

nattobrom®

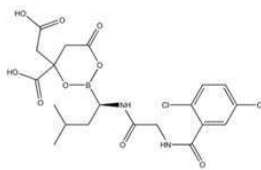


Approfondimenti e lavori scientifici

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nattobrom[®]



E' un integratore a base di nattokinasi, bromelina e curcuma la cui sinergia di azione può essere utile per la salute del sistema cardiocircolatorio.

L'enzima **nattokinasi** appartiene al gruppo delle proteasi della serina (enzimi di scissione della fibrina) ed è ottenuto dalla fermentazione dei semi di soia, da cui deriva il natto, un antichissimo cibo giapponese.

La **bromelina** è un enzima presente nel gambo e nel succo dell'ananas.

La **curcuma longa** è una spezia appartenente alla famiglia delle zingiberacee originaria del sud est asiatico, molto utilizzata nella medicina tradizionale cinese ed indiana.

Contiene 60 compresse

Peso netto 67,2 g

Ingredienti

Curcumina (Curcuma longa L.) rizoma es 95 % Curcuminoidi totali; Bromelina 2500 GDU/g; Agente di carica: Cellulosa microcristallina, Idrossipropilcellulosa; Estratto di Soia fermentata (Nattokinasi) 20.000 / 28.000 FU/g; Agenti antiagglomeranti: Latte di noce di cocco, Amido di patata, Gomma guar; Gomma di acacia; Pepe nero frutti es (Piper nigrum L.)

L'enzima **nattokinasi** deriva dalla fermentazione dei semi di soia trattati generalmente con *Bacillus subtilis var. natto*; il natto è un alimento tipico giapponese conosciuto da millenni, un *superfood* con diverse proprietà salutistiche.

In innumerevoli studi scientifici tale enzima ha dimostrato notevole efficacia nella prevenzione delle patologie cardiovascolari ed in particolare una potente attività fibrinolitica, antipertensiva, antiaterosclerotica, ipolipidemizzante, antiplastrinica e neuroprotettiva. (1)

La nattokinasi, grazie alla sua attività fibrinolitica, ha inoltre mostrato la capacità degradare la proteina Spike. (2)

Rispetto ad altri enzimi fibrinolitici (urochinasi, t-PA e streptochinasi), la nattokinasi presenta il vantaggio di non avere effetti collaterali. (3)

La **bromelina** possiede proprietà antinfiammatorie, proteolitiche e anticoagulanti.

La letteratura scientifica riporta studi riguardanti la possibile applicazione della bromelina nel trattamento di malattie cardiovascolari, disturbi della coagulazione, malattie infettive, malattie associate all'infiammazione. (4)

Pertanto la **bromelina** risulterebbe utile in casi di flebite, artrite reumatoide ed altre patologie infiammatorie su base ossidativa e autoimmune.

Bromelina e nattokinasi, grazie alla loro attività proteolitica e fibrinolitica, sono in grado di scomporre e degradare determinate proteine responsabili dei coaguli di sangue.

La **curcuma** è stata e continua ad essere oggetto di studi scientifici sia in vitro che in vivo e numerose sono le proprietà ad essa attribuibili ed in particolare spiccano l'attività antiossidante e antiinfiammatoria.

In alcuni recenti ed importanti studi scientifici, in vitro e in vivo, ognuno dei tre componenti di **nattobrom®** ha dimostrato un ampio spettro di proprietà antivirali. Tali potenzialità vengono sicuramente aumentate e amplificate dalla sinergia dei tre ingredienti congiuntamente presenti nella formula di **nattobrom®** e saranno pertanto meritevoli di ulteriori approfondimenti.

Indicazioni:

Integratore alimentare utile per completare le carenze in casi di aumentato fabbisogno dei suoi componenti.

Avvertenze: Gli integratori non sono intesi come sostituti di una dieta variata ed equilibrata e di un sano stile di vita. Non eccedere la dose consigliata. Tenere lontano dalla portata dei bambini al di sotto dei 3 anni. Conservare il prodotto in luogo fresco e asciutto. Il consumo del prodotto in combinazione con medicinali può avvenire solo sotto controllo medico. Non consumare in gravidanza ed allattamento. In caso di alterazioni della funzione epatica, biliare o di calcolosi delle vie biliari, l'uso del prodotto è sconsigliato. Non utilizzare per periodi prolungati senza consultare il medico. Per i soggetti in trattamento con farmaci anticoagulanti ed antiaggreganti piastrinici, si consiglia di sentire il parere del medico curante prima di assumere il prodotto.

Modalità d'uso: Si consiglia l'assunzione di 1 compressa al giorno preferibilmente a digiuno

Contenuti medi	Per dose giornaliera 1 compressa
Nattokinasi	100 mg 2000 FU
Curcuma lunga es	500 mg
Apporto di curcuminoidi	475 mg
Bromelina	250 mg
Attività	2500 GDU
Pepe nero es	1 mg

NATTOBROM® PER CHI E' UTILE

I componenti di **nattobrom®** sono stati scelti al fine di creare una sinergia d'azione tra loro al fine di potenziare e coadiuvare l'azione fibrinolitica tipica della nattochinasasi.



La **bromelina**, estratta dal gambo dell'ananas, è un fondamentale enzima sulfidrilico proteolitico con riconosciute numerose attività benefiche per l'organismo umano.

La **bromelina** è stata negli anni oggetto di diversi studi scientifici da cui sono emerse diverse proprietà benefiche nel trattamento di disturbi cardiovascolari con alta efficacia e bassa tossicità.

Nello specifico alla **bromelina** sono riconosciute proprietà che contrastano i "disturbi della coagulazione del sangue e della eccessiva formazione di fibrina), malattie infettive, malattie associate all'infiammazione e molti tipi di cancro". (Pawel Hikiş* et al. *"Beneficial Properties of Bromelain"* *Nutrients* 2021).

Gli enzimi proteolitici sono coinvolti in diversi processi essenziali e per le loro proprietà sono state particolarmente attenzionate dalla comunità scientifica.

Studi sistematici suggeriscono che la **bromelina**, grazie alle sue proprietà biologiche, è un composto interessante nel trattamento di diverse malattie cardiovascolari

La **bromelina** per le sue proprietà anticoagulanti e fibrinolitiche, induce la rottura del trombo, riduce l'aggregazione delle piastrine e la viscosità del sangue. (Maurer H.R. *Bromelain: Biochemistry, pharmacology and medical se. Experientia. 2001;58:1234–1245*)

Studi in vivo hanno dimostrato che la **bromelina**, grazie alle sue eccellenti proprietà fibrinolitiche, provoca la dissoluzione della placca aterosclerotica con elevata efficienza, riducendo così il rischio di malattia aterosclerotica.

Già nella seconda metà del XIX secolo la **bromelina** è stata oggetto di studio.

Gli studi ex vivo condotti su pazienti dopo infarto miocardico, ictus hanno dimostrato che la **bromelina** provoca una diminuzione dell'aggregazione piastrinica, riducendo così il rischio di trombosi arteriosa ed embolia.

La **bromelina** agisce sia sulle vie esterne che su quelle interne del sistema di coagulazione del sangue. La **bromelina** regola l'omeostasi della coagulazione del sangue inibendo la sintesi della fibrina e aumentando l'attività fibrinolitica del siero.

Gli studi dimostrano che la combinazione dell'azione antinfiammatoria e proteolitica che inibisce l'aggregazione piastrinica e la sintesi delle prostaglandine è anche caratterizzata da

una buona attività antitumorale. (Streiff, M.B.; Milentijevic, D.; McCrae, K.; Yannicelli, D.; Fortier, J.; Nelson, W.W.; Laliberté, F.; Crivera, C.; Lefebvre, P.; Schein, J.; et al. *Effectiveness and safety of anticoagulants for the treatment of venous thromboembolism in patients with cancer*. *Am. J. Hematol.* 2018, 93, 664–671. -Khorana, A.A.; Noble, S.; Lee, A.Y.Y.; Soff, G.; Meyer, G.; O'Connell, C.; Carrier, M. *Role of direct oral anticoagulants in the treatment of cancer-associated venous thromboembolism: Guidance from the SSC of the ISTH*. *J. Thromb. Haemost.* 2018, 16, 1891–1894.)

Si ritiene che le proprietà fibrinolitiche e l'attività antiplastrinica possano essere coinvolte nell'attività antitumorale della **bromelina**. (Chobotova, K.; Vernallis, A.B.; Majid, F.A.A. *Bromelain's activity and potential as an anti-cancer agent: Current evidence and perspectives*. *Cancer Lett.* 2010, 290, 148–156). ipotizzando che l'effetto inibitorio della **bromelina** sullo sviluppo del cancro sia dovuto alla prevenzione degli aggregati tumore-piastrine.

Sulla base di numerosi studi in vitro e in vivo, è stato inoltre dimostrato che la **bromelina** può influenzare la coagulazione del sangue modulando selettivamente il livello di prostaglandina E2 (PGE2) e di trombossano A2 appartenenti alla classe dei prostanoidi. La **bromelina**, in modo dose-dipendente, ha causato una diminuzione statisticamente significativa del livello di attività della PGE2 e del trombossano A2 (la loro inibizione) e ha spostato il rapporto trombossano A2/prostaciclina (PGI2) a favore della prostaciclina antinfiammatoria ((Pawel Hikisz* et al. *"Beneficial Properties of Bromelain"* *Nutrients* 2021).

Il processo coagulativo si avvale di numerosi fattori. Alcuni di questi favoriscono l'aggregabilità piastrinica e la produzione di fibrina, altri, che intervengono quando il vaso danneggiato è stato riparato, inibiscono i suddetti processi dissolvendo il coagulo

Il razionale della formulazione e l'inserimento della **curcuma longa** (titolata al 95% in *curcumina*) si riallaccia proprio al concetto della necessità di combinare le proprietà della **bromelina** ad una azione antiinfiammatoria.

La **Curcumina** (diferuloilmetano), è una molecola appartenente alla classe dei polifenoli estratta dal rizoma della *Curcuma longa*, una pianta appartenente alla famiglia delle Zingiberaceae molto diffusa nel sud-est asiatico.

Viene tradizionalmente utilizzata nella medicina Ayurvedica, Cinese e Indiana per le sue proprietà antisettiche, analgesiche, antinfiammatorie e antimalariche ed è una sostanza considerata farmacologicamente sicura a seguito di assunzione orale anche ad alti dosaggi (12gr./die).

In particolare la **curcumina** ha dimostrato, come si può evincere da una letteratura scientifica davvero imponente (più di 25000 pubblicazioni), proprietà: antitumorali, anti-infiammatorie (compresa la neuroinfiammazione), antiossidanti, antiangiogenetiche, immunomodulatrici, antitrombotiche, cardio-epato-nefro protettici, ipoglicemicizzanti, antireumatiche e antifibrotiche.

L'utilizzo dell'intero rizoma di **curcuma longa titolato in curcuminoidi al 95%** è dovuto alla necessità di creare al meglio la sinergia tra i componenti di **nattobrom®** al fine di ottenere la migliore prestazione da ogni singola sostanza senza interazioni di effetti contrastanti.

Il rizoma della curcuma longa contiene, infatti, numerosi composti chimici, tra cui gli oli essenziali *tumerone*, *atlantone* e *zingiberone*, che son in grado di potenziare la sinergia tra i vari componenti di nattobrom®.

Se prevenzione è la nostra parola d'ordine, nattobrom® rappresenta la migliore soluzione per preservare il sistema cardiocircolatorio e prevenire l'insorgere sia di patologie ad esso inerenti che di malattie che sono responsabili di situazioni cronico-degenerative multifattoriali di difficile trattabilità.

NATTOCHINASI

La **nattochinas** è un enzima naturale che viene estratto dalla fermentazione del natto, un alimento tipico della cucina giapponese derivante dalla fermentazione della soia.

Questo enzima si forma proprio durante il processo di fermentazione dei semi di soia operato con il batterio amico *Bacillus subtilis natto*.

Tale alimento ha origini antichissime le cui proprietà sarebbe già state conosciute oltre 2.000 anni fa.

L'enzima **nattochinas** appartiene al gruppo delle proteasi della serina ossia enzimi con proprietà di scissione della **fibrina**.

La comunità scientifica si è occupata molto dello studio della nattochinas e sono numerose le pubblicazioni scientifiche di lavori in vitro e clinici che hanno evidenziato gli effetti benefici di tale enzima grazie alla capacità di degradare direttamente la fibrina e di promuovere il processo fibrinolitico, ossia il meccanismo opposto alla coagulazione del sangue.

Tale beneficio può essere quindi utile nel prevenire alcune patologie cardiovascolari grazie alle proprietà antitrombotiche, senza quegli effetti collaterali che caratterizzano i farmaci anticoagulanti.

Le proprietà della nattochinas potrebbero essere sintetizzate nello schema riportato qui di seguito

ATTIVITA'	AZIONE	BENEFICI
COAGULAZIONE	<i>Fluidificante del sangue</i>	<i>Riduzione rischio trombotosi, ictus ecc. ecc.</i>
PRESSIONE SANGUIGNA	<i>Riduzione della pressione sanguigna</i>	<i>Controllo della ipertensione</i>
PROCESSI ANTINFIAMMATORI	<i>Effetto antiinfiammatorio</i>	<i>Miglioramento della salute generale</i>
CIRCOLAZIONE SANGUIGNA	<i>Miglioramento della circolazione sanguigna</i>	<i>Ottimale ossigenazione e trofismo delle cellule</i>

Systematic Review

Nattokinase Supplementation and Cardiovascular Risk Factors: A Systematic Review and Meta-Analysis of Randomized Controlled TrialsXinmin Li^{1,†}, Junzi Long^{2,†}, Qian Gao², Mengyang Pan², Jing Wang², Fangjie Yang²,
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Abstract

Background: As a fibrinolytic enzyme from fermented soybean, nattokinase has been shown to be potentially beneficial for cardiovascular health, but current clinical evidences regarding the nattokinase supplementation on cardiovascular risk factors are various. This study aims to evaluate the cardiovascular efficacy of nattokinase. **Methods:** Four electronic databases were systematically searched to collect eligible randomized controlled trials. Data were extracted and summarized in a pre-designed form by two independent reviewers. Review Manager 5.4 software (Cochrane Library Software, Oxford, U.K.) was used for meta-analysis and bias risk assessment. **Results:** Six studies were eligible for quantitative analysis with 546 participants. The overall methodological quality of included studies was high. Relatively low total dosage of nattokinase had a negative effect on blood total cholesterol (MD [mean difference] = 5.27, 95% CI [confidence intervals]: 3.74 to 6.81, $p < 0.00001$), high-density lipoprotein cholesterol (MD = -2.76, 95% CI: -3.88 to -1.64, $p < 0.00001$), and low-density lipoprotein cholesterol (MD = 6.49, 95% CI: 0.83 to 12.15, $p = 0.02$). Nattokinase supplementation significantly reduced systolic blood pressure (MD = -3.45, 95% CI: -4.37 to -2.18, $p < 0.00001$) and diastolic blood pressure (MD = -2.32, 95% CI: -2.72 to -1.92, $p < 0.00001$), and led a slight increase in blood glucose (MD = 0.40, 95% CI: 0.20 to 0.60, $p < 0.0001$) as compared to placebo. Nattokinase group with relatively high total dosage also had a higher total cholesterol (MD = 3.18, 95% CI: 2.29 to 4.06, $p < 0.00001$) than control interventions, but no significant differences were found in levels of high-density lipoprotein cholesterol and low-density lipoprotein cholesterol. No significant correlation was found between nattokinase supplementation and triglyceride ($p = 0.71$). No notable adverse events were reported in all studies due to intake of nattokinase. **Conclusions:** This study further supports that nattokinase can be used as an effective adjunctive therapy for hypertension, but relatively low-dose supplementation of nattokinase may have no significant lipid-lowering effect. More work will need to be done to determine whether the positive efficacy of nattokinase on cardiovascular risk factors is dose-dependent. **Systematic Review Registration:** This work has been registered on PROSPERO (CRD42022315020).

Keywords: nattokinase; cardiovascular disease; cardiovascular risk factor; meta-analysis**1. Introduction**

Cardiovascular diseases (CVDs) remain the most common causes of premature mortality and disability globally [1]. The major risk factors of CVD, such as coagulation abnormality, hypertension, dyslipidemia, and hyperglycemia have been well established [2]. Dietary modification is a fundamental strategy for the prevention of CVD, and adequate dietary choices may promote cardiovascular health [3]. Previous research has established that dietary intake of soybeans is negatively associated with the risks of CVD [4], which shows the promise of soy food as a dietary therapy for CVD.

Over past decades, traditional Japanese diets have attracted growing attention because of substantially low CVD morbidity and the highest life expectancy of Japanese population [5]. Natto is a famous traditional Japanese food made from fermented soybeans, which contains a variety

of functional ingredients, including nattokinase. As a serine protease produced by *Bacillus subtilis*, nattokinase has potential anti-coagulatory, thrombolytic, anti-atherosclerotic, lipid-lowering, and anti-hypertensive effects [6,7]. In addition to these favourable cardiovascular profiles, nattokinase can be orally administered with inexpensive cost, proven safety and preventative efficacy [8]. Hence, nattokinase consumption is growing in both healthy and CVD individuals around the world, especially in Asian countries.

Nattokinase has a stronger fibrinolytic activity than plasmin *in vivo*, and can even hydrolyze fibrin directly [9]. Oral administration of nattokinase not only promotes the release of tissue plasminogen activator from vascular endothelial cells, but inhibits the level of plasminogen activator inhibitors [10,11]. Recently, experimenters also provide a new insight that nattokinase is able to prevent atherosclerosis and thrombosis by exerting anti-inflammatory,



anti-oxidative stress and anti-apoptotic effects [6,12–14]. In addition, a number of *in vitro* and animal experiments have established that nattokinase suppresses hypertension via inhibiting angiotensin-converting enzyme and plasma angiotensin II level [8,15,16].

Although recent review studies suggest that nattokinase is a promising alternative in the prevention and treatment of CVD [17–19], cardiovascular benefits of nattokinase, such as its lipid-lowering effect, remain controversial [20–23]. This study thus aims to assess the efficacy of nattokinase on cardiovascular risk factors and to provide evidence-based recommendations for clinical decision-making.

2. Methods

This systematic review and meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendations [24], and the protocol had been registered on PROSPERO (CRD42022315020).

2.1 Search Strategy

Electronic searches for pertinent studies were conducted in PubMed, Web of Science, Embase, and Cochrane Library from their inception to February 14, 2023, using the keyword “nattokinase” in the title or abstract. In order to adequately identify eligible studies, no restrictions were placed on terms of cardiovascular risk factors. Two authors separately screened retrieved records, and discrepancies were resolved by consulting with other authors. Moreover, references of included literature were checked to find more eligible studies.

2.2 Selection Criteria

(I) Type of study: prospective parallel-group randomized controlled trials (RCTs) with at least a 1-month follow-up.

(II) Subjects: adults with or without established cardiovascular risk factors.

(III) Intervention and control measures: comparison between nattokinase supplementation and placebo intervention without limitations on oral dosage and frequency.

(IV) Outcome measurements: blood lipids, blood pressure, blood glucose, metabolic factors, hemorheological parameters, coagulation indexes, and adverse effects.

Studies in which the outcome measurements could not be synthetically evaluated were included for qualitative analysis but excluded from the meta-analysis. This study did not include trials that observed fermented soybeans (natto) on cardiovascular risk factors, because fermented soybean product contained a high amount of nutrients, some of which had cardiovascular benefits (not just nattokinase). Furthermore, we excluded cohort studies, case control studies, case report/series, and review studies.

2.3 Data Extraction and Quality Assessment

Collected data were summarized in a pre-designed form by two authors, and any difference was solved by consensus, which included basic information of research, population characteristics, medication administration details, and outcome measurements. Quality appraisal was undertaken by two reviewers based on the Cochrane Collaboration's tool, and another author made a final decision regarding any disagreement.

2.4 Data Synthesis and Analysis

This meta-analysis was performed using the RevMan 5.4 version software (Cochrane Library Software, Oxford, U.K.). Quantitative analyses were carried out if more than one study reported the same outcome data that were available and consistent on clinical grounds. Between-study heterogeneity was tested by the Q-test (Chi-square) and quantified by the I^2 statistic, where a significant Q-test ($p < 0.10$) and value for $I^2 > 50\%$ represented high heterogeneity among studies, and random-effect models were used. Otherwise, the fixed-effect model was utilized [25]. For continuous variables, mean difference (MD) was utilized to signify the degree of deviation between variables, and standardized mean difference (SMD) was used when mean value varied widely. We conducted sub-group analysis to investigate the potential sources of heterogeneity, and provided a narrative overview for unexplained substantial heterogeneity. The sensitivity analysis would be used to assess the robustness of our findings via excluding trials that were considered to have a high risk of bias in one or more domains. Publication bias was detected using funnel plot if more than 10 eligible studies were included in the corresponding meta-analysis [26]. The p value of <0.05 was defined as statistically significant, and 95% confidence intervals (CI) were calculated for all included data.

3. Results

3.1 Search Results

The first search yielded 1032 studies, and thirty-six remained after excluding duplicates and irrelevant studies. Based on the selection criteria, 24 studies were removed, and 7 RCTs were finally included in the qualitative analysis [27–33]. Data from the 6 RCTs could be synthesized for the meta-analysis [27–29,31–33]. The PRISMA flow chart presents the detailed retrieval process (Fig. 1).

3.2 Participant Characteristics

The main study characteristics are presented in Table 1 (Ref. [27–33]). A total of 311 participants received nattokinase and 296 received matching placebo. The mean number of participants per study was 86, ranging from 28 to 265. The average age of participants was significantly varied in each trial, and approximately 62% of the population was female. Five studies recruited subjects with cardiovascu-

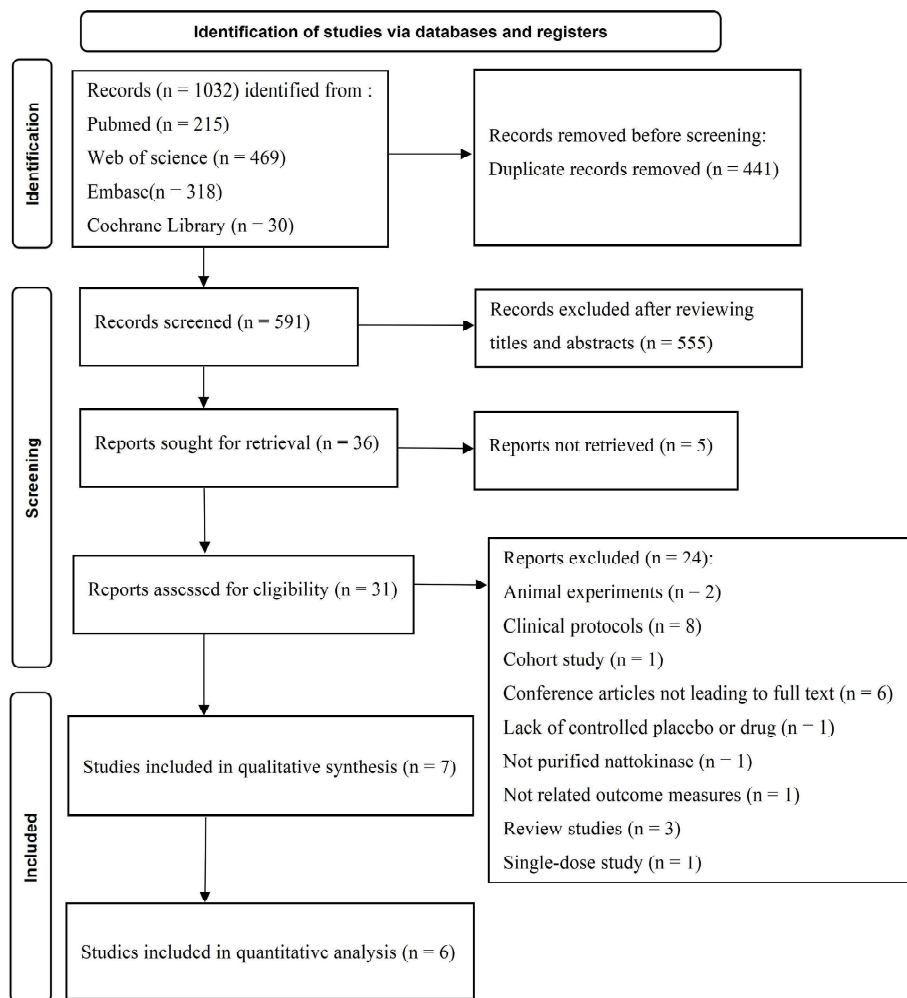


Fig. 1. The PRISMA flow diagram of study selection.

lar disease risk factors, including hypertension [28,29] and hyperlipidemia [31–33]. One trial recruited patients diagnosed with sub-acute ischemic stroke [30], and another trial included healthy subjects without any clinical evidence of cardiovascular risk factors [27]. Nearly 56% of the included participants were Americans and the others were Asians.

3.3 Intervention and Outcome Measurements

In the present meta-analysis, nattokinase supplementation as an intervention to manage cardiovascular risk factors was compared with the matching placebo. The daily dosage of nattokinase was highly variable among the included trials, ranging from 1200 to 8000 FU (a fibrin unit used to quantify the ability of nattokinase to lyse fibrin *in vitro*) [34]. Both nattokinase and placebo were produced in the same capsule form. Five studies performed a follow-up evaluation at the eighth week [28–31,33]. The follow-up time of the other two studies was 6 months [32] and 3 years [27]. All included studies evaluated potential risks of CVDs, including blood coagulation and fibrinolysis fac-

tors, blood lipids, blood pressure, and blood glucose. Six of included trials observed adverse events encountered with nattokinase and control interventions [27,28,30–33]. Four studies evaluated compliance by counting returned capsules [27,28,32,33].

3.4 Risk of Bias Assessments

All the studies were randomized and provided information about randomization and allocation concealment. Double-blinded method was reported in six trials [27–29,31–33] and one trial had a single-blinded design [30]. Two studies reported no patient drop-outs [29,30], and five studies provided numbers and reasons for dropping out [27,28,31–33], so they all were considered to have low risk of attrition bias. All included trials reported the main results as planned, and four of them were judged to have a low risk of selective reporting bias [27,29,32,33]. Table 2 (Ref. [27–33]) presents the risk of bias assessment results.

Table 1. Characteristics of included trials evaluating the effect of nattokinase on risks of cardiovascular disease.

Trial	Location	Study design	Participants		Age (year) Mean (SD)/[Range]	Nattokinase supplementation	Control	Outcomes for quantitative analysis
			N/C					
Hodis <i>et al.</i> (2021) [27]	USA	RCT; prospective; single-center; double-blinded	132/133		65.3 [60.6–72.3]	2000 FU/day 36 months	Matching placebo 36 months	Total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, blood glucose
Jensen <i>et al.</i> (2016) [28]	USA	RCT; prospective; multi-center; double-blinded	39/35		53.4 [20.8–82.8]	2000 FU/day 8 weeks	Matching placebo 8 weeks	Blood pressure
Kim <i>et al.</i> (2008) [29]	South Korea	RCT; prospective; single-center; double-blinded	39/34		N: 47.6 ± 1.78 C: 46.5 ± 1.65	2000 FU/day 8 weeks	Matching placebo 8 weeks	Blood pressure
Pham <i>et al.</i> (2020) [30]	Vietnam	RCT; prospective; single-center; single-blinded	31/30		60.1 [30–70]	1200 FU/day 60 days	Matching placebo 60 days	Blood pressure
Wu <i>et al.</i> (2009) [31]	Taiwan	RCT; prospective; single-center; double-blinded	15/15		N: 54.8 ± 9.6 C: 51.6 ± 10.1	8000 FU/day 8 weeks	Matching placebo 8 weeks	Total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol
Yang <i>et al.</i> (2009) [32]	Taiwan	RCT; prospective; multi-center; double-blinded	18/10		N: 51.6 ± 8.6 C: 56.3 ± 11.8	7000 FU/day 6 months	Matching placebo 6 months	Total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol
Yoo <i>et al.</i> (2019) [33]	South Korea	RCT; prospective; single-center; double-blinded	37/39		N: 54.3 ± 1.25 C: 53.1 ± 1.40	6000 FU/day 8 weeks	Matching placebo 8 weeks	Total cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, blood glucose

Abbreviations: N, nattokinase group; C, control group; FU, fibrinolytic unit; RCT, randomized controlled trial; SD, standard deviation.

Table 2. The summary of reviewers' judgments about each risk of bias item for included trials.

Trial	Random sequence generation		Allocation concealment		Blinding of participants and personnel		Blinding of outcome assessment		Incomplete outcome data		Selective reporting		Other bias
Hodis <i>et al.</i> (2021) [27]	L		L		L		L		L		L		L
Jensen <i>et al.</i> (2016) [28]	L		L		L		U		L		U		U
Kim <i>et al.</i> (2008) [29]	L		L		L		U		L		L		L
Pham <i>et al.</i> (2020) [30]	L		L		U		U		L		U		U
Wu <i>et al.</i> (2009) [31]	L		L		L		U		L		U		U
Yang <i>et al.</i> (2009) [32]	L		L		L		L		L		L		L
Yoo <i>et al.</i> (2019) [33]	L		L		L		L		L		L		L

Abbreviations: L, low risk of bias; U, unclear or unrevealed risk of bias.

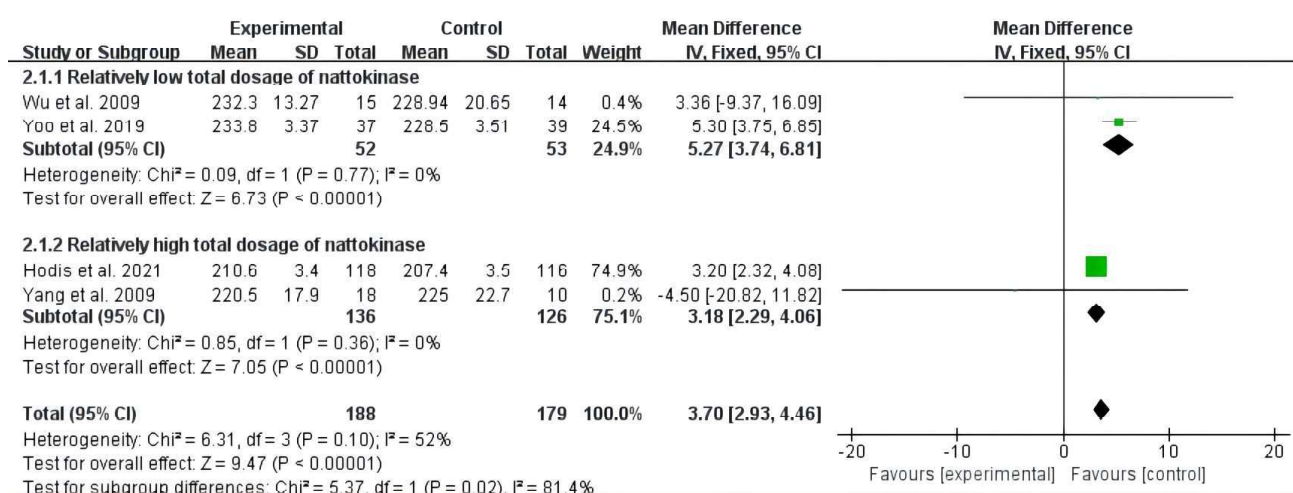


Fig. 2. Forest plot of RCTs investigating the effect of nattokinase on total cholesterol. RCTs, randomized controlled trials; SD, standard deviation; CI, confidence intervals.

3.5 Qualitative Results

Blood coagulation and fibrinolytic parameters. In a trial conducted among healthy subjects, nattokinase showed no detectable effects on any observed coagulation and fibrinolytic factor, such as prothrombin time, activated partial thromboplastin time, von Willebrand factor antigen and tissue plasminogen activator antigen, at time points of 1 week and 1, 3, and 6 months relative to placebo [27]. Jensen *et al.* [28] found that average level of von Willebrand factor was reduced by 15% in patients with hypertension after nattokinase supplementation, whereas consistent change was not found for subjects consuming placebo after 8 weeks ($p < 0.09$). In the study by Yoo *et al.* [33], nattokinase group had a greater increase in collagen–epinephrine closure time ($p = 0.001$) and activated partial thromboplastin time ($p = 0.016$) than those in placebo group.

Degree of atherosclerosis. Results of a 3-year intervention with nattokinase supplementation in healthy individuals showed that annualized rate of carotid artery intima-media thickness progression was 0.013 mm (95% CI, 0.010 to 0.015) per year in the nattokinase group, and 0.011 mm (95% CI, 0.009 to 0.013) per year in the placebo group ($p = 0.31$). In addition, the mean rate of carotid arterial stiffness was not significantly different between two groups [27].

Blood pressure and associated regulators. Pham *et al.* [30] found that nattokinase supplementation (60 days) led to statistically significant reductions ($p < 0.05$) in both systolic blood pressure (SBP) and diastolic blood pressure (DBP) compared to pre-treatment. In an 8-week trial, researchers found that patients' DBP was significantly decreased after nattokinase intervention compared with placebo intervention (nattokinase: 84 ± 1.9 mmHg; placebo: 87 ± 1.7 mmHg; $p < 0.01$). Besides, participants with normal and high baseline plasma renin activity in both two groups had plasma renin activity decreased, and for subgroups with low baseline plasma renin activity, 66%

of them in the nattokinase group had plasma renin activity improved to normal levels, but there were only 8% of subjects had plasma renin activity normalized in the placebo group [28]. During the 8-week intervention period, Kim *et al.* [29] found that the mean renin activity was increased in the control group and decreased in the nattokinase group without a statistically significant difference, but the corresponding net change was significant ($p = 0.026$).

3.6 Quantitative Data Synthesis and Analysis

3.6.1 Effect of Nattokinase on Blood Lipids

Overall, four studies with a total of 399 participants included measures of total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) [27,31–33]. There was high heterogeneity for the above three meta-analyses ($I^2 = 52\%$, 95% , and 93%). We observed that subgroup-analysis based on the total dosages of nattokinase might explain the potential heterogeneity between these studies. Two trials had an 8-week nattokinase intervention [31,33], and the other two observed nattokinase supplementation for 6 months and 3 years [27,32], so the total therapeutic dose of these two subgroups were considered to be relatively low and high, respectively.

The subgroup of relatively low total nattokinase dosage showed that there was a positive association between nattokinase supplementation and total cholesterol (MD = 5.27, 95% CI: 3.74 to 6.81, $p < 0.00001$); relatively high total dosage of nattokinase supplementation also led to an increase in total cholesterol (MD = 3.18, 95% CI: 2.29 to 4.06, $p < 0.00001$) (Fig. 2).

The aggregated results of these studies showed that nattokinase group with relatively low total dosage had more HDL-C levels reduced (MD = -2.76 , 95% CI: -3.88 to -1.64 , $p < 0.00001$) than control group. Nattokinase supplementation with relatively high total dosage showed a fa-

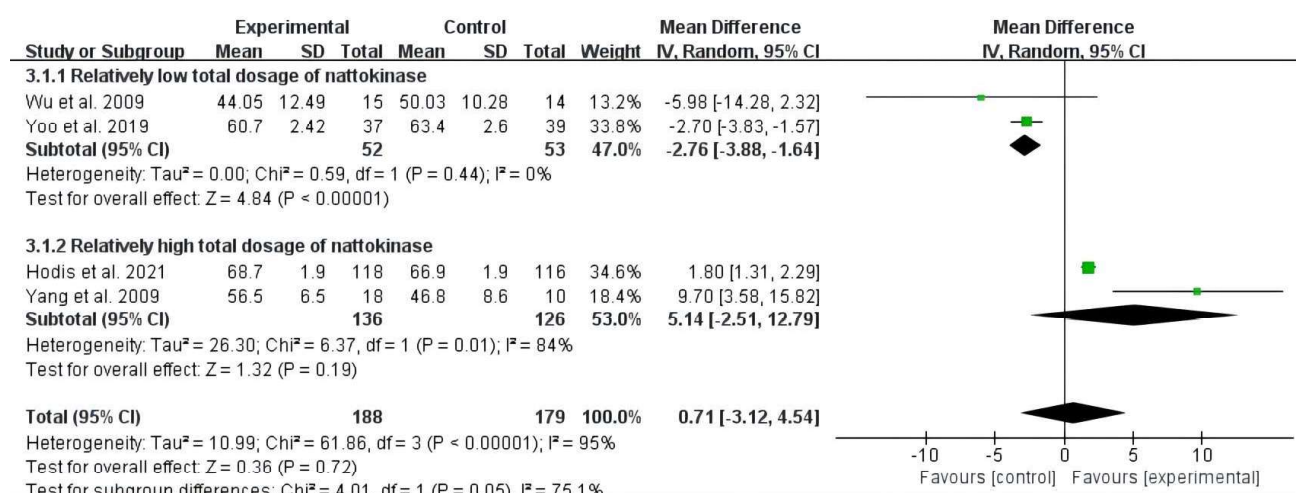


Fig. 3. Forest plot of RCTs investigating the effect of nattokinase high-density lipoprotein cholesterol. RCTs, randomized controlled trials; SD, standard deviation; CI, confidence intervals.

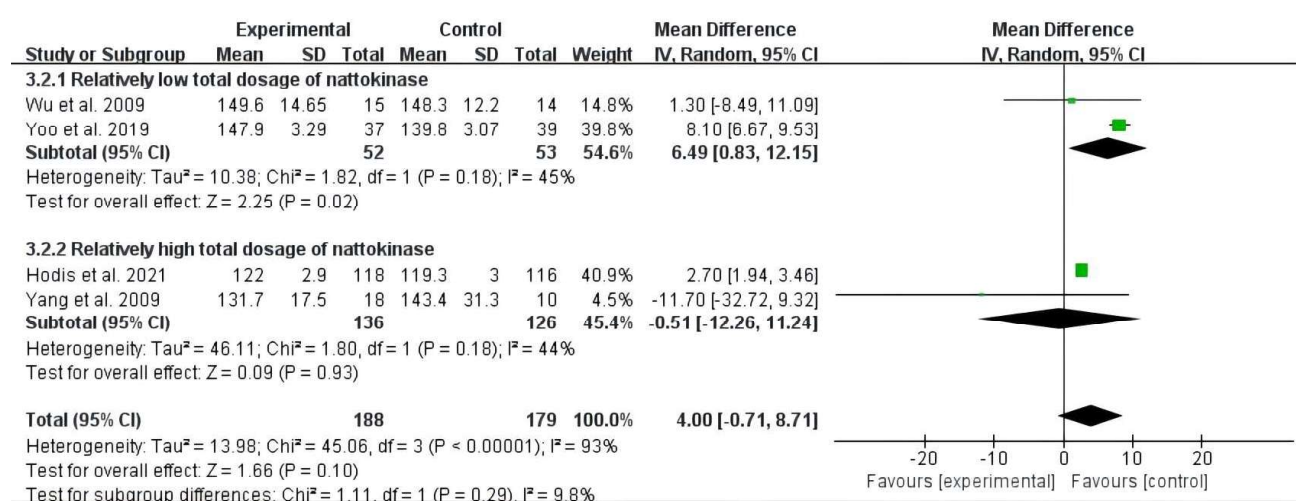


Fig. 4. Forest plot of RCTs investigating the effect of nattokinase on low-density lipoprotein cholesterol. RCTs, randomized controlled trials; SD, standard deviation; CI, confidence intervals.

