PhD COURSE in PEDIATRIC SCIENCES (XXXV c.)

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"Clinical, immunological and genetic profile of chronic neutropenia: beyond primary autoimmune and idiopathic forms"

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1.Introduction

1.1. Definitions

“Neutropenia” refers to a condition characterized by a reduction of the circulating neutrophil count (Absolute Neutrophil Count, ANC) below the threshold for ageand ethnicity.

For Caucasian infants and children younger than one year of age, neutropenia is defined for ANC below1.0 x 10^9/L while for those subjects above 1 year of ageto adulthood ANC threshold to define neutropenia is 1.5 x 10^9/L. According to current knowledge,Africans,Caribbean and Middle Eastern ethnic populations, carry lower level of nethrophils than Caucasians. [1]

In contrast, neutropenia during the first week of life and in preterm births (28-36 weeks) is defined for ANC value below 2.5 x 10^9/L,while in early preterm (<28 weeks) neutropenia is defined for values below 1.0 x 10^9/L. [2]

Based on the circulating neutrophil count, in the Caucasian population after the first year of life, different degrees of neutropenia are distinguished:

- Mild neutropenia, for ANC between 1.0 and 1.5 x 10^9/L
- Moderate neutropenia, for ANC between 0.5 and 1.0 x 10^9/L
- Severe neutropenia, for ANC below 0.5 x 10^9/L, or very severe for ANC below 0.2 x 10^9/L.

In addition, chronic neutropenia is defined as a reduction in ANC that persists for more thanthree months. [3]

1.2. Classification of neutropenias

Neutropenia is basically categorized as congenital and acquired.

Congenital neutropenia includes isolated disorders and neutropenia associated with hematologic or extrahematological symptoms (***Table 1***).Acquired forms include drug-related neutropenia, post-infectious, autoimmune and alloimmune neutropenias [4-7]

Idiopathic neutropenia isan exclusion diagnosisthat should be periodically reassessed. New elements possibly acquired during follow-up may better characterize the profile of this condition, allowing its reclassification. [8]

Table 1. Neutropenia classifications

<table>
<tr><th colspan="2">Congenital neutropenia</th><th rowspan="11">Idiopathic neutropenia</th><th colspan="2">Acquired neutropenia</th></tr>
<tr><td rowspan="2">Isolated</td><td>Severe congenital neutropenia</td><td rowspan="2">Autoimmune</td><td>Primitive</td></tr>
<tr><td>Cyclic neutropenia</td><td>Secondary</td></tr>
<tr><td rowspan="9">Associated to</td><td rowspan="2">Extra-hematological signs</td><td rowspan="2">Alloimmune</td><td>Primitive</td></tr>
<tr><td>Secondary</td></tr>
<tr><td rowspan="2">Metabolic disorders</td><td colspan="2">Associated to myeloproliferative disorders</td></tr>
<tr><td colspan="2">Associated to acquired bone marrowfailure</td></tr>
<tr><td rowspan="2">Immunodeficiency/ Immunodysregulation</td><td colspan="2">Post-infectious</td></tr>
<tr><td colspan="2">Drug related</td></tr>
<tr><td rowspan="2">Bone marrowfailure</td><td colspan="2">Related to nutritional deficiencies</td></tr>
<tr><td colspan="2">Neutropenia in pregnancy</td></tr>
</table>

1.3. Autoimmune neutropenia

Autoimmune neutropenia (AIN) is defined as a reduction in ANC associated with the presence of antineutrophil antibodies in the patient's serum. [8]

AIN can be defined as primary, which is generally an isolated condition or secondary, when neutropenia is concomitant to autoimmune disorders or other conditions like neoplasm, bone marrow transplantation etc.

Anti-neutrophil antibodies

Five classes of neutrophilsurface antigens have been described (Human Neutrophil Antigens, HNAs), with different frequency in the population. [9-11] In addition, specific type of antibodies directed against neutrophil surface antigens, drive the diagnosis towards different types of neutropenia (i.e. alloimmune or autoimmune).

In most patients with immune-mediated neutropenia, the antibodies are directed against FcgIIIB receptor on neutrophil surface, even though other target have been identified.

Antibodies directed against neutrophils cause, through antibody recognition and complement activation, an increase in peripheral phagocytosis and an inhibition of bone marrow proliferation, thereby causing a reduction in neutrophil circulating neutrophils. [12-14] The antibodies themselves also have a qualitative effect on the action of the neutrophils. Indeed, their presence causes a reduction in the production of CO2 and free radicals, interfering with the aggregation-disaggregation mechanisms of the neutrophils and with their motility.

Methods of identification of anti-neutrophil antibodies

The most widely method used for the identification of antibodises directed against neutrophils is GIFT (both indirect and direct granulocyte immunofluorescence test). Indirect test, basically detects free anti-neutrophil antibodies in the patient's serum. Heterologous donor neutrophils are incubated with the patient's serum to allow reactive antibodies to bind antigenic epitopes; subsequently neutrophils are washed and incubated with a reagent mixture of IgG globulin, IgM fluorescently labeled. The presence of antibodies bound to the surface of neutrophils is detected by flow cytometry or microscopic fluorescence.

Another frequently usedtest is the granulocyte agglutination test (GAT), which reveals a semiquantitative phenomenon of agglutination occurring after reaction between patients serum and neutrophils contained in the substrate.

According to the most recent Workshop on Granulocyte Laboratory Experts, the combination of GAT and GIFT represents the golden standard to detect antibodies against neutrophils. [15,16]

Other indirect methods are also available (monoclonal antibody-specific immobilization of granulocyte antigens, MAIGA; flow cytometric white blood cell immunofluorescence assay, Flow-WIFT; microbeads assay LabScreen® Multi), of which some are more specific (MAIGA) or more sensible (Flow-IFT) than I-GIFT. However, these methods are technically complicated and require time prolonged, so their applicability in clinical practice is limited. [17]

The direct test detect antibodies on the surface of neutrophils of the patient, is associated with a high rate of false positives. [18]

The I-GIFT test has high specificity and positive predictive value (85% and 91.8% respectively), compared with a sensitivity limited to 62.5%. [19] However, sensitivity increases to 82% by repeating the test. [20,21] Therefore, in case of negativity of the first determination and clinical suspicion of AIN, the test should be repeat at least four times over the course of 4-6 months.

Conversely, given the rarity of false positives, a positive or borderline result for anti-neutrophil IgG or IgM in at least one sample, associated with a clinical phenotype suggestive of AIN, is enough to make the diagnosis of AIN. [1,21-27].

When antibodies against neutrophils are repeatedly negative, in spite of clinical characteristic very similar to autoimmune form, neutropenia is defined as idiopathic.

Primary Autoimmune Neutropenia

Primary AIN (pAIN) has a typical onset in early childhood both in males and females. Severe infections are rare, and neutropenia in most patients spontaneously remits within 24-36 months from onset. [1]

The estimated incidence is 1/100,000 in children younger than 10 years of age. A higher incidence of 1:6300 live births has been reported in Sicily.[20] However, it's likely that this figure is bit underestimated, given the benign course of the disease and the high frequency of diagnosis done by chance (8-27%). [28,29]

Neutropenia would be probably caused by the neutrophils killing due to the specific antibodies action which do not recognized "self surface antigens" possibly modified by drugs components or by infective agents (ie molecular mimicry). An another interesting hypothesis regarding development of neutropenia is related to the T-suppressor immaturity which is not able to manage immunological "anarchy". [21,30,31]

Bone marrow smear often shows shift left of granulocyte maturation which means predominance of progenitors at an earlier stage, in the absence of a definite maturation arrest. The myeloid

hypoplasia occasionally observed could be explained by the effect of autoantibodies on bone marrow precursors. [32-34]

Over the past 30 years, several cohorts of patients affected with AIN have been published and the observations sometime overlap as shown below (***Table 2***). [19-21,30,35-39].

Table2. Characteristics of differentcohorts of children with pAIN

Author	**Number of patients**	**Age at diagnosis (months)**	**Female (%)**	**Severe infections (%)**	**Recovery (%)**	**Age at recovery/duration of neutropenia (months)**
Lazelari P 1986	121	8 (3-30)	60	---	95	---/20
Bux J 1998	240	8 (5-15)	54	12	80	---/7-24
Bruin M 1999	21	---	---	No	86	---/30 (16-52)
Chung 2004	24	9	50	10	55 at 3 years	----/28.6
Wang L 2008	55	9.8 (4-28)	45	No	100 (in 24 patients)	22.5 (13-44)/12.7
Sella R 2010	72	10 (0-42)	37	15	100 (in 53 patients)	---/4.4 (0.5-30)
Audrain M 2011	116	16 (3-59)	48	---	---	---
Farruggia P 2015	157	8 (0-54)	36	9.6	90 at 5 years	25.7/15.6

In summary, these studies have shown common characteristics: young age of onset (7-8 months), resolution in almost all cases (80-90 %) within 36 months from diagnosis, mild clinical phenotype and low incidence of severe infections (estimated around 10-15% of cases).

Secondary Autoimmune Neutropenia

Secondary AIN (sAIN) usually occurs in late childhood or adolescence, mostly in female subjects. It may be associated with infection, immunodeficiency, neoplasms, drug administration, hematopoietic stem transplantation, other autoimmune diseases (e.g. Evans syndrome, autoimmune thyroiditis, systemic lupus erythematosus, and many others). [8]

Data regarding sAIN in pediatric age are rather scarce. A recent Italian study analyzed a small case series from the Italian Neutropenia Registry, comparing the characteristics of the 26 patients affected with sAIN with a cohort of 263 patients diagnosed with pAIN. [40] The study showed significant differences between primary and secondary neutropeniaas summarized in ***Table 3***.

