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Discovery of age and blood group associated variability in nattokinase mediated thrombolysis and its relevance to cardiovascular management

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The post-COVID period has been accompanied by a notable rise in cardiovascular disease (CVD), emphasizing the importance of evaluating thrombolytic agents across heterogeneous populations. Although nattokinase (NK) is known for its fibrinolytic properties, systematic data on its clot dissolution efficiency and clot dissolution time (CDT) in the Indian population remain limited. Hence, this study examined NK-mediated thrombolysis across ABO/RhD blood groups, age strata, and gender supported by molecular docking and ultrastructural analyses. A total of 1,796 blood samples encompassing all eight ABO/RhD blood groups were collected from individuals aged 10–70 years from the Saurashtra region. In vitro clot lysis assays were used to quantify CDT following NK treatment, while scanning electron microscopy (SEM) and molecular docking & dynamic were employed to investigate NK–fibrin–RhD interactions. Significant variation in CDT was observed across blood groups, with the longest dissolution time recorded in AB− females (2660 s) and the shortest in O+ males (1510 s). Four-way ANOVA analysis revealed a significant three-way interaction effect of age group x ABO blood group x RhD factor ($p = 0.005$), whereas the four-way interaction including gender was not significant ($p = 1.000$). CDT trends were further validated using an independent cohort of 562 samples collected from nine distinct locations across India, which showed consistent patterns. Molecular interaction analysis revealed that the RhD–NK–fibrin complex displayed substantially higher binding affinity (− 1813.9 kcal/mol) than the NK–fibrin complex alone (− 733.3 kcal/mol), aligned with enhanced clot dissolution efficiency observed in SEM analyses. In conclusion, NK-mediated CDT is not uniform across blood groups and is influenced by age and blood group antigen composition. These findings recommend large-scale in vivo validation studies to optimize NK dosing across diverse populations.

Keywords ABO blood group, Cardiovascular diseases, Molecular docking, Nattokinase, RhD factor

Thrombosis is a major global health problem estimated to cause approximately 25% mortality among cardiovascular diseases (CVDs) across the globe[1]. The CVDs caused nearly 19.8 million (~ 32% of global) deaths in 2022 (latest WHO data retrieved on 13/08/2025). These thrombotic events, such as venous thromboembolism (VTE), ischemic stroke, and myocardial infarction, are the key players for increasing the CVD burden[2]. VTE solely affects approximately ~ 10 million people per year, making it one of the top acute causes of cardiovascular death[3]. Conventional anticoagulants (warfarin, heparin, and DOACs) and thrombolytics (tPA and streptokinase) remain as a first-line treatment for thrombolysis[4]. But they carry the risk of bleeding, short half-lives, and high costs, which limit their accessibility, especially in low-resource situations[5], i.e., the tPA, have a lower post-stroke therapeutic window and a 6.8% intracranial hemorrhage risk[6]. While streptokinase is limited by its high immunogenicity (inducing antibody responses after 4–5 days) and systemic fibrinogen depletion, increasing the bleeding risk (~ 1.5% major hemorrhage rate)[7]. These challenges have spurred interest in alternative fibrinolytic agents, particularly those with oral bioavailability and improved safety profiles.

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Nattokinase (NK), a serine protease enzyme derived from the *Bacillus subtilis natto* has demonstrated potent fibrinolytic activity through direct fibrin degradation and plasminogen activation[8]. It emerges as a promising solution, demonstrating comparable fibrinolytic activity without these drawbacks in preclinical models. Preclinical studies report that NK shows ~ 2.8% higher therapeutic index compared to tPA indicating enhanced safety margins while maintaining comparable thrombolytic efficacy[9]. While focusing on safety concerns, NK showed potent fibrinolysis in 153 vascular cases (aged from 22 to 92 years), neutral effects on hemostasis in 265 normotensive adults (2000 FU/day, 3 years), and a potent antihypertensive effect in 86 patients (8 weeks, with the same dose)[10–12]. Notably, a double-blinded trial (37 males and 42 females) revealed sex-divergent responses to NK (100 mg/day, 8 weeks), with males showing higher diastolic BP reductions than females. Still, no studies have yet investigated whether any pharmacodynamics or sex-specific factors were responsible for these observations, which is a critical gap for NK research[13].

Over the last decades, researchers have actively studied the relation between the ABO blood group and thrombolysis[14,15]. As the non-O blood groups have higher vWF (von Willebrand factor) and FVIII levels compared to blood group O, supporting the ABO group's influence on the key procoagulant proteins[16]. A recent population cohort analysis also supports the finding that an ABO frameshift variant (rs8176719) increases the risk of VTE (HR = 1.30; 95% CI = 1.20–1.42; $P = 3.9 \times 10^{-10}$) by approximately 30%[17]. The reported clinical trial on 1,500 patients reflected that people with the A blood group were more likely to have severe coronary artery disease, including acute coronary syndrome, triple-vessel disease, and left ventricular dysfunction[18]. The exact reason behind these results was unclear, but these studies highlight that the ABO blood group plays a crucial role in thrombosis research.

However, there is currently no concrete scientific information available on the available data bases (Scopus, PubMed, and Web of Science) focusing on the efficiency and efficacy of NK, focusing on the clot dissolution time (CDT) in different ABO blood group systems[19–21]. Furthermore, the mechanism of clot dissolution of NK in ABO groups and the role of RhD protein in thrombosis or thrombolysis remain unresolved as well.

Hence, this research aims to:

i. Compare NK-mediated CDT across ABO blood groups,
ii. Investigate the role of RhD in modulating NK's fibrinolytic activity,
iii. Establish preliminary dose-response relationships for personalized NK therapy.

By addressing these gaps, our work could pave the way for ABO-tailored NK dosing, reducing side effects and enhancing efficacy, aligning with WHO's Global CVD Roadmap and SDG 3.8 (Universal Health Coverage)[22].

Materials and methods

The blood samples used in this study were collected from the Indian Medical Scientific Research Foundation (IMSRF), Rajkot, Gujarat, India. All methods involving human blood samples were carried out in accordance with relevant guidelines, regulations and the principles of the Declaration of Helsinki. All the experimental protocols were reviewed and approved by the Indian Medical Scientific Research Foundation (IMSRF), Rajkot, Gujarat, India. All the samples were collected from healthy male and female participants who were medically screened prior to blood donation. All participants (and/or their legal guardians for minors) provided informed consent before participation. All samples were anonymized before being provided to the authors for maintaining the privacy of the donor. The screening area for sample collection was mainly the population of Rajkot (22°30′N, 70°48′E) and surrounding 100 km range (Saurashtra region) of Gujarat, India. A total of 1,796 samples were collected in the period of June 2023 to July 2024, representing all eight blood groups (Fig. 1). This study evaluated the efficiency of NK in different ages (10 to 70 years), genders (male and female), and ABO blood groups with RhD factor (A+, A−, B+, B−, AB+, AB−, O+, and O−) by evaluating the CDT in seconds and data were recorded. The results of the RhD protein groups were further analysed by molecular docking analysis to understand the mechanism of lower CDT compared to the RhD-negative group. All the healthy individuals were selected after thorough screening for this study to ensure reliable results.

