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**MOLECULAR CHARACTERIZATION OF PAEDIATRIC IDIOPATHIC
HYPEREOSINOPHILIA**

***Maria Cristina Rapanotti^{1,2,3}, *Roberta Caruso⁴, Emanuele Ammatuna^{2,3}, Serena Zaza^{2,3},
Laura Trotta⁴, Mariadomenica Divona^{2,3}, Laura Cicconi², Daria Funaro⁴, Giorgio Federici¹,
Sergio Amadori², Giulio De Rossi⁴, Francesco Lo-Coco^{2,3}**

¹ Department of Laboratory Medicine, Policlinico “Tor Vergata”, Rome, Italy

² Department of Biopathology, University of Tor Vergata

³ Laboratorio di Neuro-Oncoematologia, Fondazione Santa Lucia, Rome, Italy

⁴ Hematology Unit-“Bambino Gesù” Children Hospital, IRCCS, Rome, Italy

*These authors have equally contributed to this work

RUNNING TITLE: Molecular features of paediatric hypereosinophilia

Corresponding author:

Maria Cristina Rapanotti, PhD

Department of Laboratory Medicine

Policlinico “Tor Vergata” University of Rome

Viale Oxford 81 - 00133 - Rome - Italy

Tel: 39 06-20902169

Fax: 39 06-20902359

E-mail: cristinarapanotti@yahoo.it

*These authors have equally contributed to this work

RUNNING TITLE: Molecular features of paediatric hypereosinophilia

Summary

Hypereosinophilic syndromes (HES) include a group of heterogeneous diseases characterized by the persistent increase of the number of eosinophils in blood and bone marrow. Few cases of paediatric hypereosinophilia (pHES) have been described in the literature. Early identification of pHES that may evolve towards a lymphomyeloproliferative disease is relevant in light of prognostic and therapeutic implications. Molecular features of 10 pHES patients were analysed at presentation and during their clinical outcome, including analysis of *BCR-ABL* and *FIP1L1/PDGFR*A fusion genes, quantitation of Wilm's tumour suppressor gene copy number and clonality of T-cell receptor and immunoglobulin heavy chain (IgH). All patients had normal karyotype and germ line T-cell receptor configuration. Five children showed IgH clonality at presentation: of these, two developed a B non-Hodgkin lymphoma and a B-lineage acute lymphocytic leukaemia at six and 12 months, respectively, two spontaneously reverted to a polyclonal IgH profile during the follow-up, and the last one persisted with pHES without B-clonal evolution after 19 months. One patient had a *PDGFR*A/*FIP1L1* fusion and achieved hematologic and molecular remission after imatinib therapy. IgH rearrangement was observed to be a frequent molecular feature of pHES in children and may precede B-cell clonal expansion and evolution into B-cell malignancies.

Introduction

Hypereosinophilic syndromes (HES) include a group of heterogeneous diseases characterized by the persistent increase of eosinophil numbers in the peripheral blood and the bone marrow (Chusid *et al.*, 1975; Brito-Bapapulle *et al.*, 2003, Bain *et al.*, 2004, Gleich and Leiferman, 2009). HES is predominantly a disease of males (male:female ratio 9:1) and is usually diagnosed between the ages of 20 and 50 years. Tissue infiltration by eosinophils is responsible for the end-organ damage that results from the activity of proteins released by the eosinophil granules. A major change in the new WHO 2008 classification was the reorganization of chronic eosinophilic leukaemia CEL/HES into “CEL not otherwise specified (CEL, NOS)” and “myeloid and lymphoid neoplasms with eosinophilia associated with *PDGFRA*, *PDGFRB*, and *FGFR1* abnormalities” (Klion *et al.*, 2006; Bain *et al.*, 2009).

The relevance of identifying PDGFR-rearranged eosinophilic disorders is related to the efficacy of treatment with imatinib mesylate. (Cools *et al.*, 2004; Gotlib *et al.*, 2004; Helbig *et al.*, 2008). However, the myeloproliferative CEL, NOS are also frequently responsive to imatinib, suggesting that other as yet unrecognized mutations affecting tyrosine kinases may underlie their pathogenesis (Salem *et al.*, 2003).

