Original article

Neonatal alloimmune neutropenia: diagnosis and management of 31 Italian patients

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Background - In neonates, antibody-mediated destruction of neutrophils may occur as a consequence of auto- or isoimmune disorders. There are few studies on this topic, and particularly on neonatal alloimmune neutropenia (NAN).

Materials and methods - We retrospectively evaluated the clinical and molecular/serological findings of 83 neutropenic infants referred to our Reference Laboratory for diagnostic evaluation of NAN, from 2008 to 2021. We also genotyped 260 Italian healthy subjects for the four principal human neutrophil antigens (HNA).

Results - The diagnosis of NAN was confirmed in 31 cases. The other 52 cases were autoimmune neutropenia (n=21), neutropenia caused by maternal neutrophil autoantibodies (n=8), neutropenia of non-immune origin (n=17), and cases in which a diagnosis could not be reached (n=6). The median age at neutropenia onset and absolute neutrophil count (ANC) were significantly lower in NAN than in non-NAN cases (1 *vs* 30 days, p<0.005; 330 *vs* 580/μL, p=0.003, respectively). About 74% of NAN cases developed neutropenia within the first week of life and laboratory investigations were required within 2 weeks. In five patients the neutropenia was discovered at the end of the first month of life and they were referred to our laboratory 1-2 months later when neutropenia had already resolved. Infections were seen in 19% of NAN cases. The median time to resolution of NAN was 31 days. About 50% of NAN cases were due to alloantibodies against HNA-1b, the most frequent allele of HNA-1 in the Italian population (allele frequency 0.63). In five cases of NAN the mothers had an HNA-1 null phenotype, a frequency higher than that observed in our Italian cohort.

Discussion - NAN should be considered by clinicians in infants with neutropenia onset within 5-7 days of life, even though there can be other reasons for a low ANC. If neutropenia is detected later, benign neutropenia seems more likely, although persistence of maternal alloantibodies cannot be ruled out.

Keywords: *neutropenia, newborns, human neutrophil alloantibodies.*

INTRODUCTION

Neonatal neutropenia is usually defined in Caucasians as a peripheral blood absolute neutrophil count (ANC) <1,000/μL[1], although, during the first week of life (72-240 hours) the lowest ANC threshold is 2,500/μL[2,3] and from 2 weeks to 6 months drops to 1,100/μL[4]. Neutropenia is frequently observed in neonatal intensive care units, with an

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overall incidence of 8%; in very low birth weight neonates this percentage can increase to 50% in the first week of life[5]. Furthermore, neutropenia can be due to several variables and diseases related to pregnancy or delivery, e.g., maternal hypertension, Rh-hemolytic disease of the newborn, twin-twin transfusion syndrome, asphyxia, neonatal infections, and sepsis[1,5-6]. Neutropenia can also be caused by an immune-mediated destruction of neutrophils[5]: this is the case of the "classical" neonatal alloimmune neutropenia (NAN) and the very rare neonatal neutropenia due to pre-existing anti-neutrophil antibodies transmitted to the fetus by mothers with autoimmune neutropenia[1,7].

NAN is caused by a trans-placental transfer of maternal anti-neutrophil IgG antibodies that have developed against paternal human neutrophil antigens (HNA) expressed on the neonate's neutrophils[8]. HNA genes exhibit an extended polymorphism resulting in a variety of different antigens. As of the present, 14 HNA alleles have been officially assigned to five HNA systems[9]. The alleles most commonly involved are those of HNA-1, which includes four different alleles, -1a, -1b, -1c, -1d representing the polymorphisms of FcγRIIIb[8]. Cases of NAN due to incompatibilities to HNA-2, -3, -4, and -5, although less frequent, have also been described[10-15].

The true incidence of NAN is not known, because it is often asymptomatic and an ANC is usually not performed routinely, but only in neonates with suspected pathological conditions. Thus, in the majority of cases, neutropenia is detected incidentally during investigations performed for other reasons. According to the literature, the incidence of NAN is estimated to be roughly 1-2:1,000 neonates and may occur in as many as 25-40% of first pregnancies[16,17]. Clinical manifestations range from none (incidentally detected neutropenia) to severe omphalitis, skin infections, and, rarely, meningitis or pneumonia[16]. The median duration of neutropenia is 7-11 weeks[16,18], even though maternal antibodies can persist in the neonatal circulation for up to 6 months[19].

