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Development of *pharmaco*proteomics assays for dissecting the molecular and cellular pathways regulated by anticoagulant peptides in platelets and dendritic cells

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Research Synopsis

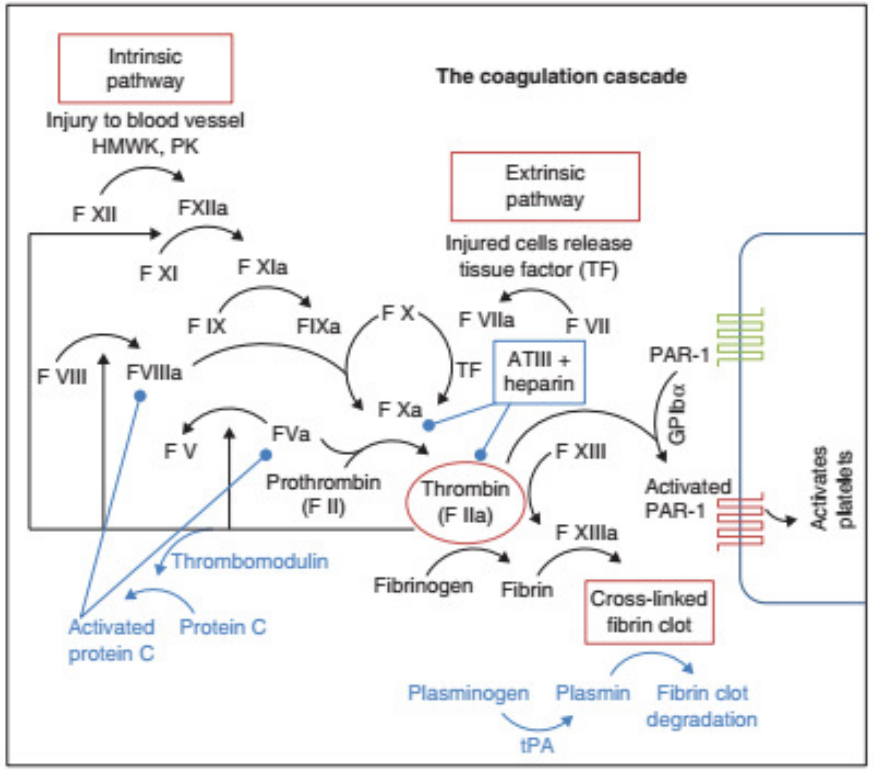
- Pharmacoproteomics uses advanced proteomic technologies for promoting drug discovery and development by highlighting protein expression profiles of diverse cellular and molecular pathways in response to different drug treatment.
- The research presented herein highlights two major achievements of such pharmacoproteomics approaches, including 1) the discovery of novel anticoagulant tetrapeptides and anti-thrombin direct inhibitors (DTI) enabled by the analysis of their effects upon the global protein expression profiles in human platelets, and 2) mapping the pro-coagulative and pro-inflammatory cellular pathways activated in the dendritic cells (DC) treated with bradykinin (BK) peptide RPPGFSPFR.
- Label free quantification (LFQ) analysis of proteomics data generated by nanoLC-MS/MS on a Q-Exactive/Orbitrap mass spectrometer was coupled with Ingenuity Pathways Analysis (IPA) bioinformatics analysis and facilitated the discovery of the downregulation of actin, integrin and RhoA signaling pathways in human platelets treated with DTI. This in turn supported the discovery of new DTIs which act as potent inhibitors of thrombin-activated platelets aggregation, i.e., peptides with drug-like properties that can be used in the treatment of acute coronary diseases (ACD).
- Using a similar pharmacoproteomic platform we observed that mouse DCs stimulated with the BK peptide (RPPGFSPFR) became active in the production of molecules involved in migration/chemotaxis, MHC-I, and MHC-II expression, antigen presentation, inflammation, and cytokines secretion, further mediating the production of coagulation factors V and VIII.
- This research highlights the advantages of employing pharmacoproteomic technologies as a reliable analytical platform that help to the discovery and development of peptide-drugs with anti-coagulant activities.

PART I: Discovery of novel anticoagulant tetrapeptides and anti-thrombin direct inhibitors (DTI) enabled by the analysis of their effects upon the global protein expression profiles in human platelets.

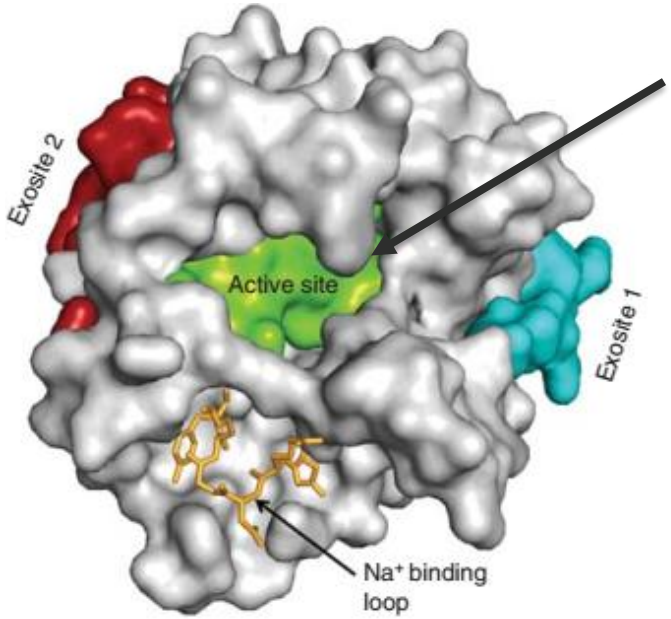
Significance of research: recent studies conducted by researchers at Columbia University Irving Medical Center (1) suggest that one of the immune system's oldest branches, the complement system, and coagulation dysfunction, may be influencing the severity of COVID-19. The team found evidence that clotting activity is linked to COVID-19 severity and that mutations in certain complement and coagulation genes are associated with hospitalization of COVID-19 patients (2). These findings suggest that using anti-coagulant therapies would help to decrease the coagulation-mediated inflammation and hyperreactive state induced by COVID-19 infection (1).

1. **Immune complement and coagulation dysfunction in adverse outcomes of SARS-CoV-2 infection** Nature Medicine | VOL 26 | October 2020 | 1609–1615 |

Figure 1 **An overview of the coagulation cascade.** In black are the procoagulant factors, while in blue are the anticoagulant factors. Thrombin (factor IIa) plays a key role in the cascade and acts as a link between the enzymes and platelets. It also provides negative feedback via its activation of protein C. **(2)**



Inhibitors of the activation of platelet aggregation were shown to be important pharmacological agents for the management of acute coronary syndrome (ACS). Thrombin, a serine protease from the coagulation cascade, induces the activation of platelets to aggregate by binding to and cleaving the extracellular N-terminal domains of protease-activated receptors 1 and 4 (PAR1 and PAR4).



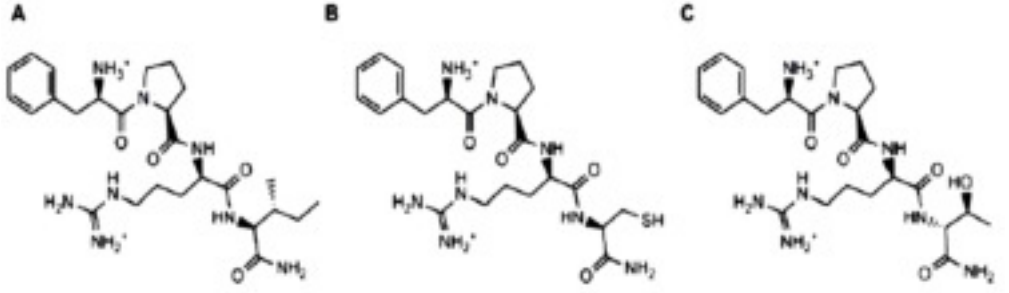
Inhibitors binding to the active site: Direct thrombin inhibitors (DTI)

The complexity of thrombin's functions is driving the design of complex anticoagulants. Advancements in formulations and production processes have attempted to make traditional DTIs (direct thrombin inhibitors) more cost effective to produce.
The literature reveals a trend to develop a thrombin 'modulator' rather than an 'inhibitor (2).

Figure 3. A crystal structure of thrombin (PDB ID: 1PPB) highlighting the active site in green, exosite 1 in teal, exosite 2 in red and the Na⁺ binding loop in orange.

(2) Expert Opin. Ther. Patents (2014) 24(1)

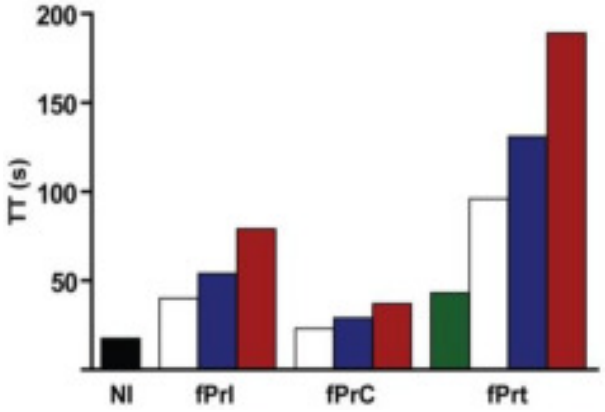
PART I Discovery and development of novel tetrapeptides direct thrombin inhibitors (DTI) lead compounds and potent inhibitors of thrombin-mediated platelets aggregation



1st generation: D-Phe-Pro-D-Arg-X-CONH2 (3)

X-varied with L and D-natural amino acids.

Anticoagulant properties measured by "Prolongation of thrombin time (TT)" was increased by the novel tetrapeptides thrombin inhibitors (DTI).

