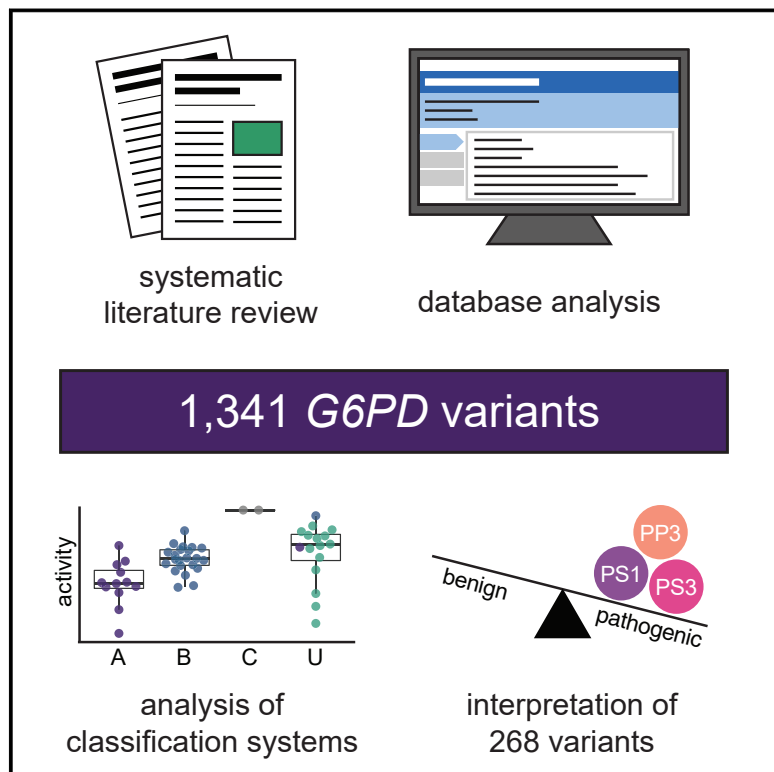


# Functional interpretation, cataloging, and analysis of 1,341 glucose-6-phosphate dehydrogenase variants

## Graphical abstract



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**Interpreting the effect of sequence variation in *G6PD* can be used to predict which individuals are at risk for adverse drug reactions. By analyzing data from publications and databases, we provided interpretations for 186 *G6PD* variants of uncertain significance, bringing the total number of interpreted variants to 400.**



# Functional interpretation, cataloging, and analysis of 1,341 glucose-6-phosphate dehydrogenase variants

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## Summary

Glucose-6-phosphate dehydrogenase (G6PD) deficiency affects over 500 million individuals who can experience anemia in response to oxidative stressors such as certain foods and drugs. Recently, the World Health Organization (WHO) called for revisiting *G6PD* variant classification as a priority to implement genetic medicine in low- and middle-income countries. Toward this goal, we sought to collect reports of *G6PD* variants and provide interpretations. We identified 1,341 *G6PD* variants in population and clinical databases. Using the ACMG standards and guidelines for the interpretation of sequence variants, we provided interpretations for 268 variants, including 186 variants that were not reported or of uncertain significance in ClinVar, bringing the total number of variants with non-conflicting interpretations to 400. For 414 variants with functional or clinical data, we analyzed associations between activity, stability, and current classification systems, including the new 2022 WHO classification. We corroborated known challenges with classification systems, including phenotypic variation, emphasizing the importance of comparing variant effects across individuals and studies. Biobank data made available by All of Us illustrate the benefit of large-scale sequencing and phenotyping by adding additional support connecting variants to *G6PD*-deficient anemia. By leveraging available data and interpretation guidelines, we created a repository for information on *G6PD* variants and nearly doubled the number of variants with clinical interpretations. These tools enable better interpretation of *G6PD* variants for the implementation of genetic medicine.

## Introduction

Glucose-6-phosphate dehydrogenase (*G6PD* [MIM: 305900]) deficiency (MIM: 300908) is the most common enzymopathy worldwide, affecting more than 500 million individuals.<sup>1</sup> *G6PD* is important in red blood cells since it is the sole source of NADPH needed for detoxification of reactive oxygen species.<sup>2</sup> Individuals with *G6PD* deficiency have variants with decreased activity, which can lead to three main clinical manifestations: neonatal jaundice, chronic non-spherocytic hemolytic anemia (CNSHA), and acute hemolytic anemia (AHA) in response to stressors such as certain foods, antibiotics, antimalarials, and infections that elevate reactive oxygen species.<sup>3,4</sup> Underlying *G6PD* deficiency is great genetic diversity, with hundreds of identified variant alleles, mostly missense variants in the coding sequence.<sup>1</sup> Interpreting the function and clinical effects of *G6PD* variants is critical to prevent adverse drug reactions, which are avoidable by prescribing alternate drugs, and to promote neonatal health by prompting increased monitoring.<sup>3,5</sup>

*G6PD* is located on the X chromosome, so variant phenotypes are most clearly observed in hemizygotes and homozygotes; heterozygotes are largely unaffected, though phenotypes vary between individuals and over time due to random X-inactivation,<sup>4</sup> but identification is important to avoid anemia and jaundice in hemizygous fetuses.<sup>1,5</sup> Some *G6PD* variants reach high frequency in specific pop-

ulations since they provide protection from severe malarial infection and thus are under balancing selection.<sup>2,6</sup> Many of these *G6PD* variants are well studied and have been reported in dozens of publications along with their activity, clinical manifestations, and distribution across local and global populations.<sup>2</sup> However, many variants are rare and have little data or conflicting reports on their connection to *G6PD* deficiency and anemia.

To aid in understanding the effects of *G6PD* variants, Yoshida and colleagues suggested a five-category classification system in 1971, based on variant activity and severity of anemia.<sup>7</sup> The classification scheme, called the World Health Organization (WHO) Classification, was modified in 1985 to contain four classes: class I variants with very low activity resulting in CNSHA, class II with very low and severe deficiency, class III with decreased activity and moderate to mild deficiency, and class IV with near normal activity.<sup>8</sup> However, even the original authors cautioned that the classification groups were for "convenience" and cutoffs made "somewhat arbitrarily."<sup>7</sup> The identification of variants associated with CNSHA that have activity over the 10% cutoff, and the overlap of clinical presentations between classes II and III, led to blurring of distinctions between classes.<sup>9</sup> Additionally, many variants were classified based on activity or clinical presentation measured in only one individual.<sup>9</sup>

Due to these issues, in 2019 the WHO issued a call to reconsider the *G6PD* variant classification system to

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standardize reporting of activity measurements.<sup>9</sup> Updated guidelines were released in 2022 to account for variability by requiring activity measurements in at least three unrelated hemizygotes and setting new thresholds for median activity to create new classes: A, variants leading to CNSHA with under 20% activity; B, AHA with less than 45% activity; C, no hemolysis and 60%–150% activity.<sup>10</sup> However, these new guidelines were developed and tested on only 17 variants, and any that do not conform to these classes or fall between 45% and 60% activity are grouped as class U, uncertain.

