Comparing bottom-up proteomics and the FIRE methodology for untargeted protein adductomics

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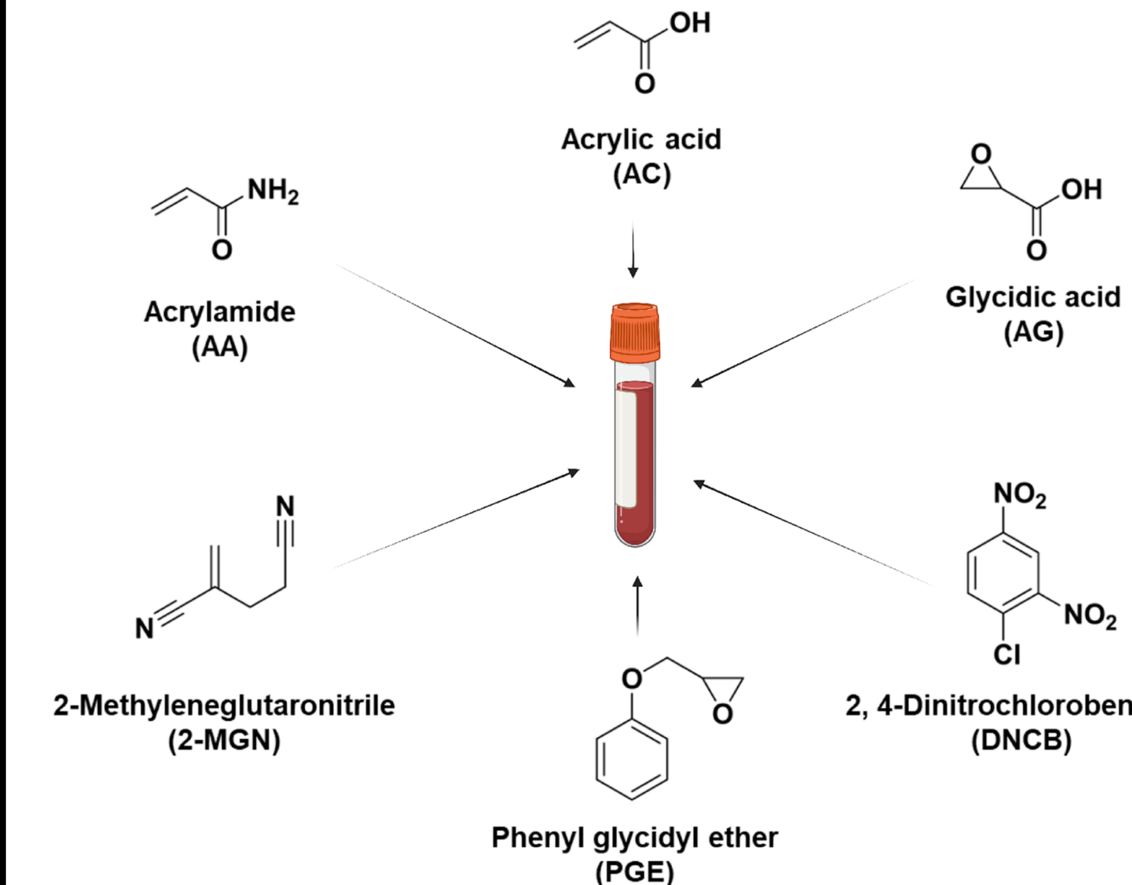
I. INTRODUCTION

