CBG Montevideo: a clinically novel SERPINA6 mutation leading to haploinsufficiency of corticosteroid-binding globulin.

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CBG Montevideo: SERPINA6 mutation.

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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 hypoglycaemia, exercise intolerance, chronic fatigue and hypotension in the proband, a 7-year-old boy, and his affected mother.

Previous reports of nine 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 hypoglycaemia, 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.

Introduction

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 synthesised chiefly by hepatocytes \(^7,^8\) and circulates in concentrations of 450-650 nmol/L \(^9-^12\).

Several heritable SERPINA6 pathogenic gene mutations have been identified following clinical inquiry into patients presenting with hypocortisolaemia 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 four 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 over-represented 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 (NGS) 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 NGS performed to evaluate a fatigue syndrome, with
hypoglycaemia in one 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 hypoglycaemic; a further episode at day 8 was associated with hypocortisolaemia (Table 1). He was treated with hydrocortisone (20 mg/m²/day, divided in three doses) with improvement. Later in childhood this was later changed to hydrocortisone 7 mg mane and 3 mg mid-afternoon. Episodes of hypoglycaemia 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 hypoglycaemia, 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. MRI 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 SureSelect V6 kit, 100x, 150PE). Quality of reads was analyzed using FastQC
and were mapped to the human reference genome (GRCh37) using the Burrows-Wheeler Alignment Tool. Only unique reads mapping in proper pairs were further considered. Variant calling was performed using GATK (best practices) and ANNOVAR was used for the annotation process. Different sets of filters were used in order to detect potentially causative mutations:

i. Homozygous variants in coding/splicing regions with a population frequency lower than 1% and;

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

iii. 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 using an in-house human reactive polyclonal rabbit antibody and a monoclonal mouse antibody, 12G2 (RRID:AB_2632404).

**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 53x. A variant in *SERPINA6* was found in heterozygous state: NM_001756.3, c.164_165del (p.V55fs, chr14: 94,780,821). The gene had 100% of its bases covered with at least 20 reads and a mean coverage of 177x. The frameshift is situated at 13% of the protein (exon 2). A premature stop codon is generated 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 (NMD) 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 (*NRSA1*)), 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, while 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), while % 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.

Discussion

We report a newly described CBG gene (SERPINA6) pathological variant, “CBG Montevideo”: a two 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 hypoglycaemia 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 as 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 hypoglycaemia has not been reported.

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

CBG is important in glucocorticoid delivery to the brain, with fast non-genomic action on neurones modulating stress-induced behaviour, learning and memory recall. CBG has been isolated from cerebrospinal fluid, hypothalamus and pituitary, and CBG mRNA from neurons suggesting local expression and supporting a role for CBG in the regulation of the hypothalamic-pituitary-adrenal axis in response to stress. Human studies have shown a relation between CBG haplotypes and chronic pain and chronic fatigue syndromes. Furthermore behavioural studies in CBG-knockout mice demonstrate learned helplessness and despair-like behaviour after prolonged uncontrollable stress, a model for depression.
Phenotypic effects for SERPINA6 haploinsufficiency and other genetic variants have been reported in homozygotes and heterozygotes. 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 hypoglycaemia. Cortisol promotes gluconeogenesis and hepatic glucose output, along with hepatic glycogen synthesis, thus is crucial for glucose homeostasis particularly in the fasting state. 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 hypoglycaemia 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 seven result in either reduced synthesis or cortisol binding function. CBG Null/Adelaide (c.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. CBG Adelaide co-segregated with CBG Lyon in a large Italian-Australian family, where chronic idiopathic fatigue, chronic pain (25% of participants) and hypotension was described prior to 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. 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 one 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. CBG Santiago (c.13delC, p.Leu5CysfsX26) and CBG A51V 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 (c.1282G>C, p.Trp393Ser) and CBG G237V with a complete loss of binding affinity. CBG Leuven (c.344T>A, p.Leu115His) and CBG Lyon (c.1165G>A, p.Asp389Asn) result in a three- and four-fold reduction in binding affinity respectively, with a partial loss seen in CBG E102G. A phenotypic spectrum has been observed in individuals harbouring SERPINA6 mutations, with chronic fatigue, chronic pain, hypotension and perhaps obesity being features.

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 hypocortisolaemia with associated hypoglycaemia, 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 due to 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% \textsuperscript{13,21}. A hyper-reactivity response to psychological stress is also seen with CBG Lyon, with elevated ACTH, salivary cortisol, epinephrine and norepinephrine levels which associated with transient muscle weakness \textsuperscript{21}.

CBG-knockout mice display learned helplessness and despair-like behaviour in response to enduring psychological stressors \textsuperscript{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 \textit{SERPINA6} pathologic variants (Table 3).

In summary we describe the clinically novel “CBG Montevideo” a \textit{SERPINA6} pathogenic variant that resulted in a 50% reduction in plasma CBG levels, low serum total cortisol and associated clinical features of hypoglycaemia, exercise intolerance, chronic fatigue and hypotension in the proband. The identification of increasing numbers of individuals with \textit{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 hypoglycaemia) 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.

\textbf{Data Availability Statement:} Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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Tables

**Table 1:** Laboratory results during critical episodes.

*In hypoglycaemia, serum cortisol should be >18 ug/dL and insulin <2 mIU/L

**Table 2:** Clonidine-Growth hormone stimulation test.

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

**Table 3.** SERPINA6 pathogenic variants detected in humans and associated with clinical outcomes. Adapted and reprinted with permission from 55.

