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Acute Intermittent Porphyria: Predicted Pathogenicity of *HMBS* Variants Indicates Extremely Low Penetrance of the Autosomal Dominant Disease

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ABSTRACT: Acute intermittent porphyria results from hydroxymethylbilane synthase (*HMBS*) mutations that markedly decrease HMBS enzymatic activity. This dominant disease is diagnosed when heterozygotes have life-threatening acute attacks, while most heterozygotes remain asymptomatic and undiagnosed. Although >400 *HMBS* mutations have been reported, the prevalence of pathogenic *HMBS* mutations in genomic/exomic databases, and the actual disease penetrance are unknown. Thus, we interrogated genomic/exomic databases, identified non-synonymous variants (NSVs) and consensus splice-site variants (CSSVs) in various demographic/racial groups, and determined the NSV's pathogenicity by prediction algorithms and in vitro expression assays. Caucasians had the most: 58 NSVs and two CSSVs among ~92,000 alleles, a 0.00575 combined allele frequency. In silico algorithms predicted 14 out of 58 NSVs as "likely-pathogenic." In vitro expression identified 10 out of 58 NSVs as likely-pathogenic (seven predicted in silico), which together with two CSSVs had a combined allele frequency of 0.00056. Notably, six presumably pathogenic mutations/NSVs in the Human Gene Mutation Database were benign. Compared with the recent prevalence estimate of symptomatic European heterozygotes (~0.000005), the prevalence of likely-pathogenic *HMBS* mutations among Caucasians was >100 times more frequent. Thus, the estimated penetrance of acute attacks was ~1% of heterozygotes with likely-pathogenic mutations, highlighting the importance of predisposing/protective genes and environmental modifiers that precipitate/prevent the attacks.

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KEY WORDS: allele frequency; allele prevalence; disease penetrance; in silico prediction; in vitro expression

Introduction

Current efforts in genomic medicine are focused on determining which non-synonymous variants (NSVs) identified by genomic/exomic sequencing are pathogenic or benign. Various approaches to evaluate the pathogenicity of these variants include an array of in silico prediction programs and for certain proteins functional expression assays. These methods allow the assessment of the numerous NSVs that are discovered for a given gene and provide the clinical relevance of these variants as well as information of the prevalence of disease causing mutations, some of which may have allele frequencies >0.001 [Xue et al., 2012; Amendola et al. 2015].

Here, we investigated the pathogenicity of all NSVs and consensus splice-site variants (CSSVs) in genomic/exomic databases for autosomal dominant acute intermittent porphyria (AIP; MIM# 176000), the most common acute hepatic porphyria [Puy et al.,2010; Anderson et al., 2014]. AIP is an inborn error of heme biosynthesis resulting from the reduced activity of the heme biosynthetic enzyme, hydroxymethylbilane synthase (HMBS; MIM# 609806, EC 4.3.1.8; also known as porphobilinogen deaminase). Heterozygotes experience potentially life-threatening acute neurovisceral attacks precipitated by certain porphyrinogenic drugs (e.g., P450 inducers), dieting or fasting, and hormonal changes which induce the hepatic expression of the first and rate-limiting enzyme in the pathway, 5′-aminolevulinic acid synthase (ALAS1; MIM# 125290, EC 2.3.1.37) [Granick, 1963, 1966; Sassa et al., 1970]. Induction of the *ALAS1* mRNA is regulated in the liver by a negative feedback repression mechanism that depends on the amount of free hepatic heme. Due to the increased ALAS1 enzymatic activity, the reduced HMBS activity becomes rate-limiting and the neurotoxic porphyrin precursors, 5′-aminolevulinic acid (ALA) and porphobilinogen (PBG), accumulate systemically and cause the acute neurovisceral attacks.

Clinically, the life-threatening acute attacks are characterized by excruciating abdominal pain, nausea, vomiting, hypertension, tachycardia, and central and peripheral nervous system manifestations, including motor/sensory neuropathy and psychiatric symptoms. If untreated by infusion of hemin to replenish the hepatic heme pool and downregulate *ALAS1*, severe attacks can progress to advanced motor neuropathy, respiratory muscle paralysis, and bulbar palsy [Puy et al.,2010; Anderson et al., 2014]. Typically, the attacks occur after puberty and can be episodic or recurrent and ~90% occurs in women. Affected women may have monthly attacks due to hormonal changes in the luteal phase of their menstrual cycles [Hift et al., 2005; Innala et al., 2010]. It is estimated that about 10% of AIP heterozygotes have acute attacks [McColl et al., 1982]. In fact, most

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patients are diagnosed during or following an acute attack, while most AIP heterozygotes remain asymptomatic throughout their lives.

