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### **► To cite this version:**

L. da Costa, Narla Mohandas, Ludivine David-Nguyen, Jessica Platon, Isabelle Marie, et al.. Diamond-Blackfan anemia, the archetype of ribosomopathy: How distinct is it from the other constitutional ribosomopathies?. *Blood Cells, Molecules and Diseases*, 2024, pp.102838. 10.1016/j.bcmd.2024.102838 . hal-04473527

**HAL Id: hal-04473527**

**<https://u-picardie.hal.science/hal-04473527v1>**

Submitted on 20 Oct 2024

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**Diamond-Blackfan anemia, the first ribosomopathy:  
how distinct from the other constitutional ribosomopathies?**

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**Running title: Diamond-Blackfan anemia, as the first ribosomopathy.**

**Keywords:** Diamond-Blackfan anemia; ribosomal protein genes; ribosome biogenesis; pre-rRNA processing.

**Abstract (247 words)**

Diamond-Blackfan anemia (DBA) was the first ribosomopathy described. DBA is a congenital hypoplastic anemia, characterized by a usually macrocytic aregenerative anemia, related to a blockade in the erythroid bone marrow maturation between the BFU-e/CFU-e erythroid progenitor stages. In 50% of the DBA cases, various malformations are associated. Strikingly, for a hematological disease with a relative erythroid tropism, DBA is due to ribosomal haploinsufficiency in one of the 20 ribosomal protein (RP) genes, involved in the disease. Only few other genes are described in the DBA syndrome and part of the so-called DBA-like disorders. Haploinsufficiency in a RP gene is associated to a defect in ribosomal RNA (rRNA) maturation, which is the functional signature of DBA. However, the link between a mutation in a RP gene and the defect in rRNA maturation is still to be fully defined, and as the link between the defect in rRNA maturation and the erythroid tropism defect in DBA. Meanwhile, some progresses have been made in that latter direction. Indeed, DBA erythroid defect has been recently shown to be related in a non-exclusive manner 1) to a defect in translation, in particular in *GATA1* erythroid gene translation, 2) to a defect in the HSP70, the *GATA1* chaperone, and 3) the free heme toxicity. In addition, p53 activation is involved in DBA pathophysiology. The DBA phenotype may be then the result of the combined effects of all these actors and explain the heterogenous phenotype of the DBA patients, even in a same family.

## Introduction

Diamond-Blackfan anemia (DBA) syndrome (OMIM #105650, #300946, #606129, #606164, #610629, #612527, #612528, #612561, #612562, #612563, #613308, #613309, #614900, #615550, #615909, #617408, #617409, #618310, #618312, #618313) )<sup>1-3</sup> is part of the inherited bone marrow failure syndrome (IBMFs), with Fanconi anemia (FA) (OMIM#227645, #227646, #227650, #300514,#600901,#603467, #609053, #609054,#610832, #613390, #613951, #614082, #614083, #615272, #616435, #617243, #617244, #617247, #617883)<sup>4-7</sup>, congenita dyskeratosis (DKC)(OMIM#305000, #613987, #613988, #613989, #613990, #615190, #616353)<sup>8-11</sup>, Shwachman-Diamond syndrome (SDS) (OMIM#260400, #617941)<sup>12-15</sup>, cartilage hair hypoplasia (CHH)(OMIM#250250)<sup>16,17</sup> for the most common. IBMFs are rare diseases, with a diagnosis made usually <10 years of age, but all along the life in the less severe cases. The diagnosis may be difficult and borderline cases between each disorder may lead to misdiagnosis or delay in diagnosis and treatment. The common characteristics are 1) an intrinsic and quantitative bone marrow failure, with maturation and differentiation defect either in a specific lineage such as DBA syndrome (erythroid defect), or SDS (granulopoiesis defect) or in more than one lineage to the progressive and complete bone marrow reduction of the cell production of the white and red blood cells, and the platelets (aplastic anemia of the FA), 2) an activation of p53 described in all of the IBMFs, besides to some p53-independent cases<sup>18-21</sup>, 3) Hematopoietic Stem Cell Transplantation (HSCT) as the only curative treatment of the IBMF so far<sup>22-27</sup>, and 4) to be considered as preleukemic or with risk of solid tumor conditions<sup>28-36</sup>. The pathophysiological mechanism responsible for the bone marrow failure is depending on the IBMF disorder: ribosomopathies in cases of Diamond-Blackfan anemia<sup>37,38</sup>, related to ribosomopathies but not the *primum movens* of the disease in case of the Shwachman-Diamond syndrome<sup>39</sup> <sup>40,41</sup> and the dyskerin (*DKC1*) mutated gene congenita dyskeratosis, telomeropathies in DKC (other than linked to *DKC1* mutated gene)<sup>42-44</sup>, and DNA repair anomalies in case of FA<sup>45-47</sup>.

In term of definition and classification: the DBA syndrome is the term, which regroup DBA and DBA-like diseases.

## DBA phenotype

DBA exhibited a moderate to severe, usually macrocytic (Mean corpuscular volume (MCV))>100fl in adults), normochromic aregenerative (reticulocyte count<20x10<sup>9</sup>/L) anemia, with normal leukocytes and platelet counts most often. At diagnosis or during DBA evolution, neutropenia, thrombocytopenia even thrombocytosis maybe reported. It is still mandatory to

perform a bone marrow aspiration or bone marrow biopsy in order to confirm the characteristic erythroblastopenia which is less than 5% of total erythroblast on the bone marrow smear or hypoplastic anemia on the bone marrow biopsy, in an otherwise normal bone marrow with no sign of myelodysplasia<sup>1,3,48</sup>. Compare to the other IBMFs, the hematological features of DBA are found only in DBA except some forms of FA, which enter into the disease by the anemia and erythroblastopenia before the complete aplasia. But in that case, the age at presentation makes the differences since FA cases are mostly diagnosed in the first decade when DBA median age at diagnosis is 2 month-old. Increased MCV is a common feature of most of the IBMFs, as such the increased percentage of HbF.

