“Lipids and mediators as smoking related biomarkers of effect“

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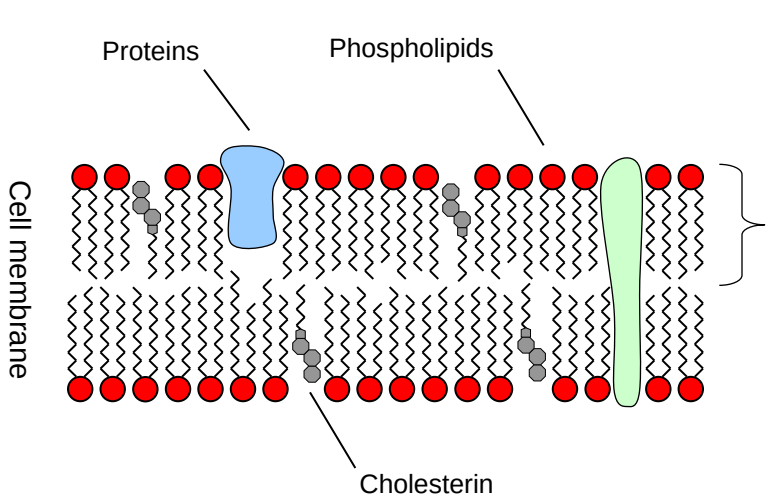
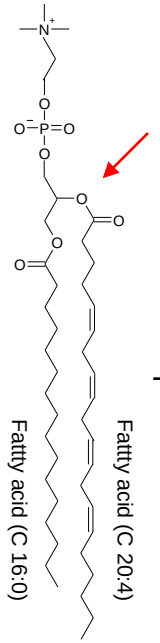
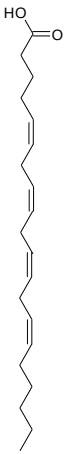
Background – Eicosanoid metabolism





Phospholipid (Phosphatidyl-choline; PC 36:4)

PLA_2 (Phospho-lipase A_2)



Arachidonic Acid (n-6; C20:4)

Aspirin
Ibuprofen

STOP

COX (Cyclo-oxygenases)
- Prostaglandins (PGD_2, PGE_2, PGI_2, $PGF_{2\alpha}$)
- Thromboxanes (TXA_2, TXB_2)

LOX (Lip-oxygenases)
- Leukotrienes (LTB_4, LTE_4)
- Hydroxyeicosa-tetraenoic acids (HETEs)

Non-enzymatic (oxid. stress)
- Isoprostanes (8-iso-$PGF_{2\alpha}$)

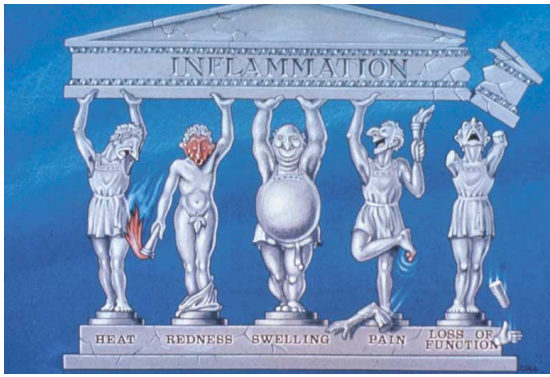
Physiological effect:

Cell membrane composition (arachidonic acid content) is:

- crucial for cellular functions
  - Ecker et al., PNAS, 2010
  - Tian et al., JCB, 2008
- cell type specific
  - Sampaio et al., PNAS, 2011
  - Ecker et al, Immunobiol., 2010
- influenced by nutrition (n-3 vs. n-6)
  - Calder et al., Br. J. Nutr., 2009
  - Ecker et al., JLR, 2010
- involved in diseases
  - Maxfield, Tabas, Nature, 2005

Eicosanoids:

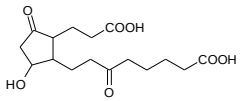
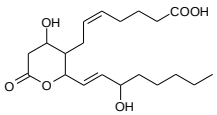
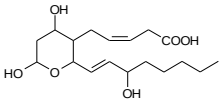
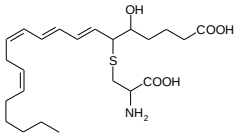
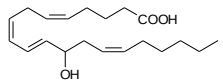
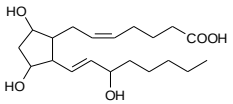
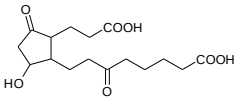
„Powerful lipid mediators involved in inflammation that are derived from mainly 20 carbon fatty acids (eicosa= 20; greek)."



