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ciclo XXIX

DIAMOND-BLACKFAN ANEMIA: IMMUNOPHENOTYPIC PROFILING OF ERYTHROID PROGENITOR-DERIVED EXTRACELLULAR VESICLES AND ANALYSIS OF NEW CANDIDATE GENES

SSD (Settore Scientifico Disciplinare) della tesi MED04

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1. Summary

I worked for my PhD in Prof. Irma Dianzani's Lab, at the Department of Health Sciences in Novara working on Diamond-Blackfan anemia (DBA). DBA is an inherited red blood cell aplasia that is usually diagnosed in early infancy. Patients with DBA exhibit a macrocytic normochromic anaemia and reticulocytopenia. Elevated fetal haemoglobin levels (HbF) and elevated erythrocyte adenosine deaminase (eADA) activity are other features of DBA. Bone marrow failure (BM) is due to the loss of erythroid progenitors (BFU-E and CFU-E) that is evident by colony assay. The occurence of other inherited bone marrow failure syndromes that share clinical symptoms make DBA a diagnosis of exclusion.

DBA was the first disease associated with defective ribosome biogenesis and rRNA processing. Heterozygous mutations in one of 17 ribosomal protein (RP) genes, both of the small (RPS) and the large (RPL) subunit, have been found in about 65% of patients. RP mutations may be inherited with an autosomal dominant pattern or may arise de novo. Recently, mutations in *GATA1* and *TSR2* have also been found in rare patients with DBA showing that also non-RP genes can cause DBA. The genes affected in approximately 35% of suspected DBA patients remain unknown leaving a degree of diagnostic uncertainty for these patients.

Recently, a rapid assay based on the analysis of rRNA processing has been proposed to ascertain the presence of a ribosomopathy. Nevertheless, this approach is not suitable for those forms of DBA caused by genes not involved in ribosome biogenesis.

Taking into account the lack of a specific diagnostic assay new strategies need to be developed to support DBA diagnosis.

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 extracellular vesicles and the screening of two new candidate genes.

During the three years of my PhD program I contributed to the establishment of a new diagnostic tool based on the immunophenotypic profiling of erythroid progenitor-derived

extracellular vesicles (EVs) (Macrì et al., 2015). EVs are membrane bound organelles released by various cell types. Their membrane displays typical markers of the parental cell of origin. Since EVs are detectable in body fluids, they have been developed as diagnostic and prognostic biomarkers in several disorders. Our approach was innovative since no data were available on erythroid progenitor-derived EVs. We chose CD34, CD71 and CD235a markers to study erythroid EVs. We characterized the EVs immunophentoypic profiles of 13 DBA patients, 22 healthy controls and 16 patients with other haematological diseases. Among the different EV clusters we found, only the CD34+/$CD71_{low}$ population showed a statistically significant difference between DBA patients and controls ($p< 0.05$). The absence of this cluster is in agreement with the low levels of BFU-E found in DBA patients. The assessment of ROC curves demonstrated the potential diagnostic value of this population. We suggested that this assay may be useful to improve DBA diagnosis as a quicker and less invasive alternative to BM BFU-E culture analysis.

In the second part of my PhD project I looked for new DBA genes in a subset of Italian DBA patients who were already screened for the 7 RP genes (*RPS10*, *RPS17*, *RPS19*, *RPS26*, *RPL5*, *RPL11*, and *RPL35A*) that are most frequently mutated in DBA patients, and for *GATA1* and resulted mutation negative. I sequenced two genes: *MCM2*, a gene involved in erythropoiesis and *WBSCR22*, a gene involved in ribosome biogenesis.

The study of *MCM2* was carried out in collaboration with Dr. David Bodine (National Human Genome Research Institute, Genetics and Molecular Biology Branch, Bethesda, MD) and Dr. Jason Farrar (University of Arkansas for Medical Sciences, Little Rock, AR) who found a *MCM2* homozygous mutation in a proband from a DBA American family showing possible recessive inheritance. They also demonstrated the role of MCM2 in erythropoiesis. Moreover they found two distinct heterozygous mutations in 2 out of 40 sporadic US patients. Thus, we selected 53 DBA patients from Italian registry and screened *MCM2* by Sanger sequencing. The only SNV that I found was a heterozygous change in

exon7: c.G1186A: p.A396T. This SNV was the same variant found by our US collaborators in a single patient out of 40. Analysis of this change led to the consideration that this SNV could be considered as a VUS (Variant of Unknown Significance). Subsequent analysis of rRNA processing in our proband suggested the involvement of a RPL and led to the previous reassessment of sequencing analysis. Finally, a splicing variant in *RPL5* was found.

In conclusion, our results on MCM2 screening show that this gene is not mutated in our patients panel.

WBSCR22 was studied because it is a key player in 40S biogenesis. Downregulation of WBSCR22 leads to an accumulation of pre-rRNA 18SE by Northern Blot. Thus, we decided to screen this gene in a single patient who showed this specific rRNA pattern but no sequence variation was found.

In conclusion, we suggest that all the DBA patients with an unusual rRNA precursor pattern should be studied by exome or whole genome sequencing.

1. Riassunto

Ho svolto il mio periodo di Dottorato nel laboratorio della Prof.ssa Irma Dianzani, presso il Dipartimento di Scienze della Salute di Novara. Il mio progetto di tesi è stato incentrato sull'anemia di Diamond-Blackfan (DBA), una rara aplasia eritroide che, solitamente, compare nel primo anno di età e spesso è associata a malformazioni. I pazienti presentano un'anemia normocromica, macrocitica con reticolocitopenia. Altre importanti caratteristiche della DBA sono elevati livelli di emoglobina fetale e un'attività elevata dell'enzima eritrocitario adenosina deaminasi.

La lesione molecolare presente nella DBA determina un blocco nel differenziamento a livello dei progenitori eritroidi nel midollo osseo. Infatti, sia BFU-E che CFU-E non si differenziano efficientemente e mostrano un fenotipo pro-apoptotico. L'analisi delle colture midollari derivate da pazienti con DBA mostra una riduzione dei progenitori eritroidi.

La diagnosi risulta essere prevalentemente clinica ed è resa difficoltosa dal fatto che i sintomi sono comuni ad altre sindromi da insufficienza midollare.

La DBA è la prima malattia descritta la cui causa risieda in difetti della biogenesi del ribosoma e del processamento dell'rRNA. Sono state descritte mutazioni in eterozigosi in 17 geni codificanti proteine della subunità piccola (RPS) o grande (RPL) del ribosoma. Complessivamente il 65% dei pazienti presenta una mutazione in uno di questi geni mentre il rimanente 35% non ha una diagnosi molecolare.

Le mutazioni nelle RP possono essere ereditate con un pattern autosomico dominante o possono essere *de novo*. Recentemente, sono state riscontrate rare mutazioni in *GATA1* e *TSR2,* dimostrando che anche geni non codificanti proteine ribosomali possono causare la DBA.

Recentemente è stato sviluppato un saggio basato sull'analisi dell'rRNA per accertare la presenza di una ribosomopatia. Questo approccio non è, però, idoneo per quelle forme di DBA causate da geni non coinvolti nella biogenesi del ribosoma.

Tenuto conto della mancanza di uno specifico test diagnostico, è stato necessario sviluppare

strategie alternative per supportare la diagnosi di DBA.

L'obiettivo del mio progetto di tesi di Dottorato è stato quello di migliorare la diagnosi di DBA con due approcci distinti: lo sviluppo di un nuovo saggio diagnostico basato sullo studio delle vescicole extracellulari e lo screening di due nuovi geni candidati.

Durante i tre anni di Dottorato ho contribuito allo sviluppo di un nuovo test diagnostico basato sul profilo immunofenotipico delle vescicole extracellulari derivate dai progenitori eritroidi (Macrì et al., 2015).

Le vescicole extracellulari sono degli organelli delimitati da membrana che vengono prodotti da diversi tipi cellulari. La loro membrana presenta marcatori tipici delle cellule da cui derivano. Poichè le vescicole extracellulari sono presenti in molti fluidi corporei, sono state sviluppate come biomarcatore diagnostico e prognostico in diverse patologie.

Il nostro approccio è stato innovativo poichè non esistevano dati sulle vescicole extracellulari derivate dai progenitori eritroidi. Come marcatori per studiare vescicole extracellulari di origine eritroide sono stati selezionati i seguenti marcatori: CD34, CD71 e CD235a. E' stato caratterizzato il profilo immunofenotipico di 13 pazienti con la DBA, 22 controlli sani e 16 pazienti con altre malattie ematologiche. Tra le diverse popolazioni di EV che sono state identificate, solamente la popolazione CD34+/$CD71_{low}$ ha mostrato una differenza statisticamente significativa tra i pazienti DBA e i controlli ($p< 0.05$). L'assenza di questo cluster è coerente con il ridotto numero di BFU-E trovato nei pazienti DBA.

La valutazione delle curve ROC ha dimostrato il potenziale valore diagnostico di questa popolazione di vescicole extracellualri. I nostri risultati suggeriscono che questo test possa essere utile per migliorare la diagnosi di DBA in quanto più veloce e meno invasivo rispetto all'analisi delle colture di BFU-E da midollo osseo.

La seconda parte del mio progetto di tesi è stata focalizzata sulla ricerca di nuovi geni DBA in un gruppo di pazienti italiani che erano già stati sequenziati per i 7 geni RP più frequentemente mutati (*RPS10, RPS17, RPS19, RPS26, RPL5, RPL11,* and *RPL35A*) e per

GATA1 e in cui non erano state riscontrate mutazioni. Ho sequenziato due geni: *MCM2*, un gene coinvolto nell'eritropoiesi e *WBSCR22*, un gene coinvolto nella biogenesi del ribosoma.

Lo studio di *MCM2* è stato condotto in collaborazione con il Dr. David Bodine (National Human Genome Research Institute, Genetics and Molecular Biology Branch, Bethesda, MD) e con il Dr. Jason Farrar (University of Arkansas for Medical Sciences, Little Rock, AR) i quali hanno identificato una mutazione omozigote in *MCM2* in un probando di una famiglia americana con una possibile ereditarietà autosomica recessiva. È stato successivamente dimostrato il ruolo di MCM2 nell'eritropoiesi. Inoltre, sono state identificate due distinte mutazioni in eterozigosi in 2 su 40 pazienti americani sporadici.

Di conseguenza sono stati selezionati 53 pazienti DBA dal registro italiano e *MCM2* è stato sequenziato con metodo Sanger. L'unica variazione a singolo nucleotide (SNV) che è stata riscontrata, è un cambiamento in eterozigosi nell'esone 7: c.G1186A: p.A396T. Questa SNV è stata identificata anche dai nostri collaboratori in un paziente americano su 40. L'analisi di questa variante ha portato alla considerazione che si tratti di una variante con un significato non noto. Successive analisi del processamento dell'rRNA nel nostro probando hanno suggerito il coinvolgimento di una RPL. Ciò ha portato alla rivalutazione della precedente analisi di sequenziamento che ha permesso di identificare una variante di splicing in *RPL5*.

In conclusione, i nostri risultati sullo screening di *MCM2* hanno mostrato che questo gene non è mutato nel nostro pannello di pazienti.

WBSCR22 è stato studiato poichè ricopre un ruolo importante nella biogenesi della subunità 40S del ribosoma. La downregolazione di WBSCR22 porta ad un accumulo del pre-rRNA 18SE visualizza mediante analisi di Northern Blot, E' stato quindi analizzato un paziente che mostrava questo specifico pattern degli rRNA, ma nessuna variazione della sequenza è stata osservata.

In conclusione, suggeriamo che tutti pazienti con la DBA con un pattern dei pre-rRNA inusuale debbano essere sottoposti al sequenziamento dell'esoma o dell'intero genoma.

2. Introduction

Erythropoiesis

Human blood contains approximately 5×10^6 erythrocytes per microliter (normal range 4.7×10^6/µl to 6.1×10^6/µl for males and 4.2×10^6/µl to 5.4×10^6/µl for females). These cells have an average life span of 120 days. New erythrocytes are constantly produced in the bone marrow (BM), which provides a niche consisting of endothelial cells of the vascular system, osteoblasts, stromal cells, hematopoietic cells, and the extracellular matrix. Production of erythrocytes at high scale requires an intricate coordination between intrinsic and extrinsic erythroid programmes.

Erythropoiesis is the process through which red blood cells (RBCs) are produced from haematopoietic stem cells (HSC). It is a tightly regulated process that can be divided into two stages: early and late (Figure 1). During the early stage of erythropoiesis, HSCs sequentially give rise to common myeloid progenitor, megakaryocyte-erythrocyte progenitor, burst-forming unit-erythroid (BFU-E), and colony-forming unit-erythroid (CFU-E) cells (Hattangadi et al. 2011). BFU-E and CFU-E cells have been traditionally defined by colony assays (DeZern et al. 2013). BFU-E have large colonies containing up to several thousand hemoglobinized cells that appear after 10–14 days in semi-solid culture. Their growth is dependent on several factors, such as stem cell factor (SCF), thrombopoietin (TPO), interleukin 3 (IL-3), IL-11, and FLT3-ligand. More mature erythroid progenitors, CFU-E, consist of small colonies of 16–125 cells that appear after 5–8 days in semi-solid culture (Liang and Ghaffari 2016). While not required for producing BFU-E and CFU-E cells from stem cells *per se*, EPO is essential for RBC production from CFU-E and acts as a strong stimulator by inducing CFU-E survival and proliferation (Wu et al. 1995; Gautier et al. 2016).

It is well known that CD34 and CD45 markers are expressed in human hematopoietic progenitor cells. Moreover, CD36 and CD71 markers are known to be earlier erythroid

markers than glycophorin A (CD235a). Recently, an exhaustive study performed by Li et al., demonstrated that BFU-E and CFU-E are characterized by $CD45^{+}CD235a^{-}IL\text{-}3R^{-}CD34^{+}CD36^{-}CD71_{low}$ and $CD45^{+}CD235a^{-}IL\text{-}3R^{-}CD34^{-}CD36^{+}CD71_{high}$ phenotypes, respectively (Li et al. 2014).

During the late stage (also named as terminal erythroid differentiation), morphologically recognizable pro-erythroblasts undergo mitosis to produce basophilic, polychromatic, and orthochromatic erythroblasts. The pro-erythroblast, the first identifiable precursor, is the most voluminous and has a large nucleus. The basophilic erythroblast is a cell with a large diameter in which the chromatin is condensed and the cytoplasm is intensely basophilic because of the abundance of free ribosomes. The polychromatic erythroblast is characterized by the disappearance of the nucleolus and the synthesis of hemoglobin whose accumulation increases acidophilia. Finally, the ortochromatic erythroblast has a small nucleus with very condensed chromatin and acidophilus cytoplasm.

The erythroid precursor maturation is characterized by a reduction in the cell volume and mitotic activity which stops at the level of ortochromatic erythroblast. Immunophenotypic profiling of erythroid precursors allowed to identify and isolate terminally differentiating erythroblasts at distinct developmental stages (Hu et al. 2013; Fajtova et al. 2013).

The enucleation by the last precursor leads to the reticulocyte (R1) that goes through the wall of the sinusoids into the bloodstream. The differentiation of reticulocyte (R2) in the mature erythrocyte is characterized by degradation of cytoplasmic organelles and the further reduction of the cell volume that is accompanied by a rearrangement of the plasma membrane through vesiculation (Griffiths et al. 2012). Reticulocytes represent approximately 0.8% of all circulating erythrocytes and their number is an index of the BM function (Buttarello 2016).

In conclusion, erythroid cells at the terminal stages of differentiation have lost their nucleus, endoplasmic reticulum, and mitochondria, and are full of hemoglobin.

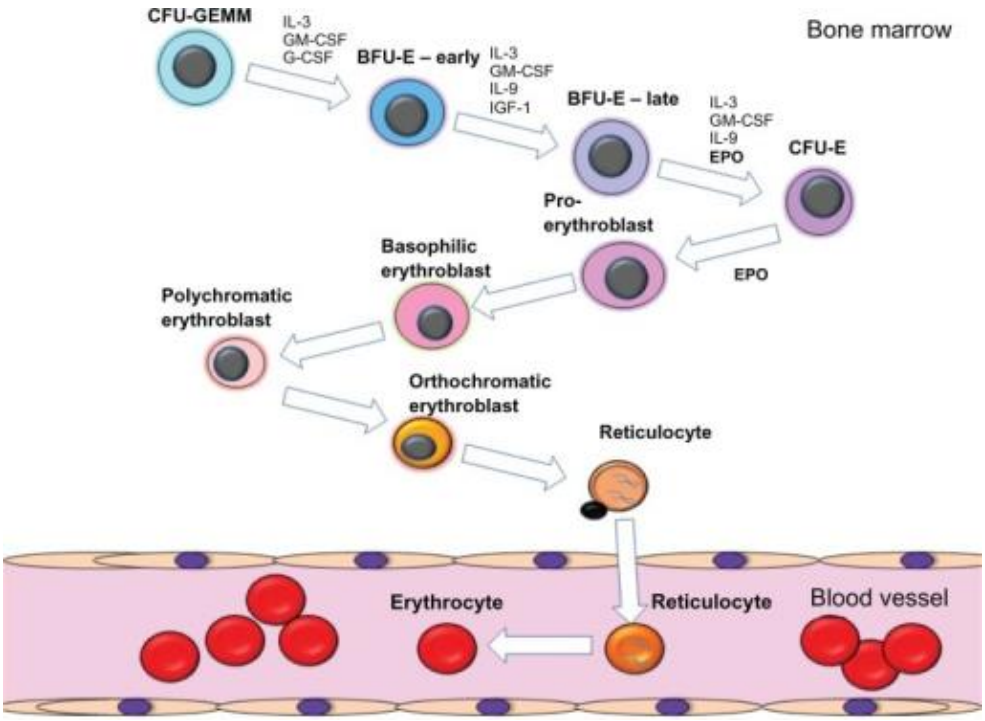


Figure 1. General scheme of erythropoiesis (Sinclair 2013).

