SCUOLA DI ALTA FORMAZIONE

PHD PROGRAM IN MEDICAL SCIENCES AND BIOTECHNOLOGY

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CYCLE: **XXIX**

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YEAR: 2013/2014

SCIENTIFIC BACKGROUND

Diamond-Blackfan Anaemia: Clinical Features

Diamond Blackfan Anaemia (DBA, OMIM 105650) is a rare inherited pure red cell aplasia that typically presents in the first year of life. It shows autosomal dominant transmission. Penetrance is incomplete and expressivity widely variable even in patients from the same family. Patients with DBA exhibit a macrocytic normochromic anaemia and reticulocytopenia [1]. Elevated fetal haemoglobin levels (HbF) and elevated erythrocyte adenosine deaminase (eADA) activity are important supporting features associated with DBA. Moreover, erythroid progenitors (BFU-E and CFU-E) in the patients' bone marrow (BM) show a pro-apoptotic phenotype and their number is reduced [2].

Like other inherited bone marrow failure syndromes, DBA is associated with congenital abnormalities such as craniofacial, thumb, kidney and heart malformations and growth retardation in approximately 40% of patients [3]. The risk of solid tumors, MDS, or leukemia is elevated in DBA. The cumulative incidence of malignancy is approximately 20% by age 46 years [4].

Corticosteroids are the first choice therapy in DBA even though the mechanism whereby they ameliorate anaemia remains unclear. The subset of DBA patients who do not respond to corticosteroids or require high doses with unacceptable toxicities receives chronic red cell transfusions. Transfusional iron overload is a major cause of morbidity and mortality in DBA. The only definitive treatment for the haematological manifestations of DBA is haematopoietic stem cell transplantation (HSCT) [5].

During the course of the disease, approximately 17% of all DBA patients enter spontaneous or drug-induced remission, defined as a state of therapy independence for at least six months with physiologically acceptable haemoglobin levels. The mechanism behind remission remains unknown and about 15% of those who enter remission relapse [6].

Diamond Blackfan Anemia: Genetics

DBA is considered as the prototype of ribosomopathies. Heterozygous mutations or single copy deletions in 13 genes that encode proteins of the 40S (*RPS7, RPS10, RPS17, RPS19, RPS24, RPS26*, *RPS29*) or 60S (*RPL5, RPL11, RPL15, RPL26*, *RPL35A, RPL31*) ribosomal subunits have been identified in about 65% of patients [7, 8]. Ribosomal gene mutations may be inherited with an autosomal dominant pattern or may arised de novo. Recently, mutations in the X-linked transcription factor GATA1, which is essential for erythropoiesis, have also been found in patients with DBA [9, 10].

Haploinsufficiency or reduced expression of a ribosomal protein results in decreased levels of the corresponding ribosomal subunit and in the defective processing of the ribosomal RNA precursors (pre-rRNA) [11]. The erythroid progenitors show reduced proliferation and undergo p53-dependent or indipendent apoptosis [12, 13].

How a defect in ribosome biogenesis specifically affects only erythropoiesis is still under study. The specific red cell aplasia may be attributed to hypersensitivity of erythroid progenitors to increased p53 levels, or to the high protein synthesis requirement of rapidly dividing erythroblasts [14]. However, other mechanisms, such as defective translation of specific transcripts, and splicing defects that affect erythroid specific genes were also reported [12]. The reduced expression of GATA1 by DBA cell models provides a possible link between ribosome defect and erythropoiesis failure [15].

DBA diagnosis

Diagnosis of DBA is hampered by the overlapping clinical presentations with other BM failure syndromes such as Fanconi Anaemia (FA), Shwachman-Diamond syndrome (SDS) and Dyskeratosis Congenita (DC) [14]. FA is excluded from diagnosis by negative results in a chromosome breakage assay while the absence of telomere shortening rules out DC. The reduced number of BFU-E and CFU-E after 15 days of BM cells culture directs to a DBA diagnosis.

The absence of unique diagnostic feature for DBA often makes DBA a diagnosis of exclusion. While the identification of the underlying molecular basis for DBA in many patients has now made diagnosis possible through genetic testing, the genes affected in approximately 35% of suspected DBA patients remain unknown leaving a degree of diagnostic uncertainty for these patients.

Further confounding a diagnosis of DBA is the increased identification through genetic testing of patients with non-classical forms of DBA including patients with malformations without anaemia or with anaemia presenting as an adult. Recently, we have proposed a rapid and convenient assay readily available in diagnostic laboratories where functional defects in ribosome synthesis linked to haploinsufficiency for large subunit ribosomal proteins could be used as a criterion for making a DBA diagnosis [6]. This approach is currently limited to large subunit ribosomal proteins and would only be supportive by exclusion for DBA caused by defects in non-ribosomal protein genes. As a consequence, a new strategy needs to be developed to support DBA diagnosis.