The WHO Genomics Initiative also identified *G6PD* variant classification as a priority for implementation of genetic and genomic medicine in low- and middle-income countries.<sup>9</sup> The Clinical Pharmacogenetics Implementation Consortium (CPIC) recently released updated guidelines for medication use in *G6PD*-deficient individuals, including seven drugs with high risk of inducing AHA in *G6PD*-deficient individuals.<sup>4</sup> To improve genetic assessment of *G6PD* deficiency and risk of AHA, we sought to collect all reports of *G6PD* variant activity measurements and clinical presentation. We also tested the implementation of the 2022 WHO classification system and applied the American College of Medical Genetics (ACMG) clinical guidelines to incorporate data from multiple individuals across studies to support clinical interpretation of *G6PD* variants. Here we provide interpretations for 186 variants previously of uncertain significance or not reported on ClinVar, and a collection of information on variant activity, stability, and clinical presentation. We found that biobank data in All of Us capture diverse *G6PD* variation in sequence and activity and align with interpretations from previous variant reports. These interpretations can increase the applicability of genetic medicine to individuals with rare *G6PD* variants.

## Material and methods

### Variant curation

Variants were curated from PubMed searches and the following databases in accordance with their data use agreements: CPIC (last updated February 25, 2021),<sup>11</sup> ClinVar,<sup>12</sup> dbSNP,<sup>13</sup> gnomAD (v.2.1.1 and v.3.1.1),<sup>14</sup> LOVD (v.3.0 build 26c),<sup>15</sup> bravo (TOPMed Freeze 8) ([bravo.sph.umich.edu](http://bravo.sph.umich.edu)), HGMD,<sup>16</sup> and All of Us (see section below). Intronic variants were included only if a clinical annotation (listed on ClinVar) or functional annotation (activity or stability) was provided; gross deletions were not included. Data on variant activity was also acquired through PubMed searches for “*G6PD* [variant name] activity”; for unnamed variants, the nucleotide change was used in place of a name. New publications were also reviewed weekly beginning in 2021 using a PubMed saved search alert for “*G6PD* deficiency.” Methods sections were viewed first to see if activity measurements were made and variants were genotyped; if not, the paper was not analyzed further, with the exception of reports of previously undescribed variants which were all included. Variants synthesized *in vitro* but not yet identified in humans are listed in Table S3 but not Table S1 or included in to-

tal variant counts. Final database searches were conducted on August 12, 2022.

Variants were denoted by nucleotide change on the negative strand of hg19/GRCh37 and cDNA position for transcript GenBank: NM\_001042351.1; cDNA position is reported throughout the manuscript. The most common name for each variant is reported in Table S1, but all alternate names are also included in Table S2. Variant types were determined by nucleotide changes in the coding region (e.g., single missense, synonymous) and noncoding variant types by VEP annotation listed in gnomAD v.2.1.1. Alternate exons from GenBank: NM\_000402.4 and NM\_001360016.2 and the 5' and 3' UTR of GenBank: NM\_001042351.1 were confirmed using the NCBI Genome Data Viewer.<sup>17</sup>

The only notable corrections made when adding variants to the list were as follows: ClinVar contains two entries for *G6PD* Puerto Limon on two different transcripts (variation IDs 10381 and 804134), so we listed the variant only once. Variant c.463C>G (p.His155Asp) was presented as *G6PD* Acrokorinthos though it contained only one of the two nucleotide changes found in the initial characterization of *G6PD* Acrokorinthos (c.[376A>G; 463C>G], p.[Asn126Asp; His155Asp]); thus we labeled it as “Acrokorinthos single.”<sup>18,19</sup> A report of c.1186C>T referenced a case study on the same individuals which listed the variant as c.1186C>G, which matched the residue change (p.Pro396Ala) reported in both studies so thus the data are used as support for c.1186C>G (p.Pro396Ala).<sup>20,21</sup> ClinVar variants 1237186 and 1280478 were not included since they do not encode a nucleotide change.

### Classifying variants according to ACMG guidelines

Criteria provided by the American College of Medical Genetics (ACMG) were applied to reports of curated variants (Table S4).<sup>22</sup> Scores were computed according to a Bayesian classifier with Prior\_P 0.1 and translated to interpretations.<sup>23</sup> Variant interpretations and evidence were submitted to ClinVar under submission name “*G6PD*\_Dunham2022” with accession numbers ClinVar: SCV002599134 to SCV002599417.

Evidence codes PVS1, PS1, PS2, PS3, PS4, PM1, PM2, PM4, PM5, PM6, PP1, PP3, PP4, and PP5 were used in support of pathogenicity. Reports of decreased activity by clinical testing in red blood cells were considered evidence for PS3 (functional study supports damaging effect); reports of decreased activity for variants expressed in model systems were decreased to moderate support (PS3\_M). As recommended for interpreting prevalence in affected individuals compared to control subjects (PS4), reports of variants in unrelated individuals were considered moderate support since case-control studies are rare (PS4\_M).<sup>22</sup> The critical domains used for PM1 (located in critical functional domain) were the NADP binding domain (GxxGDLA) from residues 38–44, substrate binding domain from residues 198–206, dimerization domain from residues 380–425, and structural NADP binding site from residues 488–489.<sup>15,24–26</sup> Other residues shown to be critical for functional domains in individual variant publications were also included with appropriate references. PM2 (absent or extremely low frequency in control subjects) was determined using gnomAD v.2.1.1 and v.3.1.1, with a conservative cutoff of 0.001 for below carrier frequency since the global prevalence estimate is 0.024–0.079 (average 0.05), but allele frequency can be below 0.01 depending on the region.<sup>27,28</sup> For variants with multiple nucleotide alterations, the lowest frequency site was used to determine if the complex variant frequency was likely below carrier frequency.

