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ORIGINAL ARTICLE

Interferences associated with the factor XIa inhibitors asundexian and milvexian in routine and specialized coagulation assays and their removal by activated charcoal-based adsorbents

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Abstract

Background: Direct inhibition of factor (F)XI/FXIa is emerging as a promising anticoagulant strategy to mitigate the risk of bleeding typically associated with antithrombotic medications, including direct oral anticoagulants (DOACs). While DOACs are known to interfere with a broad range of coagulation assays, the interferences associated with emerging FXIa inhibitors remain incompletely characterized.

Objectives: This study evaluated the interferences associated with the FXIa inhibitors asundexian and milvexian in a panel of routine and specialized coagulation assays. Additionally, we assessed the capacity of charcoal-based adsorbents—including raw activated charcoal and DOAC-Stop—to remove interferences in coagulometric assays.

Methods: Human-derived plasma was anticoagulated with increasing concentrations of asundexian or milvexian (0-10 000 ng/mL) and assayed for routine and specialized coagulation parameters before and after treatment with charcoal-based adsorbents using Sysmex CN/CS-series analyzers. Plasma was anticoagulated with both asundexian and milvexian at therapeutic and supratherapeutic concentrations representative of those reported in recent pharmacokinetic trials.

Results: Asundexian and milvexian produced significant dose-dependent interferences in FXIa-dependent coagulation assays, including markedly prolonged activated partial thromboplastin time-based clotting times and markedly reduced intrinsic coagulation factor activities. Treatment with charcoal-based adsorbents effectively removed both asundexian- and milvexian-associated interferences across all affected assays.

Conclusion: Emerging FXIa inhibitors interfere significantly with FXIa-dependent coagulation assays, potentially leading to misinterpretation of hemostatic function. However, charcoal-based adsorbents efficiently remove these interferences and enable routine and specialized coagulation testing in the presence of asundexian and milvexian.

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KEYWORDS
anticoagulation, asundexian, DOAC-Stop, factor XI inhibitors, milvexian

1 | INTRODUCTION

Anticoagulation remains a cornerstone in the prevention and treatment of thrombosis. For decades, vitamin K antagonists were the only oral agents available for long-term anticoagulation. Although effective, these agents are associated with numerous limitations, including the need for regular monitoring, dietary restrictions, and a narrow therapeutic index [1]. The recent introduction of direct oral anticoagulants (DOACs)—including small-molecule inhibitors targeting factor (F)IIa and FXa—has changed anticoagulation therapy due to their more predictable pharmacokinetics and pharmacodynamics [2,3]. However, despite pharmacologic advantages, bleeding remains a significant limitation: major bleeding occurs in approximately 2% to 4% of patients treated with DOACs per annum, and an additional 10% to 12% experience clinically relevant nonmajor bleeding [4–8]. Consequently, the clinical development of safer anticoagulants has become a major research focus.

One emerging anticoagulant strategy involves the targeted inhibition of either FXI or the activated form of FXI (FXIa). In contrast to other components of the intrinsic coagulation pathway, FXI/FXIa appears to be of limited importance in tissue factor-initiated hemostasis but plays a critical role in pathologic thrombi—FXI/FXIa primarily contributes to thrombus consolidation and enhances resistance to fibrinolysis, particularly as thrombi progress into the lumen of blood vessels and away from tissue factor complexes exposed at sites of vascular injury [9]. Targeted inhibition of FXI/FXIa is, therefore, considered a means to uncouple hemostasis from thrombosis and is supported by clinical and epidemiologic data: patients with deficiencies in FXI seldom experience spontaneous bleeding [10] and exhibit a reduced risk of thrombotic events, particularly venous thromboembolism [11].

Several approaches targeting FXI/FXIa are currently in clinical development, including small synthetic molecules, monoclonal antibodies, antisense oligonucleotides, aptamers, and naturally occurring inhibitors [12]. Among these, asundexian and milvexian are emerging oral anticoagulants that function as selective, reversible small-molecule inhibitors of FXI/FXIa. Asundexian is currently being investigated in the OCEANIC program, while milvexian is under evaluation in the LIBREXIA program—both assessing the potential of small synthetic molecules targeting FXI/FXIa in the prevention of acute cardiovascular events and venous thromboembolism [13]. Although efficacy data are still evolving [14], the possible introduction of these novel anticoagulants into clinical practice raises concerns regarding their impact on coagulation testing. DOACs are well known to interfere with a wide range of routine coagulation assays, such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), as well as specialized coagulation assays such as coagulation factor activities and thrombophilia testing [15]. DOAC-associated interferences can, therefore, complicate both diagnostic and therapeutic decision-making [15]. Given that asundexian and milvexian act in a similar pharmacologic manner—albeit on a different target—to current DOACs, direct inhibitors of FXI/FXIa are likewise expected to interfere with coagulation assays. While we have reported preliminary observations of such interferences in routine testing [16], a comprehensive evaluation across a range of routine and specialized coagulation assays remains lacking.

In this study, we evaluate asundexian- and milvexian-associated interferences in routine and specialized coagulation assays using *in vitro* plasma-based systems at concentrations representative of maximum plasma concentrations reported in pharmacokinetic studies. Furthermore, we investigated the efficacy of charcoal-based adsorbents, including raw activated charcoal (AC) and DOAC-Stop, in removing these interferences. Our findings aim to inform both clinical and laboratory practice by identifying potential limitations in assay interpretation and proposing a practical solution to enable accurate assessment of coagulation in the context of emerging anticoagulant therapies.

2 | METHODS

2.1 | Collection and preparation of human plasma samples

Commercial standard plasma was prepared, according to the manufacturer's recommendations, by reconstituting lyophilized preparations with distilled water at ambient temperature (catalog number: 10446238 ORKL17; Siemens Healthineers). Standard plasma was anticoagulated with increasing concentrations of direct FXIa inhibitors after reconstitution and analyzed within 2 hours. Additionally, platelet-poor plasma was prepared by the separation of whole blood from 25 ostensibly healthy individuals. Whole blood samples were collected into 3.2% trisodium citrate (Greiner Bio-One VACUETTE, Cruinn Diagnostics), and platelet-poor plasma was prepared at ambient temperature by double-centrifugation at 3300 × *g* for 7 minutes prior to storage at −80 °C. Platelet-poor plasma was anticoagulated with increasing concentrations of direct FXIa inhibitors after thawing in a water bath at 37 °C for 5 minutes and analyzed within 2 hours.

