RESEARCH

Molecular study of Pompe disease in Egyptian infants

Mona Essawi[1], Nagham ElBagoury[1], Engy Ashaat[2], Wessam Sharaf-Eldin[1*] and Ekram Fateen[3]

Abstract

Background: Pompe disease (PD) is a serious genetic disorder caused by deficiency of acid α-glucosidase (GAA) and subsequent glycogen accumulation inside lysosomes. This study included a cohort of 5 Egyptian infants (1–8 months old) with far lower than average normal GAA activity and clinical signs of PD in 4 of the 5 cases. The fifth case was discovered by newborn screening (NBS). Molecular analysis of the GAA gene was performed to confirm the diagnosis and identify the underlying mutation.

Results: The study identified the causative mutations [c.1193T > C (p.Leu398Pro), c.1134C > G (p.Tyr378*) & c.1431del (p.Ile477Metfs*43)] in 4 cases. However, molecular analysis reversed the expected pathologic state in the fifth infant, where his reduced enzymatic activity was related to the presence of pseudodeficiency allele c.868A > G (p.Asn290Asp) in addition to heterozygous disease-causing mutation c.2238G > C (p.Trp746Cys).

Conclusion: This study presents the first molecular analysis of *GAA* gene in Egypt and has thrown some light on the importance of PD molecular diagnosis to provide precise diagnosis and enable therapeutic commencement in affected subjects.

Keywords: Pompe disease, Acid α-glucosidase, Molecular analysis, Pseudodeficiency allele

Background

Pompe disease (PD), also called glycogen storage disease type II (GSD II) or acid maltase deficiency (AMD) (MIM# 232300), is a genetic neuromuscular disorder with an autosomal recessive mode of inheritance. It is caused by mutations in acid alpha-glucosidase gene (*GAA*) which is localized to chromosome 17q25.2- q25.3 and consists of 20 exons (exon 1 is noncoding). Gene mutations lead to deficiency of GAA enzyme (NP_000143.2) resulting in lysosomal glycogen accumulation in multiple tissues, with profound pathology in skeletal and cardiac muscles [1]. According to the disease age of onset, PD is classified into two main clinical forms: infantile-onset PD (IOPD) which begins within the first months of life, and late-onset PD (LOPD) which appears any time after the first

year of life. Childhood, juvenile and adult-onset PD are examples of LOPD. IOPD is referred to as classic or non-classic due to presence or absence of cardiomyopathy, respectively. IOPD is associated with poor prognosis as without treatment most infants die within the first year life. However, LOPD has milder phenotype with more protracted disease course, but eventually leads to respiratory failure [2]. Age of onset, organ involvement and disease severity depend on genotype and residual enzyme activity. Enzyme activity is minimal or absent in IOPD, but may be reduced to varying degrees (3–30%) in LOPD [3].

PD has been reported in most populations with a worldwide incidence of 1 in 40,000 live births [4]. However, its prevalence may considerably vary among different ethnicities, with a fluctuating range from 1 in 14,000 in African-American population to 1 in 600,000 in Portuguese population [5]. Moreover, very rare occurrence rate of PD has been documented in some countries such as Sweden and Finland [6, 7].

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The spectrum of *GAA* mutations is very heterogeneous. To date, 648 disease-associated variants have been listed at the Pompe disease GAA variant database (http://www.pompevariantdatabase.nl) [8]. All types of mutations have been described including complex rearrangements; however, missense variations are the most frequent type of mutations (˃50%). So far, only one variant in the regulatory region has been described. Although most *GAA* mutations are restricted to a small number of families, some mutations are frequently reported. Best examples are provided by c.525delT and entire deletion of exon 18 (c.2481 + 102_2646 + 31del) which are mostly occurring in white populations. Both mutations have been considered as severe variations and are mainly associated with IOPD. Also, the intronic mutation, c.-32-13T > G, is the most common mutation in patients with LOPD accounting for about 50% of cases [9]. Moreover, the variant c.1935C > A is the most frequent in Asia−Pacific [10] and the truncating mutation c.2560C > T was found to be overrepresented in Brazil, North Africa and African-Americans [9].

