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Pediatric Diamond-Blackfan anemia in the Netherlands: An overview of clinical characteristics and underlying molecular defects

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Abstract

Introduction: Diamond-Blackfan anemia (DBA) is characterized by hypoplastic anemia, congenital anomalies, and a predisposition for malignancies. Most of our understanding of this disorder stems from molecular studies combined with extensive data input from international patient registries.

Objectives: To create an overview of the pediatric DBA population in the Netherlands. Methods: Forty-three patients diagnosed with DBA from all Dutch university pediatric hospitals were included in this study, and their clinical and genetic characteristics were collected from patient records.

Results: Congenital malformations were present in 24 of 43 patients (55.8%). An underlying genetic defect was identified in 26 of 43 patients (60.5%), the majority of which were found in the *RPS19* gene (12 of 43, 27.9%) with 1 patient carrying a mutation in a novel DBA candidate gene, *RPL9*. In 31 of 35 (88.6%) patients, an initial response to glucocorticoid treatment was observed. Six patients (14.0%) underwent hematopoietic stem cell transplantation, and eleven patients (11 of 43, 25.6%) became treatment-independent spontaneously.

Conclusion: In agreement with previous reports, the Dutch pediatric DBA population is both clinically and genetically heterogeneous. National and international registries, together with more extensive genetic testing, are crucial to increase our understanding of genotype and phenotype correlations of this intriguing disorder.

KEYWORDS

bone marrow failure, Diamond-Blackfan anemia, genotype-phenotype correlation, patient registry, ribosomopathy

1 | INTRODUCTION

Diamond-Blackfan anemia (DBA) is a rare congenital disorder that is both clinically and genetically very heterogeneous, with most patients presenting with severe hypoplastic anemia in early infancy.[1] It represents a part of a group of rare genetic disorders, known as the inherited bone marrow failure syndromes (IBMFS), and is characterized by bone marrow failure, congenital anomalies, and a predisposition to cancer.[2] The majority of DBA patients carry haploinsufficient mutations in 1 of several ribosomal protein genes, classifying DBA along with several other congenital bone marrow failure syndromes as a "ribosomopathy".[3,4] DBA was first recognized as a specific clinical entity by the American pediatricians Diamond and Blackfan in 1938 and affects approximately 6 per million live births.[5,6] It is characterized by macrocytic anemia with reticulocytopenia, a normocellular bone marrow with a paucity of erythroid precursors, and clinical presentation during the first year of life in about 90% of patients.[1] Physical anomalies, including craniofacial abnormalities, thumb deformities, short stature, and malformations of the heart and urogenitals, occur in about 50% of DBA patients.[1,7] Some of these malformations appear to be linked to the specific ribosomal protein gene mutation. For example, cleft lip, cleft palate, and abnormal thumbs occur most frequently in DBA patients carrying a mutation in *RPL5*, while mutations in *RPL11* are predominantly associated with thumb defects. Furthermore, *RPL5* and *RPL11* defects are associated with an increased risk of (multiple) malformations in general when compared to other DBA genes.[8-11] DBA patients reveal very few erythrocyte progenitor cells in their bone marrow and often have an increased level of fetal hemoglobin.[12] The activity of erythrocyte adenosine deaminase (eADA), a critical enzyme involved in purine metabolism, is often elevated in the serum of DBA patients (80%-85% percent of DBA cases).[13]

The widely accepted pathophysiology of DBA is that ribosomal protein mutations render erythroid progenitor cells profoundly sensitive to apoptosis, ultimately leading to failure of the erythropoietic lineage development.[14] To date, mutations in 19 ribosomal genes, from both the small and large ribosomal subunit, RPS and RPL, respectively (*RPS7*, *RPS10*, *RPS15A*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPS27*, *RPS28*, *RPS29*, *RPL5*, *RPL11*, *RPL15*, *RPL18*, *RPL26*, *RPL27*, *RPL31*, *RPL35*, *and RPL35A*), have been identified in DBA patients, all of which result in haploinsufficiency of ribosomal protein genes [8,15-22]

This results in ribosomal stress and subsequent activation of the p53 tumor suppressor pathway, which is perceived as the primary origin for the clinical manifestations.[23]

