Structural biology in variant interpretation: Perspectives and practices from two studies

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Summary

Structural biology offers a powerful lens through which to assess genetic variants by providing insights into their impact on clinically relevant protein structure and function. Due to the availability of new, user-friendly, web-based tools, structural analyses by wider audiences have become more mainstream. These new tools, including AlphaMissense and AlphaFold, have recently been in the limelight due to their initial success and projected future promise; however, the intricacies and limitations of using these tools still need to be disseminated to the more general audience that is likely to use them in variant analysis. Here, we expound on frameworks applying structural biology to variant interpretation by examining two accompanying articles. To this end, we explore the nuances of choosing the correct protein model, compare and contrast various structural approaches, and highlight both the advantages and limitations of employing structural biology in variant interpretation. Using two articles published in this issue of *The American Journal of Human Genetics* as a baseline, we focus on case studies in *TP53* and *BRCA1* to illuminate gene-specific differences in the applications of structural information, which illustrate the complexities inherent in this field. Additionally, we discuss the implications of recent advancements, such as AlphaFold, and provide practical guidance for researchers navigating variant interpretation using structural biology.

Background

Due to practical and technological limitations, variant interpretation is often focused on changes at the DNA and mRNA levels. In cases where mRNA transcripts are not expected to be degraded by nonsense-mediated decay (NMD), the effects of genetic alterations manifest by affecting the sequence, folding, and structure of the ensuing proteins translated by these altered mRNAs. Considering this, it is unsurprising that the structure of proteins has been an active field of research for over 50 years. The functional insights attained from these studies have played a significant role in guiding the interpretation of genomic sequencing results.[1,2] To date, the experimentally determined atomic coordinates in the Protein Data Bank (PDB) represent only about 17% of the human proteome[3]; however, recent deep learning methods to predict three-dimensional (3D) protein structures have expanded this coverage to theoretically include the entire human proteome.[4–6] One study found that our baseline knowledge of protein structures improved from 48% to 76% by adding AlphaFold predictions.[7] Thus, as the power of available computational resources has grown, methods that make direct use of this structural information have provided predictive tools for investigating protein structure-function relationships and, by proxy, the effect of genetic variation on those relationships.

The American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) publish and maintain standardized guidelines for the assessment of variants.[8] The ACMG/AMP guidelines define the degrees of likelihood that a variant is pathogenic or benign (pathogenic, likely pathogenic, uncertain significance, likely benign, and benign) as well as the lines of evidence and corresponding weights for those lines of evidence that, together, are used to comprise a final interpretation. Some elements of structural biology have been incorporated into the ACMG/AMP guidelines since their inception in 2000. For example, the consideration of protein domains critical to function has been built into several evidence codes such as PVS1 (loss-of-function variants that are not prone to NMD should consider the impact to critical functional domains), PM1 (for variants located in critical functional domains), and PP3/BP4 (*in silico* predictors, some of which indirectly incorporate structural concepts).[9] However, the direct implementation of structural analysis using more complex properties, such as features derived from 3D structures, is largely absent in ACMG/AMP guidelines despite the apparent benefit for variation interpretations.[8,10]

As we continue to embrace easier-to-use, more sophisticated computational structural modeling tools, the variant interpretation community needs guidance on how to utilize these tools as well as their respective strengths and limitations. In this perspective, the nuances, applications, strengths, and limitations of these newer tools are laid out to highlight their use in two accompanying articles in this issue of *The American Journal of Human Genetics* (Rotenberg et al.[11] and Ramadane-Morchadi et al.[12]) for missense and single amino acid deletions in the cancer predisposition genes *TP53* (MIM: 191170) and *BRCA1* (MIM: 113705).

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Table 1. Comparison of methods used for solving or modeling protein structures

Method	Maximum resolution	Captures protein dynamics	Relative protein size	Preference for variant interpretation	Selected sources
X-ray crystallography	high	no	small to medium	first	PDB
Cryoelectron microscopy	medium but improving	no	large	second	PDB
Nuclear magnetic resonance	N/A	yes	small to medium	third	PDB
Homology modeling	dependent on structures used as per above rows	dependent on structures used as per above	dependent on structures used as per above	fourth	user-generated, SWISS-MODEL, MODBASE
Machine-learning-based *ab initio* modeling	–	no	–	to be determined	user-generated, PDB, AlphaFold2, AlphaFold3, RoseTTAFold, ESMFold

Good practices for using protein structure

Central to variant assessment using structural biology is the consideration of several key factors: (1) selection of the structure, (2) determining stability metrics, (3) using *in silico* predictors, and (4) filtering based on an amino acid's location within the protein structure.