Different therapeutic approaches have been adopted for treatment of PD, starting from nutrition-based therapy and ending with gene therapy approaches. Nutrition-(low carbohydrate and high protein diet) and exercise-based therapy has been employed as a palliative approach to slow muscle impairment [11]. Gene therapy has been considered especially for treatment of neural manifestations since they are not resolved by peripheral enzyme replacement [12].

In the meanwhile, enzyme replacement therapy (ERT) is the most commonly used therapeutic approach for PD. ERT should be started before presentation of symptoms and occurrence of irreversible injury, to attain optimal treatment. Early intervention of ERT in patients with IOPD markedly achieves improved survival, reduced ventilation need and better movement, resulting in enhanced quality of life [13].

The importance of the effect of early therapeutic intervention in IOPD patients throws the light on the importance of newborn screening (NBS) for early diagnosis and treatment initiation. However, *GAA* pseudodeficiency alleles can reduce the enzyme activity into the abnormal range interfering with NBS for PD. In this context, gene molecular analysis could be utilized to identify false-positive cases in NBS program for PD [13].

This study was mainly conducted to set up the molecular analysis of *GAA* in Egypt, where the protocol has been applied on a number of Egyptian patients with reduced GAA enzyme activity.

Methods

Subjects

We reported a cohort of 5 subjects with reduced GAA enzyme activity. Biochemical analysis was carried out for all subjects as they exhibited disease manifestations except for one case whose diminished enzyme activity was observed throughout NBS for inborn errors of metabolism (IEMs) carried out in USA.

The study obeyed the beliefs of the Declaration of Helsinki. Written consent was obtained from parents of all enrolled subjects. The study protocol was approved by Medical Research Ethics Committee, Cairo, Egypt (Registration Number: 11098).

Clinical diagnosis

All subjects were subjected to full history taking, family pedigree construction, clinical examination, and echocardiography.

Biochemical analysis

EDTA blood sample (5 ml) was collected from each patient. GAA enzyme activity was assayed in mixed leukocytes according to the method of Okumiya et al. (2006), in which 4-methylumbelliferone-α-glucopyranoside was used as substrate with an inhibitor acarbose to remove the interference of other glucosidases [14].

Molecular analysis

Genomic DNA (gDNA) was isolated from peripheral blood leukocytes (PBLs) using the salting out protocol [15]. Primer pairs have been designed to amplify the 19 coding exons of *GAA* gene and the flanking intronic sequences using the GenBank sequence (accession no. NM_000152.5) (Table 1). PCR was carried out in a total volume of 15 µl comprising 100 ng of gDNA, 10 pmol of each primer, 100 µM of dNTPs (Thermo Scientific, EU), 1.5 µM MgCl2, 1X Taq buffer and 2.5 U Taq polymerase (Thermo Scientific, EU). As a common variation in *GAA* gene, the entire deletion of exon 18 was firstly tested by amplification of exons 17 and 18 simultaneously using the forward primer of fragment 11 and the reverse primer of fragment 12. For sanger sequencing, successful PCR products were purified using 4 U Exonuclease I (Thermo Scientific, EU) and 0.4 U Shrimp Alkaline Phosphatase (Thermo Scientific, EU). Then, cycle sequencing PCR was carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), followed by removal of dye terminators using CENTRI-SEP purification spin columns (Applied Biosystems). Sequencing was performed using a 3500 ABI Prism DNA sequencer (Applied Biosystems). Data files obtained from the sequencer were displayed by

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Table 1 Sequences of designed primers, product sizes and annealing temperatures