The erythroblastopenia is the result of the erythroid blockade at the BFU-e/CFU-e erythroid progenitor stage. RPS19 mutated DBA patients have been described with a decreased erythroid proliferation, a normal erythroid differentiation with few apoptotic cells, while RPL5 and RPL11 erythroid cells from DBA patients exhibited a large decreased in erythroid proliferation, a defect in erythroid differentiation, increased apoptotic cells<sup>19</sup>. A cell cycle arrest has been described in both<sup>19</sup>. p53 has been involved in the reduced erythroid proliferation in DBA, inducing apoptosis and cell cycle arrest<sup>18,19</sup>, meanwhile some p53-independent pathways are described<sup>49-51</sup> even in some DBA patients for some new genes<sup>52</sup>. Recently, using single cell transcriptomic (scRNAseq) of hematopoietic stem and progenitor cells from various DBA patients mutated in RPS or RPL genes known to be involved in DBA, RPS-DBA cells exhibited an arrest of erythroid differentiation with a loss of BFU-e and megakaryocyte progenitors and a preservation of these progenitors in RPL-DBA patients<sup>53</sup>. We found different results with a normal erythroid differentiation and normal growth from BFU-e cell population in depleted RPS19 erythroid cells either from various RPS19 mutated DBA patients or after shRNA depletion of CD34<sup>+</sup> cells<sup>19,54</sup>. The most surprising and also in an opposite direction to our results<sup>54,55</sup> and V. Sankaran's ones<sup>56</sup>, GATA-1 protein expression has been found normal in RPL-DBA patients<sup>53</sup> while in all the patients mutated in RPL11 tested and after CD34<sup>+</sup> depleted erythroid cells, we obtained a large decreased of GATA-1 expression on the Western blots (same amount of cells loaded) (Figure 1A-B) or Image stream flow cytometry technology (Figure 1C) and visualized a defect in GATA-1 nucleus localization (Figure 1D). We can speculate that unicellular experiments are making the differences, without any other relevant clue.

One of the DBA biological features is an increased erythrocyte deaminase (eADA) activity in more than 84 to 90% of the DBA patients<sup>57-59</sup>. As an erythrocyte enzyme, the eADA activity need to be measured before transfusion. The sensitivity and the specificity of the increased

eADA in DBA have been evaluated respectively to 84% and 95% with a negative and positive predictive value of 91%. It is important to note that the increase in eADA is quite specific of DBA. Only rare cases of cartilage hair hypoplasia, which is the only ribosomopathy in which eADA have been rarely found elevated, myelodysplastic syndromes, paroxysmal nocturnal haemoglobinuria, and acquired immunodeficiency syndrome have been shown to exhibit an increased eADA. In comparison to other IBMFs, increased eADA has been found in only one patient affected each by FA (*FANCA* gene mutated), DKC, SBS, but misclassification may be possible in both latest patients since no gene have been identified for these IBMF<sup>60</sup>. The important value of eADA is to distinguish DBA from transient erythroblastopenia of childhood (TEC), which is the major differential diagnosis of DBA and the more difficult one, since only the evolution will make the differences between both<sup>3,48,61</sup>. However, meanwhile its important relevance in clinical DBA phenotype, the link between an increased eADA activity and DBA is still to be fully defined.

An elevated HbF percentage is also one DBA criteria. It needs to be measured at least after 6 to 24 months of age (Normal value <2%).

DBA is associated to various congenital malformation mostly in the cephalic area including microcephaly, hypertelorism, micrognathia, low set ears or hair line, short or webbed neck... and in the extremities with flat thenar eminence, the rare but quite pathognomonic triphalangeal thumb, and any abnormality in the thumbs (duplication, bifid..) should may think about DBA<sup>3,48,62</sup>. Malformations are also described in organs such as heart (ventricular or atrial septal defect, coarctation of aorta ..) and kidney (absent, horseshoe, hypospadias). Mental retardation is not a feature of DBA, and when associated it may be due to contiguous syndrome or a neurological disease association. The relation between the RP haploinsufficiency and the malformative syndrome is yet to be fully defined but some phenotype/genotype correlations have been identified such as the cardiac defect and mutation in *RPS24* gene<sup>63</sup> and the association between RPL11 mutated gene and triphalangeal thumb or the vast malformative syndrome, including very often the cleft palate associated with RPL5 mutations<sup>64,65</sup>.

Short stature is part of the DBA malformative syndrome and often at the first place (22% of the DBA cases). Short stature is a common feature of all the IBMFs, meanwhile the congenital abnormalities reported in DBA without being specific of DBA are quite characteristic. FA shares some malformations with DBA but FA is revealed later in life after 5 years usually for a median age at diagnosis 2 month-old in DBA<sup>4,7</sup>. DKC and SBS exhibit their own characteristics. In DKC, patients exhibit the classic triad: abnormal pigmentation, skin appendage and anormal skin appendage (nails and hair anomalies, such as premature hair loss,

premature white hair). In addition, are described premature osteoporosis, dental anomalies, lacrimation, cirrhosis, mental retardation before the occurrence of the medullar aplasia in the second decade, lung fibrosis and immune deficiency<sup>8,11</sup>. SBDS affected patients exhibit the classic defect in exocrine pancreas with a decreased fat-soluble vitamins and neutropenia<sup>13</sup>. In addition, large and diversly combined varieties of systemic defects are described with central nervous system anomalies, skin, liver, heart ones. These IBMFs are usually easy to differentiate with DBA except some border line cases such the cases reported with p53 mutation, who share some feature of DKC<sup>66</sup>.

### **Evolution and malignancies**

DBA evolution is characterized mostly by the occurrence of various malignancies: hemopathies and solid tumors<sup>33-36,67</sup>. All kind of hemopathies have been described: myelodysplastic syndromes, being the most frequent in the American registry (Observed to Expected (O/E) ratio: 352.1) meanwhile it is not our case in the French registry, but also acute myeloid (O/E ratio: 28.8) or lymphoblastic leukemia, myeloma, lymphomas. In the solid tumors, the most frequent cancer is the colon cancer (O/E ratio: 44.7) and since DBA is a pediatrics disease, osteosarcoma in infants is the most frequent cancer (O/E ratio: 42.6), then lung cancer (O/E ratio: 9.4)<sup>34,36</sup>. However, compare to the other IBMFS with a quite ineluctable evolution to malignancies (myelodysplastic syndromes, acute myeloid leukemia in young adults and oral cancers in FA), the risk of malignancies for an IBMF is lower in non-transplanted DBA<sup>33</sup>: 4.8% for all type of cancers, with a cumulative incidence of solid tumors and hematological malignancies of 13.8 by the age of 45 year-old in the last study from the American DBA registry<sup>36,67</sup>.

Strikingly, the European DBA consensus group (EURO-DBA) identified that depending on the mutation in a same RP gene, namely *RPL9* gene may be responsible for DBA phenotype or promote malignancies<sup>68</sup>. Somatic mutations or haploinsufficiency in RP genes have been extensively reported recently either in hemopathies, such T-acute lymphoblastic leukemia (mutations in *RPL10* and *RPL5* genes)<sup>69,70</sup>, myelodysplastic syndromes (RPS14 haploinsufficiency in minus 5q syndrome)<sup>71</sup>. RPS27a upregulation has been found in acute myeloid leukemia (AML) and chronic myeloid leukemia<sup>72</sup> and is involved in solid tumor, overexpressed in response to DNA damage and in renal, breast and colon carcinoma<sup>73</sup>. Even non coding RNAs, small nucleolar RNAs, such as the C/D box containing SNORD42A has been found involved in leukemogenesis<sup>74</sup>. SNORD42A was highly expressed in primary cells from AML affected patients and various human leukemia cell lines. Acting on the U116 18S

rRNA 2'-O-methylation, SNORD42A is involved in leukemia cell proliferation and survival<sup>74</sup>. More than 1034 articles have been published on RP and leukemia and more than 2386 in carcinoma to date.