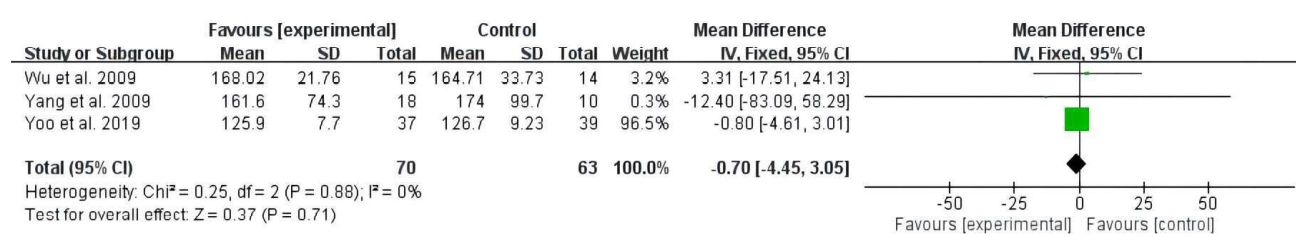


Fig. 5. Forest plot of RCTs investigating the effect of nattokinase on triglyceride. RCTs, randomized controlled trials; SD, standard deviation; CI, confidence intervals.

avorable but non-statistically significant effect in increasing HDL-C levels (MD = 5.14, 95% CI: -2.51 to 12.79, $p = 0.19$) compared with controls (Fig. 3). Furthermore, pooled analyses of the low total dosage subgroup showed that nattokinase induced higher LDL-C levels than control interventions (MD = 6.49, 95% CI: 0.83 to 12.15, $p = 0.02$), but no statistically significant effects were observed for the

group with relatively high total nattokinase dosage ($p = 0.93$) (Fig. 4).

As shown in Fig. 5, data from three studies with 134 participants were pooled to assess the effect of nattokinase on triglyceride [31–33]. No significant effect of intake of nattokinase was found in improving triglyceride levels (MD = -0.7, 95% CI: -4.45 to 3.025, $p = 0.71$).

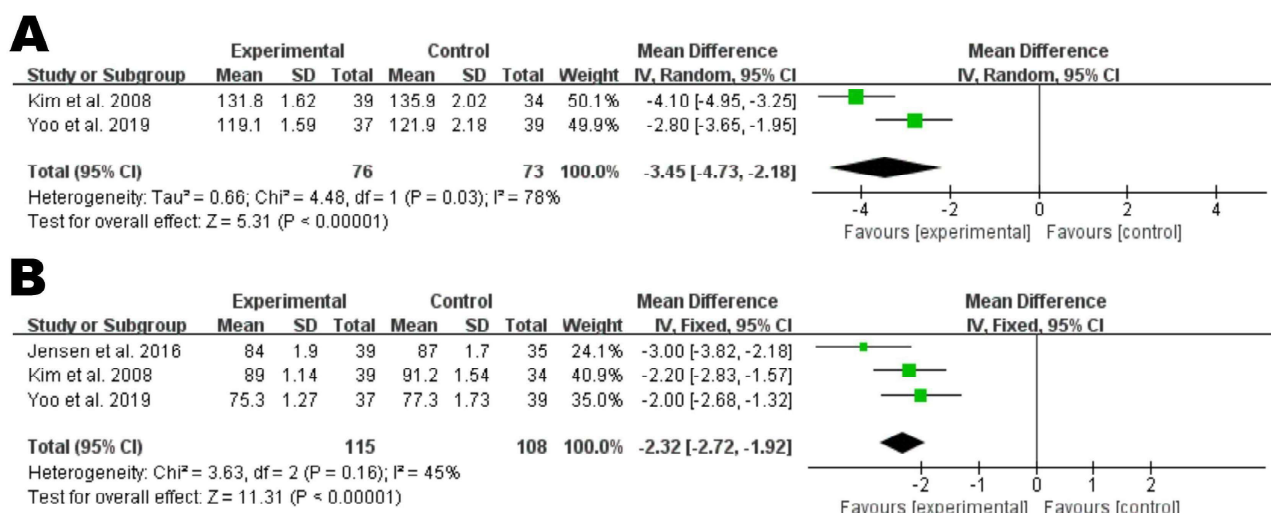


Fig. 6. Forest plot of RCTs investigating the effect of nattokinase on systolic blood pressure (A) and diastolic blood pressure (B). RCTs, randomized controlled trials; SD, standard deviation; CI, confidence intervals.

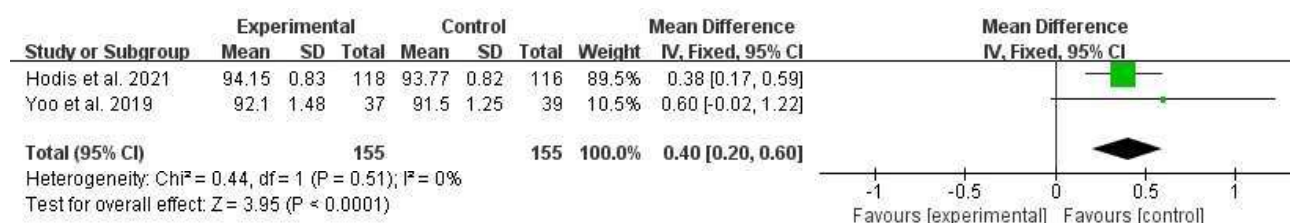


Fig. 7. Forest plot of RCTs investigating the effect of nattokinase on blood glucose. RCTs, randomized controlled trials; SD, standard deviation; CI, confidence intervals.

3.6.2 Effect of Nattokinase on Blood Pressure

Based on the results of 3 RCTs that included 115 individuals in the nattokinase group and 108 individuals in the control group [28,29,33], nattokinase supplementation was found to be associated with a significant decrease in SBP ($MD = -3.45$, 95% CI: -4.37 to -2.18 , $p < 0.00001$) and DBP ($MD = -2.32$, 95% CI: -2.72 to -1.92 , $p < 0.00001$) as compared to placebo (Fig. 6).

3.6.3 Effect of Nattokinase on Blood Glucose

The impact of nattokinase on blood glucose was evaluated in two studies, including a total of 341 participants (Fig. 7) [27,33]. The results showed that nattokinase induced a slight increase of blood sugar with no heterogeneity across studies ($I^2 = 0\%$) in comparison with control interventions ($MD = 0.40$, 95% CI: 0.20 to 0.60 , $p < 0.0001$).

3.7 Adverse Events and Compliance Rate

No notable adverse events were reported among all groups due to intake of nattokinase or placebo. The average compliance rate of participants exceeded 95% in three studies [27,28,32].

4. Discussion

To the best of our knowledge, this is the first systematic review to examine the association between nattokinase supplementation and cardiovascular risk factors. Overall, present study involving 607 participants found that nattokinase might have a beneficial influence on blood pressure, but no significant improvements were observed in blood lipids and blood glucose. Despite the high methodological quality of eligible studies in this meta-analysis, the total number of studies examining any cardiovascular risk factor was small, so these findings should be interpreted cautiously.

Since the fibrinolytic activity of nattokinase was discovered in the 1980s, a variety of animal-based studies have been made to support its strong thrombolytic activity [18, 35–38]. Even a single dose of oral nattokinase was shown to enhance fibrinolysis and anticoagulation in humans [22,39]. Moreover, nattokinase positively affected various blood rheological parameters in a dose-dependent manner, including platelet aggregation, red blood cell aggregation, whole blood viscosity and vascular tension, which could be considered as a good candidate in improving blood flow [40–43]. Clinical data on fibrinolytic and antithrombotic effects

of nattokinase were sparse in the present meta-analysis, which limits quantitative analysis of these results; however, it is worth mentioning that in recent years, nattokinase has been used on a much larger scale than previously—researchers have expanded their scopes to other cardiovascular benefits of nattokinase, such as lipid-lowering and hypotensive potentials.

In the meta-analysis of nattokinase on blood lipids, relatively low total dosage of nattokinase supplementation did not exert significant positive effects on levels of total cholesterol, LDL-C, and HDL-C, and even aggravated the dyslipidemia. A previous self-controlled clinical trial also reported no obvious effects of nattokinase (4000 FU/day, 8 weeks) on lipid parameters in both healthy volunteers and patients with cardiovascular risk factors [20]. Our findings were unexpected and inconsistent with results of several animal experiments [13,44,45]. This observation may be explained by the fact that all included trials used purified nattokinase products, whereas most previous animal studies used crude natto extracts that contained anti-cholesterol agents, such as soy isoflavones [32,46].

However, a recent clinical study of 1062 patients with hyperlipidemia showed that nattokinase supplementation at a dosage of 10,800 FU per day for 1 year significantly decreased blood total cholesterol, triglyceride, LDL-C and increased HDL-C [47]. Ren *et al.* [48] also found that high-dose nattokinase administration over a relatively long period of time (26 weeks, 6500 FU) was effective in inhibiting the progression of atherosclerotic plaques and hyperlipidemia. Noticeably, in the meta-analysis of relatively high total dosage of nattokinase supplementation, the increase in HDL-C and the decrease in LDL-C were detected among included participants. So far, the lipid-lowering mechanism of nattokinase has not been understood, and one possible explanation is that nattokinase has proteolytic activity on some certain proteins involved in lipid metabolism [32]. In general, our observations reflected those of Chen *et al.* [17] who also proposed that long-term and high-dose nattokinase supplementation seemed to have positive impacts on blood lipids. For future researches, therefore, it is an important issue to determine if the lipid-lowering efficacy of nattokinase is dose-dependent.

Pooled results of this study suggested that nattokinase produced beneficial influences on CVDs by lowering SBP and DBP levels, which matched those observed in earlier animal studies [8,15,49]. It was already known that nattokinase had high gastrointestinal stability, and it might reduce blood pressure by cleaving plasma fibrinogen after absorption in the small intestine [8,36]. More significantly, degradation products of nattokinase were demonstrated to exert different antihypertensive effects—inhibition of angiotensin I converting enzyme and plasma angiotensin II level [8,16]. Results of the experiment by Ibe *et al.* [50] also indicated that nattokinase appeared to inhibit angiotensin converting enzyme related to increases in oral

dosage. Renin is a key enzyme in renin-angiotensin system, which has long been considered as an attractive antihypertensive target [51]. Although researchers found a decrease in plasma renin activity in nattokinase group compared to controls, the results were not statistically significant [28,29], and it was still not known whether nattokinase could prevent the elevation of plasma renin activity levels against arterial hypertension.

To date, data about the impact of nattokinase on blood sugar were limited. The present study found that nattokinase consumption had very little influence on blood glucose level (MD = 0.4). Combination of nattokinase, aronia, red ginseng, and shiitake mushroom was found to improve glucose metabolism and diminish insulin resistance [52]. Several randomized crossover studies also showed that breakfast accompanied with natto suppressed blood glucose elevation and improved insulin sensitivity in the early postprandial phase, but this may be attributed to γ -polyglutamic acid and polysaccharide components contained in the natto [53–55]. In a word, there is still no direct evidence to identify the antidiabetic effect of nattokinase.

In terms of safety, no major adverse events were reported for nattokinase supplementation in all included trials at different doses. A great deal of recent works corroborated that nattokinase was a safe agent for cardiovascular risk factors with low haemorrhagic risk and no toxicologic concerns. For instance, the standard safety margin (haemorrhagic adverse effect) of nattokinase was proven to be three times that of tissue plasminogen activators [56]. Daily nattokinase consumption of 10 mg/kg for 28 days was well tolerated in human volunteers [57], and even no adverse effects were observed when the daily dose of nattokinase was 480,000 FU/kg in mice, which was 1000-fold higher than the recommended daily dose in humans [58]. Nevertheless, several case studies have recently emerged that provide contradictory findings on allergic and bleeding risks of nattokinase [59–61]. As proposed by Gallelli *et al.* [23], therefore, although the positive effect of nattokinase on CVD outweighs possible described complications, patients must be always monitored for reference parameters, including clinical condition, coagulation profile, renal function, diet, and weight, and clinicians should make timely and reasonable dose adjustments to ensure the safety of nattokinase administered as monotherapy or in pharmacological combination.

5. Limitations

Several limitations of present study should be considered. Firstly, two major limitations were the small total sample size and varied therapeutic dosage of nattokinase for the included studies. Secondly, no restrictions were placed on the cardiovascular health status of included participants. Thirdly, quantitative analysis of the antithrombotic and anticoagulant effects of nattokinase was lacking. Fourthly, the heterogeneity in the corresponding meta-analyses was diffi-

cult to estimate because of limited included studies. Fifthly, our search strategies were limited to English papers, which might be linguistically biased. Last but not least, publication bias could not entirely be ignored, since less than 10 studies were pooled in the individual analysis.

6. Conclusions

Based on the available clinical data, the most obvious finding from this study was that short-term and low-dosage ingestion of nattokinase might have no significant lipid-lowering effects. The second major finding was that nattokinase could be considered as a promising adjunctive tool in the treatment of hypertension. Due to the existing limitations of this work, however, these findings could be considered to be preliminary, and a definitive conclusion on whether nattokinase supplementation was strongly associated with the improvement of any cardiovascular risk factor could not be drawn. Moreover, we inferred that the positive effect of nattokinase on cardiovascular risk factors might be enhanced with increasing oral doses, especially the hypolipidemic effect, and further clinical trials investigating the long-term and high-dose administration of nattokinase on cardiovascular risk factors were strongly recommended.

Availability of Data and Materials

The data used to support the results of this study are available from the corresponding author upon request.

Author Contributions

Conceptualization—XL, JL, and YZ; literature search—XL and JL; data collection—QG and MP; quality assessment—JW and FY; software preparation—XL; quantitative analysis—JL and YZ; qualitative analysis—QG and MP; writing—original draft preparation—XL and JL; writing—review and editing—QG, MP, JW, FY, and YZ; tables and figures—JW and FY; supervision—YZ. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.rcm2408234>.

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Risk stratification for future cardiac arrest after COVID-19 vaccination

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Abstract

Unheralded cardiac arrest among previously healthy young people without antecedent illness, months or years after coronavirus disease 2019 (COVID-19) vaccination, highlights the urgent need for risk stratification. The most likely underlying pathophysiology is subclinical myopericarditis and reentrant ventricular tachycardia or spontaneous ventricular fibrillation that is commonly precipitated after a surge in catecholamines during exercise or the waking hours of terminal sleep. Small patches of inflammation and/or edema can be missed on cardiac imaging and autopsy, and the heart can appear grossly normal. This paper reviews evidence linking COVID-19 vaccines to cardiac arrest where unfortunately the majority of victims have had no antecedent clinical evaluation. We propose a comprehensive strategy for evaluating cardiovascular risk post-vaccination, incorporating detailed patient history, antibody testing, and cardiac diagnostics in the best attempt to detect abnormalities before sudden cardiac death. This approach aims to identify individuals at higher risk of cardiac events after COVID-19 vaccination and guide appropriate clinical management. It is prudent for each primary care physician to have a pre-established plan when addressing this issue in their practice.

Key Words: COVID-19; mRNA vaccines; Myocarditis; Cardiac arrest; Risk stratification; Cardiovascular safety

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Core Tip: This study reviews evidence linking mRNA vaccines to cardiac pathology and proposes a comprehensive risk stratification approach involving patient history, antibody testing, and cardiac diagnostics. By identifying high-risk individuals through measurable endpoints like spike protein exposure and cardiac biomarkers, this approach seeks to guide clinicians in addressing the risks of myocarditis, arrhythmias, and cardiac arrest post-vaccination. Implementing this framework in primary care settings may improve cardiovascular outcomes and reduce preventable deaths.

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INTRODUCTION

We continue to observe coronavirus disease 2019 (COVID-19) vaccinated persons suffer cardiac arrests since the inception of the mass vaccination campaign in late 2020[1-5]. **Figure 1** illustrates the likely mechanisms. Both Pfizer-BioNTech (BNT162b2) and Moderna (mRNA-1273) mRNA have been found in human heart muscle at autopsy[6]. Spike protein has been stained in endomyocardial biopsy samples of young men suffering from COVID-19 vaccine-induced myocarditis[7]. Victims have been found to have circulating Spike protein but ineffective antibodies, likely IgG4 subclass, that fail to neutralize Spike protein and allow its assault on the heart[8]. Positron emission tomography data have revealed a shift from free fatty acid metabolism to glucose metabolism in the hearts of the majority of individuals who have received a COVID-19 vaccine[9]. The positron emission tomography pattern resembles global ischemia. This could be due to vaccine Spike protein hemagglutination in myocardial capillaries or cellular changes in mitochondrial respiration and substrate metabolism[10]. Small patches of dysfunctional, inflamed, or scarred myocardium are sufficient to serve as a nidus for re-entrant ventricular tachycardia that can degrade to ventricular fibrillation and lead to cardiac arrest[11]. A surge in catecholamines (epinephrine, norepinephrine, and dopamine) that can occur during sports or the waking hours of sleep (3 AM to 6 AM) may trigger reentrant ventricular tachycardia or spontaneous ventricular fibrillation leading to a cardiac arrest in patients with COVID-19 vaccine subclinical myocarditis[12].

RATIONALE FOR RISK STRATIFICATION

Elevated numbers of sudden deaths among athletes after COVID-19 vaccination have raised concerns[13]. Alessandria *et al*[14] amplified these concerns, demonstrating higher all-cause death risks in COVID-19 vaccinated individuals compared to the unvaccinated. Participants that received 2 doses lost 37% of life expectancy compared to the unvaccinated population during follow-up. The largest COVID-19 vaccine safety study to date with approximately 99 million vaccinated individuals found that the risk of myocarditis was significantly elevated after mRNA COVID-19 vaccinations, with the risk being 510% higher following the second dose of the mRNA-1273 vaccine and 186% higher following the second dose of the BNT162b2 vaccine, compared to baseline rates[15]. Hulscher *et al*[16] have demonstrated that cardiac arrest within a few weeks of COVID-19 vaccination is likely caused by vaccine myocarditis with no prior premonitory phase that allows for detection. Rose *et al*[17] found that among individuals with clinical myocarditis shortly after COVID-19 vaccination reported to the Vaccine Adverse Event Reporting System (VAERS), the mortality rate was 2.9%.

The United States Food and Drug Administration's Center for Biologics Evaluation and Research has established a follow-up period of 5 to 15 years for novel genetic products to monitor for any long-term adverse effects that may emerge in the exposed population[18]. With the passage of time, we have learned much from the evaluation of many vaccine-injured victims who have symptoms months to years after injection[19-21]. Brogna *et al*[22] found vaccine-generated stabilized prefusion Spike protein in subjects up to 6 months after COVID-19 mRNA vaccination. Cardiac abnormalities have been observed for at least a year following the initial diagnosis of COVID-19 vaccine-induced myocarditis, suggesting the potential for long-term effects[23]. As of January 13, 2025, we are unaware of any published risk stratification approach for the prevention of COVID-19 vaccine-induced cardiac arrest. Thus, we propose a comprehensive strategy to address this concerning gap in knowledge.

RISK STRATIFICATION APPROACH

When patients are seen in clinical practice for the initial evaluation of cardiovascular symptoms following severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection or vaccination, a proposed approach is outlined in **Figure 2**. COVID-19 vaccine-induced myocarditis is caused principally by the Spike protein[7,8,16,24]. Measures of cytokine activation and inflammation are secondary to Spike protein resident in the myocardium and circulating in blood[7,8].

A detailed SARS-CoV-2 infection and vaccine administration history is essential to the evaluation. Serious COVID-19 requiring hospitalization and mechanical ventilation should be noted. Each SARS-CoV-2 infection and COVID-19 vaccine

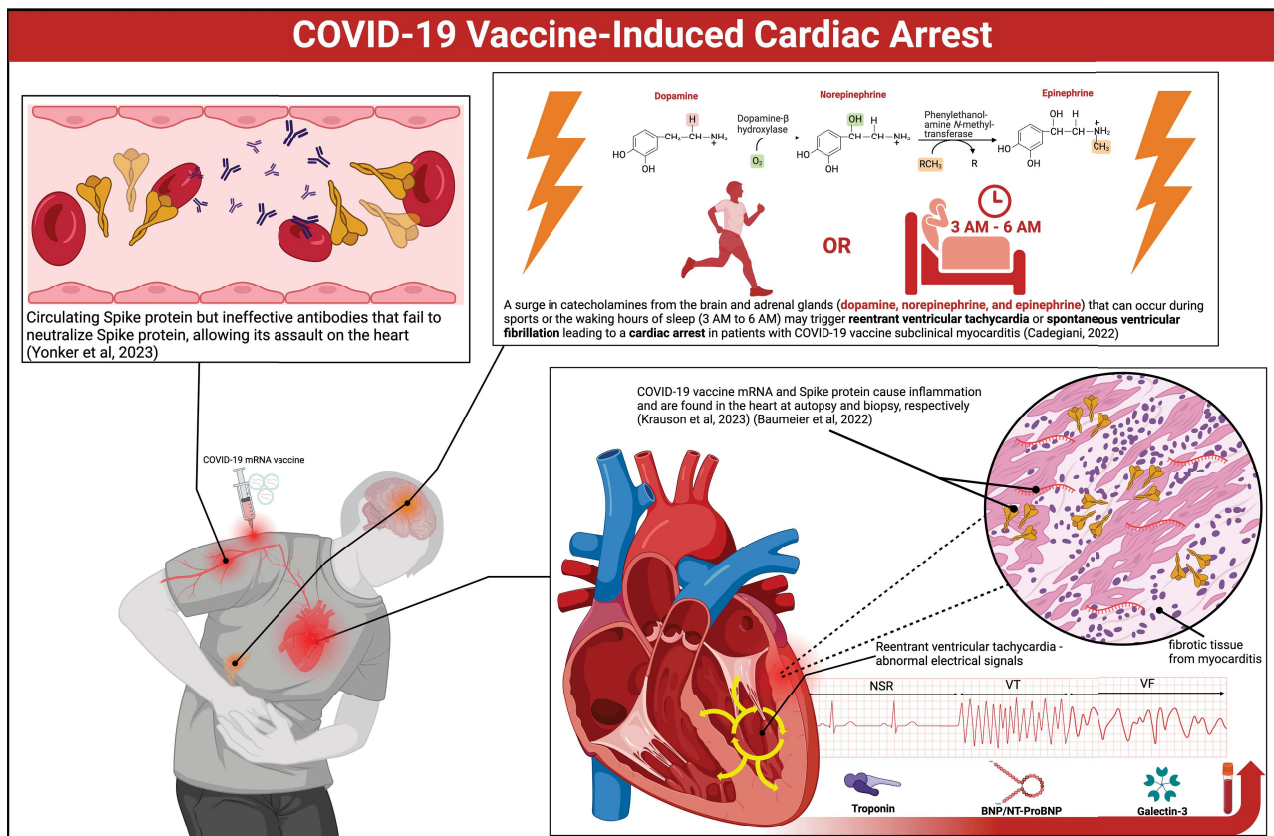


Figure 1 Coronavirus disease 2019 vaccine-induced cardiac arrest. NSR: Normal sinus rhythm; VT: Ventricular tachycardia; VF: Ventricular fibrillation; BNP/NT-proBNP: Brain natriuretic peptide and N-terminal proBNP (Created in BioRender, [Supplementary material](#)).

dose can be considered an 'exposure'. It can be reasonably assumed that the greater the number of exposures, the more likely it is to have large quantities of Spike protein in the body, where natural proteases and lysosomes seem unable to clear it[25].

An extended range total antibody against the Spike protein is a useful proxy for prior Spike exposure. For example, the Roche Diagnostics Elecsys Anti-SARS-CoV-2 Spike assay measures antibodies against the receptor binding domain[26]. Roche Elecsys Anti-SARS-CoV-2 assay has a normal value of < 0.8 U/mL, and in clinical practice, 0-1000 is low risk, and > 1000 indicates higher risk with either multiple infections or COVID-19 vaccine administrations. We have observed that it is not uncommon to find patients with > 25000 U/mL, remaining unmeasurably high even years after vaccination. Antibody concentrations can take at least 12 months to taper off. Thus, a Spike antigen test measurable in whole blood, plasma, or serum is greatly needed to provide a real-time estimate of Spike toxicity.

If (1) the number of Spike protein exposures was low; (2) there were nonserious infections; (3) Spike antibody levels are below 1000 U/mL; (4) minimal or no injection site or initial systemic reaction to vaccination[27,28]; and (5) if the vaccine was obtained from a low-risk batch[29,30] (with zero hospitalizations and deaths found in a VAERS batch analysis[31]), then additional clinical investigation may not be warranted. If there are higher risk features as indicated in the [Figure 2](#), then it is prudent to perform more formal cardiac testing and risk stratification.

It is reasonable to obtain an electrocardiogram and blood testing for high-sensitivity troponin, BNP/NT-ProBNP, galectin-3, and D-dimer. Elevated troponin may indicate ongoing myocarditis[32]. BNP/NT-proBNP are reliable indicators of cardiac pressure/volume overload and predict heart failure[33]. Galectin-3 is a chronic inflammation/fibrosis marker and when elevated predicts future heart failure[34]. D-dimer is a proxy for micro-blood clotting[35] and in our experience < 0.2 is low risk, 0.2-0.5 is moderate, and > 0.5 is high risk for thrombotic events. Patel *et al*[36] demonstrated that 17% of participants exhibited an increase in d-dimer levels from their normal baseline following COVID-19 vaccination. Genetic testing for pathogenic mutations in cardiac ion channels or major proteins is reasonable in patients with high-risk findings or resuscitated cardiac arrest. Mutations in the SCN5A sodium channel have been associated with an increased risk for COVID-19 vaccine cardiac arrest[37].

Point-of-care echocardiography or formal cardiac ultrasound and Doppler can be used to evaluate cardiac structure, function, and presence of pericardial fluid[38]. This package of diagnostics, when results are completely normal, suggests a low risk for future heart failure and cardiac death[39]. Conversely, multiple abnormalities found in electrocardiogram, blood tests, and echocardiography can prompt the use of cardiac magnetic resonance imaging (MRI) with contrast[40]. The MRI is confirmatory for left ventricular function and importantly can detect areas of inflammation/scar with late gadolinium enhancement (LGE)[41]. In other types of cardiomyopathy, LGE ≥ 15% of the left ventricle indicates high risk for cardiac arrest[42].

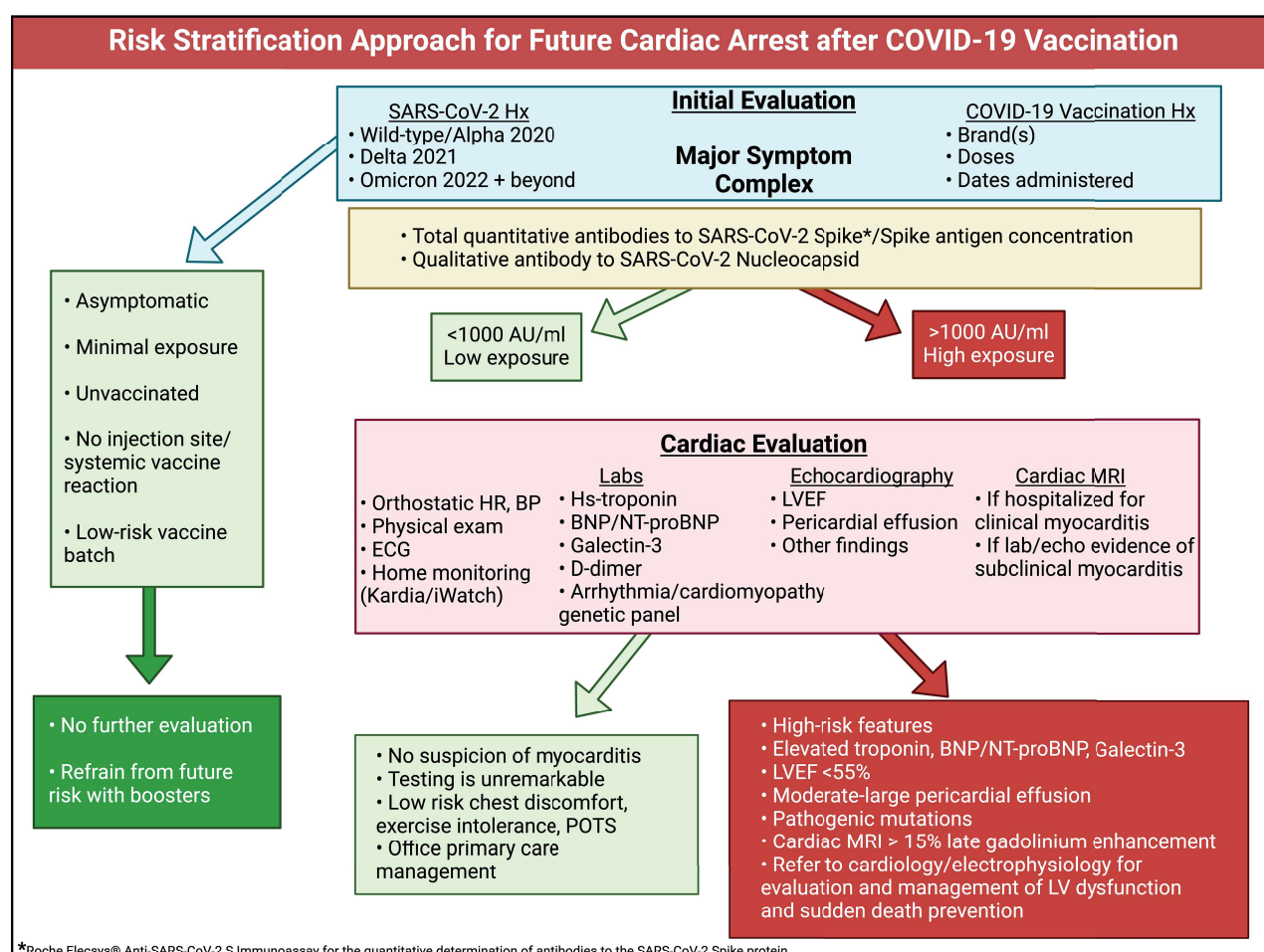


Figure 2 Risk stratification approach for future cardiac arrest after coronavirus disease 2019 vaccination. Green boxes indicate clinical features, test results, and patients at lower risk. Red and pink boxes show tests and results indicating higher risk. Hx: History; AU/mL: Antibody units per milliliter; HR: Heart rate; BP: Blood pressure; ECG: Electrocardiogram; Hs-troponin: High-sensitivity troponin; BNP/NT-proBNP: Brain natriuretic peptide and N-terminal proBNP; LVEF: Left ventricular ejection fraction; POTS: Postural orthostatic tachycardia syndrome (Created in BioRender, [Supplementary material](#)).

Small patches of myocardial inflammation, edema, or fibrosis may not be detectable by axial slices on cardiac MRI or autopsy[43,44]. Thus, the heart may appear normal on post-mortem examination and the final report may indicate death due to “natural causes” in a healthy patient with no antecedent disease. We believe these cases likely represent previously silent subclinical myocarditis and cardiac fibrosis which serves as the substrate for re-entrant ventricular tachycardia that degenerates to ventricular fibrillation and asystole in patients that do not receive prompt defibrillation.

When risk stratification indicates low-risk, primary care office management is suggested with McCullough Protocol: Base Spike Detoxification and adjunctive medications depending on the syndrome (Figure 3)[25]. For subclinical myopericarditis, oral colchicine 0.6 mg twice a day (BID) or once a day is indicated for at least one year[45]. For COVID-19 vaccine-induced postural orthostatic tachycardia syndrome, use of colchicine and nadolol 20-40 mg BID can be helpful [46]. In patients who screen as high-risk, Base Spike Detoxification[25] is also indicated. For those at very high risk for cardiomyopathy and/or ventricular arrhythmias, formal cardiology consultation is suggested with the main goals of preventing heart failure and sudden death. Additionally, some patients may require ICD implantation if there are symptomatic arrhythmias, LGE > 15%, or genetic predictors such as pathogenic *SCN5A* mutations[37,47].

While this framework is designed for the general population, it is important to recognize that specific subgroups, such as older adults and individuals with pre-existing cardiovascular conditions, may require additional considerations. Older adults, for example, may benefit from more frequent monitoring and tailored preventive strategies to mitigate heightened risks associated with age-related cardiovascular changes. Similarly, patients with a history of heart disease should undergo customized evaluations and interventions to address their unique vulnerabilities. Incorporating these subgroup-specific approaches will further enhance the practical utility of the proposed framework across diverse patient populations. While the proposed risk stratification framework is already supported by substantial indirect evidence and sound clinical principles, it is important to note that real-world implementation data are currently lacking. This represents an opportunity to validate its effectiveness through future retrospective analyses and prospective clinical studies, which will not only strengthen its utility but also refine its practical application. By bridging this gap, the framework has the potential to become an indispensable tool for improving patient outcomes and guiding evidence-based care.

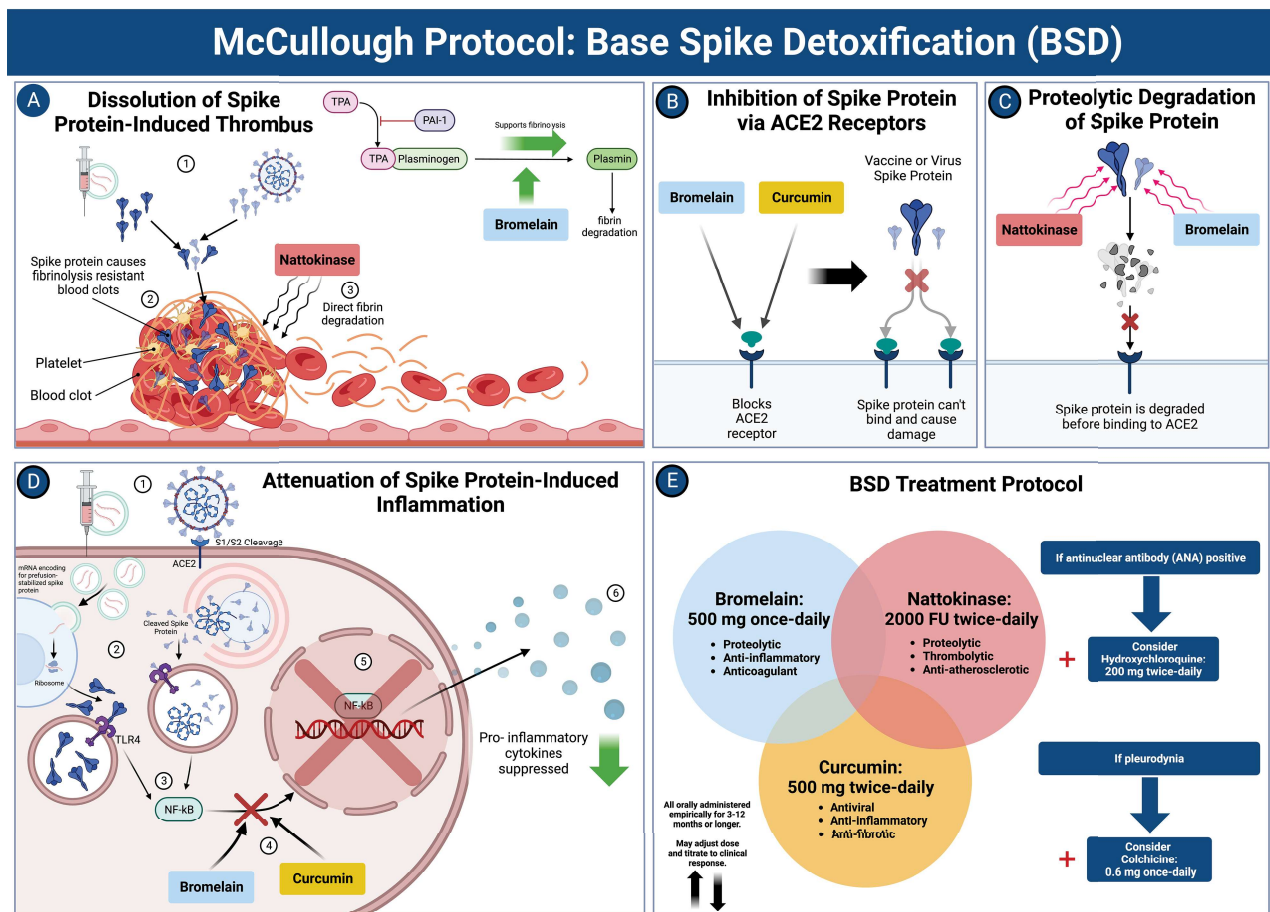


Figure 3 McCullough protocol: Base spike detoxification. A: Dissolution of spike protein-induced thrombus. Nattokinase directly degrades fibrinolysis-resistant fibrin (from spike protein), and bromelain upregulates fibrinolysis; B: Inhibition of spike protein via ACE2 receptors. Bromelain and curcumin block the ACE2 receptor, preventing spike protein from binding; C: Proteolytic degradation of spike protein. Nattokinase and bromelain degrade spike proteins, rendering them inactive; D: Attenuation of spike protein-induced inflammation. Bromelain and curcumin downregulate the nuclear factor kappa B signaling pathway induced by spike protein, leading to the suppression of inflammatory molecules; E: Base spike detoxification treatment protocol. The full treatment regimen and the addition of other compounds based on clinical indication are illustrated. TPA: Tissue plasminogen activator; PAI-1: Plasminogen activator inhibitor-1; ACE2: Angiotensin converting enzyme-2; NF-κB: Nuclear factor kappa B; S1/S2: Spike protein subunits S1/S2; TLR4: Toll-like receptor 4. Citation: Hulscher N, Procter BC, Wynn C, McCullough PA. Clinical Approach to Post-acute Sequelae After COVID-19 Infection and Vaccination. *Cureus* 2023; 15(11): e49204 Copyright ©The Author(s) 2023. Published by Springer Nature[25] (Supplementary material).

CONCLUSION

In summary, we have proposed a risk stratification approach that addresses the clinical concern of future cardiac arrest following COVID-19 vaccination. The numerous studies highlighting serious cardiovascular safety concerns related to COVID-19 vaccines have raised public and physician awareness. It is prudent for each primary care physician to have a pre-established approach when addressing this issue in their practice.

FOOTNOTES

Author contributions: McCullough PA and Hulscher N were responsible for conceptualization, investigation, project administration, validation, visualization, writing – original draft, writing – review & editing.

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Article

Nattokinase: Biological activities, pharmacological mechanisms, therapeutic applications, challenges and perspectives

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Abstract

Nattokinase (NK), a potent fibrinolytic serine protease derived from the traditional Japanese fermented soybean food natto, has garnered substantial scientific interest over the past four decades due to its remarkable cardioprotective and thrombolytic properties. First isolated and characterized in the 1980s, this enzyme (EC 3.4.21.62), also known as subtilisin NAT, is produced during the fermentation of soybeans by *Bacillus subtilis* var. *natto*. This comprehensive review synthesizes the current body of scientific literature on nattokinase, encompassing its historical discovery, molecular structure, physicochemical properties, extraction and purification methodologies, pharmacokinetic profile, and multifaceted pharmacological mechanisms. Particular emphasis is placed on the enzyme's established fibrinolytic and antithrombotic activities, its emerging antihypertensive, anti-inflammatory, antioxidant, lipid-modulating, and neuroprotective effects, as well as its potential applications in respiratory health and the management of COVID-19-associated coagulopathy. A critical appraisal of clinical trial evidence, including randomized controlled trials evaluating nattokinase alone and in combination with other nutraceuticals, is presented alongside an analysis of safety data from toxicological assessments. The review further examines the current landscape of quality control standardization, regulatory considerations, and the challenges posed by inconsistent manufacturing practices. Finally, the review identifies critical knowledge gaps, discuss the limitations of existing research, and propose future research directions to fully elucidate the therapeutic potential of this promising natural enzyme in the prevention and adjunctive management of cardiovascular, neurodegenerative, and other non-communicable diseases.

Keywords nattokinase; subtilisin NAT; fibrinolytic enzyme; cardiovascular disease; thrombolysis; hypertension; anti-inflammatory; antioxidant; *Bacillus subtilis* var. *natto*; clinical trial; fermentation.

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1 Introduction

Cardiovascular diseases (CVDs) remain the preeminent cause of premature mortality and disability on a global scale, imposing an immense burden on healthcare systems and economies worldwide (Selvarajan and

Bhatnagar, 2017; Li et al., 2023; Muric et al., 2024). The pathophysiological underpinnings of CVDs are multifactorial and complex, encompassing a constellation of interrelated risk factors including coagulation abnormalities, hypertension, dyslipidemia, endothelial dysfunction, chronic inflammation, and oxidative stress (Granito et al., 2024; Muric et al., 2024). While significant therapeutic advancements have been achieved through the development of synthetic anticoagulants, antiplatelet agents, statins, and antihypertensive medications, these pharmacological interventions are frequently associated with adverse effects, drug-drug interactions, and substantial financial costs, prompting a sustained and growing interest in identifying and validating safe, effective, and accessible natural alternatives or adjunctive therapies (Muric et al., 2024; Weng et al., 2017).

