UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE ANÁLISES CLÍNICAS
CURSO DE GRADUAÇÃO EM FARMÁCIA

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Análise *in sílico* de variantes de nucleotídeo único (SNVs) *missense* associadas ao gene GHS-R na obesidade

Florianópolis
2022

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Trabalho de Conclusão de Curso submetido ao curso de Graduação em Farmácia do Centro de Ciências da Saúde da Universidade Federal de Santa Catarina como requisito parcial para a obtenção do título de Bacharel em Farmácia.

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Florianópolis

2022

Ficha de identificação da obra elaborada pelo autor,
através do Programa de Geração Automática da Biblioteca Universitária da UFSC.

Fonseca Nunes, Bruno
Análise in sílico de variantes de nucleotídeo único (SNVs) missense associadas ao gene GHS-R na obesidade / Bruno Fonseca Nunes ; orientadora, Fabiola Branco Filippin Monteiro, 2022.
26 p.

Trabalho de Conclusão de Curso (graduação) - Universidade Federal de Santa Catarina, Centro de Ciências da Saúde, Graduação em Farmácia, Florianópolis, 2022.

Inclui referências.

1. Farmácia. 2. Obesidade. 3. Grelina. 4. Variantes. 5. Gene. I. Branco Filippin Monteiro, Fabiola. II. Universidade Federal de Santa Catarina. Graduação em Farmácia. III. Título.

Bruno Fonseca Nunes

Análise *in sílico* de variantes de nucleotídeo único (SNVs) *missense* associada ao gene GHSR na obesidade

Este Trabalho de Conclusão de Curso foi julgado adequado para obtenção do título de Bacharel em Farmácia e aprovado em sua forma final pelo Curso de Graduação em Farmácia

Florianópolis, 12 de dezembro de 2022.

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Florianópolis, 2022

Dedico este trabalho aos meus pais que me deram a vida, aos meus familiares e amigos que de alguma forma contribuíram para que chegasse até aqui, e a minha orientadora por toda dedicação e apoio.

APRESENTAÇÃO

Este Trabalho de Conclusão de Curso será apresentado na forma de artigo científico. Esta forma de escrita foi escolhida pelos autores, devido interesse real em publicar o trabalho, dado sua natureza.

A revista escolhida para publicação foi a **Gene reports**, que tem publicações com foco na regulação, expressão, função e evolução dos genes em todos os contextos biológicos, incluindo todos os organismos procarióticos e eucarióticos, bem como vírus, apresentando um Cite Score de 1.6 em 2022.

AGRADECIMENTOS

Primeiramente a Deus, pela oportunidade da vida, e pela orientação para passar por todos os percalços da vida que sugiram até o momento.

A meus familiares, em especial a meus pais que jamais desistiram de me fornecer uma educação de qualidade, valorizando e confiando sempre em meu desempeno mesmo nos momentos em que tudo parecia dar errado.

Aos amigos que fiz durante esta graduação, em especial à Ana Carolina Ibarra dos Santos e Ana Leticia Müller, que foram um grande suporte e grandes presentes que a vida e a Universidade me deram, jamais esquecerei da nossa amizade e o quanto vocês representam em minha vida. Às minhas parceiras de ônibus, Gabriela de Oliveira Rocha, Vanessa Soares, Isadora Aguiar, Luise Almeida e Ana Cecília Lopes, que compartilharam os momentos de inda e vinda de Garopaba. À Raissa Meneguzzi que mesmo distante no final do curso, esteve presente nos melhores e piores momentos da graduação.

A meus colegas do Laboratório de Imunobiologia do Tecido Adiposo - LAITA, em especial à Msc. Carolina Dumke de Siqueira, Msc. Letícia de Oliveira Souza Bratti, Daphany Marah Gorges, Ana Carolina Martins, Vinicius Matheus Knihs e Elizabeth Soeth Alves, em especial à grande Gênia Contemporânea, Msc. Maria Luiza Carneiro Buchele, que dividiu e compartilhou todo seu conhecimento, não só de cultura celular, mas de vida comigo.