Written informed consent was obtained from all individuals prior to the collection of whole blood samples. This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (review reference number: ECM 4 [f] 12/09/2023 and ECM 5 [7] 12/09/2023).

2.2 | Preparation of anticoagulated human plasma samples

Asundexian (catalog number: HY-137431) and milvexian (catalog number: HY-125856) were obtained from MedChemExpress. Stock solutions of asundexian and milvexian were prepared at a concentration of 1 mg/mL in dimethyl sulfoxide (catalog number: D8418, Sigma–Aldrich). Working solutions, further diluted in phosphate-buffered saline (pH 7.4), were subsequently spiked into reconstituted standard plasma or thawed platelet-poor plasma at increasing concentrations prior to analysis (0-10 000 ng/mL). The concentrations of the direct FXIa inhibitors used throughout were chosen to represent therapeutic, as well as supratherapeutic, plasma concentrations of both asundexian and milvexian in accordance with recent pharmacokinetic studies [17,18]. The final volumetric dilution of anticoagulant solution was the same for each increasing concentration, and the volume added never exceeded 1% of the total sample volume.

2.3 | Routine and specialized coagulation assays

Routine coagulation assays were performed on a CN-6000 analyzer (Sysmex), and specialized coagulation assays were performed on a CS-2500 analyzer (Sysmex), both according to the methodologies provided by the manufacturer. All reagents were obtained from Siemens Healthineers. The PT, APTT-FS, and von Clauss' fibrinogen assay were performed using Dade Innovin (catalog number: 10445704), Dade Actin FS (catalog number: 10445710), and Dade Thrombin (catalog number: 10445721), respectively. One-stage single-point extrinsic and intrinsic coagulation factor assays were performed using Dade Innovin (catalog number: 10445704) and Dade Actin FS (catalog number: 10445710) using appropriate coagulation factor-deficient plasma. Antithrombin functional activity was determined chromogenically using the bovine thrombin-based Berichrom Antithrombin assay (catalog number: 10446672). Protein C functional activity was determined chromogenically using the snake venom-based Berichrom Protein C assay (catalog number: 10446500). Free protein S antigen was determined using the particle-enhanced, immunoturbidimetric INNOVANCE Free Protein S assay (catalog number: 10446029). von Willebrand factor antigen (VWF:Ag) and activity were determined using the particle-enhanced immunoturbidimetric VWF:Ag Assay (catalog number: 10445967) and the BC von Willebrand assay (catalog number: 10446425), respectively. Activated protein C resistance (APC) was determined using the ProC Global screening (APC Screen) and confirmatory (APC Confirm) assays (catalog number: 10446101) and expressed as a normalized ratio (APC ratio) of the APC Screen and APC Confirm assays (APC ratio = [APC Screen/APC Confirm] × 0.37). In accordance with the methodology provided by the manufacturer, APC was determined upon predilution in coagulation FV-deficient plasma. Lupus anticoagulant testing was performed using the dilute Russell's viper venom time-based LA1 screening (LA Screen; catalog number: 10446063) and LA2 confirmatory (LA Confirm; catalog number: 10446064) assays and expressed as a normalized ratio of the LA Screen and LA Confirm assays (normalized ratio = LA Screen/LA Confirm).

2.4 | Treatment of anticoagulated human plasma samples with charcoal-based adsorbents

Two methods were evaluated to remove direct FXIa inhibitors and associated interferences from human plasma samples: (1) plasma samples were treated with raw AC (catalog number: C9157; Sigma–Aldrich), as previously described [19,20]. Briefly, 20 mg of raw acid-washed AC was weighed using a 3-dimensional (3D)-printed micromeasure (National Institutes of Health 3D: 3DPX-020736) and added to 0.5 to 1 mL of human-derived plasma. Samples were agitated for 5 minutes prior to centrifugation at 2000 × *g* for 5 minutes to clarify the plasma. Adsorbed plasma was carefully transferred to a separate tube for analysis of routine and specialized coagulation parameters without further processing. (2) Plasma samples were treated with DOAC-Stop (Haematex Research) according to the manufacturer's recommendations. Briefly, 1 DOAC-Stop tablet was added to 0.5 to 1 mL of plasma, and plasma samples were gently agitated for 5 minutes prior to centrifugation at 2000 × *g* for 5 minutes to clarify the plasma. Adsorbed plasma was carefully transferred to a separate tube for analysis of routine and specialized coagulation parameters without further processing.

2.5 | Statistical analysis

Data points were generated from separate aliquots of reconstituted standard plasma and individual thawed platelet-poor plasma samples in independent experimental assays. Data were curated using Microsoft Excel (version 2018, Microsoft Corporation). Statistical analyses were performed using GraphPad Prism (version 10.4.1, GraphPad Software). Continuous data with normal distributions, as determined by the D'Agostino–Pearson test, were expressed as means and SDs. Mean percentage differences (%Δ) were calculated after anticoagulation with asundexian or milvexian, as well as before and after treatment with AC or DOAC-Stop. Reference change values (RCVs) were used to detect clinically significant changes between measurements before and after treatment with AC [21]. RCVs were calculated with the following equation: $RCV = 100\% \times 2^{1/2} \times Z \times (CV_A^2 + CV_I^2)^{1/2}$ [21], where Z was 1.96 for a corresponding 95% CI, CV_A was the local long-term imprecision, and CV_I was the within-subject biological variation taken from Hollestelle et al. [22]. Data were compared using the Wilcoxon matched-pairs signed-rank test, one-way analysis of variance (ANOVA) test with Dunnett post hoc test for multiple comparisons, or 2-way ANOVA test with Tukey post hoc test for multiple comparisons. P value of $< .05$ was considered statistically significant ($^*P < .05$; $^{**}P < .01$; $^{***}P < .001$).