The clot Lysis assay

We performed the in vitro clot lysis method of Devi et al. (2016) with necessary modifications according to our laboratory setup[23]. The human venous blood (5 mL) was collected with a syringe and transferred 1 mL blood into the five sterile microtubes (1 mL each tube). The tubes were left undisturbed at room temperature for 30 min for a clot formation. After the incubation, the clot formation was assessed visually. If insufficient clot formation was observed, the samples were centrifuged at 2000 x g for 10 min to aid clotting. Further, the supernatant was carefully removed, and the clot was washed with phosphate-buffered saline (PBS) to remove the other fluidic materials. A 2000 FU/100 mg capsule of NK was mixed with autoclaved double-distilled water to make the NK solution. In three microtubes containing the clot, 100 µL of the NK solution was added for triplicates. There were two sets of microtubes where one tube had 100 µL of streptokinase with the same concentration added, and the other two had 100 µL of PBS (phosphate-buffer saline) added as a negative control. All the tubes were incubated at 37 °C incubator. The CDT was monitored in every 30 s until complete clot dissolution was observed. Each assay was performed in triplicate technical replicates for each biological sample and the obtained data were recorded. The data were statistically analysed using Duncan's Multiple Range Test (DMRT), with p-values < 0.01 (WASP statistical package, ICAR-Central Coastal Agricultural Research Institute)[24]. To study the effects and interactions of studied four factors (Age Group x Gender x ABO type x RhD factor), a four-way ANOVA (Analysis of Variance) was performed using IBM SPSS Statistics tool (version 31.0.1.0)[25]. The analysis was performed using mean values of CDT in three biological replicates from each group and a total of 576 samples were analysed. The selection and averaging criteria for these groups are detailed in Supplementary Table 1.

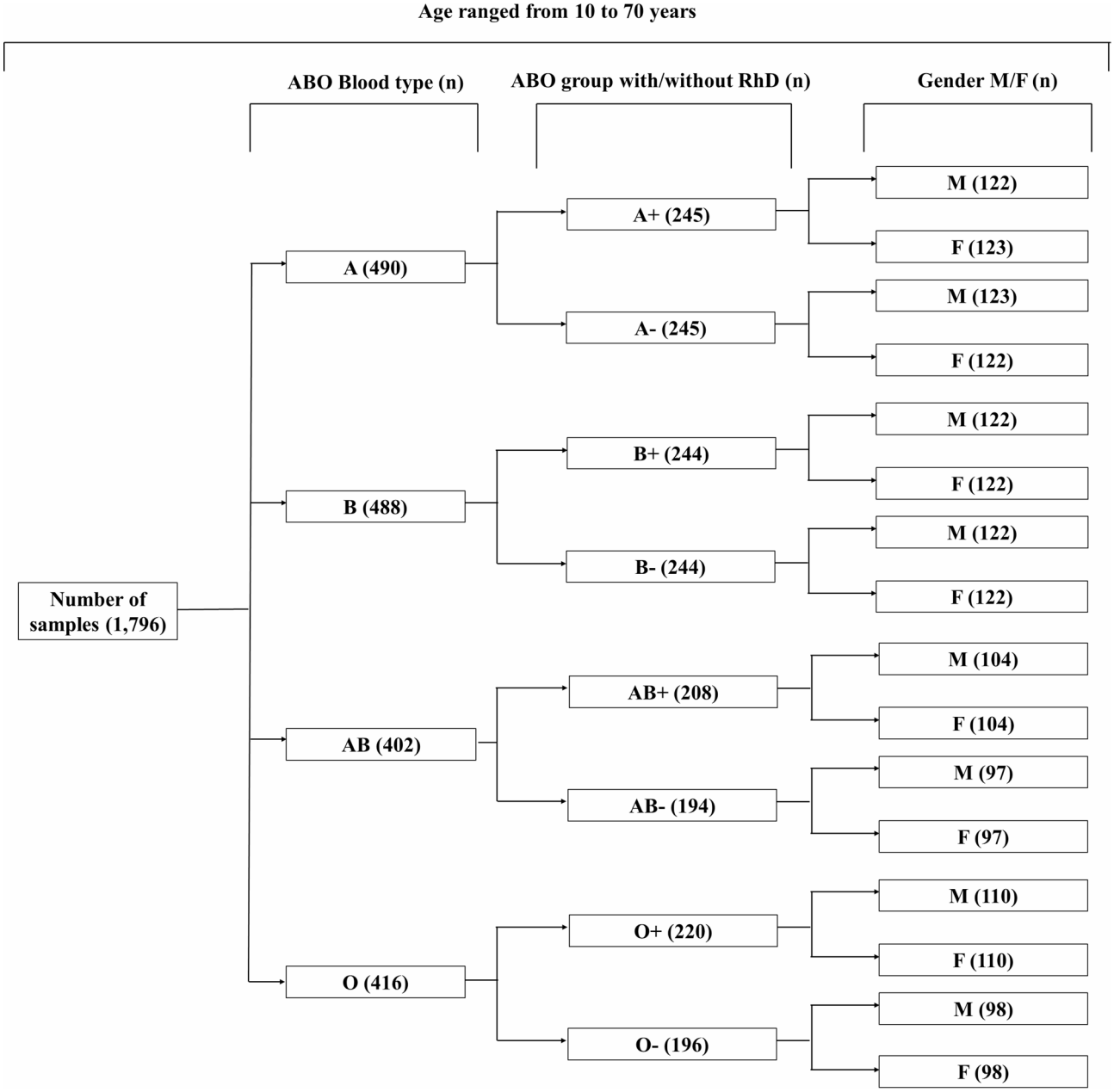

Fig. 1. Distribution of blood samples based on ABO and RhD blood groups, stratified by gender. A total of 1,796 samples were categorized into four ABO blood groups (**A**, **B**, **AB**, and **O**) and further divided based on RhD factor (positive and negative). Each subgroup was equally distributed among male (M) and female (F) participants, with an age range of 10 to 70 years.

Multi-location validation of the clot dissolution time across blood groups

To validate the initially obtained results from the Saurashtra region, a total of 562 new blood samples across ABO blood groups were collected from nine different major urban centres of India (Table 1). The blood clot lysis assay protocol was performed in triplicates to all samples using the same protocol. For the quantitative assessment to measure the consistency of our findings, a comparative analysis of CDT has been performed. Here, the results from the Saurashtra region were treated as expected results (A) and the results from the new locations was considered as observed results (B). The percentage variation between these paired result sets was calculated using the formula[26]: $\frac{|A-B|}{A} \times 100$.

Comparative SEM analysis of eight blood groups for visualizing clot response to nattokinase treatment

The venous blood sample (900 µL) of all eight blood types (4 males/4 females) with different age groups were collected into sterile 2 mL microtubes and anticoagulated using 100 µL of 3.2% sodium citrate (0.109 M),

Location	Gender		Total $N=562$ (% age of the total)
	Male ($N=281$)	Female ($N=281$)	
Gujarat			
Ahmedabad	52	44	96 (17.1%)
Surat	39	33	72 (12.8%)
Maharashtra			
Pune	28	24	52 (9.3%)
Aurangabad	21	19	40 (7.1%)
Thane	27	27	54 (9.6%)
Rajasthan			
Jodhpur	27	32	59 (10.5%)
Pali	22	29	51 (9.1%)
Madhya Pradesh			
Ujjain	33	40	73 (13.0%)
Indore	32	33	65 (11.6%)

Table 1. Comprehensive distribution profile of the blood sample collection categorised by geographic location and gender (Total number of samples $N=562$).

followed by gentle mixing. The 100 µL sample was transferred in the four sterile microtubes (coded as tube no 1st to 4th) for the SEM study. The 1st tube is considered as the control tube without any treatment to the blood sample. The clot formation was initiated by addition of 10 µL of 0.025 M calcium chloride ($CaCl_2$) in all the rest three sample (2nd to 4th samples) tubes and incubated at 37 °C for 10–15 min. The 2nd tube is considered as the clot tube for SEM study. In the 3rd and 4th tube containing the blood clot, 10 µL of NK enzyme (200 FU/10 mg) were inoculated to study the fibrinolysis. The 3rd tube was incubated for 1510 s (to compare with the O+ blood group results). The 4th tube was incubated up to the complete clot lysis (based on the results of the clot lysis assay) at 37 °C. Following results, the residual fluid was carefully removed, and the tubes were fixed in 1 mL of 2.5% glutaraldehyde (Sigma-Aldrich, USA) for 4 h at 4 °C. All the samples were washed thrice with 0.1 M phosphate buffer (10 min each) and dehydrated through a graded ethanol series of 30%, 50%, 70%, 90%, and three changes of 100%, each for 10 min. Approximately 1 mL of absolute ethanol was retained in the final step prior to critical point drying (CPD) (E3100, Quorum Technologies Ltd. UK). After performing CPD, the dried samples were mounted onto aluminium SEM stubs using carbon adhesive tape, sputter-coated with gold alloy (10–20 nm) (The Quorum SC7620, Quorum Technologies Ltd. UK), and images were taken using SEM (EVO 18, Carl Zeiss Microscopy GmbH, Germany) to analyse the fibrinolysis results. The SEM micrographs were morphometrically analysed using ImageJ software (version 1.54p)[27]. For each sample and conditions, the diameter of ten ($N=10$) clearly visible red blood cells was measured. The mean diameter and standard deviation (SD) were calculated for each sample. The percentage variation after the clot lysis was calculated as [26] :

$$\%\ Variation = \frac{[\text{Control RBC diameter} - \text{Post lysis RBC diameter}]}{Control\ RBC\ diameter} \text{ x } 100$$

