythmias, uropathy, cryptorchidism, and exocrine pancreatic dysfunction
GFI1 mutation	*GFI1*	AD	Sometimes maturation arrest, lymphopenia, increased numbers of immature myeloid cells in the peripheral blood and inner ear defect
HYOU1 deficiency	*HYOU1*	AR	Hypoglycemia and inflammatory complications
JAGN1 mutation	*JAGN1*	AR	Sometimes maturation arrest, bone and teeth abnormalities, and exocrine pancreatic dysfunction
Kostmann disease	*HAX1*	AR	Maturation arrest, mental retardation, seizures, and susceptibility to MDS/AML
P14/LAMTOR2 mutation	*LAMTOR2*	AR	Chronic neutropenia, hypogammaglobulinemia, no maturation arrest, oculocutaneous albinism, and failure to thrive
Pearson syndrome	Mitochondrial DNA deletions	Mitochondrial	Refractory sideroblastic anemia, vacuolization of bone marrow precursors, and exocrine pancreatic dysfunction
Schimke immuno-osseus dysplasia	*SMARCAL1*	AR	Spondylo-epiphyseal dysplasia, slowly progressive immune defect, and immune-complex nephritis
SEC61A1 mutation	*SEC61A1*	AD	Maturation arrest and tubulointerstitial kidney disease
SMARCD2 mutation	*SMARCD2*	AR	Dysplastic syndrome, no granules in neutrophils, chronic diarrhea, bone abnormalities, and low set ears
Specific granule deficiency	CEBPE	AR	Neutrophils with bilobed nuclei
TCIRG1 neutropenia	*TCIRG1*	AD	Variable/no maturation arrest and skin angiomatosis
VPS45 mutation	*VPS45*	AR	Myeloid hyperplasia, myelofibrosis, nephromegaly, HSM, mental retardation, epilepsy, and osteosclerosis
Wolcott-Rallison syndrome	*EIF2AK*	AR	Maturation arrest, insulin-dependent neonatal diabetes, epiphyseal dysplasia, growth retardation, hepatic and renal dysfunction, developmental delay, and exocrine pancreatic deficiency
Associated with immunodeficiency/ immune dysregulation			
Adenosine deaminase 2 deficiency	*ADA2*	AR	Severe combined immunodeficiency, vasculitis, cerebrovascular disease, pure red cell aplasia, and BMF
ALPS	*FAS, FASLG, CASP10*	AD	Lymphoproliferation and autoimmune cytopenias
CD40L/hyper IgM syndrome, type I	*CD40L*	X-linked	Severe infections, autoimmune disease, and cancer predisposition
Chédiak-Higashi syndrome	*LYST*	AR	Decreased pigmentation of hair and eyes, peroxidase-positive inclusion bodies in the myeloblasts and promyelocytes of the bone marrow, peculiar malignant lymphoma
CLPB syndrome	*CLPB*	AR	Cataracts and neurologic involvement
FHLH	*PRF1*, Perforin deficiency (*FHL2*)	AR	Fever, HSM, and cytopenias
	UNC13D, UNC13D deficiency (*FHL3*)	AR	Fever, HSM, and cytopenias
GATA2 syndrome	*GATA2*	AD	Monocytopenia, deafness, and HPV infections
Griscelli syndrome, type II	*RAB27A*	AR	Hypomelanosis and neurologic impairment
Hermansky-Pudlak syndrome type2	*AP3B1*	AR	Albinism
Reticular dysgenesis	*AK2*	AR	Severe combined immunodeficiency and sensorineural deafness
STK4 mutation	*STK4*	AR	Intermittent neutropenia, monocytopenia, T- and B-lymphopenia, atrial defect, and HPV infections

(Continued)

Table 2 (Continued)

Congenital Neutropenias			
	Genes Involved	**Type of Inheritance**	**Main Features/Notes**
WHIM syndrome	*CXCR4*	AD	No arrest of maturation, myelokathexis, and lymphopenia, cardiopathy (Tetralogy of Fallot)
Wiskott-Aldrich syndrome	*WAS* loss of function	X-linked	(WASp-XLT) Eczema, thrombocytopenia, severe infections, and bloody diarrhea
CVID	Various genes including *TNFSRF13, BAFFR, CTL4, LRBA, PI3K*	AD,AR	Infection recurrence, hypogamma globulinemia, and autoimmune cytopenias including neutropenia.
Associated with metabolic disorders and nutritional deficiency			
Gaucher disease type I	*GBA*	AR	HSM, thrombocytopenia, and osteolytic lesions
Glycogen storage disease Ib	*SLC37A4/G6PT1*	AR	Hepatomegaly, IBD, and fasting hypoglycemia
Isovaleric acidemia	*IVD*	AR	Neonatal ketoacidosis, developmental delay, lethargy, and feeding refusal
Methylmalonic acidemia	*MMUT*	AR	Lethargy, failure to thrive, recurrent vomiting, hypotonia, hepatomegaly, and developmental delay
Propionic acidemia	*PCCB, PCCA*	AR	Lethargy, cardiomyopathy, feeding difficulties, and acute encephalopathy
Transcobalamin II deficiency	*TCN2*	AR	Developmental delay, diarrhea, vomit, lethargy, and mucosal ulceration
Associated with bone marrow failure			
Fanconi anemia	*FANC* complementation group	AR X-linked (FANCB)	Congenital malformations and cancer predisposition
Ribosomopathies			
Diamond-Blackfan Anemia	*RPS7, RPS10, RPS15, RPS17, RPS19, RPS24, RPS26, RPS27, RPS27a, RPS28, RPS29 RPL5, RPL9, RPL11, RPL15, RPL18, RPL26, RPL27, RPL31, RPL35a*	AD	Erythroid hypoplasia, congenital malformations, growth retardation, osteosarcoma, MDS, and AML
	GATA1	X-linked	Early-onset anemia, thrombocytopenia, and bone marrow erythroid hypoplasia
	EPO	AR	Erythroid hypoplasia
	TSR2, HEATR3	X-linked AR	Erythroid hypoplasia, craniofacial defects, short stature, facial, and acromelic dysmorphic features, and intellectual disability
Cartilage-hair hypoplasia	*RMRP*	AR	Short stature (dwarfism) with other skeletal abnormalities; metaphyseal chondrodysplasia, ligamentous laxity; fine, sparse hair (hypotrichosis); and abnormal immune system function (immune deficiency), recurrent infections.
Shwachman-Diamond syndrome	*SBDS, EFL1, DNAJC21*	AR	Mild neutropenia, dysgranulopoiesis, mild dysmegakaryopoiesis, dyserythropoiesis, exocrine pancreas deficiency, metaphyseal dysplasia, cognitive impairment, cardiomyopathy, metaphyseal dysplasia, failure to thrive, and hair/skin/teeth abnormalities.
SAMD9/SAMD9L syndromes	*SAMD9/SAMD9L*	AD	Adrenal insufficiency, congenital malformations, cerebellar ataxia, severe invasive infections, and MDS predisposition
SRP54 mutation	*SRP54*	AD	Maturation arrest, severe neurodevelopmental delay, and exocrine pancreatic dysfunction
Telomere diseases	*DKC1*	X-linked	Mucocutaneous features, liver fibrosis, idiopathic pulmonary fibrosis, and cancer predisposition
	hTR, TERT, TINF2, DKC1, ACD	AD	
	TERT, NHP2, NOP10, WRAP53, NOLA3,TCB1, RTEL1, CTC1, PARN	AR	
U6 small nuclear RNA biogenesis	*USB1* (Clericuzio syndrome, poikiloderma with neutropenia)	AR	Retinopathy, developmental delay, facial dysmorphisms, and poikiloderma

This is based on the following references: [3,4,13,15,31–34].

