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Computational Analysis of TSHb Variants (Native, Mutated and Deleted) in Interaction with TSHR: Molecular Docking and Dynamic Simulations for Mechanistic Insights into Congenital Hypothyroidism

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Abstract

Thyroid Stimulating Hormone (TSH) is a primary protein and biomarker for the early detection of congenital hypothyroidism (CH). CH poses a serious risk for newborns as it can lead to neurological development issues, motor skill delays, and lower IQ. This study aims to computationally compare the interaction effectiveness of TSHb (native, mutation, deletion) with TSHR (Thyroid Stimulating Hormone Receptor). TSHb were constructed based on the 3D structures of fPDB proteins, specifically 7FIH_Y, 6P57_A, 6P57_B, 1HCN_B, and 1QFW_B. TSH and TSHR models were obtained from the RSCB PDB (ID: 7XW5). Molecular dynamics simulations were conducted using YASARA Dynamics. Potential energy, SASA, hydrogen bonds within the solute, hydrogen bonds between solute and solvent, RMSD, and radius of gyration analyses were obtained through the md_analyze macro. Docking and molecular dynamics simulations revealed that mutations and deletions in TSHb impact its stability, functionality, and interactions with TSHR. Furthermore, significant structural changes and reduced conformational stability were observed in the TSHb variant with a nucleotide deletion at position 114. However, this TSHb deletion variant did not alter the amino acid sequence of the active site for binding with TSHR. It is believed that this deletion variant retains sufficient affinity interaction with TSHR compared to the native TSHb.

Keywords: Congenital hypothyroidism, TSHb, TSHR, Molecular docking, Molecular dynamic

Introduction

Congenital hypothyroidism (CH) is a condition of decreased or non-functioning thyroid gland that is acquired since the newborn. This occurs due to anatomical abnormalities or metabolic disorders of thyroid hormone formation or iodine deficiency [1]. The European Society for Pediatric Endocrinology and the European Society for Endocrinology state that HK is a condition of underdevelopment of the hypothalamic-pituitary-thyroid (HPT) axis, which occurs mainly in premature newborns, and results in various complications including impaired thyroid activity and inadequate thyroid hormone secretion [2]. The Indonesian Pediatrician Association (IDAI) outlined data that 95 % of congenital hypothyroidism does not

show any symptoms or signs in newborns and most CH sufferers experience late diagnosis, resulting in impacts in the form of impaired growth, motor development, and intellectual impairment [3]. The prevalence of congenital hypothyroidism in Indonesia is estimated to be between 1:2,000 and 1:3,000 newborns [4].

TSH serves as a key biomarker for assessing thyroid hormone levels and holds significant potential for the early detection of congenital hypothyroidism [5]. American Thyroid Association (ATA) and the American Association of Clinical Endocrinologists (AACE) state that measuring TSH levels is the most important test for detecting all forms of thyroid hormone disorder [6]. TSH is a heterodimeric glycoprotein and consists of TSHa subunits and TSHb subunits, various mutations in congenital hypothyroidism originate from TSH [7]. In congenital hypothyroidism TSH and TSHR plays an important role in the thyroid hormone biosynthesis mechanism, so that if a mutation occurs it will have an impact on disrupting the thyroid gland stimulation [8]. The subunit of TSHb that plays a major role in the biological activity process of TSH, that it is able to channel specificity and stability in hormone receptor interactions. The TSH contains a "seat belt" region that is crucial for how it binds to and activates the A subunit of TSHR, as previously documented [9]. This TSHb-TSHR interaction is structural, but it also governs the influence of signaling pathways activated downstream of TSH engagement. For instance, during the mutation process the TSH subunit undergoes a conformational change that can yield a different TSHR signaling profile [10]. TSHR is also involved in the initiation several of the signalling pathways such as the Gs/Adenyl cyclase pathway that is one of the key regulatory drivers for thyroid hormone biosynthesis.

Interaction between TSH and TSHR depends on numerous factors including glycosylation pattern of subunits and presence of autoantibodies that may act as TSH agonists or antagonists [11]. In congenital hypothyroid conditions, autoantibodies against to TSHR and TSH may also lead to thyroid dysfunction [12]. Taken together, TSH subunits-TSHR interaction is complex in terms of structure, function and immunology. Knowledge of these interactions is better to understand the mechanisms involved in thyroid disorders also to identify interaction between TSH and TSHR. Using computational modeling, the study aims to compare the abilities of TSH native, mutation and deletion fragments to activate TSHR and their relevance to congenital hypothyroidism. This study will inform the TSH and TSHR design and accuracy to ensure the third TSH subunit is appropriately processed. However, comparatively little attention is paid to TSHb's physiological roles or the phenotype resulting from TSHb deletion, which this article aims to explore using a selection of TSHb deletion models. Overall, previous studies showed that various phenotypes, such as growth retardation [11] and obesity [12], could result from TSHb mutations, highlighting the need for expanded genetic screening schemes in patients presenting with hypothyroid symptoms [13]. Data were up to October 2023 In conclusion, recent discoveries have highlighted the essential role of TSHb in thyroid function and the mechanism of various mutations and deletions leading to congenital hypothyroidism.

TSHb, the β-subunit of TSH, is responsible for the regulation of thyroid function and the balance of human metabolism. The TSHb gene has various genetic variations such as mutations and deletions that can notably affect hormone-receptor interactions ultimately contributing to endocrine disorders like congenital hypothyroidism. Molecular modeling and docking simulation have been widely used as a computational study to detect the effect of variant genes in hormone connection proteins. Molecular dynamics and docking study of various mutations in other endocrine-related genes including TSHb have also been reported [7]. One such example, for instance, a recent study utilized homology modeling and molecular docking to predict the loss of ligand interactions due to deletions in the thyrotropin-releasing hormone receptor (TRHR), which demonstrated a marked loss of receptor activation potential [14].

In a recent work, Mezei *et al.* [15] examined previously described mutations in the TSHb protein and performed comparative modeling on the wild-type and mutant proteins. Indeed, their findings showed that substitutions to hydrophobic residues in the binding pocket led to marked disruption of receptor recognition. Our findings emphasize the value of computational simulations to anticipate the functional consequences of genetic variations within hormone-imposing proteins.

TSH consists of 2 subunits: Alpha and beta, with the beta subunit playing a crucial role in receptor

specificity and biological activity [16]. When TSH binds to TSHR, it activates intracellular signaling pathways, primarily through the production of cyclic adenosine monophosphate (cAMP), Which is essential for thyroid hormone synthesis and secretion [17]. This signaling cascade not only regulates the production of thyroid hormones (T3 and T4) but also influences thyroid cell proliferation and differentiation [18]. TSH and TSHR play a crucial role in maintaining homeostasis within the hypothalamus-pituitary-thyroid (HPT) axis. When thyroid hormone levels decrease, the hypothalamus releases thyrotropin-releasing hormone (TRH), Which stimulates the pituitary gland to produce TSH [19]. Research indicates that both alpha and beta isoforms of TSHR are involved in mediating the effects of TSH.

Previous work indicates that both alpha and beta isoforms of TSHR are involved in mediating the effects of TSH [20]. While TSHb is traditionally recognized for its role in stimulating thyroid hormone production, emerging evidence indicates that its function extends beyond this classical pathway. Importantly, HPT axis independent splice variants of TSHb suggest that TSHb is able to signal thyroid hormone synthesis in response to a variety of physiological stimuli, including stress and infection. Moreover, studies show that TSHb might have local regulatory actions in the thyroid gland itself, helping to modulate hormone output during immunological attacks [21]. These results emphasize the multifaceted contribution of TSHb in thyroid regulation and as a key modulator enabling the gland to adapt to various internal and external conditions.

Continued investigation of TSHb and its relationship with TSHR and other regulatory players has the potential to reveal novel mechanisms involved in thyroid pathophysiology and highlight new targeting options for treatment of associated diseases. Our research design is based on this discussion: TSHb/TSHR were selected as our study targets. The initial method utilizes a peptide array screening for TSHb to include native as well as mutated/deleted TSHb variants against TSHR, emphasizing differences in their molecular interactions. In this manner, shedding light on how TSHb variations affects the protein structure stability, and functional integrity. The second method, for conformational stability, involved molecular dynamics (MD) simulations and YASARA Dynamics. The performance metrics investigated are potential energy, SASA, hydrogen bonds, RMSD, and radius of gyration. Statistics obtained using this approach provide an estimate of the stability of each TSHb variant in a biophysical context. The study provides new insights into how TSHb mutations and nucleotide deletions impair TSHb protein folding through these approaches.

Such information provides a basis for designing highly sensitive and specific nucleic-acid based diagnostic devices for the early diagnosis of congenital hypothyroidism. The use of 3D protein structures from 7FIH_Y, 6P57_A, 6P57_B, 1HCN_B and 1QFW_B guarantees the accuracy and reliability of the computational models included in the study. Hence this study could contribute to better comprehension of the molecular interactions between TSHb variants and TSHR and provide a promising way for prompt detection and risk management of congenital hypothyroidism by employing novel computational methods. Our findings demonstrate that mutations and deletions in TSHb influence its stability, functionality, and interaction with TSHR. Specifically, a nucleotide deletion at position 114 induces structural alterations and reduces conformational stability without disrupting the active binding site for TSHR. Despite these changes, the deletion variant is likely to retain sufficient binding affinity toward TSHR, comparable to the native TSHb.