3 | RESULTS

3.1 | Asundexian and milvexian induce dose-dependent interferences in FXIa-dependent coagulation assays

To investigate the extent of the interferences associated with emerging direct FXIa inhibitors in routine and specialized coagulation assays, we first anticoagulated commercial standard plasma with either asundexian or milvexian to final concentrations of 1000 and 4000 ng/mL (n = 5). The concentrations of the direct FXIa inhibitors were chosen to represent maximum plasma concentrations reported in recent pharmacokinetic studies [17,18]. Commercial standard plasma both with and without direct FXIa inhibitors was then analyzed for routine and specialized coagulation parameters (Tables 1 and 2).

In the presence of asundexian or milvexian, no appreciable interferences were observed for assays dependent on extrinsic coagulation factors, including the PT and PT-based one-stage assays for FII, FV, FVII, and FX (Tables 1 and 2). Similarly, no appreciable interferences were observed for the natural anticoagulants antithrombin, protein C, and protein S, the lupus anticoagulant, VWF:Ag, and the VWF activity (Tables 1 and 2).

In contrast, assays dependent on intrinsic coagulation factors particularly those requiring the activity of FXI/FXIa demonstrated significant dose-dependent asundexian- and milvexian-associated interferences (Tables 1 and 2). At 1000 and 4000 ng/mL of asundexian, the APTT (mean ± SD) was prolonged from a baseline of 27.3 ± 0.3 seconds to 70.9 ± 0.3 seconds and 97.6 ± 0.2 seconds, corresponding to %Δ of 159.7% and 257.5%, respectively (Table 1). Similarly, at 1000 and 4000 ng/mL of milvexian, the APTT (mean ± SD) was prolonged from a baseline of 27.3 ± 0.3 seconds to 71.3 ± 0.8 seconds and 101.8 ± 0.8 seconds, with %Δ of 161.2% and 272.9%, respectively (Table 2). The activities of FVIII, FIX, FXI, and FXII, as measured by APTT-based 1-stage assays, were also significantly and dose-dependently reduced in the presence of asundexian and milvexian (Tables 1 and 2). In the presence of increasing concentrations of asundexian, the activities of FVIII, FIX, and FXII were significantly reduced by 54.5% and 69.6%; 35% and 60.0%; and 8.5% and 24.3%, respectively, relative to baseline (Table 1). Furthermore, in the presence of increasing concentrations of milvexian, the activities of FVIII, FIX, and FXII were also significantly reduced by 60.7% and 89.5%; 45.3% and 67.8%; and 15.6% and 32.5%, respectively, relative to baseline (Table 2). Unsurprisingly, the most pronounced reductions were observed for FXI: in the presence of increasing concentrations of asundexian, the activity of FXI was significantly reduced by 54.7% and 75.3%, respectively, relative to baseline (Table 1); in the presence of increasing concentrations of milvexian, activity of FXI was significantly reduced by 63.6% and 96.3%, respectively, relative to baseline (Table 2).

Moreover, in the APTT-based ProC Global assay for APC, the presence of asundexian and milvexian resulted in dose-dependent prolongations in both the APC Screen and APC Confirm assays (Tables 1 and 2). Notably, at a concentration of 1000 ng/mL, asundexian- and milvexian-associated prolongations were proportional between the APC Screen and APC Confirm assays; as such, the overall APC ratio remained unchanged (Tables 1 and 2). However, at concentrations of 4000 ng/mL, no coagulation was observed for the APC Screen, which rendered the APC ratio undeterminable due to the presence of asundexian and milvexian (Tables 1 and 2).

As we previously noted [15], milvexian-associated interferences in routine and specialized coagulation testing are slightly more pronounced than asundexian-associated interferences. Importantly, all observed %Δ, except for those related to FXII activity at the lower concentrations of asundexian and milvexian, were deemed clinically significant when compared with the respective RCVs of each assay (Tables 1 and 2).

3.2 | Charcoal-based adsorbents effectively remove both asundexian and milvexian *in vitro*

Given the clinically significant interferences associated with direct FXIa inhibitors, strategies for the removal of asundexian and milvexian will be required to facilitate the provision and interpretation of hemostatic testing. Therefore, we next investigated whether charcoal-based adsorbents could effectively remove asundexian and milvexian prior to routine or specialized coagulation testing, as has been reported for direct FIIa and FXa inhibitors [23]. Commercial standard plasma was anticoagulated with increasing therapeutic concentrations of asundexian or milvexian (0-4000 ng/mL), as well as a potentially supratherapeutic concentration of 10 000 ng/mL (n = 5). Standard plasma, both with and without direct FXIa inhibitors, was subsequently analyzed before and after treatment with either raw AC or DOAC-Stop to remove the direct FXIa inhibitors.

In the absence of a specific drug-calibrated assay for either asundexian or milvexian, APTT-based clotting times were employed to monitor the adsorption of direct FXIa inhibitors based on our previous findings that an APTT with Dade Actin FS is sensitive to concentrations of asundexian and milvexian ≥ 50 ng/mL [16].

Treatment with either AC or DOAC-Stop had minimal impact on the APTT in nonanticoagulated standard plasma; furthermore, treatment with both AC and DOAC-Stop corrected asundexian- and milvexian-associated prolongations in APTT-based clotting times to the untreated baseline, indicating efficient adsorption of both direct FXIa inhibitors (Figure 1, Supplementary Table S1). Complete absorption was observed for all therapeutic concentrations of asundexian and milvexian (Figure 1A–D), and complete adsorption was even observed for supratherapeutic concentrations (Figure 1E–H). In terms of adsorption efficiency, based on the APTT clotting times after treatment, both AC and DOAC-Stop appeared to demonstrate comparable efficiencies for the removal of direct FXIa inhibitors *in vitro* (Supplementary Table S1). Indeed, no statistically significant differences were observed between the clotting times of anticoagulated standard plasma posttreatment with either AC or DOAC-Stop for all concentrations of asundexian and milvexian (Supplementary Table S1).

TABLE 1 Summary of asundexian-associated interferences in routine and specialized coagulation assays.

