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Case Report

Case Report

CBG Montevideo: A Clinically Novel *SERPINA6* Mutation Leading to Haploinsufficiency of Corticosteroid-binding Globulin

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Abbreviation:CBG, corticosteroid-binding globulin.

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Abstract

Corticosteroid-binding globulin (CBG) is the main transport protein for cortisol, binding up to 90% in a 1:1 ratio. CBG provides transport of cortisol within the circulation and targeted cortisol tissue delivery.

Here, we describe the clinically novel "CBG Montevideo" a *SERPINA6* pathogenic variant that results in a 50% reduction in plasma CBG levels. This was associated with low serum total cortisol and clinical features of hypoglycemia, exercise intolerance, chronic fatigue, and hypotension in the proband, a 7-year-old boy, and his affected mother.

Previous reports of 9 human CBG genetic variants affecting either CBG concentrations or reduced CBG-cortisol binding properties have outlined symptoms consistent with attenuated features of hypocortisolism, fatigue, and hypotension. Here, however, the presence of hypoglycemia, despite normal circulating free cortisol, suggests a specific role for CBG in effecting glucocorticoid function, perhaps involving cortisol-mediated hepatic glucose homeostasis and cortisol-brain communication.

Key Words: corticosteroid binding globulin, *SERPIN A6*, haploinsufficiency, cortisol deficiency

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Corticosteroid-binding globulin (CBG) is the principal circulating cortisol transport protein [1, 2]. CBG is a serine protease inhibitor (serpin) and is encoded by a single gene, *SERPINA6*, a member of a group of highly conserved SERPIN genes at 14q; *SERPINA6* comprising 5 coding exons over 19 kilobases at 14q32.13 [3, 4]. CBG is a 383-amino acid glycoprotein with a molecular size of 52 kDa [5, 6], is synthesized chiefly by hepatocytes [7, 8], and circulates in concentrations of 450 to 650 nmol/L [9-12].

Several heritable *SERPINA6* pathogenic gene mutations have been identified following clinical inquiry into patients presenting with hypocortisolemia in association with a variety of clinical features including chronic fatigue, chronic pain, exercise intolerance, depression, hypotension, and obesity. Two such *SERPINA6* variants result in a reduction in plasma CBG levels (CBG Null/Adelaide [13, 14] and CBG Santiago [15]) and 4 variants result in a reduction in cortisol-binding affinity and/or capacity (CBG Leuven [16-18], CBG Lyon [13, 14, 19-22], CBG G237V [23], and CBG Athens [22]). Additional mutations have been identified through population screening, CBG A51V and CBG E102G within a Han Chinese population resulting in reduced plasma CBG levels and reduced cortisol-binding capacity, respectively [24]. A polymorphism, CBG A224S was overrepresented in a chronic fatigue cohort [17, 25]. Markers in the CBG gene, but not in other key hypothalamic-pituitary-adrenal axis regulatory genes, are associated with chronic widespread pain syndrome [25]. The CBG gene is expressed in brain regions that are involved in the stress response [26]. The CBG locus is important in the regulation of cortisol concentrations as evidenced in large community studies [27]. Next-generation sequencing techniques have improved diagnosis and discoveries of new pathogenic mutations in several different medical specialities, especially in rare diseases [28-30]. Here we present a novel *SERPINA6* null mutation, identified via next-generation sequencing performed to evaluate a fatigue syndrome, with hypoglycemia in 1 participant. The mutation results in a heterozygous complete loss of function; this is the fourth CBG mutation to be described to have a significant effect on circulating CBG levels and provides further evidence of a phenotype whose pathophysiology is not yet understood but may represent a selective tissue deficiency of cortisol.

Case Report

The index case was a 7-year-old boy. He has no family history other than arterial hypotension considered to contribute to presyncopal episodes affecting his mother. He has a healthy older brother.

The boy was the result of a nonconsanguineous union, with a normal pregnancy and birth. At day 2 of age, he experienced a seizure and was hypoglycemic; a further episode at day 8 was associated with hypocortisolemia (Table 1). He was treated with hydrocortisone (20 mg/m^2/d, divided in 3 doses) with improvement. Later in childhood this was later changed to hydrocortisone 7 mg mane (morning) and 3 mg mid-afternoon. Episodes of hypoglycemia continued in early childhood, occurring typically in the morning associated with presyncopal symptoms and was observed to be pale. At age 4 years, he experienced a second seizure; no further episodes occurred when regular frequent meals and avoidance of prolonged fasting were instituted.

