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Molecular approaches to diagnose Diamond-Blackfan anemia: The EuroDBA experience

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ABSTRACT

Diamond-Blackfan anemia (DBA) is a rare congenital erythroblastopenia and inherited bone marrow failure syndrome that affects approximately seven individuals in every million live births. In addition to anemia, about 50% of all DBA patients suffer from various physical malformations of the face, hands, heart, or urogenital region. The disorder is almost exclusively driven by haploinsufficient mutations in one of several ribosomal protein (RP) genes, although for ~30% of diagnosed patients no mutation is found in any of the known DBA-linked genes. Because DBA is such a rare disease with a particularly wide range of clinical phenotypes and molecular signatures, the development of collaborative efforts such as the ERARE-funded European DBA consortium (EuroDBA) has become imperative for DBA research. EuroDBA was founded in 2012 and brings together dedicated clinical and biological researchers of DBA from France, Italy, the Netherlands, Germany, Israel, Poland, and Turkey to achieve a number of goals including the consolidation of data in patient registries, establishment of minimal diagnostic criteria, and projects aimed at more fully describing the different mutations linked to DBA. This review will cover the history of the EuroDBA registries, the methods used by EuroDBA in the diagnosis of DBA, and how the consortium has successfully worked together towards the discovery of new DBA-linked genes and the better understanding their pathophysiological effects.

1. Introduction

Diamond Blackfan Anemia (DBA, OMIM #105650) is a rare congenital erythroblastopenia that is clinically and genetically very heterogeneous (Vlachos et al., 2008). It represents part of a group of rare genetic disorders known as the inherited bone marrow failure syndromes (IBMFS) (Shimamura and Alter, 2010), and is characterized as a pure red cell aplasia that is also linked to physical malformations (Vlachos and Muir, 2010). Because nearly all the genetic lesions driving DBA to date have been found in ribosomal protein (RP) genes and result in a pre-ribosomal RNA (rRNA) maturation defect, DBA is considered a "ribosomopathy" (Dianzani and Loreni, 2008; Leger-Silvestre et al.,

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2005). This term is applied to disorders in which the pathogenic mutation results in defective ribosome biogenesis and/or the ability of ribosomes to properly translate mRNAs into protein.

The first description of DBA appears in a 1936 issue of Medicine in a chapter titled, "Anaemia of Infancy and Early Childhood" written by Hugh W. Joseph (JOSEPHS, 1936). However, the recognition of DBA as a specific clinical entity is attributed to the American pediatricians Louis Diamond and Kenneth Blackfan, who published a paper describing it in 1938 (Diamond and Blackfan, 1938). In its classical form, DBA affects approximately seven per one million live births and is characterized by a clinical presentation within the first year of life, macrocytic anemia with reticulocytopenia and a normocellular bone marrow with a paucity of erythroid precursors (Clinton et al., 1993). However, by increasing the disease awareness in recent years more patients with atypical DBA manifesting later in life (or who were previously misdiagnosed) are referred to specialized DBA clinics. Physical malformations occur in roughly 50% of patients and include (among others) craniofacial and thumb deformities, short stature, cardiac and urogenital malformations (Ruggero and Shimamura, 2014). Neurological or cognitive problems are very rare in DBA. DBA patients generally exhibit increased levels of fetal hemoglobin and the activity of erythrocyte adenosine deaminase (eADA) is elevated in 80–85% of all patients (Glader and Backer, 1988; Fargo et al., 2013). The risk of DBA patients developing cancer is higher than normal, although the risk does not appear to be as high as with other inherited bone marrow failures such as Fanconi anemia, Shwachman-Diamond syndrome (SDS), or dyskeratosis congenita (Vlachos et al., 2012; Alter et al., 2003).

1.1. Genetics of DBA

The DBA genotype, similar to the phenotype, is highly heterogeneous. The vast majority of allelic variations in DBA genes are mostly sporadic or *de novo* (55% of cases) and familial in the remaining 45%. In several instances of patients inheriting the mutation from a parent, the parent will not show any overt phenotype and are considered "silent carriers". Silent carriers may also exhibit only a macrocytosis without anemia and/or an elevated eADA. The first DBA-linked gene to be identified was *RPS19* in 1999 (Draptchinskaia et al., 1999). Subsequent to this initial finding, the identification of other mutations were revealed in *RPS24, RPS17, RPL5, RPL11, RPS10,* and *RPS26* (Gazda et al., 2006, 2008; Cmejla et al., 2007; Doherty et al., 2010). Many other mutations in RP genes have been identified within the last ten years and today the list includes *RPS7, RPS10, RPS15A, RPS17, RPS19, RPS24, RPS26, RPS27, RPS28, RPS29; RPL5, RPL9* (in review), *RPL11, RPL15, RPL18, RPL26, RPL27, RPL31, RPL35,* and *RPL35A* (Boria et al., 2010; Gazda et al., 2012; Landowski et al., 2013; Farrar et al., 2014; Wang et al., 2015; Mirabello et al., 2014, 2017a; Gripp et al., 2014). This list represents 20 of the 80 functional RP genes in humans. Based on published observations (and unpublished observations of EuroDBA partners) it can be noted that the majority (> 90%) of mutations (including large deletions) occur in only 6 genes (*RPS19, RPL5, RPS26, RPL11, RPL35A*, and *RPS24*). Other genes (such as *RPS29, RPS17, RPS7, RPS10, RPL15,* etc.) are mutated only in very few DBA patients worldwide and account for less than 10% of all mutated cases.

