Development of Kallikrein-Kinin System Biomarker Assays to Investigate Bradykinin-Mediated Diseases

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Introduction

- Activation of the plasma kallikrein-kinin system (KKS) results in cleavage of high-molecular-weight kininogen (HMWK) and production of vasodilatory kinins, such as bradykinin (BK) and its metabolite BK1-5.[1] Kallidin (KD) is another biologically active kinin produced by tissue kallikrein cleavage of either HMWK or low-molecular-weight kininogen (LMWK).
- BK is involved in various physiological and pathological processes, including angioedema (AE).[2] Differentiating BK-mediated vs histamine-mediated AE and assessing other BK-mediated disorders by measuring biomarkers produced upon activation of the KKS remains a challenge due to proteolytic instability of the kinins and limitations of current analytical assays.[3]
- Establishment of a method to accurately measure BK and related peptides could aid in identifying BK-mediated AE as well as other BK-mediated diseases.*

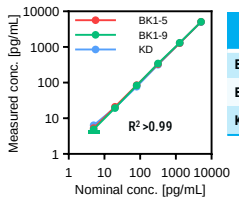
Materials and Methods

- To inhibit *ex vivo* activation of KKS proteases and proteolytic degradation of BK, a protease inhibitor (PI) cocktail was manufactured in a liquid and lyophilized form.
- Blood samples were collected from healthy volunteers (HV) by Fidelis Research AD in accordance with the Declaration of Helsinki and approved by The National Bioethics Committee of Medicines and Medical Devices (CNBMDM, protocol no. FRT-19101). All participants gave their written informed consent before enrollment.
- Plasma was collected using liquid PI or lyophilized PI form, or using ethylenediaminetetraacetic acid (EDTA) as a control.
- An ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS)/MS protocol was optimized to measure BK (BK1-9), BK1-5, and KD (Attoquant Diagnostics GmbH).
- A capillary-based immunoassay was also developed to quantify the cleaved and intact HMWK (cHMWK and iHMWK, respectively) (Charnwood Molecular Ltd).
- Qualification of the UPLC-MS/MS and capillary-based immunoassay was performed using plasma from HVs collected under different conditions.

Results

- Calibration curves were prepared by spiking different kinin peptides in surrogate blank matrix.
- For all tested kinins, the back-calculated concentrations of the calibrator standards were within ±15% of the nominal value and met qualification criteria (**Figure 1**).

Figure 1. Calibration curves of BK1-5, BK1-9, and KD



	Linearity range of quantification	
	LLoQ	ULoQ
BK1-5	5 pg/mL	10,240 pg/mL
BK1-9	5 pg/mL	10,240 pg/mL
KD	20 pg/mL	10,240 pg/mL

LLoQ: lower limit of quantification; ULoQ: upper limit of quantification

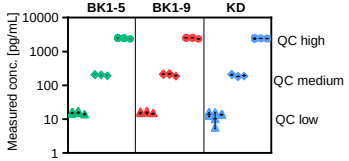
References

1. Kaplan AP, et al. *Adv Immunol.* 2014;121:41-89. **2.** Maurer M, et al. *Clin Rev Allergy Immunol.* 2021;61:40-9.
3. Kaplan AP, et al. *Front Med (Lausanne).* 2017;4:206.

Results

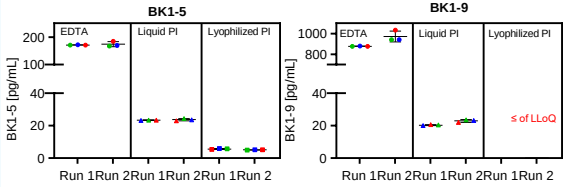
- Kinin LC-MS/MS assay in blank matrix met qualification criteria. Within- and between-run accuracy and precision. Coefficient of variation (CV) <15% (**Figure 2**).

Figure 2. Analysis of quality control (QC) samples for BK1-5, BK1-9 and KD in blank matrix



- KD levels were below the limit of detection in all types of human plasma analyzed.
- Liquid PI efficiently inhibited KKS activation and stabilized BK1-9 levels compared with EDTA alone and was superior to lyophilized PI, resulting in low inter- and intra-run variability (CV <15%) for both BK1-5 and BK1-9 (**Figure 3**).

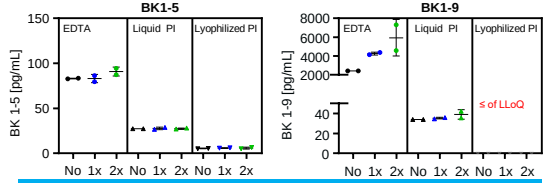
Figure 3. BK1-5 and BK1-9 inter- and intra-run variability in EDTA plasma and plasma with PI



	BK1-5		BK1-9	
Plasma	Mean (pg/mL)	CV %	Mean (pg/mL)	CV %
EDTA	172.8	<10%	925.0	<10%
Liquid PI	23.6	<10%	21.7	<10%
Lyophilized PI	5.4	<10%	Not applicable	Not applicable

- Liquid but not lyophilized PI efficiently inhibited ex vivo KKS activation and stabilized BK1-9 following 2 cycles of freeze and thaw (FT) as compared with EDTA (**Figure 4**).