Protein structure retrieval and modelling

The 3D structure of nattokinase (PDB ID: 4DWW) and the fibrin protein (PDB ID: 2HLO) were obtained from the Protein Data Bank (RCSB PDB)[28]. Due to the unavailability of the RhD-protein crystal structure, we have designed it for the current investigation. The amino acid sequences (accession number ALM96707.1) were retrieved from the NCBI database. The 3D structure of RhD protein was predicted using the I-TASSER (Iterative Threading Assembly Refinement) tool[29]. To ensure the reliability of the predicted RhD protein model, required quality assessment tools were used and validate the protein structure. The newly designed model quality was evaluated using ProSA (Protein Structure Analysis) and ERRAT from the SAVES v5.0 web server. The secondary structure validation was performed by the SOPMA (Self-Optimized Prediction Method with Alignment) web server. Additionally, the 3D model's quality was assessed using ProQ/ProQ3D, a comprehensive tool for single-model quality estimation[28]. The backbone geometry and residue-specific validation were performed using the PDBSum tool, which provided detailed insights into bond angles, torsion angles, and residue interactions[30]. The predicted RhD protein and the downloaded 3D crystal structures of NK and fibrin (6-chain structure) were prepared for docking using Biovia Discovery Studio Visualizer tool. A total of three protein-protein docking analyses were performed using the ClusPro tool. In the initial docking, the fibrin was selected as a receptor, and NK as a ligand, and uploaded on the ClusPro server[31]. In the second docking, the NK was docked with the prepared RhD protein using the same server to generate the RhD-NK complex. The highest energy complex of the RhD-NK complex was then used as a receptor in the third docking with fibrin as a ligand. The results of all docked complexes were analysed using the PDBSum database.

Results

The DMRT analysis reflects a significant difference in CDT across various ages, genders, and blood groups. We observed the longest (2660 s) and shortest (1510 s) CDTs in the 66–70 age group (AB– females) and 31–35 age

group (O+ males), respectively. It is observed that from the 31–35 age group, the dissolution time increases by 39% to the 10–15 age group and by 44% to the 65–70 age group (Fig. 2).

For better analysis, all the samples of males and females (898 of each gender) were combined and performed the DMRT analysis. The result revealed that the females exhibited slightly longer dissolution time (2094 s) compared to males (2085 s). The results are presented in Table 2. The O blood group had the shortest average dissolution time (1936 s), followed by the B blood group (2041 s) and the A blood group (2140 s). The AB blood group had the longest average dissolution time (2240 s). The final sequence of CDT from higher to lower is AB > A > B > O. Further stratification by RhD protein showed that the RhD-negative group had a higher dissolution time (2116 s) compared to the RhD-positive groups (2063 s).

Based on the in vitro results, the molecular docking hypothesis was forwarded for the docking & dynamics study to visualize the interaction between NK and RhD protein. This helped to explain why the CDT was lower in RhD-positive groups, i.e., RhD protein interacts with NK and increases the hydrolysis efficiency of fibrin. These results are matched with the SEM analysis.

Four-way ANOVA of demographic factors on clot dissolution time

To study the interactive effects of age group x gender x ABO type x RhD factors on CDT, a four-way ANOVA was effectively performed. This statistical model showed that, all the analysed four factors had an independent significant impact on CDT as showed the R^2 value of 0.999 (Table 3). The age group exhibited the strongest effect (partial $\eta^2 = 0.999$), followed by ABO blood group (partial $\eta^2 = 0.994$), RhD factor (partial $\eta^2 = 0.896$), and gender (partial $\eta^2 = 0.205$) on the CDT. The analysed results confirmed that two-way interaction effects were predominantly significant, with statistically meaningful interactions observed between age x gender, age x ABO blood group, and ABO blood group x RhD factor (all $p < 0.01$). Notably, a significant three-way interaction among age group x ABO blood group x RhD factor was detected ($p = 0.005$), indicating a combined influence of these variables on the CDT. In contrast, the four-way interaction involving age group x gender x ABO blood group x RhD factor was not statistically significant ($p = 1.000$), suggesting the absence of a higher-order synergistic effect among all factors examined (Table 3).

To delineate specific group-wise differences, post-hoc pairwise comparisons were conducted using Tukey's honestly significant difference (HSD) test. The analysis confirmed that clot dissolution time (CDT) differed significantly among all ABO blood groups ($p < 0.001$), with lytic efficiency ranked from fastest to slowest as O > B > A > AB. Post-hoc evaluation of age groups revealed a non-linear association with CDT characterized by a progressive decreasing to maximum in middle adulthood (31–35 years), followed by increase in older age groups. Notably, CDT values for the 31–35 and 36–40 years groups did not differ significantly ($p = 0.440$), indicating a plateau around the peak dissolution time.

Age	Treatment No.	Blood Group																Average
		A+		A-		B+		B-		AB+		AB-		O+		O-		
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	
10-15	T1	2380^{e}	2385^{e}	2433^{e}	2435^{e}	2281^{e}	2295^{e}	2317^{e}	2337^{e}	2496^{e}	2500^{e}	2544^{e}	2561^{e}	2126^{d}	2166^{e}	2245^{e}	2236^{e}	2359^{e}
16-20	T2	2167^{f}	2180^{f}	2212^{f}	2224^{f}	2066^{f}	2077^{f}	2103^{f}	2114^{f}	2256^{f}	2267^{f}	2311^{f}	2321^{f}	1950^{f}	1958^{f}	2012^{f}	2026^{f}	2140^{f}
21-25	T3	1984^{h}	1987^{h}	2034^{h}	2040^{h}	1885^{h}	1893^{h}	1936^{h}	1943^{h}	2082^{h}	2087^{h}	2133^{h}	2140^{h}	1784^{h}	1788^{h}	1836^{h}	1841^{h}	1962^{h}
26-30	T4	1849^{j}	1852^{j}	1893^{j}	1896^{j}	1743^{j}	1744^{j}	1805^{j}	1799^{j}	1944^{j}	1953^{j}	2003^{j}	2006^{j}	1646^{j}	1649^{j}	1697^{j}	1699^{j}	1824^{j}
31-35	T5	1725^{k}	1729^{k}	1776^{k}	1779^{k}	1629^{k}	1630^{k}	1676^{k}	1680^{k}	1820^{k}	1828^{k}	1877^{k}	1880^{k}	1510^{l}	1530^{k}	1583^{k}	1585^{k}	1715^{k}
36-40	T6	1731^{k}	1736^{k}	1781^{k}	1785^{k}	1632^{k}	1634^{k}	1680^{k}	1686^{k}	1831^{k}	1832^{k}	1879^{k}	1882^{k}	1531^{k}	1535^{k}	1580^{k}	1585^{k}	1708^{k}
41-45	T7	1903^{i}	1909^{i}	1957^{i}	1960^{i}	1802^{i}	1814^{i}	1853^{i}	1863^{i}	2008^{i}	2008^{i}	2062^{i}	2058^{i}	1706^{i}	1726^{i}	1753^{i}	1769^{i}	1884^{i}
46-50	T8	2095^{g}	2095^{g}	2149^{g}	2154^{g}	2011^{g}	2017^{g}	2061^{g}	2059^{g}	2183^{g}	2188^{g}	2257^{g}	2270^{g}	1894^{g}	1890^{g}	1940^{g}	1943^{g}	2075^{g}
51-55	T9	2242^{e}	2251^{e}	2290^{e}	2304^{e}	2143^{e}	2151^{e}	2192^{e}	2202^{e}	2341^{e}	2358^{e}	2388^{e}	2408^{e}	2036^{e}	2046^{e}	2091^{e}	2099^{e}	2221^{e}
56-60	T10	2347^{d}	2353^{d}	2399^{d}	2399^{d}	2246^{d}	2252^{d}	2296^{d}	2301^{d}	2445^{d}	2446^{d}	2493^{d}	2498^{d}	2142^{c}	2147^{d}	2201^{d}	2208^{d}	2323^{d}
61-65	T11	2424^{b}	2436^{b}	2472^{b}	2489^{b}	2326^{b}	2339^{b}	2376^{b}	2386^{b}	2522^{b}	2542^{b}	2570^{b}	2583^{b}	2221^{b}	2233^{b}	2277^{b}	2290^{b}	2405^{b}
66-70	T12	2488^{a}	2511^{a}	2537^{a}	2563^{a}	2391^{a}	2409^{a}	2437^{a}	2460^{a}	2578^{a}	2608^{a}	2637^{a}	2660^{a}	2286^{a}	2313^{a}	2342^{a}	2363^{a}	2474^{a}
	CD (0.01)	23.82	28.68	23.72	29.85	20.6	25.62	19.81	27.88	27.24	28.77	24.51	30.37	10.34	8.08	19.47	23.81	6.84
	CV (%)	0.49	0.59	0.48	0.6	0.45	0.55	0.42	0.59	0.54	0.56	0.47	0.58	0.24	0.18	0.43	0.53	0.36
Values not sharing the same letter are significantly different (P = 0.01) as determined by Duncun's Multiple Range Test																		