The exact mechanism for the isolated red cell aplasia has not been established yet. However, several different theories, including hypersensitivity of erythroblasts to elevated p53-levels, a high protein synthesis demand in the rapidly dividing erythroblasts, increased autophagy, as well as cell-specific translation- and splicing-defects, have been proposed.[3,24-27] In addition to RP genes, extremely rare mutations in non-ribosomal genes like *GATA-1*, an erythroid transcription factor that is essential for erythropoiesis, and *TSR-2* (a ribosome maturation factor) have been linked to DBA.[17,28] Genetic mutations can be found in approximately 60%-70% of DBA cases, with RPS19, the first described gene for DBA, found mutated the most frequently in about 25% of cases.[7,8,29] The inheritance pattern described for DBA is mostly autosomal dominant, with the exception of *GATA-1* and *TSR-2* being inherited in an X-linked fashion. About 40%-45% of DBA patients is thought to have inherited the mutation from 1 of their parents, whereas the remainder of cases is thought to be the result of a de novo mutation.[7]

More than 65 years after the original report of their effectiveness, glucocorticoids are still the first-line therapy for DBA and the only drug that has been proven to be effective.[1,30-32] While it has been demonstrated that glucocorticoid treatment increases erythropoietin (EPO) sensitivity of erythroid progenitors and induces *c-kit* signaling in erythroblasts, the exact mechanism by which they exert a positive clinical effect in DBA patients is still unresolved.[14,33] Around 80% of DBA patients respond to an initial course, with hemoglobin and reticulocytes usually increasing within a few weeks.[1] Glucocorticoid treatment is associated with serious side effects, including hypertension and growth retardation, which are a substantial concern for long-term treatment. In approximately 40% of DBA patients, glucocorticoid treatment is unsuccessful due to a lack of response, unacceptable doses, or side effects requiring cessation of treatment. In these cases, treatment with chronic blood transfusions becomes necessary. Since glucocorticoid treatment in very early infancy is associated with serious complications, including neuromotor dysfunction at later age, erythrocyte transfusions are the first choice of treatment during the first year of life.[34,35] Data from different international registries show that approximately 40% of DBA patients is transfusion-dependent, 40% is glucocorticoid-dependent, and the other 20% is in remission.[36,37]

The only potentially curative treatment for bone marrow failure in DBA is allogeneic hematopoietic stem cell transplantation (HSCT). While the results of HSCT for inherited bone marrow failure disorders have improved in the past 2 decades, HSCT is still associated with a considerable risk of treatment-related mortality.[36,38,39]

Recently, based on data from the DBA Registry of North America (DBAR), it was estimated that in DBA patients, the cancer incidence is 5.4 times higher than in the general population. Compared to other IBMFS, this risk is low, reflected by 39-71 times higher risk in patients with Fanconi anemia, and 11 times higher risk in patients suffering from dyskeratosis congenita.[40] DBA-associated malignancies include predominantly myelodysplastic syndrome/acute myeloid leukemia (MDS/AML) in the non-transplanted patients, colon carcinoma, and osteogenic sarcoma.[41]

While the insights into the pathophysiology of DBA are increasing, the correlation between DBA genotype and phenotype is still largely unknown. For that reason, collecting clinical and genetic data in national and international registries are crucial. Here, we give a concise overview of the Dutch pediatric DBA population, in which we describe

novel DBA genotypic and phenotypic variants, contributing to a better understanding of the DBA spectrum as a whole.

2 | PATIENTS AND METHODS

2.1 | Patient selection

After a national enquiry, clinical data from all pediatric (age of 0-19 years) patients diagnosed with DBA, and currently treated by a pediatric hematologist, were selected for further analysis.

2.2 | Data collection

Information on the phenotypic characteristics and genetics was obtained from medical records and interviews with the treating pediatric hematologists. Standardized case report forms (CRF's) were used for the data collection in all patients. CRF's were designed based on international registries and available literature, and included information on demographics, patient history, clinical and laboratory findings, genetics, clinical course, and treatment. Furthermore, the records of subsequent visits were additionally checked to follow-up on the current status of these patients until November 2016.