Extracellular vesicles

Extracellular vescicles (EVs) are membrane-bound organelles released by various cell types. Their membrane displays typical markers of the parental cell of origin. Microvesicles (MV) have a diameter of 50-1000nm and they have not an endosomal origin. The outer layer of MV membrane has been often shown to display phosphatidylserine (PS), but this may depend on the cell type from which MVs derive or on the functional cell state [16]. Apoptotic bodies (ABs) are 1-5 μm in diameter. They are released as blebs by cells undergoing apoptosis and they are PS positive. Classification of EVs, their isolation protocol and detection, molecular details of their release, clearance and biological function are still under intense investigation [17, 18].

MVs play a pivotal role in important biological processes such as membrane traffic and horizontal transfer of proteins and nucleic acids among neighboring cells [19]. With regard to the erythroid compartment, it is well known that mature red blood cells shed EVs during eryptosis (a form of erythroid cellular stress) and that reticulocytes eliminate the nucleus and other cellular compartments through vesiculation [20]. No data are available on EV production from erythroid progenitors or early precursors. Interestengly, the role of EVs derived from plasma has been developed as biomarkers for diagnostic or prognostic applications.

Search for new DBA genes

While the majority of sequencing studies focusing on all the known structural components of the ribosome identified novel DBA genes, these studies also suggested that approximately 35% of patients with a clinical diagnosis of DBA do not have an identifiable RP gene abnormality [6].

Recently, Dr. Farrar's group (University of Arkansas for Medical Sciences, Little Rock, AR) analysed three DBA American families by whole exome sequencing and identified three candidate DBA genes: *MCM2*, *FLNB,* and *SEMA7A.* Homozygous or compound heterozygous mutations in one of these three genes were found in each family proband. These three families showed an autosomal recessive inheritance. Our collaborators demonstrated a novel role in erythropoiesis of

these genes. In particular the knockdown of *MCM2* in CD34+ cells inhibits erythroid colony formation [21].

Subsequently, heterozygous very rare single nucleotide variations (SNVs) (allele frequencies of ~2/1000) in *MCM2* were found in 2 out of 40 sporadic patients. These variants were predicted to be deleterious suggesting the role of *MCM2* in DBA. Interestingly, we demonstrated a protein-protein interaction between MCM2 and RPS19 linking MCM2 directly to the ribosome [22]. We also reported that the RPS19 mutant reduced its interaction with MCM2 [23].

RATIONAL OF THE PROJECT

The aim of my PhD project was to improve DBA diagnosis working on two different approaches: the development of a new diagnostic assay based on the study of EVs and the screening of new candidate DBA genes.

AIM 1: Immunophenotypic profiling of erythroid progenitor-derived extracellular vesicles in DBA

As an alternative strategy for developing a more inclusive assay for possible use in DBA diagnosis we turned to the study of EVs whose presence may be altered as a consequence of increased apoptosis associated with many bone marrow failures and whose characteristic molecular properties may specifically define the nature of the bone marrow failure. In this part of my project we focused on the immunophenotypic characterisation of erythroid EVs from plasma of three different groups of individuals: DBA patients, patients with other red blood cell (RBC) diseases, and healthy controls. We reasoned that erythroid EVs may vary in the peripheral blood of DBA patients as a consequence of the loss of erythroid progenitor cells in the bone marrow of these patients.

We chose three markers to characterise EVs derived from cells of the erythroid lineage: CD34, CD71 and CD235a. CD34 is the main haematopoietic stem cell marker. CD71, is transferrin receptor 1, which is essential for iron uptake and consequently for haemoglobin synthesis during erythroid differentiation (late progenitors, i.e. CFU-E and early precursors, i.e proerythroblast, basophilic erythroblast, polychromatophilic erythroblast and orthochromatic erythroblast). Finally, CD235a, i.e. glycophorin A, is the erythroid specific marker and it is expressed by erythroid precursors, i.e proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatic erythroblast, reticulocytes and erythrocytes (Figure 1) [24].

AIM 2: Mutation detection in new candidate DBA genes

The only way to confirm a clinical diagnosis of DBA is mutation detection. The main purpose of this part of my PhD project was to reduce the percentage of patients who remain as yet genetically

undefined. Consequently, in collaboration with Dr. Farrar's group we decided to sequence *MCM2* in the subset of Italian DBA patients who have already been screened for 6 RP genes and *GATA1*, without the detection of mutations or single copy deletions. *MCM2* (3q21) consists of 16 exons coding for a protein involved in the pre-replication complex. We selected a cohort of about 50 patients from the Italian DBA registry with the aim to amplify the power of detecting addional cases. *MCM2* was screened by Sanger sequencing.

Subsequently we plan to sequence *SEMA7A* in order to confirm or not its role with DBA.

METHODS

METHODS AIM 1

Patients and controls

Peripheral blood samples were collected from DBA patients (*n=8*) (Table 1), patients with other RBC diseases (hereafter named non-DBA patients) (*n=10*) (Table 2) and healthy controls (*n=22*). For transfusion dependent patients, peripheral blood samples were collected after 2-5 weeks from the last transfusion, depending on their anaemia severity.

EV isolation

Blood samples were collected into 3.2% sodium citrate tubes. Platelet free plasma (PFP) was obtained by a centrifugation at 2,400*g* for 10 minutes at room temperature. PFP was then centrifuged at 1,800*g* for 30 minutes at 4°C. Supernatant was subjected to ultracentrifugation at 100,000*g* for 60 minutes at 4°C (Optima^{TM} LE-80K, Beckman Coulter; rotor SW60Ti, Beckman Coulter). Pellets containing isolated EVs were suspended in 1 mL of PBS (filtered using a 0.22 µm pore size membrane) and stored at 4°C.