### **DBA molecular diagnosis**

Diamond-Blackfan anemia (DBA) genotype as the phenotype is highly heterogenous. DBA inheritance is either *de novo* or sporadic (55% of the DBA cases) or familial (45%) inheritance. In the familial cases, dominant inheritance is the most frequent and the sporadic cases are inherited dominantly in the offspring (neodominance inheritance)<sup>1-3,75,76</sup>. In some rare DBA cases, recessive<sup>77</sup> or X-linked inheritance<sup>78-80</sup> are reported but mostly in DBA-like disease.

### ***RP genes and DBA:***

The first gene identified in 1999<sup>81</sup> was surprisingly for a disease with an erythroid tropism, the ribosomal protein S19 gene (*RPS19* gene) with all over the world 25% of the DBA patients carrying a mutation in this gene. Since then, 22 other RP genes have been found to be involved in DBA. *RPL5* (7% of DBA cases), *RPL11* (5%)<sup>64</sup>, *RPL35a* (3.5%)<sup>82</sup>, *RPS24* (2.4%)<sup>83</sup>, *RPS17* (1%)<sup>84</sup>, *RPS10* (3%), *RPS26* (7%)<sup>85</sup> genes have been identified and account with *RPS19* gene for the most frequently mutated genes with around 50% of the mutations. Other RP genes have been found mutated in few DBA mutations<sup>76</sup>: *RPS7*, *RPS15*, *RPS20*, *RPS27*<sup>86</sup>, *RPS27a*<sup>72</sup>, *RPS28*<sup>87</sup>, *RPS29*<sup>88</sup> in the small ribosomal subunit and *RPL8*, *RPL9*, *RPL15*<sup>89</sup>, *RPL17* (manuscript in preparation), *RPL18*, *RPL26*, *RPL27*<sup>86</sup>, *RPL31* genes in the large ribosomal subunit, but in low frequency of DBA patients (less than 1% for each of the RP genes). Some genes (*RPS11*, *RPL3*, *RPL10*, *RPL10A*, *RPL19*, *RPL34*, *RPL0*)<sup>76</sup> are considered as putative candidate genes since they have not been identified in multiple unrelated DBA patients and/or validated by functional tests confirming the rRNA maturation defect and ribosome biogenesis impairment, the defect in erythroid proliferation or reproducing DBA in animal models. These 7 latter genes need further experiment before validation as DBA genes. All the mutations are heterozygous and the homozygosity is supposed to be lethal. All kind of mutations have been identified with some particularities depending on the RP genes. Most of the missense mutations have been identified in *RPS19* gene while non sense mutations, small deletions or insertions, splice site mutations have been found in *RPL5*, *RPL11* genes. Large deletions have been found in 20% of the DBA cases in these RP genes mostly, in *RPS17*, *RPL35a*, *RPS19* genes<sup>90,91</sup>. Phenotype/genotype correlations are not that obvious but *RPL5* gene is associated with a large panel of malformations including cleft palate<sup>64</sup>; *RPL11* gene, with thumb anomalies, amongst them the quite pathognomonic triphalangeal thumbs<sup>64,92</sup>, and *RPS24* gene is associated with



cardiac defect<sup>63</sup>. *RPS19* gene is associated with the less malformations compared to the other RP genes.

***Other genes than RP genes but still involved in ribosome biogenesis:***

Recently, the euroDBA consortium (EJPRD RIBOEurope) identified a new gene, so called *HEATR3* gene<sup>52</sup>. Six individuals from 4 unrelated families (Netherland - 2 families, Turkey and France) exhibited anemia, growth retardation and some particular features: the 5 homozygous cases were affected by intellectual disability and brachydactyly, while the heterozygous composite French patient exhibited a mild phenotype with a MICI like phenotype in addition to the classical DBA erythroblastopenia and growth retardation. He was probably mild phenotype due to the presence of the biallelic mutations (Figure 3A). Interestingly, the father who has transmitted the splice site mutation exhibited a slight decreased of erythroid proliferation suggesting a pathogenous effect of this allelic variation even at the heterozygous stage (Figure 3B). In the *HEATR3* mutated DBA patients, the erythroid proliferation defect is associated with a normal erythroid differentiation and no apoptosis (Figure 3C). *HEATR3* is the chaperone of RPL5, co-translationated with RPL5 and involved in RPL5 nucleolar import for the biogenesis of a RPL11(uL5)/RPL5(uL18)/5S rRNA particle. The defect in rRNA maturation confirming the DBA diagnosis has been established. It is characterized by the accumulation of 32S pre-rRNAs as shown on a strong increase of 32S/41S ratios and a decrease of 12S/32S ratios, which is the feature of a delay in endonucleolytic cleavage in the internal transcribed spacer 2 (ITS2), which separates the 5.8S and the 28S rRNAs. *HEATR3* candidate gene identification reinforced DBA as the disease in which all the genes involved are ribosome protein genes or related to ribosome biogenesis. Indeed, in addition to *HEATR3*, *TSR2* gene should also be considered as a DBA gene; *TSR2* is involved in rRNA maturation process and binds RPS26<sup>87</sup>. DBA has been described as such as the first ribosomopathy in 2005<sup>38</sup> by a collaboration between a French group (Pierre-Emmanuel Gleizes, Toulouse University, France) and an American group (Steve Ellis, Louisville University, USA). Invalidating each *Rps19* gene in *Saccharomyces cerevisiae*, they have shown a specific profile of defect in ribosomal RNA maturation (rRNA). PE Gleizes and colleagues then reported the same profile in mutated RPS19 erythroid progenitors from DBA patients confirming for the first time RPS19 haploinsufficiency due to a defect in rRNA maturation was responsible for DBA<sup>37</sup>. As a matter of fact, a defect in rRNA maturation is the DBA signature and is the functional proof of the deleterious effect of an allelic variation in a RP gene.

In addition to RP or ribosome related genes responsible for DBA, few genes are responsible for DBA-like disease. So far, these genes are the *GATA-1*<sup>56,78-80,93</sup>, *EPO*<sup>77</sup> and *TP53* genes<sup>66</sup>.

*ADA2 (CECR1)* gene is responsible for erythroblastopenia with a certain degree of steroid response but the MCV, the eADA activity level, the rRNA maturation are normal. Indeed, some mutated *ADA2 (CECR1)* affected patients may be registered in DBA registries meanwhile patients mutated in *ADA2 (CECR1)* are even in a syndrome apart, the so called *ADA2* deficiency, a systemic and rheumatologic disease, which combines diversely autoinflammation features, vasculitis, and mild immunodeficiency <sup>76,94</sup>.

