ORIGINAL ARTICLE

Neonatal Alloimmune Neutropenia: 10 Year Experience in Portugal

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Abstract

Introduction: Neonatal alloimmune neutropenia is a rare condition often resulting from maternal sensitization to paternal human neutrophil antigens present on the fetal neutrophils. There is a paucity of data on neonatal alloimmune neutropenia in Portugal. This study aimed to describe the cases of neonatal alloimmune neutropenia diagnosed in a Portuguese laboratory unit of cytometry and genetics.

Methods: An observational retrospective study was conducted on 12 newborns suspected of having neonatal alloimmune neutropenia referred to our laboratory from 2010 to 2019. Descriptive statistical analysis of the laboratory results, clinical data, outcomes, and management was performed.

Results: A total of 10 patients with neonatal alloimmune neutropenia were included in this study, of whom seven were female. The mean gestation age was 34 ± 4 weeks. The median age and absolute neutrophil count at the time of diagnosis were 1 day (interquartile range 0) and 230 cells/µL (interquartile range 550). All cases had incompatible human neutrophil antigens genotypes: human neutrophil antigen 1a (three cases), human neutrophil antigens 1b (six cases), and human neutrophil antigens 3a (two cases). Moreover, two cases were incompatible with two human neutrophil antigens polymorphisms and eight infants developed infections in the first months of life. All infections involved hospitalization. The median number of infections per patient was one (interquartile range 1). Two newborns received neonatal alloimmune neutropenia directed treatment. Neutropenia resolution occurred at a median age of 58 days (interquartile range 65).

Discussion: The human neutrophil antigens maternal-fetal incompatibilities found in Portugal were consistent with the available literature. Most clinicians had a vigilant attitude, without therapy, towards neonatal alloimmune neutropenia, even when severe neutropenia and infection were present. We encourage pediatricians to investigate neonatal alloimmune neutropenia in newborns with neutropenia without other established causes.

Keywords: Autoimmune Diseases; Infant, Newborn; Infant, Newborn, Diseases; Neutropenia/diagnosis; Neutropenia/immunology; Neutropenia/therapy; Portugal

Keypoints

What is known:

- Neonatal alloimmune neutropenia can present with infections or be asymptomatic and often its resolution occurs spontaneously before 6 months of age.
- In newborns with neutropenia without an obvious cause, clinicians must consider neonatal alloimmune neutropenia.
- Even though newborns with neonatal alloimmune neutropenia reach full neutrophil recovery, a considerable proportion of them have severe and potentially life-threatening infections.

What is added:

- Laboratory investigation involving immune and genetics assays of neonatal alloimmune neutropenia should not be delayed for both parents and newborns.
- This is the first Portuguese study on neonatal alloimmune neutropenia. A national reference diagnostic laboratory and registry of neonatal alloimmune neutropenia patients would contribute to the increase of knowledge and improvement of patient care.

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Introduction

Neonatal alloimmune neutropenia (NAIN) is a rare cause of neutropenia in newborns which occurs due to the transplacental passage of maternal immunoglobulin G (IgG) antibodies directed against fetal neutrophil antigens inherited from the father. The incidence has been estimated to be between 2/1000 to 8/10 000 live births.[1,2] However, in some studies, it has been reported up to 1/120 000 live births.[3,4]

Currently, 11 human neutrophil antigens (HNA) are known in five different HNA systems (HNA-1, 2, 3, 4, and 5).[5] Alloantibodies against the HNA-1 are the ones most frequently identified.[2,4]

Infants with neonatal alloimmune neutropenia can present with infections, including sepsis, or be asymptomatic.[4,6] Usually, neutropenia is an incidental finding in blood cell counts performed for indications not related to infection. It is worth mentioning that up to 50% of these infants lack infection at presentation, despite having severe neutropenia.[4-6] Treatment with prophylactic antibiotics, granulocyte-colony stimulating factor, and intravenous immunoglobulins (IVIg) has been administered with little evidence of effectiveness.[5] Nevertheless, in the majority of cases, resolution of neonatal alloimmune neutropenia occurs spontaneously before 6 months of age.[3,4,6]

The diagnosis of neonatal alloimmune neutropenia impacts the follow-up process and treatment provided to newborns. Since neutropenia is frequently induced by sepsis in infants, sometimes it is difficult to determine whether the neutropenia has resulted in sepsis or has been a consequence of it.[7,8] Therefore, the physician has to consider neonatal alloimmune neutropenia to reach the diagnosis.

