Received: 27 May 2025 | Accepted: 28 May 2025

https://doi.org/10.1016/j.rpth.2025.102950

ORIGINAL ARTICLE

Impact of asundexian on a panel of coagulation assays

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Handling Editor: Professor Michael Makris

Abstract

Background: During the last few years, the small, oral, activated factor XI inhibitor, asundexian, has been investigated in different cardiovascular disorders. However, little is known about its impact on laboratory coagulation assays.

Objectives: To describe the effects of asundexian on a panel of laboratory coagulation assays.

Methods: The following assays were performed in normal pooled plasma spiked with increasing concentrations of asundexian (0-2000 ng/mL): activated partial thromboplastin time (aPTT), prothrombin time (PT), fibrinogen quantification (PT-derived and Clauss method), one-stage aPTT and PT-based coagulation factor assays, chronometric protein C and immunoturbidimetric protein S assays, reptilase time, chromogenic ecarin assay, dilute Russell's viper venom time assays, thrombin generation assay initiated by tissue factor (1, 5, and 20 pM) and ellagic acid (0.42 μM), rotational thromboelastometry intrinsically- and extrinsically-triggered assays, kaolin-activated clotting time, and glass bead-activated clotting time. This latter assay was also carried out in whole blood.

Results: Asundexian up to 2000 ng/mL impacted aPTT and one-stage aPTT-based coagulation factor assays, with high variability between reagents and methodologies. Asundexian reduced thrombin generation triggered by ellagic acid and 1 or 5 pM tissue factor. The rotational thromboelastometry intrinsically-triggered assays and kaolin- and glass bead-activated clotting time assays were affected by asundexian 2000 ng/mL, while overall, no significant interference was observed with any of the remaining assays.

Conclusion: Despite this study including a comprehensive panel of coagulation assays, asundexian may still affect other coagulation assays not assessed here. Further investigations in patients treated with asundexian are therefore warranted.

KEYWORDS
asundexian, FXI inhibitors, laboratory coagulation assays, LA diagnosis

Essentials

- Asundexian targets a clotting protein called factor XIa.
- Its effects on laboratory tests used to assess blood clotting were documented.
- The effects of asundexian on some assays varied across reagents and methods.
- Other assays remained unaffected, indicating reliable results in the presence of asundexian.

1 | INTRODUCTION

Direct oral anticoagulants (DOACs) constituted a significant advance in anticoagulation [1]. Their comparable efficacy, fixed dosing, reduced monitoring requirement, and improved safety profile compared with vitamin K antagonists made them the first choice in many indications, such as nonvalvular atrial fibrillation (NVAF). However, the use of DOACs in real-life conditions highlights the need for a precise measurement of their activity to manage bleeding, thrombosis, overdoses, perioperative anticoagulation, and situations associated with variable DOAC exposure (administration in patients with extreme body weight, renal and hepatic disorders, possible drug–drug interactions, or gastrointestinal malabsorption) [2,3]. In addition, the bleeding rates observed in patients treated with DOACs remain nonnegligible [4].

New anticoagulation strategies have, therefore, been explored to achieve noninferior efficacy while minimizing the bleeding risk. One of them consists of targeting (activated) factor (F)XI (FXIa), which could decouple thrombosis from hemostasis [5]. Such molecules are under exploration for a variety of indications beyond conventional anticoagulation. These include reducing thrombotic risk in patients with end-stage renal disease, as well as after ischemic stroke and myocardial infarction, and preventing postoperative venous thromboembolism [5,6].

The phase 2 PACIFIC STROKE (NCT04304508) and PACIFIC AMI (NCT04304534) trials compared asundexian 10, 20, and 50 mg once daily with placebo on top of background antiplatelet therapy for secondary prevention after noncardioembolic ischemic stroke and acute myocardial infarction, respectively [7,8]. There was no statistically significant difference in bleeding between the asundexian and placebo groups in these studies. The rate of combined ischemic stroke and covert infarcts was, however, not significantly different between the groups in PACIFIC STROKE. There was also no significant difference in secondary myocardial infarctions between asundexian and placebo in the PACIFIC AMI trial [8]. This may be due to the low number of stent thrombosis and ischemic and hemorrhagic strokes observed in these studies, which limited the ability to accurately assess efficacy based on these data [7,8]. Similarly, the phase 2 clinical trials AZALEA-TIMI 71 (NCT04755283) and PACIFIC AF (NCT04218266) demonstrated reduced bleeding rates in stroke and systemic embolism prevention in NVAF with abelacimab and asundexian compared with rivaroxaban and apixaban, respectively. However, these trials were not powered to assess efficacy, and the limited occurrence of thrombotic events did not permit drawing any conclusions about efficacy [9,10].

The phase 3 OCEANIC AF trial (NCT05643573) further compared the direct oral FXIa inhibitor asundexian (Table 1 [11,12]) 50 mg once daily with apixaban 5 mg twice daily in the prevention of stroke and systemic thromboembolism in NVAF. The trial was interrupted due to the lack of efficacy of asundexian, highlighting the need to design appropriate upstream clinical trials to provide sufficient reliance in terms of efficacy [10]. Nevertheless, this also emphasizes the need to identify optimal indications for FXIa inhibitors and to document the use of appropriate coagulation assays to assess the pharmacodynamic profiles of these agents and select the most appropriate dose regimens during clinical trial development [13]. However, asundexian is still pursuing the OCEANIC STROKE (NCT05686070) phase 3 trial, where it is compared with a placebo in the prevention of ischemic stroke in patients with a personal history of ischemic stroke or high-risk transient ischemic attack [14,15]. Although asundexian is not yet marketed, the current development pipeline of this agent could lead to its commercialization, and its potential impact on coagulation assays remains a major concern, as observed with DOACs [16].

Indeed, DOACs have been shown to interfere with routine and specialized coagulation tests, leading to diagnostic errors in evaluating thrombophilia and coagulopathies [2,17]. Based on its pharmacodynamic profile, asundexian is also expected to influence some of these tests. Nevertheless, the current literature only provides little data regarding its effects on standard laboratory assays and none on thrombophilia diagnostics [8,11,12,18–22]. Asundexian also demonstrates limited renal excretion and potential for drug-to-drug interactions involving CYP3A4 and P-gp, but, as with DOACs, the importance of these factors in daily practice cannot be fully assessed in phase 3 trials [12,23–25]. Thus, the necessity to realize the punctual assessment of asundexian's anticoagulant effect, at least in some specific populations, cannot be excluded at this time. Understanding asundexian's impact on coagulation assays and identifying methods to measure its anticoagulant activity could ensure the proper use and interpretation of coagulation assays in patients treated with this inhibitor, especially in complex clinical scenarios [2].

Prior research has provided practical guidance on the laboratory assessment of DOACs, detailing their impact on coagulation

TABLE 1 Summary of the characteristics of asundexian (BAY 2433334).

