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AHNAK2 Polymorphisms rs2582513 and rs10145032 Are Associated with Rivaroxaban Pharmacodynamics and Bleeding Risk: A Two-Stage Multicenter Cohort Study

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Abstract

Purpose

Existing studies have investigated how genetic variations in drug- metabolizing enzymes and transporters affect rivaroxaban concentrations, but the relationship between these variants and bleeding events is unclear. Moreover, other genes related to pharmacodynamics and bleeding risks need further investigation. Additionally, identifying specific genetic loci in healthy populations and validating their clinical impact are key challenges this study faces.

Method

A multicenter prospective cohort study was conducted to detect genetic polymorphisms and analyze their pharmacodynamic effects on rivaroxaban in healthy individuals. The study then validated these findings by examining clinical outcomes in patients with non-valvular atrial fibrillation (NVAF).

Results

The findings revealed that specific mutations in the AHNAK nuclear protein 2 *(AHNAK2*) gene, notably *rs2582513* and *rs10145032*, were correlated with a blunted prolongation of prothrombin time (PT) in healthy individuals undergoing rivaroxaban treatment (p = 0.0003, 0.0004). The ROC curve was employed to assess the predictive capacity of these two single nucleotide polymorphisms (SNPs) for prothrombin time, yielding an area under the curve (AUC) of 0.93. Additionally, these mutations were associated with an elevated risk of bleeding events in clinical patients with atrial fibrillation (AF). When compared to GA/GC and AA carriers, subjects with the GG genotype for *AHNAK2 rs2582513/rs10145032* exhibited a statistically significant increase in bleeding events, with a p of 0.03. The ROC curve's AUC for these two SNPs, *rs2582513* and *rs10145032*, on the *AHNAK2* gene in forecasting bleeding events was 0.85. Combined analysis with *ABCB1 rs1045642* and *CYP3A5 rs776746* improved the AUC for bleeding event prediction to 0.89.

Conclusion

This study reveals for the first time that mutations in the *AHNAK2* gene represent a hypothesis-generating predictive biomarker candidate for anticoagulation therapy. Individuals carrying the *GG* genotype exhibited weaker PT prolongation after receiving rivaroxaban, yet were associated with higher bleeding risks in clinical patients. These findings should be considered hypothesis-generating and warrant further validation in independent cohorts and functional studies before clinical application.

1 Introduction

Direct oral anticoagulants (DOACs), such as rivaroxaban, are primarily used to treat high-risk patients with conditions like atrial fibrillation (AF) and venous thromboembolism(1–3). Rivaroxaban functions by inhibiting factor Xa and blocking the coagulation cascade pathway. Although it reduces the occurrence of ischemic events, it also elevates the risk of bleeding events during clinical use(4). Recent data indicates that major bleeding affects approximately 6.62 DOAC users per 100 treatment years, with such incidents anticipated to be more prevalent in emergency departments(5). Studies have investigated the impact of genetic variations in drug metabolizing enzymes and transporters on rivaroxaban's efficacy in AF patients, with results suggesting a correlation between *ABCB1* SNP and rivaroxaban concentration levels(6, 7). However, these genetic variations have not been definitively associated with bleeding events, and other genes, beyond those involved in drug metabolism and transport, have not been thoroughly examined in relation to pharmacodynamics and bleeding risks.

While existing studies have investigated how genetic variations in drug-metabolizing enzymes and transporters (e.g., *ABCB1, CYP3A5*) affect rivaroxaban concentrations, the relationship between these variants and bleeding events remains inconsistent. Moreover, genes outside pharmacokinetic pathways, particularly those influencing pharmacodynamics, require further exploration to improve risk prediction. This study aims to address this gap by employing a two-stage design to explore and identify potential genetic loci associated with rivaroxaban response, providing hypothesis-generating evidence for subsequent pharmacogenomic research.

2 Study Methods

2.1 Study population

Healthy subjects

The study enrolled Chinese participants aged 18 to 45 with a body mass index (BMI) ranging from 18 to 26 kg/m² to receive a single dose of rivaroxaban at 10, 15, or 20 mg. Participants were administered the reference product, Xarelto (rivaroxaban), in either a fed or fasting state, depending on their assigned sequence. Participants had no history of coagulation disorders, chronic diseases (e.g., hypertension, diabetes), smoking habits, or alcohol consumption. The gender distribution was 98 males (41.3%) and 146 females (58.7%) across all dose groups. Data from the reference group was analyzed in this study, which was conducted at various research centers, including Peking University First Hospital, Beijing Huilongguan Hospital, the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine, the Affiliated Hospital of Qingdao University, and the First Affiliated Hospital of Nanchang University. Subjects were discharged 48 to 72 hours after receiving rivaroxaban.

Clinical patients

The study included adults aged ≥ 18 years with non-valvular atrial fibrillation (NVAF) who were prescribed rivaroxaban for thromboprophylaxis. Patients were excluded if they had conditions such as immunodeficiency diseases, viral hepatitis, or severe liver or renal abnormalities, if they had taken specific medications within 14 days prior to initiating rivaroxaban, or if they had contraindications such as an allergy to rivaroxaban, a recent bleeding history, recent major surgery, or a reluctance to adhere to medication schedules or blood donation regulations. The rivaroxaban dosage was 10 mg/day (n = 89), 15 mg/day (n = 112), and 20 mg/day (n = 56). The median CHA_2DS_2-VASc score was 3 (interquartile range: 2–4), and the median HAS-BLED score was 2 (interquartile range: 1–3). Concurrent medications included statins (n = 124, 48.2%), antiplatelet drugs (n = 37, 14.4%), and antihypertensive drugs (n = 158, 61.5%).