A diagnosis of CEL, NOS requires evidence of clonality of the eosinophils. Since in many cases it is impossible to define clonality, the diagnosis of idiopathic HES is still made. The diagnosis of idiopathic HES is based on exclusion criteria and frequently remains doubtful because of the lack of genetic markers (Brito-Bapapulle *et al.*, 2003). Early discrimination between idiopathic HES confined to an unidentified reactive process and hypereosinophilia accompanied by a clonal proliferation eventually evolving into a lymphoproliferative disease is relevant in light of its obvious prognostic and therapeutic implications (Smith *et al.*, 2004; Roche-Lestienne *et al.*, 2005). In the latter case, the hypereosinophilia is the result of a primitive T or B lymphoid disorder and can be considered as an epiphenomenon. Long-term evolution of this form of hypereosinophilia is often complicated by T-cell lymphoma or leukaemia, indicating that T cells are involved in the pathogenesis of these disorders (Raghavar *et al.*, 1987; Cogan *et al.*, 1994; Roufousse *et al.*, 2000; Bank *et al.*, 2001; Roumier *et al.*, 2003). Evolution into B-cell malignancies has been occasionally reported both in adults and in childhood (Blatt *et al.*, 1992; Urtasun *et al.*, 2001; Bernasconi, 2001; Hill *et al.*, 2003; Wynn *et al.*, 2003; Horigome *et al.*, 2005; Rapanotti *et al.*, 2005; Bhatti *et al.*, 2009). Finally, the expression level of *WT1* has been recently investigated as an additional marker to the standard parameters so far considered in the diagnosis of CEL, NOS in the adult population,

and it seems to be particularly useful in cases where standard molecular and cytogenetic analyses are not informative (Cilloni *et al.*, 2007).

Few cases of pHES have been described in the literature (Blatt *et al.*, 1992; Urtasùn *et al.*, 2001; Bernasconi, 2001; Hill *et al.*, 2003; Scheinfeld *et al.*, 2003; Wynn *et al.*, 2003; Chien *et al.*, 2004; Horigome *et al.*, 2005; Rapanotti *et al.*, 2005; Rives *et al.*, 2005; Farruggia *et al.*, 2009). pHES has a slight male predominance and shows a distinct biologic and clinical course compared with the adult forms. The disease in children is commonly associated with chromosomal abnormalities, including trisomy 8 and gene rearrangements that may involve *ABL* (Brito-Bapapulle *et al.*, 2003, Bain *et al.*, 2004). In addition, pHES has also been described in association with acute lymphoblastic leukaemia, while the abnormal fusion *FIP1L1/PDGFR* has rarely been found (Rives *et al.*, 2005), and evolution of idiopathic HES into a B-cell neoplasia has been only occasionally described (Blatt *et al.*, 1992; Jain *et al.*, 2000; Urtasùn *et al.*, 2001; Bernasconi, 2001; Hill *et al.*, 2003; Scheinfeld *et al.*, 2003; Wynn *et al.*, 2003; Chien *et al.*, 2004; Horigome *et al.*, 2005; Rapanotti *et al.*, 2005, D'Angelo *et al.*, 2008). Here, we report the molecular and clinical features of 10 pHES studied at diagnosis and during follow up.

Patients and methods

Patients and healthy donors

Between November 2001 and October 2007, 10 children (six males and four females) with marked hypereosinophilia lasting for at least six months were observed at the Division of Haematology, Bambino Gesù Children's Hospital of Rome. The main clinical and laboratory features at the time of presentation are summarized in Table 1. The median absolute number of eosinophils was 10.1/mm³ (range 1.5–70.8/mm³). Five patients presented with anaemia, while the platelet count was normal in all but one patient (UPN6). In three cases (UPN2, UPN4 and UPN5), there were no signs of organ involvement at diagnosis.

