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Autoimmune neutropenia of childhood secondary to other autoimmune disorders: data from the Italian Neutropenia Registry

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Running title: Secondary autoimmune neutropenia of childhood in Italy.

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Summary
This registry study analyzes the characteristics at presentation and the outcomes of 26 patients affected by autoimmune neutropenia secondary to other autoimmune disorders (s-AIN) enrolled in the Italian neutropenia registry of A.I.E.O.P. (Associazione Italiana di Onco-Ematologia Pediatrica) over a 15-year time-span: this cohort of patients, the largest ever described, was compared to the cohort of 263 patients affected by primary autoimmune neutropenia of childhood (p-AIN) enrolled in the Registry in the same period. The median age of onset of neutropenia was 0.77 year and 10.07 year in p-AIN and s-AIN respectively ($p = 1.105e-12$). The prevalence of former preterm babies among p-AIN (and not s-AIN) patients was significantly higher than in a cohort of 487 consecutively hospitalized children ($p = 0.008362$). Median value of neutrophils at onset was lower in p-AIN ($0.45 \times 10^9/L$) than in s-AIN $0.63 \times 10^9/L$ ($p =0.03$); median value of lymphocytes was reduced in s-AIN ($1.58 \times 10^9/L$) and not in p-AIN ($4.36 \times 10^9/L$) group ($p = 6.29e-11$). Leucopenia ($p = 1.80e-07$) and severe infections ($p = 0.0001$) occurred more frequently in s-AIN; monocytes ($p = 0.039$) and spontaneous remission ($p = 3.21e-11$) in p-AIN. In conclusion, p-AIN is in the vast majority of cases a benign and self-limiting disorder typically occurring under 2-3 years of age whereas s-AIN is a more severe disease, usually presenting after first 5 years of life, usually associated to lymphocytopenia and with a highly frequent tendency to become chronic.

Key words: autoimmune; neutropenia; childhood.

Introduction
Neutropenia is defined by a reduction of Absolute Neutrophil Count (ANC). Among Caucasian people the lower normal limit of ANC in children up to the age of 1 year is $1.0 \times 10^9/L$, whereas from >1 year to adulthood the limit is $1.5 \times 10^9/L$; neutropenia is defined as mild in case of ANC between 1.0 and $1.5 \times 10^9/L$, moderate with ANC between 0.5 and $1.0 \times 10^9/L$, and severe with ANC <0.5x10^9/L [YHN]. Autoimmune Neutropenia (AIN) is due to auto-antibodies against Human Neutrophil Antigens: the most frequent AIN in childhood is the primary type (p-AIN) (FFFF, EEEE,GGGG), whereas in adulthood AIN is mostly represented (DDDD) by secondary neutropenia which can be associated to infection, drug administration, immunodeficiency, neoplasms, bone marrow transplantation or other autoimmune disorders (WSWSWS, QQRR HJKL). No specific study about AIN secondary to autoimmune diseases (s-AIN) in childhood has been published yet. In the present paper we describe the natural history of 26 pediatric patients affected by s-AIN, whose clinical characteristics were compared with those of a group of 263 children affected by primary autoimmune neutropenia of childhood (p-AIN), some of which had been more succinctly presented in another report (GGGG).

**Patients and methods**

**Data collection.** Children diagnosed with p-AIN or s-AIN and recorded in the Italian Neutropenia Registry (INR) of A.I.E.O.P. (Associazione Italiana di Onco-Ematologia Pediatrica) from 01/01/2002 to 01/06/2016 were considered eligible for the study. INR institution was approved by the ethics committee of the coordinating Centre (G. Gaslini Institute, Genova, Italy). Informed consent for the collection of clinical data was obtained from the parents or legal guardians according to the Helsinki declaration and registry guidelines at the moment of enrollment in the Registry. Anonymity was guaranteed by codifying data entry.