2.3 | DNA testing

DNA testing in most patients was performed by PCR and BigDye Sanger sequencing of the coding region and flanking splice sites of 9 established DBA genes (*RPS7*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPL5*, *RPL11*, *RPL35A*). In patients tested a long time ago, only Sanger sequencing of *RPS19*, or *RPS19* and *RPS24* had been performed. Assessment of copy number variations (exon or gene deletions) was not routinely performed. In a limited number of cases, either multiplex ligation-dependent probe amplification (MLPA) or single nucleotide polymorphism (SNP) array was used. The public DBA gene database of LOVD (Leiden Open Variation Database, www.dbagenes.unito.it) was consulted to see if mutations were previously described in other patients or should be considered novel.

2.4 | Ethics

This study was conducted according to the guidelines as established by the Medical Ethics Committee of the University Medical Center Utrecht that granted a waiver for the ethical review requirement. Approval for the inclusion of patients was obtained from the treating pediatric hematologists, and all data were anonymized.

3 | RESULTS

3.1 | Baseline characteristics

We identified 43 Dutch pediatric DBA patients of which 27 females and sixteen males. The median age at inclusion was 8.4 years (0.5-19.5 year). The majority was of Dutch heritage, but a diverse spectrum of ethnicities and geographical backgrounds was recognized (Table 1). Twenty-five patients were classified as "classical" DBA patients according to the criteria presented by Diamond et al[42] in his landmark 1976 review (Figure 1), which are still used today. Additionally, twelve patients were identified as sporadic, non-classical cases with insufficient classical criteria but bearing a mutation in 1 of the reported genes that are associated with DBA. Furthermore, 6 patients were classified as "probable" DBA cases with a combination of diagnostic and supporting criteria.

3.2 | Clinical presentation

Prematurity was identified in 10 of 43 (23.3%) patients (31- to 36-week gestational age), which was related to fetal distress or other fetal complications in 5 patients. In 5 cases, prematurity was related to maternal conditions or of unknown cause. In 9 of 10 prematurely born DBA patients, severe anemia was present within the first weeks to months of life.

Twenty-eight patients (28 of 43, 65.1%) presented with anemia and a need for erythrocyte transfusions within the first 3 months of life, with 15 (15 of 28, 53.6%) of them displaying anemia and

TABLE 1 Ethnical background of Dutch Pediatric DBA population

Ethnic backgrounds	Number of patients (n)
Dutch/Caucasian	29
Mixed	5
Morocco	2
Turkey	2
Iran	1
India	1
Spain	1
Sierra Leone	1
Unknown	1
Total	43

Diagnostic criteria
Age at presentation <12 mo
Macrocytic anaemia with no other significant cytopenias
Reticulocytopenia
Normal marrow cellularity with a paucity of erythroid precursors

FIGURE 1 Classical diagnostic criteria according to Diamond et al

transfusion dependency immediately after birth. Six patients presented with anemia after the age of 1 year (6 of 43, 14.0%), of which 3 patients presented near the age of 2 years. Twenty-seven patients (27 of 43, 62.8%) displayed 1 or more congenital malformations, with the majority of them (17 of 27, 63.0%) being affected in multiple organ systems. Historically, growth retardation is classified as a "malformation" without discriminating between constitutional short stature and growth delay, which can be both constitutional (DBA-associated) or the consequence of chronic anemia, prednisone use, and/or iron overload. Here, growth retardation was defined as a trend of growing beneath −2.5 standard deviations. According to our definition, 24 of 43 patients (55.8%) with 1 or more malformation were observed excluding growth retardation and revised for conditions that could be contributed to a familial history (like familial hip dysplasia) (Table 2).

3.3 | Treatment

Thirty-five patients (35 of 43, 81.4%) were treated with glucocorticoids (prednisone) at some stage during the course of disease, the majority beginning after 1 year of age. In all cases, the initial dose was 2 mg/kg/d, followed by an attempt to decrease the dose to a minimal effective dose. Upon treatment, 31 of 35 (88.6%) had an initial complete response with increasing hemoglobin levels and reticulocytes within a few weeks and became transfusion-independent, whereas only 4 patients (4 of 35, 11.4%) did not respond from the start. Prednisone therapy was discontinued in 9 of 35 (25.7%) patients based on the requirements of unacceptable high doses, severe glucocorticoid toxicity (eg, growth impairment, weight gain, behavioral changes), a declining response, or a combination of factors. Thirteen patients (13 of 35) were treated with prednisone at the time of inclusion, the majority having acceptable hemoglobin levels at dosages below 0.5 mg/kg/d (10 of 13, 76.9%), not infrequently even in the vicinity of 0.1 to 0.2 mg/kg/d (8 of 13, 61.5%).

