Old and new faces of neutropenia in children

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In children, the term neutropenia usually identifies a group of inherited and acquired diseases characterized by a reduced number of mature circulating neutrophils and by an increased susceptibility to infections. Neutropenia is defined as severe when the absolute neutrophil count (ANC) is below $0.5x10^9$/L.[1] This article will provide an overview, with special focus on the most recent findings, on the two main forms occurring in children: severe congenital neutropenia (SCN) and primary autoimmune neutropenia (AIN).

Severe Congenital Neutropenia

SCN encompasses a heterogeneous group of often inherited disorders appearing early in infancy with a variable clinical phenotype.[2] In many cases neutropenia represents the sole disease, but sometimes it is associated to a multisystem involvement (neurological, endocrine, immune systems and other somatic districts).[3]

Mutations in different genes with various inheritance can cause SCN. In 1956 Rolf Kostman first described a cluster of neutropenia patients in a northern Swedish family in whom the disease was fatal within the first year of life because of infections. Later on this neutropenia was attributed to mutations of the *HAX1* gene that is transmitted in an autosomal recessive fashion, and is often associated to neurological symptoms.[4] The term Kostmann syndrome has sometimes been inappropriately used for neutropenia due to mutations of the *ELANE* gene, which is responsible for more than half of European and North American SCN patients.[5] The *ELANE* gene encodes for neutrophil elastase. More than 120 distinct *ELANE* mutations, either transmitted in an autosomal dominant mode or sporadically, have been described so far.[6] Some of them are shared in both cyclic and severe congenital neutropenia without a clear explanation of how a given genetic lesion may be associated to different phenotypes.[6] Mutations in other genes like *GFI1*, *WASP*, *G6PC3* and *VPS45* are also the cause of SCN, although less frequently.[7-9] *JAG1* and *TCIRG1* are the latest discovered genes resulting in isolated neutropenia.[10-11] Mutations of *COH* (Cohen syndrome), *BTK* (X linked form), *CD40L* (Hyper IgM) and *CXCR4* (WHIM syndrome) genes generate neutropenia in the context of immunodeficiencies.

Recently, mutations of the *CLBP* gene were reported as a cause of SCN associated to cataracts, neurological impairment and increased urinary excretion of 3-Methylglutaconic acid (3-MGA) within the framework of the autosomal recessive metabolic disorder MEGCANN. The *CLBP* gene encodes for a mitochondria protein that is widely expressed in human tissues including granulocytes and- to a larger extent- the brain, and interacts with other proteins like HAX1 which has a critical role in the maintenance of mitochondria transmembrane potential, thus preventing excessive cell apoptosis.[12]

It is, however, worth noting that in spite of the untiring research activity in the genetic neutropenia field, more than one third of SCN patients are still gene orphans thus far.

In many cases the lack of neutrophil production is due to a marrow maturation block at the promyelocyte stage, as occurs in *ELANE*, *HAX1*, *G6PC3*, *WAS* and *JAGN-1* gene mutations. In these cases, myeloid precursors beyond promyelocytes are not produced because of increased apoptosis[13] occurring through different mechanisms like unfolded protein response (ELANE and G6PC3) or deranged mitochondria transmembrane potential (HAX1 and CLBP). Apoptosis may affect cells other than marrow myeloid precursors, like neurons, urinary tract cells, lymphocytes and natural killer cells, thus accounting for the multisystem phenotype observed in some forms of SCN (Kostman, glycogen storage disease 1b, GATA2 and MEGCANN diseases).[14-15] In other circumstances the pathogenic mechanism resides in the lack of/scarce sensitivity to endogenous G-CSF due to the dysfunctionality of the extracellular portion of the G-CSF receptor (G-CSF3R) or to the defective mobilization of bone marrow neutrophils (WHIM syndrome).[16-17]