**Table 1. Missense variants associated with G6PD deficiency in All of Us**

Missense variants	Number of participants with deficiency without anemia	Number of participants with deficiency with anemia
None	2	5
hemizygous c.[202G>A; 376A>G]	1	6
homozygous c.[202G>A; 376A>G]	0	2
heterozygous c.[202G>A; 376A>G]	1	6
c.[202G>A; 376A>G]; [376A>G]	0	1
heterozygous c.376A>G	1	0
hemizygous c.563C>T	0	2
heterozygous c.563C>T	1	0
c.[376A>G; 968T>C]; [376A>G]	0	1

Missense variants in *G6PD* found in 29 individuals with the code “Deficiency of glucose-6-phosphate dehydrogenase” or “Glucose-6-phosphate dehydrogenase deficiency anemia” in All of Us, described by location on transcript GenBank: NM\_001042351.1. Number of individuals are shown in accordance with a Data and Statistics Dissemination Policy exception granted by the All of Us Resource Access Board.

Evidence codes BA1, BS2, BS3, BP4, BP5, and BP6 were used in support of benign classification. BA1 (>5% frequency in control subjects) was determined using gnomAD v.2.1.1 and v.3.1.1; BS1 (frequency in control subjects greater than expected) was not used since variants over the average carrier frequency of approximately 0.05 were already captured by BA1.<sup>27</sup> Reports of normal activity by clinical testing in red blood cells of hemizygotes were considered evidence for BS3 (functional study shows no damaging effect). If the only evidence supporting a pathogenic classification was PM2 (absent or extremely low frequency in control subjects) but other evidence supported a benign classification, PM2 was disregarded as recommended by previous application of the guidelines.<sup>29</sup>

### Current variant classifications

World Health Organization classifications by 1985 guidelines were taken from published variant lists or individual publications on the variant.<sup>1,15,30</sup> In rare instances when multiple classes were proposed, the lower was listed. WHO 2022 criteria were applied to classify variants with activity measurements in at least three unrelated individuals by computing weighted median activity and most severe clinical presentation.<sup>10</sup>

ClinVar classifications are given as the interpretation listed for each variant as of August 12, 2022; review status (from 0 to 4 stars with increasing support, though no *G6PD* variants had three or four stars) was noted at the same time. “Conflicting interpretations of pathogenicity” was shortened to “conflicting.” If two non-conflicting interpretations were given (e.g., “benign/likely benign”), only the first was listed.

### Variant activity and stability

All reports that measured variant activity are listed in Table S3, including activity of variants expressed in model systems. To

compare average variant activities, only reports of activity measured from red blood cells of hemizygotes were considered. If not computed by the study authors, average activity compared to the reference *G6PD* B was calculated using the provided normal range in the same study, or in rare instances the normal range from a previous publication from the same laboratory. Average variant activity reported in Table S1 was calculated as average across studies weighted by number of hemizygotes with measured activity per study.

All reports that measured variant stability are listed in Table S3. A consensus stability for each variant across studies is listed in Table S1; in case of conflicts, stability measured in red blood cells was reported, or “conflicting” listed if reports from red blood cells differed.

### Clinical presentations

The most severe clinical presentation observed for each variant is reported by study in Table S1. In decreasing severity, they are CNSHA, AHA, and asymptomatic deficiency. Nondeficiency was also recorded. In studies where individuals were reported as deficient without testing for anemia, the presentation was simply listed as “deficient;” this may also include other presentations such as jaundice without anemia, which are reported in Table S3.

### All of Us

All of Us data ([allofus.nih.gov](https://allofus.nih.gov)) were queried using the All of Us researcher workbench cloud computing environment using Python and R coding languages. To find variants that have not been previously reported in humans, we used the online All of Us data browser to identify *G6PD* variants in the full whole-genome sequencing (WGS) cohort. 118 non-intronic variants (considering exons of GenBank: NM\_001042351.1, NM\_000402.4, and NM\_001360016.2) were identified that had not been found in previous PubMed or database searches as detailed above (Table S1, “identified” column indicates “This paper (in All of Us)”).

A cohort was created for participants with WGS results available and a *G6PD* deficiency diagnosis with or without anemia (“deficiency of glucose-6-phosphate dehydrogenase” or “glucose-6-phosphate dehydrogenase deficiency anemia”) resulting in 29 participants. Genomic data were extracted from variant call format (VCF) files available to All of Us researchers and loaded into the workspace notebook using Hail and filtered to the *G6PD* region (chrX: 154,527,010–154,552,570, GRCh38). Variants comprising multiple nucleotide changes (multi-allelic variants) were split and the resulting Hail matrix was written to Plink format. The Plink files were converted to .raw format for upload into R. 101 variants were identified among these 29 people (Table S5); for Table 1 the list was then filtered to missense variants. Variant phasing for *G6PD* was done using Aldy v.4.1.<sup>31,32</sup> When multiple phasing results were returned, we chose the haplotype that matched the more commonly observed LD structure. For example, for two heterozygotes Aldy considered that the variants c.202G>A and c.376A>G were either on the same or separate alleles, but in these instances we chose the haplotype option with these variants on the same allele because that is more commonly observed. Numbers of individuals with each variant are printed in accordance with a Data and Statistics Dissemination Policy exception granted by the All of Us Resource Access Board in November 2022.

Activity data were collected from the following laboratory concept names: “Glucose-6-phosphate dehydrogenase (*G6PD*);



**Table 2. Missense variant enrichment in individuals with G6PD deficiency in All of Us**

Variant SNP	p value
c.202G>A	$<2.2 \times 10^{-16}$
c.376A>G	$9.1 \times 10^{-13}$
c.563C>T	$1.1 \times 10^{-5}$
c.968T>C	0.08

Enrichment p values by two-sided Fisher exact tests for missense variant SNPs found in 29 individuals with the code “Deficiency of glucose-6-phosphate dehydrogenase” or “Glucose-6-phosphate dehydrogenase deficiency anemia” in All of Us compared to individuals without either code.

quantitative,” “Glucose-6-Phosphate dehydrogenase [Entitic Catalytic Activity] in Blood,” “Glucose-6-Phosphate dehydrogenase [Enzymatic activity/mass] in Red Blood Cells,” “Glucose-6-Phosphate dehydrogenase [Enzymatic activity/volume] in Red Blood Cells,” “Glucose-6-Phosphate dehydrogenase [Enzymatic activity/volume] in Serum,” “Glucose-6-Phosphate dehydrogenase [Presence] in DBS,” “Glucose-6-Phosphate dehydrogenase [Presence] in Red Blood Cells,” and “Glucose-6-Phosphate dehydrogenase [Presence] in Serum.” Values were normalized to the average of the observational medical outcomes partnership (OMOP) standardized lower and upper limits of the normal range provided for each laboratory measurement and multiplied by 100 to give a percent activity of normal. For values that did not have OMOP normal ranges, but were categorized as “normal,” a value of 100% was assigned. For values without ranges or categorization, the value was normalized to the median of all values in that laboratory concept name group. For participants with multiple lab values, we used the lowest value. Individual values are shown in accordance with a Data and Statistics Dissemination Policy exception granted by the All of Us Resource Access Board in November 2022.