All patients received 1 or more erythrocyte transfusions at some point in the course of their disease. In addition, 13 of 43 (30.2%) patients were treated with chronic blood transfusions following ineffective glucocorticoid therapy. At the time of inclusion, 3 patients were treated with regular erythrocyte transfusions based on their young age (<12 months).

Six (6 of 43, 14.0%) patients were treated with an allogeneic stem cell transplantation, of which 5 of 6 (83.3%) were transplanted based on inadequate responses to glucocorticoid treatment (non-response, toxicity, or high-dosage dependence), leading to erythrocyte transfusion dependence. One patient presented with transfusion-dependent pancytopenia during early infancy, which had been diagnosed with DBA post-transplantation (*RPS19* c.185G>A). The age at transplantation varied from 1.7 years to 13.7 years of age. All patients were treated with an ablative conditioning regimen and showed successful engraftment of bone marrow stem cells from either a matched related sibling (4 of 6) or an unrelated matched donor (MUD; 2/6). In 1 patient, SCT was complicated by both acute and chronic graft-versus-host disease (GVHD) of the skin and intestines. All 6 transplanted patients

TABLE 2 Congenital malformations in Dutch pediatric DBA patients

Site	Description	Number
Craniofacial	Hypertelorism	4
	Broad flat nasal bridge	5
	Epicanthus	5
	Microcephaly	3
	Gnathopalatoschisis	1
	Ptosis	1
	Micrognathia	3
	Low set ears	2
	Prominent ears/floppy ears	1
	Frontal bossing	2
	Choanal atresia	1
	High palate	2
	Accessory auricle	1
Ophthalmological	-	0
Neck	Shorted/webbed neck	2
Thumbs	Distal implant of thumbs with accessory bone piece	1
	Adduction position	1
	Polydactyly	2
	Hypoplasia	2
	Radial dysplasia	1
	Deformity not otherwise specified	1
Urogenital	Multicystic kidney dysplasia (MKD)	1
	Hypospadia	1
Cardiac	VSD	4
	Coronary fistula	1
	Bicuspid aortic valve	1
Other	Syndactyly toes	1
	Skin: cafe au lait spots, cong. naevi, hemangioma, dermatofibroma	4
	Delayed development/ PMR	3
	Growth retardation	10
	Cryptorchidism	1
	Bilateral hip dysplasiaa	2
	Clinodactyly dig V	1
	Anal atresia	1
	Skeletal leg deformity	1
	Mental retardation	1

MKD, multicystic kidney disease; PMR, psychomotoric retardation; VSD, ventricular septal defect.
[a]Both with positive family history.

acquired full donor chimerism, maintain normal hemoglobin levels since HSCT, and are treatment-independent (Table 3).

Treatment independence, defined as being transfusion-independent for more than 6 months without any treatment, was observed in 11 of 43 patients (25.6%) that were not transplanted, and in 17 of 43 (39.5%) in total (including 6 patients post-SCT). Mean age at first clinical remission in the non-transplanted patients was 4.6 years old (1.0-10.3 years). Alternative therapeutic interventions were applied in 4 of 43 (9.3%) patients, including erythropoietin in 1 patient, and leucine in 1 patient. Due to very limited effects, these modalities were stopped after intervals varying from 1 week (EPO) to 20 months (leucine). Two patients have been treated with cyclosporine for 14 months and 8 years, respectively, which resulted in a temporarily decreased glucocorticoid-dependence. Meanwhile 3 of 4 of these patients have been treated with an allogeneic HSCT.

3.4 | Malignancies

No DBA-associated malignancies were observed in our study group, yet the follow-up time in our population is limited.

