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Novel mutations in the bone morphogenetic protein 6 gene in patients with iron overload and non-homozygous genotype for the *HFE* p.Cys282Tyr mutation

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Abstract

Background: Five main genes are associated with hemochromatosis; however, current studies show that, in addition to these genes, others may be associated with primary iron overload (IO). One of these is the bone morphogenetic protein 6 (*BMP6*), which encodes a protein that modulates hepcidin synthesis and, consequently, iron homeostasis. **Aim:** To identify *BMP6* gene pathogenic variants in patients with IO and non-homozygous genotype for the *HFE* p.Cys282Tyr mutation. **Materials and methods:** Fifty-three patients with primary IO and non-homozygous genotype for the *HFE* p.Cys282Tyr were selected. Subsequent bidirectional DNA sequencing of *BMP6* exons was performed. **Results:** Two novel variants were found. One at homozygous state p.Gln158Ter (c.472C>T) was pathogenic, the other one at heterozygous state p.Val394Met (c.1180G> A) was of uncertain significance (VUS); the third variant at heterozygous state p.Arg257His (c.770G>A) has already been described and associated with IO. No *BMP6* pathogenic variants that would explain iron overload phenotypes were detected in 94% of the studied patients. **Conclusion:** Identification of the *BMP6* pathogenic variants in Brazilian patients with primary IO might contribute to the genetic understanding of this phenotype.

Keywords: Hemochromatosis, iron overload, *BMP6*, sequencing, non-homozygous genotype for the *HFE* p.Cys282Tyr.

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1. Background

Hemochromatosis is an autosomal recessive disease, in which iron overload is related either to a decrease in hepcidin production or to a defect in hepcidin binding to ferroportin (1-2). In most cases, hemochromatosis is associated with homozygosity for the *HFE* p.Cys282Tyr mutation. Although p.C282Y/p.H63D compound heterozygosity has been associated with hemochromatosis, this genotype is mainly a risk factor for slightly higher serum iron parameters and mildly increased hepatic iron stores, and it is considered insufficient to cause hemochromatosis, according with current guidelines (3). Rare cases of hemochromatosis can also be caused by pathogenic variants in other genes, corresponding to non-*HFE* hemochromatosis (4-8). Juvenile hemochromatosis (JH) is type 2, subdivided into type 2A, related to a pathogenic variant in the *HJV (hemojuvelin)* gene, and type 2B, related to mutations in the *HAMP (hepcidin) gene*; type 3 is related to a pathogenic variant in the *TFR2* gene; and type 4 is related to a pathogenic variant in the *SLC40A1* (*ferroportin*) gene (9).

Some studies have reported that sequencing the five main genes related to hemochromatosis may not be sufficient to explain all cases of primary iron overload (IO) (10,11). A Portuguese group, using next generation sequencing (NGS), evaluated a panel of genes involved in iron metabolism (*HFE, TFR2, HJV, HAMP, SLC40A1* and *FTL (L-ferritin)*) in 87 patients with a non-classical form of hemochromatosis, and were unable, in some patients, to detect changes that could explain their phenotype, which suggests that the genetic changes possibly responsible for IO in these patients could be located in genes not addressed in this study (11). One study sequenced the *HFE, HJV, HAMP, TFR2* and *SLC40A1* genes from 51 samples from Brazilian patients with

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primary IO. The frequency of the homozygous p.Cys282Tyr genotype in the *HFE* gene in this sample was significantly lower (21.6%) than in other studies (12),