Synonym			**BAY 2433334**		
Molecular weight			**592.93 g/mol**		
Pharmacologic target			**Activated FXI**		
Clinical trials[a]					
Clinical trial phase	**Phase 2**	**Status**	**Indication**	**Dose**	**Comparator**
Phase 2	PACIFIC AF (NCT04218266)	Completed	Prevention of ischemic stroke in NVAF	20 mg or 50 mg OD	Apixaban 5 mg twice daily[b]
	PACIFIC STROKE (NCT04304508)	Completed	Secondary prevention of noncardioembolic ischemic stroke	10 mg, 20 mg, or 50 mg OD[c]	Placebo[c]
	PACIFIC AMI (NCT04304534)	Completed	Secondary prevention after acute myocardial infarction	10 mg, 20 mg, or 50 mg OD[c]	Placebo[c]
Phase 3	OCEANIC AF (NCT05643573)	Terminated	Prevention of stroke and systemic embolism in NVAF	50 mg OD	Apixaban 5 mg twice daily[b]
	OCEANIC STROKE (NCT05686070)	Recruiting	Secondary prevention after acute ischemic stroke or high-risk TIA	Not available[c]	Placebo[c]

Pharmacokinetics	
Percentage amount of unchanged drug excreted in urine	**Reference**
10.1 (4.21) to 13.5 (3.42)	Kubitza et al. 2022 [12]
12.3 (3.36)	Chen et al. 2024 [11]

Expected therapeutic plasma concentration ranges with several dose regimens (repeated administration)

Dose regimen	**Duration (d)**	**C_{max} (ng/mL)**	**$t_{1/2}$ (h)**	**Reference**
25 mg OD	5	605 (6.95)	15.6 (11.7)	Chen et al. 2024 [11]
	8	507 (12.7)	15.8 (18.7)	Kubitza et al. 2022 [12]
50 mg OD	5	1110 (8.76)	17.4 (17.8)	Chen et al. 2024 [11]
	8	963 (24.8)	17.0 (17.8)	Kubitza et al. 2022 [12]
100 mg OD	5	1970 (11.4)	17.9 (12.7)	Chen et al. 2024 [11]
	5	2560 (23.2)	17.6 (11.6)	Chen et al. 2024 [11]
	8	1950 (28.1)	17.7 (22.8)	Kubitza et al. 2022 [12]

Pharmacokinetic parameters are reported as mean (coefficient of variation).

C_{max}, maximal plasma concentration; FXI, factor XI; NVAF, nonvalvular atrial fibrillation; OD, once daily; $t_{1/2}$, terminal half-life; TIA, transient ischemic attack.

[a]Phase 2 and 3 clinical trials from the ClinicalTrials.gov website (https://clinicaltrials.gov/) using the following terms in the field "other terms": "asundexian" and "BAY 2433334."

[b]2.5 mg twice daily if 2 or more of the following were present: age ≥ 80 years, body weight ≥ 60 kg, and serum creatinine ≥ 1.5 mg/dL.

[c]On top of single or dual antiplatelet therapy.

assays and proposing strategies to mitigate interference [2,17,26–28]. These studies emphasize the importance of establishing laboratory protocols to account for anticoagulant effects, particularly in emergencies or when evaluating bleeding risks and thrombophilia.

The present study aimed to extend this previous experience with DOACs to asundexian. By systematically evaluating its impact on a comprehensive range of coagulation assays, this work aimed to provide critical insights into these agents' diagnostic challenges and offer guidance on their clinical and laboratory management. The aim is to bridge the current knowledge gap, enabling the safe and effective use of asundexian.

2 | METHODS

In line with previous investigations, asundexian was spiked at increasing concentrations in citrated platelet-poor pooled plasma or whole blood [29–33]. A summary of the tested reagents in this study is provided in Table 2.

TABLE 2 Summary of the coagulation assays performed in this study.

Coagulation assay	Reagent(s)	Manufacturer	Method	Coagulation analyzer (manufacturer)
aPTT	HemosIL SynthASil	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
	STA-C.K. Prest	Diagnostica Stago		STA R MAX (Diagnostica Stago)
	STA-PTT Automate 5			STA R MAX (Diagnostica Stago)
	Dade Actin FS	Siemens Healthineers		STA R MAX (Diagnostica Stago)
PT	HemosIL RecombiPlasTin 2G	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
	STA-NeoPTimal 5	Diagnostica Stago		STA R MAX (Diagnostica Stago)
Fibrinogen (PT-derived)	HemosIL RecombiPlasTin 2G	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
Fibrinogen (Clauss method)	HemosIL Fibrinogen-C	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
1-stage aPTT-based intrinsic coagulation factor assays	HemosIL FVIII Deficient Plasma HemosIL FIX Deficient Plasma HemosIL FXI Deficient Plasma HemosIL FXII Deficient Plasma Each in combination with HemosIL SynthASil	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
1-stage PT-based extrinsic coagulation factor assays	HemosIL FII Deficient Plasma HemosIL FV Deficient Plasma HemosIL FVII Deficient Plasma HemosIL FX Deficient Plasma Each in combination with HemosIL RecombiPlasTin 2G	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
Protein C	HemosIL ProClot HemosIL aPTT-SP	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
Protein S	HemosIL Free Protein S	Werfen	Immunoturbidimetric	ACL TOP 700 (Instrumentation Laboratory)
Reptilase time	STA-Reptilase	Diagnostica Stago	Chronometric	STA R MAX (Diagnostica Stago)
Ecarin chromogenic assay	STA-ECA II	Diagnostica Stago	Chromogenic	STA R MAX (Diagnostica Stago)
LA (dRVVT)	HemosIL dRVVT Screen	Werfen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)
	HemosIL dRVVT Confirm	Werfen		
TGA	PPP Reagent Low	Diagnostica Stago	Fluorometric	Calibrated automated thrombogram (Diagnostica Stago)
	PPP Reagent			
	PPP Reagent High			
	Dade Actin FS	Siemens Healthineers		
ROTEM	INTEM	TEM International GmbH	Viscoelastic	ROTEM delta (TEM International GmbH)
	EXTEM			
kACT	kACT Kit	Sienco, Inc	Viscoelastic	Sonoclot SCP2 (ROMED nv)
gbACT	gbACT Kit			

aPTT, activated partial thromboplastin time; dRVVT, dilute Russell's viper venom time; gbACT, glass bead-activated clotting time; kACT, kaolin-activated clotting time; LA, lupus anticoagulant; PT, prothrombin time; ROTEM, rotational thromboelastometry; TGA, thrombin generation assay.