AD = autosomal dominant; AR = autosomal recessive; ALPS = autoimmune lymphoproliferative syndrome; MDS = myelodysplastic syndrome; AML = acute myeloid leukemia; FHLH = familial hemophagocytic lymphohistiocytosis; HPV = human papilloma virus; IBD = chronic inflammatory bowel disease; HSM = hepatosplenomegaly; BMF = bone marrow failure.

diseases such as skin rashes, arthritis, vitiligo, hepatosplenomegaly, dryness of eyes or mouth, and lymphadenopathy, among others.[10–12]

What tests should be included in the initial and further levels of investigation?

Patients with acute neutropenia, particularly in the presence of symptoms/signs of infection, may require immediate investigation and even hospitalization depending on the severity of neutropenia and symptoms. For patients with chronic, isolated neutropenia without a phenotype suggestive of any underlying CN syndrome, a flowchart of basic investigation shown in Figure 1 is recommended.[10–12,15,19]

If the initial evaluation does not suggest ADAN, nor postinfectious or drug-induced neutropenias, the first level of investigation, possibly adjusted to the availability of the recommended tests, should be followed as shown in Box 3. A second line of

Table 3

Classification of Acquired Neutropenias

Acquired	
Primary or idiopathic: neutropenia as predominant, often isolated feature	Antibody-mediated Primary autoimmune Primary alloimmune Nonantibody-mediated Idiopathic neutropenia of infancy CIN/idiopathic cytopenia of undetermined significance-neutropenia (ICUS-N)
Secondary: neutropenia associated/due to	Hypersplenism (due to congestive, infiltrative, phagocytic, and reactive splenomegaly) Infections Viral (e.g., HIV, HCV, HBV, CMV, EBV, HIV, influenza, parvovirus B19, measles, and Sars-Cov-2) Bacterial (e.g., *Salmonella*, *Brucella*, *Rickettsia*, *Mycobacterium*, *Mycoplasma*, and *H. Pylori*) Parasitic (e.g., *Plasmodium spp*, visceral leismaniasis) Fungal (e.g., histoplasmosis) Autoimmune diseases Organ specific (e.g., thyroid diseases, inflammatory bowel disease, and primary biliary cirrhosis) Systemic (e.g., systemic lupus erythematosus, rheumatoid arthritis including Felty's syndrome, Sjogren syndrome, systemic sclerosis, and graft-vs-host disease) Nutritional deficiencies B12, folic acid, iron, copper, and caloric malnutrition Immuno-regulatory disorders Common variable immunodeficiency, ALPS, ALPS-like diseases, HLH, and macrophage activation syndrome Hematologic diseases Primary benign (aplastic anemia) Clonal (myeloid malignancies/lymphoid malignancies including LGL) Drug-induced -Chemotherapy -Nonchemotherapeutic drugs: analgesics and NSAIDs, antibiotics (beta-lactams, cefipime, trimethoprim-sulfametoxazole, sulfasalazine, vancomycin, rifampicin, fluconazole, ketoconazole), antidiuretics (furosemide, spironolactone), antiretroviral (HIV) therapy, antithyroids (tiamazofe, metimazole), clozapine (olanzapine), deferiprone, dipyrone (metamizole), phenothiazines (alimemazine), quinine/quinidine, IVIG, monoclonal antibodies (Rituximab), and biological therapies (Infliximab, etanercept) Extended list of drugs associated with neutropenia can be found in the following references: [35–38].
Likely acquired	
In children/adolescents when neutropenia, in the presence or absence of specific antibodies against neutrophils arises or persists beyond the age of 5 years, the definition of late-onset and long-lasting neutropenia may be appropriate. Recent articles identified this atypical neutropenia as an epiphenomenon of immune dysregulation with typical biochemical/immunological features and the presence of variants in genes regulating immunity.	

ALPS = autoimmune lymphoproliferative syndrome; CIN = Chronic idiopathic neutropenia; CMV = cytomegalovirus; EBV = Epstein-Barr virus; HCV = Hepatitis C virus; HLH = hemophagocytic lymphohistiocystosis; ICUS-N = idiopathic cytopenia of undetermined significance-neutropenia.

investigation should be followed if the first-line evaluation is inconclusive, as also shown in Box 3.

Notably, the GL-WG experts highly recommend genetic testing in children, young adults, and selected adults to exclude CN if the second level of investigation is inconclusive. Also, in young children with a family history of severe neutropenia, or with typical congenital anomalies or repeated severe infections, genetic testing should be expedited and performed after the first level of investigation.

Box 1: What is Important to Know From Patient/ Family History When We Investigate Neutropenia?

Patient history should include inquiry about occurrence of infections and their frequency, type, severity, and need for hospitalization. Specifically, history of omphalitis, gingivitis, periodontitis, skin infections, abscesses, and pneumonias as well as duration and response to antibiotics should be also investigated.

Presence of congenital malformations in the patient or family is important.

For adult patients, drug history is important, as well as work-up for autoimmune and other disorders that may be associated with neutropenias.

Detailed family history should include ethnic origin, consanguinity, occurrence of recurrent infections, and neutropenias in other family members, as well as unexplained infant death or miscarriages.

Box 2: What Should a Detailed Clinical Examination Comprise When We Investigate Neutropenia?

Careful clinical examination of skin and mucous membranes, upper and lower respiratory tract and abdomen to exclude underlying infection, lymphadenopathy, and/or hepatosplenomegaly. Clinicians should be aware that neutropenic patients might have only subtle symptoms of infection due to reduced inflammatory response.

In children and adults, clinical examination is crucial to detect congenital disorders. It should focus on growth, evidence of cognitive impairment, developmental delay, dysmorphism (mainly skeletal), nail, hair or skin abnormalities, signs of bronchiectasis due to recurrent chest infections, hepatomegaly or splenomegaly, organ malformation, evidence of superficial veins, and finally signs of photophobia, nystagmus, oculocutaneous albinism, and neuropathy. The absence of obvious clinical signs does not exclude the presence of a congenital disorder.

Cardiac function, presence of enlarged lymph nodes, joint symptoms, and symptoms compatible with autoimmune, metabolic, gastrointestinal, or nutritional diseases should also be considered.

What is the role of BM examination? When and how often?