To date, over 400 presumably pathogenic *HMBS* mutations have been reported and catalogued in the Human Gene Mutation Database (HGMD) [Stenson et al., 2014]. Although there are no documented genotype/phenotype correlations, certain mutations are more frequent among patients with multiple or recurrent attacks (e.g., those encoding p.R149X, p.R173Q) [von und zu Fraunberg et al., 2005]. A recent estimate of the prevalence of AIP, based on newly diagnosed patients in Western European countries who sought medical attention for their attack, was ~0.000005 (or ~1 in 200,000) [Elder et al., 2013], with the notable exception of the Scandinavian countries where the disease prevalence is most frequent due to a founder effect for the mutation encoding p.W198X [Mustajoki et al., 1976; Lee et al., 1991; Floderus et al., 2002; Mykletun et al., 2014]. To our knowledge, the only other systematic study of AIP prevalence was the cross-sectional study of 3,350 French healthy blood donors, which identified two unrelated healthy individuals with decreased (~50%) erythrocytic HMBS activity [Nordmann et al., 1997], who were then confirmed to have known *HMBS* pathogenic mutations encoding p.D178N and p.L244fs for a frequency of ~0.0006 (one in 1,675). Thus, these findings indicate a marked discrepancy in the estimated prevalence of *HMBS* heterozygotes and the occurrence of acute attacks in AIP patients, the penetrance, in the general Caucasian population.

To address this discrepancy, we interrogated genomic/exomic databases with a combined total of 45,955 Caucasians to identify NSVs and CSSVs. We used in silico pathogenicity prediction programs and in vitro enzyme expression studies to assess if these *HMBS* variants were likely pathogenic or benign.

Materials and Methods

Database Variant Collection

The nomenclature of the *HMBS* variants reported is based on the cDNA sequence NM_000190.3 and in accordance with the standards of the Human Genome Variation Society (HGVS; http://www.HGVS.org/varnomen). Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence. NSVs and consensus splice site *HMBS* mutations in various demographic and racial/ethnic groups including Caucasian or Western European populations were identified in public genomic/exomic databases using primarily the Diseases Variant Store (DIVAS) as the portal to other databases (Disease Variant Store, https://rvs.u.hpc.mssm.edu/divas/) [Cheng et al., 2016]. The DIVAS contains five major databases that include population ethnicity and allele frequencies: the 1000 Genomes Project [Genomes Project C et al., 2015], the NHLBI Exome Sequencing Project (ESP) (Exome Variant Server, http://evs.gs.washington.edu/EVS/), the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org), UK10K (http://www.uk10k.org/), and the Scripps Wellderly Cohort (https://genomics.scripps.edu/browser/) [Erikson et al., 2016]. Reported presumably pathogenic *HMBS* mutations causing AIP were obtained in the HGMD (HGMD Professional 2016.1, https://portal.biobase-international.com/hgmd/pro/search_gene.php), which listed 403 mutations [Stenson et al., 2014].

In Silico Prediction of Variant Pathogenicity

All 58 *HMBS* NSVs in Caucasian and Western European populations were assessed to determine if they were deleterious or benign using 16 algorithms evaluating each variant. These programs (in Supp. Table S1) included: CADD [Kircher et al., 2014], CONDEL [Gonzalez-Perez et al., 2011], I-Mutant [Capriotti et al., 2005], MAPP [Stone et al., 2005], MutationAssessor [Reva et al., 2011], MutPred [Li et al., 2009], nsSNPAnalyzer [Bao et al., 2005], PANTHER [Tang et al., 2016], PhD-SNP [Capriotti et al., 2006], PolyPhen-2 [Adzhubei et al., 2010], PON-P2 [Niroula et al., 2015], PoPMuSiC [Dehouck et al., 2011], PredictSNP [Bendl et al., 2014], PROVEAN [Choi et al., 2015], SIFT [Kumar et al., 2009], and SNAP2 [Hecht et al., 2015]. For CADD, deleterious was defined as a CADD Phred score >25 (The Phred quality score (Q) is logarithmically related to the error probability (E). $Q = -10\log E$). For I-Mutant and PoPMuSiC, a variant was considered deleterious when it decreased the structural stability. For MutationAssessor, a Functional Impact (FI) score >2.0 was considered deleterious. For MutPred, a variant with probability of deleterious mutation >0.8 was considered deleterious. For PANTHER, Pdeleterious >0.8 was considered deleterious. For SNAP2, a score >10 and expected accuracy >70% were considered deleterious. For all other tools, the recommended default setting was used for calling deleterious/pathogenic lesions. For each variant, the "Consensus Deleterious Score" was the ratio of the number of programs predicting "deleterious" over the total number of programs with a predicted result, (i.e., predicting deleterious/total number of predictions). This approach of "Consensus Deleterious Score" from all 16 in silico prediction algorithms was used since it has been shown that combining multiple tools tends to provide a more reliable prediction of the "likely pathogenicity" [Polikar, 2006]. "Unknown" was not considered a prediction. Arbitrary cutoffs of <25% and >75% for deleterious were used to define each variant as "likely benign" and "likely pathogenic," respectively, and as "ambiguous," if the score fell between 25% and 75%.