Therefore, due to the paucity of data on the disease in Portugal, the present study aimed to describe the laboratory and clinical findings of the newborns with neonatal alloimmune neutropenia diagnosed in our Portuguese laboratory unit of cytometry and genetics and compare them with the results reported in the literature to discuss some practical issues concerning the laboratory investigation of the disease.

Methods

We identified all the newborns with suspected NAIN, who were referred to the unit for diagnosis in hematology, clinical hematology department, Centro Hospitalar Universitário do Porto, from January 2010 to December 2019.

The inclusion criteria were suspected cases of neonatal alloimmune neutropenia referred to the unit and confirmed neonatal alloimmune neutropenia diagnosis and available medical records.

The laboratory results and clinical data were reviewed, followed by a descriptive statistical analysis of the laboratory and clinical findings.

To conduct flow cytometry and genetic tests, peripheral blood samples from the infant, the mother, and the father were collected into tubes containing ethylene diamine tetra acetic acid-K3 as an anticoagulant. The mother plasma was separated by centrifugation.

In our laboratory, serological / immunological tests for the diagnosis of neonatal alloimmune neutropenia were performed by flow cytometry using a NAVIOS™ benchtop flow cytometer (Beckman Coulter, California, USA). This was conducted by demonstrating the presence of IgG coated neutrophils in the newborn and IgG alloantibodies in maternal plasma against the neutrophils from the father (crossmatch testing), using in-house developed methods.

Briefly, washed newborn peripheral blood cells were incubated with anti-IgG antibodies conjugated with fluorescein isothiocyanate to evaluate the presence of IgG antibodies on the surface of the newborn neutrophils, and the median fluorescence intensity obtained on neutrophils was measured by flow cytometry. The results obtained from this process were considered negative or positive when compared with the results obtained from the neutrophils of three healthy individuals (blood donors) studied in parallel. The crossmatch test was performed by incubating the mother plasma with neutrophils from the father, followed by the detection of IgG antibodies at the father neutrophil surface as described above. The results were then considered negative or positive by comparison with those obtained from healthy individuals pool of plasma (male blood donors were selected to avoid the possibility of using plasma of women with anti-neutrophil antibodies due to previous pregnancies).

In both cases, red blood cell lysis and leukocyte fixation were carried out by processing the samples with FACS™ lysing solution (Becton Dickinson, New Jersey, USA), according to the manufacturer instructions. It should be noted that when the newborn neutrophil count is less than 100 cells/µL, it is usually impossible to determine the presence of IgG on the infant neutrophils since the recovered neutrophils are not sufficient to carry out the study. In addition, the crossmatch testing can be negative, even in the presence of anti-neutrophil IgG antibodies in maternal serum, due to the post-zone effect. We considered neonatal alloimmune neutropenia diagnosis

when both tests performed were positive or one was positive, with incompatibility in HNA genotyping.

Human neutrophil antigens incompatibility was determined by HNA genotyping, performed on the newborns and their parents, using the HNA genotyping tray (OneLambda, Thermo Fisher Scientific Inc., California, USA), which allowed for the detection of nine HNA polymorphisms (1a, 1b, 1c, 3a, 3b, 4a, 4b, 5a, and 5b). Before 2018, the HNA genotyping test available in our laboratory only allowed the detection of three HNA polymorphisms (1a, 1b, 1c). In specific cases with concomitant thrombocytopenia, incompatibility for human platelet antigens (HPA) was also determined using the ExProbe SE HPA 1~6, 15, 21 typing kit (TBG Biotechnology Corp., Texas, USA), which made it possible to identify the following HPA polymorphisms: HPA-1a/b, HPA-2a/b, HPA-3a/b, HPA-4a/b, HPA-5a/b, HPA-6a/b, HPA-15a/b, and HPA-21a/b.