Table 3. Comparisonbetween a cohort of pAINs and a cohort of sAINs [40]

	pAIN (263)	**sAIN (26)**	***p***
Gender (F%)	41%	61%	0.049
Age at onset (median)	0.77	10.07	<0.01
Age at diagnosis (years, median)	1.09	10.98	<0.01
G-CSF	6.9%	23.1%	<0.01
Severe infections	11.8%	40.0%	0.0001
Spontaneous recovery	74.9%	7.7%	<0.01
Age at recovery (median)	2.14	14.11	<0.01
Leucocytes (median) at onset	5.93 x 10^9/L	2.48 x 10^9/L	<0.01
Lymphocytes (median) at onset	4.36 x 10^9/L	1.58 x 10^9/L	<0.01
Monocytes (median) at onset	0.62 x 10^9/L	0.34 x 10^9/L	<0.01
Neutrophils (median) at onset	0.45 x 10^9/L	0.63 x 10^9/L	0.035

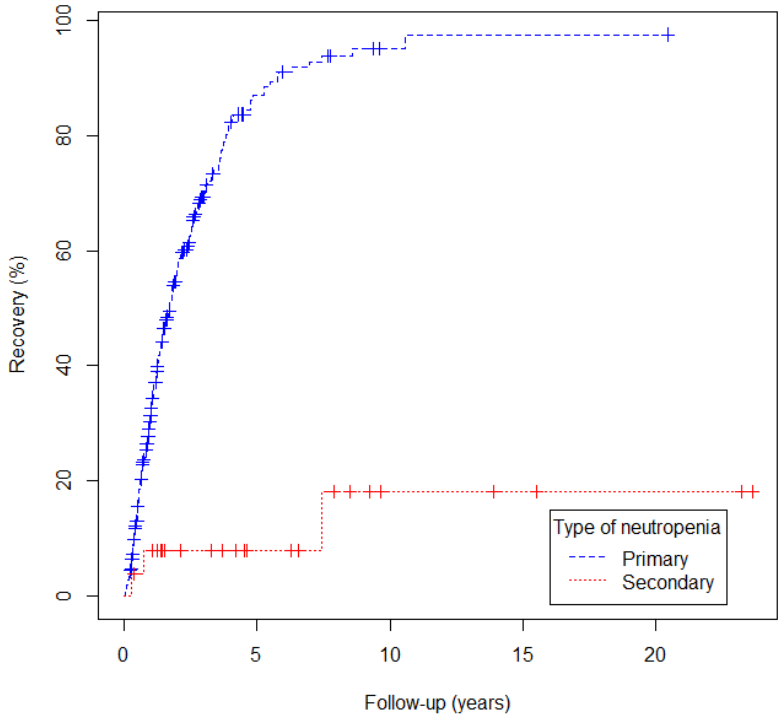
The median age at onset of neutropenia was 0.77 years for pAIN and 10.07 years for sAIN. Among patients with pAIN there was a higher proportion of ex-preterm patients. This finding is interesting because in agreement with the hypothesis that pAIN is related to the presence of still immature T-suppressor system.

The mean value of neutrophils at onset is significantly lower in pAIN than in sAIN (0.45 x 10^9/L vs 0.63 x 10^9/L), while lymphopenia is present in sAIN only (1.58 lymphocytes x 10^9/L in the sAIN vs 4.36 x 10^9/L in the pAIN). Other differences are: the higher frequency of severe infections and leucopenia in sAIN compared to pAIN. Conversely, monocytosis is far more frequent in pAIN than in sAIN.

In sAIN described in the paper cited above, neutropenia was associated with: Evans syndrome, thyroiditis autoimmune, coeliac disease, GH deficiency, diabetes, systemic lupus erythematosus, hepatitis, autoimmune encephalitis. In 42.3% of sAIN patients, symptoms of autoimmunity appear contemporary to neutropenia, while in 30.7% they preceded it, and in 26.9% followed it.

sAIN tended to resolve in a minority of cases (7.7% of patients) which is a lower percentage than the one observed in pAIN. This characteristic identifies secondary neutropenia as a condition with a tendency to become chronic, as shown in ***Figure 1***.

Figure 1. pAIN and sAIN resolution rates observed in the Italian Neutropenia Registry study [40]



In adulthood, sAIN is more frequent than pAIN, in association with infections, drug administration,

immunodeficiency, neoplasms, hematopoietic stem transplantation, other autoimmune diseases. Autoimmune diseases associated with sAIN include Evans syndrome, autoimmune thyroiditis, systemic lupus erythematosus, Sjögren's syndrome, arthritis rheumatoid, Felty's syndrome, autoimmune hepatitis, and multiple sclerosis. [41]

Secondary neutropenia in pediatric age has some features shared with AIN in adults: the prevalence of the female sex (60% in the pediatric sAIN, 70% in adult), the low frequency of spontaneous resolution (10% approximately), the frequency of leucopenia,severe infections, use of G-CSF therapy and monocytosis at onset.
These data suggest that pediatric sAIN and adult AIN, both primary and secondary, seems to be actually the same pathology.

1.4. Idiopathic neutropenia

Idiopathic neutropenia (IN) is defined as chronic neutropenia not due to genetic, or associated to infectious, inflammatory or autoimmune disorders and not even concomitant to malignancies. It's therefore a diagnosis of exclusion. Given the low sensitivity of anti-neutrophil antibody detection methods, there's an overlap between the autoimmune and idiopathic neutropenias. [42]
Within IN two different forms may be recognized: idiopathic neutropenia of childhood, phenotypically similar to primary AIN and IN of adults (Chronic Idiopathic Neutropenia, CIN). [1]

Idiopathic neutropenia of childhood

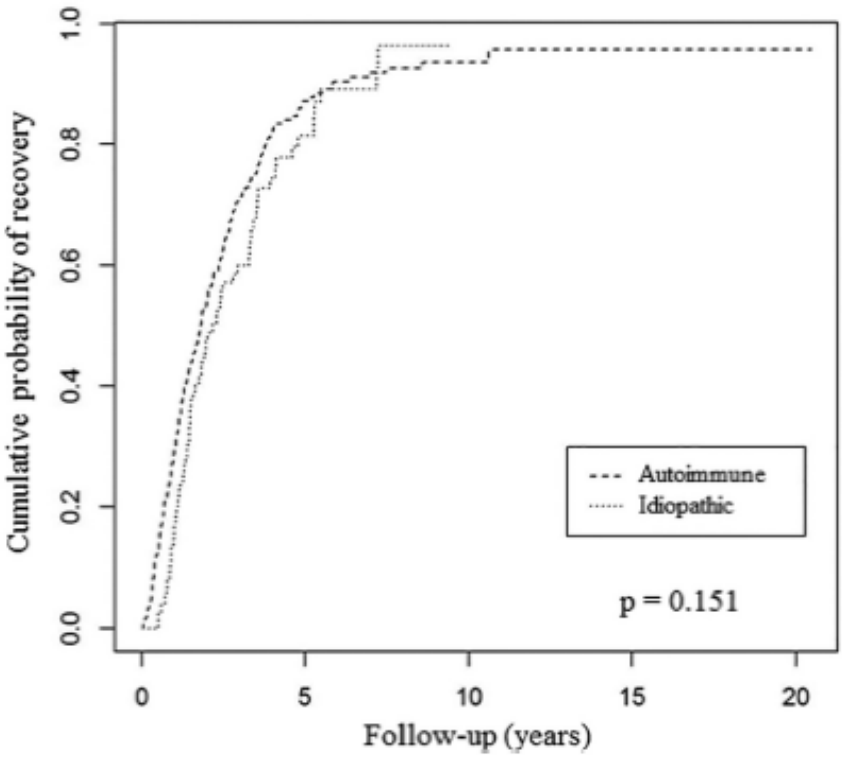
IN is defined as neutropenia rising early in life with mild infectious load and negativity of antibodies against neutrophils on several samples (at least 3-4); usually it remits after a variable period of time. In case of lack of remission, a longitudinal follow up is warranted to observe any possible appearance of new elements allowing re-definition of the original diagnosis. [1,25,27,43,44,]
To date, the largest cohort of IN described in the literature belongs to the italian registry, which collects 85 affected children. In this paper data on IN have been compared with an historical cohort of pAIN affected children. [6]
The analysis identified many similarities between pAIn and IN as shown below. (***Table 4***)

Indeed, the two groups were similar in term of clinical presentation, type and number of infections, prognostic factors, time and mode of resolution. However, there were significant differences in age of onset (0.8 years in pAIN vs 1.2 years in IN) and age at diagnosis (1.1 years vs 2 years, respectively). No significant differences were shown as for leucopenia and lymphocyte values at onset, severity of neutropenia, monocytosis, lymphocyte subpopulations, presence of trombocytosis, incidence of severe infections, hypergammaglobulinemia or DAT test positivity.
Finally, as shown in ***Figure 2***, no significant difference was observed in the two groups trend toward spontaneous resolution, which was 87.12% after 5 years follow-up for pAIN and 81.28% for IN.
Moreover, in both groups, the lower age at diagnosis, the absence of leucopenia and the presence of monocytosis correlate with early resolution.

Table 4. Comparison between the two cohorts of IN and pAIN [6]

	pAIN (336)	**IN (85)**	**p**
Gender (M%)	56.8%	50.6%	0.29
Age at neutropenia onset (years, median)	0.8	1.2	<0.001
Age at diagnosis (years, median)	1.1	2.0	<0.001
Neutrophils (median) at onset	0.45 x 10^9/L	0.38 x 10^9/L	0.47
Neutropenia degree (severe-moderate-mild)	55.2%-37.6%-7.2%	60.0%-29.4%-10.6%	0.28
Leucocytes (median) at onset	6.1 x 10^9/L	5.7 x 10^9/L	0.60
Leucopenia at onset	35.9%	42.4%	0.27
Monocytes (median) at onset	0.62 x 10^9/L	0.61 x 10^9/L	0.87
Monocytosis at onset	20.0%	29.3%	0.08
Lymphocytes (median) at onset	4.5 x 10^9/L	4.3 x 10^9/L	0.16
Trombocytosis at onset	11.1%	20%	0.06
Hyper IgG at onset	6.6%	5.9%	0.83
Isolated IgA deficiency	3.1%	3.0%	0.97
Positive direct antiglobulin test	4.4%	3.1%	0.74
Bone marrow aspirate done	31.6%	49.4%	0.003
Severe infections	11.9%	11.8%	0.97
G-CSF treatment	7.5%	2.8%	0.29

Figure 2. Resolution of pAIN and IN according to Kaplan Meyer curve [6]



Considering the obvious similarities between pAIN and IN, the authors conclude, in agreement with previous literature data, that most cases of IN of childhood are likely an immune-mediated neutropenias in which anti-neutrophil antibodies cannot be identified because of the low sensitivity of the currently used diagnostic techniques. [6,19,21,23,30,45,46] The children affected with idiopathic neutropenia with phenotypic features similar to AIN should therefore be managed as pAIN affected subjects.