A todos professores que compartilharam seus conhecimentos, e que de alguma forma marcaram minha passagem pela graduação, especialmente à Prof.ª. Drª. Fabíola Branco Filippin Monteiro, minha orientadora, que aceitou a missão de orientar este trabalho, ainda, pela oportunidade de desenvolvimento de Iniciação Científica, que com certeza é um grande ponto positivo da minha formação.

"*Ninguém ignora tudo. Ninguém sabe tudo. Todos nós sabemos alguma coisa. Todos nós ignoramos alguma coisa. Por isso aprendemos sempre.*" (FREIRE, Paulo. **A importância do ato de ler**. São Paulo: Cortez, 1989)

In silico analysis of missense single nucleotide variants (SNVs) in GHSR gene-associated obesity

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Abstract

In recent years, the search for understanding the pathophysiology of obesity has been intense, especially the satiety signs in the hypothalamus and brain regions associated with hormonal signalling in orexigenic and anorexigenic neurons. Such signs are quite complex and often associated with energy expenditure and feelings of hunger and satiety in mammals. Recent studies have shown that some key peptides promote exacerbation or inactivation of these pathways. Different phenotypes regarding body composition, however, are resulted from mutations (allelic variants) in the genes encoding these proteins, especially peptide receptors. In particular, the hormone receptor ghrelin (GHSR), located on the surface of orexigenic neurons, has been associated with the regulation of hunger. Furthermore, allelic variants of the GHSR gene may compromise significant changes in signalling provided by the GHSR, providing altered hormone-binding phenotypes and its receptor. In this sense, searching for allelic variants that can explain the different phenotypes of thinness or overweight/obesity can help understand the pathway and define new strategies for early laboratory diagnosis. Initially, mining was carried out on the dbSNP which presented 373 non-synonymous Single nucleotide polymorphisms (SNPs) located in coding regions (missense). After being filtered by minor allele frequency (MAF) lower than 1%, and submitted to 8 different *in silico* predictions tools we found eight variations: L91F, R237W, I134T, V216A, V46F, S174N, N319H, and D194Y. Variants were analyzed at the HOPE project web server and Swiss-Model database. The results and future analyses of these mutations may give us a better elucidation of the implication of mutations and their possible correlation with the pathophysiology of obesity.

Keywords: obesity; ghrelin; GHSR; receptor; variants; genes.

1. Introduction

Human ghrelin (GR) is encoded by the GHRL gene, located on the short arm of chromosome 3 (3p25-26), and contains 6 exons and 4 introns [1]. GR is a peptide hormone produced by the epsilon cells of the stomach and pancreas with an endocrine function that stimulates the release of growth hormone (GH), thus acting in growth hormone cells located in the pituitary and hypothalamus [2]. In addition to its role as a stimulator of GH release, GR is also able to stimulate gastric acid secretion in humans. Studies have shown that in rats, GR can increase food intake stimulated by gastric emptying provided by controlled fasting. The results showed that, when centrally administered, it was able to induce eating behaviour within five minutes after administration [3]. These studies were crucial to a better understanding of genetic resources and their role in hunger. Recently, studies have shown that in humans, GR affected appetite regulation, stimulated food intake [4,5,6,7], metabolic pathways, and increased adiposity, triglyceride synthesis and gluconeogenesis [8].

The GR receptor, the so-called growth hormone secretagogue receptor (GHS-R), belongs to the large family of G-coupled receptors, encoded by the gene GHSR located on chromosome 3 (3q26.2), with two introns and two exons [1]. GR and its receptor GHS-R constitute the "ghrelin axis" and play a role in regulating many metabolic outcomes, such as appetite regulation, effects on insulin and glucose homeostasis, energy balance, and lipogenesis [9]. In this sense, data on the metabolic effects of ghrelin and its hypothalamic receptors are known, and the ghrelin axis is a promising target for intervention in obesity and type 2 diabetes [9]

Neurotensin, motilin, neuromedin and GPR39 receptors, as well as GHSR, are also examples of G protein-coupled receptors (GPCR). [10]. The first report of GHSR, more specifically GHSR1a, describes its functionality through the Gα q11 subunit of protein G, increasing the intracellular concentrations of Ca^{2+} through the inositol signalling pathway 1,4,5-triphosphate [11, 12]. Studies also have shown that cAMP, phospholipase C, protein kinases A and C and AMP kinase contribute to the downstream transduction of the ghrelin pathway [13,14, 15].

