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Sources and Cellular Responses to Reactive Oxygen Species (ROS)

**Endogenous sources**
- Mitochondria
- Peroxisomes
- Lipoxigenases
- NADPH oxidases
- Cytochrome P450

**Exogenous sources**
- Ultraviolet light
- Ionizing radiation
- Chemotherapeutics
- Inflammatory cytokines
- Environmental toxins

**Antioxidant defences**
- Enzymatic systems
  - CAT, SOD, GPx
- Non-enzymatic systems
  - Glutathione
  - Vitamins (A,E,C)

**Impaired physiological function**
- Decreased cellular proliferation
- Defective host defences

**Homeostasis**
- Normal growth and metabolism

**Impaired physiological function**
- Random cellular damage
- Specific signalling pathways

**The etiology of every major disease has been linked to oxidative stress**
- Protein Modification
- DNA Damage
- Lipid Peroxidation

Source: Toren Finkel and Nikki J. Holbrook, Nature 408, 239-247(9 November 2000) doi:10.1038/35041687
Molecular Complexity of Oxidized Phospholipids (OxPLs)

**Arachidonate**

- Oxidized fatty acyl
  - -OOH
  - -OH
  - PLs with multiple oxidation sites / functional groups

- Oxidative fragmentation (Hock cleavage, β-scission)
  - α,β-Unsaturated
  - Saturated
  - Furan-containing

- Cyclization, Oxidation, rearrangement
  - Isoprostanones
  - Isolevuglandins
  - Isothromboxanes

- Non-fragmented OxPLs

- Fragmented OxPLs

R = Lyso-PC, lysoPE etc

PC, Phosphocholine

**Advanced lipid-oxidation end-products (ALE):**

- Malondialdehyde
- 4-Hydroxynonenal
Lipid Analysis Workflows

(Biological) Lipid Sample

Sample prep

Lipid Extraction (e.g. Bligh & Dyer) (SPE) (group selective)

Untargeted

Targeted

Combined Targeted / Untargeted

Untargeted

Shotgun MS/MSAll

DDA (IDA)

DIA (SWATH)

MRMHR

Targeted

SWATH

Combined Targeted / Untargeted

Shotgun MS/MSAll

DDA (IDA)

DIA (SWATH)

MRMHR

SWATH

Untargeted

Shotgun

DDA (IDA)

DIA (SWATH)

MRMHR

Targeted

Combined

Untargeted

Targeted

Combined Targeted / Untargeted

LipidView

IDA Explorer

PeakView / MultiQuant

MasterView

MS-DIAL

MasterView

MarkView (Sciex) (PCA, t-test)

SIMCA (Umetrics) (PCA, PLS, PLS-DA, OPLS-DA)
Data-Dependent Acquisition (DDA)

Most intensive precursor ions detected in survey scan are selected for isolation and fragmentation in a serial manner.

- Performance declines with complexity of samples
- Limited reproducibility (stochastic precursor selection)
- Bias towards high abundant analytes
- Low sensitivity
- Missed trigger for fragmentation
- Limited analyte coverage

Information-dependent acquisition (IDA)
UHPLC-MS/MS Method with DIA

Data-Independent Acquisition (DIA)

- Fragmentation of all detectable precursor ions systematically parallelized
- Improved reproducibility for identification
- Better sensitivity
- Improved analyte coverage
- Composite/multiplexed fragment ion spectra
- DIA (SWATH): Quantification in MS/MS mode (sensitivity and specificity gain)
- Retrospective data processing
- Data processing more elaborate, but SW for automated metabolite ID is being developed

SWATH: Sequential windowed acquisition of all theoretical fragment ion mass spectra
Heatmap IDA

Untargeted Lipidomics

Intensity 500-2000
Intensity 500-2000
Intensity 2000-5000
Intensity 2000-5000
Intensity 5000-10000
Intensity 5000-10000
Intensity 10000-30000
Intensity 10000-30000
Intensity > 30000
Intensity > 30000
SWATH 2.0 Calculation (swathTUNER)

Calculation of variable SWATH windows

Two methods for generating variable windows:
- Equal precursor ion population (PIP)
- Equalized precursor total ion current (TIC)