Physical examination was normal. He had normal growth, within the first 2 years of life height and weight was between 3rd and 15th percentile, and head circumference at the 50th percentile. Measurements later in childhood were not available. Neurological and milestone development was also normal. His parents reported that he would exhibit a certain intolerance to intensive exercise, describing that when playing vigorously with other children he would often need to "pause to recharge," after which he would continue to play. Laboratory results during critical episodes (Table 1) showed hypoglycemia, with appropriately low insulin and elevated ketones levels, and inappropriately low serum cortisol. In the early neonatal period, plasma acylcarnitines and amino acids were normal, as were IGF-1 levels, clonidine-growth hormone stimulation (Table 2), and electrocardiogram. Magnetic resonance imaging of both the pituitary and adrenal glands was unremarkable.

Methods

DNA sequencing and bioinformatics analysis

We performed exome sequencing of germline DNA from the patient on a Hiseq 4500 Illumina sequencer (Agilent

Table 1. Laboratory results during critical episodes

Age	48 h	8 d	2 y
Blood glucose level mg/dL (70-110) (mmol/L) (4-6)	17 (0.9)	34 (1.9)	15 (0.8)
Serum sodium level (mEq/L) (134-150)	-	-	135
Ketones	-	-	Elevated +++
TSH mIU/mL (0.7-4.8)	0.78	4.45	-
FT4 ng/dL (0.7-1.48)	-	1.33	-
Cortisol µg/dL (1.8-30.3)	-	7.7[a]	-
Insulin mIU/L (1.3-13)	-	2	-
Growth hormone ng/mL (<13.6)	-	7.39	18.2
Plasma renin activity ng/mL/h (1.1-22)	-	6.85	-
Aldosterone ng/dL (3-65.8)	-	31	-

[a]In hypoglycemia, serum cortisol should be ≥18 µg/dL and insulin <2 mIU/L.

Table 2. Clonidine-GH stimulation test

	GH ng/mL
Basal	18.2
30 min poststimulation	2.9
60 min poststimulation	9.13
90 min poststimulation	6.13
120 min poststimulation	3.29

Clonidine-GH stimulation test, a growth hormone of >6 ng/mL is consistent with an appropriate GH response.

SureSelect V6 kit, 100×, 150PE). Quality of reads was analyzed using FastQC [31] and were mapped to the human reference genome (GRCh37) using the Burrows-Wheeler Alignment Tool [32]. Only unique reads mapping in proper pairs were further considered. Variant calling was performed using GATK (best practices) [33] and ANNOVAR [34] was used for the annotation process. Different sets of filters were used to detect potentially causative mutations:

1. Homozygous variants in coding/splicing regions with a population frequency lower than 1%; and
2. Heterozygous variants in coding/splicing regions with at least two variants in the same gene and a population frequency lower than 1% (compound heterozygous); and
3. Heterozygous variants in coding/splicing regions with a population frequency less than 0.5%.

Sanger sequencing was used to validate causative variants.

CBG immunoassay

Plasma levels of CBG were measured via ELISA as previously described [35] using an in-house human reactive polyclonal rabbit antibody and a monoclonal mouse antibody, 12G2 (RRID:AB_2632404) (https://scicrunch.org/resources/Antibodies/source/nif-0000-07730-1/search?q=*&l=&facet%5B%5D=Target%20Antigen:human%20corticosteroid-binding%20globulin&sort=desc&column=Target%20Antigen&sort=desc).

Results

The pedigree, plasma CBG levels, and DNA sequencing data are represented in Fig. 1. We obtained a total of 55 495 070 reads, a total of 124,246 variants and medium exome coverage of 53×. A variant in *SERPINA6* was found in heterozygous state: NM_001756.3, c.164_165del (p.V55fs, chr14: 94.780.821) [36]. The gene had 100% of its bases covered with at least 20 reads and a mean coverage of 177×. The frameshift is situated at 13% of the protein (exon 2). A premature stop codon is generated

