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INTRODUCTION

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 with an incidence of 6-7 newborns per million live births.. 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 arise 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]. 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), Dyskeratosis Congenita (DC) and Transient Erythroblastopenia of Childhood (TEC) [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. Interestingly, EVs derived from plasma have been used 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].

More than 200 extra-ribosomal factors are required to synthesize ribosomes and may be involved in DBA pathogenesis. In particular, *TSR2*, which encodes a direct binding partner of *RPS26*, was characterized as a new DBA gene supporting the involvement of non-RP genes in the disease [21]. Moreover, six recent works by different groups identified *WBSCR22* as a key player in the biogenesis of 40S ribosomal subunit. The human WBSCR22 gene is located in Williams-Beuren Syndrome (WBS) critical region in chromosome 7q11, 23. WBS is a multisystem developmental

disorder associated with hemizygous deletion of a ~1.6 Mb region in the given locus. The WBS region contains more than 25 genes and the deletion of this region results in haploinsufficiency of WBS control region transcripts. Knock-down of WBSCR22 in human cells results in accumulation of pre-rRNA 18SE and a reduced level of free 40S ribosomal subunit [22].

Recently, Dr. Farrar's group (University of Arkansas for Medical Sciences, Little Rock, AR) studied three DBA American families that showed autosomal recessive inheritance. These families were studied by whole exome sequencing and two candidate DBA genes were identified: *MCM2*, and *FLNB*. Homozygous or compound heterozygous mutations in one of these genes were found in each family proband. Farrar demonstrated that these genes have a novel role in erythropoiesis. In particular the knockdown of *MCM2* in CD34+ cells inhibits erythroid colony formation [23].

Subsequently, heterozygous single nucleotide variations (SNVs) in *MCM2* were found in 2 out of 40 sporadic patients with DBA. These variants were predicted to be deleterious suggesting a role of *MCM2* in DBA. Interestingly, we demonstrated a protein-protein interaction between MCM2 and RPS19, linking MCM2 directly to the ribosome [24]. We also reported that two RPS19 mutants showed a reduced interaction with MCM2 [25].

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) [26].

AIM 2: Mutation detection in new candidate DBA genes

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 and *MCM2* was screened by Sanger sequencing.

Since analysis of rRNA processing by Northern Blot showed the accumulation of pre-rRNA 18SE in a single patient with an unknown mutation, we sequenced WBSCR22 in this patient. The human *WBSCR22* gene is located in Williams-Beuren Syndrome (WBS) critical region in chromosome 7q11,23 and consists of 13 exons. *WBSCR22* was screened by Sanger sequencing.

METHODS

METHODS AIM 1

Patients and controls

Peripheral blood samples were collected from DBA patients (*n=13*) (Table 1), patients with other RBC diseases (hereafter named non-DBA patients) (*n=16*) (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™ 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 Becton Dickinson (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. We have set the EV dimensional gate to analyse events between 500 nm and 1000 nm. Gate positioning was performed evaluating the median corresponding to 1000nm microbeads and exploiting the direct proportionality between the scattered light and the dimension (Figure 2, upper left panel; Figure S2).

The relative amount of EVs per samples was determined using the TruCount™ 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 [27].

METHODS AIM 2

Genomic DNA was isolated from peripheral blood with standard procedure and analysed for *MCM2* and *WBSCR22*. All the 16 *MCM2* and the 13 *WBSCR22* 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 AND DISCUSSION

RESULTS AND DISCUSSION AIM 1

During the first year of my PhD program I analysed 8 patients with DBA, 10 patients with other haematological diseases and 22 healthy controls. After the submission of this work to PLOSONE journal, reviewers asked us to increase the number of DBA and non-DBA patients in order to further validate our results. In this second year I collected 5 DBA patients and 6 non-DBA patients. The number of new patients is not large, but it is important to note that we are dealing with very rare diseases (the frequency of DBA is 5-7 newborns per million live births), so each pediatric haematology centre follows-up a very low number of patients. Moreover we increased the number of non-DBA haematological conditions, i.e. one patient with Fanconi Anaemia, one with CDAII, one with lymphadenitis, one with thrombocytopenia, one with ALPS.

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 the gates of interest (Figure S1).