Among the diverse array of bioactive compounds derived from traditional fermented foods, nattokinase (NK) has emerged as a particularly compelling candidate for cardiovascular health promotion and disease prevention (Chen et al., 2018; Granito et al., 2024; Weng et al., 2017). Nattokinase is an alkaline serine protease (EC 3.4.21.62), alternatively designated as subtilisin NAT, that is produced abundantly by the bacterium *Bacillus subtilis* var. *natto* during the fermentation of boiled soybeans to produce natto, a traditional Japanese food that has been consumed for over a millennium (Dabbagh et al., 2014; Lampe and English, 2016; Sumi et al., 1987). The enzyme exhibits potent fibrinolytic activity, directly degrading fibrin, the primary proteinaceous constituent of blood clots, and simultaneously enhancing endogenous fibrinolytic pathways through multiple complementary mechanisms (Sumi et al., 1987; Weng et al., 2017). The recognition of nattokinase's thrombolytic potential has catalyzed an extensive and continuously expanding body of scientific inquiry, encompassing fundamental biochemical characterization, preclinical mechanistic investigations, animal model studies, and a growing portfolio of human clinical trials (Li et al., 2023; Liu et al., 2024; Muric et al., 2024).

Epidemiological observations have provided compelling circumstantial evidence supporting the cardiovascular benefits of natto consumption. Notably, studies have demonstrated that habitual natto intake is associated with a reduced risk of mortality from cardiovascular disease, an effect attributed primarily to the food's high content of nattokinase and its attendant fibrinolytic and antithrombotic properties (Granito et al., 2024; Muric et al., 2024). These population-level findings have been buttressed by a substantial corpus of experimental and clinical investigations that have systematically documented the positive effects of nattokinase on various facets of cardiovascular health, including the thinning of blood, dissolution of existing clots, reduction of blood pressure, and attenuation of atherosclerotic processes (Selvarajan and Bhatnagar, 2017; Muric et al., 2024). Beyond its well-established cardiovascular applications, recent research has unveiled a broader spectrum of biological activities attributable to nattokinase, encompassing potent anti-inflammatory and antioxidant effects, neuroprotective actions relevant to neurodegenerative disorders including Alzheimer's disease, and even potential antiviral properties against SARS-CoV-2 (Granito et al., 2024; Li et al., 2026; Tanikawa et al., 2022; Mahakalakar et al., 2025).

The escalating global prevalence of CVDs and other non-communicable diseases (NCDs) has created an urgent imperative for innovative, cost-effective, and well-tolerated preventive and therapeutic strategies (Granito et al., 2024; Li et al., 2023). Nattokinase, with its favorable safety profile, oral bioavailability, and pleiotropic mechanisms of action, is uniquely positioned to address this unmet need (Lampe and English, 2016; Weng et al., 2017). However, despite the substantial and growing body of evidence supporting its therapeutic potential, several critical challenges remain. These include the absence of standardized manufacturing and quality control protocols, significant heterogeneity in the design and outcomes of clinical trials, incomplete elucidation of its pharmacokinetic and pharmacodynamic properties, and a paucity of large-scale, long-term clinical outcome studies (Li et al., 2023; Feng et al., 2023; Yi et al., 2025). The present comprehensive review

aims to critically synthesize the existing scientific literature on nattokinase, providing a detailed and nuanced overview of its discovery, biochemical properties, pharmacological mechanisms, clinical evidence, and safety considerations, while also identifying key knowledge gaps and charting a course for future research endeavors.

2 Discovery and History

2.1 The Serendipitous Discovery by Dr. Hiroyuki Sumi

The discovery of nattokinase stands as a quintessential example of scientific serendipity intersecting with astute observation. The enzyme was first identified in 1980 by Dr. Hiroyuki Sumi, a Japanese researcher then affiliated with the Department of Physiology at the University of Chicago Medical School, who was engaged in a systematic search for natural agents capable of effectively dissolving thrombi associated with cardiac and cerebral infarction (Sumi et al., 1987). Driven by the hypothesis that traditional fermented foods might harbor potent fibrinolytic enzymes, Dr. Sumi and his colleagues undertook an ambitious and exhaustive screening program, testing more than 173 different natural foods for their ability to degrade artificial fibrin clots *in vitro* (Dabbagh et al., 2014; Sumi et al., 1987).

The pivotal experiment occurred on a day in 1980 when Dr. Sumi, inspired by the notion that natto, a food produced through fermentation, might contain fibrin-degrading enzymes, applied an extract derived from natto to an artificial thrombus (Sumi et al., 1987). Remarkably, within a period of approximately three hours, the thrombus had undergone substantial dissolution, losing approximately two centimeters in length (Sumi et al., 1987). This rate of thrombolysis was strikingly superior to that achieved with urokinase, a standard thrombolytic agent of the era, which required nearly two days to effect comparable dissolution of an identical thrombus under similar experimental conditions (Sumi et al., 1987). This dramatic and unexpected finding provided the initial, compelling evidence for the existence of a potent fibrinolytic principle within natto, an observation that would ultimately lead to the isolation, purification, and characterization of the enzyme that was subsequently designated "nattokinase" (Sumi et al., 1987).

The formal announcement of this discovery was made in 1987, when Sumi and colleagues published their seminal paper detailing the purification and characterization of the fibrinolytic enzyme from natto, thereby establishing the scientific foundation for all subsequent research on this remarkable biocatalyst (Sumi et al., 1987). The name "nattokinase" was coined to reflect both its origin in natto and its enzymatic nature, and it has since become the universally recognized designation for this enzyme, also known by its systematic name, subtilisin NAT (EC 3.4.21.62) (Lampe and English, 2016; Sumi et al., 1987).

2.2 Traditional Use of Natto and Cultural Significance

Long before its scientific characterization, natto held an esteemed place in traditional Japanese cuisine and folk medicine. Natto is a traditional fermented food produced by inoculating steamed soybeans with *Bacillus subtilis* var. *natto* (also referred to as *Bacillus natto*) and allowing the mixture to ferment under controlled conditions of temperature and humidity for a period of approximately 18 to 24 hours (Dabbagh et al., 2014; Ashiuchi et al., 2001; Fig. 1). The resultant product is characterized by its distinctive pungent aroma, stringy and viscous texture, and unique umami flavor profile, attributes that have secured its status as a staple of the Japanese diet for well over a thousand years (Dabbagh et al., 2014; Ashiuchi et al., 2001). Traditionally, natto is consumed as a breakfast food, served over steamed rice, incorporated into sushi preparations, or spread on toast; it is even available as an ice cream flavor in contemporary Japan (Dabbagh et al., 2014).

Beyond its culinary applications, natto has been empirically valued in traditional Japanese medicine for its purported health-promoting properties. Historical accounts indicate that natto was traditionally employed for the management of heart conditions, the alleviation of physical fatigue, and as an antiberiberi agent, reflecting an early, intuitive recognition of its beneficial effects on cardiovascular and overall health (Dabbagh et al.,

2014). The longevity and consistently high consumption of natto in Japan, coupled with the comparatively low incidence of cardiovascular disease in populations with high natto intake, have provided fertile ground for epidemiological investigations exploring the potential cardioprotective effects of this traditional food (Granito et al., 2024; Weng et al., 2017).



Fig. 1 *Bacillus subtilis* var. *natto* (Longxigong, 2024).

2.3 Evolutionary Trajectory of Nattokinase Research (1980–Present)

The scientific trajectory of nattokinase research has evolved through several distinct, overlapping phases since its initial discovery in 1980. The foundational period, spanning approximately from 1980 to 1990, was characterized by efforts focused on the isolation, purification, and preliminary biochemical characterization of the enzyme, culminating in the landmark publication by Sumi et al. in 1987 (Sumi et al., 1987). During this phase, the basic enzymatic properties of nattokinase, including its molecular weight, substrate specificity, and *in vitro* fibrinolytic activity, were established, laying the essential groundwork for subsequent investigations (Sumi et al., 1987; Sumi et al., 1990).

The second phase, extending through the 1990s and into the early 2000s, witnessed a significant expansion of research into the *in vivo* efficacy of nattokinase, employing a variety of animal models to explore its antithrombotic, antihypertensive, and anti-atherosclerotic effects (Fujita et al., 1993; Suzuki et al., 2003a; Suzuki et al., 2003b). Concurrently, investigations into the molecular mechanisms underpinning nattokinase's fibrinolytic activity deepened, revealing that the enzyme not only directly degrades fibrin but also potentiates endogenous thrombolysis by enhancing the production of tissue plasminogen activator (t-PA) and catalyzing the conversion of prourokinase to its active form (Sumi et al., 1990; Yatagai et al., 2007). Furthermore, this era saw the initiation of early-stage human clinical trials, which provided preliminary evidence for the safety and efficacy of orally administered nattokinase in reducing blood pressure and improving fibrinolytic parameters in human subjects (Kim et al., 2008).

The most recent phase, commencing around 2010 and continuing to the present day, has been marked by a dramatic diversification and deepening of nattokinase research. This period has been characterized by the conduct of larger, more rigorously designed randomized controlled trials (RCTs) evaluating the effects of nattokinase, both as monotherapy and in combination with other nutraceuticals, on a broad spectrum of cardiovascular risk factors and clinical outcomes (Hodis et al., 2021; Li et al., 2023; Liu et al., 2024). Concomitantly, high-resolution structural biology techniques, including X-ray crystallography, have yielded

atomic-level insights into the three-dimensional architecture of the enzyme, facilitating a more nuanced understanding of its catalytic mechanism and substrate interactions (Yanagisawa et al., 2010; Yanagisawa et al., 2013). Perhaps most significantly, this contemporary phase has witnessed the discovery of novel, unanticipated biological activities of nattokinase extending far beyond its canonical fibrinolytic function. These newly identified properties include potent anti-inflammatory and antioxidant effects mediated through activation of the Nrf2/HO-1 signaling axis (Granito et al., 2024; Li et al., 2026), neuroprotective actions relevant to the prevention and treatment of Alzheimer's disease and other neurodegenerative disorders (Tanikawa et al., 2024; Mahakalakar et al., 2025), and antiviral activity against the SARS-CoV-2 spike protein, suggesting potential utility in the context of COVID-19 (Tanikawa et al., 2022). These recent discoveries have fundamentally broadened the conceptual framework for nattokinase's therapeutic applications, positioning it as a pleiotropic agent with potential relevance to a diverse array of human diseases beyond the cardiovascular system (Granito et al., 2024; Weng et al., 2017).

3 Biochemical and Molecular Properties

3.1 Source and Microbial Production

Nattokinase is a naturally occurring extracellular enzyme produced predominantly by the bacterium *Bacillus subtilis* var. *natto*, a Gram-positive, spore-forming, rod-shaped microorganism that serves as the principal fermentative agent in the production of natto (Dabbagh et al., 2014; Lampe and English, 2016; Wang et al., 2009). The bacterium is typically isolated from natto starter cultures or directly from the fermented soybean product itself, and it is cultivated on a variety of nutrient media under aerobic or semi-aerobic conditions to achieve optimal enzyme yields (Dabbagh et al., 2014; Wang et al., 2009). While *Bacillus subtilis* var. *natto* is the most extensively characterized and industrially utilized source of nattokinase, it is noteworthy that other *Bacillus* species, as well as microorganisms present in analogous fermented soybean foods from other Asian culinary traditions, have also been reported to produce fibrinolytic enzymes with properties similar to or identical with nattokinase (Dabbagh et al., 2014; Ashiuchi et al., 2001; Peng et al., 2005). Examples of such traditional fermented foods include *chungkook-jang* in Korea, *douchi* in China, *thua nao* in Thailand, and *tempeh* in Indonesia, each of which harbors distinct microbial consortia that contribute to the production of bioactive peptides and enzymes with potential health benefits (Dabbagh et al., 2014; Ashiuchi et al., 2001).

The production of nattokinase by *Bacillus subtilis* var. *natto* is influenced by a complex interplay of nutritional and environmental factors. Optimal enzyme yields are typically achieved through fermentation on basal media enriched with carbon sources such as glucose or starch and nitrogen sources including peptone, yeast extract, or soybean meal hydrolysates (Dabbagh et al., 2014; Jagathy et al., 2017). The incubation temperature, pH, aeration rate, and duration of fermentation are critical parameters that must be carefully optimized to maximize enzyme production while minimizing the accumulation of undesirable metabolites (Dabbagh et al., 2014; Jagathy et al., 2017). Recent advances in fermentation technology, including the development of integrated bioprocessing strategies that couple biofilm-based fermentation with the use of low-cost agricultural byproducts such as soybean meal hydrolysates, have demonstrated significant potential for enhancing nattokinase yields and improving the economic viability of large-scale production (Yi et al., 2025). Notably, a study by Zhang et al. (2025) reported the development of an integrated bioprocessing strategy that achieved a nattokinase yield of 2949.3 U/mL using soybean meal hydrolysates prepared with trypsin under biofilm-based fermentation conditions, representing a substantial improvement over conventional submerged fermentation methods (Yi et al., 2025).

3.2 Molecular Structure and Amino Acid Sequence

At the molecular level, nattokinase is a single polypeptide chain composed of precisely 275 amino acid

residues, with a calculated molecular weight of approximately 27.7 kDa (Yanagisawa et al., 2010; Yanagisawa et al., 2013; Fig. 2). The enzyme is a member of the subtilisin family of serine proteases, a large and diverse group of endopeptidases characterized by a conserved catalytic triad consisting of aspartic acid, histidine, and serine residues that orchestrate the hydrolytic cleavage of peptide bonds (Yanagisawa et al., 2010; Yanagisawa et al., 2013). The amino acid sequence of nattokinase exhibits a high degree of homology with other bacterial subtilisins, including subtilisin E from *Bacillus subtilis* and subtilisin BPN' from *Bacillus amyloliquefaciens*, reflecting the evolutionary conservation of this catalytically efficient enzyme family (Yanagisawa et al., 2010).

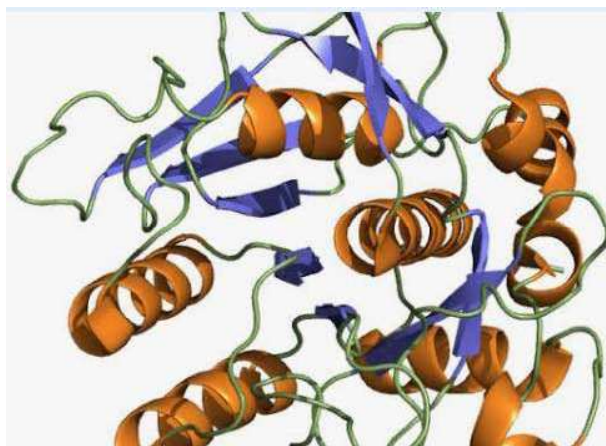


Fig. 2 Nattokinase molecule (Wikipedia, 2026).

The three-dimensional crystal structure of nattokinase has been determined at high resolution using X-ray crystallography, providing invaluable atomic-level insights into the structural determinants of its catalytic activity and substrate specificity (Yanagisawa et al., 2010; Yanagisawa et al., 2013). The overall fold of the enzyme is characteristic of the subtilisin family, featuring a central parallel β -sheet core flanked by α -helices and stabilized by multiple intramolecular interactions. The active site is located within a pronounced surface cleft and contains the canonical catalytic triad comprising residues Asp32, His64, and Ser221 (subtilisin BPN' numbering), which function in concert to effect the nucleophilic attack on the scissile peptide bond of the substrate (Yanagisawa et al., 2010). In addition to the catalytic triad, the substrate-binding pocket of nattokinase is lined by a series of hydrophobic and polar residues that confer specificity for particular amino acid sequences, thereby determining the enzyme's preference for cleavage at specific sites within target proteins such as fibrin (Yanagisawa et al., 2010; Yanagisawa et al., 2013). Notably, despite its close structural resemblance to other subtilisins, nattokinase displays distinct substrate specificity that distinguishes it from related enzymes and contributes to its unique fibrinolytic profile (Yanagisawa et al., 2013).

3.3 Physicochemical Characteristics

Nattokinase exhibits a set of well-defined physicochemical properties that are critical to understanding its stability, activity, and behavior under various environmental and physiological conditions. The enzyme functions optimally as an alkaline serine protease, exhibiting maximal catalytic activity at pH values ranging from approximately 8.0 to 10.0 (Dabbagh et al., 2014; Wang et al., 2009). The optimal temperature for nattokinase activity is typically reported to be in the range of 40°C to 50°C, although the enzyme retains substantial activity at physiological temperature (37°C) and displays a degree of thermostability that is relevant to its application in food processing and nutraceutical formulation (Dabbagh et al., 2014; Wang et al., 2009;

Lonxigong, 2024). The enzyme is soluble in water and aqueous buffer systems, a property that facilitates its extraction, purification, and incorporation into various dosage forms (Dabbagh et al., 2014).

The stability of nattokinase is influenced by a variety of environmental factors, including pH, temperature, and the presence of denaturing agents or proteolytic enzymes. The enzyme is relatively stable over a pH range of approximately 6.0 to 10.0, with significant loss of activity occurring under more acidic or alkaline conditions (Dabbagh et al., 2014). Thermal stability studies have demonstrated that nattokinase retains a substantial fraction of its activity following exposure to temperatures up to 50°C for extended periods, although irreversible denaturation and loss of activity occur at higher temperatures (Dabbagh et al., 2014). The presence of calcium ions has been shown to enhance the thermostability of nattokinase, an observation that is consistent with the known role of divalent cations in stabilizing the tertiary structure of subtilisin-family proteases (Dabbagh et al., 2014). These stability characteristics have important implications for the formulation, storage, and gastrointestinal survival of orally administered nattokinase supplements.

3.4 Extraction and Purification Methodologies

The extraction and purification of nattokinase from fermentation broths or directly from natto represent critical steps in the production of high-purity enzyme preparations suitable for research, nutraceutical, and potential pharmaceutical applications. A variety of chromatographic and non-chromatographic techniques have been developed and optimized for this purpose, with the choice of methodology dictated by considerations of yield, purity, scalability, and cost-effectiveness (Dabbagh et al., 2014; Wang et al., 2009; Yi et al., 2025).

The initial step in the purification process typically involves the removal of bacterial cells and particulate matter from the fermentation broth by centrifugation or filtration, yielding a clarified supernatant that contains the extracellular nattokinase along with a complex mixture of other proteins, peptides, polysaccharides, and small molecules (Wang et al., 2009). Subsequent purification steps are designed to selectively concentrate and isolate the nattokinase protein from this complex milieu. Ammonium sulfate precipitation is frequently employed as a preliminary fractionation technique, taking advantage of the differential solubility of proteins at varying salt concentrations to achieve a crude enrichment of nattokinase (Jagathy et al., 2017; Wang et al., 2009). This step is typically followed by one or more chromatographic separations, which exploit differences in the physicochemical properties of proteins to achieve higher degrees of purity.

Gel filtration chromatography, also known as size-exclusion chromatography, is commonly utilized to separate proteins on the basis of their molecular size and shape, effectively removing contaminating proteins of significantly different molecular weight (Wang et al., 2009; Yanagisawa et al., 2010). Ion-exchange chromatography, which separates proteins according to their net surface charge, represents another powerful purification tool that has been widely applied to nattokinase (Wang et al., 2009). A study by Wang et al. (2009) reported the purification of nattokinase from the supernatant of *Bacillus subtilis* natto B-12 culture broth to 56.1-fold homogeneity using a combination of ammonium sulfate precipitation, Sephacryl S-100 gel filtration, and S-Sepharose ion-exchange chromatography, achieving a yield of 100 mg of purified enzyme per liter of culture (Wang et al., 2009). Affinity chromatography, employing ligands that specifically bind to nattokinase, offers the potential for even higher degrees of purification, although its application is often limited by the cost and complexity of preparing suitable affinity matrices (Dabbagh et al., 2014).

Recent innovations in downstream processing have focused on the development of integrated, multi-product extraction strategies that enable the simultaneous recovery of nattokinase along with other valuable metabolites produced by *Bacillus subtilis* natto during fermentation (Yi et al., 2025). In a noteworthy study, Yi et al. (2025) described a sequential extraction system that capitalized on the differential precipitation characteristics of ammonium sulfate for various proteins and protein complexes to achieve the concurrent isolation of nattokinase, menaquinone-7 (vitamin K₂), and γ -polyglutamic acid from a single fermentation

matrix. This integrated approach achieved an overall extraction efficiency of 70% for the three target metabolites, demonstrating the feasibility and economic advantages of multi-product recovery strategies for the sustainable production of nattokinase and related bioactive compounds (Yi et al., 2025).

3.5 Pharmacokinetics and Absorption

A comprehensive understanding of the pharmacokinetic behavior of orally administered nattokinase is essential for optimizing dosing regimens, predicting therapeutic outcomes, and ensuring the safe and effective use of this enzyme in clinical and nutraceutical applications. However, the pharmacokinetics of nattokinase have been relatively understudied compared to other aspects of its pharmacology, and significant gaps in knowledge remain regarding its absorption, distribution, metabolism, and excretion (ADME) in humans.

The oral bioavailability of nattokinase, like that of many protein-based therapeutics, is constrained by the formidable barriers presented by the gastrointestinal tract, including the denaturing effects of gastric acid, proteolytic degradation by digestive enzymes, and limited permeability across the intestinal epithelium (Ero et al., 2013; Zhou et al., 2025). Despite these challenges, a substantial body of evidence indicates that biologically relevant quantities of nattokinase and/or its active metabolites are absorbed into the systemic circulation following oral ingestion. A pilot study by Ero et al. (2013) investigated the serum pharmacokinetics of nattokinase in healthy human volunteers following the oral administration of a single daily dose of 2,000 fibrinolytic units (FU) of the enzyme. Using an immunological detection method, the study demonstrated that nattokinase and/or its immunoreactive metabolites were detectable in serum between 2 and 24 hours post-ingestion, confirming that the enzyme is absorbed from the gastrointestinal tract and reaches the systemic circulation in a form that retains immunological recognition (Ero et al., 2013).

The mechanisms underlying the intestinal absorption of nattokinase have been explored using a variety of *in vitro* and *ex vivo* model systems. Liu et al. (2023) investigated the transport and internalization mechanisms of nattokinase across the intestinal epithelium using Caco-2 cell monolayers and an animal everted intestinal sac model. The results of this investigation indicated that nattokinase is a moderately absorbed biomolecule, with its transport through enterocytes being an energy- and time-dependent process (Feng et al., 2023). A more recent and detailed study by Zhou et al. (2025) employed a rat everted intestinal sac model to quantify the small intestine absorption rate of nattokinase, reporting a value of 37.0% under the experimental conditions employed. Importantly, this study further characterized the nature of the absorbed species, revealing that the peptides crossing the intestinal barrier were predominantly hydrophobic, uncharged, and shorter than 10 amino acids in length (Zhou et al., 2025). This finding suggests that nattokinase undergoes significant proteolytic processing during transit across the intestinal epithelium, and that the biologically active species reaching the systemic circulation may consist of smaller peptide fragments rather than the intact, full-length protein.

The stability of nattokinase during gastrointestinal transit is a critical determinant of its ultimate bioavailability. *In vitro* digestion simulations conducted by Zhou et al. (2025) demonstrated that nattokinase retained approximately 25.8% of its initial enzymatic activity following exposure to simulated gastric and intestinal fluids, indicating that a substantial fraction of the ingested enzyme survives the harsh conditions of the upper gastrointestinal tract. These findings underscore the importance of formulation strategies, such as enteric coating or encapsulation, to protect nattokinase from gastric inactivation and enhance its delivery to the intestinal absorptive surface (Zhou et al., 2025). Further research is urgently needed to fully elucidate the pharmacokinetic profile of nattokinase, including the identification and characterization of its active metabolites, the determination of its volume of distribution and elimination half-life, and the evaluation of potential drug-drug interactions that may influence its absorption or clearance.

4 Pharmacological Actions and Mechanistic Insights

In the view of network biology or pharmacology, nattokinase is a regulatory and controlling factor in the human biological network (Zhang, 2016b, 2018, 2026, 2027a-d). Fig. 3 provides a schematic overview of nattokinase metabolic regulation pathways.

4.1 Fibrinolytic and Thrombolytic Activities

The fibrinolytic and thrombolytic activities of nattokinase constitute its most extensively characterized and clinically relevant pharmacological properties. The enzyme's capacity to degrade fibrin, the insoluble proteinaceous matrix that forms the structural backbone of blood clots, underpins its utility in the prevention and management of thrombotic disorders, including deep vein thrombosis, pulmonary embolism, ischemic stroke, and myocardial infarction (Granito et al., 2024; Muric et al., 2024; Weng et al., 2017). The mechanisms by which nattokinase promotes fibrinolysis and thrombolysis are multifaceted and operate at multiple levels within the hemostatic system.

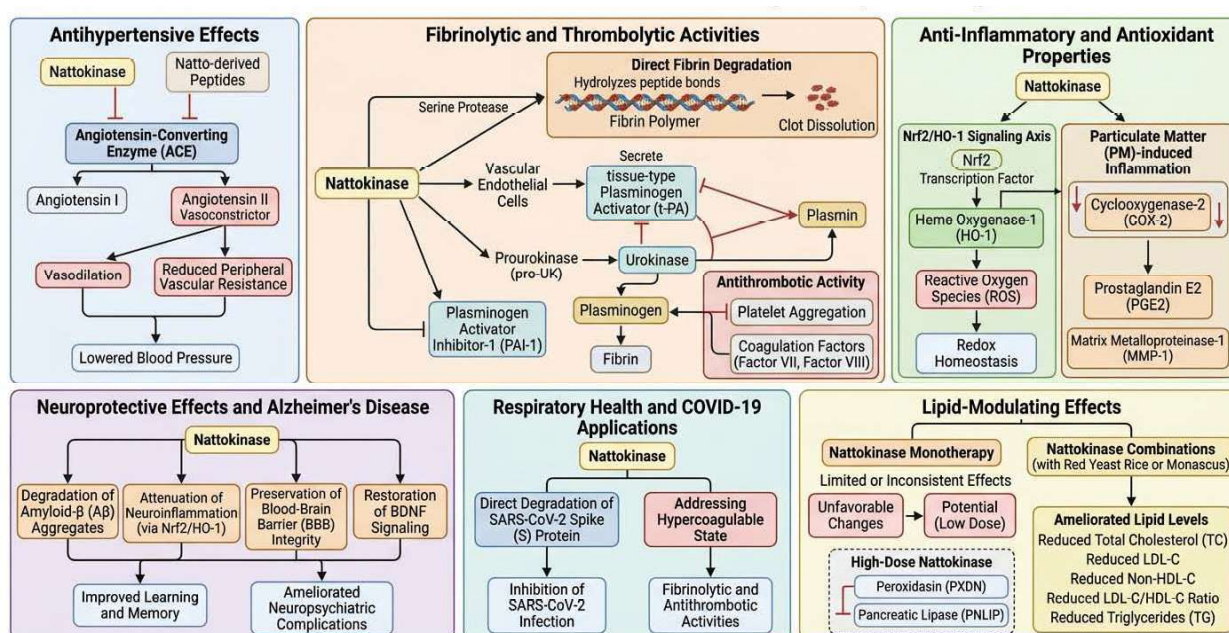


Fig. 3 Metabolic regulation pathways of nattokinase.

The most direct mechanism involves the enzymatic cleavage of fibrin itself. Nattokinase, as a serine protease, hydrolyzes peptide bonds within the fibrin polymer, thereby disrupting the structural integrity of the clot and facilitating its dissolution (Sumi et al., 1987). *In vitro* studies have consistently demonstrated the potent fibrin-degrading activity of nattokinase, with thrombolysis assays showing that the enzyme achieves fibrin clot lysis rates of 30-40% at concentrations of 5,000 µg/mL or higher (Zhou et al., 2025). Notably, this thrombolytic efficacy is observed irrespective of clot age, with nattokinase exhibiting comparable activity against both fresh and aged (stale) fibrin clots, a property that distinguishes it from some conventional thrombolytic agents whose activity diminishes as clots mature and undergo structural remodeling (Zhou et al., 2025). This characteristic may have important clinical implications, as it suggests that nattokinase could be effective in the treatment of both acute and chronic thrombotic events.

Beyond its direct fibrinolytic action, nattokinase enhances endogenous thrombolytic pathways through several complementary mechanisms. The enzyme has been shown to stimulate vascular endothelial cells to

produce and secrete tissue-type plasminogen activator (t-PA), a key endogenous fibrinolytic enzyme that catalyzes the conversion of the inactive zymogen plasminogen to the active protease plasmin, which in turn degrades fibrin (Sumi et al., 1990; Yatagai et al., 2007). By augmenting t-PA release, nattokinase indirectly amplifies the body's intrinsic capacity for fibrinolysis. Additionally, nattokinase has been reported to catalyze the activation of prourokinase (pro-UK) to its enzymatically active form, urokinase, which similarly promotes the conversion of plasminogen to plasmin (Sumi et al., 1990; Yanagisawa et al., 2010). This dual activation of endogenous plasminogen activators represents a powerful synergistic mechanism for enhancing thrombolysis.

A third important mechanism involves the degradation and inactivation of plasminogen activator inhibitor-1 (PAI-1), the primary physiological inhibitor of t-PA and urokinase (Urano et al., 2001). By reducing PAI-1 activity, nattokinase effectively removes a critical brake on the fibrinolytic system, allowing t-PA and urokinase to function with enhanced efficiency and prolonging the duration of their thrombolytic action (Urano et al., 2001). The combined effect of these multiple, convergent mechanisms—direct fibrin degradation, stimulation of t-PA production, activation of prourokinase, and inhibition of PAI-1—renders nattokinase a remarkably effective and versatile fibrinolytic agent.

In addition to its fibrinolytic properties, nattokinase exhibits antithrombotic activity through the inhibition of platelet aggregation and the reduction of coagulation factor levels. Studies have demonstrated that nattokinase can suppress platelet aggregation, a critical step in the initiation and propagation of thrombus formation (Jang et al., 2013; Kurosawa et al., 2015). Furthermore, nattokinase supplementation has been associated with reductions in plasma levels of coagulation factors, including factor VII and factor VIII, which are essential components of the intrinsic and extrinsic coagulation cascades, respectively (Hsia et al., 2009). By targeting both the cellular (platelet) and humoral (coagulation factor) arms of the hemostatic system, nattokinase provides comprehensive protection against pathological thrombus formation.

4.2 Antihypertensive Effects

Hypertension is a major modifiable risk factor for cardiovascular disease, and the identification of safe and effective blood pressure-lowering agents is a public health priority. A substantial body of evidence, derived from both preclinical animal studies and human clinical trials, indicates that nattokinase possesses significant antihypertensive properties (Li et al., 2023; Liu et al., 2024; Muric et al., 2024). The blood pressure-lowering effects of nattokinase have been consistently observed across multiple studies, with the magnitude of the reduction typically being in the range of 3-8 mmHg for systolic blood pressure and 2-5 mmHg for diastolic blood pressure (Kim et al., 2008; Li et al., 2023).

The most robust evidence for the antihypertensive efficacy of nattokinase comes from a systematic review and meta-analysis of randomized controlled trials published by Li et al. (2023). This comprehensive analysis, which included six eligible RCTs encompassing a total of 546 participants, found that nattokinase supplementation resulted in a statistically significant reduction in both systolic blood pressure (mean difference [MD] = -3.45 mmHg, 95% confidence interval [CI]: -4.37 to -2.18 mmHg, $p < 0.00001$) and diastolic blood pressure (MD = -2.32 mmHg, 95% CI: -2.72 to -1.92 mmHg, $p < 0.00001$) compared to placebo (Li et al., 2023). These findings were consistent across the included studies, which were judged to be of high methodological quality, providing strong support for the use of nattokinase as an effective adjunctive therapy for hypertension (Li et al., 2023).

The mechanisms underlying the antihypertensive effects of nattokinase are thought to involve the inhibition of angiotensin-converting enzyme (ACE), a key component of the renin-angiotensin-aldosterone system (RAAS) that plays a central role in the regulation of blood pressure (Fujita et al., 1993; Okamoto et al., 1995). ACE catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II, and its inhibition leads to vasodilation, reduced peripheral vascular resistance, and a consequent lowering of blood

pressure. Studies have demonstrated that nattokinase and natto-derived peptides can inhibit ACE activity *in vitro* and *in vivo*, providing a plausible mechanistic basis for the observed antihypertensive effects (Fujita et al., 1993; Okamoto et al., 1995). It is noteworthy that the antihypertensive action of nattokinase may be attributable not only to the intact enzyme but also to bioactive peptides generated during the fermentation process or during gastrointestinal digestion, which may exhibit potent ACE inhibitory activity (Fujita et al., 1993; Okamoto et al., 1995).

The clinical significance of the blood pressure reductions achieved with nattokinase should not be underestimated. Epidemiological studies have established that even modest reductions in blood pressure, on the order of 2-5 mmHg, are associated with clinically meaningful decreases in the risk of stroke, coronary heart disease, and all-cause mortality (Li et al., 2023). Therefore, the consistent and statistically significant antihypertensive effects of nattokinase observed in clinical trials suggest that this natural enzyme could play a valuable role in the comprehensive management of hypertension, particularly as an adjunct to lifestyle modifications and conventional pharmacotherapy.

4.3 Anti-Inflammatory and Antioxidant Properties

While the fibrinolytic and antihypertensive activities of nattokinase have historically garnered the greatest attention, a growing body of recent research has illuminated the enzyme's potent anti-inflammatory and antioxidant properties, which are now recognized as integral components of its broader cardioprotective and health-promoting effects (Granito et al., 2024; Li et al., 2026; Mahakalakar et al., 2025). Chronic inflammation and oxidative stress are fundamental pathogenic processes that underlie the development and progression of atherosclerosis, hypertension, endothelial dysfunction, and a host of other cardiovascular and non-communicable diseases (Granito et al., 2024; Zhang, 2027a-d). The ability of nattokinase to mitigate these processes represents a significant and previously underappreciated dimension of its therapeutic potential.

A comprehensive review by Granito et al. (2024) synthesized the available evidence regarding the anti-inflammatory and antioxidant effects of nattokinase, concluding that the enzyme can be employed as a novel adjuvant therapeutic strategy to mitigate inflammation and oxidative stress in non-communicable diseases, including cardiovascular disease. The authors emphasized that while nattokinase was initially recognized primarily for its fibrinolytic and antithrombotic activities, the more recent identification of its anti-inflammatory and antioxidant properties significantly expands its potential clinical applications and underscores its pleiotropic nature (Granito et al., 2024).

At the cellular and molecular level, the anti-inflammatory and antioxidant effects of nattokinase are mediated, at least in part, through the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling axis (Li et al., 2026; Mahakalakar et al., 2025). Nrf2 is a transcription factor that serves as a master regulator of the cellular antioxidant response, controlling the expression of a battery of cytoprotective genes, including HO-1, that function to neutralize reactive oxygen species (ROS) and restore redox homeostasis. A recent study by Li et al. (2025), published in the Journal of Dental Sciences, provided detailed mechanistic insights into the Nrf2/HO-1-dependent protective effects of nattokinase in human gingival fibroblasts (HGF-1 cells) exposed to particulate matter (PM)-induced inflammation and oxidative stress. The study demonstrated that nattokinase pretreatment markedly suppressed the PM-induced expression of pro-inflammatory mediators, including cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2), as well as the matrix-degrading enzyme matrix metalloproteinase-1 (MMP-1) (Li et al., 2025). Mechanistically, nattokinase was shown to enhance Nrf2 activation and HO-1 expression, thereby attenuating the PM-induced activation of NADPH oxidase-derived ROS generation and the downstream PI3K/Akt and MAPK signaling cascades (Huang and Zhang, 2012; Li and Zhang, 2013; Zhang, 2016a, 2017; Li et al., 2026). Crucially, pharmacological inhibition of Nrf2 or HO-1 abolished the protective effects of nattokinase, establishing the

essential role of this signaling pathway in mediating the enzyme's anti-inflammatory and antioxidant actions (Li et al., 2026).

These findings are further corroborated by studies conducted in other experimental systems. A research has extended the Nrf2/HO-1 activation paradigm to the context of neurodegenerative disease, demonstrating that nattokinase protects against blood-brain barrier dysfunction and neuroinflammation through mechanisms involving the Nrf2/HO-1 pathway. The convergence of evidence from diverse cellular and animal models strongly implicates Nrf2/HO-1 activation as a central and conserved mechanism by which nattokinase exerts its anti-inflammatory and antioxidant effects across multiple tissue types and disease contexts.

4.4 Lipid-Modulating Effects

The effects of nattokinase on lipid metabolism and plasma lipid profiles have been the subject of considerable investigation, yet the evidence remains somewhat equivocal and appears to be contingent upon the specific formulation, dosage, and combination with other nutraceuticals. While some studies have reported favorable effects of nattokinase on total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG), other investigations have failed to demonstrate significant lipid-lowering benefits, and a systematic review and meta-analysis has cast doubt on the efficacy of nattokinase monotherapy for improving lipid parameters (Li et al., 2023).

The meta-analysis by Li et al. (2023) provided a comprehensive and rigorous assessment of the available randomized controlled trial evidence concerning the effects of nattokinase on cardiovascular risk factors, including lipid profiles. The analysis revealed that relatively low total dosages of nattokinase were associated with a negative effect on blood total cholesterol (MD = 5.27, 95% CI: 3.74 to 6.81, $p = 0.00001$), HDL-C (MD = -2.76, 95% CI: -3.88 to -1.64, $p < 0.00001$), and LDL-C (MD = 6.49, 95% CI: 0.83 to 12.15, $p = 0.02$) (Li et al., 2023). Nattokinase groups receiving relatively high total dosages also exhibited higher total cholesterol (MD = 3.18, 95% CI: 2.29 to 4.06, $p < 0.00001$) compared to control interventions, although no significant differences were observed in HDL-C or LDL-C levels at these higher doses (Li et al., 2023). Furthermore, no significant correlation was found between nattokinase supplementation and triglyceride levels ($p = 0.71$) (Li et al., 2023). Based on these findings, the authors concluded that relatively low-dose supplementation of nattokinase may have no significant lipid-lowering effect and may even be associated with unfavorable changes in lipid profiles (Li et al., 2023).

In contrast to the equivocal findings for nattokinase monotherapy, studies evaluating nattokinase in combination with other lipid-modulating nutraceuticals have yielded more promising results. A four-month randomized, double-blind, placebo-controlled clinical trial by Liu et al. (2023) examined the effects of nattokinase-monascus supplements (NMSs) on dyslipidemia. The study demonstrated that NMSs significantly ameliorated lipid levels, including reductions in total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and non-high-density lipoprotein cholesterol (non-HDL-C), as well as a decrease in the LDL-C to HDL-C ratio (Feng et al., 2023). However, no significant effects were observed on triglyceride levels, HDL-C, or carotid intima-media thickness (CIMT) (Feng et al., 2023). The beneficial effects of the combination product are likely attributable to the inclusion of monascus, a source of naturally occurring statin-like compounds (monacolins) that are known to inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis.

Similarly, a 90-day randomized, double-blind, placebo-controlled trial by Liu et al. (2024) investigated the effects of nattokinase combined with red yeast rice (RYR) in patients with stable coronary artery disease (CAD). Red yeast rice is another natural source of monacolins and is recognized for its cholesterol-lowering properties. The study found that the combination of NK + RYR produced the maximum effect in reducing triglyceride (-0.39 mmol/L), total cholesterol (-0.66 mmol/L), and diastolic blood pressure (-7.39 mmHg),

while simultaneously increasing HDL-C (0.195 mmol/L) compared to other groups (all p for multiple group comparisons < 0.01) (Liu et al., 2024). Both the NK + RYR and NK alone groups exhibited significantly improved lactate dehydrogenase levels compared to the other groups (-29.1 U/L and -26.4 U/L, respectively) (Liu et al., 2024). The NK + RYR group also demonstrated more potent reductions in thromboxane B2 and increases in antithrombin III compared to placebo (both $p < 0.01$), suggesting that the combination may favorably alter antithrombin and COX-1 pathways, potentially reducing thrombosis risk in CAD patients (Liu et al., 2024).