In cases of infants with diagnosed neonatal alloimmune neutropenia, further data were obtained through a review of medical records. Due to the high volume of requests received to our laboratory from all over the country, it was necessary to get in touch with the infants medical assistants to obtain their medical records through the electronic patient record in each hospital. A standard clinical form with the medical information needed was sent, so that the proper clinical information was obtained.

Neutropenia was defined by an absolute neutrophil count less than 1500 cells/µL, with severe neutropenia occurring when absolute neutrophil count was equal to or less than 500 cells/µL. Resolution of neutropenia was considered the time when absolute neutrophil count first reached or exceeded 1500 cells/µL with no recurrence of neutropenia.

Descriptive statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) ® software, version 27.0 (IBM Corporation, Armonk NY, USA). Categorical variables are presented as frequencies and percentages and continuous variables as means and standard deviations (SD), or medians and interquartile ranges (IQR) for variables with skewed distributions.

Results

A total of 12 patients were referred to our laboratory for the investigation of neonatal alloimmune neutropenia during the study period (which covered nearly 10 years), 11 of whom were diagnosed with neonatal alloimmune neutropenia, one was excluded due to unavailable clinical information, and another patient was also excluded for having congenital neutropenia due to an *ELANE* gene mutation. Therefore, 10 infants with neonatal alloimmune neutropenia were included in this study and evaluated for clinical and experimental data (Tables 1, 2,3

Table 1. Clinical features of infants with neonatal alloimmune neutropenia

Case	Sex	Gestation (weeks)	Birthweight (g)	Obstetric complications	Reason for CBC	First CBC with neutropenia (days of life)	Presenting infection*	Admission	LOS (days)	Other infectious complications*	Therapeutic management	Neutropenia resolution (days of life)	Infection after discharge during the first 6 months of life
1	F	33	1890	SPB	Sepsis screening	1	No	Nursery	25	No	- Prophylactic antibiotic†	81	No
2	F	39	3230	GD	Omphalitis	14	Omphalitis	Nursery	14	No	- Directed antibiotic	56	No
3	F	29	1165	PPROM GD	Sepsis screening	1	No	NICU	33	No	- Prophylactic antibiotic	29	Bronchiolitis requiring hospital admission (RSV)
4	M	34	2180	PPROM	Sepsis screening	1	No	Nursery	21	Omphalitis	- Prophylactic antibiotic - Directed antibiotic	106	No
5	F	30	760	PE IUGR	Sepsis screening	1	Sepsis (*S. aureus*)	NICU	80	UTI (*E. coli*)	- Directed antibiotic - Prophylactic antibiotic	NA	No
6‡	M	29	950	IUGR	Sepsis screening	1	No	NICU	68	Sepsis (*S. epidermidis*)	- Prophylactic antibiotic - Directed antibiotic - IVIg 400 mg/kg/day, 5 days, D42	58	No
7	F	39	3620	(Anti-C + anti-Jka antibodies) §	Risk of HDN	1	No	Nursery	11	No	None	10	No
8	F	33	980	IUGR	Sepsis screening	1	Fever without focus	NICU	77	Omphalitis Conjunctivitis (*S. aureus*)	- Prophylactic antibiotic - Directed antibiotic - IVIg 1 g, 2 days, D31	237	Fever without focus requiring hospital admission
9	F	37	2490	No	Jaundice	2	No	Nursery	NA	Conjunctivitis (*S. aureus*)	- Prophylactic antibiotic - Directed antibiotic	66	No
10	M	37	3100	PPROM	Sepsis screening	1	Sepsis	NICU	5	No	- Directed antibiotic - Prophylactic antibiotic	29	No

CBC - complete blood count; D31 - intravenous immunoglobulins made on the 31st day of life; D51 - intravenous immunoglobulins made on the 51th day of life; *E. coli* - *Escherichia coli*; GD - gestational diabetes; HDN - hemolytic disease of the newborn; IVIg - intravenous immunoglobulins; IUGR - intrauterine growth restriction; LOS - length of stay (duration of hospitalization); NA - not available; NICU - neonatal intensive care unit; PE - preeclampsia; PPROM - preterm premature rupture of membranes; RSV - respiratory syncytial virus; *S. aureus* - *Staphylococcus aureus*; *S. epidermidis* - *Staphylococcus epidermidis*; SPB - spontaneous preterm birth; UTI - urinary tract infection.