The recommendations on the type, time, and frequency of BM investigations are summarized in Box 4. BM aspiration and trephine biopsy can give information on the cellularity, the numbers and maturation of erythroid, myeloid precursors, megakaryocytes, and on the presence of dysplastic features. It can also inform on the presence of nonmyeloid cells and contribute to the differential diagnosis, particularly in adults.

Besides morphology, a cytogenetic evaluation can give further information on acquired chromosomal abnormalities typical for MDS or AML. BM samples can also be used for genetic testing of acquired somatic gene mutation, which play a role in leukemogenesis in CN (e.g., *RUNX-1*, *CSF3R*, *TP53*)[3,53–59] or suggest increased risk of transformation to MDS/AML in acquired neutropenias (e.g., *SRSF2* and *IDH1*).[60]

In patients with severe chronic neutropenia (SCN), the BM examination may be helpful for the differential diagnosis and exclusion of MDS/AML. If not performed during the diagnostic

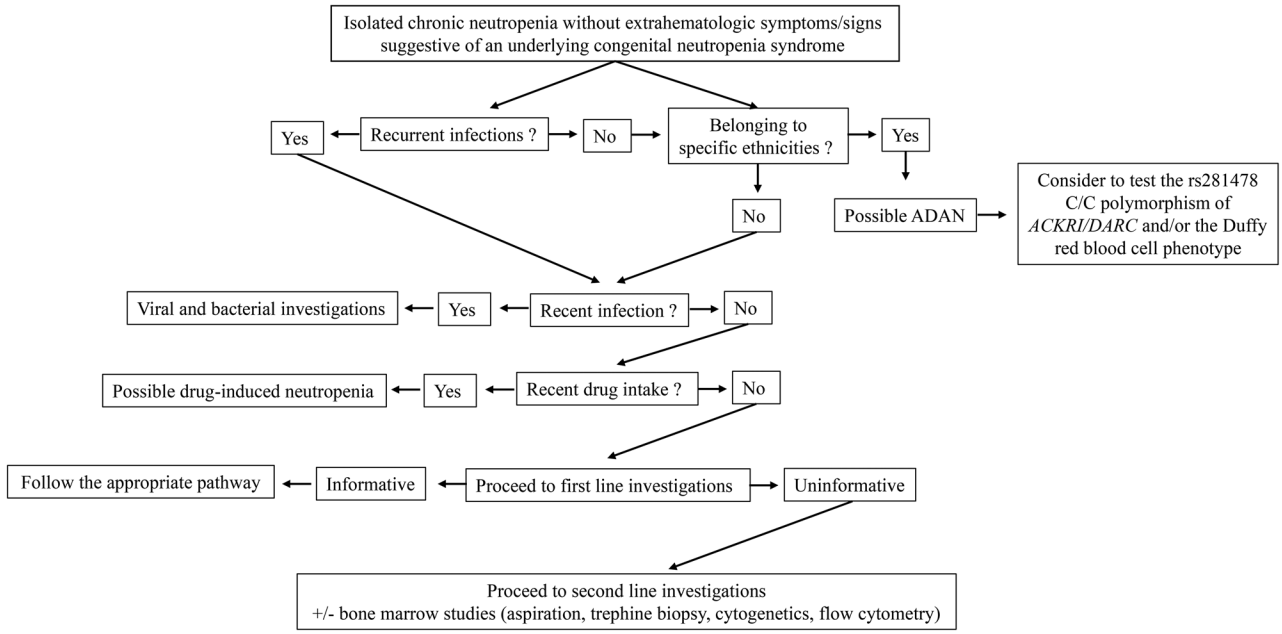

Figure 1. Flowchart for the basic evaluation of a patient with chronic neutropenia.

Box 3: What Tests Should the Different Lines of Investigation of Chronic Neutropenia Include After Referral to the Specialist?

First-line investigations

CBCs, PB smear, biochemistry tests including liver and kidney function, immunoglobulin levels, CRP, vitamin B12 and folate, flow cytometric analysis of PB lymphocyte subsets, virology antibody screening (i.e., HepB, HepC, HIV, EBV, CMV, and Parvovirus), indirect antineutrophil antibodies (GIFT, GAT, and other); thyroid hormones (FT3, FT4, TSH), antithyroid antibodies (anti-TG and anti-TPO).

Additional investigation in children: flow cytometric analysis of TCR-α/β-positive double-negative (CD4- and CD8-) CD3 PB lymphocytes.

Additional investigations in adults: antiphospholipid and anticardiolipin antibodies, flow cytometric analysis of LGL/TCR clonality in PB lymphocytes, serum ferritin, RF, ANA, ENA, ds-DNA, and ESR.

Second-line investigations

CBCs in family members, serial blood counts twice a week over a period of 6 weeks to exclude CyN, copper; ceruloplasmin, anti-tTG-IgA, deamidated gliadin peptide antibodies IgA/IgG and pancreatic isoamylase.

Additional investigation in children: RF, ANA, ENA, and ds-DNA

Additional investigation in adults: serum electrophoresis, serum complement levels, next generation sequencing of gene panels related to myeloid malignancies to identify idiopathic cases at risk to MDS/AML development.

In children, young adults, and considered for adults: genetic investigations.

AML = acute myeloid leukemia; ANA = antinuclear antibodies; anti-TG = antithyroglobulin; anti-TPO = antithyroid peroxidase; CBC = complete blood counts; CMV = cytomegalovirus; CyN = cyclic neutropenia; dsDNA = double-stranded DNA; tTG = tissue transglutaminase antibodies; EBV = Epstein-Barr virus; ENA = extractable nuclear antigen; ESR = erythrocyte sedimentation rate; GAT = granulocyte agglutination test; Hep = hepatitis; HIV = human immunodeficiency virus; GIFT = granulocyte immunofluorescence test; LGL = large granular lymphocytes; MDS = myelodysplastic neoplasms; PB = peripheral blood; RF = rheumatoid factor; TCR = T-cell receptor.

Box 4: What Is the Role of BM Examination? When and How Often?

A diagnostic BM with morphology, cytogenetics, and NGS of genes related to myeloid malignancies should be performed:

1. In pediatric patients with severe and moderate chronic neutropenia with the exception of patients with primary AIN with positive antigranulocyte antibodies and drug-induced neutropenias.
2. In patients with suggested AIN but negative granulocyte antibody test, if patients suffer from recurrent infections.
3. In any patients before G-CSF treatment.
4. In all adult patients with unexplained chronic neutropenia with the exception of those with long-standing, mild, isolated neutropenia that remains stable over time.

Annual BM and cytogenetics follow-up should be performed in patients:

1. With congenital BM failure syndromes independent of ANC and treatment with G-CSF.
2. With undefined SCN (after extensive investigation) with G-CSF treatment, may be considered.

Repeated BM follow-up should be performed in patients: With decreasing ANC or additional changes in other blood cell counts (e.g., anemia and thrombocytopenia) or erythrocyte indices.