The study's flow chart is depicted in Fig. 1. The research adhered to clinical practice guidelines and the Declaration of Helsinki, with the protocol receiving approval from the independent Ethics Committee and the Institutional Review Board of Peking University First Hospital, as well as all sub-central hospitals involved in the study.

2.2 Sample collection

In healthy individuals, pharmacodynamic (PD) parameters were assessed at specific time intervals (0h, 3h, 8h, and 12h) following drug administration, with venous blood samples obtained for genetic testing prior to drug intake. In clinical patients, PD parameters were evaluated once the drug had reached a steady-state concentration (after more than 48h of rivaroxaban intake), with blood samples taken at peak concentration (3h post-rivaroxaban administration) for DNA extraction and genotyping.

Plasma samples were promptly frozen at -70°C within six months of collection for subsequent analysis.

2.3 Sample detection

PD analysis

The PD parameters, including activated partial thromboplastin time (APTT) and prothrombin time (PT), were assessed using the Sysmex CS-2100i automatic multi-parameter analyzer, manufactured by Sysmex in Japan. The APTT and PT values were determined using validated coagulation test kits, namely Thromborel-S and Actin, produced by Siemens Healthineers in Germany. The measurement range for these parameters is 0-500 ng/mL, with an intra-assay coefficient of variation of 1.5% and an inter-assay coefficient of variation of 2.3%. Rivaroxaban calibrators and controls were used to ensure the accuracy and quality of the tests conducted.

Genetic testing

The SureSelect target enrichment system, in conjunction with the Illumina NovaSeq 6000 sequencer from Illumina in the United States, was employed for SNP detection. Following genotyping, a rigorous quality control procedure was applied to both the samples and genetic markers. SNPs meeting the criteria of a missing rate of less than 10%, a minor allele frequency (MAF) exceeding 5%, and a Hardy-Weinberg equilibrium (HWE) p-value greater than 10^{-6} were selected for subsequent analyses. Genotyping data for *ABCB1 rs1045642* and *CYP3A5 rs776746* were also obtained for combined analysis.

2.4 Study endpoint

Patients are monitored via telephone follow-ups or regular outpatient clinic visits at intervals of 1 month, 6 months, and 1 year post-clinical enrollment. The primary safety measure focuses on occurrences of bleeding, encompassing both major and minor bleeding events as per the classification system established by the Bleeding Academic Research Consortium(BARC). Major bleeding was defined as intracranial bleeding, gastrointestinal bleeding requiring hospitalization, or bleeding leading to a hemoglobin decrease ≥ 2 g/dL; minor bleeding included epistaxis, gingival bleeding, and ecchymosis. Secondary outcomes include incidents of stroke or systemic embolic events. A microembolism occurring outside the central nervous system, resulting in acute ischemia of various body parts such as limbs, kidneys, and visceral organs, is identified through the sudden onset of localized pain accompanied by symptoms like cold, pulseless limbs, or hematuria. Confirmation of this condition is achieved through diagnostic procedures such as angiography, ultrasound, or computed tomography.

2.5 Statistical analysis

Statistical analyses were conducted using the Social Sciences 21.0 software (IBM, Armonk, USA). Pearson correlation analysis was employed to examine relationships between variables. Descriptive statistics for continuous and categorical variables were presented as mean ± standard deviation (SD), frequency, and percentage. Logistic regression was utilized to evaluate the association between *AHNAK2* polymorphisms and binary bleeding outcomes (reported as odds ratios [OR] with 95% confidence intervals [CI]), while linear regression was used for continuous PT ratio. Both crude and adjusted models accounting for predefined confounders were performed. For the analysis of healthy subjects, confounders included age, gender, BMI, and fasting status. For NVAF patients, confounders were age, gender, BMI, CHA_2DS_2-VASc score, HAS-BLED score, rivaroxaban dosage, and concurrent use of antiplatelet drugs. Statistical significance was set at $p < 0.05$. The p-value was determined through the differential gene hypergeometric distribution test, while the Benjamini-Hochberg multiple test was used to calculate the false discovery rate. Gene-dose interaction analysis was performed to explore whether the effect of *AHNAK2* polymorphisms on PT varied by rivaroxaban dose. A post-hoc statistical power analysis for the primary outcome (association between *AHNAK2* polymorphisms and bleeding events) was conducted using G*Power 3.1, with a calculated power of 89% ($\alpha = 0.05$, effect size Cohen's $w = 0.20$, MAF = 0.35).

3 Results

3.1 Effect of AKNAK2 gene polymorphism in healthy subjects

The demographic attributes of the participants without health issues were similar across the various dosage groups, with the exception of age variations observed in the rivaroxaban 15 mg and 10 mg dosage cohorts, as indicated in Table 1.

Table 1
Population characteristics of the study population on rivaroxaban administration (N = 304).