### Statistics

p values for figures were computed in R using t test or one-way ANOVA with Tukey’s honestly significant difference (HSD) as noted in figure legends. p values for Table 2 were calculated using Fisher exact test in R on 2×2 contingency tables based on allele counts of the variants in alleles from individuals with versus without a diagnosis of G6PD deficiency or G6PD deficiency anemia.

## Results

### An updated catalog of G6PD variants

Through database analysis and literature searches, we identified 1,341 G6PD variants that have been reported in humans (Figure 1A and Table S1). 296 of these variants have been described in publications, 75% (221/296) in individuals with G6PD deficiency.

For 414 variants, additional detailed information on molecular function or clinical interpretation was available (Figure 1A). However, only 27% (113/414) have been characterized in multiple reports.

### Interpreting G6PD variants using ACMG guidelines

By applying the ACMG standards and guidelines for the interpretation of sequence variants<sup>22,23</sup> to our collected in-

formation, we classified 268 G6PD variants, 186 of which were previously unclassified (166) or listed as uncertain significance or other on ClinVar (20) (Figure 1B and Table S4). We also provided interpretations of 12 variants with conflicting interpretations on ClinVar, lending additional support for classification.

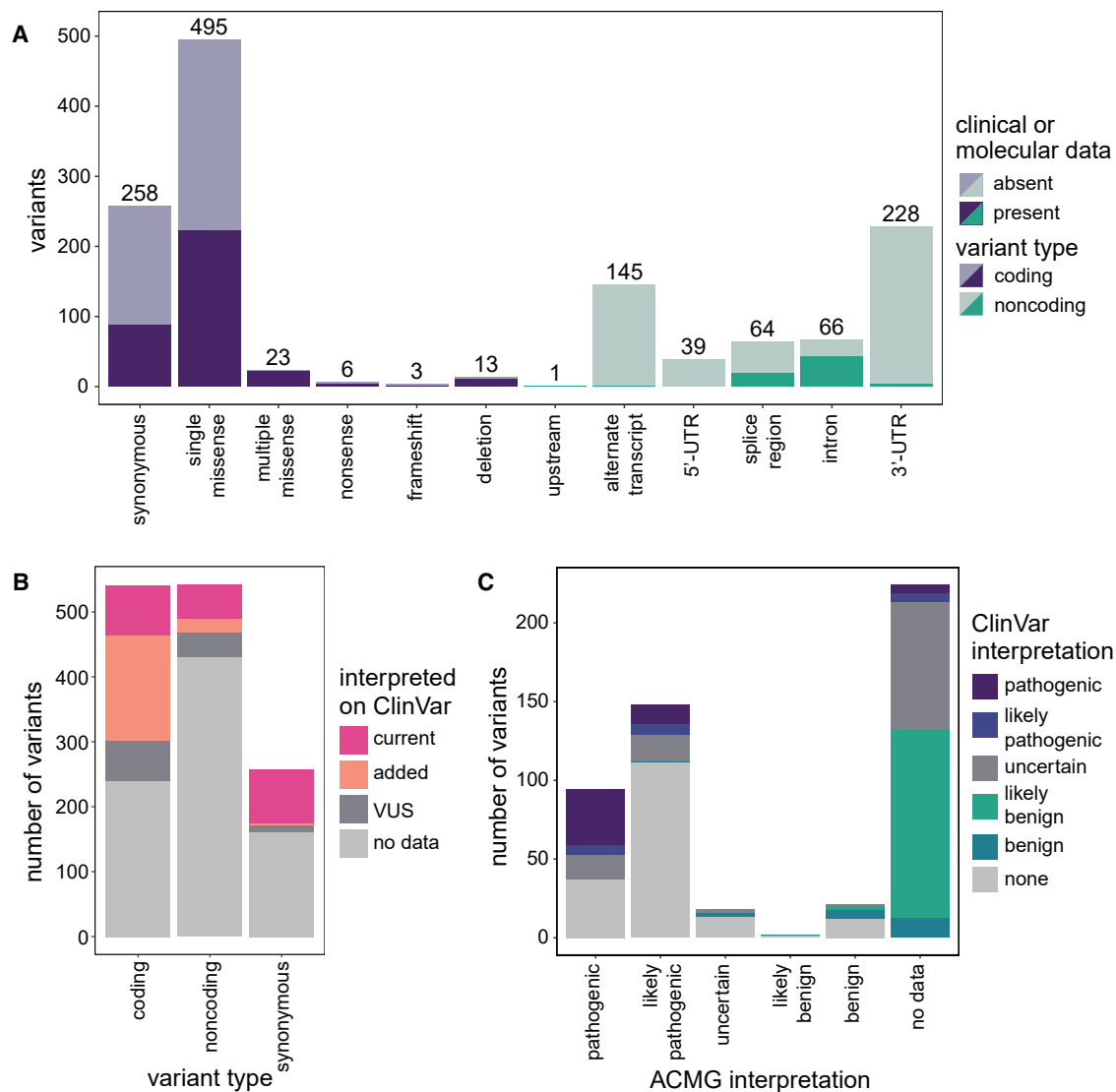
We observed a general consensus for 75 variants interpreted on ClinVar with sufficient information for us to also interpret them using ACMG guidelines (Figure 1C). The 61 variants ClinVar interpreted as pathogenic or likely pathogenic we also interpreted in one of those groups. There were far fewer published reports in support of benign interpretation, but our interpretation agreed with ClinVar for 9/14 benign and likely benign variants. For three variants interpreted on ClinVar as benign, we found conflicting reports of hemizygotes with and without deficiency and wide variation in activity (Tables S3 and S4); these conflicts led us to interpret them as variants of uncertain significance. For the other two conflicting variants, one was deposited as likely benign but without evidence to assess independently (variation ID 1546331), while we found a report of decreased activity and G6PD deficiency;<sup>33</sup> the other conflicting variant was deposited to ClinVar by OMIM as benign for its “nearly normal properties” (variation ID 10362), but investigating the referenced reports showed that activity was decreased to 20% of normal.<sup>34,35</sup> Applying ACMG guidelines to the information on these two variants suggested they are likely pathogenic.