Materials and methods

Modelling construction

The 3D structure modeling was performed using threading-based modeling via trRosetta [20] (https://yanglab.qd.sdu.edu.cn/trRosetta). The modeling samples consisted of 3 TSHB variants: Native, mutation, and deletion. The modeling process was guided by reference 3D structures from several Protein Data Bank (PDB) entries, including 7FIH_Y, 6P57_A, 6P57_B, 1HCN_B, and 1QFW_B.

Computational analysis procedure

Molecular docking method

The 3D structures of the TSHR-TSHb protein complex were retrieved from the RCSB PDB database (https://www.rcsb.org) under PDB ID: 7XW5 [22] to serve as the foundation for the molecular docking studies. Protein preparation was performed using Discovery Studio 2021, during which water molecules and non-essential ligands were removed to eliminate

potential interference during docking simulations. Hydrogen atoms were added, and energy minimization was conducted to stabilize the protein structures, ensuring accurate docking alignment. Following protein preparation, HDOCK (http://hdock.phys.hust.edu.cn/) was employed for targeted protein-protein docking between the TSHR and each of the TSHb variants (native, mutated, and deleted). Targeted docking was applied to focus on the known interaction interfaces between TSHR and TSHb, capturing the biologically relevant binding regions.

The docking simulation generated output metrics, including Docking Score, Confidence Score, and Ligand RMSD (Å). The docking score indicated the binding affinity between TSHR and the TSHb variants, with lower values corresponding to stronger interactions. The confidence score provided an assessment of the reliability of the predicted docking poses, while the ligand RMSD value reflected the precision of the docking alignment relative to reference conformations. HDOCK server is an FFT-based global docking program that can improve the shape-based pairwise scoring function. The key point of the docking scoring function is that during sampling, the score for a ligand grid will take into account the contributions not only from its nearest neighboring receptor grids but also from other receptor grids by a form of $\sim e^{-1/r2}$, where r is the distance between the ligand grid and the receptor grids. An angle interval of 15 ° is used for rotational sampling, and a spacing of 1.2 A is adopted for FFT-based translational search. The confidence score was given that the protein-protein/RNA/DNA complexes in the PDB typically have a docking score of around −200 or better. Yan et al. [23] have empirically defined a docking score-dependent confidence score to indicate the binding likeliness of 2 molecules using $1.0/[1.0 + e^{0.02 \cdot (\text{Docking Score} + 150)}]$ as formula.

After docking, the interactions of TSHb variants with TSHR were visualized and analyzed with BioVia Discovery Studio 2021 and LigPlot. Three-dimensional Visualization of Docking complexes: Detailed 3-dimensional visualization of the docking complexes was performed using BioVia Discovery Studio, which allows for the identification of key interaction residues as well as characterization of the various hydrogen bonding, hydrophobic interactions, and electrostatic forces. To enhance these analyses, LigPlot also generated 2-dimensional interaction maps, depicting the hydrogen bond networks and hydrophobic bonds responsible for the stability of the complexes.

The docking analysis provided guidance on the interaction pattern differences between native, mutated and deleted TSHb variants binding TSHR that is vital to understand the differential effects the structural variants can have on binding ligands and potential functional activities. The findings from this study help to enhance our understanding of the molecular mechanisms operating in congenital hypothyroidism, and demonstrate the promise of structural chemistries as a means of modulating protein-protein interactions.

Molecular Dynamics (MD) simulation

The simulation samples consisted of 3 types: native TSHb, mutated TSHb, and deleted TSHb. Molecular dynamics simulations were conducted using YASARA Dynamics software developed by Biosciences GmbH. The first step involved inputting each sample into the program by selecting options, followed by the Macro & Movie menu, and finally choosing Set Target to define the molecular system to be simulated. A macro was then input to perform the molecular dynamics simulation. The macro was preconfigured with physiological parameters, specifically temperature (310 K) and pH (7.4), in accordance with the NVT ensemble (constant number of particles, volume, and temperature).

The simulation runtime was set to 20,000 ps (20 ns) using the md_run macro. The simulation employed the AMBER14 force field, with snapshots saved at intervals of 25 ps. The Particle Mesh Ewald (PME) method was utilized to calculate electrostatic forces [24]. Additionally, 0.9 % Na^+ and Cl^- ions were added to neutralize the system, and the solvation box was simulated using transferable intermolecular potentials, incorporating the 3-point water model (TIP3P) [25]. The complex was computationally modeled using a cubic box with a buffer distance of 20 Å.

Post-simulation, structural and energetic properties were analyzed using the md_analyze macro. The analyses included potential energy, solvent-accessible surface area (SASA), the number of hydrogen bonds within the solute, the number of hydrogen bonds between the solute and solvent, root-mean-square deviation (RMSD), and radius of gyration to measure

the compactness of the protein structure. Subsequently, RMSF analysis was conducted using the md_analyze macro to evaluate flexibility variations of individual residues. Data interpretation and reporting results were compiled and compared for native, mutated, and deleted TSHb variants. Findings were visualized through graphs and statistical comparisons. The structural stability and interaction changes due to mutations and deletions were analyzed. This procedure provided an accurate computational simulation to assess the impact of TSHb variants on protein stability and interaction with TSHR, contributing to a better understanding of congenital hypothyroidism mechanisms.

Results and discussion

Modelling and docking analysis

Serially expressed structures of native, mutation sites on TSHb and deletion, results. Using data from Expasy, the translated amino acid sequences are used to examine how a single nucleotide variation (SNV) in the TSHb gene may modify gene function.

mRNA → native

GTCACCACAGCATCTGCTCACCAATGCAAAGT
AAGCATGACTGCTCTCTTTCTGATGTCCATGCT
TTTTGGCCTTACATGTGGGCAAGCGATGTCTTT
TTGTATTCCAACTGAGTATACAATGCACATCG
AAAGGAGAGAGTGTGCTTATTGCCTAACCATC
AACACCACCATCTGTGCTGGATATTGTATGAC
ACGGGATATCAATGGCAAACTGTTTCTTCCCA
AATATGCTCTGTCCCAGGATGTTTGCACATATA
GAGACTTCATCTACAGGACTGTAGAAATACCA
GGATGCCCACTCCATGTTGCTCCCTATTTTTCC
TATCCTGTTGCTTTAAGCTGTAAGTGTGGCAAG
TGCAATACTGACTATAGTGACTGCATACATGA
AGCCATCAAGACAAACTACTGTACCAAACCT
CAGAAGTCTTATCTGGTAGGATTTTCTGTCTAA
TAGTGATATAATTTGCAATTTGGTTAAATGTGC
TTGCCTGAAATAAAGCTAATAAAAATATTATG
TTTCACATTA

Expansy translation

MTALFLMSMLFGLACGQAMSFCIPTEYTM
HIERRECAYCLTINTTICAGYCMTRDINGKLFLPK
YALSQDVCTYRDFIYRTVEIPGCPLHVAPYFSYP
VALSCKCGKCNTDYSDCIHEAIKTNYCTKPQKSY
LVGFSV

mRNA → mutation

GTCACCACAGCATCTGCTCACCAATGCAAAGT
AAGCATGACTGCTCTCTTTCTGATGTCCATGCT
TTTTGGCCTTGCATGTGGGCAAGCGATGTCTTT
TTGTATTCCAACTGAGTATACAATGCACATCG
AAAGGAGAGAGTGTGCTTATTGCCTAACCATC
AACACCACCATCTGTGCTGGATATTGTATGAC
ACGGGATATCAATGGCAAACTGTTTCTTCCCA
AATATGCTCTGTCCCAGGATGTTTGCACATATA
GAGACTTCATCTACAGGACTGTAGAAATACCA
GGATGCCCACTCCATGTTGCTCCCTATTTTTCC
TATCCTGTTGCTTTAAGCTGTAAGTGTGGCAAG
TGCAATACTGACTATAGTGACTGCATACATGA
AGCCATCAAGACAAACTACTGTACCAAACCT
CAGAAGTCTTATCTGGTAGGATTTTCTGTCTAA
TAGTGATATAATTTGCAATTTGGTTAAATGTGC
TTGCCTGAAATAAAGCTAATAAAAATATTATG
TTTCACATTA

Expansy translation

MTALFLMSMLFGLACGQAMSFCIPTEYTM
HIERRECAYCLTINTTICAGYCMTRDINGKLFLPK
YALSQDVCTYRDFIYRTVEIPGCPLHVAPYFSYP
VALSCKCGKCNTDYSDCIHEAIKTNYCTKPQKSY
LVGFSV

mRNA → deletion (114)

GTCACCACAGCATCTGCTCACCAATGCAAAGT
AAGCATGACTGCTCTCTTTCTGATGTCCATGCT
TTTTGGCCTTGCATGTGGGCAAGCGATGTCTTT
TTGTATTCCAACTGAGTATACAATGCACATCG
AAAGGAGAGAGTGTGCTTATTGCCTAACCATC
AACACCACCATCTGTGCTGGATATTGTATGAC
ACGGGATATCAATGGCAAACTGTTTCTTCCCA
AATATGCTCTGTCCCAGGATGTTTGCACATATA
GAGACTTCATCTACAGGACTGTAGAAATACCA
GGATGCCCACTCCATGTTGCTCCCTATTTTTCC
TATCCTGTTGCTTTAAGCTGTAAGTGTGGCAAG
TGCAATACTGACTATAGTGACTGCATACATGA
AGCCATCAAGACAAACTACGTACCAAACCTC
AGAAGTCTTATCTGGTAGGATTTTCTGTCTAAT
AGTGATATAATTTGCAATTTGGTTAAATGTGCT
TGCCTGAAATAAAGCTAATAAAAATATTATGT
TTCACATTA

Expansy translation

MTALFLMSMLFGLACGQAMSFCIPTEYTM
HIERRECAYCLTINTTICAGYCMTRDINGKLFLPK
YALSQDVCTYRDFIYRTVEIPGCPLHVAPYFSYP
VALSCKCGKCNTDYSDCIHEAIKTNYVPNLRSLI
W

Native TSHb sequence encodes a full-length polypeptide, representing a reference for comparative interpretation. The triplet sequence maintains a stable conformation required for proper interaction with the Thyroid-Stimulating Hormone-Receptor (TSHR). Conversely, single nucleotide substitutions create point mutations that change the amino acid sequence and consequent local folding changes. The expasy model translation highlights the substitution of critical residues, likely causing a disruption of ligand-binding interface. On the other hand, TSHb deletions give rise to truncated or frame-shifted polypeptides, especially at nucleotide position 114. Analyses of translational reads demonstrate frameshifts that extensively change downstream amino acid sequences, which can severely disrupt TSHb function.