The common denominators of the clinical phenotype of SCN are the infections and the risk of transformation into MDS/AML.[18] After the introduction in the 1990's of G-CSF in clinical practice, infections have become generally manageable.[19-20] Conversely, the prolonged life duration achieved with G-CSF incremented the evolution towards MDS/AL whose cumulative incidence, according to the Severe Chronic Neutropenia International Registry (SCNIR) and the Severe Neutropenia French Registry (SNFR), is estimated at 22% and 10.8%, respectively, after 15 years from the start of G-CSF therapy. The risk of transformation has been correlated to the dose and the duration of G-CSF exposure, with amounts higher than 8 μg/kg/day being associated to increased risks.[21-22]

The neoplastic transformation is in part due to factors intrinsic to neutropenia and in part to pro-cancer elements acquired over time. Some neutropenia diseases are constitutively more prone to transformation. This is the case of Shwachman-Diamond syndrome, whose cumulative incidence of MDS/AL is 18–36% over a timespan of 20–30 years, according to the North American Shwachman-Diamond Syndrome Registry and the SNFR.[22-23] Some specific *ELANE* mutations (i.e. G214R or C151Y) are more frequently associated to transformation.[6]

Another factor significantly associated with the development of MDS/AML in SCN is the acquisition of a truncating mutation of *CSF3R* genes that were found in 78% of SCN/AML cases, whereas they were present in only 34% of SCN non-leukemic patients.[24] *"Per se"*,the presence of a CSF3R mutation does not automatically herald the advent of leukemia since distinct mutated clones may co-exist, and sometimes rise and disappear in a stochastic model.[25-26] Additional "cooperative events" are required, throughout the

course of transformation, after the first hit.[27] In this respect a study of SCN patients on G-CSF who underwent leukemic transformation showed, in sequential analyses, that *RUNX1* mutations appeared after CSF3R mutations in the transforming cells,[28] thus pointing to this sequence of genetic events as a novel leukemogenic pathway in SCN. Other later events like monosomy 7, trisomy 8 and/or trisomy 21 may appear just before the MDS/AL onset and can be considered as a further event in the leukemogenic evolution.[28]

The mechanisms by which the genetic events contribute to clonal transformation have not yet been fully elucidated, but it is hypothesized that mutations of the external part of CSF3R induces a sustained activation of STAT5 that leads to ROS production with consequent intracellular DNA-damage.[27] Other evoked mechanisms include the formation of RAS oncogene activating mutations and *CSF3R* gene mutations conferring proliferative advantage to transforming cells.[29]

Cyclic neutropenia (CyN), a disease characterized by oscillating neutrophil counts with a periodicity of 21-28 days,[30] has long been considered a "relatively" benign disorder based on the absence of reports of clonal evolution in the international registries.[21-22] Interestingly, a patient with CyN due to *ELANE* mutations, formerly detected in SCN but not in CyN subjects, was recently shown to evolve to AML. A *CSF3R* p.Gln741X mutation was found in leukemic cells and *RUNX1* mutation p.Asp171Asn was present in the patient's marrow cells.[31] These findings suggest that CyN subjects may also undergo clonal evolution and that this may occur through a molecular mechanism similar to that seen in other "less benign" forms of SCN. These findings might potentially change the monitoring policy in CyN patients, who will have to be more tightly monitored for clonal escape than before.

Overall, the above findings suggest a careful surveillance of the blood count and bone marrow of SCN patients with monitoring focused on *CSF3R* and *RUNX1* mutations, particularly in those subjects treated with high cumulative doses of G-CSF. The Marrow Failure Study Group of the AIEOP (Associazione Italiana Emato-Oncologia Pediatrica) recommends an extensive bone marrow study with cytogenetics and G-CSFR mutation analysis every year, to be moved to every 6 months or earlier in the case that new mutations or abnormal clones appear.[32]