To define the alloimmune basis of neutropenia with certainty, specific and sensitive investigations must be performed within a well-defined laboratory workup including anti-neutrophil antibody screening with specificity identification on maternal and neonatal sera, cross-reactivity of maternal serum with paternal granulocytes, and HNA genotyping of both parents and the neonate[1,20].

The main purpose of the present study is to describe the clinical, serological, and molecular features of an Italian cohort of NAN cases, evaluated in our laboratory between January 2008 and December 2021. We also evaluated the Italian population frequencies of the ten principal HNA alleles in a cohort of healthy subjects.

MATERIALS AND METHODS

Subjects

From the beginning of 2008 until the end of 2021, 83 neutropenic infants suspected of having NAN were referred to our reference laboratory in the Policlinico Hospital in Milan for serological and molecular investigations. Demographic and biological data of the patients and their parents were retrieved from clinical records. The date of collection of parental blood samples represented the age of NAN workup for a given infant. Laboratory diagnosis of a suspected case of NAN was made following the detection of anti-HNA alloantibodies in maternal serum, directed against an HNA epitope present on both the infant's and father's neutrophils but not on maternal ones. A positive cross-match between maternal serum and paternal neutrophils was performed to confirm the diagnosis. All samples were evaluated within 24 hours of collection. Age at resolution of neutropenia was defined as the age at which the ANC first reached or exceeded 1,000/μL with no further recurrence of neutropenia.

Finally, 260 healthy blood donors were genotyped to define HNA frequencies. The study was conducted in accordance with the Declaration of Helsinki.

Detection of HNA antibodies

To evaluate the presence and specificity of anti-HNA antibodies in an infant's and mother's sera we performed the granulocyte immunofluorescence test (GIFT) by flow cytometry (FACSCantoII with FACSDiva software, BD Biosciences, San Jose, CA, USA) on a panel of freshly isolated neutrophils from four HNA-typed healthy subjects, which had to include at least homozygous expression of HNA-1a and HNA-1b alleles. GIFT was also performed on the father's granulocytes (cross-match), if available[21-23]. Subsequently, on the basis of the results of molecular typing, we tested the infant's and mother's sera again on an additional targeted panel including cells

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homozygous for the alleles of the probably implicated antigen.

Only for cases in which the mismatch was related to HNA-3, we also performed the granulocyte agglutination test (GAT)[22] on HNA-3a and -3b homozygous donors.

Sera were also examined by a bead-based assay able to recognize HNA-1a, -1b, -1c, HNA-2, HNA-3a, -3b, HNA-4a, and HNA-5a, -5b with Luminex technology (LABScreen Multi assay, One Lambda, Los Angeles, CA, USA), for identification of antibody specificity[24]. Data were acquired and analyzed by a Luminex 200 flow analyzer using the software XPonent 4.0 and HLA Fusion 3.0.

Since the Luminex technology for evaluation of anti-neutrophil antibodies has only been available since 2015, sera collected and frozen before that date, when still available, were retrospectively tested.

HNA typing

To verify the presence of a mismatch between the patient's and maternal HNA haplotypes, HNA typing was carried out for both parents and the infant. Until the end of 2010 we only performed serological typing of HNA-1 and -2 using anti-CD16b (clone CLB-gran 11.5) and anti-CD177 (clone MEM-166) monoclonal antibodies (BD Biosciences); with the availability of the HNA-Type extra3 kit (BAGene, BAG Health Care, Lich, Germany) we genotyped for HNA-1a, -1b, -1c, HNA-3a, -3b, HNA-4a, -4b, and HNA-5a, -5b alleles. We could retrospectively genotype suspected NAN cases referred to our laboratory before 2011 using frozen samples.