Nevertheless, research regarding the interplay and function of the TSHb subunit concerning TSHR in the thyroid gland system still remains very limited. Finally, the TSHb subunit is essential to synthesize and secrete Thyroid Stimulating Hormone (TSH), which involved in regulating thyroid hormone production. Pathogenic variants causing congenital central hypothyroidism (C-CH) have been reported for the TSHb subunit resulting in decreased synthesis of the TSH leading to lower thyroid hormone levels [10,24]. It is shown that expression of TSHb depends on many factors including transcription factors, immune responses and so on which are critical for TSH production [27]. These interactions also indicate that TSHb does not operate in a vacuum but rather within an elaborate regulatory system involving numerous genes and pathways. The 3 TSHb native, mutation & deletion which achieved TM-score > 0.7 with very high confidence (**Table 1**).

Table 1 presents the TM Scores and Confidence Levels for 3 different TSHb variants (Native, Mutation, and Deletion). The results indicate that all 3 variants have very high confidence in their structural modeling, with TM Scores ranging from 0.775 to 0.781. Understanding the TM Score, the template modeling Score (TM Score) is a quantitative measure used to evaluate the similarity between a predicted protein structure and a reference structure (often an experimentally determined structure such as an X-ray crystallography model). The TM Score ranges from 0 to 1, TM Score > 0.7 → Indicates a highly reliable model, meaning the predicted structure closely resembles the actual structure. TM Score < 0.5 → Suggests low reliability, meaning the predicted model may not accurately represent the real protein structure. Since all 3 TSHb variants in **Table 1** have TM Scores above 0.7, it confirms that the modeled structures are highly reliable and closely match the expected conformation. Relationship Between TM Score and Confidence level the confidence level is a qualitative descriptor of how trustworthy a structural prediction is, which is directly influenced by the TM Score: Higher TM Scores (≥ 0.7) correspond to a very high confidence level, meaning that the structural prediction is robust and likely to be accurate. Lower TM Scores (< 0.7) would indicate moderate to low confidence, suggesting that the model may require further refinement or validation. In **Table 1**, all TSHb variants (Native, Mutation and Deletion) have TM Scores around 0.775 - 0.781, reinforcing their very high confidence levels. This implies that despite structural variations (due to mutation or deletion), the fundamental 3D conformations of the TSHb variants remain structurally sound and well-modeled. Biological Significance of the Findings Since the TSHb subunit plays a crucial role in TSH synthesis and secretion, maintaining its structural integrity is essential for normal thyroid function. The very high confidence in modeling accuracy suggests that: The native, mutated, and deleted TSHb structures maintain a stable overall fold, which may indicate functional conservation despite genetic alterations. Mutations and deletions do not drastically disrupt the structural framework, although they may still affect protein function through local conformational changes or altered interactions with TSHR. The TM Score and Confidence Level in **Table 1** strongly support the accuracy of the structural models for all 3 TSHb variants. The high TM Scores suggest that the models are structurally reliable, reinforcing confidence in further computational analyses and functional interpretations of these variants.

Table 1 Validation of the quality of modelling results.

TSHb Variants	TM Score	Confidence
Native (Blue)	0.775	Very high
Mutation (Purple)	0.781	Very high
Deletion (Orange)	0.777	Very high

Table 2 Docking scores, confidence scores, and ligand RMSD values for interactions between TSHR and TSHb variants.

	TSHR-TSHb native	TSHR-TSHb mutation	TSHR-TSHb deletion
Docking score	−227.69	−266.47	−228.24
Confidence score	0.8255	0.9113	0.827
Ligand rmsd (Å)	279.3	354.59	4.94

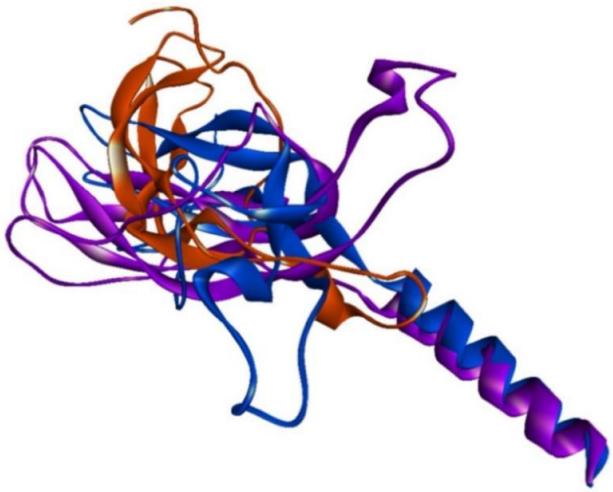

Figure 1 Superimpose the 3D structure of TSHb native (blue), mutation (purple) and deletion (orange) resulting from threading modelling using trRosetta.

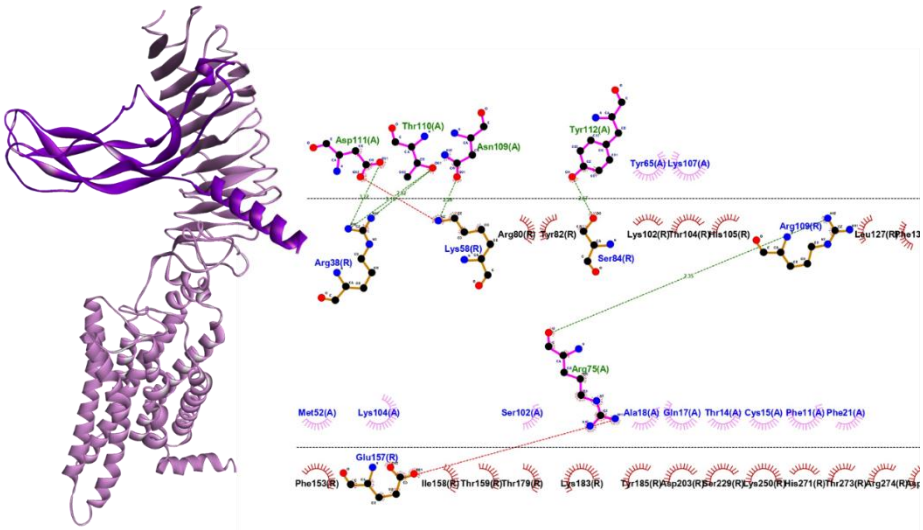

Figure 2 Visualization of the interaction between TSHR and native TSHb.

The results of **Figure 1**, molecular docking show that TSHb that has undergone mutation and deletion forms a bond with better affinity than native in (**Table 2**). This tendency is shown in the lower docking result score and higher confidence score. However, if TSHb mutation interacts with TSHR, RMSD TSHb has a larger result score and shows that the binding affinity of TSHb mutation is better than native TSHb, because TSHb mutation requires more energy to change its conformation to better bind to its receptor. While in TSHb deletion, the ligand RMSD actually becomes very small or almost does not experience a conformational change to adjust its binding to TSHR. This tendency can be caused by a significant structural change in TSHb deletion as a result of the deletion of nucleic acid at number 114.

The docking results for various TSHb variants interacting with TSHR (**Table 2**) reveal notable differences in the stability and binding affinity of these complexes. The TSHR-TSHb native complex exhibited a docking score of −227.69, indicating a relatively stable interaction under physiological conditions, with a confidence score of 0.8255, suggesting high reliability of the predicted binding pose. However, the TSHb mutation variant showed a stronger interaction, reflected by a more negative docking score of −266.47, coupled with the highest confidence score of 0.9113. This suggests that mutations in TSHb may enhance its binding affinity to TSHR, though this is accompanied by a substantial increase in ligand flexibility, as evidenced by a significantly higher RMSD value of 354.59 Å, indicating greater conformational shifts during docking.

In contrast, the TSHb deletion variant exhibited a docking score of −228.24, comparable to the native form, indicating that the deletion did not drastically reduce the interaction stability. The confidence score remained high at 0.827, further supporting the reliability of this binding prediction. Notably, the ligand RMSD for the deletion variant was remarkably low, at just 4.94 Å, indicating minimal conformational changes during docking and suggesting that the ligand maintained a highly stable interaction with TSHR despite the structural deletion. These results suggest that while mutations in TSHb enhance binding affinity through potential conformational flexibility, deletions maintain interaction stability by preserving structural rigidity, thus providing insight into the differential effects of genetic alterations on TSHR-TSHb complex formation.