Recent studies have documented that genetics plays an important role in the development of obesity, with an estimated heritability between 40% and 70% [16, 17, 18]. Obesity exists in monogenic and polygenic forms. Monogenic obesity, mainly caused by genetic mutations in a single gene, is responsible for a small number of cases of extreme early obesity [19, 20, 21]. Studies of this rare form of obesity have identified genetic variants in several genes and provided preliminary information on the pathogenesis of monogenic obesity [22]. However, recent Broad Genomic Association (GWAS) studies have so far identified 751 genetic variants (single nucleotide polymorphisms, SNPs) in genes associated with various phenotypes of overweight and obesity associated with BMI confirming polygenic obesity. However, polygenic obesity is characterized by

remission through a healthy lifestyle, including physical activity [23] and adhering to healthy eating patterns. In this sense, understanding the role of different genetic mutations and their implications in the pathophysiology and clinical practice of obesity will benefit the search for more effective methods to diagnose and mitigate metabolic diseases.

Different types of variants and their consequences on phenotype, in addition to population frequency, mediate genetic effects that have great importance on the body weight of individuals. Variants that have a lower frequency allelic (MAF) of 5% in a population are considered common, since they have between 1 and 5% are considered intermediate, and finally, as with frequency, less than 1% are considered rare [24].

Bioinformatics plays a vital role when one wishes to understand genomic variations, several computational tools have been developed to predict whether a certain variation is deleterious or not to the protein. Each platform uses different methods and principles, which are based on protein structure analysis, conservation evolutionary analysis, sequence environment, functional annotations, and biochemical and physical properties of amino acids. Platforms such as SIFT [25, 26], PANTHER [27], and PROVEAN [28] are based on evolutionary conservation, instruments such as PhD-SNP [29], PolyPhen-2 [30], SNAP2 [31] MAPP [32], and PON-P2 [33] combine evolutionary conservation data and other types of resources, while tools such as HOPE [34] and PredictSNP [35] are meta-predictors that make consensus predictions based on other tool results.

Many variants of different genes have been implicated in human disease phenotypes but, in the absence of functional assays, the related pathogenicity of many remains unclassified. Several *in silico* tools have been developed to predict the effect of missense variants. Some of these tools are used routinely by diagnostic labs to advise clinicians of disease likelihood in the absence of previous evidence.

Therefore, this study aimed to search for missense allelic variants of the GHSR gene in the public domain database that contain a large collection of simple genetic polymorphisms and associate them with loss or gain of function of GHSR protein through in silico prediction study.

2. Methods

2.1 Database collection

The Single Nucleotide Polymorphisms Database (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/) was queried for the GHSR gene (Gene ID: 2693) in May 2022. All missense variants were collected and filtered using a global MAF of 1% [36] for further analysis. MAF was also confirmed in The Genome Aggregation Database (GnomAD) (https://gnomad.broadinstitute.org/) v2 release, composed of 125,748 exomes and 15,708 genomes (GRCh37).

2.2 Amino acid substitution prediction tools

For prediction analysis, PolyPhen-2, PON-P2, SIFT, PhD-SNP, PROVEAN, PANTHER, SNAP, Predict SNP, and MAPP tools were used.

The PolyPhen-2 (Polymorphism Phenotyping v2) is a *sequence-based feature tool,* capable of predicting the possible impact that can be the replacement of given amino acid, both in the structure and function of a human protein [30]. For each amino acid substitution, there is a qualitative prediction ("probably damaging", "potentially damaging", "benign" or "unknown"), determining a score ranging from 0.0 (tolerated) to 1.0 (deleterious) [30].

PON-P2 is a random *"forest predictor"* that aims to determine the association of pathogenicity of amino acid substitution. The "*forest predictor" algorithm* classifies the resources surveyed according to the mean decrease in the Gini index (an index that aims to calculate any distribution), that is, the greater the decrease of this index, the more critical the characteristic [33]. For tolerance forecasts, we use the reliability estimate of the forecasts and group the variants as pathogenic, neutral, or unknown [33].