Chronic idiopathic neutropenia of adulthood

In adult patients, CIN is defined by ANC values <1.8 x 10^9/L persisting for at least three months, in the absence of other causes of neutropenia and negativity of anti-neutrophil antibodies. [47]

This form of neutropenia is usually isolated without cicling pattern and usually affects middle-aged women; moreover it is characterized by a low incidence of infections and shows a favourable prognosis, with rare leukemic transformation. [48-50]

As for pathophysiological mechanism, CIN seems to have a immune mediated background. [51,52]

There is growing evidence that neutropenia in these patients is due to accelerated death by apoptosis of myeloid progenitors in the context of a bone marrow microenvironment characterized by the excess of pro-inflammatory cytokines (IL-6, interferon-γ and TGFb1) and pro-apoptotic mediators (TNF-α and IL-1b). [52-55]

Study onthe bone marrow progenitors of these patients showed an accelerated apoptosis of CD34+/CD33+ cells, but not of the more primitive ones. On the same CD34+/CD33+ cells, significant up-regulation of the FAS receptor compared with the more primitive CD34+/CD33- cells has been observed. It's therefore hypothesized that FAS is involved in the depletion of CD34+/CD33+ progenitor cells by the mechanism of apoptosis.

Similarly to other autoimmune diseases, CIN is associated with lymphopenia, due to the simultaneous reduction of both CD4+ and CD8+, but not CD19+. [56]

A thorough analysis of the lymphocyte T phenotype in this category of patients has revealed a reduction in the population of naïve T lymphocytes (CD45RA+) compared with controls, an increased percentage of activated CD8+ with possible myelosuppressive action, and a reduction in serum and bone marrow IL7 levels. The authors' hypothesisis that at the origin of the lymphopenia observed in CINs is an aberrant expansion of T cells resulting in their apoptosis, associated with inadeguate thymic maturation, partly due to low levels of IL7. [57]

In CIN affected patients, lower levels of IgA and IgG compared to healthy population have been detected. Analysis of IgG subclasses shows a reduction in IgG1, IgG3 and IgG4. Increased IgM values have also been described, associated with a higher proportion of naïve B cells (IgD+/CD27-) and a lower proportion of B memory (class-switched memory, IgD-/CD27+) compared to healthy controls. [58]

Finally, in patients with CIN, monocytopenia was reported more frequently compared to the controls, with a concordant reduction in granulo-monocyte colony-forming units (GM-CFUs) in the bone marrow.

An inverse correlation between ANC and monocyte count has also been described inpatients with CIN. This could be due to a compensatory effect of the monocyte proliferation at the bone marrow in response to neutropenia. [59]

This thorough characterization of CIN affected subjects suggests that this condition is not idiopathic, but rather has an autoimmune, or otherwise immune-mediated even without specific antibodies.

1.5. Late-onset and long-lasting neutropenia

In daily clinical practice, several cases of AIN that do not have all the typical features of either pAIN nor of sAIN have been observed. These neutropenias usually do not rise in early childhood or lasts more than expected time (Late Onset Neutropenia and Long Lasting Neutropenia).

Recently our group published a study which compares these two distinct types ofAIN with the cohort of patients with pAIN: Late Onset Neutropenia, (LO-Np), with onset beyond 3 years of age, and Long Lasting Neutropenia (LL-Np), with onset within 3 years of age but lasting longer than 36 months. [60]

The main results of this study are shown in ***Table 5***.

Data from this study report a significantly higher median value of ANC in LO-Np and LL-Np than in pAIN, and more severe neutropenia in pAIN (58%), rather than in LL-Np (37%) and LO-Np (32%).

Leucopenia was more frequent in LO-Np (73%) than in LL-Np (26%) and the pAIN (11%). Analysis of lymphocyte subpopulations showed a CD19+ depletion in LO-Np (48% of cases) and LL-Np (35% of cases), not evident in the pAIN. A similar situation was evidenced for natural killer cells.

There were no significant differences among the three groups regarding the frequency of severe or recurrent infections and the type of infections. However, in LO-Np and LL-Np G-CSF therapy was performed more frequently than in pAIN.

LL-Np has a lower tendency of spontaneous resolution, lower lymphocyte and leukocyte counts compared to LO-Np; conversely autoimmunty markers are more frequently shown in LO-Np than in LL-NP (55% vs 17%).

The significant difference in the resolution rate (100% in pAINs vs 58% in LL-Np vs 13% in LO-Np), leads the authors to hypothesize that at least some of the patients with LL-Np have a residual probability of spontaneous resolution that may occur later, qualifying their condition as true pAIN. [40, 61]

In a small subgroup of patients affected with LO-Np and LL-Np, analysis of an NGS panel of 162 genes has been performed. In 3/10 patients with LO-Np and in 1/5 patients with LL-Np, variants of immune-dysregulation genes were found in the TNFRSF13B, TINF2, and LRBA genes [62-65]

These data seem to suggest these two types of neutropenia may hide a more complex immune dysregulation disorder. Indeed, these neutropenias could represent an epiphenomenon of immunodysregulation rather than an isolated event.

Table 5. Comparison betweenp AIN, Late Onset Neutropenia (LO-Np) and Long Lasting Neutropenia (LL-Np) [60]

	pAIN (135 pts)	**LO-Np (31 pts)**	**LL-Np (48 pts)**	**p**
Gender (F)	41/135 (30%)	16/31 (52%)	25/48 (52%)	0.001
Age at diagnosis (years) [median (IQR)]	0.6 (0.3-1.3)	11.5(7.6-14.6)	1.18 (0.6-2.2)	<0.001
Time of neutropenia (years) [median(IQR)]	1.03 (0.54-1.7)	2.1 (1.4-4.4)	4.5(3.5-7.09)	<0.001
Neutropenia recovery	**135/135 (100%)**	**4/31(13%)**	**28/48(58%)**	**<0.001**
ANC at onset [median (IQR)]	**430 (230-716)**	**649 (430-970)**	**552(350-790)**	**<0.001**
Leucocytes x 10^9/L at onset [median (IQR)]	6125 (5010-7920)	3180 (2670-3710)	5030 (3440-6900)	<0.001
Lymphocytes x 10^9/L at onset [median (IQR)]	4740 (3500-5880)	1680 (1240-1900)	2370 (1920-3400)	<0.001
Monocytosis at onset	15/120 (12.5%)	5/26 (19%)	10/38 (26%)	ns
CD3 reduction	7/72 (10%)	2/24 (8%)	11/39 (28%)	0.02
CD4 reduction	8/72 (11%)	4/25 (16%)	11/38 (29%)	0.06
CD8 reduction	11/71 (15%)	4/25 (16%)	7/40 (17,5%)	ns
CD19 reduction	**6/64 (9%)**	**12/25 (48%)**	**13/37 (35%)**	**<0.001**
NK reduction (CD3-CD16+CD56+)	**10/62 (16%)**	**9/23 (39%)**	**10/34 (29%)**	**0.06**
Immunoglobulin depletion	7/113 (6%)	4/26 (15%)	3/44 (7%)	ns
Infections	65/130 (50%)	14/29 (48%)	18/47 (38%)	0.4
Severe infections	16/65 (25%)	3/14 (21%)	3/18 (17%)	0.2
G-CSF treatment	7/135 (5%)	3/21 (14%)	7/42 (17%)	0.04
Autoimmune diseases/autoimmune markers	**2/135 (1%)**	**16/29 (55%)**	**8/48 (17%)**	**<0.001**

2. Aim of the study

To the best of our knowledge, data on LL-Np or LO-Np affected patients without specific antibodies against neutrophils are not so far available.

The present study aims to collect a large sample of those neutropenias which rise after the age of 3 years or lasts more than 36 months (with early onset) regardless of the presence of anti-neutrophil antibodies in serum.

Specifically, we are going to evaluate any differences in terms of the immunologic, infectious pattern, evolution to other forms of autoimmunity, and genetic background. The results of this study could allow in this way to delineate a peculiar profile for early identification of individuals who might be predisposed to complex immunodysregulation.

3. Patients and methods

3.1. Inclusion criteria and definitions

The patients included in the present study were selected from the Italian Neutropenia Registry, set up in 2004 and based at the Haematology Unit of IRCCS Giannina Gaslini Institute. The Registry includes all patients affected with chronic neutropenia, defined as ANC value of less than 1500/mmc in at least 3 samples and lasting more than 3 months. Clinical and genetic data are collected after obtaining the informed consent.

Patients registered by 31 March 2022 belonging to the following categories were considered eligible:

- patients with neutropenia that started before the age of 3 years and persistent for at least 3 years (Long Lasting Neutropenia);
- patients with neutropenia risen after the age of 3 up to 25 years and lasting for more than 12 months (Late Onset Neutropenia).

Patients with at least one of the following characteristics were excluded:

- presence of other cytopenia at onset
- presence of any autoimmune disease or immunodeficiency at the onset of neutropenia;
- presence of neoplasm at the onset of neutropenia;
- personal history of bone marrow transplantation;
- drug-associated neutropenia;
- ethnic neutropenia.

