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Original Article

In Silico Analysis of Non-Synonymous SNPs in NOS3 Gene

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ABSTRACT

Background: Hypertension, a prevalent chronic disorder characterized by blood pressure ≥130/80 mmHg, results from complex interactions between genetic and environmental factors, affecting approximately 20% of the global population and contributing to significant morbidity, including stroke and heart failure. The endothelial nitric oxide synthase (NOS3) gene, encoding eNOS critical for nitric oxide synthesis and vascular homeostasis, harbors numerous non-synonymous single-nucleotide polymorphisms (nsSNPs) potentially linked to hypertension pathogenesis, yet comprehensive characterization remains lacking. Objective: This study aimed to computationally identify and prioritize deleterious nsSNPs in the NOS3 gene using a multi-tool bioinformatics approach to enhance understanding of genetic risk factors for hypertension. Methods: A total of 614 nsSNPs were retrieved from Ensembl (Release 114, May 2025, accession ENSG00000164867) and analyzed using PolyPhen-2 (v2.2.2), SNPs&GO, PhD-SNP (v2.0.6), SIFT (v6.2.1), and PANTHER (v18.0) to predict deleterious effects. Further assessments included stability (I-Mutant v2.0, MUpro v1.1), function (MutPred2 v2.0), conservation (ConSurf v2016), structure (I-TASSER v5.2, TM-align v20220412), and interactions (STRING v12.0, GeneMANIA v3.6.0). Results: Thirty nsSNPs (4.9% of total) were predicted deleterious by all tools, with most reducing protein stability (ΔΔG <0), occurring in conserved regions (ConSurf score 7-9), and inducing structural deviations (RMSD >1 Å). Functional analyses suggested potential disruptions in NO synthesis, supported by interaction networks with genes like NOSIP and CAV1. Conclusion: These findings identify 30 high-confidence deleterious nsSNPs in NOS3, providing a prioritized list for experimental validation and population studies to elucidate their role in hypertension, with implications for personalized risk assessment and therapeutic development.

Keywords: Hypertension, NOS3, nsSNPs, in silico analysis, bioinformatics, endothelial nitric oxide synthase, genetic risk factors

INTRODUCTION

Hypertension is a common, chronic, age-related disorder typically characterized by elevated blood pressure (systolic ≥130 mmHg or diastolic ≥80 mmHg) (1). It arises from intricate multifactorial interactions between numerous genetic risk factors and environmental influences (2). The global prevalence of hypertension is increasing due to population growth, aging, and shifts in behavioral risk factors (3). In North America, hypertension contributes significantly to approximately 500,000 strokes (resulting in 250,000 deaths) and 1,000,000 myocardial infarctions (resulting in 500,000 deaths) annually (4). In Pakistan, the prevalence stands at 33% among individuals over 45 years and 18% among adults aged 18-35 years (5).

Multiple genes are implicated in blood pressure regulation and hypertension pathogenesis, including the endothelial nitric oxide synthase (eNOS) gene, also known as NOS3 (6). The NOS3 gene encodes eNOS, which plays a crucial role in synthesizing nitric oxide (NO), a key molecule involved in vascular functions such as regulating smooth muscle tone, inhibiting lipid and cholesterol accumulation in arteries, preventing platelet aggregation, and reducing lipoprotein oxidation—collectively contributing to cardiovascular disease prevention (7, 8). Mammals express three NOS isoforms: neuronal NOS (nNOS, encoded by NOS1, primarily in neurons), inducible NOS (iNOS, encoded by NOS2, mainly in macrophages), and eNOS (encoded by NOS3, predominantly in endothelial cells) (9). NOS3 is essential for vasodilation, with NOS3-knockout mice exhibiting vascular abnormalities (10). Located on chromosome 7q35-36, NOS3 serves as a key marker in the vascular system and is linked to essential hypertension (11, 12). Variants in NOS3, particularly non-synonymous single-nucleotide polymorphisms (nsSNPs), have been associated with hypertension across diverse populations; these nsSNPs can alter protein structure, stability, or function, potentially disrupting NO production and endothelial homeostasis (10, 13, 14). However, no comprehensive study has characterized all nsSNPs in NOS3 to date. In silico approaches offer an efficient, cost-effective means to screen and prioritize large sets of genetic variants for potential deleterious effects before resource-intensive experimental validation, leveraging predictive algorithms grounded in sequence conservation, structural modeling, and functional annotation (15, 16). Building on recent advancements in computational genomics and prior studies on NOS3 variants (17, 18), this study aims to

computationally analyze all 614 nsSNPs in NOS3 to identify those predicted to be most deleterious, thereby prioritizing candidates for future wet-lab and population-based investigations into hypertension risk.