- Improvement of MS/MS spectra quality, esp. of compounds with low intensity
- Improved identification score
- Improved quantitative reproducibility
MS-DIAL: Data-independent MS/MS deconvolution

Open-source software pipeline (for data processing & automated metabolite identification)

a) Data import
Raw data or mzML → Conversion → ABF

b) Peak spotting (Rt versus MS1 peak detection)

Isolation window for precursor ion selections
Focused spot

MS²Dec (MS/MS deconvolution)

Raw MS/MS spectrum

Deconvoluted MS/MS spectrum

Compound identification

Deconvoluted MS/MS versus reference MS/MS

MSP format
MassBank
LipidBlast

Relative abundance (%)

9 | Oliver Fiehn & Masanori Arita, et al., Nature Methods, 2015, doi:10.1038/nmeth.3393
Benefits of MS-DIAL

- User-friendly display of results
- Alignment results can be viewed
- Deconvoluted spectra with better spectral quality
- Deconvoluted MS/MS chromatogram with enhanced assay specificity
- Automated lipid identification (of lipids in LipidBlast database)
- Supporting documentation (no black box system)

Mass accuracy in all samples within ±4 mDa

Peak in all samples within ±0.04 min
### Platelet Lipidomics

<table>
<thead>
<tr>
<th>Acute Coronary Syndrome (STEMI)</th>
<th>Stable Angina Pectoris</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS 1</td>
<td>SAP K2</td>
<td>R1</td>
</tr>
<tr>
<td>ACS 2</td>
<td>SAP K3</td>
<td>R2</td>
</tr>
<tr>
<td>ACS 4</td>
<td>SAP K4</td>
<td>R3</td>
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<tr>
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<td>SAP K5</td>
<td>R4</td>
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<td>SAP K6</td>
<td>R5</td>
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<td>R7</td>
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<tr>
<td>ACS 14</td>
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</table>

- Analysis in randomized run order
- QC sample every 5th injection to validate stable method performance
- Analysis in positive and negative ionisation mode
- Software based database search with over 40000 lipid entries (LipidMaps) or MS-DIAL (LipidBlast)
- 7000-8000 features per sample

#### Data pre-processing:
- Retention time correction to internal standards
- Normalization to internal standards
- Normalisation to total area sum of peaks

#### Statistical evaluation:
- Principal component analysis (PCA)
- Supervised PCA
- t-test -- > „vulcano“ plot
  → Statistics used to find significant molecular features varying between groups of sample set
Assay stability as assessed by QC

CVs of retention times:
0.4% for LPC(17:1)
0.2% for PC(17:0/20:4)

CVs of raw signal intensities:
12% for LPC(17:1)
10% for PC(17:0/20:4)
Platelet Lipidomics

Score Plot

- Responses are log transformed and autoscaled
- Multivariate statistics: PCA-DA

Platelets from patients with SAP and ACS can be distinguished from healthy ones by their lipid profiles
Volcano Plots

*p*-Value vs log Fold Change

**t-Test: ACS vs SAP**

Statistical Significance

Decreased compared to control

Increased compared to control

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**t-Test: ACS vs Control (Healthy)**

**t-Test: SAP vs Control (Healthy)**
Identification of Significant Features

- Hits with \( p < 0.05 \) were checked for identification (MS-DIAL)
- Positive identification were boxplotted

Matching reference spectrum for PE 38:4; (18:0/20:4(5Z,8Z,11Z,14Z))

Neutral loss of Phosphoethanolamine
Heatmap of Lipids Identified with MS-DIAL

- **ESI(+)**
- **7,500 molecular features**
- **664 lipids with \( p < 0.05 \) (ACS vs control)**
- **162 of them unidentified by MS-DIAL**
- **Shown are identified ones (502)**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Metabolite name</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PC 38:3; PC(18:3(6Z,9Z,12Z)/20:0); [M+H]+</td>
</tr>
<tr>
<td></td>
<td>SQDG 35:5; SQDG(16:2/19:3); [M+NH4]+</td>
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<tr>
<td></td>
<td>TG 53:7; TG(16:2/18:0/19:5); [M+NH4]+</td>
</tr>
<tr>
<td></td>
<td>lysoPE 16:0; PE(O-16:0/0:0); [M+H]+</td>
</tr>
</tbody>
</table>