EV immunophenotypic profiles

100 µL of isolated EVs were incubated for 15 minutes at 4°C in the dark with the following combinations of antibodies: 1) anti-IgG2A-FITC/IgG1-PE (isotypic control); 2) anti-CD71-FITC and anti-CD34-PE (this mixture was expected to identify vesicles from CFU-E to ortochromatic erythroblast); 3) anti-CD71-FITC, anti-CD34-PerCP and anti-CD235a-PE (this mixture was expected to identify vesicles from late progenitors to mature erythrocytes). After a washing step with filtered PBS, EVs were resuspended in 400 µL of Annexin-V buffer and 2.5 µL of Annexin-V were added to the mixes 2 and 3. All reagents for the immunophenotypic analysis were purchased from BD. A FACSCanto II flow cytometer (BD) with FacsDiva software (BD) was used for data acquisition. Standard size micro beads of 1 and 2 µm (Flow Cytometry Size Calibration Kit,

Invitrogen) were used to calibrate the instrument. The EV dimensional gate was set to analyze events between 500 nm and 1000 nm (Figure 3, upper left panel).

The relative amount of EVs per samples was determined using the TruCount^{TM} tubes (BD), according to the formula: (number of events in specific gate/ number of TruCount events) * (number of TruCount beads per test/ test volume)* dilution factor. Statistical analyses were performed using Mann-Whitney and Kruskal-Wallis tests. The potential diagnostic value of the assay was evaluated with the assessment of Receiver Operation Curve (ROC). An AUC of 1 represents an excellent test; an AUC of 0.5 represents a test that fails to discriminate between the two groups under study. A rough guide for classifying the accuracy of a diagnostic test is the traditional academic point system: 90-100% = excellent, 80-90% = good, 70-80% = fair, 60-70% = poor, 50-60% = fail [25].

METHODS AIM 2

Genomic DNA was isolated from peripheral blood with standard procedure and analysed for *MCM2*. All the 16 *MCM2* exons were screened for mutations in our cohort of patients using standard PCR-based Sanger sequencing. Coding sequences and exon-intron boundaries were PCR amplified using Go Taq® Flexi DNA Polymerase (Promega). The same set of primers was used for both PCR amplification from genomic DNAs and for Sanger sequencing (IGA Technology Services). Primer sequences are shown in Table 4.

RESULTS

RESULTS AIM 1

The aim of this study was to evaluate if EV populations in the peripheral blood of DBA patients can be leveraged as a potential diagnostic tool. We studied EVs by flow cytometry, which provides information both on vesicle dimensions and immunophenotypic properties. Vesicles in the size range of 500nm to 1000nm (EV dimensional gate) were used in this study (Figure 3, upper left panel). This size range, due to the instrument sensitivity limit, includes a heterogeneous population of EVs including both microvesicles and relatively small apoptotic bodies.

The markers we used were chosen to identify EVs derived from early progenitors including BFU-E (CD34), CFU-E (CD34, CD71), erythroid precursors (CD71, CD235a) and cells in the late stage of erythroid maturation, i.e reticulocytes and erythrocytes (CD235a) (Figure 1). An isotypic control was used to set positive and negative discrimination limits of fluorescence (data not shown).

Figure 2 shows EVs from healthy controls grouped into the following categories: EVs shed from

late erythroid progenitors (CD34+/CD71$_{low}$/CD235a+/PS+), erythroid precursors (CD71+/CD34-/CD235a$_{low}$/PS-), and cells of late erythroid stages, e.g. reticulocytes and erythrocytes (CD71-/CD235a+/CD34-. About 60% of this population was PS+). Since the anti-CD235a-PE was prone to aggregation we evaluated whether the same EV populations could be defined without using this antibody. Comparison of experiments with and without anti-CD235a antibody showed that CD71+/CD34-/PS- and CD34+/CD71$_{low}$/PS+ populations corresponded to the CD71+/CD34-/CD235a$_{low}$/PS- and CD34+/CD71$_{low}$/CD235a+/PS+ populations, respectively (vesicles obtained using or not the antibody against CD235, were counted and their numbers corresponded) (Figure 3, Table 3).

When we tested DBA patients, only the CD34+/CD71$_{low}$/PS+ population (hereafter named CD34+/CD71$_{low}$) was significantly different when compared to other groups. This population, representing late erythroid progenitors was substantially reduced in 7/8 DBA patients relative to healthy controls. Among patients with other red blood cell disorders, 9/10 patients showed a proportion of EVs derived from late erythroid progenitors similar to controls (Figure 3). As shown in Figure 4A, the median difference between DBA patients and all the other individuals (non-DBA patients + healthy controls) was statistically significant ($p<0.05$, Mann-Whitney Test) as was the difference between DBA patients and each of the other groups when compared separately ($p<0.05$, Kruskal-Wallis Test; Figure 3B). As expected, the difference of medians comparing non-DBA patients *vs* healthy controls was not statistically significant (Figure 4B).