Eosinophil infiltration in the bone marrow at presentation ranged from 21.5% to 80% (median 35%). Cardiac involvement was documented by echocardiography and electrocardiography (EKG) in four patients who presented with chronic cardiac tamponade and pleural effusion, sinus tachycardia, restrictive cardiomyopathy and pancarditis with mitral valvular insufficiency, respectively (UPN3, UPN6, UPN7 and UPN8). Three patients (UPN3, UPN7 and UPN9) presented with pulmonary involvement with interstitiopathy, pleural effusion and asthmatic bronchitis. All but two patients (UPN1 and UPN10) started therapy with steroids (prednisone 1 mg/kg/day) followed

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3 by α -interferon up to 1 MIU three times a week. All patients were followed up at regular intervals
4 with periodic clinical controls and standard laboratory and instrumental evaluation.
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8 9 *Clinical and laboratory exams*

10 Bone marrow (BM) aspirate and complete clinical chemistry, including serum immunofixation and
11 IgE count assessed by the PRIST method, and allergic tests (RAST method), were performed in all
12 patients together with blood culture, virological investigations and immunophenotype. Instrumental
13 examinations including chest radiography, abdomen ultrasound, total body computed tomography
14 scan, EKG and echocardiography were carried out to exclude the causes of secondary
15 hypereosinophilia and to evaluate end-organ damage. Immunophenotype was performed by flow
16 cytometry at onset in all cases. The following monoclonal antibodies were used for the
17 characterization of potential abnormal T or B clonal population: CD19, CD10, CD20, CD22, CD24,
18 HLADR, CD3, CD7, CD5, CD4, CD8, CD79a, CD13, CD33, CD34 and TdT.
19

20 Genetic studies included conventional karyotyping on bone-marrow-derived G-banded metaphases.
21 Molecular biology analysis was performed by RT-PCR to identify the presence of the *BCR-ABL*
22 and *FIP1L1/PDGFR* fusion genes (vanDongen *et al.*, 1999; Bacher *et al.*, 2006). In order to assess
23 the clonality of IgH and TCR repertoires, BM genomic DNA was extracted from bone marrow
24 mononuclear cells. TCR clonality was performed by DHPLC sequencing of the TCR β genomic
25 region (Cave *et al.*, 1994). IgH clonality analysis was performed on BM genomic DNA by
26 amplifying CDR1, CDR2 and CDR3 regions of the heavy chain immunoglobulin genes (IgH-
27 PCR). Amplification of the CDR3 region was performed using CA1 and CA2 primers,
28 respectively, the “consensus primers” for variable and joining regions of the heavy chain (Nizet *et*
29 *al.*, 1991). Amplifications of CDR1 and CDR 2 were performed using oligo-degenerate sequence
30 primers mapping the frameworks FR2 and FR3, as previously described (Diss *et al.*, 1993).
31

32 Quantitative *WT1* assessment (RQ-PCR) and fluorescence measurement were performed on the
33 ABI PRISM 7700 Sequence Detection System (PE Applied Biosystem, Epster City, CA, USA) as
34 described by Cilloni and co-workers (2007).
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46 47 48 49 50 51 52 53 *Control populations*

54 Blood samples from 30 healthy donors were taken from the Transfusion Centre as a negative
55 control population. Bone marrow aspirates from 25 B-ALL and peripheral blood from 25 chronic
56 lymphocytic leukaemia (CLL) patients were used as positive controls, all of which had a
57 monoclonal IgH profile and a polyclonal TCR β repertoire. To assess the prevalence of IgH
58 rearrangement in chronic myeloid neoplasms, we analysed a cohort of 10 patients affected by
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3 chronic myeloid leukaemia (CML) (five patients), essential thrombocytemia (three patients) and
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5 chronic myelomonocytic leukaemia (two patients), all of which demonstrated polyclonal IgH.
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8 9 **Results**