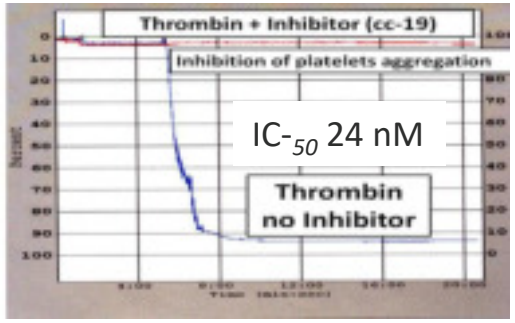


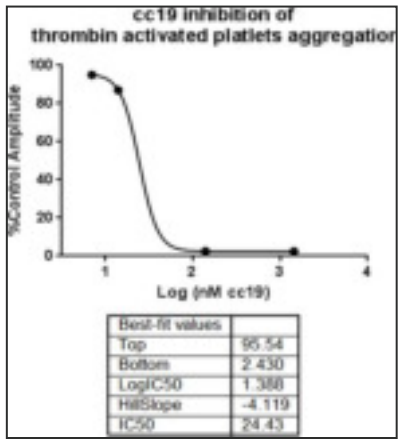
1st generation: *D-Phe*-Pro-D-Arg-X-CONH2 (3)

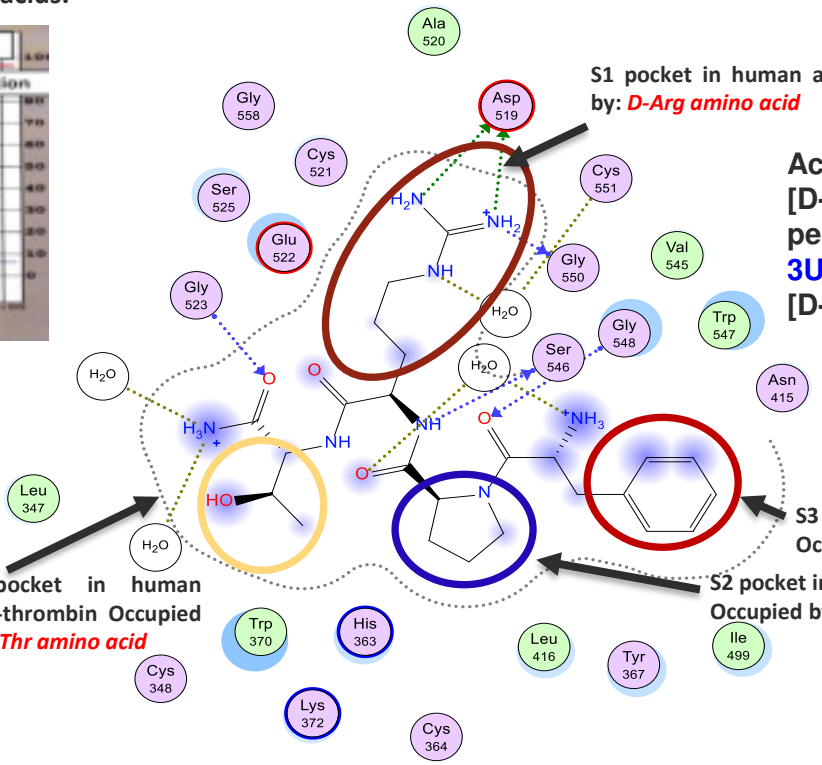
f is D-Phe
t is D-Thr
r is D-Arg
Capital letters are the L-amino acids

Human plasma thrombin times were measured in the absence of inhibitor (NI) and in the presence of :

0.10 mM (green bar) : 100 uM
0.25 mM (white bars),
0.5 mM (blue bars) or
1 mM (red bars) of the indicated tetrapeptide.







S1 pocket in human alpha-thrombin Occupied by: *D-Arg amino acid*

Active site of thrombin in complex with original [D-Phe-Pro-D-Arg-D-Thr-CONH2] anticoagulant peptide.

3U8O.pdb: Alpha-Thrombin in complex with DTI [D-Phe-Pro-D-Arg-DThr-CONH2 (3)

S3 pocket in human alpha-thrombin Occupied by: *D-Phe amino acid*

S2 pocket in human alpha-thrombin Occupied by: *L-Pro amino acid*

S1' pocket in human alpha-thrombin Occupied by: *D-Thr amino acid*

Emerging lead compound

D-Phe-Pro-D-Arg-D-Thr-CONH2:
Ki for thrombin is 850 nM (based on S2238 substrate hydrolysis and in vitro enzyme inhibition assay).

(3) Figueiredo AC, Clement CC, Zakia S, Gingold J, Philipp M, Pereira PJ. Rational design and characterization of D-Phe-Pro-D-Arg-derived direct thrombin inhibitors. PLoS One. 2012;7(3):e34354. Shared authorship and corresponding author

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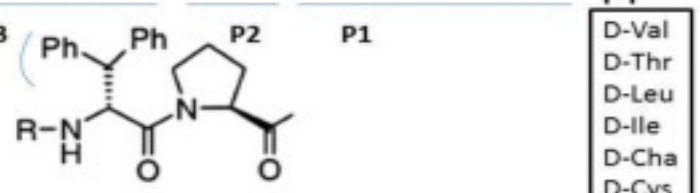
PART I Discovery and development of novel tetrapeptides direct thrombin inhibitors (DTI) lead compounds and potent inhibitors of thrombin-mediated platelets aggregation

- During last decade (2010-2020) my research group reported the further development of new peptidic DTI and performed an ***optimization for P3 position by replacing D-Phe with different un-natural Phe-analogs*** (such as ***D-3,3-di-Phenylalanine***, D-3,5-difluorophenylalanine, trans and dihydrocinnamic acids, (L)/(D)-Tic [1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid], (L)/(D)-Thi [Thienylalanine], D-Naphthylalanine (D-Nal) and 1,2,3,4-tetrahydronorharman-3-carboxylic acid (D-Tpi) among others).
- The **SAR (structure-activity relationship) at P3 position showed that the compounds with D-3,3-di-Phenylalanine in P3 are the new lead DTIs with inhibitory constant in the lowest 50 nM range.**
- All the novel **D-3,3-di-Phenylalanine-based tetrapeptides DTIs** are characterized by an improved inhibition of thrombin activation of platelets aggregation (4).

2nd generation of tetrapeptides DTIs (2012-2020)

New direct thrombin inhibitors with different P3-unnatural amino acids developed from the P3-Pro(P2)-D-Arg(P1)-P1'-CONH2 *DTI scaffold*

D-3,3Diphenylalanyl | L-Pro | D-Arg-P1'-CONH2



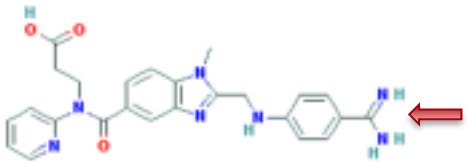
P1'
- D-Val
- D-Thr
- D-Leu
- D-Ile
- D-Cha
- D-Cys
- D-Ala
- D-Ser
- D-Thi

Table 1. Summary of some of the lead peptidic DTIs and the corresponding experimental and predicted Ki (nM).

Peptide sequence (ID)	*Experimental Ki (nM)*	*Predicted (AutoDock Vina) Ki (nM)*
D-3,3-Diphenylalanine-Pro-D-Arg-D-Cys-CONH$_2$	65.5 ± 0.3	40.2
D-3,3-Diphenylalanine-Pro-D-Arg-D-Ala-CONH$_2$	130.6 ± 0.5	85
D-3,3-Diphenylalanine-Pro-D-Arg-D-Thr-CONH$_2$	104.4 ± 1.5 **CC-19**	50.2
D-3,3-Diphenylalanine-Pro-D-Arg-D-Val-CONH$_2$	102.2 ± 5.5	45
D-3,3-Diphenylalanine-Pro-D-Arg-D-Ile-CONH$_2$	64.2 ± 2.5	35
D-3,3-Diphenylalanine-Pro-D-Arg-D-Leu-CONH$_2$	540.2 ± 2.4	350
D-3,3-Diphenylalanine-Pro-D-Arg-D-Thi-CONH$_2$	312.5 ± 0.8	120

Anticoagulants properties for the 2nd generation of DTI: all assays performed in human plasma or blood.

1. ***TEG (***thromboelastography) ***assays* showing the potency for inhibition of thrombin activated platelets aggregation in the whole blood.**
2. ***TT time (thrombin time).***



Compound ID	Molarity in the final assay	TT (sec)
1st generation lead	250 uM	90
2nd generation cc19	4.5 uM	64
PRADAXA (DABIGATRAN) (FDA approved)	0.4 uM	75

Proceedings of the 24th American Peptide Symposium
Ved Srivastava, Andrei Yudin, and Michal Lebl (Editors)
American Peptide Society, 2015

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Structure-Based Design, Synthesis and Evaluation of Novel Peptidic Inhibitors of Thrombin-Induced Activation of Platelets Aggregation

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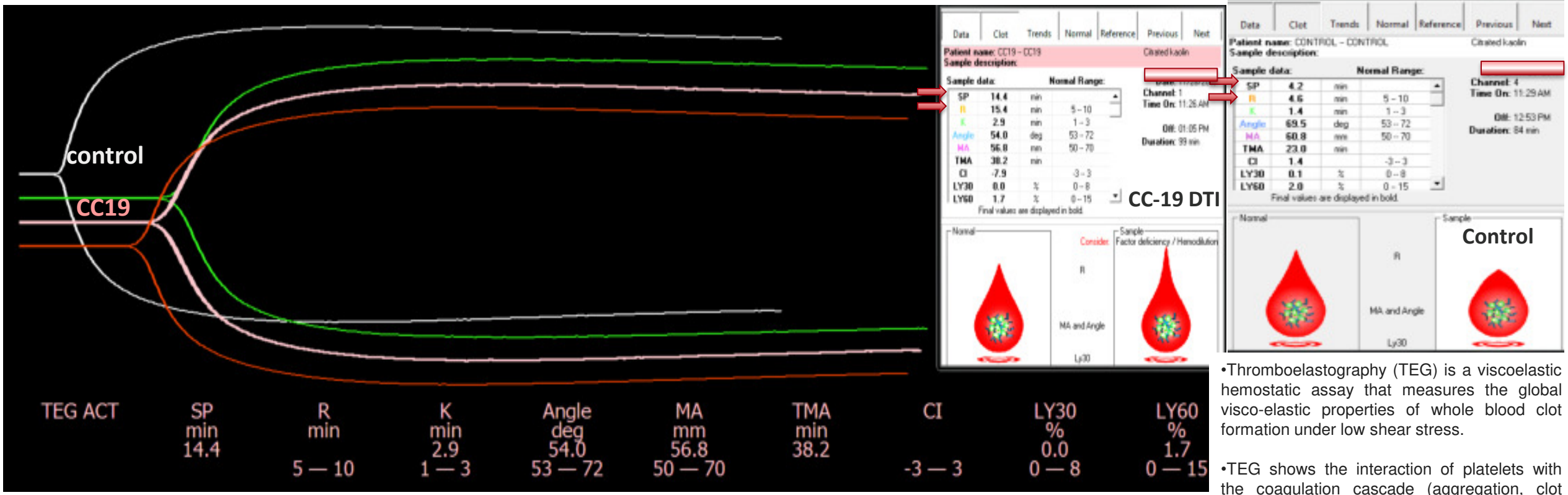
PART I Discovery and development of novel tetrapeptides direct thrombin inhibitors (DTI) lead compounds and potent inhibitors of thrombin-mediated platelets aggregation

✓ -Synthetic inhibitors of the general sequence D-Phe-Pro-D-Arg-P1'-CONH2 bind in a substrate-like orientation to the active site of thrombin.
-All inhibitors can escape thrombin proteolysis and act as inhibitors due to the presence of D-Arg at position P1 with concomitant formation of an unfavorable geometry for the nucleophilic attack by the catalytic serine residue.
-For each inhibitor, its binding affinity correlates well with the extent of interactions established by the residue at position P1'.
-**Specific amino acid substitutions required for activity against platelet aggregation** have been identified, and lead compounds, containing the D-3,3-D-phenylalanine in the P3 position have been developed.
-These lead compounds completely inhibited threshold alpha-thrombin-induced platelet aggregation at concentration 10uM-30nM **(measured with purified platelets and thrombin-mediated aggregation assays and by thromboelastography (TEG))**.