In patients poorly/non responding to G-CSF (i.e., requiring>10 μg/kg/day and > 20 μg/kg/day, respectively) or already transformed to MDS/AL, stem cell transplantation (SCT) is an indicated treatment option.[33-34] The outcome of SCT in 136 SCN patients was analyzed in a collaborative study from EBMT and SCETIDE.[34] The three year probability of OS and EFS were 82% and 71%, respectively. In multivariate analysis, factors associated to a better outcome were transplant before age 10 years from an HLA-matched, related or unrelated, donor. Late post-SCT tumors were not reported. Transplant related mortality was 17%, thus suggesting that this procedure should not be offered to patients who can be effectively and easily managed with standard doses of G-CSF (5 μg /kg/day or less).[34]

Research on new therapies are limited by the lack of animal models recapitulating the human severe neutropenia phenotype. Recently, induced pluripotent stem cell (iPSC) lines from SCN subjects have been generated and might become a helpful tool to screen possible new drugs.[35]

Autoimmune Neutropenia

The most clinically relevant form of acquired neutropenia in children is primary autoimmune neutropenia (AIN), a disease typically appearing in early infancy due to the production of specific antibodies against human neutrophil antigens, that is regarded as due to the immaturity of the immune-suppression system allowing the autoantibody production as a consequence of a "surveillance escape event". The clinical characteristics and outcome of AIN have been recently described in a large cohort of 157 patients.[36] The median ANC and age at diagnosis were $0.45x10^9$/L and 1.06 years, respectively, and the median time of resolution was 2.14 years. Severe infections occurred in 9.6% of cases, and recovery from neutropenia in about 89.9% within 5 years from diagnosis. No antibiotic prophylaxis was used, and G-CSF was administered in 7.1% of patients only during severe infections. In multivariate analysis, factors associated with a favorable outcome were early age at onset and lack of monocytosis. Although primary AIN generally appears as a substantially benign and self-limiting condition, some subcategories of patients, such as those with later onset or with disease persisting for many years or who develop involvement of further cell lineage need to be thoroughly assessed for secondary forms. Indeed, a multilineage immune-mediated cytopenia including neutropenia might be the manifestation of a wider autoimmune disorder or an epiphenomenon of an immune dysregulation,[37] like autoimmune lymphoproliferative syndrome (ALPS) which is caused by defective FAS-mediated apoptosis signalling.[38] Autoimmune cytopenias may also be secondary to common variable immune deficiency (CVID) or to "ALPS-like syndrome", a clinical phenotype close to ALPS but with a still undefined genetic background.[37]

Of note, patients formerly diagnosed with one of the above diseases were recently found to have an abnormal T-cell hyperproliferation due to pathogenic mutations upregulating mTor (mammalian target of rapamycin),[39] as in the case of the defective function of phosphoinositide 3-kinase δ (*PI3Kδ*).[40] Other mutations may either generate deficiency of the suppressing T-cell molecule CTLA-4,[41] or impair its exposure on the cell membrane because of the deficiency of another anchor protein named LRBA (LPS-Responsive beige-like anchor protein deficiency).[42] These immune dysregulation diseases have a heterogeneous clinical phenotype which may include neutropenia. For these reasons children with atypical AIN, due to late onset, late recovery and involvement of additional cell lineage over time, require extensive immunological workup, including the evaluation of lymphocyte subsets with double negative T-cells and immunoglobulin serum level measurement. Clinical surveillance focused on organs which are potentially the target of immune dysregulation like the lungs, bowels and joints is also recommended. Finally, the identification of these non pure AIN patients has some important clinical implications. First, they have an increased risk of lymphomas, that in the case of ALPS is estimated to be 14- and 50-fold higher for non Hodgkin

and Hodgkin disease, respectively, that requires close monitoring. Second, patients resistant to first-line therapies like steroids and intravenous immunoglobulins, may respond to immunosuppressive drugs such as MMF and sirolimus,[43] normally not indicated in classical AIN.

In conclusion, future efforts should concentrate on the definition of pathogenic and leukemogenic mechanisms and to further refine treatment to minimize infection and clonal evolution risks. Children with neutropenia should undergo a comprehensive diagnostic and monitoring program in specialized pediatric hematology centers.

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