Demographics, history, clinical and laboratory data, consisting of an initial registration form and a subsequent yearly follow-up, were extracted from the INR. The age at initial presentation of AIN was 0-18 years though patients could be over 18 years of age at the time of diagnosis or inclusion in the analysis. Diagnosis of AIN was performed according to the guidelines published by our group
that require the positivity of indirect anti-neutrophil test in at least one evaluation over a series of 4, over a time-span of 6 months or more. In the analysis only patients affected by p-AIN and s-AIN were included: all cases of AIN associated to infection, neoplasm, bone marrow transplantation or drug administration were not included. Patients with AIN in a context of immunodeficiency other than selected IgA deficiency (SIgAD) were also excluded. Thrombocytopenia was defined as a platelet count of <150x10⁹/L, sustained or intermittent, for at least 3 months and autoimmune hemolytic anaemia (AIHA) as Haemoglobin (Hb) level below the normal range for age with positive direct anti-globulin test (DAT) and evidence of haemolysis.

**Details on infections.** Data on documented infections were collected from neutropenia onset until neutropenia recovery or the last follow-up. Each infectious episode was reviewed and an infection was arbitrarily defined as "severe" in the presence of a final diagnosis of sepsis, pneumonia, skin/soft tissue abscesses, osteomyelitis, otomastoiditis or meningitis/encephalitis: other types of possible severe infections were evaluated on a case by case basis. No cases of periodontitis, oral abscess, deep abdominal infections or fungemia were reported.

**Laboratory evaluation.** Samples were analyzed in parallel in three different Italian laboratories located in Genoa, Turin and Milan. Detection of circulating anti-granulocyte antibodies was performed by the indirect Granulocyte Immunofluorescence Test (GIFT). A panel of purified granulocytes obtained by density gradient separation (Dextran and Ficoll) from healthy donors was used: the donors were unselected and not genotyped for HNA. Briefly, 2x10⁵ paraphormaldehyde-fixed (1% for 2') granulocytes from 4-10 healthy male donors were individually incubated with a pool of AB sera from non-transfused male donors (negative control), with patients’ sera and with a positive control serum (30’ at 37°C). After washing with phosphate-buffered saline (PBS) containing 0.1% NaN₃ and 1% Fetal Calf Serum, cells were incubated with fluorescein isothiocyanate (FITC)-labeled F(ab’)_₂ fragments of rabbit anti-human IgG (30’ at RT) and then acquired by a FACSCanto II flow-cytometer. GIFT was considered positive in the laboratory of Milan if the difference between the Median Channel of Fluorescence 1 (MCF-1) of negative control
serum and patient’s MCF-1 on the same donor’s cells was equal to or greater than an internal cut-off value determined on a wide population of negative control sera (n = 300), and in the laboratory of Genova and Turin if it was >2sd of the mean of one hundred control sera from healthy subjects.

**Statistical analysis.** In order to investigate the association between children who were born preterm (≤37 weeks of gestational age) and then developed AIN, we compared the prevalence of former preterm babies in the p-AIN and s-AIN groups with that of preterm newborns in a group of 487 control children consecutively hospitalized for various reasons during 2014 in a paediatric center of Sicily (chi square test for independency). The prevalence of leucopenia (for age), monocytosis (>1.0x10^9/L), SIgAD (confirmed in at least 2 dosages) and increased immunoglobulin G (IgG) for age were also evaluated. Since SIgAD appeared to be more frequent in our patients than in the general population, we compared its prevalence in the p-AIN and s-AIN groups with 470 laboratory controls in hospitalized and outpatient children (chi square test for independency) for a quantitative evaluation of how significant this finding was. Bivariate recovery curve analysis was performed on the whole data sample, using Kaplan Meier curves and Cox proportional hazard models. All p values were two-sided, and values <0.05 were considered statistically significant. Statistical analysis was performed using the open source statistical software R [QAZ]. Recovery curve analysis was performed using the R package Survival [WSX].

