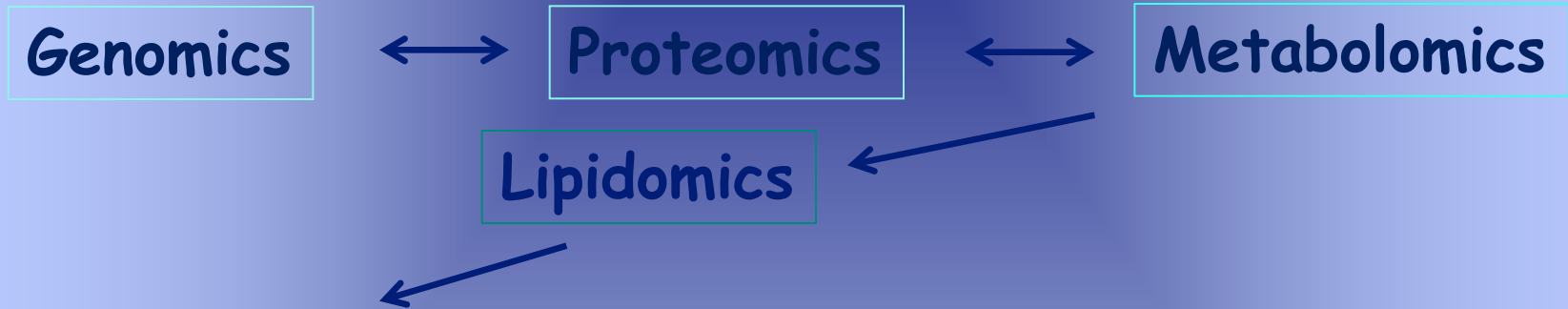


LIPIDOMIKA

1. Co jsou lipidy, buněčné membrány, buněčná signalizace za účasti lipidů
2. Metody stanovení lipidů (chromatografické analýzy, enzymové aktivity, inhibitory, vizualizace lipidů)
3. Fyziologický/patofyziologický/toxikologický význam lipidů, interpretace dat z lipidomických analýz (účast v diferenciaci, změny lipidomu v karcinogenezi, lipidomické změny po expozici cizorodými látkami)

LIPIDOMICS



Structural lipids, lipid storage, cell signaling

- phospholipids, triglycerides, PIPs
- sphingolipids (SLs)
- fatty acids (FAs)
- eicosanoids (PGs,...)
- sterols (bile acids, cholesterol, steroid hormones)



Lipid mediators (PIPs, AA, SLs, FAs, PGs,...):

- intracellular signaling
- auto/paracrine signaling

LIPIDY: IZOLACE, DETEKCE, SEPARACE A KVANTITATIVNÍ ANALÝZA

- ◆ FOSFOLIPIDY - chemické složení v buněčných membránách; metody stanovení fosfolipidů (TLC, HPTLC, HPLC/MS), DIACYLGLYCEROL - stanovení hladin a fosfolipázových aktivit, TRIGLYCERIDY (akumulace v lipidních dropletích) - flurometrická detekce; FOSFATIDYLINOSITOLFOSFÁTY (PIPs) - intracelulární koncentrace (HPLC)
- ◆ SFINGOLIPIDY (SL) - koncentrace SL v buňkách, extracelulárním prostoru, v plasmě; enzymy metabolismu SL (genová exprese, proteiny, enzymové aktivity) - RT-PCR, Western blotting, ICC, IHC barvení, HPLC/MS, GC/MS
- ◆ MASTNÉ KYSELINY - chemické složení mastných kyselin (GC/MS); exprese genů metabolismu FAs; EICOSANOIDY (prostaglandiny, leukotrieny, ...) stanovení hladin, stanovení enzym. aktivit LOX, COX, CYP, PG syntáz - HPLC/MS
- ◆ STEROLY (cholesterol, žlučové kyseliny, steroidní hormony a jejich metabolity) - stanovení hladin (HPLC), komplementární stanovení genové exprese, proteinů a enzymových aktivit enzymů metabolismu sterolů..)

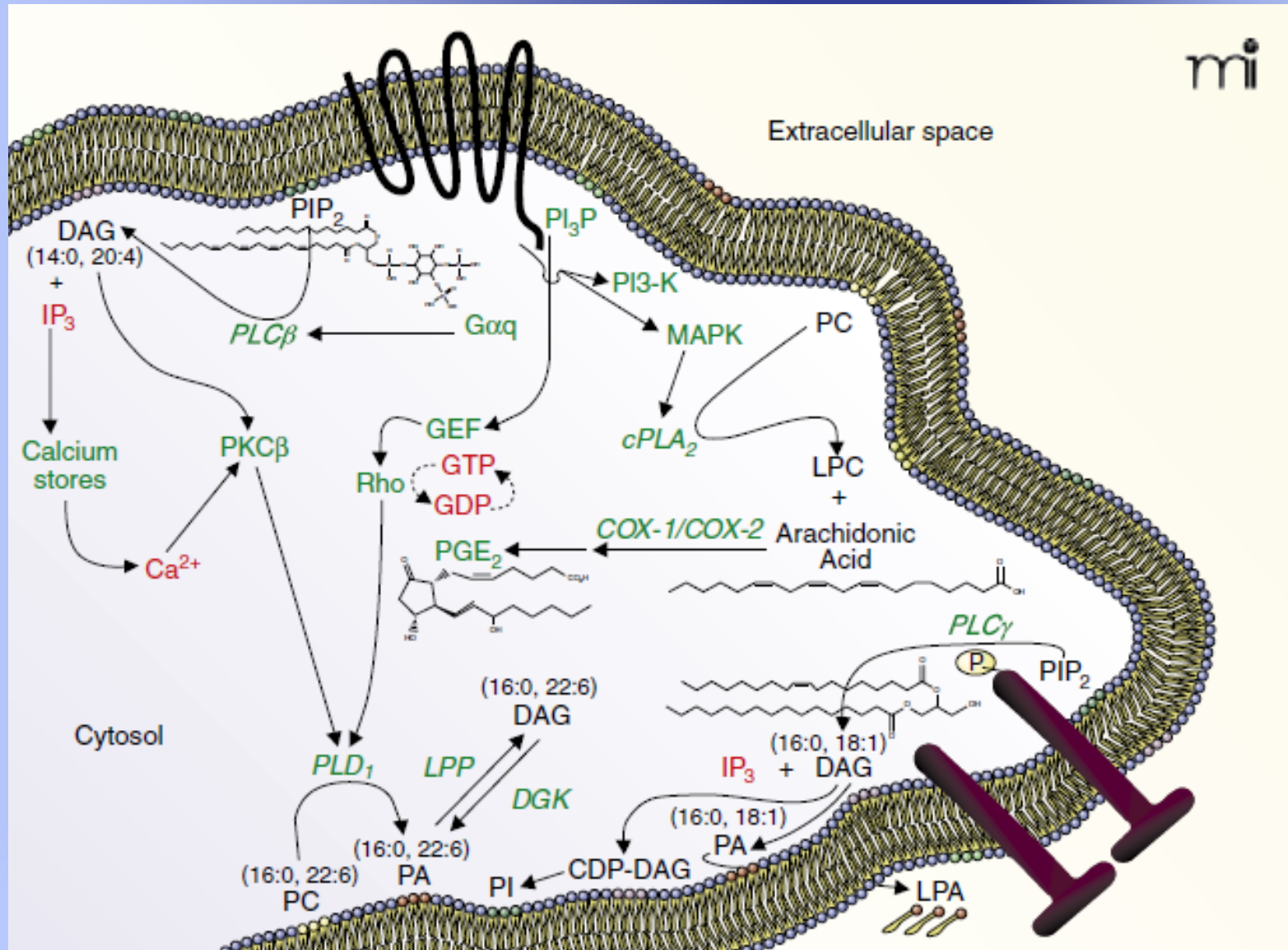
Lipidové buněčné membrány

“Cell membranes are not just homogenous mixtures of lipids and proteins; they are membrane domains with specific lipid and protein compositions regulating vesicular traffic, cell polarity and cell signaling pathways.” (Bieberich, Chemistry and Physics of Lipids, 2018)

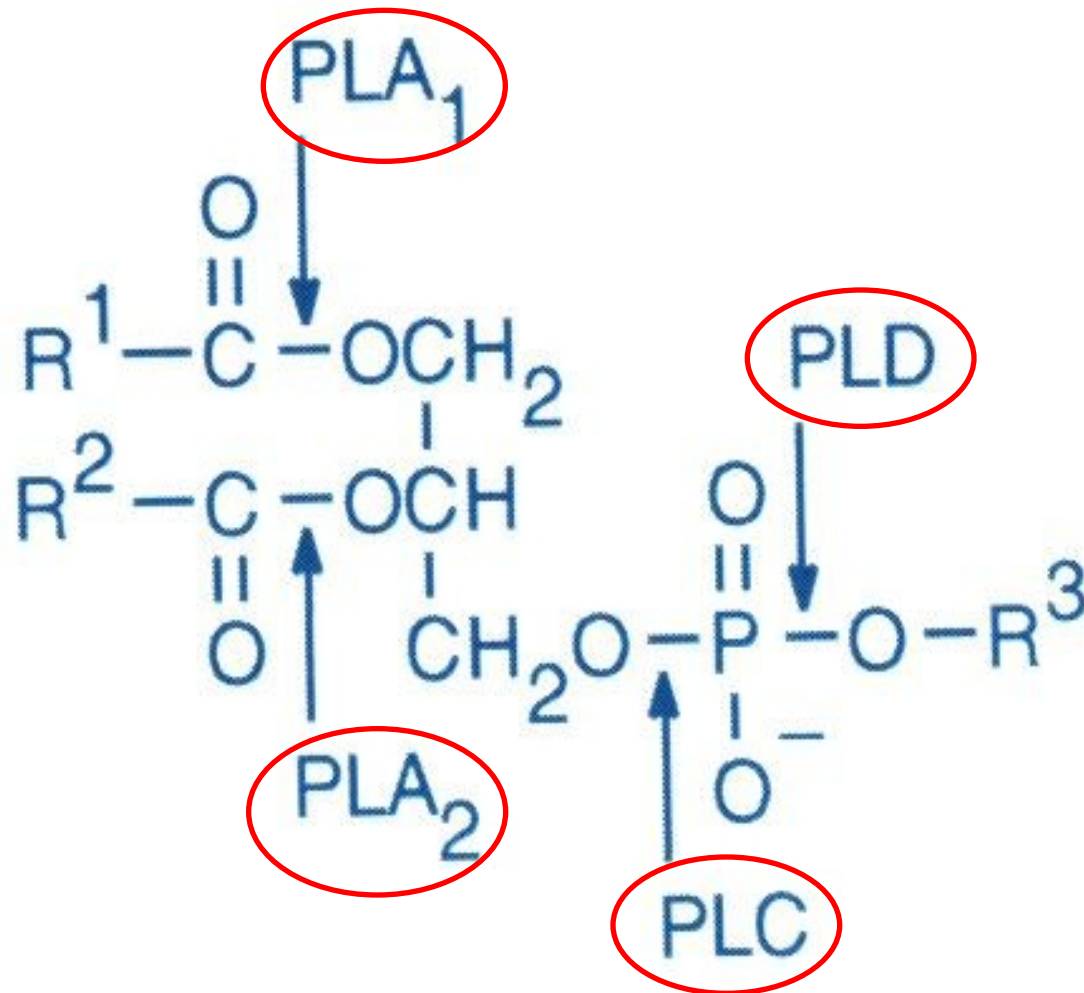
Různé fáze v lipidové membráně:

- “liquid ordered phase” (Lo) = lipidové rafty, velmi dynamické, obohacené o cholesterol a sfingolipidy (SM, GSLs), které jsou asociovány s raft. proteiny; ceramid a lysosfingolipidy indukují srůstání raftů do větších domén; toto srůstání (“coalescence”) pravděpodobně indukováno enzymaticky (SM konverze na ceramid sfingomyelinázami, hydrolýza GSLs glykosidázami);
- “liquid disordered phase” (Ld); přítomnost nenasycených fosfolipidů podporuje Ld; zde přítomny, „izolovány“ neraftové proteiny.

INTRACELULÁRNÍ SIGNALIZACE (černě lipidy, zeleně proteiny)



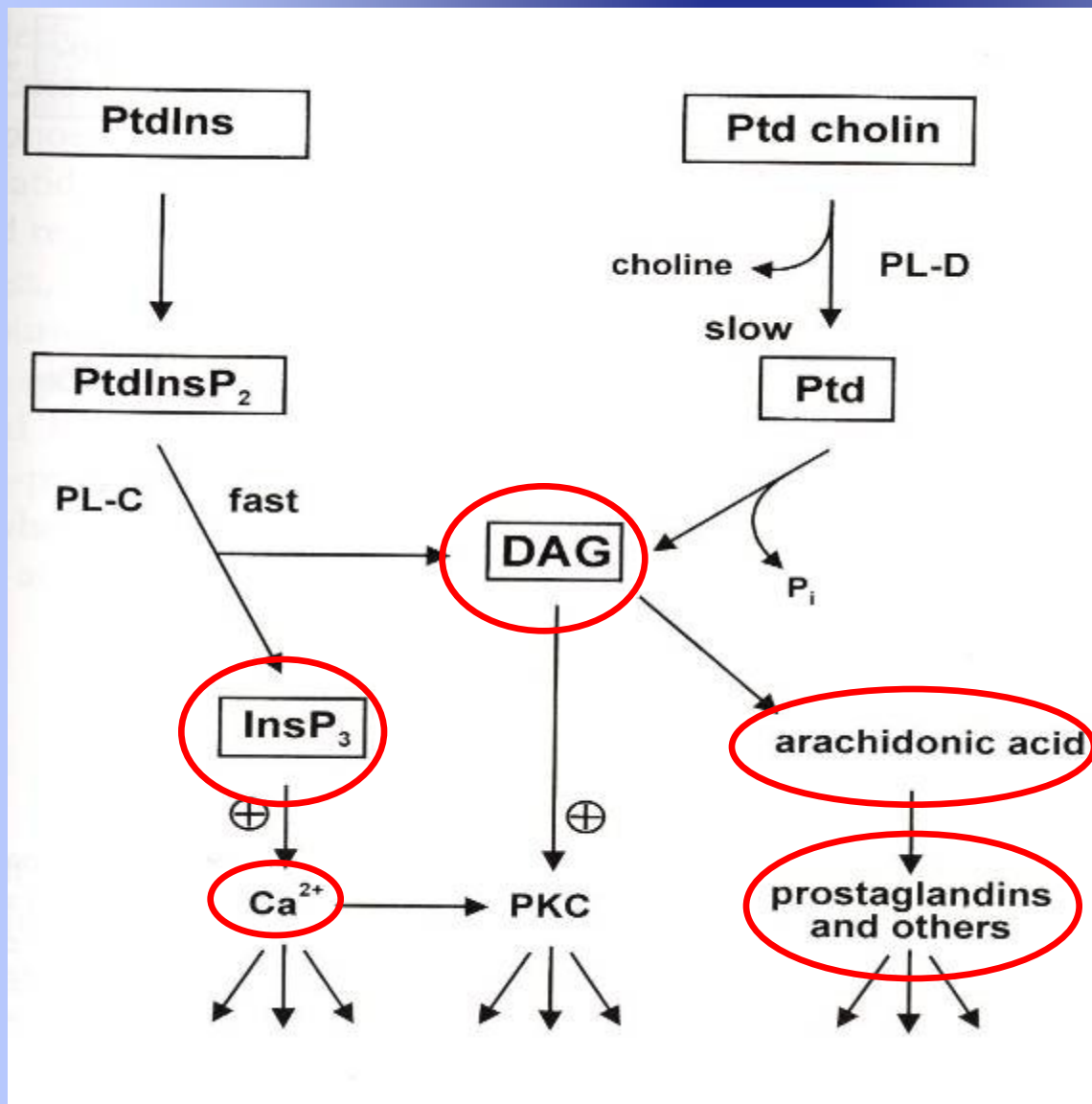
SLOŽENÍ GLYCEROLFOSFOLIPIDŮ / FOSFOLIPÁZY (PLA)



R1, R2:
mastné kyseliny

R3:
cholin
etanolamin
serin
inositol
aj.