In Vitro Expression Studies: Activity and Thermostability

HMBS cDNA constructs for each of the 58 *HMBS* NSVs were individually generated using the QuikChange Lightning Single-Site Mutagenesis kit (Agilent Genomics, Santa Clara, CA) to alter the *HMBS* wild-type (WT) cDNA in the pKK223 vector [Chen et al., 1994]. All constructs were re-sequenced to confirm their respective authenticities. Since HMBS is a cytosolic enzyme that does not undergo any post-translational modifications, WT and mutant constructs were each expressed in *E. coli* strain BL21(DE3)pLysS (Promega, Madison, WI), which had low endogenous HMBS background activity and produced large quantity of the recombinant human enzyme as used previously for mutational and crystallization analyses [Chen et al., 1994; Gill et al., 2009; Song et al., 2009]. The enzymatic activity of each mutant enzyme in the lysate was calculated as the percent of the expressed WT activity, which was expressed in the same experiment, as described previously [Chen et al., 1994]. All results are presented as the mean activities and standard deviations of at least three independent experiments. For the enzyme thermostability studies, the expressed recombinant enzyme in the lysate was assayed for HMBS activity after incubation at 65°C for 90 min at pH 8.0. The mean thermostability of each mutant HMBS enzyme was calculated as the percent of the initial expressed WT activity after heat treatment and was based on at least three independent experiments.

Table 1. Total *HMBS* Non-Synonymous and Consensus Splice-Site Variants in Genomic/Exomic Databases in Various Demographic/ Racial Groups

	Caucasians[a] (91,919 alleles)	Latinos (11,578 alleles)	Africans (10,406 alleles)	South Asians (16,512 alleles)	East Asians (8,654 alleles)
A. Non-synonymous variants					
Total number of variants	58	16	20	20	10
Listed in HGMD (*N*)	12	3	6	4	1
Allele frequency <0.0002 (*N*, % of Total)	55 (95%)	13 (81%)	14 (70%)	13 (65%)	7 (70%)
Allele frequency range	0.00192–0.00001	0.00052–0.00009	0.0017–0.0001	0.00367–0.00006	0.000462–0.00012
Total non-synonymous allele frequency	0.00573	0.00271	0.00624	0.007	0.00176
B. Consensus splice-site variants[b]					
c.422+1G>A	0.00001	0	0	0	0
c.613-1G>T	0	0	0.0001	0	0
c.652-2delA	0.00001	0	0	0	0
Total variant frequency:	0.00575 (1 in 174)	0.00271 (1 in 369)	0.00634 (1 in 157)	0.007 (1 in 140)	0.00176 (1 in 568)

[a]Caucasian sequences include European and Finnish data.
[b]Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in NM_000190.3.