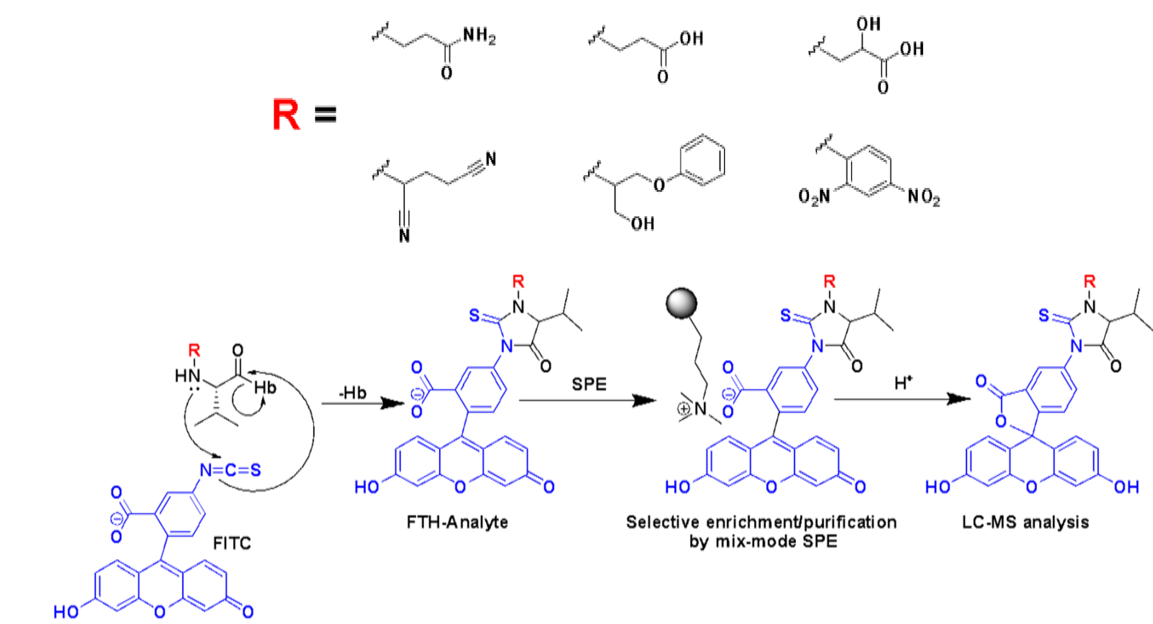
- Humans are exposed to large numbers of endogenous and exogenous electrophilic compounds which form covalent adducts at nucleophilic sites in biomolecules
- Interrogating these adducts gives a record of the exposome of an individual and can be indicative of underlying health problems
- While DNA adducts are detectable and directly impact gene replication and translation, these adducts are relatively short-lived and of variable abundance in tissues
- Protein adducts in the blood form an attractive source of information on the exposome
- The Törnqvist lab has developed the FIRE methodology to interrogate N-terminal adducts in hemoglobin
- This research compares the ability of FIRE methodology and bottom-up proteomics to detect hemoglobin adducts in an untargeted fashion and begins the development of adductomics tools in Galaxy

II. SAMPLE PREPARATION



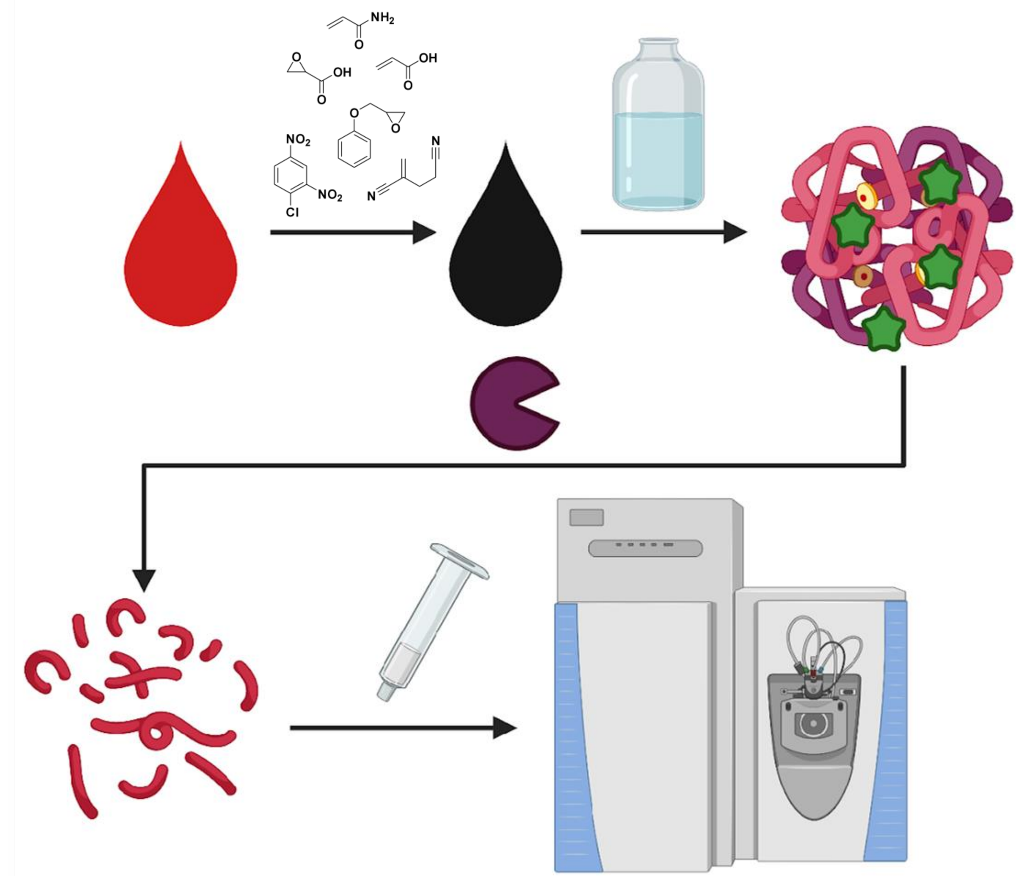
- Donor blood was aliquoted and incubated with industrial contaminants (AA, AC, and AG) and contact allergens (2-MGN, PGE, and DNCB)
- Blood samples were incubated at 37°C with individual of electrophile stock solutions for variable concentrations (100 µM or 5 mM) and incubation times (1 or 21 hours)
- Pools of electrophiles were added to individual blood aliquots and incubated for 21 hours to examine their relative reactivity
- Following incubation with electrophiles, erythrocytes were isolated from blood and washed with saline solution
- Hemoglobin was isolated from erythrocytes via hypotonic lysis with centrifugation, after which samples were split and subjected to either the FIRE procedure or bottom-up proteomics