Fig. 2. Heatmap of blood clot dissolution times (seconds) by ABO/RhD blood group, age, and sex (colour green to red indicates the increasing pattern of CDT).

Variable	Total *N* = % (1796)	Blood clot dissolution time (in seconds)
Gender		
Male	50 (898)	2085[b]
Female	50 (898)	2094[a]
CV%	0.276	
CD (0.01)	2.189	
ABO Group		
A+	13.6 (245)	2115[d]
A−	13.6 (245)	2165[c]
B+	13.6 (244)	2017[f]
B−	13.6 (244)	2065[e]
AB+	11.6 (208)	2213[b]
AB−	10.8 (194)	2268[a]
O+	12.3 (220)	1908[h]
O−	10.9 (196)	1965[g]
CV%	0.348	
CD (0.01)	5.407	
RhD group		
RhD +	51 (917)	2063[b]
RhD −	49 (879)	2116[a]
CV%	0.293	
CD (0.01)	2.326	

Table 2. DMRT analysis of demographic factors using the blood CDT values. The mean values having different superscript letters (a to f) represents different statistically significant ($P < 0.01$) demographic factors with each other. Values not sharing the same letters are significantly different ($P < 0.01$) as determined by DMRT (Duncan's Multiple Range Test).

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.	Partial Eta Squared (η^2)
Corrected Model	47074498.944[a]	191	246290.570	2098.036	< 0.001	0.999
Intercept	2511594440.304	1	2511594440.304	21395119.105	0.000	1.000
Age Group	39125036.561	11	3556821.506	30298.928	< 0.001	0.999
Gender	11651.964	1	11651.964	99.258	0.000	0.205
ABO	7343977.728	3	2447992.576	20853.324	< 0.001	0.994
RhD	390337.183	1	390337.183	3325.103	< 0.001	0.896
Age Group x Gender	5543.440	11	503.949	4.293	< 0.001	0.110
Age Group x ABO	14826.246	33	449.280	3.827	< 0.001	0.247
Age Group x RhD	1225.375	11	111.398	0.949	0.493	0.026
Gender x ABO	101.670	3	33.890	0.289	0.834	0.002
Gender x RhD	1.918	1	1.918	0.016	0.898	0.000
ABO x RhD	1966.141	3	655.380	5.583	0.001	0.042
Age Group x Gender x ABO	1930.686	33	58.506	0.498	0.992	0.041
Age Group x Gender x RhD	280.694	11	25.518	0.217	0.996	0.006
Age Group x ABO x RhD	6990.014	33	211.819	1.804	0.005	0.134
Gender x ABO x RhD	40.319	3	13.440	0.114	0.952	0.001
Age Group x Gender x ABO x RhD	936.670	33	28.384	0.242	1.000	0.020
Error	45078.144	384	117.391			
Total	2562077844.548	576				
Corrected Total	47086577.088	575				

Table 3. Results of four-way ANOVA analysis for a CDT. Here, the values include: type III sum of Squares, degrees of freedom (df), mean Square, F-statistic, significance (*p*-value), and partial Eta squared (η^2) as a measure of effect size. a. R Squared = .999 (Adjusted R Squared = .999)

Multi-location validation of the clot dissolution time across blood groups

Multi-location data exhibited trends in clot dissolution time (CDT) that were consistent with those observed for the Saurashtra region (Fig. 2). As anticipated, the longest CDT was recorded for the AB− blood group (2670 s), while the shortest CDT was observed for the O+ blood group (1510 s) (Supplementary Table 2). The validation analysis indicated a high level of agreement between the predicted and experimentally observed values. Across all demographic and serological categories, the percentage variation was consistently low, ranging from 0.2% to 1.6%, with an average deviation of 0.7%. Such limited variability demonstrates the stability of the clot lysis measurements and supports their applicability across different study locations. A comprehensive comparison of all eight blood groups is presented in Supplementary Table 2.

SEM analysis

SEM analysis was successfully performed to visualize and quantitatively assess the NK-induced fibrinolysis. Here, the results of the O+ and AB− (the fastest and slowest blood types, according to clot lysis results, respectively) show the comparative study between two blood groups as a representative of all the blood group results. The SEM analysis supports the results of the clot lysis assay following the distinct clot dissolution patterns between the O+ and AB− blood groups. The untreated RBCs (control sample) of O+ and AB− have been observed with the diameters of 7.22 ± 0.34 µm and 6.89 ± 0.25 µm, which are in the normal range of 6.8 µm to 7.8 µm[32] (Fig. 3A and B; Table 4). When the clot formation was initiated, the diameter of RBCs was decreased up to 5.89 ± 0.52 µm and 5.53 ± 0.69 µm in both of the blood samples, respectively[33] (Fig. 3C and D). After the treatment of NK at 1510 s, the result shows complete clot dissolution in the O+ blood sample (Fig. 3E). The diameter of the RBCs was 6.96 ± 0.60 µm, which is close to the original size of the RBCs. In the AB− blood type, the partial clot dissolution (dissolved and undissolved clots) was observed at the same time with the diameter of the RBCs 6.26 ± 0.32 µm (Fig. 3F). While at 1877 s, both blood samples showed almost restoration of the RBCs' diameter, 7.21 ± 0.36 µm and 6.88 ± 0.47 µm, respectively, with the percentage variation of 0.14% and 0.20% (Fig. 3G and H). These results suggest faster fibrinolytic efficiency in O+ blood compared to AB−. Additional SEM micrographs for the other six blood groups were also following the same fashion and were presented in Supplementary Fig. 1 to 3. A comprehensive quantitative assessment of red blood cell (RBC) diameter across all eight major blood groups is summarized in Table 4.