All the RP gene mutations identified in DBA patients to date are heterozygous. Homozygosity is largely suspected to be lethal, a suspicion supported by the lethality of homozygous RP gene mutations in several animal models including zebrafish and mice (Amsterdam et al., 2004; Matsson et al., 2004). A wide range of mutation types is evident and at least in some cases appears to depend on the particular RP gene. Most of the missense mutations have been identified in the *RPS19* gene while predominantly nonsense mutations, small deletions or insertions, and splice site mutations are found in *RPL5* and *RPL11* (Gazda et al., 2008; Willig et al., 1999a). Partial- and whole-gene deletions have been detected (depending on the study cohort) in 10–20% of DBA patients using various copy-number methods (quantitative PCR, multiplex sequencing [MLPA], CGH and SNP arrays), mostly in *RPS17*, *RPL35A*, and *RPS19* genes (Farrar et al., 2011; Kuramitsu et al., 2012; Quarello et al., 2012).

While DBA is considered almost exclusively linked to RP gene mutations, two non-RP genes have been reported in patients including *GATA1* and *TSR2* (Klar et al., 2014; Parrella et al., 2014; Sankaran et al., 2012; Wegman-Ostrosky and Savage, 2017). However these genetic lesions are extremely rare; only five DBA patients have been reported carrying a *GATA1* mutation and only one with a *TSR2* mutation. The *TSR2* gene is related to ribosome biogenesis since it is involved in pre-rRNA processing and binds to eS26 (RPS26) protein. The *GATA1* gene encodes for the major erythroid transcription factor GATA1 and is not reported to be involved in ribosome biogenesis.

In a substantial number of patients (approximately 30%) the underlying genetic defects remain unknown despite the routine screening of the known RP genes linked to DBA. However, with the increasing availability and diagnostic role of next generation sequencing methods, including multiplex gene sequencing and whole exome sequencing (WES), novel genetic defects are being slowly but steadily identified (Wegman-Ostrosky and Savage, 2017).

1.2. History of European DBA registries

The rarity of diseases like DBA makes it difficult for one institute or clinician to become the centralized point of patient care. This difficulty exacerbates collecting the already sparse amount of clinical and biological data and using them to generate meaningful genotype:phenotype correlations. Thus the key to success when it comes to understanding and ultimately defeating DBA, or any other rare disease, is collaboration. Although national and international collaborations can be challenging, extraordinary progress has been made in developing, funding, and maintaining groups of clinical and biological researchers who share the same goal: To better understand and ultimately cure a specific rare disease such as DBA.

DBA networks preceded the creation of formal patient registries in Europe. The pioneering group in 1995 included clinicians from France, Germany, Italy, England, Sweden, and Switzerland under the umbrella of the European Society for Pediatric Haematology and Immunology (ESPHI) and the Société d'Hématologie et d'Immunologie Pédiatrique (SHIP). Their goals were simple and straightforward: To share DBA clinical data and samples, to build registries, and to test new drugs (Ball et al., 1996; Bastion et al., 1994; Gustavsson et al., 1998; Ramenghi et al., 1999; Willig et al., 1999b). By working together this group shared a major achievement in 1999 with the discovery of *RPS19* as the first known DBA-linked gene (Draptchinskaia et al., 1999). This gene today remains the most commonly mutated gene found in ~25% of DBA patients and as such is routinely the first gene candidate sequenced when genotyping a patient.

The first observational DBA patient registries were initiated in the Czech Republic in 1988 and officially announced in 1992 (Pospisilova et al., 2012). This was shortly followed by registries in the USA (DBAR, 1993) (Pospisilova et al., 2012; Vlachos et al., 2001), Germany (1993), France (1995) and Italy (1995). The Italian registry is maintained as an online registry freely accessible to clinicians (Campagnoli et al., 2004). In a similar strive to create greater transparency and openness the Italian group developed the publically available online RP gene database including mutational data from countries worldwide in 2008 (http://www.dbagenes.unito.it). This registry regularly updates DBA mutation data and remains the standard go-to database when researchers are querying the novelty of recently identified mutations in their patients (Boria et al., 2008).

While the registries mentioned above contain the majority of European DBA patients due to the size of the host country's population, it is not necessary for a country to be highly populated in order to establish a meaningful registry. This is illustrated by the Israeli registry,

which was founded in 2007 (Tamary et al., 2010). Although Israel has a relatively low population, the respective registry contains virtually all known DBA patients in the country. This allows the registry data to be used for very precise statistical measurements of disease and phenotype frequencies that are far more difficult in larger countries. Another example is the incipient Dutch DBA registry, which was founded this year. The fact that there are a limited number of clinics in close proximity that treat DBA patients in the Netherlands resulted in the establishment of a substantially sized registry (43 patients) in a very brief period of time (van Dooijeweert et al., in press European Journal of Haematology).

In contrast, the initiation of patient registries in large or heavily populated countries can seem like a daunting task. This is especially true in countries that may not have access to or funding for state-of-the-art molecular diagnostics. An example of this is Poland, before it became a EuroDBA member in 2016. From 1998 to 2016 genotyping of Polish patients had been performed in collaboration with Boston Children's Hospital, which contributed to the discovery of *RPL5, RPL11, RPS10,* and *RPS26* as DBA-linked genes (Gazda et al., 2008; Doherty et al., 2010). Another example is Turkey. An estimated 100 patients were diagnosed with DBA in the various hematology clinics around the country. However, there was no centralized point of care until 2014 with the development of the Inherited Bone Marrow Failure Center at Hacettepe University in Ankara. Today, both Turkey and Poland are members of EuroDBA and have the necessary funding required to build their own patient registries and systematically genotype their own patients.