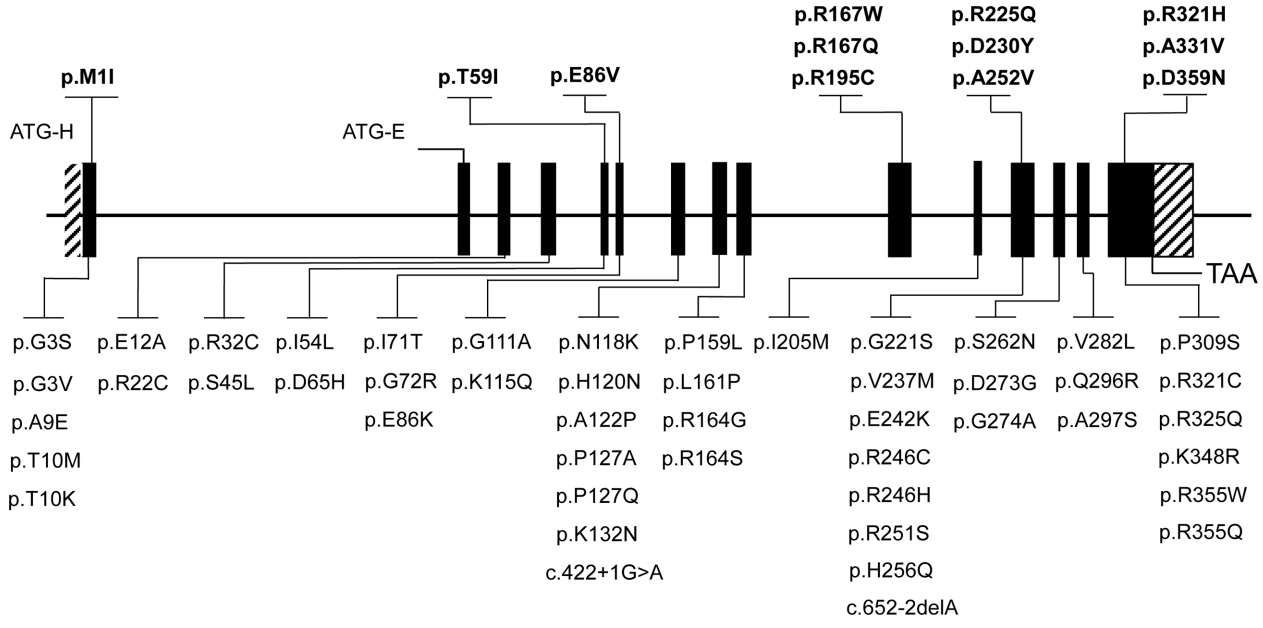

Figure 1. *HMBS* non-synonymous and consensus splice-site variants identified in Caucasians in genomic/exomic databases. Variants in the upper panel have been listed in HGMD as causing AIP. Variants in the lower panel are novel and identified only in the genomic/exomic databases.

Results

Identification and Characterization of *HMBS* Variants in Genomic Databases

Genomic databases that included 69,530 individuals from various demographic and ethnic/racial groups, including 45,955 Caucasians, were interrogated for *HMBS* non-synonymous and CSSVs (Table 1). All 101 identified *HMBS* variants (98 non-synonymous and three CSSVs) are listed in Supplementary Materials (Supp. Table S2) by demographic/racial group. The CSSVs c.422+1G>A and c.652-2delA were identified only in Caucasians, and c.613-1G > A was present only in Africans (Table 1). Overall, a high total allele frequency of non-synonymous *HMBS* variants was found in each demographic/ racial group (Table 1): 58 non-synonymous and two CSSVs with a total allele frequency of ~0.00575 (~1 out of 174) for Caucasians (Fig. 1); 16 NSVs with a total allele frequency of ~0.00271 (~1/369) for Latinos; 20 non-synonymous and one CSSV with a total allele frequency of ~0.00634 (~1/157) for Africans; 20 NSVs with a total allele frequency of ~0.007 (~1/140) for South Asians; 10 NSVs with a total allele frequency of ~0.00176 (~1/568) for East Asians. Notably, only one variant (encoding p.R321H) occurred in all five groups, ranging from 0.00012 in East Asians to 0.00192 in Caucasians. The variant encoding p.R246C was present in four groups, variants encoding p.P127Q and p.R225Q were shared by three groups, and 16 others were present in two groups (see Supp. Table S2). Of the 20 NSVs shared among two or more groups, nine (45%) were at CpG dinucleotides, hotspots for mutation [Cooper et al., 1988] (Supp. Table S2).

To determine the predicted pathogenicity of the 58 non-synonymous *HMBS* variants in Caucasians, as a relevant group for an estimate of AIP prevalence, each NSV was evaluated by in silico

pathogenicity prediction programs and by in vitro expression assays. Most (95%) of the Caucasian variants had an allele frequency 0.0002 or less, while three variants encoding p.S45L, p.E86V, p.R321H, had allele frequencies of 0.0004, 0.001, and 0.0019, respectively.

Predicted Pathogenicity of the Caucasian *HMBS* NSVs

The functional impact of each of the 58 NSVs on HMBS activity and stability was assessed by 16 different in silico pathogenicity prediction programs (Supp. Table S1) and the results were combined to generate a "Consensus Deleterious Score" or "CDS." Based on the CDS, each variant was classified as likely "deleterious," "benign" or, if most results were unknown or could not assess the variant, they were classified as "ambiguous." Supplementary Table S3 summarizes the results of each prediction program for each variant. Of the 58 variants, 12 were previously listed in HGMD as causing AIP, and five were predicted as "deleterious" (variants encoding p.R167W [Llewellyn et al., 1992], p.R167Q [Delfau et al., 1990], p.R195C [Kauppinen et al., 1995], p.R225Q [Floderus et al. 2002], and p.A331V [Bonkovsky et al., 2014]), while seven were considered "ambiguous" (Table 2). Of the remaining 46 NSVs, nine were predicted as "deleterious" (variants encoding p.R22C, p.D65H, p.I71T, p.G72R, p.A122P, p.L161P, p.R251S, p.V282L, and p.R355W), 12 were "benign," and the remaining 25 were designated as "ambiguous" and classified as unknown (Table 2).