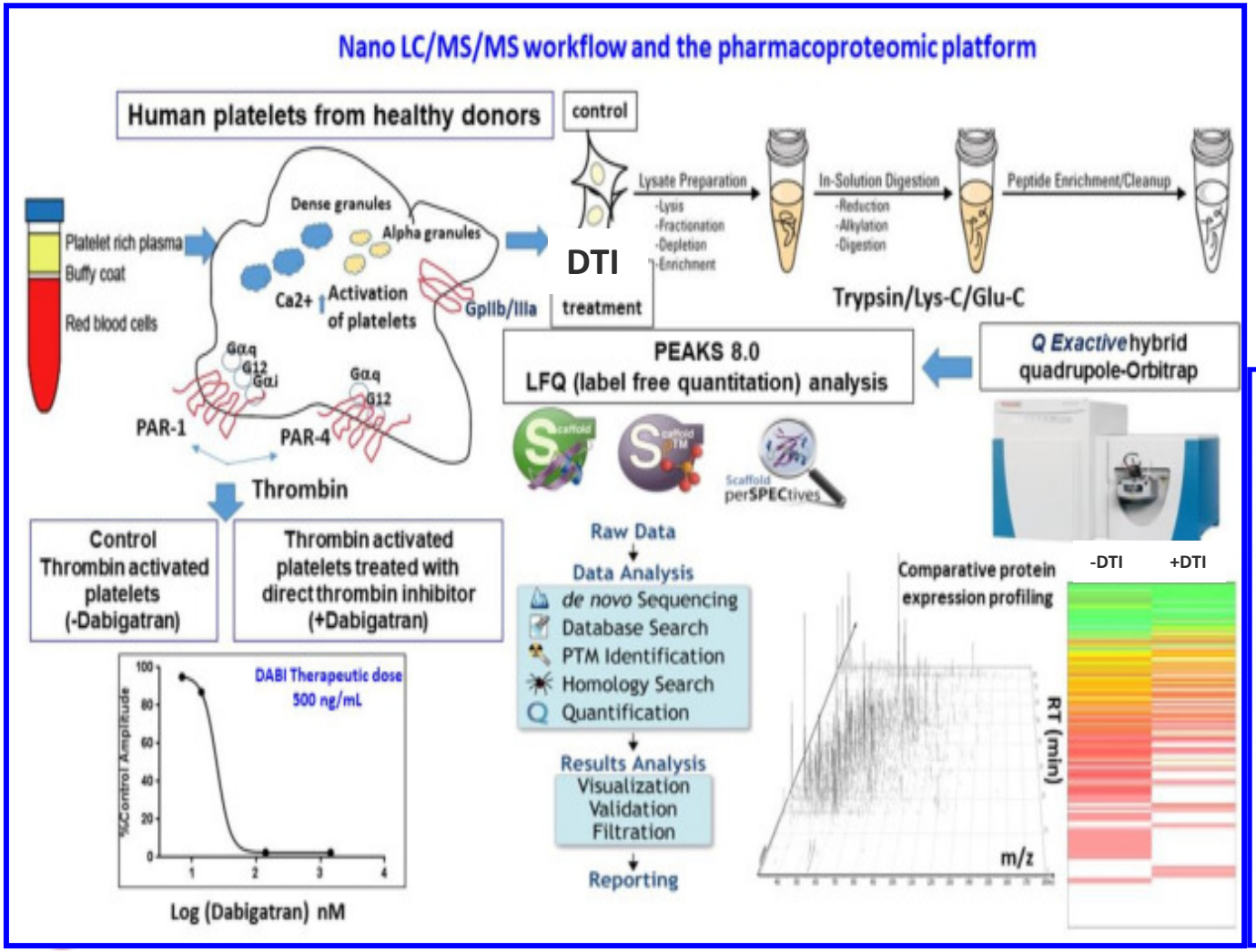


•Thromboelastography (TEG) is a viscoelastic hemostatic assay that measures the global visco-elastic properties of whole blood clot formation under low shear stress.

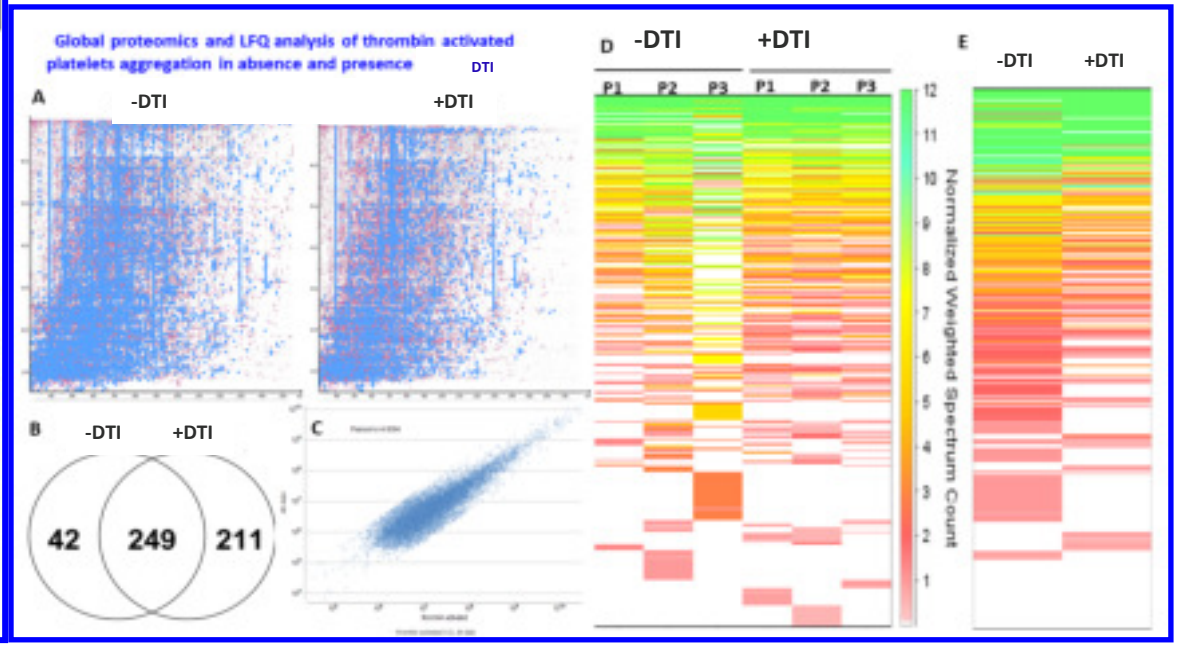
•TEG shows the interaction of platelets with the coagulation cascade (aggregation, clot strengthening, fibrin cross-linking and fibrinolysis).

PART I: Discovery of novel anticoagulant tetrapeptides and anti-thrombin direct inhibitors (DTI) enabled by the analysis of their effects upon the global protein expression profiles in human platelets.

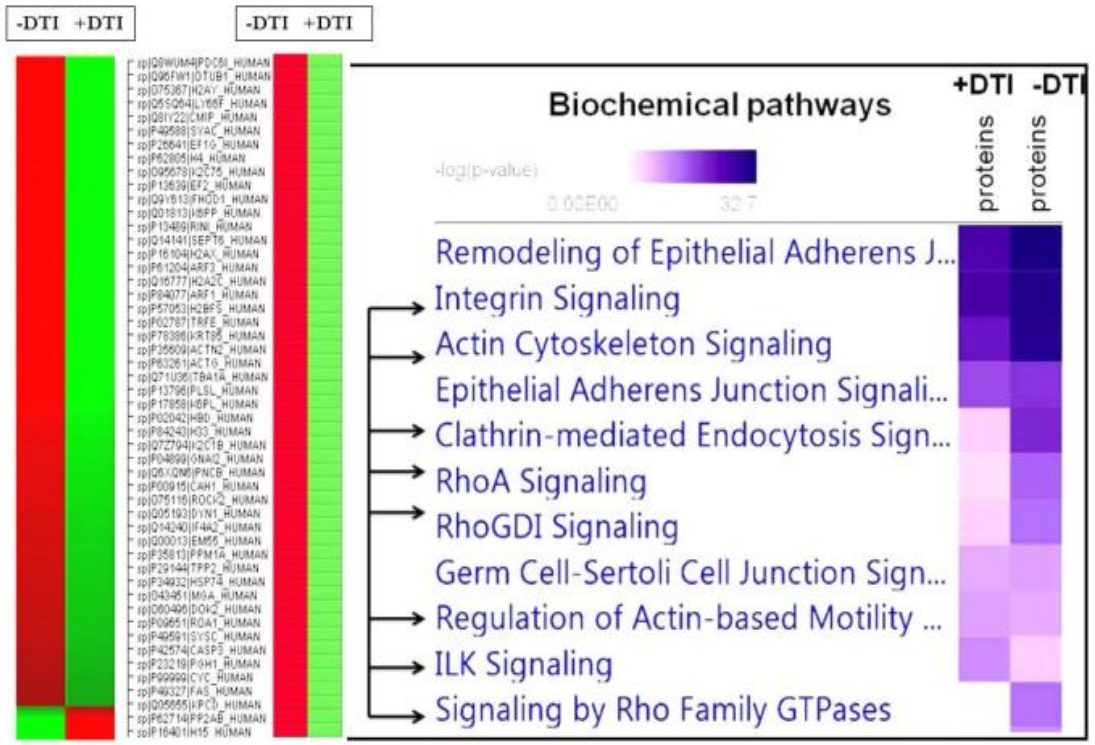
Label free quantitative proteomics (LFQ) analysis of clinic proteomic samples from three different platelets pools from healthy patients treated *ex-vivo* with *tetrapeptide DTI cc-19.*