Figure S1 shows EVs from healthy controls grouped into the following categories: EVs shed from late erythroid progenitors (CD34+/CD71$_{low}$/CD235a$_{low}$/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+). 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$_{low}$/PS+ populations, respectively (vesicles obtained using or not the antibody against CD235, were counted and their numbers corresponded) (Figure 2, Table 3). Consequently, we reasoned that the mixture without CD235a was more suitable for diagnostic purposes because it was not influenced by these technical concerns. When we started this work PS was considered as the main marker of EVs. Current data indicate that EVs populations derived from different cells may be PS+ or PS- [17]. In our experiments, its presence was not helpful in the discrimination of EV population derived from DBA patients compared to controls.

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 8/13 DBA patients relative to healthy controls. Among patients with other haematological disorders, 15/16 patients showed a proportion of EVs derived from erythroid progenitors similar to controls (Figure 2). 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 3). As expected, the difference of medians comparing non-DBA patients *vs* healthy controls was not statistically significant (Figure 3). Finally the CD71-/CD34-/CD235a+ EV population, that is shed from the late stage of maturation, represents the bulk of events. It should be noted that in all transfusion dependent patients, either DBA or non-DBA, this population includes also EVs shed from donor cells. Consequently, we could not compare this population among the three groups analysed.

The area under the ROC curve (AUC) that compares DBA patients *vs* healthy controls was 0.84 (= good) while the AUC of DBA patients *vs* all the other individuals was 0.80 (= good). Moreover, comparison between DBA patients and non-DBA patients produced an AUC of 0.75 (= fair). 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 0.66 (= poor), consequently this assay cannot discriminate between these groups (Figure 4).

AUC values suggest that this test has a good accuracy to discriminate DBA patients from healthy controls, and/or from non-DBA patients. The $CD34+/CD71_{low}$ EV population is expected to be shed from BFU-E progenitors and our results are in agreement with the low levels of BFU-E found in DBA patients (Table 1).

Actually, this population seems to reflect more the bone marrow BFU-E numbers than the level of anaemia or the disease type. According to this hypothesis, 3 DBA patients (#7, #10, #12), who show this population, had normal erythropoiesis at the time of this analysis (Table 1). In contrast, we found a single DBA patient (#11) with anaemia at the time of this analysis who showed normal levels of this EV population without an evident explanation.

On the other hand, all but one patient with other haematological diseases clustered in the healthy control range. This cluster includes six patients with congenital anaemias characterised by erythroid cell loss at a stage that is later than BFU-E (i.e. HbS, spherocytosis, CDA II and Beta thalassemia), and five patients with acquired conditions. We also analysed a patient with Fanconi Anaemia which can have overlapping features with DBA.

Patient #26 is the only non-DBA patient who did not show this population. He is affected by an iron refractory anaemia with severe microcytosis. We expect that this patient also has a defect of

erythroid progenitors, but a BM evaluation has not been performed so far.

Overall these data suggest that the EV assay we devised may be useful to improve DBA diagnosis as a quicker and less invasive alternative to BFU-E cultures. It should be noted that this assay is performed from peripheral blood, is amenable to transfused patients and requires only two working days, whereas BFU-E cultures require 15 working days and needs to be performed using a bone marrow sample in DBA patients.

Finally, this assay could be a useful tool to select erythroid cell-derived EVs in order to study their content and their functional role in erythroid differentiation. It may also be modified to isolate EVs derived by other lineage specific progenitors and used to study other types of bone marrow failure syndromes.

This work has been recently accepted for publication on PLOS ONE journal.

RESULTS AND DISCUSSION AIM 2

In the first year of my PhD project 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. During this year, I sequenced other 36 DBA patients. The only SNV of interest I found, was an heterozygous coding sequence change in exon7: c.G1186A: p.A396T. This SNV was also found by our American collaborators in a single patient out of 40. Thus, this SNV was found in 2 out of 92 DBA patients with an allele frequency of 1,08%. This SNV has been reported in the 1000genomes project and in the ESP database with an allele frequency of 0.002%. Although this variant is uncommon, the prediction algorithms are not univocal in their interpretation (SIFT: tolerated, Polyphen: possibly damaging, Mutation Assessor: function impact medium, Mutation Tasting: disease causing). The wild type sequence is not conserved through the species. Missense variants may modify splicing. Human Splicing Finder showed that this SNV disrupts an Exonic Splicing Regulatory Sequences (Goren et al.) that is not a classical splicing regulatory element. In conclusion, this SNV can be considered as a Variant of Unknown Significance (VUS).

Analysis of WBSCR22 showed a SNV in the third intron c.256-11A>G. Analysis of this variant by Human Splicing Finder did not show any splicing alteration. Other patients with accumulation of pre-rRNA 18SE will be studied for this gene.