IIIA. FITC FOR THE MEASUREMENT OF ADDUCTS (R) VIA MODIFIED EDMAN PROCEDURE (FIRE)



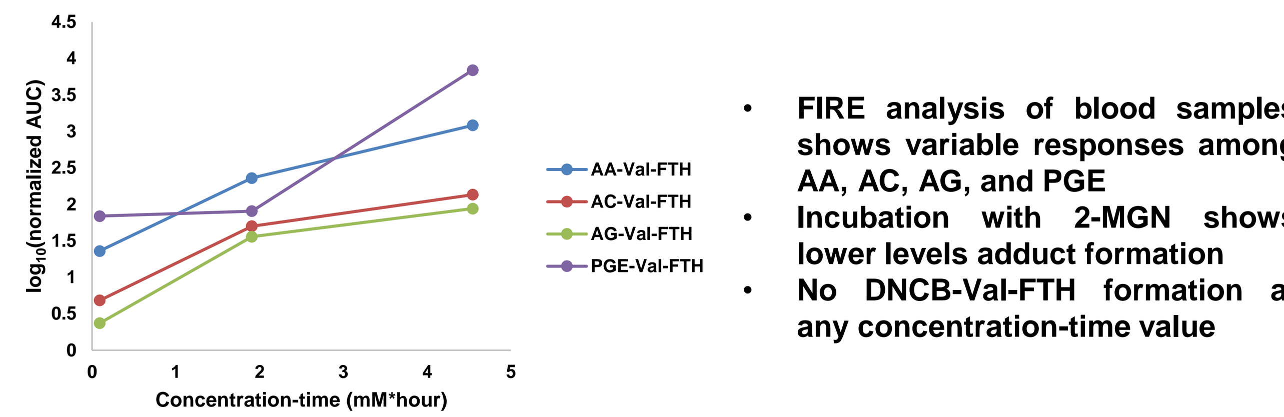
- N-terminal protein adducts are isolated via a modified Edman degradation
- Isolated hemoglobin was incubated overnight at 37°C with fluorescein isothiocyanate (FITC) and potassium carbonate, releasing the N-terminal valine
- FTH-analytes are immobilized and washed via mixed-mode SPE
- Purified FTH-analytes are dried down and analyzed via LC-MS on a QExactive Orbitrap Quadrupole Hybrid Mass Spectrometer