The establishment of these patient registries represents a crucial step in creating a global DBA network. Beyond Europe, many other countries around the world have in recent years successfully established their own DBA patient registries (Table 1). The populations of countries initiating these registries range from over a billion (China) to fewer than 3 million (Lithuania). Thus the size and population density of any given country should not be considered a deterrent when deciding to establish a patient registry for a rare disease.

Table 1
Numbers of DBA patients reported in countries worldwide. Patient numbers were retrieved from recent literature, personal communications, and presentations from the 2014 DBA Global Bridges Meeting. Population numbers were retrieved from http://www.worldometers.info/world-population/ on the 18th of May 2017. 🗁 = Formal registry in progress. DBAR = Diamond Blackfan Anemia Registry of North America. *DBAR is loosely affiliated with Canada, Australia, and Mexico.

Geographical Location	Population (in millions)	Number of Patients	Formal Registry
Europe, EuroDBA members			
Germany/Austria/ Switzerland	80.6/8.6/8.5	330/20/30	✓
France	64.9	356	✓
Italy	59.8	265	✓
The Netherlands	17	43	✓
Israel	8.3	46	✓
Poland	38.6	36	🗁
Turkey	80.4	65	🗁
Europe, other			
Czech Republic	10.6	61	✓
Greece	10.9	17	✓
United Kingdom	65.5	104	✓
Lithuania	2.8	4	
Denmark	5.7	17	
Sweden	9.9	40	
Norway	5.3	22	
Finland	5.5	10	
Spain	46.1	45	
Internationally reported cohorts			
United States (DBAR)	326.2	750	✓
Egypt	95.2	22	
China	1400	104	
South Korea	50.7	60	
Japan	126	68	
Russia	143.4	90	
Iran	80.3	30	
Saudi Arabia	32.3	30	

1.3. History of the European DBA consortium

In 2012, the European Union's ERA-Net for Research Programs on Rare Diseases (ERARE) issued a transnational call specifically for Young Researchers. This call led to the founding and successful funding of the European Diamond-Blackfan Anemia consortium, EuroDBA. The original EuroDBA members were clinical/biological scientists in hematology who were organizers of the two largest DBA patient registries in Europe at the time (Germany and France) together with the coordinator, a biological researcher from the Netherlands with expertise in ribosomal proteins. This consortium was initiated with three major goals. One was to identify and characterize novel genetic lesions in the registered patients who did not have a mutation in any of the known DBA-linked genes. The second was to fully clarify and disseminate up-to-date clinical treatments and guidelines for patient care. The third was to develop molecular and cellular methods, including the use of zebrafish models and patient cell lines, to more fully understand the pathophysiology of DBA.

The EuroDBA network over the next years expanded to include as associated partners other European countries that hosted DBA patient registries, such as Poland, the Czech Republic, Italy, Spain, and Israel. In 2015 the funding for EuroDBA was renewed and the consortium was able to formally include many of the aforementioned countries. Moreover, the renewal allowed for the inclusion of the clinical groups in Poland, Turkey, as well as another group of biological researchers in France with expertise in pre-rRNA processing and how it is impaired by RP gene mutations.

1.4. Initial DBA diagnostics

DBA patients, usually young infants (median age at diagnosis 2 month-old), typically present at the clinic with the basic hallmarks of anemia including pale pallor, failure to thrive, and feeding difficulties. After collecting the familial history of the patient, the first test is typically a blood smear and a Complete Blood Cell count together with a reticulocyte count. DBA may be suspected if hemoglobin (Hb) concentration is low for the age of the patient, with absent or low reticulocyte count ($< 20 \times 10^9$/L) and often a macrocytosis (which is age-adjusted). Fetal Hb might also be increased, however this is an unspecific marker that is also elevated in other bone marrow disease states. Most groups include a supportive eADA analysis of blood prior to transfusion. This DBA-specific marker is elevated in 80–90% of DBA patients who are not regularly transfused. A high erythropoietin level may help with the diagnosis, reflecting the intrinsic defect of bone marrow in DBA patients. A positive family history for anemia, and/or syndromic features (present in at least 50% of DBA patients) is also indicative of DBA. Bone marrow aspiration is performed to determine the content of erythroid precursors, which in DBA is typically low (below 5%). In cases of late-onset DBA (or delayed diagnosis) e.g. in adolescents or adults, marrow might display hypocellularity with dysplasias and megaloblastic changes resembling low grade MDS or 5q-syndrome. During the initial workup, other differential diagnoses should be considered. These include parvovirus B19-associated pure red cell aplasia (identified by cytology, IgM/IgG serology and in immunodeficient patients, by a PCR analysis of blood and/or bone marrow samples), which is rare and also might present with additional pancytopenia. Other differential diagnosis is transient erythroblastopenia (TEC), which however usually manifests beyond the first year of age (and patients show normal MCV, eADA and HbF values). Unlike DBA, both parvovirus B19 infection and TEC are neither associated with positive family history nor with congenital anomalies. Sometimes, in unclear cases, clinicians might want to rule out other

Table 2
A description of the routine and molecular steps taken by different EuroDBA partners in the diagnosis of DBA. [a] tested in EDTA-blood prior initial transfusion or at least 4–6 weeks after last transfusion. *Testing performed not routinely but rather in cases with atypical presentation. [‡] Genes sequenced only when following scenarios are met: GATA1, RP-genes are negative and the proband is male; TSR2: RP-genes are negative and the patient has typical facial anomalies. Abbreviations: HbF, fetal hemoglobin; eADA, erythrocyte adenosine deaminase; BM bone marrow; IBMFS, inherited bone marrow failure syndromes such as Fanconi anemia and Shwachman-Diamond syndrome; Sanger, Sanger-based sequencing; RP, ribosomal protein; CGH, comparative genomic hybridization; SNP, single nucleotide polymorphism; MLPA, multiplex ligation-dependent probe amplification. DE = Germany, FR = France (Paris), IL = Israel, IT = Italy, PL = Poland, TR = Turkey.

