Targeted vs non-targeted analysis of metabolic biomarkers of blood activation

Evgenii Serebriakov[1]; Chau Tran[1]; Daniil G. Ivanov[1]; Sina Farzaneh[2]; Juan M. Jiménez[2]; Igor A. Kaltashov[1]

Department of Chemistry[1], Department of Biomedical Engineering[2]

University of Massachusetts - Amherst, Amherst, MA

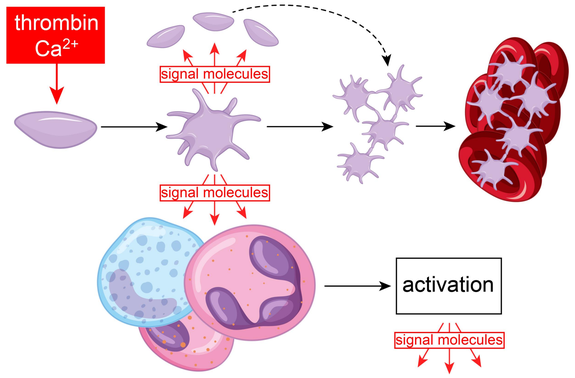
Overview

- Non-targeted LC-MS/MS (DIA) data analysis shows significant fold change of 25 features between *in vitro* platelet-activated and non-activated blood plasma.
- 14 peptides and serotonin (5-hydroxytryptamine, 5-HT), found in activated blood plasma samples only, can be used as a markers of the blood activation cascade process.
- Non-targeted lipidomic RP LC-MS/MS analysis does not yield reliable biomarkers of thrombosis process. Negative fold change of lipids signals level after *in vitro* blood activation less than 2 was found. Phosphatidylserine (PS) species signals potentially useful as markers of platelet activation were not detected.
- Fatty acid amides, reported as signaling molecules in the cardiovascular system, cannot be used as biomarkers in targeted or non-targeted analysis due to the significant signal level in non-biological blank samples.

Introduction

Thrombogenesis is a multifactorial process with unpredictable outcomes[1]. Metabolome profiling can offer insights into the dynamic network of signaling compounds that are associated with thrombogenesis.

During thrombogenesis, thromboxane A2, ADP and other secondary mediators amplifies the platelet response and subsequent activation of the coagulation pathway. Platelets release serotonin stored in platelets' dense granules[2], and other signaling molecules upon their activation[3].

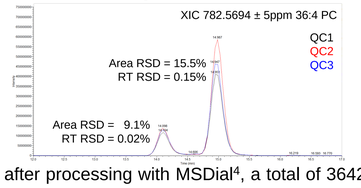


Untargeted LC-MS/MS DIA analysis of *in vitro* activated blood plasma allows us to find biomarkers for quantitative and qualitative characterization of stent thrombosis and complement flow cytometry measurements.

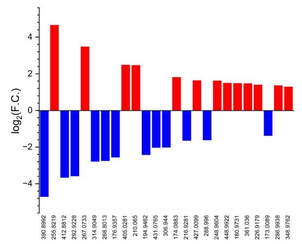
Non-targeted analysis

LC-HRMS non-targeted metabolomic workflow was developed to investigate differences between non-activated and activated blood plasma. Relative standard deviation (RSD)% values of retention time (RT RSD) and XIC peaks area (Area RSD) for 10 features from the HILIC and RPLC QC samples were calculated from triplicated pooled QC sample.

RT RSD were between 0.02% and 0.29%.
Average Area RSD was 0.1%
Area RSD were between 4.7% and 16.4%.
Average Area RSD was 9.3%



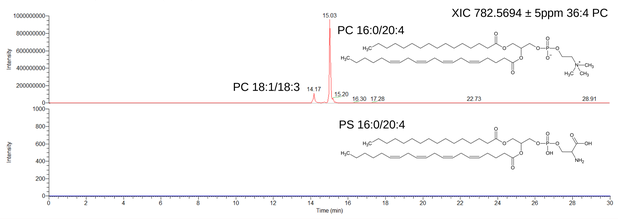
For HILIC and RPLC samples, after processing with MSDial[4], a total of 3642 features were obtained and used for data analysis. Using Metaboanalyst[5], fold change threshold of 2 was applied to select compounds that show differences between non-activated and activated samples. A total of 102 features had statistically significant differences between the activated and non-activated samples. 57 features were upregulated in activated plasma, (fold up to 3.9); 45 features were downregulated, (fold changes reaching 4.7).