The potential diagnostic value of the analysis of CD34+/CD71$_{low}$ EVs was assessed using the ROC curve test. The area under the ROC curve (AUC) that compares DBA patients *vs* healthy controls was 0.97 (= excellent) while the AUC of DBA patients *vs* all the other individuals was 0.95 (= excellent). Moreover, comparison between DBA patients and non-DBA patients produced an AUC of 0.90 (= excellent). In all comparisons the cluster was able to distinguish the groups under study with a p-value< 0.01. As expected the AUC that plotted non-DBA patients and healthy controls was 0.64 (= fair), consequently this assay cannot discriminate between these groups (Figure 5).

AUC values suggest that this test has an excellent accuracy to discriminate DBA patients from healthy controls, and/or non-DBA patients.

The CD34+/CD71$_{low}$ EV population is shed from the CFU-E progenitors and our results are in agreement with the low levels of CFU-E found in DBA patients (Figure 1, Table 1).

Actually, this population seems to reflect more the bone marrow CFU-E numbers than the level of anaemia or the disease type. According to this hypothesis, the only DBA patient (#7), who did not show this population, had normal erythropoiesis at the time of this analysis. This patient presented with classical DBA in childhood and is now in complete haematological remission (Hb, 11.7 g/dL).

On the other hand, 9 out of 10 patients with other RBC diseases were characterised by a

hypercellular bone-marrow at the time of the analysis and clustered in the healthy control range. Among them five patients had congenital anaemias characterised by erythroid cell loss at a stage later that CFU-E (i.e. HbS, spherocytosis, CDA II and Beta thalassemia), and four patients had acquired conditions (i.e. iron deficiency anaemia, acquired erythrocytosis and aplastic anaemia). Patient 26, who did not show the CD34+/$CD71_{low}$ population, is affected by an iron refractory anaemia with severe microcytosis. We expect that also this patient has a defect of erythroid progenitors, but a BM evaluation has not been performed so far.

These data overall suggest that the EV assay we devised may be useful to improve DBA diagnosis as a quicker and less invasive alternative to CFU-E cultures. It should be noted that this assay is performed from peripheral blood, is amenable to transfused patients and requires two working days, whereas CFU-E cultures require 15 working days and need to be performed using a bone marrow sample collected usually from very young patients.

This work has been recently submitted to PLOS ONE journal.

RESULTS AIM 2

Until now, I sequenced the *MCM2* gene in 17 DBA patients among the 50 presently available. None of them showed mutations in any of the 16 exons of MCM2. Taking into account that the expected SNV frequence in the American cohort is very low, this result was not surprising. Currently, I am sequencing the others DBA patients included in our selected cohort.

FIGURES

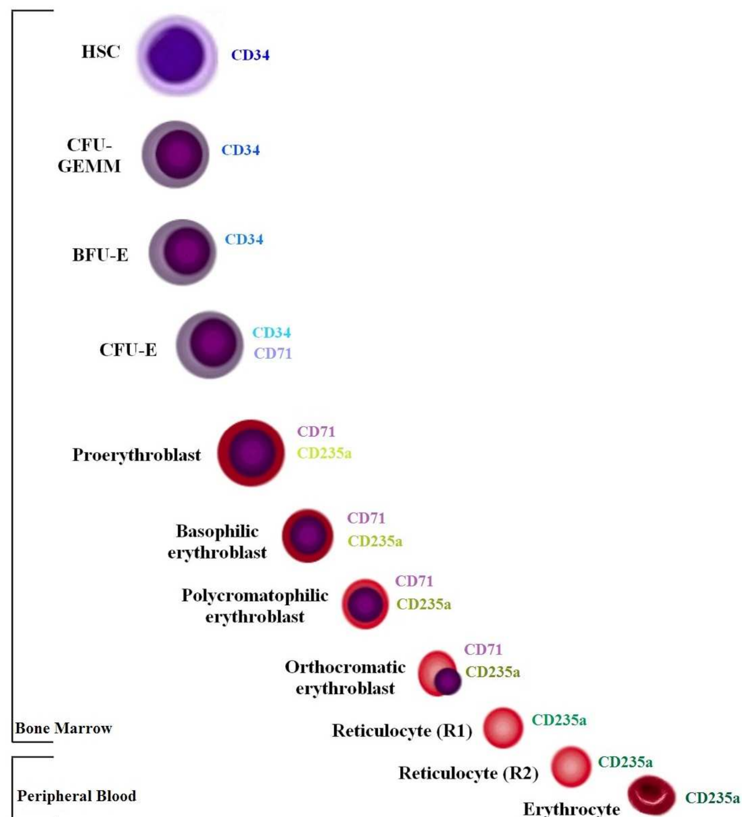

Figure 1: Simplified scheme of erythropoiesis. The most representative markers expressed during erythroid maturation steps are indicated. The intensity colour variation corresponds to expression levels.