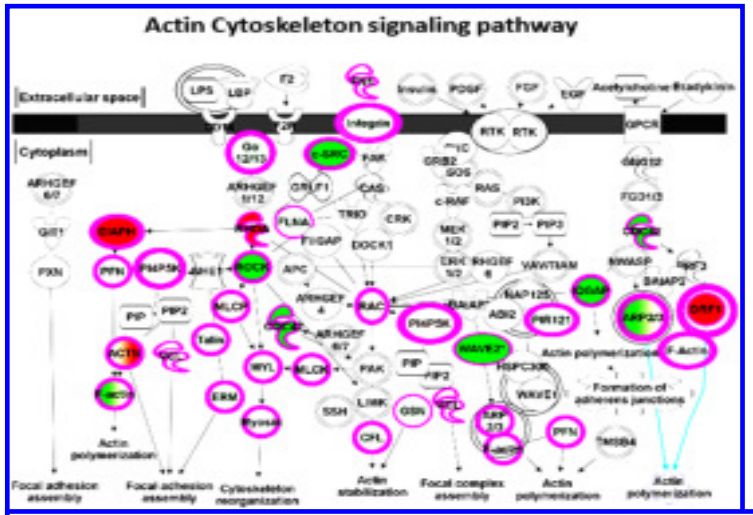
A) 2D-map of m/z vs RT (min) for two representative LC/MS/MS experiments performed on peptidic digests from GluC/Lys/trypsin digestion of protein extracts from human thrombin-activated platelets in presence or absence of DTI cc-19. *nanoLC*-UPLC were developed with 90 min or 180 min gradients. B) Venn diagram showing one set of representative proteomic output from platelets from healthy patients (average of three technical replicates and two biological replicates) under two treatment conditions mentioned in a); C) representative reproducibility analysis plot (Pearson=0.86) for two technical replicates; D) Unclustered Heat Map generated with Scaffold perSPECtive analysis of combined technical replicates for each of the three biological replicates (P1-P3). MS/MS weighted, and normalized spectra counts were used to generate the heat maps; E) same as in D but represented as "pooled" biological replicates for the proteomic samples of platelets in the absence or presence of DTI cc-19.



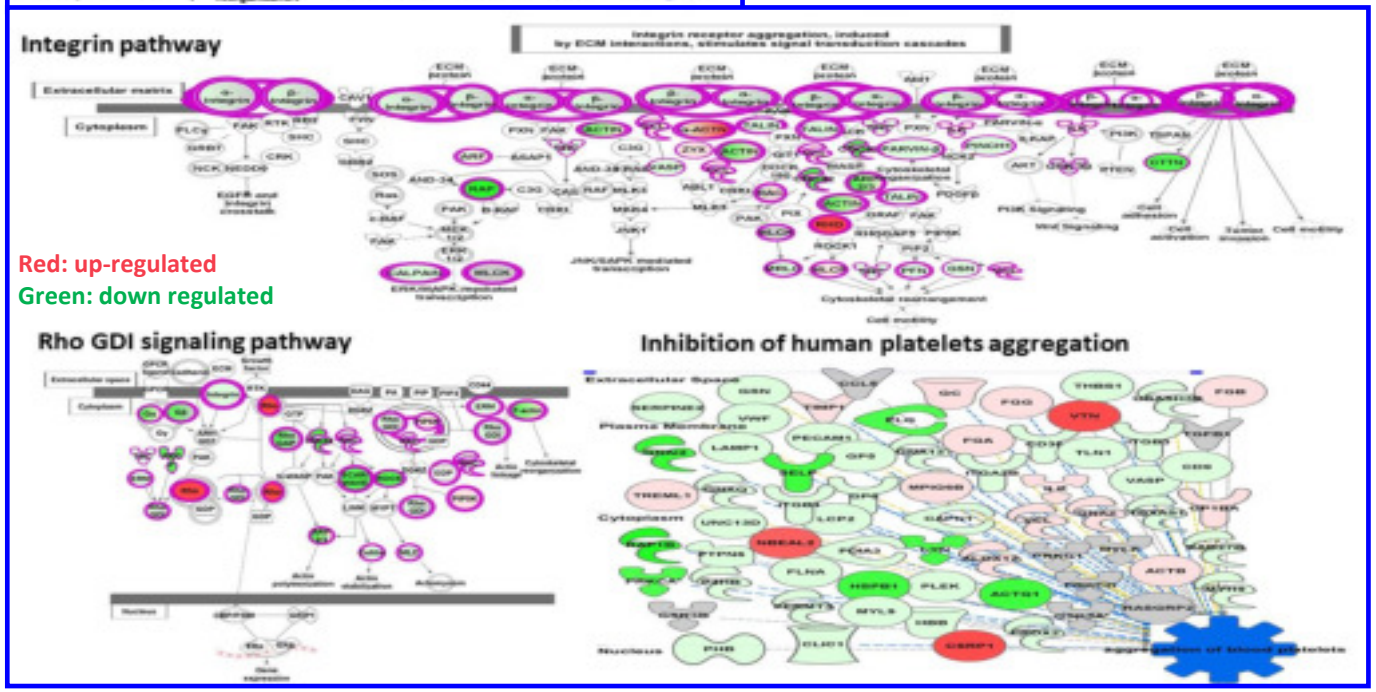
PART I: Discovery of novel anticoagulant tetrapeptides and anti-thrombin direct inhibitors (DTI) enabled by the analysis of their effects upon the global protein expression profiles in human platelets.



Protein Profile Heatmap and LFQ (label-free quantitation performed in PEAKS X LFQ module) of the proteomes of human platelets in absence and presence of the DTI thrombin inhibitor. The representative proteins were clustered if they exhibited a similar expression trend across the samples. The hierarchical clustering is generated using neighbor joining algorithm with a Euclidean distance similarity measurement of the log2 ratios of the abundance of each sample relative to the average abundance. Cell color represents the log2(ratio) to the average area across different samples (left). The heat map corresponding to the main biochemical pathways affected by drug treatment was generated by IPA analysis (right).



Bioinformatics and IPA analysis of LFQ data from the proteomics profiling of platelets treated with DABI vs control (untreated) predicted significant suppression of many cellular pathways involved in cell-cell adhesion (integrin), cell movement and aggregation (i.e. inhibition of platelets aggregation), cytoskeleton rearrangement and acting and Rho A signaling.



PART I: Discovery of novel anticoagulant tetrapeptides and anti-thrombin direct inhibitors (DTI) enabled by the analysis of their effects upon the global protein expression profiles in human platelets.

CONCLUSION

- We developed a *pharmaco*proteomics platform that monitor changes in the protein expression profiles of platelets, purified from healthy human donors and ex-vivo activated with thrombin in presence or absence of our novel developed tetrapeptides DTIs.

- The quantitative analysis of the biochemical pathways was accomplished with ingenuity pathway analysis (IPA; Ingenuity Systems) using the protein ratios extracted from LFQ analyses.

- The bioinformatics analysis predicted that many proteins involved in ILK and integrin signaling pathways, the actin-mediated cell signaling and cellular movement, including rhoA and rhoGDI signaling were at least two-fold statistically significant down-regulated ($p<0.05$) in the DTI-treated platelets than in the control, untreated, and thrombin-activated human platelets.
  Remarkably, the LFQ analysis validated the dabigatran (an FDA-approved anticoagulant) mediated downregulation of some of the already published biomarkers of the integrin mediated signaling pathways (such as of GPIIb/IIIa) (5,6), a finding which could explain the tetrapeptide DTI mediated inhibition of thrombin activated platelets aggregation.

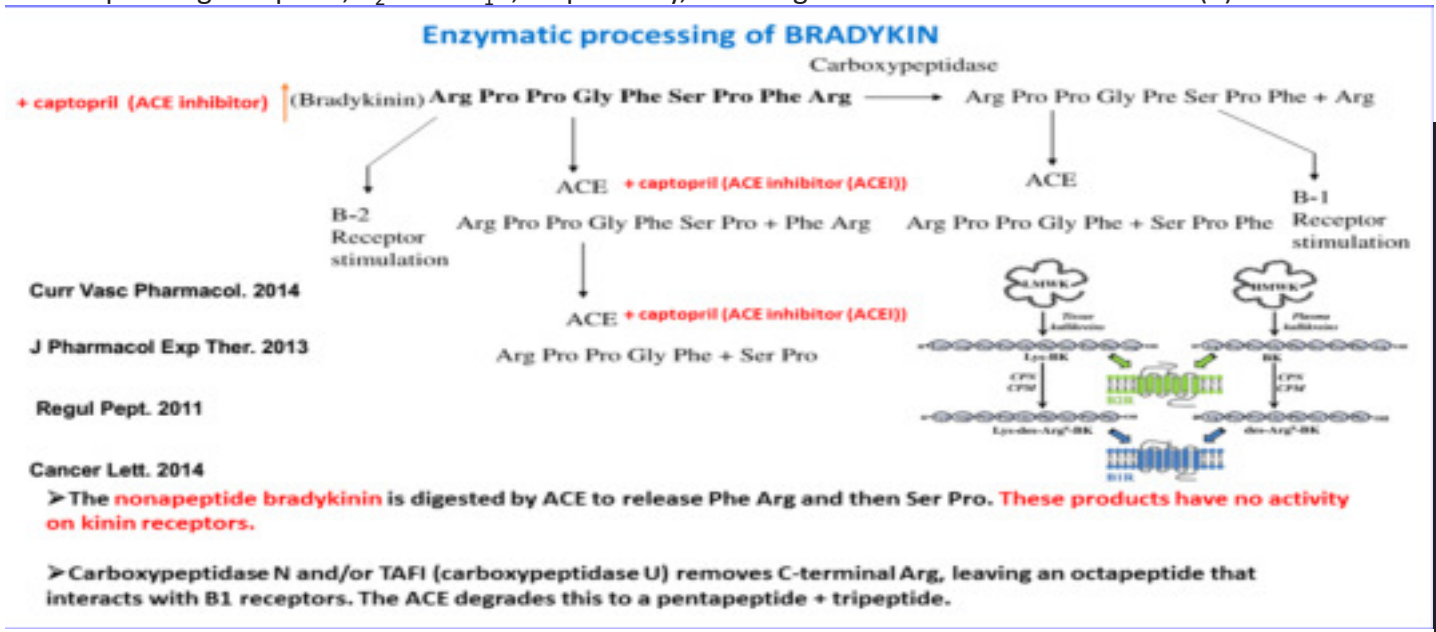
- Our results further advocate for the implementation of platelets proteomics profiling as a reliable assay for monitoring the efficacy of selected drug treatment during Acute Coronary Syndrome (ACS) management and for other diseases where the coagulation-mediated inflammation is hyperactivated (including COVID-19 infection).