By combining the 219 variants already interpreted on ClinVar with our additional interpretation of 186 variants and five benign variants called into question, 400 G6PD variants now have proposed interpretations other than uncertain or conflicting (Figure 1B). Most are nonsynonymous coding variants (60%, 239/400), and most of those are single missense variants (85%, 202/239).

### Evaluating G6PD activity in the context of variant classifications

Since G6PD deficiency results from insufficient G6PD activity, we used our collected data on G6PD variant activity to assess different strategies that infer or categorize G6PD variant function by activity. Most G6PD variants associated with deficiency alter the amino acid sequence, and few regulatory variants have been characterized.<sup>1</sup> We confirmed that variants not affecting the coding sequence had significantly higher activity and were rarely associated with anemia (Figure 2A). Variants altering the coding sequence led to a range of activity and clinical presentations, but all nonsense variants and deletions led to less than 10% normal activity and CNSHA (Figure 2B).

Using the 1985 WHO classification scheme, we found that G6PD variant activity increases as class levels increase (Figure 2C), but our data corroborate known issues with the 1985 WHO classification method.<sup>9,10</sup> Several class I variants associated with CNSHA have over 10% activity, and for some reported as class I we were unable to find clinical reports to support the diagnosis of CNSHA. Classes



**Figure 1. G6PD variant interpretation**

(A) A total of 1,341 *G6PD* variants found from variant database and PubMed queries. Clinical or molecular data indicate the variant is interpreted on ClinVar (as benign, likely benign, likely pathogenic, or pathogenic) or has activity or stability data available from published studies.

(B) Of 1,341 variants, 214 variants have interpretations other than VUS currently available on ClinVar, and an additional 186 were interpreted by applying ACMG guidelines to published reports. VUS includes variants interpreted as uncertain or with conflicting interpretations, including five previously interpreted on ClinVar.

(C) Concordance between variant interpretations for all variants reported on ClinVar, including VUSs, and by our application of ACMG guidelines for 510 total *G6PD* variants.

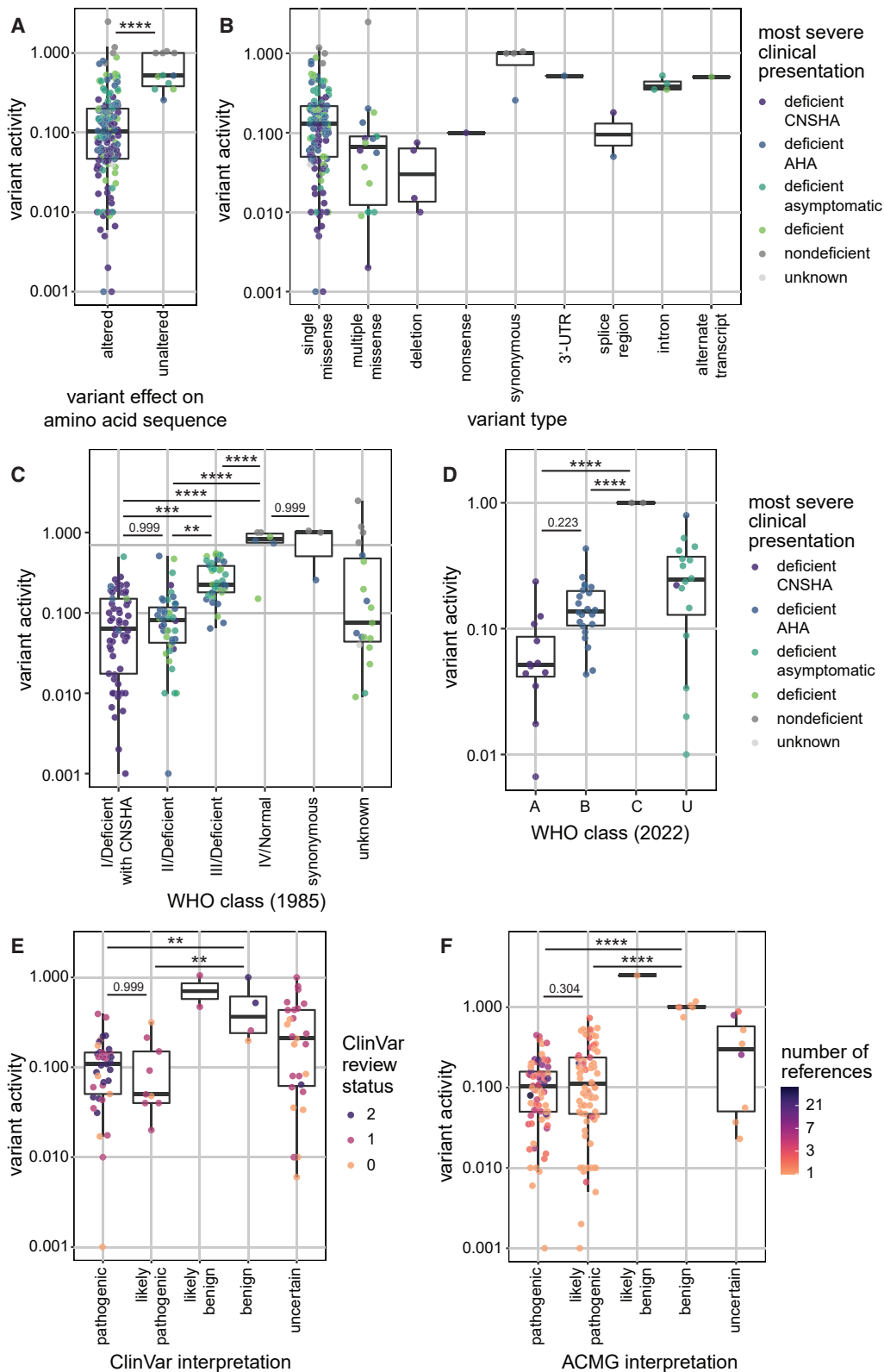
II and III both contained variants that led to AHA, rendering them redundant. Applying the 2022 WHO classification method, which requires variants to have measured activity in at least three unrelated hemizygotes,<sup>10</sup> we observed clearer separation between variants leading to CNSHA and AHA (Figure 2D). However, many variants that have not been reported to lead to anemia also have activity below 60% of normal and thus were classified as uncertain.

Although the interpretations provided by ClinVar do not require reporting of variant activity, pathogenic and likely pathogenic variants have significantly lower activity than

benign variants (Figure 2E). Variant activity is considered when applying the ACMG guidelines, so pathogenic and likely pathogenic variants have significantly lower activity than benign variants (Figure 2F).