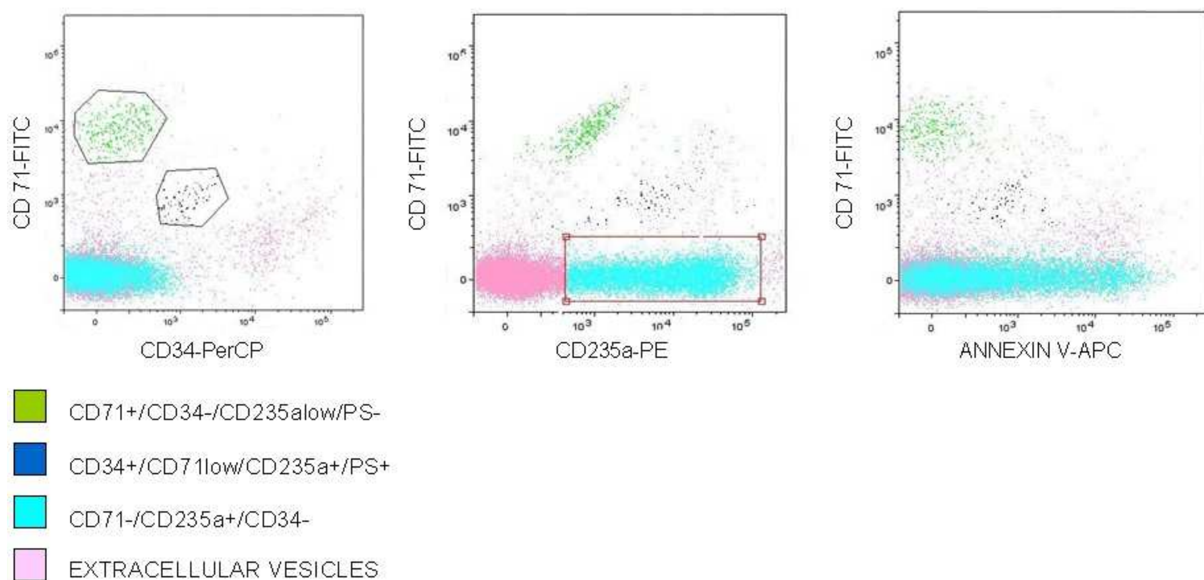

Figure 2: EV distribution in dot plot graphs obtained from the acquisition of the samples incubated with anti-CD71-FITC, anti-CD34-PerCP, anti-CD235a-PE and Annexin V-APC. Only the events occuring in the EV dimensional gate were included. Three EV clusters were identified and indicated with different colours.

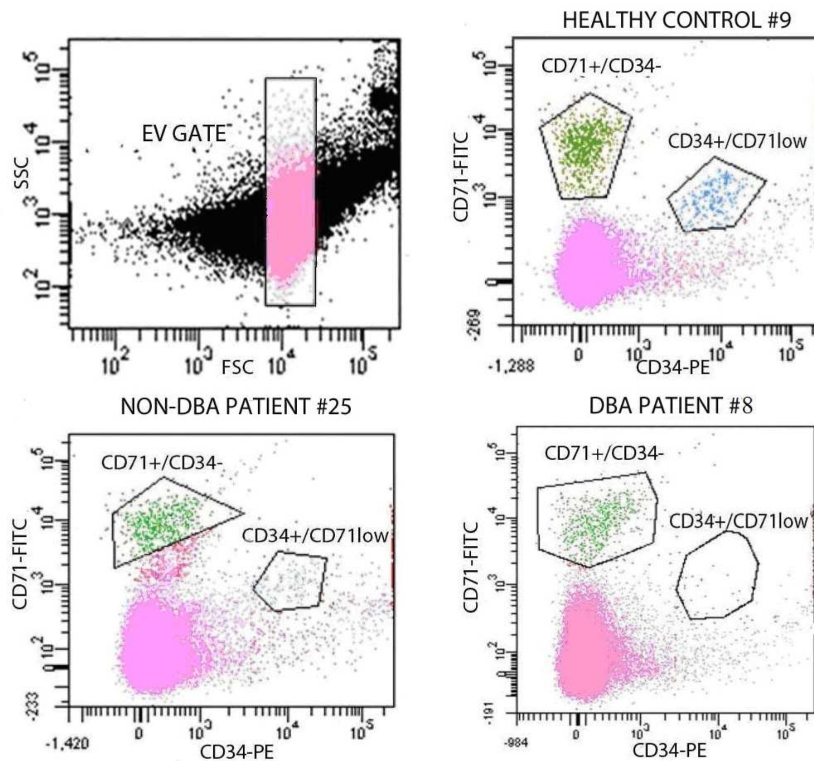

Figure 3: EV distribution in a dot plot graph comparing CD71 and CD34 markers of the events occuring in the EV dimensional gate. The dot plots were obtained from the acquisition of the samples not incubated with the anti-CD235a-PE. CD71+/CD34- (corresponding to CD71+/CD34-/CD235a$_{low}$/PS-) and CD34+/CD71$_{low}$ (corresponding to CD34+/CD71$_{low}$/CD235a+/PS+) populations are shown in representative individuals from the groups of healthy controls, DBA patients and non-DBA patients.