Fragment	Exon	Primer sequence	Product size (bp)	Annealing (°C)
1	2	F: 5'CTT TGA GAG CCC CGT GAG TG 3'	718	63
		R: 5'TTG TGA GGT GCG TGG GTG TC 3'		
2	3	F: 5'TCA GAG AGG CTG AAT GTG CTG 3'	306	60
		R: 5'GCA CAG AGC CCA GAA CTC AC 3'		
3	4 & 5	F: 5'GGT GCG CCT CTT CTG ATA TG 3'	625	60
		R: 5'GGA AAT GAG TCC TAT GGG CTG 3'		
4	6, 7 & 8	F: 5'TGG GGA GAG AGC CTC AAC TC 3'	653	62
		R: 5'GCA CAC ACC ACG ATC ATC ATG 3'		
5	9	F: 5'GCT GTA CAC ACG CAT GAT GTC 3'	269	60
		R: 5'GCC TCT GCT TTC TAA CCC CC 3'		
6	10 & 11	F: 5'TCA GGC TGA GGC TCA GTG GG 3'	465	63
		R: 5'CAG AGA TGA GGG TGC TAA GTC 3'		
7	12	F: 5'TAA CGC CAG CCC CAC AGA GG 3'	380	60
		R: 5'TCC GCT TTT CCT CCT CCC TG 3'		
8	13 & 14	F: 5'CAT CAG GTG GCC CAG ACA G 3'	688	62
		R: 5'GAG AGT CTT GGG TGG GTG G 3'		
9	15	F: 5'ATG CTG GGT GGC TGA GAA G 3'	390	59
		R: 5'CCA GGC CCA AAT GTT GTC TC 3'		
10	16	F: 5'CCA GCC CCA TCC CAT TCA T 3'	298	59
		R: 5'GTT CCT CTT TCC GCC ACC T 3'		
11	17	F: 5'AGA TGG AGA GCG TGG TTC C 3'	287	55
		R: 5'CCC CAG CTC TGC AGT GTG 3'		
12	18 & 19	F: 5'TAG TTA CTG GCA GCC TGG TG 3'	821	57
		R: 5'ATC CTC TGT TCC TGG CAT CG 3'		
13	20	F: 5'CAC AAT GAC GAC CTC TGA GC 3'	369	56
		R: 5'GGA ACC AGC CTC CCT CTG 3'		
14*	17, 18 & 19	F: 5'AGA TGG AGA GCG TGG TTC C 3'	Wild-type: 1386	56
		R: 5'ATC CTC TGT TCC TGG CAT CG 3'	Mutant: 848	

* Amplification of this fragment was carried out to investigate the entire deletion of exon 18 (538 bp)

Finch TV, version 1.4.0. Query sequences were aligned against those present in BLAST/Basic Local alignment search tool (http://blast.ncbi.nlm.nih.gov) [16].

In silico analysis

Several in silico tools were employed to predict the effect of the firstly reported missense variation including Blosum62 (Amino acid substitution matrices from protein blocks) [17], MutationTaster (http://www.mutationtaster.org/) [18], PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) [19], AGVGD (http://agvgd.hci.utah.edu/agvgd_input.php) [20], SNPs&GO (http://snps-and-go.biocomp. unibo.it/snps-and-go/) [21], MUTRED software (http://mutpred.mutdb.org/) [22], SIFT & PROVEAN (http://provean.jcvi.org/index.php) [23] and Hope (https://www3.cmbi.umcn.nl/hope/) [24].

Results

Here, we reported a cohort of 5 subjects from 4 unrelated families (3 males and 2 females). All of them showed reduced GAA enzyme activity with mean $= 0.47 \pm 0.227$ (normal enzyme activity is >3 μmol/gpt/h). Their ages ranged from 1 to 8 months. All patients showed positive consanguinity except one whose diminished enzyme activity was detected throughout NBS for IEMs without observation of any disease manifestations. Other study subjects showed typical picture of IOPD including cardiomegaly, cardiomyopathy, hepatomegaly, feeding difficulties, failure to thrive, developmental delay, macroglossia, muscle weakness, hypotonia and respiratory distress (Table 2).

The entire deletion of exon 18 was detected in none of the enrolled cases. In 4 patients, sanger sequencing succeeded to identify homozygous pathogenic mutations confirming their diagnosis. The detected mutations

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included a frameshift [c.1431del (p.Ile477Metfs*43)], a nonsense [c.1134C > G (p.Tyr378*)] and a missense [c.1193T > C (p.Leu398Pro)] mutations, where the latter was reported in 2 related patients. Moreover, only one heterozygous mutation was detected in one subject who showed reduced enzyme activity during NBS; however, he was recruited without any disease manifestations. Interestingly, gene molecular analysis identified a pseudodeficiency allele (inherited from father), besides the pathogenic heterozygous mutation (inherited from mother) in this subject explaining the low detected enzyme level and denoting for the importance of gene molecular testing to confirm the diagnosis (Table 3). A novel missense mutation (p.Leu398Pro) has been identified in two siblings and already submitted to the ClinVar database (Fig. 1). Performed in silico analysis favored a pathogenic effect for this novel missense mutation. Importantly, the mutated residue is located in the enzyme catalytic domain (347–726 residues). Possible structural alterations induced by this