Great advances in the molecular diagnosis of hemochromatosis have already been observed, but it remains hemochromatosis cases for whom no changes are found in the main genes related to iron homeostasis. Thus, it is important to explore further genes involved in iron metabolism, in order to better understand the molecular basis of hemochromatosis). Hamdi-Rozé et al reported that rare *HFE* variants may be a frequent cause of hemochromatosis in non homozygous p.Cys282Tyr hemochromatosis patients. BMPs (bone morphogenetic proteins) are proteins that belong to the Transforming Growth Factor Beta (TGF-beta) superfamily, which is involved in cell differentiation and cell proliferation (13,14). The BMP / SMAD signalling pathway is strongly involved in systemic iron regulation through its impact on the hepcidin gene (*HAMP*) transcription (17 - 16). In animal models, *BMP6* expression is highly related to iron homeostasis; in wild-type mice, iron-enriched diet leads to an increase in both *BMP6* expression and SMAD1/5/8 phosphorylation, which was correlated with an increase in *HAMP* transcription (18,19). In another study, it was found that mice with a deficiency in *BMP6* expression showed a reduction in hepcidin levels and a phenotype similar to patients diagnosed with hemochromatosis (20,21). In a human study, hepcidin levels were markedly low in patients who had pathogenic variants in the *BMP6* gene (22). Liver cell isolation experiments indicates that non parenchymal cells (NPCs), considering Kupffer cells (KCs), sinusoidal endothelial cells (SECs), and hepatic stellate cells (HSCs) rather than hepatocytes, are the main source of BMP6 in the liver (23). Taken together, these data suggest that *BMP6* plays an important role in the regulation of hepcidin; thus, mutations in this gene may lead to iron-related disorders, as iron overload.

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Therefore, our aim was to search for *BMP6* gene pathogenic variants in patients with IO and non-homozygous genotype for the *HFE* p.Cys282Tyr mutation.

2. Methods

2.1 Patients

The study protocol was approved by the Ethics Committee (EPM-Unifesp - 1233/2018 and HC-FMUSP - 4027/14/007, Brazil) and consent form was obtained from the patients prior to entering the study. Fifty-three patients were selected from hematology ambulatory clinics (Hematology Outpatient Clinic of Hospital das Clínicas - São Paulo, Outpatient Clinic of the Blood Center of Santa Casa - São Paulo, and Naoum Institute of Hematology - São José do Rio Preto) in Brazil. Inclusion criteria were: age greater than or equal to 18 years, plasma transferrin saturation (TS) $\geq$ 45 % and serum ferritin (SF) $\geq$ 200 ng/mL for women and $\geq$ 300 ng/mL for men. The exclusion criteria were patients with homozygosity for the *HFE* p.Cys282Tyr mutation, positive serology for hepatitis C or B, alcoholic liver disease, high alcohol consumption (>20g per day), haemolytic anaemia, repeated blood transfusions, metabolic syndrome or insulin resistance not resulting from hemochromatosis (24).

2.2 DNA extraction and genotyping

The blood samples of the patients were collected using the BD Vacutainers System (Becton Dickinson, NJ, USA), and genomic DNA (DNAg) was extracted from the leukocytes using the QIAamp DNA Blood Mini Kit commercial extraction method (QIAGEN), in accordance with the protocol described by the manufacturer (23). *HFE* p.Cys282Tyr (c.845G>A; rs1800562) and p.His63Asp (c.187C>G, rs1799945)

mutations were genotyped using Real Time Polymerase Chain Reaction (PCR 7500 Applied Biosystem real-time PCR, USA - TaqMan assays).

2.3 Primer design, Sanger sequencing and analysis

Eigth primer pairs were designed to explore the 7 exons of the *BMP6* gene, expanding to 20 bps of intronic regions, (supplementary Table 1). The PCR products were purified with the ExoSAP-IT reagent (GE Healthcare, NJ, USA) and the Sanger methodology was applied using the Applied Biosystems 3130 / 3130xl Genetic Analyzers, the analysis of the results in electropherogram format was carried out by the Geneious software (Biomatters).

2.4 Data analysis

To classify the changes found in the *BMP6* gene, the lowest allele frequency indicated for rare diseases (MAF <0.01%) was used (25). Population data were consulted: EVS (Exome Variant Server), gnomAD (Genome Aggregation Database) and ABraOM (Brazilian Online Archive of Mutations). In addition, pathogenicity prediction software was used: Polyphen-2 (Polymorphism Phenotyping v2), which estimates structural and functional effects of human variants and analyses the evolutionary conservation of the affected protein. The predictions were calculated for all resulting amino acid residue substitutions in human UniProtKB proteins with the maximum CDS sequence overlap and identity. Provean (protein variation effect analyser), which seeks to impact the biological function of a protein, was used. The Provean scores are computed based on the homologs collected from a database. Thus, the prediction results may slightly vary with the protein database used and also the versions of PSI-BLAST and CD-HIT programs. Currently, the PROVEAN web server