IIIB. BOTTOM-UP PROTEOMICS



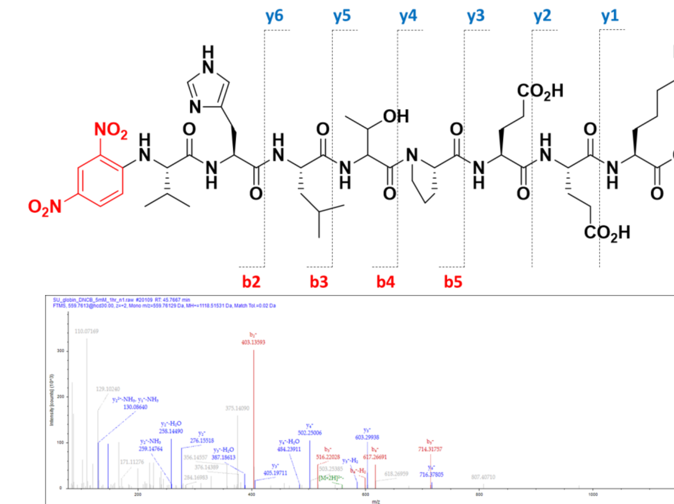
- 100 µg of hemoglobin from each sample was diluted in alkaline buffer followed by overnight tryptic digestion and desalting
- 1 µg of hemoglobin peptides were injected onto a QExactive Orbitrap Quadrupole Hybrid Mass Spectrometer for LC-MS analysis
- Raw data was searched using Proteome Discoverer with added variable modifications for AA (71.03711 m/z), AC (72.02113 m/z), AG (88.01605 m/z), 2-MGN (106.05309 m/z), PGE (150.0681 m/z), and DNCB (166.00145 m/z) at nucleophilic sites within the alpha and beta hemoglobin side chains
- Initial global MS data for N-terminal and sidechain adducts was used to populate an inclusion list for targeted validation