2.1 | Preparation of platelet-poor plasma and collection of whole blood

This study was performed in accordance with the Declaration of Helsinki, and the protocol was approved by a local ethics committee (Centre Hospitalier Universitaire Université Catholique de Louvain, CHU UCL, Namur, Yvoir, Belgium; approval number: B03920096633). Platelet-poor plasma aliquots were provided by our institutional biobank, the Namur Exchange Biobank (Nab-X, BB190116). Two different pools of platelet-poor plasma were used: one from 25 volunteers for the thrombin generation assay (TGA; batch number: I2212) and another from 48 healthy volunteers for all other assays (batch number: I2304). The exclusion criteria were a personal and familial history of thrombotic and/or hemorrhagic events, antiplatelet and/or anticoagulant medication, any other medicines impacting hemostasis, and FV Leiden or G20210A prothrombin mutation. Whole blood was drawn using a 21-gauge needle (Vacuette Preanalytics, Greiner Bio-One) via venipuncture from the antecubital vein and collected in tubes containing 0.109 M sodium citrate (9:1 v/v, Vacuette Preanalytics). Whole blood underwent double centrifugation (2 × 15 minutes, 2500 × *g* at 20-25 °C), and the supernatant was collected to obtain platelet-poor plasma. Platelet-poor plasma from all donors was pooled together to prepare normal pooled plasma (NPP). NPP was aliquoted and frozen at −80 °C within 4 hours following blood collection.

The Nab-X also supplied whole blood from 1 healthy donor. For whole blood collection, citrate collection tubes were replaced by plain tubes (Sarsted AG & Co KG) and did not undergo centrifugation. Whole blood was then used within 2 minutes following its collection.

All donors provided written informed consent prior to blood collection.

2.2 | Preparation of plasma and whole blood solutions

Asundexian (CAS number: 2064121-65-7; purity: 99.93%) was purchased from Bio-Connect. The molecule was diluted in dimethyl sulfoxide 25% (v/v) to prepare 1 mg/mL stock solutions. Stock solutions underwent serial dilutions in phosphate-buffered saline (PBS) to yield intermediate solutions. NPP aliquots were thawed at 37 °C for 10 minutes, and each intermediate solution was added to NPP (dilution factor [F] = 20) to obtain final plasma concentrations of asundexian ranging from 0 to 2000 ng/mL. The final concentration of dimethyl sulfoxide in plasma was, therefore, ≤0.05% (v/v), which did not influence coagulation [34]. The tested concentration range of asundexian was based on pharmacokinetic data showing that repeated administration of asundexian 50 mg once daily for 8 and 5 days led to maximum plasma concentrations of 963 and 1110 ng/mL, respectively [11,12]. A maximum concentration of 2000 ng/mL was selected to account for possible accumulation of asundexian (ie, impaired metabolism, renal failure, coadministration of CYP3A4, and P-glycoprotein, P-gp, inhibitors), to anticipate a potential adaptation of the currently investigated dose regimens, and to detect a possible shift in the concentration-response models beyond a certain threshold. The 0 ng/mL plasma solution was prepared by spiking NPP with PBS (F = 20). Before analysis, the spiked plasma underwent a second 10-minute incubation at 37 °C.

Whole blood was spiked with PBS or intermediate solutions of asundexian (final concentrations of asundexian in whole blood: 0 and 2000 ng/mL, F = 20) and analyzed within 2 minutes following its collection.

2.3 | Coagulation assays

This study assessed the impact of asundexian on activated partial thromboplastin time (aPTT), prothrombin time (PT), PT-derived and Clauss fibrinogen measurements, one-stage aPTT-based and one-stage PT-based coagulation factor assays (sample dilution: 10-fold for all assays, except for the FII assay in which a 4-fold dilution was performed), proteins C and S measurements, reptilase time, an ecarin chromogenic assay, lupus anticoagulant (LA) assays based on the determination of the dilute Russell's viper venom time (dRVVT), TGA, rotational thromboelastometry (ROTEM), and kaolin- and glass bead-activated clotting time assays (kACT and gbACT, respectively; Table 2). All assays were performed in NPP (platelet-poor plasma), except for the gbACT assay, which was also performed in whole blood. Plasma and whole blood were obtained according to the above-mentioned procedure. Assays involving HemosIL Reagents (Werfen) were performed on an ACL TOP 700 (Instrumentation Laboratory), and assays using STA reagents (Diagnostica Stago) or Siemens Healthineers reagents were performed on a STA R MAX (Diagnostica Stago), except for the TGA, which was performed on a calibrated automated thrombogram (Diagnostica Stago). For the TGA with Dade Actin FS (Siemens Healthineers), a 40-fold dilution of this reagent was performed (ellagic acid 0.42 μM, final concentration in wells).

The ROTEM assays were carried out on a ROTEM delta (TEM International GmbH), while the kACT and gbACT assays were performed with a Sonoclot Coagulation & Platelet Function Analyzer System, model SCP2 (ROMED nv). For the kACT and gbACT tests in NPP, 120 μL of each plasma solution was added to a cuvette containing 240 μL of 50 mM calcium chloride solution. When these in-house assays were performed in whole blood, 360 μL of whole blood spiked with asundexian or PBS was added directly to the cuvettes.

2.4 | Statistical analysis

Statistical analyses were computed using the GraphPad Prism software version 10.3.1. Baseline measurements were defined as measurements of the 0 ng/mL plasma solution (NPP spiked with PBS, F = 20). Results of the aPTT assays were expressed as an aPTT ratio, calculated as the ratio of the aPTT in the presence of a specified concentration of asundexian and the aPTT of the 0 ng/mL condition (NPP spiked with PBS, F = 20). A comparison of the sensitivity of the

4 reagents was performed based on the concentration of asundexian required to double the aPTT ratio (ie, double the clotting time [CT]). Results of the PT assays were reported as international normalized ratios and PT ratios, computed as the ratios of the PT in the presence of asundexian and the PT of the 0 ng/mL condition. Results of the one-stage aPTT-based and one-stage PT-based coagulation factor assays were presented as the variation in clotting factors. Details about these calculations are reported in Supplementary Methods.

Results of the LA testing based on the measurement of the dRVVT were reported as dRVVT screen ratios (HemosIL dRVVT Screen), dRVVT confirm ratios (HemosIL dRVVT Confirm), and normalized dRVVT ratios (formulas are provided in Supplementary Methods).

The following parameters were determined to describe the impact of asundexian on TGA: 2-fold lag time (the concentration of asundexian needed to double the lag time), 2-fold time to peak (the concentration of asundexian required to double the time to peak), peak half-maximal inhibitory concentration (IC_{50}; the concentration of asundexian to halve the peak), endogenous thrombin potential IC_{50} (the concentration of asundexian to halve the endogenous thrombin potential), and the mean velocity rate index (mVRI) IC_{50} (the concentration of asundexian to halve the mVRI). Statistical significance was assessed using Tukey's multiple comparisons test following one-way analysis of variance, with the conventional P value threshold of .05 ($P < .05$).

The reference change value (RCV) was used as a threshold for analytical variation. The detailed methodology and corresponding results are provided in Supplementary Methods and Supplementary Table 1.

3 | RESULTS

A summary of the impacts of asundexian on the different coagulation assays investigated in this study can be found in Table 3.