		DE	F	IL	IT	PL	TR
INITIAL NON-GENETIC WORKUP	aCBC + MCV, Retic count	+	+	+	+	+	+
	aHbF/eADA	+/+	+/+	+/+	+/+	-/+	+/+
	BM morphology	+	+	+	+	+	+
	BM karyotyping	-	-	+	-	+	-
	Parvo-B19 BM PCR	-*	+	+	+	+	+
	Test other IBMFS	-*	+	+	+	-*	+
	Syndromic workup(echo/ ultrasound/ x-ray)	+	+	+	+	+	+
MOLECULAR DIAGNOSTICS	Sanger RPS19	+	+	+	+	+	+
	Sanger/targeted NGS:						
	Common RP genes	+	+	+	+	+	+
	Uncommon RP genes	+	+	+	+	-	+
	GATA1/TSR2	+[‡]	+[‡]	+	+[‡]	-	+
	CGH/SNP array	+	+	+	-	-	+
	RP-specific MLPA	-	-	+	+	-	-
	Whole Exome Seq	+	+	+	+	-	+

IBMFS such as Fanconi anemia or SDS. Finally, in the rare patients diagnosed later in life (young adult and adults), an immune erythroblastopenia needs to be ruled out. The steps that the different clinical partners of EuroDBA undertake in this initial non-genetic workup are shown in Table 2.

1.5. Molecular DBA diagnostics

Because the *RPS19* gene is by far the most frequently mutated gene in DBA (25% of cases), most screening analysis begins with targeted Sanger sequencing of *RPS19* (Table 2). This approach uses PCR amplification and sequencing of each RP gene exon and promoter region by specific forward and reverse primers in both directions. The subsequent genetic diagnostics does not fit a “one for all” approach to identify mutations, intra-exonic, full exon or whole gene deletions. Based on the availability of routine and sophisticated genomic methods, different approaches were developed in different countries (Table 2). The first goal is to identify the most common genetic defects using routinely available methods such as Sanger sequencing or CGH array. Next generation sequencing (either targeted, or whole exome) might not yet be accessible to all laboratories, however recent developments in clinical diagnostics will likely lead to routine use of NGS instead of Sanger sequencing. Additional novel non-genetic techniques have been developed that reduce the time and cost of the molecular diagnosis of DBA.

One newly developed method takes advantage of the fact that rRNA in cells with small RP mutations typically reveals an increased 28S/18S ratio, while rRNA in cells with large RP mutation reveals a decreased 28S/18S ratio (Farrar et al., 2014). To read these ratios, a Bioanalyzer can be used to measure the levels of different rRNA strands. Ethidium bromide gels are also used to visualize if a visible 32S band exists, which is typically indicative of an RPL gene mutation. EBV-immortalized cells (if available) can be used for this technique, so may T cells isolated from patient blood that are subsequently activated with phytohemagglutinin. This method, used in Italy, can rapidly determine the presence of large or small ribosomal subunit defect. Such results can be helpful in deciding which common candidate DBA genes should then be sequenced by Sanger.

Since the routine use of NGS, many samples can be investigated for the presence of mutations in multiple RP genes at once (along with any others such as *GATA1* and *TSR2*). This approach was developed within the French group of EuroDBA, and serves as a standard platform for other consortia members. The approach uses Roche “NimbleGen SeqCap EZ” library and an Illumina flowcell (Flowcell standard 2 × 150) with a library of 144 genes including 74 genes for red cell disorders. The sequences are run on a Miseq or a Nextseq, analyzed on a CLC Biomedical workbench (Qiagen), and the allelic variations are then verified by Sanger technique. Other approaches (e.g. initiated in Germany and Turkey) include sequencing of the few most commonly mutated RP genes using Sanger sequencing, rapidly followed by commercial exome sequencing in case of negative results.

In case of a negative mutational result, large deletions are screened either by RP-specific MLPA such as in Italy or by high-resolution CGH array such as in Germany, France, and Israel. New bioinformatics algorithms allow for the use of whole exome data to compile copy number maps that can also identify microdeletions encompassing RP genes. Commercial probes and kits need to be verified before use since the probes might not sufficiently cover most of the RP genes. The percentage of DBA patients with unknown genetic cause is similar in all registries and can be estimated at approximately 30%. If an RP mutation or other deleterious mutation has not been found, exome sequencing should be performed. Ideally, this requires the availability of trios (both biological parents and the patient) to reduce as much as possible the number of variants of unknown significance that exome sequencing unfailingly reveals. Consanguinity is a very rare facet of DBA inheritance, and the search for novel gene mutations in children of consanguineous marriages usually focuses on monoallelic alterations. In index patients who are the only affected family members, the analysis naturally targets potentially pathogenic *de novo* variants, which however will exclude the scenario of novel mutations associated with silent carrier status in the parents. After exome sequencing studies, many (if not the majority, according to unpublished observations from the French and German DBA registries) may still have unresolved genotypes. It is possible that such patients may carry mutations in promoter/enhancer or deep intronic regions of RP or other erythropoiesis-specific genes, other structural genomic anomalies might be present, or multigenetic causes underlie disease manifestation. As mentioned above, most patients analyzed using exome sequencing will carry multiple potentially pathogenic variants of unknown significance, requiring lengthy molecular and cellular analysis to be performed before any conclusions can be drawn.