*GATA1* gene encodes for the major erythroid transcription factor GATA1. Only few DBA patients in the world have been described with a mutation in *GATA1* gene on the X chromosome <sup>56,78-80,93</sup>. The mutation, in the close vicinity or in the exon 2 splicing site identified in DBA or DBA-like patients is responsible for the loss of the long form of GATA1 protein (loss of the transactivating domain) since the mutation leads to the exon 2 skipping. *GATA-1* DBA-like mutation is usually associated with a congenital dyserythro/dysmegakaryo-poiesis in a hypocellular bone marrow <sup>95</sup>. *EPO* gene mutation (homozygous missense allelic variation in exon 5, g.100,320,704G>A;p.(Arg150Gln)) has been described in a male infant from a consanguineous Turkish family and exhibited a decreased erythroid proliferation and delayed erythroid differentiation <sup>77</sup>. The inheritance is autosomal recessive in the *EPO* mutated affected patient. Finally, but not the last, recently have been described 2 different gain of function mutations in exon 10 of *TP53* gene (c.1083delG or c.1077delA leading to the same *TP53* mutated protein, p.(Ser362Alafs\*8) in two unrelated DBA-like individuals <sup>66</sup>.

Targeted-next generation sequencing (t-NGS), whole exome (WES) and now whole genome (WGS) sequencing identify the mutated allele (s) in the DBA and DBA-like patients in more than 80% of the cases. The Whole genome sequencing and other future innovative will hopefully fill the gap of the remaining DBA syndrome patients with no identified mutation after a deep sequencing using the easiest techniques.

### **Treatment and steroid responsiveness in DBA, as the major signature compare to the other ribosomopathies and IBMFs**

The particularities of DBA are the steroid response in more than 60 to 80 % of the DBA patients, but only 40% of them remain steroid responder on the long term. The effect of the steroid on raised hemoglobin level occurred in two weeks to one month <sup>96</sup>. Still the reasons of the steroid response are still fully to be defined but it has been shown that steroids increase the erythroid cell survival and proliferation. Indeed, glucocorticoids (GC) increases the sensibility of the erythroblast to erythropoietin (EPO) <sup>97</sup>. The GR binds the EPO-receptor, which merges the c-kit (SCR-receptor), which synergizes the cell signaling in late erythroid progenitors and

precursors to increase specific erythroid genes, erythroid proliferation and of the GC-receptor (GC-R) dimerization leads to inhibition of the stress erythropoiesis (hemolysis) in mice, suggesting a major role of the GC in the activation of the stress erythropoiesis<sup>98</sup>. Dexamethasone (DXM), a GC has been shown to increase erythroid cell proliferation in vitro. Recently, a study in normal CD34<sup>+</sup> cells pointed out the difference of DXM effect depending on the CD34<sup>+</sup> tissue origin: DXM being efficient in erythroid progenitor expansion when the CD34<sup>+</sup> were harvested from peripheral blood or bone marrow, but not from cord blood CD34<sup>+</sup><sup>99</sup>. p57Kip2, a Cip/Kip cyclin-dependent kinase inhibitor has been found specifically upregulated. It suggests that GC-R transcriptional response needs to be blocked during the fetal life, which makes sense with the results from Iskander et al.,<sup>100</sup>. Another good reason for not using GC during pregnancy. In addition, it exists a cross-talk between GC-R and p53. It has been shown that DXM, after GC-R binding is able to increase erythroid proliferation by inhibiting p53 activation after p53 proteasomal degradation<sup>101</sup>. In a mice model invalidating *Rps19* gene, GC (DXM or prednisolone, which is used in DBA patients regularly) treatment restored erythroid BFU-e and CFU-e proliferation and differentiation, with concomitant decreased p53-dependent gene transcription<sup>102</sup>. Study in primary erythroid progenitors from bone marrow of DBA patients, either regularly transfused or under steroid treatment revealed that the number of BFU-e and CFU-e were reduced in the group of the transfused DBA patients but not in the steroid treated DBA patients<sup>103</sup>. GC-R binds its receptor, which dimerizes, translocates to the nucleus, where it regulates the transcription of various genes, which contain a specific DNA sequence binding, so called «glucocorticoid receptor responsive element» (GRE)<sup>104</sup>. As such an example the *Zfp36l2* gene, a glucocorticoid responsive gene critical for GC-mediated differentiation delay and increased liver erythropoiesis in mice has been found important for BFU-e self-renewal<sup>105</sup> or *Myb* gene for the erythroblast proliferation<sup>106</sup>. Recently, it has been shown that *ZFP36L2* gene expression was reduced in primary DBA erythroid cells and specific CRISPR/Cas9 invalidation of RPL11 in K562 cell lines. DXM was able to increase *ZFP36L2* expression with a loss of erythroid differentiation marker GYPA. Studying a patient before and after that GC has been introduced as DBA treatment, revealed an increased *ZFP36L2* gene expression. Interestingly, after lentiviral wild type *ZFP36L2* gene infection in RPL11 depleted K562 cells recapitulated the delay in the erythroid differentiation (decreased GYPA<sup>+</sup> cells)<sup>53</sup>. The GC role in DBA seems to block erythroid cell differentiation to maintain a pool of erythroid progenitors by increasing *ZFP36L2* expression. The GC seems to play a role in the stress erythropoiesis restoration in DBA. Indeed, the authors identified a defect in endogenous GC-R transcriptional signature to stress erythropoiesis in DBA meanwhile some feature of

stress erythropoiesis such as increased HGB2 and GDF15 were found in DBA erythroid progenitors.

### **Other IBMFs with a ribosome machinery defect versus DBA:**

#### **Shwachman-Bodian-Diamond Syndrome (SBDS):**

Shwachman-Bodian-Diamond Syndrome (SBDS) is a rare (live birth incidence of 1/77 000<sup>13,14,107</sup> autosomal recessive IBMF associated with bi-allelic mutation in *SBDS* gene in the vast majority of the SBDS patients<sup>108</sup>. The first description has been reported in 1961 and then in 1964 by Harry Shwachman, Martin Bodian and Louis Diamond<sup>109</sup>. SBDS is a multi-systemic disorder with the classical neutropenia and exocrine pancreatic insufficiency, growth retardation, skeletal abnormalities (metaphyseal chondrodysplasia, rib cage dysplasia, osteopenia) and cognitive retardation. As other IBMF, SBDS is associated with a risk of malignancies, and much higher compared to DBA, with a cumulative incidence of 18.8% to develop MDS/AML at 20 years and 36.1% at 30 years<sup>110</sup>. As DBA, SBDS is associated with solid tumors. The clonal hematological evolution in SBDS seems to be associated to the loss of function<sup>111</sup>. The defect in ribosome biogenesis has been characterized and as in DBA patients, SBDS cells exhibit a defect in rRNA maturation, with an impairment of the 60S subunit of the ribosome. However, Moore and colleagues have shown after deletion of yeast SBDS ortholog, *sdo1* compared to the yeast DBA Rpl33A mutant that the ribosomal defect was different in both eukaryote models: *rpl33A* mutant leading to decay of early ribosomal precursors, 5S rRNA and other large subunit rRNA accumulated as extra-ribosomal entities, when *sdo1* mutant showed accumulation of 60S subunit precursors in the nucleoplasm at a later stage of maturation, leading to relatively stable pre-particles 60S with associated 5S rRNA into the nucleus<sup>112</sup>. These differences may also explain the different phenotype between both diseases.

