

CONTINUING EDUCATION IN TOXICOLOGIC PATHOLOGY RESPIRATORY AND CARDIOVASCULAR SYSTEM

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PHOSPHOLIPIDOSIS - TOXICOLOGIC PATHOLOGY PERSPECTIVE

**Forth Conference of Society for Toxicologic
Pathology in India**

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- Regulatory perspective
- Risk management strategy

○ Summary and conclusion

PL – Phospholipid ; PLD - Phospholipidosis



INTRODUCTION - PHOSPHOLIPID

Phospholipid (PL): A lipid that contains one or more phosphate groups.

PL are;

- i. Amphipathic in nature, i. e. each molecule consists of a hydrophilic and a hydrophobic portion.
- ii. Essential and dynamic components of plasma and intracellular membranes

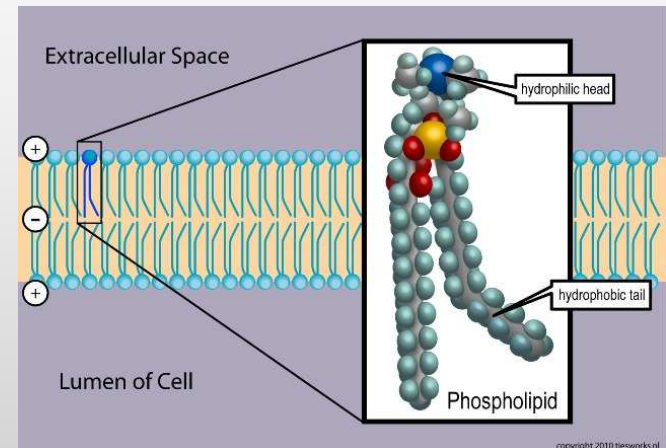
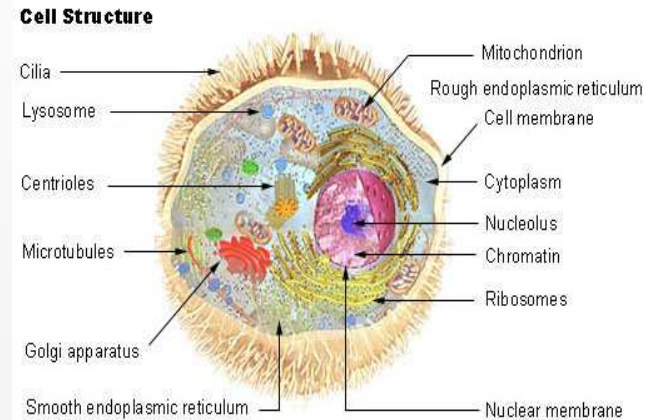
Functions of PL