1.6. DBA genotypes and phenotypes

As the number of registered patients grows the more readily researchers are able to identify and characterize DBA clinical and cellular phenotypes in a way that would be impossible with small patient cohorts. Reported correlations between clinical phenotypes and mutations in specific RP genes include *RPL5* mutations associated with physical malformations such abnormal thumbs, cleft palate, and cardiac defects. *RPL11* mutations are reported to associate with thumb abnormalities (including triphalangeal thumbs) and mutations in *RPS26* with skeletal defects (Boria et al., 2010; Pospisilova et al., 2012). The EuroDBA network recently identified a group of unrelated patients with truncating *RPL15* mutations who all presented with very severe and early

onset of anemia (hydrops fetalis in most cases). Even more remarkable was that within this group, the patients carrying the same point mutation in *RPL15* all became treatment independent (in revision). Although it is widely known that between 20 and 25% of DBA become treatment-independent at some point during their lives, this is the first indication of a genetic association with this clinical phenomenon. The challenge of drawing meaningful genotype:phenotype correlations is present in any rare disease patient cohort, and is especially true with DBA where often there are fewer than five reported patients with a mutation in the same RP gene. However, with the increasing cooperation of clinics with DBA patient registries, such as those within the EuroDBA network, the challenge is no longer insurmountable.

One surprising feature of RP gene mutations is that by no means are all RP genes equal, despite their past reputation as “housekeeping genes”. In addition to driving DBA, inherited haploinsufficient mutations of RP genes are starting to be reported in non-hematopoietic congenital disorders. Disorders such as intellectual disability, autism, and dysmorphism are linked to *RPL10* and *RPS23*; congenital asplenia is linked to *RPSA;* and hereditary nonpolyposis colorectal carcinoma is linked to *RPS20* in patients who have no evidence of anemia (Paolini et al., 2017; Thevenon et al., 2015; Zanni et al., 2015; Bolze et al., 2013; Nieminen et al., 2014). Additionally there are a number of parents who carry the same RP mutation as their DBA-afflicted child yet have no clinical features at all, known as “silent carriers”. Although the reasons behind these differences are not well understood, there do seem to be some similarities in terms of the molecular consequences. For examples, the inherited mutations in *RPS23, RPS20,* and every DBA-linked RP mutation studied to date result in ribosome biogenesis defects, including impaired pre-rRNA processing and abnormal ribosomal subunit formation (Paolini et al., 2017; Nieminen et al., 2014; Pereboom et al., 2014; Choesmel et al., 2007).

The defects in ribosome biogenesis by RP gene mutations have been proposed to activate the TP53 tumor suppressor pathway by inducing stress in the nucleolus, the cellular organelle where ribosome biogenesis originates (Horos and von Lindern, 2012). However, one of the great puzzles of DBA is why, if RPs are expressed in every cell in the body, are erythrocytes so specifically affected when one copy of an RP gene is mutated? The specificity of the defects to erythroid cells has not been satisfactorily explained, although theories ranging from hypersensitivity of erythroblasts to elevated TP53 levels, a high protein demand in rapidly dividing erythroblasts, cell-specific translation and splicing defects and the induction of autophagy have also been proposed as mechanisms that result in the reduction of erythrocyte progenitor cells (Dianzani and Loreni, 2008; Horos and von Lindern, 2012; Paolini et al., 2016; Dutt et al., 2011; Fumagalli and Thomas, 2011; Heijnen et al., 2014; Horos et al., 2012; Narla et al., 2011; Moniz et al., 2012). The role of Heat Shock Protein 70 (HSP70), a chaperon of GATA1 (Gastou et al., 2017) and the impairment of GATA1 translation may also add to understanding the erythroid tropism of DBA (Ludwig et al., 2014).

The collaboration between the clinical and biological researchers has allowed for advancement in the pathophysiology studies of DBA that would be next to impossible for any one group to achieve alone. EuroDBA routinely provides examples of this level of cooperation. In one instance, a study published by the consortium demonstrated that DBA-linked RP gene mutations induce cellular autophagy. Here the German EuroDBA group generated EBV-immortalized lymphoblast cell lines (LCLs) from patients in their registry that were then analyzed by the EuroDBA group of biological researchers in the Netherlands, who were performing the same molecular analyses on their zebrafish models of RP loss using either mutants (if available) or knocking down the RP of interest transiently with morpholinos. Simultaneously, the EuroDBA group in France performed red cell culture assays with erythroblasts infected with shRNAs to knock down *RPS19,* the resulting colonies being sent to the Netherlands for the same cellular analysis as was being used for the patient-derived LCLs and the zebrafish (see Fig. 1) (Heijnen et al., 2014).

Another example of the consortium's capacity to cooperate on unraveling the pathophysiology of DBA began with the identification of a patient in the Netherlands whose exome sequence revealed a mutation in *RPL9*, a gene not previously linked to DBA. A blood sample from this patient was sent to the German EuroDBA partners for establishment of LCLs, a second blood sample was sent to the French EuroDBA partners who performed a red cell culture assay on isolated erythroblasts. The LCLs were subsequently sent to the biological EuroDBA partners in France who performed pre-rRNA analysis, and to the partners in the Netherlands who performed other cellular analyses including polysome profiles, TP53 analysis, proliferation and *de novo* protein synthesis measurements. The resulting colonies from the red cell culture assays were also analyzed for proliferation and differentiation defects as well as for TP53 analysis. Analysis of the Netherlands group into zebrafish models of *rpl9* loss confirmed the impairment of red cell development in mutant embryos. Taken all together, the EuroDBA consortium was able to determine that *RPL9* is a bona fide DBA gene in a swift and systematic manner that would have otherwise been laborious and time-consuming (in submission).