FIGURES

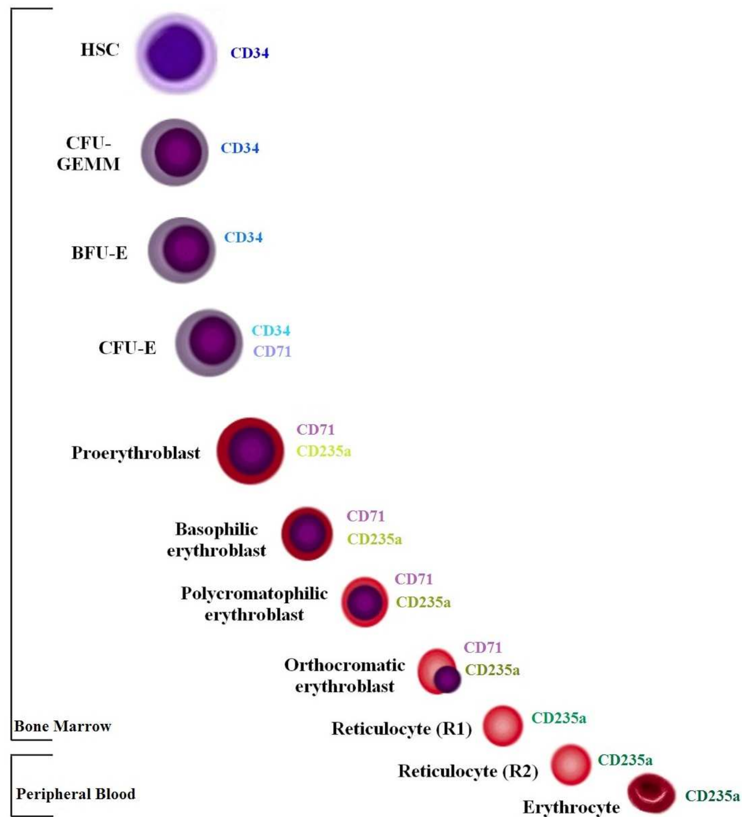

Figure 1. Simplified scheme of erythropoiesis. The most representative markers expressed during erythroid maturation steps are indicated. The intensity colour variation corresponds to expression levels.