Mechanistic insights into the potential lipid-modulating effects of nattokinase have been provided by a recent study by Zhou et al. (2025), which employed a high-fat diet-induced atherosclerosis model in ApoE^{-/-} mice. Proteomic analysis of mouse liver tissues identified that high-dose nattokinase treatment (900 FU/kg body weight) down-regulated the expression of peroxidasin (PXDN) and pancreatic lipase (PNLIP), proteins that are involved in promoting lipid oxidation and intestinal lipid absorption, respectively (Zhou et al., 2025). The down-regulation of these proteins may contribute to the amelioration of atherosclerosis and dyslipidemia, providing a plausible molecular basis for the observed effects. Collectively, the available evidence suggests that while nattokinase as a standalone therapy may have limited or inconsistent effects on lipid profiles, its combination with other lipid-modulating nutraceuticals, such as red yeast rice or monascus, produces more robust and clinically meaningful improvements in lipid parameters.

4.5 Neuroprotective Effects and Alzheimer's Disease

An exciting and rapidly evolving frontier in nattokinase research concerns its potential neuroprotective effects and its application in the prevention and treatment of neurodegenerative disorders, most notably Alzheimer's disease (AD) (Tanikawa et al., 2024; Mahakalakar et al., 2025). Alzheimer's disease is a progressive and devastating neurodegenerative condition characterized by the accumulation of amyloid- β (A β) plaques and neurofibrillary tangles, chronic neuroinflammation, oxidative stress, and synaptic dysfunction, culminating in profound cognitive decline and dementia. The identification of safe and effective agents capable of targeting these pathogenic processes is a critical unmet need. Emerging evidence from preclinical studies suggests that nattokinase may possess a constellation of properties that render it a promising candidate for neuroprotection in AD and related disorders.

A landmark study by Tanikawa et al. (2024) investigated the effects of nattokinase in a rat model of Alzheimer's disease induced by the combined administration of D-galactose and aluminum chloride. The results of this investigation were highly encouraging, demonstrating that oral administration of nattokinase to AD model rats resulted in increased levels of free-form β -amyloid in the cerebrospinal fluid (CSF), a finding that is consistent with the mobilization and clearance of A β deposits from the brain parenchyma (Tanikawa et al., 2024). Furthermore, nattokinase treatment was associated with improved aluminum and amyloid plaque accumulation in the brain, suggesting that the enzyme may facilitate the removal of these neurotoxic aggregates (Tanikawa et al., 2024). The authors concluded that nattokinase has potential therapeutic applications in the treatment of Alzheimer's disease (Tanikawa et al., 2024).

Complementary and convergent findings have been reported by Mahakalakar et al. (2024), who investigated the neuroprotective effects of nattokinase in a mouse model of A β 1-42-induced neuropsychiatric complications, neuroinflammation, and disruption of brain-derived neurotrophic factor (BDNF) signaling. The study demonstrated that nattokinase administration improved learning and memory impairment and ameliorated neuropsychiatric complications, effects that were attributed, at least in part, to the downregulation of neuroinflammatory pathways and the restoration of BDNF signaling (Mahakalakar et al., 2025). BDNF is a critical neurotrophin that supports the survival, differentiation, and synaptic plasticity of neurons, and its disruption is implicated in the pathophysiology of AD and other neurodegenerative conditions. The ability of

nattokinase to preserve BDNF signaling represents a potentially important mechanism of neuroprotection.

The neuroprotective effects of nattokinase are further supported by a comprehensive review by Mahakalakar et al. (2024), which synthesized the available preclinical evidence regarding the potential of nattokinase to prevent blood-brain barrier (BBB) dysfunction in neurodegenerative disorders. The review concluded that there is sufficient evidence from experimental animal studies to indicate that nattokinase supplementation can alleviate BBB dysfunction, reduce brain inflammation, and improve cognitive ability (Mahakalakar et al., 2024). The blood-brain barrier is a highly specialized and tightly regulated endothelial interface that protects the brain from circulating pathogens, toxins, and inflammatory mediators. Disruption of BBB integrity is a common feature of many neurodegenerative diseases and contributes to the propagation of neuroinflammation and neuronal injury. The observation that nattokinase can preserve BBB function and mitigate neuroinflammation positions it as a potentially valuable therapeutic agent for a spectrum of neurodegenerative conditions.

The mechanisms by which nattokinase exerts its neuroprotective effects are likely multifaceted and may include: (1) the degradation of amyloid- β aggregates, as suggested by the increased CSF A β levels observed in the study by Tanikawa et al. (2024); (2) the attenuation of neuroinflammation through the Nrf2/HO-1 pathway, as demonstrated by Hsu et al. (2023) and Mahakalakar et al. (2024); (3) the preservation of BBB integrity and the prevention of vascular dysfunction, which may involve the enzyme's fibrinolytic and antithrombotic properties; and (4) the enhancement of neurotrophic support through the restoration of BDNF signaling (Mahakalakar et al., 2024). Further research, including well-designed clinical trials in human subjects with AD or mild cognitive impairment, is urgently needed to validate these promising preclinical findings and to fully elucidate the therapeutic potential of nattokinase in the realm of neurodegenerative disease.

4.6 Respiratory Health and COVID-19 Applications

The emergence of the COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has catalyzed intense interest in identifying safe and effective therapeutic agents capable of mitigating viral infection, reducing disease severity, and preventing the development of long-term complications. A notable and unexpected development in this context has been the investigation of nattokinase as a potential antiviral agent with activity against SARS-CoV-2 (Tanikawa et al., 2022). The rationale for exploring nattokinase in the context of COVID-19 is grounded in two complementary lines of evidence: its direct degradative effect on the SARS-CoV-2 spike protein and its potential to address the hypercoagulable state and microvascular thrombosis that characterize severe COVID-19.

A pivotal study by Tanikawa et al. (2022), published in the journal *Molecules*, examined the effect of nattokinase on the spike (S) protein of SARS-CoV-2. The S protein is a critical structural component of the viral envelope that mediates viral attachment to host cell receptors (angiotensin-converting enzyme 2, ACE2) and facilitates viral entry into target cells. The study demonstrated that nattokinase is capable of degrading the S protein of SARS-CoV-2 *in vitro*, an effect that was observed under physiologically relevant conditions (Tanikawa et al., 2022). The authors concluded that their findings suggest that nattokinase exhibits potential for the inhibition of SARS-CoV-2 infection via S protein degradation (Tanikawa et al., 2022). By disrupting the structural integrity of the S protein, nattokinase may interfere with the virus's ability to bind to and enter host cells, thereby limiting the establishment and propagation of infection.

In addition to its direct antiviral potential, nattokinase may confer benefit in COVID-19 through its well-established fibrinolytic and antithrombotic activities. Severe COVID-19 is frequently complicated by a profound hypercoagulable state, characterized by elevated levels of D-dimer and fibrinogen, increased thrombin generation, and impaired fibrinolysis, which can lead to the formation of microvascular thrombi and the development of acute respiratory distress syndrome (ARDS) and multi-organ failure (Okeahialam, 2024).

Nattokinase, with its potent fibrinolytic properties and its ability to enhance endogenous thrombolysis, has been proposed as a potential therapeutic agent to address the coagulopathy associated with COVID-19 (Okeahialam, 2024). By degrading fibrin clots, inhibiting platelet aggregation, and reducing coagulation factor levels, nattokinase could theoretically improve microvascular perfusion, alleviate tissue hypoxia, and mitigate the systemic inflammatory response that drives disease progression.

The hypothesis that nattokinase may be beneficial in COVID-19 has been articulated in several publications. Okeahialam (2024), writing in the Santosh University Journal of Health Sciences, proposed nattokinase as a "low-hanging fruit" for the treatment of COVID-19, emphasizing its capacity to counter plasminogen activator inhibitor-1 (PAI-1) and thereby liberate tissue plasminogen activator (t-PA) to lyse fibrin deposits that obstruct the vasculature (Okeahialam, 2024). The author argued that the fibrinolytic properties of nattokinase could be particularly valuable in addressing the microvascular thrombosis that contributes to the morbidity and mortality of severe COVID-19.

It is important to emphasize that the evidence supporting the use of nattokinase for COVID-19 is currently limited to *in vitro* studies and theoretical considerations; rigorous clinical trials evaluating its efficacy in human subjects with COVID-19 have not been reported. Furthermore, caution is warranted, as some sources have noted that there is no solid proof that nattokinase effectively removes spike proteins in real human scenarios (THIP Media, 2023). The translation of *in vitro* findings to clinical benefit requires careful validation through well-designed, randomized, placebo-controlled trials. Nevertheless, the existing data provide a compelling rationale for further investigation and underscore the potential of nattokinase to contribute to the armamentarium of strategies for combating COVID-19 and its associated complications.

4.7 Additional Biological Activities

Beyond the extensively studied cardiovascular, anti-inflammatory, antioxidant, neuroprotective, and antiviral properties detailed above, nattokinase has been reported to exhibit a range of additional biological activities that further underscore its pleiotropic nature and expand the potential scope of its therapeutic applications.

Improvement of Blood Rheology and Microcirculation. Nattokinase has been shown to improve blood rheological properties, including the reduction of blood viscosity and the enhancement of red blood cell deformability, which collectively promote more efficient microcirculatory blood flow (Pais et al., 2006). Improved microcirculation is essential for adequate tissue oxygenation and nutrient delivery and may contribute to the observed benefits of nattokinase in conditions such as peripheral artery disease and diabetic microangiopathy.

Ocular Health and Retinal Protection. Emerging evidence suggests that nattokinase may confer benefits in the realm of ocular health, particularly through the reduction of pathological retinal neovascularization (Weng et al., 2017). Retinal neovascularization is a hallmark of several vision-threatening conditions, including diabetic retinopathy and age-related macular degeneration. The mechanisms underlying this protective effect are not fully elucidated but may involve the anti-inflammatory, antioxidant, and anti-angiogenic properties of nattokinase.

Enhancement of Skin Temperature Recovery. An intriguing study by Nara et al. (2023) investigated the effects of a single oral dose of nattokinase on skin temperature recovery following cold water immersion. This double-blind, placebo-controlled crossover study conducted in nine healthy men found that nattokinase accelerated the recovery of skin temperature after cold exposure, an effect that may reflect improved peripheral blood flow and microcirculatory function (Nara et al., 2023). While the clinical significance of this finding remains to be determined, it provides additional evidence for the vascular and thermoregulatory effects of nattokinase.

Gastrointestinal and Intestinal Health. Nattokinase has been suggested to have applications in regulating

gastrointestinal and intestinal function, including potential antibacterial actions (Core Pure Bio & Tech, 2021). The enzyme may contribute to the maintenance of a healthy gut microbiota and the prevention of gastrointestinal infections, although rigorous studies in this area are currently lacking.

5 Clinical Trials and Therapeutic Efficacy

5.1 Clinical Applications and Formulations

Nattokinase is currently marketed and consumed primarily as a dietary supplement and functional food ingredient, rather than as a regulated pharmaceutical agent. It is available in a variety of oral dosage forms, including powders, pills, capsules, and softgels, and is frequently formulated alone or in combination with other bioactive ingredients, such as red yeast rice, coenzyme Q10, omega-3 fatty acids, and various vitamins and minerals (Dabbagh et al., 2014; Liu et al., 2024). The enzyme is promoted for a range of health applications, most notably for the support of cardiovascular health, the maintenance of healthy blood pressure and circulation, and the prevention of thrombotic events (Dabbagh et al., 2014; Muric et al., 2024). However, it is important to acknowledge that clinical trials to definitively support such uses are still evolving, and the level of evidence varies across different indications (Drugs.com, 2026).

The clinical dosing of nattokinase has not been standardized, and optimal dosing regimens remain to be firmly established through rigorous clinical investigation. Clinical studies have employed a wide range of nattokinase doses, typically expressed in fibrinolytic units (FU), with daily doses ranging from 2,000 FU to 10,000 FU or higher (Ero et al., 2013; Li et al., 2023). A commonly used dosage in clinical trials evaluating thrombolytic/fibrinolytic and blood pressure effects has been 100 mg/day of nattokinase, which is equivalent to 2,000 FU, administered for a duration of 8 weeks (Drugs.com, 2026). However, the optimal dose for specific indications and patient populations may vary and requires further investigation.

5.2 Overview of Clinical Research Studies

The clinical evidence base for nattokinase has expanded considerably over the past two decades, encompassing a growing number of randomized controlled trials (RCTs) that have evaluated its effects on a variety of cardiovascular risk factors and clinical outcomes. The most comprehensive and rigorous synthesis of this evidence is provided by the systematic review and meta-analysis of randomized controlled trials conducted by Li et al. (2023). This analysis, which included six eligible RCTs with a total of 546 participants, concluded that nattokinase can be used as an effective adjunctive therapy for hypertension, but that relatively low-dose supplementation may have no significant lipid-lowering effect (Li et al., 2023). The methodological quality of the included studies was assessed as high, lending credibility to the findings (Li et al., 2023).

In addition to the trials included in the Li et al. (2023) meta-analysis, several more recent RCTs have further expanded the clinical evidence base. A four-month randomized, double-blind, placebo-controlled trial by Liu et al. (2023) evaluated the effects of nattokinase-*monascus* supplements (NMSs) in patients with dyslipidemia. The study enrolled 104 participants (56 in the NMS group and 58 in the placebo group), of whom 48 and 45, respectively, completed the intervention (Feng et al., 2023). The results demonstrated that NMSs significantly ameliorated lipid levels, including reductions in TC, LDL-C, and non-HDL-C, as well as a decrease in the LDL-C to HDL-C ratio (Feng et al., 2023). The study was well-designed and the results were clear and consistent, although the authors noted the need for further research with larger sample sizes and longer follow-up periods (Feng et al., 2023).

A 90-day randomized, double-blind, placebo-controlled trial by Liu et al. (2024) investigated the effects of nattokinase combined with red yeast rice (RYR) in 178 patients with stable coronary artery disease (CAD). The study employed a four-group design (NK + RYR, NK alone, RYR alone, and placebo), which allowed for the evaluation of both individual and combined effects. The findings were striking: the NK + RYR

combination produced the maximum effect in reducing triglyceride (-0.39 mmol/L), total cholesterol (-0.66 mmol/L), and diastolic blood pressure (-7.39 mmHg), while simultaneously increasing HDL-C (0.195 mmol/L) compared to all other groups (all p for multiple group comparisons < 0.01) (Liu et al., 2024). Furthermore, the NK + RYR group showed more potent reductions in thromboxane B2 and increases in antithrombin III compared to placebo (both $p < 0.01$), suggesting that the combination may favorably alter antithrombin and COX-1 pathways, potentially reducing thrombosis risk in CAD patients (Liu et al., 2024). No adverse effects due to the interventions were reported (Liu et al., 2024).

An ongoing clinical trial, registered on ClinicalTrials.gov (NCT05200234), is evaluating the effects of nattokinase on cerebral blood flow and cognitive function in patients with asymptomatic intracranial/carotid stenosis (ClinicalTrials.gov, 2023). This randomized controlled trial will provide valuable insights into the potential neuroprotective and cerebrovascular benefits of nattokinase in a high-risk population and will contribute to the evidence base supporting its use for brain health.

5.3 Quality Control and Standardization

The quality control and standardization of nattokinase products represent critical challenges that have significant implications for the safety, efficacy, and reproducibility of clinical research findings and consumer experiences. The absence of universally accepted and enforced standards for nattokinase manufacturing, purity, and potency has resulted in substantial variability in the composition and biological activity of commercially available supplements (Li et al., 2023; Yi et al., 2025).

The primary metric used to quantify the potency of nattokinase products is enzymatic activity, which is typically expressed in fibrinolytic units (FU) or international units (IU) (Demeter Herb, 2026; Nutriavenue, 2025). The fibrin plate method is widely regarded as the gold standard for determining nattokinase activity, as it provides a direct and visual measure of the enzyme's ability to degrade fibrin. However, alternative assay methods, including HPLC-based assays such as the S-2444 test, are also employed by some manufacturers to ensure consistent potency and reliable thrombolytic performance across production batches (Nutriavenue, 2025). The lack of harmonization across different assay methodologies can complicate the comparison of product potencies and the interpretation of clinical study results.

In addition to enzymatic activity, other quality indicators that are relevant to the characterization and standardization of nattokinase products include molecular weight and amino acid sequence confirmation, which can be achieved through methods such as SDS-PAGE electrophoresis, HPLC chromatography, or mass spectrometry (Demeter Herb, 2026). The purity of the enzyme preparation, the presence of potential contaminants (such as residual bacterial cells, endotoxins, or fermentation byproducts), and the stability of the enzyme under various storage conditions are also important quality parameters that require careful monitoring and control (Yi et al., 2025).

Regulatory frameworks for nattokinase vary considerably across different global markets. In the United States, nattokinase is regulated as a dietary supplement under the Dietary Supplement Health and Education Act (DSHEA) of 1994, which places the responsibility for ensuring product safety and accurate labeling on the manufacturer but does not require pre-market approval or demonstration of efficacy (Demeter Herb, 2026). In other jurisdictions, such as the European Union, nattokinase may be subject to more stringent regulations, particularly if it is intended for use as a novel food ingredient or if specific health claims are made (Demeter Herb, 2026). The development and adoption of internationally recognized quality standards and reference materials would greatly facilitate the rigorous evaluation of nattokinase products and enhance the credibility of the clinical evidence base.

5.4 Safety Profile and Toxicological Assessment

The safety of nattokinase has been evaluated in a comprehensive series of toxicological studies and has been

monitored in numerous human clinical trials. The preponderance of available evidence indicates that nattokinase is of low toxicological concern and is well-tolerated when administered orally at the doses typically employed in clinical practice (Lampe and English, 2016).

The most thorough and authoritative toxicological assessment of nattokinase was conducted by Lampe and English (2016) and published in the journal Food and Chemical Toxicology. This investigation included a battery of GLP-compliant studies in rodents and human volunteers. *In vitro* assays demonstrated that nattokinase was non-mutagenic in bacterial cells (Ames test) and non-clastogenic in mammalian cells (chromosomal aberration test), indicating a lack of genotoxic potential (Lampe and English, 2016). *In vivo* studies conducted in Sprague-Dawley rats revealed no adverse effects in 28-day and 90-day subchronic toxicity studies at doses up to 167 mg/kg-day and 1,000 mg/kg-day, respectively (Lampe and English, 2016). The 90-day oral subchronic no-observed-adverse-effect level (NOAEL) for nattokinase in male and female Sprague-Dawley rats was determined to be 1,000 mg/kg-day, the highest dose tested (Lampe and English, 2016). Furthermore, mice inoculated with 7.55×10^8 CFU of the enzyme-producing bacterial strain (*Bacillus subtilis* var. *natto*) showed no signs of toxicity or residual tissue concentrations of viable bacteria (Lampe and English, 2016). Finally, the consumption of 10 mg/kg-day nattokinase for 4 weeks was well-tolerated in healthy human volunteers, with no indication of adverse effects (Lampe and English, 2016). Based on these comprehensive data, the authors concluded that the oral consumption of nattokinase is of low toxicological concern (Lampe and English, 2016).

The favorable safety profile of nattokinase is further corroborated by the findings of numerous human clinical trials. A systematic review and meta-analysis of randomized controlled trials by Li et al. (2023) reported that no notable adverse events were observed in any of the included studies due to the intake of nattokinase. Similarly, the randomized controlled trials by Liu et al. (2023) and Liu et al. (2024) reported no adverse effects attributable to the nattokinase interventions.

Despite this strong safety record, certain precautions and contraindications must be recognized. The European Food Safety Authority (EFSA) has noted that nattokinase at commonly used doses is not associated with adverse effects (Examine.com, 2026). However, due to its fibrinolytic and antithrombotic properties, nattokinase has the potential to increase the risk of bleeding, particularly when administered in conjunction with anticoagulant and antiplatelet agents such as warfarin, aspirin, clopidogrel, and direct oral anticoagulants (Drugs.com, 2026; MSKCC, 2022). There is a theoretical risk that nattokinase could cause an existing clot to dislodge, resulting in a stroke or embolus at a distant location (MSKCC, 2022). Consequently, patients with a history of deep vein thrombosis, ischemic stroke, peptic ulcer disease, or coagulation disorders, as well as those in the perioperative period, are advised to avoid the use of nattokinase (Drugs.com, 2026; MSKCC, 2022).

An additional consideration relates to the potential presence of vitamin K2 (menaquinone-7) in natto-derived products. Natto is naturally rich in vitamin K2, which is also produced by *Bacillus subtilis* var. *natto* during fermentation. High concentrations of vitamin K2 can antagonize the anticoagulant effects of warfarin, leading to a reduction in the international normalized ratio (INR) and an increased risk of thrombotic events (Drugs.com, 2026). This interaction may also occur with nattokinase supplements if vitamin K2 is not adequately removed during the manufacturing process (Drugs.com, 2026). Therefore, patients receiving warfarin therapy should exercise caution and consult with their healthcare provider before initiating nattokinase supplementation.

Rare cases of late-onset anaphylaxis associated with natto consumption have been reported and have been attributed to poly-gamma-glutamic acid (γ -PGA), a product of the fermentation process that may be present in nattokinase supplements (Drugs.com, 2026). While these reactions are exceedingly uncommon, they

underscore the importance of vigilance for hypersensitivity reactions in susceptible individuals.

In summary, nattokinase exhibits a favorable safety profile when used appropriately, with a low incidence of adverse effects reported in both preclinical toxicological studies and human clinical trials. The primary safety concern relates to its potential to increase bleeding risk, particularly in the context of concomitant anticoagulant or antiplatelet therapy. Prudent clinical practice dictates that nattokinase supplementation should be undertaken under the guidance of a qualified healthcare professional, with careful consideration of individual patient risk factors and potential drug interactions.

6 Unresolved Issues, Limitations, and Challenges

Despite the substantial and growing body of evidence supporting the therapeutic potential of nattokinase, several critical issues remain unresolved, and significant challenges must be addressed to fully realize the clinical promise of this natural enzyme.

6.1 Heterogeneity of Clinical Trial Design and Outcomes

A major limitation of the current clinical evidence base is the considerable heterogeneity in trial design, including variations in patient populations, inclusion and exclusion criteria, nattokinase dosage and formulation, duration of intervention, and choice of outcome measures (Li et al., 2023; Muric et al., 2024). This heterogeneity complicates the synthesis and interpretation of findings across studies and limits the ability to draw definitive conclusions regarding the optimal use of nattokinase for specific clinical indications. The meta-analysis by Li et al. (2023) represents an important step toward synthesizing the available evidence, but the authors themselves acknowledged that more work is needed to determine whether the positive efficacy of nattokinase on cardiovascular risk factors is dose-dependent. Future clinical trials should strive for greater standardization in design and reporting to facilitate robust meta-analyses and to inform evidence-based clinical practice guidelines.

6.2 Lack of Standardized Quality Control and Product Consistency

As discussed in Section 5.3, the absence of universally accepted and enforced quality control standards for nattokinase products poses a significant challenge to both clinical research and consumer safety. The potency, purity, and composition of commercially available nattokinase supplements can vary widely, making it difficult to compare results across studies and to ensure that consumers receive a consistent and reliable product (Li et al., 2023; Yi et al., 2025). The development and adoption of internationally recognized reference standards and validated analytical methods for assessing nattokinase activity, purity, and identity are urgently needed to address this critical gap.

6.3 Incomplete Understanding of Pharmacokinetics and Metabolism

While important progress has been made in elucidating the pharmacokinetic behavior of nattokinase (Ero et al., 2013; Feng et al., 2023; Yi et al., 2025), significant gaps in knowledge persist. The precise nature of the biologically active species that reach the systemic circulation following oral ingestion remains to be fully characterized. The absorbed fractions appear to consist primarily of small hydrophobic peptides (Zhou et al., 2025), but the identity, pharmacokinetic properties, and biological activities of these peptides have not been comprehensively defined. Furthermore, the distribution, metabolism, and elimination of nattokinase and its metabolites have not been systematically investigated in humans. A more complete understanding of nattokinase's ADME profile is essential for optimizing dosing regimens, predicting potential drug interactions, and ensuring the safe and effective use of this enzyme.

6.4 Need for Large-Scale, Long-Term Outcome Trials

The vast majority of clinical trials evaluating nattokinase have been of relatively short duration (typically 8-24 weeks) and have focused on surrogate markers of cardiovascular risk, such as blood pressure, lipid profiles,

and fibrinolytic parameters, rather than on hard clinical outcomes, such as the incidence of myocardial infarction, stroke, or cardiovascular mortality (Li et al., 2023; Feng et al., 2023; Liu et al., 2024). While these surrogate markers are valuable and provide important mechanistic insights, they do not constitute definitive proof of clinical benefit. Large-scale, long-term randomized controlled trials with hard clinical endpoints are required to definitively establish the efficacy of nattokinase for the primary and secondary prevention of cardiovascular events and to justify its broader adoption in clinical practice.

6.5 Underdeveloped Research on Non-Cardiovascular Applications

The investigation of nattokinase's potential applications beyond the cardiovascular system, including its neuroprotective, antiviral, and anti-inflammatory effects, is still in its nascent stages. The promising findings from preclinical studies in models of Alzheimer's disease (Tanikawa et al., 2024; Mahakalakar et al., 2025) and the *in vitro* evidence of activity against SARS-CoV-2 (Tanikawa et al., 2022) provide a strong rationale for further investigation, but these findings have not yet been translated into human clinical trials. Rigorous clinical research in these emerging areas is needed to validate the preclinical observations and to determine whether nattokinase can offer tangible benefits for patients with neurodegenerative disorders, viral infections, and other non-communicable diseases.

6.6 Potential for Interactions and Contraindications

While nattokinase has a generally favorable safety profile, the potential for clinically significant drug interactions, particularly with anticoagulant and antiplatelet agents, requires further investigation and careful clinical monitoring (Drugs.com, 2026; MSKCC, 2022). Well-designed pharmacokinetic and pharmacodynamic interaction studies are needed to quantify the magnitude of the bleeding risk associated with the concomitant use of nattokinase and antithrombotic medications and to develop evidence-based guidance for the safe co-administration of these agents.

7 Future Research Directions and Perspectives

The field of nattokinase research is dynamic and rapidly evolving, with numerous exciting opportunities for future investigation. Based on the current state of knowledge and the identified limitations and challenges, several key research directions can be prioritized.

7.1 Conduct of Large-Scale, Pivotal Clinical Outcome Trials

The highest priority for future nattokinase research is the design and execution of large-scale, multicenter, randomized, double-blind, placebo-controlled trials evaluating the effects of nattokinase on hard clinical outcomes, including the incidence of major adverse cardiovascular events (MACE), in high-risk patient populations. Such trials would provide the definitive evidence needed to establish nattokinase as a first-line adjunctive therapy for the prevention of cardiovascular disease.

7.2 Comprehensive Pharmacokinetic and Pharmacodynamic Characterization

Detailed pharmacokinetic studies in humans are needed to fully characterize the absorption, distribution, metabolism, and elimination of nattokinase and its active metabolites. The application of advanced analytical techniques, such as mass spectrometry-based proteomics and peptidomics, should be employed to identify and quantify the specific peptide species that reach the systemic circulation and to correlate their plasma concentrations with pharmacodynamic effects.

7.3 Elucidation of Molecular Mechanisms in Diverse Disease Contexts

Continued investigation into the molecular mechanisms by which nattokinase exerts its pleiotropic effects is essential. Particular emphasis should be placed on delineating the role of the Nrf2/HO-1 signaling pathway in mediating the anti-inflammatory and antioxidant effects of nattokinase across different tissues and disease states, as well as on identifying additional molecular targets and signaling pathways that may be modulated by

the enzyme.

7.4 Clinical Translation of Neuroprotective and Antiviral Findings.

The promising preclinical data supporting the neuroprotective and antiviral activities of nattokinase should be translated into well-designed human clinical trials. Pilot studies in patients with mild cognitive impairment or early Alzheimer's disease could evaluate the effects of nattokinase on biomarkers of amyloid pathology, neuroinflammation, and cognitive function. Similarly, clinical trials in patients with mild to moderate COVID-19 could assess the impact of nattokinase on viral load, disease severity, and the incidence of thrombotic complications.

7.5 Development of Standardized Quality Control Protocols and Reference Materials

Concerted efforts should be directed toward the development and validation of standardized, internationally recognized methods for the assessment of nattokinase activity, purity, and identity. The establishment of certified reference materials and the harmonization of regulatory standards across global markets would greatly enhance the reliability and comparability of nattokinase products and research findings.

7.6 Investigation of Synergistic Formulations and Personalized Approaches

The evidence suggests that nattokinase may be most effective when used in combination with other bioactive nutraceuticals, such as red yeast rice and monascus. Future research should systematically evaluate the optimal combinations and ratios of nattokinase with other cardioprotective agents and should explore the potential for personalized, biomarker-guided approaches to nattokinase therapy.

7.7 Long-Term Safety Surveillance and Pharmacovigilance

Although the available toxicological and clinical data support the safety of nattokinase, ongoing post-marketing surveillance and pharmacovigilance are warranted to detect any rare or long-term adverse effects that may emerge with widespread and prolonged use.

8 Conclusion

Nattokinase, a fibrinolytic serine protease derived from the traditional Japanese fermented food natto, has emerged as a remarkably versatile and promising natural enzyme with a broad spectrum of biological activities and therapeutic applications. Since its serendipitous discovery by Dr. Hiroyuki Sumi in 1980, a substantial and continuously expanding body of scientific research has established nattokinase as a potent fibrinolytic and antithrombotic agent, an effective adjunctive therapy for hypertension, and a pleiotropic modulator of inflammation, oxidative stress, lipid metabolism, and neuroprotection. The enzyme's favorable safety profile, oral bioavailability, and multiple convergent mechanisms of action position it as a valuable candidate for the prevention and management of cardiovascular disease and a range of other non-communicable diseases.

The evidence supporting the cardiovascular benefits of nattokinase is robust and is derived from a combination of compelling epidemiological observations, mechanistic *in vitro* and *in vivo* studies, and a growing portfolio of randomized controlled clinical trials. Systematic review and meta-analysis have confirmed the antihypertensive efficacy of nattokinase, while recent clinical trials have demonstrated that its combination with lipid-modulating nutraceuticals such as red yeast rice and monascus produces clinically meaningful improvements in lipid profiles and other cardiometabolic parameters. Beyond its cardiovascular applications, exciting new research has unveiled the enzyme's potential to protect against neurodegeneration, degrade the SARS-CoV-2 spike protein, and mitigate inflammation and oxidative stress through activation of the Nrf2/HO-1 signaling axis.

Despite this remarkable progress, significant challenges remain. The heterogeneity of clinical trial designs, the lack of standardized quality control and product consistency, incomplete understanding of nattokinase's pharmacokinetic properties, and the absence of large-scale, long-term clinical outcome trials constitute critical

barriers to the full realization of nattokinase's therapeutic potential. Addressing these limitations through rigorous, well-designed future research is an urgent priority.

In conclusion, nattokinase represents a compelling example of the untapped therapeutic potential harbored within traditional fermented foods. With continued scientific investigation and the translation of promising preclinical findings into rigorous clinical trials, nattokinase is poised to make a significant and lasting contribution to the prevention and management of cardiovascular disease and other chronic conditions that afflict humanity. The journey that began with a simple observation in a Chicago laboratory in 1980 has evolved into a rich and multifaceted scientific enterprise that holds the promise of improving human health on a global scale.

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Nattokinase-driven remodeling of tumor microenvironment enhances the efficacy of MSLN-targeted CAR-T cell therapy in solid tumors

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Abstract

Chimeric antigen receptor T cell (CAR-T) therapy has revolutionized the treatment of hematological malignancies but faces significant challenges in solid tumor therapy. The tumor microenvironment (TME) of solid tumors, including the dense extracellular matrix (ECM) and immunosuppressive factors, creates physical and biochemical barriers that hinder CAR-T cell infiltration, function, and persistence. Despite advances in CAR-T cell engineering, overcoming these TME barriers remains a major obstacle. The ECM in solid tumors restricts CAR-T cell penetration and limits their antitumor efficacy. Recent studies have explored ECM modulation as a strategy to enhance CAR-T therapy. Nattokinase (NKase), a natural fibrinolytic enzyme, has shown potential in degrading ECM components, promoting TME remodeling, and improving therapeutic responses. In this study, we evaluate the therapeutic efficacy of NKase in combination with MSLN-targeted CAR-T cells for solid tumors. We hypothesize that NKase will reduce tumor fibrosis by degrading ECM components, facilitating CAR-T cell infiltration and enhancing their cytotoxic activity. In vitro and in vivo experiments will assess the effects of NKase on CAR-T cell infiltration, activation, memory phenotype maintenance, and antitumor activity. This study aims to optimize CAR-T therapy for solid tumors and supports the clinical potential of NKase as an adjunctive therapeutic agent.

Keywords Chimeric antigen receptor T cell (CAR-T) therapy · Solid tumors · Tumor microenvironment (TME) · Extracellular matrix (ECM) · Nattokinase (NKase) · Mesothelin (MSLN) · Tumor fibrosis · Immune infiltration · Antitumor activity · Immunotherapy

Introduction

Chimeric antigen receptor T cell (CAR-T) therapy has revolutionized the treatment of hematological malignancies [1–3], demonstrating remarkable success in the clinical setting [4, 5]. However, its application in the treatment of solid tumors remains a significant challenge [6–8]. The efficacy of CAR-T cells in solid tumors is limited by various factors [8, 9], including the immunosuppressive tumor microenvironment (TME) [10, 11], inadequate infiltration of CAR-T cells into tumor tissue, and the mechanisms of immune escape

employed by tumors [12]. Solid tumors are characterized by a dense extracellular matrix (ECM) [13–15] and a hostile immunosuppressive microenvironment [16–18], both of which create physical and biochemical barriers that hinder CAR-T cell infiltration [19, 20], function, and persistence, thereby restricting therapeutic effectiveness [20, 21].

Although substantial progress has been made in improving CAR-T cell functionality, particularly in enhancing their antitumor activity [22], overcoming the barriers imposed by the TME remains a major challenge [23, 24]. The ECM in solid tumors plays a crucial role as a physical barrier [25], impeding CAR-T cell penetration into the tumor core [26, 27]. Recent studies have begun to focus on modulating the tumor ECM as a potential strategy to improve the efficacy of immunotherapies, including CAR-T therapy [28, 29].

The ECM of solid tumors is a complex network composed of various components [30, 31], mainly including structural proteins (such as collagen and elastin) [32], adhesion glycoproteins (such as fibronectin and laminin) [33], proteoglycans and glycosaminoglycans (such as hyaluronic

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acid and versican) [34], ECM-modifying enzymes [35], and growth factors bound to the matrix [36, 37]. These components not only provide structural support for tumor cells but also regulate cell adhesion, migration, proliferation and signal transduction, promoting tumor growth, invasion, and metastasis [38, 39]. At the same time, the dense and stiff nature of tumor ECM also hinders drug penetration and the entry of immune cells, thereby leading to drug resistance and immune escape [40, 41]. Nattokinase (NKase), a natural fibrinolytic enzyme derived from *Bacillus subtilis* during the fermentation of soybeans (a process used to make natto), has garnered attention due to its ability to degrade ECM components, promote remodeling of the TME, and enhance the overall therapeutic response [42].

In prior studies, our research team successfully developed CAR-T cells targeting mesothelin (MSLN) [43], which demonstrated promising therapeutic effects in MSLN-positive tumor models, such as ovarian cancer. Building on this success, we hypothesize that combining NKase with MSLN CAR-T cells can improve tumor cell infiltration and enhance CAR-T cell-mediated antitumor effects. Specifically, we propose that NKase, through its ability to degrade fibrin and other ECM components within the tumor microenvironment, may reduce tumor fibrosis and facilitate CAR-T cell penetration into the tumor, thereby amplifying their cytotoxic activity.

The aim of this study is to investigate the impact of NKase on the therapeutic efficacy of MSLN-targeted CAR-T cells in solid tumors. Specifically, we aim to assess how NKase influences CAR-T cell infiltration, activation, exhaustion, memory phenotype maintenance, and antitumor activity. Through both *in vitro* and *in vivo* experiments, this study will explore how NKase-induced remodeling of the tumor microenvironment enhances CAR-T cell function, offering new strategies to optimize CAR-T cell therapy for solid tumors.

Materials and methods

Cells and culture conditions

HEK293 (human embryonic kidney), Ovarcar3 (ovarian cancer), and HCC1806 (breast squamous carcinoma) lines were obtained from ATCC and cultured in DMEM, RPMI-1640 with 10% fetal bovine serum (FBS). PBMCs from healthy donors (TPCS#PB025C, Miles-bio, Shanghai) were isolated using Lymphoprep (Stemcell, Canada), and T cells were purified by negative selection with the EasySep™ human T Cell Isolation Kit (Stemcell, Canada). On day 0, T cells were activated with antihuman CD3 and CD28 beads (Miltenyi, Germany) and cultured in X-VIVO medium (Ronsa, Switzerland) supplemented with recombinant IL-2 (300 IU/mL) and

10% fetal bovine serum (FBS). CAR-T cells were infected with lentivirus, and the cell phenotype and positive rate were assessed after 48 h.

Lentivirus production

Lentivirus for CAR-T cells was produced by co-transfecting HEK293T cells (ATCC) with lentiviral and packaging plasmids (pMDLg/pRRE, pRSV-Rev, pMD2.G). Supernatants were collected 48 and 72 h post-transfection, concentrated by ultracentrifugation, and stored at -80°C .

Flow cytometry

Flow cytometry was performed on a Beckman Coulter machine, and data were analyzed using software CytExpert. The cells were stained at room temperature for 25 min, washed twice with PBS, and then tested on the machine. The detailed information of the antibodies is listed in Table S1.

CCK-8 cell viability assay

HCC1806, Ovarcar3 tumor cell lines and MSLN CAR-T cells were, respectively, seeded at 2×10^4 cells per well in 96-well plates. NKase was purchased from Shaanxi Zerang Biology (Catalog No. 1147; 20,000 FU/g). NKase solutions of gradient concentrations of 0, 62.5, 125, 250, 500, 1000, and 2000 FU/100 μl were prepared using the corresponding culture media for HCC1806, Ovarcar3 tumor cell lines, and MSLN CAR-T cells to treat the cells for 24 h. After treatment, 20 μl of CCK-8 reagent (CCK-8 kit purchased from Beyotime, Catalog No. C0038) was added to each well and incubated in the incubator for 2–3 h before detection on a microplate reader.

CAR-T cytotoxicity assay

Short-term Killing: CAR-T cells were co-cultured with luciferase-expressing Ovarcar3, HCC1806 cells at E/T ratios of 1:1, 1:2, and 1:4 for 8 h. After incubation, luminescence was measured, and cytotoxicity was calculated as $[(\text{background luminescence} - \text{sample luminescence}) / \text{background luminescence}] \times 100\%$.

Long-term killing: Tumor cells and CAR-T cells were co-incubated for 24 h at the specified effector-to-target ratio (1:1, 1:2, 1:4, 1:8), and the lysis of target cells was measured. Then, new target cells were added to the effector cells at the same effector-to-target ratio, and four rounds of co-incubation and target cell lysis detection were repeated.

Real-time killing: Tumor cells were spread on a 96-well electrode plate coated with L-cysteine. After the tumor cells were fully covered, effector cells were added successively at

the specified effector target ratios (1:1, 1:2, 1:4). Continuously collect data.