1.7. Functional validation of DBA mutations

The functional validation of DBA-linked RP gene mutations may be achieved by analyzing the maturation of ribosomal RNA precursors by northern blot (Gazda et al., 2008; Doherty et al., 2010; Landowski et al., 2013; Farrar et al., 2011, 2014; Mirabello et al., 2014; Choesmel et al., 2007; Choesmel et al., 2008; Mirabello et al., 2017b). Mutations in DBA-linked RP genes invariably lead to haploinsufficiency of the corresponding protein. Since most ribosomal proteins are progressively incorporated into pre-ribosomal particles concomitantly to pre-ribosomal RNA maturation, lack of a given RP impairs processing of pre-ribosomal precursors (pre-rRNAs) in a specific manner (Henras et al., 2015). Modifications of the pre-rRNA pattern can thus be visualized by northern blot and used as a “molecular signature” for the defect of this RP. This characteristic pre-rRNA pattern can be determined by northern blot analysis of RNAs extracted from a patient's cells in order to validate the functional impact of a mutation. In case of a mutation in a new RP gene suspected to be pathogenic, the patient pre-rRNA profile is compared to that obtained after knocking down expression of the corresponding RP with siRNAs in a cell line such as HeLa (see Fig. 1A). Control samples from unrelated individuals, and/or unaffected parents or siblings are used for comparison. Because ribosome processing is affected ubiquitously in DBA patient cells, a variety of cell types can be used to prepare total RNAs including peripheral blood lymphocytes, LCLs, or fibroblasts. This technique is also useful to examine whether ribosome biogenesis is affected in patients for whom sequencing failed to reveal any mutation/deletion among the known DBA genes.

A complementary approach consists of analyzing ribosomes from cytoplasmic fractions on sucrose gradients. By providing the relative abundance of small and large ribosomal subunits and the distribution of polysomes (translating ribosomes), this technique reveals to which extent a RP defect not only impairs either pathway, but also impacts translation. Fig. 1 provides an example of coupling these techniques for a DBA patient from the EuroDBA registry, for whom no RP gene defect was found by sequencing. Fig. 1A reveals a clear ribosome biogenesis dysfunction in patient LCLs, with an accumulation of both 30S and 32S pre-rRNAs (precursors to rRNA constitutive of the small and the large ribosomal subunits, respectively). Quantifications of product to precursor ratios relative to the controls further ascertained these findings (Fig. 1A), which strongly support the diagnosis of DBA despite the lack of a candidate gene. Fig. 1B illustrates how polysome profiling revealed a substantial loss of 60S subunits in the patient LCLs compared to the healthy control cells, suggesting a defective RP from the large ribosomal subunit. Fig. 1C illustrates the results of a typical o-dianisidine stain of 2-day old zebrafish embryos. In these experiments a mutant