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uses the NCBI nr database (September 2012), BLAST v2.2.24+, and CD-HIT v4.5.4. Sift (Intolerant Classification of Tolerant), which is based mainly on the evolutionary condition of the protein, uses a query string and various alignment information to predict tolerated and harmful substitutions for all positions in the query string. To obtain the result, there is a procedure with several steps, among which we have the search for similar sequences, choice of closely related strings that can share a function similar to the query string, the alignment of these chosen sequences is obtained and normalized probabilities are calculated for all possible alignment substitutions. Positions with normalized probabilities less than 0.05 are predicted to be harmful, those greater than or equal to 0.05 are predicted to be tolerated. The mutation taster that performs a series of *in silico* tests to estimate the impact of the variant on the protein and to determine its disease-causing potential. The changes were compared with the HGMD database (Human Gene Mutation Database) and ClinVar, which aggregate information on genomic variation and its relationship to human health, as well as articles found on Pubmed. For pathogenic variant p.Gln158Ter (c.472C> T), we also used the CADD software, which has C-scores for all 8.6 billion possible single nucleotide variants (SNVs) of the reference genome, a selection of short insertions / deletions, as well as a few sets of large variants (e.g., gnomAD, ExAC, 1000 genomes, ESP). It also provides a simple search for SNVs and allows the scoring of short insertions / exclusions. Finally, the changes found were classified according to the ACMG (American College of Medical Genetics and Genomics) criteria (26).

3. Results

3.1 Identified genetic variants

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Of the fifty-three patients, we identified three *BMP6* variants (3/53, 6%) with potential for pathogenicity. They are a novel pathogenic variant in patient #01, a novel variant of uncertain significance (VUS) in patient #02, and a pathogenic variant, already described, in patient # 03. The novel pathogenic variant, at homozygous state, found in patient #01, was p.Gln158Ter (c.472C>T, exon 1). The novel VUS, at heterozygote state, found in patient #02, was p.Val394Met (c.1180G>A, exon 4). Patient #03 has the pathogenic variant p.Arg257His (c.770G>A, exon 2) at the heterozygous state.

The rs2241669 polymorphism, present in exon 2, was observed in 4 patients (allelic frequency 3%). In exon 4, the rs17557 polymorphism was found in 29 patients (17%), and the rs17558 in 4 patients (3%).

No *BMP6* mutation that would explain iron overload phenotypes was detected in 94% (50/53) of the studied patients.

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3.2 Patients data

We enrolled fifty-three patients with mean age of 49±13, 79% of males. The mean values of plasma TS and serum ferritin were 65±19 %, and 1,375±840 μg/L, respectively.

Table 1 shows data of the patients. Patient #01 was a 61-year-old Brazilian man, with TS of 82% and SF of 1,505 μg/L. Genetic analysis for pathogenic variants in the *HFE* gene, p.Cys282Tyr and p.His63Asp, revealed a heterozygous genotype for both. The *BMP6* sequencing revealed the homozygous genotype for the novel pathogenic variant at position p.Gln158Ter (c.472C>T). It is a nonsense mutation, and causes a premature stop codon in the protein according to the mutation taster.

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Patient #02 is 46-year-old Brazilian man. TS was 61% and SF 1,096 μg/L. In addition to the sequencing of the *BMP6* gene, sequencing was also performed for *HFE, HJV, HAMP, TFR2,* and *SLC40A1* genes, and no variants were found. The patient reported that his father, mother and two sisters were diagnosed with hemochromatosis. One of his sisters died at the age of 41 because of complications from iron overload. The *BMP6* sequencing revealed heterozygosity for the novel p.Val394Met, classified as VUS (variant of uncertain significance).

A 32-year-old Brazilian man is patient #03, with SF of 301.4 μg/L, TS of 46.4% and secondary IO excluded. The patient reported that his father was diagnosed with hemochromatosis. Genetic analysis revealed a compound heterozygous genotype for *HFE* p.Cys282Tyr and p.His63Asp. The *BMP6* sequencing revealed the heterozygous genotype for the pathogenic variant p.Arg257His, described in the literature (27).

3.2 Population frequency, *in silico* prediction, classification according to ACMG

The population frequency and *in silico* prediction of the alterations are listed in Table 2. After evaluating the genetic results of the alterations, they were classified into levels of pathogenicity according to the ACMG criteria that we included also in Table 2.