MATERIALS AND METHODS

This study employed an in silico predictive modeling approach to screen and prioritize non-synonymous single-nucleotide polymorphisms (nsSNPs) in the NOS3 gene for potential deleterious effects on protein structure, function, stability, and interactions. All analyses were conducted using publicly available bioinformatics tools between January and June 2024. The workflow integrated multiple complementary algorithms to enhance prediction robustness, with variants selected for further analysis only if deemed deleterious by all initial screening tools. For reproducibility, the NOS3 protein sequence and variant data are available from Ensembl (Release 114, May 2025; gene accession ENSG00000164867). Input files, detailed parameters, and custom scripts (where applicable) are available from the corresponding author upon reasonable request.

Data Collection

Missense variants (nsSNPs) for the human NOS3 gene were retrieved from the Ensembl database (Release 114, May 2025) (19). The protein sequence encoded by NOS3 (UniProt accession P29474) was obtained in FASTA format for use in downstream analyses.

Screening of Deleterious nsSNPs

To identify potentially deleterious nsSNPs affecting the structure and function of the NOS3 protein, five bioinformatics tools were employed: PolyPhen-2 (version 2.2.2, HumDiv model) (20), SNPs&GO (web server, default parameters) (21), PhD-SNP (version 2.0.6) (22), SIFT (version 6.2.1) (23), and PANTHER-PSEP (version 18.0) (24). The NOS3 FASTA sequence and amino acid substitutions for each of the 614 nsSNPs were submitted as input. Thresholds for deleterious predictions were as follows: PolyPhen-2 score ≥0.85 (probably damaging); SNPs&GO probability >0.5 (disease); PhD-SNP prediction as "disease"; SIFT score ≤0.05 (affecting protein function); PANTHER subPSEC ≤-3 (probably damaging). Only nsSNPs predicted as deleterious by all five tools were advanced for further analysis.

Functional Analysis of NOS3 Variants

MutPred2 (version 2.0) was used to predict the molecular mechanisms underlying structural and functional alterations caused by the selected nsSNPs in the NOS3 protein (25). The FASTA sequence and amino acid changes were input, with scores >0.5 considered potentially harmful and >0.75 indicating high-confidence damaging effects.

Stability Analysis

The impact of nsSNPs on NOS3 protein stability was assessed using I-Mutant (version 2.0) (22) and MUpro (version 1.1) (26). The NOS3 protein sequence was submitted, with parameters set to physiological conditions (temperature 25°C, pH 7.0). Stability changes were quantified by the change in Gibbs free energy (ΔΔG), where ΔΔG <0 indicates decreased stability and ΔΔG >0 indicates increased stability.

Evolutionary Conservation Analysis

ConSurf (version 2016) was utilized to evaluate the evolutionary conservation of amino acid positions in the NOS3 protein (27, 28). The FASTA sequence was submitted, and conservation scores were calculated on a scale of 1 (variable) to 9 (highly conserved), using default Bayesian inference and multiple sequence alignment parameters.

Post-Translational Modification (PTM) Site Prediction

Potential PTMs in the NOS3 protein were predicted using GPS-MSP (version 3.0) for methylation (29) and a combination of GPS (version 6.0) (30) and NetPhos (version 3.1) (31) for phosphorylation at serine, threonine, and tyrosine residues. The FASTA sequence and variant positions were input, with phosphorylation thresholds set at >0.5 for NetPhos (predicted phosphorylated) and default scores for GPS.

3D Structure Prediction and Comparison

The three-dimensional (3D) structures of wild-type and mutant NOS3 proteins were modeled using I-TASSER (version 5.2) (32). The FASTA sequence and amino acid substitutions were submitted to generate models. Structural similarity between wild-type and mutant models was evaluated using TM-align (version 20220412) (33), with template matching (TM) scores (range 0–1, where 1 indicates perfect alignment) and root mean square deviation (RMSD) values calculated to quantify deviations (higher RMSD indicates greater structural variation).

Protein-Protein and Gene-Gene Interaction Networks

Protein-protein interactions involving NOS3 were analyzed using STRING (version 12.0) (34), with the protein identifier submitted to generate a network based on experimental, database, and text-mining evidence (confidence score threshold ≥0.7 for high-confidence interactions). Gene-gene interactions, including co-expression, co-localization, shared domains, and pathways, were explored using GeneMANIA (version 3.6.0) (35), with NOS3 as the query gene and default weighting methods applied.

RESULTS

A total of 38,669 single-nucleotide polymorphisms (SNPs) associated with the NOS3 gene were retrieved from the Ensembl database (Release 114, May 2025). Among these, 614 were classified as missense (nsSNPs), 1,564 as synonymous, 211 as 5' untranslated region

(UTR) variants, 705 as 3' UTR variants, 1,636 as non-coding transcript exon variants, and 23,464 as intronic. The remaining variants fell into other categories (Figure 1).