Table 3 shows the interactions formed between the TSHR protein and each TSHb variant. Amino acids in bold font are active site amino acids from the PDB structure reference (7XW5) which are maintained as binding sites. While the amino acids in blue in **Figure 1** are active site conserved regions that are not affected by mutations or deletions from TSHb. In general, native TSHb maintains 5 aa TSHR and 3 aa TSHb. Length of time mutated TSHb only maintains 4 aa conserved regions from TSHR, there is not a single amino acid active site for TSH retained. This result is consistent with the high RMSD value of the mutated TSHb ligand. Although its binding affinity is much lower both from native TSHb side, the binding and conformation of the ligand of TSHb and TSHR are quite different. This causes the binding effect to be different from native TSHb. Furthermore, TSHb deletion becomes a variant with the most amino acids retained, namely as much as10 aa in TSHR and 7 aa in TSHb. However, the 3D structural conformation of the deleted TSHb is very different from the wild type (**Figure 1**).

Table 3 Interactions of amino acid residues produced between TSHR and TSHb variants.

Ligand	Amino Acid Binding Sites	
	TSHR	**TSHb**
TSHb PDB	R:ALA204	Y:ASP111
	R:ARG109	Y:ASP114
	R:ARG38	Y:CYS115
	R:ASN110	Y:GLU118
	R:ASP203	Y:ILE120
	R:GLU251	Y:LYS59

Ligand	Amino Acid Binding Sites	
	TSHR	**TSHb**
	R:ILE152	Y:LYS64
	R:ILE60	Y:THR110
	R:LEU230	Y:TYR65
	R:LYS209	
	R:LYS58	
	R:PHE153	
TSHb Native	**R:ARG109**	
	R:ARG38	A:ARG54
	R:ASP151	A:ARG75
	R:ASP160	A:ASN109
	R:GLU107	**A:ASP111**
	R:GLU157	A:ASP76
	R:HIS105	A:CYS103
	R:LYS102	A:CYS15
	R:LYS209	A:LYS104
	R:LYS250	A:LYS107
	R:LYS58	A:MET52
	R:PHE134	A:PHE11
	R:PHE153	**A:THR110**
	R:SER84	A:TYR112
	R:TYR185	**A:TYR65**
	R:TYR82	
TSHb Mutation	**R:ARG109**	
	R:ARG38	
	R:ARG80	A:ALA100
	R:ASN135	A:ARG54
	R:CYS390	A:ARG80
	R:GLU107	A:ASP76
	R:LYS183	A:CYS15
	R:LYS209	A:GLU26
	R:LYS250	A:ILE23
	R:LYS58	A:LEU101
	R:PHE130	A:MET9
	R:PHE134	A:PHE21
	R:SER84	A:PRO24
	R:TYR185	A:SER102
	R:TYR206	
	R:TYR387	
	R:TYR82	
TSHb Deletion	**R:ALA204**	A:ARG34
	R:ARG109	A:ARG54
	R:ARG274	**A:ASP111**
	R:ARG38	**A:ASP114**
	R:ARG80	**A:GLU118**

Ligand	Amino Acid Binding Sites	
	TSHR	**TSHb**
	R:ASP203	**A:ILE120**
	R:ASP382	A:LYS121
	R:ASP43	**A:LYS59**
	R:GLU251	**A:LYS64**
	R:GLU30	A:TYR124
	R:GLU34	**A:TYR65**
	R:GLU61	
	R:HIS32	
	R:ILE60	
	R:LEU230	
	R:LYS209	
	R:LYS58	
	R:PHE153	
	R:TYR385	
	R:TYR82	

The intricate interactions between TSHR and various thyroid-stimulating hormone beta (TSHb) variants (**Tabel 3**) elucidate critical insights into the structural and functional implications of these molecular complexes. Using a detailed residue-level approach, the binding dynamics of the TSHR-TSHb complexes were examined, revealing unique patterns of stabilization and conformational adaptability across native, mutated, and deleted TSHb variants. These interactions, predominantly governed by electrostatic forces and hydrophobic contributions, demonstrate significant variability influenced by genetic alterations.

When TSHb was docked into the TSHR (PDB ID: 6EJY), the key residues mentioned above formed strong electrostatic and hydrogen bond interactions with TSHb residues (ASP111 and CYS115). It's worth noting that charged residues, particularly ARG and ASP, have a key role in stabilizing the complex since the electrostatic interactions are quite strong. This primary interaction highlights the contribution of conserved residues to the structural fidelity required for efficient receptor-ligand interaction. The TSHb native variant is also significantly preserved at critical engagements, were these are mainly IMP/GLU with ARG109 of TSHR, which we show to heretofore also engages consistently with ASP109, PHE153 and TYR82. Additionally, the hydrophobic interaction involving TYR65 in TSHb further enhances the electrostatic stabilization and serves for the built-in structural conformation and functional stability of the native complex at physiological conditions.

In contrast, the mutation variant shows notable binding pattern changes such as loss of existing contact profiles and formation of alternative contacts. In particular, the mutation at ARG109 breaks its conventional binding interactions and forms new contacts with ARG54 and ASP644 in TSHb. The presence of binding flexibility is further confirmed by additional engagements with residues including ARG638 from TSHR (absence from native complex). The enhanced flexibility is evident by the increased root-mean-square deviation (RMSD) value for the ligand, indicating significant conformational reorganization. These changes are additionally driven by i, which shift the signalling dynamics of the receptor. The greater negative docking score achieved upon mutation of C767 indicates a higher affinity compound relative to the wild type, presenting a paradox of observed increased strength, but loss of overall structural stability resulting in loss of function due to mis-folding and thus conformationally unstable receptor activation or inhibition.

The deletion variant of TSHb displays a unique interaction landscape compensatory binding profile to the WT variant that compensates for the loss of key

residues. Interaction of GLU118 and ILE120 in TSHb with GLU107 and GLY34 in TSHR shows the formation of stabilizing contacts from despite the structural deletion. The hydrophobic and polar interactions between TSHb residues LYS64 and TYR65 seems to compensate for the absence of native residues, contributing to the overall stability of the hybrid complex. Notably, this deletion variant maintains docking scores with low deviation from the native form, evidenced by a low RMSD value, attesting to structural reliability achieved through compensatory mechanisms. This structural plasticity implies that deletion events can maintain receptor functional activity by retaining canonical interactions at the binding site even in the face of extensive conformational divergence of the ligand.

Advanced molecular visualizations using LigPlot provide additional granularity to these findings by categorizing the nature and strength of intermolecular interactions. Hydrogen bonds, represented by green lines in the visualizations, were observed at distances of 2 - 3.5 Å, while hydrophobic interactions - depicted by black and blue lines - occurred at distances exceeding 3.5 Å. Unfavorable interactions, highlighted in red, were also identified, suggesting potential destabilizing effects at inappropriate binding sites. In the native TSHb complex (**Figure 2**), residues such as A:ASP111, A:THR110, A:ASN109, A:TYR112, and A:ARG75 formed hydrogen bonds, with ASP111 being conserved from the PDB reference. Conversely, the mutation variant (**Figure 3**) demonstrated entirely new hydrogen bond formations, involving residues such as A:ALA100, A:GLU26, A:PRO24, and A:CYS15, without retaining any residues from the original PDB structure. The deletion variant (**Figure 4**) retained the highest number of conserved residues, including A:GLU118, ASP111, A:LYS64, and A:TYR65, contributing to the overall stability of the complex.

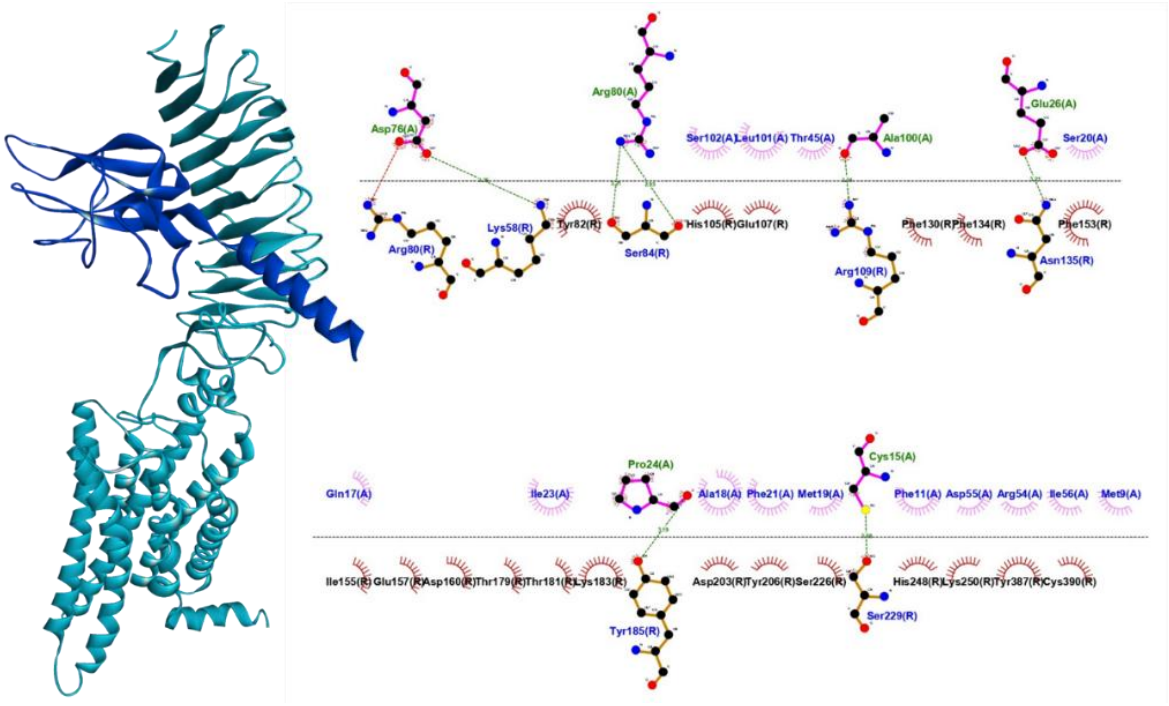

Figure 3 Visualization of the interaction between TSHR and mutated TSHb.