* The infectious agent is presented below the infection episodes in the cases it was identified.

† The newborns under prophylactic antibiotics had the same regimen: gentamicin and ampicillin.

‡ This case was previously published.[14]

§ Anti-C e anti-Jka antibodies were detected, without clinical signs of hemolytic disease in the newborn. It was not considered an obstetric complication.

Table 2. Blood counts and absolute neutrophil count evolution

Case	First complete blood count with neutropenia				Neutropenia nadir		Formal work-up at a reference laboratory		Neutropenia resolution*
	Time (days of life)	ANC (cells/μL)	Hemoglobin (g/dL)	Platelets (cells/μL)	Time (days of life)	ANC (cells/μL)	Time (days of life)	ANC (cells/μL)	Time (days of life)
1	1	210	19.4	201 000	9	110	9	< 100†	81
2	14	900	16.2	198 000	35	0	24	< 100	56
3	1	110	12.9	76 000	2	30	15	< 100	29
4	1	230	17.1	315 000	28	80	3	< 100	106
5	1	200	16.4	119 000	1	200	78	523	NA
6†	1	230	16.0	121 000	3	60	16	NA	58
7	1	1000	16.2	155 000	3	100	7	781	10
8	1	200	16.8	135 000	2	0	22	313	237
9	2	700	20.0	197 000	6	100	12	639	66
10	1	400	17.0	238 000	2	200	3	300	29

ANC - absolute neutrophil count; NA - not available.
* The age at which absolute neutrophil count first reached or exceeded 1500 cells/μL without recurrence of neutropenia.
† This case was previously published.[14]

and Figure 1v).

Out of 10 infants, seven (70%) were female and six (60%) were premature (Table 1). The mean ± SD of gestational age at birth and birth weight were 34 ± 4 weeks and 2037 ± 1054 g, respectively, and the median Apgar score at five minutes was 9 (IQR 1). A total of six (60%) infants were born via vaginal delivery. All pregnancies were regularly monitored, and eight (80%) pregnant women had obstetric complications. Anti-C e anti-Jka antibodies were detected in case 7, without clinical signs of neonatal hemolytic disease. Therefore, it was not considered an obstetric complication. One mother had a son with neonatal alloimmune neutropenia (case 2).

The hematological findings are presented in Table 2. The median absolute neutrophil count at presentation was 230 cells/μL (IQR 550) at a median age of one day (IQR 0). The mean ± SD nadir absolute neutrophil count was 88 ± 71 cells/μL at a median age of 3 days (IQR 12). Neutropenia resolution was achieved at a median age of 58 days (IQR 65). A total of four patients also presented mild to moderate thrombocytopenia (< 150 000 platelets/μL), with a mean ± SD platelet count of 175 500 ± 69 209 platelets/μL, ranging from 76 000 to 315 000 platelets/μL (Table 2, cases 3, 5, 6 and 8).

In total, four (40%) newborns had infections at presentation. We identified 10 infection episodes during the first hospitalization, four episodes at presentation, and six infection episodes in the cases of five newborns during a hospital stay, with a median number of one infection episode per newborn (IQR 1). Complications beyond the initial presentation were encountered in five patients (Table 1). An infectious agent was isolated in five infection episodes: two sepsis, one caused by *Staphylococcus aureus* and another by *Staphylococcus epidermidis*, two conjunctivitis caused by *Staphylococcus aureus* and a urinary tract infection caused by *Escherichia coli*. Moreover, three (30%) newborns developed no infection while neutropenic (cases 1, 3, and 7).

Five (50%) newborns were admitted to a neonatal intensive care unit (NICU) after birth, but the admission was not directly due to neutropenia (premature rupture of membranes, prematurity, and sepsis were the main causes). The mean ± SD duration of the hospitalization was estimated to be 37 ± 30 days. It was found that four (40%) infants had neutropenia resolution before discharge. None of the newborns, including those having concomitant thrombocytopenia, had hemorrhagic manifestations. There were no cases of death.