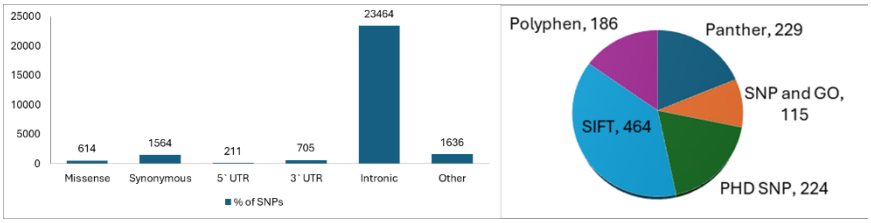

Figure 1 & 2. Distribution of SNPs in the NOS3 gene, with intronic variants most abundant (23,464) and 5′ UTR least (211), and deleterious nsSNPs predicted by SIFT (464), PANTHER (229), PhD-SNP (224), PolyPhen-2 (186), and SNPs&GO (115).

Identification of Deleterious nsSNPs

The 614 nsSNPs were screened using five bioinformatics tools to predict deleterious effects on NOS3 protein structure and function. PolyPhen-2 identified 186 as probably damaging (score ≥0.85), 77 as possibly damaging (0.5 < score < 0.85), and 351 as benign (score ≤0.5). PhD-SNP classified 224 as disease-related and 390 as neutral. SIFT predicted 464 as damaging (tolerance score ≤0.05) and 150 as tolerated (score >0.05). SNPs&GO identified 115 as disease-associated (probability >0.5) and 499 as neutral. PANTHER classified 229 as probably damaging (subPSEC ≤-3), 357 as possibly damaging, and 95 as probably benign (Figure 2).

To enhance prediction robustness, only nsSNPs deemed deleterious by all five tools were selected, resulting in 30 variants (approximately 4.9% of the 614 nsSNPs). These represent the high-confidence intersection of predictions across tools, minimizing false positives from individual algorithm biases (Table 1). No formal sensitivity analysis was conducted, but the multi-tool concordance approach aligns with established practices for reducing prediction variability.

Table 1. Identification of 30 nsSNPs predicted as deleterious by all five tools.

SNP ID	Allele Change	Amino Acid Change	SNPs&GO (Probability >0.5: Disease)	PANTHER (Probably Damaging)	SIFT (≤0.05: Affecting Function)	PolyPhen-2 (≥0.85: Probably Damaging)	PhD-SNP (Disease Prediction Score)
rs753853899	G>A	R179H	Disease (0.509)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (1)
rs1315177061	G>A>C	R183P	Disease (0.635)	Probably Damaging (0.85)	Affecting (0.01)	Probably Damaging (1)	Disease (7)
rs760308142	G>A	G222D	Disease (0.648)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (0.998)	Disease (2)
rs1563215842	T>G	W244G	Disease (0.544)	Probably Damaging (0.85)	Affecting (0.01)	Probably Damaging (1)	Disease (5)
rs1423301071	C>A	Q247K	Disease (0.674)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (6)
rs368296624	C>T	R250C	Disease (0.649)	Probably Damaging (0.78)	Affecting (0)	Probably Damaging (1)	Disease (2)
rs1204347173	G>T	G278V	Disease (0.579)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (5)
rs772146460	C>T	P334L	Disease (0.528)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (2)
rs201023253	T>G	Y357D	Disease (0.818)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (2)
rs751192053	A>G	Y357C	Disease (0.831)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (1)
rs41508746	C>G>T	R372G	Disease (0.843)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (4)
rs759717803	G>A	R372H	Disease (0.834)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (2)
rs1265731365	A>G	N374S	Disease (0.711)	Probably Damaging (0.85)	Affecting (0.01)	Probably Damaging (0.998)	Disease (6)
rs1312085340	T>C	L393P	Disease (0.768)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (3)
rs1356457482	A>G	K395E	Disease (0.694)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (1)
rs1266365289	G>A	A398T	Disease (0.803)	Probably Damaging (0.86)	Affecting (0.01)	Probably Damaging (0.998)	Disease (2)
rs1370195438	A>C>G	T416P	Disease (0.781)	Probably Damaging (0.85)	Affecting (0.01)	Probably Damaging (1)	Disease (7)
rs1312402859	C>T	T416I	Disease (0.722)	Probably Damaging (0.85)	Affecting (0.03)	Probably Damaging (0.995)	Disease (3)
rs371369842	C>G>T	H421Q	Disease (0.854)	Probably Damaging (0.85)	Affecting (0.02)	Probably Damaging (1)	Disease (4)
rs563793183	G>A	E434K	Disease (0.774)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (0)
rs369543769	G>A>C>T	G440C	Disease (0.688)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (0.988)	Disease (1)