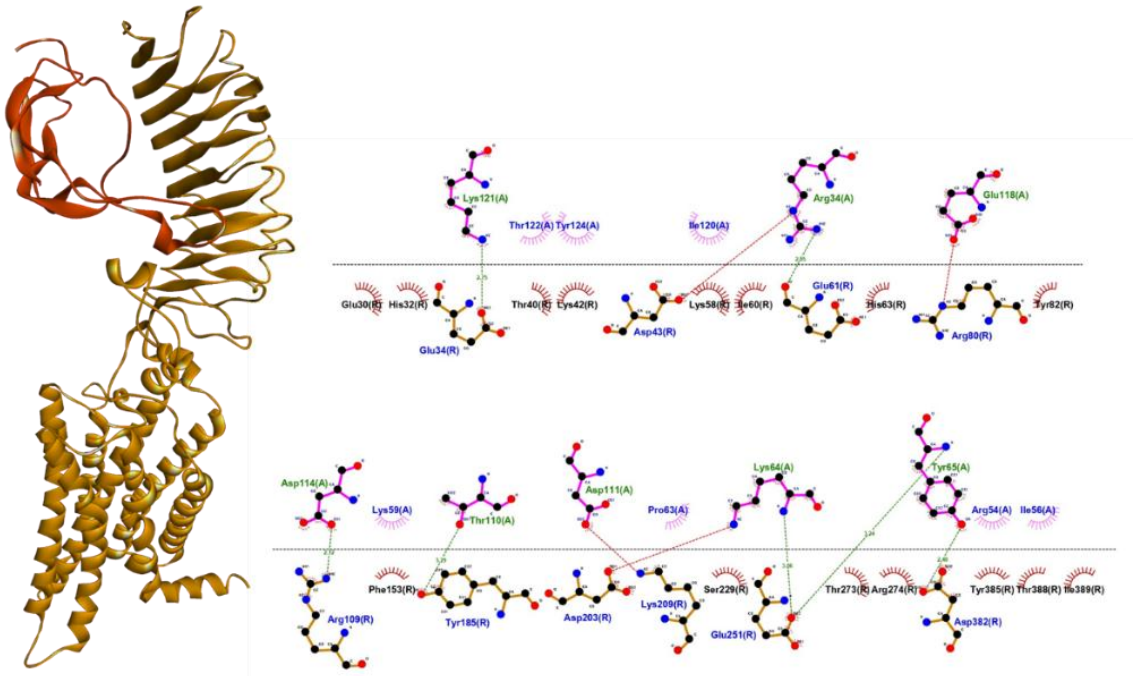

Figure 4 Visualization of the interaction between TSHR and deleted TSHb.

Together, these in-depth analyses emphasize the importance of electrostatic forces, hydrophobic interactions, and structural compensations in tuning TSHR-TSHb binding dynamics. The mutation and deletion variants expose opposite mechanisms, as mutations expand binding flexibility at the cost of stability, whereas deletions preserve structural integrity via such extended adaptive compensatory interactions. Such residue-specific interactions elucidated here have important implications for providing the molecular basis of thyroid-related disorders like congenital hypothyroidism. Moreover, these insights will lay the foundation for targeted therapeutic approaches that harness the differential TSHb variant binding properties to influence TSHR activity.

Molecular dynamic analysis

Molecular dynamics simulations were performed using the YASARA Dynamics suite to evaluate the stability and energetic profiles of the TSHb protein in its native, mutated, and deleted forms, both as isolated proteins and in complex with ligands. Simulations were conducted at physiological conditions - 310 K (37 °C), with a NaCl concentration of 0.9 % and a pH of 7.4 - to closely mimic the *in vivo* environment. The potential energy profiles of the systems were tracked over a 20 ns timescale to assess both the initial relaxation and the subsequent equilibrium phases.

The superimposition of TSHb structures, encompassing native, mutated, and deletion variants, before (green) and after (purple) molecular dynamics (MD) simulations, provides a comprehensive visualization of the structural deviations induced by mutations and deletions (**Figure 1**). This analysis offers an advanced perspective on the conformational changes and stability of TSHb, complementing quantitative metrics such as RMSD, radius of gyration (Rg), solvent-accessible surface area (SASA), and hydrogen bond (H-bond) analyses.

The increase in potential energy in the first 0.025 ns reflects the energy activation needed to make structural rearrangements on the way to a stable conformation towards the final state, as shown in **Figure 5**. This swift energy level elevation illustrates the system's acclimatization, encompassing solvent and ionic interactions, to the established conditions. Aside from this activation phase, all TSHb variants exhibited canonical energy stabilization, with potential energy fluctuations throughout the equilibrium phase. Well, these variations are inherent in the molecular dynamics process, which is a representation of the continuous interactions of the atoms, the flexibility of the protein,

and the brief binding between the protein and solvent molecules.

Importantly, TSHb mutation only minimally perturbed the potential energy of the system, indicative of structural robustness and minor disruption of the energetic profile of the protein. In comparison, the energy landscape for the deletion variant of TSHb had a marked difference. Notably, the change in energy at the equilibrium potential transitioned from −1,350,000 kJ/mol in the native state to −1,420,000 kJ/mol in the deletion mutant, resulting in a substantial decrease in energy. This major change is a consequence of extensive structural rearrangements in the deletion TSHb that likely disrupt stabilizing intramolecular interactions and modify the folding dynamics of the protein. Such a loss of intensity in energy would indicate a less compact and more flexible (and potentially more unstable) conformation that can affect the functional component of the protein.

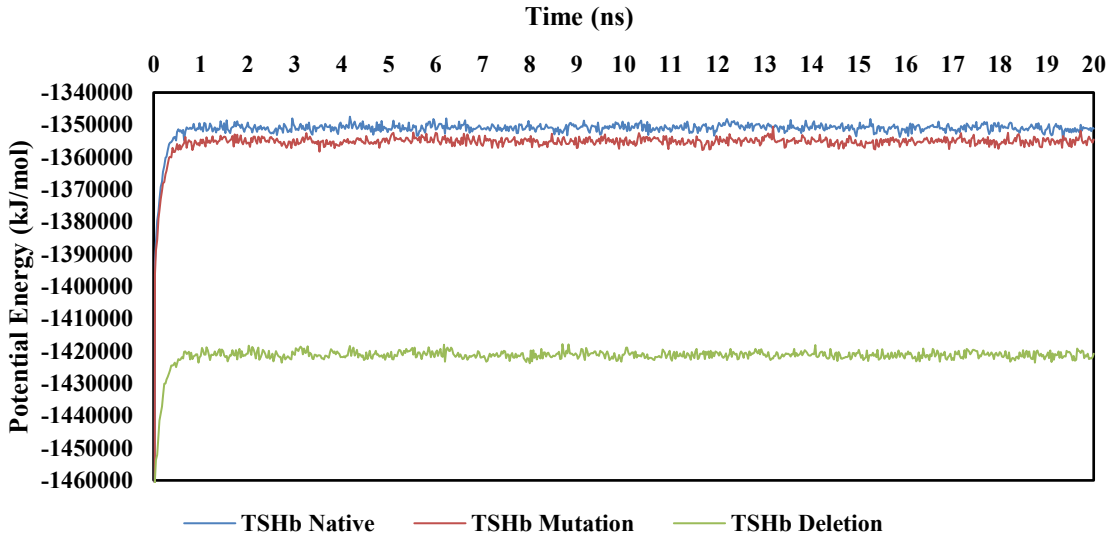

Figure 5 Potential energy of native, mutation and deletion TSHb**.**

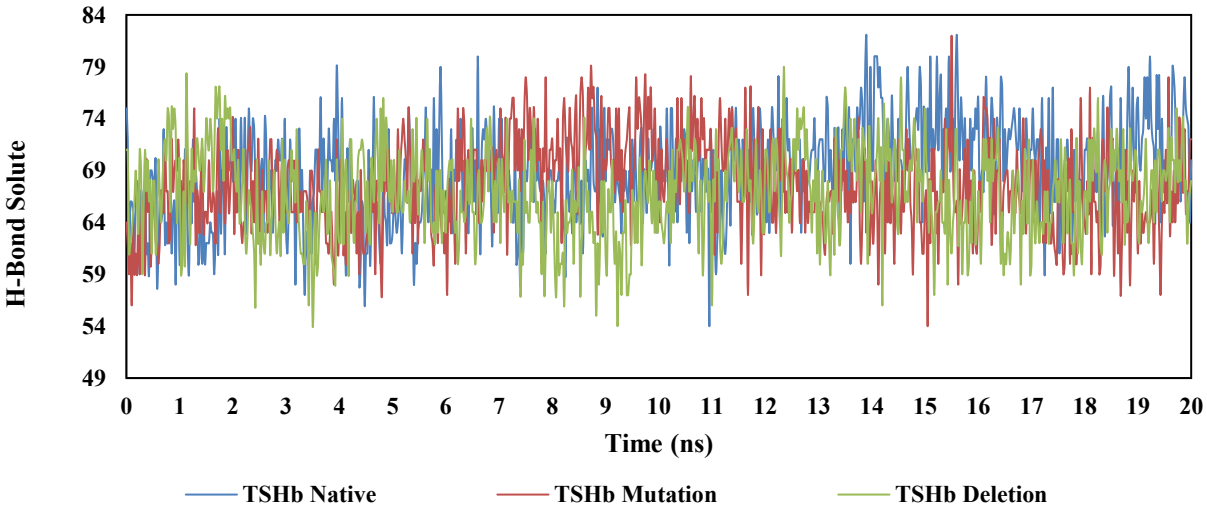

Figure 6 Number of H-Bonds in the sample protein complex (solute).