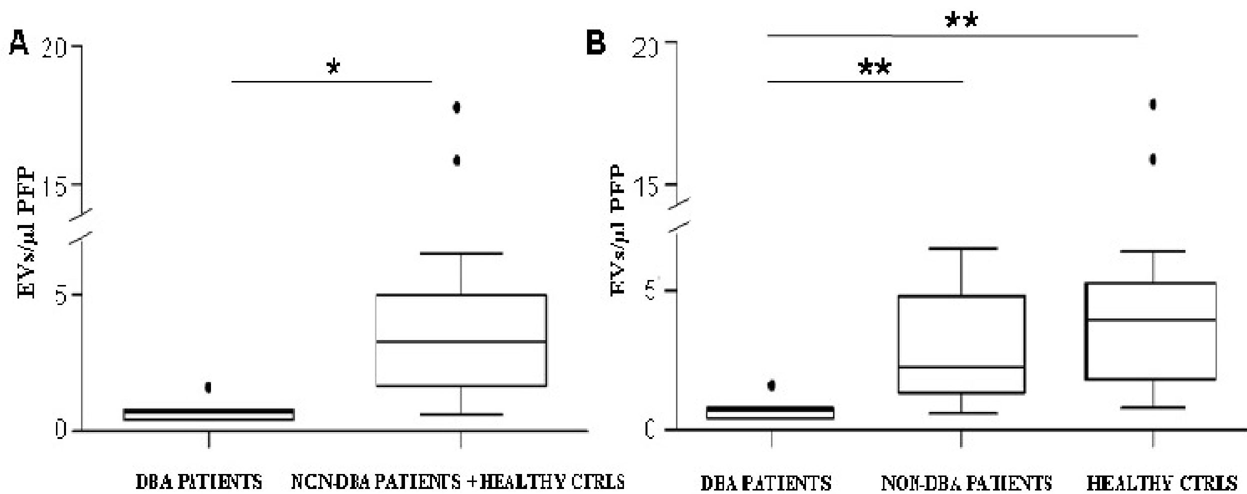

Figure 4: Box plot of Absolute number of events included in the CD34+/CD71$_{low}$ gate. Outliers are shown in black spots. **(A)** Comparison between DBA patients and all the other individuals (non-DBA patients + healthy controls) *The difference of medians is statistically significant (p<0.05, Mann-Whitney test). **(B)** Comparison between DBA patients, non-DBA patients and healthy controls. **The difference of medians is statistically significant (p<0.05, Kruskall-Wallis te

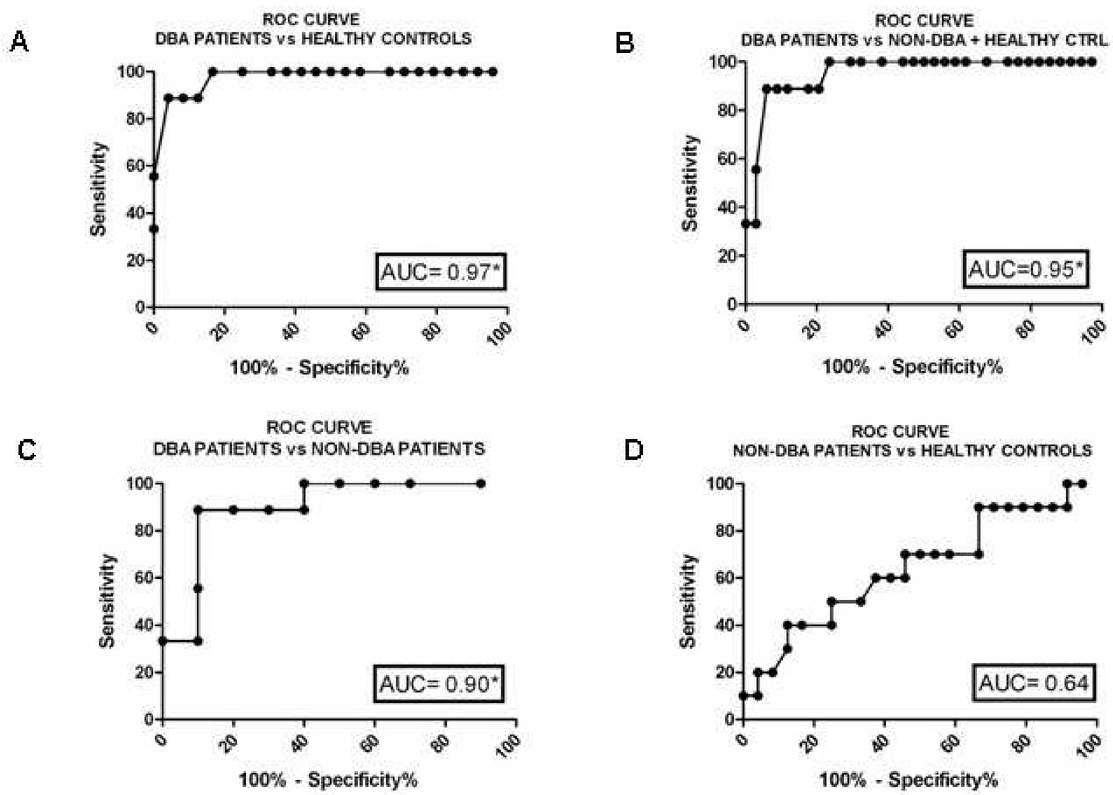

Figure 5: ROC curves evaluating the accuracy (AUC) of the CD34+/CD71$_{low}$ analysis in the discrimination of (**A)** DBA patients *vs* healthy controls (**B)** DBA patients *vs* all the others (healthy controls + non-DBA patients) (**C)** DBA patients *vs* non-DBA patients (**D)** non-DBA patients *vs* healthy controls. * p-value <0.01.