Diamond-Blackfan Anemia and other IBMFS

Inherited bone marrow failure syndromes (IBMFS) are a heterogeneous group of rare hematological disorders characterized by the impairment of hematopoiesis and are characterized by specific clinical presentation and pathogenic mechanism. IBMFS include Fanconi Anemia (FA), Dyskeratosis Congenita (DC), Diamond Blackfan Anemia (DBA) and Shwachman-Diamond syndrome (SDS) (Collins and Dokal 2015).

The basic defect in Fanconi Anemia involves one of multiple components of the nuclear protein complex that recognizes, excises and repairs inter-strand DNA crosslinks that arise from the activity of reactive oxygen species and other endogenous DNA cross-linkers as

well as toxic exogenous DNA crosslinking agents (Brosh et al. 2017).

Dyskeratosis Congenita is a syndrome that results in bone marrow failure with macrocytic anemia due to inability to maintain the chromosome telomeres (Perdigones et al. 2016). Fanconi Anemia and Dyskeratosis Congenita affect multiple hematopoietic lineages, whereas the erythroid lineage at the BFU-E and CFU-E stages are specifically affected in Diamond–Blackfan Anemia. Lastly, also Schwachmann-Diamond syndrome may show macrocytic anemia together with pancreas insufficiency (Roach et al. 2015).

These inherited anemias are mainly diagnosed in infancy and are often associated with malformations (Koury 2014).

The presence of normochromic anemia with severe reticulocytopenia and marked reduction or absence of erythroid progenitors from the BM is defined as pure red cell aplasia (PRCA). There are two main clinical entities of PRCA in paediatric patients: DBA and transient erythroblastopenia of childhood (TEC). TEC is idiopathic and self-limiting and occurs in previously haematologically normal children, whereas DBA is an inherited disorder (van den Akker et al. 2014).

DBA clinical features and diagnosis

Diamond-Blackfan Anemia (DBA, OMIM 105650), was first reported by Josephs in 1936 and redefined as a distinct clinical entity by Diamond and Blackfan in 1938 (Lipton and Ellis 2009). DBA is usually diagnosed during the first months after birth and has an incidence of 6-7 newborns per million live births (Vlachos et al. 2008).

The primary hematological feature of DBA is a normochromic and macrocytic anemia with normal leukocytes and platelets and the presence of reticulocytopenia (Vlachos et al. 2008). Elevated fetal haemoglobin levels (HbF) and elevated erythrocyte adenosine deaminase

(eADA) activity are other features of DBA (Alter et al. 2013; Narla et al. 2016) (Table 1).

DBA needs to be distinguished by other IBMFS which can have overlapping clinical presentations (Dianzani and Loreni 2008). FA is excluded by negative results in a chromosome breakage assay, while the absence of telomere shortening rules out DC. SDS is characterized by pancreatic insufficiency and is often associated with skeletal malformations and neutropenia. Distinguishing between DBA and TEC, especially in the first year of life, can be challenging (van den Akker et al. 2014).

The absence of unique clinical features often makes DBA a diagnosis of exclusion.

Diagnostic criteria
Age less than 1 year
Macrocytic anemia with no other significant cytopenias
Reticulocytopenia
Normal marrow cellularity with a paucity of erythroid precursors
Supporting criteria
Major
Identification of a pathogenic variant in one of the genes known to be associated with DBA
Family history of DBA consistent with autosomal dominant inheritance
Minor
Elevated erythrocyte adenosine deaminase activity (eADA)
Congenital anomalies described in ‘‘classical’’ DBA
Elevated HbF concentration
No evidence of another IBMFS
One or more congenital anomalies described in classic DBA

Table 1. Diagnosing Diamond-Blackfan anemia (Vlachos et al. 2008; Clinton and Gazda 2016).

Features of non-classic DBA
Mild or absent anemia with only subtle indications of erythroid abnormalities such as macrocytosis, elevated eADA, and/or elevated HbF concentration
Onset later in life
Congenital anomalies or short stature consistent with DBA and minimal or no evidence of abnormal erythropoiesis

Table 2. Features of non-classic DBA (Clinton and Gazda 2016).

Fifty per cent of DBA patients show congenital abnormalities such as craniofacial, thumb, kidney and heart malformations, whereas 30% of patients show growth retardation (Vlachos and Muir 2010; Lipton et al. 2006). The phenotypic spectrum of DBA is broad.

DBA shows autosomal dominant transmission. Penetrance is incomplete and expressivity widely variable even in patients from the same family. A non-classic form includes mild anemia or no anemia with only subtle erythroid abnormalities, or physical malformations without anemia. In non-classic DBA, anemia develops later than one year of age (Vlachos et al. 2008) (Table 2).

DBA is a cancer predisposition syndrome with a preponderance of hematological malignancies, but also solid tumors such as osteogenic sarcoma may occur. The cumulative incidence of malignancy is approximately 40% by age 46 years (Vlachos et al. 2012).

Approximately 20% of patients show a spontaneous improvement of anemia resulting in independence of therapy for at least 6 months with acceptable hemoglobin levels (Clinton and Gazda 2016). The mechanism behind remission remains unknown and about 15% of those who enter remission relapse (Dianzani and Loreni 2008).

Genetics

DBA is considered as the prototype of ribosomopathies. The first DBA gene identified was *RPS19* (MIM# 603474), that is mutated in ~27% of cases. To date, mutations in other 16 ribosomal protein (RP) genes either of the small (*RPS7* MIM# 612563, *RPS10* MIM# 603632, *RPS15A* MIM# 603674, *RPS17* MIM# 180472, *RPS24* MIM# 602412, *RPS26* MIM# 603701, *RPS27* MIM#603702, *RPS28* MIM#603685, *RPS29* MIM#603633) or the large (*RPL5* MIM# 612561, *RPL11* MIM# 612562, *RPL15* MIM #604174, *RPL26* MIM #603704, *RPL27* MIM #607626, *RPL31*, *RPL35A* MIM# 180468) subunit have been detected. In total, 65% of patients carry a mutation in one of these17 genes (Danilova and Gazda 2015).

Ribosomal gene mutations may be inherited with an autosomal dominant pattern or may arise de novo. Most DBA genetic lesions lead to loss of function (LOF), but a dominant negative mechanism was proposed to explain the effect of a missense mutation (RPS19; R62W) (Devlin et al. 2010).

Approximately 35% of patients with a clinical diagnosis of DBA do not have an identifiable RP gene abnormality (Danilova and Gazda 2015; Gerrard et al. 2013).

More than 200 extra-ribosomal factors are required to synthesize ribosomes and may be involved in DBA pathogenesis. In this regard, *TSR2* which encodes a direct binding partner of *RPS26*, has been proposed as a new DBA gene. This supports the involvement of non-RP genes in the disease (Gripp et al. 2014). Mutations in the X-linked transcription factor *GATA1*, which is essential for erythropoiesis, have been described in a small number of DBA patients (Sankaran et al. 2012; Parrella et al. 2014).

These findings not only increase the growing repertoire of DBA genes, but potentially challenge the current view that failure to adequately synthesize ribosomes leads, *per se*, to

the erythroid and possibly the non-hematologic manifestations of DBA (Farrar 2014) .

Ribosome Biogenesis and rRNA processing

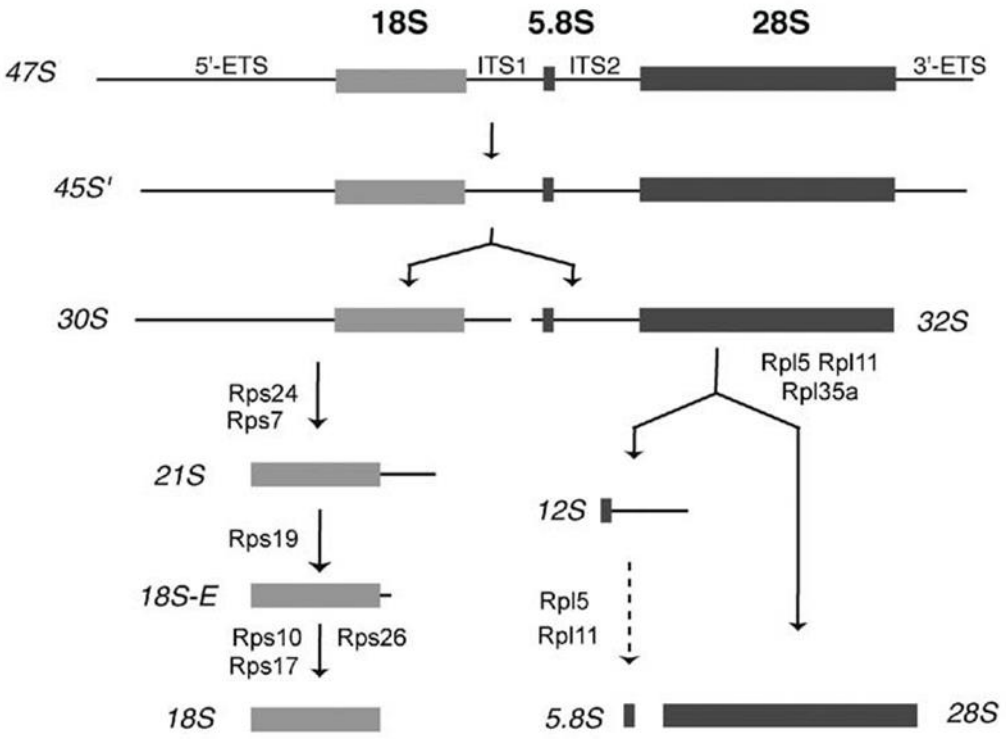


Figure 2. Scheme of rRNA processing (Ellis and Massey 2006).

Ribosome biogenesis involves over 200 different factors, around 80 ribosomal proteins and more than 100 small nucleolar (sno)RNAs.

Mammalian cells have hundreds of copies of tandemly repeated ribosomal DNA (rDNA) that are simultaneously transcribed to pre-ribosomal RNA (pre-rRNA) in the nucleolus. The 47S pre-rRNA is transcribed by RNA polymerase I and processed to mature 28S, 18S and 5.8S rRNA. The 5S rDNA is transcribed by RNA polymerase III in the nucleus. Pre-rRNA processing is driven by endonucleases and exonucleases that remove ITS and ETS, respectively (Figure 2). During these steps, the pre-47S rRNA is associated with ribosomal

proteins, ribonuclease, RNA helicases and other extra-ribosomal factors, to form the pre-90S ribosome. Then, the pre-90S ribosome evolves into pre-40S containing the pre-21S rRNA, and pre-60S containing the pre-32S rRNA. The pre-rRNA 21S matures in 18SE rRNA and then in 18S, and pre-rRNA 32S matures in 28S and 5.8S rRNA (Figure 2). The two ribosomal subunits are then independently exported to the cytoplasm, where the 40S subunit interacts with the mRNA, joins with the 60S and perform protein synthesis (Henras et al. 2015).

Haploinsufficiency or reduced expression of a ribosomal protein results in the defective processing of the ribosomal RNA precursors and in decreased levels of the corresponding ribosomal subunit.

The RPS19 protein was demonstrated to play an important role in 18S rRNA maturation in yeast and in human cells (Leger-Silvestre et al. 2005; Choesmel et al. 2007; Flygare et al. 2007; Idol et al. 2007). In particular, haploinsufficiency of RPS19 leads to an accumulation of pre-rRNA 21S, a reduction of 40S subunits and an increase of free 60S subunits (Flygare et al. 2007).

Alterations of pre-RNA processing and small or large ribosomal subunit synthesis were demonstrated in human cells with RPS24, RPS7, RPL35A, RPL5, RPL11, and RPL26 deficiency, respectively (Choesmel et al. 2008; Farrar et al. 2008; Gazda et al. 2008; Gazda et al. 2012). Finally, mutations in *RPL5* and *RPL11* cause an accumulation of pre-rRNA 32S which is visible even in ethidium bromide gel staining and capillary electrophoresis (Figure 3) (Farrar et al. 2014).

The role of each RP is crucial in different steps of rRNA processing and can be evaluated using Northern Blot with specific probes for each pre-rRNAs (Flygare et al. 2007; Farrar et al. 2008; Farrar et al. 2011) (Figure 3).

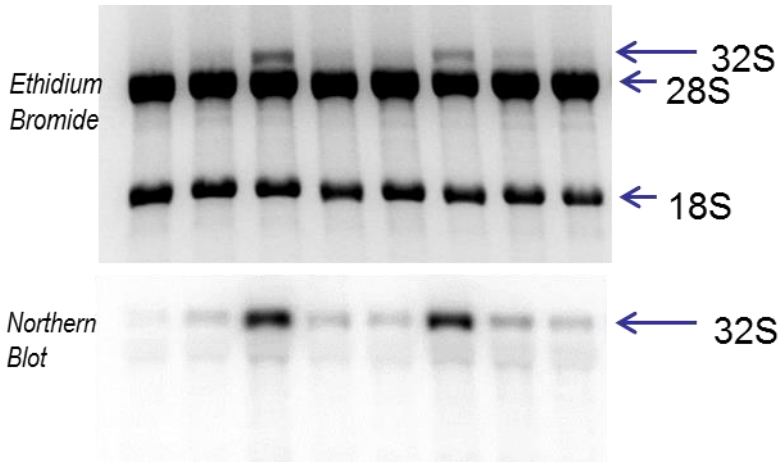


Figure 3. Northern blot analysis of pre-rRNA processing. Total RNA was extracted from activated lymphocytes, resolved by electrophoresis and visualized by ethidium bromide staining. Northern blot with a probe specific for 32S pre-rRNA was performed to validate the result obtained by gel staining.

A rapid and convenient assay based on capillary electrophoresis was proposed to support DBA diagnosis (Quarello et al. 2016). 28S and 18S rRNAs represent the major peaks resolved by capillary electrophoresis (Figure 4). It was reported that the ratio of 28S/18S is higher in patients with *RPS* mutations whereas it is lower in patients with *RPL* mutations (Quarello et al. 2016). In addition, a prominent peak corresponding to pre-rRNA 32S was visible in patients with RPL deficiency (Figure 4). Thus, analysis of rRNAs 28S/18S and 32S/28S ratios by capillary electrophoresis, was proposed to address the molecular screening in the first step of DBA diagnosis (Quarello et al. 2016) (Figure 4).

This approach would only be supportive by exclusion for DBA caused by defects in non-ribosomal protein genes.

Finally, the depletion of RPS19 causes a reduction of rRNA synthesis in cell lines of both erythroid and non-erythroid origin (Juli et al. 2016).

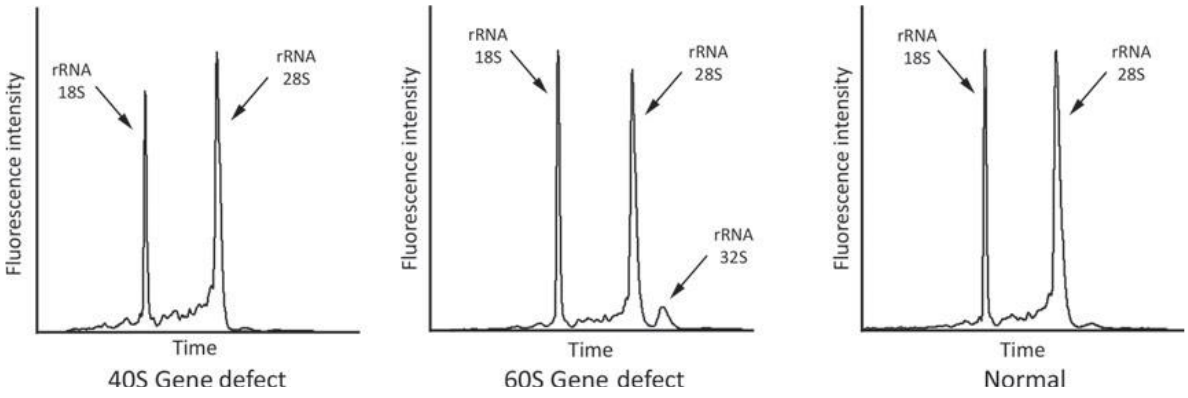


Figure 4. Agilent Bioanalyzer 2100 electropherograms obtained from total RNA of peripheral blood mononuclear cells of DBA patients carrying *RPS* and *RPL* gene mutations and a healthy control (Quarello et al. 2016).

DBA models

To reproduce the pathological conditions and understand the pathogenetic mechanism underlying the disease, different DBA models were developed.

The first attempt to obtain a murine model by knock-out of *RPS19* failed as the homozygous embryo did not survive the first stages of development. However, although the homozygous loss of *RPS19* was lethal before implantation, the mouse carrying an heterozygous deletion, exhibited no pathological phenotype (Matsson et al. 2004). This was probably due to the onset of compensatory mechanisms (Ellis and Lipton 2008).