3.1 | aPTT

Asundexian increased the aPTT ratio in a concentration-dependent way. The relationship between the concentration of asundexian and the aPTT ratio was described by simple linear regression models for Dade Actin FS, STA-C.K. Prest, and STA-PTT Automate 5 and by a one-phase association model for HemosIL SynthASil (Figure 1). The concentration of asundexian needed to double the aPTT to obtain an aPTT ratio of 2.0 varied across reagents. For Dade Actin FS and STA-C.K. Prest, these concentrations were 1542.6 ng/mL and 1711.5 ng/mL, respectively, while for HemosIL SynthASil and STA-PTT Automate 5, these concentrations fell out of the tested concentration range (>2000 ng/mL; Figure 1 and Table 4). The plasma concentrations of asundexian needed to observe a significant prolongation of the aPTT (aPTT ratio of 1.2) were highly variable depending on the reagent used. In our study, these concentrations ranged from 319.2 ng/mL (113.7-613.6 ng/mL; STA-C.K. Prest) to 928.6 ng/mL (684.4-1228.4 ng/mL; HemosIL SynthASil; Table 4).

3.2 | PT

Asundexian up to 2000 ng/mL had no significant impact on the PT measured with the STA NeoPTimal and HemosIL RecombiPlasTin 2G reagents (Supplementary Figure 1).

3.3 | Fibrinogen measurement

Asundexian up to 2000 ng/mL had no significant impact on the measurement of fibrinogen by the PT-derived and Clauss methods (Supplementary Figure 2 and Supplementary Table 1).

3.4 | One-stage clotting factor assays

The absolute levels of clotting factors in the 0 ng/mL condition (NPP spiked with PBS) are provided in Supplementary Table 2. Asundexian modestly impacted the measurement of clotting factors in the intrinsic pathway. At a concentration of 2000 ng/mL, the inhibitor decreased the baseline measurements of FVIII, FIX, FXI, and FXII by 16.7%, 16.9%, 14.7%, and 20.5%, respectively. None of these reductions was statistically significant (Figure 2A). However, the reduction in the measurement of FVIII, FIX, and FXII observed in the presence of asundexian at a concentration of 2000 ng/mL exceeded the analytical variability threshold, defined by the RCV, indicating a significant change from baseline (RCV of 16.2, 11.4, and 15.6, respectively; Figure 2A and Supplementary Table 1). Asundexian had no significant impact on the measurement of clotting factors in the extrinsic and common pathways. All variations from the baseline measurements were <10%, and none exceeded the expected analytical variability as defined by the RCV (Figure 2B and Supplementary Table 1). The absolute measurements of the clotting factors are provided in Supplementary Figure 3.

3.5 | Proteins C and S measurement, reptilase time, and ecarin chromogenic assay

No significant impact of asundexian up to 2000 ng/mL was observed on these assays (Supplementary Figure 4 and Supplementary Table 1).

3.6 | dRVVT

Asundexian, ranging from 100 to 500 ng/mL, only minimally, yet statistically significantly, reduced the dRVVT screen CT and dRVVT screen ratio measured with HemosIL dRVVT Screen. As a result, no meaningful change was observed in the dRVVT screen and confirm assays (Supplementary Figure 5).

TABLE 3 Summary of the impacts of asundexian on coagulation assays.

Coagulation assay	Reagent(s)	Method	Coagulation analyzer (manufacturer)	Impact (Y/N)
aPTT	HemosIL SynthASil	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	Y
	STA-C.K. Prest		STA R MAX (Diagnostica Stago)	Y
	STA-PTT Automate 5		STA R MAX (Diagnostica Stago)	Y
	Dade Actin FS		STA R MAX (Diagnostica Stago)	Y
PT	HemosIL RecombiPlasTin 2G	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	N
	STA-NeoPTimal 5		STA R MAX (Diagnostica Stago)	N
Fibrinogen (PT-derived)	HemosIL RecombiPlasTin 2G	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	N
Fibrinogen (Clauss method)	HemosIL Fibrinogen-C	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	N
1-stage aPTT-based intrinsic coagulation factor assays	HemosIL FVIII Deficient Plasma HemosIL FIX Deficient Plasma HemosIL FXI Deficient Plasma HemosIL FXII Deficient Plasma Each in combination with HemosIL SynthASil	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	Y
1-stage PT-based extrinsic coagulation factor assays	HemosIL FII Deficient Plasma HemosIL FV Deficient Plasma HemosIL FVII Deficient Plasma HemosIL FX Deficient Plasma Each in combination with HemosIL RecombiPlasTin 2G	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	N
Protein C	HemosIL ProClot	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	N
Protein S	HemosIL Free Protein S	Immunoturbidimetric	ACL TOP 700 (Instrumentation Laboratory)	N
Reptilase time	STA-Reptilase	Chronometric	STA R MAX (Diagnostica Stago)	N
Ecarin chromogenic assay	STA-ECA II	Chronometric	STA R MAX (Diagnostica Stago)	N
LA (dRVVT)	HemosIL dRVVT Screen	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	Y
	HemosIL dRVVT Confirm	Chronometric	ACL TOP 700 (Instrumentation Laboratory)	N
TGA	PPP Reagent Low	Fluorometric	Calibrated automated thrombogram (Diagnostica Stago)	Y
	PPP Reagent			Y
	PPP Reagent High			N
	Dade Actin FS			Y
ROTEM	INTEM	Viscoelastic	ROTEM delta (Instrumentation Laboratory)	Y
	EXTEM			N
kACT	kACT Kit	Viscoelastic	Sonoclot SCP2 (ROMED nv).	Y
gbACT	gbACT Kit			Y

aPTT, activated partial thromboplastin time; dRVVT, dilute Russell's viper venom time; gbACT, glass bead-activated clotting time; kACT, kaolin-activated clotting time; LA, lupus anticoagulant; PT, prothrombin time; ROTEM, rotational thromboelastometry; TGA, thrombin generation assay.

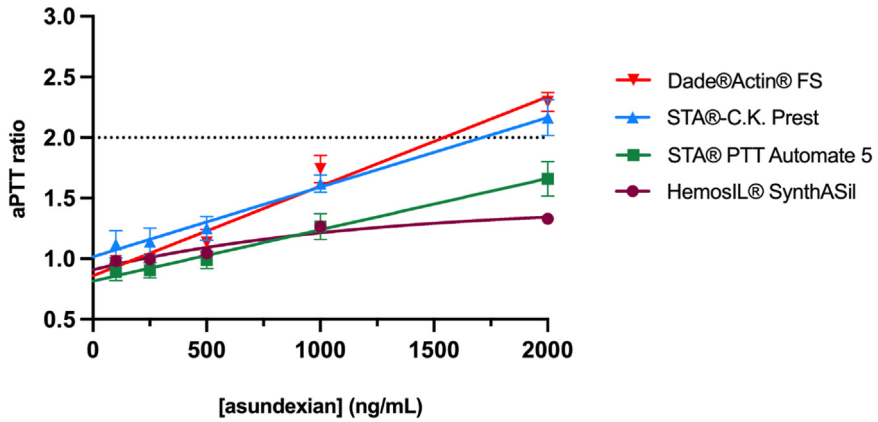

FIGURE 1 Impact of asundexian on the activated partial thromboplastin time (aPTT) ratio. The aPTT ratio was calculated as the ratio between the aPTT in the presence of asundexian and the baseline aPTT (0 ng/mL: plasma spiked with phosphate-buffered saline, dilution factor = 20). The relation between the concentration of asundexian and the aPTT ratio is described by simple linear regression models for Dade Actin FS, STA-C.K. Prest, and STA-PTT Automate 5 and by a one-phase association model for HemosIL SynthASil.