For the alteration p.Gln158Ter (c.472C>T) no population frequency data was found and the only *in silico* predictor that has information about this alteration was a mutation taster that was highlighted as a "prediction disease causing" alteration. According to the CADD tool, which is the most complete for annotating variants and can analyze nonsense variants, the program's authors suggest a cut-off of 15 to consider the change as harmful (in your case, it was 34). Another interesting point about the variant is that it is not present in gnomad. This site gathers genetic information from

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individuals selected to be "control population" (theoretically without Mendelian diseases in the family). GnomAD is the largest bank today. If we look at nonsense changes (or loss of function), we see that they only appear in heterozygosity in gnomad, and it was found in homozygosity in our analysis. According to the ACMG, it was classified as a pathogenic alteration.

The population frequency found for p.Val394Met change was 0.0821% in ABraOM and 0.05129% in GenomAD. Regarding predictors *in silico*: Provean proved it to be a “tolerated” alteration, PolyPhen-2 described the variant as “benign”, Sift “tolerates” the variant, and the mutation taster described it as “disease causing”. This change was not found in ClinVar or HGMD; according to ACMG, it was classified as a VUS type, as it did not score enough to be pathogenic or possibly pathogenic.

The p.Arg257His pathogenic variant had a population frequency of 0.4936% in ABraOM and of 0.1375% in GenomAD. Predictors *in silico* reported that: in Provean the variant is considered “Damaging”, in PolyPhen-2 it is “Probably damaging”, in Sift the variant is described as “deleterious” and in mutation taster it is said to be “disease causing”. This change was not found in ClinVar and in HGMD. According to ACMG classification, it is a pathogenic alteration.

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4. Discussion

In our study, we identified a known pathogenic variant (*BMP6* p.Arg257His), as well as two novel changes not yet described in the literature, namely p.Gln158Ter (c.472C>T), classified as pathogenic and p.Val394Met, classified as VUS.

The latter variant, classified as VUS, does not have data of population frequency in the majority of predictors *in silico*. There are data that indicate that the VUS might be pathogenic. Our patient had a first-degree relative with iron overload. Nevertheless,

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according to ACMG, there was not enough score to classify it as pathogenic and, therefore, it is necessary to improve the data related to these changes described as VUS.

In 2016, Daher et al sequenced the *BMP6* gene in samples from 70 patients with the IO phenotype who did not carry any genetic alteration for the major hemochromatosis-related genes (28). In this study, three different *BMP6* propeptide heterozygous pathogenic variants were identified (p.Pro95Ser, p.Leu96Pro and p.Gln113Glu). According to their analyses, these pathogenic variants showed a strong association with the IO phenotype observed in the studied patients. In addition, they also performed *in vitro* studies to investigate the possible functional alterations caused by the identified pathogenic variants. They showed that these pathogenic variants were able to impair both the *BMP6* protein secretion and the SMAD1/5/8 signalling pathway activation, thereby leading to reduced hepcidin production by the liver. Taken together, these data suggest that *BMP6* propeptide pathogenic variants may be responsible for causing IO in some patients (28).There was a strong association of heterozygosity for three different *BMP6* propeptides with the IO phenotypes observed in the corresponding patients (28).

The same p.Leu96Pro alteration, previously described by Daher et al (28), was also identified by an Italian group in 2017, along with two other novel BMP6 propeptide pathogenic variants (p.Glu112Gln and p.Arg257His), in patients with IO (27). According to *in silico* analysis, the amino acid substitutions caused by the p. Leu96Pro and p.Glu112Gln pathogenic variants are located at key regions related to interactions between monomers, while the amino acid change caused by the p.Arg257His pathogenic variant may affect the interaction between *BMP6* and extracellular matrix proteins. Therefore, the data suggest that these pathogenic variants could lead to inappropriate *BMP6* protein assembly, as well as affect its cellular localization. However, no experimental data have been reported yet to verify this hypothesis (27).

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While previous studies lead us to believe that *BMP6* propeptide pathogenic variants are associated with protein changes that can cause IO (27,28), a recent study suggests that attributing the cause of the disease to these propeptide alterations should be considered with caution (29). This study evaluated three clinical cases of unexplained IO carrying the propeptide *BMP6* pathogenic variant p.Gln118_Leu119insGlnGlnHis. *In vitro* data showed no difference in BMP6 secretion or localization between the wild type and the p.Gln118_Leu119insGlnGlnHis *BMP6* alteration, indicating that this alteration does not affect the *BMP6* protein function. Moreover, the functional impact prediction using bioinformatics tools confirmed the benign nature of this alteration. Thus, the authors concluded that there is no functional relationship between the *BMP6* propeptide p.Gln118_Leu119insGlnGlnHis variation and IO, in contrast to what was proposed in previous studies.