In Vitro Activity and Thermostability of HMBS Variant Enzymes

All 58 NSVs were expressed in vitro and their enzymatic activities and thermostabilities were determined (Table 2). Of the 12 variants listed in HGMD as pathogenic, only four (encoding p.M1I [Chen et al., 1994], p.R167W, p.R167Q, and p.R195C) had enzymatic activities of ≤3% of the expressed WT activity. Notably, the eight variants encoding p.T51I [Schneider-Yin et al., 2008], p.E86V [Floderus et al., 2002], p.R225Q, p.D230Y [Whatley et al., 2009], p.A252V [Mgone et al., 1993], p.R321H [Schuurmans et al., 2001], p.A331V, and p.D359N [Di Pierro et al., 2006] listed in HGMD all had ∼60%–100% of the expressed WT activity. Of the remaining 46 variants in genomic/exomic databases, three encoding p.A122P, p.L161P, and p.R251S had expressed enzymatic activities of ≤2% of expressed WT activity (but have not been reported in AIP patients to date) and 41 had normal or near normal enzymatic activities (62%–130% of expressed WT activity), while the variants encoding p.G3V and p.G3S had ∼35% and ∼200% of expressed WT activity, respectively (Table 2).

The 51 variants with residual HMBS activities >10% of expressed WT activity were subjected to heat inactivation at 65°C, pH 8.0 for 90 min. While 48 variants were relatively thermostable compared with the expressed WT enzyme (Table 2), three encoding p.N118K, p.A252V, and p.A331V had markedly reduced enzyme activities after heat inactivation (2%, 10%, and 12% of initial expressed WT activity, respectively), indicating that their respective amino acid substitutions destabilized the HMBS mutant protein, and suggesting that they may be pathogenic. Of note, variants encoding p.A252V and p.A331V were found in AIP patients and listed in HGMD as pathogenic.

Thus, the likely pathogenic NSVs with <3% of expressed WT activity included four variants listed in HGMD, and three novel NSVs from the genomic/exomic databases with a combined allele frequency of ∼0.000477. The addition of the two CSSVs increased the likely pathogenic *HMBS* allele frequency to ∼0.000504. Finally, when the three variants with markedly decreased thermostabilities were included, the total likely pathogenic allele frequency, or prevalence of autosomal dominant AIP, was ∼0.00056 or 1 in ∼1,782 (Table 3).

Discussion

The acute hepatic and erythropoietic porphyrias are among a unique group of monogenic disorders (e.g., G6PD deficiency, malignant hyperthermia, dihydropyrimidine dehydrogenase deficiency) that require additional genetic and/or environmental triggering factors (e.g., drugs, metabolites, etc.) for their clinical expression, which has led to their being referred to as pharmacogenetic and/or ecogenetic disorders [Meyer, 2004; van Kuilenburg, 2004; Stowell, 2008; Puy et al.,2010; Anderson et al., 2014]. For AIP and the other acute hepatic porphyrias, the potentially life-threatening acute attacks are precipitated by porphyrinogenic drugs (typically P450 inducers), dieting/fasting or hormonal changes. Because of these triggering factors, most probands are diagnosed only during or after an acute attack, while the disease remains clinically asymptomatic and undiagnosed in most heterozygotes. Thus, the actual incidence, prevalence, and penetrance of autosomal dominant AIP remain unknown.

To date, the estimated prevalence of AIP in Western European countries, based on the frequency of newly diagnosed symptomatic patients was at ∼0.000005 or 1 in ∼200,000 individuals [Elder et al.,2013]. Estimates of the penetrance of AIP range from ∼10% in Western Europe to 30%–50% in Scandinavia [Andersson et al., 2000, Schuurmans et al., 2001; Bylesjö et al., 2009; Mykletun et al., 2014]. But since the prevalence of all individuals carrying a pathogenic mutation for AIP has not been determined, the penetrance of the disease remains unknown. Therefore, we determined the prevalence of pathogenic *HMBS* mutations by interrogating the non-synonymous and CSSVs in genomic/exomic databases that included ∼46,000 Caucasians and assessed which were likely pathogenic. A total of 58 *HMBS* NSVs were identified with a combined allele frequency of ∼0.00575 (1 in ∼174 individuals). In vitro expression of each variant identified only seven with expressed activities that were <3% of expressed WT activity. In addition, there were two pathogenic CSSVs and three thermolabile NSVs, yielding a combined allele frequency, or prevalence of autosomal dominant AIP, of ∼0.00056 or 1 in ∼1,782. Interestingly, the estimated prevalence of healthy French blood donors (3,350 individuals) with reduced HMBS activity was similar, 1 in 1675 [Nordmann et al., 1997].

Over 400 reported *HMBS* mutations causing AIP patients have been listed in HGMD, including 162 (∼40.2%) non-synonymous (missense) mutations [Stenson et al., 2014]. Of particular note, 12 of the 162 NSVs listed in HGMD were present in genomic/exomic databases. However, only six of these had <3% of WT expressed activity or reduced thermostability. Review of the original reports for the other six variants identified 13 reportedly symptomatic AIP patients who had variants encoding p.T59I [Schneider-Yin et al., 2008], p.E86V [Floderus et al., 2002], p.R225Q [Floderus et al., 2002; von Brasch et al., 2004], or p.R321H [Schuurmans et al., 2001; von Brasch et al., 2004; Anyaegbu et al., 2012; Cerbino et al., 2015]. However, the pathogenicity of most of these patients was not verified by elevated urinary ALA or PBG levels with the exception of (1) three patients with p.R321H who also had a second and pathogenic *HMBS* mutation [von Brasch et al., 2004; Cerbino et al., 2015]; and (2) one patient with p.R321H who had significantly increased urinary ALA or PBG concentrations, suggesting the presence of an unidentified second and pathogenic mutation [Anyaegbu et al., 2012] (Supp. Table S4).