Western blot analysis

HCC1806 cells before and after 125FU NKase treatment were homogenized in cold RIPA lysis buffer containing PMSF, and the supernatant was collected after centrifugation. Protein quantification was performed using a bicinchoninic acid (BCA) protein assay kit. Thirty micrograms of total protein was subjected to SDS-PAGE gel separation and transferred onto a 0.22- μ m PVDF membrane. The membrane was blocked with blocking solution (5% skim milk in TBST: 10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween 20) at room temperature for 1 h, then incubated overnight at 4 °C with primary antibodies, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 1–2 h (anti-fibronectin antibody was purchased from Abcam; anti-alpha smooth muscle actin antibody was purchased from Abcam.). Finally, the membrane was developed using an ECL Western blotting detection kit. The detailed information of the antibodies is listed in Table S1

Immunofluorescence staining

HCC1806 cell lines and mouse tumor tissues before and after 125FU NKase treatment were fixed in 4% paraformaldehyde at 4 °C overnight, embedded in paraffin, and sliced. After deparaffinization and hydration, tissues were pretreated with sodium citrate solution for antigen retrieval. Next, the tissue sections were blocked with 5% goat serum for 30 min at room temperature and incubated with the primary antibody at 4 °C overnight, followed by incubation with the secondary antibody for 1 h at 37 °C in the dark (anti-fibronectin antibody was purchased from Abcam, catalog number ab2413). After counterstaining with DAPI to reveal the cell nucleus, tissue sections were photographed using a Nikon 80I 10–1500 $\times$ microscope. Quantification of colocalization was analyzed by the Image J colocalization finder plugin.

3D microsphere infiltration experiment

3D tumor microsphere models were constructed using Corning® 96-well transparent round-bottomed spherical microplates for HCC1806 cells with green fluorescent protein (GFP). After each well of 2E4 tumor cells formed into spheres and were cultured for 48 h, after 48 h, add the prepared 125FU NKase solution, after 24 h, add MSLN CAR-T cells at a 1:1 effector-to-target ratio to the corresponding wells. After 24 h, immunofluorescence photography showed the killing of 3D microspheres.

Mouse tumor model

Female B-NKG mice (6 weeks, Biocytogen, Beijing) were subcutaneously implanted with 2×10^6 HCC1806 cells. On day 10, the mice were randomly assigned to three experimental groups (n = 6 per group). The experimental group received intratumoral injections of 125 FU NKase into tumor tissues. Twenty four hours later, either PBS or 1×10^6 MSLN CAR-T cells were administered via tail vein injection. The size of the tumor and the body weight of the mice was measured every two days, tumor volumes calculated as $\text{volume} = (\text{length} \times \text{width}^2) \times 0.5$.

Cytokine measurement

Cell supernatant: After co-incubating HCC1806 cells with MockT, MSLN CAR-T, and NKase-added MSLN CAR-T cells at a 1:2 effector-to-target ratio for 24 h, collect the cell culture supernatant and centrifuge (1000 $\times$ g, 10 min) to remove cell debris. **Plasma supernatant:** Collect 200 μ l of peripheral blood from each group of mice, centrifuge at 3000g for 10 min, and aspirate the supernatant for testing. The BD™ Cytometric Bead Array (CBA) Mouse/Rat Soluble Protein Master Buffer Kit (Cat: 558266) is used to detect the release of cytokines. Mix the fluorescently encoded microspheres coated with specific antibodies with the test supernatant, add PE-labeled detection antibodies, incubate at room temperature in the dark for 3 h to form a "capture microsphere—cytokine—PE antibody" sandwich complex. After the antibody incubation is completed, add washing buffer for centrifugation to remove unbound antibodies, resuspend the precipitate with flow cytometry PBS. Use a flow cytometer (such as BD FACSCalibur) to analyze the fluorescence signals of the microspheres to quantitatively determine the concentrations of IL-10, TNF- α , and IFN- γ cytokines. Process and output the results through software FCAP.GUI.

Histopathological examination

On the tenth day after CAR-T cell reinfusion, one mouse in each group was randomly sacrificed to obtain tumor tissue samples. All mice were sacrificed on the 28th day after CAR-T reinfusion, and the heart, liver, spleen, lung, kidney, and brain tissues were collected. The tissue specimens were fixed with 4% buffer solution formaldehyde. Paraffin sections were used for hematoxylin and eosin (H&E) staining and immunohistochemical analysis. Both the primary antibody and the secondary antibody were rabbit antihuman antibody CD3 antibody and enzyme-labeled goat anti-rabbit IgG H&L separate (Abcam). Then, the cell nucleus was

stained with DAPI (Abcam). Tumor staining of mice treated with PBS was used as the control. The infiltration quantity of IHC CD3⁺T cells was analyzed using ImageJ.

Statistical analysis

All experiments were performed at least three times. Data were analyzed using GraphPad Prism 10.0 software and presented as $SD \pm$. Student's *t* test was used for two-group comparisons, and one-way or two-way ANOVA was used for multiple-group analysis. Statistical significance was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns, not significant.

Results

Effects of NKase concentration on tumor cells and MSLN CAR-T

To investigate whether NKase could enhance the antitumor efficacy of MSLN-targeted CAR-T cells by modulating the tumor microenvironment, we first evaluated its potential cytotoxic effects on tumor and immune cells, as well as its impact on CAR-T functionality.

We began by confirming the successful construction and surface expression of the chimeric antigen receptor in MSLN CAR-T cells. Flow cytometric analysis demonstrated robust expression of the CAR construct in transduced T cells, confirming the generation of functional MSLN CAR-T cells (Fig. 1A–B). Next, HCC1806 and Ovarc3 tumor cells, as well as MSLN CAR-T cells, were treated with a gradient of NKase concentrations ranging from 0 to 2000 FU, and cell viability was assessed. The data indicated that NKase exerted no significant effect on the viability of either HCC1806 or Ovarc3 tumor cells within the concentration range of 0 to 500 FU, nor did it influence the viability of MSLN CAR-T cells within the range of 0 to 2000 FU (Fig. 1C–E). To identify the minimum effective concentration of NKase for tumor treatment, we further examined the cytotoxic activity of MSLN CAR-T cells against HCC1806 cells after treatment with different concentrations of NKase, employing luciferase reporter assays at various effector-to-target (E/T) ratios. Notably, after 8 h of treatment with NKase concentrations ranging from 62.5 to 2000 FU, a significant increase in the lysis rate of HCC1806 cells was observed across all tested E/T ratios (Fig. 1F–H). Similarly, a significant enhancement in the lysis rate of Ovarc3 cells by MSLN CAR-T cells was noted with NKase concentrations ranging from 125 to 2000 FU at different E/T ratios (Fig. 1I–K). These findings suggest that NKase significantly enhances the antitumor activity of MSLN CAR-T cells, thereby improving their cytotoxic potential against target tumor cells. Based on these results, a concentration of 125

FU was selected as the optimal concentration for subsequent experiments.

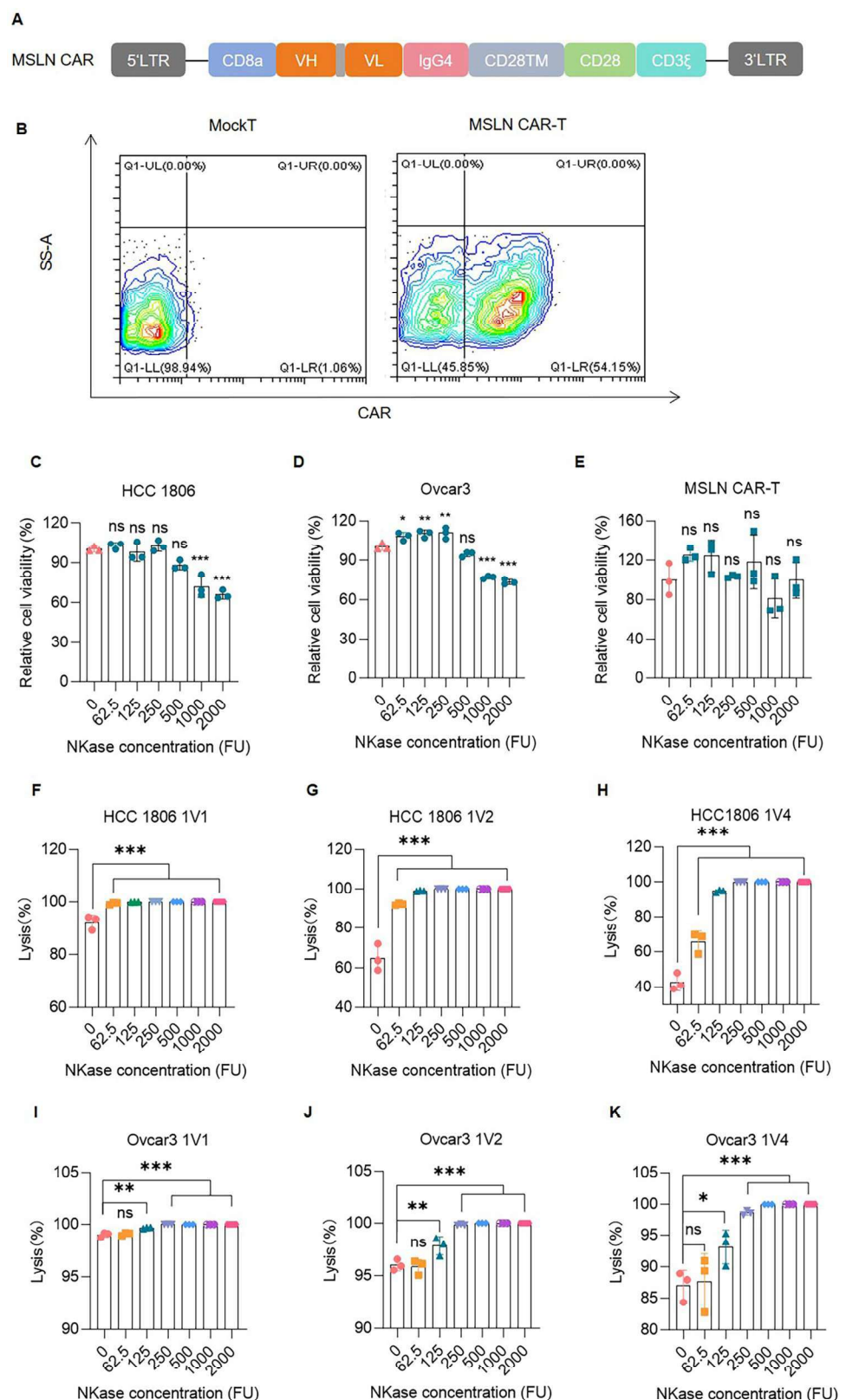
NKase potentiates MSLN CAR-T cell activation and antitumor response

Since NKase was always present in the environment of MSLN CAR-T during the *in vitro* killing experiments, therefore, to evaluate the influence of NKase on the functional state of MSLN CAR-T cells, we assessed the expression of activation, exhaustion, and apoptosis-related markers using flow cytometry. CBA was used to measure the release of cytokines. MSLN CAR-T cells were stimulated either with untreated HCC1806 tumor cells or HCC1806 cells pretreated with NKase, and changes in marker expression were analyzed before and after stimulation. Flow cytometry analysis showed that compared with MSLN CAR-T cells stimulated with HCC1806, the expression of activation markers CD25 and CD69 in MSLN CAR-T cells stimulated with HCC1806 cells treated with NKase was significantly increased (Fig. 2A, B), indicating that the presence of the NKase environment promoted T cell activation. In addition, the expression of the exhaustion markers PD-1, LAG-3, and TIM-3 was markedly decreased in CAR-T cells in the NKase-treated condition (Fig. 2C–E), suggesting that NKase attenuates T cell exhaustion within the tumor microenvironment. Furthermore, the apoptotic biomarkers Annexin V and 7-AAD were significantly downregulated in MSLN CAR-T cells following stimulation with NKase-treated tumor cells, relative to those stimulated with untreated tumors (Fig. 2F, G). CBA results showed that the cytokine release of MSLN CAR-T cells co-incubated with NKase-treated HCC1806 cells was higher, indicating an enhanced killing effect (Fig. 2H–J). These results indicate a decrease in apoptotic signaling in the presence of NKase. Taken together, these findings suggest that NKase regulates the functional phenotype of MSLN CAR-T cells by promoting activation, reducing exhaustion, and decreasing apoptosis. This modulation of CAR-T cell behavior by NKase may contribute to enhanced persistence and improved antitumor efficacy in the tumor microenvironment.

NKase promotes long-term cytotoxicity and memory phenotype in MSLN CAR-T cells

To further evaluate the cytotoxic effect of MSLN CAR-T cells on tumor cells treated with NKase, we conducted real-time cell killing experiments using HCC1806 and Ovarc3 tumor cell lines. The tumor cells were first pretreated with 125 FU NKase, and then the lysis of these cells by MSLN CAR-T cells was evaluated. The lysis rate varied with the ratio of effector cells to target cells (E/T) (1:1, 1:2, 1:4) (Fig. 3A–F). In all tested E/T ratios, the lysis rate of tumor

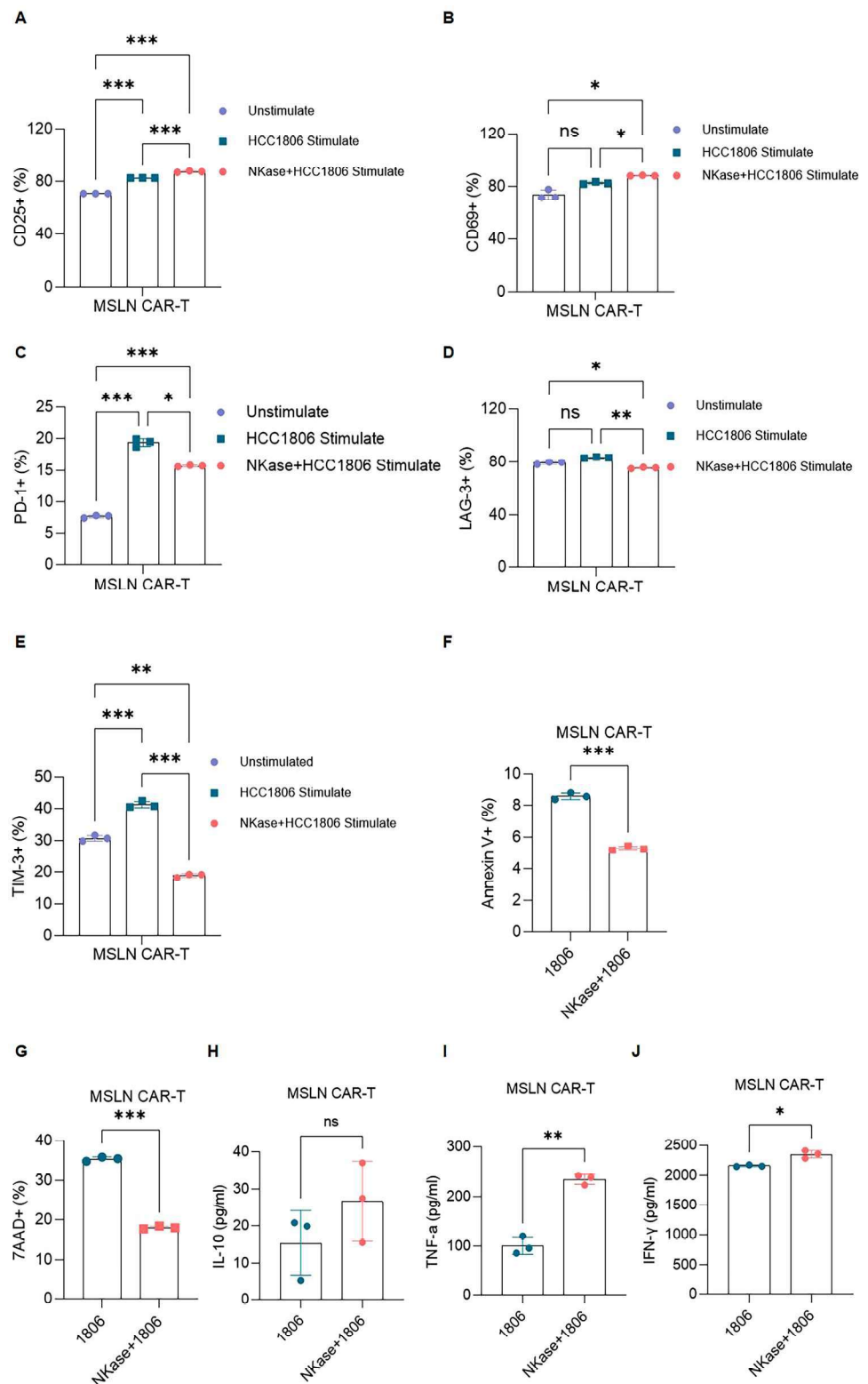
Fig. 1 Effects of NKase Concentration on Tumor Cells. **A** Schematic representation of the CAR structure in MSLN CAR-T cells. The CAR construct includes a signal peptide, anti-MSLN scFv, IgG4 hinge region, CD28 transmembrane domain, CD28 co-stimulatory domain, and CD3 ζ intracellular signaling domain. **B** Flow cytometric analysis of CAR-positive expression in MSLN CAR-T cells. **C–E** Cell viability of HCC1806, Ovarc3, and MSLN CAR-T cells following treatment with NKase at concentrations ranging from 0 to 2000 FU was assessed using the CCK-8 assay. **F–K** Luciferase reporter assays demonstrated the cytotoxic activity of MSLN CAR-T cells against HCC1806 **F–H** and Ovarc3 **I–K** tumor cells at effector-to-target ratios of 1:1, 1:2, and 1:4 after 8 h of exposure to NKase at concentrations ranging from 0 to 2000 FU. Data represent the mean $\pm$ SEM from three independent in vitro experiments; ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; one-way ANOVA



cells in the NKase pretreatment group was significantly higher than that of the untreated control group (NC group), indicating that NKase pretreatment of tumors significantly

enhanced the tumor-killing ability of MSLN CAR-T cells. To investigate whether NKase would affect the differentiation state of CAR-T cells, we analyzed the expression of

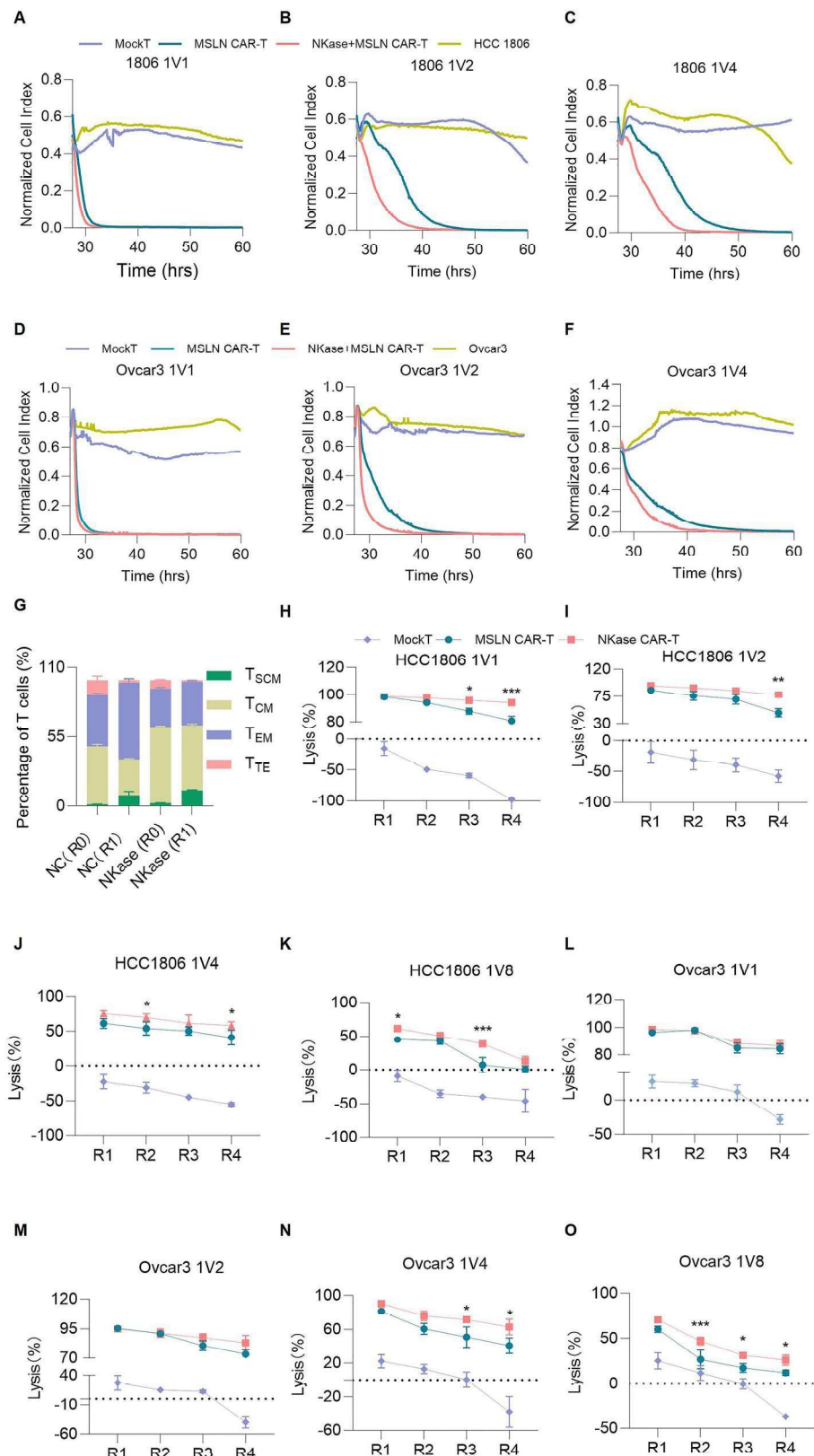
Fig. 2 NKase Potentiates MSLN CAR-T Cell Activation and Antitumor Response. **A–E** The expression levels of activation markers (CD25, CD69) and exhaustion markers (TIM-3, LAG-3, and PD-1) in MSLN CAR-T cells were analyzed before and after stimulation with HCC1806 tumor cells and NKase-treated HCC1806 tumor cells. **F–G** Flow cytometric analysis was conducted to assess the expression of early apoptosis marker (Annexin V) and late apoptosis marker (7-AAD) in MSLN CAR-T cells. **H–J** Following the co-incubation of HCC1806 tumor cells and NKase-treated HCC1806 tumor cells with MSLN CAR-T at an effector-to-target ratio of 1:2 for 24 h, the concentrations of IL-10, TNF- α , and IFN- γ in the cell culture supernatants were quantified. Data are presented as mean $\pm$ SEM from three independent in vitro experiments; ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; two-way ANOVA



CD45RO and CD62L by flow cytometry to distinguish the initial/memory T cell subsets, including stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and terminal effector T cells (T_{TE}) (Fig. 3G).

Notably, NKase led to an increase in the proportion of TSCM and TCM subgroups, while the population of effector memory T cells (T_{EM}) and terminal effector T cells (T_{TE}) decreased. These results indicate that NKase promotes the

Fig. 3 NKase Promotes Long-Term Cytotoxicity and Memory Phenotype in MSLN CAR-T Cells. **A–F** Real-time cytotoxicity assays revealed the lysis of HCC1806 and Ovar3 target cells by MSLN CAR-T cells in both the negative control (NC) group and the 125 FU NKase treatment group at effector-to-target ratios of 1:1, 1:2, and 1:4. **G** Flow cytometric analysis was performed to assess the expression levels of CD45RO and CD62L on MSLN CAR-T cells in the NC and 125 FU NKase treatment groups. The proportions of stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and terminally differentiated effector T cells (T_{TE}) were determined based on distinct cell populations. **H–O** In vitro multi-round cytotoxicity assays were carried out to evaluate the persistence of MSLN CAR-T cell cytotoxicity in the NC and 125 FU NKase treatment groups. Effector and target cells were co-incubated at defined effector-to-target ratios (1:1, 1:2, 1:4, 1:8) for four consecutive rounds (R1–R4), each lasting 24 h, with target cell lysis assessed in each round. The experiments were repeated three times, and p values were calculated using two-way ANOVA. ns, not significant; *, $p < 0.05$; ***, $p < 0.001$



memory-like phenotype of MSLN CAR-T cells, which may help prolong cell lifespan and maintain the persistence of antitumor function. To assess the persistence of the cytotoxicity of MSLN CAR-T cells, we conducted repeated in vitro tumor-killing experiments with different E/T ratios (1:1, 1:4, 1:8) over multiple rounds (Fig. 3H–O). Compared to the control group, MSLN CAR-T cells co-cultured with NKase-treated tumor cells exhibited superior killing ability in all stimulation rounds. These findings suggest that NKase pretreatment of tumors enhances the persistence and long-term cytotoxic function of MSLN CAR-T cells, thereby improving their potential efficacy in the tumor microenvironment.

NKase enhances the infiltration of MSLN CAR-T cells and the antitumor effect in vivo

To evaluate the impact of nattokinase (NKase) on the infiltration and antitumor activity of mesothelin-targeted CAR-T (MSLN CAR-T) cells in vivo, A total of 2×10^6 HCC1806 cells were subcutaneously injected into six-week-old female NSG mice. The animals were randomly assigned to three groups: the PBS control group, the MSLN CAR-T group, and the NKase + MSLN CAR-T group. Ten days after tumor cell implantation, the experimental group received intratumoral injection of 125 FU NKase for tumor tissue pretreatment. Twenty four hours later, each group was intravenously administered with either PBS or 1×10^6 MSLN CAR-T cells per mouse ($n = 6$). Thereafter, tumor size and body weight were monitored every two days, and peripheral blood samples were collected every four days for flow cytometric analysis (Fig. 4A). Like the in vitro experiments, CD69 expression was significantly upregulated in the NKase-treated group compared to controls, indicating enhanced T cell activation (Fig. 4B), while PD-1 expression was markedly reduced, suggesting alleviation of T cell exhaustion (Fig. 4C). Analysis of memory T cell subsets demonstrated a shift toward a memory phenotype, with increased proportions of T_{SCM} and T_{CM} and decreased frequencies of T_{EM} and T_{TE} in the NKase group (Fig. 4D), indicative of improved persistence potential. No significant difference in body weight was observed between groups, confirming good tolerability (Fig. 4E), whereas tumor volume and tumor size were significantly lower in the NKase-treated group (Fig. 4F–G). Immunohistochemical staining further showed increased infiltration of CD3 + T cells into tumor tissues following NKase treatment (Fig. 4H, I). The CBA results also showed that the MSLN CAR-T + NKase group had a higher level of IFN- γ release (Fig. 4J). Importantly, hematoxylin and eosin (H&E) staining of major organs revealed no pathological abnormalities or tissue damage in either group (Fig. 4K), indicating that NKase does not induce off-target toxicity. Collectively, these results demonstrate that NKase enhances the in vivo activation, infiltration, and antitumor function of

MSLN CAR-T cells while maintaining a favorable safety profile.

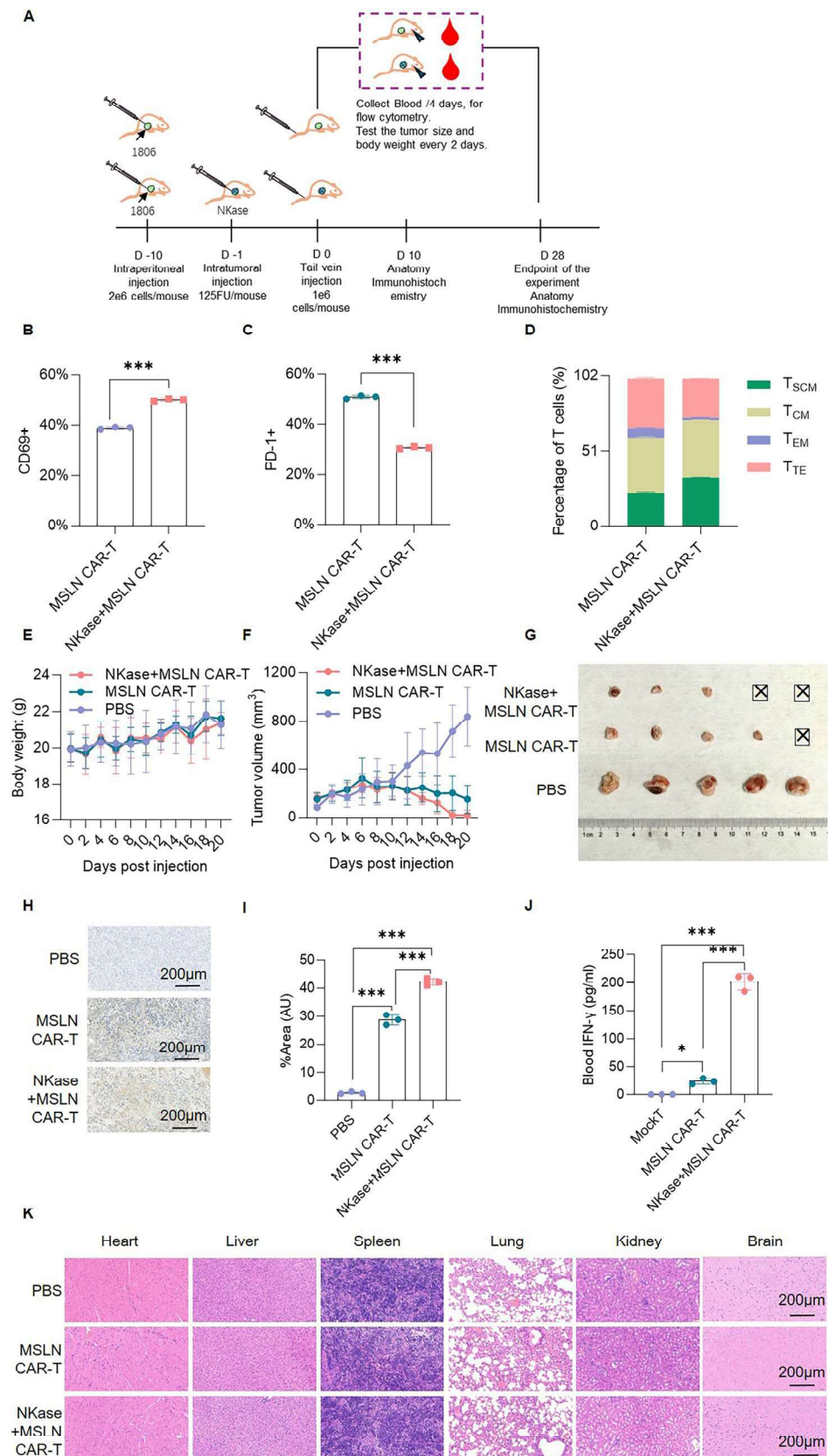
Analysis of gene expression differences in tumor cells treated with NKase

To investigate the impact of nattokinase (NKase) on tumor cell gene expression, we first performed transcriptomic analysis on HCC1806 tumor cells before and after NKase treatment. A volcano plot of differentially expressed genes (DEGs) revealed substantial transcriptomic changes following NKase exposure, with numerous genes showing significant upregulation or downregulation (Fig. 5A), indicating that NKase broadly influences tumor gene expression through diverse molecular mechanisms. Gene ontology (GO) enrichment analysis was subsequently conducted to explore functional implications of these changes, and the results, visualized through a bubble chart, revealed that NKase treatment significantly affected multiple biological processes, including cell adhesion, extracellular matrix (ECM) degradation, fibrinolysis, and apoptosis (Fig. 5B). These findings suggest that NKase may exert antitumor effects by modulating key cellular functions and microenvironmental interactions. We analyzed the expression of apoptosis-related genes using a heatmap. Multiple pro-apoptotic genes were significantly upregulated following NKase treatment (Fig. 5C), indicating that NKase may promote tumor cell apoptosis and thus potentiate the cytotoxic activity of MSLN CAR-T cells. GO interaction network analysis of DEGs (Fig. 5D) further demonstrated significant downregulation of genes associated with tumor cell migration and adhesion (Fig. 5E), as well as genes involved in ECM structure and organization (Fig. 5F), suggesting that NKase may impair tumor invasiveness by disrupting cell-ECM interactions. Additionally, analysis of genes related to ECM degradation revealed that NKase significantly upregulated several matrix-degrading enzymes (Fig. 5G), indicating enhanced ECM remodeling. These transcriptomic changes collectively suggest that NKase contributes to tumor microenvironment reprogramming by promoting apoptosis, inhibiting invasion, and facilitating ECM degradation, which may synergize with MSLN CAR-T therapy to improve antitumor efficacy.

NKase disrupts tumor-associated fibronectin to enhance MSLN CAR-T cell infiltration

By analyzing the gene expression of tumor cells treated with NKase, we found that the gene *FN1* was decreased, which indicated a reduction in the ECM component fibronectin protein [44, 45]. In order to further investigate whether NKase affects the expression of fibronectin in tumor cells and thereby regulates the tumor microenvironment, we first evaluated the remodeling of the ECM

Fig. 4 NKase enhances the infiltration of MSLN CAR-T cells and the antitumor effect in vivo. **A** Schematic representation of the in vivo experimental design. **B** Flow cytometric analysis of CAR-T cell activation in mouse peripheral blood. **C** Flow cytometric analysis of CAR-T cell exhaustion in mouse peripheral blood. **D** Assessment of memory marker expression (CD45RO and CD62L) on CAR-T cells in mouse peripheral blood. The proportions of stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and terminally differentiated effector T cells (T_{TE}) were determined based on distinct cell populations. **E–F** Graphical representation of body weight and tumor volume measurements in mice at various time points. **G** Tumor growth curves depicting changes in tumor size across experimental groups. **H–I** Immunohistochemical staining results showing infiltration of CD3+ cells in tumor tissues on day 10 post-MSLN CAR-T cell infusion. **J** The expression level of IFN- γ in the plasma supernatant of mice. **K** At the conclusion of the experiment, major organs, including heart, liver, spleen, lung, kidney, and brain, were harvested from mice and subjected to hematoxylin and eosin (**H & E**) staining to evaluate the safety profile of MSLN CAR-T cells in nontarget tissues. The experiment was repeated three times, and p values were calculated using one-way ANOVA. ns, not significant; *, $p < 0.05$; ***, $p < 0.001$



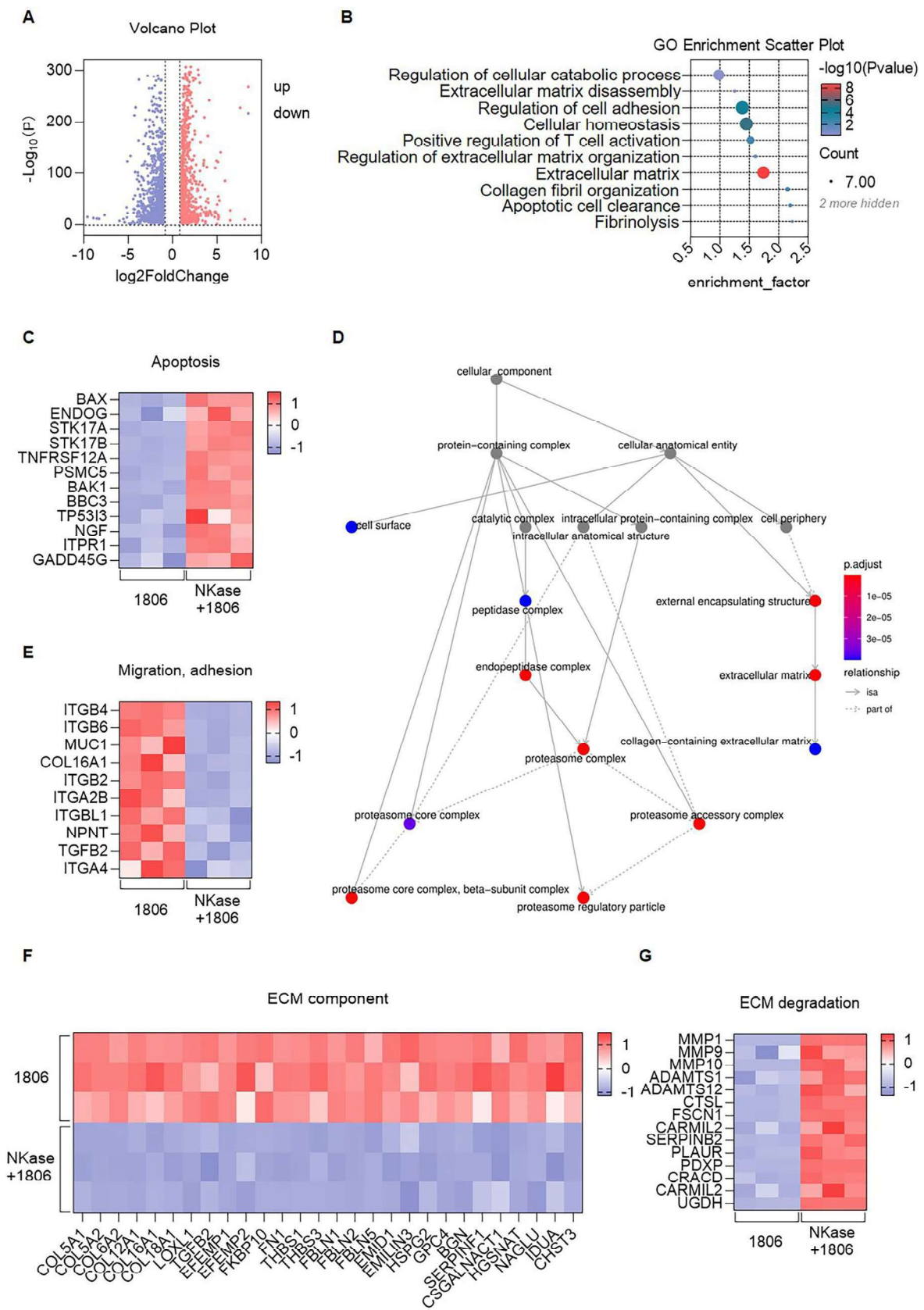


Fig. 5 Analysis of Gene Expression Differences in Tumor Cells Treated with NKase. **A** Volcano plot illustrating the 1,806 differentially expressed genes (DEGs) identified in tumor cell lines before and after NKase treatment. **B** GO enrichment bubble chart depicting the functional annotation of differentially expressed genes in tumor cell lines before and after NKase treatment. **C** Heatmap displaying the expression profiles of apoptosis-related differentially expressed genes in HCC1806 tumor cells before and after NKase treatment. **D** Protein–protein interaction (PPI) network analysis of differentially expressed genes in HCC1806 tumor cells before and after NKase treatment. **E** Heatmap showing the expression patterns of migration- and adhesion-related differentially expressed genes in HCC1806 tumor cells before and after NKase treatment. **F** Heatmap presenting the expression levels of extracellular matrix (ECM) component-related differentially expressed genes in HCC1806 tumor cells before and after NKase treatment. **G** Heatmap summarizing the expression changes of ECM degradation-associated differentially expressed genes in HCC1806 tumor cells before and after NKase treatment

by detecting the expression of fibronectin in HCC1806 tumor cells treated with 125 FU NKase. Immunofluorescence analysis revealed a marked reduction in fibronectin levels after NKase treatment (Fig. 6A–B), and quantitative fluorescence intensity analysis using ImageJ confirmed the significant decrease, suggesting that NKase effectively promotes ECM degradation. To validate this observation, we performed Western blot analysis to evaluate the expression of fibronectin and α -smooth muscle actin (α -SMA), a marker of fibrotic response. The results demonstrated significantly lower expression of both fibronectin and α -SMA in the NKase-treated group compared to controls (Fig. 6C–E), and densitometric analysis using ImageJ further supported these findings, indicating that NKase suppresses the fibrotic activity of tumor cells. To assess whether NKase-mediated ECM modulation enhances CAR-T cell infiltration, we conducted a 3D tumor microsphere infiltration assay using HCC1806 spheroids. MSLN CAR-T cells exhibited significantly greater infiltration in the NKase-treated group than in the control group (Fig. 6F–G), and quantitative analysis revealed a substantial decrease in green fluorescence intensity, confirming enhanced CAR-T cell penetration. Finally, to validate these findings in vivo, we performed immunofluorescence analysis of tumor tissues harvested 10 days after MSLN CAR-T cell infusion. Tumors from NKase-treated mice exhibited significantly reduced fibronectin expression compared to controls (Fig. 6H–I), consistent with enhanced ECM degradation. ImageJ-based quantification further confirmed these results. Collectively, these findings demonstrate that NKase improves the tumor microenvironment by promoting fibronectin degradation, reducing fibrosis, and facilitating the infiltration of MSLN CAR-T cells, thereby contributing to enhanced antitumor efficacy.

Conclusion

In summary, NKase remodels the tumor microenvironment by degrading fibronectin, reducing fibrosis, and promoting the infiltration of MSLN CAR-T cells. Moreover, it enhances the antitumor activity of MSLN CAR-T cells by improving their activation, reducing exhaustion and apoptosis, and promoting a memory-like phenotype. These effects result in stronger and more durable tumor killing in vitro and in vivo without causing off-target toxicity. NKase is a promising adjuvant that can improve CAR-T cell therapy for solid tumors.