From Lawrence et al., Nat.Rev.Immunol., 2002

Urinary eicosanoids as biomarkers of effect

Aim: "Comprehensive quantification of key urinary eicosanoids with patho-physiological relevance by LC-MS/MS ."

Chemical Structure	Compound	MW (g/mol)	Patho-physiological Relation	Disease relation / Biomarker for
	tetranor PGE-M	328.4	Inflammation	Cancer (e.g. lung)
	11-dehydro-TXB_2	368.5	Platelet activation	Diabetes
	2,3-dinor-TXB_2	342.4	Platelet activation	Diabetes
	LTE_4	439.6	Inflammation	Asthma
	12-HETE	320.5	Platelet activation	Hypertension, Diabetes
	8-iso-$PGF_{2\alpha}$	354.5	Oxidative Stress, Free radical generation	Atherosclerosis, CVD, Diabetes, Alzheimer
	2,3-dinor-8-iso-$PGF_{2\alpha}$	326.4	Oxidative Stress, Free radical generation	Atherosclerosis, CVD, Diabetes, Alzheimer

Eicosanoid quantification – a real analytical challenge

"What methods are available ?"

- LC-MS/MS (often with derivatization)
- Sample preparation: SPE (solid phase extraction)
- But, most methods are „single analyte/single eicosanoid class methods"

"What are our aims ?"

"What could be improved ?"

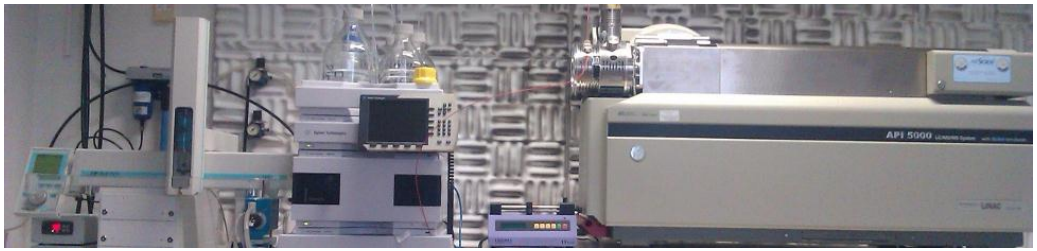
- Multiple analytes in one method
- Sample preparation (too much „sample-on-bench-time")
- Eicosanoid separation („more separation power in shorter running times")
- Sensitivity (lower limits of detection and quantification)
- Robustness and data quality (better validation data, GLP!)

How can we solve this problems ?

What approaches are reasonable ?