GLYCEROLFOSFOLIPIDY a bioaktivní lipidy



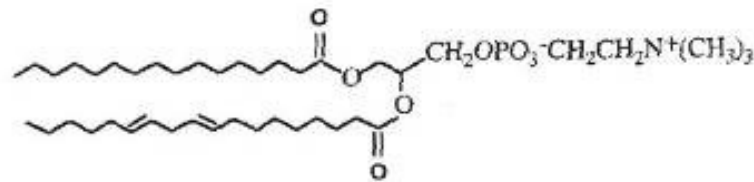
Fosfatidylinositol-
specifická fosfolipáza C
a fosfolipáza D
(reakce fosfatidylcholin-
specifické fosfolipázy C
není znázorněna)

Co stanovujeme:

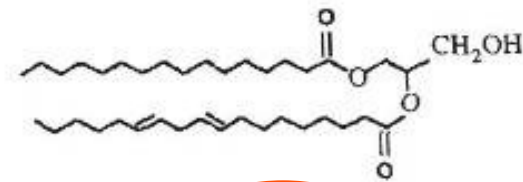
- složení fosfolipidů;
- hladiny DAG, PIPs..;
- produkci mastných kys.
a metabolitů mastných
kys. (AA, prostaglandinů,
leukotrienů aj.)

SFINGOFOSFOLIPIDY - další bioaktivní lipidy

Funkce DAG:
(aktivace PKC,
proliferační signály aj.)



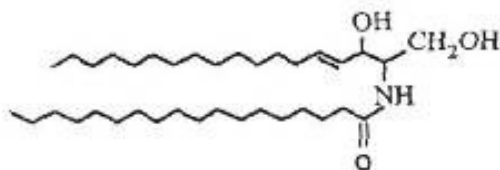
Phosphatidylcholine



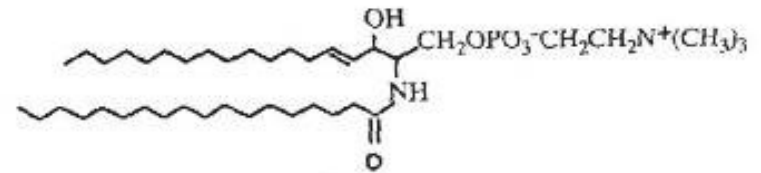
Diacylglycerol

PC-PLC
SMsyntáza

SMáza



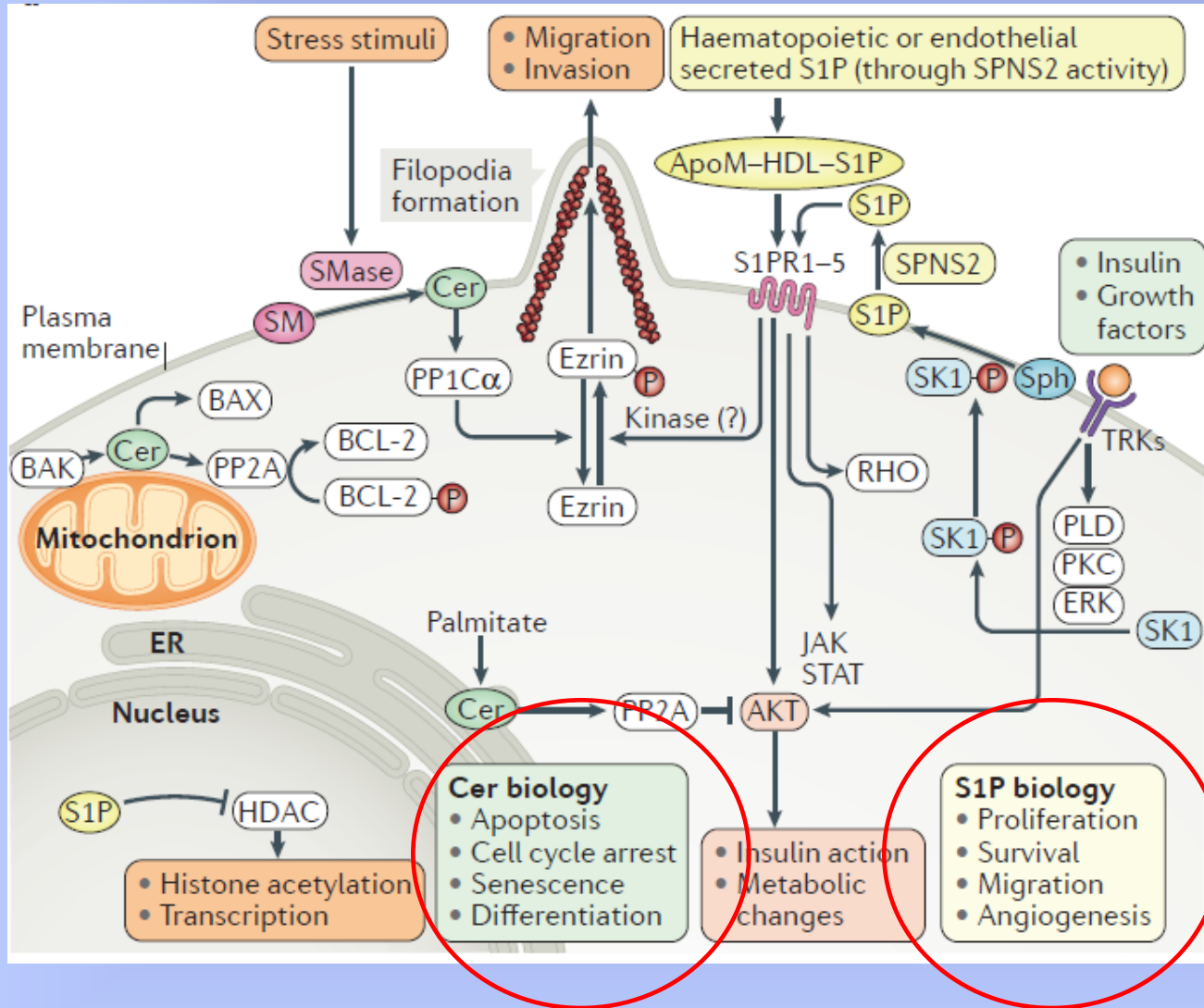
Ceramide



Sphingomyelin

**Různé funkce ceramidu
v různých typech buněk
(aktivace fosfatáz, apoptotické
signály, funkce v membránách)**

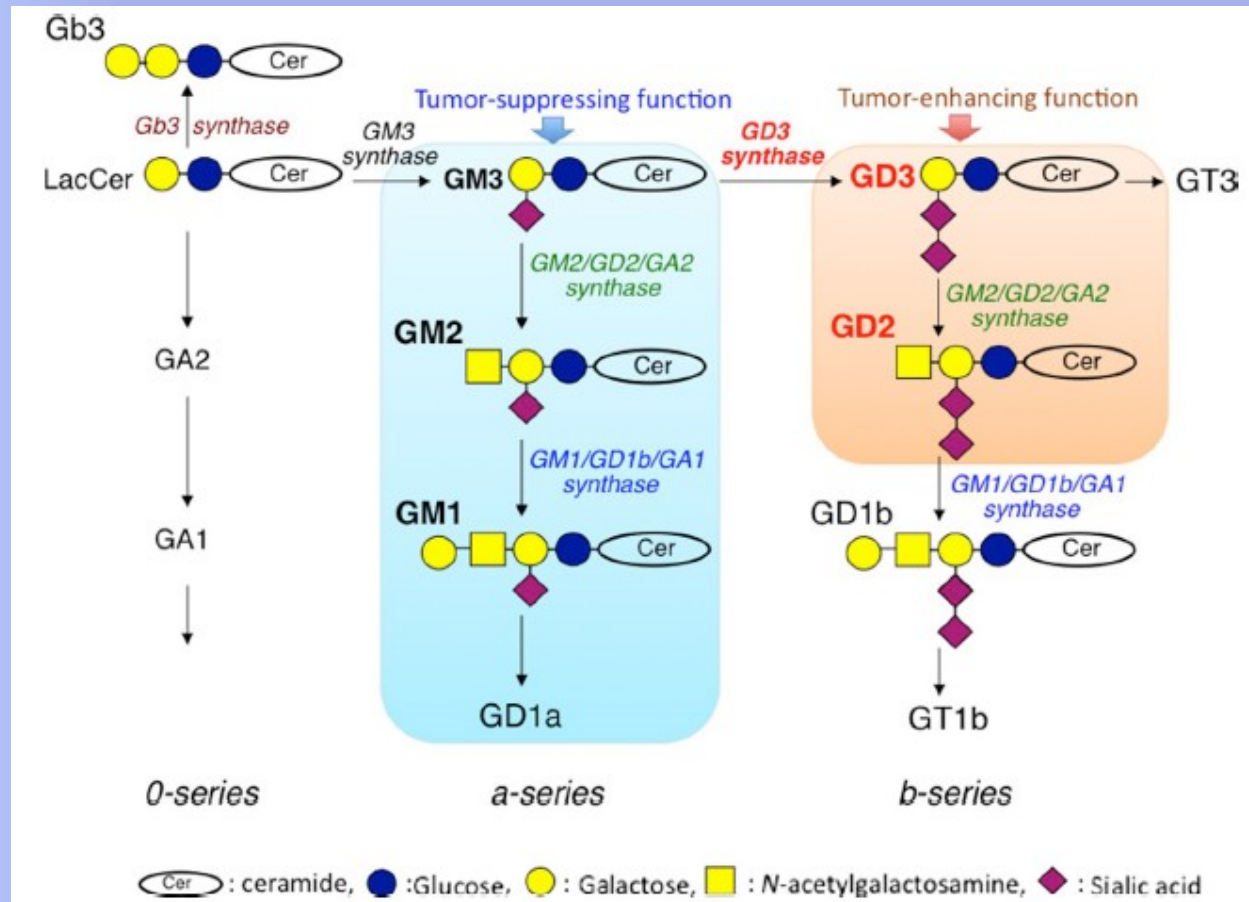
Fyziologické a patofyziologické role SLs ("S1P/Cer rheostat")



Activation of protein kinases (ERK1/2, Akt):
Sph-S1P-S1P receptors, proliferation, cell survival, migration, ...

Stress stimuli:
Cer, dhCer, ...:
apoptosis, cell cycle arrest, autophagy leading to cell death, senescence,...

Fyziologické a patofyziologické role GSLs



GD3 and GD2 enhance the malignant properties of cancer cells such as cell proliferation, invasion, migration, and metastasis; GM3, GM2 and GM1 often suppress malignant properties (Furukawa et al., *Cancer Sci*, 2019, 110, 1544-1551).

STANOVENÍ FOSFOLIPIDŮ

Tenkvrstvá chromatografie (TLC, HPTLC):

Fosfolipidy odvozené od glycerolu - skupinová analýza;

Sfingolipidy - skupinová analýza;

- izolace skupiny pro další analýzu individuál. lipidů (zde 2. krokem je metoda LC/MS)

LC/MS:

Stanovení jednotlivých fosfolipidů

- analýza celého vzorku (lyzátu buněk, liposomů apod.); separace jednotlivých tříd na kolonce SPE + analýza LC/MS
- izolace HPTLC + analýza LC/MS;

TLC STANOVENÍ LIPIDŮ (výběr mobilních fází)

LIPIDS	Solvent System (RF)					
	1	2	3	4	5	6
Triglycerides					0.70	0.96
Free Fatty Acid					0.51	0.16
Diglycerides 1,2					0.70	0.24
Diglycerides 1,3						0.32
Monoglycerides						
Phosphatidylethanolamine	0.79	0.55		0.43		
Phosphatidyl (Monomethylethanolamine)	0.71	0.41		0.33		
Cardiolipin	0.67	0.56	0.38			
Phosphatidylglycerol	0.60	0.50	0.31	0.36		
Phosphatidyl (Dimethylethanolamine)	0.58	0.56		0.27		
Phosphatidic Acid	0.55	0.05	0.58			
Phosphatidylinositol	0.39	0.10				
Phosphatidylcholine	0.34	0.30		0.17		
Phosphatidylserine	0.33	0.12				
Cerebrosides	0.94	0.55				
Ceramides	s [*]	s [*]				
Sphingosine	0.28	0.75				
Sphingomyelin	0.28	0.13				
Lyso-Phosphatidylglycerol	0.54	0.20				
Lyso-Phosphatidylethanolamine	0.45	0.20				
Monolysocardiolipin	0.45	0.34				
Lyso-Phosphatidic Acid	0.40	0.01				
Dilysocardiolipin	0.32	0.21				
Lyso-Phosphatidylinositol	0.29	0.03				
Lyso-Phosphatidylcholine	0.22	0.08				
Lyso-Phosphatidylserine	0.18	0.02				

* s^f - solvent front.

SOLVENT SYSTEMS

	Ratio (V:V)
Chloroform : Methanol : Water	65 : 25 : 4
Chloroform : Methanol : Ammonium Hydroxide	65 : 25 : 4
Chloroform : Hexane : Methanol : Acetic Acid	50 : 30 : 10 : 5
Toluene : Pyridine : Water	60 : 60 : 10
Cyclohexane : Ethyl Acetate	3 : 2
Toluene : Chloroform : Methanol	85 : 15 : 5

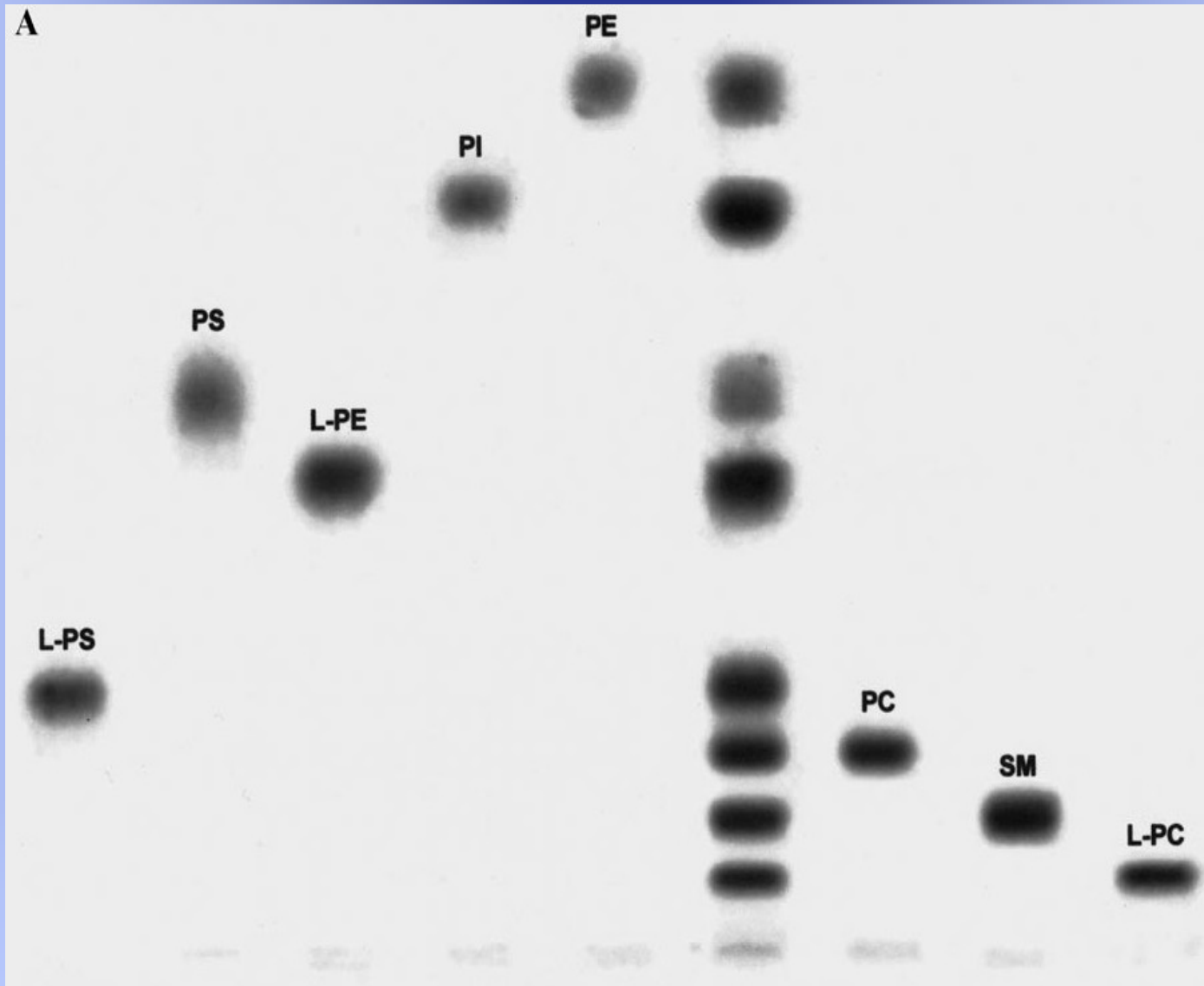
Proč stanovujeme fosfolipidy:

- složení buněčných membrán
- složení fosfolipidů v liposomech (=medicínské nanonosiče léčiv)
- stanovení enzymových aktivit (PI-PLC, PC-PLC, PLD)

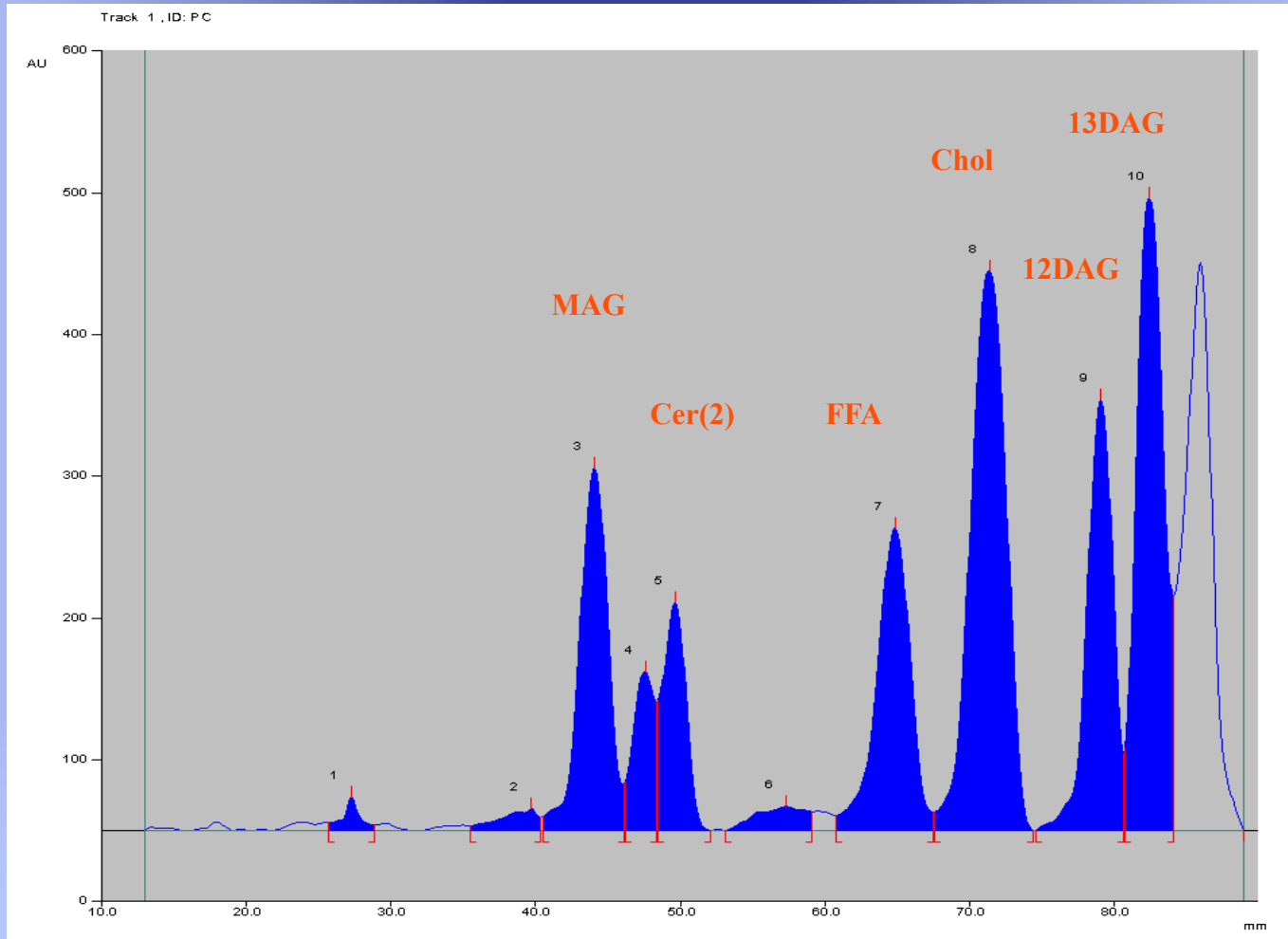
Příklad stanovení PC-PLC:

- buňky značeny [¹⁴C] cholinem a [³H] kyselinou myristovou (inkorporace do PC)
- extrakce lipidů (CHCl₃:MeOH)
- separace TLC
- popř. spray s barvičkou (primulin)
- separace TLC v 2. mobilní fázi

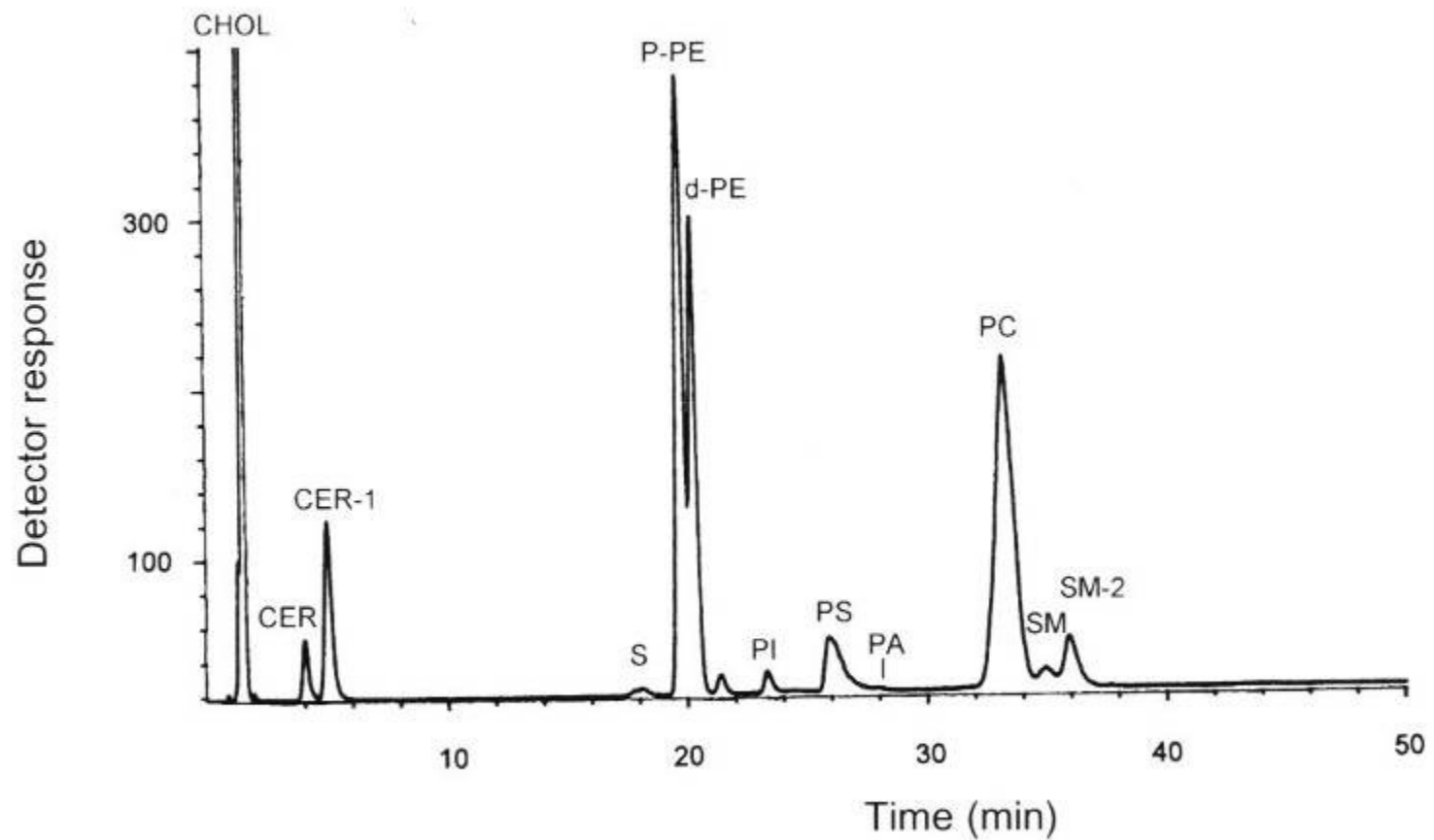
„PHOSPHOLIPIDOMICS“ (HPTLC)



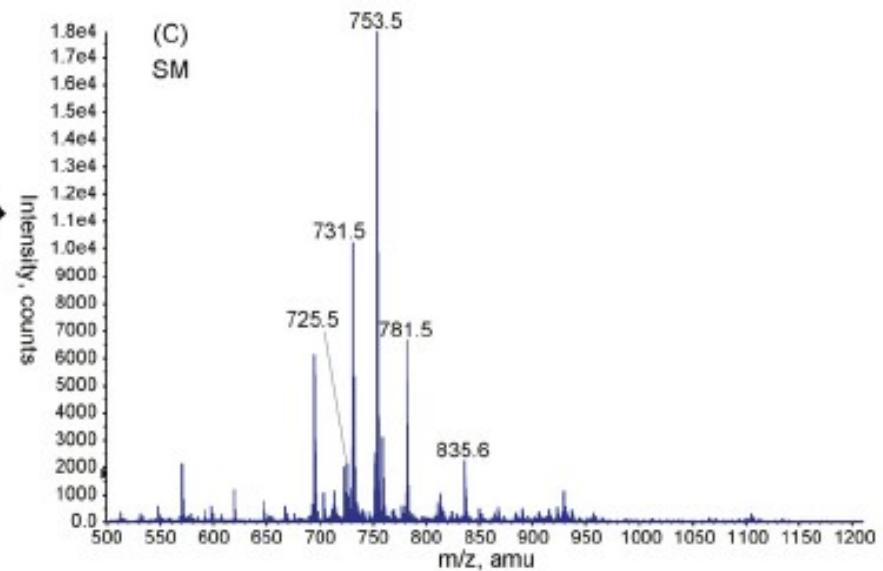
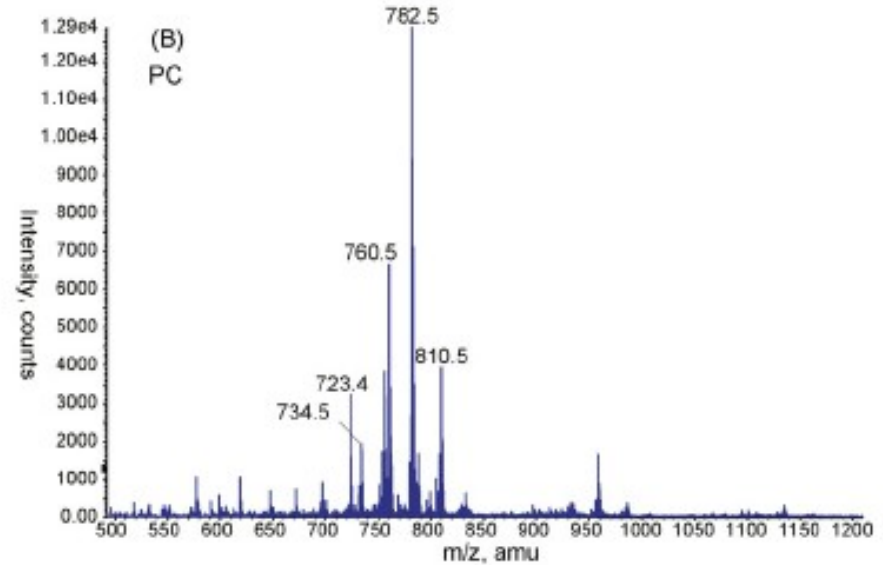
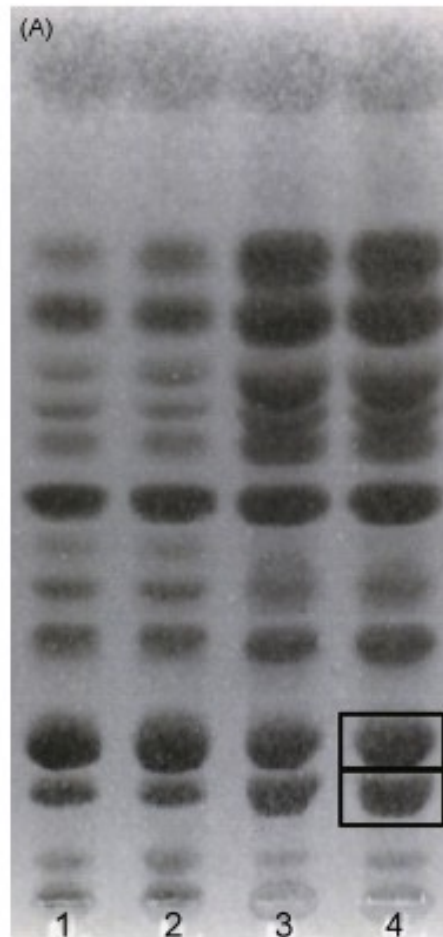
STANOVENÍ CERAMIDŮ A DAG METODOU HPTLC (detekce densitometricky)



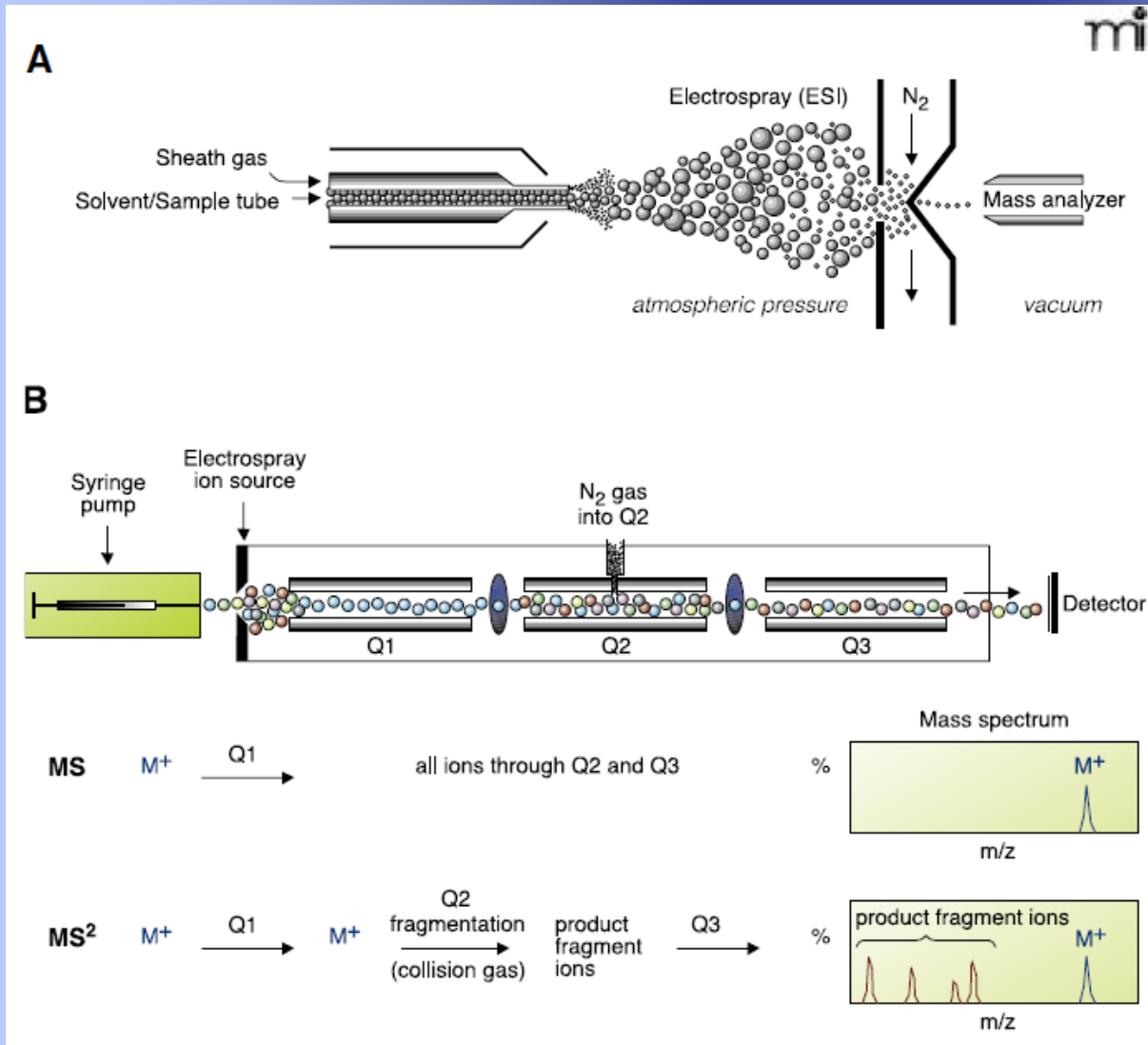
HPLC (SKUPINOVÉ) STANOVENÍ FOSFOLIPIDŮ



„PHOSPHOLIPIDOMICS“ (TLC + LC/MS)