The quantitative analysis of RBC morphology under these four conditions (mentioned in the Table 4) found a consistent pattern of deformation, followed by recovery. Under normal conditions, the mean of the RBC diameter across all the blood group samples ranged within the expected physiological values of 6.16 ± 0.37 µm to 7.22 ± 0.34 µm. Upon clot formation, a major decrease in RBC diameter was observed, with values ranging from 3.51 ± 0.33 µm to 5.89 ± 0.52 µm. It indicates significant cellular deformation and compaction within the fibrin network. Following treatment with NK and clot dissolution over 1510 s, the RBC diameter started recovering, with the diameter ranging from 6.14 ± 0.42 µm to 7.21 ± 0.36 µm, and the RBC reached its almost original size after complete clot lysis. This near-complete restoration of morphology was quantified by a minimal percentage variation between the post-lysis and control diameters, which ranged from 0.10% to 0.32% across all individuals.

Protein prediction and validation

The analysis and validation of the RhD protein before using for the docking study were mention in Table 5. The predicted RhD model were demonstrated good quality, accuracy, and high stability.

Docking results analysis

NK-Fibrin analysis: The normal fibrin structure (A to F chains) contains a total of 20 salt bridges, 52 H-bonds, and 779 non-bonded contacts. The ClusPro web server generated 30 docked complexes of NK-Fibrin as a result of the initial docking. We selected the first NK-Fibrin complex with the lowest energy of -733.3 kcal/mol for further analysis (Table 6). This complex contains a total of 30 salt bridges, 117 H-bonds, and 1122 non-bonded contacts. Here, A↔I contains 6 H-bonds and 32 non-bonded contacts, while B↔I contains 12 H-bonds and 100 non-bonded contacts. The interacted amino acid residues of NK were TYR I:256, ASN I:184, GLN I:185, LYS I:12, GLN I:271, THR I:130, TYR I:167, SER I:161, THR I:162, SER I:159, TYR I:263, TYR I:262, GLY I:160, SER I:158 with the A chain of ARG A:141, GLU A:186, ALA A:190, LYS A:19 and B chain of GLU B:363, LYS B:396, CYS B:407, HIS B:408, TRP B:424, GLY B:430, ASP B:432, SER B:443, TRP B:444. The result shows how NK cells and fibrin interact in Rh negative blood groups (Fig. 4; Table 6).

NK-RhD analysis: Using the ClusPro web server, we obtained 30 docked complexes for the second docking step, which involved docking the predicted RhD with NK. The complexes with the lowest energy of – 1285.4 kcal/mol were used for further analysis. This complex contains 5 H-bonds and 116 non-bonded contacts. For NK, ASN I: 56, GLU I: 112, ASN I: 109, and SER I: 105 amino acid residues interacted with RhD protein TYR J: 178, TYR J: 311, and ARG J: 318 (Fig. 5).

NK-RhD-Fibrin analysis: The ClusPro generated a total of 23 RhD-NK-Fibrin complexes, where the first complex with the lowest energy, – 1813.9, was selected for further analysis. This complex contains 38 salt bridges, 160 H-bonds, and 1700 non-bonded contacts. In this case, A↔I has 8 hydrogen bonds and 54 non-bonded contacts, while B↔I has 5 hydrogen bonds and 108 non-bonded contacts. More salt bridge formation shows that NK binds to fibrin more strongly. RhD-NK's amnio acid residues that interacted with fibrin were LYS I:12, ASN I:184, SER I:252, THR I:255, GLN I:275, SER I:159, SER I:163, GLN I:185, ARG I:186, and ASN I:259. They were connected to the A chain of GLN A:187, GLU A:186, ARG A:141, ALA A:190, LYS A:191, and B chain of ARG B:406, TRP B:424, ASP B:432, and HIS B:429 (Fig. 6).

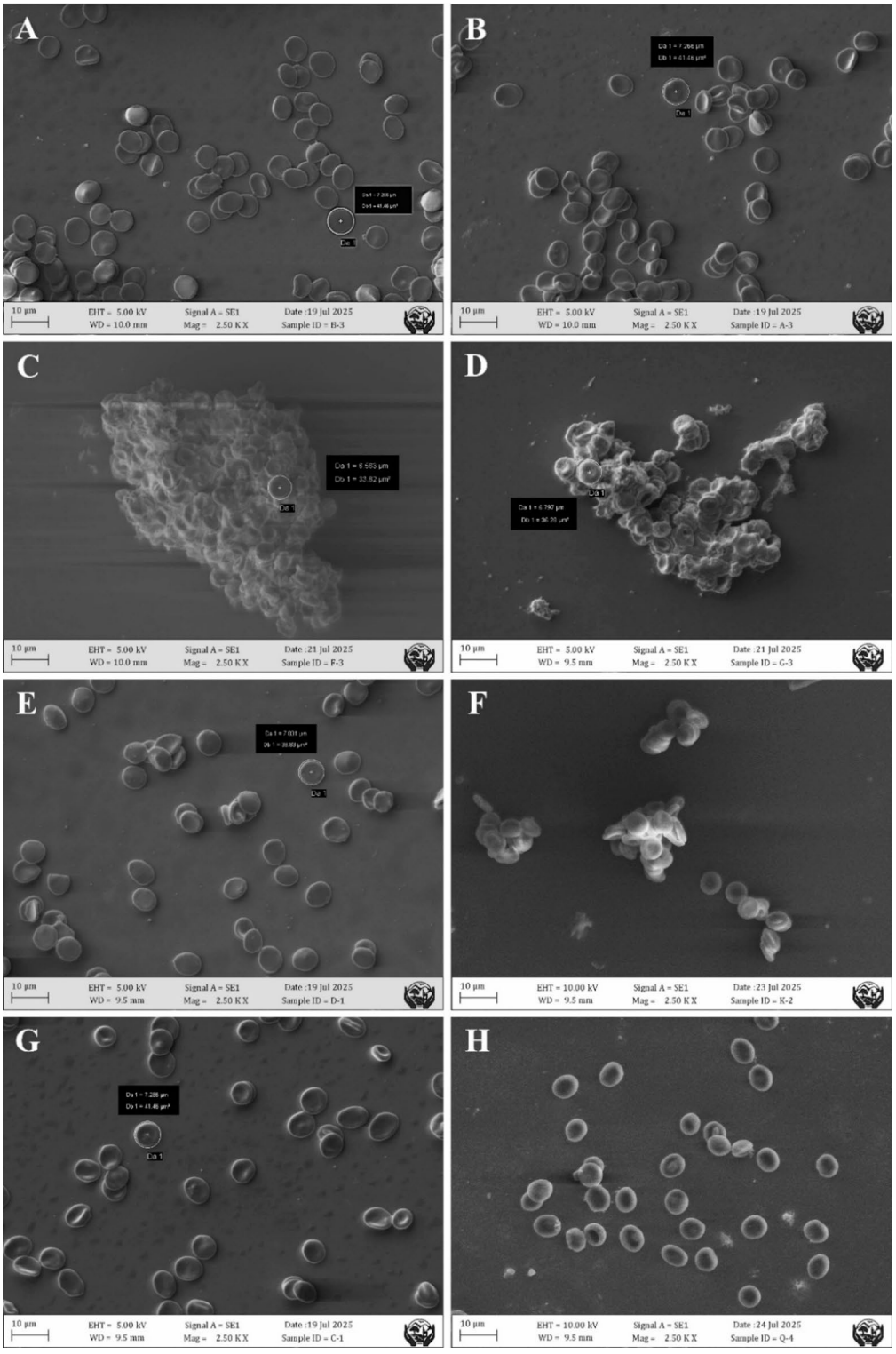

Fig. 3. SEM analysis of clot formation and NK-mediated fibrinolysis in O + and AB− blood groups. **A** and **B**: Morphology of red blood cells of O + and AB− blood samples, respectively. **C** and **D**: RBCs during clot formation show morphological compression with O + and AB− blood samples. **E** and **F**: Post-NK treatment at 1510 s of O + and AB− blood samples. **G** and **H**: Complete clot lysis is confirmed at 1877 s O + and AB− blood samples.