Approach 1 – High end LC-MS/MS system for detection

API 5000

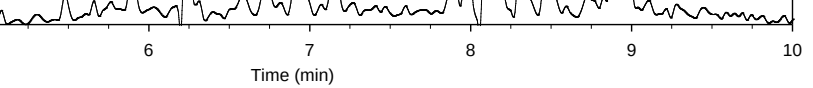


- More Sensitivity
- More Robustness

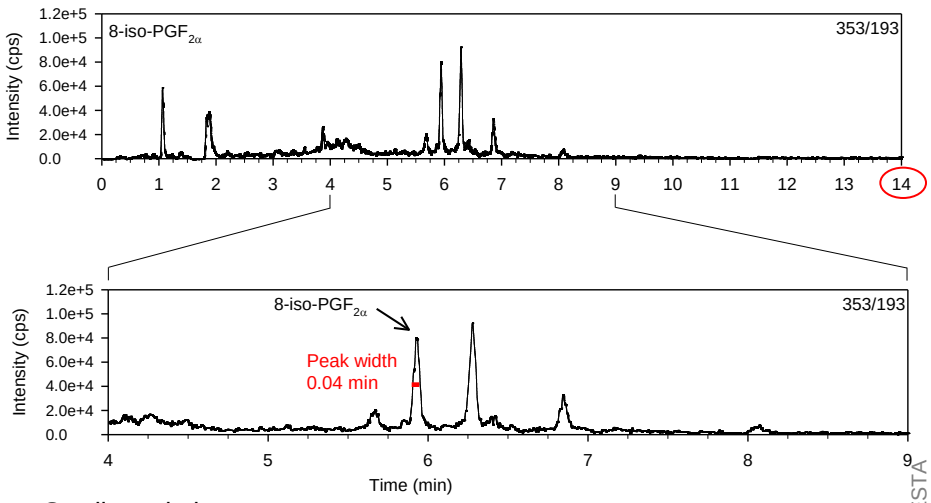
Waters XEVO TQ-S

Approach 2 – UPLC and small particle columns for separation

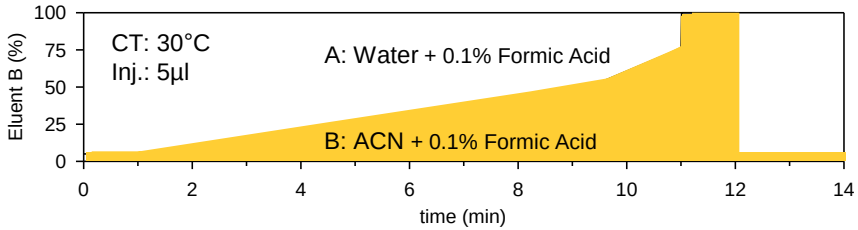
HPLC-column:
3 x 100 mm; 3.5 µm
(Agilent Zorbax SB-C18)



UPLC-column:
2.1 x 50 mm; 1.7 µm
(Waters BEH C18)



Gradient elution:



- More separation power, all 7 analytes in one run
- Shorter run-times
- Smaller column particles → sharper peaks → better S/N → more sensitivity

Approach 3 – LLE for sample preparation instead of SPE

C18-RP-SPE (500mg)

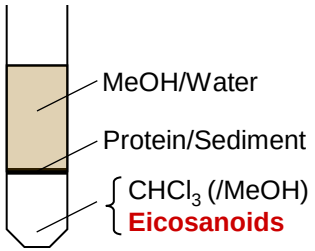
Typical protocol:

- 5 ml MeOH (conditioning)
- 5 ml Water (conditioning)
- 3 ml Sample
- 3 ml 5 % MeOH (rinse)
- Dry (vacuum; >30 min))
- 4 ml MeOH (elution)
- Evaporate
- Re-dissolve in 100 µl Solvent

Liquid-liquid extraction (LLE)

Protocol:
(mod. Bligh & Digher)

- Mix 3 ml acidif. Sample with 11.35 ml (MeOH/$CHCl_3$; 2/1)
- 30 min RT
- Add 7.5 ml ($CHCl_3$/Water; 1/1)
- Centrifuge 10 min at 2500 rpm
- Recover $CHCl_3$-Phase
- Evaporate
- Re-dissolve in 100 µl Solvent



- Not ideal for all analytes (e.g. PGE-M, 12-HETE)
- Also tested: pol.RP-SPE, pol.RP-A^--SPE

- Allows solid extraction of all 7 analytes
- Less matrix interferences
- Easier practical viability
- Less "sample-on-bench-time"