**Results**

**Characteristics at presentation.** Some results of our analysis comparing p-AIN and s-AIN cohorts are shown in Table I, whereas more specific characteristics of s-AIN patients are presented in Table II; the median follow-up in p-AIN and s-AIN cohort was 1.30 years and 4.10 years respectively. At appearance in p-AIN cohort the median ANC was 0.45 x 10^9/L (range 0-1.49 x10^9/L), and the neutropenia was severe in 56.3%, moderate in 36.1% and mild in 7.6%, whereas in s-AIN cohort the median ANC was 0.63 x 10^9/L (range 0.30- 1.40 x10^9/L) and the neutropenia was severe in
42.3%, moderate in 46.2% and mild in 11.5%: the difference of ANC at onset between p-AIN and s-AIN was barely significant (p = 0.035) but there was no significant difference in terms of type neutropenias (from severe to mild) (p = 0.3787). Median white blood cells count (WBC) and median absolute lymphocyte count (ALC) at onset were 5.93 x 10^9/L vs. 2.48 x 10^9/L (p = 2.81e-11) and 4.36 x 10^9/L vs. 1.58 x 10^9/L (p = 6.29e-11) in p-AIN and s-AIN patients respectively. In s-AIN all lymphocytes sub-classes (CD3, CD19, CD4, CD8 and CD56) were reduced as compared to pAIN, with CD19 lymphocytes more decreased than CD3 lymphocytes: the median CD19 count was 0.242 x 10^9/ in s-AIN group vs. 0.934 x 10^9/ in p-AIN group (p = 8.13e-10) whereas the median CD3 count was 1.385 x 10^9/in s-AIN group vs. 2.824 x 10^9/L in p-AIN group (p = 2.32e-06). Leucopenia for age was more frequent in s-AIN vs. p-AIN (92.3% vs. 39.0%; p = 1.80e-07) whereas monocytosis for age occurred more often in p-AIN vs. s-AIN (20.6% vs. 3.8%; p = 0.039).

The frequency of children who were born preterm and then developed p-AIN or s-AIN was 12.85% and 3.84% respectively: the prevalence of former preterm babies among p-AIN patients was significantly higher than in a cohort of 487 children consecutively hospitalized for various reasons during 2014 in a pediatric Italian center: 12.85% vs. 6.98% (p = 0.008362). On the contrary there was no significant difference comparing s-AIN and the same group of control children (p = 0.5367).

The prevalence of SIgAD was 3% in p-AIN and 13.6% in s-AIN children and both prevalences were significantly higher than that observed (0.21%) in a group of 470 laboratory controls (p = 0.0009 in p-AIN and p = 7.239e-12 in s-AIN), thus pointing to a potential association between these two findings: the co-existence with SIgAD was significantly higher in s-AIN than in p-AIN (p = 0.015). Increased level of IgG was found in 6.5% of p-AIN and 4.5% of s-AIN respectively (p = 0.71).

Bone Marrow (BM) examination was done in 35.1% of p-AIN patients and in 65.4% of s-AIN patients (p = 0.0024). In all but 3 patients of p-AIN group it showed normal or increased cellularity with or without a relative paucity of the more mature stages of granulocyte development: in these 3 p-AIN children a moderate decrease of myeloid cellularity was observed.
Neutropenia appeared contemporarily to other autoimmune manifestations in 11/26 s-AIN patients (42.3%), appeared firstly in 8/26 patients (30.7%) (median and mean time of appearance of other autoimmune signs: 440 and 987 days respectively) and later in 7/26 patients (26.9%) (median and mean time of appearance of s-AIN: 558.5 and 865.3 days respectively).

Outcome. At the time of the analysis 16/263 p-AIN patients were lost at follow-up: 185 out of 247 evaluable p-AIN patients (74.9%) and 2 out of 26 s-AIN patients (7.7%) had recovered from neutropenia ($p = 2.26e-12$). The Kaplan Meier recovery curve of both groups is shown in Figure 1. Among 119 children with p-AIN and at least 5 years of FUP 112 (94.1%) had recovered from neutropenia.

Overall 11.8% of the p-AIN and 40.0% of the s-AIN children suffered from severe infections ($p = 0.0001$). Antibiotic prophylaxis was never administered. Granulocyte Colony Stimulating Factor (GCSF) was used in 6.9% of p-AIN and 23.1% of s-AIN patients respectively ($p = 0.0045$): it was always used sporadically a part from a girl suffering from s-AIN who, after some recurrent bronchopneumonias, started the continuous administration at the age of 12 years. Among s-AIN patients intravenous immunoglobulin (IVIG) and intravenous or oral steroids were used in 10 patients with the goal of treating autoimmune disorders other than AIN. In the group of children affected by Evans Syndrome (ES) two were treated also with mycophenolate mofetil, one with 4 doses of rituximab and another one, with splenectomy (performed 5 years after the onset of disease). Furthermore a girl presenting an autoimmune hepatitis was treated with cyclosporine and mycophenolate mofetil.