Membrane PL

1. A reservoir for intracellular messengers.
2. Serve as anchors to cell membranes for some proteins.

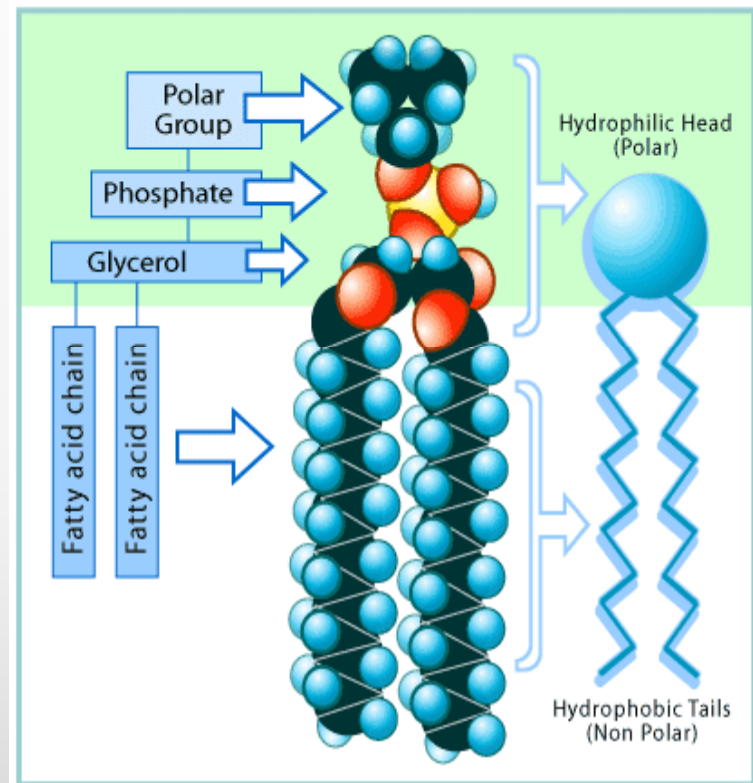
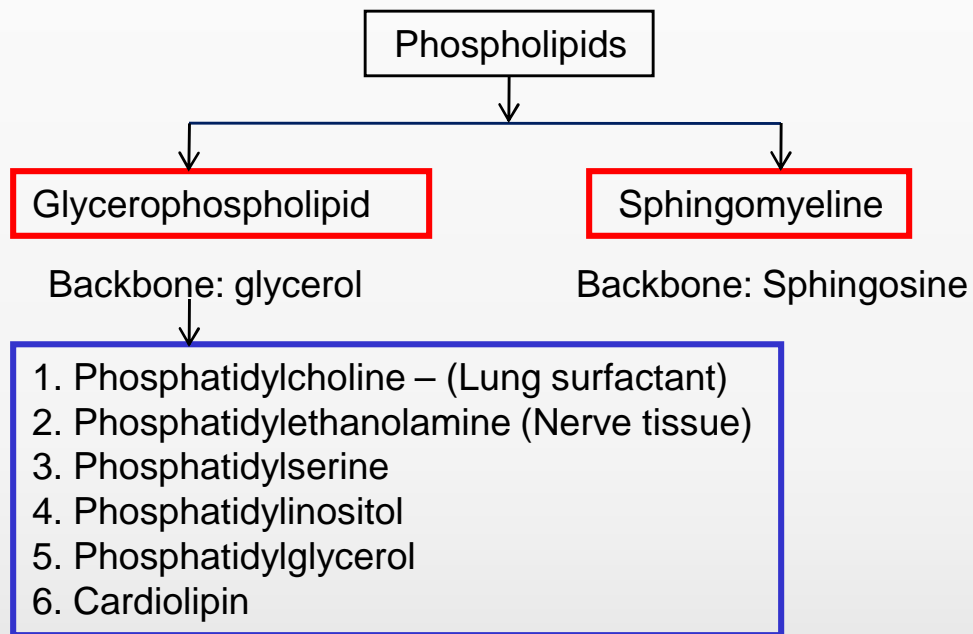
Non- membrane PL

1. Components of lung surfactant.
2. Essential components of bile, where their detergent properties aid in solubilization of cholesterol.