Another mouse model carrying an heterozygous *RPS19* missense mutation (Y54N) similarly showed only mild anemia. Even in this case, the homozygous mutation was lethal (McGowan et al. 2008). Furthermore, a transgenic mouse model with an *RPS19* missense mutation (R62W) showed retarded growth and mild anemia together with reduced numbers of erythroid progenitors (Devlin et al. 2010).

The first mouse model that recapitulated the disease was obtained using transgenic RNA interference that allowed an inducible downregulation of RPS19. This mouse developed macrocytic anemia, leukocytopenia and variable platelet count (Jaako et al. 2011).

Several zebrafish DBA model were generated using the technique of "Morpholino" for *RPS19* (Danilova, Sakamoto, and Lin 2008; Uechi et al. 2008), *RPL11* (Danilova, Sakamoto, and Lin 2011), *RPS29* (Taylor et al. 2012), *RPS24* (Song et al. 2014), *RPS27* (Wang, Yoshida, et al. 2015), *RPL27* (Wang, Yoshida, et al. 2015) and *RPL5* (Wan et al. 2016).

In order to reproduce the condition of protein haploinsufficiency, different cell culture models, characterized by silencing of a RP by RNA interference, were developed. Knock-down of *RPS19* using a tetO system in TF1 erythroid leukemia cells caused reduced proliferative ability and cell differentiation (Miyake et al. 2005; Miyake et al. 2008). In TF1 cells other RP were silenced, including RPL5 and RPL11 (Aspesi et al. 2014; Moniz et al. 2012).

Finally, downregulation of *RPS19* in human CD34+ cells induced decreased proliferation and differentiation of erythoid progenitors cells and cell cycle arrest (Ebert et al. 2005; Flygare et al. 2005; Kuramitsu et al. 2008).

Pathogenetic mechanisms

Ribosome synthesis is cell cycle controlled: it is sensitive to growth factors and nutrient signalling and inhibited upon stress signals. Haploinsufficiency of a RP can cause ribosomal stress leading to a pro-apoptotic phenotype of erythroid progenitors.

One of the first indications that p53 is involved in ribosome stress was the finding that a mutation in *Bop1*, which is involved in maturation of rRNAs, leads to p53-dependent cell cycle arrest in mouse cells (Pestov, Strezoska, and Lau 2001). Moreover, RPS6 haploinsufficiency activates p53 in mouse embryos and T cells (Panic et al. 2006).

Studies in zebrafish and mouse established p53 activation as a general response to RP

haploinsufficiency (Dutt et al. 2011; Taylor et al. 2012; McGowan et al. 2008). Several hypotheses were suggested to explain the activation of p53 in DBA. The nucleolus was proposed to be a universal stress sensor that is responsible for the maintenance of a low level of p53 in the cell. However, RP deficiency does not always lead to nucleolar disruption, suggesting that other mechanisms sense ribosomal stress as well (Fumagalli et al. 2009). Moreover, some RPs were shown to bind MDM2 and to inhibit its binding to p53, leading to p53 stabilization and cell cycle arrest (Danilova and Gazda 2015) (Figure 5). Decreased RP synthesis activates p53, inducing downstream events that result in cell cycle arrest or apoptosis (Figure 5).

Another way of p53 activation in RP-deficient cells might be the altered nucleotide metabolism (Figure 5).

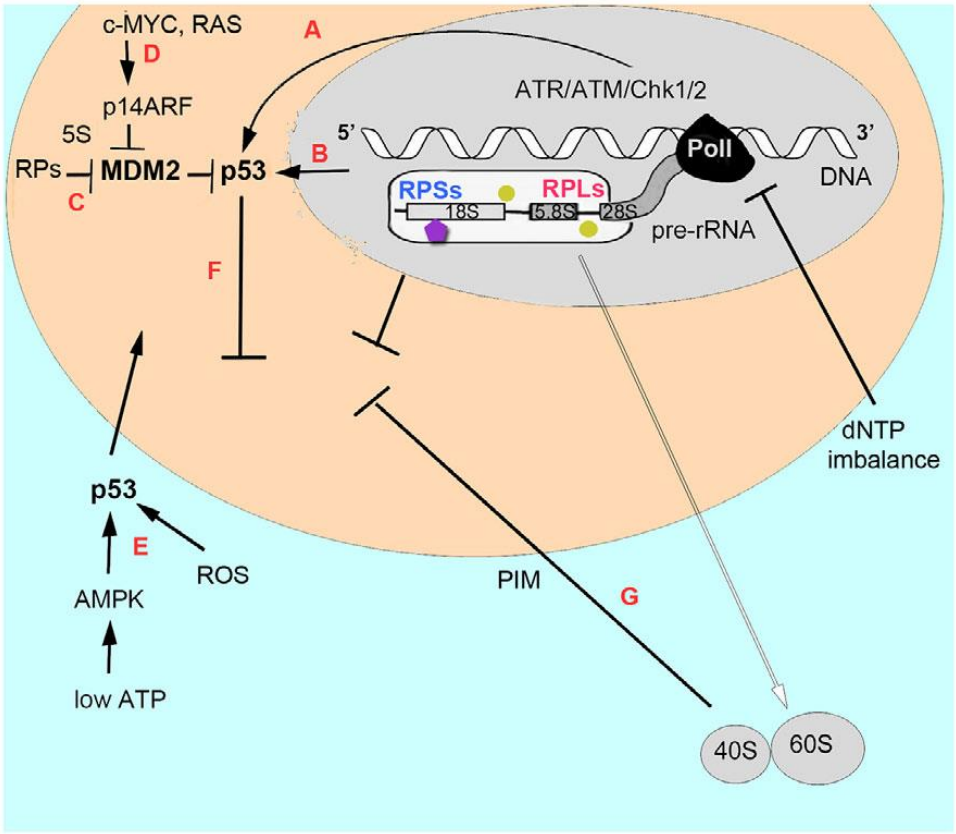


Figure 5. Defects in ribosomal biogenesis activate p53 and other stress-response mechanisms (Danilova and Gazda 2015).

p53 might also be activated by increased levels of ROS (Reactive Oxygen Species) in RP-deficient cells (Heijnen et al. 2014) (Figure 5). Finally, the different hypotheses of p53 activation are not mutually exclusive.

Conversely p53-independent pathways may contribute to apoptosis of erythroid progenitors (Singh et al. 2014). Studies of transcriptional responses to RP deficiency performed in p53-negative human cell lines revealed changes in the expression of genes involved in metabolism, proliferation, apoptosis and cell redox homeostasis (Aspesi et al. 2014).

Different hypotheses were proposed to explain how a defect in ribosome biogenesis specifically affects only erythropoiesis. The tissue-specific expression of RPs could make the haploinsufficiency of certain RPs as limiting factor in a specific tissue (Ellis and Massey 2006). Erythroid committed cells may be more sensitive to p53 activation as compared to other hemopoietic lineages (Dutt et al. 2011).

Furthermore, several RPs are known to have extra-ribosomal functions. It is speculated that their mutations cause the loss of hypothetical functions, that are specific for erythroid maturation, possibly in combination with ribosomal stress (Lipton and Ellis 2009).

The reduction in ribosomes causes a defect in general protein synthesis, emphasized in a rapidly proliferating tissue. In addition, erythroblast cells undergo chromatin condensation and enucleation, and it is likely that a great number of ribosomes must be generated in a very short time, to allow the synthesis of globins, also when cells will be less efficient to make new ribosomes (Lipton and Ellis 2009).

It was also demonstrated that the alteration of heme metabolism could play a role in the pathogenesis of DBA. The insufficient production of hemoglobin and the accumulation of the excess of heme might contribute to oxidative stress in erythroid progenitors (Ellis 2014). The heme overload could also contribute to defective erythropoiesis in DBA by affecting the proliferation, apoptosis, and differentiation of early erythroid progenitors (Mercurio et al. 2016; Yang et al. 2016).

Finally, the reduced expression of GATA1 by DBA cell models provides a possible link between ribosome defect and erythropoiesis failure (Ludwig et al. 2014).

Standard treatment

DBA patients are first treated with steroids that may carry many side effects (Sjogren et al. 2015). The subset of DBA patients who do not respond to corticosteroids or require high doses of steroids with unacceptable toxicities receives chronic red cell transfusions. Transfusional iron overload is a major cause of morbidity and mortality in DBA and iron chelation is needed to avoid secondary hemochromatosis (Roggero et al. 2009).

The only curative option for hematological manifestations of DBA is allogenic hematopoietic stem cell transplantation (HSCT). The avoidance of iron overload due to regular transfusions is the main reason to consider HSCT from either related or HLA-compatible unrelated donors in DBA children (Fagioli et al. 2014).

In the absence of HLA compatible donors, haploidentical parents may be considered as donors, although a more intensive conditioning regimen is required and patients show a high risk of both acute and chronic graft-versus-host disease (GvHD). Moreover, the risk of using silent carriers as donors should be considered.

Taking into account all these issues, the assessment of alternative therapies is needed in order to develop a definitive cure for DBA.

New drugs

Recently, important advances have been made in the assessment of small molecules as potential drugs to treat DBA. The BM failure is due to BFU-E erythroid progenitors that

undergo apoptosis. As BFU-E are not sensitive to EPO, the attempt to use this cytokine in DBA failed (Flygare et al. 2011). Otherwise CFU-E, that express EPO receptor, are too few in number to sustain erythropoiesis. Therefore, another approach is the development of drugs that selectively target BFU-E.

It was demonstrated that glucocorticoids increased red blood cell production stimulating BFU-E self- renewal (Flygare et al. 2011). A crucial gene that is upregulated by glucocorticoids is PPAR-α (Lee et al. 2015). The use of PPAR-α agonists such as fenofibrate was shown to synergize with glucocorticoids, promote BFU-E self- renewal and improve erythropoiesis. This effect might reduce the dose of glucocorticoids and their many adverse effects. Interestingly, fenofibrate is already approved by FDA to treat hypercholesterolemia and hypertriglyceridemia. Discussions are currently underway to set up a clinical trial to test this drug in DBA patients.

The important discovery of TGF-β pathway involvement in DBA lead the way to new drugable targets (Ge et al. 2015). Importantly, blocking of TGF-β signaling by receptor kinase inhibition, enhanced BFU-E self-renewal and total erythropoiesis in a glucocorticoid-independent manner. Thus, TGF- β inhibitors like Galunisertib (LY2157299 H2O, Lilly Oncology), might be used, as alternative to steroids to treat EPO-unresponsive anemias such as DBA. Whether this small molecule is a suitable candidate for the treatment of DBA patients remains to be determined (Gao et al. 2016).

Finally, in 2011 a group of DBA patients have been enrolled for a clinical trial (NCT01464164) to test the efficacy of Sotatercept (ACE-011, Celgene).

This drug is an activin receptor type IIA ligand trap and inhibits signaling downstream of TGF-β superfamily members. Previous clinical trials for other clinical conditions reported that women treated with this drug produced increased numbers of erythrocytes and hemoglobin level (Sherman et al. 2013). Moreover, anemia has been ameliorated in murine models after administration of RAP-011 (murine orthologous of ACE-011) (Carrancio et al.

2014). RAP-011 improved erythropoiesis in DBA zebrafish model further supporting the use of Sotatercept in patients (Ear et al. 2015).

All of these drugs were already employed in humans for other clinical needs and this means that their adverse effects are well known. However, detailed studies have to be carried out to evaluate the efficacy of these small molecules as innovative therapeutic options for DBA patients.

Gene therapy

Important results have been reported in gene therapy of HSC to treat hematological and neurodegenerative storage disorders (Naldini 2015). It is well known that HSC have a great therapeutic potential due to their self-renewal ability. Thus, this population represents an ideal target for stable genomic integration of therapeutic genes and also a valid alternative to HSCT especially when no matched donor is available. Recent progress has been reached in gene therapy of hemoglobinopathies such as Beta-Thalassemia and Sickle Cell Disease (Cavazzana-Calvo et al. 2010; Cavazzana et al. 2015). Finally, in 2012 the beginning of Phase I/II Gene Therapy Trial of Fanconi Anemia Patients with lentiviral vectors (LV) carrying the FANCA gene was reported. This result supports the translation from bench to the clinic of gene therapy in BM failure syndromes ('Phase I/II Gene Therapy Trial of Fanconi Anemia Patients with a New Orphan Drug Consisting of a Lentiviral Vector Carrying the FANCA Gene: A Coordinated International Action (EuroFancolen)' 2015).

Gene therapy in DBA may avoid a lot of critical aspects of allogenic HSCT such as intensive post transplant immunosuppressive treatment to prevent GvHD, a condition that results in a poor patients quality of life. Autologous cells that have been corrected by *ex vivo* transduction should display a selective proliferative advantage in BM repopulation and

this may reduce the preconditioning regimen dose and its side effects.

In the past different investigators tried to assess the feasibility of gene therapy in DBA. In particular Hamaguchi et al. reported that transduction of *RPS19* cDNA using oncoretroviral or LV vectors into RPS19-deficient CD34+ cells from DBA patients improves erythroid colonies formation both in solid and liquid cultures (Hamaguchi et al. 2002; Hamaguchi et al. 2003). Moreover, Flygare et al. showed that high levels of RPS19 are required to increase erythroid differentiation in RPS19-deficient DBA CD34+ cells *in vitro*. Corrected DBA patient cells displayed a proliferative advantage *in vivo* and consequently an improved engraftment (Flygare et al. 2008).

The use of animal models that recapitulate DBA is imperative to investigate the feasibility, the therapeutic efficacy and the safety of gene therapy. The mouse model produced by Jaako et al., developed macrocytic anemia and BM failure that were restored *in vitro* by *RPS19* gene transfer using LV (Jaako et al. 2011). Moreover, transplant of corrected RPS19-deficient cells in recipient mice cured the anemia and the lethal BM failure without signs of vector-mediated toxicity (Jaako et al. 2014).

To minimize the issues concerning *ex vivo* gene delivery, the *in situ* gene transfer in BM was proposed. In particular, a Fanconi Anemia animal model has been treated with intraosseous infusion of LV encoding *FANCC* gene, resulting in phenotypic correction (Habi et al. 2010). It has been reported that intraosseous delivery of LV targeting Factor VIII in megakaryocytes becoming platelets corrects murine Hemophilia A (Wang, Shin, et al. 2015). This method should be evaluated in DBA mouse models to provide a more targeted approach.

The breakthrough of reprogramming mature adults cells to pluripotency represents a revolution towards personalized therapy because the risk of immune rejection and the ethical concerns of using embryonic cells are eluded. Induced Pluripotent Stem Cells

(iPSCs), as an unlimited source of cells, are easier than HSC to be genetically manipulated and entirely characterized before transplant. Garcon et al., for the first time, obtained iPSCs from DBA patients fibroblasts carrying mutation in *RPL5* or *RPS19* and provided a renewable reservoir of cells that display DBA pathological features (Garcon et al. 2013). The same investigators performed transcriptome analysis in these cells and demonstrated the striking dysregulation of TGF-β pathway (Ge et al. 2015). The authors also proved that providing a wild-type copy of the RP gene that was haploinsufficient results in the phenotype rescue. DBA iPSCs were corrected via complementary DNA transfer into the safe harbor AAVS1. It is well known that integrated trans-genes are stably expressed using this locus without epigenetic silencing and avoiding the insertional mutagenesis issues. This work represented the proof of concept that DBA iPSCs recapitulate the disease and that they are amenable to genetic correction.

The clinical application of iPSCs obtained from DBA patients and genetically restored to regenerate the defective tissue is attractive. Nevertheless, the efficiency of DBA fibroblasts reprogramming to iPSCs is very low and this limits the efficacy and future use of this strategy. Moreover, it has to be considered that fibroblasts may show an intrinsic genetic instability due to the UV exposition, hampering the clinical translation of these reprogrammed cells (Martincorena et al. 2015).

More in general, ten years after Yamanaka's discovery (Takahashi and Yamanaka 2006), iPSCs therapies have been proved challenging to develop. However, iPSCs have made their mark in human disease modeling.

Taking into account these considerations, today DBA iPSCs represent an essential tool to investigate the molecular mechanisms involved in this disorder although restricted to patient mutations. Along with editing technologies these cells may be useful to study the effect of a single mutation and to translate the consequent findings to a personalized medicine (Maeder and Gersbach 2016).

3. Aim

Because of the difficulty of establishing the diagnosis of DBA, in some instances the diagnosis may only be performed after other disorders have been ruled out. Further confounding a diagnosis of DBA is the increased identification of patients with non-classical forms of DBA including patients with malformations without anemia or with anemia presenting as an adult. Molecular testing for identification of a heterozygous pathogenic variant in one of the known DBA genes confirm the diagnosis if clinical features are inconclusive (Danilova and Gazda 2015).

The recent assay based on the analysis of pre-rRNA processing is useful to confirm the status of ribosomopathy and to address the sequencing screening to the large or small ribosomal subunit. Nevertheless, this approach is not suitable for those forms of DBA caused by defects in non-RP genes.

The genes affected in approximately 35% of suspected DBA patients remain unknown leaving a degree of diagnostic uncertainty for these patients. The study focused on the screening of all 80 ribosomal proteins in a subset of DBA patients did not identify new RP genes (Gerrard et al. 2013). Furthermore, the paradigm of DBA as ribosomopathy was undermined by the discovery of non-RP genes like *GATA1* and *TSR2*.

Taking into account the lack of a specific diagnostic assay and the need of genetic confirmation in a percentage of patients, new strategies need to be developed to support DBA diagnosis.