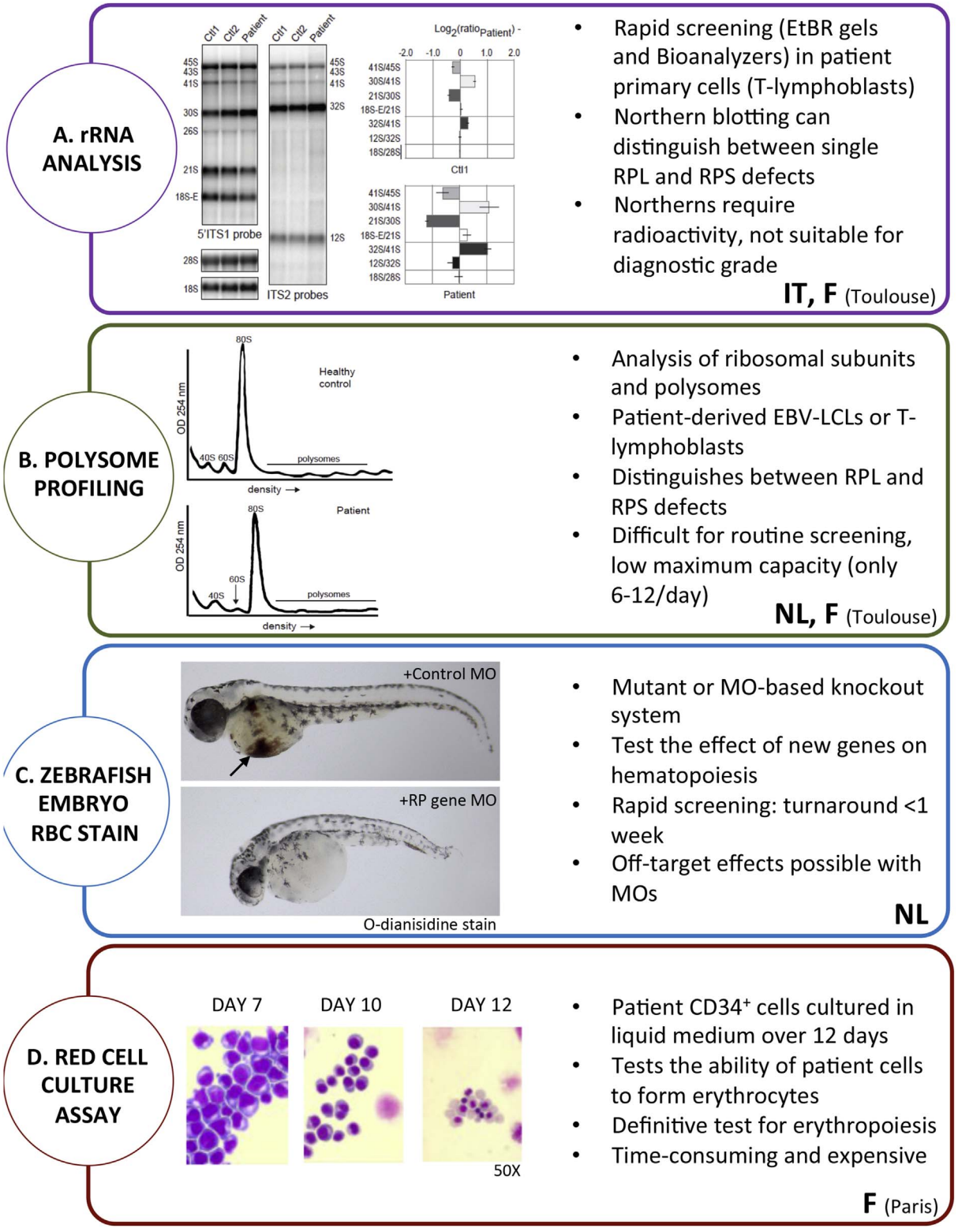

Fig. 1. Functional analyses used for informal diagnostics. A) Northern blot analysis of LCLs from the same patient as above compared with two healthy controls lines (Ctl1 and Ctl2) reveals multiple pre-rRNA processing defects that are not consistent with known RPL mutations. Includes quantification by Ratio Analysis of Multiple Precursors (RAMP[20]). **B)** Polysomes profiles of healthy control LCLs (upper) show equal 40S and 60S ribosome subunit peaks while the profile from a patient diagnosed with DBA (lower, with no mutations in known DBA-linked RP genes) reveals a severe reduction of the 60S peak, consistent with an impairment of biogenesis of the large ribosomal subunit. **C)** 2-day old zebrafish embryos injected at the one-cell stage with control MOs or MOs targetting a RP gene and then stained with o-dianisidine to reveal hemaglobin-expressing red blood cells (arrow). **D)** Red cell culture assays plate $CD34^+$ cells derived from patients and helathy controls and plated in liquid culture medium (+EPO, SCF, IL3) for 12 days.

zebrafish line is used, if available, or morpholinos (MOs) that target the gene of interest are injected into the embryos at the one-cell stage. The o-dianisidine stain at 2 days of age reveals hemoglobin-expressing red blood cells (Fig. 1C, upper), which are clearly absent in the embryos injected with the MOs targeting an RP gene (Fig. 1C, lower). As a final validation that patient $CD34^+$ cells are impaired in forming

erythrocytes, a red cell culture assay is performed (Fig. 1D). Here blood is drawn from the patient, $CD34^+$ cells are isolated and cultured in liquid medium containing erythropoietin (EPO), stem cell factor (SCF), and interleukin-3 (IL3). After 12–13 days in culture the cells are analyzed for colony formation, cell surface determinant markers of differentiation by FACS analysis, protein expression by western blotting, and/or gene expression by qPCR.

So far, northern blot analysis remains the method of choice to validate new DBA mutations or to support diagnosis of DBA. However, it is labor-intensive and not easily transposable in clinics due to the use of radiolabeled probes. Various commercial solutions using capillary electrophoresis (e.g. Agilent BioAnalyzer, Biorad Experion) exist to quantify 18S and 28S rRNAs that compose respectively the backbones of the small and the large ribosomal subunits. This straightforward technique can be used to detect biases in the 28S/18S rRNA ratio in DBA patient cells and determine which of the two ribosomal subunits is affected (Quarello et al., 2016). This is best observed in RNAs extracted from peripheral blood lymphocytes (PBMCs) subjected to activation by phytohemagglutinin. Although this technique is not sensitive enough to see the vast majority of rRNA precursors, it allows detection of an increase of 32S pre-rRNAs when the large subunit production is impaired. Assessment of the 18S/28S ratio is now routinely used by the Italian members of the consortium prior to Sanger sequencing in order to determine which of the small or the large subunit pathway is affected, and to prioritize the RP genes to be sequenced. In future years, sensitive analytical approaches adapted to pre-ribosomal precursor analyses need to be adapted to clinical environments, in order to routinely validate ribosome biogenesis defects and help diagnosis of DBA.

1.8. Improving treatment and aiming for a cure

The registering of DBA patients, systematic genotyping, and the continued efforts in the laboratory have already been invaluable for establishing important genotype:phenotype relationships such as those discussed above. The molecular signatures of the different RP gene mutations are already beginning to be used to improve diagnostics. The continuing inclusion of more clinical and genetic data in patient registries means at this rate it won't be long before the results may be translated into meaningful patient management protocols. The hope is that in the future a patient's genetic information will be able to single-handedly predict, for example, a successful steroid treatment, the susceptibility to iron overload upon chronic blood transfusions, the likelihood of undergoing treatment independence one day, or the likelihood of developing cancer.

The other more obvious hope for the future of any rare disease is a cure. In terms of current treatment for DBA, steroids and blood transfusions can keep the disease at bay but these approaches have considerable side effects. This is especially true for chronic blood transfusions, which can lead to iron overload and organ failure (Roggero et al., 2009). The only present day cure for the bone marrow failure phenotype of DBA is hematopoietic stem cell transplantation (HSCT), which can be a risky procedure especially if a matched sibling donor is not available (Peffault de Latour et al., 2015). The present day recommendation is to perform a BMT if there is an intra-familial HLA-identical donor, and after elimination of the possibility that the donor is a “silent carrier” (Dalle and Peffault de Latour, 2016). Metoclopramide may be used in addition to steroids in order to decrease the amount of the prescribed steroid dose under the maximum dose of 0.3 mg/kg/day (Abkowitz et al., 2002; Vlachos et al., 2008). Although the supplement leucine is currently in clinical trials (ClinicalTrials.gov identifier NCT02386267), to date there are no other pharmaceutical options for DBA patients.