Dose	Age (years)	BMI (kg/m²)	Crea (μmol/L)	ALT (U/L)	AST (U/L)	ALP (U/L)	HGB (g/L)	RBC (×10¹²/L)	TG (mmol/L)	TCHO (mmol/L)	LDL (mmol/L)	HDL (mmol/L)	Gender (M/F)
10 mg	34.96 ± 9.18 (185)	23.00 ± 1.84 (185)	67.60 ± 14.38 (185)	15.98 ± 9.13 (185)	18.31 ± 4.63 (185)	66.94 ± 17.10 (185)	146.23 ± 15.22 (185)	4.91 ± 0.49 (185)	1.26 ± 0.61 (185)	4.34 ± 0.75 (185)	2.54 ± 0.62 (56)	1.25 ± 0.22 (56)	52/133
15 mg	27.76 ± 5.95 (72)	22.66 ± 1.83 (72)	96.52 ± 14.83 (72)	17.58 ± 8.25 (72)	16.33 ± 3.63 (72)	63.13 ± 17.81 (72)	151.25 ± 12.73 (72)	4.96 ± 0.40 (72)	0.83 ± 0.30 (72)	4.20 ± 0.69 (72)	2.44 ± 0.59 (72)	1.38 ± 0.27 (72)	28/44
20 mg	25.68 ± 6.48 (47)	22.00 ± 1.99 (47)	72.88 ± 13.73 (47)	14.77 ± 7.27 (47)	18.57 ± 4.42 (47)	71.23 ± 16.71 (47)	148.34 ± 14.41 (47)	5.01 ± 0.47 (47)	1.04 ± 0.40 (47)	4.24 ± 0.66 (47)	2.44 ± 0.56 (47)	1.26 ± 0.28 (47)	18/29

Data are presented as mean ± SD (n). Body mass index (BMI), Serum creatinine (Crea), Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Hemoglobin concentration (HGB), Red blood cells (RBC), Triglycerides (TG), Total cholesterol (TCHO), Low density lipoprotein (LDL), High density lipoprotein (HDL), Male (M), Female (F).

Note: All baseline characteristics were balanced across genotype groups (all $p > 0.05$), except for age differences between dose groups as indicated. No significant differences were observed in other demographics or laboratory values.

This research examined the effect of genetic variation on the PT ratio at the 3-hour mark, utilizing the PLINK software with a significance threshold of $p < 0.005$. The study pinpointed two SNPs, *rs2582513* and *rs10145032*, situated on the *AHNAK2* gene, which markedly affected the PT ratio of rivaroxaban (with p of 0.0003 and 0.0004, as illustrated in Table 2 and Fig. 2A-2B). Participants possessing the *GG* genotype at the *rs2582513* locus of the *AHNAK2* gene demonstrated a lower PT ratio compared to those with *GA* and *AA* genotypes, with a p of 0.0003. Likewise, individuals with the *GG* genotype at the *rs10145032* locus of the *AHNAK2* gene also showed a reduced PT ratio in contrast to those with *GC* and *CC* genotypes, with a p of 0.0004. These results indicate that individuals with the *GG* genotype at the *rs2582513* and *rs10145032* loci of the *AHNAK2* gene undergo less alteration in PT after taking rivaroxaban, suggesting a hypercoagulable state and indicating a potential necessity for dosage modification in clinical practice.

Table 2
Effects of AHNAK2 gene polymorphisms on prothrombin time ratio in healthy subjects (N = 304).

Gene	SNP	P	A1	A2	Mean ± SD (A1A1)	Mean ± SD (A1A2)	Mean ± SD (A2A2)	GENO(n)
AHNAK2	rs2582513	0.0003	G	A	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	40/139/110
AHNAK2	rs10145032	0.0004	G	C	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	40/137/112
Prothrombin time (PT) ratio = PT at 3 h post-dose / baseline PT.								
A1: minor allele; A2: major allele; GENO: number of subjects with genotype A1A1/A1A2/A2A2.								
p-values were derived from linear regression analysis adjusted for age, gender, BMI, and fasting status.								

Furthermore, Receiver Operating Characteristic (ROC) curve analysis was employed to assess the predictive value of prothrombin levels based on the two SNPs, *rs2582513* and *rs10145032*, within the *AHNAK2* gene. The analysis revealed an Area Under the Curve (AUC) of 0.93, a sensitivity of 1.0, and a specificity of 0.66, indicating that the presence of the G allele could serve as a predictive marker for pharmacodynamic responses (as depicted in Fig. 2C).

It is worth noting that while *GG* carriers showed less PT prolongation, this finding in healthy volunteers did not directly translate to a reduced anticoagulant effect in clinical patients, as discussed in Section 4.2.