TABLES

DBA PATIENTS	SEX	AGE	TREATMENT	Hb (g/dL)	MCV (fL)	RBC (10^6/uL)	Reticulocytes (10^9/L)	BM	BM BFU-E BASAL	BM BFU-E WITH SCF	PB BFU-E BASAL	PB BFU-E WITH SCF
1	M	24	TRANSFUSION	8.0	88.8	3.04	13.5	ERYTHROID APLASIA	0	46	0	1
2	F	43	STEROIDS	10.7	89.8	3.54	N/D	ERYTHROID HYPOPLASIA	N/D	N/D	1	7
3	M	7	TRANSFUSION	6.8; 6.8**	80.9; 70.8**	2.34; 2.39**	2.8; 5.1**	ERYTHROID HYPOPLASIA	23	26	1	8
4	M	5	TRANSFUSION	9.5	79.8	3.20	10.7	ERYTHROID APLASIA	0	0	N/D	N/D
5	F	27	NONE*	10.9	83.8	3.78	45.8	ERYTHROID APLASIA	0	7	N/D	N/D
6	F	4	TRANSFUSION	9.6	82.1	3.36	6	ERYTHROID APLASIA	0; 5; 20†	2; 74; 46†	0; 0▲	0; 2▲
7	M	37	NONE*	11.7	106.8	3.27	35.7	ERYTHROID HYPOPLASIA	7	41	N/D	N/D
8	F	5	TRANSFUSION	6.2	81.8	2.12	11.6	N/D	N/D	N/D	N/D	N/D

Table 1: Clinical characteristics of DBA patients (*patient in clinical remission; **two analysis performed in two independent samples; †three different analysis a few years apart; ▲two different analysis a few years apart). The ranges of PB BFU-E BASAL and BM BFU-E BASAL of healthy individuals are 16.0+/-8.0 and 57.0+/-28, respectively.

NON-DBA PATIENTS	SEX	AGE	TREATMENT	Hb (g/dL)	MCV (fL)	RBC (10^6/uL)	Reticulocytes (10^9/L)	DISEASE
23	F	7	NONE	13.3	67.6	5.74	44.8	Heterozygous for Beta Thalassemia
24	M	4	IRON	12.0	80.5	4.16	69.9	Iron deficiency anaemia
25	M	1	NONE	12.2	70.6	4.96	55.7	Heterozygosis for Hb S
26	M	5	NONE	9.0	62.3	4.45	47	Iron refractory anaemia
27	M	9	TRANSFUSION	9.9	88.0	2.98	33.5	Congenital dyserythropoietic anemia type II (CDA II)
28	M	12	NONE	14.4	85.6	4.69	110.5	Spherocytosis (Splenectomized)
29	M	20	NONE	17.5	88.9	5.26	80.4	Acquired erytrocytosis
30	M	4	NONE	10.8	67.8	4.85	118.1	Iron deficiency anaemia
31	F	7 months	NONE	8.2	76.3	3.19	64.9	Homozygous for Beta thalassemia
32	M	15	CYCLOSPORIN, ATGT, STEROIDS	6.7	92.0	2.09	28.9	Aplastic anaemia

Table 2: Clinical characteristics of Non-DBA Patients (ATGT: Anti Thymocyte Globulin Treatment).

HEALTHY CONTROLS	CD34+/CD71low
1	0,8
2	2,6
3	17,8
4	5,9
5	1,6
6	2,2
7	4,9
8	1,7
9	5,2
10	2,2
11	3,8
12	1,3
13A	6,2
13B	1,4
14	4,2
15A	15,9
15B	2,9
16	4,5
17	6,4
18	2,4
19	5,3
20	1,7
21	4,1
22	4,5
NON-DBA PATIENTS	**CD34+/CD71low**
23	6,5
24	4,8
25	1,2
26	0,6
27	3,6
28	1,4
29	2,1
30	1,5
31	2,4
32	4,8
DBA PATIENTS	**CD34+/CD71low**
1A	0,7
1B	0,4
2	0,7
3	0,8
4	0,8
5	0,4
6	0,4
7	1,6
8	0,8

Table 3: Absolute number of CD34+/CD71$_{low}$ population in the three groups analyzed. (*A, B indicate the analysis performed in two independent samples on the same subject).