Analysis of hydrogen bonding dynamics from the YASARA simulations indicates a clear difference in the solute and solute-solvent interactions between the TSHb variants. These 2 categories were investigated, namely

hydrogen bonds in solute (**Figure 6**) and solute−solvent (**Figure 7**). Interestingly, while the same minimum number of solute 54 H-bonds occurs with all TSHb variants, the TSHb deletion variant presents a lower maximum of 79 H-bonds, compared to the native and mutation forms. The native protein has much higher average solute H-bonds than after both mutation and deletion. In contrast, analysis of the solute-solvent interaction profile demonstrates opposite trends; the TSHb mutation promotes H-bonding with the solvent, whilst the deletion generates less in comparison to the native structure. This increase in solute-solvent H-bonds suggests a change in the physicochemical properties of the protein, which is likely, in the case of the mutated TSHb, a shift to a more hydrophilic character. Such evidence reveals the complex molecular disturbances caused by mutation and deletion, that may dramatically alter the stability of TSHb variants aqueous solutions in a manner important to their functional contexts.

Solvent Accessible Surface Area (SASA) is a fundamental property that qualifies the total surface area of a protein that is accessible to solvent molecules, highly relevant to the understanding of both the protein's conformational flexibility and interaction dynamics [26,27]. SASA is a representative of spatial distribution of amino acid residues and provides important information for facilitatory protein-ligand and protein-protein interactions. It also constitutes a major factor in protein stability, folding, and unfolding studies, as it indicates the degree of exposure of protein surface to solvent molecules surrounding it. SASA calculations utilize a hypothetical solvation sphere that makes van der Waals contacts with the molecular surface of the protein.

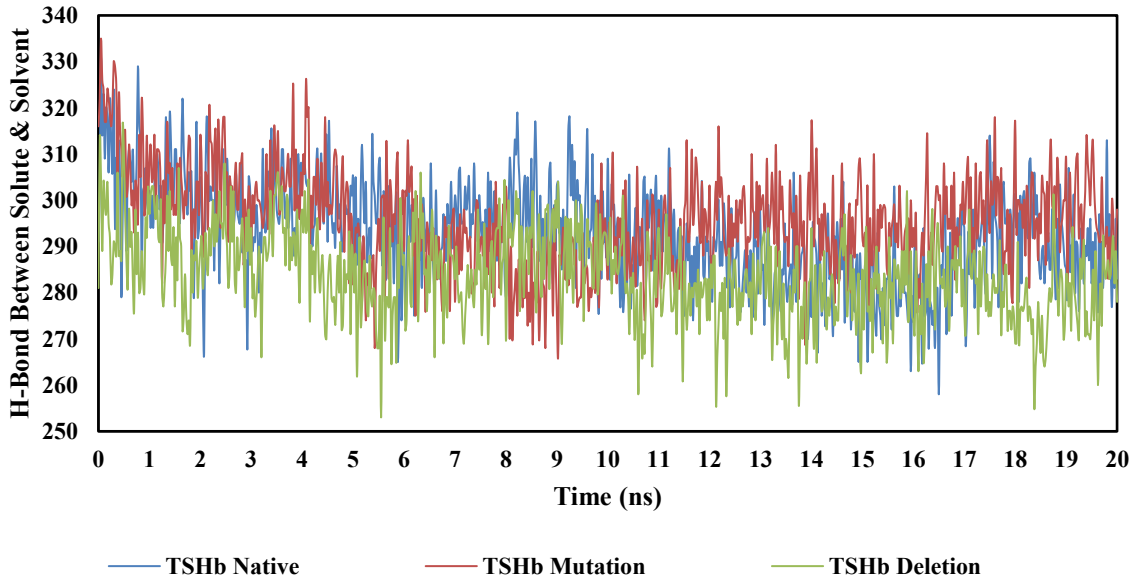

Figure 7 Number of H-Bonds between the protein complex and the solvent.

SASA directly relates to the protein's structural compactness as well. Larger values of SASA correspond to stretched, unfolded protein structures, while smaller SASA values are indicative of compact structures. Consistently low fluctuations of SASA values during molecular dynamics simulations should be anticipated as long as the system remains conformationally stable without encountering unfolding events, including those that involve significant rearrangement of the protein backbone [30]. Thus, large changes in SASA can indicate significant changes in protein dynamics, such as opening of buried hydrophobic cores or refolding events.

The trends from the SASA analysis (**Figure 8**) closely match those of the hydrogen bond (H-bond) analysis. And SASA, which resulted in the highest SASA average values for the whole simulation for the TSHb mutant variant compared to its wildtype, indicating that the mutation makes the structure more expanded and less compact. This broadening may be due to disruption of important stabilizing interactions leading to increased solvent exposure. This increase in

SASA of the mutated TSHb also reinforces the hypothesis of increased flexibility and potential unfolding compared to the native form of TSHb.

Meanwhile, the SASA value of the TSHb deletion variant was even lower than that of the native TSHb. There are well-established studies that show that that, with the surface area exposed, the protein may start to take on more contracted or collapsed structure as driven by hydrophobic forces. The decreased SASA implies that deletant has undergone structural conferring reduced solvent accessibility, which may be an indication of enhanced hydrophobicity and possible compromised functional dynamics.

Overall, these observations offer significant insights into the structural impact of the identified mutations and deletions in TSHb, and compare with the similar degree of flexibility between TSHb mutations and deletions observed in the SASA attain an important metric for evaluating the overall conformation stability, relative exposure and accessibility for the TSHb variants, in a total physiological environment. These insights can guide subsequent investigations into the downstream biological significance of the observed effects of genetic modifications on protein folding and functional integrity.

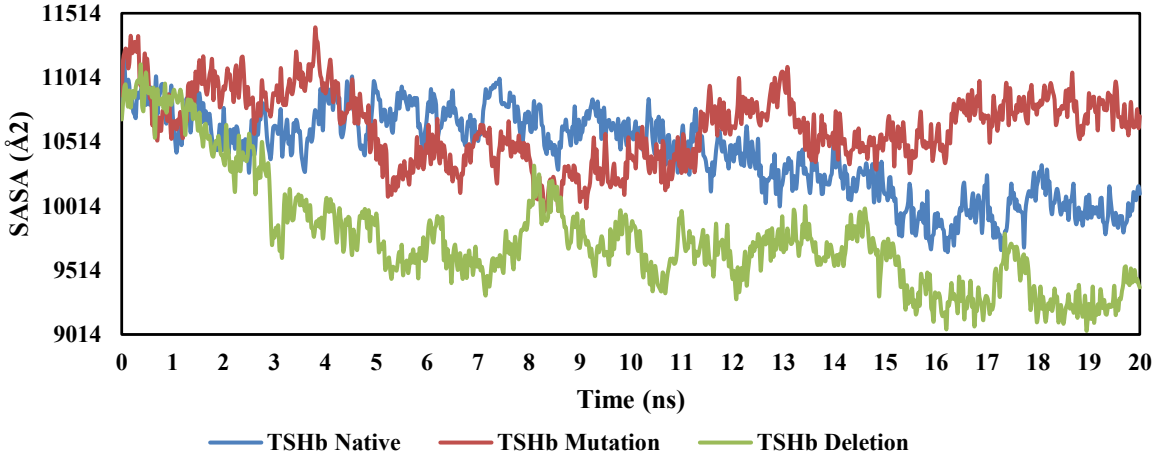

Figure 8 SASA of native TSHb, mutations and deletions**.**

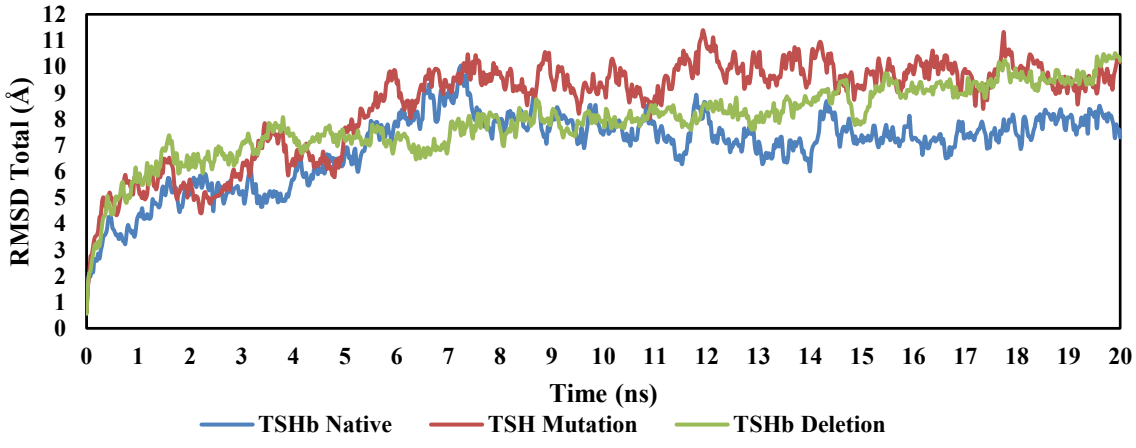

Figure 9 Total RMSD of native, mutated and deletion TSHb**.**

RMSD (Root Mean Square Deviation) is a critical parameter used to evaluate the conformational dynamics and stability throughout molecular dynamics (MD) simulations of macromolecules. It measures how far, on average, the atom positions differ over time from a specific reference structure, therefore offering insight into structural fidelity and the influence of external or mutational perturbations. Root mean square deviation

(RMSD) values are often calculated to assess the stability of a complex over time in docking and MD studies, such that 2 Å indicates marked structural rearrangement [31].