The diagnosis of AIN was defined on the basis of a positive finding of anti-neutrophil antibodies by indirect test (I-GIFT).

IN was definied when a minimum of three determinations of anti-neutrophil antibodies were negative.

3.2. Data Collection

Data collected at diagnosis and/or during follow-up are:

- personal data and family history to search for the recurrence of haematological and/or autoimmune diseases;
- detailed infectious history: number and type of infections (mouth ulcers, gingivitis, periodontitis, skin infections, abscesses, sinusitis, pharyngitis, bronchitis, otitis, pneumonia, bacteremia, liver abscesses, urinary tract infections, other);
- presence of clinical stigmata (facial dysmorphisms, heart disease, malformations, hepatomegaly, skin abnormalities, skeletal abnormalities, neurological symptoms, immunological abnormalities) and signs of immune activation such as lymphadenomegaly and splenomegaly attributable to autoimmune diseases;
- CBC examination and leukocyte count at the onset of the disease and during follow-up: the values at onset, the value at the last follow-up and the median value were taken into account for the analysis (N.B. the median values for leucocytes, lymphocytes and neutrophils were calculated excluding the blood counts during infectious episodes);
- indirect anti-neutrophil antibody assay directed against membrane antigens of neutrophils, identified by indirect GIFT with cytofluorimetry reading;
- immunoglobulin and immunoglobulin G subclasses assay (IgG, IgA, IgM, IgG1, IgG2, IgG3, IgG4), analyzed through turbidimetric method;
- markers of autoimmunity and significant autoimmune patterns (acquired both at diagnosis and during follow-up): ANA, ENA, anti-cardiolipin antibodies, anti-phospholipids antibodies, anti-ds-DNA antibodies, RA-test, p-ANCA, c-ANCA, anti-transglutaminase and anti-endomysium antibodies, direct and indirect antiglobulin test;
- treatment data concerning the use of neutrophil growth factor (Granulocyte colony-stimulating factor, G-CSF) or other therapies;
- analysis of lymphocyte subpopulations by flow cytometry, including extensive analysis of B-cell maturation. Cytometric evaluation was routinely performed during the diagnostic work up by an eight-colour immunostaining panel and with the lyse and wash procedure. All instruments and reagents were Becton Dickinson (BD), NJ, USA products. Briefly, 100µl of EDTA anticoagulated whole peripheral blood were incubated with fluorochrome labelled mAbs (20 min at 4°C) then lysed with FACS Lysing solution (10 min at RT). Sample acquisition was performed on a FACS Lyric flow cytometer equipped with three lasers, a blue (488-nm), a red (633-nm) and a violet (405-nm). Data analysis was based on FACS SuiteTM software. Surface immunostaining was performed using the following RUO mAbs: CD3, CD4, CD8, CD16-56, CD19, CD20, CD27, TCRab, TCRgd,

HLA-DR, CD25, CD45, CD45RO, CD45RA, CD45B220, CD10, CD38, CD21, IgM, IgD. Fluorochromes differently mixed for antibody panels were: APC, APC-H7, FITC, PE, PE-Cy7, PerCP-Cy5.5, V450 and V500. Thanks to various combinations and different expression of the listed antibodies, we analysed some subpopulations inside T and B compartment: cytotoxic T lymphocytes (CD3+ CD8+), B lymphocytes (CD19+), Natural Killer (NK) lymphocytes (CD3- CD56+ CD16+), Naïve T lymphocytes (CD3+ CD45RA+), Memory T lymphocytes (CD3+ CD45RO+), regulatory T lymphocytes (CD3+ CD4+ CD25br CD45RA+), Gamma-Delta T lymphocytes (CD3+ TCRγδ+), activated T lymphocytes (CD3+ HLADR+), Naive memory T/TH/TC cells (CD3+ CD4+/CD8+ CD45RA+ CD27+), Central memory T/TH/TC cells (CD3+ CD4+/CD8+ CD45RA- CD27+), Effector T/TH/TC cells (CD3+ CD4+/CD8+ CD45RA- CD27-), Terminal differentiated T/TH/TC cells (TEMRA, CD3+ CD4+/CD8+ CD45RA+ CD27-), Memory B lymphocytes (CD19+ CD27+), transitional B lymphocytes (CD27- CD10+ CD38+), Naïve B lymphocytes (CD27- CD10+- CD38+- IgD+), Marginal zone B lymphocytes (CD27+ IgD+ IgM+), Switched memory B lymphocytes (CD27+ IgD- IgM-), pre-switched memory B lymphocytes (CD27+ IgD- IgM+), IgD Memory B lymphocytes (CD27+ IgD+IgM-), CD21low cells (CD21low, CD38low), double negative B lymphocytes (CD27- IgD-), DN2 population (CD21lowCD27- IgD-). To define any reduction/increase in the values of specific subclasses of lymphocytes, age reference values were used in terms of absolute values (cells/mmc) for the main lymphocyte classes (T lymphocytes, T helper, cytotoxic T, B, NK). [66,67] For the maturational B lineage and subclasses of T lymphocytes, percentage values were considered instead. [68,69];

- genetic analysis by Next Generation Sequencing (NGS) technique using a panel comprising in the first instance 58 genes (carried out in 9 patients), and subsequently a larger panel comprising 162 genes causing neutropenia, bone marrow failure or immunodeficiency, carried out in the majority of patients. The variants found were then confirmed by Sanger PCR technique. The pathogenicity of the variants found was defined according according to the The American College of Medical Geneticsand Genomics (ACMG) as follow: (1) pathogenic, (2) likely pathogenic, (3) uncertain significance, (4) likely benign, or (5) benign. [70]

The genes included in the panel used are:

AIRE, CARD11, CASP10, CASP8, CD19, CD20, CD40, CD40L, CD70, CTLA4, CTPS1, DCLRE1C, FADD, FAS, CECR1/ADA2, FASL, FOXP3, GATA2, GBA, GORASP1, IKZF1, IL10, IL10RB, IL2RA, ITK, KRAS, LRBA, NCKAP1L/HEM1, NEMO, NFKB1, NRAS, PIK3CB, PIK3CD, PIK3R1, PRKCD, RAG1, RAG2, RASGRP1, SOCS1, STAT1, STAT3, STAT5B, MPL, TLR8, TNFRSF13B, TNFRSF13C, MAGT1, ACD/TPP1, CTC1, DKC1, MYSM1, NAF1, NHP2,

NOP10, PARN, POT1, RPL11, RPL15, RPL26, RPL27, RPL35A, RPL5, RPS10, RPS17, RPS19, RPS24, RPS26, RPS27, RPS28, RPS29, RPS7, RTEL1, STN1, TERC, TERT, TINF2, TSR2, WRAP53/TCAB1, RPS14, ERCC6L2, SRP72, TP53, C16orf57/USB1, DNAJC21, EFL1, SBDS, SRP54, ATM, BLM/RECQL3, LIG4, NBN, NHEJ1, AP3B1, BLOC1S6, CD27, LYST, PRF1, SH2D1A, SLC7A7, STX11, STXBP2, UNC13-D, XIAP/BIRC4, ANKRD26, ASXL1, ATG2B, CD79B, DDX3X, DDX41, EOMES, ERAP1, GATA3, GSKIP, IL13, MKL1, MYD88, PVT1, RBBP6, REL, TCF3, ETV6, ACKR1/DARC, AK2, AK3, CLPB, CSF3R, CXCR2, CXCR4, DNM2, EIF2, ELA2, G6PC3, GFI1, HAX1, JAGN1, LAMTOR2, RAC2, RMRP, SEC61A1, SLC37A4, SMARCAL1, SMARCD2, STK4, TAZ, TCIRG1, TCN2, VPS13B, VPS45, WAS, WIPF1, RAB27A, GATA1, ADAR1, CBL, CEBPA, MECOM, NPM1, RUNX1, SAMD9, SAMD9L.

In the case of the detection of possibly pathogenic variants of specific genes, have been carried out:
- telomere length analysis (performed in the case of variants in genes involved in the regulation of telomere length);
- FAS-mediated apoptosis analysis (performed in the case of variants of genes involved in regulating the mechanism of FAS-mediated apoptosis).

3.3 Statistical methods

Descriptive statistics were performed in terms of absolute frequencies and percentages for qualitative data. Quantitative data were described in terms of median values and interquartile range (IQR) values due to their non-normal (Gaussian) distribution.

Bivariate analysis was applied to compare the variables of interest in patients belonging to AIN *vs.* IN. The Pearson's chi-square test or Fisher's exact test, for expected frequencies < 5, were applied to compare proportions, while comparisons of quantitative variables between groups were performed by the non-parametric Mann-Whitney test.

The non-parametric Wilcoxon matched-pairs signed-rank test was used to compare distributions of the quantitative variables atonset and last visit. The distributions of the quantitative variables of interest were plotted by boxplot in relation to the times of onset, during the follow-up and last visit.

The cumulative risk to develop signs or symptoms of autoimmunity from onset to last follow-up was estimated by the Kaplan-Meier method and was expressed as percentage with 95% confidence interval (95%CI).

All tests were two-tailed and a p value < 0.05 was considered statistically significant. All analyses were performed using Stata (Stata Corp. Stata Statistical Software, Release 16.1 College Station, TX, StataCorporation, 2019).

4. Results

4.1 Population characteristics

From 1 January 2005 to 30 September 2022, 63 patients were enrolled, with an equal distribution between males and females (M 49% vs. F 51%).

Patients with AIN were 32/63 patients (49%), those with IN 25/63 (40%). In 6 patients the diagnosis is still poorly defined due to an insufficient number of negative tests. Patients affected with LO and LL neutropenia were 71% (45/63) and 29% (18/63), respectively.

The median age of onset was 9.2 years (IQR 2 - 15.6 years) and the median follow up of 5.2 years (IQR 4 - 8.2 years).