Sl. No.	Blood group	Age (Age group)	Gender	RBC diameter (in µm) Mean ± SD (N = 10 cells/image) (in the mentioned 4 conditions)				% age variation over control $\left(\frac{B}{A}\right) \times 100$
				(1) In control (A)	(2) In clot	(3) In 1510 s (O+ ve CDT)	(4) After Clot lysis (B)	
1.	A+	38 (36–40)	Female	6.16 ± 0.37	3.84 ± 0.38	5.49 ± 0.27	6.14 ± 0.42	0.27
2.	A−	55 (51–55)	Male	6.45 ± 0.58	3.51 ± 0.33	3.92 ± 0.31	6.43 ± 0.25	0.25
3.	B+	26 (26–30)	Female	6.61 ± 0.52	4.17 ± 0.45	4.66 ± 0.52	6.59 ± 0.33	0.28
4.	B−	19 (16–20)	Male	6.78 ± 0.29	3.71 ± 0.31	4.49 ± 0.57	6.77 ± 0.39	0.18
5.	AB+	65 (61–65)	Female	6.38 ± 0.42	5.51 ± 0.50	5.52 ± 0.34	6.37 ± 0.39	0.10
6.	AB−	33 (31–35)	Male	6.89 ± 0.25	5.53 ± 0.69	6.26 ± 0.32	6.88 ± 0.47	0.20
7.	O+	33 (31–35)	Male	7.22 ± 0.34	5.89 ± 0.52	6.96 ± 0.60	7.21 ± 0.36	0.14
8.	O−	45 (41–45)	Female	6.77 ± 0.55	5.55 ± 0.38	6.10 ± 0.46	6.75 ± 0.35	0.32

Table 4. Quantitative analysis of red blood cell (RBC) following nattokinase treatment under normal, clot and clot-lysis conditions.

Method/Tool		Result/Value	Interpretation/Significance
Analysis Type			
Structural Quality (Z-score)	PROSA Tool	− 6.33	Acceptable range for X-ray and NMR-derived structures.
Interaction Energy	PROSA Tool	99% negative residues	Indicates favourable interactions across the protein's residues.
Quality Factor	ERRAT	95.67%	Significant quality factor, indicating high structural integrity.
Secondary Structure Composition	SOPMA	Alpha helices: 51.08%, Random coils: 29.50%, Extended strands: 15.59%, Beta turns: 3.84%	Higher alpha helices suggest a strong structural framework, with flexibility and binding energy in coils.
3D Model Confidence (LGScore)	ProQ	8.063	Indicates good atomic-level confidence in the predicted structure.
3D Model Confidence (ProQ3D)	ProQ3D	0.569	Supports the accuracy and confidence at the atomic level.
Backbone Geometry Analysis	PDBSum Webserver	84.4% in most favoured regions, 14.2% in allowed regions, 0.8% in generously allowed regions	High proportion of residues in the most favourable regions indicates a well-folded model.

Table 5. Validation of the predicted RhD protein structure using multiple computational assessment tools.

Complex	Lowest Docking Energy (kcal/mol)	Total Hydrogen Bonds (H-bonds)	Total Non-Bonded Contacts	Key Interacting NK Residues (Chain I)	Key Interacting Partner Residues
NK-Fibrin	−733.3	18 (A↔I: 6, B↔I: 12)	1122	TYR I:256, ASN I:184, GLN I:185, LYS I:12, GLN I:271, THR I:130, TYR I:167, SER I:161, THR I:162, SER I:159, TYR I:263, TYR I:262, GLY I:160, SER I:158	Fibrin Chain A: ARG 141, GLU 186, ALA 190, LYS 19 Fibrin Chain B: GLU 363, LYS 396, CYS 407, HIS 408, TRP 424, GLY 430, ASP 432, SER 443, TRP 444
NK–RhD	−1285.4	5	116	ASN 56, GLU 112, ASN 109, SER 105	RhD (Chain J): TYR 178, TYR 311, ARG 318
RhD–NK–Fibrin	−1813.9	13 (A↔I: 8, B↔I: 5)	1700	LYS 12, ASN 184, SER 252, THR 255, GLN 275, SER 159, SER 163, GLN 185, ARG 186, ASN 259	Fibrin Chain A: GLN 187, GLU 186, ARG 141, ALA 190, LYS 191 Fibrin Chain B: ARG 406, TRP 424, ASP 432, HIS 429

Table 6. Summary of molecular Docking interactions of nattokinase with RhD and fibrin complexes.

Discussion

Clot Dissolution Time is influenced by ABO blood group, RhD factor, age and gender confirmed by DMRT and ANOVA analyses. The observed decrease in CDT among younger individuals (10–35 years) and its increase in older individuals (36–70 years) reflect age-related variations in fibrinolytic activity. These results are in agreement with published data from healthy adult populations demonstrating a gradual age-associated increase in hypercoagulability, with notable changes occurring between 40 and 57 years[34]. The CDT reached its lowest values during early middle age (31–35 years), suggesting that fibrinolytic activity may decline during this period, consistent with age-associated changes in coagulation and thrombolysis reported in previous studies. Favaloro et al. (2014) reviewed that the fibrinolytic activity progressively declines from middle age to older age as the tPA level decrease and PAI-1 level increases which are a key contributor to increase thrombotic risk observed with