Concerning the therapeutic options, nine (90%) patients received at least one course of antibiotics and eight (80%) received prophylactic antibiotics (gentamicin and ampicillin), mainly due to premature rupture of membranes and prematurity, as well as neutropenia in cases 5, 9, and 10. Moreover, three (30%) newborns had infections (omphalitis, sepsis, and conjunctivitis) while under treatment with antibiotics prophylactic (cases 4, 6, and 9), of whom two with infectious agents (a sepsis caused by *Staphylococcus epidermidis* and conjunctivitis caused by *Staphylococcus aureus*) were isolated. As indicated in Table 1, none of the newborns received granulocyte-colony stimulating factor, and intravenous immunoglobulins was administered to two infants (cases 6 and 8), with neutropenia resolution observed in one of them (case 8).

After discharge and in the first six months of life, two infants had one more infection episode requiring

hospitalization (cases 3 and 8), without and with neutropenia, respectively.

In the neonatal alloimmune neutropenia investigation, the median age at which formal laboratory investigation was undertaken was 14 days (IQR 17) (Table 2). In five cases (100% of the cases tested with success), increased levels of IgG antibodies were detected at the membrane of the newborns neutrophils (Table 3). We also found that four of the newborns had absolute neutrophil count < 100 cells/μL, at the time of the study. Therefore, enough neutrophils could not be recovered to test the presence of IgG coated neutrophils in the newborns. Eight (80%) cases had a positive crossmatch test, demonstrating the presence of IgG antibodies in maternal plasma, against paternal neutrophils. Among the five cases with available test results (the mentioned two tests), three (60%) were positive for both (Table 3). In cases having a negative crossmatch test (cases 5 and 9), the newborns had increased levels of IgG at the neutrophil surface, documenting an immune etiology

Table 3. Laboratory features of infants with neonatal alloimmune neutropenia

Case	IgG on newborns neutrophils*	Crossmatch test*		HNA genotyping†				HNA incompatibility‡									
				1	3	4	5	Type	1a	1b	1c	3a	3b	4a	4b	5a	5b
1	NA§	Positive	Mother	bb	bb	aa	aa										
			Father	aa	aa	ab	aa	M/F	Yes			Yes			Yes		
			Newborn	ab	ab	aa	aa	M/N	Yes			Yes			No		
2	NA§	Positive	Mother	null	NA	NA	NA										
			Father	ab	NA	NA	NA	M/F	Yes	Yes							
			Newborn	a	NA	NA	NA	M/N	Yes	No							
3**	NA§	Positive	Mother	aa	ab	ab	ab										
			Father	bb	ab	aa	ab	M/F		Yes							
			Newborn	ab	aa	aa	ab	M/N		Yes							
4	NA§	Positive	Mother	bb	aa	aa	ab										
			Father	ab	ab	aa	aa	M/F	Yes				Yes				
			Newborn	ab	aa	aa	ab	M/N	Yes				No				
5**	Positive	Negative¶	Mother	ab	aa	aa	aa										
			Father	bb	bb	ab	aa	M/F					Yes		Yes		
			Newborn‖	NA	NA	NA	NA	M/N					NA		NA		
6††	NA‖	Positive	Mother	ac	NA	NA	NA										
			Father	abc	NA	NA	NA	M/F		Yes							
			Newborn	abc	NA	NA	NA	M/N		Yes							
7	Positive	Positive	Mother	aa	bb	aa	ab										
			Father	bb	ab	ab	ab	M/F		Yes		Yes			Yes		
			Newborn	bb	ab	aa	bb	M/N		Yes		Yes			No		
8	Positive	Positive	Mother	null	NA	NA	NA										
			Father	bb	NA	NA	NA	M/F		Yes							
			Newborn	b	NA	NA	NA	M/N		Yes							
9	Positive	Negative¶	Mother	aa	aa	ab	aa										
			Father	bb	aa	ab	ab	M/F		Yes							Yes
			Newborn	ab	aa	ab	aa	M/N		Yes							No
10	Positive	Positive	Mother	aa	ab	ab	aa										
			Father	bb	aa	ab	ab	M/F		Yes							
			Newborn	ab	ab	aa	aa	M/N		Yes							

ATB - antibody; HNA - human neutrophil antigens; IgG - immunoglobulin G; NA - not available; M/F – mother / father HNA incompatibility; M/N - mother / newborn HNA incompatibility.