Electrospray ionization and triple quadrupole mass spectrometer (ESI-MS)

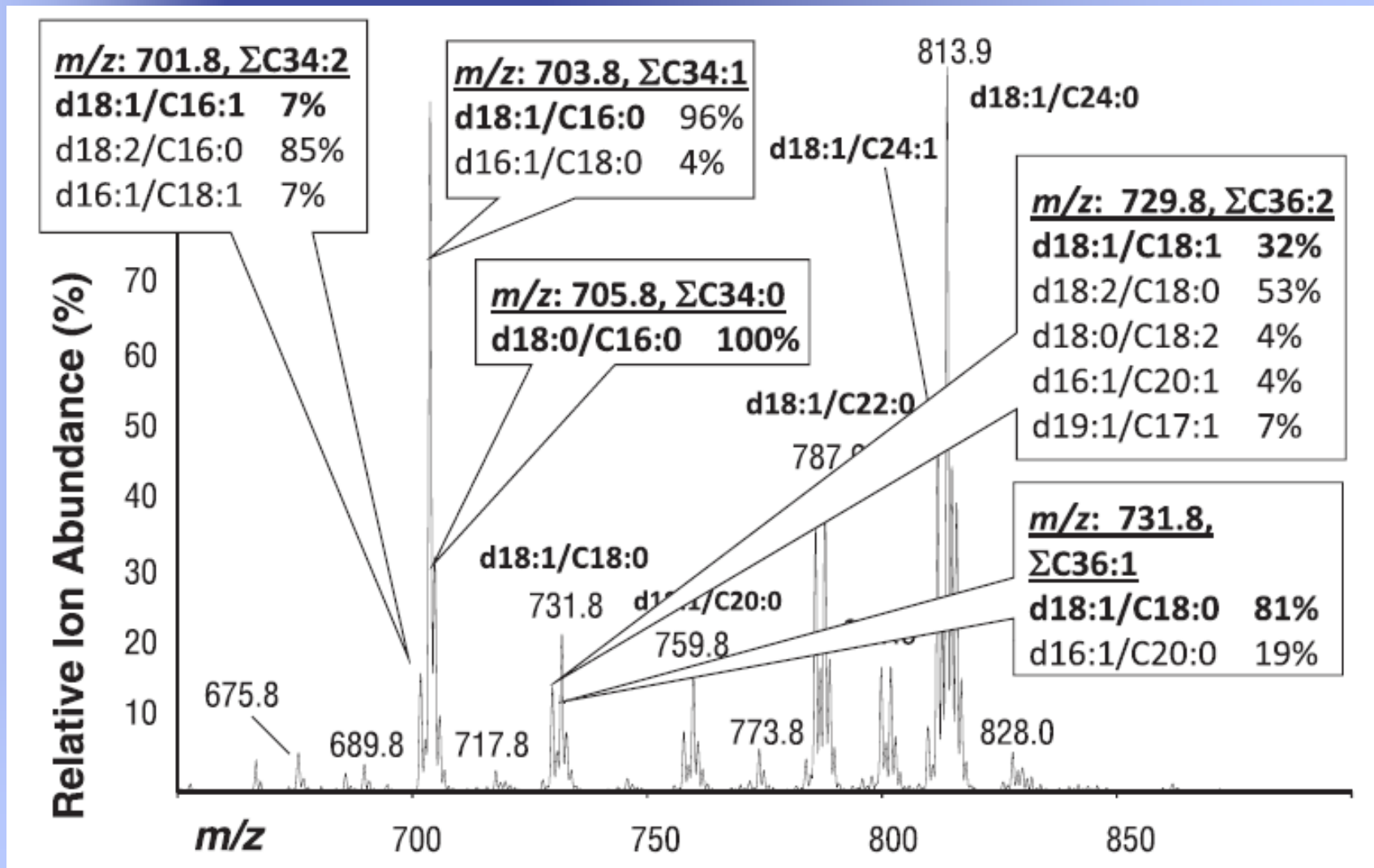


molekulární ionty

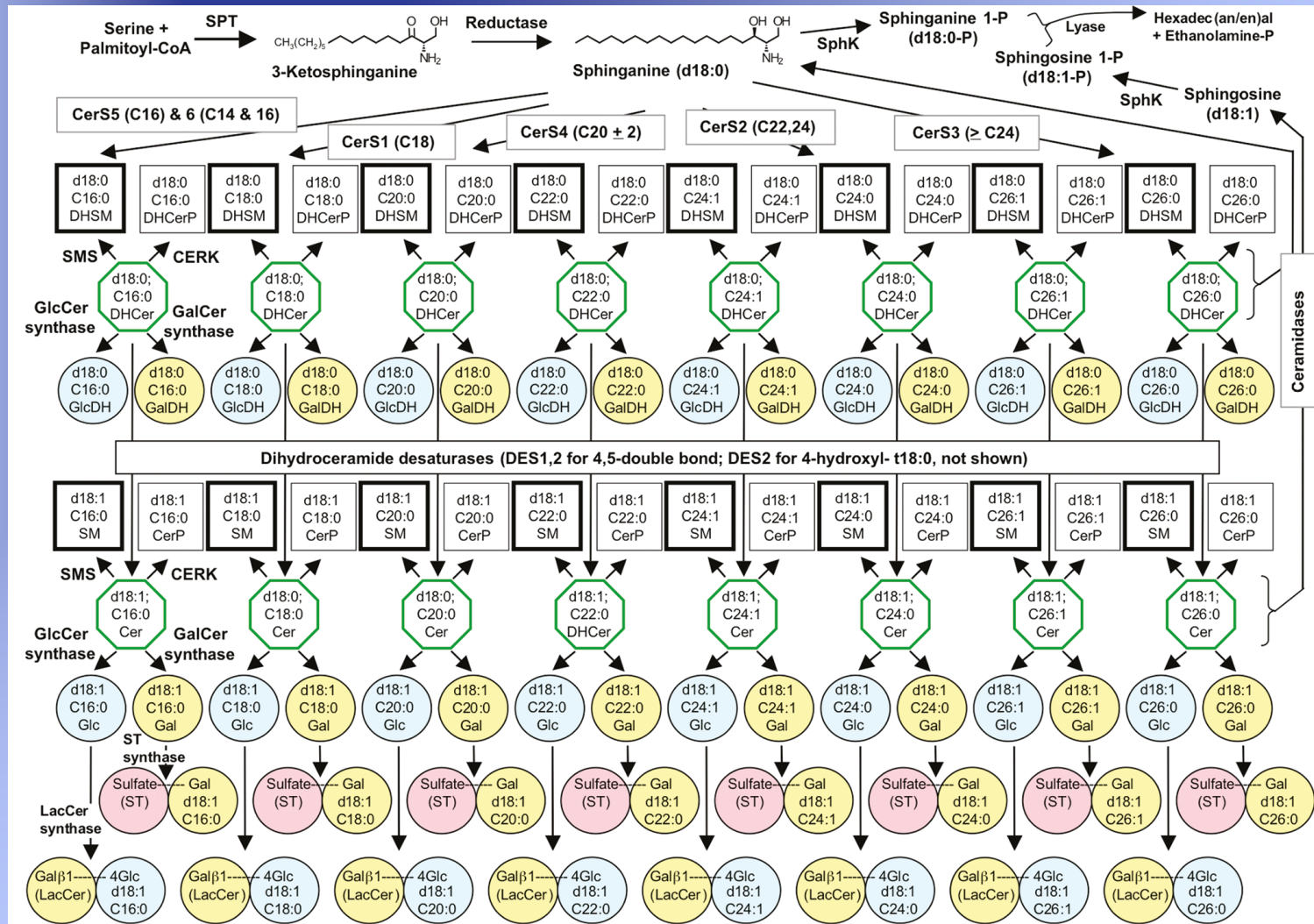
fragmentační spektrum

STANOVENÍ SFINGOLIPIDŮ

Typické spektrum SLs (stejné složení, jiné vlastnosti):



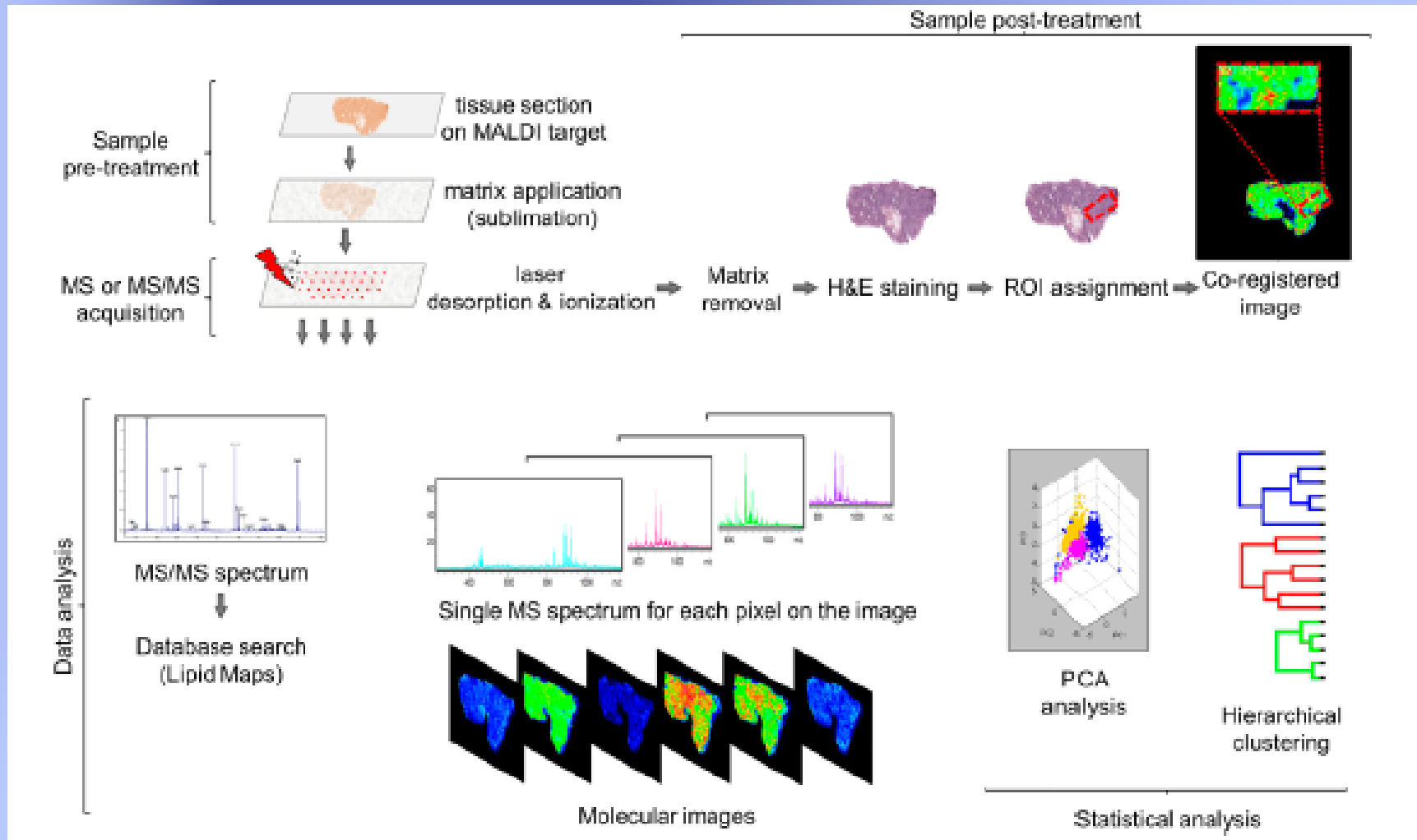
STANOVENÍ SFINGOLIPIDŮ (LC/MS/MS)



Key: Lipid backbone (DH)Cer SM Cer1P GlcCer GalCer LacCer Sulfatide
 Sphingoid base (dx:y)
 Fatty acyl chain (cx:y)

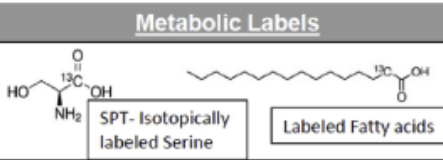
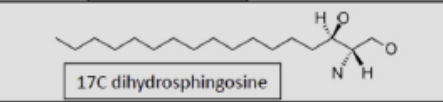
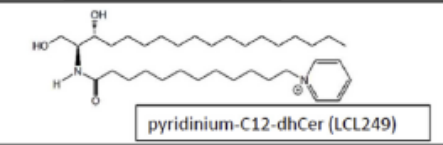
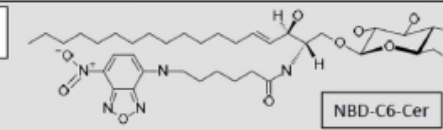
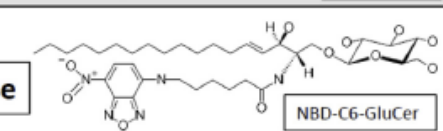
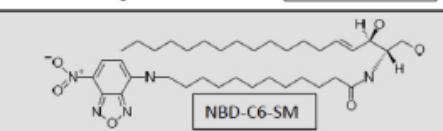
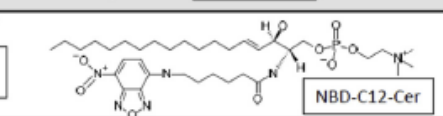
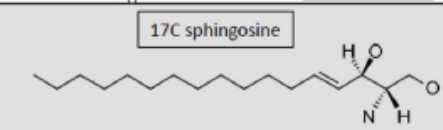
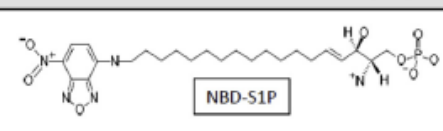
Alternativní stanovení SLs metodou MALDI-IMS

zamražený vzorek tkáně je potažen homogenní vrstvou organického roztoku; laserový paprsek poté scanuje specifickou oblast vzorku a sbírá hmotnostní spektrum pro každou koordinátu:

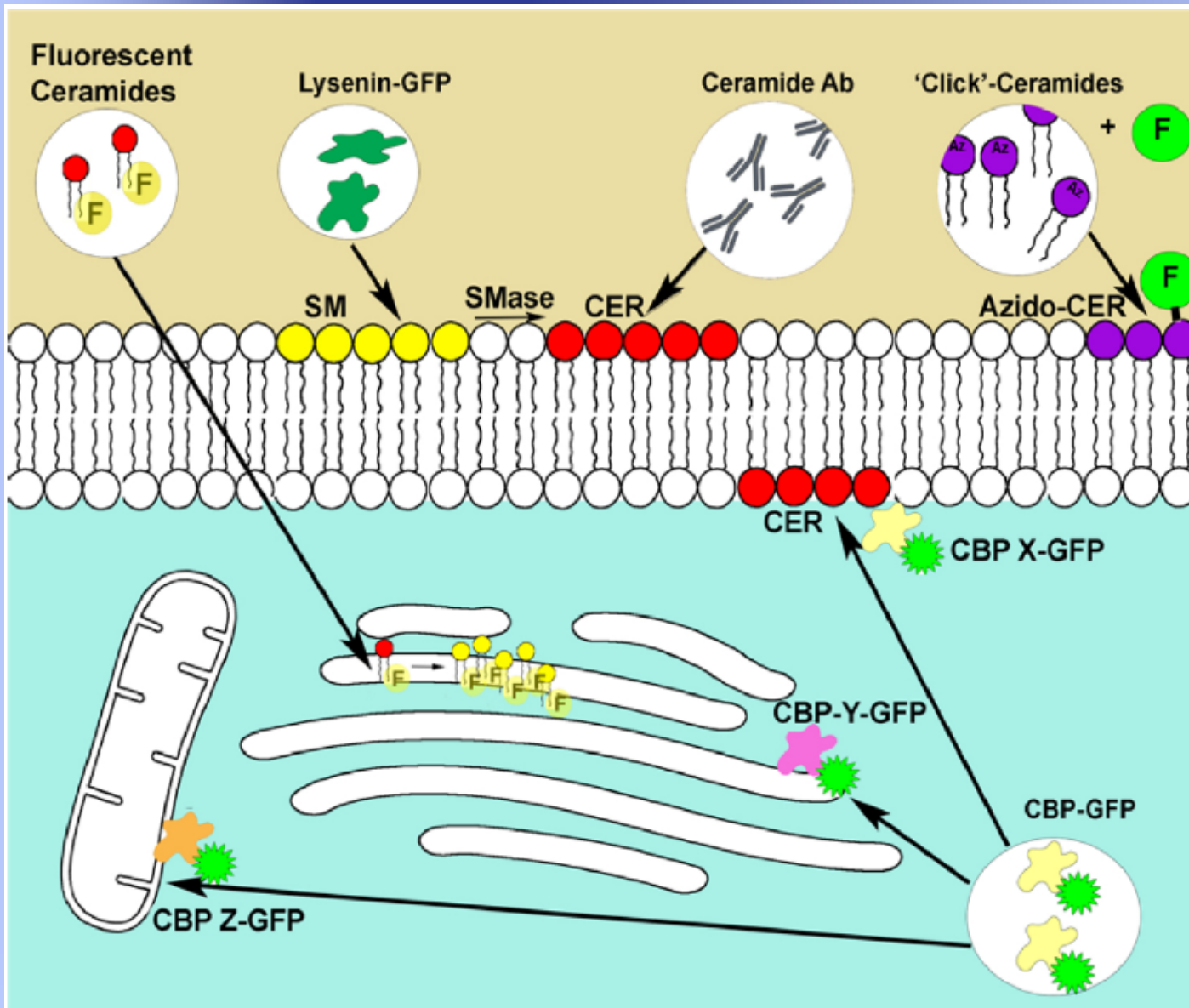


STANOVENÍ SFINGOLIPIDOVÉHO METABOLISMU

- 1) Stanovení hladin SLs v buňkách / tkáni; poté kalkulace poměru SLs (např. Cer/dhCer, Cer/S1P, GM3/GD3 aj.)
- 2) Proby pro studium SL metabolismu (Snider et al., Anal. Biochem, 2019): C13, deuterované nebo fluoresc. značené substráty
- 3) Využití specifických chem. inhibitorů enzymových aktivit nebo silencing genů těchto enzymů