In addition, during the study period, we genotyped 260 healthy Italian subjects to define the local HNA-1, -3, -4, and -5 allelic frequencies. Since no consistent polymorphism responsible for the absence of HNA-2 on neutrophils has been identified, so far[9], in the current study HNA-2 typing of healthy subjects was not included.

Statistical analysis

A descriptive statistical analysis was performed. The Mann-Whitney U test was used to assess differences among groups. Categorical variables were analyzed using Fisher's exact test. Differences were considered statistically significant when a p value <0.05 was obtained. The analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

RESULTS

During the study period, 83 requests for laboratory testing of patients with suspected NAN were evaluated in our clinical laboratory in Milan and 31/83 (37%) cases were confirmed as NAN. **Figure 1** represents a clinical and laboratory algorithm useful for characterizing neonatal

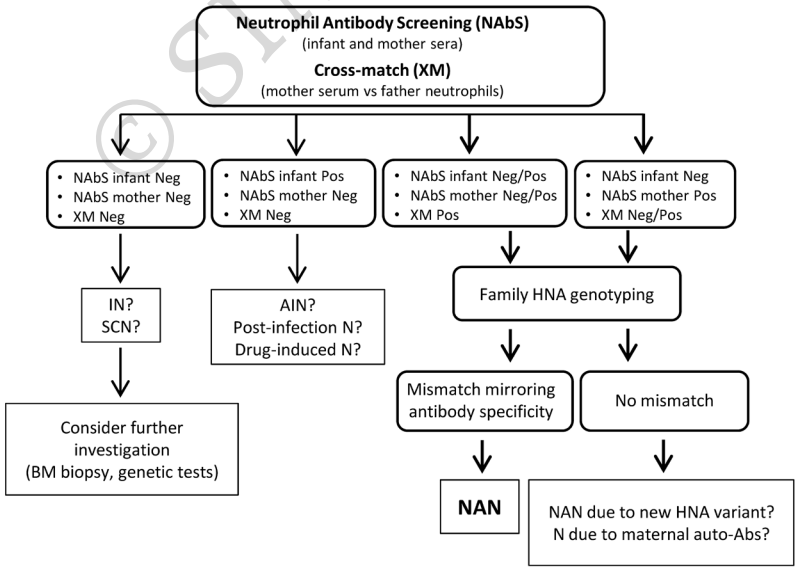

Figure 1 - Algorithm for the laboratory evaluation of neonatal alloimmune neutropenia
N: neutropenia; NAN: neonatal alloimmune neutropenia; SNC: severe congenital neutropenia; AIN: autoimmune neutropenia; IN: idiopathic neutropenia; HNA: human neutrophil antigen; BM: bone marrow; Abs: antibodies; Neg: negative; Pos: positive.

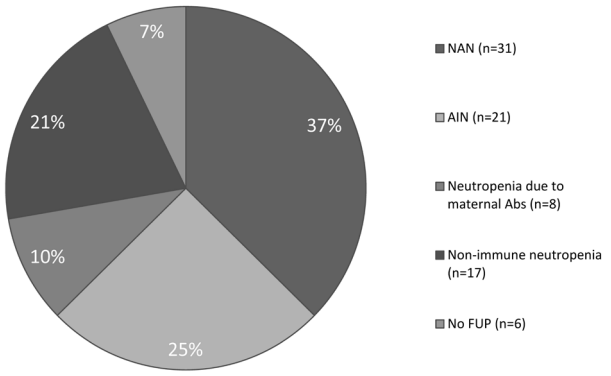

Figure 2 - Algorithm for the investigation of suspected cases of neonatal alloimmune neutropenia
NAN: neonatal alloimmune neutropenia; AIN: autoimmune neutropenia; FUP: follow-up.

neutropenias. Among the 52 non-NAN cases, 21 were autoimmune neutropenia, and 8, including 3 brothers in one family and 2 in another, were neutropenia in neonates from mothers suffering from autoimmune neutropenia. Of the remaining, 17 were finally diagnosed as neutropenias of non-immune origin (post-infection, genetic, etc.) and in 6 cases a diagnosis could not be reached (**Figure 2**).