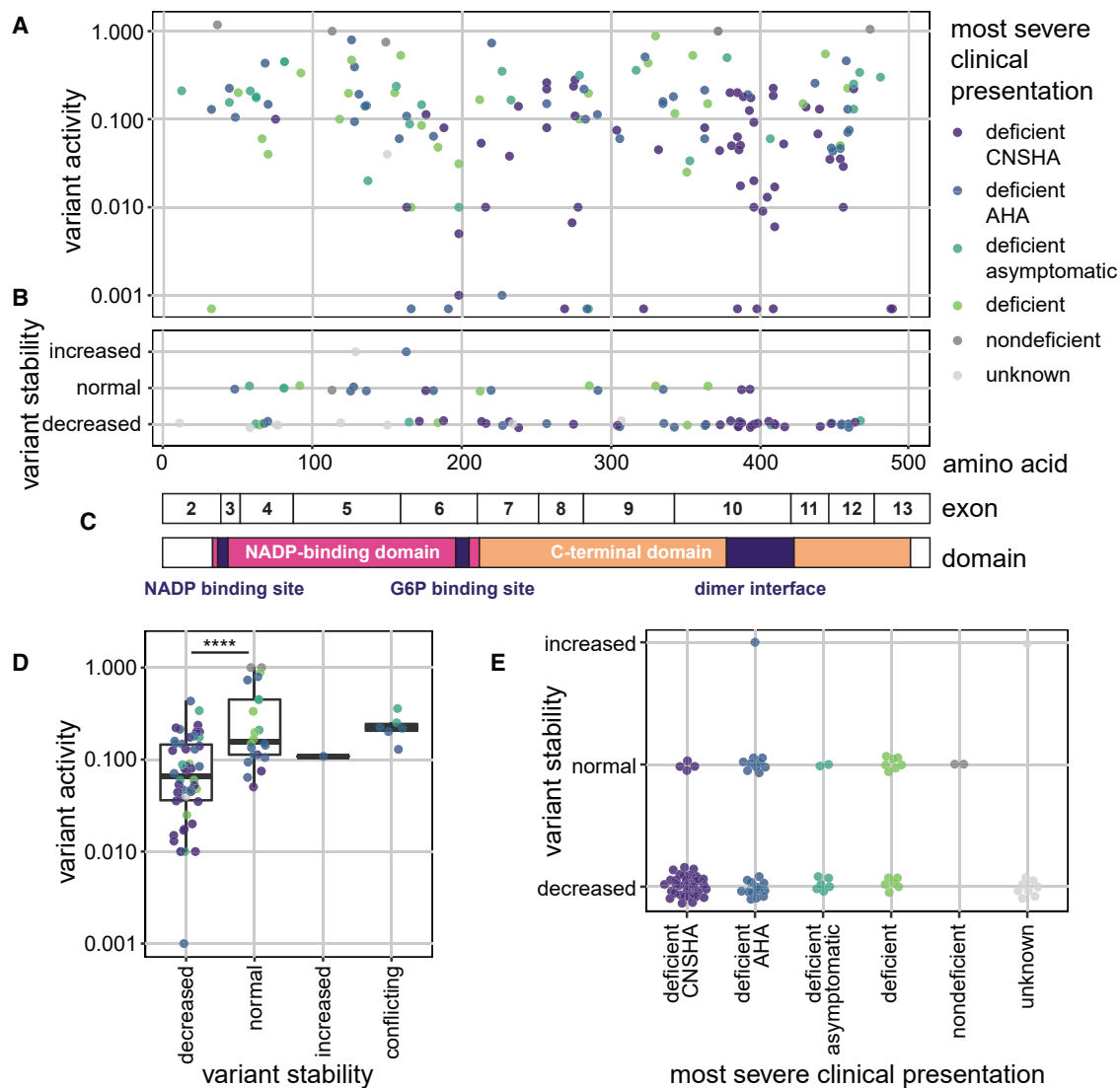
### G6PD structure impacts variant function and clinical presentation

*G6PD* contains several domains required for its function, including NADP binding sites, a substrate binding site, and dimerization domain.<sup>26</sup> The dimerization domain is especially important since *G6PD* is active only as a dimer and tetramer.<sup>1</sup> Variants that disrupt the dimerization



**Figure 2. Classification systems for G6PD variants**

(A and B) Average G6PD activity by effect on the coding sequence (A) and by type of genetic alteration in the variant (B), with most severe reported clinical presentation associated with each variant (“deficiency” indicates further information on anemia was not collected). (C–F) Average G6PD variant activity by 1985 (C) and 2022 (D) WHO classification, ClinVar interpretation (E), and by our application of ACMG guidelines (F). In ClinVar review status, 0 indicates least supporting evidence, and increasing numbers indicate increasing support. All average activities are from red blood cell extracts and weighted by number of hemizygotes per study. p value by one-way ANOVA with Tukey’s HSD, except (A) by t test, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.



**Figure 3. G6PD structure and variant effects**

(A–C) G6PD variant activity (A) and stability (B) across the structural domains (C), with most severe clinical presentation associated with each variant (“deficiency” indicates further information on anemia was not collected). Only single missense and synonymous variants shown.

(D and E) Variant stability compared to activity (D) and phenotypic severity (E).

All average activities are from red blood cell extracts and weighted by number of hemizygotes per study. *p* value by one-way ANOVA with Tukey’s HSD, \*\*\*\**p* < 0.0001.

domain are often predicted to be detrimental, which is evident in our collected data by a cluster of variants in the dimer interface with low activity and stability associated with CNSHA (Figures 3A–3C).