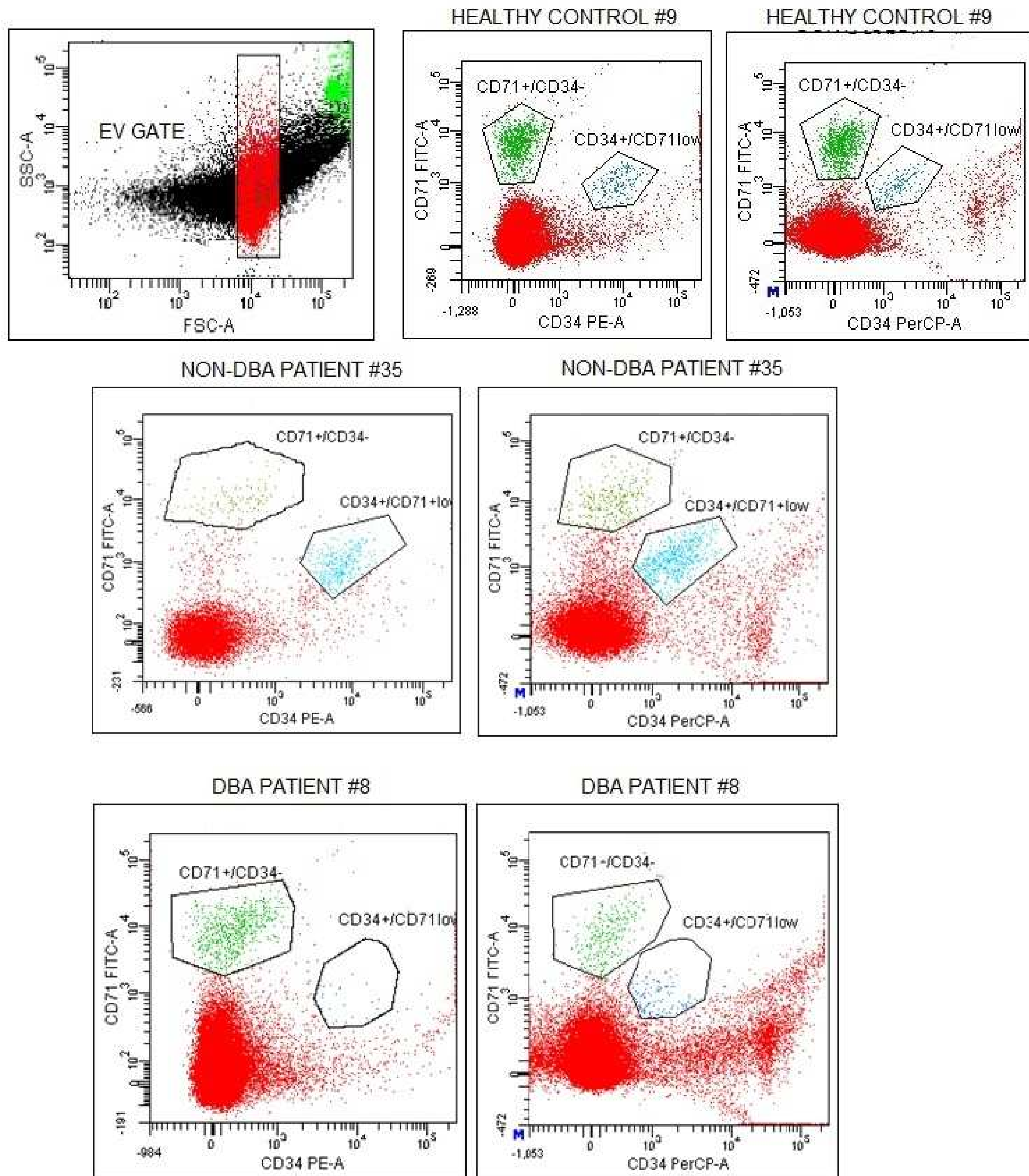

Figure 2. EV distribution in a dot plot graph comparing CD71 and CD34 markers of the events occuring in the EV dimensional gate. CD71+/CD34- (corresponding to CD71+/CD34-/CD235a_{low}/PS-) and CD34+/CD71_{low} (corresponding to CD34+/CD71_{low}/CD235a+/PS+) populations are shown in representative subjects (healthy controls, DBA patients and non-DBA patients). Comparison of EV populations obtained with or without CD235a staining is shown.

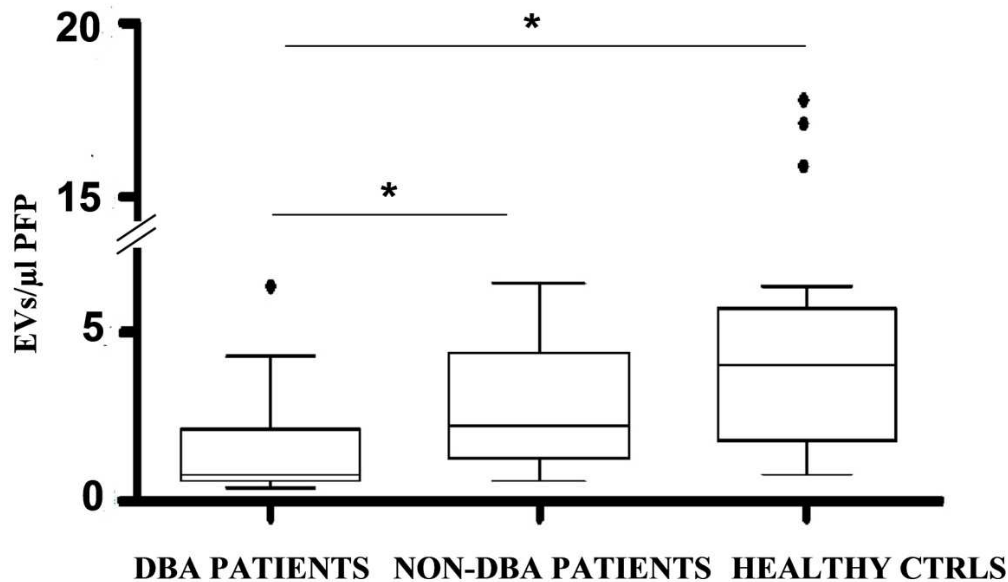

Figure 3. Box plot of Absolute number of events included in the CD34+/CD71$_{low}$ gate. Outliers are shown in black spots. Comparison between DBA patients, non-DBA patients and healthy controls. *The difference of medians is statistically significant (p<0.05, Kruskall-Wallis test).