3.2 Validation of the role of AHNAK2 gene in clinical patients

A total of 257 patients diagnosed with NVAF were included in the study. The baseline characteristics of these patients can be observed in Table 3. All baseline variables were comparable across *AHNAK2* genotype groups (all $p > 0.05$). The research aimed to investigate the influence of genetic variations on clinical outcomes, specifically bleeding, embolism, and other events, following treatment with rivaroxaban in NVAF patients. The analysis revealed that two SNPs, namely *rs2582513* and *rs10145032* located on the *AHNAK2* gene, had a significant impact on bleeding events occurring one-month post-rivaroxaban administration. Individuals carrying the *GG* genotype of *AHNAK2 rs2582513* exhibited a notably higher incidence of bleeding events compared to those with *GA* and *AA* genotypes (adjusted OR for *GG* vs. *GA/AA* = 2.45, 95% CI: 1.12–5.38, p = 0.03; Fig. 3A, Table 4). Similarly, subjects with the *GG* genotype of *AHNAK2 rs10145032* also experienced increased bleeding events in contrast to *GC* and *CC* carriers(adjusted OR for *GG* vs. *GC/CC* = 2.43, 95% CI: 1.10–5.67, p = 0.03; Fig. 3B, Table 4). Long-term follow-up data showed that the association between GG genotype and bleeding events remained significant at 6 months (p = 0.02), and 1 year (p = 0.03) (Table 4).The study suggests that the *GG* genotype of *AHNAK2* gene *rs2582513* and *rs10145032* could serve as

potential predictive biomarkers for rivaroxaban-treated patients, offering valuable insights for personalized clinical management of NVAF patients. Although the association between *GG* genotype and elevated bleeding risk was statistically significant, the effect size was modest and the 95% confidence intervals were relatively wide, suggesting that these results need to be interpreted with caution.

Table 3
Baseline characteristics of patients with non-valvular atrial fibrillation (NVAF) (N = 257).

Characteristics	Value
Age (years), mean ± SD	69 ± 10
Gender (male), n (%)	133(51.8%)
BMI (kg/m²), mean ± SD	25.3 ± 3.8
CHA_2DS_2-VASc score, median (IQR)	3 (2–4)
HAS-BLED score, median (IQR)	2 (1–3)
Rivaroxaban dosage, n (%)	10 mg/day:89(34.6%); 15 mg/day:112(43.6%); 20 mg/day:56(21.8%)
Comorbid diseases, n (%)	
Hypertension	176(68.5%)
Diabetes	66(25.7%)
Hyperlipidemia	76(29.6%)
Coronary heart disease	86(33.5%)
Heart failure	36(14.0%)
Apoplexy	46(17.9%)
Concurrent medications, n (%)	
Statins	124(48.2%)
Antiplatelet drugs	37(14.4%)
Antihypertensive drugs	158(61.5%)
IQR: Interquartile range. These data confirm baseline balance across genotype groups (all p > 0.05).	

Table 4
Long-term follow-up results of bleeding events by AHNAK2 genotype in NVAF patients (N = 257).

Follow-up time	SNP	Genotype comparison	Bleeding event rate (%)	Adjusted OR (95%CI)	p
1 months	rs2582513	GG vs. GA/AA	23.8 vs. 10.8	2.45 (1.12–5.38)	0.03
1 months	rs10145032	GG vs. GC/CC	21.4 vs. 11.2	2.43 (1.10–5.67)	0.03
6 months	rs2582513	GG vs. GA/AA	28.6 vs. 12.3	2.71 (1.25–5.87)	0.02
6 months	rs10145032	GG vs. GC/CC	29.5 vs. 12.8	2.78 (1.28–6.04)	0.02
1 year	rs2582513	GG vs. GA/AA	32.1 vs. 14.5	2.63 (1.21–5.72)	0.03
1 year	rs10145032	GG vs. GC/CC	33.3 vs. 15.1	2.69 (1.23–5.87)	0.03
Bleeding events were defined according to BARC criteria (type 2, 3, or 5). OR: odds ratio; CI: confidence interval. Adjusted for age, gender, BMI, CHA_2DS_2-VASc score, HAS-BLED score, rivaroxaban dosage, and concurrent use of antiplatelet drugs. p-values were derived from logistic regression analysis.					

Furthermore, ROC curve analysis was used to evaluate the predictive capacity of the two SNPs, *rs2582513* and *rs10145032*, located on the *AHNAK2* gene, for bleeding events. The analysis revealed an AUC of 0.85, a sensitivity of 0.86, and a specificity of 0.56, indicating the potential of these genetic mutations as predictive markers for pharmacodynamic outcomes (refer to Fig. 3C). However, these models were derived from a single dataset without external validation. As such, the predictive accuracy may be overestimated, and independent replication in diverse populations is essential before any clinical application can be considered. These findings should therefore be interpreted as exploratory and hypothesis-generating.

3.3 Gene-dose interaction analysis

To explore whether the effect of *AHNAK2* genotype on PT varied by rivaroxaban dose, we performed an interaction analysis. Significant interaction between the *GG* genotype and reduced PT change was observable (p = 0.02, Supplementary Table S1): *GG* carriers had lower PT ratios in the 10 mg group (p < 0.001), attenuated differences in the 15 mg group (p = 0.003), and no difference in the 20 mg group (p = 0.06). This provides personalized dosing guidance (e.g., *GG* carriers may require lower doses). This combined analysis offers hypothesis-generating support for *AHNAK2* as a complementary biomarker to established pharmacogenetic markers.