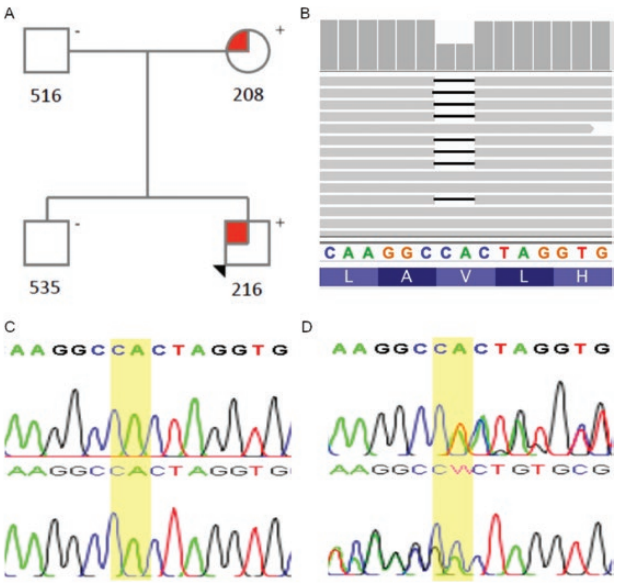

Figure 1. The CBG Montevideo kindred. (A) Pedigree showing the proband (arrow) and his immediate family members. Mutation status is indicated in the top right-hand corner: +, positive for *SERPINA6* 2-bp deletion: -, negative for *SERPINA6* 2-bp deletion. Red shading indicates the presence of symptoms consistent with hypocortisolism. Serum CBG levels (nmol/L) represented under individuals. (B) The familial *SERPINA6* 2-bp deletion identified by next-generation sequencing in the proband as seen in Integrated Genomics Viewer. The top bar illustrates an approximately 50% decrease in coverage at the position of the variant, consistent with the heterozygous state of the deletion. (C,D) Electrophoretograms from *SERPINA6* bidirectional Sanger sequencing validation studies showing the wild-type sequence in panel C in the father and brother and confirming the 2-bp deletion in panel D in the proband and mother. The yellow bar highlights the location of the variant, with downstream frameshift shown to the right-hand side of the sequence in the top forward-reading sequence and to the left-hand-side in the bottom reverse reading sequence of panel D.

after 43 amino acids. Therefore, it should be considered a loss of function variant, whether by generating a truncated protein or via nonsense mediated decay, which is predicted to occur. Allelic frequency in controls is 0.000003977 (European non-Finnish) in gnomAD v2.1.1 and no homozygotes have been reported. No other variants in this gene nor other variants in other genes potentially involved in the phenotype were found. Those explored included: genes associated with familial glucocorticoid deficiency: melanocortin 2 receptor (*MC2R*), melanocortin 2 receptor accessory protein (*MRAP*); congenital adrenal hypoplasia (DAX1), steroidogenic factor 1 (*NR5A1*), those coding for steroidogenic enzymes (*CYP21A2*, *CYP11B1*, *CYP17A1*, *HSD3B2*, *STAR*, *POR*, *CYP11A1*), pituitary transcription factors (*PROP1*, *PIT1*, *LHX3*, *LHX4*, *HESX1*), signalling molecules (*KAL*), and pituitary hormones and receptors (*GH1*, *GHRH*, *KISS1R*). The proband and his mother were heterozygous for the *SERPINA6* variant, whereas his unaffected father and brother were homozygous for the wild-type allele. Plasma CBG levels were reduced approximately 50% in the proband and mother (202-209 nmol/L

[reference range 450-650 nmol/L]), consistent with an inactivating mutation in heterozygous state in *SERPINA6*. Morning total serum cortisol levels were significantly reduced in the proband during critical episodes (Table 1) and mother (1.96 µg/dL, normal 10-20 µg/dL), whereas % free cortisol remained normal at 15% and 14%, respectively.

Using the American College of Medical Genetics and Genomics variant classification criteria, the *SERPINA6* variant detected in the proband and mother was classified as pathogenic (class 5) as it fulfils PVS1, PS3, PM2, and PP1 [37].

Discussion

We report a newly described CBG gene (*SERPINA6*) pathological variant, CBG Montevideo: a 2 base-pair deletion leading to a frameshift and a premature stop codon with a 50% reduction in plasma CBG levels in heterozygotes. The pathogenic variant, predicted to produce a complete loss of function of the allele, was found in heterozygous state in a child (proband) with associated hypocortisolism manifesting with episodes of hypoglycemia and exercise intolerance. The CBG Montevideo variant was subsequently identified in the mother, who also has hypocortisolism with chronic fatigue, hypotension, and presyncope. No phenotype has been reported in association with the mutation responsible for CBG Montevideo because it appears to be very rare in gene databases. The association with fatigue and hypotension has been seen with other loss of function mutations; however, hypoglycemia has not been reported.

CBG binds cortisol in a 1:1 molar ratio within a single binding pocket [1, 38] and undergoes a permanent conformational change upon proteolysis by neutrophil elastase, liberating bound cortisol [39-41]. CBG binding affinity for cortisol is also reduced with increases in temperature [42, 43] and in acidosis [43] demonstrating the modifiable binding and delivery characteristics of cortisol to alleviate inflammation.