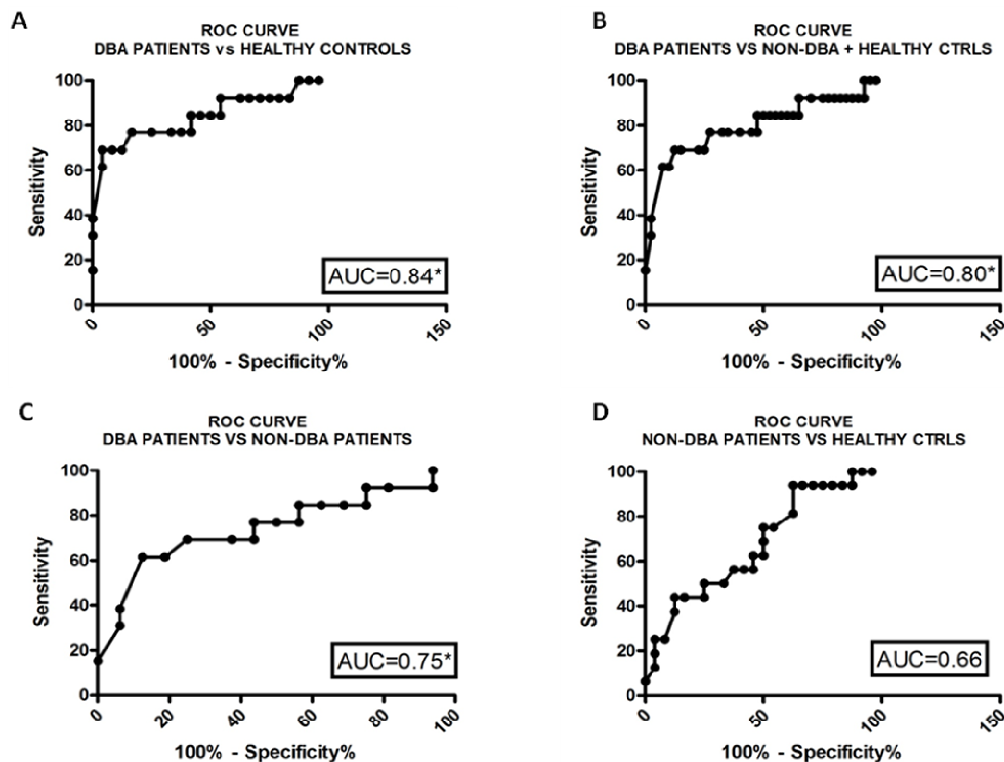

Figure 4. ROC curves analysis of CD34+/CD71$_{low}$ population. ROC curves evaluating the accuracy (AUC) of the CD34+/CD71$_{low}$ analysis in the discrimination of (**A**) DBA patients *vs* healthy controls (**B**) DBA patients *vs* all the others (healthy controls + non-DBA patients) (**C**) DBA patients *vs* non-DBA patients (**D**) non-DBA patients *vs* healthy controls. * p-value <0.01.

POPULATION HIERARCHY

- All Events
  - EV GATE
    - ANNEX +
      - CD71+/ CD34-
      - CD34+/CD71+ low
    - CD71+/CD34-
    - CD34+/CD71+low
  - TRUCOUNT

CD71+/CD34-/CD235alow/PS-

CD34+/CD71low/CD235a low/PS+

CD71 /CD235a+/CD34-

EXTRACELLULAR VESICLES

Figure S1: 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.