5. Pernille Just Vinholt et al; J Thromb Thrombolysis 44:216–222, (2017).
6. A. ACHILLES et al; Journal of Thrombosis and Haemostasis, 15: 473–47 (2016).

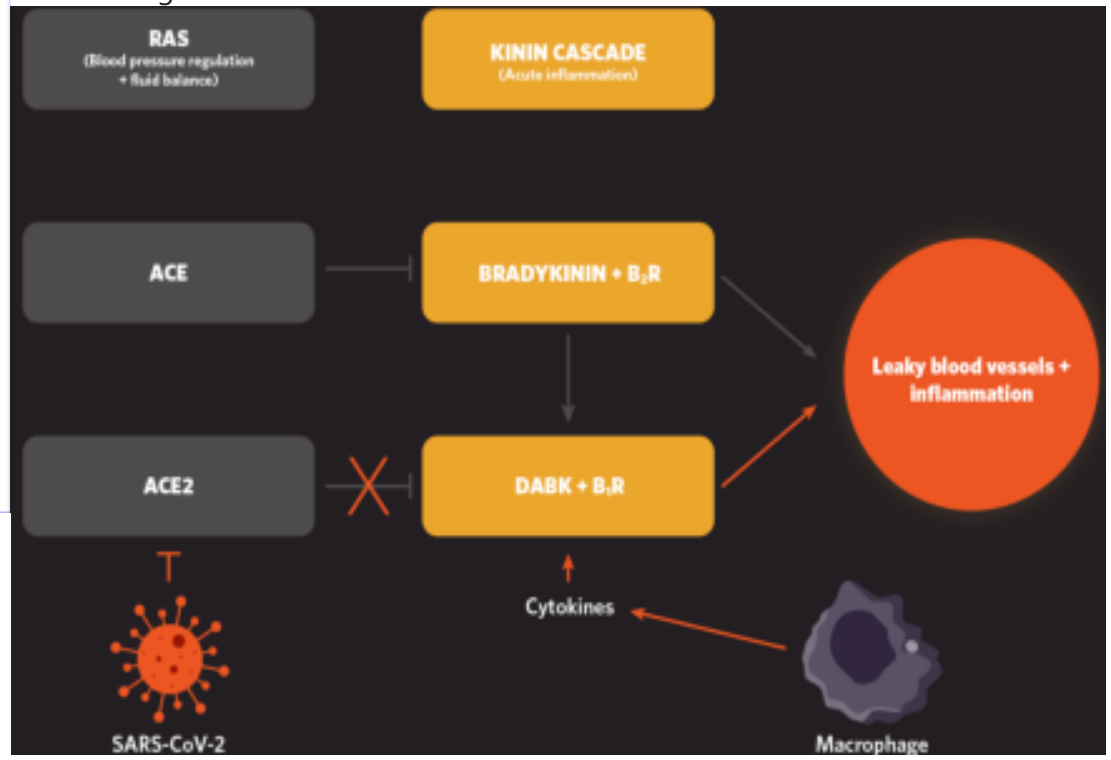
PART II: Development of pharmacoproteomic approaches for mapping the pro-coagulative and pro-inflammatory cellular pathways activated in the dendritic cells (DC) treated with bradykinin (BK) peptide RPPGFSPFR.

Significance of research: Excess of the inflammatory molecule bradykinin may explain the fluid build-up in the lungs of patients with coronavirus infections. ***Clinical trials of inhibitors are putting this hypothesis to the test.***

Activation of the kinin system in the context of blood clotting—which can be dysregulated in some COVID-19 patients—**generates bradykinin and related peptides through two distinct pathways: 1) the plasma kallikrein pathway (activated by a clotting factor called the Hageman factor)** and **2) the tissue kallikrein pathway (activated by tissue enzymes and plasmin, an enzyme in the fibrinolytic system that breaks down clots in the bloodstream).** Bradykinin is then converted to des-Arg9-bradykinin (DABK). When bradykinin and DABK bind to their corresponding receptors, B_2R and B_1R, respectively, fluid begins to leak from blood vessels (**7**).



The leaky blood vessels and lung fluid build-up in some COVID-19 patients might be explained by the virus's corruption of an inflammation safeguard, namely, ACE2's degradation of DABK



- Researchers propose that the kinin cascade—in which bradykinin and des-Arg9-bradykinin (DABK) are major proteins—goes into overdrive to cause these effects during COVID-19. Ordinarily, the RAS—in which angiotensin-converting enzyme (ACE) and ACE2 are key enzymes—keeps the kinin cascade under control. ACE breaks down the protein bradykinin, preventing it from binding to its receptor B_2R, while ACE2 degrades DABK and stops it from binding to B_1R. When SARS-CoV-2 hooks up with ACE2 as a means of entering cells, some of the brakes are removed, according to the model, thereby permitting DABK to bind to its receptor and trigger blood vessel leakage and inflammation.
- Activated macrophages secrete proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-alpha, which in turn induce B_1R to amplify the inflammatory milieu seen in COVID-19.

7. Alakananda Dasgupta; The Scientist, *Aug 26, 2020*

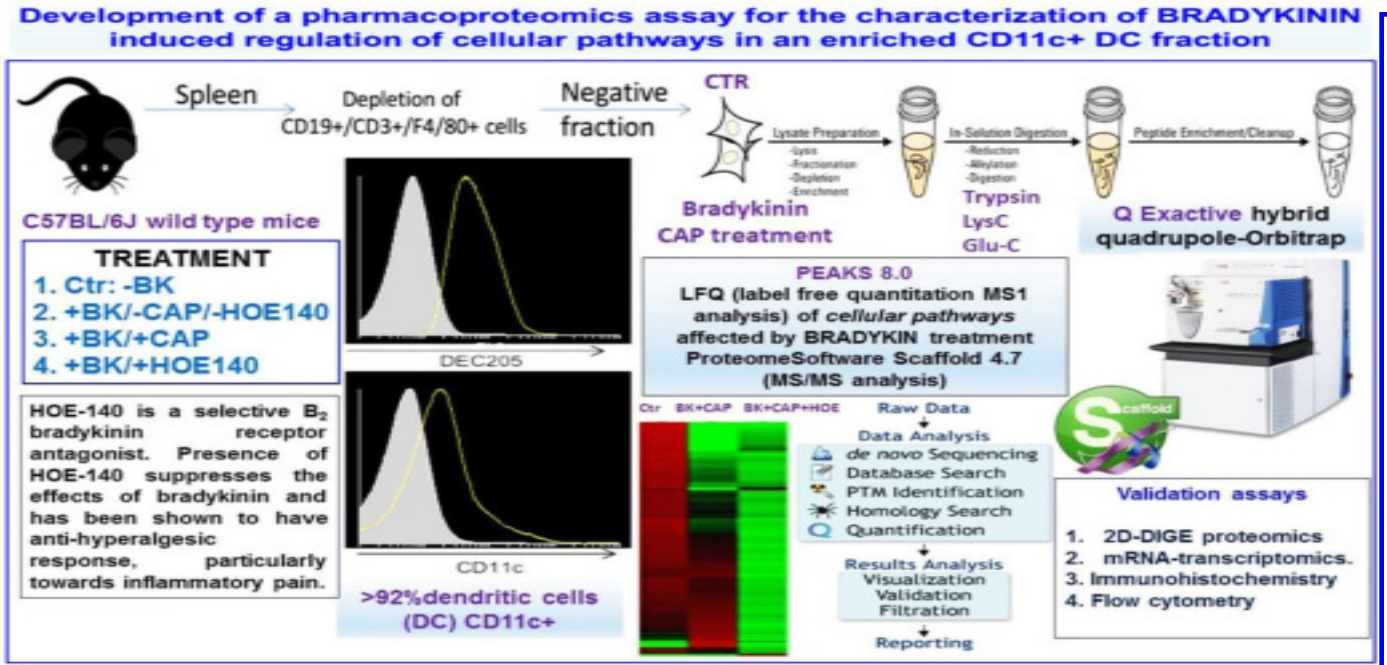
PART II: Development of pharmacoproteomic approaches for mapping the pro-coagulative and pro-inflammatory cellular pathways activated in the dendritic cells (DC) treated with bradykinin (BK) peptide RPPGFSPFR

Research Synopsis

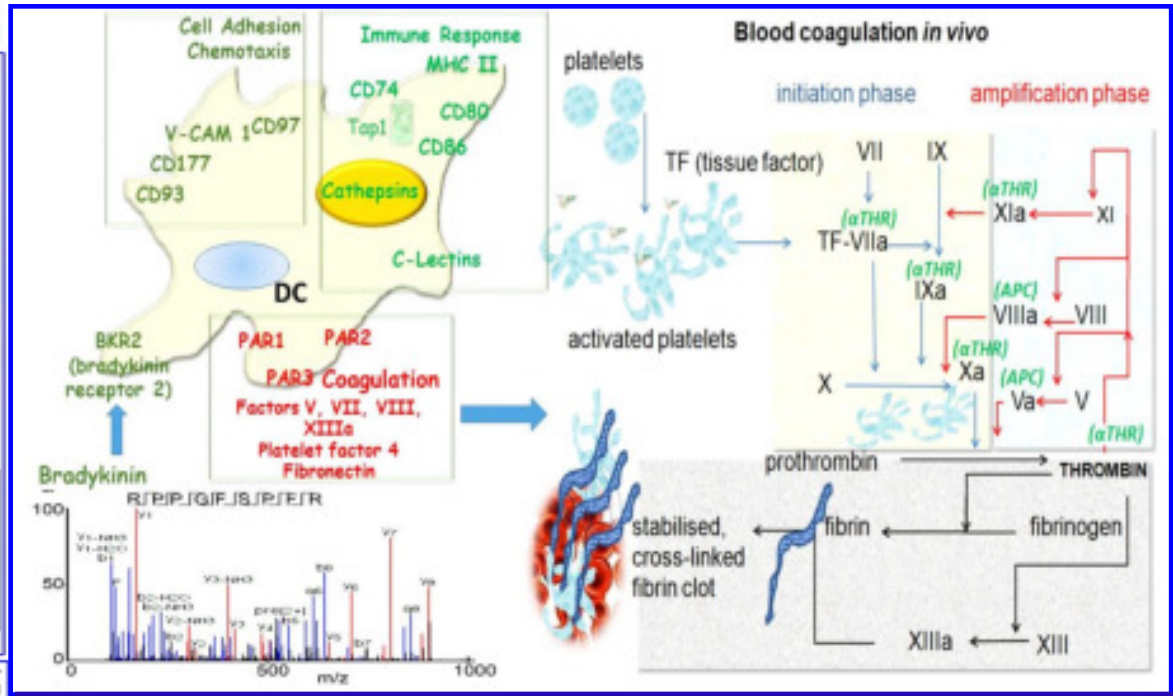
Evidence from independent research studied proved that kinins, which constitute a family of octa- to decapeptides structurally related to bradykinin (BK), have modulatory effects on cells that mediate many immune responses, including macrophages, dendritic cells (DC), and T and B lymphocytes.