Table 6. Cohort characteristics

	Total, n = 63
Female, n (%)	31 (49)
Type of neutropenia, n (%)	
AIN	32 (49)
IN	25 (40)
Poorly defined IN	6 (11)
LO-Np	45 (71)
LL-Np	18 (29)
Median age at onset (years) (IQR) min-max	9.2 (2.0-15.6) 0-23.9
Median age at diagnosis (years) (IQR) min-max	12.9 (5.2-19.2) 0.3-50.9
Median follow-up time (years) (IQR) min-max	5.2 (4.0-8.2) 1.3-30.7

4.2 Haematological parameters

The median value of total leucocytes is below normal ranges even at the first finding of neutropenia, and remains stably reduced during follow-up up. The median leucocytes value at onset is 3.15 $x10^9$/L and at the last follow-up 3.23 $x10^9$/L (p = 0.099).

The value of neutrophils has a tendency to increase from a median value at onset of 0.73 $x10^9$/L (IQR 0.30-1.07) to a median value at last follow-up of 1.03 $x10^9$/L (p <0.05).

Otherwise, the absolute number of lymphocytes decreased progressively from a median value at onset of 1.71 $x10^9$/L to a median value at last follow-up of 1.54 $x10^9$/L (p <0.05).

In ***Table 7***, the median value atonset, during the follow-up and at last visit of leucocytes, neutrophils and lymphocytes are shown.

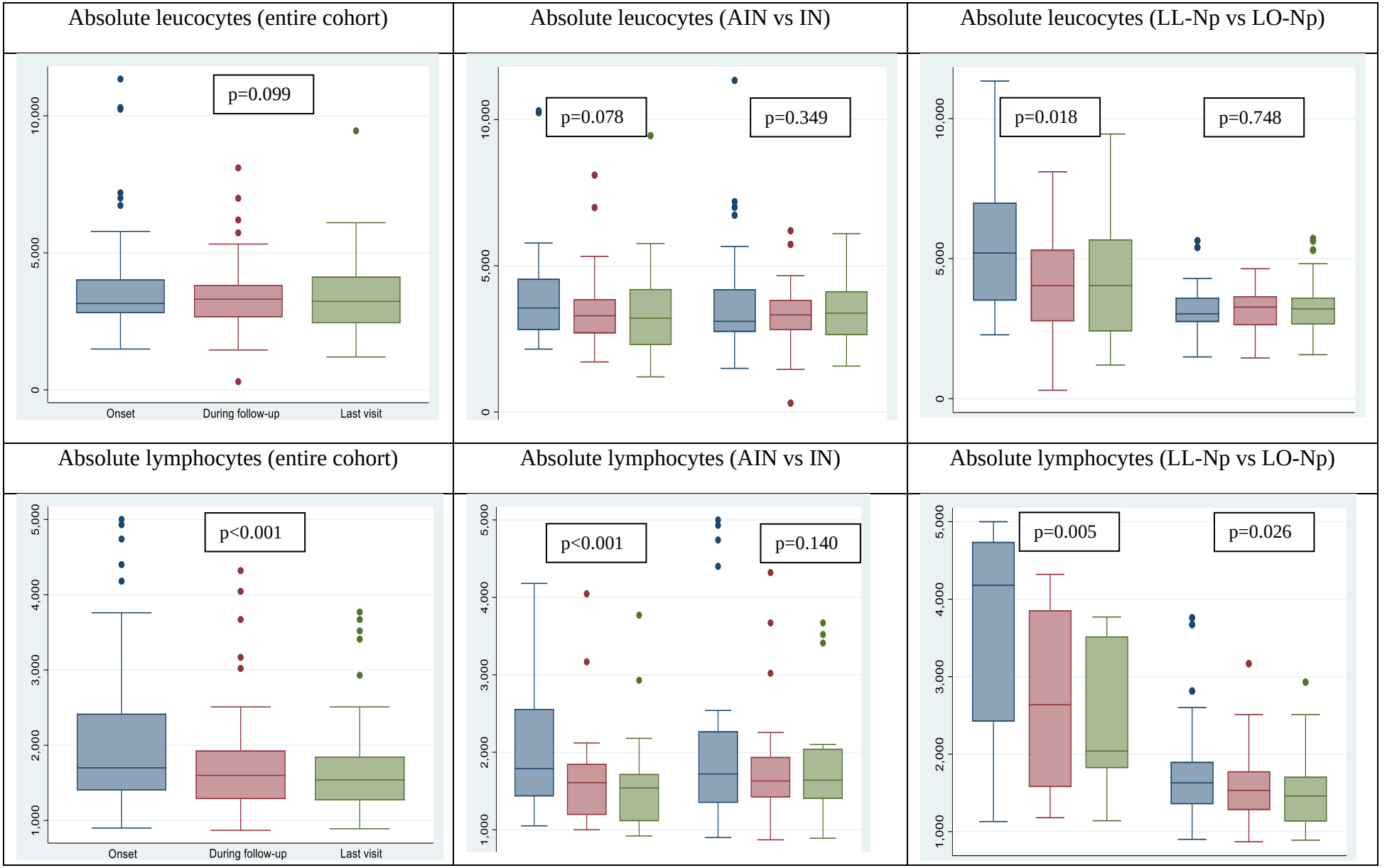
Table 7. Changes in haematological parameters during follow-up

	Onset	**Median follow-up**	**Last visit**	**p**
Leucocytes $x10^9$/L (median, IQR)	3.15 (2.80-4.03)	3.31 (2.65-3.83)	3.23 (2.43-4.13)	0.099
Neutrophyls $x10^9$/L (median, IQR)	0.73 (0.30-1.07)	0.91 (0.51-1.25)	1.03 (0-45-1.46)	<0.05
Lymphocytes $x10^9$/L (median, IQR)	1.71 (1.40-2.54)	1.64 (1.33-1.93)	1.54 (1.14-1.85)	<0.05

The analysis by neutropenia subtypes (autoimmune vs idiopathic and LL-Np vs LO-Np) (***Figure 3***) shows a decreasing trend in absolute leucocyte counts more evident in AIN vs IN, without reaching a statistical significance (p = 0.078). A statistically significant reduction in leucocyte values is shown in the LL neutropenia group vs LO neutropenia, with a median value at onset of 5.20 $x10^9$/L and at last follow-up of 4.04 $x10^9$/L (p = 0.018).

Conversely, a progressive decline in absolute lymphocyte counts is observed in autoimmune neutropenias (p < 0.05) but not in idiopathic neutropenias (p = 0.140). On the other hand, the decline in lymphocyte values over time is confirmed in both LL and LO neutropenias (p < 0.05).

Figure 3. Changes in leucocyte and lymphocyte values over time by subgroups



4.3 Clinical data

Infectious pattern

71% of the patients (45/63) had at least one infectious episode during follow-up.

The most frequent types of infection were: upper respiratory infections (occurred in 60% of patients who had at least one infectious episode), infections of the oral cavity (aphthous stomatitis, gingivitis/periodontitis, 49% of patients), skin infections (29% of patients), otitis (24% of patients), pneumonia (18% of patients) and urinary tract infections (UTI, 13% of patients).

Table 8. Type and frequency of infectious episodes in the entire cohort

Type of infection among patients with at least one infectious event n= 45 (45/63=71.4%), **n (%)**		**Patients with recurrent infections (%)**
Upper respiratory infections	27 (60.0)	18/27 (66.7)
Aphthae, gingivitis, periodontitis	22 (48.9)	19/22 (86.4)
Skin infections	13 (28.9)	8/13 (61.5)
Fever of unknown origin (FUO)	16 (35.6)	11/16 (68.7)
Otitis	11 (24.4)	7/11 (63.6)
Pneumonia	8 (17.8)	2/8 (25.0)
Urinary tract infections	6 (13.3)	1/6 (16.7)
Severe/opportunistic infections	9 (20.0)	
Sepsis/bacteriemias	3	
Meningitis	1	
Recurrent pneumonias (>1)	2	
Opportunistic infections (Campylobacter, Herpes Zoster)	3	

Among recurrent infections (defined as infections occurring >1 episode in a single patient) the most frequent were infections of the oral cavity (86% of the cases) followed by fevers of unknown origin and upper respiratory tract infections (69% and 67% of cases respectively).
Severe infectionswere observed in 20% of the cases (defined as the presence of sepsis, meningitis, opportunistic infection or recurrent pneumonia). Considering the entire study population, patients with a history of severe infections correspond to 14%. Infections considered 'opportunistic' (recurrent VZV infections and Campylobacter Jejuni gastroenteritis) showed no correlation with lymphopenia, immunoglobulin deficiency, and low T Helper (CD4+) lymphocyte values (data not show).