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 extracellular vesicles and the screening of two new candidate DBA genes.

4. Immunophenotypic profiling of erythroid progenitor-derived extracellular vesicles in DBA

Aim

As an alternative strategy for developing a more inclusive assay for possible use in DBA diagnosis I turned to the study of extracellular vesicles (EVs) whose presence may be altered as a consequence of increased apoptosis associated with BM failure.

In this part of my PhD project I focused on the immunophenotypic characterization of erythroid EVs in ribosomal stress condition. This study was carried out in collaboration with the Chemical Clinical Analysis laboratory, SCDU, Azienda Universitaria Ospedaliera Maggiore della Carità, Novara. The aim of my study was to establish the method in our DBA cell model and then to analyse plasma EV of three different groups of individuals: DBA patients, patients with other hematological diseases, and healthy controls. We reasoned that erythroid EVs may vary both *in vitro* cell model and in the peripheral blood of DBA patients as a consequence of the increased apoptosis caused by ribosomal stress.

Rationale of the project

Extracellular vesicles

Extracellular vescicles (EVs) are membrane-bound organelles produced by various cell types. Cells release EVs either constitutively or in a regulated manner and these vesicles harbor a specific subset of proteins, mRNAs, miRNAs, lipids and metabolites reflecting their parental cells and conditions.

The exponential findings in this field demonstrated that EVs have specialized functions and play a key role in cell biology. In particular, EV-mediated intercellular communication has been reported as an evolutionarily conserved phenomenon (Deatherage and Cookson 2012). EVs can be detected in body fluids including blood, plasma, urine, saliva, amniotic fluid, milk, pleura ascites (Mathias et al. 2009; Lasser et al. 2011). For this reason, the first clinical application was the assessment of EVs as biomarkers for non-invasive diagnosis and prognosis of various human diseases (Chaput, Taieb, Schartz, et al. 2005; Choi et al. 2013; D'Souza-Schorey and Clancy 2012; Mullier et al. 2014; Sarlon-Bartoli et al. 2013; Shedden et al. 2003). Different therapeutic approaches were pursued to utilize EVs and their mimetics for vaccine, chemotherapeutic drug and siRNA delivery (Alvarez-Erviti et al. 2011; Chaput, Taieb, Andre, et al. 2005; Jang et al. 2013; Kordelas et al. 2014; Lai, Chen, and Lim 2011).

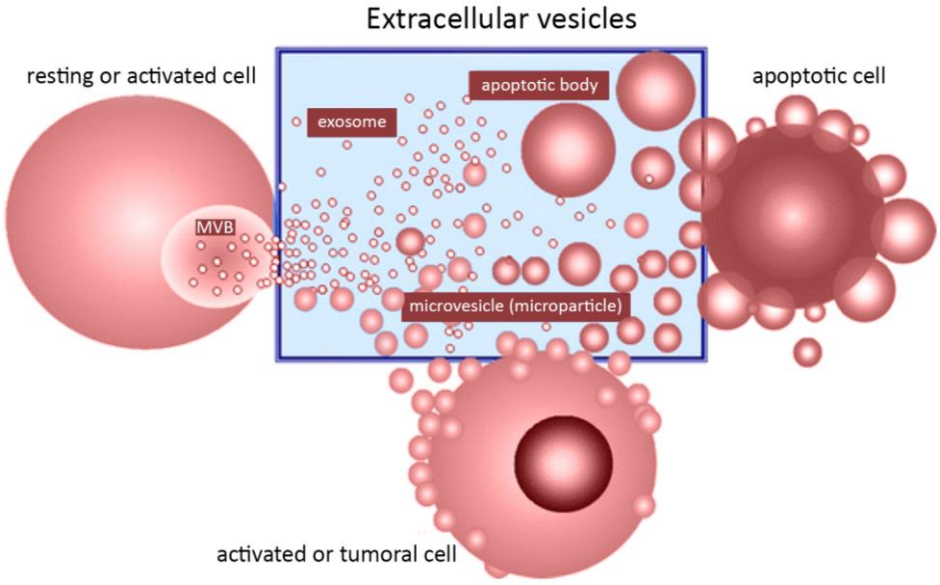


Figure 6. Schematic representation of EVs (Gyorgy et al. 2011).

The explosion of scientific data has justified the need of databases that tried to categorize EVs on the base of different parameters such as size, production and content. Although advances in this fast growing field were made, the nomenclature of EVs is still controversial. The most important position statement has been achieved by The International Society for Extracellular Vesicles with the creation of EVpedia (http://evpedia.info) in 2013 (Kim et al. 2013; Kim et al. 2015). Nevertheless, to date more than twenty different classifications converge to the term EVs.

The most important three main categories in which EVs can be broadly divided are exosome, microvesicles and apoptotic bodies.

The term exosome (EXO) comprises preformed vesicles which are smaller than 100nm with a density between 1.13 and 1.19 g/ml. EXO originate from intracellular multivesicular bodies (MVB). They are released via exocytosis, when MVB leave the lysosomal pathway and fuse with the plasma membrane (Gyorgy et al. 2011).

EXO were mainly characterized in immunity and cancer. They are involved in antigen

presentation, immunostimulation, tumour growth, metastasis and angiogenesis (Thery, Ostrowski, and Segura 2009; Weidle et al. 2017; van der Pol et al. 2012). They can protect tumour cells inhibiting antitumor drug accumulation and contribute to multidrug resistance (Ciravolo et al. 2012).

Microvesicles (MV) are surrounded by phospholipid bilayer and have a diameter of 50-1000nm. They have not an endosomal origin and are directly shed from budding of plasma membrane (Gyorgy et al. 2011). The outer layer of MV membrane was often shown to display phosphatidylserine (PS), but this may depend on the cell type from which MVs derive or on the functional cell state (Connor et al. 2010).

MVs are released during cell stress conditions after activation of cell surface receptors or during apoptosis following intracellular Ca^{2+} increase (van der Pol et al. 2012; Baroni et al. 2007; Kahner, Dorsam, and Kunapuli 2008). MV play a pivotal role in important biological processes such as membrane traffic and horizontal transfer of proteins and nucleic acids among neighboring cells. Indeed, MV specifically interact with cells to transfer receptors, ligands, mRNAs and miRNAs (Gyorgy et al. 2011). These vesicles are involved in the pathogenesis of rheumatoid arthritis (Boilard et al. 2010), in tumour pro-invasive activity (Giusti et al. 2008) and in neoplastic transformation (Antonyak et al. 2011).

MV were deeply studied in coagulation. Upon exposure to collagen, platelets release MV coated with tissue factor which binds its surface ligand (for example P-selectin1) on macrophages (Polgar, Matuskova, and Wagner 2005), neutrophils (Pluskota et al. 2008) and other platelets.

An increased amount of MV was observed in plasma from patients with anti-phospholipid syndrome (Asherson’s syndrome) (Ardoin, Shanahan, and Pisetsky 2007) as well as in inflammatory conditions (Distler et al. 2005).

Cells dying by apoptosis undergo several stages: they start with condensation of the nuclear chromatin, followed by membrane blebbing and progress to disintegration of the cellular

content into membrane vesicles known as apoptotic bodies (AB). AB are 1-5 μm in diameter similarly to the size range of platelets (Hristov et al. 2004). They are PS positive and they have a density between 1.16 to 1.29 g/ml, partially overlapping with exosome density and a morphology that is heterogeneous when compared with other vesicles visualized by TEM (van der Pol et al. 2012).

Isolation and characterization methods

EV studies are hampered by lack of a standardized protocol to isolate the different vesicle types both from cell culture and biological fluids.

The widely accepted protocol is based on differential centrifugations which represents the gold standard method. In general, the isolation procedure includes a first centrifugation step (200-1,500 × *g*) to eliminate cells and cellular debris followed by a stronger centrifugation (10,000-20,000 × *g*) to eliminate vesicles with a bigger size (100-800 nm). A higher speed centrifugation (100,000 – 200,000 × *g*) is able to pellet EXO (Mathivanan, Ji, and Simpson 2010; Thery et al. 2006).

To better purify pellets, EVs can be loaded on sucrose gradient or cushions. Other systems include immunoaffinity isolation and nanomembrane ultrafiltration concentrator (Cheruvanky et al. 2007; Grant et al. 2011). Since vesicles are heterogeneous, there is no system able to accurately separate a specific type. Furthermore, isolation methods are based on size, sedimentation, density, and surface molecules, but there is no consensus about these specific characteristics for each subpopulation of EVs. The isolation efficiency depends on the shape and viscosity of solution, as well as on temperature, centrifugation time and the rotor type used for the centrifugation (van der Pol et al. 2012). The study of EV with TEM (transmission electron microscope) allows to more specifically identify the

different EV, but the sample preparation can alter their morphology (van der Pol et al. 2012). Some studies proposed the use of atomic force microscope to detect a higher number of vesicles (Ashcroft et al. 2012).

The most spread technique to characterize EV is the immunophenotyping by flow cytometry. Despite being limited by instrumental sensitivity, flow cytometry remains the most widely used method to simultaneously assess physical parameters and the presence of membrane markers. Using a specific panel of antibodies, this method is able to evaluate the cellular origin of the different populations. The use of flow citometry needs precise instrument calibration before EV analysis. Size beads of different diameters can be used to evaluate EV size (Simak and Gelderman 2006).

The relative amount of EVs in a sample can be determined using TruCount^{TM} tubes (Becton Dickinson) containing a known number of beads.

Characterization of plasma EVs and sources of variability

Different subpopulations of extracellular vesicles were reported in blood. Many studies documented the EV number variation in different pathologies and support their use as diagnostic or prognostic biomarkers. 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 (Griffiths et al. 2012; Ghashghaeinia et al. 2012). No data were available on EV production from erythroid progenitors or early precursors.

Previous studies have shown different causes of variability that may affect the characterization of the EV obtained from plasma (Ayers et al. 2011). The pre-analytical variables that need to be considered are: the diameter of needle used for sample collection,

way of blood collection (vacutainer, syringe, tube) and type of anticoagulant, storage temperature, freeze-thaw cycles (Gyorgy et al. 2011; Simak and Gelderman 2006). It is well known that the use of needles with different diameters may influence the cellular stress with a consequent alteration in EV production.

Among the different anticoagulants the most used is the sodium-citrate which is a reversible inhibitor of Ca^{2+}.

The main factor that influence the amount of EVs and the expression of certain markers, is the elapsed time between collection of peripheral blood and the first centrifugation to get free platelets plasma. To avoid the increase of EVs, mainly from platelets, the sample has to be processed in the shortest possible time (Jayachandran et al. 2012).

Although limited variations of temperature does not heavily hamper EV isolation, freezing alters both number and the immunophenotype of already isolated EVs. EV sample remains stable for 3-4 days at room temperature (Jayachandran et al. 2012). Moreover, the number of Annexin-V positive EVs increases after several freeze-thaw steps (Connor et al. 2010).

Besides pre-analytical also analytical variables have to be considered. The differential centrifugation based protocol allows to better separate the different EV populations but the increase of steps can have a negative impact on their numbers.

In conclusion, in order to obtain comparable samples the precise standardization of the pre-analytical and analytical components is needed.

Methods

DBA cell model

CD34+ cell culture and transduction

Cord blood samples were collected from healthy donors after informed consent (Department of Public Health and Pediatric Sciences, University of Turin).

Peripheral blood mononuclear cells were isolated with Ficoll-Paque™ Premium (GE Healthcare). Human CD34+ cells were isolated from mononuclear cord blood cells according to MACS® CD34 MicroBeads Kit protocol (Miltenyi). The CD34+ cells were maintained in culture for 4 days in SFEM medium (StemSpan® Serum-Free Expansion Medium) with the addition of the following cytokines to induce erythroid differentiation: SCF (20ng / ml), IL-3 (10ng / ml) and EPO (2U / ml) (ImmunoTools).

On day 4, CD34+ cells were transduced with third-generation lentiviral vectors (Follenzi et al. 2000) expressing shRNA SCR or shRNA RPS19 (Miyake et al. 2005) with an MOI equal to 70. Transduction efficiency was evaluated after three days by detection of GFP with a FACSCalibur flow cytometer (BD Biosciences). On day 4 from transduction the cells were collected and processed for RNA and proteins extraction to evaluate RPS19 haploinsufficiency. Furthermore, the supernatant of cell culture was processed to isolate EVs.

Western Blot analysis

Cells were lysed in Lysis Buffer (50mM Tris-HCl pH 7.5, 1mM EDTA, 150mM NaCl, 1% NP40) supplemented with protease inhibitors (1mM PMSF, 1µg/ml Leupeptin, 0,5µg/ml Aprotinin, 1µg/ml Pepstatin). Cell debris was removed by centrifugation at 13,000 g for 10 min and the supernatant was collected. Protein concentration was measured by Bradford

reagent (Bio-rad). Protein samples were prepared by addition of Laemli Sample buffer and separated on 12% SDS–PAGE, transferred on nitrocellulose membrane (*BioRad*) and incubated with primary monoclonal antibodies specific for RPS19 (Abnova), CD71 (Abnova), p21, p53 and GAPDH (Santa Cruz Biotechnology). Detection of immunoblots was carried out with Western Lightning® Plus-ECL (PerkinElmer). Downregulation or overexpression of the proteins of interest was estimated after normalization to the intensity of GAPDH. Quantification analysis was performed by using *ImageJ 1.43u* software.

qRT-PCR

Total RNA was isolated from CD34+ cells using RNeasy Plus Mini kit (*Qiagen*) and reverse transcribed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). TaqMan® Gene Expression assay (*Applied Biosystems*) was performed to evaluate gene expression of *RPS19*, *TFRC* (CD71) and *CDKN1A* (p21) using *GAPDH* as normalizator.

Extracellular vesicles

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 anemia 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 or cellular surnatant were 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 BFU-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 500nm and 1000nm. Gate positioning was performed evaluating the median corresponding to 1000nm microbeads and exploiting the direct proportionality between the scattered light and the dimension (Figure 7).

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 1 (Zhou and Harezlak 2002)

Results and Discussion

Immunophenotypic analysis of EVs in ribosomal stress

The aim of this part of the thesis was to develop a simple and rapid assay which might support DBA diagnosis. As an innovative approach, we studied immunophenotypes of erythroid EVs.

We studied EVs by flow cytometry, which provides information both on vesicle dimensions and immunophenotypic properties. Our differential centrifugation-based protocol allows us to obtain a heterogeneous population composed by EXO, MV and the smallest AB (Vizziello et al., 2009). Vesicles in the size range of 500nm to 1000nm (EV dimensional gate) were used in this study (Figure 7). This size range, due to the instrument sensitivity limit, comproses a heterogeneous population of EVs including both MV and relatively small AB and excluding EXO .

To characterize eryhtroid EVs, the following markers were chosen: CD34, CD71 e CD235a (Figure 12). Moreover, Annexin V was used to evaluate the presence of phospatidilserine. Isotypic control IgG2A/IgG1 was used as negative control to set the fluorescence.

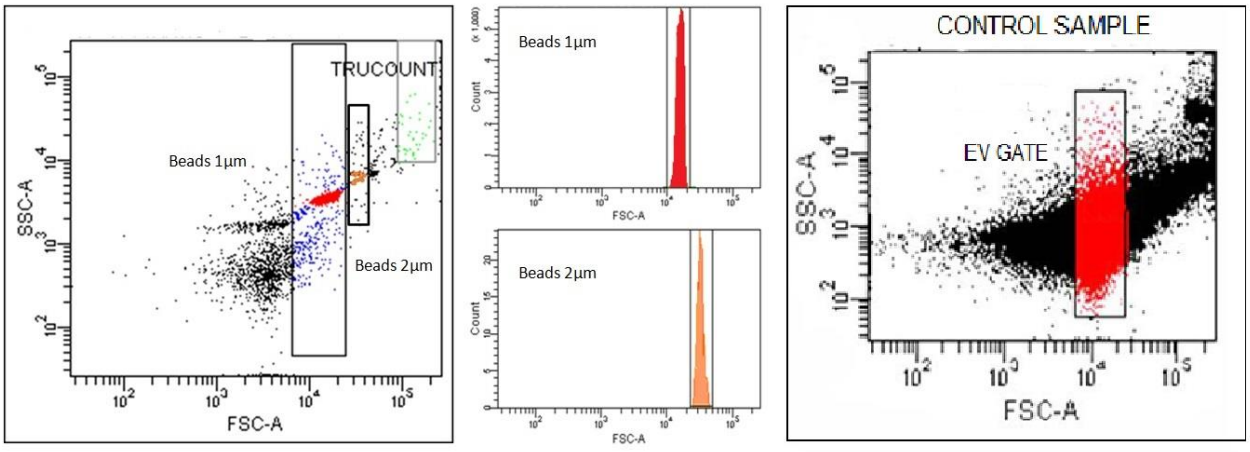


Figure 7. EV dimensional gating strategy. Beads with two diameters (1 and 2 µm) are shown. The beads were analysed in FSC vs SSC plot and in FSC vs number of events histogram. The right panel show the application of the EV dimensional gate on a representative control sample. Standard size beads were not intermixed with the sample.

In the first phase of the project this procedure was set up on our DBA cell model, and subsequently it was translated on plasma EVs from DBA patients compared to non-DBA patients and healthy controls.