3.7 | TGA

Asundexian ranging from 250 to 2000 ng/mL reduced thrombin generation triggered by 1 pM tissue factor (TF; PPP Reagent Low, Diagnostica Stago). When thrombin generation was triggered by 5 pM TF (PPP Reagent; Diagnostica Stago), the inhibition of thrombin generation by asundexian was less pronounced. No significant reduction in thrombin generation was observed with the 20 pM TF reagent (PPP Reagent High; Diagnostica Stago; Figure 3). Asundexian delayed and decreased thrombin generation triggered by ellagic acid (Dade Actin FS). The peak and mVRI of thrombin generation triggered by PPP Reagent Low were reduced by 50% within the tested concentration range of asundexian, as well as the mVRI with the Dade Actin FS Reagent. No doubling of the lag time or time to peak was observed in this study (Figure 3 and Table 4). When falling within the tested concentration range, the IC_{50} was computed based on the one-phase decay models describing the relationship between the concentration of asundexian and the peak or mVRI (Supplementary Methods).

3.8 | ROTEM assays

In the intrinsically-triggered (INTEM) assay, asundexian at a 2000 ng/mL concentration prolonged the CT from 220 seconds (baseline) to 241 seconds. No impact on the clot formation time, alpha angle, maximum clot firmness, or amplitude 5 and 10 minutes after clot formation started was observed. Asundexian did not impact any of these 5 parameters in the extrinsically-triggered (EXTEM) assay.

3.9 | kACT and gbACT assays

In NPP, asundexian at a concentration of 2000 ng/mL prolonged the activated CT (ACT) from 404 seconds (baseline) to 571 seconds in the kACT assay and from 456 seconds to 1588 seconds in the gbACT assay. The clot rate (CR) was reduced from 3.6 to 2.1 in the kACT assay and from 3.0 to 1.0 in the gbACT assay. In whole blood, the ACT was prolonged from 178 seconds to 220 seconds, while the CR and platelet function decreased from 16.0 to 12.3 and from 3.0 to 2.7, respectively, in the presence of asundexian 2000 ng/mL (gbACT).

4 | DISCUSSION

This study aimed to document the effect of asundexian on a panel of routine and specific coagulation assays and to provide guidance for informed use of these assays in patients receiving this FXIa inhibitor. NPP was spiked with different solutions of asundexian to mimic therapeutically plausible plasma concentrations of this molecule [11,12]. This approach did not consider *in vivo* processes, such as drug metabolization, but it is unlikely that asundexian's metabolites interfere with coagulation assays. Indeed, the M10 metabolite, accounting for a total drug-related exposure of 16.4% in human plasma, has been demonstrated to be pharmacologically inactive [23]. Importantly, a similar approach was successfully used to describe the impact of DOACs on coagulation assays [1,2,29–33].

Our investigations showed a concentration-dependent prolongation of the aPTT in the presence of asundexian, with variable sensitivity across reagents. The concentration of asundexian required to double the aPTT differed by around 500 ng/mL between some reagents (Table 4). A similar phenomenon has been documented *in vitro* with milvexian [35]. Previous investigations showed that the sensitivity of aPTT reagents to mild to moderate clotting factor deficiencies, including FXI deficiency, is highly variable [36,37]. The variability of aPTT results has also been described with heparins and explained by different factors (contact activator, lipid profile, total phospholipid concentration, and incubation time) [38,39]. Taken together, these factors could explain the variable sensitivity of aPTT reagents observed in the presence of FXIa inhibitors.

Buckley et al. [19] performed an aPTT assay with the Dade Actin FS Reagent and observed a concentration-dependent prolongation of the CT. Nevertheless, in their experiments, a much lower concentration of asundexian was needed to obtain an aPTT ratio of 2.0 (between 250 and 500 ng/mL) compared with our result, ie, 1542.6 ng/mL. Heitmeier et al. [20], who performed the aPTT assay

TABLE 4 Intrapolated concentrations of asundexian needed to significantly prolong the activated partial thromboplastin time, to double the activated partial thromboplastin time, lag time, and time to peak, and to decrease by half the peak, endogenous thrombin potential, and mean velocity rate index.

Assay	Reagent	Parameter	Value, ng/mL (95% CI)
aPTT	HemosIL SynthASil	1.2-fold aPTT (aPTT ratio = 1.2)	928.6 (684.4-1228.4)
	STA-PTT Automate 5		907.0 (580.8-1398.5)
	STA-C.K. Prest		319.2 (113.7-613.6)
	Dade Actin FS		460.1 (252.0-748.9)
aPTT	HemosIL SynthASil	2-fold aPTT (aPTT ratio = 2.0)	n.c. (>2000 ng/mL)
	STA-PTT Automate 5		n.c. (>2000 ng/mL)
	STA-C.K. Prest		1711.5 (1294.6 to >2000)
	Dade Actin FS		1542.6 (1183.5 to >2000)
TGA	PPP Reagent Low	2-fold lag time	n.c. (>2000 ng/mL)
	PPP Reagent		n.c. (>2000 ng/mL)
	PPP Reagent High		n.c. (>2000 ng/mL)
	Dade Actin FS		n.c. (>2000 ng/mL)
	PPP Reagent Low	Peak IC_{50}	1876.0 (1598.1 to >2000)
	PPP Reagent		n.c. (>2000 ng/mL)
	PPP Reagent High		n.c. (>2000 ng/mL)
	Dade Actin FS		n.c. (>2000 ng/mL)
	PPP Reagent Low	ETP IC_{50}	n.c. (>2000 ng/mL)
	PPP Reagent		n.c. (>2000 ng/mL)
	PPP Reagent High		n.c. (>2000 ng/mL)
	Dade Actin FS		n.c. (>2000 ng/mL)
	PPP Reagent Low	2-fold time to peak	n.c. (>2000 ng/mL)
	PPP Reagent		n.c. (>2000 ng/mL)
	PPP Reagent High		n.c. (>2000 ng/mL)
	Dade Actin FS		n.c. (>2000 ng/mL)
	PPP Reagent Low	mVRI IC_{50}	1428.4 (1053.6-1855.3)
	PPP Reagent		n.c. (>2000 ng/mL)
	PPP Reagent High		n.c. (>2000 ng/mL)
	Dade Actin FS		1435.3 (1214.9-1743.4)

The concentrations required to significantly prolong and double the baseline aPTT (ie, to reach an aPTT ratio of 1.2 and 2.0, respectively) were calculated using the models describing the relationship between the concentration of asundexian and the aPTT ratio (simple linear regression models for Dade Actin FS, STA-C.K. Prest, and STA-PTT Automate 5 and a one-phase association model for HemosIL SynthASil; Figure 1A). When calculable (falling within the tested concentration range), the peak IC_{50} and mVRI IC_{50} were computed using the one-phase decay model, which describes the relationship between the concentration of asundexian and the peak or mVRI, respectively.