Discussion

Despite the remarkable progress achieved by CAR-T cell therapy in hematological malignancies [46, 47], its clinical efficacy in treating solid tumors remains significantly constrained by various factors [48, 49]. Among these, the extracellular matrix (ECM) of tumor cells is widely recognized as a critical barrier limiting the effectiveness of CAR-T cells [50, 51]. The ECM not only provides structural support as an essential component of solid tumors [52], but also forms a dense physical barrier that restricts the infiltration of immune cells, including CAR-T cells, into the tumor mass [53, 54]. Moreover, components such as fibronectin, collagen, and laminin within the ECM can activate immune-suppressive pathways by interacting with integrin receptors [55, 56], thereby further impeding CAR-T cell functionality and inducing cellular exhaustion and inactivation [57]. Consequently, overcoming the ECM barrier and reshaping the tumor microenvironment have emerged as pivotal strategies to enhance the therapeutic efficacy of CAR-T cells in solid tumors [58, 59].

NKase, a serine protease derived from *Bacillus subtilis* natto during the fermentation process of natto, exhibits natural fibrinolytic and thrombolytic properties [60]. Several studies have demonstrated that NKase can degrade fibrin and other ECM components, facilitating matrix remodeling and thereby reducing interstitial adhesiveness and mechanical barriers [60]. Furthermore, NKase has been shown to have an excellent safety profile [61], with no significant toxicity reported in both nutritional and clinical studies [62], which supports its potential for use in cancer therapy.

The present study represents the first comprehensive investigation into the synergistic effects of NKase in enhancing the efficacy of MSLN-targeted CAR-T cell therapy in MSLN-positive solid tumors. Our findings indicate that NKase significantly augments the cytotoxic

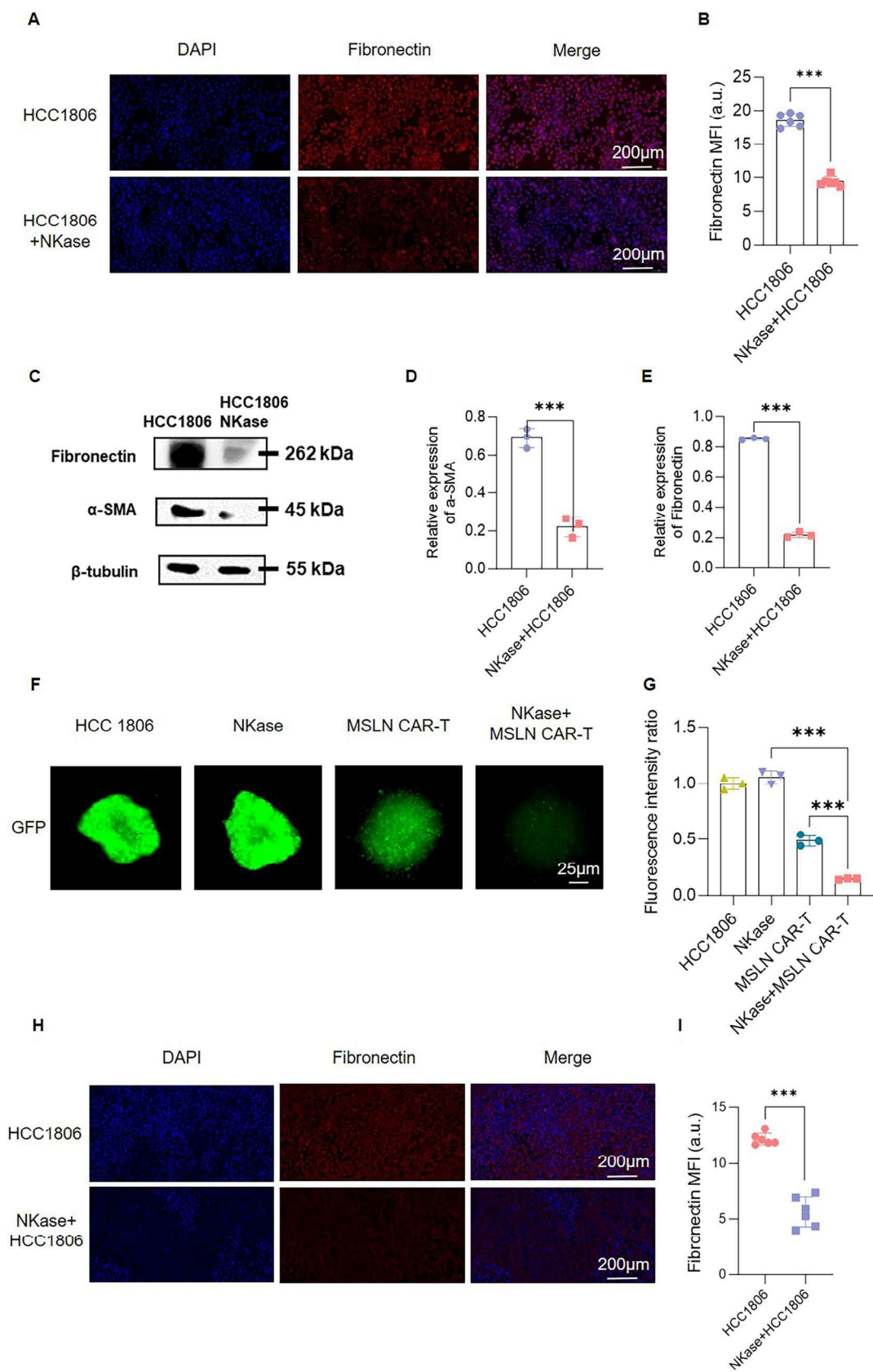


Fig. 6 NKase Disrupts Tumor-Associated Fibronectin to Enhance MSLN CAR-T Cell Infiltration. **A–B** Immunofluorescence staining demonstrated fibronectin degradation in the HCC1806 cell line following treatment with 125 FU NKase. The expression level of fibronectin was quantified using ImageJ software. **C–E** Western blot analysis confirmed the expression levels of fibronectin and α -smooth muscle actin (α -SMA) in the HCC1806 cell line after exposure to 125 FU NKase. Band intensities were quantified using ImageJ software. **F–G** Three-dimensional microsphere infiltration assays evaluated the in vitro infiltration capacity of MSLN CAR-T cells in both the negative control (NC) group and the 125 FU NKase treatment group. Green fluorescence intensity derived from HCC1806 cells was quantified using ImageJ software. **H–I** Immunofluorescence staining revealed reduced fibronectin expression in tumor tissues from mice on day 10 post-infusion of MSLN CAR-T cells. Fibronectin levels were quantified using ImageJ software. Data are presented as mean $\pm$ SEM from three independent in vitro experiments; ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; one-way ANOVA

activity of CAR-T cells against tumor cells and improves their functional status at noncytotoxic concentrations. In vitro analyses confirmed that NKase-treated tumor cells exhibited higher lysis rates across multiple effector-to-target (E/T) ratios. Mechanistic studies revealed that NKase can increase the excessive activation of CAR-T cells, as evidenced by the increase in the expression of activation markers such as CD25 and CD69. Moreover, NKase was found to inhibit the exhaustion phenotype by reducing PD-1, LAG-3, and TIM-3 expression, while also preventing apoptosis, thus prolonging CAR-T cell functional persistence. Additionally, NKase facilitated the differentiation of CAR-T cells into T stem cell memory (T_{SCM}) and central memory (T_{CM}) phenotypes, indicating its potential to support the maintenance of CAR-T cell memory. Transcriptomic analysis further corroborated these observations, showing that NKase treatment significantly upregulated the expression of genes involved in ECM degradation, inhibition of cell adhesion, and promotion of apoptosis, suggesting its multifaceted role in improving CAR-T cell therapeutic outcomes. In vivo experiments additionally confirmed that NKase markedly enhanced CAR-T cell tumor infiltration and antitumor activity, with no apparent systemic toxicity.

Although the present study demonstrates the promising potential of combining NKase with MSLN CAR-T cells for the treatment of solid tumors, several limitations exist. First, the study was conducted primarily using two MSLN-positive tumor models, HCC1806 and Ovar3. Whether similar outcomes can be achieved in other solid tumor models requires further investigation. Second, the pharmacokinetics, metabolic stability, and long-term immunogenicity of NKase have not been thoroughly explored, and further studies focusing on these aspects are essential for assessing the clinical applicability of NKase in tumor therapy. Additionally, the present study did not extensively examine the direct effects of NKase on ECM-producing

cells, such as tumor-associated fibroblasts (CAFs), an area that warrants further exploration.

In conclusion, this study provides compelling evidence that the combination of NKase with MSLN CAR-T cells enhances CAR-T cell infiltration, functional persistence, and antitumor efficacy in solid tumors. The results suggest a promising new approach for improving CAR-T cell therapy, offering novel insights into ECM modulation for cancer immunotherapy. These findings not only broaden the therapeutic potential of ECM-targeting strategies but also provide a solid theoretical foundation for enhancing CAR-T cell effectiveness in solid tumors. Future investigations will be required to further validate the clinical relevance of NKase as an adjunctive treatment in CAR-T cell therapy, with the goal of expanding its application to a wider range of solid tumor indications.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00262-025-04167-0>.

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Author contributions Lf. Z.: Conceptualization, data curation, methodology, statistical analysis, writing—original draft and writing—review & editing. Y. Z.: Data curation, methodology, and writing—review & editing. Xh. Y.: Data curation, methodology and statistical analysis. Ty. C.: methodology and statistical analysis. Jq. L.: Data curation, investigation and statistical analysis. Zg. G.: Conceptualization, funding acquisition, project administration, writing—original draft and writing—review & editing.

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Data availability The raw RNA-seq data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under Bio-Project accession number PRJNA1314171. All data are available in the main text or the supporting information.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval and consent to participate Animal experiments were conducted at the animal laboratory of Nanjing Normal University in accordance with protocols approved by the Animal Welfare Committee of the university (Approval number: IACUC-20230311).

Consent for publication All authors hereby unanimously agree to publish this article.

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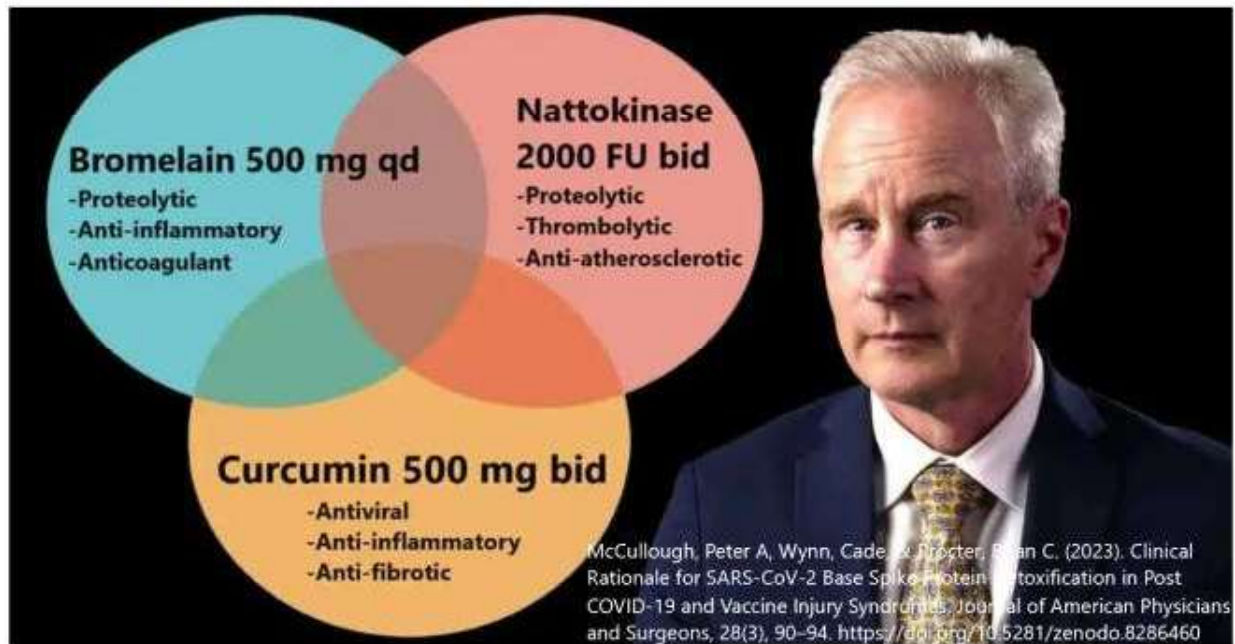
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Clinical Rationale for SARS-CoV-2 Base Spike Protein Detoxification in Post-COVID-19 and Vaccine Injury Syndromes

by Dr. Peter McCullough | Aug 26, 2023 | Feature 3, Health



- Nattokinase 2000 FU (100) mg orally twice a day without food
- Bromelain 500 mg orally once a day without food
- Curcumin 500 mg orally twice a day (nano, liposomal, or with piperine additive suggested)

Spike Protein Detoxification Protocol of Dr Peter McCullough

by Jeffrey Dach MD

Mike is a 62 year old hedge fund manager who took the multiple COVID vaccines over the past three years. He was rushed to the hospital Emergency Room with chest pain, and quickly transferred to the Cardiac Catheter Laboratory where coronary angiography failed to show the cause of his chest pain. The doctors were perplexed. In any event, a stent was placed into one of the coronary arteries which was thought to be narrowed slightly. He went home and a few weeks later had another bout of chest pain. The above scenario was repeated with a quick trip back to the Cardiac Cath Lab, and again coronary angiogram showed very little findings. A second stent was placed anyway. He went home and a few weeks later, everything was repeated again. A third bout of chest pain sent him back to the hospital and a third stent was placed. And again, after he went home, he still complained of intermittent chest pain despite the three stents. The doctors were perplexed, and had nothing to further to offer the patient.

Header Image: Courtesy of McCullough, Peter A., et. al. "[Clinical rationale for SARS-CoV-2 base spike protein detoxification in post Covid-19 and vaccine injury syndromes.](#)" *J Am Physicians Surg* 28 (2023): 90-94.(1)

Autopsy Studies Confirm spike Protein Accumulates in Myocardium

Mike's story is typical for chest pain from myocarditis (inflammation of the heart muscle) induced by the Covid Vaccine which causes accumulation of spike protein in the myocardium. (12-36)

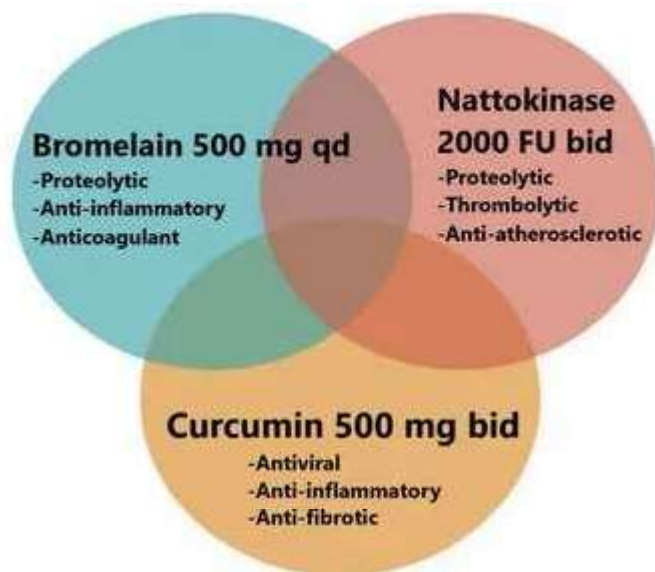
Symptoms of can include chest pain, as well as the more serious cardiac arrhythmia and sudden cardiac arrest. Myocardial function can be compromised leading to congestive heart failure with reduced ejection fraction. Cardiologists are willfully blind to these adverse effects of the Covid Vaccines, thus explaining the futile repeated procedures in the Cath Lab for many patients like Mike suffering from chest pain caused by Covid Vaccine induced myocarditis. (12-36)

The Covid Vaccine is not really a Vaccine. It is genetic therapy which programs our cells with messenger RNA which instructs our cells to manufacture the spike protein, an extremely toxic substance which induces an inflammatory reaction. There is no way to turn off this production, There is no "Off Switch". In 2023, Dr. Daniel Santiago reviewed all cause mortality during the pandemic finding that five doses of the Covid Vaccine are evidently lethal to nearly all medicare participants. (37-40)

A commonly reported symptom after Covid Vaccination is pain and tenderness within [enlarged axillary lymph nodes](#) which may last for months. This is caused by spike protein accumulation within axillary lymph nodes. Unfortunately, spike protein is produced and accumulates in many other organ systems because of the lipid nanoparticle delivery vehicle. Thus, the messenger RNA delivered to the body by the Covid Vaccine travels freely throughout the body and is incorporated into cells of many other organ systems, the heart, brain, nervous system, and ovaries etc., accounting for the extensive varied adverse side effects.(41-44)

The next obvious question is, what can be done about all this spike protein accumulating in the heart and other organs? What can we do to get rid of it? Unfortunately conventional medicine is of no help. Mainstream cardiologists simply throw up their hands and shrug their shoulders and have nothing useful to offer.

There is one eminent, highly published cardiologist who has offered a treatment protocol which, in my opinion, is highly effective for removing spike protein. This is the Dr. Peter McCullough's spike protein detoxification protocol. This is the protocol Mike should have been taking from the beginning. (1)



All orally administered empirically for 3-12 months or longer

Base Spike Detoxification Protocol from Dr Peter McCullough

Left Image: courtesy of McCullough, Peter A., Cade Wynn, and Brian C. Procter. "[Clinical rationale for SARS-CoV-2 base spike protein detoxification in post Covid-19 and vaccine injury syndromes.](#)" *J Am Physicians Surg* 28 (2023): 90-94. (1)

IN 2024, Dr Peter McCullough writes:

The spike protein is responsible for the pathogenicity of the SARS-CoV-2 infection and drives the development of adverse events, injuries, disabilities, and death after vaccination through immunologic and thrombotic mechanisms. The long-lasting spike protein has been found in the brain, heart, liver, kidneys, ovaries, testicles, and other vital organs at autopsy in cases of death after vaccination. In the case of vaccine-induced thrombotic injury, the spike protein has been found within the blood clot itself. Thus, there is strong rationale for considering residual SARS-CoV-2 spike protein as a treatment target in post Covid-19 and vaccine injury syndromes. The spike protein participates directly in pathophysiology, incites inflammation, and propels thrombosis. While specific syndromes (cardiovascular, neurological, endocrine, thrombotic, immunological) will require additional therapies, we propose the clinical rationale for a base detoxification regimen of oral nattokinase, bromelain, and curcumin for patients with post-acute sequelae from SARS-CoV-2 infection and COVID-19 vaccination. The empiric regimen can be continued for 3-12 months or more and be guided by clinical parameters:

- Nattokinase 2000 FU (100) mg orally twice a day without food
- Bromelain 500 mg orally once a day without food
- Curcumin 500 mg orally twice a day (nano, liposomal, or with piperine additive suggested)

No therapeutic claims can be made for this regimen because it has not been tested in large, prospective, double-blind, placebo controlled randomized trials. No such studies are planned or funded currently by federal or institutional sponsors. The main caveats are bleeding and allergic reactions. The regimen can be used in addition to anti-platelet and antithrombotic agents, however, caution is advised with respect to monitoring bleeding risks. (1)

How to Test for Spike Protein Antibodies

Anyone can order the Spike Protein Antibody test online at [Labcorp on Demand](#). No need for a doctor's prescription. The price is \$69.00 dollars.

Here is a quick guide of interpretation of your results:

<1000 U/ml very low level past exposure, negligible risk.

1000-5000 U/ml symptoms or medical problems could be related to Spike protein.

>5000 U/ml Spike protein likely circulating in your bloodstream.

10,000-25,000 U/ml very high risk for cardiac damage, blood clots, neurologic problems, autoimmunity, and potentially cancer

Remember, the antibodies are an indirect reflection of the Spike protein.

Thanks and Credit for this information goes to [Dr. Peter McCullough](#).

How to Order Supplements On-Line

Nattokinase, Bromelain and Curcumin are nutritional supplements which can be purchased over the counter without a prescription. For your convenience, here are links to these products at my Pure Encapsulations Store for on-line ordering:

[NSK-SD™ \(Nattokinase\)](#) – each vegetable capsule contains: nattokinase (2000 FU) (soy) 100 mg

Nattokinase is a highly purified fibrinolytic enzyme that has demonstrated encouraging potential for supporting endogenous fibrinolytic activity and promoting healthy blood flow, circulation, and blood vessel function. Pure Encapsulations NSK- SD™ is standardized to contain a minimum of 20,000 FU (fibrinolytic units) per gram.

[Bromelain 2400](#) Each vegetable capsule contains Bromelain (2,400 gdu/gram) 500 mg Bromelain enhances digestion and maintains healthy musculoskeletal tissue. Bromelain is a proteolytic enzyme from the stem of the pineapple plant. Taken with food, bromelain aids in the healthy functioning of the digestive system. Taken between meals, bromelain maintains healthy kinin and fibrin production, supporting healthy musculoskeletal function. Bromelain 2400 nutritionally supports the body's innate ability to digest food and to maintain musculoskeletal comfort at the cellular level.

[Curcumin 500 with Bioperine®](#): each vegetable capsules contains: turmeric (Curcuma longa) extract (root)† (standardized to contain 95% curcuminoids) 500 mg. – Bioperine® (Piper nigrum) extract (fruit) †† 5.3 mg. Turmeric may be contra-indicated with blood thinning medications.

Curcumin 500 with Bioperine® provides powerful support for maintaining a healthy inflammatory response, promoting cellular health, and supporting healthy liver, colon and musculoskeletal function. Bioperine® is a black pepper extract that contains the alkaloid piperine. Research reveals that it has the potential to enhance the bioavailability of curcumin, promoting its absorption.

Empiric Therapy can be Pursued

In April 2024, Dr Frank Yap, M.D. discussed the lack of clinical trials for spike protein detoxification protocols. However Dr Frank Yap feels that empiric therapy can be pursued, writing:

Patients should push their doctors to refer them to clinical trials, and when that is not feasible, **then empiric therapy can be pursued.** (9)

Update on [Ĉovĭd Văccĭnē Myocarditis from Dr Peter McCullough](#)

<https://twitter.com/i/status/1784007781193830799>

Link to the above video on twitter see reference (47).

Here is the link to Nakahara's myocarditis PET study:

Nakahara, Takehiro, et al. "[Assessment of Myocardial 18F-FDG Uptake at PET/CT in Asymptomatic Ĉovĭd Văccĭnated and NonĈovĭd Văccĭnated Patients.](#)" Radiology 308.3 (2023): e230743. *Conclusion: When compared with nonĈovĭd Văccĭnated patients, asymptomatic patients who received their second Ĉovĭd Văccĭnation 1–180 days prior to imaging showed increased myocardial 18F-FDG uptake on PET/CT scans. (45)*

Here is the link to a single manuscript with 18,204 cases of myocarditis and pericarditis after Ĉovĭd Văccĭnation: Lane, Samantha, Alison Yeomans, and Saad Shakir. "[Reports of myocarditis and pericarditis following mRNA Ĉovĭd Văccĭnation: a systematic review of spontaneously reported data from the UK, Europe and the USA and of the scientific literature.](#)" BMJ open 12.5 (2022): e059223.(46)

Take a Look at the "Big Picture"

For those of you interested in the "Big Picture" of what is really happening behind the scenes, the pandemic was a carefully planned psychological operation run by the intelligence community and department of defense in collaboration with "Big Pharma", various Government Agencies (FDA, CDC, NIH, HHS etc.), and a captured mass media to create mass hysteria and fear, and to turn society against itself. For more clarification, I suggest these two free articles by David A. Hughes:

[Mass Paranoia and Hysteria: Turning Society Against Itself](#) David A. Hughes

["Ĉovĭd-19," Psychological Operations, and the War for Technocracy](#) David A. Hughes Volume 1, **Here is a Book Review:**

Camouflaged by "Ĉovĭd-19," an undeclared global class war was initiated in 2020, aimed at replacing liberal democracy with technocracy, a novel, biodigital form of totalitarianism. The opening campaign involved the **largest psychological operation in history**, intended to demoralise, disorientate, and debilitate the public. This volume deals with the application of shock and stress, trauma-based mind control, the use of fear and threat, cognitive attack, weaponised deception, and techniques for turning society against itself....David A. Hughes is Senior Lecturer in International Relations at the University of Lincoln. He received his undergraduate and master's degrees from Oxford University and holds doctorates in German Studies (Duke University) and International Relations (Oxford Brookes University). His research focuses on psychological warfare, "9/11," "Ĉovĭd-19," the deep state, technocracy, global class relations, and resurgent totalitarianism.

Conclusion: Fibrinolytic and proteolytic enzymes have a long history of use in medicine. Spike protein detoxification is one of the more recent uses. Curcumin is a well known and popular anti-inflammatory supplement used for centuries. Thanks to Dr. Peter McCullough for making this spike protein detoxification protocol available for public use. Who should be taking this? Some doctors have suggested that everyone should be taking this protocol on a regular basis. This protocol is used on an empiric basis, as there are no validating long term medical studies as yet, and probably never will be.

Although nutritional supplements in this spike protein detoxification protocol are generally regarded as safe (GRAS), the reader is advised to consult and work closely with a knowledgeable physician before starting. (1-10)

The reader is advised to read this [DISCLAIMER](#).

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Articles with related interest:

[Fibrinolytic and Proteolytic Enzymes for Treatment of Various Conditions](#)

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spike protein detoxification

- 1) McCullough, Peter A., Cade Wynn, and Brian C. Procter. "[Clinical rationale for SARS-CoV-2 base spike protein detoxification in post COVID-19 and vaccine injury syndromes.](#)" J Am Physicians Surg 28 (2023): 90-94.
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8) [Solving the Čøvřđ challenge. Base Spike Detoxification Protocol by Dr. Peter McCullough](#)

The spike protein is responsible for the pathogenicity of the SARS-CoV-2 infection and drives the development of adverse events, injuries, disabilities, and death after vaccination through immunologic and thrombotic mechanisms. The long-lasting spike protein has been found in the brain, heart, liver, kidneys, ovaries, testicles, and other vital organs at autopsy in cases of death after vaccination. In the case of vaccine-induced thrombotic injury, the spike protein has been found within the blood clot itself. Thus, there is strong rationale for considering residual SARS-CoV-2 spike protein as a treatment target in post Čøvřđ-19 and vaccine injury syndromes. The spike protein participates directly in pathophysiology, incites inflammation, and propels thrombosis. While specific syndromes (cardiovascular, neurological, endocrine, thrombotic, immunological) will require additional therapies, we propose the clinical rationale for a base detoxification regimen of oral nattokinase, bromelain, and curcumin for patients with post-acute sequelae from SARS-CoV-2 infection and Čøvřđ-19 vaccination.

The empiric regimen can be continued for 3-12 months or more and be guided by clinical parameters:

- Nattokinase 2000 FU (100) mg orally twice a day without food
- Bromelain 500 mg orally once a day without food
- Curcumin 500 mg orally twice a day (nano, liposomal, or with piperine additive suggested)

9) [Dr Peter McCullough: How to Detox Spike Protein from Body](#)

By Dr Frank Yap, M.D. – April 23, 2024

According to Dr Peter McCullough (in a Twitter post – July 2023): Base Spike Detox is what I am currently using in my practice for those who have had Čøvřđ-19 multiple times, one or more of the Čøvřđ-19 vaccines, or both and believe persistent SARS-CoV-2 Spike protein could be causing problems in their body. A major publication is under review and editing; however, the information is far too important to hold back.

Update (Nov 2023): Published on August 25, 2023 in *Journal of American Physicians and Surgeons*. [CLICK HERE FOR THE COMPLETE PAPER](#) > Base Spike Protein Detoxification and on November 21, 2023 in *Cureus* (PubMed – November 2023)

I have arrived, based on the emerging scientific literature and my clinical observation, that three OTC products are essential as a triple base combination:

Nattokinase 2000 FU (100 mg) twice a day
Bromelain 500 mg once a day
Nano/Liposomal Curcumin 500 mg twice a day

Additional products can be added, including NAC, IVM (Ivermectin), HCQ (Hydroxychloroquine), fluvoxamine, low-dose naltrexone, and blood thinners, depending on the clinical evaluation and the syndrome. The therapeutic objective is to start treatment and allow the body to clear Spike and its

fragments with the natural reticuloendothelial system. I believe this triple combination is the best approach.

Patients can get a big head start if they self-initiate Base Spike Detox as they get organized for appointments. I have found three months is a minimum duration, and some require more than a year. Don't expect instant results, be patient. I have a major manuscript under review for publication that summarizes the clinical rationale and evidence supporting Base Spike Detox.

Important safety warnings include bleeding for those on blood thinners or who have bleeding disorders (e.g., hemophilia), soy allergy, allergies to any component of the combination, and gastrointestinal intolerance. Women of childbearing potential without contraception, pregnant, breastfeeding, and children should not take this combination unless directed by a doctor.

COVID-19 Vaccine Myocarditis Research Wins Award

We're thrilled to announce that Dr Peter McCullough, won first place on Preprints.org for a study he co-authored on vaccine-induced myocarditis.

Preprints.org is a vital platform for the early dissemination of scholarly research before standard peer review and publication in scientific journals. By allowing research findings to be shared early with the scientific community, Preprints.org facilitates scientific communication and collaboration, enabling researchers to receive feedback and engage with the broader scientific community.

Autopsy Proven Fatal COVID-19 Vaccine-Induced Myocarditis claimed the top spot in the Medicine and Pharmacology category, specifically focusing on cardiac and cardiovascular systems. This research paper delves into a critical issue surrounding COVID-19 vaccination — the potential risk of myocarditis, particularly in cases where it leads to fatal outcomes.

Nattokinase and Spike Protein

Tanikawa et al. examined the effect of nattokinase on the spike protein of SARS-CoV-2. In the first experiment, they demonstrated that spike was degraded in a time and dose-dependent manner in a cell lysate preparation that could be analogous to a vaccine recipient. The second experiment demonstrated that nattokinase degraded the spike protein in SARS-CoV-2 infected cells. This was reproduced in a similar study done by Oba and colleagues in 2021.

Tanikawa T, Kiba Y, Yu J, Hsu K, Chen S, Ishii A, Yokogawa T, Suzuki R, Inoue Y, Kitamura M. Degradative Effect of Nattokinase on Spike Protein of SARS-CoV-2. *Molecules*. 2022 Aug 24;27(17):5405.

Nattokinase is dosed in fibrinolytic units (FU) per gram and can vary according to purity. Kurosawa and colleagues have shown in humans that after a single oral dose of 2000 FU D-dimer concentrations at six, and eight hours, and blood fibrin/fibrinogen degradation products at four hours after administration elevated significantly ($p < 0.05$, respectively).

Thus an empiric starting dose could be 2000 FU twice a day. Full pharmacokinetic and pharmacodynamic studies have not been completed, but several years of market use as an over-the-counter supplement suggests nattokinase is safe with the main caveat being excessive bleeding and cautions with concurrent antiplatelet and anticoagulant drugs.

Editor's Note: We are aware of a fact-check article by AFP titled "Experts rubbish COVID vaccine 'detoxification' supplement". In my humble opinion, the title may come across as excessively confident and strongly worded.

The article makes a claim that 'There is no evidence that nattokinase dissolves the spike protein of SARS-CoV-2 in the human body,' while also citing evidence from the Tanikawa study. It's important to acknowledge that the Tanikawa study is a small lab study, which is a common starting point in the research process before moving on to larger studies.

Nevertheless, we agree that nattokinase is not a miracle drug, and further research is necessary. **It's worth noting that there are more than 200 studies related to nattokinase published on PubMed.**

Moreover, it's essential to recognize that there are multiple approaches to managing COVID-19. We encourage you to consult with your trusted healthcare provider to thoroughly discuss and assess the potential benefits and risks of various possible treatments.

Ivermectin and Spike Protein

Former NIH researcher David Scheim, PhD, early in the pandemic proposed that SARS-CoV-2 Spike protein was acting like a grappling hook pulling together circulating red blood cells (RBCs) into long chains and clumps in a process called hemagglutination (HA).

This explained why the red blood cells could not carry oxygen normally and was congruent with the finding of micro blood clots in the lungs. Boschi et al have provided additional support for this mechanism (source). By reversing the clumping of red blood cells, ivermectin enabled the patient's proper respiratory function to return.

According to the authors:

"Ivermectin blocked HemAgglutination when added to RBCs prior to spike protein and reversed HA when added afterwards."

In another spectacular publication, Stone et al, describes the prompt improvement of oxygenation in patients with ivermectin (source).

The published oxygenation curves from multiple studies clearly show this physiological effect of ivermectin occurs so rapidly, it must be explained by a direct anti-Spike protein effect of ivermectin. An anonymous video of a critically ill man demonstrates the very effect that Scheim, Stone, Hazan, and Babalola have described in the Figure above. So for the next critically ill patient with COVID-19, if the opportunity presents itself, push for the administration of ivermectin. This is the only published therapy for COVID-19 that improves oxygen saturation while the patient mounts a recovery. As in this man, it may be the critical factor for a turnaround and a chance to walk out of the hospital.

Key Takeaway

Patients should push their doctors to refer them to clinical trials, and when that is not feasible, **then empiric therapy can be pursued.** It is important to realize that in the absence of completed large randomized placebo controlled randomized trials, which are easily 5 or more years away in the future, no therapeutic claims can be made. In the meantime we must be perceptive as patients and open-minded as clinicians to come up with reasonable approaches that can be used to help those sick now with post-COVID syndromes.

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62% Normalization in SpO2 within 24 Hours for severe COVID-19 patients after Ivermectin-Based Combination Treatment.

Peter A. McCullough's Substack.

Clinical Approach to Post-acute Sequelae After COVID-19 Infection and Vaccination

Nicolas Hulscher¹, Brian C. Procter², Cade Wynn², Peter A. McCullough^{3,4}

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Abstract

The spike protein of SARS-CoV-2 has been found to exhibit pathogenic characteristics and be a possible cause of post-acute sequelae after SARS-CoV-2 infection or COVID-19 vaccination. COVID-19 vaccines utilize a modified, stabilized prefusion spike protein that may share similar toxic effects with its viral counterpart. The aim of this study is to investigate possible mechanisms of harm to biological systems from SARS-CoV-2 spike protein and vaccine-encoded spike protein and to propose possible mitigation strategies. We searched PubMed, Google Scholar, and 'grey literature' to find studies that (1) investigated the effects of the spike protein on biological systems, (2) helped differentiate between viral and vaccine-generated spike proteins, and (3) identified possible spike protein detoxification protocols and compounds that had signals of benefit and acceptable safety profiles. We found abundant evidence that SARS-CoV-2 spike protein may cause damage in the cardiovascular, hematological, neurological, respiratory, gastrointestinal, and immunological systems. Viral and vaccine-encoded spike proteins have been shown to play a direct role in cardiovascular and thrombotic injuries from both SARS-CoV-2 and vaccination. Detection of spike protein for at least 6-15 months after vaccination and infection in those with post-acute sequelae indicates spike protein as a possible primary contributing factor to long COVID. We rationalized that these findings give support to the potential benefit of spike protein detoxification protocols in those with long-term post-infection and/or vaccine-induced complications. We propose a base spike detoxification protocol, composed of oral nattokinase, bromelain, and curcumin. This approach holds immense promise as a base of clinical care, upon which additional therapeutic agents are applied with the goal of aiding in the resolution of post-acute sequelae after SARS-CoV-2 infection and COVID-19 vaccination. Large-scale, prospective, randomized, double-blind, placebo-controlled trials are warranted in order to determine the relative risks and benefits of the base spike detoxification protocol.

Categories: Internal Medicine, Infectious Disease, Therapeutics

Keywords: medicine, treatment protocol, long covid, post-acute sequelae, covid-19 vaccines, covid-19 treatment, spike glycoprotein, spike protein detoxification, sars-cov-2 spike protein, sars-cov-2

Introduction And Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly contagious and pathogenic virus that emerged in late December 2019. It causes an acute respiratory disease named "coronavirus infectious disease-2019" (COVID-19). The World Health Organization (WHO) declared the outbreak of SARS-CoV-2 a pandemic on March 11, 2020 [1]. As of November 15, 2023, there have been over 700 million reported cases of COVID-19 and almost seven million deaths [2]. To combat this virus, novel gene-based vaccines were developed that utilize viral vector (AstraZeneca - ChAdOx1 nCoV-19; Johnson and Johnson - Ad26.CoV2.S) and mRNA (Moderna - mRNA 1273; Pfizer-BioNTech - BNT162b2) platforms to encode for spike protein, which elicits an immune response that generates antibodies [3]. At the time of writing, about 70% of the global population have received one or more doses of a COVID-19 vaccine [2].

The SARS-CoV-2 spike protein plays a critical role in the virus's infection process. Spike protein emerges from the viral surface with a clove-like shape, facilitating SARS-CoV-2's binding to host cells. This protein, which is a class I fusion transmembrane glycoprotein, is cleaved into two main sections by host proteases: the S1 and S2 subunits. The S1 subunit has a receptor-binding domain that is responsible for recognizing and binding to the host cell receptor ACE-2. Moreover, the S1 subunit is a site of drastic evolutionary pressure. Mutations of the spike protein lead to new strains that can re-infect those who have already recovered from a previous SARS-CoV-2 variant. These new variants can be more infectious than previous ones due to a higher affinity for the ACE-2 receptor. The S2 subunit is responsible for the fusion of host and viral membranes. Most of the vaccines deployed to prevent COVID-19 illness utilize a full-length spike protein stabilized in a pre-fusion state to generate immunity against SARS-CoV-2 [4].

Post-acute sequelae (long COVID) after SARS-CoV-2 infection or COVID-19 vaccination is characterized by new or persisting multi-system symptoms months to years after initial infection or vaccination [5,6]. These symptoms can range from generalized anxiety disorder and difficulty concentrating to fatigue and muscle weakness. The true incidence and prevalence of long COVID after infection are not fully known, but it has

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been approximated to be quite common. It is been estimated that 50% of those who were infected with SARS-CoV-2 develop long COVID, which equates to hundreds of millions of individuals [7]. Research on the prevalence of post-COVID vaccination syndrome is limited; however, a prospective cohort study found that 52.8% of individuals developed post-COVID vaccination syndrome for at least one month after primary COVID-19 vaccination. In this study, the prevalence increased to 83.9% among those who received booster doses [8]. A retrospective cross-sectional study found a post-COVID vaccination syndrome prevalence of 26.2% after COVID-19 vaccination, with higher rates seen in individuals who received Moderna vaccines compared to Pfizer vaccines [9]. These data indicate that post-acute sequelae after COVID-19 vaccination are clinically relevant; however, more research is required to find the true incidence and prevalence.

Concerns have been raised that suggest spike protein is involved in various pathologies, including post-acute sequelae after SARS-CoV-2 infection and COVID-19 vaccination [10-12]. This may be due to spike protein exhibiting harmful properties, including inflammation, endothelial tissue damage, and brain damage [10]. Moreover, spike protein has been associated with thrombotic endothelialitis, endothelial inflammation, hyperactivated platelets, and fibrinoid microclots in those with long COVID [11]. Parry et al. found that spike protein is detrimental to many organ systems by analyzing autopsies and biopsies of spike-infected tissues [12]. If spike protein is found to be harmful to human health, it could lead to the development of new treatments that aim to reduce tissue damage caused by COVID-19. Additionally, this discovery could prompt research into ways to prevent or treat health issues resulting from COVID-19 vaccines. This is particularly relevant since COVID-19 vaccines work by using spike protein to trigger an immune response. The aim of this study is to investigate possible mechanisms of harm on biological systems from SARS-CoV-2 spike protein and vaccine-encoded spike protein for the purpose of exploring causes of COVID-19-associated harm, COVID-19 vaccine injury syndromes, and potential therapeutic strategies.