Assessment of DBA cell model in RPS19-depleted CD34+ cells

As a proof of concept we assessed EV production in RPS19-depleted CD34+ cells. Healthy donors' CD34+ cells, cultured in the presence of EPO, were transduced with a third generation lentiviral vectors expressing shRNA SCR or shRNA RPS19 with an MOI of 70. Transducion efficiency, measured as GFP expression, was on average 80%.

Gene expression of *RPS19*, *TFRC* and *CDKN1*A were evaluated to validate the DBA cellular model. Downregulation of RPS19 was evaluated in qRT-PCR and Western Blot analysis (Figure 8 and Figure 9). The level of RPS19 transcript and protein was reduced to about 50% thus, mimicking the RP haploinsufficiency showed by DBA patients. As shown in Figure 8, *TFRC* gene expression was halved in RPS19-downregulated cells compared to the control. Accordingly, Western Blot analysis showed a reduction of 50% of CD71 (Figure 9). These findings were in agreement with previous data indicating that RPS19 haploinsufficiency induced a defect in erythroid differentiation (Dutt et al. 2011). Moreover, the densitometry analysis of Western Blot revealed that p53 was enhanced 1,5 fold in RPS19-downregulated cells compared to the control (Figure 9). The increased stability of p53 was consistent with the pro-apoptotic phenotype due to the ribosomal stress. On the other hand, the activation of p53 was evaluated by analyzing its target p21. In particular, *CDKN1A* showed a fold change of 3 by qRT-PCR corresponding to an increase about 2,2 fold of p21 by densitometry analysis of Western Blot (Figure 8 and Figure 9).

In conclusion, we validated our DBA cell model by confirming RPS19-downregulation, the increase of p53 and its target p21 and the block of erythroid maturation.

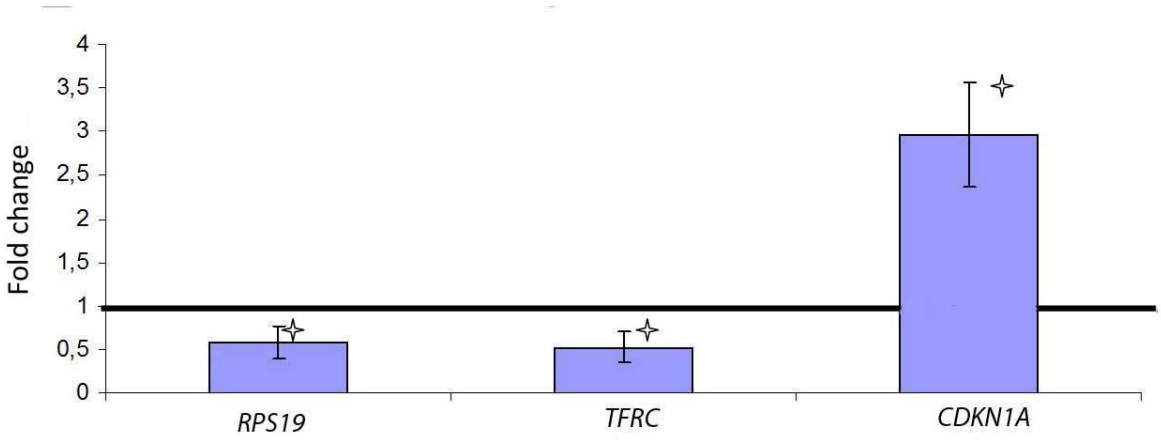


Figure 8. Validation results by qRT-PCR. Fold change of the expression of three altered genes in RPS19-depleted CD34+ cells compared to scrambled control (set equal to 1). Data were obtained by qRT-PCR measurement and normalized to GAPDH. *p value < 0.05. Average of three experiment is shown.

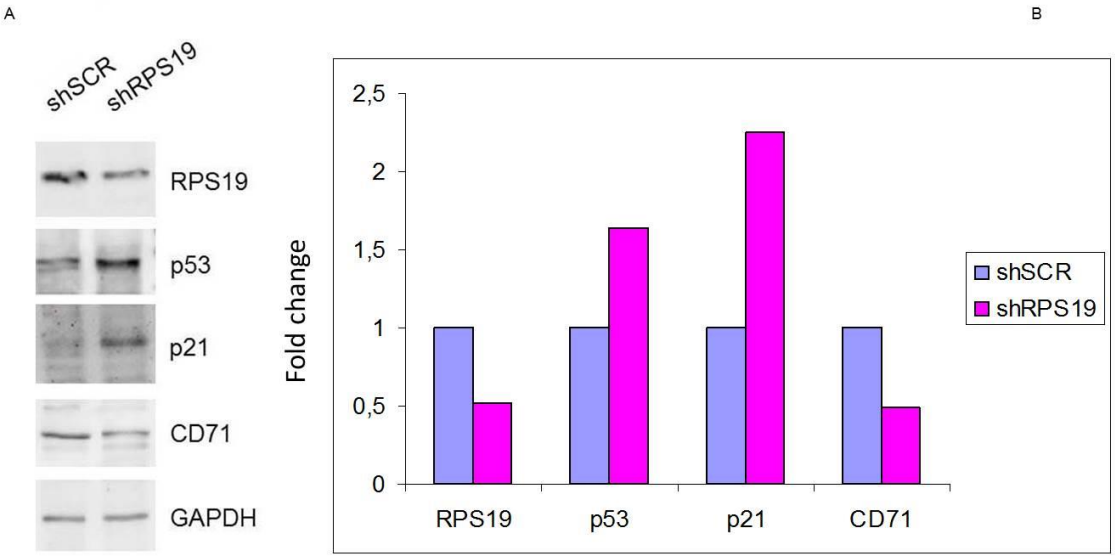


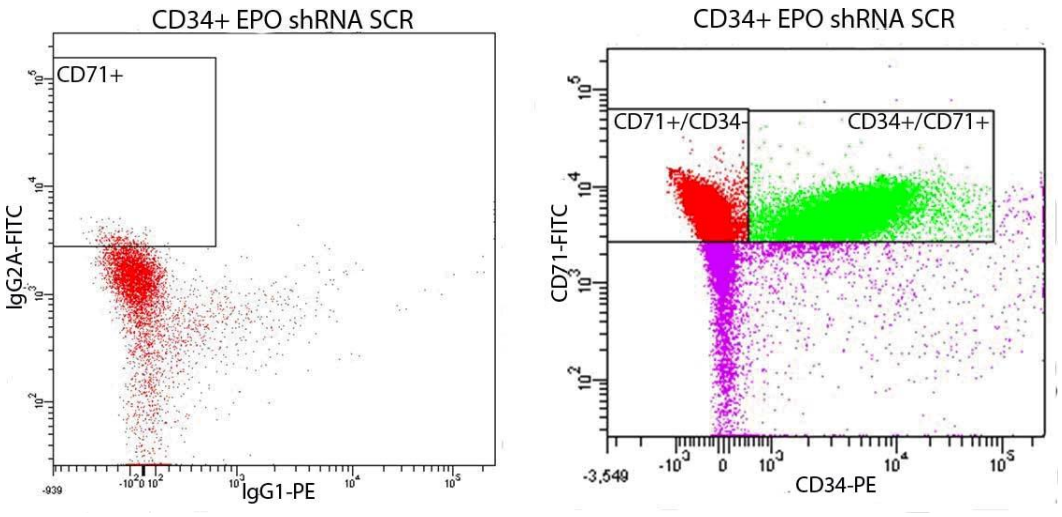
Figure 9. A Western Blot analysis in CD34+ cells treated with EPO and transduced with LV shRNA SCR or shRNA RPS19. 15µg of total proteins were loaded on each lane. **B** Densimotery analysis of Western Blot performed by using *ImageJ 1.43u* software. The signal of protein of interest was normalized to GAPDH and compared to SCR control.

Immunophenotypic analysis of EVs in RPS19-depleted CD34+ cells

Immunophenotypic analysis of EVs isolated from DBA cellular model revealed two distinct populations: CD71+/CD34- and CD34+/CD71+ (Figure 10 and Figure 11). These EV pupulations derived from distinct stages of erythroid differentiation (BFU-E e CFU-E, Figure 1 and Figure 12). Our DBA cell model was maintained in culture less than 10 days. This time was not sufficient to complete erythroid differentiation and this is consistent with the early maturity of EV we observed.

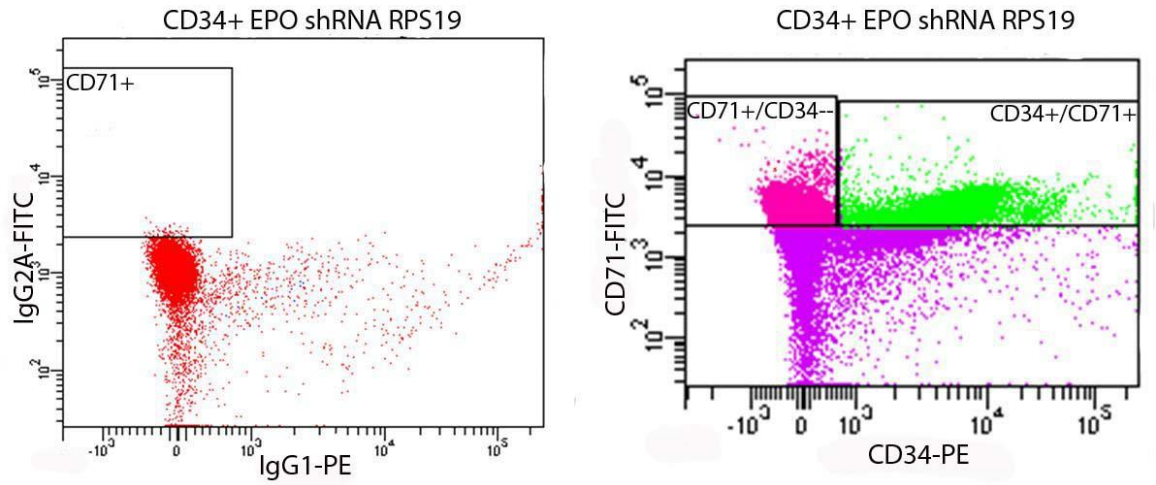
The number of events CD71+/CD34- and CD34+/CD71+ was double in the DBA cell model (EPO shRNA RPS19) compared to the control (EPO shRNA SCR) (Figure 10 and Figure 11).

During pro-apoptotic stress, EV production often anticipates the final stages of cell death and the formation of AB (Simak & Gelderman, 2006). Thus, this enhancement may be due to the increased production of AB by cells undergoing ribosomal stress.



A **B**

Figure 10. Flow citometry analysis of EVs isolated from CD34+ cells treated with EPO and transduced with LV shRNA SCR. **A** Analysis of mixture 1 containing isotype control. The gate show positivity to CD71. **B** Analysis of mixture 2. Two distinct EV populations are gated: CD71+/CD34- and CD34+/CD71+.



A **B**

Figure 11. Flow citometry analysis of EVs isolated from CD34+ cells treated with EPO and transduced with LV shRNA RPS19. **A** Analysis of mixture 1 containing isotype control. The gate show positivity to CD71. **B** Analysis of mixture 2. Two distinct EV populations are gated: CD71+/CD34- and CD34+/CD71+.

These findings supported the presence of quantitative differences between two EV erythroid populations in the RPS19-downregulated cells compared to the control.

Immunophenotypic profiling of erythroid progenitor-derived EVs in DBA: a new diagnostic strategy

The evidence of distinct EV populations in our DBA cell models supported the analysis of erythroid EVs in plasma derived from three groups of individuals: DBA patients, non-DBA patients and healthy controls (Table 4, Table 5, Table 6). 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.

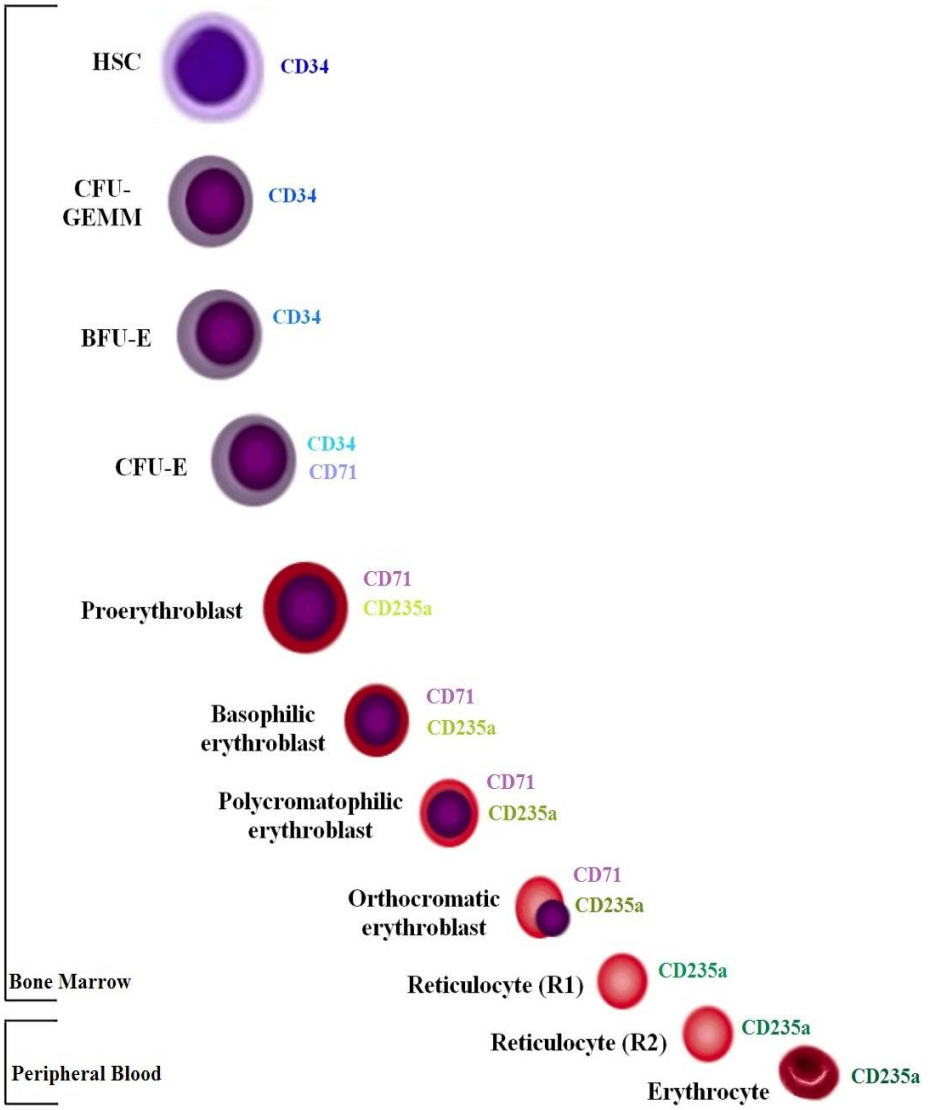


Figure 12. Simplified scheme of erythropoiesis. The most representative markers expressed during erythroid maturation steps are indicated. The intensity colour variation corresponds to expression levels.

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 4: 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. The normal range of e-ADA is 0,8-1,2 U/g Hb.

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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 anemia
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 anemia
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 anemia
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 anemia
33	F	8	STEROIDS	9.9	85.4	3.4	44.7	Aplastic anemia
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 Anemia
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 5: Clinical characteristics of non-DBA patients (ATGT: Anti Thymocyte Globulin Treatment).

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The markers we used were chosen to identify EVs derived from erythroid progenitors (CD34, CD71), erythroid precursors (CD71, CD235a) and cells in the late stage of erythroid maturation, i.e reticulocytes and erythrocytes (CD235a) (Figure 1 and Figure 12). Figure 13 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+). The bulk of these EVs are likely derived from the peripheral blood because if the detected EVs derived also from BM, EV populations derived from all types of erythroid progenitors and precursors would be expected. For example, we have not detected a CD71$_{high}$/CD235a$_{low}$ population, expected to be shed from orthochromatic erythroblasts.

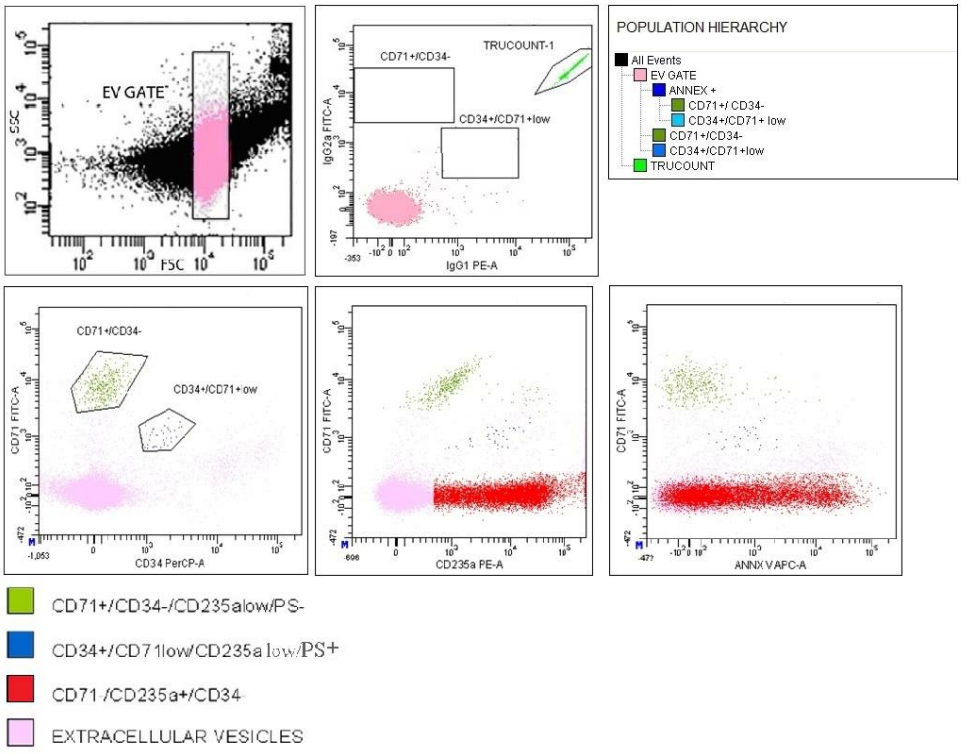

Figure 13. EV distribution in dot plot graphs obtained from the acquisition of the samples incubated with anti-CD71-FITC, anti-CD34-PerCP, anti-CD235a-PE and Annexin V-APC. Only the events occuring in the EV dimensional gate were included. Three EV clusters were identified and indicated with different colours.