Parameter	Reference range	Standard plasma	Asundexian						RCV (%)
			+1000 ng/mL	*P* value	%Δ	+4000 ng/mL	*P* value	%Δ	
PT (s)	9.5-11.1	11.6 ± 0.2	11.8 ± 0.1	ns	1.7	11.9 ± 0.0	ns	2.6	10.4
APTT (s)	21-29	27.3 ± 0.3	70.8 ± 0.3	**<.001**	**159.7**	97.6 ± 0.2	**<.001**	**257.5**	**9.0**
Fibrinogen (g/L)	1.7-4.1	2.6 ± 0.0	2.5 ± 0.0	ns	−3.8	2.5 ± 0.1	ns	−3.8	29.1
Antithrombin (%)	80-120	90.9 ± 0.4	89.5 ± 2.3	ns	−1.5	88.5 ± 2.3	ns	−2.6	12.1
Protein C (%)	70-120	92.2 ± 0.8	90.4 ± 2.9	ns	−2.0	91.1 ± 2.5	ns	−1.2	20.1
Protein S (%)	60-114	83.2 ± 0.1	83.1 ± 0.2	ns	−0.1	81.4 ± 0.8	ns	−2.2	13.2
APC Screen (s)	–	153.9 ± 1.8	216.8 ± 16.7	**<.001**	**40.9**	NC[a]	–	–	**12.3**
APC Confirm (s)	–	66.1 ± 1.2	88.7 ± 1.2	**<.001**	**34.2**	122.7 ± 3.6	**<.001**	**85.6**	**12.3**
APC Ratio	≥0.7	0.9 ± 0.0	0.9 ± 0.1	ns	0.0	ND[b]	–	–	12.3
LA Screen (s)	31-43	36.1 ± 0.7	34.9 ± 0.2	ns	−3.3	36.0 ± 0.2	ns	−0.3	20.7
LA Confirm (s)	30-38	35.0 ± 0.5	35.3 ± 0.5	ns	0.9	36.0 ± 0.6	ns	2.9	20.3
LA Ratio	0.8-1.2	1.0 ± 0.02	1.0 ± 0.02	ns	0.0	1.0 ± 0.02	ns	0.0	13.9
VWF:Ag (%)	50-160	84.3 ± 0.9	86.7 ± 1.6	ns	2.8	86.7 ± 1.1	ns	2.8	36.5
VWF:RCo (%)	55-156	86.2 ± 0.9	83.1 ± 1.8	ns	−3.6	87.2 ± 1.3	ns	1.2	48.5
FII (%)	70-146	88.6 ± 2.5	84.3 ± 2.9	ns	−4.9	85.2 ± 1.6	ns	−3.8	16.4
FV (%)	62-150	88.9 ± 0.7	84.0 ± 2.2	ns	−5.5	86.7 ± 1.8	ns	−2.5	17.5
FVII (%)	67-143	81.7 ± 0.7	84.5 ± 4.4	ns	−3.2	85.1 ± 1.0	ns	−1.3	24.9
FX (%)	70-152	114.4 ± 1.1	110.7 ± 1.8	ns	−4.9	112.9 ± 1.9	ns	−3.8	26.2
FVIII (%)	50-149	89.9 ± 1.2	40.9 ± 0.8	**<.001**	**−54.5**	27.3 ± 1.9	**<.001**	**−69.6**	**21.1**
FIX (%)	55-163	103.0 ± 0.6	67.0 ± 1.1	**<.001**	**−35.0**	41.2 ± 1.2	**<.001**	**−60.0**	**18.0**
FXI (%)	67-127	80.3 ± 1.1	36.4 ± 0.8	**<.001**	**−54.7**	19.8 ± 7.8	**<.001**	**−75.3**	**19.0**
FXII (%)	52-164	138.3 ± 0.5	126.6 ± 5.1	**<.001**	−8.5	104.7 ± 3.6	**<.001**	**−24.3**	**16.1**

Data are presented as mean ± SD (n = 5). Data in **bold** indicate either a statistically significant difference compared with baseline if the P value is < .05 or a clinically significant difference compared with baseline if %Δ is greater than the RCV. Statistical significance was determined using one-way ANOVA with Dunnett post hoc test.

%Δ, mean percentage difference; APC, activated protein C resistance; APC Confirm, APC confirmatory assay; APC Ratio, normalized ratio of the APC Screen and APC Confirm assays; APC Screen, APC screening assay; APTT, activated partial thromboplastin time; F, factor; LA, lupus anticoagulant; LA Confirm, LA2 confirmatory assay; LA Ratio, normalized ratio of the LA Screen and LA Confirm assays; LA Screen, LA1 screening assay; NC, no coagulation; ND, not determined; ns, not significant; PT, prothrombin time; RCV, reference change value; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor.

[a] NC observed due to the presence of asundexian.

[b] Ratio not determinable due to the presence of asundexian.

3.3 | DOAC-Stop effectively removes asundexian- and milvexian-associated interferences in a simulated patient cohort

To further investigate whether asundexian- and milvexian-associated interferences were concomitantly removed by treatment with charcoal-based adsorbents under more physiological conditions, platelet-poor plasma from ostensibly healthy individuals (N = 25) was anticoagulated with increasing concentrations of asundexian or milvexian (0-4000 ng/mL) to experimentally simulate a patient cohort in receipt of direct FXIa inhibitors. Platelet-poor plasma samples, both with or without direct FXIa inhibitors, were subsequently analyzed before and after treatment with DOAC-Stop, focusing on FXI/FXIa-dependent assays identified as sensitive to asundexian- and milvexian-associated interferences.

As expected, asundexian and milvexian induced similar dose-dependent interferences in platelet-poor plasma as those observed in commercial standard plasma (Figures 2 and 3, Supplementary Tables S2 and S3). Treatment with DOAC-Stop corrected asundexian- and milvexian-associated prolongations to baseline in all sensitive assays at all concentrations tested (Figures 2 and 3, Supplementary Tables S2 and S3); moreover, treatment with a charcoal-based adsorbent resulted in neither statistically nor clinically significant differences in all sensitive assays after treatment compared with

TABLE 2 Summary of milvexian-associated interferences in routine and specialized coagulation assays.