AIN = autoimmune neutropenia; ANC = absolute neutrophil count; BM = bone marrow; G-CSF = granulocyte colony stimulating factor; NGS = next generation sequencing; SCN = severe chronic neutropenia.

work-up, BM examination should be initiated before granulocyte-colony stimulating factor (G-CSF) treatment to exclude MDS/AML and as a baseline for monitoring the risk for malignant transformation. In many CN patients, the diagnostic BM shows a maturation arrest of neutrophil precursors at an early stage (promyelocyte/myelocyte) with few cells of the neutrophilic series beyond the promyelocyte stage.[61] The number of promyelocytes is slightly increased. Marrow eosinophilia is common. Cellularity is usually normal or slightly decreased.

Megakaryocytes are normal in number and morphology. The in vitro growth of granulocyte colonies in granulocyte-macrophage colony-forming unit assays, if performed, may also show a maturation arrest that mimics the disease.

BM aspiration and trephine biopsy in combination with cytogenetics and flow cytometry (FC) should be considered in all adult patients with unexplained neutropenia to exclude MDS or other hematologic diseases with BM involvement (e.g., leukemia, lymphoma, myeloma, and myelofibrosis), or even infiltration of BM by nonhematologic malignancy.[62,63] In patients with long-standing, isolated, mild neutropenia that remains longitudinally stable, the BM examination might be omitted and if performed, there is no need to repeat it if the initial evaluation does not reveal any underlying disease, unless there is a significant change in CBCs.[1,12]

Annual BM examination (aspiration, trephine biopsy, and cytogenetic evaluation) is recommended for CN and other BM failure syndromes independent of ANC and treatment with G-CSF. It may be also considered for idiopathic neutropenia (IN) that is not clearly congenital, but requires G-CSF treatment.

What is the role of antineutrophil antibody testing? What are the recommended tests? How should we interpret positive results?

Evidence on the role and significance of antineutrophil antibodies in the diagnosis of neutropenias comes mainly from pediatric data.[40,41] The identification of autoantibodies in children verifies the diagnosis of AIN, an entity with typically early onset and benign clinical course with few infections, spontaneous resolution within a few years, and no risk for leukemic development.[64–71] The specificity of autoantibodies is against FcγRIIIb (CD16b) in the majority of patients. Studies on the presence of antineutrophil antibodies in neutropenic adult patients are far fewer compared with those in children. The main target still appears to be FcγRIIIb (CD16b) but a broader antibody specificity has been described compared with children.[72–74]

The most widely used and validated assay for detection of antineutrophil antibodies is the indirect granulocyte immunofluorescence test (GIFT), which relies on incubation of test granulocytes with patient serum followed by the detection of bound antibody using a fluorescent antihuman immunoglobulin reagent. Other available assays such as the granulocyte agglutination test and monoclonal antibody immobilization of granulocyte antigen (MAIGA) have been found to be inferior to GIFT as screening assays in the diagnostic of autoantibodies.[40,65,75] However, they can be used to determine antibody specificity or as additional methods in selected cases. Recently, high-throughput bead-based assays such as the LabScreen Multi have been used for alloantibody detection, but it is yet to be evaluated in a larger series of AIN patients as a screening test.[76] Notably, most clinical laboratories use only one of these methods, which may have a higher or lower false-positive or false-negative rate depending on the technology, so antineutrophil antibody testing should not be used alone to make a diagnosis, and should be interpreted with caution.[77]

Positive antibody identification makes the diagnosis of AIN likely, especially in children of the right age group and with a typical medical history. Although less emphasis can be put on considering alternative diagnoses in such cases, the possibility of false-positive and false-negative results must always be considered. However, positive antibody testing has been reported in patients with genetically proven severe CN, so the result should not be used to exclude that diagnosis.[78,79] Several studies have shown that small children without detectable antibodies but with a typical medical history have a very similar disease course as patients with AIN.[41] Following thorough investigations to exclude other disease entities, a diagnosis of IN can be given in these patients. IN and AIN have overlapping clinical features, although the former may present at older age with longer disease duration and distinct immunologic profile as mentioned in the Classification section of the text.[41,42] Repeated antibody testing in reference laboratories is recommended in cases of suspected AIN with negative tests.

In adults, antineutrophil antibodies can be found in primary and secondary AIN.[1,12,35] It is particularly important to exclude the presence of alloantibodies again HLA class I, which are common in transfused patients and previously pregnant women and can give false-positive GIFT results.[19] An independent analysis of HLA class I antibodies, and/or confirmation of the specificity of the granulocyte antibodies using MAIGA test or GIFT with genotyped neutrophils, is therefore recommended before positive antibody results are conclusively interpreted.

The summary of recommendations on the autoantibody screening for the diagnosis of AIN is presented in Box 5.

What is the role of genetic testing: which genes, methods, and cell source? What is its position in the diagnostic algorithm?

Genetic testing and analysis of CN patients is important to confirm diagnosis, to estimate the relative risk of late complications such as MDS/AML, and to offer genetic counseling to affected patients and family members. Because of the variability of the clinical picture, unaffected related donors for those patients who are candidates for hematopoietic stem cell (HSC) transplantation should always undergo genetic screening.

Following negative results of first-level investigations (see Box 3), all patients with SCN and recurrent infections and/or family history of severe neutropenia and typical anomalies should undergo genetic work-up either by single-gene Sanger sequencing or by multigene next generation sequencing (NGS) methods. Sanger sequencing of *ELANE* (mutated in ~45% of patients with severe CN) is recommended as the first approach to genetic diagnosis of typical cases.[3,4] However, family history or clinical findings may suggest another specific neutropenia-associated gene to be sequenced (Table 2). For example, in the presence of cardiomyopathy, *TAZ* (Barth syndrome) sequencing may be diagnostic, while in the presence of cardiac and genitourinary malformations sequencing of *G6PC3* may lead to diagnosis. Poor growth, malabsorption, fatty stool, and bone malformation suggest *SBDS* mutations that are found in most patients with Shwachman-Diamond syndrome (SDS).[80] Overall, Sanger sequencing of the suspected genes is relatively easy and cost effective. Following identification of the responsible gene(s), Sanger sequencing is also recommended for mutation screening of the members of affected families.[3]

Multigene NGS or whole exome sequencing (WES) ideally should include patient and parental DNA (trio analysis). A targeted NGS panel including all genes known to be mutated in CN (>30) is a reasonable first step that provides uniform sequencing coverage for all genes of interest and requires simpler bioinformatic analysis. The choice of genes within the panel should include not only all those that strictly cause neutropenia when mutated but also genes resulting in diseases in which neutropenia is a secondary feature (immunodeficiency/immune dysregulation, metabolic and nutritional deficiency, and other BMF syndromes) (Table 2).[3,4,42] WES can also be used in cases where no mutations

Box 5: Recommendations on Antineutrophil Antibody Testing

As shown in Box 3, antineutrophil antibody testing should be performed as first-line investigation in both children and adults.

Indirect granulocyte immunofluorescence test (GIFT) is recommended as a first-line assay in reference laboratories.