SNP ID	Allele Change	Amino Acid Change	SNPs&GO (Probability >0.5: Disease)	PANTHER (Probably Damaging)	SIFT (≤0.05: Affecting Function)	PolyPhen-2 (≥0.85: Probably Damaging)	PhD-SNP (Disease Prediction Score)
rs371990302	C>T	S453L	Disease (0.903)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (2)
rs748837695	C>A>T	S455R	Disease (0.825)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (6)
rs774225083	C>T	P471L	Disease (0.812)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (6)
rs1402465169	G>T	W480C	Disease (0.731)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (1)
rs1311885654	T>C	V503A	Disease (0.373)	Probably Damaging (0.85)	Affecting (0.05)	Probably Damaging (1)	Disease (1)
rs1211937328	A>G	Y524C	Disease (0.643)	Probably Damaging (0.86)	Affecting (0)	Probably Damaging (1)	Disease (2)
rs757144684	G>A	E527K	Disease (0.784)	Probably Damaging (0.85)	Affecting (0.01)	Probably Damaging (1)	Disease (2)
rs148554851	G>A	E564K	Disease (0.756)	Probably Damaging (0.85)	Affecting (0.01)	Probably Damaging (0.999)	Disease (3)
rs1248252681	G>A	S572N	Disease (0.604)	Probably Damaging (0.85)	Affecting (0)	Probably Damaging (1)	Disease (1)

Legend: SNPs&GO probabilities >0.5 predict disease association; PANTHER scores ≥0.85 indicate probably damaging; SIFT scores ≤0.05 suggest effects on protein function; PolyPhen-2 scores ≥0.85 denote probably damaging; PhD-SNP scores predict disease likelihood (higher values indicate stronger prediction). All listed nsSNPs met deleterious criteria across tools.

Protein Stability Analysis of nsSNPs in NOS3

The stability effects of the 30 predicted deleterious nsSNPs were evaluated using I-Mutant and MUpro. Most variants were predicted to decrease protein stability (ΔΔG <0), potentially disrupting NOS3 folding or function. However, G440C, S453L, P471L, and Y524C showed increased stability by I-Mutant, while T416I and P471L showed increases by MUpro (Table 2). These predictions suggest variable impacts, with no confidence intervals calculated due to deterministic tool outputs.

Table 2. Predicted effects of nsSNPs on NOS3 protein stability by I-Mutant and MUpro.

No.	SNP ID	Allele Change	Amino Acid Change	I-Mutant Prediction (ΔΔG)	MUpro Prediction (ΔΔG)
1	rs753853899	G>A	R179H	Decrease (-0.15)	Decrease (-0.976)
2	rs1315177061	G>A>C	R183P	Decrease (-0.84)	Decrease (-0.933)
3	rs760308142	G>A	G222D	Decrease (-0.61)	Decrease (-0.669)
4	rs1563215842	T>G	W244G	Decrease (-1.83)	Decrease (-1.628)
5	rs1423301071	C>A	Q247K	Decrease (-1.83)	Decrease (-1.156)
6	rs368296624	C>T	R250C	Decrease (-0.77)	Decrease (-0.353)
7	rs1204347173	G>T	G278V	Decrease (-0.97)	Decrease (-0.245)
8	rs772146460	C>T	P334L	Decrease (-1.97)	Decrease (-0.173)
9	rs201023253	T>G	Y357D	Decrease (-1.83)	Decrease (-0.623)
10	rs751192053	A>G	Y357C	Decrease (-1.19)	Decrease (-0.513)
11	rs41508746	C>G>T	R372G	Decrease (-1.05)	Decrease (-1.430)
12	rs759717803	G>A	R372H	Decrease (-1.29)	Decrease (-1.158)
13	rs1265731365	A>G	N374S	Decrease (-1.05)	Decrease (-1.034)
14	rs1312085340	T>C	L393P	Decrease (-0.62)	Decrease (-2.706)
15	rs1356457482	A>G	K395E	Decrease (-0.74)	Decrease (-0.659)
16	rs1266365289	G>A	A398T	Decrease (-2.74)	Decrease (-1.287)
17	rs1370195438	A>C>G	T416P	Decrease (-1.33)	Decrease (-1.229)
18	rs1312402859	C>T	T416I	Decrease (-0.8)	Increase (0.120)
19	rs371369842	C>G>T	H421Q	Decrease (-0.67)	Decrease (-0.676)
20	rs563793183	G>A	E434K	Decrease (-0.45)	Decrease (-1.424)
21	rs369543769	G>A>C>T	G440C	Increase (0.48)	Decrease (-0.308)
22	rs371990302	C>T	S453L	Increase (1.13)	Decrease (-0.036)
23	rs748837695	C>A>T	S455R	Decrease (-1.73)	Decrease (-1.002)
24	rs774225083	C>T	P471L	Increase (0.46)	Increase (0.212)
25	rs1402465169	G>T	W480C	Decrease (-0.49)	Decrease (-0.868)
26	rs1311885654	T>C	V503A	Decrease (-3.23)	Decrease (-1.905)
27	rs1211937328	A>G	Y524C	Increase (0.80)	Decrease (-0.753)
28	rs757144684	G>A	E527K	Decrease (-1.29)	Decrease (-0.473)
29	rs148554851	G>A	E564K	Decrease (-1.3)	Decrease (-1.385)
30	rs1248252681	G>A	S572N	Decrease (-0.19)	Decrease (-0.686)

Legend: ΔΔG <0 predicts decreased stability; ΔΔG >0 predicts increased stability. Values are in kcal/mol.