Review

Methods

Article Layout

First, we categorized the findings of spike protein's deleterious effects based on the organ system targeted: cardiovascular, hematological, neurological, respiratory, gastrointestinal, and immunological systems. Additionally, we detailed the differences between viral spike protein and vaccine-generated spike protein. Lastly, we explored potential detoxification strategies, including the McCullough protocol: base spike detoxification.

Search Strategy

We performed a literature search using the PubMed and Google Scholar databases. We also searched the grey literature, which is research produced by organizations that are not affiliated with traditional commercial or academic publishing and distribution channels. The primary search terms were "SARS-CoV-2 spike protein", "spike protein effects", "COVID-19 vaccine spike protein", "cardiovascular", "hematological", "neurological", "respiratory", "gastrointestinal", "immunological", "detoxification", "treatment", "degradation", "curcumin", "bromelain", and "nattokinase". These terms were used in varying combinations with filters applied for English language articles. All references cited in selected articles were manually searched to identify any additional relevant studies.

Study Selection and Eligibility Criteria

For investigating the harmful effects of spike protein, articles that specifically assessed the direct or indirect impacts of the spike protein on biological systems, in isolation or in relation to the SARS-CoV-2 virus or COVID-19 vaccination, were considered. We also included papers that helped assess the difference between viral spike protein and vaccine-generated spike protein. Lastly, we sought to include any published treatment protocols or compounds that had signals of benefit and acceptable safety profiles that may assist in spike protein detoxification. Original articles, in vitro studies, animal studies, and clinical studies published were included. Case reports, editorials, letters to the editor, and reviews were excluded unless they provided original results or synthesized available evidence.

Results

Deleterious Effects of Spike Protein by Organ System

Cardiovascular system: The cardiovascular system has been identified as one of the primary targets of the SARS-CoV-2 spike protein. Disruptions in human cardiac pericytes, a type of cell that wraps around the endothelial cells of capillaries and venules, have been observed by the exposure of the spike protein. These disruptions include increased migration, secretion of inflammatory molecules that are seen in the cytokine storm, and creation of apoptotic factors that cause cell death [13]. Furthermore, spike protein has been found to hinder mitochondrial functions in human cardiomyocytes, potentially leading to energy deficits and reduced cardiac output. This effect may be due to spike protein inducing negative mitochondrial

membrane potential, increasing intracellular calcium, and increasing levels of reactive oxygen species (ROS). Figure 1 illustrates this process [14]. The spike protein may also induce cardiomyocyte fusion, which may amplify the risk of arrhythmias, and irregular heart rhythms that can be life-threatening. In this study, spike protein induced delayed depolarizations, altered beating frequency, and induced calcium influx problems [15]. In the context of vaccination, there have been reports of spike protein's detection in patients diagnosed with COVID-19 vaccine-induced myocarditis [16,17]. From the available evidence, spike protein displays obvious toxic effects on the cardiovascular system.

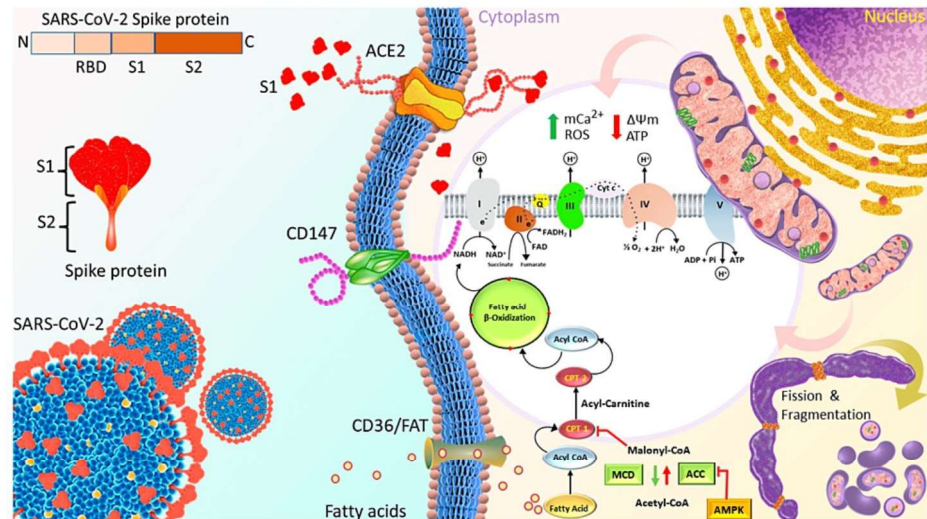


FIGURE 1: Proposed Mechanisms Through Which S1 of Spike Protein Induced Cardiac Mitochondrial Dysfunction, Which Leads to Cardiac Injury in COVID-19 Patients.

Spike protein is the glycosylated protein that covers the surface of SARS-CoV-2 and binds to the host ACE2 receptor to mediate the viral cell entry. It is composed of S1 and S2 subunits that are responsible for ACE2 binding and membrane fusion, respectively. S1 possibly binds to ACE2 on the AC16 membrane and is then internalized into the cytosol and localized in organelles, such as mitochondria, which induces the transient increase in fatty acids transport and uptake for biogenetics, $\Delta\Psi_m$, and permanent mCa^{2+} , and disrupts $\Delta\Psi_m$ later, finally impairing mitochondrial function and promoting ROS production. In turn, ROS further exacerbates mitochondrial function and mitochondrial fragmentation. Moreover, S1 causes downregulation of TOM20; this effect might inhibit the pathways leading to mitochondrial biogenesis.

Abbreviations: ACE2 = angiotensin-converting enzyme 2, FAT = fatty acid translocase, PCT1/2 = Carnitine palmitoyltransferase 1/2, MCD = Malonyl-CoA decarboxylase, ACC = acetyl-CoA carboxylase, AMPK = AMP-activated protein kinase, mCa^{2+} = mitochondrial calcium, $\Delta\Psi_m$ = mitochondrial membrane potential, ROS = reactive oxygen species.

*Figure and legend reprinted from Huynh et al. [14]. The legend title has been slightly adapted. Permission to use this figure has been granted in accordance with the open access Creative Common CC BY 4.0 license.

Hematological system: The hematological system, which includes blood and its components, is also targeted by the spike protein. The protein has been shown to incite inflammation in endothelial cells through a cascade involving integrin $\alpha 5\beta 1$ and the NF- κ B signaling pathway. This effect was also seen in vivo by intravenous inoculation of spike protein, which resulted in an increased expression of leukocyte adhesion molecules, coagulation factors, and proinflammatory cytokines in the lung, liver, kidney, and eye [18]. This inflammation can promote clot formation and hyperpermeability of the endothelial cell monolayer. Adding to the toxic effects, spike protein may transform fibrin into a form that resists fibrinolysis, making it harder for the body to dissolve blood clots. To do this, spike protein may induce structural changes to fibrin, complement 3, and prothrombin proteins [19]. These dangerous alterations can help explain the mechanism behind microclotting seen in COVID-19 patients. The spike protein's affinity to bind competitively to heparan sulfate, an important glycoprotein involved in coagulation, further aggravates coagulation and thrombosis pathologies [20]. In this study, researchers injected a zebrafish model with spike protein at a similar level as in a critically ill COVID-19 patient. They found that this directly induced blood coagulation and thrombosis [20]. Perhaps most concerning is the finding that free spike protein, in the absence of SARS-CoV-2 RNA and nucleocapsid, was found within blood clots in patients diagnosed with acute ischemic stroke (Figure 2) [21]. As with the cardiovascular system, spike protein exhibits a wide array of harmful effects on the hematological system.

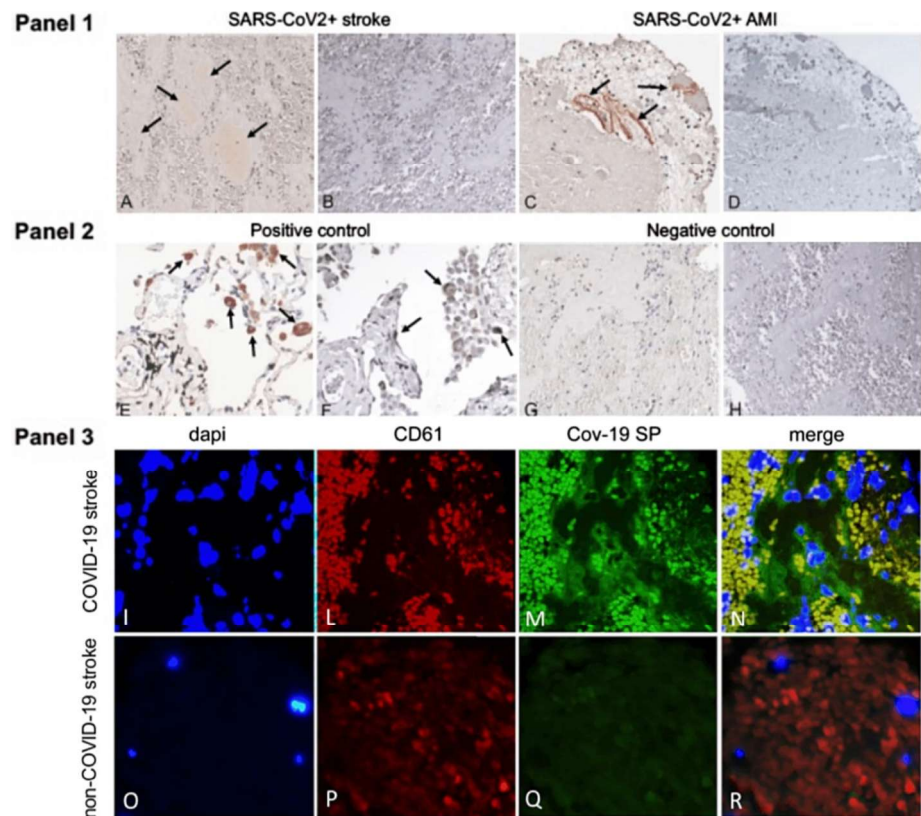


FIGURE 2: Arterial Thrombi From COVID-19 + Patients Contain SARS-CoV-2 Spike Protein But Not Nucleocapsid Protein.

Panel 1. Immunostaining positive for SARS-CoV-2 spike protein (SP) (arrows) in representative thrombotic material from COVID-19 + patients, retrieved from cerebral (A) and coronary (C) arteries. Immunohistochemistry for nucleocapsid protein (NP) was negative in the same samples (B-D). Panel 2. Representative immunohistochemical staining positive for SP (E) and NP (F) (arrows) in the lung of a patient affected by COVID-19 (positive control). Representative immunostaining negative for SP (G) and NP (H) in a thrombus retrieved from the middle cerebral artery of a patient not affected by COVID-19 (negative control). Original magnification 20X. Panel 3. Double immunofluorescence of thrombotic material retrieved from COVID-19 and non-COVID-19 patients' cerebral arteries. In the COVID-19 thrombus, platelets are co-stained with anti-CD61 (red-L,P) and anti-SARS-CoV-2 spike protein (SP) antibodies (green-M,Q), emitting yellow signals in the merged panel (N), while in the control (non-COVID thrombus), only the red CD61 signal is observed (R).

*Figure and legend reprinted from De Michele et al. [21]. The legend title has been slightly adapted. Permission to use this figure has been granted in accordance with the open access Creative Common CC BY 4.0 license.

Neurological system: Neurological implications of the spike protein are particularly concerning. Spike protein has been demonstrated to compromise the blood-brain barrier's integrity through RhoA activation, which can potentially allow harmful substances from the bloodstream to enter the brain [22]. This disruption is possibly achieved by upregulated ACE2 expression. Animal studies have revealed cognitive deficits and anxiety-like behaviors in mice following exposure to the spike protein, possibly due to non-cell autonomous death of neurons in the hippocampus [23]. Cell death of neurons was not directly caused by exposure to spike protein, but rather by spike protein's activation of glial cells, leading to neurotoxicity [23]. On a cellular level, there are indications of spike protein possibly causing deleterious changes in molecular delivery and metabolic functions in brain endothelial cells [24]. In this paper, spike protein induced mitochondrial damage in brain cells, as also seen in cardiomyocytes [14,24]. Some experimental models even suggest that spike protein might be a contributing factor in long-term cognitive dysfunctions that mimic post-COVID-19 syndromes [25]. Corroborating the study mentioned earlier [23], Fontes-Dantas et al. found that brain infusion of spike protein induced neuroinflammation and hippocampal microgliosis, or over-activation of glial cells, by engulfment of synapses [25]. These data indicate that spike protein exerts potent harmful effects on the neurological system by damaging blood-brain barrier function and destroying neurons.

Respiratory system: Spike protein also targets the respiratory system. Spike protein has been shown to stimulate inflammatory pathways in human lung cells, specifically through MAPK and NF- κ B activation [26]. This inflammatory cascade occurred independent of the virus, indicating that spike protein may have been the sole cause. Spike protein's influence extends to lung macrophages, where it triggers their activation [27]. The activation of lung macrophages was coupled with the promotion of efficient phagocytosis and

dysfunction of intracellular calcium concentrations [27]. Such activations can exacerbate inflammatory responses in the lungs and may cause lung injury. Moreover, spike protein can induce dysfunctions in lung endothelial cells, leading to thrombo-inflammation, largely contingent on the C3a/C3a receptor signaling [28]. These dysfunctions include loss of thrombomodulin, von Willebrand factor increase, vascular and epithelial C3 deposits, and increased C3a receptor expression [28]. Spike protein is likely detrimental to the respiratory system, which is not surprising given that SARS-CoV-2 is a respiratory virus.

Gastrointestinal system: The gastrointestinal tract is not immune to spike protein's effects either. The protein can activate inflammatory signaling and cytokine production in intestinal epithelial cells [26]. This may result in gastrointestinal injury and digestive problems. Further, spike protein has been implicated in provoking intestinal inflammation by promoting VEGF production in enterocytes [29]. VEGF production was increased by activating Ras-Raf-MEK-ERK signaling and inducing intestinal permeability [29]. By triggering increased permeability and inflammation, spike protein may lead to leakage of toxins into circulation, resulting in negative systemic effects. Moreover, there has been a clinical case where the spike protein was identified in the intestinal cells of a patient diagnosed with COVID-19 multisystem inflammatory syndrome [30]. This finding provides evidence that spike protein may play a vital role in inducing inflammation in COVID-19 patients, particularly in the gut.

Immunological system: SARS-CoV-2 spike protein also exerts damaging effects on the immune system, particularly in monocytes, which are part of the innate immune system and are a type of white blood cell that helps destroy infected cells and regulate cellular homeostasis [31]. Recombinant spike protein induces significant monocyte structural abnormalities, including cytoplasmic intrusions, aberrant nuclei, dysmorphia, granulation, vacuolization, and platelet phagocytosis [32]. The deleterious effects were more severe with Alpha and Delta variant SARS-CoV-2 recombinant spike proteins compared to Omicron, indicating a correlation with clinical disease severity [32]. Moreover, spike protein may cause cytokine release syndrome, a hyper-inflammatory response, by activating human monocytes to produce cytokines [33]. This may lead to inflammatory conditions that cause multiple organ dysfunction. Ait-Belkacem et al. found that spike protein induced differing effects on monocytes between children and adults [34]. In this study, the authors concluded that spike protein may cause lower monocyte and B cell activation, along with higher inflammation, in adults compared to children [34]. Concerningly, spike protein can persist in monocytes for an indefinite period of time. In patients with post-acute sequelae SARS-CoV-2 infection (PASC or long COVID) and previous severe COVID-19 infection, spike protein was found in CD16+ monocytes for at least 15 months after initial infection [35]. The researchers used mass spectrometry techniques to confirm the presence of spike protein. Finally, spike protein was found to damage hematopoietic stem and progenitor cells (HSPCs) through hyperactivation of Nlrp3 inflammasome, resulting in pyroptosis, or cell death triggered by inflammation [36]. From the available data, it can be concluded that SARS-CoV-2 spike protein is detrimental to the immunological system by possibly causing long-term immune dysfunction.

Differentiating the SARS-CoV-2 Spike Protein From Vaccine-Generated Spike Protein

Vaccine-encoded spike protein differs from the viral version by the replacement of the amino acids lysine and valine with two or six proline amino acids, which stabilizes the spike conformation in an inactive prefusion state [37,38]. The purpose of this stabilization is that it makes the spike protein more immunogenic than the viral version, thus making it more effective at inducing antibody production. Even though stabilized prefusion spike protein is effective at invoking an immune response, it may not be safer than its viral counterpart. Spike protein from the virus is cleaved at the S1/S2 boundary, thus causing smaller fragments. mRNA vaccines encode for the full-length prefusion protein that is not cleaved [4]. These structural differences may contribute to spike protein pathogenicity. Yonker et al. [16] showed that a vaccine-generated spike was found unbound by antibodies in those with post-vaccine myocarditis and not in controls. Furthermore, Baumeier et al. [17] found stabilized prefusion spike protein in cardiomyocytes via biopsy in patients with vaccine-induced myocarditis while ruling out infectious causes. Brogna et al. [39] found the stabilized prefusion spike protein in human subjects for up to six months after vaccination using mass spectrometry techniques. This extensive persistence of vaccine-generated spike protein in humans is also seen with COVID-19 infection spike protein, as shown by Patterson et al.'s [35] and Craddock et al.'s [40] findings that spike protein can be found in human subjects for at least a year after acute infection in those with post-acute sequelae. Perry et al. [12] concluded that spike protein is pathogenic from both SARS-CoV-2 and vaccine mRNA. These data indicate that the stabilized prefusion spike proteins can persist in the human body, be difficult to break down by natural human mechanisms, and may possess similar harmful characteristics to the viral type.

Rationale for SARS-CoV-2 and COVID-19 Vaccine Spike Protein Detoxification

Given the deleterious effects of spike protein [13-36] and its persistence [35,39,40] in those with post-acute sequelae after SARS-CoV-2 infection and COVID-19 vaccination, strategies to eliminate spike protein from the human body may be warranted to reduce the burden of disease from COVID-19 and vaccine injury syndromes. As revealed by autopsy, some severe COVID-19 vaccine injury syndromes that may be directly or indirectly caused by spike protein include sudden cardiac death, myocardial infarction, myocarditis, pericarditis, pulmonary embolism, vaccine-induced immune thrombotic thrombocytopenia, brain

hemorrhage, multi-organ failure, respiratory failure, and cytokine storm [12,41]. Some complications from the SARS-CoV-2 virus and COVID-19 that may be caused by spike protein include myocardial infarction, pulmonary embolism, deep vein thrombosis, aortic thrombosis, acute limb ischemia, and disseminated intravascular coagulation [42]. As mentioned earlier, spike protein was found residing in heart tissue in patients with post-vaccination myocarditis [17] and in blood clots in patients with acute ischemic stroke [21]. Most of the injury syndromes involve cardiac and thrombotic events that have been associated with spike protein, revealing it as a prime target for treatment. At the time of writing, specific and widely established treatment protocols to remove spike protein from the human body are non-existent. Thus, it is essential to conduct further investigation and create effective treatment strategies for the management of spike protein-related pathologies.

McCullough Protocol: Base Spike Detoxification

McCullough et al. recently published (2023) the first rationale for spike protein detoxification, called the McCullough protocol: base spike detoxification [43], that holds considerable promise. The protocol includes a natural triple-agent oral regimen of nattokinase, bromelain, and curcumin that provides four putative, primary mechanisms of action: 1) proteolytic degradation of spike protein, 2) inhibition of inflammation from spike protein and its fragments in tissues, 3) dissolution of microthrombi, and 4) anticoagulation. Figure 3 illustrates the base spike detoxification protocol and its mechanisms.

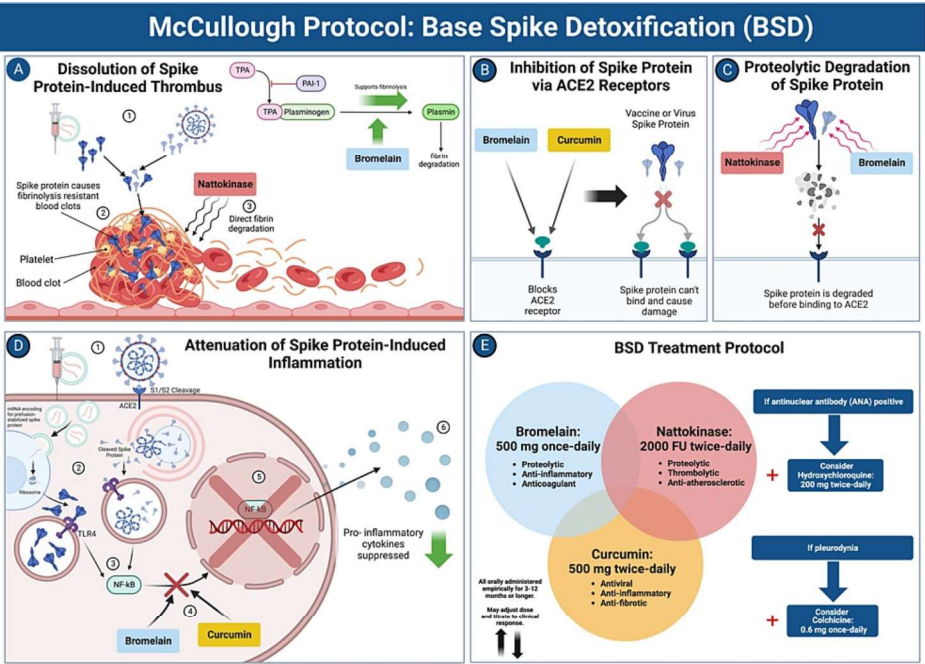


FIGURE 3: McCullough Protocol: Base Spike Detoxification (BSD).
A: Dissolution of spike protein-induced thrombus. Nattokinase directly degrades fibrinolysis-resistant fibrin (from spike protein), and bromelain upregulates fibrinolysis. B: Inhibition of spike protein via ACE2 receptors. Bromelain and curcumin block the ACE2 receptor, preventing spike protein from binding. C: Proteolytic degradation of spike protein. Nattokinase and bromelain degrade spike proteins, rendering them inactive. D: Attenuation of spike protein-induced inflammation. Bromelain and curcumin downregulate the NF-κB signaling pathway induced by spike protein, leading to the suppression of inflammatory molecules. E: BSD treatment protocol. The full treatment regimen and the addition of other compounds based on clinical indication are illustrated.

Abbreviations: TPA = tissue plasminogen activator, PAI-1 = plasminogen activator inhibitor-1, ACE2 = angiotensin converting enzyme-2, NF-κB = nuclear factor kappa B, S1/S2 = spike protein subunits S1/S2, TLR4 = toll-like receptor 4.

*Created with BioRender.com. Panel E adapted from McCullough et al. [43].

The full base spike detoxification protocol is as follows [43]:

Bromelain 500 mg once a day, nattokinase 2,000 FU twice a day, and curcumin 500 mg twice a day. The regimen is to be followed for 3-12 months or more, depending on disease resolution progress. These are initial dosages and may be adjusted in accordance with the tolerability and severity of injury syndrome. Because doses are far below known limits of safety, dose escalation would be reasonable if there are residual symptoms after three months of therapy. If ANA is positive and an autoimmune disease is suspected, prescribed hydroxychloroquine 200 mg twice

a day should be added to the regimen. If pleurodynia or atypical chest pain is present, prescribed colchicine 0.6 mg once a day should be used in addition.

Nattokinase, a proteolytic enzyme derived from the fermentation of soybeans by *Bacillus subtilis* natto [44], has been traditionally used in Japan for cardiovascular benefits and possesses direct fibrinolytic activity by hydrolyzing fibrin and plasmin substrate, meaning it can be used to dissolve blood clots [45]. This makes nattokinase a vital tool to dissolve spike protein-induced blood clots, as they may contain fibrin that is resistant to fibrinolysis [19]. Additionally, nattokinase has a potent degrading effect on the spike protein of SARS-CoV-2 [46,47]. Figure 4 shows the degradative effect of nattokinase on spike protein on the cell surface [47]. The extensive persistence of spike protein [35,39,40] indicates that external drugs, specifically protein-degrading enzymes such as nattokinase, may be needed to degrade it in the human body. Kurosawa et al. demonstrated that, in humans, D-dimer levels were notably increased at both six and eight hours, while blood fibrin/fibrinogen breakdown products showed a significant rise four hours post the intake of a one-time oral dose of 2,000 FU (100 mg) ($p < 0.05$) [48]. Based on these findings, a suggested initial dosage might be 2,000 FU administered twice daily. Nattokinase has been shown to be largely safe other than possible excessive bleeding especially when combined with other medications [48].

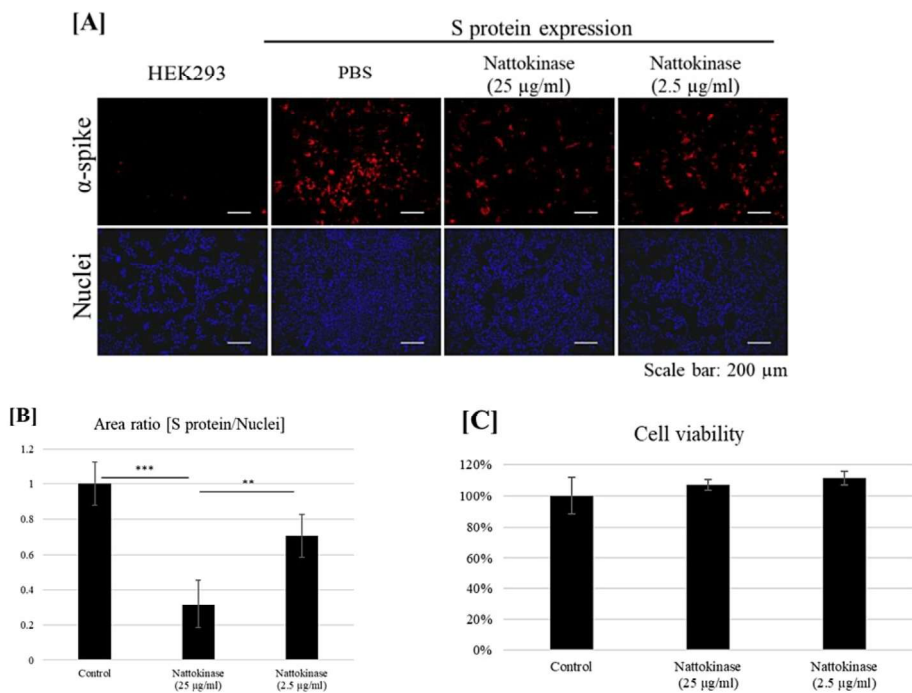


FIGURE 4: Degradative Effect of Nattokinase on Spike Protein.

(A) Degradative effect of nattokinase on spike protein on the cell surface. Spike-pcDNA3.1 was transfected with HEK293 cells and incubated for 9 h. After incubation, nattokinase (25 and 2.5 µg/mL) was added to the culture medium and further incubated for 13 h. Cells were fixed, and immunofluorescent analysis was performed. S protein on the cell surface was stained with an anti-spike protein antibody (red), and the nucleus was stained with DAPI (blue). (B) Ratio of the S protein area to the nucleus positive area. Three images per sample were captured, and S protein/nucleus positive areas were calculated. Data are shown as mean + SD, and the p-value was determined by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using R software (R-3.3.3 for Windows) (** $p < 0.01$; *** $p < 0.001$). (C) Cell viability was evaluated by an MTT assay. Indicated nattokinase was added to the culture medium and incubated for 13 h; an MTT assay was performed.

*Figure and legend reprinted from Tanikawa et al. [47]. The legend title has been slightly adapted. Permission to use this figure has been granted in accordance with the open access Creative Common CC BY 4.0 license.

Bromelain, a proteolytic enzyme sourced from the stem of pineapples [49], has been traditionally hailed for its healing and anti-inflammatory capabilities, particularly in cases of arthritis and injury. Of significance is bromelain's anticoagulant activity. It downregulates PGE-2 and thromboxane A2, promoting a relative prostacyclin abundance in platelets. Furthermore, it aids in fibrinolysis by promoting plasminogen conversion to plasmin and inhibiting platelet aggregation [50]. Kritis et al. demonstrated that bromelain can obstruct SARS-CoV-2's entry into cells by cleaving its spike protein and reducing ACE2 and TMPRSS2 expression [51]. This enzyme can also hydrolyze glycosidic linkages, which comprise spike protein's glycosidic shield that helps protect it from immune responses [52]. To attenuate inflammation, bromelain, in part, downregulates the pro-inflammatory prostaglandin E-2 (PGE-2) through inhibition of NF- κ B and cyclooxygenase 2 and inhibits inflammatory mediators [51]. Thus, bromelain exerts multiple mechanisms of

action against spike protein's toxic effects and persistence. Bromelain has been used as a daily dosage of 200-2,000 mg; thus, 500 mg is a suggested initial dose [53]. Bromelain is mainly safe with low toxicity, but it can amplify bleeding risk and affect the absorption rate of several medications, potentially leading to drug interactions [54].

Curcumin, a polyphenol extracted from turmeric, is renowned for its anti-inflammatory properties and its ability to modulate inflammation during viral infections. Curcumin also supports fibrinolysis and the process of anticoagulation [51]. Beyond its traditionally recognized benefits, curcumin has shown promising antiviral actions against a wide range of viruses, including influenza, hepatitis, and notably, SARS-CoV-2 [55]. It achieves this by obstructing the spike protein's binding sites (ACE2 receptors and TMPRSS-2). Curcumin's anti-inflammatory effects are realized through inhibiting NF- κ B signaling [56]. An in-silico study found that curcumin can inhibit the spike protein of the Omicron variant through interaction with its amino acids [57]. Randomized trials have consistently indicated decreases in high-sensitivity C-reactive protein (hs-CRP) and other markers of inflammation in situations involving spike protein-induced infections or injuries [58,59]. Curcumin is non-toxic at doses up to 8,000 mg a day [60]. Large doses, particularly with ill-absorbed formulations, can lead to gastric complications [61]. Enhanced absorption of curcumin is achieved in combination with piperine, or with nano or liposomal formulations, which are available as over-the-counter oral supplements. Doses vary widely depending on the formulation, but 500 mg twice a day has been shown to be a common and safe dosage regardless of curcumin type [61].

Hydroxychloroquine, a well-known FDA-approved antimalarial and anti-inflammatory, adds additional support for immunocompromised patients by inhibiting the binding of spike protein to human cells [62]. A real-time meta-analysis of 413 published peer-reviewed studies for hydroxychloroquine as a treatment for COVID-19, including a total of 529,687 patients, shows a statistically significant lower risk for mortality and hospitalization, along with accelerated viral clearance [63]. This effect was the strongest when patients were treated early, indicating the importance of early treatment. Since hydroxychloroquine accelerates viral clearance, it subsequently assists in spike protein removal, and it may be a great addition to base spike detoxification. This compound has been found to be well-tolerated, safe, and not associated with a risk of ventricular arrhythmia at a dose of 200 mg twice a day provided that the expected prolongation of QTc is managed along with other drugs with serial ECGs. Gastrointestinal symptoms may occur [64].

Colchicine, an FDA-approved alkaloid found in the plants *Colchicum autumnale* and *Gloriosa superba*, has been traditionally used in therapeutics for its anti-inflammatory properties [65]. This compound can reduce the risk of myocardial infarction and stroke [66]. Moreover, colchicine may reduce myocardial injury in the presence of spike protein [67]. Pleurodynia has been diagnosed post-COVID-19 vaccination and may be indicative of cardiac inflammation [68]. The COLCORONA trial demonstrated that colchicine was safe and had a favorable impact on COVID-19 and its immediate post-acute sequelae. In patients with PCR-confirmed COVID-19, colchicine lowered the rate of hospitalization and death compared to placebo [69]. A meta-analysis of five randomized trials, including a total of 16,048 patients, found that colchicine decreased COVID-19 severity and decreased C-reactive protein (CRP), indicating its potent anti-inflammatory effect in the presence of spike protein [70]. Thus, the addition of colchicine is indicated when a patient presents with pleurodynia post-COVID-19 vaccination or post-infection. Moreover, 0.5 mg twice daily has been shown to be a safe and effective dosage for the treatment of COVID-19 [65,69,70].

Additional compounds that may assist in spike protein detoxification and degradation include the following:

N-Acetylcysteine (NAC): It dissolves spike protein through the destruction of disulfide bonds and prevents binding at ACE2 [52,71,72].

Glutathione: It disrupts spike protein disulfide bonds [72].

Ivermectin: It binds and inhibits spike protein [73].

Quercetin: It binds and inhibits spike protein [74].

Apigenin: It binds and inhibits spike protein [74].

Nicotine: It disrupts glycosylation on spike protein and blocks possible spike protein-nicotinic cholinergic receptor interaction [75,76].

Emodin: It blocks the spike protein-ACE2 interaction [77].

Fisetin: It binds and inhibits spike protein [78].

Rutin: It binds and inhibits spike protein [79].

Silymarin: It binds and inhibits spike protein [80].

Discussion

We found abundant evidence that SARS-CoV-2 spike protein may cause biological damage in the cardiovascular, hematological, neurological, respiratory, gastrointestinal, and immunological systems [13-36]. Mechanistically, spike protein has been shown to cause dysfunction in many cell types by causing metabolic deteriorations, leading to cell death. Stabilized prefusion (vaccine-derived) spike protein may possess similar harmful mechanisms as viral spike protein. Spike protein is found, in the absence of nucleocapsid, directly in cardiomyocytes and blood clots in patients diagnosed with myocarditis and acute stroke, respectively [17,21]. Vaccine-derived and viral spike proteins have been found in humans for at least 6-15 months after vaccination or infection [35,39,40] in those with post-acute sequelae, indicating spike protein as a possible primary contributing factor to long COVID, with substantial persistence in human systems due to the absence of an innate clearing mechanism. However, more research is needed to further investigate the effect of spike protein in patients with post-acute sequelae after COVID-19 and COVID-19 vaccination. Meanwhile, the current data points to a strong signal to urgently develop spike protein detoxification protocols.

Although methods to degrade or block spike protein have been investigated [46,47,51,52,56,57,62,71-80], there are currently no widely accepted protocols to do this in human subjects. The McCullough protocol: base spike detoxification is the first protocol established to help remove spike protein derived from SARS-CoV-2 infection and vaccination in humans. The three-drug regimen of nattokinase, bromelain, and curcumin was chosen due to their proven safety records, as well as their anti-inflammatory and anti-coagulant properties combined with their synergistic and potent effects in degrading and inhibiting spike protein [44-61]. This protocol may be useful in the attenuation of COVID-19 vaccine-induced injury syndromes and long-term COVID-19 complications.

The addition of other agents that can further assist in the detoxification of spike protein [62-80] may be indicated based on clinical outcomes. Most notably, the addition of N-acetylcysteine (NAC) may add an extra mechanism of action against spike protein to the protocol. NAC has been shown to disrupt disulfide bonds of the spike protein, rendering it unable to bind to the ACE2 receptor [71,72]. Moreover, the combination of NAC with bromelain has been shown to synergistically disrupt spike protein by breaking glycosidic linkages and disulfide bonds [52]. NAC has a well-established safety profile and is commonly taken at a dose of 600 mg twice a day [81]. Thus, the addition of NAC to the regimen of bromelain, curcumin, and nattokinase may be useful for individuals with severe treatment-resistant COVID-19 or COVID-19 vaccine-related complications. Since all of these compounds can reduce blood clotting, patients should be counseled and monitored for bleeding complications, including easy bruising, nasal mucosal bleeding, and gastrointestinal hemorrhage. Self-administration is not advised without physician oversight.

The base spike detoxification protocol was devised based on the best evidence currently available. No therapeutic claims can be made until large-scale, prospective, randomized, double-blind, placebo-controlled trials are completed. On November 15, 2023, we searched clinicaltrials.gov and found no planned or ongoing trials with nattokinase or bromelain in the treatment of post-acute sequelae after COVID-19 or vaccination. However, there is a planned trial to assess the effect of curcumin, in conjunction with boswellia and vitamin C, on long COVID [82]. Thus, base spike detoxification is an important advancement in the development of testable hypotheses for future trials assessing treatments for SARS-CoV-2 infection and post-COVID-19 vaccination injury syndromes.

Conclusions

SARS-CoV-2 spike protein is a highly persistent, potentially pathogenic substance that may incite inflammation and tissue damage in almost all organ systems, resulting in post-acute sequelae. The vaccine-generated spike protein is different from the viral type, but both have been associated with deleterious effects and persistence in biological systems. Thus, therapeutics that target spike protein may be essential in treating COVID-19, its long-term effects, and possibly COVID-19 vaccine injury syndromes. Base spike detoxification is a promising proposal designed to theoretically attenuate spike protein and its associated damage. However, more pre-clinical and clinical research is needed culminating with large-scale, prospective, randomized, double-blind, placebo-controlled randomized trials to fully assess safety and efficacy. Moreover, further investigation is essential to ensure vaccine-produced, stabilized prefusion spike protein safety and half-life in humans and that it does not possess the same deleterious effects as the viral spike protein.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Acquisition, analysis, or interpretation of data: Nicolas Hulscher, Peter A. McCullough

Drafting of the manuscript: Nicolas Hulscher, Peter A. McCullough

Critical review of the manuscript for important intellectual content: Nicolas Hulscher, Brian C. Procter, Cade Wynn, Peter A. McCullough

Disclosures

Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** PM receives partial salary support and holds an equity position in The Wellness Company, Boca Raton, Florida, which had no role in this study.

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Clinical Approach to Post-acute Sequelae After COVID-19 Infection and Vaccination

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Abstract

The spike protein of SARS-CoV-2 has been found to exhibit pathogenic characteristics and be a possible cause of post-acute sequelae after SARS-CoV-2 infection or COVID-19 vaccination. COVID-19 vaccines utilize a modified, stabilized prefusion spike protein that may share similar toxic effects with its viral counterpart. The aim of this study is to investigate possible mechanisms of harm to biological systems from SARS-CoV-2 spike protein and vaccine-encoded spike protein and to propose possible mitigation strategies. We searched PubMed, Google Scholar, and 'grey literature' to find studies that (1) investigated the effects of the spike protein on biological systems, (2) helped differentiate between viral and vaccine-generated spike proteins, and (3) identified possible spike protein detoxification protocols and compounds that had signals of benefit and acceptable safety profiles. We found abundant evidence that SARS-CoV-2 spike protein may cause damage in the cardiovascular, hematological, neurological, respiratory, gastrointestinal, and immunological systems. Viral and vaccine-encoded spike proteins have been shown to play a direct role in cardiovascular and thrombotic injuries from both SARS-CoV-2 and vaccination. Detection of spike protein for at least 6-15 months after vaccination and infection in those with post-acute sequelae indicates spike protein as a possible primary contributing factor to long COVID. We rationalized that these findings give support to the potential benefit of spike protein detoxification protocols in those with long-term post-infection and/or vaccine-induced complications. We propose a base spike detoxification protocol, composed of oral nattokinase, bromelain, and curcumin. This approach holds immense promise as a base of clinical care, upon which additional therapeutic agents are applied with the goal of aiding in the resolution of post-acute sequelae after SARS-CoV-2 infection and COVID-19 vaccination. Large-scale, prospective, randomized, double-blind, placebo-controlled trials are warranted in order to determine the relative risks and benefits of the base spike detoxification protocol.

Categories: Internal Medicine, Infectious Disease, Therapeutics

Keywords: medicine, treatment protocol, long covid, post-acute sequelae, covid-19 vaccines, covid-19 treatment, spike glycoprotein, spike protein detoxification, sars-cov-2 spike protein, sars-cov-2

Introduction And Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly contagious and pathogenic virus that emerged in late December 2019. It causes an acute respiratory disease named "coronavirus infectious disease-2019" (COVID-19). The World Health Organization (WHO) declared the outbreak of SARS-CoV-2 a pandemic on March 11, 2020 [1]. As of November 15, 2023, there have been over 700 million reported cases of COVID-19 and almost seven million deaths [2]. To combat this virus, novel gene-based vaccines were developed that utilize viral vector (AstraZeneca - ChAdOx1 nCoV-19; Johnson and Johnson - Ad26.CoV2.S) and mRNA (Moderna - mRNA 1273; Pfizer-BioNTech - BNT162b2) platforms to encode for spike protein, which elicits an immune response that generates antibodies [3]. At the time of writing, about 70% of the global population have received one or more doses of a COVID-19 vaccine [2].