A positive GIFT in combination with laboratory tests and clinical picture can support diagnosis of autoimmune neutropenia (AIN) but does not exclude the diagnosis of other types of neutropenia.

With a negative indirect GIFT, if the clinical suspicion of AIN remains high, GIFT should be repeated several times.

are identified in targeted genes, extending the analysis to all coding regions in the genome. In all NGS applications, the bioinformatics interpretation of the pathogenicity of genetic variants is challenging and should be based on consensus guidelines.[2,81] The pathogenicity of variants of unknown significance should rely, as much as possible, on family segregation studies and on functional studies if relevant. If suspicion of CN is high and both NGS panels and WES analysis are negative, whole genome sequencing and RNA-sequencing should be considered. DNA for detection of potential germline mutations is extracted from PB; however, in the presence of leukemic blasts in PB, nonhematopoietic cells should be used, preferably skin fibroblasts, hair follicles keratinocytes, or cells from a buccal swab (although the last carries the risk of blood contamination). Leukemia-associated somatic mutations that are acquired over time and possibly involved in leukemic transformation can be detected by a designated NGS somatic panel.[42,53] Except in cases of severe neutropenia/monocytopenia, PB and BM are considered equivalent sources for the detection of somatic mutations in myeloid-specific genes.[82,83] For RNA-sequencing, RNA should be extracted from PB or BM myeloid cells, preferably BM promyelocytes or PB neutrophils.

Genetic testing can be also used for the confirmation of ADAN in patients of Middle Eastern, African, Western India, and Yemenite and Ethiopian Jewish descent presenting with mild-to-moderate neutropenia (less frequently ANC $<0.5 \times 10^9$/L) without infection propensity.[23–25,84] An accurate genetic diagnosis will save an extensive unnecessary work-up. The investigation can identify the rs2814778 polymorphism in the *ACKR1/DARC* gene as described in the Definition section of this text. Notably, the same polymorphism has also been identified in a cohort of European patients (mostly Greeks) with chronic idiopathic neutropenia (CIN).[85] It is thus reasonable to include the investigation for the presence of the rs2814778 *ACKRI/DARC* polymorphism in all patients with chronic and mild neutropenia. All above recommendations are summarized in Box 6.

Box 6: What Is the Role of Genetic Testing: Which Genes, Methods, and Cell Source? What Is Its Position in the Diagnostic Algorithm?

Genetic diagnosis is important to confirm the diagnosis of CN, estimate the risk for MDS/AML, support stem cell donor selection for patients, and family counseling.

When the clinical picture, inheritance, or bone marrow features (i.e., block at the promyelocyte stage) are indicative of a specific gene mutation, single-gene analysis by Sanger sequencing technique could be applied.

For CN where the clinical picture does not suggest a specific genetic cause, we recommend the use of NGS techniques such as multigene panels or targeted WES.

For patients for whom a genetic cause is not identified by the above methods, WGS and RNA-sequencing may be powerful diagnostic tools.

NGS analysis of bone marrow or peripheral blood for acquired somatic variants is recommended for patients with chronic unexplained neutropenia.

Screening for known mutations is recommended in family members.

It is important to validate germline mutations mainly in fibroblasts or hair follicles keratinocytes (cells from buccal swab are less indicated for possible blood contamination), in the presence of leukemic blasts in PB.

AML = acute myeloid leukemia; CN = congenital neutropenia; MDS = myelodysplastic neoplasms; NGS = next generation sequencing; WES = whole exome sequencing; WGS, = whole genome sequencing.

The role of FC for the diagnosis of chronic neutropenias

FC of PB and/or BM cell populations is a supportive tool for the diagnosis of specific types of chronic neutropenia as shown in Box 7. Further to the quantification of cell populations from different lineages, it may evaluate cell surface, intracellular and intranuclear proteins, as well as the biological functions and immune characteristics of neutrophils and lymphocytes. FC is particularly useful in the following neutropenia-associated diseases.

Large granular lymphocyte-leukemia

FC analysis of PB lymphocytes is important for the diagnosis of large granular lymphocyte (LGL)-leukemia. The T-LGL subtype is characterized by the $CD3^+/CD8^+/CD57^+$ expression, whereas the NK-LGL subtype by the $CD3^-/CD8^+/CD16^+/CD56^+$ phenotype.[86] For T-LGL, diagnosis of clonality is confirmed by FC-based T-cell receptor Vβ (TCRVβ) repertoire and/or polymerase chain reaction-based TRB and/or TRG gene rearrangement analysis assays.[86] Recently, a single antibody (TRBC1-binding monoclonal antibody, clone JOVI–1) against 1 of the 2 mutually exclusive TCR β chain constant domains (TRBC1 and TRBC2) randomly selected during rearrangement of the TRB gene has been proposed as a potential FC marker for the assessment of Tαβ-cell clonality.[87] For NK-LGL, assessment of clonality is difficult to perform; restricted expression of activating isoforms of killer immunoglobulin-like receptors has been used as a surrogate marker for a monoclonal expansion.[86]

Myelodysplastic neoplasms

FC can be helpful for the identification of myeloid precursor lesions or MDS cases in patients with unexplained neutropenia, with a high specificity (up to 95%) and sensitivity (up to 75%).[62,63] FC evaluation of the BM myeloid lineage using specific panels proposed by International FC Scientific Societies can help exclude MDS.[88,89] Abnormalities of the myeloid series suggestive of MDS include the following: quantitative and qualitative alterations of cells in the $CD34^+$ blast gate; abnormal distribution of immature and mature granulocytic cells; abnormal pattern of expression of CD11b, CD13, CD33, CD16, CD10, CD5, CD7, and CD56 granulocytic cells; and low granulocytic cell granularity based on their side scatter characteristics.[62,63,88,89] FC is also indicated for the identification of paroxysmal nocturnal hemoglobinuria clones.[90,91]

Primary immunodeficiencies

Although the definitive diagnosis of primary immunodeficiencies is generally ascertained by genetic analysis, FC is particularly helpful in the diagnosis of the following: (a) autoimmune lymphoproliferative syndrome, which is characterized by increased

Box 7: The Role of Flow Cytometry for the Diagnosis of Chronic Neutropenias

FC of PB lymphocytes and BM granulocytic cells should be included in the diagnostic algorithm of adult patients with chronic neutropenias to support LGL leukemia and MDS diagnosis, respectively.

FC is an important tool in the diagnosis of neutropenia associated with PID syndromes such as ALPS, CVID, and HIGM syndrome.

Assessment of a PNH clone by FC testing is also recommended.

Flow FISH is recommended when a telomere biology disorder is suspected.