Figure 4. Effects of freeze and thaw on BK peptide stability

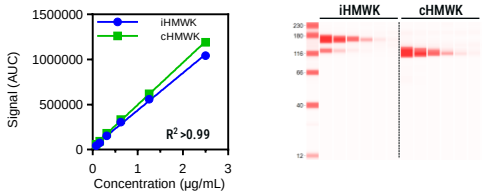


	BK1-5 (pg/mL)			BK1-9 (pg/mL)		
Plasma	No FT	1xFT	2xFT	No FT	1x FT	2xFT
EDTA	83.1	82.9	90.8	2417.3	4260.9	5942.8
Liquid PI	27.4	27.7	27.4	34.0	35.3	38.9
Lyophilized PI	5.3	5.8	5.9	<5.0	<5.0	<5.0

Results

- Standard curves were prepared by spiking iHMWK or cHMWK in kininogen-deficient plasma
- For both proteins, the back-calculated concentrations of the standards met qualification criteria (**Figure 5**).

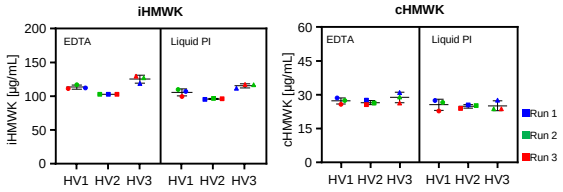
Figure 5. Capillary immunoblotting assay qualification for iHMWK and cHMWK from human plasma



	Linearity range of standard curve	Concentration range accessible in human plasma
i/cHMWK	0.078–2.5 µg/mL	26–140 µg/mL

- Comparable levels of iHMWK and cHMWK were observed in EDTA and liquid PI plasma.
- Plasma with lyophilized PI was not analyzed as it was shown not to stabilize kinins in plasma.
- Acceptable inter-run variability (CV <15%) for both iHMWK and cHMWK in both liquid PI plasma and control EDTA plasma (**Figure 6**).

Figure 6. iHMWK and cHMWK inter-run variability in EDTA plasma and plasma with PI

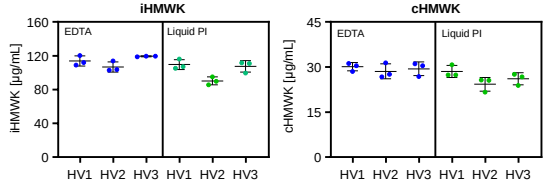


	iHMWK		cHMWK	
Plasma	Mean (µg/mL)	CV %	Mean (µg/mL)	CV %
EDTA	113.4	5.5%	29.4	2.6%
Liquid PI	102.5	10.4%	26.3	8.1%

Results

- Acceptable intra-individual variability (CV <15%) for both iHMWK and cHMWK in both liquid PI plasma and control EDTA plasma (**Figure 7**).

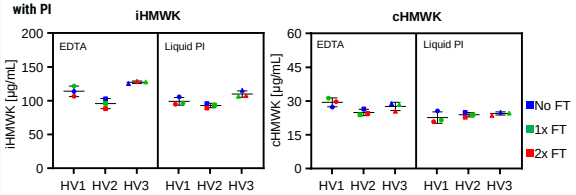
Figure 7. iHMWK and cHMWK intra-individual variability in EDTA plasma and plasma with liquid PI



	iHMWK (µg/mL)				cHMWK (µg/mL)			
Plasma	HV 1	HV 2	HV 3	CV %	HV 1	HV 2	HV 3	CV %
EDTA	113.8	106.8	119.4	<10%	30.1	28.6	29.4	<10%
Liquid PI	109.6	90.3	107.5	<10%	28.5	24.3	26.1	<10%

- Stability of iHMWK and cHMWK was analyzed in EDTA control and liquid PI plasma following 1 or 2 cycles of FT.
- Liquid PI had no apparent effect on levels of iHMWK & cHMWK following FT as compared with EDTA (**Figure 8**).

Figure 8. Effects of freeze and thaw on iHMWK and cHMWK levels in EDTA plasma and plasma with PI



	iHMWK (µg/mL)			cHMWK (µg/mL)		
Plasma	No FT	1xFT	2xFT	No FT	1x FT	2xFT
EDTA	113.9	115.2	107.7	27.5	27.8	26.6
Liquid PI	105.6	99.0	97.2	25.2	23.4	22.4

Conclusions

- **The liquid PI cocktail was shown to be more efficacious in inhibiting non-specific activation of KKS and generation of BK1-9 as compared to EDTA without PI.**
- **Liquid PI was shown to be superior to lyophilized PI in inhibiting degradation of BK1-9.**
- **The established qualified KKS biomarker assays can be used to reliably measure KKS biomarkers in human plasma.**
- **These assays could become key tools for identifying, studying, and managing BK-mediated diseases, including BK-mediated AE.**

COI: E.P.: employee of Pharvaris, holds stock in Pharvaris; **J.N.:** former employee of Pharvaris; **O.D.:** CEO of Attoquant Diagnostics GmbH; **G.V.:** former employee of Pharvaris; **A.L.:** employee of GrayMatters Consulting and consultant to Pharvaris, holds stocks/stock options in Pharvaris; advisor to Kosa Pharma.

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