EXON 1	**F**:CTCCGTGTCCCTTCTGGTC; **R**:CCAGGTCAGTGGTAGGTAGGC
EXON 2	**F**:AGAGAAAGAAGGGAAGGCCC; **R**:AATGCCATCGATTCATCTGC
EXON 3	**F**:GAACCTTAGGGAACAGGCCC; **R**:AAGAGACACCTGCTACTGCGG
EXON 4	**F**:GAAGCTGGGAAGGAGTCTGG; **R**:TCACGTGTTCACTCGGCTC
EXON 5	**F**:GGTAGGCCTTGCTTCTCACAC; **R**:AATCGCTCTTCAGGAATCACG
EXON 6	**F**:GTGTGTTGGGACACTCTCGTC; **R**:CACACTGGTCTTCCACAGCC
EXON 7	**F**:ATTCCTTGCTGTCTTTGGCTC; **R**:AAACAGATCCTAGCCCGAGTG
EXON 8	**F**:GTGTGGAGCACTTGCAATAGG; **R**:TAAGGCAAGGTTTGAGCAGTG
EXON 9	**F**:CTGTGGAAGTGGGTGTCTTTG; **R**:CCTTGAAGAATGAGTCACGCC
EXON 10	**F**:AGGGACTGTGCCTTACCATTC; **R**:CATTAACACCATTCCCGTCG
EXON 11	**F**:CGACGGGAATGGTGTTAATG; **R**:CTGCTGCACAATGACTTCCTC
EXON 12	**F**:ATGTCTTCCTCTTCCACTGCC; **R**:GGACATGAGAAGAGACCGAGG
EXON 13	**F**:CTTCTCCAGCCACTGACCTC; **R**:TTTAATGCCTCCCTGCTCTG
EXON 14	**F**:AGATGGGTCTTCTTGGCTCTG; **R**:CCACACTCCCTTTACCCTACG
EXON 15	**F**:GTGCTGGCACGTAGGGTAAAG; **R**:TAAATGGCTGTCAACCAAGGC
EXON 16	**F**:GGTGATGGTGTCTGAAAGTGC; **R**:AGCGGAAGTCCAGGTTACTTAC

Table 4: Primer sequences used for PCR amplification and Sanger sequencing. (**F**: Forward primer; **R**: Reverse primer)

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MEETINGS

Simposio: Il ruolo emergente delle vescicole extracellulari in fisiopatologia: da mediatori cellulari biomarker. Dipartimento di Scienze della Salute, Novara, 12/05/2014.

ESHG (European Society of Human Genetic) CONFERENCE: Milan 31/05/2014 – 03/06/2014

CONGRESSO ANEMIA DIAMOND BLACKFAN (ISTITUTO PIEMONTESE PER LA RICERCA ANEMIA DIAMOND-BLACKFAN): Peschiera del Garda 10/10/2014- 12/10/2014

ORAL COMUNICATIONS/ POSTER PRESENTATION

Poster presentation: Immunophenotypic profile of erythroid extracellular vesicles obtained from peripheral blood of patients with Diamond-Blackfan Anaemia (Simposio and ESHG CONFERENCE).

Oral comunications: NUOVI SVILUPPI DI DIAGNOSI: LE VESCICOLE EXTRACELLULARI NELLA DBA (CONGRESSO ANEMIA DI DIAMOND-BLACKFAN).

SEMINARS

TITLE	DATE	SPEAKER	AFFILIATIONS
Epigenetic modifications that controls stem cell differentiation	19/02/14	Salvatore Oliviero	Department of Life Sciences and System Biology & HuGeF University of Torino
Role of Phosphoinositides-3-kinase C2-alpha, a Class II PI 3-kinase, in development and cancer"	19/03/14	Emilio Hirsch	Dip. Biologia Molecolare e Cellulare e Genetica Molecolare,Universita' di Torino
Simposio: Il ruolo emergente delle vescicole extracellulari in fisiopatologia: da mediatori cellulari a biomarker	12/05/14		Dipartimento di Scienze della Salute, Università del Piemonte Orientale
Methods for the analysis of the exposure-time-response relationship in epidemiology”	23/05/14	Francesco Barone-Adesi	Division of Population Health Sciences and Education,St George's, University of London
"Assessment of cervical cancer control in Rwanda and Buthan"	09/06/14	Iacopo Baussano	Dipartimento di Scienze della Salute, Università del Piemonte Orientale
Ribosome alteration in cancer: effect or cause?	11/06/14	Fabrizio Loreni	Dipartimento di Biologia Università Tor Vergata, Roma
Metformin rewires the signaling network of breast cancer cells and changes their sensitivity to growth and apoptotic stimuli”	12/06/14	Gianni Cesareni	Dipartimento di Biologia Università Tor Vergata, Roma
"A functional link between ARX and KDM5C genes linked to neurophenotypes defines a crucial epigenetic disease path"	21/06/14	Dott. Miano	Institute of Genetics and Biophysics ABT CNR - Napol

SESSIONS

TITLE	SPEAKER	DATE	AFFILIATIONS

The C-value paradox, junk DNA and ENCODE	Dott. Diego Cotella	30/06/14	Dipartimento di Scienze della Salute, Università del Piemonte Orientale
Gene Therapy application	Prof. A. Follenzi	19/06/14; 15/07/14	Dipartimento di Scienze della Salute, Università del Piemonte Orientale
The Borghese Sessions"	Prof. S.R. Ellis	8-22 September 2014	Department of Biochemistry, University of Louisville, Kentucky