CBG is important in glucocorticoid delivery to the brain, with fast nongenomic action on neurones modulating stress-induced behavior, learning, and memory recall [44]. CBG has been isolated from cerebrospinal fluid, hypothalamus, and pituitary [45-47], and CBG mRNA from neurons [48] suggesting local expression and supporting a role for CBG in the regulation of the hypothalamic-pituitary-adrenal axis in response to stress [46]. Human studies have shown a relation between CBG haplotypes and chronic pain and chronic fatigue syndromes [25, 49]. Furthermore behavioral studies in CBG-knockout mice demonstrate learned helplessness and despair-like behavior after prolonged uncontrollable stress, a model for depression [50-52].

Phenotypic effects for *SERPINA6* haploinsufficiency and other genetic variants have been reported in homozygotes and heterozygotes [13, 21]. In the present study, CBG concentration was reduced by approximately 50% in heterozygous family members, similar to that previously observed for other null *SERPINA6* pathogenic genetic variants (Table 3). A novel phenotypic observation in CBG Montevideo was the associated morning fasting hypoglycemia. Cortisol promotes gluconeogenesis and hepatic glucose output, along with hepatic glycogen synthesis, thus is crucial for glucose homeostasis particularly in the fasting state [53]. Furthermore, CBG may play an important role in facilitating the hepatic regulation of glucose homeostasis with loss of targeted hepatic glucocorticoid delivery, compounding morning fasting hypoglycemia as observed in this case.

The discovery of several human *SERPINA6* pathogenic variants detected through clinical enquiry have revealed unexpected phenotypic implications. Nine *SERPINA6* pathogenic variants have been described in humans, of which 7 result in either reduced synthesis or cortisol-binding function. CBG Null/Adelaide (c0.32G > A, p.Trp11X) results in a premature stop codon and complete loss of synthesis, with CBG levels reduced by 50% in heterozygotes and completely devoid in the rare CBG null homozygotes [13]. CBG Adelaide cosegregated with CBG Lyon in a large Italian-Australian family, where chronic idiopathic fatigue, chronic pain (25% of participants) and hypotension was described before sequencing family members—the presence of these clinical features corresponded to the presence of either CBG mutation, whether inherited in heterozygous, compound heterozygous, or homozygous form [13]. A blinded study of 495 individuals living in the Calabrian village from where the CBG Adelaide family originated revealed the presence of chronic pain having precedence over fatigue in the 18 participants with 1 of the Adelaide or Lyon mutations, suggesting an environmental influence on the expression of pain or fatigue, both considered related symptoms in the spectrum of chronic fatigue syndrome-fibromyalgia symptomatology, as is disturbances in blood pressure regulation [16]. CBG Santiago (c0.13delC, p.Leu5CysfsX26) [15] and CBG A51V [24] affect hepatic synthesis with up to 50% reduction in plasma CBG concentrations in heterozygotes. Five mutations affect CBG:cortisol binding affinity, the most severe seen in CBG Athens (c0.1282G > C, p.Trp393Ser) [22] and CBG G237V with a complete loss of binding affinity. CBG Leuven (c0.344T > A, p.Leu115His) [18] and CBG Lyon (c0.1165G > A, p.Asp389Asn) [13, 19, 20] result in a 3- and 4-fold reduction in binding affinity, respectively, with a partial loss seen in CBG E102G [24]. A phenotypic spectrum has been observed in individuals harboring *SERPINA6* mutations, with chronic fatigue, chronic pain, hypotension, and perhaps obesity being features [54].

Table 3. *SERPINA6* pathogenic variants detected in humans and associated with clinical outcomes