Table 2. *HMBS* Non-Synonymous and Consensus Splice-Site Variants in Caucasians in Genomic/Exomic Databases

Genomic position (GRCh37/hg19)	cDNA[a]/Amino acid[b] changes	Allele frequency	In silico consensus deleterious score[c]/prediction		Percent of expressed WT activity (mean ± SD)	Thermostability: % of initial expressed WT activity after 65°C, 90 min: (mean ± SD)	Predicted pathogenicity
A. *HMBS* non-synonymous variants published in HGMD reported causing AIP: arranged by in vitro expressed activity							
118955746	c.3G > A / p.M1I	0.00001	0.38	Ambiguous	0 ± 1%	–	Pathogenic
118962124	c.500G > A / p.R167Q	0.0002	0.94	Deleterious	1 ± 1%	–	Pathogenic
118962123	c.499C > T / p.R167W	0.0001	0.94	Deleterious	3 ± 1%	–	Pathogenic
118962207	c.583C > T / p.R195C	0.0001	1	Deleterious	3 ± 1%	–	Pathogenic
118963217	c.755C > T / p.A252V	0.00001	0.64	Ambiguous	61 ± 12%	10 ± 2%	Pathogenic
118963899	c.992C > T / p.A331V	0.00003	0.79	Deleterious	62 ± 5%	12 ± 2%	Pathogenic
118959807	c.176C > T / p.T59I	0.00004	0.29	Ambiguous	77 ± 3%	51 ± 16%	Benign
118963150	c.688G > T / p.D230Y	0.00001	0.5	Ambiguous	88 ± 4%	50 ± 16%	Benign
118963982	c.1075G > A / p.D359N	0.00001	0.29	Ambiguous	86 ± 14%	42 ± 13%	Benign
118959973	c.257A > T / p.E86V	0.00113	0.43	Ambiguous	94 ± 16%	45 ± 15%	Benign
118963136	c.674G > A / p.R225Q	0.00026	0.93	Deleterious	102 ± 19%	31 ± 10%	Benign
118963869	c.962G > A / p.R321H	0.00188	0.38	Ambiguous	122 ± 24%	31 ± 10%	Benign
B. Non-synonymous *HMBS* variants identified in genomic/exomic databases: arranged by in vitro expressed activity							
118960959	c.482T > C / p.L161P	0.00001	1	Deleterious	1 ± 1%	–	Pathogenic
118963215	c.753G > C / p.R251S	0.00001	1	Deleterious	1 ± 0%	–	Pathogenic
118960719	c.364G > C / p.A122P	0.00001	0.93	Deleterious	2 ± 0%	–	Pathogenic
118960709	c.354C > G / p.N118K	0.00001	0.57	Ambiguous	73 ± 14%	2 ± 1%	Pathogenic
118955751	c.8G > T / p.G3V	0.00001	0.54	Benign	34 ± 6%	25 ± 8%	Benign
118960967	c.490A > G / p.R164G	0.00022	0.47	Ambiguous	61 ± 4%	27 ± 9%	Benign
118959928	c.212T > C / p.I71T	0.00003	1	Deleterious	62 ± 13%	62 ± 20%	Benign
118959930	c.214G > A / p.G72R	0.00004	1	Deleterious	69 ± 1%	32 ± 10%	Benign
118963123	c.661G > A / p.G221S	0.00001	0.67	Ambiguous	69 ± 15%	38 ± 12%	Benign
118959417	c.160A > C / p.I54L	0.00013	0.29	Ambiguous	71 ± 25%	52 ± 17%	Benign
118963517	c.821G > C / p.G274A	0.00001	0.27	Ambiguous	72 ± 9%	35 ± 11%	Benign
118963663	c.844G > C / p.V282L	0.00003	0.86	Deleterious	73 ± 2%	20 ± 6%	Benign
118960469	c.343A > C / p.K115Q	0.00008	0.29	Ambiguous	76 ± 10%	40 ± 13%	Benign
118960458	c.332G > C / p.G111A	0.00002	0.43	Ambiguous	76 ± 3%	32 ± 10%	Benign