In addition, it has been shown that SBDS function as a cofactor for elongation factor like GTPase 1 (EFL1), allowing the release of eIF6 from the large 60S ribosomal subunit. This process acts as a last quality control for ribosome biogenesis (the 80S assembly occurring only after eIF6 release from 60S in mammalian cells<sup>40,41,113</sup>. Mutations<sup>12</sup> have been identified in few SBDS patients (less than 3%) on the elongation factor-like 1 (*EFL1*) gene<sup>114,115</sup> or DNAJ heat shock protein family (HSP40) member 21 (*DNAJC21*) gene, a 60S ribosome assembly factor<sup>116,117</sup> or signal recognition particule 54 (*SRP54*)<sup>118</sup> genes, reinforcing the signature of SBDS as an impairment in maturation of the large 60S subunit. In DBA, both ribosomal subunits may be altered depending on the mutated RP gene and the defect in rRNA maturation

on northern blot is different with the mutated RP genes<sup>37,38</sup>. As in DBA for the erythroid tropism and for a disease as well which impaired the rRNA maturation and translation, the major issue in SBDS is how to explain the pancreas and granulocytic tropism in SBDS, meanwhile other cytopenia may be associated. As in DBA for GATA-1 translation defect<sup>56,119</sup>, the reduced amount of normal ribosomes may affect selectively the translation of some specific mRNA with upstream reading frame (uORFs) or complex 5' or 3' untranslated regions (UTRs)<sup>120</sup>.

### **X-linked Congenita dyskeratosis (X-DKC):**

Genetic mutations of dyskerin (*DKC1* gene) are associated with dyskeratosis congenita and the related Hoyerall-Hreidarrson Syndrome<sup>8,121</sup>. Both syndromes are linked to deficient telomere maintenance<sup>122</sup>. DKC is a rare IBMF with skin pigmentation, skin appendage, mucous membrane abnormalities and cytopenia with a risk of malignancies (SMD/AML) and lung fibrosis.

Dyskerin is a component of the telomerase holoenzyme complex, binding telomerase RNA (TER) H/ACA domain and protect *in vivo* the stability of TER. Dyskerin is a pseudouridine synthase that catalyzes the isomerization of uridine to pseudouridine in noncoding RNAs. Uridine forms heterotrimers with Nop10 and Nhp2. They are located onto the small nucleolar RNA (snoRNA) and small Cajal body RNA (scaRNA) to form H/ACA complexes. Binding with snoRNA and scaRNA, allows dyskerin to target site specific modifications on enzymatic RNA molecules (ribosomal RNA modification in the nucleolus for the snoRNA-H/ACA complexes and specific residue modification on spliceosomal RNA at Cajal bodies for the scaRNA H/ACA complexes). RNA interference reduction of dyskerin expression experiment is responsible for a loss of internal ribosomal entry site-dependent mRNA translation<sup>123</sup>, that mutations in *DKC1* gene in Human may be responsible of the same ribosome defect. However, different studies with X-DKC models corrected for telomere maintenance defects pointed out, that in X-linked DKC, the levels of pseudouridine modification and maturation kinetics of newly transcribed rRNA were normal<sup>124</sup>, and that in consequence there were no rRNA processing defects. In addition, the ribosomal protein content was maintained, suggesting that the ribosome biogenesis was not affect by *DKC1* mutation<sup>44</sup>. These results obtained in fibroblast cell lines from human X-DKC patients, have been validated in an immortalized pluripotent cells (IPS) model generating from 3 different mutations in *DKC1* gene (*DKC1*<sup>A353V</sup>,

*DKC1<sup>Q31E</sup>*, and *DKC1<sup>AL37</sup>*)<sup>43</sup>. Finally, the primary etiology of X-DKC is not the loss of pseudouridine modification capacity, but the telomere dysfunction<sup>43,44</sup>.

### **Cartilage Hair Hypoplasia (CHH) - Anauxetic dysplasia (CHH-AD) spectrum**

CHH is a rare autosomal recessive IBMF described by McKusick in 1965<sup>125</sup> and characterized mainly by short-limbed dwarfism, sparse hypoplastic hair, defective T-cell immunity, hypoplastic anemia and as the other IBMFs an evolution risk of developing malignancies. The differential diagnosis with DBA on the phenotype is usually easy. The molecular defect has been identified in 2001, as mutations in RNA component of RNase MRP (*RMRP*) gene<sup>126</sup>. *RMRP* is a long non-coding RNA (lncRNA) that contributes to the formation of the RNase MRP complex with various proteins involved in the RNase P complex and tRNA maturation. *RMRP* is an endonuclease with multiple functions, one of them being the cleavage of the precursor rRNA in the ITS1 sequence leading to the generation of the 5' end of 5.8 rRNA<sup>127</sup>. CRISPR/Cas9 *RMRP* invalidation in HeLa cells reproduced this phenotype with accumulation of uncleaved ITS1<sup>128</sup>. As such, CHH is considered as a ribosomopathy. Of note, as for X-DKC, *RMRP* is linked to the telomerase complex via the telomerase-associated reverse transcriptase (TERT). However, the causal relation between the mutations in *RMRP*, the defect in ribosome biogenesis and the CHH phenotype is yet to be fully defined.

### **Other ribosomopathies without an erythroid signature:**

#### **▪ The Treacher Collins Syndrome (TCS)**

The Treacher collins syndrome is a rare (1/50,000 live births) IBMFs and a ribosomopathy but not associated with an anemia<sup>129</sup>. It is characterized with craniofacial defects (micrognathia, maxillary hypoplasia, microtia or ears unusually formed or missing, coloboma, undersized cheekbones). This is an autosomal dominant disorder due to mutation in *TCOF1* gene, encoding the Treacle protein, which is located into the nucleolus reinforcing its involvement into the ribosome biogenesis<sup>130</sup>. *TCOF1* colocalizes with Upstream Binding Factor (UBF) and PolII and is involved in the transcription of ribosomal DNA. In addition, mutations in *POLR1D*, *POLR1C*, *POLR1B* genes, which are also involved in rRNA transcription and thus ribosome biogenesis, have been identified in TCS<sup>131,132</sup>. Animal models confirmed the defect in ribosome biogenesis and the direct effect of *tcof1* mutations on the decreased proliferation in craniofacial skeleton cells (neural crest cells). p53 activation, apoptosis and ROS production and DNA damage response pathway involvement are the effectors of the TCS.