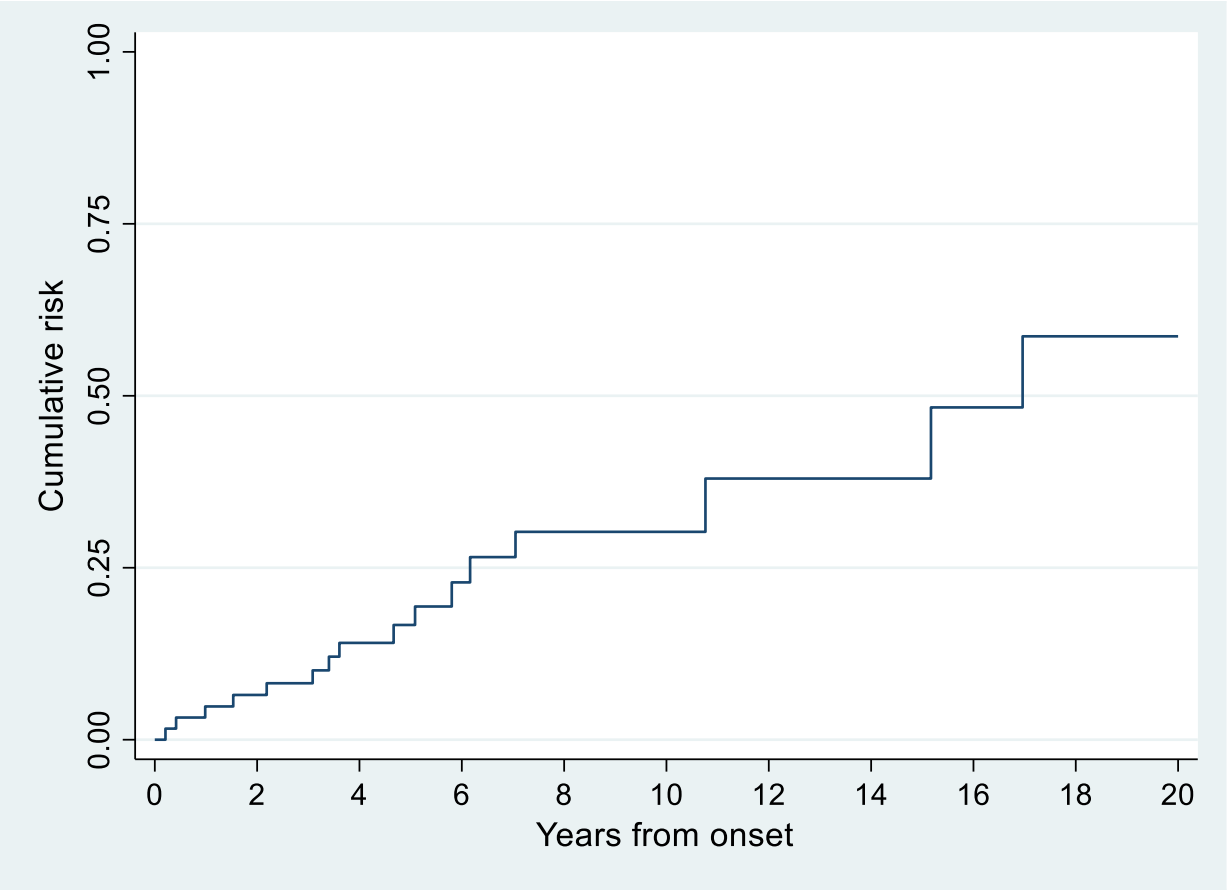
Concerning the general frequency of infectious events, no significant differences werw retrieved neither between the group of autoimmune and idiopathic neutropenias, nor between LL and LO neutropenia.
As for infections type, no significant differences were found in the prevalence of oral cavity infections between patients with autoimmune and idiopathic neutropenia.
G-CSF therapy was given in 9/63 patients (14%). In all cases G-CSF was used exclusively on-demand during infectious episodes and not continuously. No statistically significant correlation was found between the use of G-CSF and the type of neutropenia (AIN vs IN; LL-Np vs LO-Np). Lymphopenia and/or reduced immunoglobulin levels did not influence an increased nee of G-CSF use.

Signs and symptoms of autoimmunity

During the follow-up 19/63 patients (30%) showed signs and/or symptoms of autoimmunity. The most frequent signs and symptoms of autoimmunity were: thyroiditis, coeliac disease, ALPS, ANA and ENA positivity, arthritis, bone pain, chronic fatigue.

The cumulative incidence of signs and symptoms of autoimmunity was 23% (95% CI 12.9-38.5) at 6 years after onset, and 38% (95% CI 21.9-60.3) at 12 years of follow-up, showing a progression also in the following years. Signs and symptoms of autoimmunity were more frequent in the group of AIN compared to IN, with a cumulative incidence respectively 25% (95% CI 12.6-45.9) vs 16.7% (95%CI 5.2-46%) at 6 years, but without a statistically significant difference (p=ns). The cumulative incidence after 6-year follow-up was higher in Late Onset compared to Long Lasting neutropenias, corresponding to 50% (95% CI 29.1-75.2) in LO-Np vs 9% (95% CI 1.3-49.2) in LL-Np (p=ns).

Figure 4. Cumulative risk of occurrence of signs and/or symptoms of autoimmunity



4.4 Immunological profile

The immunological study includedlymphocyte subpopulations, immunoglobulins dosage and their subclasses (IgG, IgA, IgM, IgG1, IgG2, IgG3, IgG4).

In the majority of patients, IgG, IgM and IgA values were in the normal range for age (94%, 85.5% and 90% of patients respectively).

In a small proportion of patients, 11.5% of the patients, the IgM value was above normal limits. Of these patients, 60% had IN, while 40% had AIN. All patients with hyper IgM belonged to the LO neutropenia group.

Table 9. Immunoglobulin G, A and M values in the cohort

	Below normal range	**Normal values**	**Above normal range**
IgG	2 (3%)	58 (94%)	2 (3%)
IgM	2 (3%)	53 (85.5%)	7 (11.5%)
IgA	5 (8%)	56 (90%)	1 (2%)

The assesment of the lymphocyte subpopulations is shown in ***Table 10***. The value of B lymphocytes (CD19+) CD3+CD8+ T lymphocytes and NL cells were below normal in 25% of the cohort (in 8 patients with reduction of CD8 and B cells, while in 4 patients simultaneously of the 3 cell subsets).

Within theT lymphocyte subset, Naïve T cells (CD3+ CD45RA+) were above normal limits in 27/60 (45%) of the group, while regulatory T lymphocytes were below normal in 40/60 subjects (67%).

Values of activated T lymphocytes (γδ T lymphocytes and HLA-DR+) were increased respectively in 37/60 (62%) and 27/60 (45%). B-cell memory was below the normal limit in 38% of patients (23/60).

A more extensive analysis of B profile was performed in 39 patients (62% of the population), and generally showed a slight increase of Marginal Zone B lymphocytes, found in 15 out of 39 patients (38%) and a reduction in Switched Memory B lymphocytes, found in 59% of the subjects tested (23/39). The value of double negative B lymphocytes was above normal limits in 23 out of 39 patients (59%), with an increase in the DN2 and CD21low subpopulations in 10 (26%) and 12 (31%) of 39 patients analyzed.

Table 10. Subpopulation of lymphocytes in the cohort of neutropenic patients according to the age (*William T. Shearer, J Allergy Clin Immunol 2003* and *Leslie R. Bisset, Eur J Haematol 2004*)

	Low (< 2 SDS) N (%)	**Normal N (%)**	**High (> 2SDS) N(%)**
T-profile			
CD3+§	12 (19)	50 (79)	1 (2)
CD3+CD4+§	13 (21)	50 (79)	0
CD3+CD8+§	**16 (25)**	47 (75)	0
CD3CD4CD25brCD45RA+ (T regulatory)^	**40 (67)**	15 (25)	5 (8)
Gamma/delta T cells^	3 (5)	20 (33)	**37 (62)**
HLADR+^	10 (17)	23 (38)	**27 (45)**
B-profile			
CD19+§	**16 (25)**	45 (71)	2 (4)
CD19+CD27+ (Memory)^	**23 (38)**	37 (62)	0
CD27-CD10++CD38++ (Transitional)*	11 (28)	22 (57)	6 (15)
CD27-CD10+-CD38+-IgD+ (Naïve)*	13 (33)	14 (36)	12 (31)
CD27+IgD+IgM+ (Marginal zone)*	9 (24)	15 (38)	**15 (38)**
CD27+IgD-IgM- (Switched memory)*	**23 (59)**	10 (26)	6 (15)
CD27+IgD-IgM+ (Pre-switched)*	0	34 (87)	5 (13)
CD27+IgD+IgM- (IgD memory)*	0	37 (95)	2 (5)
CD27-IgD- (Double negative)*	0	16 (41)	**23 (59)**
CD27-IgD-CD21low (DN2)*	0	29 (74)	**10 (26)**
CD21lowCD38lowCD19+*	0	27 (69)	**12 (31)**
NK cells (CD3-CD56+CD16+)	**16 (25)**	46 (73)	1 (2)

§absolute count
*****B profile (CD19+ excluded) has been performed in 39/63 patients, T-cell maturation has been performed in 36/63 patients
^ 60/63 data available

The study of T-cell maturation showed a tendency to increased values of Central Memory and Effector Memory T cell respectively in 41%/53% for T CD4+ and 56%/31% for T CD8+ with a relative reduction in Naive cells (39% for T CD4+ and53% for T CD8+ respectively) while no significant alterations were observed in TEMRA (both for CD4+ and CD8+) compared to the normal reference ranges.

This “shift” toward the memory compartment in either CD4+ and CD8+ subsets was most relevant when the values were compared with the benchmark median by subclass, as shown in ***Table 11***,

with the tendency toward an increase in TEMRA CD8+ (19/36 patients) and a decrease in TEMRA CD4+ (29/36 patients).

Table 11. Maturative T-profile in 36 neutropenic patients of the cohort according to the age

(*Van Gent R., Clin Immunol 2008*)

	Low (< 2 SDS) N (%)	**Normal N (%)**	**High (> 2SDS) N(%)**
T-profile			
CD4CD45RA+CD27+ (TH Naïve)	**14 (39)**	21 (58)	1 (3)
CD4CD45RA-CD27+ (TH Central Memory)	1 (3)	20 (56)	**15 (41)**
CD4CD45RA-CD27- (TH Effector)	1 (3)	16 (44)	**19 (53)**
CD4CD45RA+CD27- (TH TEMRA)	6 (17)	29 (80)	1 (3)
CD8CD45RA+CD27+ (TS Naïve)	**19 (53)**	17 (47)	0
CD8CD45RA-CD27+ (TS Central Memory)	0	16 (44)	**20 (56)**
CD8CD45RA-CD27- (TS Effector)	0	25 (69)	**11 (31)**
CD8CD45RA+CD27- (TS TEMRA)	1 (3)	30 (83)	5 (14)

As for lymphocyte subsets, no significant differences were found between autoimmune and idiopathic neutropenia groups. Conversely, the comparison between late-onset and long-lasting neutropenia showed lower absolute T lymphocytes in LL-Np group compared to LO-Np (6/18, 33% vs 6/45, 13%) ($p<0.05$) as well as lower B lymphocytes in LL-Np (7/18, 39%) versus LO-Np (9/45, 20%) ($p<0.05$). Marginal Zone B lymphocytes values, were more frequently higher in LO-Np (12/25, 48%) rather than in LL-Np group (2/9, 22%) ($p<0.05$).