Table 2 Enzyme activity and main clinical features in the studied subjects

| Case no	Sex	Age (month)	Consanguinity #	1,4-α glucosidase activity (µmol/gpt/h)*	Cardiomyopathy	Hypotonia	Hepatomegaly	Respiratory distress	Feeding difficulties
1	M	1	+ve	0.56	+	+	+	+	+
2	F	1	+ve	0.32	+	+	+	+	+
3	M	4	+ve	0.47	+	+	+	+	+
4	F	8	+ve	0.4	+	+	+	+	+
5	M	4	−ve	0.92	−	−	−	−	−

* µmol/gpt/h: micromole/gram protein/hour—normal enzyme activity is ˃3 µmol/gpt/h; # all patients showed positive consanguinity. The only exception is the infant whose diminished enzyme activity was detected throughout NBS

Table 3 Mutations identified in the enrolled subjects:

Case no	Base change	Amino acid change	Exon	Variant type	Allele	Clinical significance
1	c.1193T > C	p.Leu398Pro	7	Missense*	Homozygous	Pathogenic
2	c.1193T > C	p.Leu398Pro	7	Missense*	Homozygous	Pathogenic
3	c.1431del	p.Ile477Metfs*43	9	Frame shift	Homozygous	Pathogenic
4	c.1134C > G	p.Tyr378*	7	Nonsense	Homozygous	Pathogenic
5	c.868A > G	p.Asn290Asp	5	Missense	Heterozygous	Pseudo deficiency
	c.2238G > C	p.Trp746Cys	16	Missense	Heterozygous	Pathogenic

*Novel mutation

c.1193 T>C (SCV000998899)
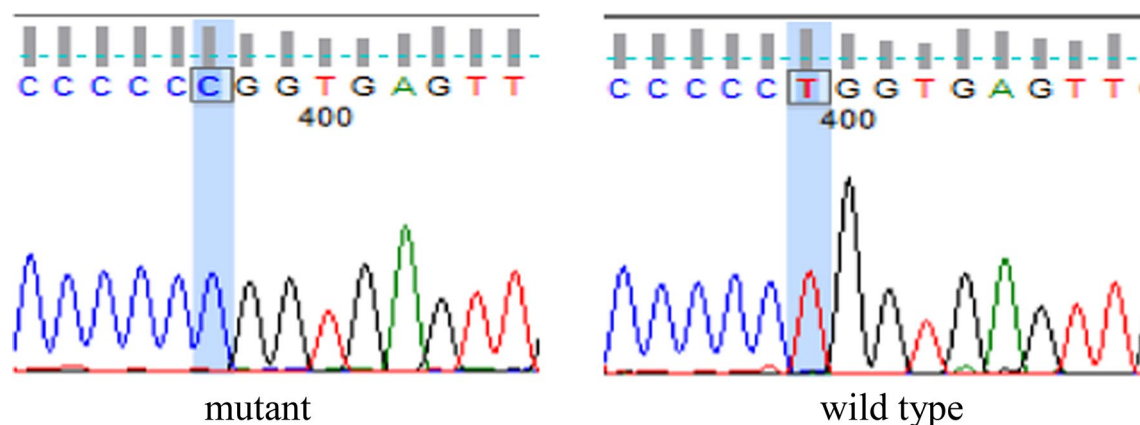
Fig. 1 Sequencing electropherogram of the novel missense mutation. ClinVar accession is written between brackets

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mutation was also assessed using HOPE webserver [24] (Fig. 2). It has been predicted that the mutation will cause an empty space in the core of the protein due to the smaller size of the mutant residue, and this mutation can affect the proper protein interaction and function. It is noteworthy that the used in silico tools also defined the pseudodeficiency allele as pathogenic (Table 4), denoting for the importance of experimental functional analysis to distinguish between pseudodeficiency alleles and real pathogenic mutations.