3.5 | Genetics

Genetic defects were found in 26 of 43 patients (60.5%). Two mutations were not identified in the genes, but the disease-causing ribosomal protein gene was proposed based on haplotyping with markers for *RPS19* in a family where many family members were affected. Furthermore, we have identified a novel, highly likely pathogenic mutation predicted to cause aberrant splicing in *RPL9* in 1 patient, a gene that has not been previously linked to DBA. All of the patients were heterozygous for the identified mutation, which is comparable with all previous reports of DBA-linked mutations. In line with other reports, we most frequently found *RPS19* mutations: 46.2% (12 of 26) in patients with known genetic defects, and in 27.9% (12 of 43) of total, followed by mutations in *RPS26* in 5 of 26 (19.2%, 11.6% of total) and mutations for both *RPL11* and *RPL5* in 2 of 26 (7.7%, 4.7% of total). Mutations in *RPL35A*, *RPL9*, and *RPS7* were all identified once in our population. Two patients were diagnosed with genetic defects involving *RPS17* and *RPL35A* as a result of chromosomal deletions of the long arms of chromosome 3 and chromosome 15, respectively (Table S1). In addition to the *RPL9* mutation, twelve mutations were not previously described on the Leiden Open Variation Database (LOVD), yet most of them were considered to be pathogenic. This will be confirmed in further studies.

3.6 | Family members

For fourteen patients, details on parental genetic testing were available. De novo mutations were confirmed in 9 of these cases when neither parent carried the mutation identified in the patient. In 5 patients, the mutation was inherited from 1 of the parents. Some mutations were identified in multiple affected family members following an autosomal dominant mode of inheritance. In 4 of these 5 families, silent carriers (ie, individuals with the same mutation but not displaying any phenotype) could be identified, indicating incomplete penetrance expression.

4 | DISCUSSION

The aim of our study was to create an overview of the Dutch pediatric DBA population, which resulted in assembled data of 43 patients. In agreement with previous studies, the Dutch population displays a large variety in both clinical characteristics as well as underlying genetic aberrations. More females than males were included with a male-to-female ratio of 1.7:1, but since no gender preponderance was ever described in comprehensive reviews, this might be due to the limited number of patients.[1,8,37] Congenital malformations were seen in 55.8% of patients, which is significantly lower than the incidence in a Greek group of patients (71%), yet higher than the incidence of congenital defects in a large cohort of Chinese patients (19.7%).[43,44] In comparison with previously reported large patient cohorts including the North American Registry (DBAR), a comparable incidence of congenital malformations (47% vs 55.8%) was observed.

Most likely, this can be explained by the incidence of specific genetic defects within ethnic subgroups, yet this needs to be investigated.

In contrast with previous reports, prematurity (in 10 of 44 patients) in our study cohort was not associated with inferior outcome so far.[37] The proportion of patients responding to an initial course of prednisone was 88.6% (31/35), which is significantly higher than what has been reported in previous studies (average 80%).[1,36,45] This could be explained by the limited size of the Dutch population, the alternative use of international criteria for effective treatment (improved

TABLE 3 Characteristics of SCT in Dutch pediatric DBA patients

N	Sex	Age (y)	Age at SCT (y)	Source	Conditioning	Complications	Current status
1	F	16.8	13.7	BM, matched sib	ATG, Bu, Flu	None	Alive, remission
2	M	19.7	6.6	BM, matched sib	ATG, Bu, Cy	None	Alive, remission
3	M	12.4	11.3	BM, matched sib	ATG, Bu, Flu	None	Alive remission
4	M	7.8	3.5	BM, MUD	ATG, Bu, Flu	AGVHD/cGVHD	Alive, remission
5	M	11.3	2.2	BM, MUD	ATG, Bu, Cy	None	Alive, remission
6	F	10.8	1.7	BM, matched sib	ATG, Bu, Cy	None	Alive, remission

ATG, antithymocyte globulin; BM, bone marrow; Bu, busulfan; Cy, cyclophosphamide; Flu, fludarabine; GVHD, graft-versus-host disease; MUD, matched unrelated donor.

erythropoiesis and/or the ability to taper the prednisone dose below 0.5 mg/kg/d), or reflects a population-specific characteristic. The same might account for the interpretation of transfusion dependency (30%), and hematological remission (25%), with respect to what has been reported in the international literature (respectively 40%, and 20%).[1,7,36,37]