SIFT (Sorting Intolerant From Tolerant) classifies intolerant and tolerant substitutions as deleterious or tolerated. To predict whether the change will affect protein function, the platform considers the position in which the change occurs. Based on sequential homology and physical properties of amino acids, it calculates the tolerance of a given substitution with a tolerance index, and mutations above 0.05 are tolerated [25, 26].

PhD-SNP (Predictor of human Deleterious Single Nucleotide Polymorphisms) predicts whether a certain amino acid substitution, classifying it as pathogenic or benign. The classification is made from a probabilistic score between 0 and 1, when this score is >0.5 the mutation is pathogenic, otherwise, it is given as benign [29].

PROVEAN predicts how the variation of a given amino acid affects protein function. The analysis of PROVEAN is based on two steps, first performs the collection of a set of homologous and distant sequences using the NCBI database. In the second stage, for each sequence of the set, a delta

score calculated by the platform itself is calculated [28]. The variation is considered "exclusion" if the score is equal to or super to a threshold established by the platform, already if the score is above the threshold is considered "neutral" [28].

PANTHER (Protein Analysis Through Evolutionary Relationships) classifies proteins according to their function. For this it uses the probabilities of amino acids of a specific position to establish an SNP score. Thus, it can determine whether the amino acid substitution performed is deleterious or neutral [27].

SNAP (Screening For Nonacceptable Polymorphisms) from the input sequence, using a neural network-based method (it is a computational model inspired by an animal's Central Nervous System, having the ability to perform machine learning and recognize patterns). The tool enables us to predict the functional effects of "*Single Nucleotide Polymorphisms (SNPs)*" not synonymous [31].

PredictSNP can be defined as a consensus classifier capable of combining some of the best-developed tools (MAPP, SNPAnalyzer, PANTHER, PhD-SNP, PolyPhen-1, PolyPhen-2, SIFT, and SNAP), thus predicting the expected effects of the mutation chosen in the protein function [35].

MAPP (Multivariate Analysis of Protein Polymorphism) quantifies the physical-chemical variation of mutations. Also calculating the deviation of substitutions of candidate amino acids from this required mutation. Therefore, the higher the calculated deviation, the greater the probability that this substitution will impair the protein function, thus generating a may effect on protein function [32].

2.3 3D modelling analysis and HOPE analyses

For biochemical and conservation analyses, the platform chosen was HOPE project web server (https://www3.cmbi.umcn.nl/hope/). It can collect structural information from different sources, ranging from calculations of the 3D protein structure and annotations on the protein sequence in UniProt, in addition to these, provides data on hydrophobicity, the size of mutant residues, and conservation of wild-type residues. Finally, based on this information, HOPE compiles these data and identifies an analysis of the effect of a given mutation on the protein structure [34]. The HOPE tool was used for 3D modelling of the wild-type and variants to analyze the molecular surface of the wild-type and protein variants, as well as the formation of intramolecular hydrogen bridges. Homology modelling of the receptor domain was performed using the SWISS-MODEL software (https://swissmodel.expasy.org/).

3. Results

3.1 General information

The GHSR gene is located at chromosome 3q26.31, and is a protein-coding gene that consists of 2 exons. GHSR has 366 nucleotides in length (NCBI Gene ID: 2693) and two transcripts (ensemble.org). This gene encodes a peptide receptor, a member of the G-protein coupled receptor family, called growth hormone secretagogue receptor transcript. The transcript 1a (GHSR1a) encodes the functional protein, the receptor for the GR ligand, and defines a neuroendocrine pathway for growth hormone release. The second transcript (1b) retains the intron and does not function as a receptor.

3.2 SNPs in the GHSR gene

The analysis of the dbSNP revealed a total of 3,001 variants in the GHSR gene, 803 (26.7%) of them were classified as intronic. Among coding variants, 207 (25.8 %) were classified as synonymous, and five were inframe deletions (0.62%). Missense substitutions accounted for 373 (46.4 %) of all reported mutations. Further, the percentages of transitions for these variants were assessed and for G > A transition was 22.8%, A > G, 11.3%, C > T 21.4%, and T > C 12.6% while transversions such as A > C were 4.8% and C > G, 11.3%, C > A, 12.9%, G > C, 8.6%, T > A, 2.9%, T > G, 4.5%, G > T, 10.4%, and A > T, 2.9%.