	Metabolic Labels	Concentration	Duration
SPT	 SPT- Isotopically labeled Serine Labeled Fatty acids	Serine- 0.1-2 mM Fatty Acid- 0.1 mM	1-4 h 3 h
CerS	 17C dihydrosphingosine	0.5-2 µM	5min – 4h
DeS	 pyridinium-C12-dhCer (LCL249)	0.5 µM	2-6 h
GCS	 NBD-C6-Cer	GCS 1-50 µM SMS 1-5 µM CERK 10 µM	1-8 h 0.5-3 h 0.5 h
SMS	 NBD-C6-GluCer	5-50 µM	0.5-3 h
Gcase	 NBD-C6-SM	2-4 µM	0.5-1 h
SMase	 NBD-C12-Cer	5-50 µM	0.5-3 h
CDase	 17C sphingosine	0.5-2 µM	15- 30 min
SK	 NBD-S1P	5-20 uM	5-40 min
SPL			

VIZUALIZACE SFINGOLIPIDŮ



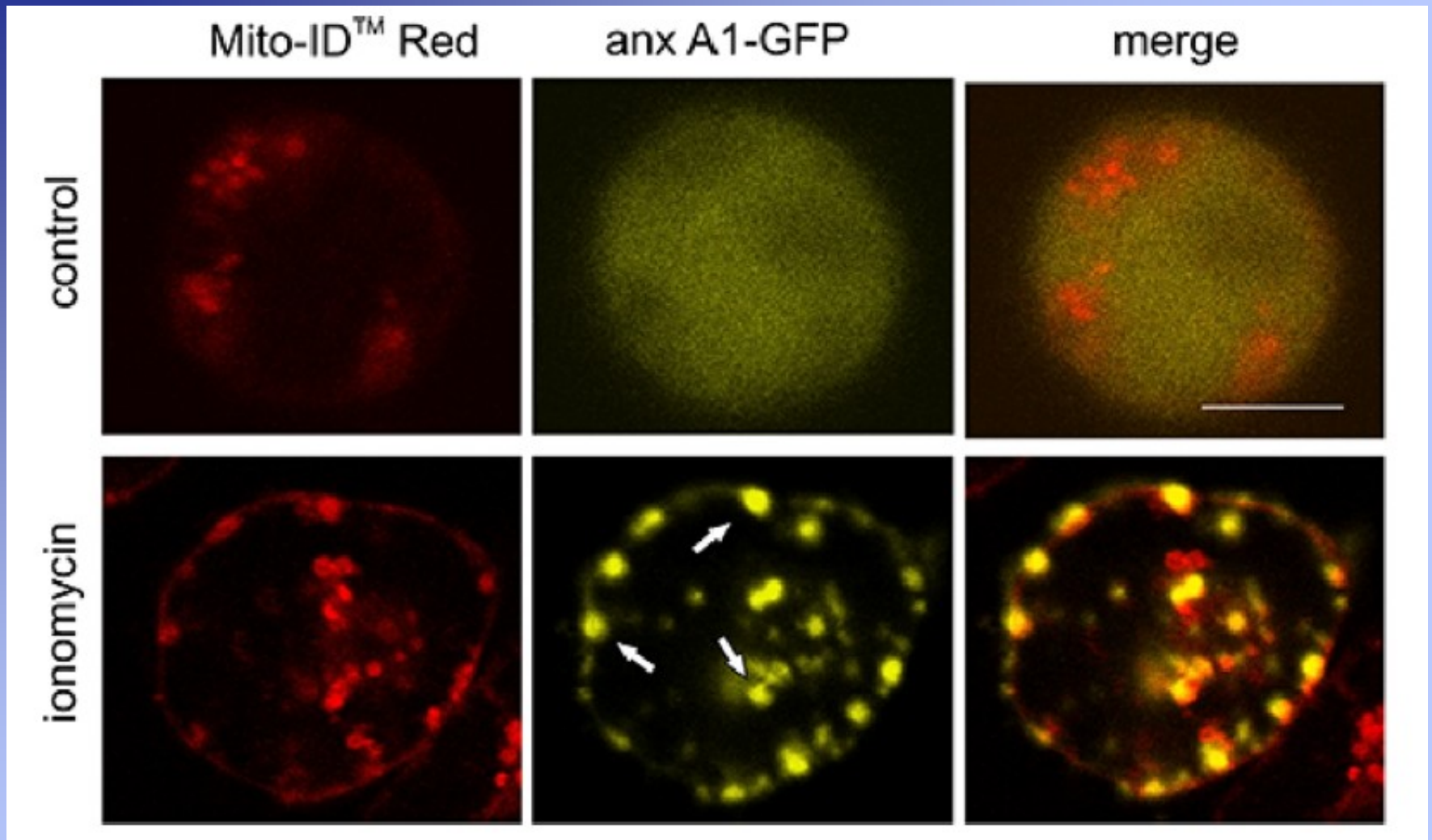
- lipidní fluoresc. analoga SLs

- proteiny vázající se specificky na SL (lysenin-SM, Shiga toxin-Gb3, cholera toxin-GM1)

- protilátky proti SLs (př. Cer Ab)

- fluoresc. sondy vázající se na lipid. rafty (C-laurdan)

Vizualizace ceramidu v mitochondriích



Využití jevu, že annexin A1 se váže specificky na ceramid
(Canals, Chem Phys Lipids, 2019)

MASTNÉ KYSELINY a metody stanovení

NASYCENÉ MASTNÉ KYSELINY („SFA“):

- máselná (4:0)
- laurová (12:0)
- myristová (14:0)
- palmitová (16:0)
- stearová (16:0)

NENASYCENÉ MASTNÉ KYSELINY („MUFA“, „PUFA“):

- olejová (18:1)
- linolová (18:2, příklad n-6 kyseliny)
- linolenová (18:3)
- arachidonová (20:4)
- eikosapentaenová (20:5)
- dokosahexaenová (22:6)

METODY STANOVENÍ:

HPLC / fluorimetrická derivatizace
HPLC / radiometrická nebo
refraktometrická detekce
GC po derivatizaci (methylaci) /MS

HPLC / fluorimetrická detekce
nebo GC/MS

DERIVATIZACE MASTNÝCH KYSELIN

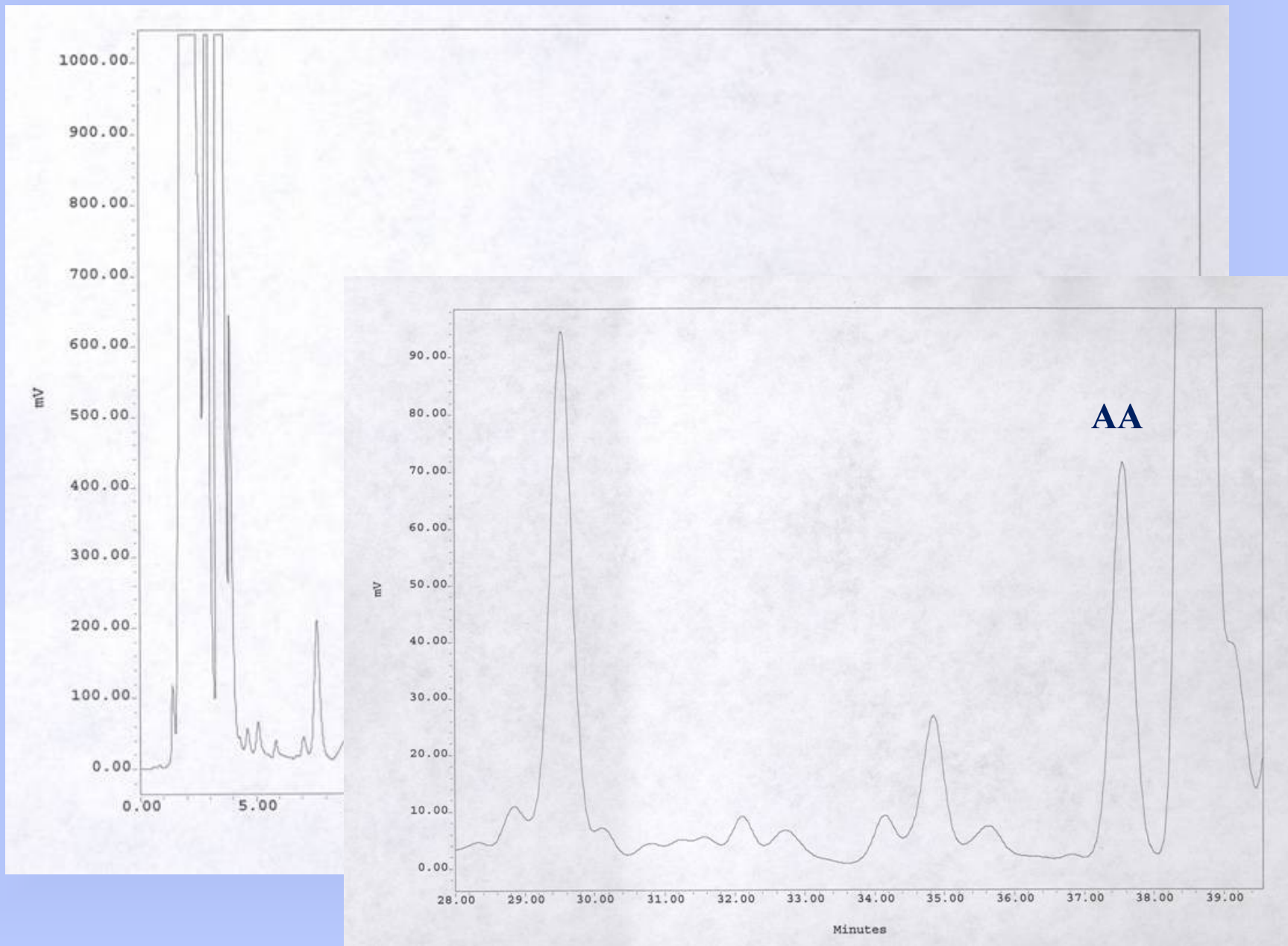
Derivatizace v HPLC se používá z následujících důvodů:

- zvýšení citlivosti nebo **umožnění detekce vůbec**
- zvýšení rozlišení nebo umožnění separace vůbec
- zamezení nežádoucí sorpce látek na koloně.

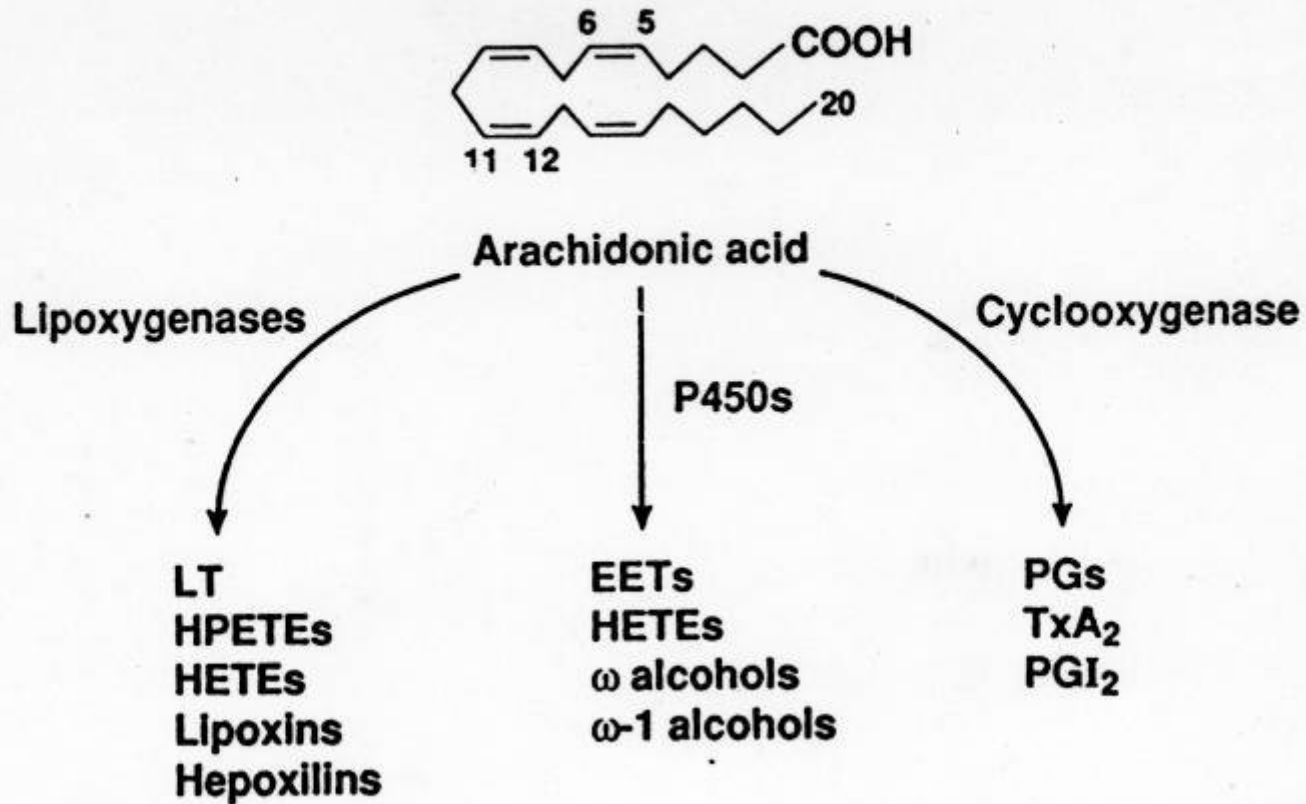
Způsoby derivatizace:

- Předkolonová derivatizace (pre-column chromatography); chemická reakce probíhá před kolonou
- Postkolonová derivatizace (post-column chromatography); chemická reakce probíhá za kolonou
- Derivatizace na koloně; chemická reakce probíhá přímo v koloně