Selection of the structure

The selection of high-quality structures is particularly important, as the available computational tools are highly sensitive to the resolution of the structure.[13] There are three primary methods for the experimental determination of protein structures: X-ray crystallography, nuclear magnetic resonance (NMR), and cryoelectron microscopy (cryo-EM). Experimentally resolved structures can be found in online databases, such as the PDB.[3] Each of these methods has strengths and weaknesses that should be considered when selecting a structure for use in variant analysis (Table 1). X-ray crystallography is an industry standard and can produce structures of high resolution.[14] NMR provides conformational ensembles of a protein in solution state, facilitating the interrogation of protein dynamics, but is limited by resolution and protein size.[15] Cryo-EM allows easier and consistent access to the structures of larger proteins and complexes, which are more difficult to crystallize, but has historically been hampered by much lower resolution compared to X-ray crystallography.[16] However, this limitation has been waning with improvements in methods and technology.[17] For the purposes of variant interpretation, the high resolution provided by X-ray crystallography makes it the preferred source of structures, followed by high-quality cryo-EM structures. While powerful in its ability to interrogate protein dynamics, the conformational complexity of structures produced by NMR makes them less useful when using protein stability as a metric, as we discuss below.

When protein structures are unavailable, models can be built using the structures of similar proteins in a process called homology modeling, whereby the construction of the 3D coordinates of proteins is facilitated by using experimentally determined coordinates of related proteins as a guide. Homology modeling relies on the observation that peptides with similar amino acid sequences form similar 3D (3D-secondary and -tertiary) structures.[18] While a comprehensive review of homology modeling is outside the scope of this perspective, we direct the readers to recent reviews on the process of discovering and selecting related proteins as templates[19,20] and the process of modeling target structures from those templates.[21] Homology-based protein structural models predicted by sequence similarity to known structures are available in model databases such as the PDB, the SWISS-MODEL repository,[22] and MODBASE.[23] However, in most cases, the native structures of proteins should be preferred over homology models in variant interpretation when available.[24]

A spate of recently developed tools utilizing various machine learning techniques allows for the wholesale prediction of protein structures using only an input protein sequence, including AlphaFold2,[5] AlphaFold3,[25] RoseTTAFold,[4] and ESMFold.[6] The use of these *ab initio* structures in variant interpretation shows initial promise, specifically when used as drop-in replacements for experimentally determined structures or models or for proteins that lack experimentally determined structures. Note that for the purposes of this perspective, we define *ab initio* modeling tools to mean tools that create structures without the use of homology modeling. Studies show that AlphaFold2 structures can be used to compute stability metrics with similar accuracy to experimentally determined structures, at least for regions of high confidence.[26] This is supported by analysis of the BRCA1 RING domain in this issue in Ramadane-Morchadi et al.,[12] which demonstrates similar performance for AlphaFold2 structures compared to NMR (RING domain) or X-ray crystallography (BRCT domain) structures. The recently released AlphaFold3[25] promises better and more comprehensive modeling of protein complexes, which can provide greater resolution and positive predictive power for its use in variant interpretation, but these improvements have yet to be validated in terms of its performance with stability calculations. Importantly, high-quality, experimentally determined structures and models still outperform AlphaFold structures in computing stability metrics (see below)[27] and should be preferred when available.