4.5 Genetics

Genetic analysis by NGS panel was performed in 92% of the patients (58/63). The 58-gene panel was performed in 9 patients, and the 162-gene panel in the remaining 49.

Among patients who underwent genetic analysis, 60% (35/58) were negative for the presence of significant variants in the genes analysed. In the remaining 23 patients corresponding to 40% of the analysed patients, 27 different variants were not found in the general population, divided according to the major genetic databases between pathogenic (P), likely pathogenic (LP), variants of uncertain significance (VUS), and benign variants (B) or probably benign (likely benign, LB). In 4 patients, 2 different variants (double heterozygosity) was detected. The data regarding the variants found are detailed in ***Table 12***.

Within the study population, 5 P/LP variants were found in 5 different patients, including 4 on the TNFRSF13B (TACI) gene and 1 on the CARD11 gene. The VUS documented were 16, out of 14 patients (2 carrying 2 VUS and another one 1 VUS and pathogenic variant of TACI). The genes on which VUS were found were: CARD11 (2), TNFRSFR13B (1), PIK3CD (1), DDX41 (1), SAMD9L (1), TSR2 (1), DNM2 (1), RTEL1 (1), RUNX1 (1), TINF2 (2), TERT (1), LYST (2, double heterozygosity) and FASL deletion.

There were 6 rare variants classifiedas B in 5 patients. These were found in the CASP10 gene.

No differences were found in the frequency of variants of anytype in the two categories autoimmune and idiopathic and for LL-Np and LO-Np (p = ns).

Table 12. Geneticvariantsfoundwithin the cohort

Patient	Gene	Genotype / Variant	Inheritance	Varsome	GnomAD (allelic frequency %)	Type of neutropenia	Clinical signs	Age at onset (years)	Autoimmunity /disimmunity
1	CASP10	c.1337A>G / p.(Tyr446Cys)	AD	B	3.00	AIN/LO-Np	Leucopena, neutropenia, fibromyalgia, recurrent abdominal pain	11.5	no
2	CARD 11	c.1178_1181delinsTCTT / p.(Tyr393_Ser394delinsPheLeu)	AD/AR	VUS	ND	IN/LO-Np	Neutropenia, recurrent herpes	13.2	no
3	TERT	c.1317-1319del / p.(Glu441del)	AD	VUS	0.0016	IN/LL-Np	Leucopena, neutropenia, upper airway infections	0.8	no
	TNFRSF13B	c.204dup / p.(Leu69Thrfs*12)	AD/AR	P	0.040				
4	PIK3CD	c.2291G>A / p.(Ser764Asn)	AD	VUS	ND	AIN/LO-Np	Leucopena. Neutropenia, monocytopenia, asymptomatic	8.9	no
5	CASP10	c.1228G>A / p.(Val410Ile)	AD	B	4.22	**IN***/LO-Np	Leucopena, neutropenia, asymptomatic	15.6	no
6	TNFRSF13B	c.579C>A / p.(Cys193Ter)	AD/AR	P	0.0056	AIN/LO-Np	Leucopena, neutropenia, allergic asthma	18.9	no
7	TINF2	c.734C>A /p.(Ser245Tyr)	AD	VUS	0.11	IN/LL-Np	Leucopena, neutropenia, upper airway infections, splenomegaly	0.1	no
8	CASP10	c.1216A>T / p.(Ile406Leu)	AD	B	0.45	IN/LO-Np	Neutropenia, ALPS, recurrent infections treated with G-CSF and antibiotics	18.9	ALPS
9	CASP10	c.1216A>T / p.(Ile406Leu)	AD	B	0.45	AIN/LO-Np	Leucopena, neutropenia, asymptomatic	11.0	no
	CASP10	c.1228G>A / p.(Val410Ile)	AD	B	4.22				
10	TSR2	c.410A>C / p.(Glu137Ala)	XL	VUS	ND	AIN/LO-Np	Leucopena, neutropenia, recurrent skin infections	7.9	no

11	TNFRSF13B	c.260T>A / p.(Ile87Asn)	AD/AR	LP	0.046	**IN*/**LO-Np	Leucopena, neutropenia, recurrent fever	11.7	no
12	CARD11	c.3G>A / p.(Met1Ile)	AD/AR	P	ND	AIN/LL-Np	Leucopena, neutropenia, airway infections	3.2	no
13	DDX41	c.829C>G / p.(Leu277Val)	AD	VUS	ND	AIN/LO-Np	Leucopena, neutropenia, cardiopathy, psychomotor delay	14.3	Abs ENA+, ds-DNA+
14	RUNX1	c.1034C>G / p.(Pro345Arg)	AD	VUS	0.0032	IN/LO-Np	Leucopena, neutropenia, recurrent infection	23.8	no
15	TNFRSF13B	c.118T>C / p.(Trp40Arg)	AD/AR	VUS	0.0044	AIN/LO-Np	Leucopena, neutropenia, upper airway infections	9.0	no
	RTEL1	c.1262C>G / p.(Gln421Glu)	AD/AR	VUS	0.059				
16	SAMD9L	c.505G>C / p.(Asp169His)	AD	VUS	0.0099	IN/LO-Np	Leucopena, neutropenia, asymptomatic	8.5	no
17	TNFRSF13B	c.542C>A / p.(Ala181Glu)	AD/AR	LP	0.54	AIN/LO-Np	Leucopena, neutreopenia, celiac disease, recurrent infections	9.3	Coeliac disease
18	DNM2	c.2179C>T / p.(His727Tyr)	AD	VUS	0.026	AIN/LO-Np	Leucopena, neutreopenia, lymphoproliferation	8.9	no
19	CASP10	c.1216A>T / p.(Ile406Leu)	AD	B	0.45	AIN/LL-Np	Leucopena, neutropenia, ecurrent infections treated with G-CSF and antibiotics	0.6	no
20	CARD11	c.1316C>T / p.(Ser439Phe)	AD/AR	VUS	0.0028	IN/LL-Np	Leucopena, neutropenia, sepsis, hepatomegaly	1.5	no
21	FASL	Del ex 1→4	AD	VUS	ND	AIN/LO-Np	Neutropenia, ALPS	12.4	ALPS
22	TINF2	c.734C>A/ p.Ser245Tyr	AD	VUS	0.11	AIN/LO-Np	Leucopena, neutropenia, short telomere lenght	17.3	no
23	LYST	c.10235G>A / p.(Arg 3412His)	AD	VUS	0.069	IN/LL-Np	Leucopena, neutropenia	1.3	no
	LYST	c.1738A>T / p.(Ile580Phe)	AD	VUS	ND				

Legend to Table 11.ND: not determined, IN*: only two negative samples of antibodies against neutrophils, in this two cases neutropenia is defined tentatively Idiopathic

5. Discussion

The present study describes the clinical, immunological and genetic pattern of a cohort of patients suffering from chronic neutropenia that does not present the classical features of autoimmune or idiopathic neutropenia due to the long duration or the late age at onset. [20,21,60]

In this population, the most relevant haematological finding is the presence at diagnosis of mild-to-moderate neutropenia, accompanied by leucopena and progressive lymphopenia during follow-up. These features are present in both the idiopathic and autoimmune groups of neutropenias.
Neutropenia at onset is in most cases of moderate type (ANC 500-1000/mmc), and the absolute neutrophil count tends to increase in the course of the follow-up. This finding is probably due to the fact that a small proportion of patients required G-CSF therapy, which the patients seem to be sensible.

The presence of neutropenia isn't associated with a high frequency of severe infections. The proportion of patients with severe infections was 14%, similar to the 12% reported in 2 studies performed on patient populations with pAIN. [20,21]
The most frequently reported infections were upper respiratory tract infections (60%), followed by oral cavity infections (45%), fever of unknown origin (36%) skin infections (29%) and otitis (24%). Oral cavity infections have the highest recurrence rate (86%), followed by fever of unknown origin (69%).
The incidence of severe infections was not correlated with the presence of concomitant factors such as low immunoglobulin values, lymphopenia or reduced absolute T absolute values of T helper lymphocytes (CD3+ CD4+) (p = ns). It is possible that the pattern of infections related to the age of the subjects
Infectious episodes are in most cases paucisymptomatic or totally absent (18% of patients presented no infections during follow-up and 80% infections considered not severe). This mild phenotype appears consistent with an under diagnosed neutropenia that can be often detected later in life of neutropenia.

Autoimmune signs and symptoms were absent at the onset of neutropenia, but appeared overtime in a consistent part of the cohort (30%) with a cumulative incidence of 23% after 6 year and 58% at 20 years of follow-up. The latter finding is probably influenced by the small number of patients with a prolonged follow-up (4 patients followed for more than 15 years) and therefore needs future confirmation.

Overall these findings suggest that LL/LO AIN and IN may have a mild phenotype characterized by low infectious risk, and by the tendency to develop autoimmune manifestations over time. In this scenario, the finding of leuco-neutropenia and lymphocytopenia, in patients with minor infection load, might be an anticipatory signs of the onset of autoimmune manifestations.