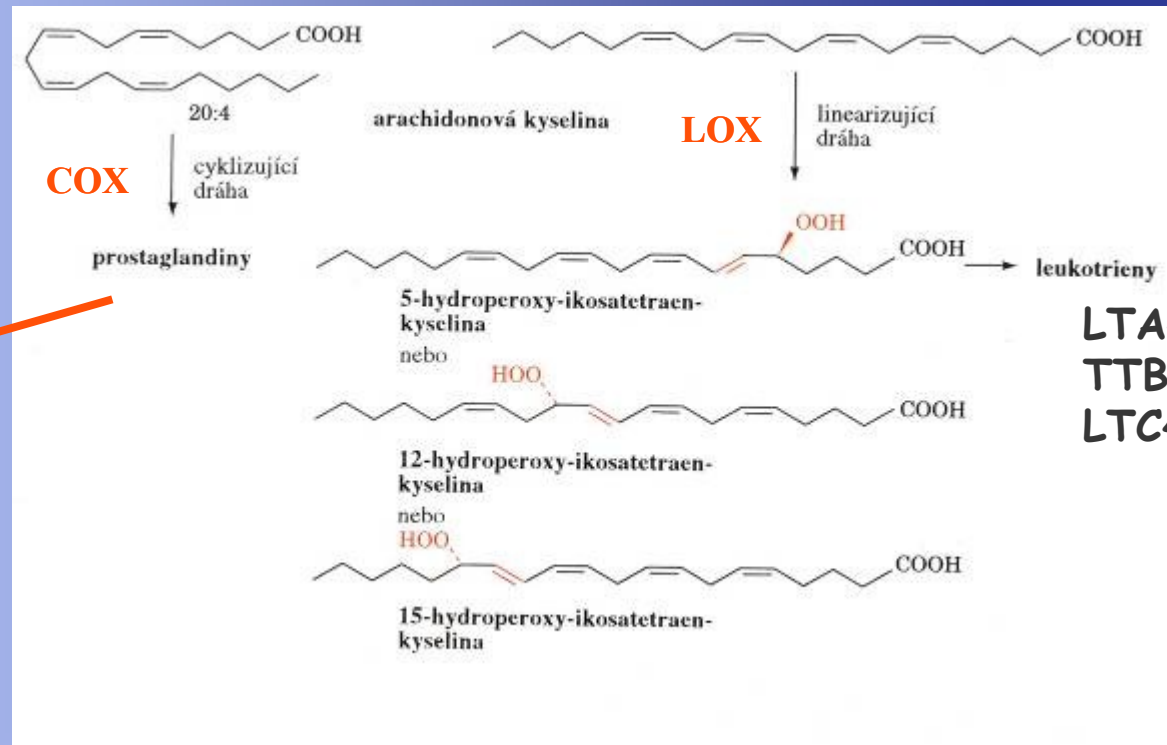
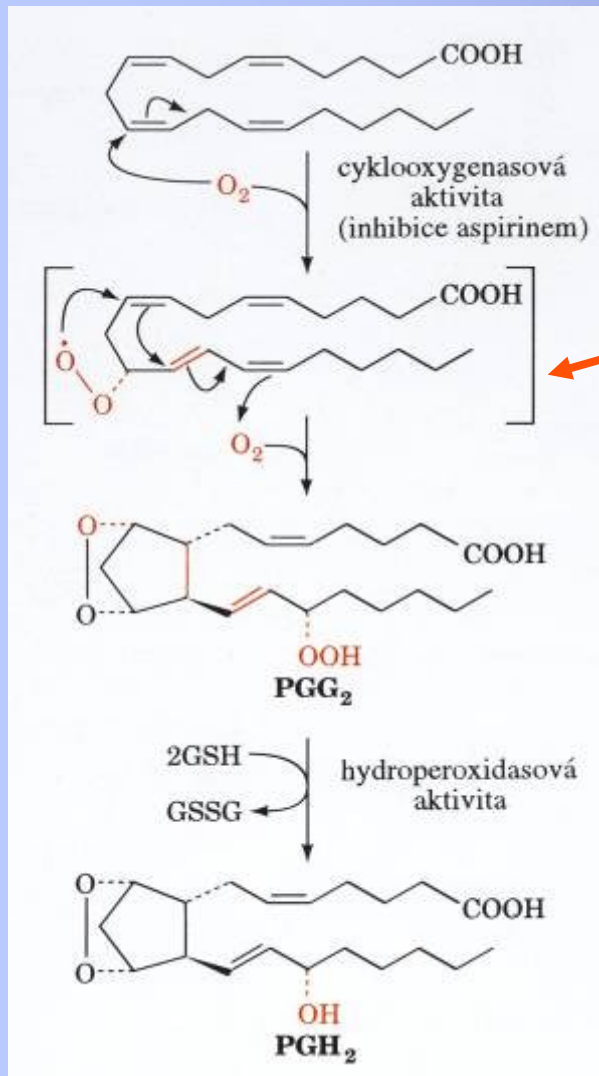
HPLC analýza karboxylových kyselin po derivatizaci pyrenyldiazomethanem



EIKOSANOIDY



KYSELINA ARACHIDONOVÁ = PREKURSOR



Stanovení koncentrací PGs, LTs, HETEs;
stanovení aktivit COX, LOX a CYP:
GC/MS nebo **HPLC/MS** analýza metabolitů AA

PGE₂ PGD₂ TXA₂

→ pro-/protizánětlivé aj. efekty

Fyziologický/patofyziologický/toxikologický význam lipidů

SIGNAL TRANSDUCTION PATHWAYS

CELLULAR EVENTS (SPECIFIC PROTEIN TARGETS)

Membrane Receptors

Ras, Rho, PLA₂, PLC

Arachidonic Acid, Reactive Oxygen Species, Ca²⁺, DNA Damage, Sphingolipids, PGs, LTs

Protein Kinases (ERK1/2, JNK, p38, PKB/Akt, PKC, PKA, c-Src, Ras, Rho), Protein Phosphatases

Transcription Factors, Modulators of Gene Expression (AhR, ERs, CAR, NF- κ B, AP-1, c-Jun, c-Fos, c-Myc, p53)

Mitogenic signal transduction
(e.g. ERK1/2, ERs, c-Myc)

Cell cycle modulation
(p53, p21, pRb, CDKs, GADDs)

Regulation of apoptosis
(TNFR, Fas, p53, Bax, Bcl-2, Casp)

Modulation of intercellular communication
(Cx 43, Cx32)

Cell survival, proliferation, cell death, senescence, cell differentiation, cancer promotion, progression

Jaký je biologický význam změn v lipidomu?

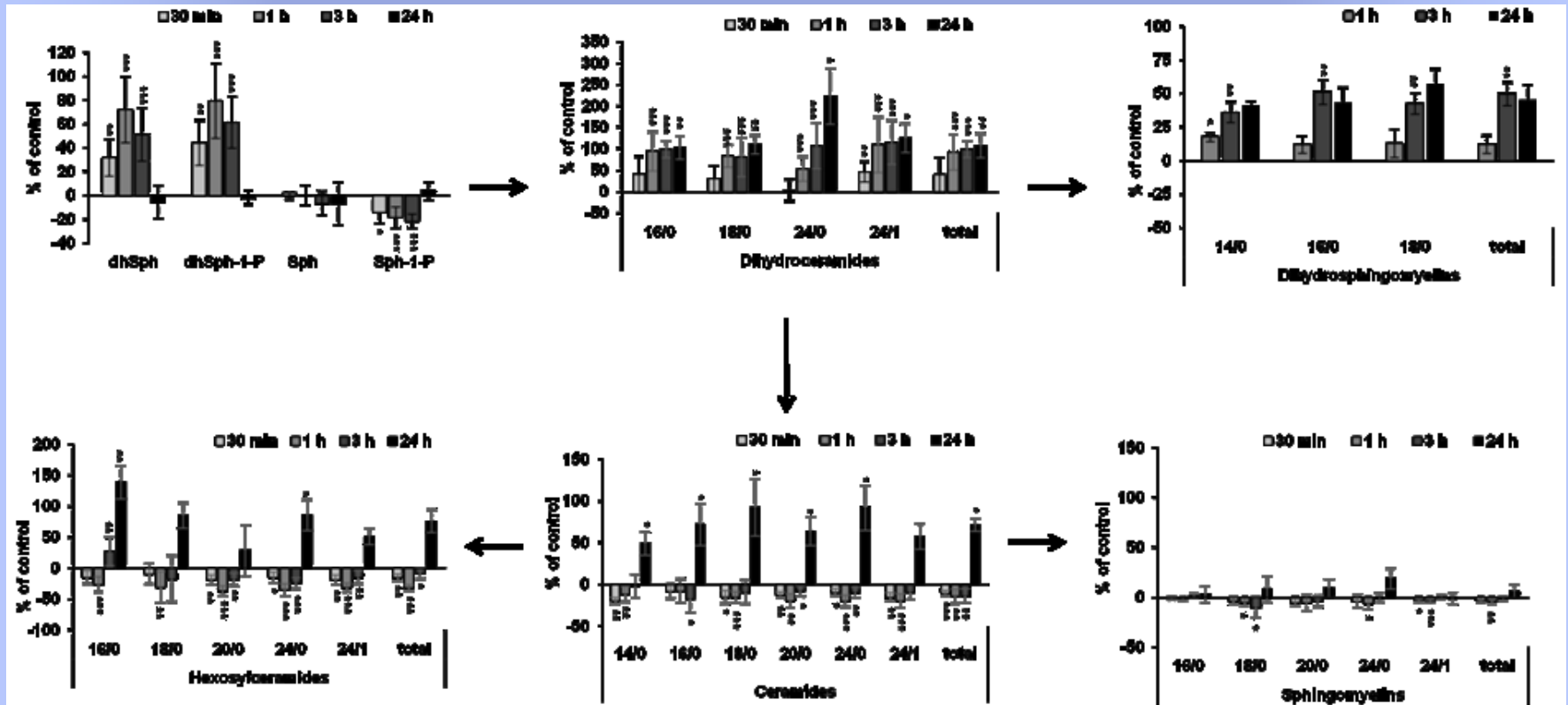
- změny vlastností plasmatických membrán nebo jejich mikrodomén (lipidových raftů);
- změny v mezibuněčné komunikaci a mezibuněčné interakci (mezerové spoje - GJIC, adherentní spoje atd.);
- změny aktivity membránově vázaných proteinů (povrchových receptorů - kináz apod.);
- změny v intracelulární komunikaci (MAPK, PKC aj. přímo ovlivněny lipidními signálními molekulami, např. sfingolipidy)



- embryogeneze, diferenciací tkáně;
- role v karcinogenezi (SLs - přežívání a proliferace buněk, migrace, resistance, změny v energet. metabolismu rakovinných buněk atd.)
- infekce - GSLs = povrchové receptory
- regulace prozánětlivých a protizánětlivých procesů (eikosanoidy)
-

Lipidomické změny po expozici cizorodými látkami

Time course of effects of 25 μ M PCB 153 on SL metabolism in liver WB-344 cells



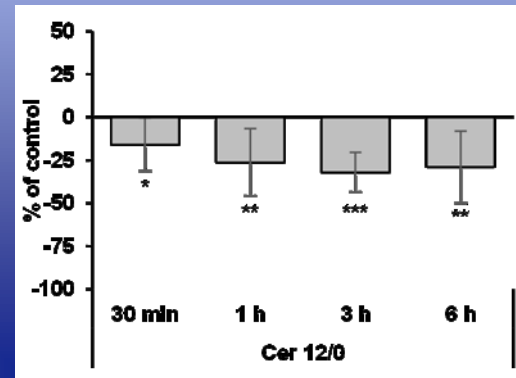
- PCB 153 increased significantly the concentrations of dhSph, dhSph-1-P, dhCer and dhSM during short-term exposure time; dhCer and dhSM were further increased after the 24-h exposure;
- PCB 153 slightly suppressed Cer and HexCer levels during 30-min. to 3-h exposure and it induced Cer, HexCer and LacCer after 24-h exposure.

Měření aktivity enzymů lipidového metabolismu a jejich genové exprese

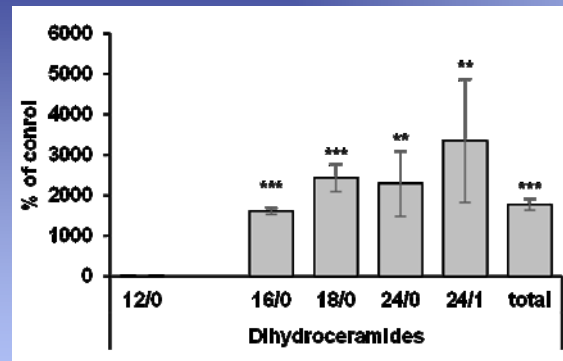
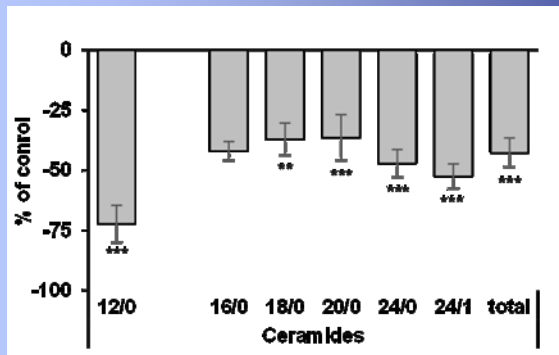
PCB 153 inhibits DES1 activity

(DES1 activity determined as production of Cer 12/0 in the cells pretreated with artificial DES1 substrate dhCer C12/0)

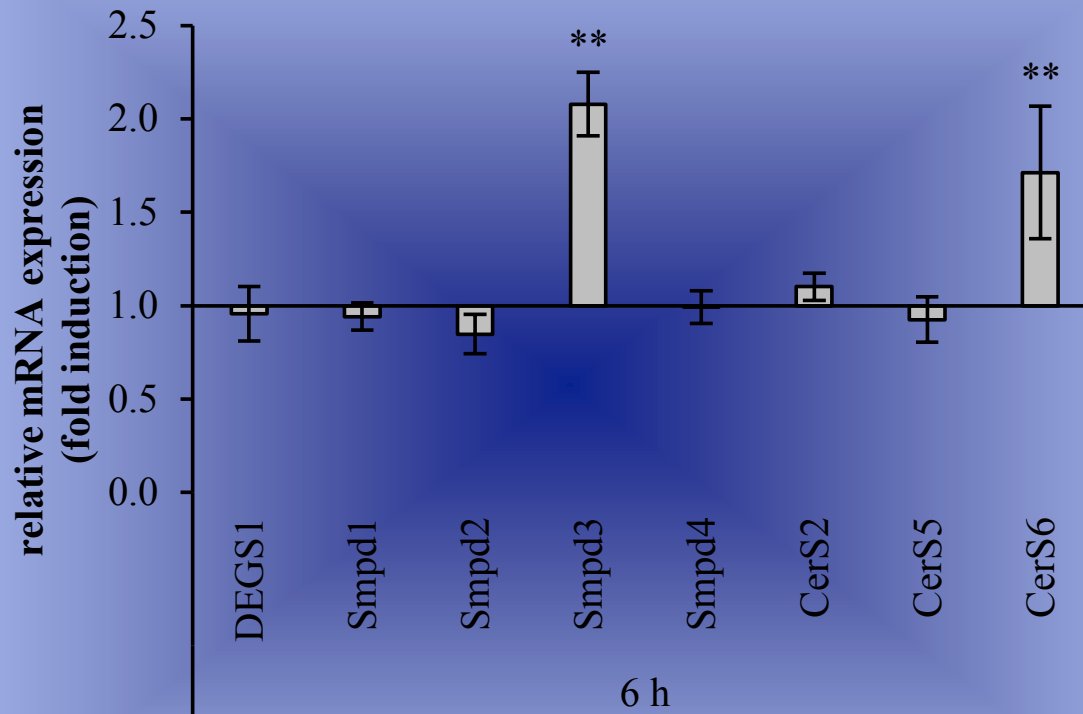
- PCB 153 treatment suppressed the Cer C12/0 production