ALPS = autoimmune lymphoproliferative syndrome; BM = bone marrow; CVID = common variable immunodeficiency; FC = flow cytometry; HIGM = hyper IgM; LG = large granular lymphocytes; MDS = myelodysplastic neoplasms; PB = peripheral blood; PID = primary immunodeficiency; PNH = paroxysmal nocturnal hemoglobinuria.

frequency of TCR-α/β-positive double-negative ($CD4^-$ and $CD8^-$) T cells; (b) common variable immunodeficiency, which is characterized by the low frequency of $CD19^+$ B cells due to the low number of the switched memory ($CD27^+IgD^-IgM^-$) B-cell subpopulation, low expression of B-cell activating factor receptor (BAFFR) on B cells in patients with BAFFR mutations, and low expression of the inducible costimulator (ICOS) on activated T cells in patients with ICOS deficiency; (c) hyper IgM syndrome (HIGM) characterized by the absence of expression of CD40 ligand (CD40L; CD154) on activated $CD4^+$ T cells in the majority of patients with X-linked HIGM and lack of CD40 expression on B cells in patients with the autosomal recessive HIGM.[92]

Telomere biology disorders

Flow FISH (or other clinically validated telomere lengths measurement technique) is recommended where available, to rule out a telomere biology disorder. Given that this group of diseases cannot be entirely excluded by genetic testing, telomere length measurement may help to reduce the diagnostic gap.

NATURAL HISTORY: FOLLOW-UP

Which groups of patients need follow-up (CBC, BM, cytogenetics, and somatic gene NGS) and how often?

The importance of baseline BM evaluation including morphology, cytogenetics, and NGS-based analysis of somatic leukemia-associated gene mutations has been discussed in the previous section and is summarized in Box 8. Exceptions for baseline BM evaluations are summarized as follows: (a) newborns with a family history of CN with a known gene mutation, where the genetic analysis may be performed first, but does not replace subsequent BM evaluation; (b) infants with positive antineutrophil antibodies and no history of bacterial infections, where a BM evaluation may be omitted but should be considered if the patient develops severe or recurrent bacterial infections or additional abnormalities in CBCs; and (c) adult patients with unexplained, long-standing, mild, and isolated neutropenia that remain stable over time.

Recommendations for follow-up are also summarized in Box 8. Annual BM evaluation is highly recommended in children with CN to recognize cytogenetic abnormalities, high-risk somatic mutations, or MDS before the development of frank leukemia. Morphological assessment of BM smears, cytogenetic analysis, and somatic NGS-based sequencing of a leukemia gene panel or, at least, deep sequencing of *CSF3R*, *RUNX1*, and *TP53* should be performed.[54–58] Although a high sensitivity of *CSF3R* mutation detection has been demonstrated using NGS-based deep sequencing of PB neutrophils, evaluation of BM morphology and cytogenetics are essential for the diagnosis of MDS or leukemia. The sensitivity of detection of other somatic leukemia-associated mutations in PB compared with BM is still being evaluated. Annual NGS analysis allows assessment of the acquisition of new HSC clones carrying somatic mutations and the associated variant allele frequencies (VAF). It also enables monitoring the temporal behavior of HSC clones with acquired mutations. Notably, some CN patients with a very high frequency of *CSF3R* HSC clones do not develop leukemia for years. In addition, recent evidence suggests that CN patients with different genetic backgrounds, independent of inherited CN-causing mutations, may acquire somatic leukemia-associated mutations at a higher frequency compared with healthy individuals of the same age.[59] The same study has shown that acquisition of multiple genetic lesions, especially in *RUNX1*, *SETBP1*, *ASXL1*, *TP53*, *PTPN11* in association with CSF3R mutation might be a strong indicator of a preleukemia stage in CN patients at neutropenia stage and requires closer patient follow-up.[59]

A small group of CN/AML patients do not have either *CSF3R* or *RUNX1* mutations but develop other leukemogenic events. SDS patients have an increased frequency of clonal hematopoiesis from a young age, with the acquisition of mutations in *TP53* and *EIF6*, in particular.[7,93] Patients with overt leukemia acquired biallelic alterations of the *TP53* locus due to deletion, CN-LOH, or point mutation.[93] Therefore, annual NGS leukemia predisposition myeloid panel analysis and SNP array should be performed in the BM, or in PB myeloid cells if BM is not available.

Regarding adults with CIN, the importance of baseline NGS analysis of BM or PB cells to identify mutations in genes related to myeloid malignancies has already been highlighted (Box 6). CIN patients with clonal disease, also characterized as patients with clonal cytopenia of undermined significance (CCUS),[20] display increased risk of transformation to MDS/AML depending on the type and number of mutations and the size of clone(s).[53,94] Although studies with CCUS patients with isolated neutropenia are limited, reports from CCUS patients with any type of cytopenia have shown that gene mutations in splicing factors, *TP53*, *IDH1/2*, or in DTA (*DNMT3A, TET2, ASXL1*) in combination with mutations in other myeloid genes, have an increased risk of progression to myeloid neoplasms.[60,94,95] We recommend that patients with mutations in 1 or more MDS/leukemia-associated genes, particularly with a high VAF (>10%) and especially younger patients where the frequency of clonal hematopoiesis with indeterminate potential is low,[96–98] need to have a closer follow-up (>4 times a year) with CBC, differential count, and morphological examination of PB smear. BM examination including aspiration, trephine biopsy, karyotype, and NGS analysis should be performed as indicated, that is, worsening of cytopenias, macrocytosis, and/or morphological abnormalities.

Box 8: Which Groups of Patients Need Follow-up (CBC, BM, Cytogenetics, Somatic Genes NGS) and How Often?

As stated in Box 4, annual BM with cytogenetics and NGS of genes related to myeloid malignancies should be performed in all patients with congenital BM failure syndromes, independent of ANC and treatment with G-CSF, to assess clonal hematopoiesis and for early diagnosis of MDS or leukemia.

In chronic neutropenia patients, we recommend performing CBC with differential white blood cell counts and morphological evaluation every 3–4 months.

When approaching adulthood, CN patients should be transferred to a dedicated hematology specialist.

Particularly for adults:

As already stated in Box 4, a diagnostic BM with morphology and cytogenetics should be performed in all adult patients with unexplained chronic neutropenia with the exception of those with long-standing, mild, isolated neutropenia that remains stable over time.

As already stated in Box 6, NGS analysis of BM or peripheral blood for acquired somatic variants is recommended for patients with chronic unexplained neutropenia.

Patients with mutations in 1 or more MDS/leukemia-associated genes, particularly with high VAF (>10%) and especially younger patients where the frequency of CHIP is low, need to have a closer follow-up (>4 times a year) with CBC, differential white blood cell count, and morphological examination. BM examination including aspiration, trephine biopsy, karyotype, and NGS analysis should be performed when indicated, that is, worsening of cytopenias, macrocytosis, and/or morphological abnormalities.

ANC = absolute neutrophil count; BM = bone marrow; CBC = complete blood counts; CHIP = clonal hematopoiesis of indeterminate potential; CN = congenital neutropenia; G-CSF = granulocyte colony stimulating factor; MDS = myelodysplastic neoplasms; NGS = next generation sequencing; VAF = variant allele frequency.