**Diagram:**
- **TG**
- **SM**
- **PE**
- **PC**
- **PlasmenylPC**
- **MGDG**
- **MG**
- **LysoPE**
- **LysoPC**
- **DGTS**
- **DGDG**
- **DG**
- **CE**

**Response Scale:**
- 0.02000
- 0.4000
- 1.000
- 1.750
- 4.000
- 200.0
Platelets of SAP and ACS Patients Show Altered OxPL Levels

- **PLPC-(OOH)**
  - m/z 790.5593

- **PAPC-(OOH)**
  - m/z 814.5593

- **PLPC-(OOH)₂**
  - m/z 822.5491

- **PC 33:1-(OH)**
  - m/z 762.5643

- Not identified by MS-DIAL
- Target list of 40,000 lipids from LipidMaps db complemented by target list of OxPLs
- Loaded into MasterView
- Level of metabolite Id:
  - ○ accurate mass
  - ○ isotope pattern
  - ○ fragmentation
- Standards for verification not available currently

PLPC: 1-Palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
Combined Targeted / Untargeted Analysis

- Targeted and nontargeted analysis in single workflow supported by SWATH
- Quantitative analysis based on precursor or fragments
- Improved selectivity and sensitivity

**Graphs:**
- Peak Area vs. Concentration (ng/mL)
- LODs (matrix): 0.2 - 2.0 ng/ml
- LOQs (matrix): 1 - 6 ng/ml
- Good repeatabilities

**Chemical Structures:**
- **PONPC:** 1-palmitoyl-2-(5’-oxovaleroyl)-sn-glycero-3-phosphocholine
- **POVPC:** 1-palmitoyl-2-(9’-oxononanoyl)-sn-glycero-3-phosphocholine
- **PAzPC:** 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine
- **PGPC:** 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine
**Combined Targeted / Untargeted Analysis**

**IDA (20 MS/MS per cycle)**
- XIC of PONPC 650.439 +/- 0.010 Da
- MS/MS triggered at RT = 12.1281

**SWATH**
- XIC of PONPC 650.439 +/- 0.010 Da
- Fragment XIC of Phosphocholine 184.073 +/- 0.01 Da

**PONPC**
1-palmitoyl-2-(5'-oxovaleroyl)-sn-glycero-3-phosphocholine

**IDA:** Quantification by TOF-MS (via precursor ion only)

**SWATH:** Supports quantification in MS/MS mode (via fragment ions)
- Enough data points across peak

**SWATH without deconvolution:**
- Limited assay specificity
- Several lipids with same/similar tR may yield the same fragment (interference)
Quantification based on Deconvoluted MS/MS Chromatogram

**IDA**
- Lyso-PC(18:1), 522.3554 +/-0.01 Da
- MS/MS triggered at RT = 10.3210

**SWATH, deconvoluted**
- Lyso-PC(18:1), 522.3554 +/-0.01 Da
- Fragment XIC 184.073 +/- 0.010 Da

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**MS2 chromatograms Precursor: 522.3556**
- Deconvoluted MS/MS Chromatograms
  - > 183.9935
  - > 184.0739
  - > 184.072
  - > 184.07
  - > 184.1068
  - > 184.0758
  - > 104.1054
  - > 184.0681
  - > 104.1083
  - > 104.104
  - > 184.0777

**Raw MS/MS spectrum**
- 184.072
- 522.3542

**Deconvoluted MS/MS spectrum**
- 184.0739
- 522.3542
Summary / Conclusions

Data-independent acquisition (DIA) with SWATH

- Better coverage of analytes as compared to DDA (IDA) profiling
- MS-DIAL a powerful SW for automated lipid identification
- Oxidized PLs not successfully identified, therefore target list approach used
- In future, focus on combined targeted/untargeted profiling
- Ion mobility and 2D-LC as further selectivity dimensions to resolve complexity

Platelet lipidomics

- SAP and ACS show significant differences in lipid profiling (regulation via signaling cascades)
- Several oxidized PLs showed increased levels in SAP and ACS patients (oxidative stress or controlled regulation upon platelet activation)
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