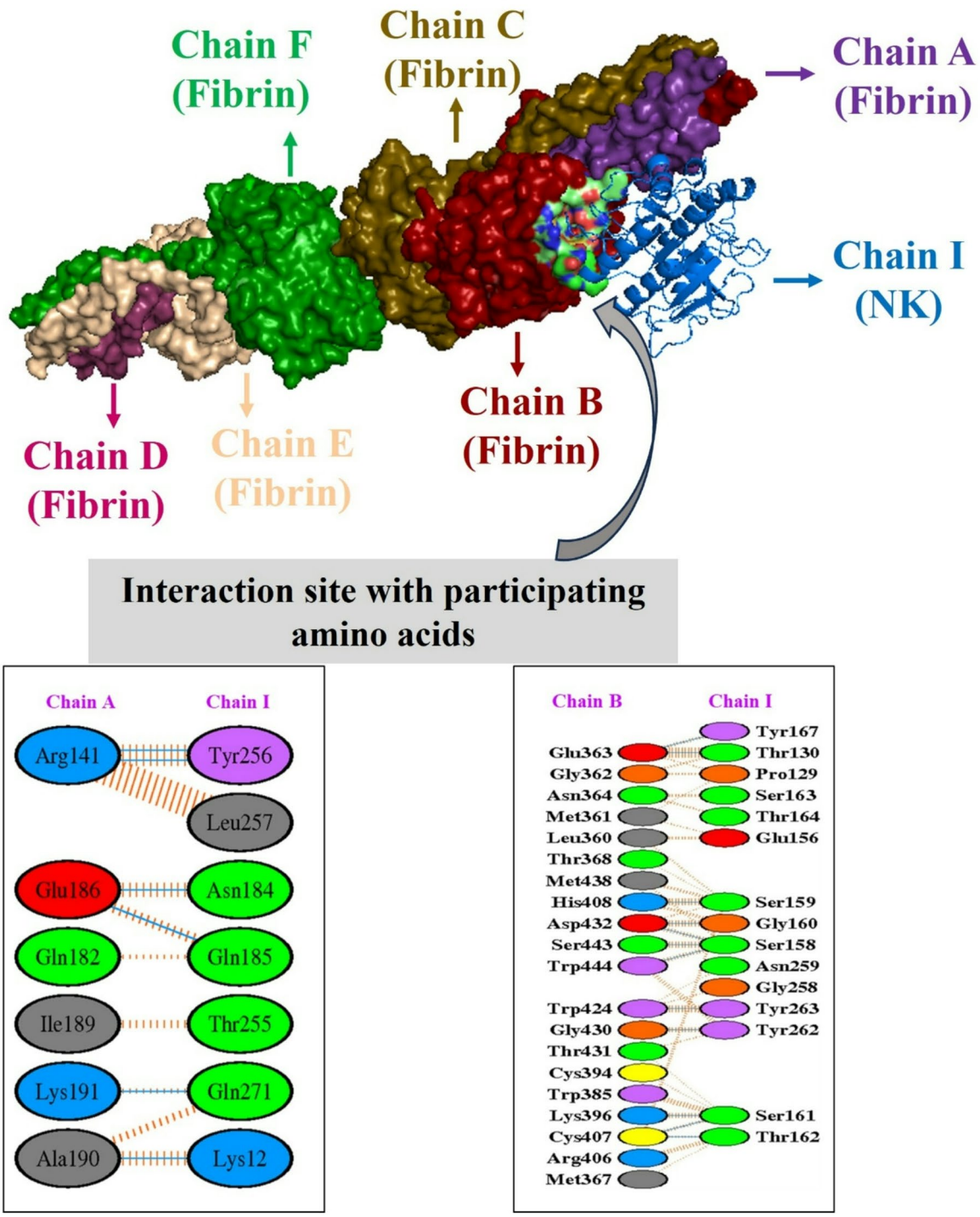

Fig. 4. The three-dimensional docking results of the interaction between NK and fibrin (a multichain complex composed of six individual fibrin chains). The chain **A** to **F** represents the fibrin chain while the chain I represents the NK shown in a blue-ribbon structure. The binding orientation of NK and fibrin is highlighted in green-red colour, with an arrow indicating the primary interaction interface between NK and fibrin.

aging[35]. A four-way ANOVA and post-hoc analyses further clarify the hierarchy of factors affecting CDT, with age and ABO blood group exerting the strongest effects, RhD factor showing a moderate effect, and gender contributing a smaller or no effect. Significant two-way interactions between age × blood group and blood group × RhD factor indicate that the effect of one factor depends on the other. The three-way interaction among age × blood group × RhD factor was also significant ($p = 0.005$), while inclusion of gender in a four-way interaction showed no additional effect on CDT, suggesting that gender does not further modify the combined influence of age, ABO blood group, and RhD factor[36]. Further validation using samples from multiple locations showed

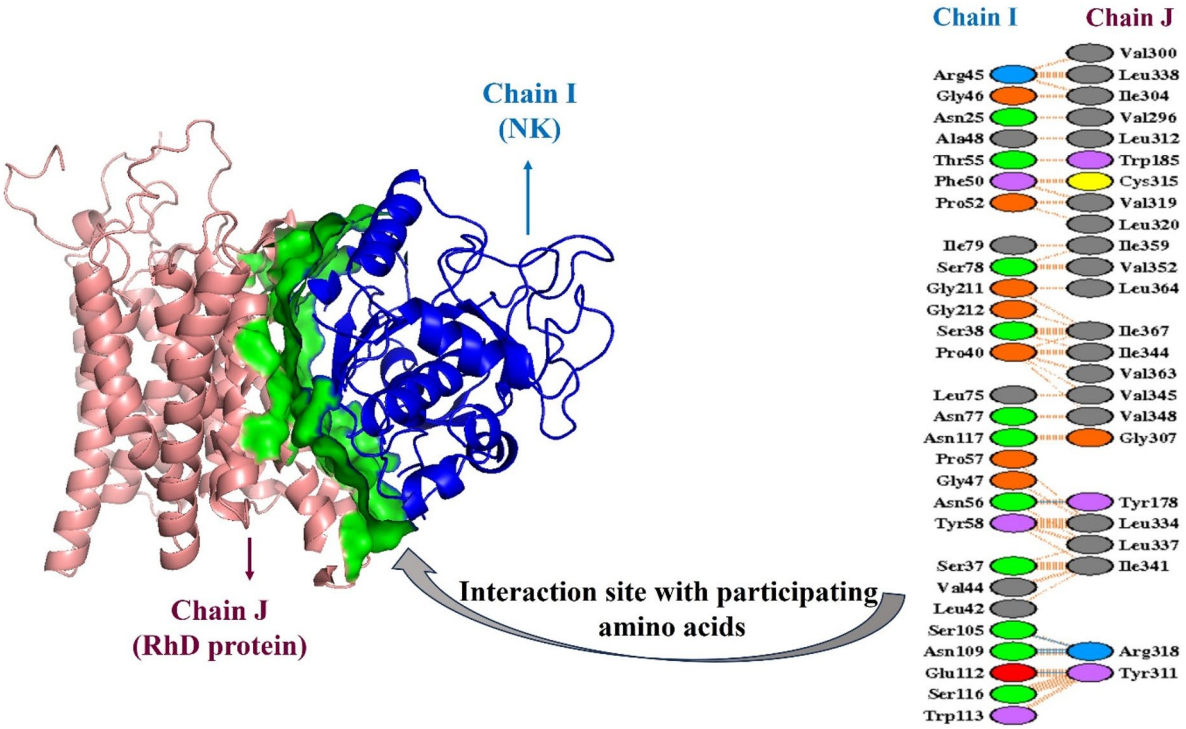

Fig. 5. The three-dimensional docking results of the interaction between NK (chain I) and RhD protein (chain J). The binding orientation of NK and RhD is highlighted in green colour, with an arrow indicating the interaction interface between NK and RhD protein.

≤ 1.6% variation with the anticipated results in clot lysis parameters, demonstrating high reproducibility. These findings suggest that the observed effects reflect conserved physiological mechanisms rather than being population-specific[37].

In the male and female comparative analysis, it was found that females had a slightly longer dissolution time than males. The reason might be the testosterone present in males, which can prevent excessive platelet aggregation and maintain higher tPA levels[38]. This tPA level can help to initiate the fibrinolysis process by activating the plasmin (a natural clot dissolving enzyme presents in the human body). While in females, an estrogen decreases the tPA and elevates the PAI-1 levels, make the clot stronger which initiates high dissolution time[39]. The results of the ABO blood group analysis show that the O+ blood group requires less time for CD, while the AB− blood group requires more time. Furthermore, within the samples of the same age group, the dissolution time also varies; for example, when comparing the dissolution time of A− and O− female samples in the 31–35 age group, the difference comes to 194 s. It indicates that A− females require a higher dosage compared to O− females for reducing the CDT. Previous studies also highlighted that non-O blood groups account for over 30% higher chances of venous thromboembolisms compared to O blood group individuals[40]. In agreement of this, previous studies also reported that individuals with blood group O exhibit the highest clot dissolution rates (61–61%) compared to A, B, and AB blood groups[41]. The possible reason might be that the vWF, present in all the blood groups, plays a key role in blood clotting[42]. According to Favaloro and group, the O blood group has lower vWF than non-O blood groups which is also supported by Rahman and group that O blood group individual 25–30% lower vWF compared to non-O blood types[43,44]. Mourant et al. (1971) reported that individuals with blood group A exhibit higher levels of antihemophilic globulin, which may increase susceptibility to thromboembolic conditions, including thrombosis and coronary infarction, compared with those having O blood group[45]. Subsequent studies have shown that prolonged CDT in non-O blood groups (A, B, and AB) provides functional support for genome-wide association studies identifying a protective effect of the O blood group against myocardial infarction. These findings suggest that reduced efficiency of NK-mediated thrombolysis may contribute to the increased cardiovascular risk observed in non-O individuals[46].