- **New identified constitutional ribosomopathies**

Since DBA, more and more constitutional human diseases have been found related to RP haploinsufficiency, but with no evidence of anemia. The question is should these defects be considered as ribosomopathies per se or should be considered as distinct phenotypes of DBA with anemia, which has been spontaneously resolved and not seen. Indeed, they have been included not in DBA disease but into the DBA syndrome. Since the description of these ribosomopathies without anemia, none of these patients have been requalified as DBA suggesting that they are really distinct ribosomopathies without anemia. This reinforces the tissue specificity for some RP haploinsufficiencies. However, the question remains what control or which pathways direct these specificities. We can argue as for DBA erythroid tropism related non extensively to the defect of GATA-1 5' TOP or 3' mRNA translation<sup>56,119</sup> that also, in these other ribosomopathies, the translation of specific subsets of mRNA may be preferentially altered by the impaired ribosome machinery and give rise to the specific tissue defect. However, if it has been well characterized in DBA, it is still to be fully defined in the case of these ribosomopathies.

Here are some examples of these ribosomopathies, with a normal hemoglobin level. Interestingly, in all of them, no defect in the pre-rRNA maturation has been identified either, reinforcing the fact that impairment in rRNA maturation in DBA is the main defect responsible for the disease.

- **RPSA and isolated congenital asplenia (ICA)**

In 2013, Bolze and colleagues<sup>133</sup> described for the first time the gene responsible for the isolated congenital asplenia, the so-called *RPSA* gene. Congenital asplenia is the only development defect in these children and no anemia is reported in these cases. Haploinsufficiency in *RPSA* is responsible for the disease and seems to be essential for spleen development. *RPSA* is involved in pre-rRNA processing. However, a defect in rRNA maturation has not been found in activated lymphocytes from *RPSA*-mutated ICA patients and the relation between *RPSA* haploinsufficiency and asplenia is still to be fully defined.

- **RPL13**

*RPL13* haploinsufficiency has been associated with spondyloepimetaphyseal dysplasia, chondrodysplasia, bone dysplasia, and short stature. The four individuals reported with splice

site mutation or missense allelic variation in *RPL13* gene share common features including normal birth length, early postnatal growth deficiency, severe short stature, and genu varum, with platyspondyly and severe epiphyseal and metaphyseal changes for lower limbs on the radiography. As the other non anemic ribosomopathies, neither of the 4 individuals exhibited any episode of anemia in their life and they had a normal blood count at last follow up. The pre-rRNA processing was normal <sup>134</sup>.

- **And in some cases, some RP genes are both described in DBA and in diseases non related to hypoplastic anemia such as RPS10 and RPS20.**

Haploinsufficiency in *RPS10*, meanwhile also a DBA gene <sup>85</sup> or *RPS23* gene are responsible for autism, intellectual disability, and dysmorphism <sup>135</sup>. *RPS20* haploinsufficiency is responsible for hereditary non polyposis colorectal carcinoma, meanwhile also recently described as a DBA gene <sup>136</sup>.

In conclusion, we hope that we convinced you that DBA is a fascinating disease with still some issues to solve but with more and more mechanisms to explain the quite specific erythroid tropism in this particular ribosomopathy. The next step now will consist to offer to the DBA affected patients, some innovative therapies, which we may replace one the burden of iterative transfusions and the side effects of long-term steroid therapy. No doubt that the progress made in gene therapy <sup>137</sup> or with targeted therapies will fill probably this gap in the next decade.



**No disclosure to make. No conflict of interest.**

**Author contributions:**

L.D.C wrote the manuscript.

**Acknowledgements**

Our special thanks go to the affected individuals, their families, and to the DBA French patient association (AFMBD, president Marcel Hibert). I sincerely thank my mentors, Gil Tchernia and Mohandas Narla for their support, friendship and many fruitful scientific discussions. I deeply thank Mohandas Narla for his careful reading and the constructive suggestions he made. I thank Dr T. Leblanc and I. Marie for our fruitful collaboration in DBA registry and Pr PE Gleizes and MF O'Donohue for both topic "erythropoiesis and ribosome biogenesis" collaboration since more than 25 years. I would like to thank all our collaborators in France and around the world and people involved in the mutational screening analysis in France (Cécile Masson, Christine Bole and the Team from the Genomics Core Facility, Institut Imagine-Structure Fédérative de Recherche Necker and Ludivine David, from the Hematology lab in R. Debré hospital, Paris). LDC is supported by #ANR EJPRD/ANR-19-RAR4-0016 (European Union's Horizon 2020 research and innovation programme under the EJP RD COFUND-EJP N° 825575), the Laboratory of Excellence for Red Cells [(LABEX GR-Ex)-ANR Avenir-11-LABX-0005-02], and The French National PHRC OFABD (DBA registry).

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## Figure legend:

### Figure 1:

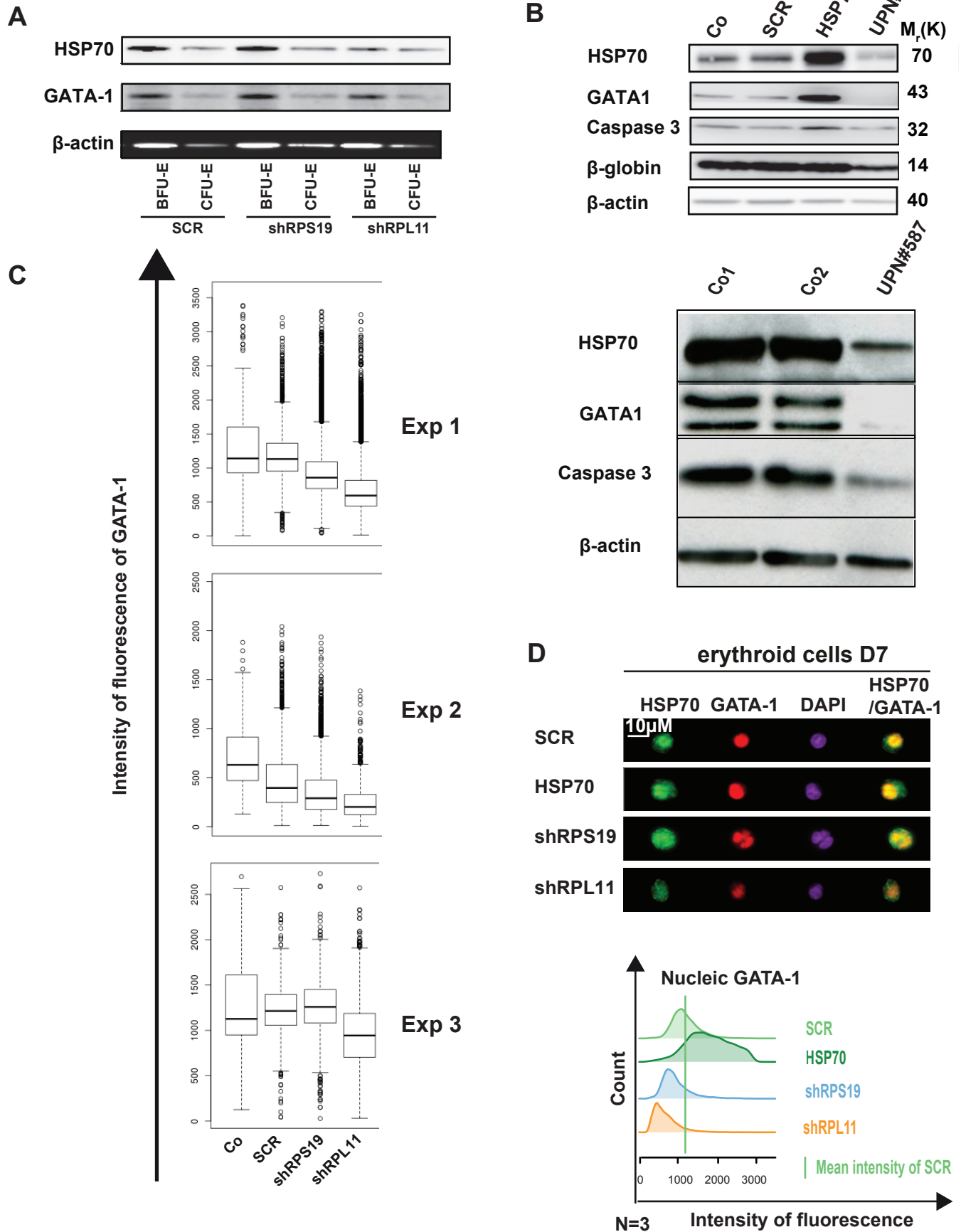
- A.** Immunoblot of HSP70 and GATA-1 expression normalized to  $\beta$ -actin at day 6 of synchronized BFU-E and CFU-E progenitors (20,000 cells/lane) obtained from cord blood CD34<sup>+</sup> infected with various shRNA against RPS19 (shRPS19), and RPL11 (shRPL11) compared to non-infected CD34<sup>+</sup> (Co) and Scramble (SCR). Molecular weight ( $M_r$ ).
- B.** Top: HSP70, GATA1, procaspase 3 and  $\beta$ -globin expression compared to  $\beta$ -actin in erythroid cells (20,000 cells) at day 10. Cells have been obtained from peripheral blood of a DBA patient UPN#479 compared to non infected cells obtained from a healthy control (Co), or healthy control cells infected with a scramble (SCR) or with wild type cDNAHSP70 (HSP70). Bottom: HSP70, GATA1, and procaspase 3 expression compared to  $\beta$ -actin in erythroid cells (20,000 cells) at day 10. Cells have been obtained from peripheral blood of a DBA patient UPN#587 compared to non infected cells obtained from two healthy controls (Co). Both DBA affected patients, UPN#479 and UPN#587 are mutated in *RPL11* gene.
- C.** Nuclear expression level of GATA1 in deficient RPS19 (shRPS19) and RPL11 (shRPL11) erythroid cells at day 10 of synchronized erythroid culture compared to non infected CD34<sup>+</sup> (Co) or infected with the scramble (shSCR) in three independent experiments. The results are presented in mean fluorescence intensity (MFI) obtained by using imaging flow cytometry (ImageStream).
- D.** Top: Imaging flow cytometry (ImageStream) analysis of GATA1 and HSP70 immunostainings in synchronized erythroid cells at day 7 of culture after depletion of RPS19-shRNA or RPL11-shRNA. Shown HSP70, GATA1, DAPI and merged HSP70 and GATA1 stainings. Magnificence X60. Scale bar= 10 $\mu$ m ; Bottom: Expression level of nuclear expression of GATA1 in deficient RPS19 (shRPS19) and RPL11 (shRPL11) erythroid cells at day 10 of synchronized erythroid culture compared to CD34<sup>+</sup> infected with shSCR or infected with wild type cDNAHSP70 (HSP70). The results are presented in mean fluorescence intensity (MFI) obtained by using imaging flow cytometry (ImageStream). The vertical green line represented the mean fluorescence intensity of GATA1 expression in erythroid cells infected with shSCR.

**Figure 2:** Repartition (%) of all the mutated genes identified in DBA affected patients.

**Figure 3:**

- A. Family tree of the French DBA family (UPN#603 and relatives) carrying the composite heterozygous allelic variations in *HEATR3* gene. Familial segregation has been verified (recessive inheritance).
- B. Erythroid culture time course from Day 0 to day 7 from UPN#603 (P4)(yellow erythroid proliferation curve), his mother (blue curve), his father (grey curve) compared to a healthy control (orange curve).
- C. Percentage of cells CD34<sup>+</sup>/CD36<sup>-</sup> (BFU-e) and CD34<sup>-</sup>/CD36<sup>+</sup> (CFU-e) (left dot plots) and Annexin V<sup>+</sup>/DAPI<sup>-</sup> (apoptotic erythroid cells) (right dot plots) from P4 compared to a healthy control.