Kinins exert their biological functions by activating two pharmacologically different G-protein coupled receptors: B1R, and/or B2R.

Herein, we report the activation of an independent pathway that links the coagulation with inflammation, in which BK peptide (RPPGFSPFR) induces the production of coagulation Factor VIII by CD11c+ mouse DCs (dendritic cells) in response to the activation of B2R receptors.



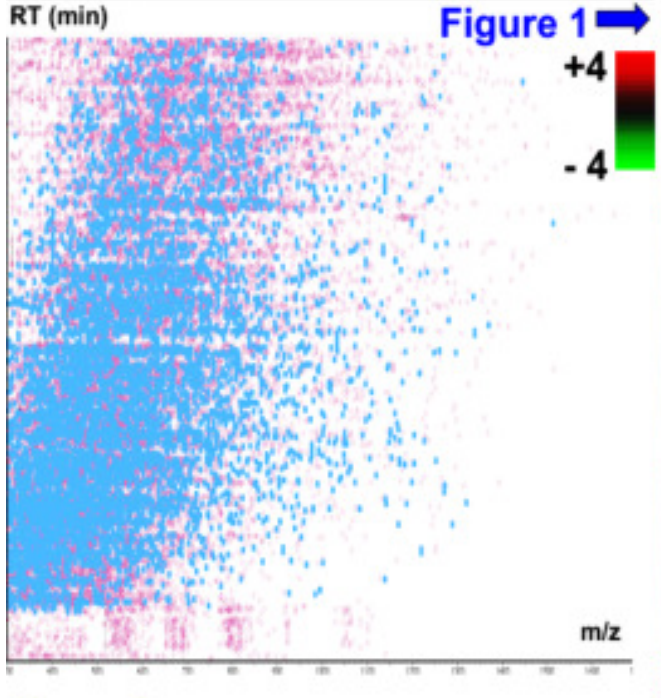
C57BL/6J wild type mice were treated with: 1) BK, or 2) Captopril, an ACE inhibitor that enhances the endogenous BK, or 3) a combination of both drugs (BK+CAP). Following treatment, an enriched the DCs (CD11c+) cells fraction was purified by immunomagnetic sorting. FACS analysis was used to check the purity of CD11C+ and the possible contamination with lymphatic endothelial and blood capillary endothelial cells. The enriched CD11+ DC cells were more than 92% pure as assed by FACS analysis. The total proteome of each sample was reduced with TCEP followed by alkylation with iodoacetamide, and further digested in solution using a combination of trypsin/Lys-C and Glu-C enzymes. The tryptic/Lys-C/Glu-C peptides digests were analyzed on a Q Exactive quadrupole orbitrap mass spectrometer coupled to an Easy nLC 1000 UHPLC. The MS/MS spectra were searched with PEAKS DB (version 8.0), while the protein abundances were generated with the built-in LFQ algorithm. 2D-DIGE and ingenuity pathway analysis (IPA) accompanied the LFQ analysis.



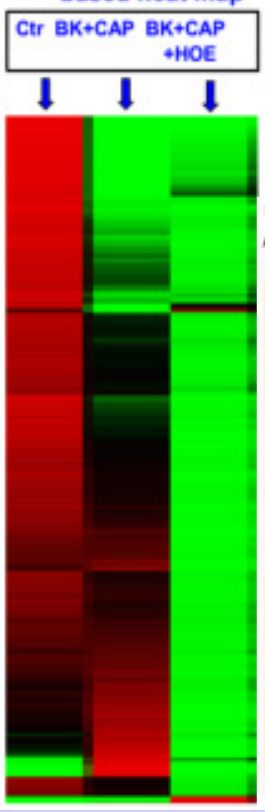
BKR2/PAR1/PAR2/PAR3 coordinated cross-talk signaling in BK activated DC plays a major role in linking the inflammation with the coagulation and amplifies the innate/adaptive immune responses mediated by DC

PART II: Development of pharmacoproteomic approaches for mapping the pro-coagulative and pro-inflammatory cellular pathways activated in the dendritic cells (DC) treated with bradykinin (BK) peptide RPPGFSPFR^{-3} $^{+3}$

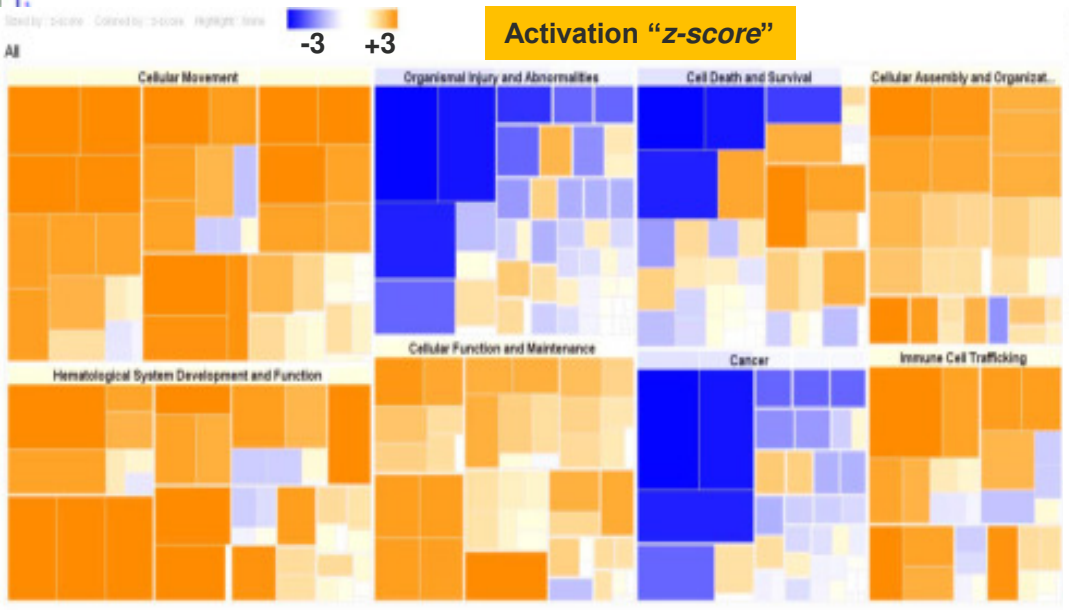
2D-MAP LC/MS of one representative treatment (one out of 3 technical and biological replicates) run using a 110 minutes gradient on a Q-Exactive quadrupole orbitrap mass spectrometer coupled with the label free quantification (LFQ) method provided by PEAKS 8.0 "Q" module. Blue: identified peptides; Purple: identified features (m/z, RT, area).



PEAKS 8.0 generated LFQ based heat map



IPA predicted **increased** immunological trafficking, cell-cell signaling, inflammatory response (including coagulation pathways), cell movement, gene expression (transactivation) and mRNA processing in the mouse DC proteome treated with BK+CAP



This research highlights the development and optimization of a label free global proteomic profiling assay coupled with 2D-DIGE analysis aimed to reveal the intracellular and molecular pathways mediating the bradykinin activation of mouse DCs. The activation of DCs in response to the BK/CAP/BK+CAP treatment was supported by two comparative proteomics approaches: 1) 2D-DIGE protein expression profiling and 2) label-free 1D SDS-PAGE coupled with nanoLC/MS/MS analysis of tryptic peptides derived from DCs protein lysates. The differential protein expression profiles were assessed by the protein abundance index that was generated with the LFQ analysis provided by the "Q" module in PEAKS 8.0 (Bioinformatics Solutions Inc.). Comparative Ingenuity Pathway Analysis (IPA) identified the upregulated cellular pathways in the BK/CAP/BK+CAP treated DCs and highlighted a wide spectrum of molecules involved in the DC's migration/chemotaxis; MHC-I and MHC-II expression; antigen presentation pathways; inflammation; and cytokines secretion. Moreover, the comparative proteomics data were validated by transcriptomic (mRNA) analysis, showing a significant upregulation of the coagulation pathway that was positively correlated with the activation of DCs. Coagulation factor VII was observed to be upregulated by any of the BK/CAP/BK+CAP treatments using proteomics approaches. Additionally, coagulation factors V, VIII, and XIIIa, together with thrombin receptors PAR2 and PAR3-were determined to be upregulated by the same treatment using transcriptomic analysis. Production of coagulation factors VIII and V by BK/BK+CAP-activated DCs was validated by RT-PCR, and by immunofluorescence studies. The activation of DCs was partially reversed by the treatment with HOE140 (a selective B2R receptor antagonist) suggesting that bradykinin activation of DCs is partially triggered by signaling through B2R. The global proteomics analysis retrieved about 1060-1200 proteins (FDR <0.8% for proteins and <1.0% for peptides) for each sample set, and represents one of the most elaborate label-free proteomics profiling of mouse DCs reported to date.

