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.