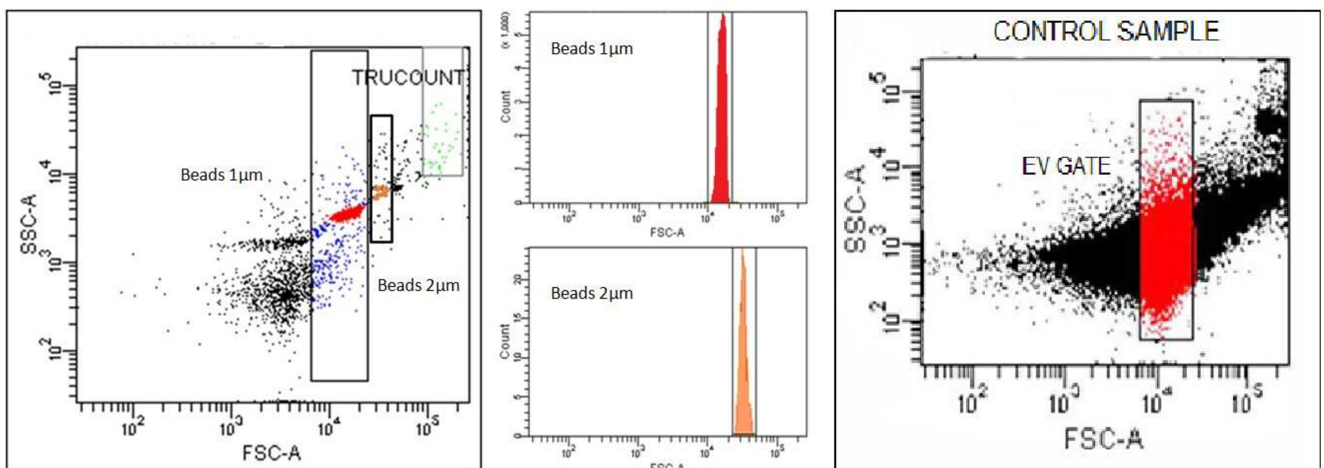

Figure S2: EV dimensional gating strategy. Beads with two diameters (1 and 2 µm) are shown. The beads were analysed in FSC *vs* SSC plot and in FSC *vs* number of events histogram. The right panel show the application of the EV dimensional gate on a representative control sample. Standard size beads were not intermixed with the sample

TABLES

DBA PATIENTS	SEX	AGE	AGE AT ONSET	TREATMENT	Hb (g/dL)	MCV (fL)	RBC (10^6/uL)	Reticulocytes (10^9/L)	eADA (U/g Hb)	BM	BM BFU-E BASAL	BM BFU-E WITH SCF	PB BFU-E BASAL	PB BFU-E WITH SCF
1	M	24	2 months	TRANSFUSION	8.0	88.8	3.04	13.5	3.2	ERYTHROID APLASIA	0	46	0	1
2	F	43	At birth	STEROIDS	10.7	89.8	3.54	N/D	3.9	ERYTHROID HYPOPLASIA	N/D	N/D	1	7
3	M	7	1 month	TRANSFUSION	6.8; 6.8**	80.9; 70.8**	2.34; 2.39**	2.8; 5.1**	N/D	ERYTHROID HYPOPLASIA	23	26	1	8
4	M	5	1 year	TRANSFUSION	9.5	79.8	3.20	10.7	N/D	ERYTHROID APLASIA	0	0	N/D	N/D
5	F	27	2 months	NONE*	10.9	83.8	3.78	45.8	5.3	ERYTHROID APLASIA	0	7	N/D	N/D
6	F	4	At birth	TRANSFUSION	9.6	82.1	3.36	6	N/D	ERYTHROID APLASIA	0; 5; 20†	2; 74; 46†	0; 0▲	0; 2▲
7	M	37	1 year	NONE*	11.7	106.8	3.27	35.7	4.1	ERYTHROID HYPOPLASIA	7	41	N/D	N/D
8	F	5	6 months	TRANSFUSION	6.2	81.8	2.12	11.6	2	N/D	N/D	N/D	N/D	N/D
9	F	17	3 months	STEROIDS	11.5	99.6	3.30	42.8	2.6	ERYTHROID APLASIA	0; 5▲	62; 54▲	N/D	N/D
10	M	13	2 months	STEROIDS	12.6	91.4	4.06	105.0	3.4	ERYTHROID APLASIA	12	89	N/D	N/D
11	M	12	2 months	TRANSFUSION	9.0	81.9	3.20	17.7	N/D	NORMAL	N/D	N/D	N/D	N/D
12	M	20	1 year	NONE	15.2	89.7	4.8	62.3	5	ERYTHROID HYPOPLASIA	0; 0▲	2; 52▲	N/D	N/D
13	M	25	4 months	TRANSFUSION	10.1	85.6	3.4	45	N/D	ERYTHROID APLASIA	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, STEROIDS	6.7	92.0	2.09	28.9	Aplastic anaemia
33	F	8	STEROIDS	9.9	85.4	3.4	44.7	Aplastic anaemia
34	F	14	NONE	9.1	85.0	3.19	56.4	Congenital dyserythropoietic anemia type II (CDA II
35	M	10	NONE	13.0	95.3	3.86	82.1	Fanconi Anaemia
36	M	10	NONE	13.0	77.1	4.97	77.3	Lymphadenitis
37	M	12	NONE	13.4	75.4	4.88	N.D	Thrombocytopenia
38	M	13	MYCOPHENOLATE	14.7	77.0	5.50	64.6	Autoimmune lymphoproliferative syndrome (ALPS)