3.4 Combined analysis with established pharmacogenetic markers

To evaluate the incremental value of *AHNAK2* beyond known DOAC-related genes, We performed combined analyses of *AHNAK2* with *ABCB1 rs1045642* and *CYP3A5 rs776746* (classic DOAC-related genes)(7, 8). The AUC for bleeding event prediction increased from 0.85 (*AHNAK2* alone) to 0.89 (combined), demonstrating *AHNAK2*'s incremental predictive value (Supplementary Table S2). This addresses single-marker limitations and supports *AHNAK2* as a complementary biomarker.

4 Discussion

The AHNAK2 protein, a member of the AHNAK protein family, is located within interphase nuclei and interacts with calcium channels to modulate the excitation and contraction of cardiomyocytes(9). To date, fewer than 100 scholarly articles have been identified through a search of the PubMed database, focusing on *AHNAK2* gene polymorphisms in the context of diagnosing and treating clinical diseases. These studies predominantly center on the gene's involvement in cancer(10–12). For instance, in papillary thyroid carcinoma (PTC), the most prevalent endocrine neoplasm with an increasing incidence, *AHNAK2* has been validated as a valuable biomarker for both the detection and prediction of outcomes in PTC cases. Furthermore, it has been shown to promote the progression of thyroid cancer by influencing pathways related to cell adhesion, cell junctions, and the immune system(13). Additionally, the *AHNAK2* gene exhibited a significant increase in expression levels within lung adenocarcinoma (LUAD) tumor tissues. The expression of *AHNAK2* was found to be associated with various clinical parameters, including disease stage, differentiation status, tumor size, and the presence of lymph node metastasis(10). *AHNAK2* has been demonstrated as a standalone prognostic factor for patients with lung adenocarcinoma (LUAD). Research has also identified *AHNAK2* as a novel prognostic indicator and oncogenic protein in cases of clear cell renal cell carcinoma(14), a potential novel biomarker for immune checkpoint inhibitors in non-small cell lung cancer(15), and a potential therapeutic target of adenocarcinomas(16). This is the first study to identify potential therapeutic targets and novel prognostic indicators within the field of antithrombotic therapy, through mutations in the *AHNAK2* gene.

4.1 Plausible biological mechanisms

The AHNAK protein, encoded by this gene, is a substantial structural scaffold protein with a molecular weight of 700 kDa and includes a central domain with 128 amino acid repeats. This protein is involved in various biological processes, including the formation of the blood-brain barrier, cellular architecture and movement, regulation of cardiac calcium channels, and the progression of tumors. An examination of the GeneCards database revealed that the *AHNAK* gene is active in multiple bodily systems, such as the blood, immune, nervous, skeletal muscle, endocrine, secretory, and reproductive systems. While *AHNAK2* has not been previously directly linked to coagulation, its established roles in calcium homeostasis and cell adhesion provide a plausible indirect mechanistic link. Based on the established roles of *AHNAK2* in calcium homeostasis and cell adhesion, we propose a speculative hypothesis that these polymorphisms may alter the binding affinity of *AHNAK2* to L-type calcium channels, disrupting intracellular calcium homeostasis in platelets (coagulation initiation) and hepatocytes (coagulation factor synthesis). Since calcium is essential for factor Xa activation (rivaroxaban's target), this dysregulation modulates pharmacodynamic responses and bleeding risk(17–19). This potential mechanistic link is entirely hypothetical, with no direct causal evidence from the present study, and requires further in vitro and in vivo functional experiments to verify.

Notably, the novelty of our findings is limited by the lack of prior evidence linking *AHNAK2* to coagulation. Combined analysis with established DOAC-related genes (*ABCB1, CYP3A5*) demonstrated that *AHNAK2* provides incremental predictive value, supporting its role as a complementary biomarker rather than a standalone target. This addresses the limitation of single-marker prediction and highlights the potential of multi-gene panels for personalized anticoagulation therapy.

4.2 An unresolved biological paradox between pharmacodynamic and clinical outcomes

An intriguing and unresolved biological paradox was observed in this study: the *AHNAK2 GG* genotype was associated with a blunted PT prolongation (suggesting a weaker anticoagulant effect) in healthy volunteers, but concurrently linked to a significantly higher bleeding risk in NVAF patients. This apparent discrepancymay be tentatively attributed to, but not verified by, the fundamental differences between the two cohorts. NVAF patients are typically older, have multiple comorbidities (e.g., hypertension, vascular fragility), and often take concomitant medications (e.g., antiplatelets) that independently increase bleeding risk. The pharmacodynamic measure in a controlled healthy setting may not fully capture the complex hemostatic balance in a diseased state. Thus, the *AHNAK2* genotype might predispose patients to bleeding through mechanisms not solely reflected by PT, such as affecting vascular integrity.

Notably, the current study data cannot elucidate the exact biological mechanism underlying this paradox, and this remains a key unresolved question that requires further in-depth research

4.3 Clinical implication and comparison with existing markers

Our two-stage study design translates the association of *AHNAK2* polymorphisms from a pharmacodynamic endpoint in healthy volunteers to a clinically relevant bleeding outcome in NVAF patients. This finding expands the pharmacogenomic landscape of rivaroxaban beyond genes primarily influencing pharmacokinetics (PK) to include those potentially modulating pharmacodynamics (PD).