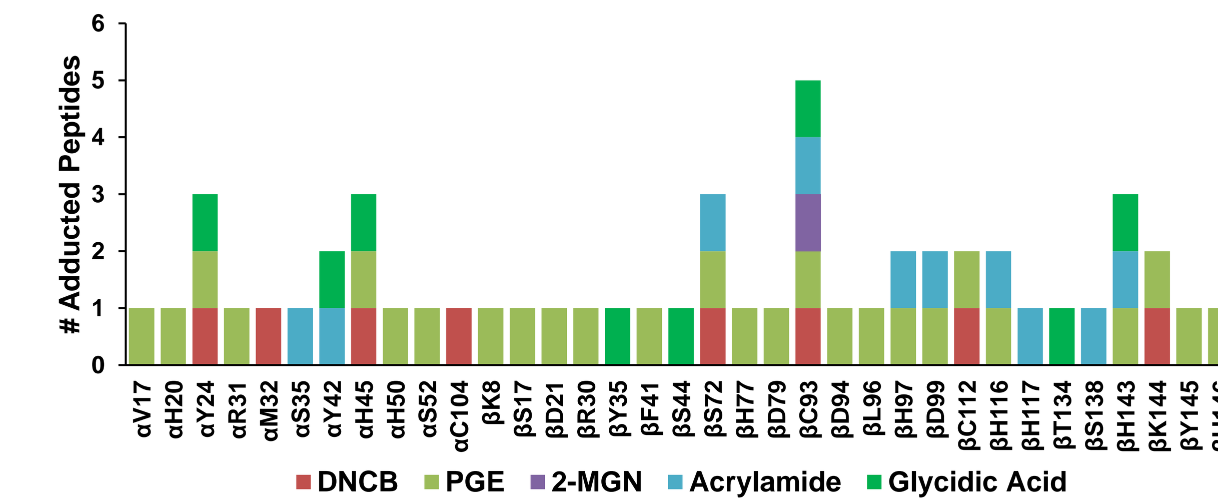
IV. BOTTOM-UP PROTEOMICS DETECTS MORE ADDUCTS THAN FIRE



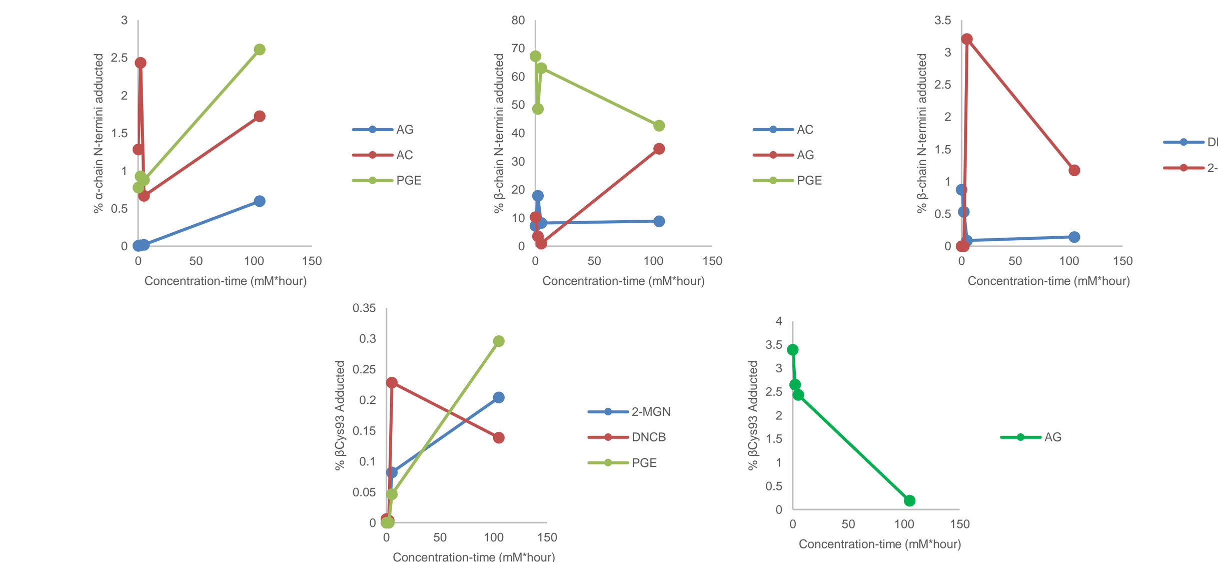
- FIRE analysis of blood samples shows variable responses among AA, AC, AG, and PGE
- Incubation with 2-MGN shows lower levels adduct formation
- No DNCB-Val-FTH formation at any concentration-time value

	N-termini	
	Alpha	Beta
DNCB	Not Validated	Validated
PGE	Validated	Validated
2-MGN	No peptide	Validated
Acrylamide	Validated	Not Validated
Acrylic Acid	Validated	Validated
Glycidic Acid	Validated	Validated





- N-terminal adduction seen in hemoglobin with bottom-up proteomics, including with DNCB
- Side chain adducts observed forming at multiple residues, with βC93 being the most reactive

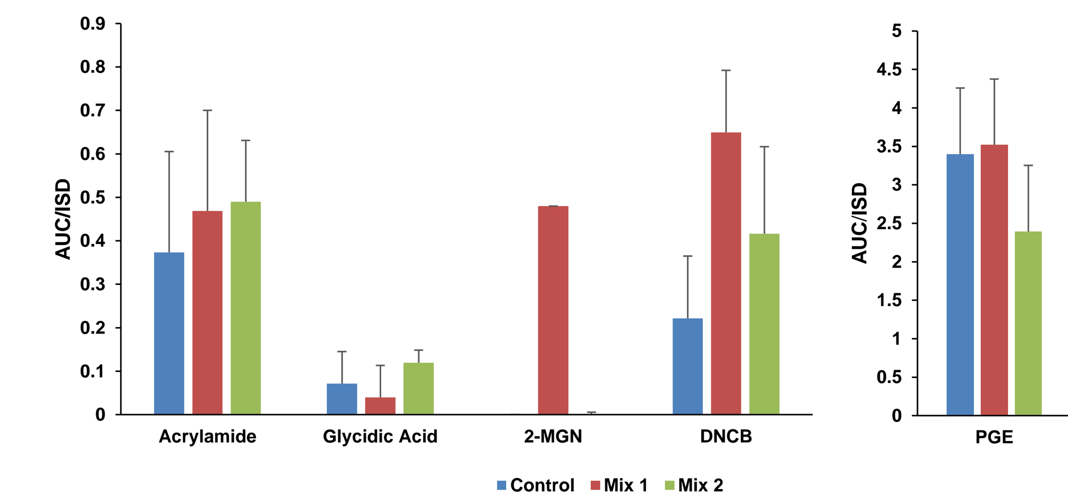


- Targeted mass spectrometry analyses demonstrate greater adduction at lower concentration–time values
- N-termini show greater levels of adduction than the most reactive sidechain βC93