Parameter	Reference range	Standard plasma	Milvexian						RCV (%)
			+1000 ng/mL	*P* value	%Δ	+4000 ng/mL	*P* value	%Δ	
PT (s)	9.5-11.1	11.6 ± 0.2	11.7 ± 0.1	ns	0.9	12 ± 0.0	ns	3.4	10.4
APTT (s)	21-29	27.3 ± 0.3	71.3 ± 0.8	**<.001**	**161.2**	101.8 ± 0.8	**<.001**	**272.9**	**9.0**
Fibrinogen (g/L)	1.7-4.1	2.6 ± 0.0	2.6 ± 0.0	ns	0.0	2.5 ± 0.1	ns	−3.8	29.1
Antithrombin (%)	80-120	90.9 ± 0.4	88.5 ± 2.3	ns	−0.7	88.5 ± 2.3	ns	−2.3	12.1
Protein C (%)	70-120	92.2 ± 0.8	91.1 ± 2.5	ns	−1.8	91.1 ± 2.5	ns	−3.0	20.1
Protein S (%)	60-114	83.2 ± 0.1	81.4 ± 0.8	ns	0.0	81.4 ± 0.8	ns	0.0	13.2
APC Screen (s)	–	153.9 ± 1.8	236.8 ± 5.4	**<.001**	**53.9**	NC[a]	–	–	**12.3**
APC Confirm (s)	–	66.1 ± 1.2	91.0 ± 3.1	**<.001**	**37.7**	135.8 ± 4.8	**<.001**	**105.4**	**12.3**
APC Ratio	≥0.7	0.9 ± 0.0	1.0 ± 0.0	ns	11.1	ND[b]	–	–	12.3
LA Screen (s)	31-43	36.1 ± 0.7	36.2 ± 1	ns	0.2	37.9 ± 0.2	ns	4.1	20.7
LA Confirm (s)	30-38	35.0 ± 0.5	35.5 ± 0.4	ns	1.4	36.6 ± 0.5	ns	4.6	20.3
LA Ratio	0.8-1.2	1.0 ± 0.02	1.0 ± 0.02	ns	0.0	1.0 ± 0.02	ns	0.0	13.9
VWF:Ag (%)	50-160	84.3 ± 0.9	88.3 ± 0.6	ns	4.7	86.6 ± 1.9	ns	2.7	36.5
VWF:RCo (%)	55-156	86.2 ± 0.9	84.0 ± 1.7	ns	−2.6	87.8 ± 1.9	ns	1.9	48.5
FII (%)	70-146	88.6 ± 2.5	85.0 ± 1.9	ns	−4.1	87.6 ± 2.5	ns	−1.1	16.4
FV (%)	62-150	88.9 ± 0.7	85.8 ± 3.1	ns	−3.5	86.1 ± 0.7	ns	−3.1	17.5
FVII (%)	67-143	81.7 ± 0.7	82 ± 1.4	ns	−4.8	81.6 ± 1.4	ns	−2.9	24.9
FX (%)	70-152	114.4 ± 1.1	108.9 ± 1.6	ns	−4.1	111.1 ± 1.7	ns	−1.1	26.2
FVIII (%)	50-149	89.9 ± 1.2	35.3 ± 1.5	**<.001**	**−60.7**	9.4 ± 0.8	**<.001**	**−89.5**	**21.1**
FIX (%)	55-163	103.0 ± 0.6	56.3 ± 0.0	**<.001**	**−45.3**	33.2 ± 2.5	**<.001**	**−67.8**	**18.0**
FXI (%)	67-127	80.3 ± 1.1	29.2 ± 0.6	**<.001**	**−63.6**	3.0 ± 0.8	**<.001**	**−96.3**	**19.0**
FXII (%)	52-164	138.3 ± 0.5	116.7 ± 0.9	**<.001**	−15.6	93.4 ± 4.2	**<.001**	**−32.5**	**16.1**

Data are presented as mean ± SD (n = 5). Data in **bold** indicate either a statistically significant difference compared with baseline if the P value is < .05 or a clinically significant difference compared with baseline if %Δ is greater than the RCV. Statistical significance was determined using one-way ANOVA with Dunnett post hoc test.

%Δ, mean percentage difference; APC, activated protein C resistance; APC Confirm, APC confirmatory assay; APC Ratio, normalized ratio of the APC Screen and APC Confirm assays; APC Screen, APC screening assay; APTT, activated partial thromboplastin time; F, factor; LA, lupus anticoagulant; LA Confirm, LA2 confirmatory assay; LA Ratio, normalized ratio of the LA Screen and LA Confirm assays; LA Screen, LA1 screening assay; NC, no coagulation; ND, not determined; ns, not significant; PT, prothrombin time; RCV, reference change value; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor.

[a] NC observed due to the presence of milvexian.

[b] Ratio not determinable due to the presence of milvexian.

baseline. Collectively, these findings demonstrate the efficacy of charcoal-based adsorption in removing direct FXIa inhibitors from plasma samples and affirm the potential utility of DOAC-Stop in clinical laboratory practice for the accurate interpretation of coagulation results in patients receiving asundexian or milvexian.

4 | DISCUSSION

Inhibition of FXI/FXIa represents a novel approach to anticoagulation, with the potential to reduce thrombotic risk while preserving physiological hemostasis. However, DOACs are known to cause extensive interferences in clinical laboratory practice [15]. Given the mechanistic similarities among DOACs, it is reasonable to anticipate that emerging direct FXIa inhibitors, such as asundexian and milvexian, may similarly interfere with routine and specialized coagulation assays. Therefore, we evaluated the extent of interferences associated with emerging direct FXIa inhibitors in a panel of assays. Furthermore, we investigated the effectiveness of charcoal-based adsorbents, including AC and DOAC-Stop, as a means of removing asundexian- and milvexian-associated interferences from human-derived plasma.