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VALIDATION assays of LFQ analysis

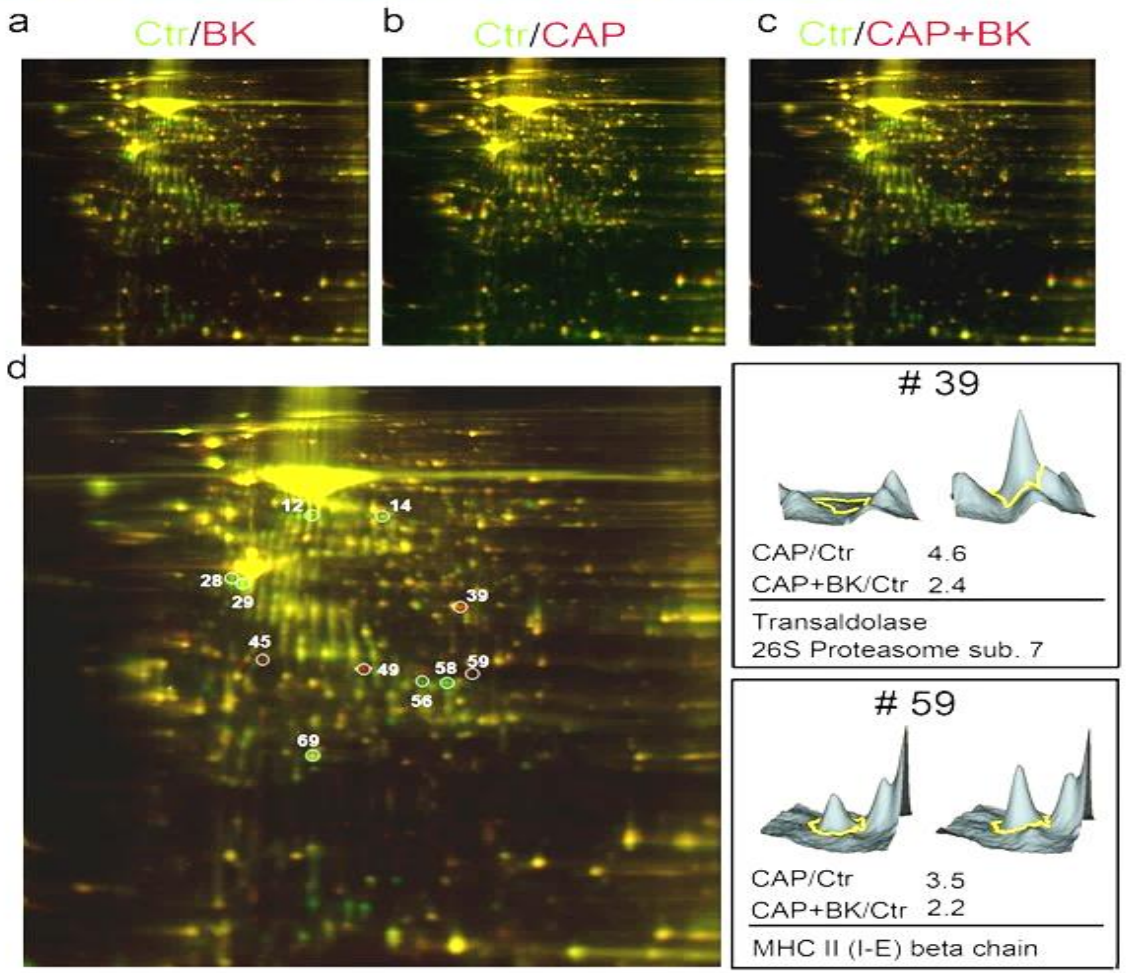
Pharmacological modulation of DC by BK and captopril (ACEI)

Comparative proteomics profiling by 2D-DIGE electrophoresis followed by nanoLC MS/MS

a,b,c) 2D-DIGE protein expression profiling from Cy-dye-labeled protein lysates derived from freshly purified dendritic cells collected from untreated mice (CTR) or mice injected with Bradykinin (BK), Captopril (CAP) or both (BK/CAP).

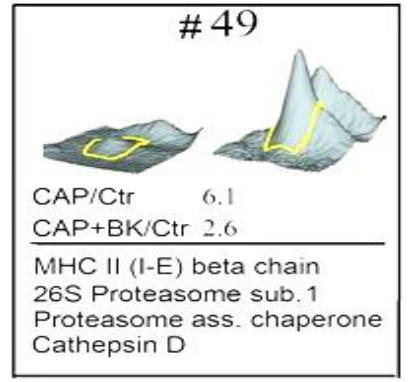
d) The same 2D-DIGE expression profile as presented in (a); circled spots select areas of major differences (> threefold) between the two samples; selected spot, fitting the fold difference were collected from each gel and analyzed by mass spectrometry (Supplement Table 1).

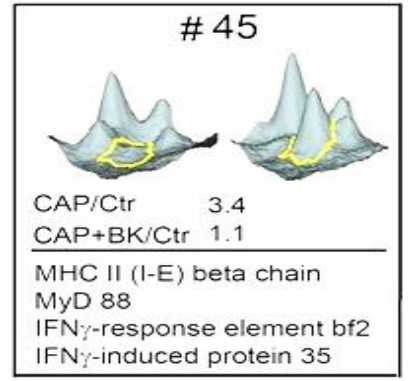
e) 3D-view analysis of some of the protein spots, using 2DCyder software to graphically represent the most significantly up-regulated proteins following BK treatment.



BK+CAP: increases the level of endogenous BK

e





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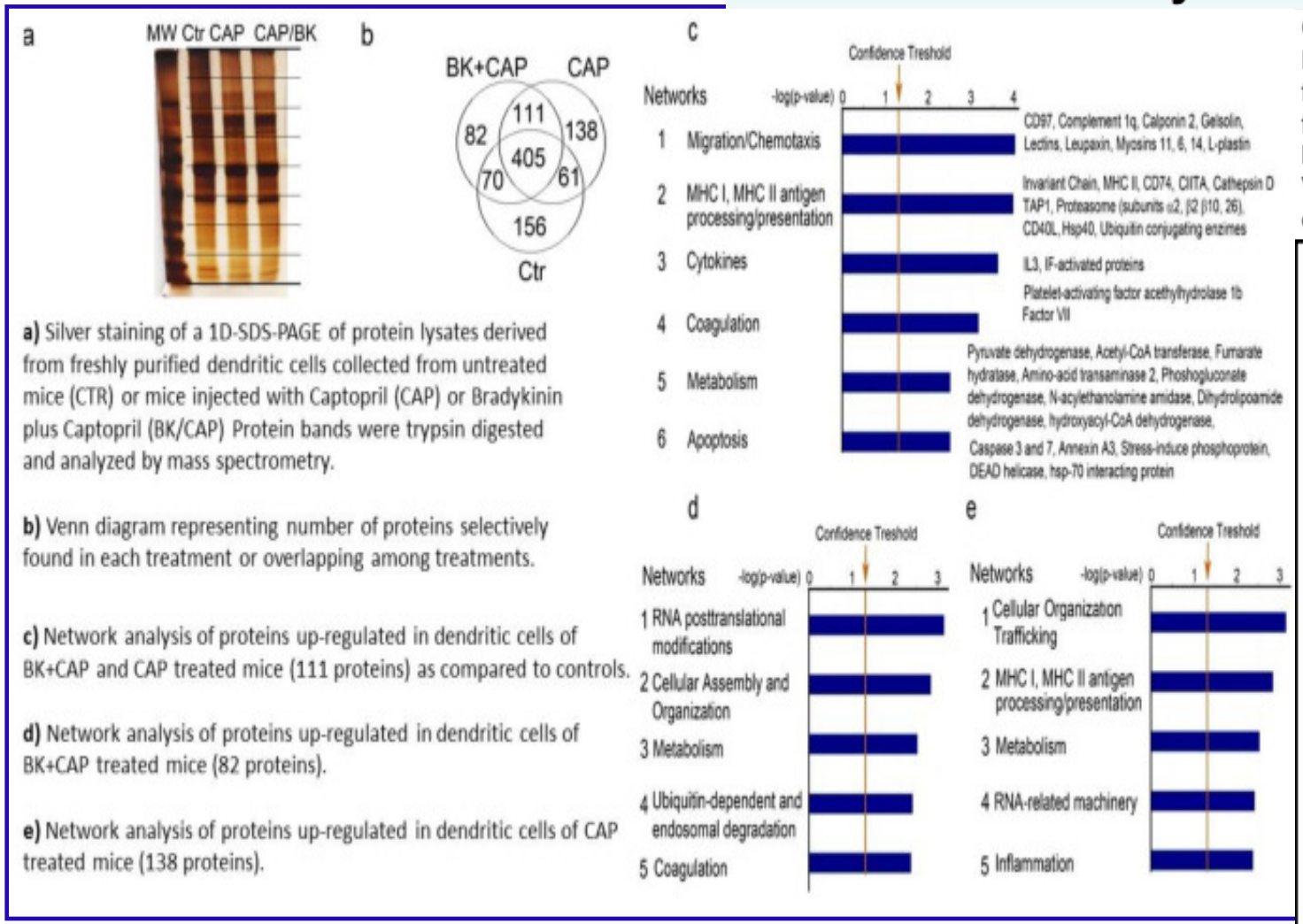
VALIDATION assays of LFQ analysis



a) Silver staining of a 1D-SDS-PAGE of protein lysates derived from freshly purified dendritic cells collected from untreated mice (CTR) or mice injected with Captopril (CAP) or Bradykinin plus Captopril (BK/CAP) Protein bands were trypsin digested and analyzed by mass spectrometry.

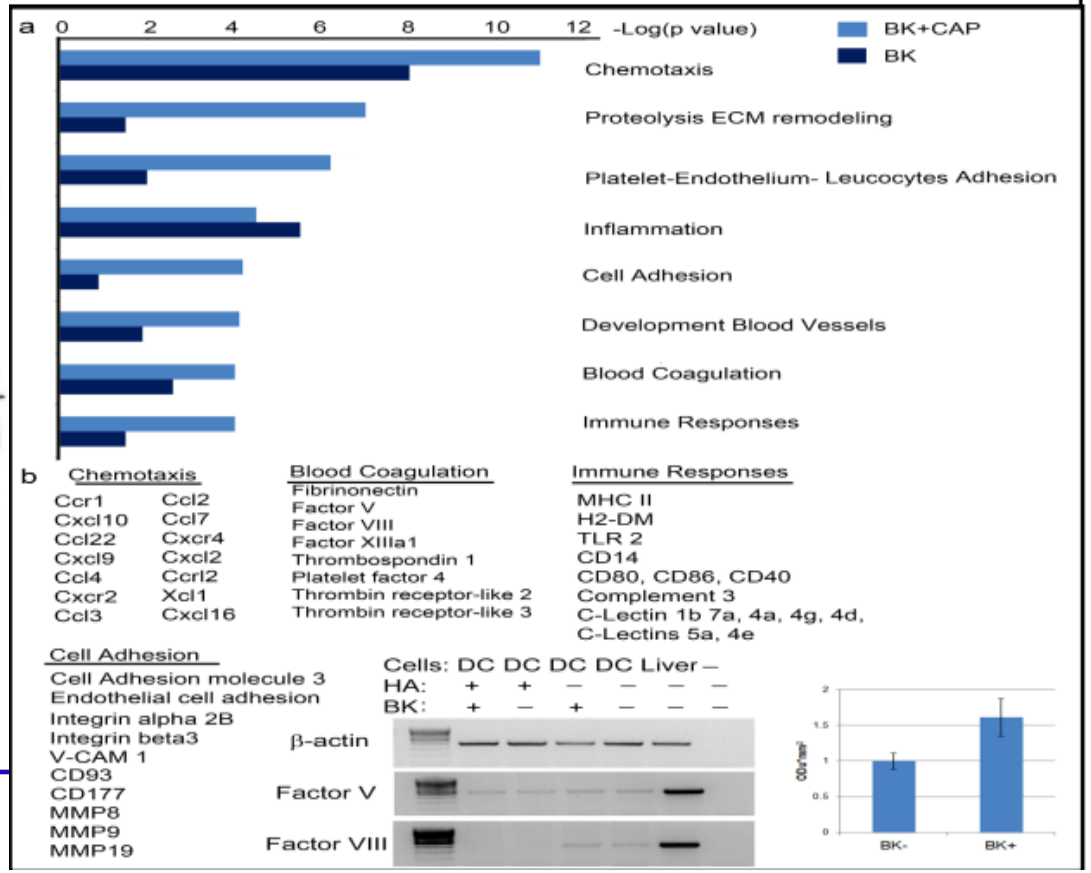
b) Venn diagram representing number of proteins selectively found in each treatment or overlapping among treatments.