Functional Analysis of NOS3 Variants

MutPred2 predicted potential functional disruptions for the 30 nsSNPs, with scores >0.75 indicating high-confidence damaging effects for most variants (e.g., R372G: 0.953). These scores suggest possible alterations in molecular mechanisms, such as loss of catalytic sites or changed interactions, though experimental validation is required (Table 3).

Table 3. MutPred2 scores for the 30 predicted deleterious nsSNPs.

Amino Acid Change	MutPred2 Score (>0.75: Damaging)	High-Confidence	Amino Acid Change	MutPred2 Score	Amino Acid Change	MutPred2 Score

R179H	0.890	R372G	0.953	G440C	0.833
R183P	0.934	R372H	0.919	S453L	0.783
G222D	0.940	N374S	0.818	S455R	0.859
W244G	0.872	L393P	0.949	P471L	0.804
Q247K	0.934	K395E	0.776	W480C	0.762
R250C	0.862	A398T	0.780	V503A	0.685
G278V	0.897	T416P	0.818	Y524C	0.816
P334L	0.933	T416I	0.752	E527K	0.808
Y357D	0.952	H421Q	0.794	E564K	0.865
Y357C	0.900	E434K	0.827	S572N	0.923

Legend: Scores >0.5 indicate potentially harmful; >0.75 high-confidence damaging.

Evolutionary Conservation Analysis of NOS3 by ConSurf

ConSurf analysis revealed that 18 of the 30 nsSNPs (e.g., R183P, W244G, S572N) occur at highly conserved positions (score 9), suggesting these sites are functionally important and substitutions may disrupt protein activity (Figure 3).

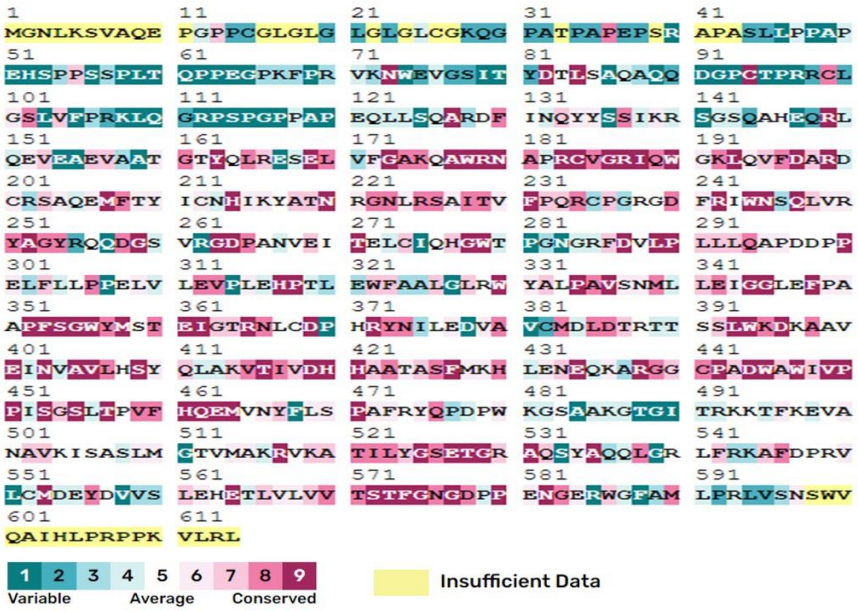

Figure 3: ConSurf output showing conservation scores for NOS3 amino acids, with deleterious nsSNP positions highlighted in highly conserved regions (red/magenta, scores 7-9).

3D Modeling of NOS3 Protein

I-TASSER generated 3D models for wild-type and mutant NOS3 proteins based on the 30 nsSNPs. TM-align comparisons showed variable structural deviations, with TM scores <1 and RMSD >1 Å for most, predicting potential conformational changes (e.g., R372H: TM 0.224, RMSD 7.95) (Table 4; Figure 4).

Table 4. TM-align scores and RMSD values for selected predicted deleterious nsSNPs relative to wild-type NOS3.