Our findings demonstrate that both asundexian and milvexian, even at the maximum plasma concentrations reported in pharmacokinetic studies [17,18], do not significantly interfere with routine and

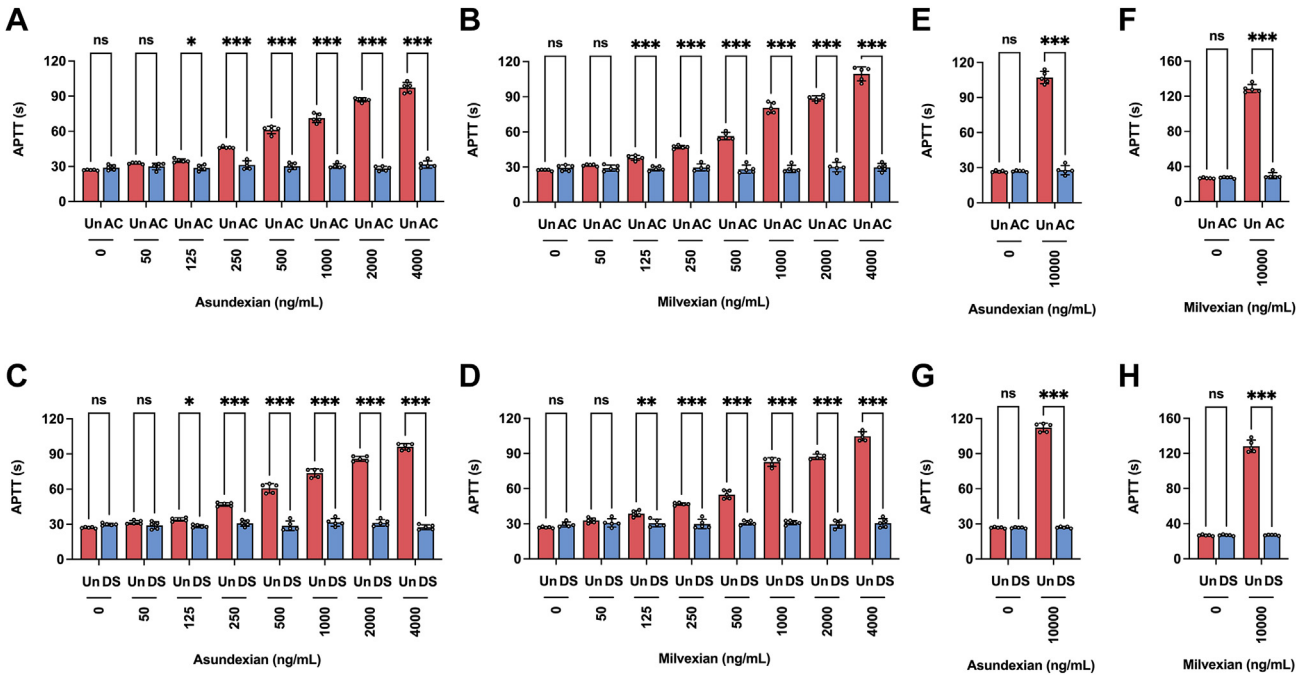

FIGURE 1 Charcoal-based adsorbents remove direct factor (F)XIa inhibitors *in vitro*. (A, B) Removal of increasing therapeutic concentrations of (A) asundexian and (B) milvexian spiked in standard plasma, either untreated (Un) or treated with raw activated charcoal (AC). (C, D) Removal of increasing therapeutic concentrations of (C) asundexian and (D) milvexian spiked in standard plasma, either Un or treated with DOAC-Stop (DS). (E, F) Removal of supratherapeutic concentrations of (E) asundexian and (F) milvexian spiked in standard plasma, either Un or treated with raw AC. (G, H) Removal of supratherapeutic concentrations of (G) asundexian and (H) milvexian spiked in standard plasma, either Un or treated with DS. Data are presented as the mean with SD using scatter dot plots. For each assay, 5 independent replicates of plasma spiked with asundexian or milvexian were assayed (n = 5). Note: no statistically significant differences were observed between platelet-poor plasma with or without raw AC or DS for all concentrations of asundexian and milvexian ($P > .05$; Supplementary Table S1). APC, activated protein C resistance; APC Confirm, APC confirmatory assay; APC Ratio, normalized ratio of the APC Screen and APC Confirm assays; APC Screen, APC screening assay; APTT, activated partial thromboplastin time; ns, not significant. Probability value (2-way ANOVA with Tukey post hoc test): ns, $P > .05$; $^{*}P < .05$; $^{***}P < .001$.

specialized assays dependent on extrinsic coagulation factors, assays employing particle-enhanced immunoturbidimetry, or chromogenic assays used in thrombophilia testing. These findings indicate that clinical laboratories may continue to conduct these tests without major concern for preanalytical interference in patients receiving FXIa inhibitors. In contrast, routine and specialized assays dependent on intrinsic coagulation factors, particularly those highly dependent on FXI/FXIa, exhibited significant dose-dependent interferences. In both commercial standard plasma and platelet-poor plasma from ostensibly healthy individuals, asundexian- and milvexian-associated interferences were sufficient to cause apparent moderate-to-severe hemostatic deficiencies in FVIII, FIX, FXI, and FXII.

Notably, direct FXIa inhibitors were associated with interferences in fewer routine and specialized coagulation assays than direct FIIa and FXa inhibitors. The extent of interference in the APTT assay for both asundexian and milvexian was similar to that we recently reported for the direct FIIa inhibitor dabigatran at concentrations expected in patient-derived plasma and more pronounced than those observed for the direct FXa inhibitors apixaban, edoxaban, and rivaroxaban [24]. However, it is important to note that the APTT does not correlate reliably with the concentration of either direct FIIa and FXa inhibitors [25], and it remains to be seen whether the APTT can be used for monitoring the concentration of either asundexian or milvexian *in vivo*.

While this study included a broad panel of routine and specialized coagulation assays with clinical relevance, we acknowledge that direct FXIa inhibitors may or may not interfere with other assays not assessed in this investigation, including global coagulation assays. Clinicians and laboratorians must, therefore, carefully consider any coagulometric assay in use, particularly with respect to interference from direct FXIa inhibitors. For instance, we observed significant asundexian- and milvexian-associated interferences in an APTT-based assay for APC. However, alternative assays, such as those employing the activation of FV or FX [26], may be resistant to interference. Assay-specific interferences are commonly reported for DOACs [27]; as such, it is important to maintain effective communication between clinicians and laboratorians when providing and interpreting routine and specialized coagulation assays in patients potentially in receipt of direct FXIa inhibitors.