In HILIC LC-MS, among the top 25 features manually checked, 13 features were upregulated in activated plasma, while 12 features were downregulated. Moreover, HILIC-ESI+/- measurements led to the detection of 11 peptides ions only present in activated but not in non-activated plasma.

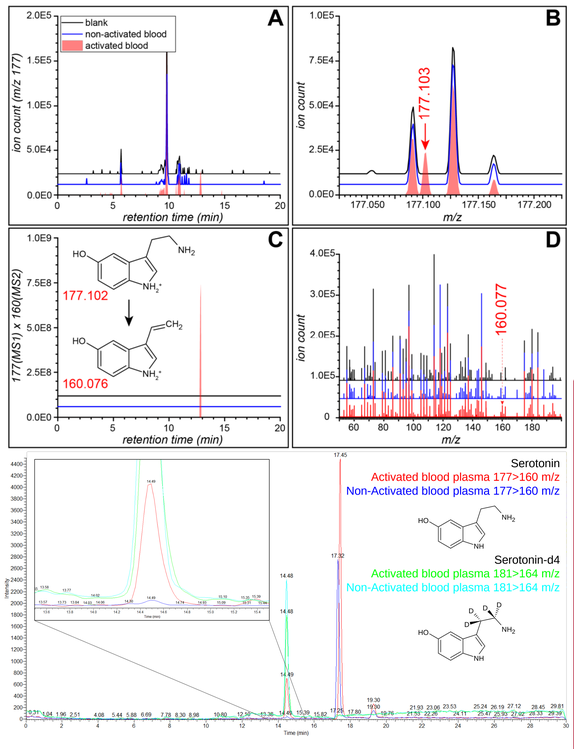
For RPLC samples, there were no ions only present in activated but not in non-activated plasma.

There also were no significant fold changes between activated and non-activated blood plasma samples. Among the top 12 features manually checked, 8 features identified as triacylglycerol (TAG) and phosphatidylcholine (PC) lipid species were upregulated in activated plasma, while 4 features identified as sphingolipid species were downregulated. There was no found any signals can be related with most abundant phosphatidylserine (PS) species in human metabolome.



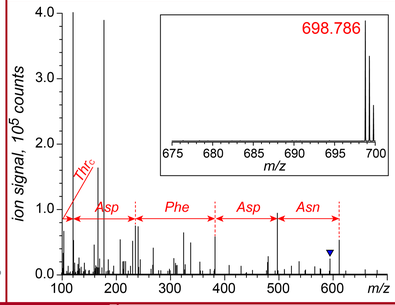
Targeted analysis of serotonin

Non-targeted analysis with reasonable signal threshold settings failed to reveal serotonin as a compound that had significant fold change between non-activated and activated plasma. However, with targeted approach, we were able to identify serotonin as an important thrombosis biomarker released by platelets in the activated blood plasma using ESI+ HILIC-MS/MS experimental data.



A: XIC corresponding to the serotonin molecular ion (m/z 177.1022 ± 0.0009) for the activated blood plasma (red), the negative control (non-activated blood plasma, blue) and the non-biological blank for activated blood plasma (black). B: MS data acquired for all three samples at the RT=12.85 min. C: products of the XIC corresponding to the mass of serotonin in MS1 (as shown in panel A) and its most abundant fragment (m/z 160.076) in DIA MS2. D: MS2 data (precursor ion isolation window 175-200 m/z) for the three samples acquired at the retention time 12.85 min (the red arrow identifies the most abundant fragment ion produced by deamination of the serotonin molecular ion in the gas phase). Quantitation of serotonin levels was carried out using serotonin-d4 as internal stable isotope-labeled standard. Serotonin levels in blood plasma samples were measured ranging from 10 to 1000 ng/mL using serotonin-d4 as internal standard (RT=14.5 min). The concentrations of serotonin in activated blood plasma sample was 19±5 ng/mL. S/N ratio of serotonin in non-activated blood plasma sample was below 3 (<1 ng/mL).