Amino Acid Change	TM Score (0-1: Alignment Quality)	RMSD (Å: Structural Deviation)
R179H	0.804	3.48
R183P	0.755	3.02
G222D	0.844	3.50
W244G	0.833	3.80
Q247K	0.848	3.50
R250C	0.753	3.04
G278V	0.722	2.67
P334L	0.706	1.62
Y357D	0.705	1.40
Y357C	0.706	1.39
R372G	0.722	2.73
R372H	0.224	7.95
N374S	0.222	8.36
L393P	0.221	7.49
S572N	0.710	1.77
T416P	0.221	7.58

Legend: TM scores closer to 1 indicate better alignment; higher RMSD values suggest greater structural differences. Only nsSNPs with notable deviations are shown.

GeneMANIA analysis indicated that NOS3 interacts with other genes primarily through physical interactions (77.84%), co-expression (8.01%), predicted associations (5.37%), co-localization (3.63%), genetic interactions (2.87%), and pathways (1.88%). The interaction networks highlight how NOS3 is functionally connected with several regulatory partners through diverse interaction modes. In one

network, genes such as NOSIP, NOSTRIN, and CAV1 emerged as major nodes, with connections color-coded to represent interaction categories including physical interactions, co-expression, co-localization, genetic associations, and shared protein domains. The majority of these were physical interactions, reflecting direct binding relationships.

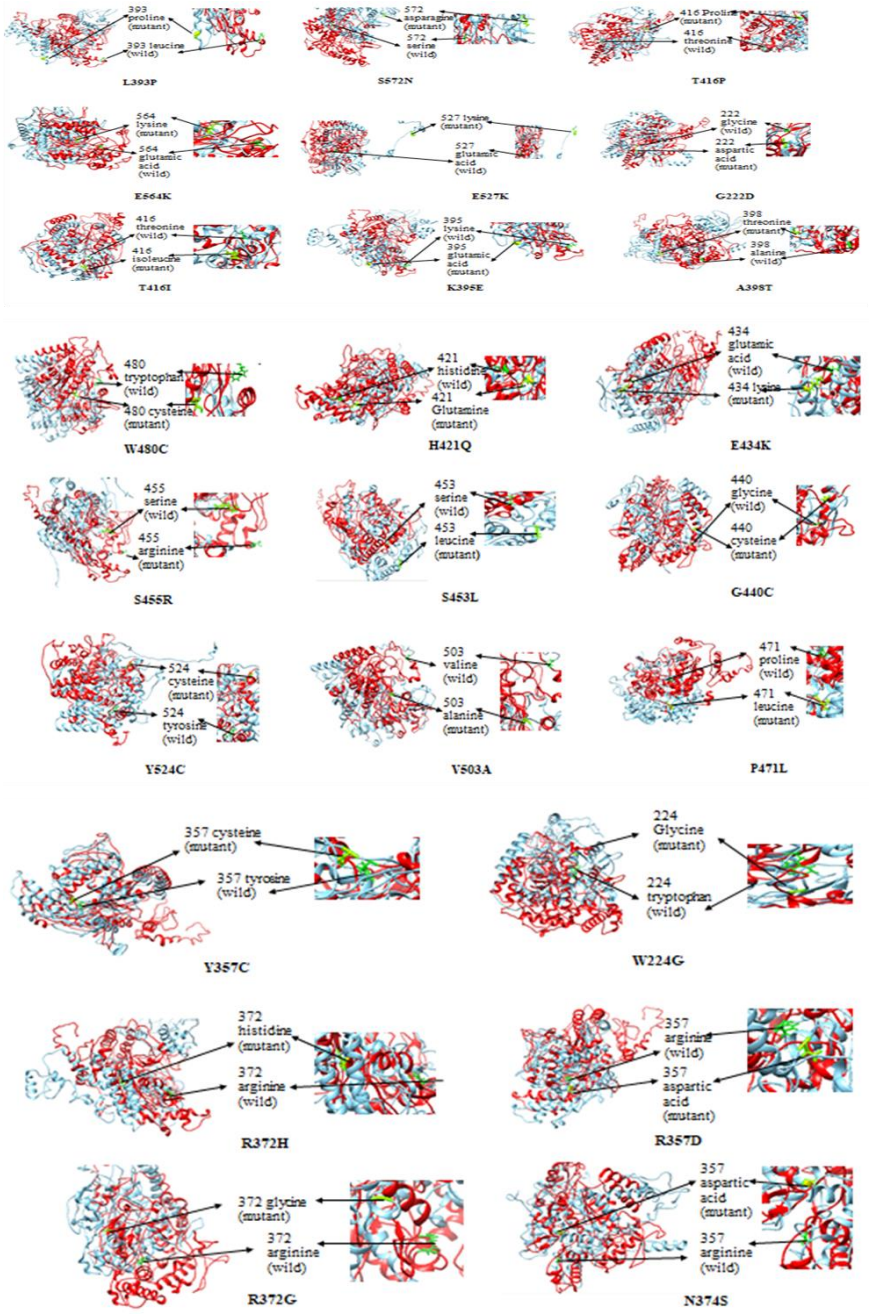

Figure 4: Gallery of 3D structural models comparing wild-type and mutant NOS3 proteins for selected nsSNPs, highlighting changes in conformation (red: mutant, blue: wild-type)