1.2/2-fold aPTT, concentration of inhibitor needed to significantly prolong or double the aPTT (to yield an aPTT ratio of 1.2/2.0); 2-fold lag time, concentration of asundexian required to double the lag time; 2-fold time to peak, concentration of asundexian required to double the time to peak; aPTT, activated partial thromboplastin time; ETP, endogenous thrombin potential; IC_{50}, half-maximal inhibitory concentration; mVRI, mean velocity rate index; n.c. (>2000 ng/mL), a 50% inhibition or a 2-fold time the concentration fell outside the tested concentration range; TGA, thrombin generation assay.

using the STA-C.K. Prest reagent, observed a concentration-dependent prolongation of the CT and reported a 1.5-fold concentration of asundexian of 0.61 μM (361.7 ng/mL). In our study, the corresponding concentration was 841.4 ng/mL (Figure 1). Compared with the published studies assessing the aPTT in plasma spiked with asundexian, we systematically observed a less pronounced prolongation of the CT. This variation between investigations using the same aPTT reagent might arise from the use of plasmas from different origins. Buckley et al. [19] reported the spiking of several commercial reconstituted human-derived plasmas (from Siemens Healthineers and HYPHEN BioMed), Heitmeier et al. [20] used commercial human citrated plasma (from Octapharma), and we

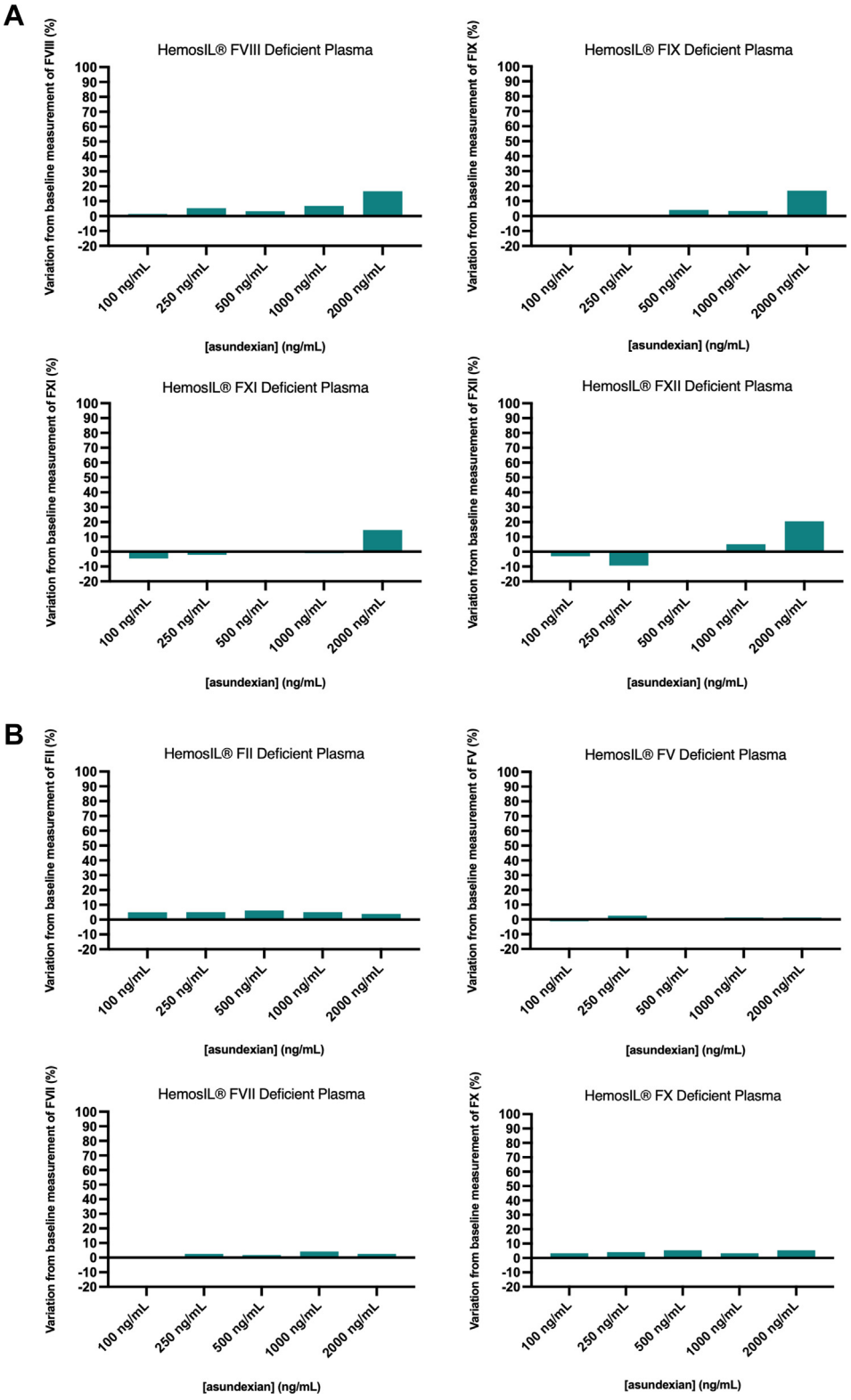

FIGURE 2 Impact of asundexian on one-stage activated partial thromboplastin time-based (A) and one-stage prothrombin time-based (B) coagulation factor assays. The results are expressed as the variation from the baseline measurements of coagulation factors. The baseline condition was defined as normal pooled plasma spiked with phosphate-buffered saline (dilution factor = 20). F, factor.