Examination of the RhD factor revealed a clear association with clot dissolution, with RhD-positive samples exhibiting shorter CDT compared to RhD-negative samples. As the mechanistic involvement of RhD in fibrinolysis remains poorly defined in existing literature, molecular docking was performed to explore potential molecular interactions, with the results further corroborated by SEM-based ultrastructural analysis. SEM analysis revealed dynamic alterations in RBCs morphology during the clot dissolution process. During initial clot formation, RBCs exhibited a pronounced reduction in diameter, attributable to water loss and mechanical compression within the contracting fibrin network[47]. At 1510 s, progressive fibrin degradation was accompanied by RBC rehydration and partial recovery of cell dimensions, although complete relaxation of the fibrin mesh appeared limited, likely due to residual osmotic stress[48]. By 1877 s (complete clot dissolution), RBCs had largely returned to near-physiological dimensions, coinciding with extensive fibrin breakdown. The consistently low

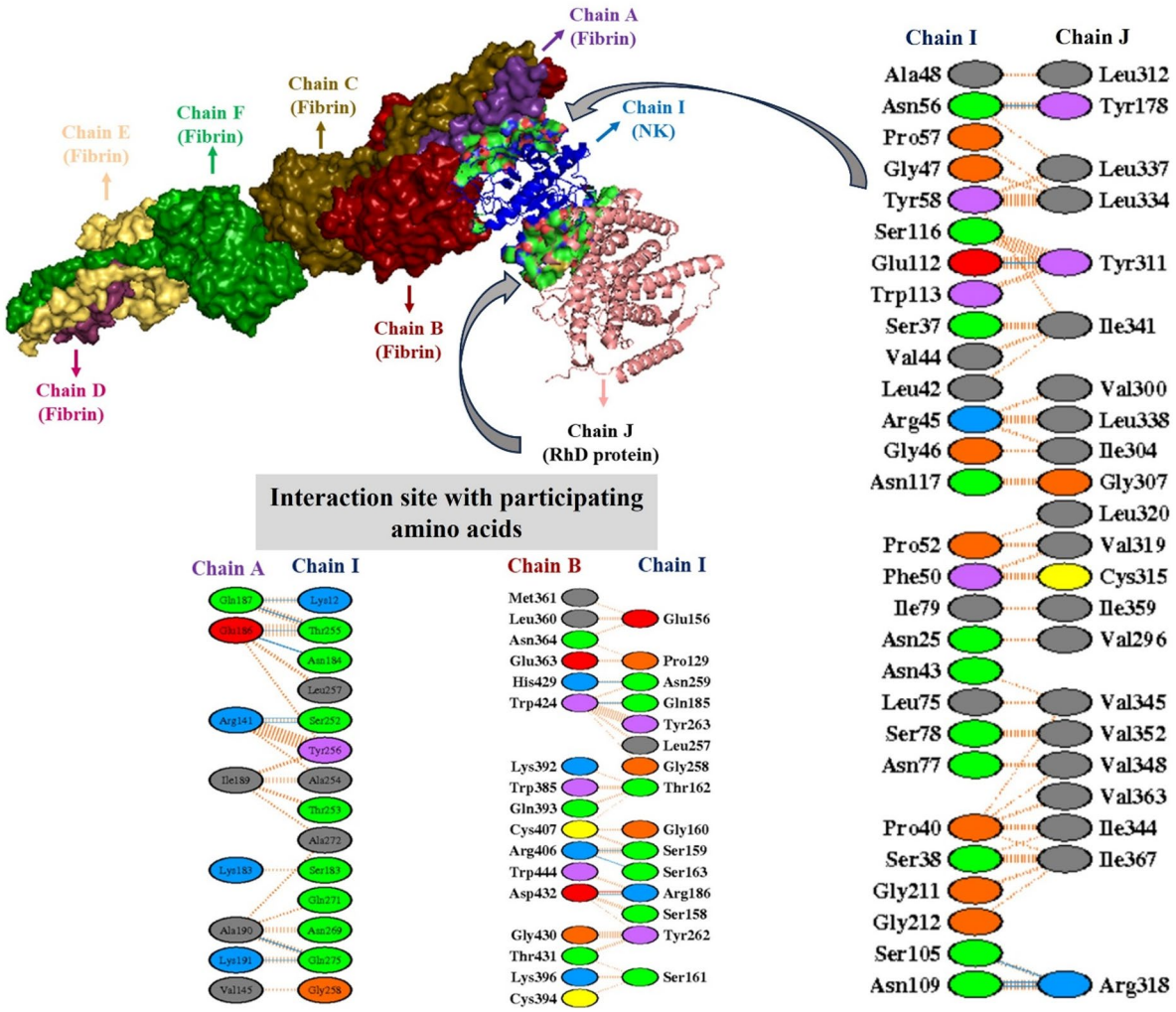

Fig. 6. The three-dimensional docking results of the interaction between RhD-NK complex with **A** and **B** chain of fibrin. The binding orientation of NK-fibrin and RhD-fibrin are highlighted in green-red colour, with an arrow indicating the primary and secondary interaction interface between the fibrin chains, NK and RhD proteins.

percentage variation in RBC diameter (< 0.5%) as compare to the control size (before clot formation) across all blood groups indicates that thrombolytic activity not only disrupts the fibrin scaffold but also permits elastic restoration of entrapped RBCs to their native biconcave discoid morphology. Such morphological recovery is essential for the re-establishment of normal blood flow and efficient oxygen transport. Variations in the extent of initial deformation among different blood groups may reflect differences in clot architecture or membrane properties of RBC[49].

Earlier investigations by Askin et al. (2018) demonstrated no significant association between ABO/RhD blood group systems for in vivo thrombus formation (clot), including thrombus burden in patients with ST-segment elevation myocardial infarction (STEMI)[50]. In contrast, our in vitro findings indicate that both ABO and RhD factors significantly influence the efficiency of clot dissolution following clot establishment. Collectively, these observations suggest that the influence of ABO and RhD blood group determinants may be more pronounced during the fibrinolytic phase rather than during primary clot formation.

Our docking results were found quite similar to the plasmin enzyme, which is also a serine protease enzyme and interacts with the A and B chains of fibrin to initiate the clot dissolution by changing the gamma chain (C-F interaction) of fibrin[51]. An effective confirmation changes in the gamma chain of fibrin in the NK-Fibrin complex, where only non-bonded contacts were present. The number of non-bonded contacts increased from 30 to 33 in the gamma chain of fibrin. But the major changes were the changes highly interacting amino acid sequence. The amino acids that interacted the most with each other in normal fibrin were SER, ASN, and ALA. In the NK-Fibrin complex, however, it is now PHE and TYR. Notably, this interaction between PHE and TYR is not as stable as the original form, and it may help start the breakdown of fibrin. While in the RhD-NK-Fibrin complex, every fibrin chain exhibits a higher number of interactions compared to the NK-Fibrin complex, with the exception of the gamma chain non-bonded contacts, which also get decreased from 30 to 18 in between the gamma chain of fibrin, which makes it less stable and easier to break down. This could potentially explain

why the RhD-positive blood group experiences faster clot dissolution compared to the RhD-negative blood group[48–51]. Overall, these findings suggest that nattokinase may effectively reverse thrombosis-associated biophysical alterations and promote consistent RBC recovery across blood groups; however, this proposed potential mechanism remains hypothetical and requires further validation through comprehensive in vitro and in vivo studies.

Limitations

These in vitro clot lysis results need further large-scale in vivo analysis focusing on the criteria of blood flow dynamics, endothelial interactions, physiological inhibitors or cellular interactions in clot dissolution. The predicted RhD-protein structure lacks the biological accuracy of experimentally derived models, which may affect docking results and its validation. Further, the obtained molecular docking and dynamics study requires more experimental validation to confirm interactions between NK-fibrin-RhD proteins. These limitations pave the way for future research scope on the blood groups with experimental validation, utilizing sophisticated techniques such as X-ray crystallography or cryo-EM for the cardio care aspects in the healthcare sector.

Conclusion

This investigation concludes that CDT is significantly influenced by the combined effects of age, ABO blood group, and RhD factor, independent of gender. Faster clot dissolution was observed in younger individuals (31–40 years), particularly those with blood group O. RhD-positive individuals showed shorter CDT than RhD-negative persons, likely due to structural variations facilitating fibrin interactions. These findings suggest that nattokinase's thrombolytic efficacy is modulated by ABO and RhD antigens rather than being uniform across the blood groups. The results highlight the need for individualized thrombolytic strategies and further multicentric in vitro and in vivo studies to optimize blood group–specific nattokinase dosing, improving its translational potential in cardiovascular disease management.

Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author contributions

TCB: Investigation, methodology, data curation, analysis & interpretation, writing the original draft. AKB: Conceptualization, supervision, validation & interpretation, reviewed and edited the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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