The demographic and clinical characteristics of NAN and non-NAN patients are shown in **Table I**. The median age and ANC at the onset of neutropenia were significantly lower in cases of NAN than in non-NAN cases (p=0.0002 and p=0.003, respectively). In 23 (74%) of the 31 NAN patients neutropenia developed within the first week of life and laboratory investigations were required within 2 weeks. In five patients neutropenia was discovered at the end of the first month of life and the babies were referred to our laboratory 1-2 months later when the neutropenia had already resolved. In non-NAN infants, both the onset of neutropenia and the execution of diagnostic tests occurred later (**Table I**). We did not find significant differences between the two groups for gender and non-Caucasians ethnicity (p=0.36 and p=0.53, respectively) (**Table I**). With regard to prematurity, 10/31 (32%) NAN cases were born before 37 weeks of gestation; five of them were premature with other clinical complications (3 respiratory distress, 1 umbilical infection, and 1 intraventricular hemorrhage). Among the non-NAN cases, there were seven premature babies (13.5%), five of whom had other complications (2 primary apnea, 2 necrotizing enterocolitis, and 1 sepsis). We observed three pairs of twins in both the NAN and non-NAN groups. Interestingly, in one of these NAN cases pregnancy was achieved through embryo donation. In the NAN group, there were two pairs of siblings who both developed NAN. **Table II** reports the incidences of prematurity and twins in NAN and non-NAN cases compared to those observed in the Italian neonatal population[25]. Expectedly the rates of prematurity and twins were higher in both the NAN and non-NAN groups than among Italian neonates.

Analyzing the 31 NAN cases in more detail, we observed that only six infants (19.4%) had signs of infection as a clinical manifestation, while in the remaining cases, neutropenia was diagnosed incidentally from a blood count that had been performed for different clinical reasons (e.g., premature birth, small for gestational age, family history of neutropenia, twin births, respiratory distress). All infections were severe and required hospitalization together with administration of granulocyte colony-stimulating factor (G-CSF). Another two neonates received G-CSF as prophylaxis. Two patients were treated with intravenous immunoglobulin, one who was not responsive to G-CSF treatment and one with ABO incompatibility with a positive direct antiglobulin

Table I - *Demographic characteristics of cases of neonatal neutropenia divided according to whether the cause was alloimmunity or not*

Characteristics	NAN (n=31)	Non-NAN (n=52)
Male to female ratio	17/14	34/18
Age, in days, at onset of neutropenia; median (range)	1 (1-30)	30 (1-90)
Age, in days, at laboratory testing; median (range)	16 (3-120)	60 (1-180)
ANC/μL at the time of onset of neutropenia; median (range)	330 (40-1,090)	580 (60-1,790)
Caucasian to the non-Caucasian ratio	21/6*	44/8

*Data available for 27 patients only. NAN: neonatal alloimmune neutropenia; ANC: absolute neutrophil count.

Table II - *Frequencies of prematurity and twins among the neutropenic babies investigated for neonatal alloimmune neutropenia*

	NAN (n=31)	Non-NAN (n=52)	Italian neonates*
% prematurity	32	13.5	6.9
% twins	19.3	11.5	1.6

*Source: Childbirth Assistance Certificate (CeDAP) data Report 2018, pre-COVID-19 pandemic. NAN: neonatal alloimmune neutropenia.

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test. The median time to recover from neutropenia was 31 days (8-180), with only one infant, born before 27 weeks of gestation, requiring 180 days.

Serological investigations showed the presence of anti-neutrophil antibodies in all 31 cases diagnosed as NAN. Antibody specificity was identified in 30 of the 31 NAN cases.

A mismatch for HNA-3 was found in only three cases (2 HNA-3b and 1 HNA-3a). In two of these cases another mismatch on HNA-1 (1 HNA-1a and 1 HNA-1b) was detected together with cognate antibodies in the mothers' sera. Moreover, in these two cases, pan-reactive HLA-class I antibodies were also detected, which probably determined a broad positive reaction on both HNA-3a and -3b neutrophils in the GAT. In the third case, reported in early 2008, HNA genotyping was done retrospectively and only a mismatch for HNA-3b was evidenced. Unfortunately, serum samples to look for antibody specificity were no longer available. However, despite the positivity in GIFT and cross-match, it was not possible to define neutrophil antibody specificity.