The novel pathogenic variant p.Gln158Ter (c.472C> T), causes a premature stop codon in the protein, and, in our homozygote patient for this variant, the expression of the gene is expected to be totally absent. It was not possible to perform *in vitro* tests for documenting the loss of the BMP6 protein, but through the analysis done in the CADD software, which is a most complete tool for annotating variants and analysing nonsense variants, we found a PHRED value of 34, much higher than the cut-off value of 15 above which the change can be considered as harmful. We also used gnomad, which gathers genetic information from individuals selected to be "control population" (theoretically without Mendelian diseases in the family). GnomAD is the largest database today, and when we searched for nonsense (or loss of function) changes we found that the variant so far has not been observed in healthy individuals, and that all nonsense variants in *BMP6* reported to date in control populations were at the

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heterozygous state. This reinforces the hypothesis that homozygous nonsense variants in *BMP6* can lead to the observed phenotype.

Our study also found a further novel variant p.Val394Met in the BMP6 gene that was classified as VUS, whose pathogenicity is possible. The identification of novel pathogenic variants associated with IO is important to provide the basis for further research aiming at elucidating the role of this gene in the spectrum of genetic iron overload diseases.

In conclusion, identification of the *BMP6* pathogenic variants in Brazilian patients with primary IO might contribute to the genetic understanding of this phenotype.

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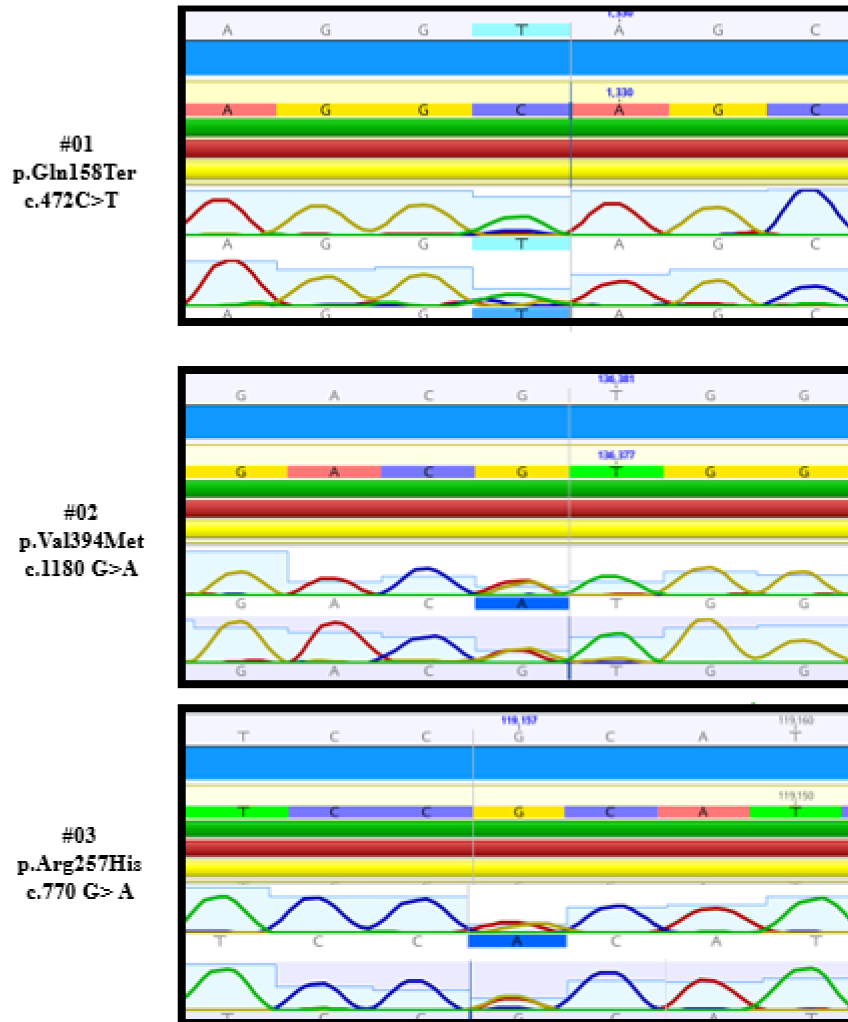