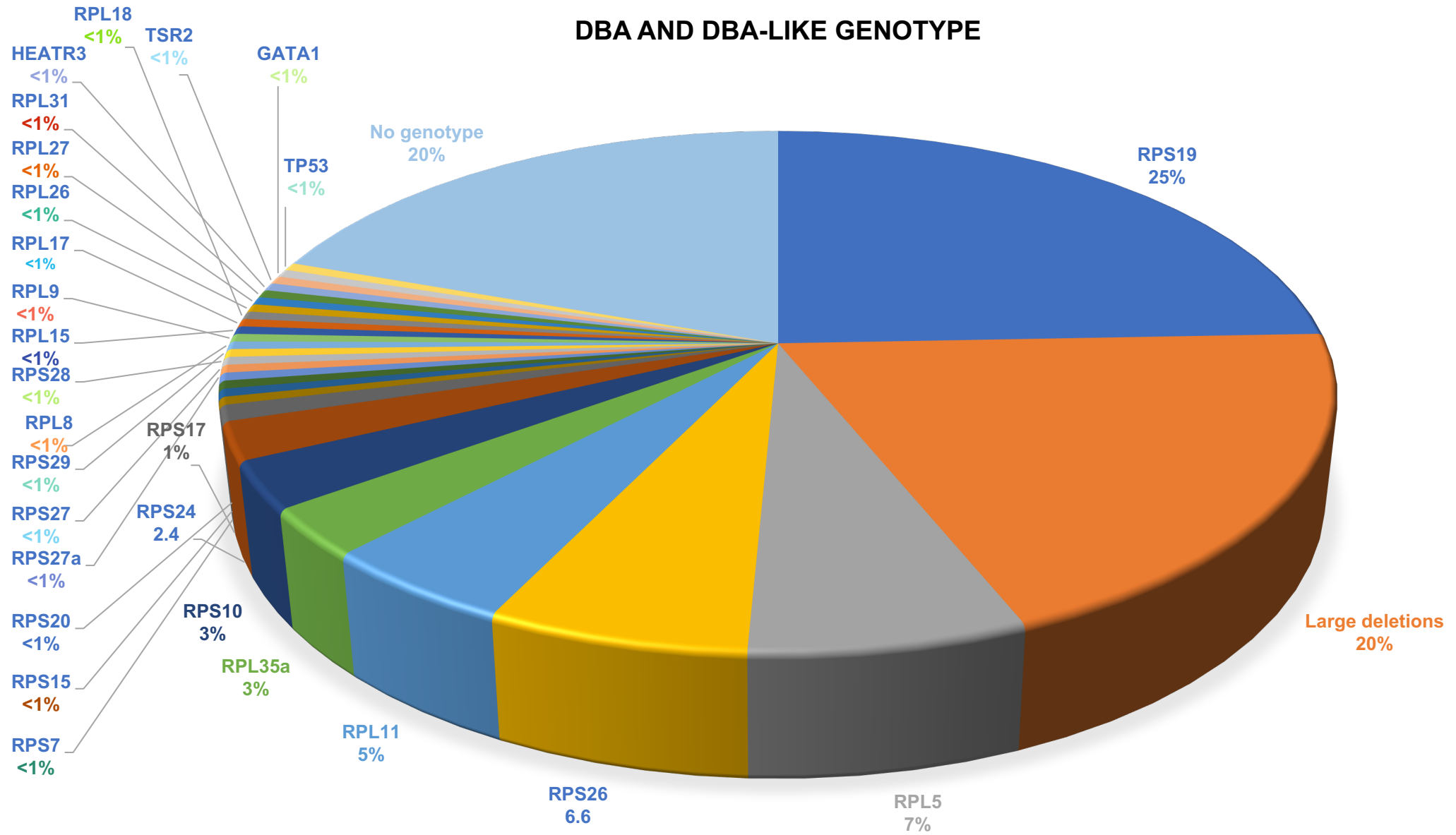
**Figure 1**



From Gastou M et al., Blood advance, 2017 with some modifications

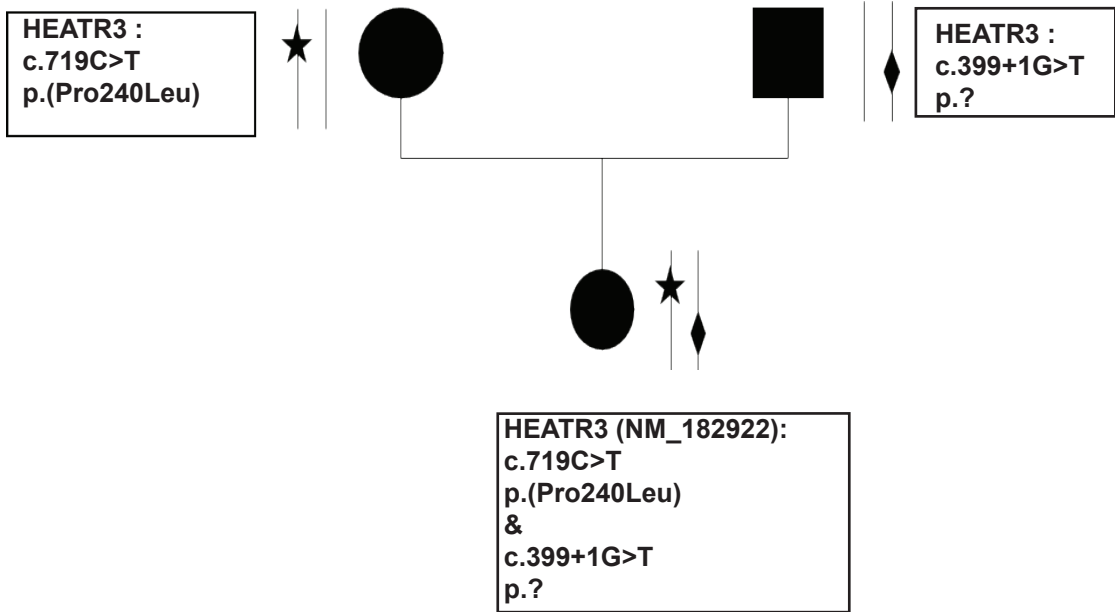
Figure 2

### DBA AND DBA-LIKE GENOTYPE

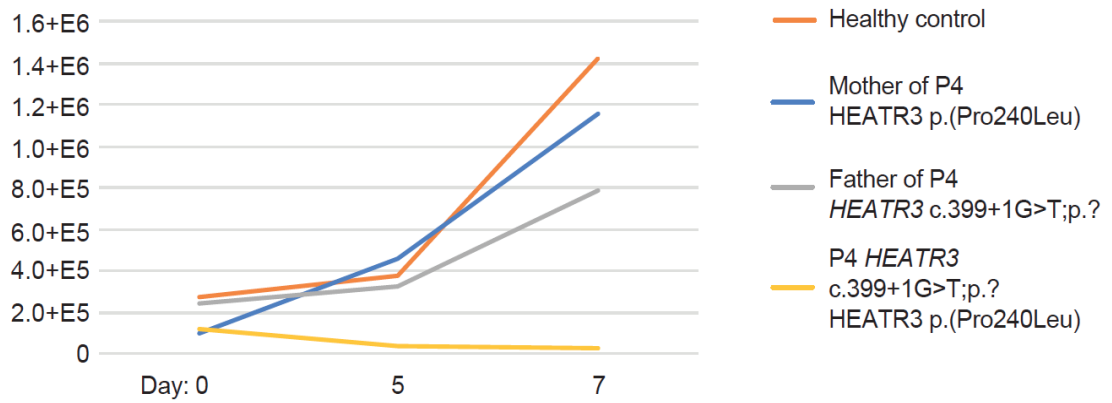


**Figure 3**

**A**



**B**



**C**