A complementary analysis further revealed extensive protein-protein associations of NOS3 with calmodulin family members (CALM3, CALML4, CALML5, CALML6) and molecular chaperones such as HSP90. These high-confidence edges underscore the central role of NOS3 within endothelial nitric oxide signaling and its dependence on calcium/calmodulin and chaperone-mediated regulation. Together, the networks emphasize NOS3 as a hub protein integrated into diverse signaling and regulatory modules.

STRING analysis generated a network with 11 nodes and 49 edges (average node degree 8.91, clustering coefficient 0.952, PPI enrichment p-value <0.001), predicting functional associations with proteins like CAV1 and AKT1. GPS-MSP identified no methylation sites in NOS3. For phosphorylation, NetPhos predicted 4 sites (2 serine, 2 threonine) above threshold (>0.5), while GPS identified 48 potential sites (33% tyrosine, 40% serine, 27% threonine). Common sites from both tools are listed (Table 5; Figure 5).

The analysis shows that all phosphorylation events in NOS3 are attributed to a single kinase family, with serine residues representing the largest share of predicted phosphorylation sites (40%), followed by tyrosine (33%) and threonine (27%). When comparing structural contexts, serine phosphorylation is more frequent in disordered regions, whereas tyrosine and threonine sites predominate in ordered regions, indicating residue-specific preferences linked to protein structural organization.

Table 5. Common phosphorylation sites in NOS3 predicted by GPS 6.0 and NetPhos 3.1.

Residue Type	Position	NetPhos Score (>0.5: Phosphorylated)	Kinase (NetPhos)	GPS Peptide Sequence	Kinase (GPS)	GPS Score
Threonine	60	0.556	DNAPK	SPPSSPLTQPPEGPK	AGC	0.045
Threonine	495	0.971	Unsp	TGITRKKTFKEVANA	CAMK	0.095
Serine	85	0.571	PKC	SITYDTLSAQAQQDG	CK1	0.211
Serine	125	0.628	ATM	PAPEQLLSQARDFIN	CK1	0.126

Legend: Scores indicate prediction confidence; kinases are predicted modifiers.

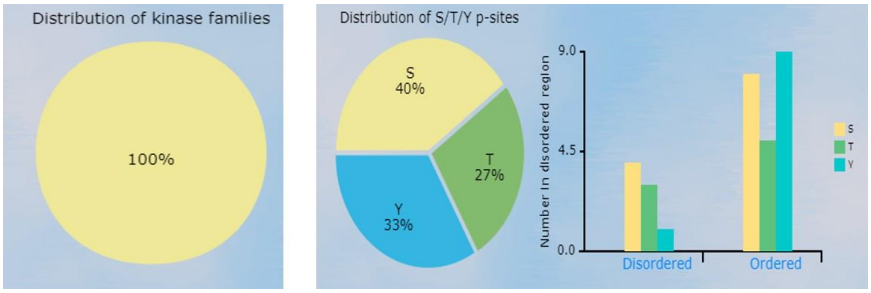

Figure 5: Diagrams showing distribution of kinase families and S/T/Y phosphorylation sites in ordered/disordered regions of NOS3.

DISCUSSION

This in silico study systematically screened 614 non-synonymous single-nucleotide polymorphisms (nsSNPs) in the NOS3 gene using multiple bioinformatics tools, identifying 30 variants predicted to be deleterious across all five primary screening algorithms (SIFT, PolyPhen-2, PhD-SNP, SNPs&GO, and PANTHER). These high-confidence predictions were further supported by assessments of protein stability (via I-Mutant and MUpro), functional impacts (MutPred2), evolutionary conservation (ConSurf), structural deviations (I-TASSER and TM-align), and interaction networks (GeneMANIA and STRING). Notably, most variants were forecasted to decrease protein stability, occur in conserved regions, and induce structural changes, potentially disrupting NOS3 function. However, these computational predictions inherently carry uncertainties, such as algorithm-specific biases and false-positive rates, emphasizing the need for empirical validation to confirm causality (15, 16).