Clinical laboratories can support the assessment of underlying hemostatic and thrombotic potential in patients receiving direct FXIa inhibitors by potentially removing direct FXIa inhibitors *ex vivo*. Consistent with previous studies evaluating the removal of both direct FIIa and FXa inhibitors [28], our findings demonstrate that the

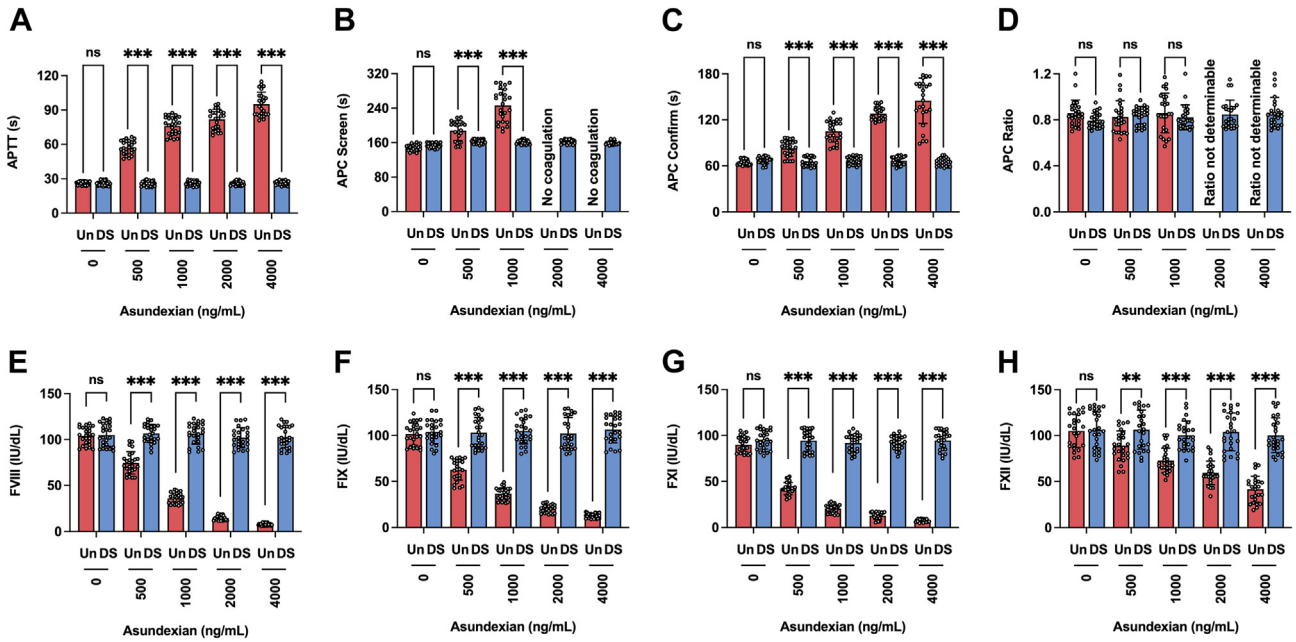

FIGURE 2 DOAC-Stop (DS) removes asundexian-associated interferences in factor (F)XIa-dependent assays. The effect of increasing concentrations of asundexian, before and after treatment with DS, on the (A) activated partial thromboplastin time (APTT), (B) activated protein C resistance (APC) screening assay (APC Screen), (C) APC confirmatory assay (APC Confirm), (D) APC normalized ratio (APC Ratio), (E) one-stage FVIII activity assay, (F) one-stage FIX activity assay, (G) one-stage FIX activity assay, and (H) one-stage FXII activity assay. Data are presented as the mean with SD using scatter dot plots. For each assay, independent replicates of platelet-poor plasma from healthy individuals spiked with asundexian or milvexian were assayed ($N = 25$). Note: no statistically significant differences were observed between platelet-poor plasma with or without DS treatment for all assays ($P > .05$). ns, not significant; Un, untreated. Probability value (2-way ANOVA with Tukey post hoc test): ns, $P > .05$; $^{***}P < .001$.

addition of charcoal-based adsorbents effectively removes asundexian- and milvexian-associated interference in routine and specialized coagulation testing. Both AC and DOAC-Stop were effective in removing interferences in the APTT from standard human plasma across a wide range of therapeutic and supratherapeutic concentrations (0-10 000 ng/mL), and treatment-corrected FXIa prolonged APTT-based clotting times to near-baseline results. In a simulated patient cohort, DOAC-Stop was highly efficacious in removing asundexian- and milvexian-associated interferences from routine and specialized coagulation assays identified as sensitive to direct FXIa inhibitors. Our findings indicate that DOAC-Stop would allow for reliable evaluation of routine and specialized coagulation assays in patients receiving emerging direct FXIa inhibitors.

While we appeared to achieve near-complete adsorption of asundexian and milvexian from commercial standard plasma and platelet-poor plasma using charcoal-based adsorbents, previous studies have reported incomplete removal of dabigatran, apixaban, and rivaroxaban using both AC [29] and DOAC-Stop [30,31]. We have previously demonstrated that APTT with Dade Actin FS is sensitive to concentrations of asundexian and milvexian ≥ 50 ng/mL [16]. While concentrations of 50 ng/mL for DOACs are considered clinically relevant [32], the threshold for clinical relevance of asundexian and milvexian remains undefined. As such, we cannot entirely exclude the possibility that asundexian- and milvexian-associated interferences in routine and specialized coagulation assays may persist after treatment with charcoal-based adsorbents, especially in the absence of a specific drug-calibrated assay for direct FXIa inhibitors. Moreover, although the impact of charcoal-based adsorbents on routine and specialized coagulation parameters appears minimal, clinically significant differences may occasionally be observed in patients with results near diagnostic cutoffs [29,33–36]. Consequently, the results of routine and specialized coagulation assays performed on plasma treated with charcoal-based adsorbents to remove direct FXIa inhibitors should be accompanied by appropriate interpretative comments to support clinical decision-making [37,38].