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 14, Table 6).

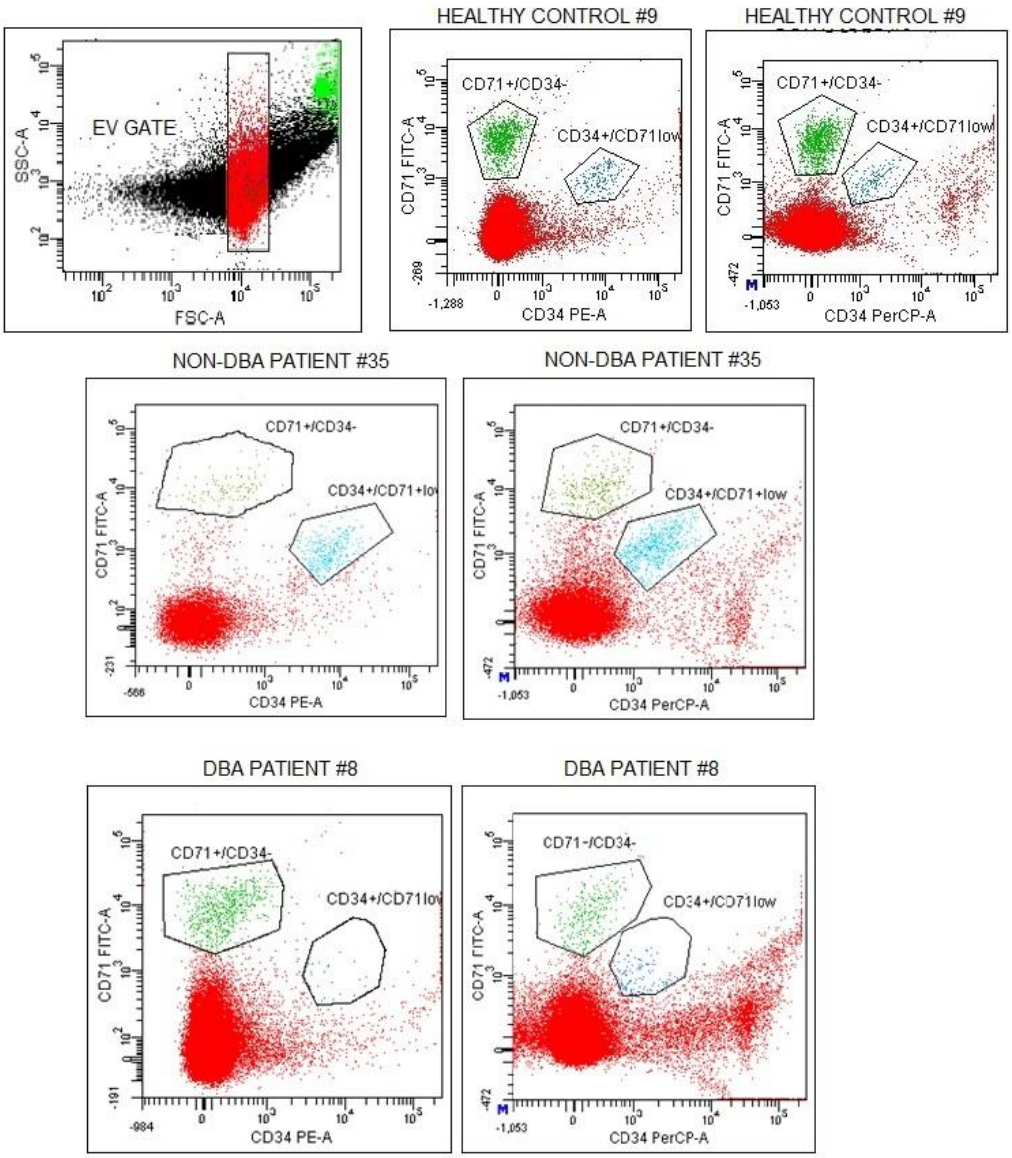

Figure 14. EV distribution in a dot plot graph comparing CD71 and CD34 markers of the events occuring in the EV dimensional gate. CD71+/CD34- (corresponding to CD71+/CD34-/$CD235a_{low}$/PS-) and CD34+/$CD71_{low}$ (corresponding to CD34+/$CD71_{low}$/CD235a+/PS+) populations are shown in representative subjects (healthy controls, DBA patients and non-DBA patients). Comparison of EV populations obtained with or without CD235a staining is shown.

Consequently, we reasoned that the mixture without CD235a was more suitable for diagnostic purposes because it was not influenced by these technical concerns.

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 6: 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).

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- (Ayers et al. 2011). 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 14). 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 15). As expected,

the difference of medians comparing non-DBA patients *vs* healthy controls was not statistically significant (Figure 15). 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.

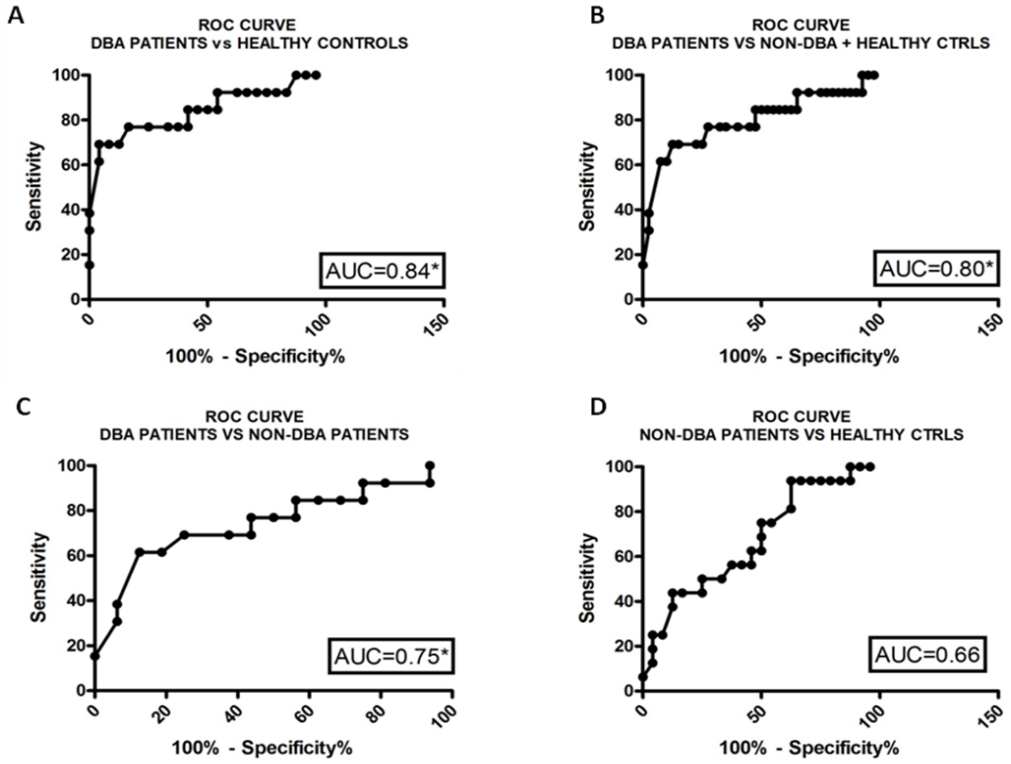

Figure 15. ROC curves analysis of CD34+/CD71$_{low}$ population. ROC curves evaluating the accuracy (AUC) of the CD34+/CD71$_{low}$ analysis in the discrimination of (**A)** DBA patients *vs* healthy controls (**B)** DBA patients *vs* all the others (healthy controls + non-DBA patients) (**C)** DBA patients *vs* non-DBA patients (**D)** non-DBA patients *vs* healthy controls. * p-value <0.01.

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 15).

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 4).

Actually, this population seems to reflect more the bone marrow BFU-E numbers than the level of anemia 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 4). In contrast, we found a single DBA patient (#11) with anemia 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 anemias 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 (Table 5). We also analysed a patient with Fanconi Anemia 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 anemia 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.

This study was focused on the only EV population that was able to distinguish between the three groups under study. Nevertheless we could also draw interesting information from the

other EV populations we characterize. As expected, the majority of EVs derived from circulating reticulocytes and erythrocytes. It was demonstrated that transition between reticulocyte R1 and R2 comprised plasma membrane rearrangements with loss of EV CD235a+ (Griffiths et al. 2012). Surprisingly, in some individuals (DBA patients: #1,#3,#4 and healthy controls: #15) this EV populations also showed a variable positivity to CD71 (Figure 16).

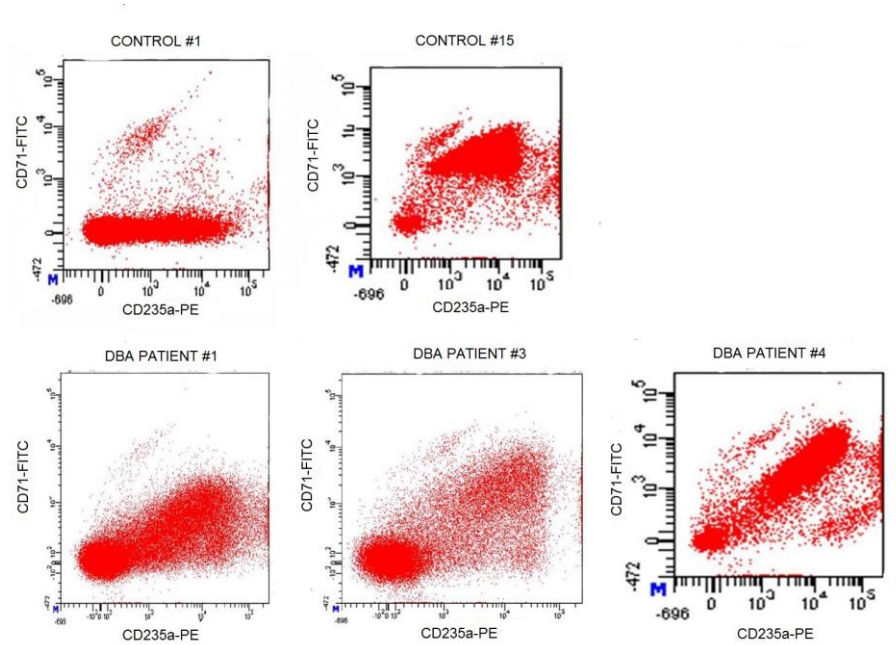

Figure 16. Qualitative analysis of EVs from late erythroid differentiation. Dot plot graphs comparing CD71 and CD235a markers (mixture 3) are reported. The variable positivity to CD71 of EV CD235a+ is shown in control #15 and DBA patients #1, #3, #5 compared to control #1.

It is well known that during the reticulocyte maturation, CD71 is downregulated through EV production because iron uptake is not required anymore (Harding and Stahl 1983; Ney 2011). In conclusion, we speculated that the circulating cells in the last stages of differentiation might not completely loose CD71 in some individuals.

DBA model vs DBA patients

The results obtained from DBA cell model and from plasma of patients showed some differences that may be mainly attributable to *in vitro* and *in vivo* environments,

respectively. We found an increased number of events in our DBA cell model compared to the control in contrast with the results found in DBA patients. It is likely that the increased number of EV produced by CD34+ shRNA RPS19 compared to CD34+ shRNA SCR was due to the enhanced production of smaller AB. *In vivo,* the majority of these AB may be fastly fagocitated by the reticuloendothelial system. Nonetheless, we cannot exclude that LV transduction may contribute to the enhancement of AB observed in DBA model.

Moreover, the model system was able to evaluate the first phase of erythroid differentiation whereas the analysis performed on plasma was the result of the entire differentiation process.

In conclusion, our DBA cell model was useful to set the EV analysis but did not recapitulate the *in vivo* situation.

PLOS ONE

RESEARCH ARTICLE

Immunophenotypic Profiling of Erythroid Progenitor-Derived Extracellular Vesicles in Diamond-Blackfan Anaemia: A New Diagnostic Strategy

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Abstract

Diamond-Blackfan Anaemia (DBA) is a rare inherited anaemia caused by heterozygous mutations in one of 13 ribosomal protein genes. Erythroid progenitors (BFU-E and CFU-E) in bone marrow (BM) show a proapoptotic phenotype. Suspicion of DBA is reached after exclusion of other forms of BM failure syndromes. To improve DBA diagnosis, which is confirmed by mutation analysis, we tested a new approach based on the study of extracellular vesicles (EVs) isolated from plasma by differential centrifugations and analysed by flow cytometry. We chose CD34, CD71 and CD235a markers to study erythroid EVs. We characterised the EVs immunophentoypic profiles of 13 DBA patients, 22 healthy controls and 16 patients with other haematological diseases. Among the three EVs clusters we found, only the CD34+/$CD71_{low}$ population showed statistically significant differences between DBA patients and controls ($p< 0.05$). The absence of this cluster is in agreement with the low levels of BFU-E found in DBA patients. The assessment of ROC curves demonstrated the potential diagnostic value of this population. We suggest that this assay may be useful to improve DBA diagnosis as a quicker and less invasive alternative to BM BFU-E culture analysis.

Introduction

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

Competing Interests: The authors have declared that no competing interests exist.

births. 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]. Approximately 40% of patients display additional clinical abnormalities such as craniofacial, thumb, kidney and heart malformations and growth retardation [2]. Erythroid progenitors (BFU-E and CFU-E) in the patients' bone marrow (BM) show a pro-apoptotic phenotype and their number is reduced in most patients. The erythrocyte adenosine deaminase (eADA) activity is elevated in 80–85% of patients, but cannot be performed in transfused patients [3].

DBA is considered as the prototype of ribosomopathies. Heterozygous mutations in one of 13 ribosomal protein (RP) genes have been found in about 65% of patients [4]. Haploinsufficiency of ribosomal proteins leads to ribosomal stress and activation of p53–dependent and independent pathways, which result in apoptosis and decreased proliferation [5, 6, 7]. A non-ribosomal form of DBA due to mutations in the GATA1 erythroid-specific transcription factor has also been reported [8, 9].

During the course of the disease, approximately 17% of all DBA patients enter spontaneous or steroid-induced remission, defined as a state of transfusion 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 [3].

Diagnosis of DBA is hampered by the presence of other BM failure syndromes such as Fanconi Anaemia (FA), Shwachman-Diamond syndrome (SDS), Dyskeratosis Congenita (DC) and Transient Erythroblastopenia of Childhood (TEC), which can have overlapping clinical presentations [10]. FA is excluded from a diagnosis by negative results in a chromosome breakage assay while the absence of telomere shortening rules out DC. SDS is characterised by pancreatic insufficiency and often associated with skeletal malformations and neutropenia.

The absence of a 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 [4]. 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 an alternative strategy for developing a more inclusive assay for possible use in DBA diagnosis we turned to the study of extracellular vesicles (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. 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. MVs have not an endosomal origin and they are enclosed by plasma membrane fragments [11;12]. 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 [13]. Apoptotic bodies (ABs) are 1–5 μm in diameter. They are released as blebs by cells undergoing apoptosis and they are PS positive.

MVs play a pivotal role in important biological processes such as membrane traffic and horizontal transfer of proteins and nucleic acids among neighboring cells [14]. 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 [15]. No data are available on EV production from erythroid progenitors or early precursors. Classification of EVs, their isolation protocol and detection, molecular details of their release, clearance and biological function are still under intense investigation [16, 17].

In the present study we focused on the immunophenotypic characterization of erythroid EVs from plasma of three different groups of individuals: DBA patients, patients with other haematological 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 marrow of these patients. To our knowledge, this is the first attempt to use EVs in the peripheral blood as an assay for marrow failure and to use EVs as a potential diagnostic tool for Diamond-Blackfan Anaemia.

Three markers were used 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 (progenitors, i.e. BFU-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 (Fig 1) [18, 19]. Additionally, the presence of PS (e.g a marker of ABs and certain MV types) was tested by Annexin-V binding.

Methods

Ethical Statement

The ethical committee of Regina Margherita Children's Hospital (Turin) approved the use of blood samples for diagnostic procedures. A written informed consent was signed by all the individuals under study or their parents, caretakers, or guardians on behalf of the minors/children.

Patients and controls

Peripheral blood samples were collected from DBA patients (*n = 13)* (Table 1), patients with other haematological diseases (hereafter named non-DBA patients) (*n = 16*) (Table 2) and healthy controls (*n = 22*) (S1 Table). For transfusion dependent patients, peripheral blood samples were collected after 2–5 weeks from the last transfusion, depending on their anaemia severity.