The SARS-CoV-2 spike protein plays a critical role in the virus's infection process. Spike protein emerges from the viral surface with a clove-like shape, facilitating SARS-CoV-2's binding to host cells. This protein, which is a class I fusion transmembrane glycoprotein, is cleaved into two main sections by host proteases: the S1 and S2 subunits. The S1 subunit has a receptor-binding domain that is responsible for recognizing and binding to the host cell receptor ACE-2. Moreover, the S1 subunit is a site of drastic evolutionary pressure. Mutations of the spike protein lead to new strains that can re-infect those who have already recovered from a previous SARS-CoV-2 variant. These new variants can be more infectious than previous ones due to a higher affinity for the ACE-2 receptor. The S2 subunit is responsible for the fusion of host and viral membranes. Most of the vaccines deployed to prevent COVID-19 illness utilize a full-length spike protein stabilized in a pre-fusion state to generate immunity against SARS-CoV-2 [4].

Post-acute sequelae (long COVID) after SARS-CoV-2 infection or COVID-19 vaccination is characterized by new or persisting multi-system symptoms months to years after initial infection or vaccination [5,6]. These symptoms can range from generalized anxiety disorder and difficulty concentrating to fatigue and muscle weakness. The true incidence and prevalence of long COVID after infection are not fully known, but it has

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been approximated to be quite common. It is been estimated that 50% of those who were infected with SARS-CoV-2 develop long COVID, which equates to hundreds of millions of individuals [7]. Research on the prevalence of post-COVID vaccination syndrome is limited; however, a prospective cohort study found that 52.8% of individuals developed post-COVID vaccination syndrome for at least one month after primary COVID-19 vaccination. In this study, the prevalence increased to 83.9% among those who received booster doses [8]. A retrospective cross-sectional study found a post-COVID vaccination syndrome prevalence of 26.2% after COVID-19 vaccination, with higher rates seen in individuals who received Moderna vaccines compared to Pfizer vaccines [9]. These data indicate that post-acute sequelae after COVID-19 vaccination are clinically relevant; however, more research is required to find the true incidence and prevalence.

Concerns have been raised that suggest spike protein is involved in various pathologies, including post-acute sequelae after SARS-CoV-2 infection and COVID-19 vaccination [10-12]. This may be due to spike protein exhibiting harmful properties, including inflammation, endothelial tissue damage, and brain damage [10]. Moreover, spike protein has been associated with thrombotic endothelialitis, endothelial inflammation, hyperactivated platelets, and fibrinoid microclots in those with long COVID [11]. Parry et al. found that spike protein is detrimental to many organ systems by analyzing autopsies and biopsies of spike-infected tissues [12]. If spike protein is found to be harmful to human health, it could lead to the development of new treatments that aim to reduce tissue damage caused by COVID-19. Additionally, this discovery could prompt research into ways to prevent or treat health issues resulting from COVID-19 vaccines. This is particularly relevant since COVID-19 vaccines work by using spike protein to trigger an immune response. The aim of this study is to investigate possible mechanisms of harm on biological systems from SARS-CoV-2 spike protein and vaccine-encoded spike protein for the purpose of exploring causes of COVID-19-associated harm, COVID-19 vaccine injury syndromes, and potential therapeutic strategies.

Review

Methods

Article Layout

First, we categorized the findings of spike protein's deleterious effects based on the organ system targeted: cardiovascular, hematological, neurological, respiratory, gastrointestinal, and immunological systems. Additionally, we detailed the differences between viral spike protein and vaccine-generated spike protein. Lastly, we explored potential detoxification strategies, including the McCullough protocol: base spike detoxification.

Search Strategy

We performed a literature search using the PubMed and Google Scholar databases. We also searched the grey literature, which is research produced by organizations that are not affiliated with traditional commercial or academic publishing and distribution channels. The primary search terms were "SARS-CoV-2 spike protein", "spike protein effects", "COVID-19 vaccine spike protein", "cardiovascular", "hematological", "neurological", "respiratory", "gastrointestinal", "immunological", "detoxification", "treatment", "degradation", "curcumin", "bromelain", and "nattokinase". These terms were used in varying combinations with filters applied for English language articles. All references cited in selected articles were manually searched to identify any additional relevant studies.

Study Selection and Eligibility Criteria

For investigating the harmful effects of spike protein, articles that specifically assessed the direct or indirect impacts of the spike protein on biological systems, in isolation or in relation to the SARS-CoV-2 virus or COVID-19 vaccination, were considered. We also included papers that helped assess the difference between viral spike protein and vaccine-generated spike protein. Lastly, we sought to include any published treatment protocols or compounds that had signals of benefit and acceptable safety profiles that may assist in spike protein detoxification. Original articles, in vitro studies, animal studies, and clinical studies published were included. Case reports, editorials, letters to the editor, and reviews were excluded unless they provided original results or synthesized available evidence.

Results

Deleterious Effects of Spike Protein by Organ System

Cardiovascular system: The cardiovascular system has been identified as one of the primary targets of the SARS-CoV-2 spike protein. Disruptions in human cardiac pericytes, a type of cell that wraps around the endothelial cells of capillaries and venules, have been observed by the exposure of the spike protein. These disruptions include increased migration, secretion of inflammatory molecules that are seen in the cytokine storm, and creation of apoptotic factors that cause cell death [13]. Furthermore, spike protein has been found to hinder mitochondrial functions in human cardiomyocytes, potentially leading to energy deficits and reduced cardiac output. This effect may be due to spike protein inducing negative mitochondrial

membrane potential, increasing intracellular calcium, and increasing levels of reactive oxygen species (ROS). Figure 1 illustrates this process [14]. The spike protein may also induce cardiomyocyte fusion, which may amplify the risk of arrhythmias, and irregular heart rhythms that can be life-threatening. In this study, spike protein induced delayed depolarizations, altered beating frequency, and induced calcium influx problems [15]. In the context of vaccination, there have been reports of spike protein's detection in patients diagnosed with COVID-19 vaccine-induced myocarditis [16,17]. From the available evidence, spike protein displays obvious toxic effects on the cardiovascular system.

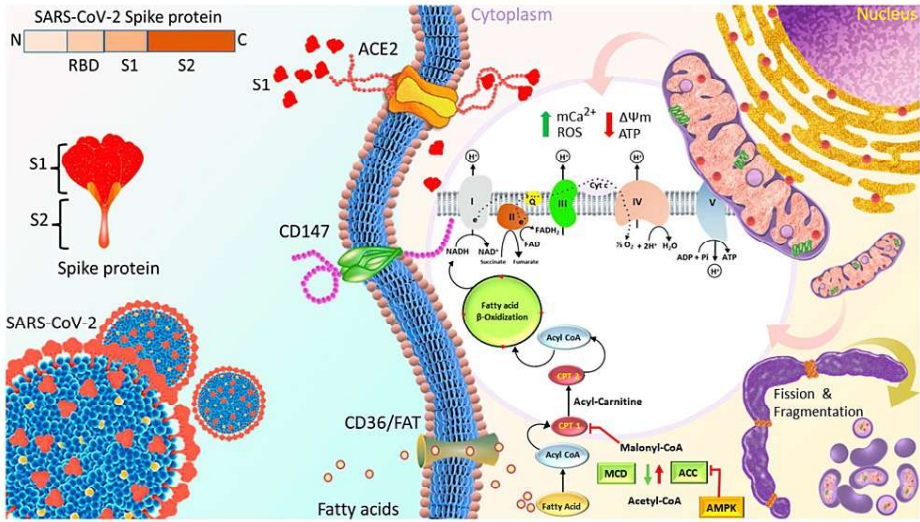


FIGURE 1: Proposed Mechanisms Through Which S1 of Spike Protein Induced Cardiac Mitochondrial Dysfunction, Which Leads to Cardiac Injury in COVID-19 Patients.

Spike protein is the glycosylated protein that covers the surface of SARS-CoV-2 and binds to the host ACE2 receptor to mediate the viral cell entry. It is composed of S1 and S2 subunits that are responsible for ACE2 binding and membrane fusion, respectively. S1 possibly binds to ACE2 on the AC16 membrane and is then internalized into the cytosol and localized in organelles, such as mitochondria, which induces the transient increase in fatty acids transport and uptake for biogenetics, $\Delta\Psi_m$, and permanent mCa²⁺, and disrupts $\Delta\Psi_m$ later, finally impairing mitochondrial function and promoting ROS production. In turn, ROS further exacerbates mitochondrial function and mitochondrial fragmentation. Moreover, S1 causes downregulation of TOM20; this effect might inhibit the pathways leading to mitochondrial biogenesis.

Abbreviations: ACE2 = angiotensin-converting enzyme 2, FAT = fatty acid translocase, CPT1/2 = Carnitine palmitoyltransferase 1/2, MCD = Malonyl-CoA decarboxylase, ACC = acetyl-CoA carboxylase, AMPK = AMP-activated protein kinase, mCa²⁺ = mitochondrial calcium, $\Delta\Psi_m$ = mitochondrial membrane potential, ROS = reactive oxygen species.

*Figure and legend reprinted from Huynh et al. [14]. The legend title has been slightly adapted. Permission to use this figure has been granted in accordance with the open access Creative Common CC BY 4.0 license.

Hematological system: The hematological system, which includes blood and its components, is also targeted by the spike protein. The protein has been shown to incite inflammation in endothelial cells through a cascade involving integrin $\alpha 5 \beta 1$ and the NF- κ B signaling pathway. This effect was also seen in vivo by intravenous inoculation of spike protein, which resulted in an increased expression of leukocyte adhesion molecules, coagulation factors, and proinflammatory cytokines in the lung, liver, kidney, and eye [18]. This inflammation can promote clot formation and hyperpermeability of the endothelial cell monolayer. Adding to the toxic effects, spike protein may transform fibrin into a form that resists fibrinolysis, making it harder for the body to dissolve blood clots. To do this, spike protein may induce structural changes to fibrin, complement 3, and prothrombin proteins [19]. These dangerous alterations can help explain the mechanism behind microclotting seen in COVID-19 patients. The spike protein's affinity to bind competitively to heparan sulfate, an important glycoprotein involved in coagulation, further aggravates coagulation and thrombosis pathologies [20]. In this study, researchers injected a zebrafish model with spike protein at a similar level as in a critically ill COVID-19 patient. They found that this directly induced blood coagulation and thrombosis [20]. Perhaps most concerning is the finding that free spike protein, in the absence of SARS-CoV-2 RNA and nucleocapsid, was found within blood clots in patients diagnosed with acute ischemic stroke (Figure 2) [21]. As with the cardiovascular system, spike protein exhibits a wide array of harmful effects on the hematological system.

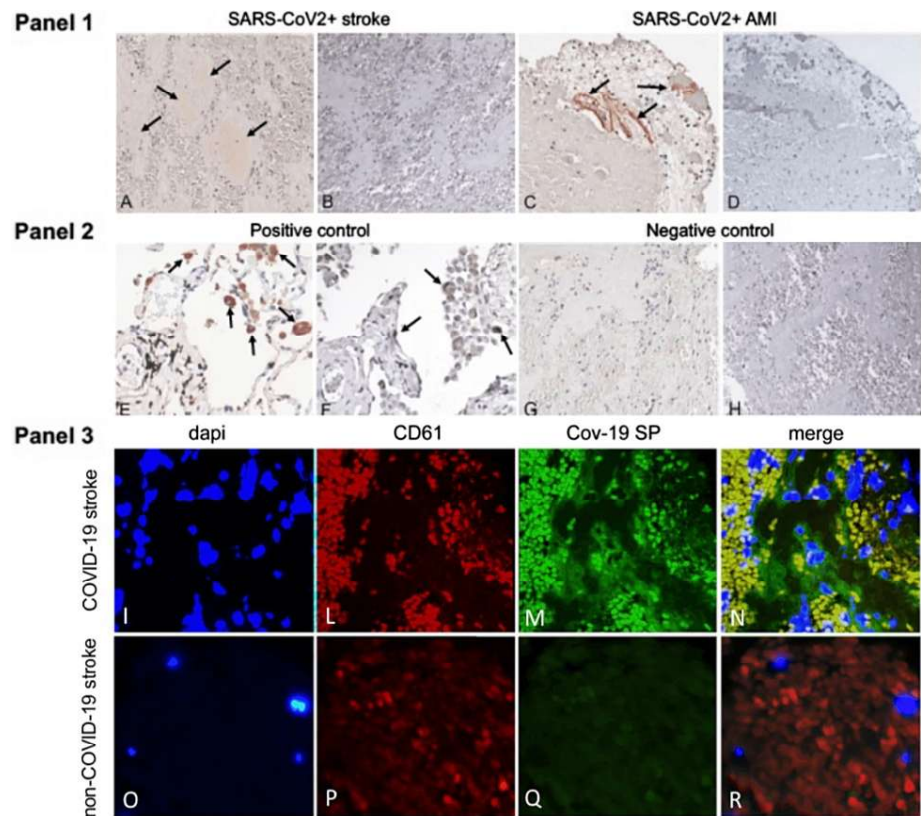


FIGURE 2: Arterial Thrombi From COVID-19 + Patients Contain SARS-CoV-2 Spike Protein But Not Nucleocapsid Protein.

Panel 1. Immunostaining positive for SARS-CoV-2 spike protein (SP) (arrows) in representative thrombotic material from COVID-19 + patients, retrieved from cerebral (A) and coronary (C) arteries. Immunohistochemistry for nucleocapsid protein (NP) was negative in the same samples (B-D). Panel 2. Representative immunohistochemical staining positive for SP (E) and NP (F) (arrows) in the lung of a patient affected by COVID-19 (positive control). Representative immunostaining negative for SP (G) and NP (H) in a thrombus retrieved from the middle cerebral artery of a patient not affected by COVID-19 (negative control). Original magnification 20X. Panel 3. Double immunofluorescence of thrombotic material retrieved from COVID-19 and non-COVID-19 patients' cerebral arteries. In the COVID-19 thrombus, platelets are co-stained with anti-CD61 (red-L,P) and anti-SARS-CoV-2 spike protein (SP) antibodies (green-M,Q), emitting yellow signals in the merged panel (N), while in the control (non-COVID thrombus), only the red CD61 signal is observed (R).

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Neurological system: Neurological implications of the spike protein are particularly concerning. Spike protein has been demonstrated to compromise the blood-brain barrier's integrity through RhoA activation, which can potentially allow harmful substances from the bloodstream to enter the brain [22]. This disruption is possibly achieved by upregulated ACE2 expression. Animal studies have revealed cognitive deficits and anxiety-like behaviors in mice following exposure to the spike protein, possibly due to non-cell autonomous death of neurons in the hippocampus [23]. Cell death of neurons was not directly caused by exposure to spike protein, but rather by spike protein's activation of glial cells, leading to neurotoxicity [23]. On a cellular level, there are indications of spike protein possibly causing deleterious changes in molecular delivery and metabolic functions in brain endothelial cells [24]. In this paper, spike protein induced mitochondrial damage in brain cells, as also seen in cardiomyocytes [14,24]. Some experimental models even suggest that spike protein might be a contributing factor in long-term cognitive dysfunctions that mimic post-COVID-19 syndromes [25]. Corroborating the study mentioned earlier [23], Fontes-Dantas et al. found that brain infusion of spike protein induced neuroinflammation and hippocampal microgliosis, or over-activation of glial cells, by engulfment of synapses [25]. These data indicate that spike protein exerts potent harmful effects on the neurological system by damaging blood-brain barrier function and destroying neurons.

Respiratory system: Spike protein also targets the respiratory system. Spike protein has been shown to stimulate inflammatory pathways in human lung cells, specifically through MAPK and NF- κ B activation [26]. This inflammatory cascade occurred independent of the virus, indicating that spike protein may have been the sole cause. Spike protein's influence extends to lung macrophages, where it triggers their activation [27]. The activation of lung macrophages was coupled with the promotion of efficient phagocytosis and

dysfunction of intracellular calcium concentrations [27]. Such activations can exacerbate inflammatory responses in the lungs and may cause lung injury. Moreover, spike protein can induce dysfunctions in lung endothelial cells, leading to thrombo-inflammation, largely contingent on the C3a/C3a receptor signaling [28]. These dysfunctions include loss of thrombomodulin, von Willebrand factor increase, vascular and epithelial C3 deposits, and increased C3a receptor expression [28]. Spike protein is likely detrimental to the respiratory system, which is not surprising given that SARS-CoV-2 is a respiratory virus.

Gastrointestinal system: The gastrointestinal tract is not immune to spike protein's effects either. The protein can activate inflammatory signaling and cytokine production in intestinal epithelial cells [26]. This may result in gastrointestinal injury and digestive problems. Further, spike protein has been implicated in provoking intestinal inflammation by promoting VEGF production in enterocytes [29]. VEGF production was increased by activating Ras-Raf-MEK-ERK signaling and inducing intestinal permeability [29]. By triggering increased permeability and inflammation, spike protein may lead to leakage of toxins into circulation, resulting in negative systemic effects. Moreover, there has been a clinical case where the spike protein was identified in the intestinal cells of a patient diagnosed with COVID-19 multisystem inflammatory syndrome [30]. This finding provides evidence that spike protein may play a vital role in inducing inflammation in COVID-19 patients, particularly in the gut.

Immunological system: SARS-CoV-2 spike protein also exerts damaging effects on the immune system, particularly in monocytes, which are part of the innate immune system and are a type of white blood cell that helps destroy infected cells and regulate cellular homeostasis [31]. Recombinant spike protein induces significant monocyte structural abnormalities, including cytoplasmic intrusions, aberrant nuclei, dysmorphia, granulation, vacuolization, and platelet phagocytosis [32]. The deleterious effects were more severe with Alpha and Delta variant SARS-CoV-2 recombinant spike proteins compared to Omicron, indicating a correlation with clinical disease severity [32]. Moreover, spike protein may cause cytokine release syndrome, a hyper-inflammatory response, by activating human monocytes to produce cytokines [33]. This may lead to inflammatory conditions that cause multiple organ dysfunction. Ait-Belkacem et al. found that spike protein induced differing effects on monocytes between children and adults [34]. In this study, the authors concluded that spike protein may cause lower monocyte and B cell activation, along with higher inflammation, in adults compared to children [34]. Concerningly, spike protein can persist in monocytes for an indefinite period of time. In patients with post-acute sequelae SARS-CoV-2 infection (PASC or long COVID) and previous severe COVID-19 infection, spike protein was found in CD16+ monocytes for at least 15 months after initial infection [35]. The researchers used mass spectrometry techniques to confirm the presence of spike protein. Finally, spike protein was found to damage hematopoietic stem and progenitor cells (HSPCs) through hyperactivation of Nlrp3 inflammasome, resulting in pyroptosis, or cell death triggered by inflammation [36]. From the available data, it can be concluded that SARS-CoV-2 spike protein is detrimental to the immunological system by possibly causing long-term immune dysfunction.

Differentiating the SARS-CoV-2 Spike Protein From Vaccine-Generated Spike Protein

Vaccine-encoded spike protein differs from the viral version by the replacement of the amino acids lysine and valine with two or six proline amino acids, which stabilizes the spike conformation in an inactive prefusion state [37,38]. The purpose of this stabilization is that it makes the spike protein more immunogenic than the viral version, thus making it more effective at inducing antibody production. Even though stabilized prefusion spike protein is effective at invoking an immune response, it may not be safer than its viral counterpart. Spike protein from the virus is cleaved at the S1/S2 boundary, thus causing smaller fragments. mRNA vaccines encode for the full-length prefusion protein that is not cleaved [4]. These structural differences may contribute to spike protein pathogenicity. Yonker et al. [16] showed that a vaccine-generated spike was found unbound by antibodies in those with post-vaccine myocarditis and not in controls. Furthermore, Baumeier et al. [17] found stabilized prefusion spike protein in cardiomyocytes via biopsy in patients with vaccine-induced myocarditis while ruling out infectious causes. Brogna et al. [39] found the stabilized prefusion spike protein in human subjects for up to six months after vaccination using mass spectrometry techniques. This extensive persistence of vaccine-generated spike protein in humans is also seen with COVID-19 infection spike protein, as shown by Patterson et al.'s [35] and Craddock et al.'s [40] findings that spike protein can be found in human subjects for at least a year after acute infection in those with post-acute sequelae. Perry et al. [12] concluded that spike protein is pathogenic from both SARS-CoV-2 and vaccine mRNA. These data indicate that the stabilized prefusion spike proteins can persist in the human body, be difficult to break down by natural human mechanisms, and may possess similar harmful characteristics to the viral type.

Rationale for SARS-CoV-2 and COVID-19 Vaccine Spike Protein Detoxification

Given the deleterious effects of spike protein [13-36] and its persistence [35,39,40] in those with post-acute sequelae after SARS-CoV-2 infection and COVID-19 vaccination, strategies to eliminate spike protein from the human body may be warranted to reduce the burden of disease from COVID-19 and vaccine injury syndromes. As revealed by autopsy, some severe COVID-19 vaccine injury syndromes that may be directly or indirectly caused by spike protein include sudden cardiac death, myocardial infarction, myocarditis, pericarditis, pulmonary embolism, vaccine-induced immune thrombotic thrombocytopenia, brain

hemorrhage, multi-organ failure, respiratory failure, and cytokine storm [12,41]. Some complications from the SARS-CoV-2 virus and COVID-19 that may be caused by spike protein include myocardial infarction, pulmonary embolism, deep vein thrombosis, aortic thrombosis, acute limb ischemia, and disseminated intravascular coagulation [42]. As mentioned earlier, spike protein was found residing in heart tissue in patients with post-vaccination myocarditis [17] and in blood clots in patients with acute ischemic stroke [21]. Most of the injury syndromes involve cardiac and thrombotic events that have been associated with spike protein, revealing it as a prime target for treatment. At the time of writing, specific and widely established treatment protocols to remove spike protein from the human body are non-existent. Thus, it is essential to conduct further investigation and create effective treatment strategies for the management of spike protein-related pathologies.

McCullough Protocol: Base Spike Detoxification

McCullough et al. recently published (2023) the first rationale for spike protein detoxification, called the McCullough protocol: base spike detoxification [43], that holds considerable promise. The protocol includes a natural triple-agent oral regimen of nattokinase, bromelain, and curcumin that provides four putative, primary mechanisms of action: 1) proteolytic degradation of spike protein, 2) inhibition of inflammation from spike protein and its fragments in tissues, 3) dissolution of microthrombi, and 4) anticoagulation. Figure 3 illustrates the base spike detoxification protocol and its mechanisms.

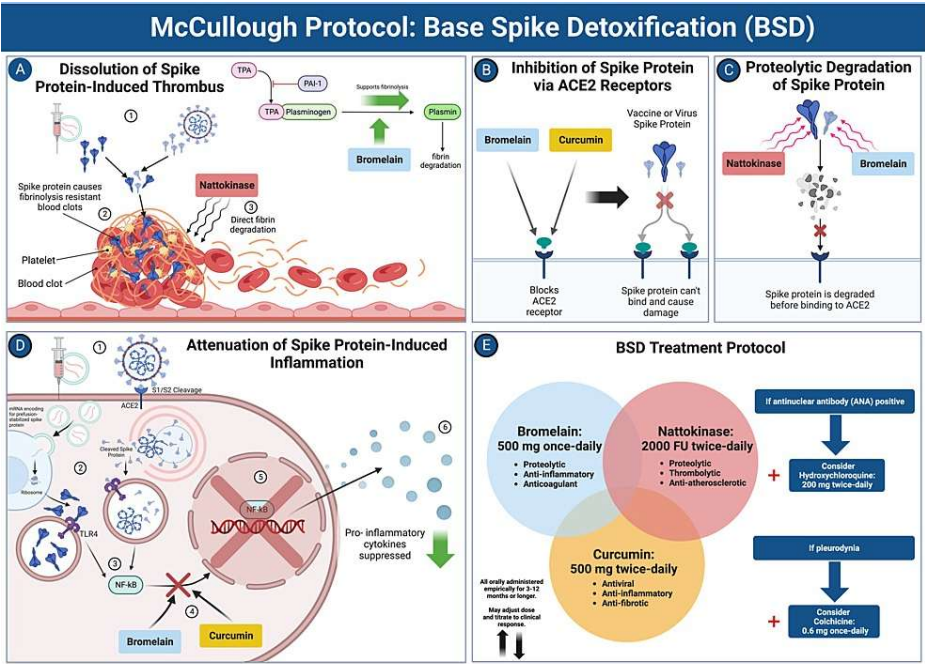


FIGURE 3: McCullough Protocol: Base Spike Detoxification (BSD).

A: Dissolution of spike protein-induced thrombus. Nattokinase directly degrades fibrinolysis-resistant fibrin (from spike protein), and bromelain upregulates fibrinolysis. B: Inhibition of spike protein via ACE2 receptors. Bromelain and curcumin block the ACE2 receptor, preventing spike protein from binding. C: Proteolytic degradation of spike protein. Nattokinase and bromelain degrade spike proteins, rendering them inactive. D: Attenuation of spike protein-induced inflammation. Bromelain and curcumin downregulate the NF- κ B signaling pathway induced by spike protein, leading to the suppression of inflammatory molecules. E: BSD treatment protocol. The full treatment regimen and the addition of other compounds based on clinical indication are illustrated.

Abbreviations: TPA = tissue plasminogen activator, PAI-1 = plasminogen activator inhibitor-1, ACE2 = angiotensin converting enzyme-2, NF- κ B = nuclear factor kappa B, S1/S2 = spike protein subunits S1/S2, TLR4 = toll-like receptor 4.

*Created with BioRender.com. Panel E adapted from McCullough et al. [43].

The full base spike detoxification protocol is as follows [43]:

Bromelain 500 mg once a day, nattokinase 2,000 FU twice a day, and curcumin 500 mg twice a day. The regimen is to be followed for 3-12 months or more, depending on disease resolution progress. These are initial dosages and may be adjusted in accordance with the tolerability and severity of injury syndrome. Because doses are far below known limits of safety, dose escalation would be reasonable if there are residual symptoms after three months of therapy. If ANA is positive and an autoimmune disease is suspected, prescribed hydroxychloroquine 200 mg twice

a day should be added to the regimen. If pleurodynia or atypical chest pain is present, prescribed colchicine 0.6 mg once a day should be used in addition.

Nattokinase, a proteolytic enzyme derived from the fermentation of soybeans by *Bacillus subtilis natto* [44], has been traditionally used in Japan for cardiovascular benefits and possesses direct fibrinolytic activity by hydrolyzing fibrin and plasmin substrate, meaning it can be used to dissolve blood clots [45]. This makes nattokinase a vital tool to dissolve spike protein-induced blood clots, as they may contain fibrin that is resistant to fibrinolysis [19]. Additionally, nattokinase has a potent degrading effect on the spike protein of SARS-CoV-2 [46,47]. Figure 4 shows the degradative effect of nattokinase on spike protein on the cell surface [47]. The extensive persistence of spike protein [35,39,40] indicates that external drugs, specifically protein-degrading enzymes such as nattokinase, may be needed to degrade it in the human body. Kurosawa et al. demonstrated that, in humans, D-dimer levels were notably increased at both six and eight hours, while blood fibrin/fibrinogen breakdown products showed a significant rise four hours post the intake of a one-time oral dose of 2,000 FU (100 mg) ($p < 0.05$) [48]. Based on these findings, a suggested initial dosage might be 2,000 FU administered twice daily. Nattokinase has been shown to be largely safe other than possible excessive bleeding especially when combined with other medications [48].

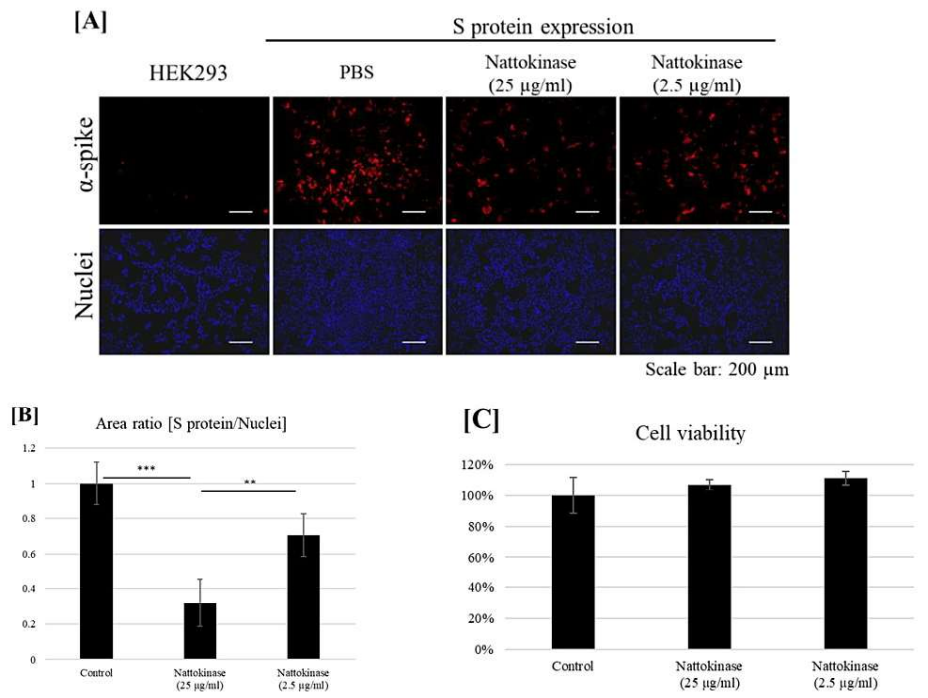


FIGURE 4: Degradative Effect of Nattokinase on Spike Protein.

(A) Degradative effect of nattokinase on spike protein on the cell surface. Spike-pcDNA3.1 was transfected with HEK293 cells and incubated for 9 h. After incubation, nattokinase (25 and 2.5 µg/mL) was added to the culture medium and further incubated for 13 h. Cells were fixed, and immunofluorescent analysis was performed. S protein on the cell surface was stained with an anti-spike protein antibody (red), and the nucleus was stained with DAPI (blue). (B) Ratio of the S protein area to the nucleus positive area. Three images per sample were captured, and S protein/nucleus positive areas were calculated. Data are shown as mean + SD, and the p-value was determined by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using R software (R-3.3.3 for Windows) (** $p < 0.01$; *** $p < 0.001$). (C) Cell viability was evaluated by an MTT assay. Indicated nattokinase was added to the culture medium and incubated for 13 h; an MTT assay was performed.

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Bromelain, a proteolytic enzyme sourced from the stem of pineapples [49], has been traditionally hailed for its healing and anti-inflammatory capabilities, particularly in cases of arthritis and injury. Of significance is bromelain's anticoagulant activity. It downregulates PGE-2 and thromboxane A2, promoting a relative prostacyclin abundance in platelets. Furthermore, it aids in fibrinolysis by promoting plasminogen conversion to plasmin and inhibiting platelet aggregation [50]. Kritis et al. demonstrated that bromelain can obstruct SARS-CoV-2's entry into cells by cleaving its spike protein and reducing ACE2 and TMPRSS2 expression [51]. This enzyme can also hydrolyze glycosidic linkages, which comprise spike protein's glycosidic shield that helps protect it from immune responses [52]. To attenuate inflammation, bromelain, in part, downregulates the pro-inflammatory prostaglandin E-2 (PGE-2) through inhibition of NF- κ B and cyclooxygenase 2 and inhibits inflammatory mediators [51]. Thus, bromelain exerts multiple mechanisms of

action against spike protein's toxic effects and persistence. Bromelain has been used as a daily dosage of 200-2,000 mg; thus, 500 mg is a suggested initial dose [53]. Bromelain is mainly safe with low toxicity, but it can amplify bleeding risk and affect the absorption rate of several medications, potentially leading to drug interactions [54].

Curcumin, a polyphenol extracted from turmeric, is renowned for its anti-inflammatory properties and its ability to modulate inflammation during viral infections. Curcumin also supports fibrinolysis and the process of anticoagulation [51]. Beyond its traditionally recognized benefits, curcumin has shown promising antiviral actions against a wide range of viruses, including influenza, hepatitis, and notably, SARS-CoV-2 [55]. It achieves this by obstructing the spike protein's binding sites (ACE2 receptors and TMPRSS-2). Curcumin's anti-inflammatory effects are realized through inhibiting NF- κ B signaling [56]. An in-silico study found that curcumin can inhibit the spike protein of the Omicron variant through interaction with its amino acids [57]. Randomized trials have consistently indicated decreases in high-sensitivity C-reactive protein (hs-CRP) and other markers of inflammation in situations involving spike protein-induced infections or injuries [58,59]. Curcumin is non-toxic at doses up to 8,000 mg a day [60]. Large doses, particularly with ill-absorbed formulations, can lead to gastric complications [61]. Enhanced absorption of curcumin is achieved in combination with piperine, or with nano or liposomal formulations, which are available as over-the-counter oral supplements. Doses vary widely depending on the formulation, but 500 mg twice a day has been shown to be a common and safe dosage regardless of curcumin type [61].

Hydroxychloroquine, a well-known FDA-approved antimalarial and anti-inflammatory, adds additional support for immunocompromised patients by inhibiting the binding of spike protein to human cells [62]. A real-time meta-analysis of 413 published peer-reviewed studies for hydroxychloroquine as a treatment for COVID-19, including a total of 529,687 patients, shows a statistically significant lower risk for mortality and hospitalization, along with accelerated viral clearance [63]. This effect was the strongest when patients were treated early, indicating the importance of early treatment. Since hydroxychloroquine accelerates viral clearance, it subsequently assists in spike protein removal, and it may be a great addition to base spike detoxification. This compound has been found to be well-tolerated, safe, and not associated with a risk of ventricular arrhythmia at a dose of 200 mg twice a day provided that the expected prolongation of QTc is managed along with other drugs with serial ECGs. Gastrointestinal symptoms may occur [64].

Colchicine, an FDA-approved alkaloid found in the plants *Colchicum autumnale* and *Gloriosa superba*, has been traditionally used in therapeutics for its anti-inflammatory properties [65]. This compound can reduce the risk of myocardial infarction and stroke [66]. Moreover, colchicine may reduce myocardial injury in the presence of spike protein [67]. Pleurodynia has been diagnosed post-COVID-19 vaccination and may be indicative of cardiac inflammation [68]. The COLCORONA trial demonstrated that colchicine was safe and had a favorable impact on COVID-19 and its immediate post-acute sequelae. In patients with PCR-confirmed COVID-19, colchicine lowered the rate of hospitalization and death compared to placebo [69]. A meta-analysis of five randomized trials, including a total of 16,048 patients, found that colchicine decreased COVID-19 severity and decreased C-reactive protein (CRP), indicating its potent anti-inflammatory effect in the presence of spike protein [70]. Thus, the addition of colchicine is indicated when a patient presents with pleurodynia post-COVID-19 vaccination or post-infection. Moreover, 0.5 mg twice daily has been shown to be a safe and effective dosage for the treatment of COVID-19 [65,69,70].

Additional compounds that may assist in spike protein detoxification and degradation include the following:

N-Acetylcysteine (NAC): It dissolves spike protein through the destruction of disulfide bonds and prevents binding at ACE2 [52,71,72].

Glutathione: It disrupts spike protein disulfide bonds [72].

Ivermectin: It binds and inhibits spike protein [73].

Quercetin: It binds and inhibits spike protein [74].

Apigenin: It binds and inhibits spike protein [74].

Nicotine: It disrupts glycosylation on spike protein and blocks possible spike protein-nicotinic cholinergic receptor interaction [75,76].

Emodin: It blocks the spike protein-ACE2 interaction [77].

Fisetin: It binds and inhibits spike protein [78].

Rutin: It binds and inhibits spike protein [79].

Silymarin: It binds and inhibits spike protein [80].

Discussion

We found abundant evidence that SARS-CoV-2 spike protein may cause biological damage in the cardiovascular, hematological, neurological, respiratory, gastrointestinal, and immunological systems [13-36]. Mechanistically, spike protein has been shown to cause dysfunction in many cell types by causing metabolic deteriorations, leading to cell death. Stabilized prefusion (vaccine-derived) spike protein may possess similar harmful mechanisms as viral spike protein. Spike protein is found, in the absence of nucleocapsid, directly in cardiomyocytes and blood clots in patients diagnosed with myocarditis and acute stroke, respectively [17,21]. Vaccine-derived and viral spike proteins have been found in humans for at least 6-15 months after vaccination or infection [35,39,40] in those with post-acute sequelae, indicating spike protein as a possible primary contributing factor to long COVID, with substantial persistence in human systems due to the absence of an innate clearing mechanism. However, more research is needed to further investigate the effect of spike protein in patients with post-acute sequelae after COVID-19 and COVID-19 vaccination. Meanwhile, the current data points to a strong signal to urgently develop spike protein detoxification protocols.

Although methods to degrade or block spike protein have been investigated [46,47,51,52,56,57,62,71-80], there are currently no widely accepted protocols to do this in human subjects. The McCullough protocol: base spike detoxification is the first protocol established to help remove spike protein derived from SARS-CoV-2 infection and vaccination in humans. The three-drug regimen of nattokinase, bromelain, and curcumin was chosen due to their proven safety records, as well as their anti-inflammatory and anti-coagulant properties combined with their synergistic and potent effects in degrading and inhibiting spike protein [44-61]. This protocol may be useful in the attenuation of COVID-19 vaccine-induced injury syndromes and long-term COVID-19 complications.

The addition of other agents that can further assist in the detoxification of spike protein [62-80] may be indicated based on clinical outcomes. Most notably, the addition of N-acetylcysteine (NAC) may add an extra mechanism of action against spike protein to the protocol. NAC has been shown to disrupt disulfide bonds of the spike protein, rendering it unable to bind to the ACE2 receptor [71,72]. Moreover, the combination of NAC with bromelain has been shown to synergistically disrupt spike protein by breaking glycosidic linkages and disulfide bonds [52]. NAC has a well-established safety profile and is commonly taken at a dose of 600 mg twice a day [81]. Thus, the addition of NAC to the regimen of bromelain, curcumin, and nattokinase may be useful for individuals with severe treatment-resistant COVID-19 or COVID-19 vaccine-related complications. Since all of these compounds can reduce blood clotting, patients should be counseled and monitored for bleeding complications, including easy bruising, nasal mucosal bleeding, and gastrointestinal hemorrhage. Self-administration is not advised without physician oversight.

The base spike detoxification protocol was devised based on the best evidence currently available. No therapeutic claims can be made until large-scale, prospective, randomized, double-blind, placebo-controlled trials are completed. On November 15, 2023, we searched clinicaltrials.gov and found no planned or ongoing trials with nattokinase or bromelain in the treatment of post-acute sequelae after COVID-19 or vaccination. However, there is a planned trial to assess the effect of curcumin, in conjunction with boswellia and vitamin C, on long COVID [82]. Thus, base spike detoxification is an important advancement in the development of testable hypotheses for future trials assessing treatments for SARS-CoV-2 infection and post-COVID-19 vaccination injury syndromes.

Conclusions

SARS-CoV-2 spike protein is a highly persistent, potentially pathogenic substance that may incite inflammation and tissue damage in almost all organ systems, resulting in post-acute sequelae. The vaccine-generated spike protein is different from the viral type, but both have been associated with deleterious effects and persistence in biological systems. Thus, therapeutics that target spike protein may be essential in treating COVID-19, its long-term effects, and possibly COVID-19 vaccine injury syndromes. Base spike detoxification is a promising proposal designed to theoretically attenuate spike protein and its associated damage. However, more pre-clinical and clinical research is needed culminating with large-scale, prospective, randomized, double-blind, placebo-controlled randomized trials to fully assess safety and efficacy. Moreover, further investigation is essential to ensure vaccine-produced, stabilized prefusion spike protein safety and half-life in humans and that it does not possess the same deleterious effects as the viral spike protein.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Acquisition, analysis, or interpretation of data: Nicolas Hulscher, Peter A. McCullough

Drafting of the manuscript: Nicolas Hulscher, Peter A. McCullough

Critical review of the manuscript for important intellectual content: Nicolas Hulscher, Brian C. Procter, Cade Wynn, Peter A. McCullough

Disclosures

Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** PM receives partial salary support and holds an equity position in The Wellness Company, Boca Raton, Florida, which had no role in this study.

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