* Both tests were done by flow cytometry; the crossmatch testing was performed by incubating the mother serum with neutrophils from the father.

† The human neutrophil antigens genotyping test earlier than 2018 available in our laboratory only allowed the detection of the HNA-1 polymorphisms.

‡ For clarity, only human neutrophil antigens polymorphisms in which incompatibility between the mother and the father (M/F) and/or the mother and the newborn (M/N) were detected are presented.

§ Not enough neutrophils were recovered to carry out the test (newborn absolute neutrophil count <100 cells/mm^3).

‖ No sample was available to carry out the test.

¶ The crossmatch between maternal serum and paternal neutrophils can be negative, even in the presence of anti-neutrophil antibodies, due to the post-zone effect.

** Human platelet antigens (HPA) genotyping performed in these cases revealed no incompatibility for the HPA polymorphisms studied. Case 3: mother: HPA-1aa, -2aa, -3ab, -4aa, -5ab, -6aa, -15ab, -21aa; father: HPA-1aa, -2aa, -3ab, -4aa, -5aa, -6aa, -15ab, -21aa; newborn: HPA-1aa, -2aa, -3ab, -4aa, -5ab, -6aa, -15bb, -21aa. Case 5: mother: HPA-1aa, -2aa, -3ab, -4aa, -5ab, -6aa, -15ab, -21aa; father: HPA-1aa, -2aa, -3aa, -4aa, -5aa, -6aa, -15ab, -21aa.

†† This case was previously published.[14]

(Table 3).

Furthermore, HNA genotyping of the mother and the father was performed in all cases, with three cases (30%) being tested only for HNA-1 (Table 3, Fig. 1). A potential couple incompatibility was found in all cases: HNA-1a in three (30%) cases, HNA-1b in seven (70%) cases, HNA-3a in two (20%) cases, HNA-3b in another two (20%) cases, HNA-4b in three (30%) cases, and HNA-5b in one (10%) case. Two of the mothers (cases 2 and 8) had an HNA-1-null phenotype and, consequently, complete deficiency of Fc gamma receptor IIIb (FcγRIIIb) (CD16) on neutrophils, as documented by flow cytometry. A total of nine newborns were genotyped for the HNA polymorphisms, confirming incompatibility for HNA-1a, HNA-1b, and HNA-3a in three (33%), six (67%), and two cases (22%), respectively. Two cases had incompatibility for two HNA polymorphisms (HLA-1a + HNA-3a and HLA-1b + HNA-3a, respectively). No incompatibility between the mothers and the newborns tested was observed for HNA-3b, HNA-4a, HNA-4b, HNA-5a, and HNA-5b (Table 3, Fig. 1). The only case in which the newborn HNA testing was missed (case 5) had a possible incompatibility for HNA-3b and/or HNA-4b, according to the HNA genotyping results of the parents.

Furthermore, HPA genotyping was performed in two (cases 3 and 5) out of four cases in which the newborn had concomitant moderate thrombocytopenia and no incompatibility was found for the polymorphisms studied (Table 2).

Discussion

In our study, the severity of neutropenia was compared with previous neonatal alloimmune neutropenia studies.[3,4,6] The neutropenia diagnosis was observed more incidentally on blood tests rather than in the presence of overt infection (40% of the cases), which was consistent with the literature.[3-6] Even though all newborns reached full recovery and there was no fatal disease, 70% of them had infections (omphalitis, conjunctivitis, sepsis, and urinary tract infection).

These rates are somewhat higher than those obtained in other studies, in which infection was observed in as many as 60% of infants with NAIN.[3,4,6] There are some explanations for this worse outcome.