10 11 *Diagnostic findings*

12 Biological and clinical features at onset are summarized in Table 1. Immunophenotype analysis was
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14 normal in all but one patient (UPN9), who showed a very low proportion of T-helper lymphocytes,
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16 with a CD4/CD8 ratio of 0.5%. The CD4/CD8 ratio was normal in all other patients. All patients
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18 expressed CD33/CD13 antigens in their myeloid compartments, whereas the CD34 marker was
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20 never expressed. All patients had a normal karyotype.
21

22 Molecular analysis documented a B-clonal disorder showing a monoclonality of the IgH-CDRII
23
24 region (framework FR2) in 5/10 patients (UPN1, UPN2, UPN7, UPN8 and UPN9).
25
26 *FIP1L1/PDGFR*A transcript was found in one patient (UPN6) who was diagnosed as having a
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28 myeloid neoplasm with eosinophilia and abnormalities of *PDGFR*A. TCR rearrangements were not
29
30 detected in any of the patients. Quantitative *WT1* analysis was performed in 6/10 patients at
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32 presentation (UPN4, UPN5, UPN6, UPN8, UPN9 and UPN10). Over-expression of *WT1* (cut-off \geq
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34 90 copies) was detected in 3/6 patients (UPN 4, UPN5 and UPN8).
35

36 37 *Patient follow-up*

38 A spontaneous reduction of eosinophils with normalization of total blood cell count was observed
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40 in two patients (UPN1 and UPN10). Two patients (UPN 4 and UPN 5) were treated with steroid
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42 therapy that resulted in a reduction of the eosinophil count within 15 and 20 days, respectively,
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44 from treatment initiation, while in another two patients reversion was achieved after the withdrawal
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46 of a neurological drug (Clobazam) and surgical correction of a congenital anomaly of the thoracic
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48 duct, respectively (UPN2 and UPN3, 7 and 2 months, respectively). B-cell clonality was detected at
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50 onset in patients UPN1 and UPN2 and converted spontaneously to a polyclonal profile in
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52 subsequent follow-up bone marrow aspirates.

53 Patient UPN6 started therapy with Imatinib (400 mg/day) and is now in continuous complete
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55 molecular and haematological remission with a follow-up of 36 months. Six months after diagnosis,
56
57 patient UPN7 developed a B- NHL showing the same monoclonal rearrangement IgH-CDRII
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59 (framework FR2) documented at the onset.

60 Patient UPN8 was treated for one year with steroids and α -interferon, achieving a decrease of
hypereosinophilia prior to development of B-lineage ALL. The patient was treated according to the

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3 AIEOP LAL 2006 protocol, eventually obtaining complete remission, which is currently maintained
4 at a follow-up of 24 months. Karyotype and T-cell configuration were normal and polyclonal,
5 respectively, in both UPN7 and UPN8. Patient UPN9, who showed monoclonality in the IgH-
6 CDRII region both at onset and in follow-up marrow samples without any evidence of a B-
7 lymphoid malignancy, is still under treatment with α -interferon because of skin and lung
8 involvement at onset. She had complete regression of organ involvement after a follow-up of 19
9 months.
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17 Discussion