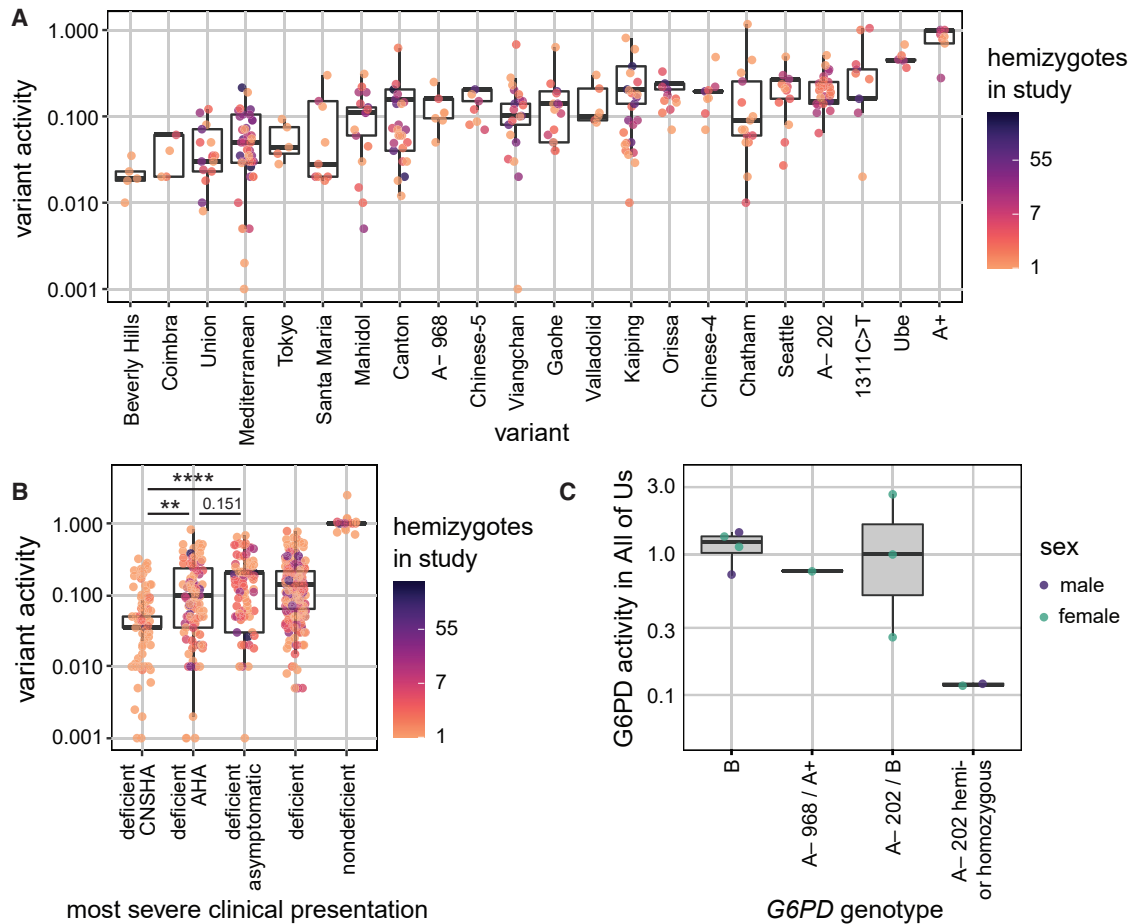
Loss of stability is a major mechanism of G6PD deficiency,<sup>1</sup> and we observed that most variants associated with CNSHA were unstable (89%, 34/38) (Figures 3D–3E). Some variants had decreased activity but normal stability, suggesting a decrease in specific activity (Figure 3D).

#### Variation across reports contributes to challenges in variant interpretation

A challenge in interpreting *G6PD* variant effects is the variability between individuals sharing the same genotype,

even when only considering hemizygotes with deficiency.<sup>10</sup> This was part of the rationale for requiring activity to be measured in three unrelated individuals for the 2022 WHO variant classification, and the variability is clear when observing reported activity for variants with five or more studies (Figure 4A). Some variants associated with anemia have reports of activity ranging from less than 2% to over 60% of normal (G6PD Chatham c.1003G>A [p.Ala335Tyr], Canton c.1376G>T [p.Arg459Leu], and Kaiping c.1388G>A [p.Arg463His]). Separating variants by clinical presentation still reveals high variability across variants and studies (Figure 4B), and the activity of variants reported with AHA is not significantly different from ones reported without symptoms.





**Figure 4. Variation in activity and clinical presentation for *G6PD* variants across studies**

(A) Average *G6PD* activity (normalized to *G6PD* B activity) per study for all variants with at least 5 independent reports.

(B) Average *G6PD* variant activity by most severe clinical presentation in each study. Nondeficient group is significantly different from each other group ( $p < 0.0001$ ). All average activities are from red blood cell extracts of hemizygotes, and boxplots weighted by number of hemizygotes per study.  $p$  value by one-way ANOVA with Tukey's HSD, \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

(C) *G6PD* activity measured in blood or serum 10 individuals with the code "Deficiency of glucose-6-phosphate dehydrogenase" or "Glucose-6-phosphate dehydrogenase deficiency anemia" in All of Us. Some individuals with B variant have noncoding variation. Individuals are grouped by nonsynonymous coding variants. Data are shown in accordance with a Data and Statistics Dissemination Policy exception granted by the All of Us Resource Access Board.

### Diverse biobank data provide additional support for genotype-phenotype relationships

The majority of *G6PD* variants with publications supporting their link to deficiency have been reported in fewer than three individuals (70%, 167/240, Table S3). The All of Us Research Project has collected clinical codes and whole-genome sequencing from thousands of diverse individuals, enabling us to connect variants with clinical presentations. During variant curation, our analysis of whole-genome sequencing data identified 118 variants not found in any of our other database or literature searches, including 18 missense and 1 frameshift variant (Table S1). Through analysis of biobank data, we found that 29 individuals were reported as diagnosed with *G6PD* deficiency, with or without anemia, and carried four *G6PD* missense variants in different combinations (Tables 1 and 2). This included rarer alleles such as A-968 (c.[376A>G; 968T>C], p.[Asn126Asp; Leu323-

Pro]), which had previously only been reported with anemia in three individuals, one homozygote and two heterozygotes.<sup>36,37</sup> Three missense variants in *G6PD* (c.202G>A [p.Val68Met], c.376A>G [p.Asn126Asp], and c.563C>T [p.Ser188Phe]) were significantly enriched in All of Us alleles from participants with a diagnosis of *G6PD* deficiency ( $p$  values  $< 0.0001$ ) (Table 2). Occurrence of common A-202 variant c.[202G>A; 376A>G], p.[Val68Met; Asn126Asp] in both *G6PD*-deficient individuals who were asymptomatic and who had AHA was in agreement with other reports (Table S3).

*G6PD* activity data were available for a subset of the 29 individuals with *G6PD* deficiency and agreed with previous reports. Individuals hemizygous or homozygous for the common A-202 variant had decreased activity (mean 12%, median 12% of normal), while heterozygotes had near-normal activity (mean 131%, median 100% of normal) but ranged widely (Figure 4C). The heterozygous combination

of A– 968 and A+ variants (c.[376A>G; 968T>C][376A>G], p.[Asn126Asp; Leu323Pro][Asn126Asp]) led to slightly decreased activity (mean 76%, median 76% of normal), similar to activity previously reported in A– 968 heterozygotes with deficiency (44%–66%).<sup>38</sup>

There were 21 variants present in individuals with a G6PD-deficient anemia diagnosis without any previously known or suspected missense variants causative of G6PD deficiency. These included 1 synonymous, 15 intronic, and 5 downstream variants (two in the 3' UTR). One of the 3' UTR variants (c.\*357G>A) and the synonymous variant (c.1311C>T [p.Tyr437=]) have been previously reported with deficiency and anemia in the absence of missense variation, but also in many individuals without deficiency (Table S3). However, despite a diagnosis of G6PD deficiency, individuals without missense variants for whom activity measurements were available did not have decreased G6PD activity (mean 115%, median 124% of normal) (Figure 4C).