Surrogate markers of transformation into MDS/leukemia

The key markers of malignant transformation to MDS or leukemia in CN patients are the following: typical dysplastic features in PB (pseudo Pelger-Huët anomaly, hypogranulation, hypersegmentation, reticulated nucleus, and ringed-shaped nuclei) and BM (defective granulation, maturation arrest at myelocyte stage, and increase in monocytoid forms); cytogenetic abnormalities; and, high frequency of somatic mutations in leukemia-associated driver genes.[99] The most common chromosomal defects in patients with CN at the MDS stage are trisomy 21 and monosomy 7. In CN patients, there is a clear correlation between the high frequency of somatic *CSF3R* and *RUNX1* mutations and leukemia development.[52–59] Typical leukemia-associated *CSF3R* mutations in CN patients are truncation mutations in the intracellular part of the receptor, leading to the absence of 1, 2, or 3 tyrosine phosphorylation sites.[56,100] *RUNX1* missense or truncation mutations are characteristic in de novo AML.[54,55] In SDS patients, the acquisition of biallelic alterations of the *TP53* locus is associated with progression to MDS and AML.[93,101]

In patients with CIN, clonal hematopoiesis involving spliceosome gene mutations and comutation patterns involving epigenetic regulators such *TET2*, *DNMT3A*, *ASXL1*, or *IDH1/2* have a positive predictive value for MDS/leukemia transformation.[60,94,95] The surrogate markers for MDS/AML transformation are summarized in Box 9.

SPECIAL SITUATIONS

Pregnancy

Long-term G-CSF treatment has been shown to be safe and effective in severe CN resulting in prolonged life expectancy of patients. For patients who have reached adulthood the desire for parenthood has become an emerging issue. Two questions are asked frequently in this respect.

Can I continue using G-CSF for the treatment of neutropenia during pregnancy?

Data on the use of G-CSF during pregnancy is limited (see list of publications in Suppl. Table S1).[102–111] However, in all publications, the use of G-CSF throughout pregnancy has been documented as safe and well tolerated with no noticeable side effects.

What is the risk of having an affected child?

With an increasing number of adult patients suffering from genetic neutropenia subtypes, genetic counseling before conception and supportive care of mothers during pregnancy are crucial. It is important that the patient is informed that the acceptance of having affected children is reasonable given the high quality of life obtained due to the treatment with G-CSF.

The recommendation for the use of G-CSF during pregnancy is summarized in Box 10. Available data support the continuation of G-CSF treatment in women with different types of SCN throughout the pregnancy to prevent major infections and newborn complications. The recommendation is based on the reported high risk for bacterial infections and septic death in severe CN. The risk may be lower for patients suffering from CyN or IN. However, because there is no better predictor for this risk than the ANC, we recommend offering G-CSF treatment to all patients with ANC below 0.5×10^9/L if they were on G-CSF treatment before pregnancy; for patients who were not on treatment, G-CSF treatment might be also considered. Based on experts' experience, we recommend frequent evaluation of ANCs during pregnancy, particularly in patients with AIN, because an ANC increase can be seen during pregnancy.

Box 9: Prognostic Indicators of Transformation into MDS/Leukemia

Typical morphology for MDS in peripheral blood and/or bone marrow, cytogenetic abnormalities (trisomy 21, and monosomy 7), somatic leukemia-associated mutations (e.g., CSF3R, RUNX1, and ASXL1), and biallelic TP53 mutations in Shwachman-Diamond syndrome are prognostic indicators of development into MDS/leukemia.

Neonatal neutropenia

As already mentioned in Definition and Diagnosis section, the lower normal limit of ANC in Caucasian children up to the age of 1 year is 1.0×10^9/L. However, this limit is different in neonates <14 days of life where there is a great variability, with the gestational age being the principal variable affecting the normal range.[16] In healthy and at term newborns between 6 and 24 hours of age, neutropenia is defined as an ANC $<6.0–7.0 \times 10^9$/L; after that, there is a slow decrease and the limit is $<3 \times 10^9$/L at about 72 hours of life. However, the situation is very different in preterm newborns. For nonextreme preterms, the lower limit of ANC is $<3.0 \times 10^9$/L at 24 hours of age and $<1.0 \times 10^9$/L at 72 hours of life (less than half of the at term newborn cutoff); in extremely preterm newborns, the fifth percentile of normal ANC is about 1.0×10^9/L immediately after birth.[16] Moreover, there are many other variables and diseases, related or not to pregnancy or delivery, capable of interfering with the ANC. Examples of variables not related to pregnancy or delivery are the following: (a) females have ANC counts on average 2.0×10^9/L higher than males;[16] (b) ANC in capillary blood is on average $1.5–2.0 \times 10^9$/L higher than in cord blood;[112,113] (c) ANC is on average higher at altitude than at sea level;[114] and (d) severe necrotizing enterocolitis in the newborn, especially if preterm, is frequently associated with transiently low ANCs.[115] Examples of variables related to pregnancy or delivery include the following: (a) maternal tobacco smoking is associated with lower ANC;[116] (b) maternal chemotherapy results in neutropenia in 5%–33% of the newborns;[117] (c) maternal antiretroviral therapy results in neutropenia in about 20%–50% of the newborns;[118] (d) maternal hypertension during pregnancy results is neutropenia in about half of newborns;[119] (e) prenatal growth retardation is an independent risk factor for neutropenia;[120] (f) labor before delivery results in higher ANC;[16] (g) Rh-hemolytic disease of the newborn is associated with neutropenia in about 50% of newborns whether or not combined with severe anemia and thrombocytopenia;[121] (h) twin-twin transfusion syndrome is a rare condition with neutropenia always present in the donor twin (the anemic one);[122] and (i) neutropenia is present in 67% of infants with asphyxia.[123]

The most common types of neonatal neutropenia are discussed below.

Infection-induced neutropenia

Infection-induced neutropenia is probably the most common neutropenia in newborns and infants.[124] The duration of neutropenia is frequently short, typically <10 days, and therefore, a diagnosis of infection-related neutropenia can be considered

Box 10: Pregnancy

The panel recommends G-CSF treatment to all patients who were on G-CSF treatment before pregnancy and all patients with severe congenital neutropenia.

For patients who were not on treatment, G-CSF treatment might be considered.

It also suggested to frequently evaluate ANCs during G-CSF therapy in pregnancy, particularly in patients with autoimmune neutropenia, because a physiological increase can be seen.

After delivery, the neutrophil count of the newborn should be checked.