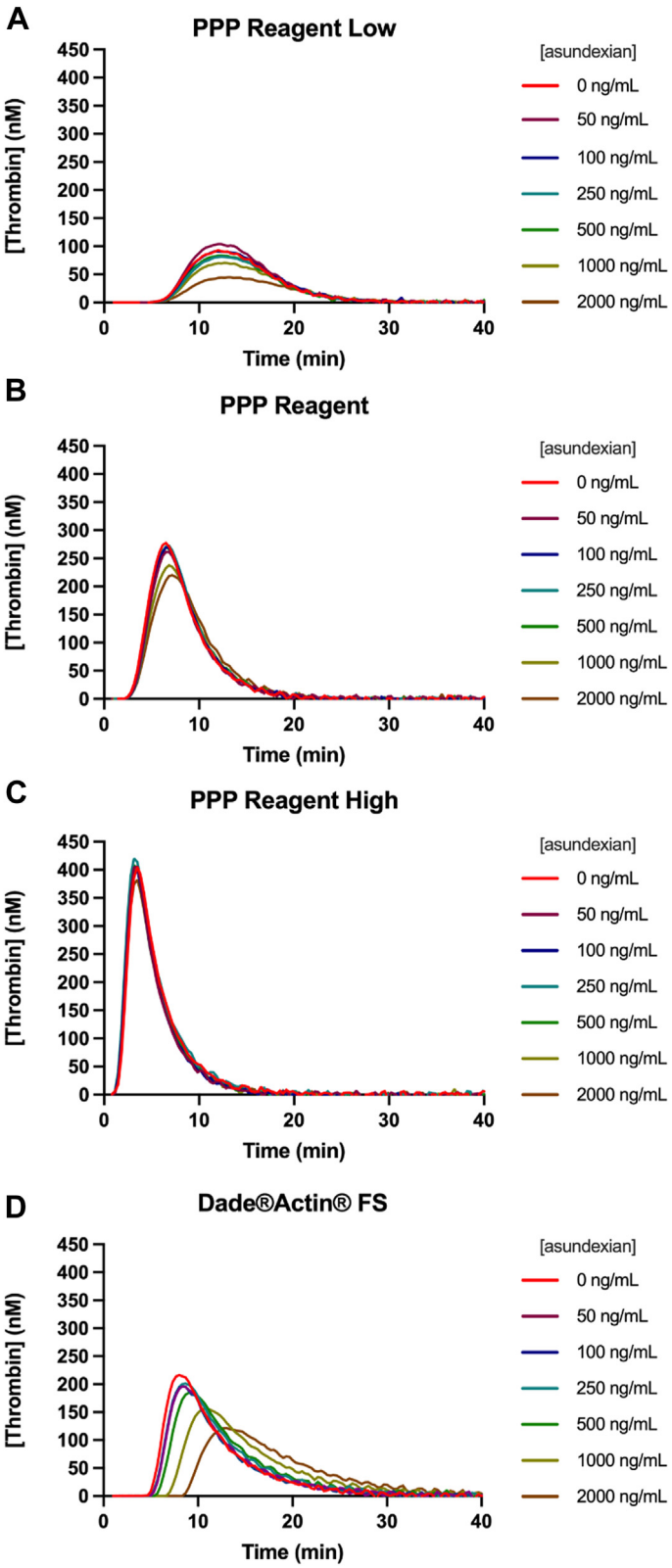

FIGURE 3 Impact of asundexian on the thrombin generation assay. A 40-fold dilution of the Dade Actin FS Reagent was performed for this experiment.

analyzed pooled normal citrated human plasma from the Nab-X biobank (n = 48), which is collected and treated according to the same conditions as clinical samples. Other factors could explain the variability, such as different incubation times of the aPTT reagent with plasma or the use of different analyzers (Buckley et al. [19] performed the aPTT assay on a CN-6000 analyzer [Sysmex], Heitmeier et al. [20] used an AMAX 200 [Trinity Biotech], and we carried out these assays on an ACL TOP 700

[Instrumentation Laboratory] and a STA R MAX [Diagnostica Stago]; Table 2).

When comparing our aPTT results with the available data from pharmacokinetic-pharmacodynamic studies, we also observed a systematically lower prolongation of the aPTT assay (Supplementary Table 3). Nevertheless, Supplementary Table 3 reports the maximum plasma concentrations of asundexian, and the time to maximum plasma concentration sometimes differs from the time to maximum aPTT ratio [11,12,21]. Moreover, these studies reported ratios to baseline instead of aPTT ratios, which may compensate for interindividual variability [11,12,21].

We, therefore, performed a subsequent set of aPTT analyses with a second batch of asundexian (Catlag-Medsystems) to exclude any manufacturing or handling issues regarding the inhibitor. Details are provided in Supplementary Table 4. When comparing the aPTT results obtained with the 2 batches of asundexian, all coefficients of variation were <5.0% (Supplementary Table 4), which confirms our first findings.

We conclude that differences in aPTT methodologies and different plasmas led to the variability observed between studies. As a result, the aPTT should not be used to estimate plasma levels of asundexian.

Moreover, the plasma concentrations of asundexian needed to observe a significant prolongation of the aPTT (aPTT ratio of 1.2) were highly variable depending on the reagent used, and a normal aPTT could not systematically exclude significant plasma levels of asundexian (Figure 1 and Table 4). Consequently, no clinical decision (ie, whether to perform an invasive surgical procedure or not) should rely on the results of aPTT assays in patients treated with asundexian. This finding contrasts with the recent recommendations for the management of bleeding and invasive procedures in patients treated with anti-FXI(a) anticoagulants provided by Godier et al. [40], where a normal aPTT is considered sufficient to rule out high doses and high plasma levels of asundexian.

The measurements of the clotting factors in the intrinsic pathway by one-stage aPTT-based assays were also impacted by asundexian. Although none of these variations from baseline measurements of the clotting factors were statistically significant, the reductions in FVIII, FIX, and FXII levels observed in the presence of asundexian at a concentration of 2000 ng/mL exceeded the expected analytical variability (Figure 2A and Supplementary Table 1). Buckley et al. [19] showed a more pronounced reduction in FXI measurement by a one-stage aPTT-based assay using the Dade Actin FS Reagent. At a concentration of 2000 ng/mL asundexian, they measured a residual activity of FXI of around 20% [19]. Our investigation yielded a value of 85%, which is consistent with the lower prolongation of the aPTT described above. No significant difference in the baseline measurement of FXI was observed between the 2 studies (between 90% and 100% for Buckley et al. [19] and 99.9% in our case). Another investigation, using the HemosIL SynthASil Reagent on an ACL TOP analyzer, was performed by Thomas et al. [21]. They measured a minimum residual level of FXI close to 75% 4 hours after administering a single 50 mg tablet of asundexian to healthy volunteers, corresponding to a peak plasma concentration of 617 ng/mL (3 hours after drug administration) [21]. In our experiments, the level of FXI observed in the presence of 500 ng/mL asundexian was 100.4% (Supplementary Table 3). The high variability between these results could be explained, at least partially, by methodological differences. This finding emphasizes again the need for standardization of aPTT-based coagulation assays. Interestingly, some fluorogenic assays developed to assess the residual activity of FXIa in the presence of asundexian, implying smaller plasma dilutions, generally showed (nearly) complete suppression of this activity. Thomas et al. [21] measured FXIa activity using a proprietary fluorogenic substrate assay in which "the level of FXIa activity was derived from a calibration curve constructed from native FXI protein." This assay was carried out at the same time point as the aPTT (4 hours after drug intake) and showed a residual activity of FXIa close to 0% [21]. No detailed methodology was described for this FXIa activity assay. These results were further confirmed by other groups that also reported the use of a proprietary fluorogenic substrate assay [11,12]. A similar assay yielded consistent results in patients treated with asundexian 50 mg once daily in the PACIFIC AF (NCT04218266) and PACIFIC AMI (NCT04304534) trials, with a >90% reduction in baseline FXIa activity. This assay was reported as a "kaolin trigger and fluorogenic substrate readout" method in both articles [8,18]. This methodology was explained by Heitmeier et al. [20]. Briefly, plasma samples were incubated with the STA-C.K.Prest Reagent (Diagnostica Stago; 1:1, 3 minutes, 37 °C) before the addition of an aqueous solution containing a FXIa fluorogenic substrate (the peptidic aminomethylcoumarin derivative Bachem I-1575 at a final concentration of 400 μM) and a plasma kallikrein inhibitor (BAY2292992 at a final concentration of 5.6 μM). Fluorescence was then measured for 40 minutes at 37 °C (excitation 360 nm and emission 460 nm). Heitmeier et al. [20] also used this assay in human plasma spiked with asundexian and demonstrated an IC_{50} of 0.14 μM (SD, 0.04; 83.0 ng/mL [SD, 23.7]).