Firstly, the high prematurity rate (60%) we found compared to other studies.[6,9] Secondly, the median age at which formal neonatal alloimmune neutropenia investigation was undertaken was higher in this study, compared to other studies (14 *vs* 7 days of life), resulting in a delay in the diagnosis.[6] Thirdly, not only we had a smaller number of newborns receiving neonatal alloimmune neutropenia directed treatment (20% *vs* 44%),[6] but we also found a difference between the first-line choice and the timing of treatment, compared to other studies (intravenous immunoglobulins *versus* granulocyte colony stimulating factor and 31-42 *vs* 1-12 days of life, respectively).[3,5,6,10]

Moreover, the median time to resolution of neutropenia was two months (58 days of life), which was higher than some studies,[6] but comparable to others.[3,4]

Our infants had neonatal alloimmune neutropenia mediated by antibodies against HNA-1b (67%), HNA-1a (33%), and HNA-3a (22%). Two cases had incompatibility for two HNA polymorphisms (HLA-1a + HNA-3a and HLA-1b + HNA-3a). This result was in agreement with the literature.[2,5,6,11-14]

We want to highlight the importance of both immune and genetics assays and the fact that both parents and infants need to be tested to ensure the correct neonatal alloimmune neutropenia diagnosis. Moreover, it should be noted that a negative crossmatch test does not exclude an HNA incompatibility (Table 3, cases 5 and 9), and a potential mother / father HNA incompatibility, based on HNA genotyping, does not necessarily imply a mother / newborn incompatibility (Table 3, cases 1, 2, 4, 7, 9 and Fig. 1). Therefore, we maintain that it is important to collect peripheral blood samples from the infant, the mother, and the father, for a proper laboratory diagnosis.

We also found that four of our newborns had absolute neutrophil count < 100 cells/μL at the time of laboratory study, preventing the investigation of the presence of IgG antibodies neutrophil membrane of the newborns. In these cases, we suggest repeating the test as soon as neutropenia begins to recover, as in our experience. Based on the study of cases suspected of neonatal alloimmune neutropenia or autoimmune neutropenia, a neutrophil count of 200-300 cells/μL is usually enough to perform the test. As for the planning of blood collection for serological / immunological studies, it should be

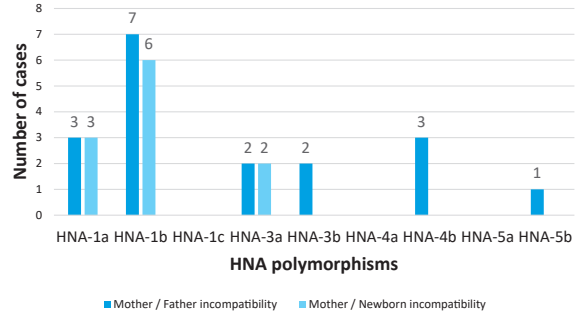

Figure 1. Anti-human neutrophil antigens specificities in 10 cases of neonatal alloimmune neutropenia.

performed before initiating neonatal alloimmune neutropenia-directed therapies, especially high doses of intravenous immunoglobulins, since they may influence the results obtained.

In general, we want to emphasize that clinicians need to maintain a high index of suspicion for neonatal alloimmune neutropenia in newborns with neutropenia in the absence of another obvious cause. Moreover, delay in laboratory diagnosis should be avoided, since a correct diagnosis of neonatal alloimmune neutropenia allows for a proper clinical and therapeutic approach. The low diagnosis of neonatal alloimmune neutropenia reported in Portugal and the example of case 2 evolution, where despite the existence of a previous sibling with the disease diagnosed, it took 24 days to make a formal neonatal alloimmune neutropenia investigation shows that efforts should be made to sensitize clinicians toward this entity.

Most clinicians have a vigilant attitude, without therapy, towards neutropenia, even in the presence of severe neutropenia and infection. We report frequent administration (80%) of antibiotics to infants at risk of developing an infection. This reported prevalence of antibiotic therapy can be explained by the presence of a conservative approach to infants at risk of sepsis, which is consistent with other studies.[6] Nevertheless, 30% of our newborns had infections while on prophylactic treatment.