Box 1. Definitions of commonly used structural/bioinformatic terms

(1) ΔΔG (stabilization): ΔΔG is a measure of the change in Gibbs free energy associated with an amino acid variant. It reflects the impact of the variant on the stability of a protein, where positive ΔΔG values indicate destabilization and negative values suggest stabilization. Destabilization is an important metric for proteins that have loss of function as a mode of pathogenicity.
(2) AlphaMissense: AlphaMissense is a deep learning tool that predicts the pathogenicity of missense variants by incorporating structural context and offering insights into how specific amino acid variants might affect protein function based on predicted or known structures.
(3) BayesDel: BayesDel is an *in silico* tool that combines multiple predictive features, including evolutionary conservation and structural characteristics, to estimate the likelihood that a variant is pathogenic.
(4) Align-GVGD: Align-GVGD is an *in silico* tool that combines protein multiple sequence alignment with a physicochemical model to classify missense variants, helping to infer the potential impact of a variant based on sequence conservation and similarity across species.
(5) Relative solvent exposure (RSA): a measure of whether a variant position is buried within the protein's three-dimensional structure or on the surface, exposed to the solvent.

Determining stability metrics

Much of the current use of structural biology in variant assessment leverages the computation of an alteration's effect on protein stability as a predictor of pathogenicity.[28] Stability refers to the ability of the protein to retain specific conformational shapes corresponding to the native structures presumed to be necessary for proper function, which is particularly informative for gene-disease relationships driven by loss of function. For proteins or regions of proteins that natively adopt well-defined conformations, the loss of the native structure typically leads to loss of function, such as by adversely impacting functionally important structures or sequestration, aggregation, or degradation of the protein.[29] Conversely, an alteration can stabilize the protein structure such that it is prevented from adopting its active conformation (loss-of-function effect) or constitutively adopts the native, or an altogether different, active conformation (a gain-of-function effect).[30] Additionally, proteins are in a constant state of flux between these different conformational states, e.g., active and inactive conformations. Alterations can impact the equilibrium of these conformational changes, biasing the system to one state or the other. We point the reader to recent reviews on this subject,[31,32] as an in-depth discussion of protein dynamics is outside the scope of this perspective.

Tools for predicting the change in stability of proteins from structures, measured as the change in Gibbs free energy (ΔΔG; see Box 1), have continued to improve over the past two decades, and many tools are now available for predicting energies of destabilization.[33] An industry "gold-standard" tool for protein stability prediction for variant assessment is FoldX, which uses an energy function that includes contributions of van der Waals, solvation, hydrogen bonding, electrostatics, and entropy effects on the free energies of stability.[34] These terms can quantitatively predict the change in unfolding energy between the native and variant conformations. Though there is a partial correlation between destabilization energies and the pathogenicity of the variant,[35] better predictions can likely be achieved when the computed energies of target variants are compared to the computed energies of nearby variants of known classification, especially when used in combination with other biophysical information about the position and surrounding region.

Using *in silico* predictors

In variant assessment, the concept of a disrupted structure through genetic variation has led to the development of widely used *in silico* models that predict pathogenicity scores, many of which incorporate limited structural features.[36] For the purposes of this perspective, we define *in silico* tools to mean computational tools that primarily use 1D sequence information to predict variant pathogenicity. *In silico* tools such as SIFT,[37] BayesDel (see Box 1),[38] and REVEL[39] primarily rely on sequence-based features and often return values interpreted as a binary outcome, i.e., deleterious or tolerated based on upper and lower thresholds, with little or no mechanistic explanation for the assignment of boundaries. The performance of these *in silico* methods shows wide variability, making calibration essential to inform the selection of algorithms to produce the most accurate predictions.[40]

Another group of *in silico* prediction methods incorporates protein structural features to infer the potential effect of sequence changes on the protein, either directly using known 3D structures or indirectly through sequence conservation and structural similarity. Some approaches, such as SNAP2,[41] predict local structural features, e.g., secondary structures such as α helices and β sheets, but not 3D protein structures. Others, including PolyPhen-2,[42] PopMusic,[43] and DUET,[44] explicitly use the protein structure, employing knowledge-based or machine-learning-derived parameters from the amino acid substitution on the protein structure. More recently, the inclusion of AlphaFold-predicted structures to create structurally aware

in silico predictors, such as in AlphaMissense (see Box 1),[45] has shown great promise in predicting variant pathogenicity, as can be seen in the results shown in Rotenberg et al.[11] and Ramadane-Morchadi et al.[12] In the context of structural biology, tools that incorporate protein structure and machine learning can be used to explain or predict the effect of alterations on the function of native proteins, which are either impractical or impossible to determine experimentally, and do so with high throughput.