EV enrichment

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 EVs were suspended in 1 mL of PBS (filtered using a 0.22 μm pore size membrane) and stored at 4°C.

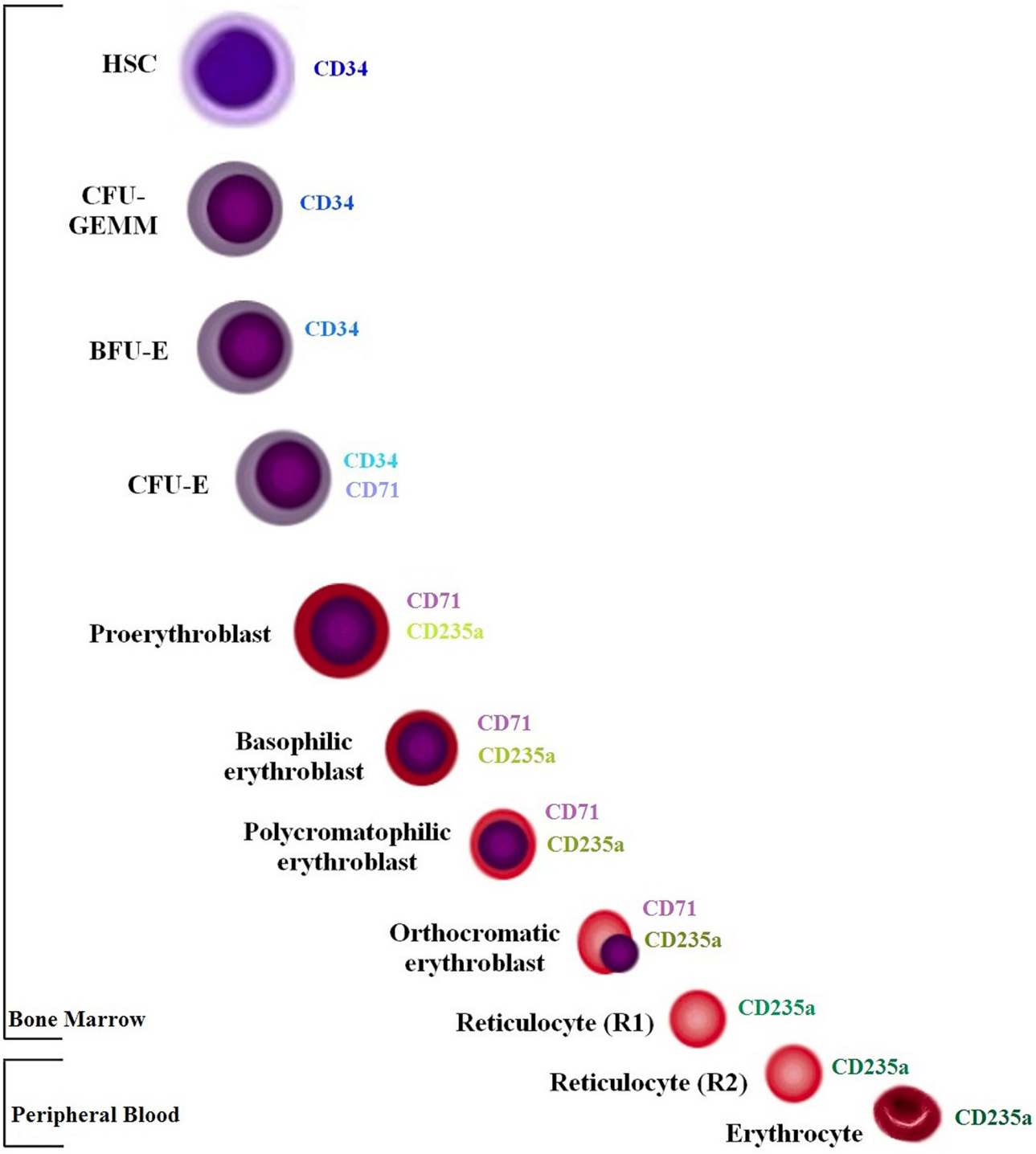

Fig 1. Simplified scheme of erythropoiesis. The most representative markers expressed during erythroid maturation steps are indicated. The intensity colour variation corresponds to expression levels.

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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. The normal range of e-ADA is 0,8–1,2 U/g Hb.

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

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Table 2. Clinical characteristics of Non-DBA Patients.

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)

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EV immunophenotypic profiles

100 μL of resuspended 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 BFU-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-APC 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. 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 (Fig 2, upper left panel and S2 Fig).

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.

ROC curves

Receiver operating characteristic (ROC) curves were established to evaluate the potential diagnostic value of the EV analysis. In a ROC curve the true positive rate (Sensitivity) is plotted in

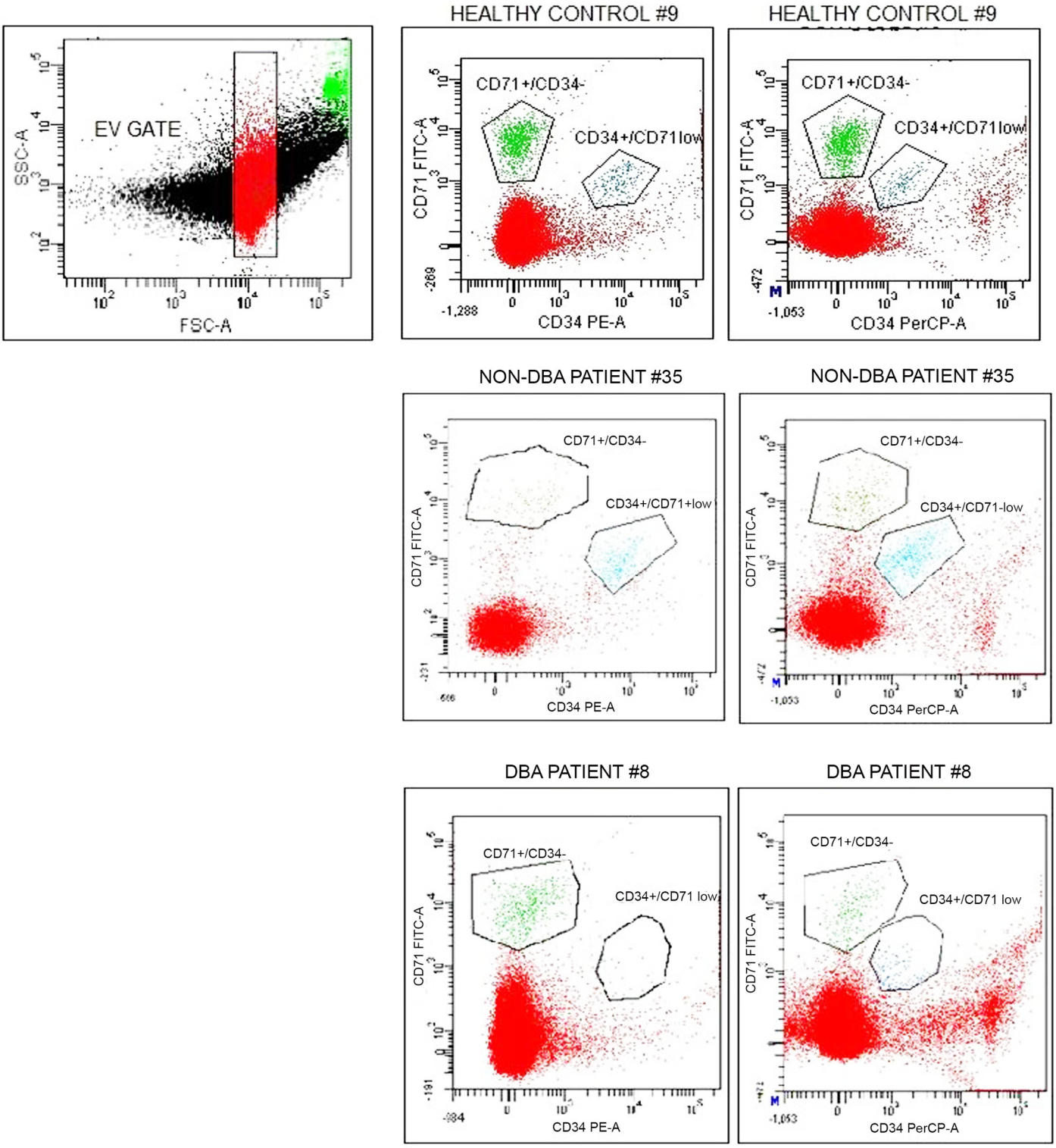

Fig 2. EV distribution in a dot plot graph comparing CD71 and CD34 markers of the events occuring in the EV dimensional gate. CD71+/CD34- (corresponding to CD71+/CD34-/CD235a$_{low}$/PS-) and CD34+/CD71$_{low}$ (corresponding to CD34+/CD71$_{low}$/CD235a+/PS+) populations are shown in representative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

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function of the false positive rate (100-Specificity) for different cut-off points of a parameter. The Area Under the ROC Curve (AUC) is a measure of the accuracy of a parameter in the discrimination of two groups under study. 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 [20].

Results

EV immunophenotypic profile

The markers we used were chosen to identify EVs derived from early progenitors including BFU-E (CD34, CD71), CFU-E (CD71), erythroid precursors (CD71, CD235a) and cells in the late stage of erythroid maturation, i.e reticulocytes and erythrocytes (CD235a) (Fig 1). An isotypic control was used to set the gates of interest (S1 Fig). The immunophenotypic analysis was performed on the events occurring only within the EV dimensional gate (Fig 2, upper panel and S2 Fig).

S1 Fig 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+). 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+/$CD71_{low}$/$CD235a_{low}$/PS+ populations, respectively (vesicles obtained using or not the antibody against CD235, were counted and their numbers corresponded) (Fig 2, and S2 Table). 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- [15]. 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 (Fig 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; Fig 3). As expected, the difference of medians comparing non-DBA patients *vs* healthy controls was not statistically significant (Fig 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.

ROC curves

The assessment of Receiver Operating Characteristic (ROC) curves demonstrated the potential use of the CD34+/$CD71_{low}$ population as a diagnostic tool. The area under the ROC curve

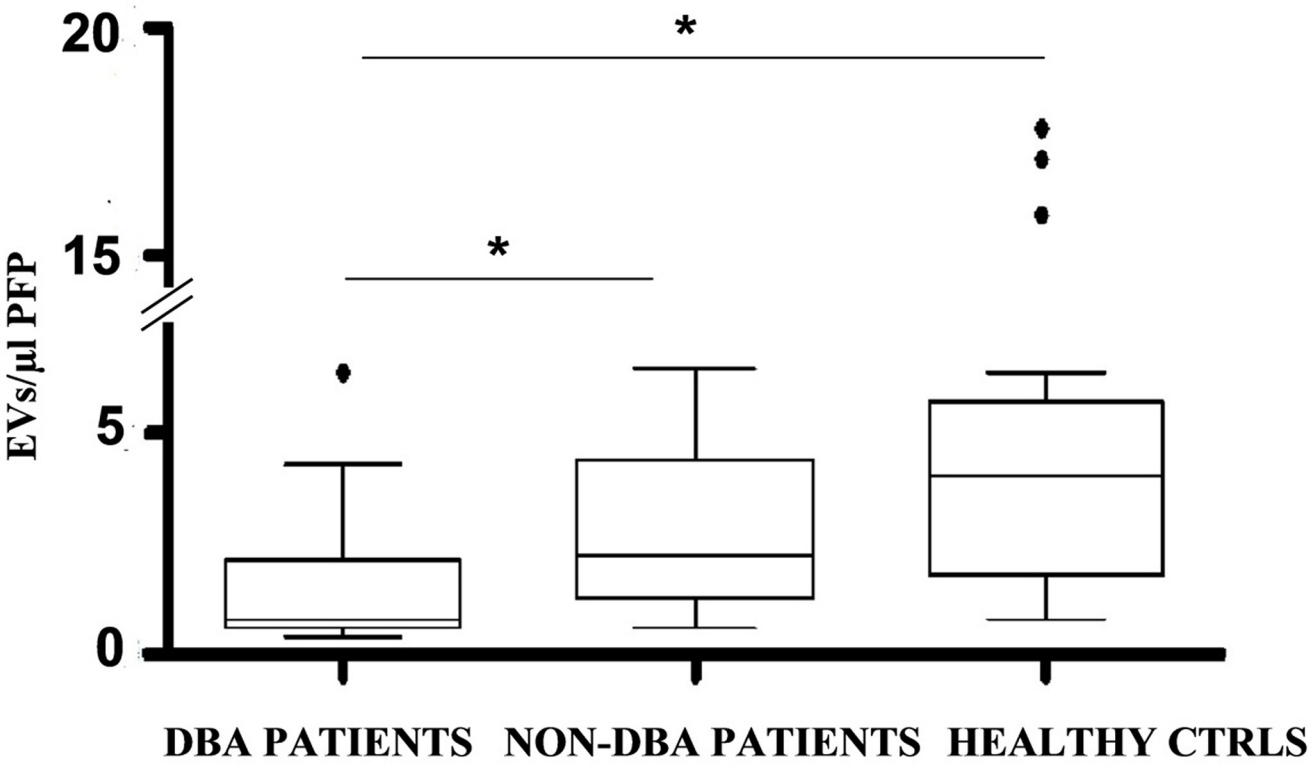

Fig 3. Box plot of Absolute number of events included in the CD34+/CD71$_{low}$ gate. Outliers are shown in black spots. Comparison between DBA patients, non-DBA patients and healthy controls. *The difference of medians is statistically significant (p<0.05, Kruskall-Wallis test).

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(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 (Fig 4).

Discussion

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 were used in this study. This size range includes a heterogeneous population of EVs including both microvesicles and relatively small apoptotic bodies.

Using our approach we were able to identify EVs ranging from early erythroid progenitors to mature erythrocytes. The bulk of these EVs are likely derived from the peripheral blood, because if the detected EVs derived also from bone marrow EV populations, vesicles derived from all types of erythroid progenitors and precursors would be expected.

Importantly, of the vesicle populations studied, the CD34+/CD71$_{low}$ cluster showed statistically significant differences between DBA patients and healthy controls or patients with other haematological diseases (Fig 3).

The potential diagnostic value of the analysis of CD34+/CD71$_{low}$ EVs was assessed using the ROC curve test. AUC values suggest that this test has a good accuracy to discriminate DBA

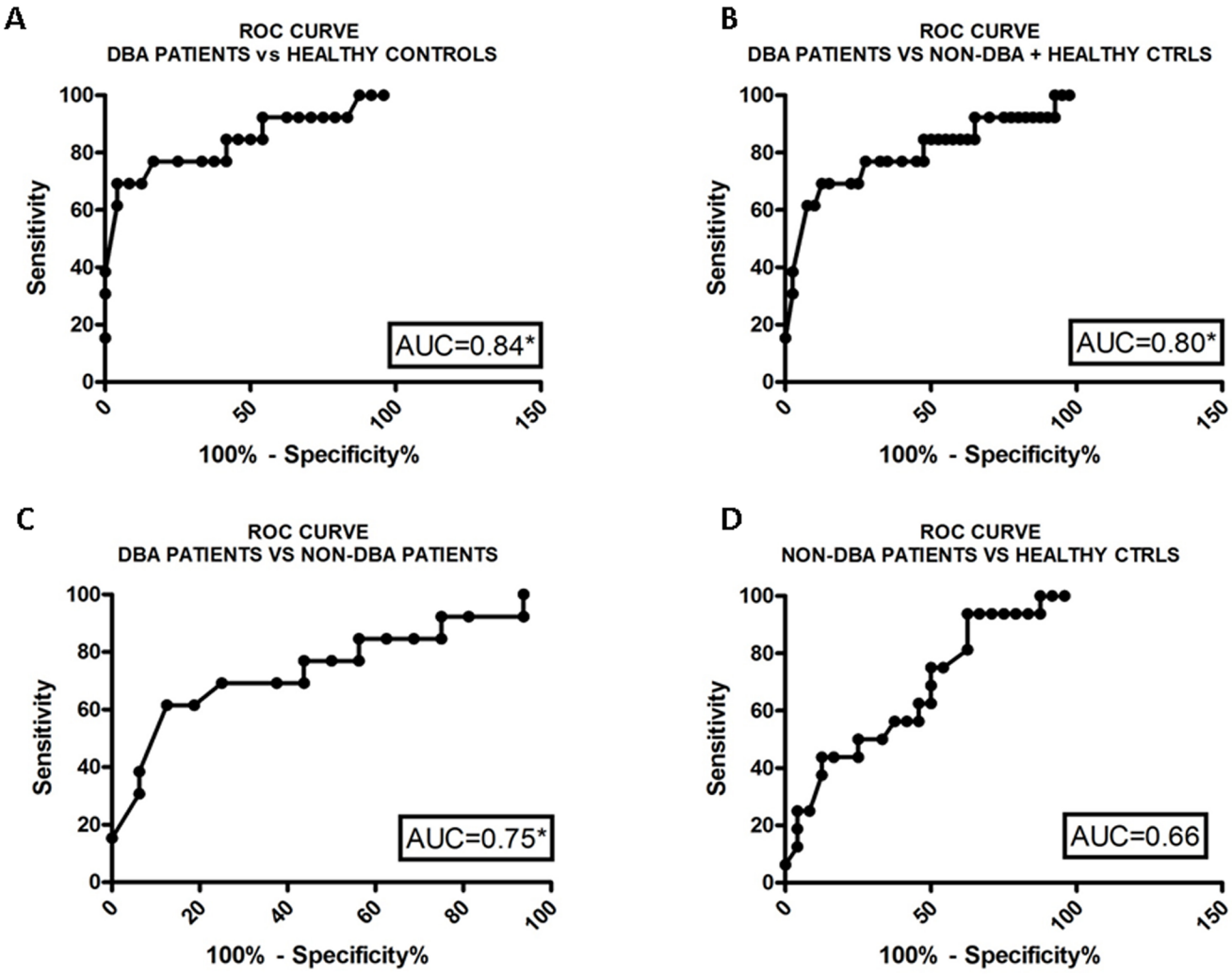

Fig 4. ROC curves analysis of CD34+/CD71$_{low}$ population. ROC curves evaluating the accuracy (AUC) of the CD34+/CD71$_{low}$ analysis in the discrimination of (**A)** DBA patients *vs* healthy controls (**B)** DBA patients *vs* all the others (healthy controls + non-DBA patients) (**C)** DBA patients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

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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.