118963708	c.889G > T / p.A297S	0.00001	0.2	Benign	76 ± 8%	49 ± 16%	Benign
118963868	c.961C > T / p.R321C	0.00001	0.27	Ambiguous	77 ± 12%	33 ± 11%	Benign
118963514	c.818A > G / p.D273G	0.00003	0.21	Benign	78 ± 15%	38 ± 12%	Benign
118955769	c.26C > A / p.A9E	0.00008	0.2	Benign	79 ± 0%	53 ± 17%	Benign
118963832	c.925C > T / p.P309S	0.00007	0.13	Benign	79 ± 6%	59 ± 19%	Benign
118955772	c.29C > T / p.T10M	0.00002	0.33	Benign	79 ± 12%	45 ± 14%	Benign
118963970	c.1063C > T / p.R355W	0.00006	0.87	Deleterious	80 ± 8%	26 ± 8%	Benign
118958995	c.64C > T / p.R22C	0.00003	1	Deleterious	80 ± 13%	38 ± 12%	Benign
118960969	c.492G > T / p.R164S	0.00003	0.27	Ambiguous	81 ± 18%	30 ± 10%	Benign
118960953	c.476C > T / p.P159L	0.00001	0.73	Deleterious	81 ± 20%	32 ± 10%	Benign
118962837	c.615C > G / p.I205M	0.00003	0.43	Ambiguous	83 ± 9%	24 ± 8%	Benign
118963186	c.724G > A / p.E242K	0.00001	0.36	Ambiguous	83 ± 13%	22 ± 7%	Benign
118960734	c.379C > G / p.P127A	0.00001	0.2	Benign	84 ± 19%	37 ± 12%	Benign
118955772	c.29C > A / p.T10K	0.00003	0.2	Benign	86 ± 25%	60 ± 19%	Benign
118960735	c.380C > A / p.P127Q	0.0002	0.27	Ambiguous	87 ± 2%	47 ± 15%	Benign
118960751	c.396G > C / p.K132N	0.00001	0.29	Ambiguous	87 ± 13%	43 ± 14%	Benign
118959391	c.134 C > T / p.S45L	0.00043	0.19	Benign	88 ± 17%	63 ± 20%	Benign
118963881	c.974G > A / p.R325Q	0.00003	0.25	Ambiguous	88 ± 4%	22 ± 7%	Benign
118963706	c.887A > G / p.Q296R	0.00001	0.21	Benign	92 ± 6%	50 ± 16%	Benign
118959824	c.193G > C / p.D65H	0.00001	0.77	Deleterious	93 ± 25%	38 ± 12%	Benign
118963230	c.768C > A / p.H256Q	0.00001	0.27	Ambiguous	93 ± 6%	40 ± 13%	Benign
118960713	c.358C > A / p.H120N	0.00001	0.33	Ambiguous	95 ± 2%	45 ± 15%	Benign
118963481	c.785G > A / p.S262N	0.00001	0.6	Ambiguous	96 ± 28%	73 ± 23%	Benign
118959351	c.94 C > T / p.R32C	0.00003	0.73	Ambiguous	98 ± 18%	43 ± 14%	Benign
118963971	c.1064G > A / p.R355Q	0.00001	0.6	Ambiguous	98 ± 10%	31 ± 10%	Benign
118963950	c.1043A > G / p.K348R	0.00004	0.13	Benign	100 ± 8%	35 ± 11%	Benign
118959972	c.256G > A / p.E86K	0.00001	0.43	Ambiguous	104 ± 27%	40 ± 13%	Benign
118958966	c.35A > C / p.E12A	0.00003	0.2	Benign	106 ± 23%	53 ± 17%	Benign
118963199	c.737G > A / p.R246H	0.00006	0.5	Ambiguous	122 ± 24%	29 ± 9%	Benign
118963171	c.709G > A / p.V237M	0.00001	0.36	Ambiguous	128 ± 6%	45 ± 15%	Benign
118963198	c.736C > T / p.R246C	0.00001	0.43	Ambiguous	130 ± 1%	53 ± 17%	Benign
118955750	c.7G > A / p.G3S	0.00003	0.21	Benign	211 ± 36%	150 ± 48%	Benign
C. Consensus splicing variants identified in genomic/exomic databases							
118960778	c.422 + 1G > A	0.00001	–	–	–	–	Pathogenic
118963111	c.652-2delA	0.00001	–	–	–	–	Pathogenic