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

HEALTHY CONTROLS	CD34+/CD71low (without CD235a staining)	CD34+/CD71low (with CD235a staining)
1	0,8	1,0
2	2,6	3,0
3	17,8	14,4
4	5,9	3,2
5	1,6	2,8
6	2,2	2,8
7	4,9	3,4
8	1,7	2,4
9	5,2	3,1
10	2,2	2,0
11	3,8	5,4
12	1,3	1,0
13A	6,2	5,3
13B	1,4	1,7
14	4,2	2,8
15A	15,9	13,2
15B	2,9	2,5
16	4,5	3,1
17	6,4	3,5
18	2,4	2,6
19	5,3	2,5
20	1,7	2,1
21	17,1	10,4
22	4,5	3,6
NON-DBA PATIENTS	**CD34+/CD71low (without CD235a staining)**	**CD34+/CD71low (with CD235a staining)**
23	6,5	4,3
24	4,8	3,4
25	1,2	2,4
26	0,6	1,3
27	3,6	4,3
28	1,4	1,5
29	2,1	3,1
30	1,5	2,1
31	2,4	3,6
32	4,8	4,4
33	0,8	1,3
34	1,1	0,7
35	4,0	4,9
36	1,4	1,9
37	4,1	3,2
38	4,5	4,9
DBA PATIENTS	**CD34+/CD71low (without CD235a staining)**	**CD34+/CD71low (with CD235a staining)**
1A	0,7	0,6
1B	0,4	0,8
2	0,7	0,8

3	0,8	1,2
4	0,8	0,8
5	0,4	0,6
6	0,4	0,9
7	1,6	1,2
8	0,8	0,7
9	0,6	0,6
10	4,3	2,6
11	1,2	1,4
12	6,4	4,3
13	2,7	3,6

Table 3: Absolute number of CD34+/CD71$_{low}$ population obtained from the three groups analyzed without or with CD235a staining, respectively. (*A, B indicate the analysis performed on the same control or patient in two independent samples).

A

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

B

EXON 1-2	**F**: ATGAGAGGAGTCGGTTGGTC; **R**: GAGACAGAAATCGGCACC
EXON 3-4	**F**:TAGAGCAGCCCAGCCTAAT; **R**:GGTGCCAGACAGGACATA
EXON 5	**F**:AACTGACCCTGAGTGTCTGGT; **R**: CCTGGTGACAGAGCAAGACTCC
EXON 6	**F**: GGACGGATGATGTCAAGAGGC; **R**: CAGTGCTAGGGAGGGACAG
EXON 7	**F**: CAGCCCTAACAATGCCTCAC; **R**: CTCCTTTTCCCGCCAATCTG
EXON 8-9	**F**:CCGCTGCCTTTCTTTCCTCAG; **R**:CAGTGTTCTCCCAGCAGGCTCTG
EXON 10	**F**:GTGGGGCAGATGGTTTGAGAAC; **R**: GGTGAGGGCAGAGAGAAAGT
EXON 11-12	**F**: TGCCCACCCAACAACCTCTGT; **R**: TCAAAGCAGCCCTGGACCCTTC
EXON 13	**F**:CTGTGGAAGTGGGTGTCTTTG; **R**:CAGCCCAAGACATCCAAGCACTGG

Table 4: **A)** MCM2 primers sequences used for PCR amplification and Sanger sequencing. **B)** WBSCR22 primers sequences used for PCR amplification and Sanger sequencing (**F**: Forward primer; **R**: Reverse primer).

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MEETINGS

EHA (European Haematology Association) CONFERENCE: Vienna 11/06/2015 – 14/06/2015

ORAL COMUNICATIONS/ POSTER PRESENTATION

Poster presentation: Immunophenotypic profiling of erythroid progenitor-derived extracellular vesicles in Diamond-Blackfan Anaemia: a new diagnostic strategy (EHA CONFERENCE).

PUBLICATIONS

S. Macrì, E. Pavesi, R. Crescitelli, A. Aspesi C. Vizziello, C. Botto, P. Corti, P. Quarello , P. Notari, U. Ramenghi, S.R. Ellis, I. Dianzani "Immunophenotypic profiling of erythroid progenitor-derived extracellular vesicles in Diamond-Blackfan Anaemia: a new diagnostic strategy" *PlosOne*. **10(9)**:e0138200.