CBG protein	*SERPINA6* nucleotide change	Discovery	CBG effect	Biochemical findings	Clinical features
CBG Montevideo p.V55fs	c0.164_165del Single base deletion → frameshift Premature stop codon	Isolated from 2 individuals from the same pedigree	Complete loss of CBG synthesis	• 50% reduction in CBG in heterozygotes • Hypocortisolemia • Hypoglycemia	• Exercise intolerance • Hypotension • Seizures
CBG Leuven p.Leu115His [16-18]	c0.344T > A	Isolated in 3 unrelated individuals from a population study; subsequently detected in 1 of 22 patients from a septic cohort	3-fold reduction in CBG-cortisol binding affinity	• Normal CBG levels	Not described. Blood donor cohort
CBG Lyon p.Asp389Asn [13, 14, 19-22]	c1165G > A	Isolated from at least 5 pedigrees and an isolated (de novo) case	4-fold reduction in CBG-cortisol binding affinity	• Low TC • Normal FC • Increased %FC • Normal ACTH and 24-hour UFC • Low CBG	• Chronic fatigue • Chronic pain • Weakness • Depression • Hypotension • Obesity
CBG Null/Adelaide p.Trp11X [13,14]	c0.32G > A Premature stop codon	Isolated from a large Italian-Australian kindred and in pedigrees from the Italian village of origin. Some also carried CBG Lyon D37N	Complete loss of CBG synthesis	• Normal 24-h UFC • Low TC • Elevated FC • Increased %FC • 50% reduction in CBG in heterozygotes • Undetectable CBG in homozygotes	• Hypotension • Chronic fatigue • Chronic pain • Obesity
CBG p.Ala246Ser [17, 55]	c0.825G > T	Found with increased frequency from a candidate gene study in an Australian chronic fatigue cohort; also seen in conjunction with other CBG mutations [15, 17, 22]	No apparent effect on binding affinity or production	• Increased plasma CBG • Trend to low TC and FC	• Chronic fatigue
CBG p.Gly237Val [23]	c0.776G > T	Isolated from a single kindred	Complete loss of CBG-cortisol binding affinity	• Very low TC • Normal FC • Low CBG • Increased %FC • Increased cortisol pulsatility	• Hypotension • Fatigue
CBG Santiago p.Leu5CysfsX26 [15]	c0.13delC Single base deletion → frameshift Premature stop codon	Isolated from a 9-y-old Spanish male, also heterozygous for A224S	Decreased CBG synthesis	• Low TC • 50% reduction in CBG • Normal ACTH	• Chronic fatigue • Chronic headaches, weakness

Table 3. Continued

CBG protein	*SERPINA6* nucleotide change	Discovery	CBG effect	Biochemical findings	Clinical features
CBG p.Ala73Val [24], 56	c0.218C > T	CBG polymorphism screening study in Han Chinese, prevalence 1:35 frequency	Decreased synthesis and/or secretion of CBG in vitro in CHO cells	• 30%-50% reduction in CBG in heterozygotes • Higher female-to-male live birth rate	NA
CBG E102G p.Glu123Gly [24]	c0.371A >G	CBG polymorphism screening study in Han Chinese	Reduced CBG-cortisol binding capacity in vitro in CHO cells	NA	NA
CBG Athens p.Trp393Ser [22]	c0.1282G > C	Isolated from a single Greek kindred, also heterozygous for CBG Lyon D367N and CBG A224S	Complete loss of CBG-cortisol binding affinity	• Normal CBG levels • Low TC • Normal FC • Increased %FC • Normal 24-h UFC	• Obesity

Adapted and reprinted with permission from [55].
Abbreviations: CBG, corticosteroid-binding globulin; CHO, Chinese hamster ovary; FC, free cortisol; NA, not available; TC, total cortisol; UFC, urinary free cortisol.

The most commonly reported clinical features of *SERPINA6* pathogenic variants include hypotension, chronic fatigue, and exercise intolerance, all of which were present in the proband and his mother (Table 3). Interestingly, the proband experienced morning hypocortisolemia with associated hypoglycemia, not previously described in known *SERPINA6* pathogenic variants. Hydrocortisone treatment and regular frequent meals in the proband were effective in this case.

In the CBG Lyon variant, where a functional loss of CBG is observed because of a 4-fold reduction in cortisol-binding affinity while CBG levels remain unaffected, total cortisol levels within the first hour after awaking was reduced by 50% [13, 21]. A hyperreactivity response to psychological stress is also seen with CBG Lyon, with elevated ACTH, salivary cortisol, epinephrine, and norepinephrine levels that associated with transient muscle weakness [21].

CBG-knockout mice display learned helplessness and despair-like behavior in response to enduring psychological stressors [50-52]. Taken together, this suggests CBG has an antinociceptive function in response to stress, perhaps at the level of the central nervous system, which may relate to the clinical features of depression, exercise intolerance, chronic fatigue, and chronic pain observed in *SERPINA6* pathologic variants (Table 3).

In summary, we describe the clinically novel CBG Montevideo a *SERPINA6* pathogenic variant that resulted in a 50% reduction in plasma CBG levels, low serum total cortisol, and associated clinical features of hypoglycemia, exercise intolerance, chronic fatigue, and hypotension in the proband. The identification of increasing numbers of individuals with *SERPINA6* variants affecting CBG function (concentration or cortisol binding reducing) and symptoms consistent with attenuated features of hypocortisolism (chiefly fatigue and hypotension, but here with hypoglycemia) despite normal circulating free cortisol, suggests a specific role for CBG in glucocorticoid function, perhaps involving cortisol regulation of hepatic glucose homeostasis and cortisol-brain communication.

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Additional Information

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