The study of lymphocyte subpopulations allowed, in a proportion of patients, to underline a characteristic pattern already partially identified in a previous study by our group, which described a cohort of patients with 'atypical' autoimmune neutropenia. [60] A reduced proportion of B-lymphocytes (CD19+) and NK-lymphocytes (CD3- CD16+ CD56+) compared to reference ranges was found, which was more evident and statistically significant in patients with LL versus LO neutropenia ($p=0.017$). Conversely, there were no significant differences in the percentage of patients with reduced B- or NK-lymphocytes between autoimmune and idiopathic neutropenias.
Concerning the B-cell maturation profile, carried out in 62% of the cohort, we observed an increase in Marginal zone B cells (CD27+ IgD+ IgM+), a reduction in B memory (CD19+ CD27+) and switched memory B lymphocytes (CD27+ IgD- IgM-) associated with an increase in double negative B lymphocytes (CD27- IgD-) and a slight increase in the DN2 subset. A mild increase in CD21low lymphocytes was also observed in a minority of patients.
On the other hand, the T lymphocytes subclasses analysed showed in a significant proportion of the patients an increase in gamma-delta T lymphocytes (CD3+ TCRγδ+) and activated T lymphocytes (HLA-DR+) and a reduction in regulatory T cells (CD3+ CD4+ CD25br CD45RA+).
The study of T cell maturation showed a “shift” toward Central Memory and Effector Memory T cells compartment with a relative reduction of Naive T CD4+ and CD8+ cells. No significant alterations in the terminnaly differentiated T cells were observed. This pattern partially matches that what has been described for patients with CVID, especially with autoimmune cytopenia and lymphoproliferative pattern [71,72], and in general for IEI/PIRD, suggesting a more activated T cell phenotype with propensity toward autoimmunity [73,74].

A similar immunological pattern has also been described in patients suffering from certain autoimmune diseases, such as Sjögren's syndrome [75], rheumatoid arthritis [76,77] and systemic lupus erythematosus (SLE). [78] Regarding T lymphocytes profile, an activation pattern characterised by an increase in HLA DR+ T lymphocytes and gamma-delta T lymphocytes, and a reduction in regulatory T lymphocytes has been observed in patients with Sjögren's syndrome [79], which are features found in considerable proportion of our cohort too.

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Some immunological characteristics of our patients are also comparable to those described in a cohort of patients suffering from autoimmune cytopenias, including autoimmune neutropenia, in which a reduction in Switched Memory B lymphocytes (CD19+ CD27+ IgD-), Naïve CD4+ T lymphocytes, recent thymic emigrants (RTEs), Naïve CD8 and regulatory T lymphocytes was described. [73] The increase in CD21low and DN2 that we observe in a minority of patients is described in several studies associating with CVID and tendency to autoimmunity [80- 88].

These fidings contribute to increasing support for the hypothesis that immunological changes are caused, at least to a large extent, by congenital immune dysregulation diseases. This concept is reiterated in the aforementioned study on immunological cytopenias, and is also strongly supported by the results of the present study.

In fact, in our cohort, the genetic analysis carried out using an NGS panel of 162 immunodysregulation and bone marrow failure genes, has showed that in 9% of cases inborn error of immunity are detected (Primary Immuno-Regulatory Disorders, PIRDs) mostly as common variable immunodeficiency (CVID).
Furthermore, in a significant portion of our sample (14/58, 24%) variants of uncertain significance are found, for which a possible role in the genesis of neutropenia cannot be ruled out to date.

Among the pathogenic variants, those at the TNFSRF13 gene are the most common. In this setting, neutropenia is thought to be related to reduced central B-cell tolerance resulting in a failure to recognize self-antigens. In these cases, the initial single lineage cytopenia (neutropenia in case of our patients) can evolve into refractory multilineage cytopenia after many years. This looks in keeping with the depletion of the B cellular reserve that can take up to decades, as reported by studies supporting the causative role of these TNFSFR13 variants. [89]

The pathogenic variant of the CARD11 gene (p.Met1Ile) found in patient n. 12 is a Loss of Function (LOF) germline mutation that results in reduced expression of a key signalling protein of the NFKB pathway. Its hypo-expression leads to the appearance of autoimmunity by inhibition of the domain controlling the self, of which in ourspecific case the autoimmune neutropenia is a hallmark of this. [90]
In our cohort, we also identified two other variants of the CARD11 gene (in patients n. 2 and 20, Table 11) classified as VUS, (p.Tyr393_Ser394delinsPheLeu and p.Ser439Phe, respectively). These variants are both located in a 'hotspot' area of the gene, where there is a high probability to cause a

loss of function.[90] In line with this the immunological picture of the patient n. 2 and 20 is very similar to that of patient n. 12 including the deficiency of regulatory T lymphocytes and B memory cells). Based on these observations it can be postulated that these VUS may play a pathogenic role in our patients.

Among the found variants that are classified as non-pathogenic (VUS/LB/B), some may deserve further considerations.
In patients n. 7 and n. 22, the same variant of the TINF2 gene p.Ser245Tyr reported as VUSby reference databases was detected. In one of the subjects (n. 22), telomere length assesed by flow-FISH technique was reduced [91], whereas in patient no. 7 was normal.
Since this variant has also been described in the literature in two patients with bone marrow aplasia [92,93], its role remains debatable.

A variant of the TERT (p.Glu441del) and RTEL (p.Gln421Glu) genes were found in patients n. 3 and 15; in both cases a double heterozygosity with a TNFSRF13 variants likely pathogenic and VUS respectively were shown. It is known that some pathogenic variants of RTEL1 may be pathogenic even in the absence of telomere shortening, as is the case of patient n.15. In patient n. 3 the flow fish is not so far available. [94] Overall, it can speculated that immune dysregulation be an initial or the sole sign of hypomorphyc telomeropathy and that telomere gene variants, possibly in combination with other variants, may contribute to the this phenotype.

CASP10 variants were identified in patients n. 1, 5, 8, 9 (double) and 19. These variants, although classified as benign and in some cases even defined as polymorphisms due to their high frequency in the population, could have a predisposing role in immune-dysregulation, as described by a previous study by our group. [95]
The CASP10 p.Val410Ile variant (found in patients n.5 and 9), initially considered to be pathogenic and later as a polymorphism (3-5% of the population), has a discussed role in the detection of a defect in the apoptosis pathway by means of a functional test performed on some affected patients. [95-98]
Similar is the p.Tyr446Cys variant, detected in patient n. 1. Although present in 1-6% of Caucasian subjects, this variant has been shown to be responsible for a defective transmission of the apoptosis signal. [98]

Finally, considering the p.Ile406Leu variant, found in patients n. 8, 9 and 19, it was initially described as pathogenic, due to a dominant negative effect exerted by the mutated protein, although not all individuals with this variant show alterations at a functional level. [96,97,99]
The CASP10 gene encodes for an enzyme (caspase 10) involved in the apoptosis pathway. An alteration in the apoptosis process can lead to autoimmune phenomena, including autoimmune and idiopathic neutropenia, as shown in all our patients.

In patient no. 4, a variant of the PIK3CD gene was identified, which encodes a kinase selectively expressed on lymphocytes. Gain-of-function mutations in this gene cause PI3Kδ activation syndrome (APDS), characterised by the presence of senescent T cells, lymphadenopathy and immunodeficiency. Loss-of function mutations of PIK3CD are also associated with immunodeficiencies. [100] The variant found in our patient (p.Ser764Asn) is not described in the common data base and its pathogenetic potential role hasto be established.

A variant of the SAMD9L gene was found in patient n. 16 (p.Asp169His) causing a missense mutation. The SAMD9/SAMD9L products are involved in antiproliferative mechanism; mutations in the genes may be linked to a haematological phenotype characterised by pancytopenia or myelodysplasia with neoplastic predisposition. [101,102] The variant reported in our patient is not reported in the literature in association with immunodeficiency and for this reason its pathogenetic role remains to be determined.

Finally, DDX41is the most common mutation predisposing to MDS/AML in adults, and patient n. 13 is actually carrying a VUS in this gene. The causal role of DDX41 is not clear so far even studied in a wide cohort of affected patients. [103]The same for germinal RUNX1 carried by patientn. 14 who has borderline values of platelets; indeed the germinal RUNX 1 is described in familial thrombocytopenia.[104]
Segregation analysis of many patients carrying VUS are actually in progress.

6. Conclusions

In conclusion, even with the intrinsic limitsof a retrospective analysis wich surely deserve more completions and numerosity,our study shows that long-lasting and late-onset neutropenia represents a peculiar category without any major differences between those subjects carrying specific antibodies against neutrophils or not.

In this context, the anti-neutrophil antibodies detection seems to be of secondary importance and the two categories autoimmune and idiopathicseem to be basically comparable in terms of biochemical, immunological, clinical and even genetic aspects.

This 'atypical' neutropenias, would probably deserve a definition of 'probably acquired' rather than “acquired” according to the renewed classification proposed in the International Guidelines on Neutropenia. [105] Suspecting an underlying PIRD below these neutropenias re-news the outdated idea that congenital disorders are always and necessarily accompanied by severe and precocious clinical features. [74]

A paradigmis represented by CVID which may have a mild phenotype at onset but could progressively worsen over time. In the present color some features appear over time like the slight reduction of lymphocytes and the progressive appearance of autoimmunity. In this view, a tailored follow-up of affected patients is warrented maily in those subjects who seem to show “anticipatory signs” (i.e. leucopena, worsening lymphocytopenia and progressive B-cell depletion).

The interpretation of the role of geneticvariants, especially VUS, is an evolving matter which needs continuous update of existing databases. Moreover, the application of ad hoc functional tests, when feasible, would be helpful for a more detailed interpretation of their role.

It’s also possible that in some of the patients in whom the genetic analysis has proved to be negative, pathogenic variants may be detected in genes not included in the panel used, which may be too 'narrow' and not include some implicated ones. Therefore, expanded investigations through modern tecniques (WES or WGS) would suggest additional variants responsible for this phenomenon.

In summary, autoimmune and idiopathic long-lasting and late-onset neutropenias with or without anti-neutrophil antibodies represent a condition that is often paucisymptomatic and characterised by haematological and immunological stigmata that make them quite recognisable from their onset. In such individuals genetic investigations to identify immunoregulatory genetic diseases are indicated in order to tailor appropriate follow-up and identify possible autoimmune diseases at an early stage and to ensure the best treatment by targeted therapies. Long term follow-up and in-depth genetic investigation are certainly indicated to define better underlyng genetic disorder.

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