Supporting Information

S1 Fig. 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.
(TIF)

S2 Fig. 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.
(TIF)

S1 Table. Characteristics of healthy controls.
(DOC)

S2 Table. 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).
(DOC)

Author Contributions

Conceived and designed the experiments: ID PN. Performed the experiments: SM EP RC CV. Analyzed the data: SM EP RC PN. Contributed reagents/materials/analysis tools: AA CB PQ UR PC. Wrote the paper: SM EP RC SRE ID.

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Mutation detection in new candidate DBA genes

Aim

The main purpose of this part of my PhD project was to look for new DBA genes in a subset of Italian DBA patients who were already screened for the 7 RP genes (*RPS10, RPS17, RPS19, RPS26, RPL5, RPL11,* and *RPL35A)* that are most frequently mutated in DBA patients, and for *GATA1* and resulted mutation negative.

I sequenced two genes: *MCM2,* a gene involved in erythropoiesis and *WBSCR22,* a gene involved in ribosome biogenesis.

Rationale of the project

MCM2

This study was performed in collaboration with Dr. David Bodine (National Human Genome Research Institute, Genetics and Molecular Biology Branch, Bethesda, MD) and Dr. Jason Farrar (University of Arkansas for Medical Sciences, Little Rock, AR) who studied a DBA American family by next generation sequencing. The healthy parents, a DBA child and a healthy sibling were studied.

A homozygous missense mutation on *MCM2* (exon9: c.1501G>A; p.G501R) was found in the patient, whereas her brother was wild type and the parents heterozygous (O'Brien et al. 2013). This family could represent a new autosomal recessive form of DBA.

MCM2 (3q21) is a part of the highly conserved mini-chromosome maintenance proteins (MCM) that are involved in the initiation of eukaryotic genome replication (Simon and Schwacha 2014). It forms a complex with MCM4, MCM6 and MCM7 and regulates the helicase activity of the complex. This protein is phosphorylated, and thus regulated by protein kinases CDC2 and CDC7 (Simon and Schwacha 2014).

RNA-Seq analysis of normal erythroid cells at defined stages of differentiation revealed that *MCM2* mRNA levels are low in CD34+ progenitor cells, increase significantly in BFU-E, CFU-E, pro-erythroblasts, early and late basophilic erythroblasts, then decrease significantly in polychromatic and orthochromatic erythroblasts (O'Brien et al. 2013).

O'Brien et al., demonstrated that *MCM2* depletion resulted in significant reductions in the number of CD41-/CD235+ erythroid cells, indicating that this gene play an important role in erythropoiesis. Furthermore, when MCM2 downregulated CD34+ cells were plated in semi-solid medium, CFU-GM colony numbers were normal, but BFU-E colony formation was significantly reduced, suggesting an erythroid specific role for MCM2 (O'Brien et al. 2013).

The same authors found two heterozygous *MCM2* SNVs (single nucleotide variations)

(exon7:c.G1186A:p.A396T and exon13:c.G2179A:p.A727T) in 2 out of 40 American sporadic patients with DBA (Bodine & Farrar, personal communication). These variants were predicted to be deleterious by the following considerations: analysis of prediction tools.

Interestingly, a previous paper of our group showed that RPS19 interacts with MCM2 and with the other components of the DNA helicase complex (MCM6 and MCM7) (Orru et al. 2007). We also found that this interaction is lost for RPS19 mutants (Caterino et al. 2014). Two DBA missense mutations (R62W and R101H) were used in this assay (Caterino et al. 2014). Overall, these results suggested the contribution of this complex in RPS19 function.

WBSCR22

The human *WBSCR22* gene is located in Williams-Beuren Syndrome (WBS) critical region on chromosome 7q11, 23 and consists of 13 exons (Doll and Grzeschik 2001). WBS is a multisystem developmental disorder associated with hemizygous deletion of a ~1.6 Mb region in the given locus (Doll and Grzeschik 2001). The WBS region contains more than 25 genes and the deletion of this region results in haploinsufficiency of WBS control region transcripts (Merla et al. 2006). WBS patients display multiple clinical symptoms including cardiovascular malformations, connective tissue abnormalities, intellectual disability (usually mild), growth and endocrine abnormalities (Schubert 2009).

Different studies showed that the WBSCR22 expression is upregulated in different cancer types, including invasive breast cancer, multiple myeloma cells and hepatocellular carcinoma. Moreover, it was demonstrated that the cell growth is inhibited by treatment with *WBSCR22* siRNA (Nakazawa, Arai, and Fujita 2011; Tiedemann et al. 2012).

WBS patients' lymphoblastoid cell lines show a reduction approximately 2.5 fold of WBSCR22 protein level compared to healthy controls (Ounap et al. 2013). However, it is

currently not known whether this decrease has any contribution to the development of the disorder.

Five recent works identified WBSCR22 as a key player in ribosome biogenesis (Ounap et al. 2013; Ounap et al. 2015; Zorbas et al. 2015; Haag, Kretschmer, and Bohnsack 2015; Letoquart et al. 2014). The human WBSCR22 protein is a 18S rRNA methyltransferase involved in pre-rRNA processing and ribosome 40S subunit biogenesis. Recent studies have shown that the role of WBSCR22 in 40S subunit biogenesis is independent on its function as an rRNA methyltransferase (Zorbas et al. 2015). Indeed, the protein itself, rather than its enzymatic activity, is required for small ribosomal subunit synthesis. Knockdown of WBSCR22 in human cells resulted in accumulation of nuclear pre-rRNA 18SE and a reduced level of free 40S ribosomal subunit (Ounap et al. 2013) (Figure 17).

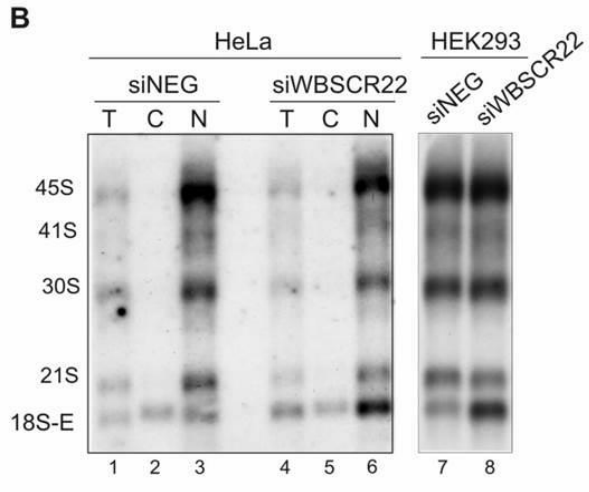


Figure 17. (B) Northern Blot analysis of HeLa (lanes 1-6) and HEK293 (lanes 7, 8) cells transfected with siWBSCR22 or siNeg. with the 5'-ITS1 probe. RNA was extracted 72 h after transfection and 3 µg of total (T), 6 µg of cytoplasmic (C) and 3 µg of nuclear (N) RNA were loaded on each lane. Lanes 7 and 8 show nuclear RNA. The positions of precursor rRNAs are indicated (Ounap et al. 2013).

I reasoned that this gene could represent a good candidate for DBA patients, that showed a similar rRNA precursor pattern. I also considered that the complex clinical phenotype of WBS could not be compared to that of DBA, because it was due to a large chromosomal deletion encompassing more than 25 genes.

Methods

Sanger sequencing

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 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 7 and Table 8. Results were analysed using Chromas Lite 2.1.1. Sequences were read by aligning with the reference sequence, using the LALIGN tool.

In silico analysis

Allele frequency for each SNV was evaluated using 1000 Genomes Project, ExAC and ESP databases. Disease-causing potential was analyzed using six different prediction tools (Mutation taster, SIFT, Provean, Polyphen-2, Mutation assessor, Phyre2). The wild type sequence conservation was reported as a GERP (Genomic Evolutionary Rate Profiling) score as reported on ESP database (GERP score ranges from -12.3 to 6.17, with 6.17 being the most conserved).

Splice site prediction for each SNV was assessed using seven different tools (Human Splicing Finder, MaxEnt, BDGP, NetGene2, GeneScan, FGENESH 2.6, FSPLICE 1.0).

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

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 8. *WBSCR22* primers sequences used for PCR amplification and Sanger sequencing. (**F**: Forward primer; **R**: Reverse primer)

Results and Discussion

The second part of my PhD project was focused on the screening of two new candidate DBA genes: *MCM2* and *WBSCR22*.

For *MCM2* screening from the Italian DBA registry we selected a cohort of 53 DBA patients (22 females and 32 males) who were already screened for the 7 RP genes (*RPS10, RPS17, RPS19, RPS26, RPL5, RPL11,* and *RPL35A)* that are most frequently mutated in DBA patients and for *GATA1*.

The only SNV of interest that I found, was a heterozygous change in exon7: c.G1186A: p.A396T. This SNV was also found by our US collaborators in a single US patient out of 40. Overall, this SNV was found in 2 out of 93 DBA patients with an allele frequency of 0,01.

This SNV was reported in the 1000genomes project with an allele frequency of 0.008 in the European population. The ExAC database reported an allele frequency of 0.01 in the European population. Similarly the ESP database reported an allele frequency of 0.01 referred to European-American population. This allele frequency is consistent with a common polymorphism.

The prediction algorithms reported the following outputs:

SIFT	Damaging
PolyPhen-2	Possibly damaging
Mutation Assessor	Function impact medium
Mutation Tasting	Disease causing
Provean	Neutral
Phyre 2	Unlikely to affect function

The wild type sequence is conserved across the species being GERP score 5.31.

Missense variants may modify splicing. Human Splicing Finder showed that this SNV disrupts an Exonic Splicing Regulatory Sequence (Goren et al.).

This SNV did not affect any functional domain of MCM2 (Figure 18).

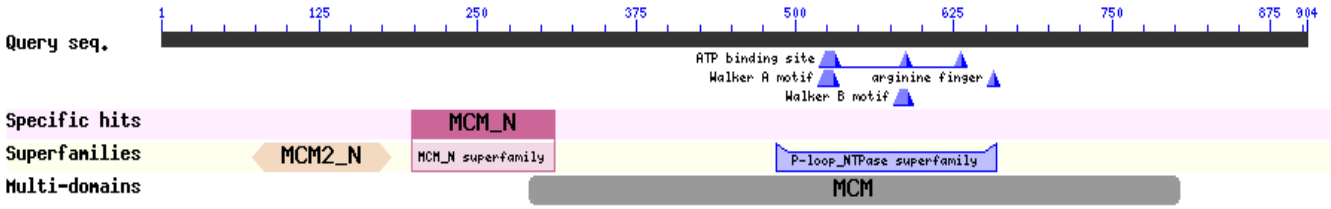


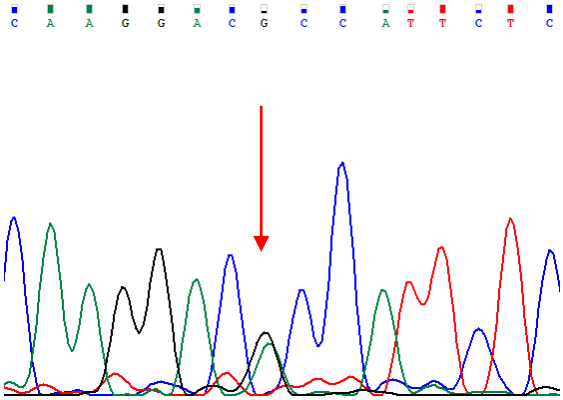
Figure 18. Conserved domains on MCM2 protein sequence (Marchler-Bauer et al. 2015).

We also decided to analyze the proband's parents who did not show any symptoms of anemia. Sanger sequencing on the parents revealed that SNV of interest was inherited from the proband's mother (Figure 19).

In conclusion, this SNV could be considered as a VUS (Variant of Unknown Significance).

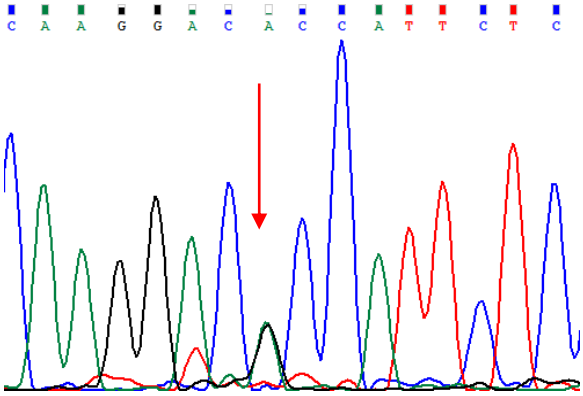
PROBAND

G/A



MOTHER

G/A



FATHER

G/G

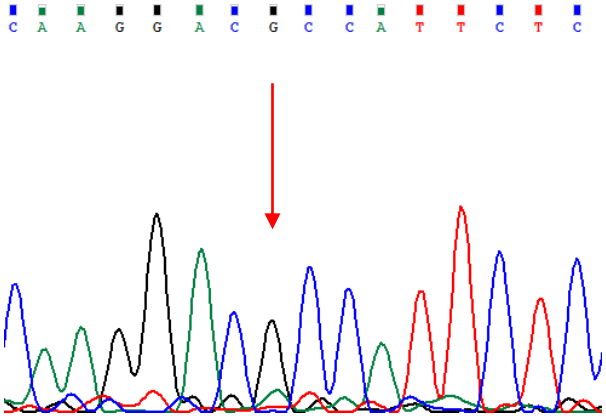


Figure 19. Electropherogram showing c.1186G>A within *MCM2* gene in the proband and parents.

This patient was subsequently evaluated by rRNA processing by Bioanalyzer. The identification of rRNA 28S/18S ratio of 1,2 and the presence of a peak corresponding to

pre-rRNA 32S (Emanuela Garelli, Department of Public Health and Paediatric Sciences, University of Turin, personal communication) induced a reassessment of the patient's sequencing data. This finding suggested the impairment of a ribosomal protein of the large subunit. Thus, resequencing of *RPL* genes was performed in the proband. This led to the discovery of splicing variant in *RPL5*: c.3+5G>A (Emanuela Garelli, personal communication). Analysis of splice site prediction tools (see Methods) revealed that this SNV alters the wild type donor site, most probably affecting splicing.

Taking into account rRNA analysis, this SNV is considered as pathogenic.

In conclusion, our results do not either support or deny a role for *MCM2* in DBA, because we have not found any patients with homozygous or compound heterozygous mutations and the effect of the single VUS we found needs to be studied in a functional assay.

Analysis of *WBSCR22* showed a SNV in the third intron c.256-11A>G. Analysis of this variant by different splice site prediction tools (see Methods) did not predict any splicing alteration. In conclusion, our results do not show a role for WBSCR22 at least in the single patient that was tested, that showed a suggestive rRNA precursor accumulation.

6. Conclusions

The difficulties in clinical diagnosis of DBA and the absence of specific assays were the main focus of my PhD thesis. With the aim to establish a simple and routine test which might support the diagnosis, I turned to the study of EVs. This was an innovative approach since no data were available on erythroid progenitors derived EVs. We reasoned that EVs might alter their properties in response to the reduced number of erythroid progenitors in BM patients'. Among the different erythroid EV populations we found, the only one able to distinguish between DBA patients and the other groups under study was the CD34+/$CD71_{low}$ population. Our finding is in agreement with the low number of BFU-E progenitors in the BM of patients.

The method we devised is reproducible, sensitive and easily translatable to laboratory routine. 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 need to be performed using a bone marrow sample in DBA patients.

The second aim of my project was to analyze to new candidate DBA genes. Although preliminary data strongly suggested *MCM2* as potential causative gene for DBA, our results were not conclusive.

Since the downregulation of *WBSCR22* leads to an accumulation of pre-rRNA 18SE, we screened this gene in a patient with the same rRNA phenotype by Northern Blot, but no sequence variation was found.

7. Future Perspectives

We propose to use our EV-based assay to support the diagnosis of new suspected DBA patients.

It should be interesting to analyze the same membrane markers in peripheral blood cells but this would be hampered by the reduced number of these cells in peripheral blood.

The recent immunophenotypic characterization of each stage of erythroid differentiation paved the way to many applications. In this point of view, we could implement our markers panel to individuate other interesting EVs subpopulations.

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.

Finally, 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.

In the future all the DBA patients with an unusual rRNA precursor pattern should be studied by exome or whole genome sequencing.

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