FIGURE 1. *BMP6* sequencing results. #01: homozygous genotype for the *BMP6* p.Gln158Ter pathogenic variant. #02: heterozygous genotype for the *BMP6* p.Val394Met genetic alteration. #03: Heterozygous genotype for the *BMP6* p.Arg257His pathogenic variant.

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TABLE 1. General and clinical data of the patients.

ID	Age/sex	TS (%) / and SF (μg/L)	LIC	*BMP6* variants	Reference of alteration
#01	61 Male	82 / 1,505		p.Gln158Ter exon 1	Novel
#02	46 Male	61 / 1,096		p.Val394Met exon 4	Novel
#03	32 Male	46.4 / 301.4	1.2 mg/g	p.Arg257His exon 2	Piubelli C., et al.2017

ID: identification; TS: plasma transferrin saturation; SF: serum ferritin; IO: Iron overload. Notes: for patient #01 no changes in the *HFE, HJV, HAMP, TFR2, SLC40A1* or *FTL* genes. In patient # 02, no changes were found in the *HJV, HAMP, TFR2, SLC40A1* or *FTL* genes. Father, mother, 2 sisters with IO. In patient # 03 no changes in the *HFE, HJV, HAMP, TFR2, SLC40A1* or *FTL* genes. Father was diagnosed with IO.

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TABLE 2. Population frequency, *in silico* prediction of alterations found in the *BMP6* gene and classification of variants according to American College of Medical Genetics and Genomics (ACMG).

		Population frequency				*In silico* prediction						
ID	*BMP6* mutation	ABraOM (%)	GnomAD (%)	Provean	PolyPhen-2	SIFT	HGMD	Mutation taster	ClinVar	Change Type	ACMG criteria	ACMG
#01	p.Gln158Ter							Prediction disease causing	not reported	Intronic region	PVS, PM2 e PP4, other criteria shown above are not met	Pathogenic
#02	p.Val394Met	0.000821	0.0005129	tolerated	benign	tolerated		disease causing	not reported	Missense	PM2. PP2 e PP4 , other criteria shown above are not met	VUS
#03	p.Arg257His	0.004926	0.001375	damaging	probably damaging	deleterious		disease causing	not reported	Missense	PM2. PP2, PP3, PP4 e PP5	Pathogenic

PVS – pathogenicity very strong, PM – pathogenicity moderate, PP – pathogenicity supplementary, VUS - variant of uncertain significance

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Supplementary table 1. Primers, hybridization temperature for the application of exons of the *BMP6* gene.

Primer pairs	Sequence	A.T. (Cº)
BMP6 1A F	GGGACTTGCTCACGCCAAGG	59.6
BMP6 1A R	CGGACAGGGCGTTGTACAGAT	
BMP6 1B F	CTCAAGACGCAGGGAAGCG	61.8
BMP6 1B R	GGGAAATGTTCTCGTCATTACGTT	
BMP6 2 F	ATCCACCTATGAAGTCAAACGGG	56.7
BMP6 2 R	GGAGTTATCTGATCACAAAAGATGCTT	
BMP6 3 F	GGCATGTTTTATCCTGACGCTGAGA	56.7
BMP6 3 R	GTCCTCCCATCGCCATCCTCTT	
BMP6 4 F	AGGAAACTAGGAAGTATCCCTTAACTG	63.4
BMP6 4 R	CTGAGTCTTCAGGTAACAAGTGTTC	
BMP6 5 F	GCTGTTGGGTGACCATACTTGTATC	59.6
BMP6 5 R	GGCTTTGAAAGGATGAGGTGAGAAT	
BMP6 6_7 F	CACATATTGCTGGCCAACCTACG	56.7
BMP6 6_7 R	CCGAGATGGCATTTAGCTTAGTTG	
BMP6 7 F	AACCAACCACGCGATTGTGC	56.7
BMP6 7 R	GGAGGGTAGGAAGAGCTTCACGG	

A.T.: annealing temperature.

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