While novel studies demonstrate promising results concerning potential new drugs,[46] hematopoietic stem cell transplantation is the only potentially curative treatment for bone marrow failure in DBA until now. Historically, HSCT in DBA has been controversial with significant treatment-related mortality risks and a favorable outcome for younger children (<10 years) who were transplanted with an HLA-identical sibling donor in particular.[47,48] In this study, HSCT was successful in 6 of 6 patients, including patients older than 10 years of age, and with the use of unrelated donor stem cells. While long-term outcome data in our patient population are not available yet, and our sample size is small, these data do suggest that allogeneic HSCT in pediatric DBA can be performed successfully with acceptable risks. This is in agreement with recent outcome data of allogeneic HSCT in other congenital bone marrow failure disorders.[39,48-50]

Genetic defects were identified in 26 of 43 (60.5%) of our patients, compared to approximately 65%, detected in eighteen DBA genes reported in the extensive genetic review by Clinton and Gazda[7] (Table S1). This could be explained by the fact that the majority of the Dutch DBA patients was subjected to a standardized ribosomal protein gene panel, which covers *RPS7*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, *RPL5*, *RPL11*, and *RPL35A*, thus not covering all associated RP genes. In addition, 22 of 43 to date had only been tested for a limited number of genes, *RPS19*, *RPS24*, and *RPS26* in particular. In line with previous studies, *RPS19* was the most frequently mutated gene, representing 46.2% of genetic defects, and 27.9% (12 of 43) of our patient population. These data have to be interpreted carefully since not all known DBA genes were tested in all patients, thereby possibly overestimating the prevalence of *RPS19* mutations in the Dutch DBA population. This is being currently investigated. Interestingly, 2 of our patients with identified genetic defects have a parent with DBA-associated congenital anomalies (thumb abnormalities, osteosarcoma in early adulthood) in the absence of the DBA mutation and a hematological phenotype. This could be the result of mosaicism in the parent, or a coincidental finding not related to the DBA genotype. Other families were characterized by DBA-associated abnormalities (predominantly hematological), while no genetic defect had been identified in the index DBA patient so far.

We observed no specific genotype-phenotype correlations other than a more severe and similar phenotype in both patients with genetic defects in *RPL11*. While both *RPL11* and *RPL5* mutations in DBA have been linked to a very high incidence of congenital malformations (73% and 83%, respectively),[9,10] we observed no congenital anomalies in 1 patient, and a very mild dysmorphic feature (downslanting of palpebral fissures) together with mild developmental delay in our other patient diagnosed with a *RPL5* mutation. In addition, we found no urogenital malformations in the 2 patients with a *RPL35A* defect, which has been correlated by Boria et al[8] in an Italian cohort of DBA patients.

In summary, our data provide a comprehensive overview of the complete Dutch pediatric DBA population, characterized by heterogeneity in genotypes, and phenotypes, and a generally good response to glucocorticoid treatment. In more detail, our pediatric population includes a relatively large number of patients with a *RPS19* mutation, which were non-identical in all cases and generally were associated with a relatively mild phenotype. Intriguingly, we have identified a novel DBA candidate gene (*RPL9*) in a patient with a complex phenotype, including colitis. Functional studies were conducted and confirmed the pathogenic nature of this mutation (full report in review). Six of our patients were successfully treated with an allogeneic HSCT including unrelated transplants. In concordance with other DBA patient registries, our data illustrate that there is a large variety of DBA characteristics and clinical manifestations, which can generally not be explained by underlying genetic defects. In addition to prospective registration in DBA patient databases, advanced genetic testing and functional analysis in human DBA models will be necessary to further increase our understanding of DBA pathophysiology.

FINANCIAL DISCLOSURES

The authors declare to have no (potential) conflicts of interest regarding the submitted article.

AUTHOR CONTRIBUTION

BD performed research, analyzed data, and wrote manuscript. CO, FS, RT, ML, AD, MP, BG, and JG performed research and reviewed manuscript. MBi analyzed data and wrote manuscript. AM and MBa designed research, analyzed data, and wrote manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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