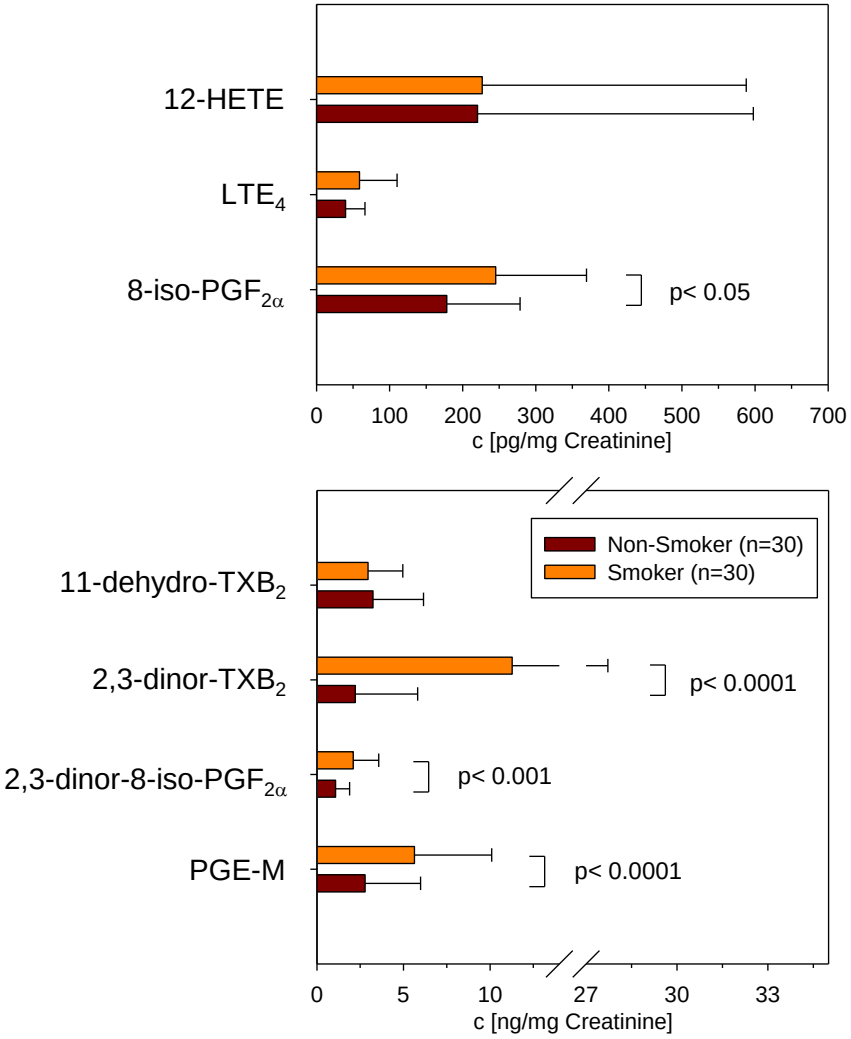
Method validation data

∑ *LLE; UPLC column; ESI, negative ion mode; No derivatization; High end mass spectrometer; Quantification by stable isotope dilution (deuturated IS).*

Summary (Validation according to FDA guidelines):

- Calibrations: linear; $R^2 > 0.99$
- Accuracy: 95 % to 113 % (3 levels)
- Intra-day precision: CVs < 11% (3 levels)
- Inter-day precision: CVs < 12 % (3 levels)
- No carry-over
- Matrix effects: -13.9 % to 10.7 % (3 levels; 3 diff. urines matrices)
- Sample stability:
  - 30 h RT: all stable, except PGE-M (-25 %); LTE4 (-28 %), 12-HETE (-29 %)
  - 6 freeze/thaw cycles: all stable, except PGE-M (- 15 %)
  - 14 d at 10°C in autosampler: all stable

Method application – Smokers vs. non-smokers



Experiment:

- Smokers (n=30) vs. non-smokers (n=30)
- Nutrition controlled
- Smoking activates COX-2 (PGE_2, TXA_2)