Filtering based on an amino acid's location within the protein structure

There are many structural features that can contribute to the impact of a variant on a protein. The two studies in this issue by Rotenberg et al.[11] and Ramadane-Morchadi et al.[12] prioritized two aspects related to protein structure: (1) intrinsic order/disorder, i.e., does the region of the protein containing the variant fold into an ordered secondary structure, and (2) relative solvent-accessible surface area (relative solvent accessibility [RSA]; see Box 1), which is a measure of whether the variant position is buried within the folded protein or exposed to solvent, the former being more likely to cause structural destabilization. These features are relatively easy to compute and are at the core of how a variant can impact monomeric protein stability. Other informative features may include structural or functional motifs, such as metal- or ligand-binding residues; post-translational modification sites/conserved residue-level interactions; and protein interaction interfaces with DNA or RNA. However, these features depend on the function of the region (better suited to specific proteins or protein classes) and may only be interpretable by those with expert knowledge of the structures and biological systems.

It is important to address the limitation of employing filtering based on intrinsic disorder and solvent accessibility. While many proteins in the human genome are structured and fold into defined tertiary structures, more than 40% of all human proteins contain large, disordered regions of greater than 30 residues.[46] These intrinsically disordered regions, which lack defined secondary or tertiary structures, are of particular interest in variant assessment, as they often contain aforementioned protein interaction interfaces important to their function, notably cellular recognition, trafficking, and signaling. Given that no fixed set of 3D coordinates can represent these regions, interpreting the tertiary and quaternary structures of their parent protein is limited, and the impact of variants on the stability of these regions is neither easy to compute nor necessarily informative. Predicting disordered regions using methods such as IUPred2A[47] (used by Rotenberg et al.[11]) provides opportunities to assess the functional effects of variants in ordered regions, and filtering out intrinsically disordered regions improves the predictive power of computed protein destabilization energies. While the performance of these disorder predictors remains unclear,[48] they can be used as a first-pass tool in determining if a region is likely to be structured and if time should be invested in determining the impact of variants of the region on the stability of the protein.

Perspectives from two recent studies

Two studies published in this issue—Rotenberg et al.[11] and Ramadane-Morchadi et al.[12]—explore the use of features generated from 3D structural models to enhance the predictive power of *in silico* tools for the classification of variants of two genes, *TP53* and *BRCA1* (Table 2). The studies recommend a comprehensive evaluation of computational evidence stratified by structural features and advocate for the use of AlphaFold2 models for ΔΔG predictions in the absence of experimental structures. Both studies emphasize the importance of integrating protein folding stability metrics and modern *in silico* tools like AlphaMissense to enhance the prediction accuracy of variant impact, particularly for genes in which loss-of-function variants are associated with disease. They collectively demonstrate that current *in silico* tools used in the ACMG framework, such as BayesDel and Align-GVGD (see Box 1), which rely heavily on sequence alignment, do not fully capture the impact of variation in protein folding stability. By incorporating structure-based evidence, including RSA, ΔΔG, and AlphaMissense, both studies propose enhancements to improve the predictive power of the *in silico* lines of evidence currently outlined in gene-specific guidelines for *TP53* and *BRCA1*.[49,50]

It is important to note that since the mechanism of pathogenicity does not completely overlap for these two genes, the strategies recommended by both studies are not identical, which highlights the importance of tailoring the use of structural evidence on a per-gene basis, if possible. Due to the complexity of gene-specific structure-function relationships and the specialized nature of the tools used to interrogate them, it is important to consult experts in structural biology when considering the use of structural information to enhance variant effect prediction. For example, certain proteins are more tolerant to changes in stability, as is indicated by the heterogeneous nature of the correlation between the computed ΔΔG values and functional data across genes.[59] Other factors that are not necessarily covered in the companion papers, such as reduced penetrance variants, require nuanced structural considerations when expanding these guidelines to other genes. With this in mind, the general strategies and frameworks established in the companion papers can be used as a starting point for the integration of structural information into the interpretation of variants of other genes (review Box 1).

Rotenberg et al.