Serological typing performed for HNA-2 identified only one mismatch in a family with two twins. In this particular case only Luminex allowed us to identify the HNA-2 specificity. Indeed, although we serologically screened our panel's donors (about 50 healthy subjects who we call periodically) with anti-CD177 monoclonal antibody, we did not find any HNA-2 negative subjects.

Table III - *Specificities of HNA alloantibodies implicated in 31 cases of neonatal alloimmune neutropenia*

Alloantibodies	Number	%
Anti-HNA-1a	10	33
Anti- HNA-1b	13	42
Anti-HNA-1c	1	3
Anti-FcγRIIIb	4	13
Anti-HNA-2	2	6
Antibody not identified	1	3

HNA: human neutrophil antigen.

In three infants alloantibodies were not detected, although specific alloantibodies against the fathers' and neonates' HNA epitopes were present in the mothers' sera. Moreover, in 17 mothers anti-HLA class I and/or class II antibodies were also detected.

HNA molecular typing confirmed the presence of a mismatch between neonates and mothers for the allele inherited from the father. Five mothers of NAN patients showed an HNA-1 null phenotype. In particular, in four of the five sera of these mothers we found pan-reactive anti-HNA-1 antibodies, detected by GIFT and/or Luminex, while in one mother's serum only anti-HNA-1a antibodies were found. **Table III** reports the specificities of the HNA alloantibodies implicated in NAN cases.

Cross-matching between paternal neutrophils and maternal serum was possible in only 27/31 cases, due to the unavailability of EDTA samples for the remaining four cases: the test resulted positive in 74% of cases.

The frequencies of HNA alleles in 260 Italian subjects are reported in **Table IV**.

DISCUSSION

In this study, we retrospectively analyzed 83 cases of neutropenic infants referred to our laboratory for granulocyte serological and molecular investigations in the last 14 years. For all of these cases, the clinical suspicion was neonatal immune-mediated neutropenia. Considering the severity of neutropenia and its persistence usually for more than a week, clinicians requested detailed tests for the detection and identification of the causative granulocyte-specific antibodies. This is important for performing a good differential diagnosis, which can point the clinician in the right direction regarding the necessary investigations and treatment[20].

An early form of autoimmune neutropenia as well as neutropenia due to maternal neutrophil autoantibodies are very rare[26,27] and NAN is the most frequent cause of immune neutropenia in neonates[28]. This was quite

Table IV - *Frequencies of HNA -1, -3, -4, and -5 alleles in 260 healthy Italian subjects and in the German population*

Population	HNA-1				HNA-3		HNA-4		HNA-5	
	1-a	1-b	1-c	Null	3-a	3-b	4-a	4-b	5-a	5-b
Italian (our study)	0.334	0.630	0.025	1.9#	0.757	0.242	0.834	0.165	0.723	0.271
German*	0.373	0.627	0.025	0.2-0.8#	0.79	0.21	0.903	0.097	0.659	0.341

#Antigen frequencies %; *From Xia W *et al.*[29] and Bux J[30].

different in our cohort in which NAN, autoimmune neutropenia, and immune neutropenia in the neonate due to maternal autoimmune neutropenia accounted for 37%, 25%, and 10% (precisely 5 mothers for 8 babies) of all cases studied, respectively. In our cohort the youngest patient with autoimmune neutropenia was a 30-day-old infant, confirming that the appearance of autoimmune neutropenia before 1 month of age is unusual[27,31]. The relatively high percentage of autoimmune neutropenia in our cohort could depend on the approach of clinicians who refer a case for laboratory investigations mainly to exclude a possible NAN more than because of a real suspicion. In about 75% of our non-NAN cases the child was older than 2 months at the time of laboratory tests, an age at which most NAN cases have usually already recovered. The delay in reporting cases is probably due to different factors including the presence of other causes of neutropenia in the baby, i.e. prematurity and low birth weight, which could make NAN hard to suspect at first[20]. Moreover, since the neutrophil count is partly dependent on gestational age, birth weight, gender, and ethnicity[2], its variation may give rise to doubt if further investigation is needed. Finally, neutropenia is often found belatedly and casually as a blood cell count is not usually performed at birth. In this regard, as just suggested by Dale[8], the ANC of a neonate should always be determined if the mother has neutropenia and when there is a family history of a previous sibling with immune neutropenia.