Current pharmacogenetic research on rivaroxaban has predominantly focused on genes affecting its absorption, distribution, and metabolism. Preclinical studies have established the critical role of efflux transporters like *ABCB1 (MDR1)* in rivaroxaban's disposition(20). Correspondingly, clinical investigations have shown that polymorphisms in genes such as *CYP3A5* and *ABCB1* can significantly influence rivaroxaban plasma concentrations(21). However, as observed in this and some previous reports, the association between these PK-based genetic markers and clinical bleeding events is often inconsistent or modest(22). This suggests that inter-individual variability in drug exposure alone may not fully explain the heterogeneity in bleeding risk.

It is within this context that the significance of our finding on *AHNAK2* emerges. The polymorphisms *rs2582513* and *rs10145032* were not only significantly associated with rivaroxaban-induced PT change—a direct PD measure—in healthy subjects but also independently predicted bleeding risk in NVAF patients. Crucially, our combined analysis demonstrated that adding *AHNAK2* genotypes to a prediction model containing established PK genes (*ABCB1, CYP3A5*) significantly improved the predictive performance for both PT response and bleeding risk This underscores that *AHNAK2* provides substantial incremental predictive value, independent of traditional PK markers, positioning it as a novel candidate PD biomarker.

The evolution of pharmacogenomics is moving from single-gene markers towards integrated polygenic risk scores combined with clinical factors(23). Our findings align perfectly with this direction. The potential clinical utility of *AHNAK2* may lie in its incorporation into a composite risk assessment model. Such a model could integrate: 1) PK genetic variables (e.g., *CYP3A5, ABCB1*) to estimate individual drug exposure; 2) PD genetic variables (e.g., *AHNAK2*) to assess individual drug sensitivity; and 3) key clinical risk factors (e.g., age, renal function, concomitant antiplatelet therapy). Developing such integrated tools could enable more precise identification of patients at high risk of bleeding on rivaroxaban, informing personalized dosing strategies.

The 2021 European Heart Rhythm Association (EHRA) Practical Guide emphasizes the need to balance thromboembolic and bleeding risks when using NOACs and acknowledges considerable inter-patient variability(2). The exploration of PD biomarkers, as undertaken in this study, is a direct response to this clinical need. While prospective validation in independent cohorts and elucidation of the underlying biological mechanism are required before *AHNAK2* can be considered for clinical application, this study provides a novel genetic clue and a strong rationale for developing more refined, personalized strategies for rivaroxaban therapy.

5 Conclusion

Mutations *rs2582513* and *rs10145032* in the *AHNAK2* gene were significantly associated with blunted pharmacodynamic responses to rivaroxaban in healthy subjects and elevated bleeding risks in NVAF patients, providing hypothesis-generating evidence for *AHNAK2* as a potential predictive biomarker candidate for rivaroxaban therapy. An unresolved biological paradox exists between the pharmacodynamic and clinical findings, and the underlying biological mechanism cannot be elucidated by the current study data. The predictive performance of these SNPs for bleeding events was derived from a single dataset without external validation, and thus may be overestimated. Additionally, the association between *AHNAK2 GG* genotype and bleeding risk was statistically significant but characterized by a modest effect size and wide confidence intervals, requiring cautious interpretation. These findings highlight the potential role of genetic factors in anticoagulation therapy but remain hypothesis-generating rather than definitive; further validation in large, independent multi-center cohorts, functional experiments to clarify the mechanistic role of *AHNAK2* in coagulation and bleeding, and external verification of predictive models are urgently needed before any clinical translation can be considered.

Declarations

6.1 Ethics approval and consent to participate

The protocol has been approved by an independent ethics committee and is registered under the identifier NCT03161496 in the Clinical Trials database (Study Registration Dates, 2012-05-19). Prior to their involvement in the study, all participants were informed about the study's objectives, duration, potential risks, and were required to provide written consent.

6.2 Consent for publication

The manuscript has been approved by all authors for publication.

6.3 Availability of data and material

All data related to the study have been shown in the article, and other relevant data can be communicated with the author.

6.4 Author Contributions

Zhiyan Liu was responsible for sample collection, data analysis, and article writing. Guangyan Mu were responsible for patient enrollment. Yunlong Tan, Yu Cao and Wenping Wang were responsible for multi-center patient management and coordination. Xiaohua Wei were responsible for sample testing and analysis. Yimin Cui and Qian Xiang were responsible for project design, supervision, and article guidance.