We also acknowledge that our study has limitations. First, although *in vitro* plasma-based systems are commonly employed in studies of DOACs [39–42], an important limitation is that both standard plasma and platelet-poor plasma anticoagulated with asundexian and milvexian *in vitro* may not effectively represent the complexity of samples taken from patients under anticoagulation therapy with direct FXIa inhibitors. Indeed, further studies involving patient-derived plasma samples are warranted to validate our findings. Second, variability across reagents and instrumentation means that our observations may not extrapolate to all available reagents and/or instrumentation. Lastly, since no established therapeutic ranges currently exist for asundexian or milvexian, the tested concentrations reflect the maximum plasma levels reported in clinical studies [17,18]. Although likely, it remains uncertain whether our findings will be maintained, specifically at concentrations outside the ranges

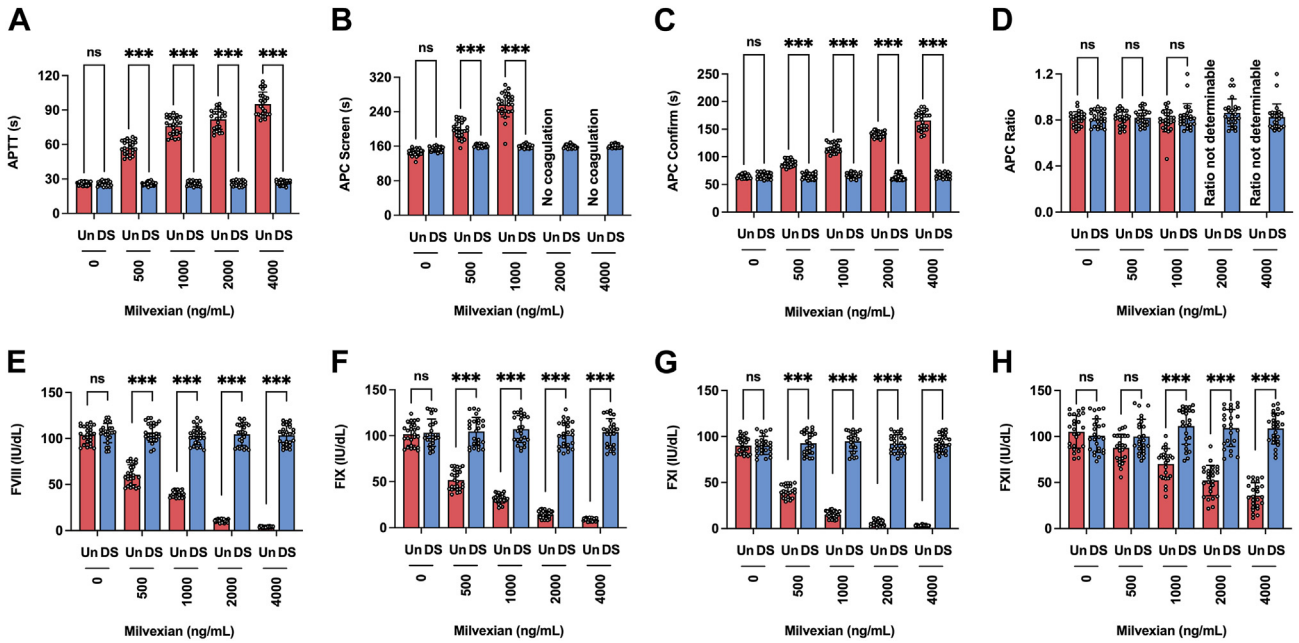

FIGURE 3 DOAC-Stop (DS) removes milvexian-associated interferences in factor (F)XIa-dependent assays. The effect of increasing concentrations of milvexian, before and after treatment with DS, on the (A) activated partial thromboplastin time (APTT), (B) activated protein C resistance (APC) screening assay (APC Screen), (C) APC confirmatory assay (APC Confirm), (D) APC normalized ratio (APC Ratio), (E) one-stage FVIII activity assay, (F) one-stage FIX activity assay, (G) one-stage FIX activity assay, and (H) one-stage FXII activity assay. Data are presented as the mean with SD using scatter dot plots. For each assay, independent replicates of platelet-poor plasma from healthy individuals spiked with asundexian or milvexian were assayed ($N = 25$). Note: no statistically significant differences were observed between platelet-poor plasma with or without DS treatment for all assays ($P > .05$). ns, not significant; Un, untreated. Probability value (2-way ANOVA with Tukey post hoc test): ns, $P > .05$; ***$P < .001$.

evaluated, as direct FXIa inhibitors progress through late-stage clinical development.

5 | CONCLUSIONS

Our findings demonstrate that the direct FXIa inhibitors asundexian and milvexian are associated with substantial dose-dependent interferences in FXIa-dependent routine and specialized coagulation assays. In contrast, extrinsic and common pathway assays remain largely unaffected. Our findings further demonstrate that external charcoal-based adsorbents, both AC and DOAC-Stop, can effectively remove direct FXIa inhibitors from human-derived plasma and represent a viable strategy for mitigating asundexian- and milvexian-associated interferences. Additional clinical validation in patient populations treated with asundexian and milvexian is warranted as direct FXIa inhibitors progress through late-stage clinical development.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.P.C. and J.V.H.; methodology, G.T.B., M.P.C., and J.V.H.; investigation, G.T.B. and J.V.H.; validation, G.T.B. and J.V.H.; formal analysis, G.T.B., M.P.C., and J.V.H.; visualization, G.T.B. and J.V.H.; writing–original draft preparation, G.T.B., M.P.C., and J.V.H.; writing–review and editing, G.T.B., M.P.C., and J.V.H.; supervision, J.V.H. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF COMPETING INTERESTS

DOAC-Stop was generously gifted for research by Haematex Research; however, the authors have received no instructions or directions of any kind from Haematex Research. Reagents for analysis were generously gifted for research by Sysmex UK and Ireland; however, the authors have received no instructions or directions of any kind from Sysmex UK and Ireland. J.V.H. also declares travel support from Sysmex UK and Ireland. The authors declare no other known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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