- fenretinide (chem. inhibitor of DES1) mimicked effects of PCB 153

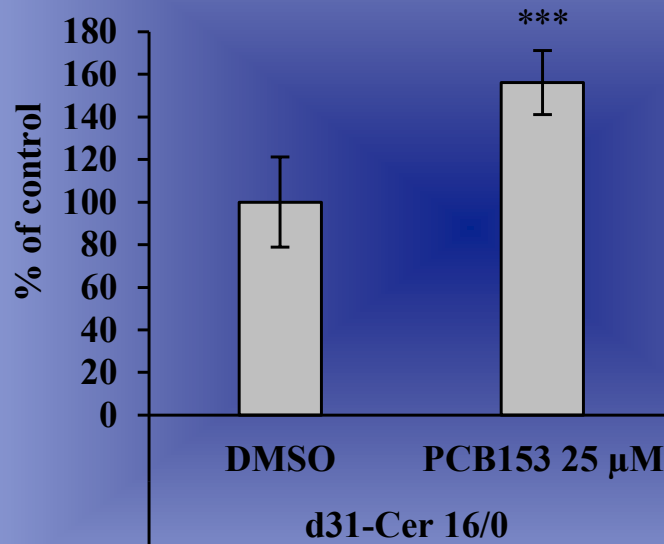


Effects of PCB 153 on expression of major enzymes involved in Cer production



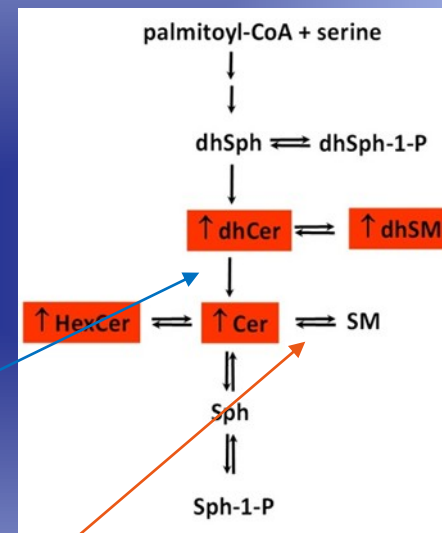
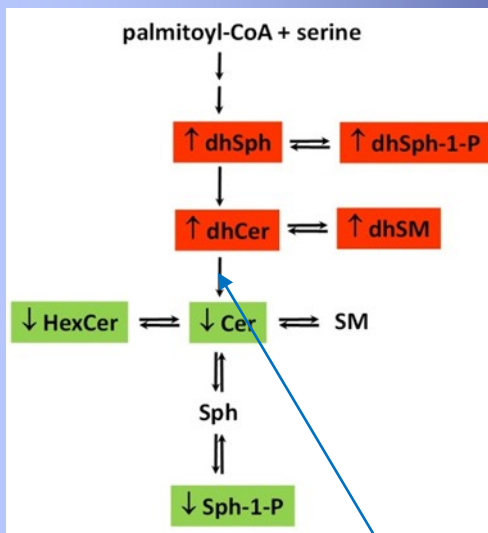
- RT-PCR analysis: the Cer increase might be linked to changes in gene expression of major enzymes responsible for Cer production after 6-h exposure to PCB 153

Modulation of sphingomyelinase activity by PCB 153 (the cells were pretreated with deuterated SM 16/0 for 6 h and then exposed to PCB 153 for 24 h)



- our data suggests a possible role of SMase activity in PCB-induced increase of Cer concentrations

Summary of changes in SL biosynthetic and remodeling pathways in WB-F344 cells following short-term and 24-h exposure to PCB 153

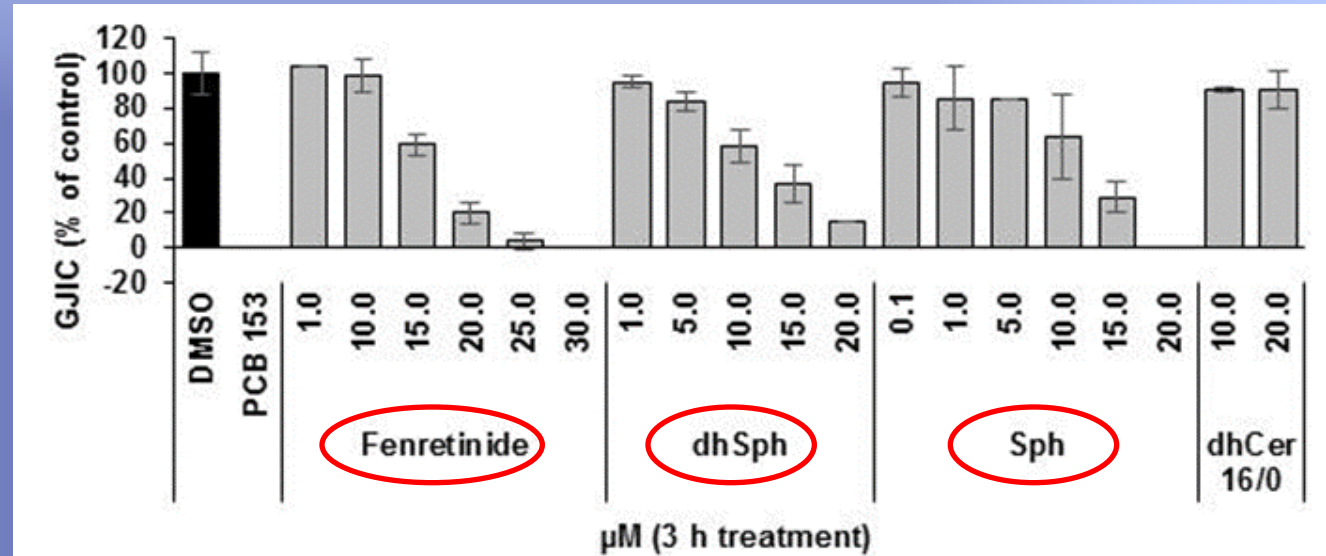
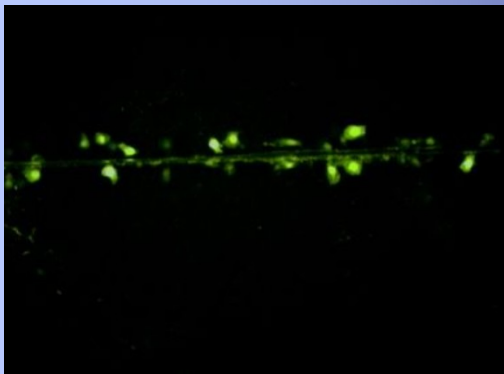
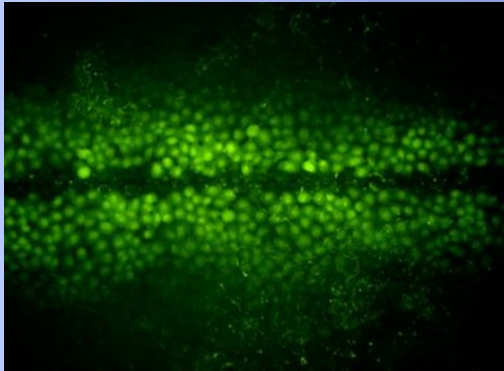


- inhibition of DES1 activity;

- induction of SMase and/or also CerS6 activity after 24-h exposure

(red, increased concentrations; green decreased concentrations)

Acute inhibition of gap junction intercellular communication (rat liver progenitor WB-F344 cells)



Inhibition of GJIC by fenretinide (DEGS1 inhibitor) or external exposure to dhSph (sphinganine), Sph (sphingosine) and dhCer (dihydroceramide): external addition of dhSph, but not of dhCer, inhibited GJIC (suggesting dhSph as a possible candidate effector in WB-F344 cells).

Změny lipidomu v karcinogenezi

Modulations of phospholipid and sphingolipid metabolism in colon cancer epithelial cells?

Metabolic reprogramming, including dysregulation of lipid metabolism is a hallmark of cancer.

(Nomura D., Cravatt B., *Biochim. Biophys. Acta*, 2013)

Many of cell biological processes are critically modulated by bioactive sphingolipids and other lipid signaling molecules, including growth regulation, cell migration, adhesion, apoptosis, senescence and inflammatory responses.

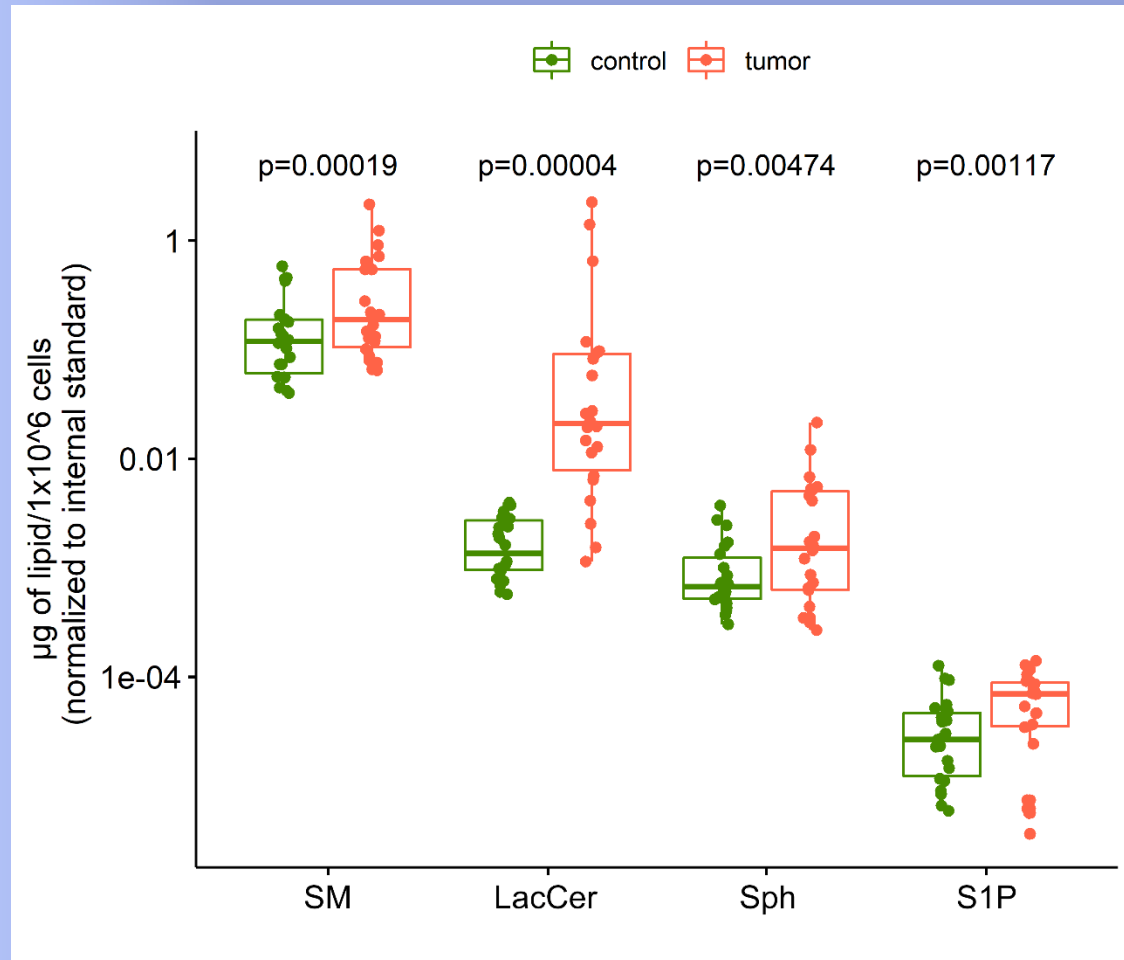
(Hannun Y.A., Obeid L.M., *Nature Reviews Mol. Cell Biol.* 19, 175-191, 2018)

Introduction: the main goals of these studies

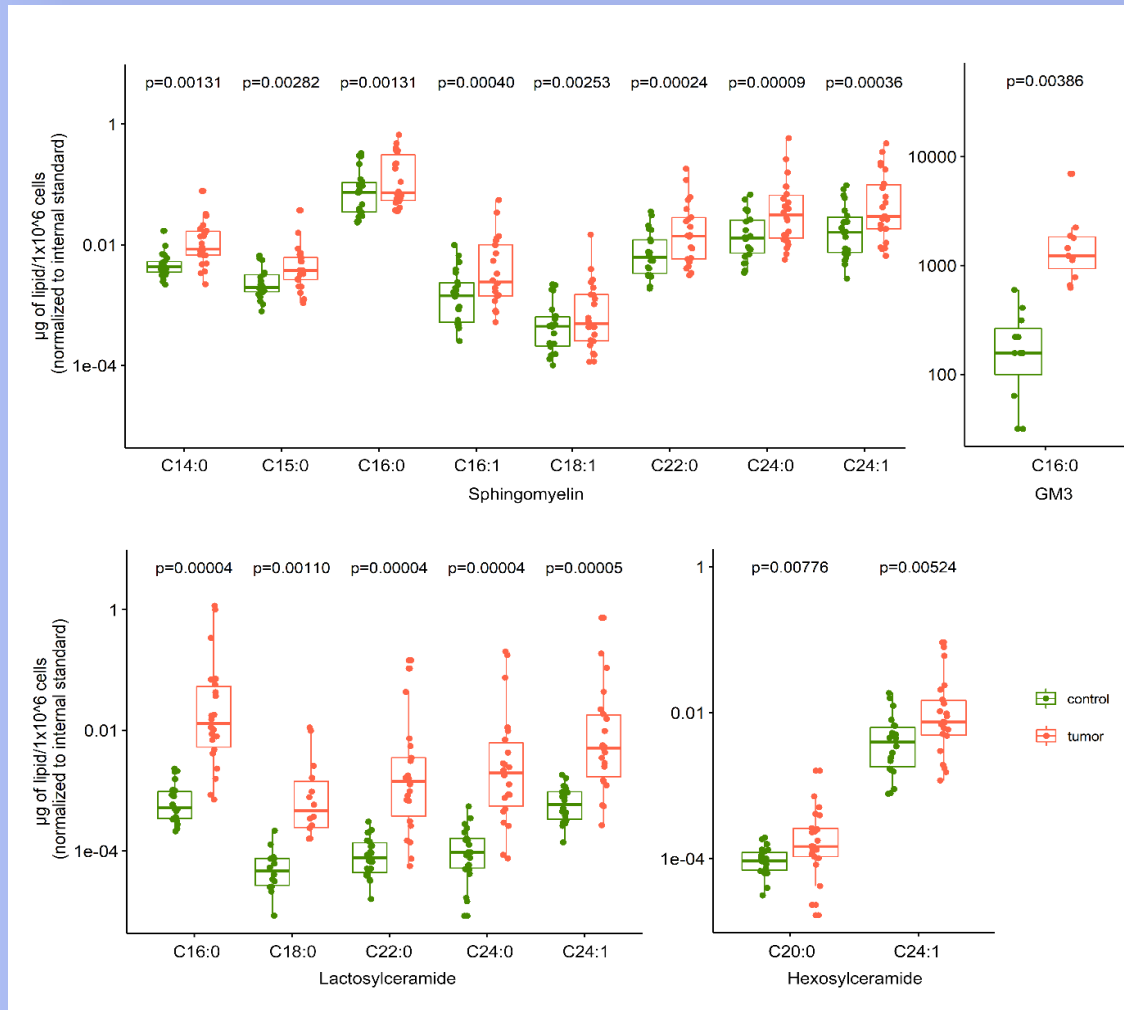
- to identify deregulation in metabolism of phospholipids, sphingolipids in subpopulation of colon cancer epithelial cells, using LC-tandem MS;
-
- to find a link between deregulation of lipid metabolism genes and altered ratio in tumor and adjacent nontumor cells;
- to compare lipidomics data in plasma samples of patients and „healthy“ people
- to identify potential markers of colon adenocarcinoma cells

Lipidomické změny v rakovinné vs. normální tkáni

Levels of SLs in the EpCAM+ epithelial cells isolated from colorectal adenocarcinoma and adjacent non-tumor colon tissue



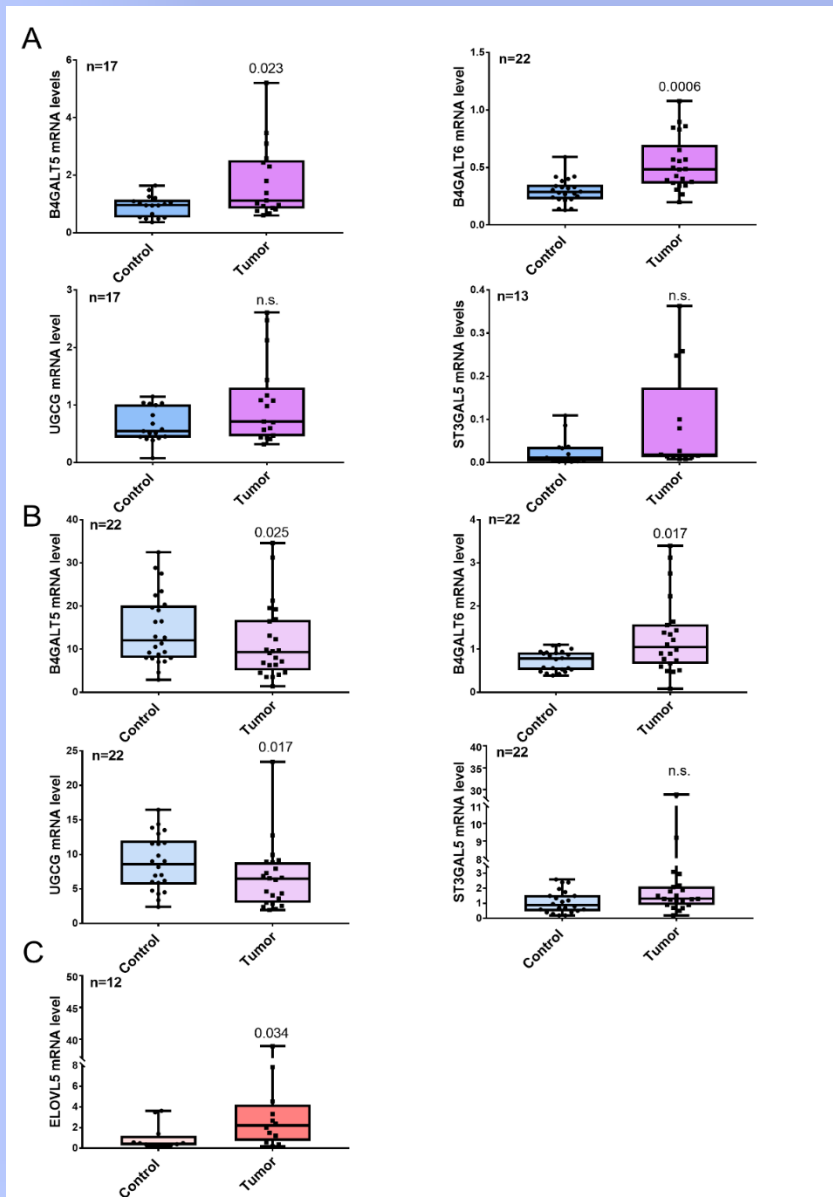
Increased levels of SLs (SM and LacCer) contain both long-chain and very long-chain fatty acids (T vs. N cells)



The data suggested a significant possible role of elongase ELOVL5. GSLs might be also deregulated in CRC (see significant increase in GM3).