RMSD analysis was performed in this study to assess the structural stability of native, mutated, and deletion variants of TSHb. The RMSD was plotted as a function of time using md_analyze macro (**Figure 9**). The TSHb was more conformationally stable than the native, as indicated by the smaller RMSD values (6 - 10 Å), which reflected small deviations and a more structurally stable framework during MD simulation [24]. The deletion mutant, on the other hand, showed higher RMSD values, reflecting greater structural flexibility and reduced stability. This corroborates the idea that deletions in the hydrophobic core destabilize proteins and lead to noticeable deviations from the native conformation. The mutation variant exhibited intermediate stability, with RMSD values lying between those for the native and deletion forms.

The results highlight the importance of hydrophobic interactions in the preservation of TSHb structural integrity. The enhanced RMSD observed for the deletion mutant illustrates alterations in molecular compactness and local interactions that maintain protein stability. Collectively, the native TSHb displayed close to energetic stability (i.e. conformational fidelity) over the course of the 20 ns on-going simulation making it likely the most stable structural variant out of all forms analyzed. The radius of gyration (Rg) is an important structural parameter that is used in evaluating the compactness and dynamic equilibrium of macromolecular systems in molecular dynamics (MD) simulations. It is the square-root of the mass-weighted average of the positions of all atoms in a system relative to their center of mass and works as a descriptor for the studies of protein solubility and folding states in aqueous environments solution [32]. A low Rg value therefore indicates a compact, folded state, while a high Rg value indicates an extended or unfolded state [33]. Fluctuations in Rg offer important information about the structural stability and the effects of mutations or deletions on the organization of macromolecules.

This analysis encompassed the analysis of all data up to a maximum of 20 ns of simulation time, where the Rg profiles of the native TSHb, its mutant and deletion mutant were evaluated (**Figure 10**), revealing the relative compactness of the data measured, and assisting with folding determination over time. Native TSHb showed the most stable Rg values of 20 - 21.5 Å, suggesting a stable and well-populated folded conformation. The mutation variant had higher Rg fluctuations compared to the wild type indicating moderate perturbations in its structure which destabilized the fold but was not sufficient to cause complete unfolding. In contrast, the deletion mutant showed significant heterogeneity and greater Rg values indicative of a more unfolded conformation and a significant loss of structural integrity.

These results suggest that deletion-induced compaction of TSHb is destabilizing due to the loss of important intramolecular contacts and hydrophobic packing. The elevated Rg for the deletion mutant is consistent with its reduced conformational stability indicated by RMSD analyses and discusses an important relationship between protein folding and sequence variation. The Rg analyses also indicate that, consistent with RMSD results, native TSHb shows greater structural stability and compactness followed by mutated/deletion forms. This highlights the importance of hydrophobic interactions and integrity of tertiary structure in keeping TSHb functional.

The very small deviations between the pre- and post-simulation conformations of the native TSHb (**Figure 11(a)**), imply high structural stability and conformational fidelity. The alpha-helical and beta-sheet formations are retained, demonstrating stability against major perturbations through the MD simulation. In comparison, the mutated TSHb (**Figure 11(b)**) displays localized variations, particularly in loop regions and termini (black arrows). These structural alterations indicate that the mutation affects specific stable intramolecular interactions causing moderate destabilization due to compromise of interfacial stability, while not affecting folding overall.

The deletion variant (**Figure 11(c)**) shows the most extreme deviations, with significant structural rearrangements noted following simulation. Notably, the black arrows point out major disturbances in the alpha-helical and loops regions, indicating that the deletion has a destabilizing ahem effect on the protein tertiary structure. The loss of compactness of the deletion mutant (as demonstrated through Rg and SASA analasyes) correlates with the retention of an H-bond

reserve that maintains stability in its complex something that is not replicated in the deletion mutant.

Overall, the results of superimposition support the quantitative results showing that mutations/deletions destabilize TSHb conformational stability. Although the mutation introduces localized destabilization, the deletion results in global structural perturbations - modifying the native fold and leading to structural compromise. It also highlights the importance of sequence integrity for TSHb structural and functional stability as genomic deletions have much larger effects than point mutations. These results offer significant insights regarding the molecular basis for TSHb destabilization as well as the insights afforded through MD simulations and structural overlays into protein stability.

The Root Mean Square Fluctuation (RMSF) which reflects the flexibility and dynamic motion of each amino acid residue in a protein over the course of MD simulations is thus a key parameter that helps validate MD simulations. Through this, it provides residue-sort reaction insight into conformational fluctuations reflecting atomic movement and the stability of particular pieces of the protein construction. RMSF values are particularly useful for investigating fluctuations around terminal regions or flexible loops, because they tend to be more dynamic than the core regions of the protein.

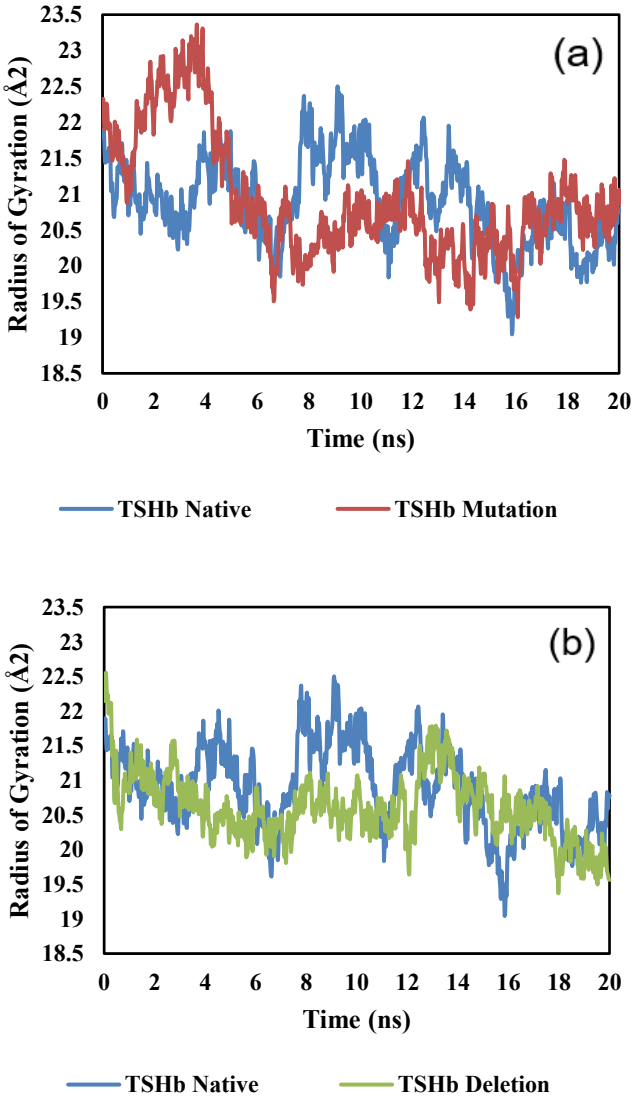

Figure 10 Comparison of radius gyration patterns between native TSHb (a) mutated TSHb and (b) deletion TSHb.

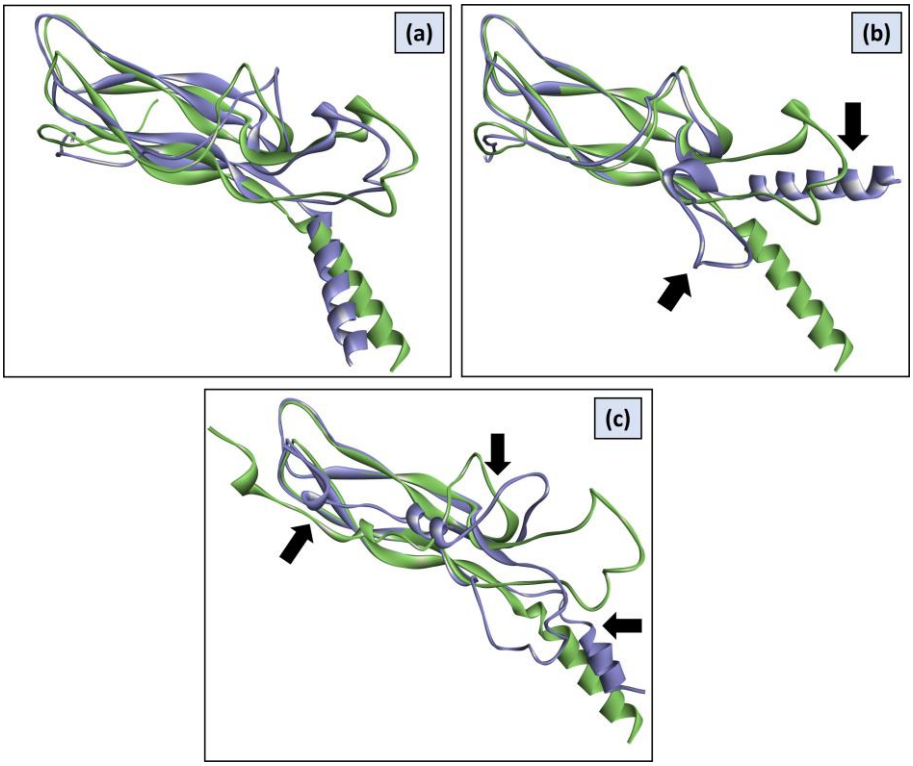

Figure 11 Superimpose of samples (a) native TSHb, (b) mutated TSHb, (c) and deleted TSHb before (green) and after (purple) molecular dynamics simulations.