CBC = complete blood counts; G-CSF = granulocyte colony stimulating factor.

appropriate if, some days after a proven infection, the ANC has normalized.[125] Nevertheless, it must be emphasized that occasionally some infections, for example, those caused by HIV or human hepatitis C virus, can cause a long-lasting neutropenia.[126,127]

Immune neutropenias

Neonatal immune neutropenias can be subclassified as the following:

1. AIN, which is unusual, but not impossible, at <1 month of age.[41,128]
2. Neonatal alloimmune neutropenia (NAN) in which a genetic mismatch for a polymorphism in one of the genes encoding human neutrophil antigens (HNA) between mother and fetus leads to immunization of the pregnant woman, passage of alloantibodies over the placenta and neutropenia in the baby.[129] Indirect antineutrophil antibodies are positive in the mother and in the newborn. The diagnostic confirmation may be obtained through a positive cross-match between maternal sera and paternal granulocytes (even if not routinely indicated). Although fetomaternal incompatibility is found in about 20% of pregnant women, alloimmunization in 0.6%–1% of pregnant women, NAN occurs only in 1:6000 newborns.[129,130]

Serious infections, mainly of skin and umbilical cord, occur in 1 out of 5 patients with NAN and the duration of neutropenia is on an average 1–4 months.[129] There are not many studies on therapy but a beneficial effect of a dose of 10 μg/kg of G-CSF has been reported.[130,131] There is no agreement about the duration of G-CSF therapy but, considering that neutropenia lasts only a few weeks and that there are some reports of serious infectious complications, G-CSF administration up to recovery of ANC should probably be considered.[131] Finally, there are a few cases of total inefficacy of G-CSF[132–134] and in some of them intravenous administration of immunoglobulins at 0.8–1 g/kg was effective.[132]

3. NAN secondary to maternal AIN is the rarest immune neutropenia of early infancy. The duration of this neutropenia is on an average the same as the classic NAN.[135] Among the reported patients, there are 2 pairs of brothers:[136,137] in the first pregnancy both the mothers and the neonates were not treated with G-CSF and both infants suffered from severe infections. During the second pregnancies, both mothers were given low-dose G-CSF and both newborns did not have neutropenia, with an excellent outcome. We, therefore, suggest that G-CSF should be offered to the mothers.

In conclusion, there are 3 different types of neonatal immune neutropenia: (1) NAN: in this case, it is fundamental to prove the HNA mismatch between the mother and the newborn as well as the presence of indirect antineutrophil antibodies in both of them; (2) NAN secondary to maternal AIN: it is easy to diagnose, being the mother affected by AIN; and (3) AIN with appearance at <1 month of age: this situation is extremely rare and requires exclusion of a false-positive indirect antineutrophil antibody and,

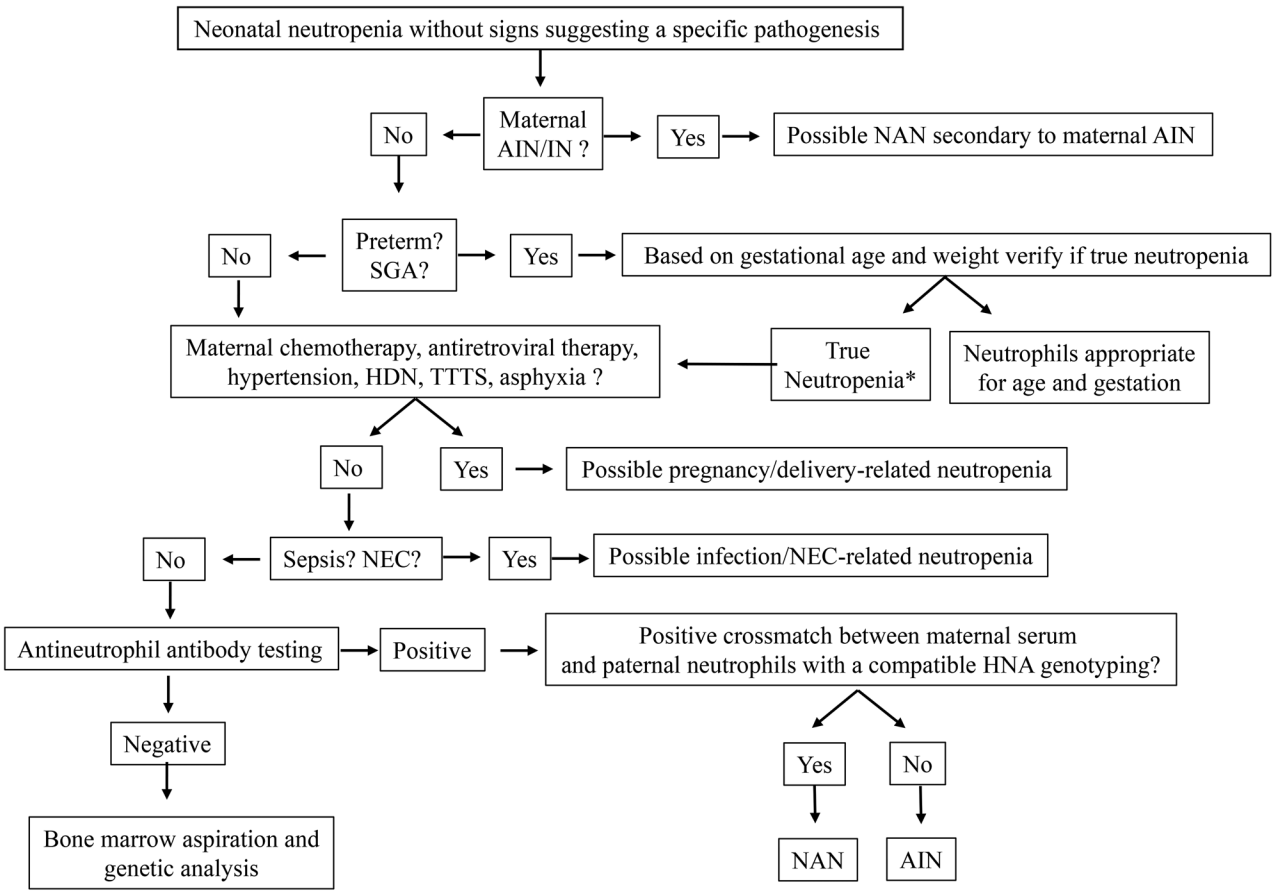

Figure 2. Flowchart for the investigation of isolated neutropenia in neonates. The flowchart is recommended in cases of isolated neutropenia without a phenotype suggestive of any specific disease/syndrome. *The neutropenia is defined as true if absolute neutrophil counts are out of range for the corresponding gestational age and there is no a prenatal growth retardation. AIN = autoimmune neutropenia; HDN = Rh-hemolytic disease of the newborn; HNA = human neutrophil antigens; IN = idiopathic neutropenia; NAN = neonatal alloimmune neutropenia; NEC = necrotizing enterocolitis; SGA = small for gestational age; TTTS = twin-twin transfusion syndrome.

especially, if neutropenia is associated with concurrent infection, genetic analysis should also be performed to exclude CN.

Transient neutropenia of infants

Transient neutropenia of infants is typically observed at ~3–4 weeks of life in former preterm newborns who suffered from anemia of prematurity.[138–140] It is probably related to HSC competition between compensatory erythropoiesis and granulocytopoiesis.[138–140]

Severe CN

Severe CN syndromes can obviously have their clinical presentation in neonatal age. For the investigation of isolated neutropenia in neonates without a phenotype suggestive of any specific disease/syndrome, it is advisable to pursue the pathway described in the flowchart of Figure 2.

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