SEMINARS

TITLE	DATE	SPEAKER	AFFILIATIONS
Stem cell in the regeneration and repair of the tissues and organs	21/11/14	Maria Prat	Department Health Sciences, UPO
Dysregulated antigen receptor signaling:molecular lessons from two congenital lymphoproliferative disorders	06/11/14	Snow	Department of Pharmacology, Uniformed Services University of the Health Sciences Bethesda, USA
Humoral responses to HCV infection and clinical outcomes	28/11/14	Arvin Patel	PhD Programme Leade rMRC Centre for Virus ResearchUniversity of Glasgow (UK)
Tissue engineering: the state of the art"	14/11/14	Francesca Boccafoschi	Department Health Sciences, UPO
La scoperta del bosone di Higgs	25/11/14	Roberta Arcidiacono e Marta Ruspa	Department Health Sciences, UPO
Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori	27/11/14	Laura Baglietto	Centre for Research in Epidemiology and Population Health, Parigi Unit: Nutrition, Hormones and Women's Health
"Uncovering the role of β-HPV in field cancerization: a collaboration in progress"	04/12/14	Girish Patel	European Cancer Stem Cell Research Institute, Cardiff (UK).
"From the legend of Prometheus to regenerative medicine"	16/12/14	Antonio Musarò	DAHFMO-Unit of Histology and Medical Embryology Sapienza University of Rome
"Microglia microvesicles: messengers from the diseased brain"	17/12/2014	Roberto Furlan	Università San Raffaele, Milano
Anticancer strategy Targeting cancer cell metabolism in ovarian cancer	19/01/2015	Prof. Dr Yong-Sang Song	Director, Cancer Research Institute
Different molecular mechanisms regulate hepatocyte differentiation during the transitions between epithelial and mesenchymal states	20/01/2015	Dr Tonino Alonzi	Lab. Espressione Genica ed Epatologia Sperimentale Istituto Nazionale per le Malattie Infettive "L. Spallanzani" IRCCS (Roma)
Targeting the liver to cure myocarditis: a lesson from a model of STAT3-dependent auto-immune myocarditis	27/01/2015	Prof. Valeria Poli	Department of Molecular Biotechnology and Health Sciences Molecular Biotechnology Center University of Turin

Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells	11/03/2013	Darko Bosnakovski	Associate Professor University "Goce Delcev" Stip Faculty of Medical Sciences
“High -tech Product preservation and Operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach”		Ing. Marco Fadda	Responsabile Ricerca e Sviluppo COMECER SPA
'Signal control in iNKT cell development and function'	09/04/2015	Dr. Xiaoping Zhong, MD, PhD	Associate Professor Department of Pediatrics-Allergy and Immunology Duke University Medical Center
"Actin-based mechanisms in the control of gene expression and cell fate"	07/05/2015	Prof. Percipalle	Associate Professor, Department of Cell and Molecular Biology, Karolinska Institute
“An Integrated Approach to the Diagnosis and Treatment of Ovarian Cancer	14/05/2015	Prof John McDonald	Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Bioscience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, US)
"Ribosomopathies"	28/05/2015	Prof. Steven Ellis	Medical School, University of Louisville, Kentucky
Recent Developments in (cutaneous) Human Polyomavirus Research	05/06/2015	Prof. Mariet Feltkamp	Assoc Prof of Medical Virology Dept of Medical Microbiology, Leiden University Medical Center Leiden, The Netherlands
"Basis of scientific research"	10/06/2015	Prof. Nicoletta Filigheddu	Dep. of Translational Medicine, University of Piemonte Orientale
BIOTECHNOLOGY FOR DERMATOLOGY”	09/07/2015	Dr Gwenaël ROLIN and Thomas LIHOREAU	Clinical Research Engineer Clinical Investigation Center, INSERM UMR1098, FED4234 IBCT, University of Franche-Comté, Besançon, France and UMR1098, FED4234 IBCT, University of FrancheComté, Besançon, France
Le cellule staminali nel danno renale acuto e nel trapianto di rene	28/07/2015	Dott. Vincenzo Cantaluppi	Dep. of Translational Medicine, University of Piemonte Orientale
Cell based models for studying molecular mechanism of Facioscapulohumeral Muscular Dystrophy and Toward animal model for Facioscapulohumeral Muscular Dystrophy (FSHD)	03/09/2015	Prof. Darko Boshnakovski	University "Goce Delcev" Stip, Faculty of Medical Sciences, Stip, R. Macedonia