[a]Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in NM_000190.3.
[b]Amino acid numbering uses +1 as the first amino acid of the NP_000181.2.
[c]Consensus deleterious score = (number of programs predicting deleterious/total number of programs with a predicted result), See *Materials and Methods* for details.

Table 3. Summary of Known and Predicted Pathogenic *HMBS* Variants in Caucasians in Genomic/Exomic Databases

cDNA changes[a]	Amino acid changes[b]	CpG dinucleotide	Reported in HGMD	HMBS activity	Allele frequency
c.3G > A	p.M1I	–	+	0 ± 1%	0.000014
c.499C > T	p.R167W	+	+	1 ± 1%	0.0001
c.500G > A	p.R167Q	+	+	3 ± 1%	0.0002
c.583C > T	p.R195C	+	+	3 ± 1%	0.0001
c.755C > T	p.A252V	–	+	10 ± 2%[c]	0.000014
c.992C > T	p.A331V	–	+	12 ± 2%[c]	0.000027
c.364G > C	p.A122P	–	–	1 ± 1%	0.000014
c.482T > C	p.L161P	–	–	1 ± 0%	0.000014
c.753G > C	p.R251S	–	–	2 ± 0%	0.000014
c.354C > G	p.N118K	–	–	2 ± 1%[c]	0.000014
c.422 + 1G > A	–	–	–	–	0.000014
c.652-2delA	–	–	–	–	0.000014
Total variant allele frequency =					**0.00056**

[a] Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in NM_000190.3.
[b] Amino acid numbering uses +1 as the first amino acid of the NP_000181.2A.
[c] Variants encoding p.N118K, p.A252V, and p.A331V are thermolabile and activities reported are percent of initial WT activity after 65°C at 90 min, pH 8.0.

Major sources of DNA sequence variations are replication errors and deamination at CpG dinucleotides, the latter being hotspots for mutation [Cooper et al., 1988]. Of the 58 *HMBS* non-synonymous genomic/exomic variants in Caucasians, 18 (~31%) occurred at CpG dinucleotides, including the most common benign variants p.R321H (allele frequency = 0.0019). Of the *HMBS* 1,086 base-pair coding sequence, there are 31 CpG dinucleotides. Interestingly, among the pathogenic variants causing biochemically confirmed AIP, the most common NSVs in unrelated probands occurred at CpG dinucleotides that encoded p.R167, p.R173, p.R225, and p.R325, representing ~27% of all identified *HMBS* mutations causing AIP (unpublished data, Desnick, RJ and Doheny, D, Mount Sinai Porphyria Diagnostic Laboratory).

A current focus of human genomics is to identify which NSVs in each disease-causing gene are pathogenic or benign. Many genomicists use in silico predictive tools to evaluate pathogenicity, as Whately and Badminton previously did to identify four putative pathogenic non-synonymous *HMBS* variants in the 1,092 individuals of all ethnic/demographic groups from the 1000 Genomes Project [Whatley and Badminton, 2013]. Although the in silico approach has proven useful to predict if a missense variant is damaging, several studies have demonstrated their limitations when used as standalone tools [e.g., Dorfman et al., 2010; van der Velde et al., 2015]. Specifically, a performance plateau of ~80% in their success rates [Riera et al. 2014] has been noted, a value below that required for clinical use [Richards et al., 2015]. In addition, in silico predictability may be gene/disease-specific [e.g., Leong et al., 2015; Adebali et al., 2016]. To circumvent these limitations, various studies suggest that prediction performance can be improved by combining the predictions of a number of tools [e.g., Chan et al., 2007; Konig et al., 2016]. Here sixteen predictive tools were employed to evaluate the *HMBS* NSVs. The "consensus deleterious score" predicted 14 variants to be deleterious, and 12 to be benign, but was unable to classify 32 variants, which were designated as "ambiguous" or "unknown." Of the 14 variants classified as deleterious, only nine (43%) had <3% expressed enzyme activities, while three (considered ambiguous) had markedly decreased thermostability in vitro. Based on these results, the functional in vitro expression and thermostability studies were more predictive of variant pathogenicity than the combined in silico analyses and should be used when appropriate to validate all new NSVs in patients with or without biochemical evidence of AIP.

In conclusion, our studies using genomic/exomic sequencing data from 45,955 Caucasians identified 10 non-synonymous and two consensus splice-site pathogenic variants for a combined prevalence of ~0.00056. Since the estimated prevalence of acute attacks is ~0.000005, and the estimated frequency of clinical pathogenic variants is ~0.00056, the penetrance of AIP is a surprising low 1% of all AIP heterozygotes. As AIP is a monogenic disorder, this extremely low penetrance suggests a critical role for modifying factors (environmental and/or genetic) in predisposing heterozygotes to acute attacks.

Abbreviations:

AIP, acute intermittent porphyria; ALA, 5′-aminolevulinic acid; ALAS1, 5′-aminolevulinate synthase; DIVAS, Disease Variant Store; ESP, Exome Sequencing Project; ExAC, Exome Aggregation Consortium; HGMD, Human Gene Mutation Database; HMBS, hydroxymethylbilane synthase; PBG, porphobilinogen

Author Contributions

R.J.D. conceived and supervised the project. B.C. and C.S. designed and performed all in vitro experiments. B.C., J.H., W.Q., and R.S. interrogated the genomic/exomic databases and/or performed the bioinformatic and in silico pathogenic prediction analyses. M.Y., M.B., D.D., I.P., and R.C. contributed to the epidemiological and bioinformatic studies and B.C. and R.J.D. wrote the manuscript with input from all authors.

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