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Figures

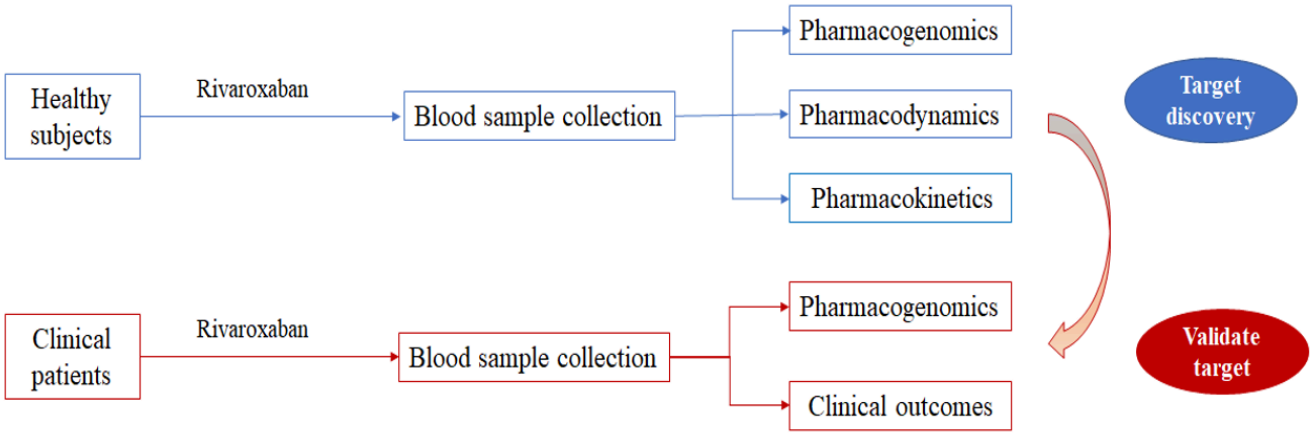


Figure 1

Study flow diagram of the two-stage cohort design.
This diagram illustrates the sequential design of the study. In Stage 1 (Discovery Phase), 304 healthy subjects receiving a single dose of rivaroxaban (10, 15, or 20 mg) were genotyped, and the association between genome-wide polymorphisms and the pharmacodynamic endpoint (prothrombin time ratio at 3 h post-dose) was analyzed to identify candidate loci. In Stage 2 (Validation Phase), the identified candidate single nucleotide polymorphisms (SNPs) within the AHNAK2 gene were analyzed in an independent cohort of 257 patients with non-valvular atrial fibrillation (NVAF) on rivaroxaban therapy, with the primary clinical endpoint being the occurrence of bleeding events (classified by BARC criteria) during follow-up (1, 6, and 12 months). This design validates the clinical relevance of the pharmacogenetic associations found in healthy volunteers.