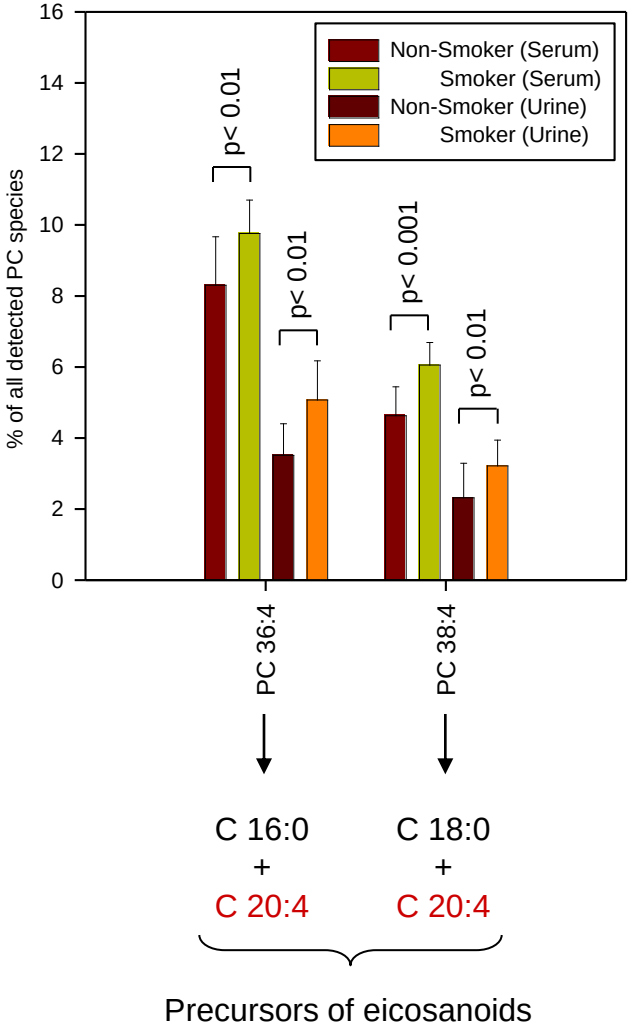
  Huang et al., BBA, 2011
- Increases oxidative stress (Isoprostanes)

  Yan et al., JLR, 2007
- Levels normalized for creatinine (urine dilution)
- Statistics: Mann-Whitney U test

- Significantly elevated PGE-M, isoprostane and 2,3-dinor-TXB_2 levels (LTE_4 by trend).
- 12-HETE not changed
- 11-dehydro-TXB_2 not changed (although elevated levels have been described).

  Ikonomidis et al., Am. Heart J., 2005

Eicosanoid precursors - Phospholipids



Do eicosanoid precursors also show a differential regulation in smokers and non-smokers?

- Precursors are C 20:4 (arachidonic acid) containing phospholipids
- The major phospholipid class in human plasma is phosphatidyl-choline (PC; ~76%)
  Quehenberger et al., JLR, 2010
- The major C20:4 containing PC species are PC 36:4 and PC 38:4

- Significantly different PC 36:4 and PC 38:4 levels.
- Results supported by data from a other group; elevated C 20:4 containing PL levels in smokers.
  Wang-Sattler et al., Plos one, 2008
- Results confirm our eicosanoid data.
- PC 36:4 and 38:4 have been associated to CVD and cancer.
  Ecker, J. Sep. Science, 2012

Conclusion

- The developed UPLC-MS/MS is robust and very sensitive for quantification of urinary eicosanoids.
  (Lowest LODs and LOQs described yet.)
- Significantly elevated PGE-M, isoprostane, and 2,3-dinor-TXB_2 levels in smokers.
- Significantly elevated levels of eicosanoid precursors PC 36:4 and PC 38:4 in smokers.

Proposal:

"A combined profiling of eicosanoids and precursors gives a more comprehensive picture on smoking-related (patho-) physiological processes and diseases
+
more valid results than analysis of eicosanoids alone."

Relevant Publications:

- Katharina Sterz; Gerhard Scherer; Josef Ecker; *J. Lipid Res.* **2012**, 53, 1026-1036
- Josef Ecker; *J. Sep. Science* **2012**, 35, 1227-1235

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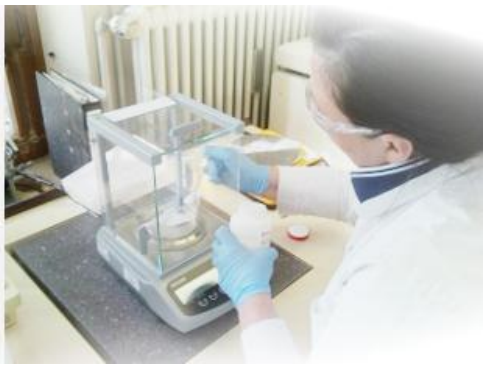

Thank you for your attention !