Discussion

The most common type of AIN in children is p-AIN, which is isolated, characterized by low risk of severe bacterial infection [DDDD,EDC], tendency to spontaneously resolve [DDDD] and usual occurrence under the age of 4-5 years [EEEE,CCCC,RFV]. In contrast, AIN in adulthood is frequently associated with other autoimmune diseases or phenomena and is characterized by heavier
infection load and rare spontaneous remission [QQRR, MMMM, PPPP, QQQQ, RRRR, SSSS, UUUU, HJKL]. The present one is the largest study ever conducted about AIN secondary to other autoimmune diseases in childhood and it highlights the heterogeneity of presentations and many aspects of clinical features and final outcomes.

In our series a significantly higher prevalence of the female sex in s-AIN was observed ($p = 0.049$): this is in accordance to what previously reported in adulthood AIN (PPPP, ZXCV, HJKL) and to the common knowledge of female prevalence in autoimmune disorders.

In pediatric s-AIN the only published report (FFFF), analyzing 7 patients, signaled an age at onset of 0.5-15 years, in agreement to the present study where the median age of appearance was significantly higher among s-AIN patients ($p = 1.12e-12$): six out of 26 s-AIN (23%) and 253 out of 263 p-AIN (96.2%) patients presented neutropenia onset at less than 5 years of age.

Leucopenia, in accordance to previous papers [EEEE,GGGG], is much less frequent among p-AIN (39%) than s-AIN patients (92.3%), probably as a consequence of associated lymphocytopenia. Monocytosis for age, previously reported [EEEE,UJM, GGGG, IIII] in about ¼ of p-AIN patients, and interpreted as possible compensatory anti-infection mechanism (IIII, LLLL), is significantly less frequent in s-AIN (3.8%) than in p-AIN (20.6%). Lymphocytopenia and the rarity of monocytosis might contribute to the significantly higher infection load of s-AIN over p-AIN, as already observed, although not in a statistically significant mode, by others (MMMM, PPPP). We found, in accordance to another report from our group [GGGG], that the prevalence of former preterm newborns among p-AIN patients was significantly higher than in hospital controls ($p$ value = 0.008362). This is in accordance with the hypothesis that p-AIN is linked to transient immaturity of the suppressor T-cell function and that the remittance corresponds to the complete development of the suppressor network whereas s-AIN, due to a more severe and persistent perturbation of immune system, has much less possibility of spontaneous recovery. SIgAD, a condition predisposing to autoimmunity [POI, VVVV], was significantly more frequent in p-AIN (3.0%; $p = 0.0009347$) and s-AIN (13.6%; $p = 7.239e-12$) patients over controls (0.21%) and in s-AIN than in
p-AIN (p= 0.015). Increased IgG level, already reported by others [GGGG, OLP], is probably a consequence of enhanced immune stimulation.

Spontaneous recovery occurred in 74.9% of p-AIN and 7.7% of s-AIN and the difference was highly significant (p = 2.26e-12); our data is in accordance to much smaller series where the remission from s-AIN was absent (FFFF) or highly infrequent (IIII, WWWW). In our series only 2 out of 26 children recovered from s-AIN: one was a patient with autoimmune thyroiditis and celiac disease who, after starting gluten-free diet recovered from AIN (and not from thyroiditis) and the second one was a boy who recovered from ES.

ES is a disease characterized by the simultaneous or sequential appearance of at least two among autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP) and immune neutropenia (YYYY, VVVV, IIII, LLLL, OOOO, PPPP, WWWW, HJKL). According to our data ES, among autoimmune disorders, is the most common presentation of s-AIN (11 patients): 5/11 patients suffered from a tri-lineage cytopenia and 6/11 patients from a bi-lineage cytopenia. The association of AIN with autoimmune thyroiditis (7 patients) has only occasionally been described in children (HHHH, WWWW) and it seems much more common in adults: Weitzman et al (PPQQ) found anti-neutrophils autoantibodies in about half of patients suffering from autoimmune thyroiditis and Kyritsi et al (MNBV) detected anti-neutrophil antibodies in 37% of patients suffering from thyroid disorders.