Discussion

PD is a multisystemic metabolic disorder caused by deficiency of GAA enzyme leading to progressive accumulation of glycogen inside lysosomes. In turn, this results in lysosomal swelling and rupture all over the human body [25]. It represents one of the most frequent causes of metabolic-based cardiomyopathy in infants [26].

Importantly, PD signs share considerable similarity with several other conditions. Differential diagnoses (DD) of PD mainly include the upper and lower motor neuron diseases. Other DD of IOPD are congenital
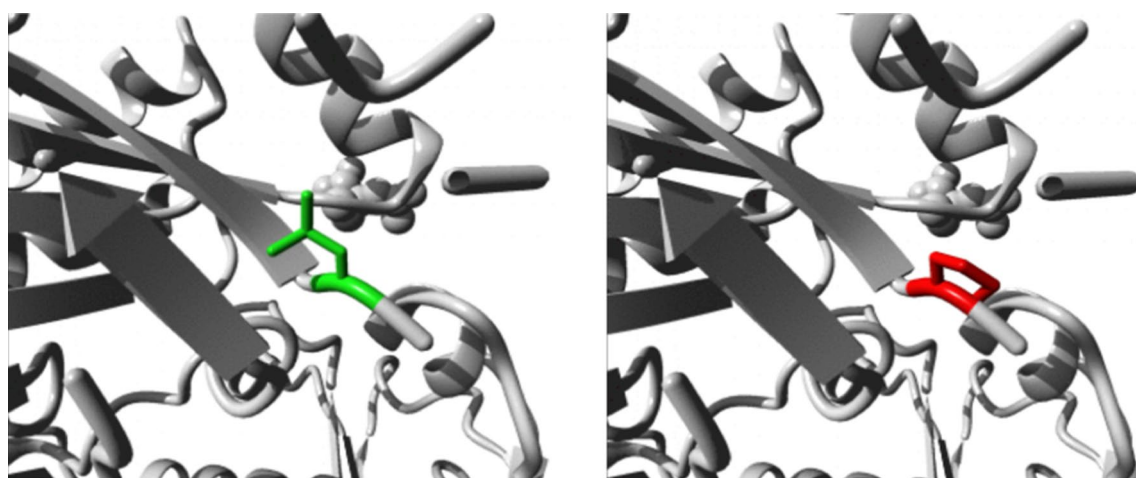
Fig. 2 The three-dimensional model of the protein with close-up of the mutation. The protein is colored gray, and the side chains of both the wild-type and the mutant residue are shown and colored green and red, respectively

Table 4 In silico analysis for the novel missense variant and the pseudodeficiency allele

Tool	c.1193 T > C (p.Leu398Pro)	c.868A > G (p.Asn290Asp)
MutationTaster (score)[a]	Disease causing (98)	Disease causing (23)
PROVEAN (score)[b]	Deleterious (− 3.108)	Deleterious (− 4.525)
SIFT (score)[c]	Damaging (0.019)	Damaging (0.009)
PolyPhen2 (score)[d]	probably damaging (0.998) Sensitivity: 0.27 Specificity: 0.99	Probably damaging (1.000) Sensitivity: 0.00 Specificity: 1.00
AGVGD[e]	Class C65	Class C15
SNPs&GO (Reliability Index)[f]	Disease-related (10)	Disease-related (10)
MUTRED software (probability)[g]	Deleterious (0.911)	Deleterious (0.595)
Blosum62[h]	− 3	1

[a] MutationTaster score ranges from 0.0 (polymorphism) to 215 (disease causing)

[b] Variants with score $\leq$ − 2.5 are "damaging" and variants with score > − 2.5 are "neutral"

[c] Variants with score $\leq$ 0.05 are "deleterious" and variants with score > 0.05 are "tolerated"

[d] PolyPhen2 score ranges from 0 (tolerated) to 1 (deleterious)

[e] AGVGD classes: C0 (least likely to interfere with function), C15, C25, C35, C45, C55, C65 (most likely to interfere with function)

[f] The SNPs&GO reliability index extends from 1 (neutral) to 10 (disease-related)