CBG – corticosteroid-binding globulin. TC – total cortisol. FC – free cortisol. UFC – urinary free cortisol. ATCH – adrenocorticotrophic hormone. CHO – Chinese hamster ovary. NA – not available.

Figures

**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 2bp deletion; -, negative for SERPINA6 2bp deletion. Red shading indicates the presence of symptoms consistent with hypocortisolism. Serum CBG levels (nmol/L) represented under individuals.

B. The familial SERPINA6 2bp deletion identified by next generation sequencing in the proband as seen in Integrated Genomics Viewer. The top bar illustrates an approx. 50% decrease in coverage at the position of the variant, consistent with the heterozygous state of the deletion.

C and D. Electrophoretograms from SERPINA6 bidirectional Sanger sequencing validation studies showing the wild-type sequence in C in the father and brother and confirming the 2bp deletion in 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 D.
|                        | 48 hours | 8 days | 2 years |
|------------------------|----------|--------|---------|
| Blood glucose level    | 17 (0.9) | 34 (1.9)| 15 (0.8) |
| Serum sodium level     | -        | -      | 135     |
| Ketones                | -        | -      | Elevated +++ |
| TSH miU/ml             | 0.78     | 4.45   | -       |
| FT4 ng/dL              | -        | 1.33   | -       |
| Cortisol ug/dL         | -        | 7.7*   | -       |
| Insulin miU/L          | -        | 2      | -       |
| Growth hormone ng/mL   | -        | 7.39   | 18.2    |
| Plasma Renin activity  | -        | 6.85   | -       |
| Aldosterone ng/dL      | -        | 31     | -       |

**Table 1**
| Time Point                          | Growth hormone ng/mL |
|-----------------------------------|----------------------|
| Basal                             | 18.2                 |
| 30 minutes post stimulation       | 2.9                  |
| 60 minutes post stimulation       | 9.13                 |
| 90 minutes post stimulation       | 6.13                 |
| 120 minutes post stimulation      | 3.29                 |

**Table 2**
| CBG protein       | SERPINA6 nucleotide change | Discovery effect | CBG effect | Biochemical findings                                      | Clinical features         |
|-------------------|-----------------------------|-----------------|------------|----------------------------------------------------------|---------------------------|
| CBG Montevideo    | c.164_165del                | Isolated from 2 individuals from the same pedigree | Complete loss of CBG synthesis | • 50% reduction in CBG in heterozygotes  
• Hypocortisolaemia  
• Hypoglycaemia | • Exercise intolerance  
• Hypotension  
• Seizures |
| p.V55fs           | Single base deletion → frameshift Premature stop codon |                  |            |                                                          |                           |
|                   |                             |                  |            |                                                          |                           |
| CBG Leuven        | c.344T>A                    | Isolated in 3 unrelated individuals from a population study; subsequently detected in one out of 22 patients from a septic cohort | 3-fold reduction in CBG-cortisol binding affinity | • Normal CBG levels | Not described. Blood donor cohort. |
| p.Leu115His       | 16-18                       |                  |            |                                                          |                           |
|                   |                             |                  |            |                                                          |                           |
| CBG Lyon          | c.1165G>A                   | Isolated from at least 5 pedigrees and an isolated (de) | 4-fold reduction in CBG-cortisol binding | • Low TC  
• Normal FC  
• Increased %FC  
• Normal ACTH and 24-hour UFC | • Chronic fatigue  
• Chronic pain  
• Weakness  
• Depression  
• Hypotension |
| p.Asp389Asn       | 13,14,19,22                 |                  |            |                                                          |                           |
| CBG Null/Adelaide p.Trp11X | c.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 | - Low CBG | - Obesity
| CBG p.Ala246Ser | c.825G>T | Found with increased frequency from a candidate gene study in an Australian chronic fatigue cohort; also seen in conjunction with | No apparent effect on binding affinity or production | - Increased plasma CBG | - Hypotension
|       |       |       |       | - Trend to low TC and FC | - Chronic fatigue

Complet e loss of CBG synthesis
- Normal 24-hour UFC
- Low TC
- Elevated FC
- Increased %FC
- 50% reduction in CBG in heterozygotes
- Undetectable CBG in homozygotes

Increased plasma CBG

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| CBG                  | Mutation/Description                                                                 | Other CBG mutations | Characteristics                                                                 | Other Characteristics |
|---------------------|--------------------------------------------------------------------------------------|---------------------|---------------------------------------------------------------------------------|----------------------|
| CBG p.Gly237Val     | c.776G>T Isolated from a single kindred                                              | 15,17,22            | Complet e loss of CBG-cortisol binding affinity                                | • Very low TC  
• Normal FC  
• Low CBG  
• Increased %FC  
• Increased cortisol pulsatility |
| CBG Santiago        | p.Leu5Cysfs X26 c.13delC Single base deletion frameshift Premature stop codon          | 15                  | Decreased CBG synthesis                                                          | • Low TC  
• 50% reduction in CBG  
• Normal ACTH |
| CBG p.Ala73Val      | c.218C>T CBG polymorphism screening study in Han Chinese, prevalence 1:35 frequency  | 24,56               | Decreased CBG 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 |
| CBG E102G           | p.Glu123Gly c.371A>G CBG polymorphism screening study in Han                           | 24                  | Reduced CBG-cortisol binding capacity in vitro                                  | NA  
NA |
| CBG Athens p.Trp393Ser<sup>22</sup> | c.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-hour UFC | Obesity |