c) Network analysis of proteins up-regulated in dendritic cells of BK+CAP and CAP treated mice (111 proteins) as compared to controls.

d) Network analysis of proteins up-regulated in dendritic cells of BK+CAP treated mice (82 proteins).

e) Network analysis of proteins up-regulated in dendritic cells of CAP treated mice (138 proteins).

Comparative mRNA analysis of Dendritic cells activated by Bradykinin. a) Network analysis of mRNAs significantly (> 3 folds) up-regulated in dendritic cells from BK and BK+CAP treated mice as compared to controls. **b)** Specific mRNAs found to be up-regulated in the following pathways; chemotaxis, cell adhesion, blood coagulation and immune responses are reported. **c)** PCR analysis for Factor V and Factor VIII in dendritic cells (DC) of hemophilic (Factor VIII deficient) (HA) or wild type mice. Liver RNA was used as positive control.



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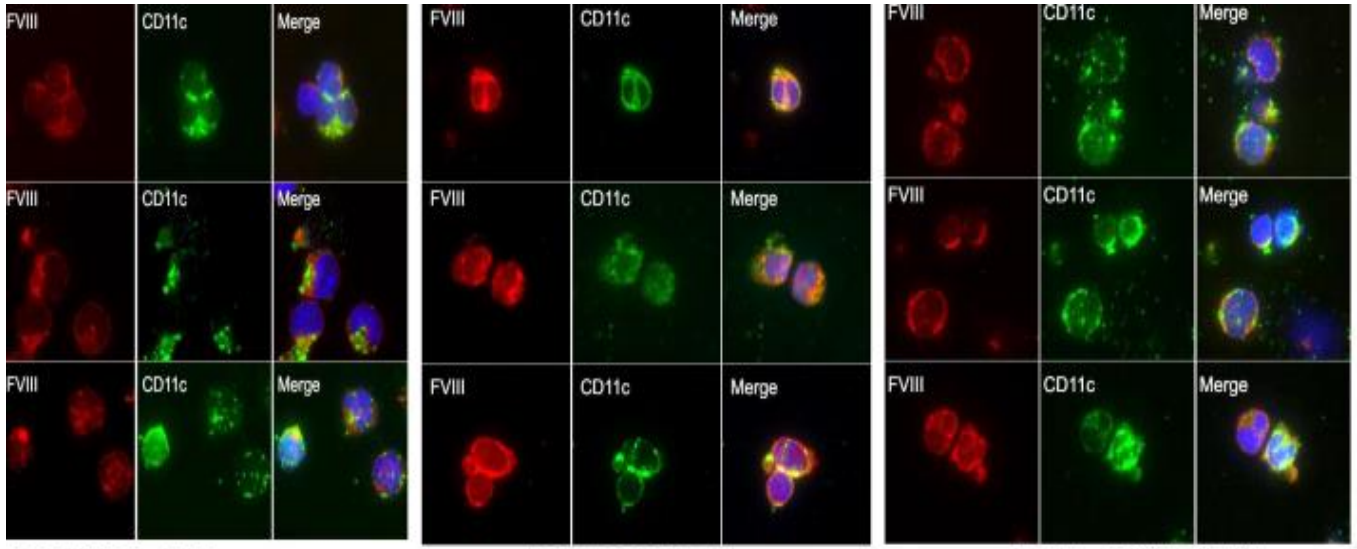
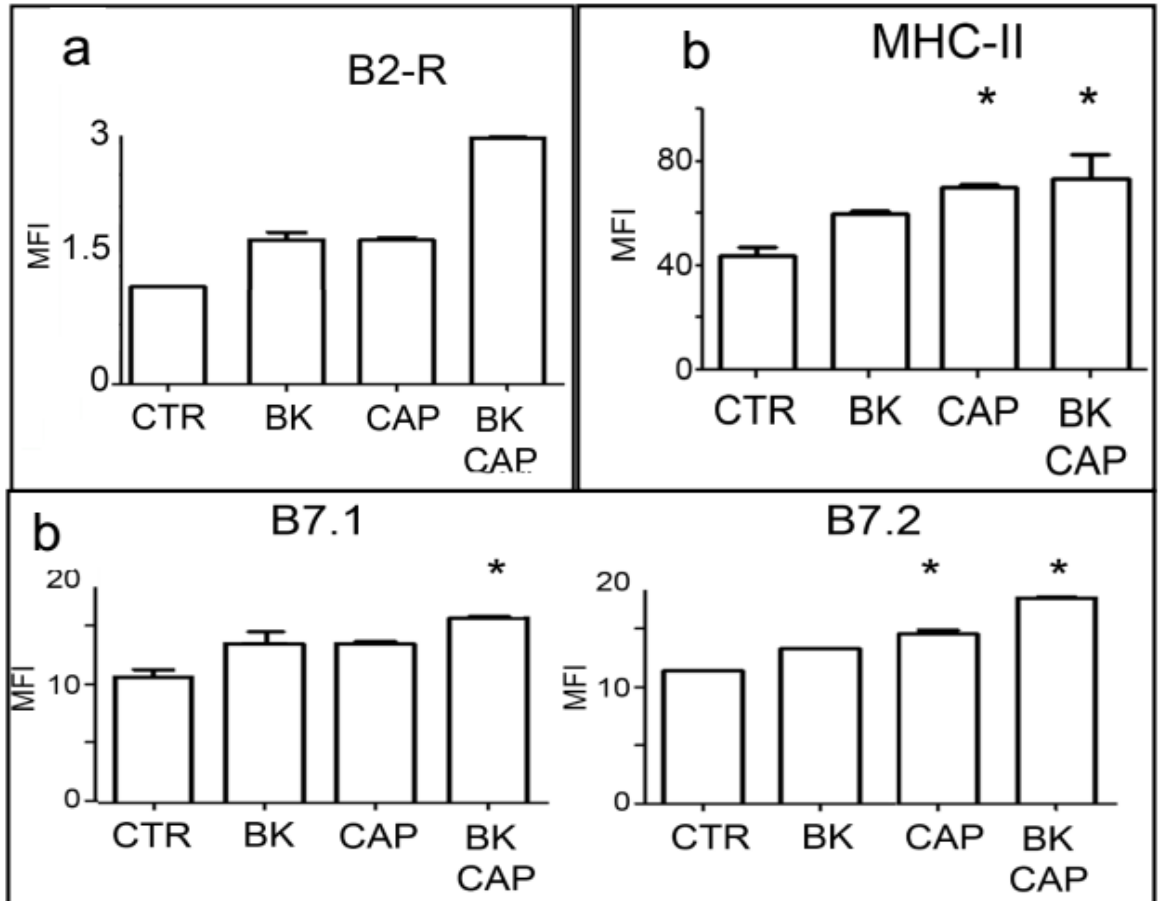


Dendritic Cells production of Factor VIII is mediated by the bradykinin receptor (2) activation *in vivo*. Immunofluorescence analysis of Factor VIII and Factor V secretion by DCs purified from mice control (untreated) or treated with BK or with BK in the presence of the B2R selective inhibitor HOE140. CD11c-purified splenic dendritic cells from mice untreated (Ctr) or treated with BK (+ BK) and BK+HOE140 for 12 hours (400nM) were stained with a rabbit polyclonal antibodies specific for Factor VIII and CD11c. DAPI staining for nuclei in blue.

***In vivo* activation of Dendritic Cells by Bradykinin:** a) Mean fluorescence index of the Bradykinin receptor 2 (B2-R) present on freshly purified dendritic cells from untreated mice (CTR) or mice injected with Bradykinin (BK), captopril (CAP), to increase endogenous levels on bradykinin, or CAP plus BK (BK, CAP). b) Mean fluorescence index of MHC class II molecules (MHC-II) and the co-stimulatory molecules B7-1 and B7-2 on freshly purified dendritic cells from mice treated as in figure 1.



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CONCLUSION

1. Pharmacoproteomics complements pharmacogenomics by highlighting protein expression profiles of diverse cellular and molecular pathways in response to different drug treatment. The valuable data obtained by pharmacoproteomics offers a wide assistance to molecular diagnostics, which is the foundation of personalized medicine.
2. The research presented herein highlights the mapping of the pro-coagulative and pro-inflammatory pathways activated in the dendritic cells (DC) treated with bradykinin (BK). Using pharmacoproteomic and transcriptomics platforms we discovered that mouse DCs stimulated with the BK peptide (RPPGFSPFR) became active in the production of molecules involved in migration/chemotaxis, MHC-I, and MHC-II expression, antigen presentation, inflammation, and cytokines secretion, further mediating the production of coagulation factors V and VIII.
3. Further research will be needed to explore the importance of BKR2/PAR1/PAR2/PAR3 coordinated cross-talk signaling in BK activated DC and the major role played by this signal transduction pathway in linking the inflammation with the coagulation. The production of factor VIII by BK-activated DCs will be further tested for its potential to ameliorate hemophilia (HA) in a factor VIII (-/-) deficient mouse (model of HA).

- **This research highlights the advantages of employing pharmacoproteomic technologies as a reliable analytical platform that help to the discovery and development of peptide-drugs with anti-coagulant activities.**