As a starting point for filtering out the missense variants, MAF >1% (0.001) was used. For additional classification of the variants that could be considered obesity-related mutations, missense substitutions were investigated more precisely. Table 1 shows all the 20 missense alterations after the filter global MAF 0.001 was applied.

Table 1. Data extracted from the dbSNP platform

ID variant	Nucleotide change	Amino acid change	Allelic frequency
rs79053943	c.271C>T	p.Leu91Phe	0.00091 (gnomAD)
rs140224509	c.124C>G	p.Leu42Val	0.00027 (gnomAD)
rs149430564	c.68C>T	p.Ala23Val	0.00009 (gnomAD)
rs150344113	c.1072G>A	p.Ala358Thr	0.00042 (gnomAD)
rs199588904	c.709A>T	p.Arg237Trp	0.00006 (gnomAD)
rs202112906	c.1070G>A	p.Arg357Gln	0.00015 (gnomAD)
rs4988511	c.401T>C	p.Ile134Thr	0.00002 (gnomAD)
rs34273140	c.829G>C c.829G>A	p.Ala277Pro p.Ala277Thr	0.0002 (1000G)
rs141596022	c.817A>G c.817A>C	p.Ile273Val p.Ile273Leu	0.00064 (gnomAD)
rs150332148	c.840C>T	p.Ile280Met	0.00329 (gnomAD)

	c.840C>G		
rs200019512	c.193A>G	p.Met65Val	0.00000 (gnomAD)
rs200380996	c.97G>A	p.Glu33Lys	0.00005 (gnomAD)
rs200570638	c.919G>T c.919G>A	p.Val307Leu p.Val307Met	0.00000 (gnomAD)
rs200619653	c.647T>C	p.Val216Ala	0.00005 (gnomAD)
rs201085948	c.440T>A	p.Phe147Tyr	0.00004 (gnomAD)
rs201558252	c.136G>T c.136G>C	p.Val46Phe p.Val46Leu	0.0002 (1000G)
rs201988616	c.521G>A	p.Ser174Asn	0.00000 (gnomAD)
rs537833793	c.955A>C	p.Asn319His	0.0002 (1000G)
rs538035493	c.580G>T	p.Asp194Tyr	0.00000 (gnomAD)
rs547944988	c.1033C>A	p.Gln345Lys	0.00003 (gnomAD)
rs554096465	c.472G>A c.472G>C	p.Gly158Arg p.Gly158Arg	0.0004 (1000G)
rs565546689	c.838A>G	p.Ile280Val	0.0002 (1000G)
rs150332148	c.840C>G	p.Ile280Met	0.00329 (gnomAD)

A = adenine; C = cytosine; G = guanine; T = thymine; Ile = Isoleucine; Met = methionine; Val = valine; Gly = glycine; Arg = Arginine; Lys = lysine; Ser = serine; Tyr = tyrosine; His = histidine; Asn = asparagine; Phe = phenylalanine; Leu = leucine; Ala = alanine; Thr = threonine; Trp = tryptophan; Gln = glutamine; Glu = glutamine; Pro = proline; > = indicate the replacement.

3.3 The predicted impact of SNPs on protein function

Out of 373 variants, 20 variants were finally selected based on allele frequencies. After that, variants were submitted to nine predicted SNP tools (PredictSNP, MAPP, PhD-SNP, PolyPhen-1, PolyPhen-2, SIFT, SNAP, PANTHER, and PROVEAN) and only deleterious mutations were selected. The selection criterion used was to present a deleterious/pathogenic result in four or more of the tools, suggesting the pathogenic nature of these mutants. Additionally, the pathogenic strength and stability of eight variants were further inspected for 3D modelling and HOPE analysis. These 8 variants were: L91F (rs79053943), R237W (rs199588904), I134T (rs4988511), V216A (rs200619653), V46F (rs201558252), S174N (rs201988616), N319H (rs537833793) and D194Y (rs538035493), are show in a 2D -snake plot of GHS-R.