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21 According to the scarce existing literature on pHES, most patients with persistent hypereosinophilia
22 do not show known molecular lesions at presentation. Hence, diagnostic refinement of the disease
23 remains difficult, and frequently malignancy of hypereosinophilia may be defined only after a
24 careful clinical and biologic follow-up (Blatt *et al.*, 1992; Jain *et al.*, 2000; Urtasun *et al.*, 2001;
25 Bernasconi, 2001; Hill *et al.*, 2003; Scheinfeld *et al.*, 2003; Wynn *et al.*, 2003; Chien *et al.*, 2004;
26 Horigome *et al.*, 2005; Rapanotti *et al.*, 2005; Rives *et al.*, 2005, D'Angelo *et al.*, 2008). Moreover,
27 to the best of our knowledge, no studies have systematically analysed TCR and IgH clonality in a
28 sizable number of pHES.
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35 In the present series, 6/10 patients with hypereosinophilia fulfilling the criteria of persistent (> 6
36 mos) and elevated (> $1.5 \times 10^9/L$) eosinophil count had molecular evidence of WBC clonality,
37 including monoclonal IgH rearrangement in five patients and *FIP1L1/PDGFR*A fusion in one. The
38 remaining four patients showed polyclonal B- and T-cell repertoires and had no detectable
39 cytogenetic and molecular aberrations. We note however that lesions other than the
40 *FIP1L1/PDGFR*A fusion have been reported in adults with hypereosinophilia. These may involve
41 *PDGFR*A, *PDGFR*B and *FGFR*1 through other fusions and point mutations (Klion *et al.*, 2006;
42 Teffery *et al.*, 2006; Bain *et al.*, 2009). Unfortunately, we were not able to search for these
43 alterations in the present series because of the lack of adequate material.
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51 B-cell clonality was the most frequently detected alteration in our pHES patients (50%). In
52 particular, in two cases (UPN7 and UPN8) a monoclonal rearrangement of the IgH-CDRII was
53 already detectable in the marrow at six and 12 months, respectively, prior to the development of a
54 B-cell lymphoproliferative disorder, clearly indicating clonal evolution. While the origin of the
55 neoplastic proliferation from the same clone detected at diagnosis is documented by the same IgH
56 rearrangement, it remains unclear whether a second undetected alteration might have occurred that
57 contributed to successive clonal expansion and overt lymphoproliferative disease. However, our
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3 results also suggest that detection of B-cell clonality at presentation does not invariably imply
4 subsequent development of a neoplastic lymphoproliferative disease, as shown by the two patients
5 with clonal IgH that did not show any progression in successive clinical and molecular evaluations.
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7 In an attempt to interpret these latter findings, we can hypothesize that B-cell clonality might,
8 in these patients,
9 remain under the control of the immune system and be extinguished after the
10 stimulus has ceased. Interestingly, as shown in UPN2, IgH clonality might accompany a clearly
11 reactive and transient pHES,
12 whereby a drug used for the underlying neurological disease was most
13 likely responsible for the prolonged hypereosinophilia. Precursor B-acute lymphoblastic
14 leukaemia/lymphoma with exaggerated eosinophilia is a rare entity, with less than 50 cases
15 including adult and paediatric populations reported since 1973, when it was first described by
16 Spitzer and Garson (Spitzer *et al.*, 1973; D'Angelo *et al.*, 2008). The most common cytogenetic
17 abnormality encountered in ALL with eosinophilia is the t(5;14), which is characterized by
18 overproduction of interleukin 3 (IL-3) (Meeker *et al.*, 1990). The latter entity is now included as "B
19 lymphoblastic leukaemia/lymphoma with t(5;14); IL-3-IgH" in the new WHO classification of
20 lymphoid neoplasms published in 2008 (Meeker *et al.*, 1990, Swerdlow *et al.*, 2008). In this case,
21 eosinophilia is secondary to overproduction of IL-3 by the lymphoblasts, resulting from activation
22 of the IL-3 gene on chromosome 5 after its translocation adjacent to the IgH gene on chromosome
23 14 (Meeker *et al.*, 1990).

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26 IgH rearrangement was present in five of our cases at the time of diagnosis of hypereosinophilia,
27 was associated with a normal karyotype and antedated the development of a B-cell disease by
28 several months in the two cases (UPN7 and UPN8) where this occurred.

29
30 In our study, we were not able to find a correlation between the level of hypereosinophilia at
31 presentation and its possible aetiology. In addition, even if the WHO classification does not include
32 WT1 analysis in the diagnostic screening of hypereosinophilic syndrome, a supplementary role of
33 this assay has recently been reported for the adult population (Cilloni *et al.*, 2007). With respect to
34 the question of whether WT1 expression in children is different from that in adults, we did not find
35 any correlation between WT1 expression levels and the differential diagnosis of HES, CEL, NOS or
36 reactive disease.