Two patients of our s-AIN cohort (lines 2 and 5 of table II) showed a not previously reported association with coeliac disease (plus other autoimmune disorders) and one of them presented, after starting gluten-free diet, a remission from neutropenia (but not from autoimmune thyroiditis). Also two patients of p-AIN cohort presented an isolated association with coeliac disease: both children suffered from typical (for age and clinical manifestations) p-AIN which appeared before the diagnosis of coeliac disease and remitted a few weeks after starting gluten-free diet. So it seems that coeliac disease can be accompanied by both p-AIN and s-AIN and that, in many cases, the gluten-free diet may contribute to recovery from neutropenia. In 3 s-AIN patients an association with
growth hormone (GH) deficiency was found: autoimmune pituitary diseases may occur along with other autoimmune features (RRSS) but these appear to be the first patients with this type of association. Two s-AIN children presented diabetes, an association rarely reported both in adults (MMMM, UUUU) and in children (FFFFFF, WWWW), and another patient systemic lupus erythematosus (SLE), a disease where neutropenia and leukopenia seem to be more common in adult (MMMM, NNNN, VVVV, IILL, HJKL) than in paediatric patients (OOPP). Furthermore a s-AIN child presented an association with autoimmune hepatitis, a feature exceptionally reported up till now in adulthood (VBN), and, finally, in a girl the AIN predated the development of autoimmune encephalitis, an association not previously reported.

In the s-AIN cohort 14 patients showed positivity of antinuclear antibodies (ANA) higher than 1:80, 6 children presented s-AIN associated with more than one defined autoimmune disorder and in 4 children (the last ones of table II) s-AIN was in a context of not defined autoimmune disease characterized by arthralgia and ANA positivity. Interestingly in 8 s-AIN patients neutropenia remained isolated, sometimes for 6-7 years, before the appearance of other autoimmune manifestations. The appearance of non-hematologic autoimmune manifestations with follow-up of some years has been described rarely in children originally affected by isolated AIN (TTTT): it seems impossible to predict if, and when, in a child suffering from isolated AIN other autoimmune signs or defined autoimmune diseases could appear.

Corticosteroids, intravenous immunoglobulins, splenectomy, rituximab, Campath-1H, cyclosporine A, sirolimus and other immunosuppressors have been used to treat both p-AIN and s-AIN [EEEE, POIU, MMMM, OOOO, PPPP, RRRR, VVVV, AABB, BBCC, DDEE, QQRR, FFGG, GGHH, HJKL], but nowadays G-CSF is much preferred (EEEE, CCDD, NNOO, QQRR, HJKL). According to this approach, G-CSF was administered in 19.2% of our s-AIN patients and in all of them a rise in ANC was obtained.

In conclusion, our report, although retrospective, is based on a reasonably sized cohort of patients and provides new insight into the clinical presentation and the outcome of children affected by AIN.
associated to other autoimmune phenomena: s-AIN in childhood is part of a scenario of profound deregulation of the immune system, with challenges in both diagnosis and management and with a really infrequent tendency to spontaneous recovery. There is no consensus about which laboratory tests should be performed in patients with AIN to look for an underlying disease: based on this analysis and some previous papers on p-AIN (EEEE, GGGG) we suggest that an AIN appearing at lower than 2 years of age and without any severe infection is probably a p-AIN and time can be taken before performing additional diagnostic evaluation. On the contrary, if the diagnosis of AIN is made at more than 2-3 years of age and/or the infectious load is heavy, we suggest that a screening for an underlying autoimmune disorder, including at least ANA, direct anti-globulin test (DAT), antitransglutaminase and antithyroid antibodies, should be performed. Finally, since various other autoimmune disorders can be associated to s-AIN, treatment can be difficult but, with the goal of correcting neutropenia, G-CSF seems to be, at least in the short term, a reasonable approach.

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Contribution
PF was the principal investigator and takes primary responsibility for the paper. PF, GP, FF and CD conceptualized and designed the study and the data collection instruments. PF and GP coordinated and supervised data collection. PF, FF, CD and GP carried out the initial analyses, participated in the interpretation of data, and drafted the initial manuscript. TL and LP contributed essential reagents or tools. PF, FF, UR, AB, SB, AF, RG, SL, NM, BM, LDN, DO, MP, GR, FT, FV and CD were involved in clinical management of the patients and contributed to the enrollment, diagnosis and sample collection. All authors critically reviewed the paper, approved the final manuscript as submitted, and agree to be accountable for all aspects of the work.
Conflict of interest disclosure
The authors report no potential conflicts of interest, including specific financial interest, relationships, or affiliations relevant to the subject of this manuscript.

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