All 8 mutations were submitted on the HOPE project platform using the FASTA sequence obtained from Uniprot (https://www.uniprot.org/uniprotkb/Q92847/entry), to acquire the biochemical and structural analysis of the wild-type protein and with mutations.

The 8 variants had their structures evaluated in the SWISS-MODEL platform (https://swissmodel.expasy.org/), analysing both the wild-type and the mutation. The illustrative comparison between wild type and mutation allows visualizing the difference in the region of the amino acid when mutated. In variant L91F, both amino acids are non-polar. Similar results were

obtained in V216A and V46F. In R237W there was a change between a non-polar amino acid (phenylalanine) for a basic (arginine). In I134T there was the substitution of a non-polar amino acid (isoleucine) for a polar (threonine). In S174N the alteration was between two polars amino acids, serine and asparagine. In N319H, the polar asparagine was replaced by a basic histidine. Finally, in D194Y, there was a change of aspartic acid (acidic) by tyrosine (polar).

4. Discussion

The GHSR gene is a potential candidate for causing metabolic diseases, many variants are related in tentative to explain the several phenotypes associated with short stature due to growth hormone secretagogue receptor deficiency and obesity/adipose tissue-related diseases. After subjecting them to platforms capable of building their prediction, analyzing missense variants can be useful to compose a group of diagnostic tests and understand diseases involving both metabolism and hunger.

More recent studies have been able to elucidate the relationship between *GHSR gene variants,* correlated with the regulation of body weight or short stature in humans [37]. In these studies, it was not possible to establish a relationship between SNPs and the regulation of weight or GH secretion. However, other studies [38] seek to establish the pharmacological consequences of known missense mutations in the receptor, demonstrating that changes in a single amino acid in GHSR can result in a wide range of pharmacological changes.

When the GHSR of children with idiopathic short stature (ISS) was analyzed [39], 5 variations in a subgroup of patients with constitutional growth retardation and puberty (CDGP), 4 of them (p.Ser84Ile (c.251G>T), p.Ala169Thr (c.505G>A), p.Val182Ala (c.545 T>C) and p.Ala358Thr (c.1072G>A)), were in highly conserved positions except for p.Ala358Thr, were missense. The five new variants of GHSR in patients with constitutional delay of growth and puberty (CDGP), or ISS, these were absent in a large population ethnically paired, using prediction and in vitro analysis, instigating the possibility that there was an association between the GHSR mutations observed and the CDGP phenotype.

Another study [40] attempted to associate an SNP in the GHSR gene with the alcohol use disorders identification test (AUDIT) score and smoking, reflecting that the association with smoking was not mediated by the association with the AUDIT and vice versa. Findings showed associations between AUDIT scores, smoking, and an SNP in the GHSR gene, supported by preclinical data showing the role of GHSR-1a in drug reward and it might be associated with the hedonic regulation of feeding promoted by GR [41].

Regarding the prevalence of the GHSR mutation, in one study [39], based on a German cohort with 326 patients, a prevalence of 1.1% of pathogenic mutations in the GHSR gene was observed in severely obese individuals. In a study with 127 Japanese individuals of short stature, or short idiopathic stature [43] 4 pathogenic mutations in the GHSR gene, ΔQ36, P108L, C173R and D246A were found, and these mutations were found in 6 patients, with a prevalence of 4.7%.

Of the 8 variants obtained, only one was previously reported (rs199588904, R237W). All the reports [39, 42, 43] about the variant R237W, seek to establish the relationship between the variant at the short stature (SS). Until now, there is no study in the literature with the same objectives as this work, to establish the correlation between the variant and the SS, not obesity.

Only two (rs199588904 and rs79053943) variants were submitted in Clinvar presenting a clinical condition of short stature due to growth hormone secretagogue receptor deficiency, and the inborn genetic disease, respectively. Inborn genetic diseases are diseases caused by genetic mutations present during embryo or fetal development, although they may be observed later in life.

In this study we found 8 variants with a high risk for dysfunction of the GHSR, this dysfunction may be compromising the binding of GR with its receptor and contributing to phenotypes of obesity and short stature, or both. Future analyses of these mutations may give us a better elucidation of the implication of mutations and their possible correlation with the pathophysiology of obesity.

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