Změny exprese genů
zapojených do metabolismu lipidů

Expression qRT-PCR analysis of genes involved in LacCer metabolism and in LCFA-/VLCFA-sphingolipid subspecies accumulation (T/N)



A: B4GALT5 and B4GALT6 increase preferentially in EpCAM+ cells isolated from CRC

B: B4GALT6 but not B4GALT5 is upregulated in total colon tissue samples

C: ELOVL5 mRNA is upregulated in total colon tissue samples

Deregulated expression of lipid metabolism genes in CRC (total tissues, T/N ratio, Custom PCR Array)

GENE	DESCRIPTION	MEAN	SD	PAIRED t-TEST
NEU3	sialidase 3 (membrane sialidase)	7.08	8.35	0.0039
ST3GAL2	ST3 beta-galactoside alpha-2,3-sialyltransferase 2	5.02	4.19	0.0020
S1PR2	sphingosine-1-phosphate receptor 2	4.91	4.91	0.0488
PTGES	prostaglandin E synthase	4.46	5.28	0.0250
PLA2G4B	phospholipase A2, group IVB (cytosolic)	4.39	3.38	0.0121
SPHK1	sphingosine kinase 1	4.38	3.88	0.0098
SMPD4	sphingomyelin phosphodiesterase 4, neutral membrane (neutral sphingomyelinase-3)	3.22	2.18	0.0059
NSMAF	neutral sphingomyelinase (N-SMase) activation associated factor	3.10	2.60	0.0163
GLA	galactosidase, alpha	3.08	3.12	0.0098
AHR	aryl hydrocarbon receptor	2.64	1.52	0.0097
CSNK1G2	casein kinase 1, gamma 2	2.31	1.76	0.0329
SGMS1	sphingomyelin synthase 1	1.92	1.21	0.0431
PTGES3	prostaglandin E synthase 3 (cytosolic)	1.86	1.21	0.0246
ST6GALNAC6	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N- acetylgalactosaminide alpha-2,6-sialyltransferase 6	0.80	1.55	0.0371
PTGER4	prostaglandin E receptor 4 (subtype EP4)	0.64	0.93	0.0488
ST6GALNAC3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N- acetylgalactosaminide alpha-2,6-sialyltransferase 3	0.60	0.51	0.0195
SMPDL3B	sphingomyelin phosphodiesterase, acid-like 3B	0.60	0.53	0.0469
PLPP3	phospholipid Phosphatase 3	0.53	0.32	0.0047
GBA2	glucosidase, beta (bile acid) 2	0.53	0.32	0.0025
LARGE	like-glycosyltransferase	0.50	0.19	0.0002
FA2H	fatty acid 2-hydroxylase	0.47	0.50	0.0072
SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	0.44	0.36	0.0022
SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal	0.34	0.39	0.0039
CYP4F12	cytochrome P450, family 4, subfamily F, polypeptide 12	0.32	0.32	0.0039
SPTSSA	serine palmitoyltransferase, small subunit A	0.31	0.30	0.0104
PLA2G10	phospholipase A2, group X	0.22	0.21	0.0020

SPHK1, S1PR2, PLPP3

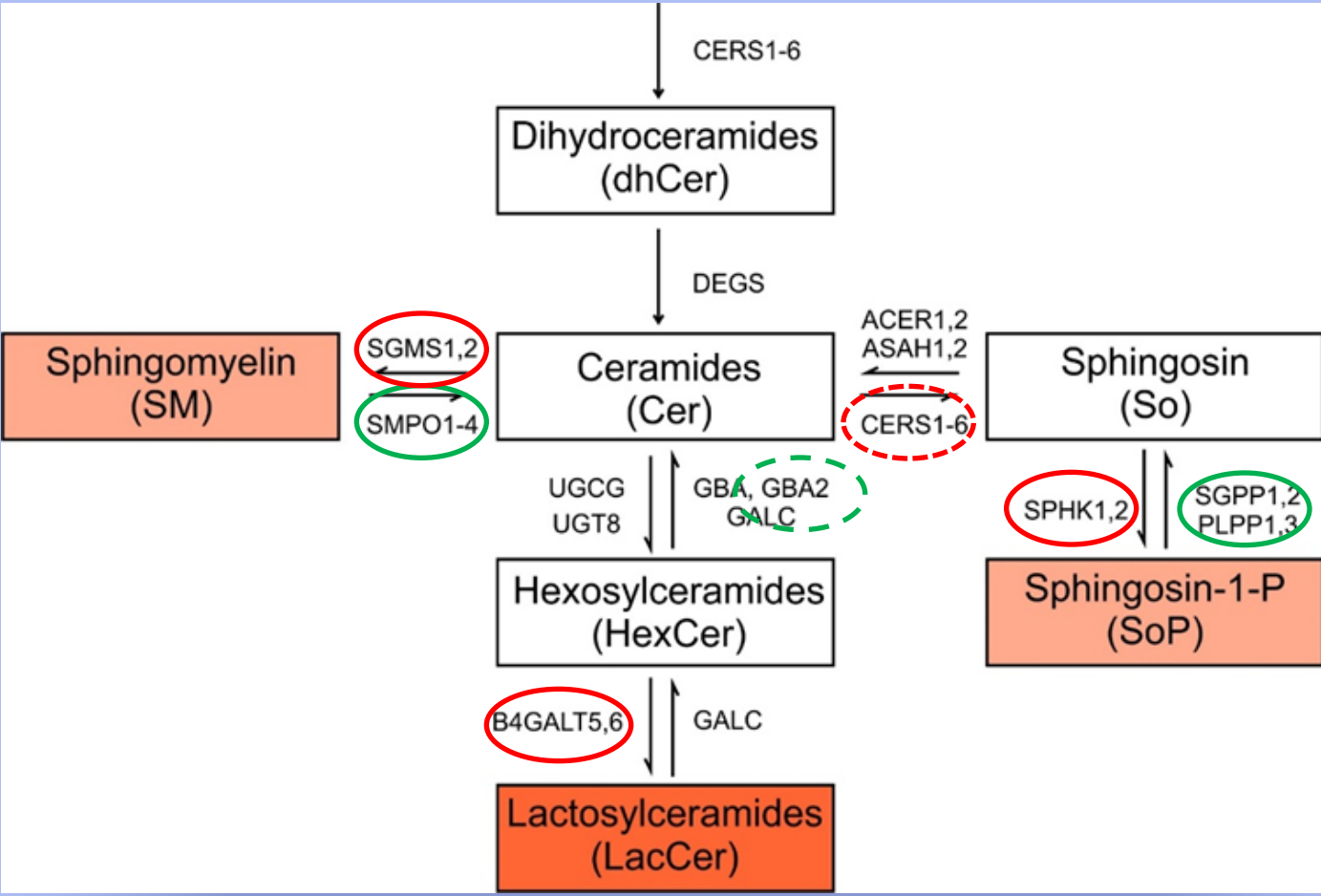
**SGMS1, SMPD4,
SMPD1, SMPDL3B,
SMPD3**

NEU3, GLA, GBA2

**ST3GAL2,
ST6GALNAC3,
ST6GALNAC6**

**PTGES, PTGES3,
PLA2G4B, PLA2G10**

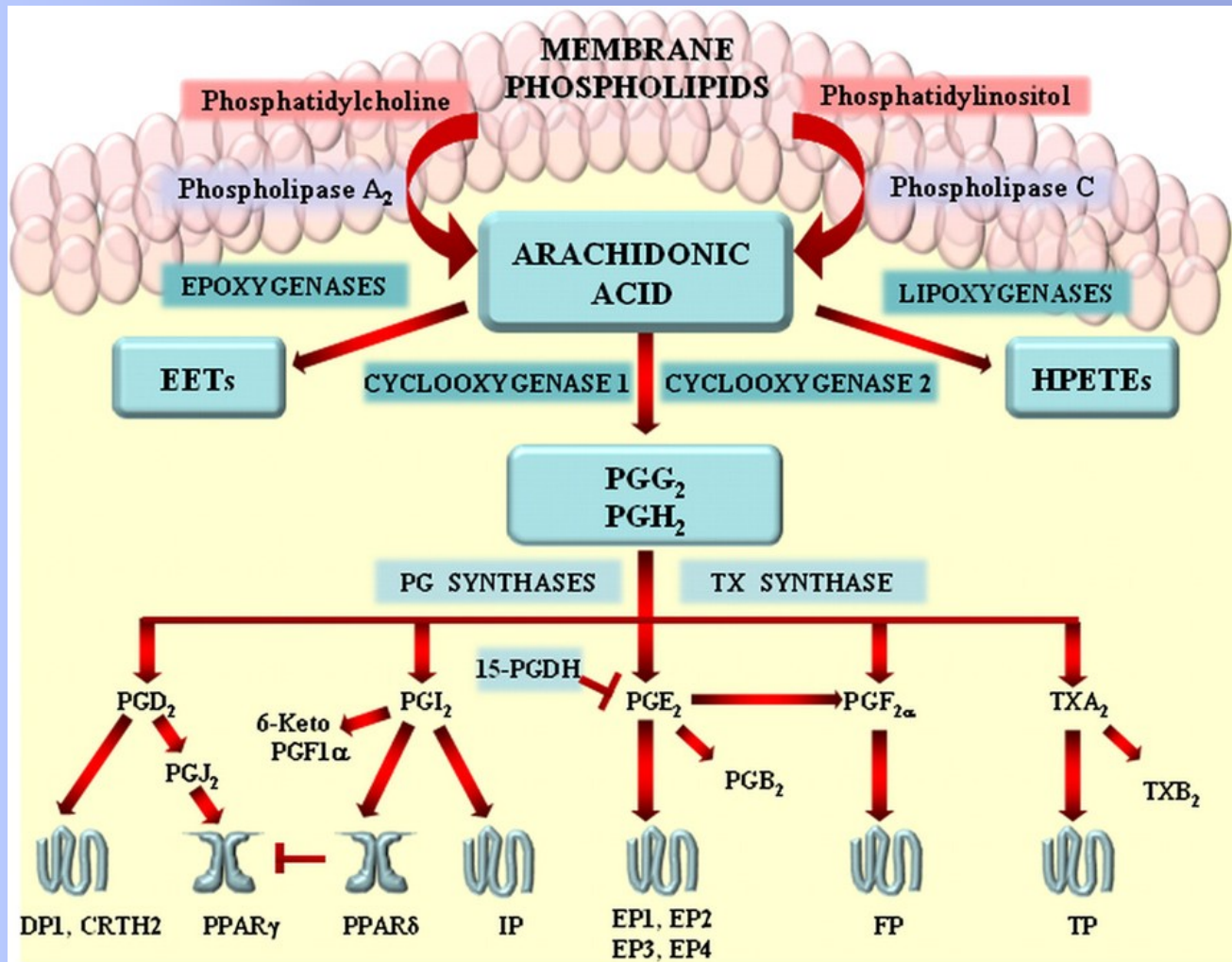
sphingolipid metabolism altered in colorectal adenocarcinoma



Other glycosphingolipid-metabolizing enzymes (NEU3, GLA)

Změny lipidomu v plasmě
(potenciální biomarkery karcinogeneze?)

Concentration of prostaglandins in colon adenocarcinoma patients vs. "healthy population"



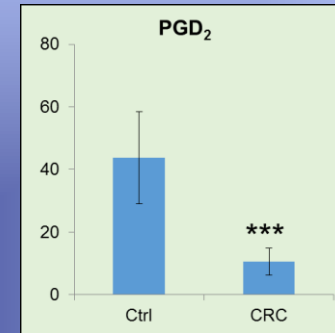
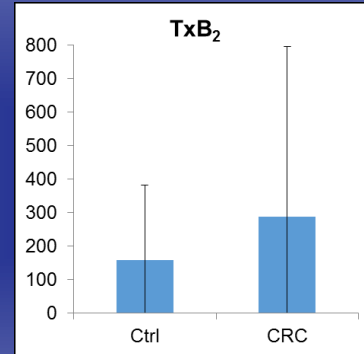
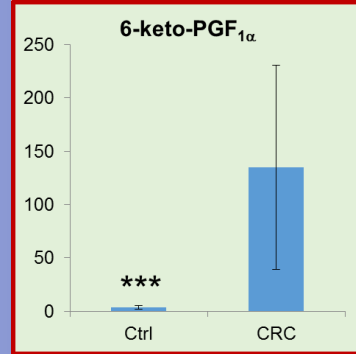
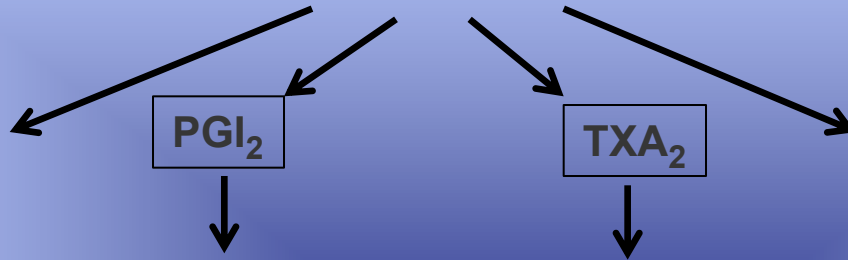
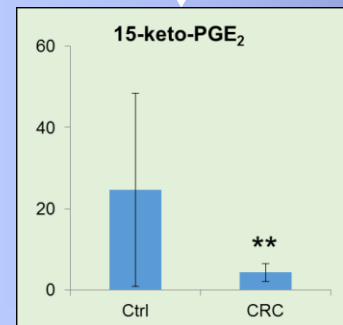
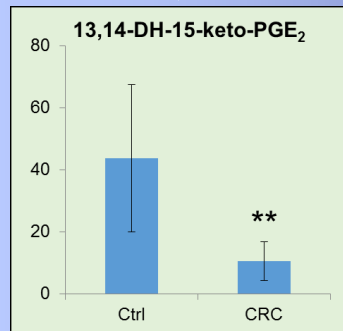
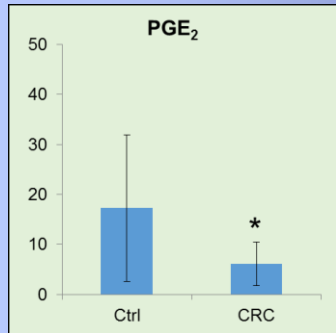
PGE₂:
pro-inflammatory and cancer-promoting effects;

PGD₂: suppressor of cell invasion and generally was reported as anti-tumorigenic

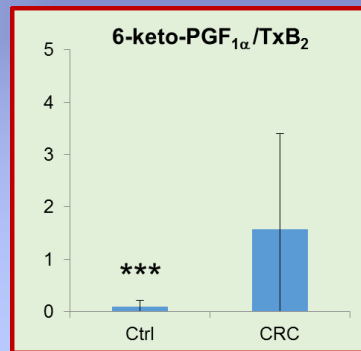
PGI₂: anti-proliferative, anti-thrombotic, vasodilator,....

TXA₂: platelet activation and aggregation

Concentration of eicosanoids in colon adenocarcinoma patients vs. "healthy population"



Diagnostic ratio:



Markers of oxidative damage