Our findings align with and extend prior computational and experimental research on NOS3 variants in hypertension and related disorders. For instance, similar in silico approaches have linked specific NOS3 nsSNPs, like c.894G>T (rs1799983), to altered endothelial nitric oxide synthase (eNOS) activity and increased risk of metabolic syndrome, with predictions of disrupted protein interactions in the eNOS oxygenase domain (36). Consistent with this, population-based studies have associated NOS3 polymorphisms, including rs1799983 and rs3918188, with essential and resistant hypertension across diverse ethnic groups (7, 11, 37). Experimental evidence from NOS3-knockout models further corroborates these associations, demonstrating vascular abnormalities and impaired vasodilation due to reduced nitric oxide (NO) production (10). In African populations, bioinformatics analyses of hypertension-related genes, including NOS3, have highlighted shared pathways involving endothelial dysfunction, reinforcing the gene's role in multifactorial disease etiology (38). While our comprehensive screening of all 614 nsSNPs addresses a gap in prior studies, which often focused on select variants (e.g., rs1799983 or rs7830), the multi-tool concordance here enhances prediction reliability compared to single-algorithm approaches (13, 14, 39).

Biologically, the 30 predicted deleterious nsSNPs may influence NOS3-encoded eNOS by altering protein stability, conserved residues, or interaction interfaces, potentially impairing NO synthesis—a critical regulator of vascular tone, platelet aggregation, and lipoprotein oxidation (7, 8). For example, variants like R372G and L393P, with high MutPred2 scores (>0.9), are forecasted to induce gain of acetylation or loss of methylation, which could disrupt enzymatic activity or binding to partners such as CAV1 and AKT1, as evidenced by STRING networks. In conserved regions (ConSurf scores 7–9), substitutions like W244G or S572N might compromise the enzyme's catalytic domain, contributing to endothelial dysfunction and hypertension pathogenesis (9, 12). Clinically, these predictions suggest a possible role in elevating disease susceptibility, particularly in populations with high hypertension prevalence, such as in Pakistan (4, 5). However, such links remain associative, as in silico models cannot fully capture post-translational or environmental modifiers (18).

Despite these insights, several limitations must be acknowledged. Predictive tools like SIFT and PolyPhen-2 exhibit accuracies of approximately 70–80%, with potential overprediction of deleterious effects due to reliance on sequence conservation and evolutionary assumptions (20, 23). Dataset biases in Ensembl, primarily derived from reference genomes, may underrepresent population-specific variants, limiting generalizability to diverse ethnicities (19). Additionally, the absence of wet-lab validation precludes definitive conclusions on functional impacts, and no sensitivity analyses were performed to test threshold variations. Phosphorylation predictions, while informative, overlook dynamic cellular contexts. Future research should prioritize experimental validation, such as site-directed mutagenesis and NO production assays in endothelial cell lines, to assess the functional consequences of these 30 nsSNPs (10). Genome-wide association studies (GWAS) in high-risk cohorts, like Pakistani populations, could evaluate their prevalence and associations with hypertension phenotypes (4, 40). Refining computational pipelines with machine learning integration may also improve prediction precision (16). Overall, this study contributes to the genetic landscape of hypertension, underscoring NOS3 as a promising target for personalized risk assessment and therapeutic strategies. The limitations of this study encompass several key areas. Predictive tools like SIFT and PolyPhen-2, while valuable, have accuracy limitations and may overpredict deleterious effects due to their reliance on sequence conservation and evolutionary assumptions. Dataset biases in Ensembl, primarily derived from reference genomes, potentially

underrepresent population-specific variants, which could limit the generalizability of findings to diverse ethnicities. The absence of wet-lab validation and sensitivity analyses further constrains the ability to draw definitive conclusions about functional impacts and the robustness of results to threshold variations. Additionally, while phosphorylation predictions provide useful insights, they do not fully capture the dynamic cellular contexts in which these processes occur.

To address these limitations and advance the field, future research directions are proposed. Experimental validation, such as site-directed mutagenesis and NO production assays in endothelial cell lines, is crucial to assess the functional consequences of the identified nsSNPs. Genome-wide association studies in high-risk cohorts, particularly in populations like Pakistan with elevated hypertension risk, could evaluate the prevalence of these variants and their associations with hypertension phenotypes. Refining computational pipelines through the integration of machine learning techniques may improve prediction precision. Despite these limitations, this study makes a significant contribution to understanding the genetic landscape of hypertension, highlighting NOS3 as a promising target for personalized risk assessment and therapeutic strategies in the future.

CONCLUSION

The in silico analysis of 614 non-synonymous single-nucleotide polymorphisms (nsSNPs) in the NOS3 gene successfully identified 30 variants predicted to be deleterious, aligning with the study's objective to characterize potentially pathogenic variants using bioinformatics tools, thereby laying a foundation for understanding their role in hypertension risk. These findings suggest that such nsSNPs may disrupt endothelial nitric oxide synthase (eNOS) function, potentially contributing to cardiovascular diseases, with implications for human healthcare through enhanced genetic risk stratification. Clinically, these insights could guide personalized screening and management strategies for at-risk populations, while research implications include the urgent need for experimental validation through functional assays and population-based studies to confirm these predictions and refine therapeutic targets, advancing the field toward precision medicine in hypertension management.

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