This study[11] addresses the complex challenges associated with the clinical classification of germline missense variants and single amino acid deletions within p53, which is encoded by *TP53* and associated with Fraumeni syndrome, a

Table 2. Comparison of methods and results of two recently published papers using structural methods and features to enhance the predictive power of prediction tools for variant classification

Step	Rotenberg et al.[11]	Ramadane-Morchadi et al.[12]
Discovery		
Protein	p53	BRCA1
Variant types	missense and single amino acid deletion	missense
Mechanism of disease	LoF	LoF
Disease	Li-Fraumeni (cancer predisposition)	hereditary breast and ovarian cancer (HBOC) (cancer predisposition)
Penetrance	high	high
Existing gene-specific ACMG/AMP guidelines	yes[49]	yes[50]
Functional data comparison set	Giacomelli et al.,[51] Kato et al.,[52] Kotler et al.[53]	Findlay et al.[54]
Protein structure selection	X-ray crystallography (1TSR[55] and 1C26[56]), AlphaFold2	X-ray crystallography (1T15[57]), solution NMR (1JM7[58]), AlphaFold2
Feature generation		
ΔΔG calculations (missense)	FoldX5	FoldX5
ΔΔG calculations (deletions)	AlphaFold2 + RosettaRelax	N/A
Protein feature filtering	intrinsic disorder and RSA	RSA
In silico tools	BayesDel, aGVGD, AlphaMissense	BayesDel, AlphaMissense
Results		
Missense	evidence strength increased when using the original *TP53* VCEP rules and combining • ΔΔG scores • AlphaMissense filtering RSA	• AlphaMissense outperforms ΔΔG and BayesDel individually • Combining AlphMissense and ΔΔG increased evidence strength • Benign evidence is highly dependent on RSA
Deletions	ΔΔG integration outperformed BayesDel, particularly for buried residues: ΔΔG score $\geq$ 4.8 rosetta energy units (REU) is a line of evidence toward pathogenicity	N/A

LoF, loss of function.

hereditary cancer predisposition syndrome. Given the high penetrance of *TP53* pathogenic variants and the extensive body of functional research surrounding it, accurate classification of variants in this gene is critical. However, as discussed above, existing bioinformatic tools used for variant classification often fail to account for changes in protein folding stability, a factor that is crucial for understanding how these variants impact protein function.

To address this gap, this study examined how the integration of structural biology information (ΔΔG scores), AlphaMissense, and *in silico* tools used by the current *TP53* variant curation expert panel (VCEP) impacts predictive power. The methodology involved calculating ΔΔG scores for missense variants using FoldX. For deletions, AlphaFold2 combined with RosettaRelax was employed because the structure of the protein backbone is fundamentally altered by deletions, which requires regeneration of the structure. Residues were categorized based on their RSA, a key structural feature, and the study assessed the predictive value of ΔΔG, AlphaMissense, BayesDel, and Align-GVGD using Boruta feature selection and logistic regression analysis.

The results demonstrated that integrating ΔΔG scores into variant assessment enhanced the specificity and strength of evidence that can be applied to missense variants, particularly those in buried residues. For single amino acid deletions, ΔΔG scores, specifically in buried residues, outperformed BayesDel. Based on these findings, the study suggests the optimal ΔΔG cutoff scores for both missense and deletion variants, proposing a revised bioinformatic prediction framework for *TP53* variants. Overall, this study underscores the critical importance of considering both protein folding stability and structural context in refining the computational prediction of variant impacts in *TP53*.

Ramadane-Morchadi et al.

This study[12] focuses on the challenges of classifying *BRCA1* missense substitution variants within the framework of the ACMG/AMP guidelines, which is particularly important in cases where rare missense variants have limited additional evidence. Typically, computational prediction for missense variant classifications relies heavily on sequence conservation and computational evidence codes, which may not fully capture the nuances of variant impact. To

address these limitations, the study evaluates the incorporation of structure-based evidence, including RSA, folding stability (ΔΔG), and AlphaMissense pathogenicity scores, into computational prediction for the classification of *BRCA1* missense variants.

Using functional scores as proxies for pathogenicity and benignity, the study rigorously compared the performance of these structural features against existing classification methods. The findings reveal that AlphaMissense consistently outperforms ΔΔG and BayesDel individually, offering similar levels of evidence strength but with a lower proportion of variants falling into the uninformative score range. Additionally, AlphaFold2-generated models were shown to be of sufficient quality for ΔΔG computations using FoldX, at least for the two domains on which the study focused. The study highlights that BP4 evidence (benign *in silico* evidence) is highly dependent on RSA, with meaningful evidence provided primarily for variants in buried or partially buried residues. The combination of AlphaMissense with ΔΔG scores was found to increase the granularity and specificity of evidence strength, leading to improved overall assessments of pathogenicity and benignity for missense variants in *BRCA1*. RSA was particularly emphasized as playing a critical role in the evaluation process, underscoring the importance of structural context in variant classification.