V. EXAMINING THE RELATIVE REACTIVITY OF ELECTROPHILES USING TARGETED PROTEOMICS

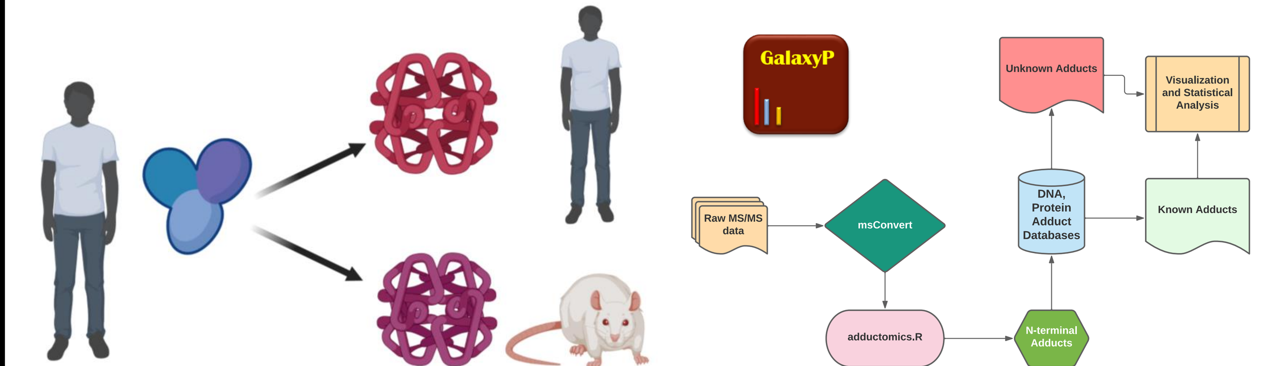
	Acrylamide	Glycidic Acid	2-MGN	PGE	DNCB
Control	-	-	-	-	-
Mix 1	100 µM	100 µM	5 mM	5 mM	5 mM
Mix 2	5 mM	5 mM	100 µM	100 µM	100 µM

- Mixtures of electrophiles were combined with aliquots of blood to examine competition between electrophiles in adduct formation
- 100 fmol of $^{13}C_5$-VLSPADKTNVK were added as an internal standard
- N-terminal peptides were examined for adducts, with the MS traces normalized against the internal standard



- Acrylamide, glycidic acid show increased abundances in Mix 2, while Mix 1 samples show an increase in 2-MGN, DNCB, and PGE
- Despite a 50x lower concentration in Mix 2, DNCB adduct abundance is observed to be equal to or greater than acrylamide and glycidic acid, respectively, indicating an overall higher level of reactivity
- Lack of 2-MGN adducts seen at 100 µM may indicate a kinetic barrier in the formation of N-terminal adducts
- Background levels of all electrophiles seen in untreated blood except for 2-MGN

V. AUTOMATED ADDUCT DISCOVERY IN GALAXY



- Detection of unknown adducts in biological samples necessitates an agnostic, untargeted data analysis technique
- Originally developed by Josie L. Hayes in the Rappaport Group, adductomicsR is an R package that allows for untargeted detection and relative quantitation of protein adducts in serum albumin
- AdductomicsR compares the spectra of hemoglobin samples to known masses of standards (in this case, the N-terminus of the beta strand and a housekeeping peptide)
- Through the modification of functions within the package, adductomicsR has been expanded to detect N-terminal hemoglobin adducts in rats and humans
- At present, adductomicsR is being wrapped into the Galaxy MSI instance at the University of Minnesota
- By joining adductomicsR with msConvert, adduct databases, and visualization software researchers can identify biomarkers rapidly with little extraneous processing and analysis

VII. SUMMARY

- The FIRE methodology and bottom-up proteomics were compared for the untargeted detection of hemoglobin adducts
- FIRE had a more consistent dose response, though proteomics was able to detect more adducts
- Targeted proteomics demonstrates variable degrees of reactivity of electrophiles in mixtures
- AdductomicsR is being wrapped into Galaxy MSI for automated adduct detection

VIII. FUTURE DIRECTIONS

- Complete the assembly and test automated bioinformatics workflows for the detection and quantitation of novel hemoglobin adducts with
- Optimize FIRE, bottom-up proteomics methodologies for high though-put analyses of large sample cohorts
- Pilot studies for combined FIRE-proteomics approach
- Analyze blood samples from human patients to characterize the exposome of chronic smoke consumption
- Validate novel hemoglobin adducts in smoking exposome through targeted mass spectrometry experiments

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