37
38 In conclusion, although though the study of a limited series, our results suggest that HES in children
39 has distinct biologic and clinical features compared with adult cases, indicating a different
40 underlying disease and molecular pathogenesis. In addition, we suggest that, besides the
41 identification of T-cell clonality, IgH rearrangement should be taken into consideration during the
42 diagnostic characterization of paediatric hypereosinophilia.

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Table 1. Patient characteristics and biological features of bone marrow at presentation and clinical evolution.

U P N	Sex/Age (years)	Hb (g/dL)	WBC 10 ³ /mm ³	Eos. count 10 ³ /mm ³	PLT count 10 ³ /mm ³	Eos. (%)	Signs of organ involvement at diagnosis	Karyotype	<i>BCR-ABL</i> and <i>FIP1L1/PDGFR</i> analysis	TCRβ clonality	<i>WT1</i> expression (cut-off < 90)	IgH clonality	Final diagnosis	Clinical evolution and treatment	IgH status at last follow up
1	M/2	10	63.2	45,496	248	80	Mild hepato- splenomegaly	46, XY	Negative	Polyclonal	N.A.	CDRII-IgH (FR2/JH)	Hypereosinophilia associated with IgH clonality	Normalization No treatment	Polyclonal
2	M/6	12.4	26	15,340	288	60	Absent	46, XY	Negative	Polyclonal	N.A.	CDRII-IgH (FR2/JH)	Hypereosinophilia associated with IgH clonality	Normalization Stop neurological drug/steroid α-interferon treatment	Polyclonal
3	M/10	12.7	4.5	1,512	274	30	Chronic cardiac tamponade and pleural effusion	46, XY	Negative	Polyclonal	N.A.	Polyclonal	Idiopathic HES	Normalization Surgical correction/steroid α-interferon treatment	Polyclonal
4	M/5	14.1	94.9	70,830	281	70	Absent	46, XY	Negative	Polyclonal	503	Polyclonal	Idiopathic HES	Normalization steroid α-interferon treatment	Polyclonal
5	F/15	12.8	13.9	6,950	130	80	Absent	46, XX	Negative	Polyclonal	240	Polyclonal	Idiopathic HES	Normalization steroid α-interferon treatment	Polyclonal
6	M/16	8.5	13.5	5,284	171	34	Lympho- adenomegaly splenomegaly, restrictive cardiomyopathy	46, XY	<i>PDGFR</i> / <i>FIP1L1</i> positive	Polyclonal	< 30	Polyclonal	MPN associated with <i>PDGFR</i> rearrang ement	Myeloid neoplasm Imatinib therapy ↓ Complete remission + 36 mos	Polyclonal
7	M/1.4	7.8	52.6	31,022	18	30	Hepato- splenomegaly, sinus tachycardia, asthmatic bronchitis	46, XY	Negative	Polyclonal	N.A.	CDRII-IgH (FR2/JH)	Hypereosinophilia associated with IgH clonality	NHL-B High-grade NHL treatment ↓ Complete remission + 60 mos	Polyclonal
8	F/9	12.8	19.6	10,100	401	35	Pancarditis with mitral valvular insufficiency	46, XX	Negative	Polyclonal	1876	CDRII-IgH (FR2/JH)	Hypereosinophilia associated with IgH clonality	ALL-B AIEOP2006 treatment ↓ Complete remission 24 mos	Polyclonal
9	F/7	10.3	8.31	4,603	834	40	Interstitial pneumonia	46, XX	Negative	Polyclonal	< 30	CDRII-IgH (FR2/JH)	Hypereosinophilia associated with IgH clonality	Persistent HES α-interferon therapy + 19 mos	CDRII-IgH (FR2/JH) α-interferon therapy
10	F/1	10.3	67.6	49,920	301	22	Hepatomegaly	46, XX	Negative	Polyclonal	< 30	Polyclonal	Idiopathic HES	Normalization No treatment	Polyclonal

N.A.: not available; MPN: Myeloid neoplasm with eosinophilia and PDGFR rearrangement