## Discussion

By conducting a systematic review of literature and databases, we compiled a highly comprehensive catalog of *G6PD* variants reported in humans. Applying the ACMG guidelines to information in published reports enabled us to classify 268 variants, 186 of which were previously unreported or of uncertain significance on ClinVar, bringing the total number of *G6PD* variants with functional interpretations to 400. We are continuing to maintain an updated catalog of *G6PD* variants and their effects, available at <https://github.com/reneegeck/G6PDcat>, so that data on variant function and clinical presentation are available for research and interpretation.

For the variants with data on activity and stability, our collected findings generally support the WHO classification systems that group variants by activity and phenotypic severity,<sup>9,10</sup> but we note that many variants with low activity have not been reported to lead to anemia and thus do not fit the WHO classification thresholds. The data also highlight that while lower variant activity is associated with more severe clinical manifestations, there is a range of activities in each variant class such that one cannot always be accurately predicted from the other. To further confound this, reports of activity and clinical presentation for one variant can vary greatly between individuals and over time.<sup>4</sup> The 2022 WHO classification system takes inter-individual variation into account, requiring that classification is based on clinical reports of the presence or absence of anemia and the activity from at least three unrelated hemizygotes.<sup>10</sup> Only 53 variants met these criteria, so we encourage clinicians to continue reporting individuals' variant activity and whether they present with anemia. Currently, the variability of activity for many variants emphasizes that point-of-care activity tests remain critical to diagnose deficiency and inform drug prescription and dosage

for each individual. Activity tests are also necessary for heterozygotes since their activity varies based on X-inactivation and may further vary over time as their red blood cells turn over, but knowing that a variant can lead to deficiency in hemizygotes is valuable to support testing in heterozygotes. However, since activity tests can be misleading in individuals with anemia, hematological disorders, or recent transfusions, measuring both *G6PD* genotype and activity can identify discordance between results and prompt increased monitoring or follow-up tests.<sup>39</sup>

Despite efforts to interpret the effects of *G6PD* variants, the function of most identified variants remains unknown. There is a particular dearth of molecular evidence on variants not associated with anemia or other symptoms. Many of these have been reported through population sequencing, but since individuals with *G6PD* deficiency can be healthy unless they encounter a trigger, presence of a variant in a population database does not preclude decreased activity. Diverse biobank data, such as that provided by All of Us, enabled us to find 118 *G6PD* variants not previously reported in other genetic variation databases, 18 of which were rare missense variants. This biobank data will increase our ability to interpret and classify variants, especially when data are provided on *G6PD* deficiency status or *G6PD* activity. While most data on *G6PD* activity and genotype-phenotype correlations have been collected through studies focused on *G6PD* deficiency, our analysis of All of Us highlights the role that large, diverse biobank data can play in the future of variant interpretation. Recent successes in functionalizing variants with multiplexed assays of variant effect are also being applied to coding variation in *G6PD*, which will be particularly useful for extremely rare variants with little to no clinical data.<sup>29,40</sup>

While most noncoding variants in *G6PD* are presumed to be benign or of little effect, this merits further investigation, particularly for variants observed in multiple individuals with *G6PD* deficiency. Noncoding variants found in individuals with *G6PD* deficiency but without missense variation, as is the case for some individuals in All of Us, could be a starting point for functional assessment of noncoding variation. However, discordance between diagnostic codes of *G6PD* deficiency and activity measurements presents a challenge for interpretation. Activity measurements taken during a hemolytic crisis or following a transfusion can be elevated due to high percentages of reticulocytes or donor cells, and lack of that information can confound analysis of biobank data.<sup>4</sup> Reporting multiple measurements over time compared to nondeficient controls aids in interpretation of *G6PD* activity tests.<sup>1,4</sup>

Our efforts to classify *G6PD* variants using available data benefited from the abundant literature published since *G6PD* deficiency was described in 1956.<sup>28</sup> Other genes with considerable variation and many reports with clinical and functional data could be similarly reviewed and analyzed to provide variant interpretations. Determining the gaps between numbers of identified variants and number of characterized variants could highlight genes that

would benefit from multiplexed functional studies to provide information on many variants of unknown or uncertain function.

By making use of published studies of *G6PD* variants, especially those reporting clinical effects, our interpretations can be used to inform clinical decision making. With our addition of 182 interpretations, the number of *G6PD* variants with data available to aid interpretation has increased to 400 variants. Current guidelines for drug use group variants according to the 1985 WHO classification system, so knowledge of activity and association with CNSHA are important to inform medication use.<sup>4</sup> The ACMG levels of pathogenicity are useful for identifying which variants lead to deficiency based on multiple avenues of evidence, and inclusion in class A or B by the WHO Classification is a useful indication of phenotypic severity and association with CNSHA or AHA. Although there is variability of effects between individuals, adding to the number of variants with proposed interpretations increases the information available to clinicians as they make decisions on how to treat and advise individuals with *G6PD* deficiency. Coupled with robust drug prescribing recommendations,<sup>4</sup> these variant interpretations enable the application of genetic medicine for individuals with *G6PD* variants.

#### Data and code availability

The most current versions of variant lists are available at <https://github.com/reneegeck/G6PDCat>. ClinVar entries are available under submission name “G6PD\_Dunham2022” with accession numbers ClinVar: SCV002599134 to SCV002599417. Plots, weighted averages and medians, and significance statistics for figures were produced using R code which is also available on GitHub. Code used for All of Us analyses is saved in the All of Us workbook and will be gladly shared with approved users upon request. Individual data from All of Us are shown in accordance with a Data and Statistics Dissemination Policy exception granted by the All of Us Resource Access Board in November 2022.

#### Supplemental information

Supplemental information can be found online at <https://doi.org/10.1016/j.ajhg.2023.01.003>.

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#### Declaration of interests

The authors declare no competing interests.

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#### Web resources

Genbank, <https://www.ncbi.nlm.nih.gov/genbank/>  
OMIM, <https://www.omim.org/>

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