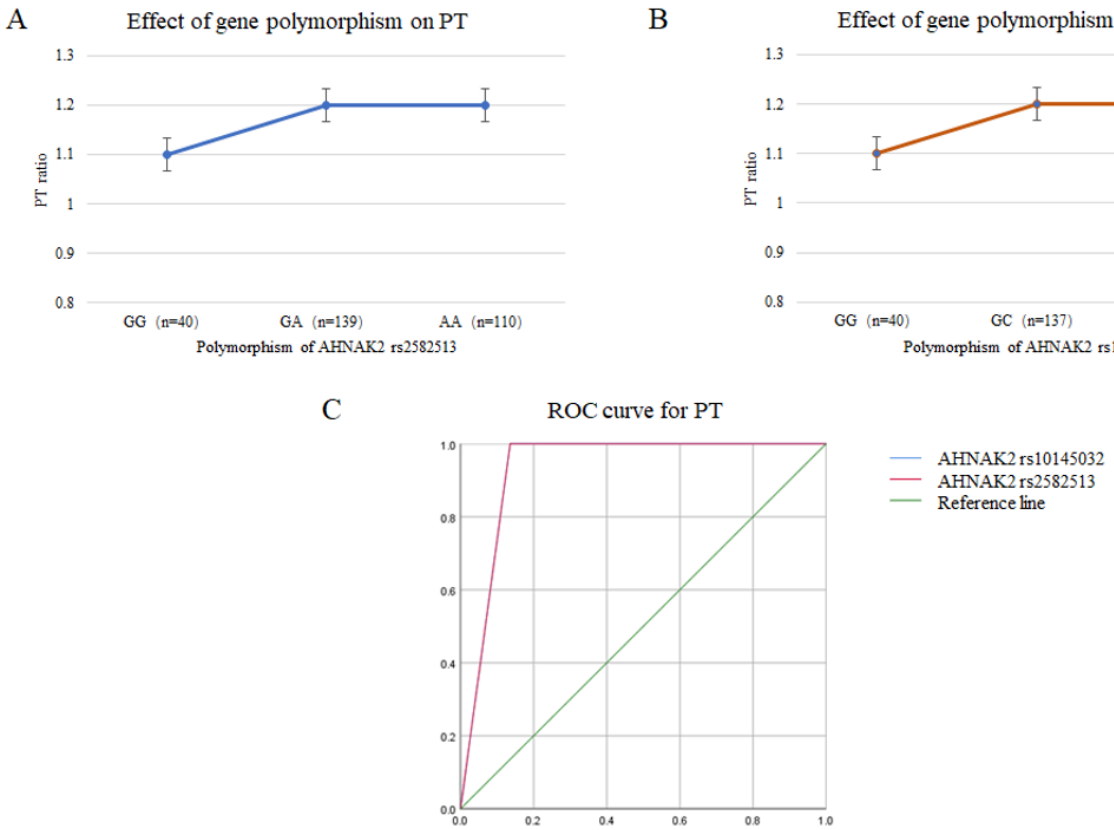


Figure 2

Association of AHNAK2 genotypes with rivaroxaban pharmacodynamic response in healthy subjects.
(A, B) Box plots showing the prothrombin time (PT) ratio (3 h post-dose PT divided by baseline PT) stratified by genotype for rs2582513 (A) and rs10145032 (B) in 304 healthy subjects receiving rivaroxaban. Carriers of the GG genotype for both SNPs exhibited a significantly lower PT ratio compared to carriers of the A or C allele (GA/AA and GC/CC, respectively), indicating a blunted anticoagulant response. Data are presented as mean ± SD; ***p < 0.001 (ANOVA with post-hoc test). Numbers of subjects per genotype: rs2582513: GG = 40, GA = 139, AA = 110; rs10145032: GG = 40, GC = 137, CC = 112. **(C)**Receiver operating characteristic (ROC) curve analysis evaluating the predictive ability of the AHNAK2 rs2582513 and rs10145032 genotypes for an elevated PT ratio response (defined as PT ratio > 1.2). The combined area under the curve (AUC) of 0.93 (95% CI: 0.90–0.96) demonstrates high predictive accuracy for this pharmacodynamic endpoint, supporting the role of AHNAK2 as a potential biomarker for rivaroxaban effect.

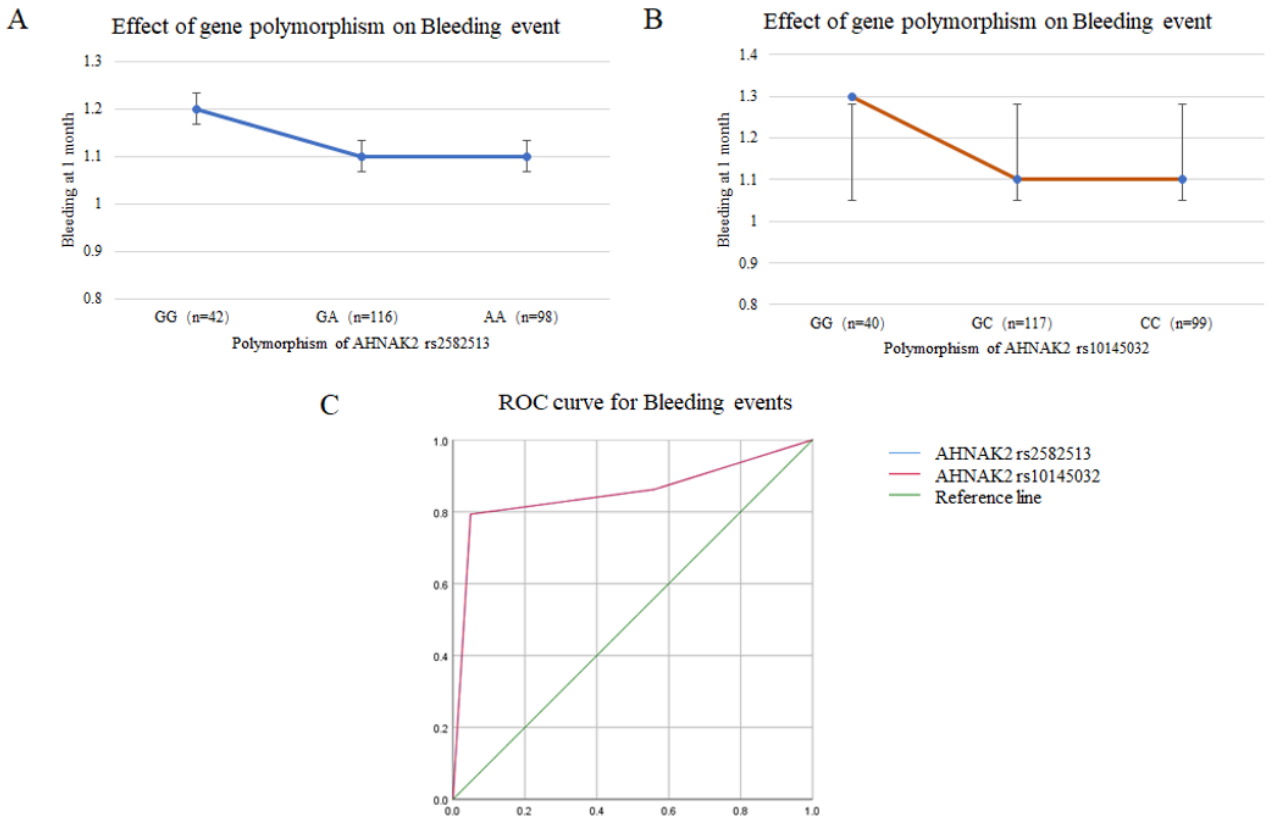


Figure 3

Association of AHNAK2 genotypes with bleeding risk in patients with non-valvular atrial fibrillation (NVAF).
(A, B) The incidence of bleeding events within one month of rivaroxaban initiation stratified by genotype for rs2582513 (A) and rs10145032 (B) in the NVAF patient cohort (n = 257). Bleeding events were defined according to BARC criteria (type 2, 3, or 5). Patients homozygous for the G allele (GG genotype) had a significantly higher incidence of bleeding compared to those carrying at least one A or C allele. **(C)** Receiver operating characteristic (ROC) curve analysis evaluating the predictive ability of the *AHNAK2* genotypes for bleeding events. The area under the curve (AUC) of 0.85 (95% CI: 0.79–0.91) indicates good predictive discrimination for clinically relevant bleeding risk, translating the pharmacogenetic finding from healthy subjects to a patient-relevant outcome.

Supplementary Files

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- SupplementaryTable.docx