Peptides



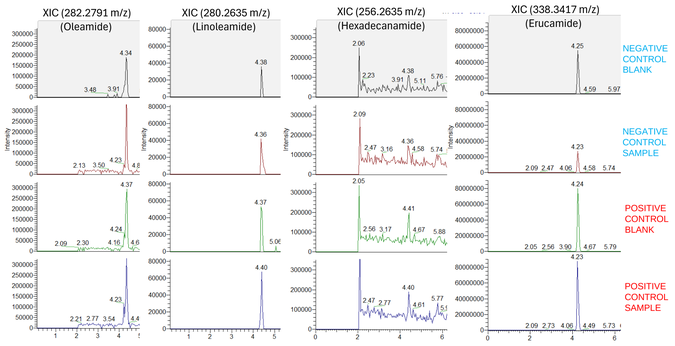
Among polar upregulated unidentified features were 11 peptides.

Peptides identification is in progress.

X...XNDFDT?

Fatty acids amides

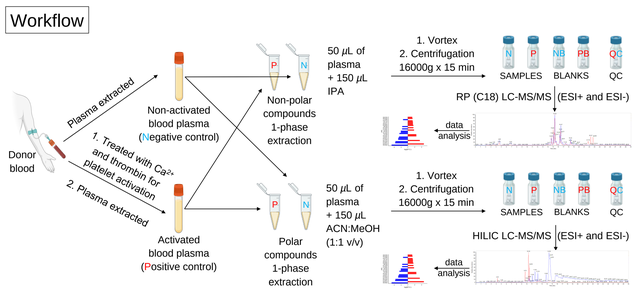
Oleamide, a fatty acid amide, has been suggested as a biogenic signaling molecule in the cardiovascular system[6] is a widely used industrial chemical substance well-known as MS contaminant[7]. We identified 4 fatty acid amides in both blanks and samples.



Also, we found several contaminants and background ions with high fold change shown by automatic data processing as up- and down-regulated features. This was discovered during a manual review of the automatic data processing results.

Methods

Workflow



LC-MS settings

Non-targeted MS analysis
Instrument: Thermo Orbitrap Fusion
MS1: 100.00-1000.00 m/z; R=30000
MS2: 50.00-1000.00 m/z; R=15000
DIA (SWATH): 36 isolation windows of 25 m/z, 2.58 s cycle time

5-HT targeted MS quantitation
Instrument: Finnigan LTQ
MS1: 159-210 m/z
MS2 of 177.00 m/z: 110.00-165.00 m/z, CE=35%
MS2 of 181.00 m/z: 110.00-170.00 m/z, CE=35%

RPLC separation
Polaris 3 Amide-C18 100x2.0mm, 3µm
Flowrate 0.2 mL/min. T = 40˚C
Mobile phase: A: 60:40 ACN:H_2O (10mM AmFA, 0.1% FA); B: 85:10:5 IPA:ACN:H_2O (10mM AmFA, 0.1% FA)
Gradient program: 1 min (30%) - 22 min (100%) - 26 min (30%)

HILIC separation
SeQuant ZIC-cHILIC 150x4.6mm, 3µm
Flow rate: 0.4 mL/min. T = 40˚C
Mobile phase: A: H_2O (0.1% FA); B: ACN (0.1% FA)
Gradient program : 1 min (95%)- 22 min (5%)- 26 min (5%)

Conclusion

- Non-targeted metabolomics based on LC-MS/MS was performed, providing different profiles of non-activated and activated blood plasma samples.
- Compounds that have potential as thrombosis biomarker candidates were established using fold change analysis.
- Targeted analysis of serotonin (5-HT) level in platelet-activated blood plasma was performed > 20-fold increase.

Future work

- Work is still ongoing for the identification of thrombosis biomarker candidates.
- Identification and quantitation of thrombosis biomarker candidates will be performed.

References

1. Koupenova, M. et al. *Eur. Heart Journal* **2017**, *38* (11), 785–791
2. Skop, B. et al. *Psychosomatics* **1996**, *37* (1), 12–16
3. Cloutier, N, G. et al. *Proceedings of the National Academy of Sciences* **2018**, *115* (7), E1550–E1559
4. Tsugawa, H. et al. *Nat Methods.* **2015** Jun;12(6):523-6
5. Pang, Z. et al. *Nucleic Acids Research*, **2024**; gkae253
6. Hiley, R. et al. *Cardiovasc. Drug Rev.,* **2007** *V.25,No.1, pp. 46–60* 7. Jug, U. et al. *Sci Rep* **2020**, *10* (1), 2163

The authors declare no competing financial interest

Acknowledgments

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