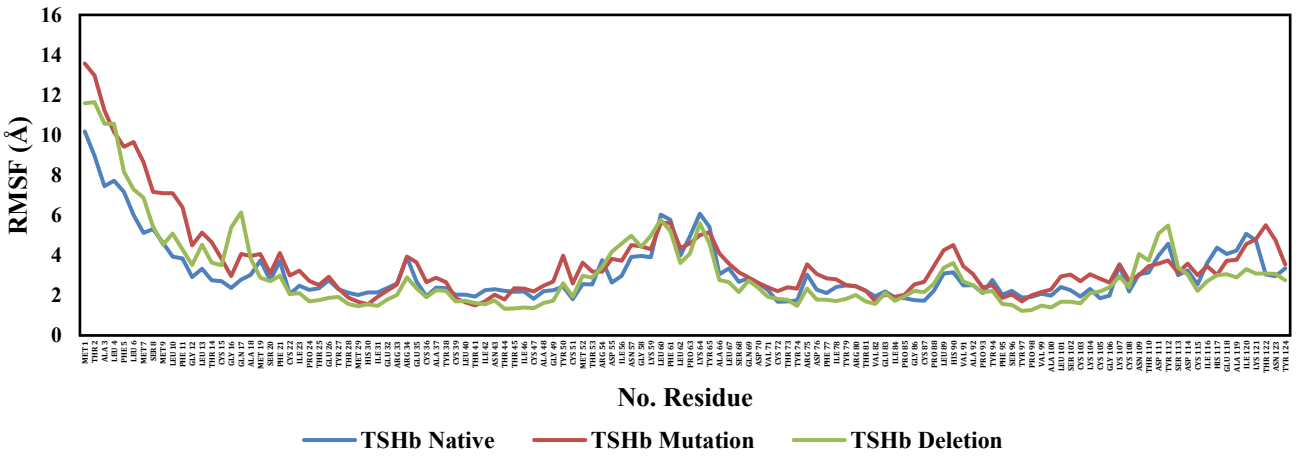

Figure 12 RMSF of amino acid residues of native, mutation and deletion TSHb protein structure**.**

RMSF analysis of TSHb wild forms reveals different fluctuation patterns for both the mutated and the deletion variants compared to the native variant (**Figure 12**). The N-terminal and C-terminal residues displayed the maximum values of RMSF (RMSF ~ 10 Å), showing that these regions remained a source of greater conformational flexibility. This is true for all 3 variants, as termini tend to be less constrained than the protein core. Central residues, mainly beta-sheets and alpha-helices, show lower RMSF, indicating their role in preserving structure.

RMSF values (**Figure 13**) for the native TSHb remain relatively low (< 5 Å) across most residues, signifying stable interactions and minimal flexibility. The mutation variant exhibits a moderate increase in RMSF for residues proximal to the mutation site,

suggesting localized destabilization and increased flexibility in these regions. In contrast, the deletion variant shows a distinct pattern due to its altered sequence and reduced amino acid count. Interestingly, despite these changes, the deletion sequence demonstrates enhanced binding stability in its secondary structure regions, as evidenced by reduced fluctuations in certain critical residues compared to the native and mutated forms.

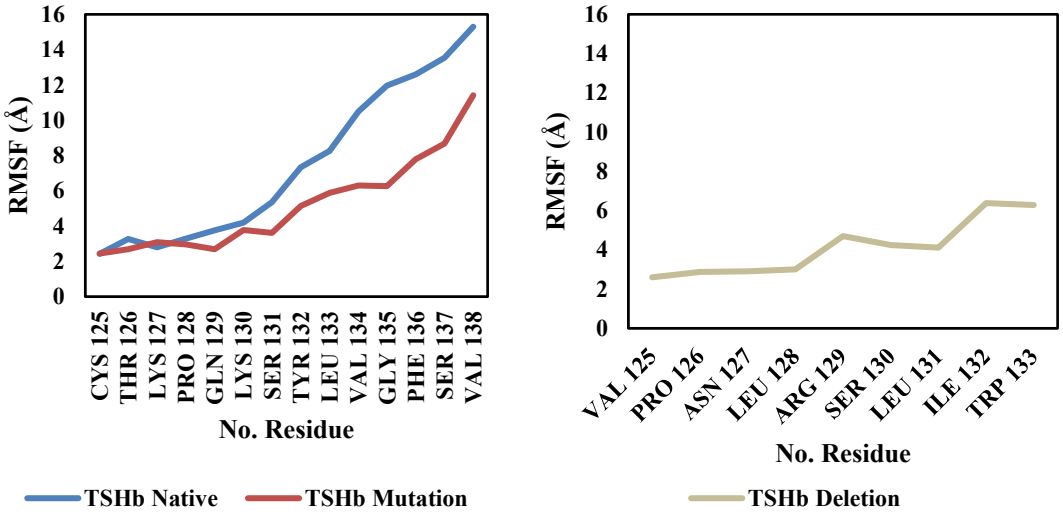

Figure 13 RMSF of amino acid residues active side interaction of native, mutation (left) and deletion (right) TSHb protein structure with the receptor protein.

This observation suggests that while the deletion introduces global destabilization, it may also facilitate stabilization in specific regions, possibly due to compensatory rearrangements or enhanced hydrophobic interactions. The reduced RMSF in key regions of the deletion variant aligns with its improved local binding stability, a finding that complements the results from radius of gyration (Rg), RMSD, and hydrogen bond analyses.

Overall, the RMSF results provide a nuanced understanding of the residue-level dynamics of TSHb variants, highlighting how sequence modifications influence flexibility and stability. The findings emphasize the complexity of structural adaptations in response to mutations and deletions, with implications for the functional integrity and binding properties of the TSHb protein. This analysis reinforces the utility of RMSF as a robust tool for probing molecular stability and flexibility in protein engineering and structural biology studies.

Comparison with previous studies

When compared to previous *in silico* studies, this research presents a unique and complementary perspective. In previous studies *in silico*, the focus was on the structure and activation of TSHR via exoloop-specific antibodies. These antibodies effectively "locked" the receptor and inhibited its downstream signaling activity [34]. In contrast, the current study uniquely emphasizes the ligand side TSHb, and investigates how its structural alterations (mutations and deletions) impact interaction with TSHR. Notably, our findings demonstrate that even in the presence of sequence deletions, TSHb retains the ability to bind TSHR, albeit with altered dynamics. This underscores 2 distinct therapeutic paradigms: receptor inhibition through antibodies versus ligand-based structural adaptation.

In previous studies *in silico*, the researchers focused on identifying and characterizing TSHR mutations in patients with congenital hypothyroidism (CH), with an emphasis on receptor side genetic

alterations [35]. While both studies highlight the importance of genetic screening, the present study extends this approach by modeling the biochemical consequences of TSHb mutations using biophysical simulations. This allows for a deeper understanding of the molecular impact of ligand variations and supports the potential for personalized diagnostics and TSHb specific therapeutic strategies.

Meanwhile, previous studies *in silico* introduced a synthetic small-molecule antagonist (VA-K-14) targeting TSHR for therapeutic intervention in autoimmune conditions such as Graves' disease. This approach demonstrates the promise of receptor-targeted drug design [36]. In contrast, our study focuses on naturally occurring mutations or deletions in TSHb that disrupt its interaction with TSHR, contributing to congenital hypothyroidism, a condition rooted in hormone deficiency rather than autoimmunity.

These 2 approaches highlight distinct clinical frameworks: one aimed at inhibiting TSHR overactivation in hyperthyroid disorders (e.g., Graves' disease), and the other elucidating the consequences of impaired TSHb function leading to CH-related hypoactivity. Clinical and therapeutic relevance, this study provides compelling evidence that TSHb mutations and deletions exert differential effects on TSHR binding. Mutations may lead to increased binding affinity but carry the risk of misfolded or dysfunctional protein states. In contrast, deletions may preserve receptor engagement through compensatory conformational adaptations, despite structural disruptions.

These insights are highly valuable for: Designing nucleic acid-based diagnostic tools that are sensitive to specific TSHb variants, developing variant-specific therapeutic strategies, such as stabilizing flexible TSHb mutants with molecular chaperones or designing mimetic peptides to restore function in deletion cases. Enhancing genotype–phenotype correlation models for congenital hypothyroidism by integrating data from both TSHR and TSHb variant analysis.

Conclusions

Thus, given the pivotal role of TSHR in the pathophysiology of congenital hypothyroidism, this study yields important findings regarding the different effects that genetic variations in TSHb - mutations versus deletions - have on the interaction with TSHR. The molecular docking results show that the mutations in TSHb significantly increased TSHb binding affinity to TSHR, with lower docking score and higher confidence score. This higher interaction is offset by decreased structural flexibility and, consequently, potentially a different biological response. On the contrary, TSHb deletions show increased binding stability similar to that of the native form (conformational changes very small and docking scores well preserved), thus indicating that these deletions would not impair the lig-Receptor interaction greatly. These results provide important mechanistic insights that can guide future diagnostic and therapeutic strategies for TSHR-related disorders. In addition, the study highlights the relevance and significance of in silico strategies to predict interactions at the molecular level, paving a new way for novel experimental research directions and studies aimed at precision and personalized medicine towards thyroid diseases.

Acknowledgements

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