[g] MUTPRED predictions are considered "deleterious" if the score is > 0.5

[h] In Blosum62 matrix, a positive score implies that substitution is more likely than any random substitution and vice versa

muscular dystrophy, spinal muscular atrophy I (acute Werdnig-Hoffman disease), congenital muscular dystrophy, mitochondrial/respiratory chain disorders, glycogen storage diseases type IIIa, idiopathic hypertrophic cardiomyopathy, Danon disease and peroxisomal disorders [27]. It is noteworthy that in the last year, about 25 cases with provisional diagnosis of PD were recruited to our institute for biochemical analysis of GAA. However, low enzyme activity was only detected in the 5 studied patients. This may reflect the phenotypic overlapping of PD with other disease conditions.

This study represents the first report of molecular analysis of *GAA* in Egypt, in which 5 cases have been reported. Four subjects have been clinically diagnosed with IOPD, showed reduced enzymatic activity and revealed homozygous pathogenic mutations confirming their diagnosis. Mutations in exon 6 were detected in 3 cases from 2 unrelated families. One missense mutation has been firstly reported which could be defined as a disease-causing variation using several in silico approaches. Interestingly, none of the mutations detected in this study has been reported as a frequent pathogenic *GAA* variation in another population, except for c.2238G > C (p.Trp746Cys) which represented the frequently reported mutation in mainland China accounting for about 27% of total mutant alleles [28].

One case was included in the study as he exhibited diminished GAA level during enzymatic assay. However, subsequent molecular analysis recommended that he would not develop PD and attributed his low enzymatic activity to pseudodeficiency. This phenomenon occurs due to using synthetic substrates (such as 4-methylumbelliferyl α-D-glucoside and 4-methylumbelliferyl-α-D-glucopyranoside) instead of the natural substrate (glycogen) in laboratory enzyme assay. Enzymes containing pseudodeficiency alleles are known to impair the enzymatic activity on these artificial substrates leading to false-positive results upon enzyme tests. However, they can process natural substrate normally, or at a level that does not result in disease. Interestingly, low enzyme level (0.36 μmol/gpt/h) was also reported in the subject's father (completely normal) who was found to be homozygous for the pseudodeficiency allele (p.Asn290Asp).

Importantly, p.Asn290Asp variant was firstly reported as a variant with uncertain significance. Later, Kroos and colleagues examined 69 *GAA* mutations including D290N by site-directed mutagenesis and transient expression in COS-7 cells or HEK293T cells and defined p.Asn290Asp as presumably non-pathogenic [29]. However, in silico tools applied in this study supposed a pathogenic effect for this variant. This in turn denotes for the importance of experimental

functional analysis to distinguish between pathogenic mutations and pseudodeficiency alleles, especially in subjects that exhibit none of disease manifestations (Table 3). Generally, a comprehensive NBS program for inherited metabolic disorders is highly recommended worldwide. The key factor is that early diagnosis and management are crucial for the survival and well-being of affected children [30]. Importantly, Egypt and other countries of Middle East have relatively high prevalence for IEMs due to increased rates of parental consanguinity and first cousin marriages [31]. It is noteworthy that national NBS program for congenital hypothyroidism has been already established in Egypt since April 2000 [31] and some pilot studies for expanded metabolic screening have been conducted [32, 33]. As enzyme deficiency can also be reported in healthy individuals due to pseudodeficiency, mutation characterization is a critical step in subjects with decreased enzyme activity to explore whether an enzyme deficiency is due to pathogenic mutation or harmless pseudodeficiency [34].

Conclusion

This study identified the pathogenic mutations in a cohort of Egyptian patients with PD including a novel missense mutation. On the other hand, it highlights that molecular procedures should be an essential part of the NBS program for PD and other LSDs in newborns who had abnormal results in the biochemical analysis. This provides an accurate diagnosis, relieving parental anxiety in case of false-positive infants and contributing for immediate therapeutic intervention in case of true-positive newborns.

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Declarations

Ethics approval and consent to participate
Written consent was obtained from parents of all enrolled subjects. The study protocol was approved by Medical Research Ethics Committee of National Research Centre, Cairo, Egypt. Approval Number is 11098.

Consent for publication
Not applicable.

Competing interests
The authors declare that there are no competing interests.

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