Regarding the one-stage PT-based assays, no significant impact of asundexian was observed with plasma levels up to 2000 ng/mL (Figure 2B and Supplementary Table 1). This is in line with the absence of impact of such concentrations of asundexian on the PT assays performed in this study and indicates a possible use of the unaffected methodologies in patients treated with asundexian. Previous investigations also showed no impact of asundexian on PT assays [11,12,19,20].

As asundexian did not exert any meaningful effect on the dRVVT screen and confirm assays, no influence on the diagnosis of LA should be expected in patients with plasma levels of asundexian up to 2000 ng/mL. Indeed, no significant impact was observed on the dRVVT LA ratio (screen/confirm; Supplementary Figure 5). As a result, the use of procedures to overcome the effect of asundexian on the dRVVT screen and confirm assays is currently not justified, unlike with DOACs [41].

The impact of asundexian on the TGA triggered by TF decreased with increasing concentrations of TF, which is consistent with the mechanism of action of asundexian. By inhibiting the amplification of coagulation, FXIa inhibitors are expected to have more impact on coagulation in the presence of low levels of TF. A previous study by Heitmeier et al. [20] showed a more pronounced, but still moderate,

reduction in thrombin generation triggered by TF at a concentration of 5 pM. Asundexian moderately reduced thrombin generation initiated by ellagic acid. This effect was less pronounced than the results of Heitmeier et al. [20]. However, methodological factors could be involved (notably, the concentration of inducer in wells, as we diluted the trigger reagent 40-fold, while Heitmeier et al. [20] did not report any dilution). The only impact of asundexian on the ROTEM INTEM assay was prolonging the CT. No impact on the ROTEM EXTEM assay was observed. Thomas et al. [21] performed a similar experiment in whole blood (with the same analyzer and intrinsically activated test) and reported a CT prolongation. Our investigation led to higher CT values as they were carried out in plasma instead of whole blood.

Asundexian affected both kACT (plasma) and gbACT assays (plasma and whole blood). The ACT prolongation and the CR reduction in plasma were more pronounced with gbACT than with kACT. All ACTs were shorter in whole blood, and all CRs were lower in plasma.

As expected, asundexian up to 2000 ng/mL did not impact the following assays: PT-derived and Clauss fibrinogen assays, chronometric protein C and immunoturbidimetric protein S assays, reptilase time, and ecarin chromogenic assay (Supplementary Figures 2 and 4, and Supplementary Table 1). Buckley et al. [19] also showed no effect of asundexian on a Clauss fibrinogen assay. Therefore, no interference with the abovementioned assays should be observed in patients treated with asundexian and exhibiting such plasma levels of this inhibitor. However, the definitive exclusion of laboratory interferences in these patients must rely on clinical data to rule out any impact of asundexian's metabolites or a potential impact of higher concentrations of asundexian.

In our study, the contribution of platelets was not evaluated. Not all available commercial reagents were assessed, which reduces the generalizability of the results. Additionally, no inhibitor, such as corn trypsin inhibitor, was added to the collection tube to prevent activation of the contact system. Our methodology preserved the pressurization of the collection tubes and maintained the citrate-to-blood ratio. While the use of such inhibitors is not feasible in routine clinical practice, this nevertheless represents a limitation. Indeed, the absence of corn trypsin inhibitor leads to FXII activation during sample collection and handling. The resulting artefactual generation of FXIa can influence the inhibitory effect of the asundexian observed in coagulation assays sensitive to the intrinsic pathway.

Finally, none of the assays performed in this study allows for direct quantification of asundexian anticoagulant activity. FXIa assays (chromogenic or fluorogenic) can determine residual FXIa activity in the presence of asundexian. However, as the severity of FXI deficiency does not correlate with bleeding phenotype, residual FXIa activity alone is unlikely to reliably predict bleeding risk in patients treated with asundexian [42].

5 | CONCLUSIONS

The present study evaluated the impact of asundexian on a panel of coagulation assays, building on the lessons learned from DOACs. Asundexian impacted aPTT and one-stage aPTT-based coagulation factor assays, exhibiting a high variability between reagents and methodologies. Therefore, these assays should not be used to evaluate the anticoagulant activity of asundexian. Asundexian reduced thrombin generation triggered by ellagic acid and 1 and 5 pM TF. No relevant effect of asundexian was observed in the dRVVT screen and confirm assays. As a result, asundexian up to 2000 ng/mL is not expected to influence the diagnosis of LA. The ROTEM INTEM, kACT, and gbACT assays were impacted by asundexian, while no significant effect was observed on the PT, one-stage PT-derived clotting factor assays, PT-derived and Clauss fibrinogen assays, chronometric protein C and immunoturbidimetric protein S assays, reptilase time, ecarin chromogenic assay, and ROTEM EXTEM assay.

This study also highlights the ongoing need for a multidisciplinary approach integrating novel anticoagulants into routine practice. With FXIa inhibitors representing a frontier in anticoagulation, their characterization will be pivotal in shaping their role within the therapeutic armamentarium.

ACKNOWLEDGMENTS

The investigations were carried out at QUALIblood s.a. (Liège, Belgium) and the Department of Clinical Biology of Clinique Saint-Luc Bouge (SLBO; Bouge, Belgium). The authors would like to thank the members of both teams for their support.

FUNDING

This research did not receive any external funding.

AUTHOR CONTRIBUTIONS

J.D.: conceptualization, supervision, project administration, modelization, data curation, and writing the original draft. J.V.: investigation, formal analysis, data curation, and writing the original draft. D.B.N.: investigation, formal analysis, and data curation. M.D.: investigation. L.M.: review and editing. C.B.: investigation. L.J.: review and editing. F.D.: review and editing. A.L.: review and editing. F.M.: review and editing. J.F.: review and editing. M.H.: review and editing. J.-M.D.: review and editing.

RELATIONSHIP DISCLOSURE

J.D. is founder, CSO, and chairman of the board of QUALIblood s.a. (Liège, Belgium) and reports personal fees from Daiichi Sankyo, Diagnostica Stago, Gedeon Richter, GyneBio Pharma, Mithra Pharmaceuticals, Norgine, Roche, Roche Diagnostics, Technoclone, Werfen, and YHLO, all outside the submitted work. A.L. reports grants/research support from Sobi, Takeda, LFB, Novo Nordisk, Octapharma, Roche/Chugai, Pfizer, and Stago, and speakers' bureau or advisory board memberships from Sobi, Octapharma, Pfizer, and Roche/Chugai. F.M. reports speakers' fees from Diagnostica Stago. The other authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.rpth.2025.102950