Very similarly to what was found in a Dutch study[28], serological investigations for suspected NAN were requested in our study for one in 30,000 births, which led to the identification of one NAN case in 82,000 births. This equated to 31 NAN cases over 14 years. As already suggested by other authors[32] the exact incidence of NAN is probably much lower than the incidences found in retrospective studies such as ours and the Dutch study. In a prospective study conducted about 20 years ago, Zupanska *et al.* reported an expected NAN frequency of one per 6,000 neonates, although this estimation only concerned severe cases and the involvement of only HNA-1 antigen.

About 32% of the neonates with NAN did not show any symptoms, in line with the findings of the Dutch study[28]. The median time for resolution of neutropenia was about 1 month. Only 19% of NAN infants had evidence of infections at presentation, a result similar to that of another study[33] but lower than that reported by Van Den Tooren-de-Groot[28].

We found a relevant percentage of premature births among our NAN cases, higher than that among non-NAN cases and, as expected, much higher than that observed in Italian neonates[25]. There were three twin pregnancies in our NAN group, which is a frequency almost 20 times higher than that observed in Italian neonates[25]. This is not surprising, since a blood count is routinely performed in pre-term babies and/or twins, thus enabling the detection of possible cases of neonatal neutropenia.

In the last 2 years, we observed, for the first time, two NAN cases related to a pregnancy achieved with embryo donation. As this procedure, increasingly used, probably exposes the mother to immunogenic stimuli, it would be advisable to perform a blood count on the neonates in all cases.

In line with other international studies[9,28] in our NAN series, about 90% of cases were due to maternal alloantibodies directed against HNA-1, the most immunogenic neutrophil antigen[34]. In our cohort, the majority of alloantibodies were against HNA-1b, which is the most frequent allele of HNA-1 in the Italian population. In three children, anti-neutrophil alloantibodies were not detected probably due to the low titer, as they were investigated about a month after birth. Interestingly, in one of these cases, the genotypic maternal-fetal incompatibility was HNA-4, and anti-HLA class I and II antibodies were detected in both the mother's and the neonate's sera. This peculiar situation was also observed by Abbas and colleagues in their neutropenic Brazilian babies[35]. However, anti-HLA antibodies are commonly found in multiparous women and whether they can rarely cause NAN or not is still a matter of debate[16,18,36-38]. However, we found no correlation between the course of the disease and HNA antibody specificity.

In our Italian cohort, we found a fairly high frequency of subjects with FcγRIIIb deficiency as compared to the frequency in other European studies (1.9% *vs* 0.1%)[28,39,40]. This may explain the high percentage of mothers with HNA-1 null phenotype in our NAN cases, since HNA-1 is highly immunogenic.

No other significant differences in HNA allelic frequencies, except for the distribution of HNA-4 alleles[41], were observed in comparison with those detected in other Caucasian populations[28,39,40].

CONCLUSIONS

This is the first report on Italian NAN cases. With some limitations regarding its retrospective nature, the majority of our results, such as incidence, clinical conditions, and HNA antibody specificities are in line with those reported for other Caucasian cohorts. From a laboratory point of view, although serological investigation for suspected NAN is laborious and needs specific equipment and reagents together with good experience in the field, we highlight the importance of a rapid request for laboratory tests in a neonate with a low neutrophil count in the absence of a definite diagnosis, even if other causes seem likely and neutropenia is identified several weeks after birth.

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AUTHORSHIP CONTRIBUTIONS

AC, PF and LP contributed to the concept of the study, formal analysis and writing the original draft. PF, LP and AR critically revised the results. ET designed the figures. All Authors contributed to the final version of the manuscript.

The Authors declare no conflicts of interest.

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