In this study, the frequency of therapeutic interventions directed to neonatal alloimmune neutropenia was lower, compared to other studies (20% *vs* 44%),[6] and higher use of granulocyte-colony stimulating factor was found in the literature.[3-6] However, the best neonatal alloimmune neutropenia directed treatment is still to be determined.[5,10,15,17]

We acknowledge the limitations of the methodology used in this study, based on a retrospective analysis. The rarity of neonatal alloimmune neutropenia affects the case series design. The previous reporting of incidence, laboratory findings, and clinical outcomes in infants with neonatal alloimmune neutropenia were limited due to small population sizes and few international studies. To the best of our knowledge, no other studies on this subject were previously published with Portuguese data, except for one case report of neonatal alloimmune neutropenia published in 2012 as part of this series.[14] We believe that a national reference laboratory for the diagnosis of neonatal alloimmune neutropenia and a national patient registry for the disease would help make an efficient diagnosis, provide epidemiological data, enrich the literature available to the medical and scientific communities, and improve patient care.

Author Contribuitions

RP and ML participated in the study conception or design. RP, JR, SF, CL, MAT and ML participated in acquisition of data. RP, JR, SF, CL, MAT and ML participated in the analysis or interpretation of data. RP and ML participated in the drafting of the manuscript. RP, JR, SF, CL, MAT and ML participated in the critical revision of the manuscript. All authors approved the final manuscript and are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest

The authors declare that there were no conflicts of interest in conducting this work.

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Protection of human and animal subjects

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Confidentiality of data

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Neutropenia Aloimune Neonatal: Experiência de 10 Anos em Portugal

Introdução: A neutropenia aloimune neonatal é uma doença rara que resulta da sensibilização materna aos antigénios dos neutrófilos humanos de origem paterna presentes nos neutrófilos fetais. Os dados sobre a doença em Portugal são escassos. O objetivo deste estudo é descrever os casos de neutropenia aloimune neonatal diagnosticados numa unidade laboratorial de citometria e genética portuguesa.

Métodos: Realizou-se um estudo retrospetivo dos dados clínicos e laboratoriais, incluindo a terapêutica instituída e evolução, de 12 casos referenciados por suspeita de neutropenia aloimune neonatal entre 2010 a 2019.

Resultados: Foram avaliados 10 recém-nascidos. Sete eram do género feminino. A idade gestacional média foi de 34 ± 4 semanas. A mediada da idade e da contagem absoluta de neutrófilos ao diagnóstico foram, respetivamente, 1 dia de vida (amplitude interquartil 0) e 230 células/µL (amplitude interquartil 550). Todos os casos tinham genotipagem para antigénios dos neutrófilos humanos incompatível: antigénio dos neutrófilos humanos 1a (três casos), antigénio dos neutrófilos humanos 1b (seis casos) e antigénio dos neutrófilos humanos 3a (dois casos). Dois casos tinham incompatibilidade para dois antigénios dos neutrófilos humanos. Oito recém-nascidos tiveram infeções nos primeiros meses de vida e todas implicaram hospitalização. A mediana do número de infeções por recém-nascido foi um (amplitude interquartil 1). Dois recém-nascidos receberam tratamento dirigido para a neutropenia aloimune neonatal. A resolução da neutropenia ocorreu com uma mediana de idade de 58 dias de vida (amplitude interquartil 65).

Discussão: As incompatibilidades para os antigénios dos neutrófilos humanos materno-fetais encontradas em Portugal foram consistentes com as descritas na literatura. Os clínicos privilegiaram uma atitude vigilante, sem terapêutica dirigida à neutropenia aloimune neonatal, mesmo em casos de neutropenia severa e infeção. Reforça-se a importância de investigar a neutropenia aloimune neonatal nos recém-nascidos com neutropenia, sem outra causa estabelecida.

Palavras-Chave: Doenças Autoimunes; Recém-Nascido; Doenças do Recém-Nascido; Neutropenia/diagnóstico; Neutropenia/imunologia; Neutropenia/tratamento; Portugal