As a monogenic blood disorder that can be cured by HSCT, one of the most exciting areas of future treatment of DBA is gene therapy and editing. In theory, since to date none of the RP gene mutations linked to DBA have been classified as gain-of-function, increasing expression of the wild type allele should rescue the phenotype even in the presence of the mutant allele. Mouse models of doxycycline-induced knockdown of *Rps19* have used this approach, whereupon irradiated mice are transplanted with c-Kit^+ cells transduced with lentiviral vectors expressing *Rps19* under the human elongation factor 1α promoter (EF1α) (Debnath et al., 2017). The authors of this particular study showed that after 2–3 months after the transplantation and 14 days of doxycycline administration, the bone marrow cells derived from mice receiving cells transduced with the *Rps19* vectors, but not a spacer sequence, increased the number of erythroid colonies grown in methylcellulose by three-fold. These results recapitulated an earlier successful study by the same group that used lentiviruses driving *Rps19* expression from the spleen focus-forming virus (SSFV) instead of EF1α (Jaako et al., 2014). A collaborative effort within EuroDBA recently demonstrated that using lentiviruses to express a wild type transgene of *RPS19* in *RPS19*$^{+/-}$ human LCLs corrects pre-rRNA processing defects and reduces TP53 activation (Aspesi et al., 2017). However, the use of retro- or lentiviruses in gene therapy is not without risk. Insertional mutagenesis of the virus remains a major safety concern, and the associated risks of leukemia are not insignificant (Hacein-Bey-Abina et al., 2003). Moreover, the use of non-endogenous promoters allows for the possibility of deregulated overexpression, which for some RP genes such as *RPL36A* has been associated with malignancy (Kim et al., 2004).

CRISPR/Cas9 is a novel kind of gene editing therapy that holds great promise for the correction of point mutations and small indels in patient cells. For a complete review of how CRISPR/Cas9 functions, see reference (Sander and Joung, 2014). In short, this technique harnesses the cell's own machinery to target specific sequences of DNA and generate small deletions which the cell then tries to repair. Introducing an exogenous template for this DNA repair theoretically allows the user to reintroduce a wild-type gene sequence at the exact position where the deletion was introduced, ideally at the exact site of the endogenous mutation. The beauty of this system is that it theoretically allows for any accessible patient cell type, such as fibroblasts, bladder epithelial cells, or blood-derived $CD34^+$ cells, to be first corrected *in vitro* then dedifferentiated into HSCs that could be used for HSCT. Alternately, HSCs could first be generated with one of the patients' cell types mentioned above, then the HSCs get corrected by CRSIPR/Cas9. The downside of this technique at the present day is that while CRISPR/Cas9 is indeed very efficient at generating deletions at or very near the user-specified DNA sequence, generating the exact desired sequence by introducing an exogenous template of wild-type DNA remains technically extremely challenging.

Instead of correcting a mutant gene, another approach uses CRISPR/Cas9 to direct integration of a new wild type gene. The “Safe Harbor” approach uses the CRISPR/Cas9 system to introduce an exogenous gene specifically into the *AAVS1* locus on the long arm of human chromosome 19 (Maggio et al., 2014). Many reports have demonstrated high specificity of this integration site for the successful expression of minigenes with no adverse recombination events or fitness reduction (De Ravin et al., 2016; Rio et al., 2014; Zou et al., 2011). In the context of DBA, this technique has already been used to introduce and drive exogenous wild type *RPS19* expression in inducible pluripotent stem cells (iPSCs) derived from a patient carrying a truncating mutation in one allele of *RPS19* (Garcon et al., 2013). This approach was able to successfully revert the ribosome biogenesis defects of the mutant cells. All this said it should be kept in mind that CRSIPR/Cas9 technology is still very incipient. However when one considers its potential, especially for curing monogenic inherited blood disorders, there is no question that this technology will advance quickly in the near future.

2. Discussion

The advent of genome sequencing has resulted in significant advances in rare disease research this past decade. While researchers are now more likely than ever able to identify disease-causing genes, the

lack of understanding the pathophysiological mechanisms underlying these mutations remains a cumbersome bottleneck in terms of finding a therapeutic cure. In fact, according to the Kakkis EveryLife Foundation, 95% of the approximately 7000 rare diseases known today do not have a single approved drug treatment (Miyamoto and Kakkis, 2011). The funding of rare disease networks, such as ERARE's EuroDBA, represents an important step forward in developing the foundation that will ultimately loosen this bottleneck and hasten the advance of successful treatment methods.

DBA represents a rare disease that has amassed a worldwide network of dedicated researchers and patient support groups helping to fund their work. While the genetics underlying DBA are now very well studied, the pathophysiology still remains not well elucidated. As such, the treatment options are rather outdated and limited. Some of the major questions that surround DBA include why is it that some patients and not others manifest physical malformations? How is it that “silent carriers” have the same pathogenic RP-mutation as their affected child yet remain symptomless? And what are the determinants that can lead to patients undergoing treatment independence, or developing cancer? The establishment of cooperative global networks for DBA is a crucial step in being able to shed light on the drivers of this rare disease.

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