Limitations of the study

While both papers show the benefit of using structural data to enhance *in silico* predictions, they are not without limitations. One caveat is that both papers evaluate the performance of structural modeling against functional studies. A gold-standard approach would be to evaluate them against final clinical interpretations; however, publicly available interpretations may already include structural components either through select *in silico* tools or expert-level protein analyses, and the use of structural data in an interpretation is not always transparent in public databases (e.g., ClinVar). The use of protein functional data is a litmus test intended to avoid potential circularity in the interpretation. Recent efforts, such as MaveDB,[60] have begun to centralize large datasets of protein functional data that could be used for this type of analysis. Another caveat is the potential overlap between the information used in *in silico* tools and the structural predictions themselves, as some *in silico* predictors incorporate a limited subset of structural information into their predictions, as discussed above. This is somewhat mitigated by determining the weight of evidence after combining structural information and *in silico* evidence, as overlapping weights of the pieces of evidence will not be additive, as seen in the two companion papers.

In addition, there are several things to note about the use of destabilization as a predictor of pathogenicity: first, it is only consistently useful for gene-disease relationships that have loss of function as a mechanism, and second, destabilization can be evidence toward pathogenicity, but lack of destabilization is not necessarily evidence toward benignity, as alterations that do not impact stability may still adversely impact key binding sites and motifs leading to pathogenicity (this is mitigated in both studies by the use of RSA to filter out these trickier solvent-exposed variants). Furthermore, all *in silico* tools share a subset of features, with some including limited structural information. Measuring the extent of overlap and establishing limits for the weight of these evidence types is largely an open question.

A final consideration is that these papers represent exemplars in a new protocol for adding structural evidence to existing frameworks. There are growing numbers of structural tools that can be calibrated for use in variant classification in a similar fashion as outlined in these two papers. Individual iterations of similar analyses may be dependent on protein, protein class, or even mode of inheritance. These papers provide a foundation upon which future work can build to understand if these methods can be extrapolated to other situations, such as different protein classes, or to genes involved in rare diseases.

Conclusion

In summary, structural biology offers additional evidence toward variant interpretation by providing detailed insights into how genetic alterations encoding missense changes impact protein structure and function. The two studies explored in this paper, each with a focus on a clinically important cancer predisposition gene, underscore the critical importance of integrating protein folding stability metrics and modern *in silico* tools like AlphaMissense to enhance the predictive accuracy of variant impact. These studies demonstrate that while traditional *in silico* tools like BayesDel and Align-GVGD, which rely heavily on sequence alignment, have historically been instrumental in variant classification, they fall short in fully capturing the impact of changes in protein folding stability. By integrating structure-based evidence, such as RSA and ΔΔG scores, with modern *in silico* tools such as AlphaMissense predictions, the studies propose significant enhancements to existing computational models. This approach not only improves the granularity and specificity of variant classification but also offers additional mechanistic interpretability, especially for variants of uncertain significance. Moreover, advancements in computational tools, particularly the emergence of deep learning models like AlphaFold and AlphaMissense, have democratized access to structural analysis, making it more accessible to the broader scientific community. However, as these tools continue to evolve, there remains a pressing need to disseminate their nuances and limitations to ensure they are applied effectively and judiciously. The integration of structural biology into the

ACMG/AMP guidelines marks a shift toward more accurate and reliable variant interpretation, paving the way for its broader adoption in clinical settings.

As we continue to embrace these sophisticated computational models, it is crucial for the variant interpretation community to receive clear guidance on the strengths and limitations of these tools. The lessons learned from these studies serve as a valuable foundation for future research and clinical applications, emphasizing the need for ongoing refinement and validation of structural biology methods in the field of genetic variant interpretation.

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Declaration of interests

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT in order to edit the manuscript for concision. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Web resources

OMIM, http://www.omim.org

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