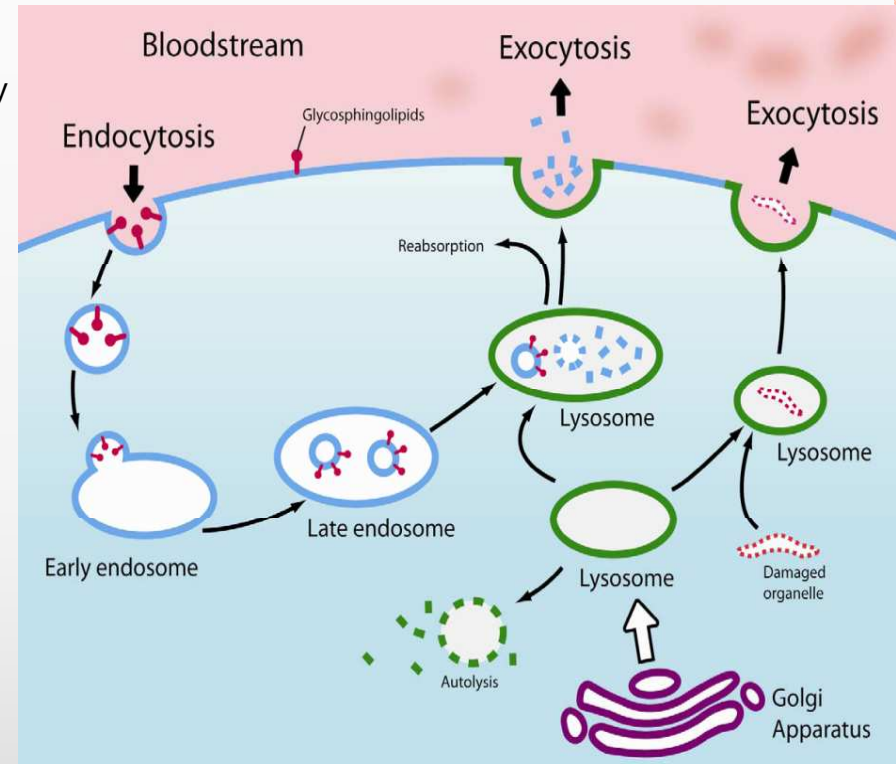
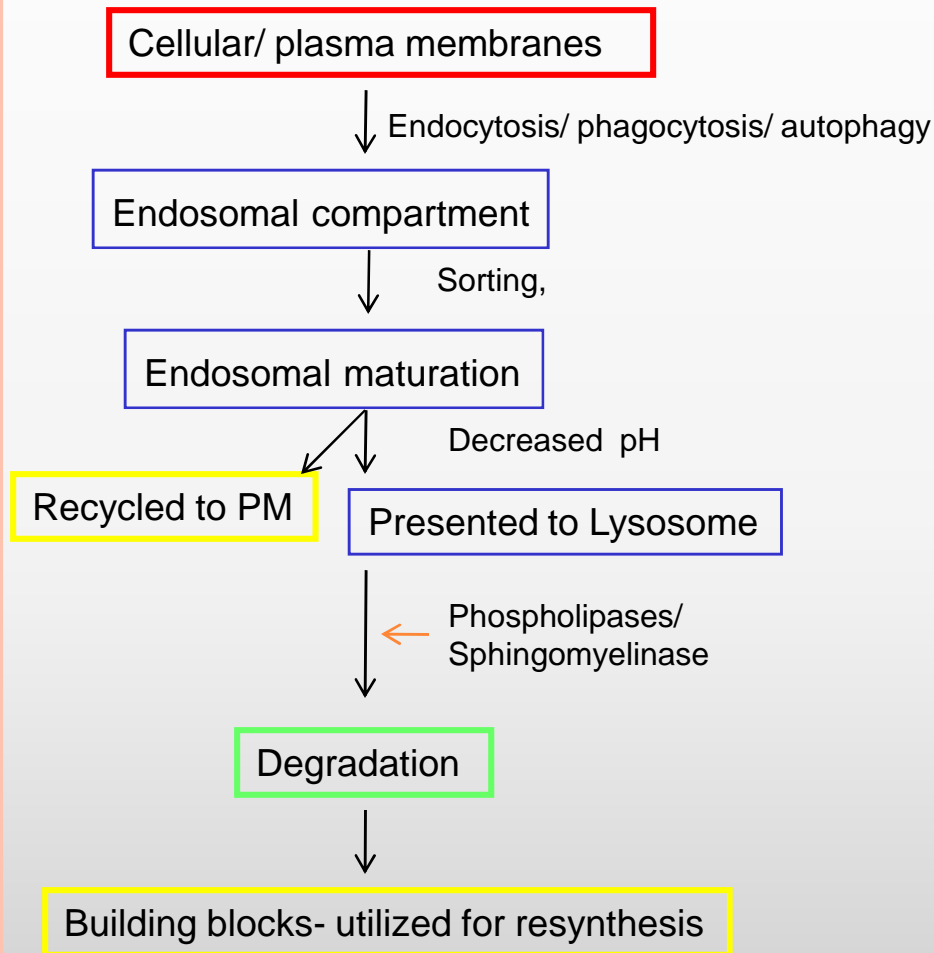
PL play important role from physiological and clinical relevance

INTRODUCTION – PHOSPHOLIPID CLASSES



Lysosomal membrane degradation of phospholipids is a complex process

LYSOSOMAL MEMBRANE DEGRADATION



Different classes of PL play important role in diseases ch'ed by membrane damage

LYSOSOMAL STORAGE OF PL - CAUSES

- Alteration in the metabolism of cell cause lysosomal storage of PL.
- The genetic disorders such as; Niemann-Pick and Tay-Sachs disease.
- Drug induced phospholipidosis; Drugs, Xenobiotics, chemicals, Hormones, cofactors and other agents



PHOSPHOLIPIDOSIS - FEATURES

- There is excessive accumulation of one or more polar phospholipids in cells.
- There is the appearance of membrane bound cytosolic inclusions with a lamellar bodies.
- The inducing drug and excess phospholipid accumulates in lysosomes.



DRUG INDUCED PHOSPHOLIPIDOSIS - MILE STONES

Notable Publications

- 1948 Nelson & Fitzhugh described the occurrence of foamy macrophages in several tissues of rats fed chloroquine for 2 years. *Arch. Pathol.* 45:454-462.
- 1966 *Greselin* reported that a cholesterol synthesis inhibiting drug trans-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dichloride (AY-9944), induced increased numbers of foam cells in the pulmonary alveoli of rats
- 1968 Gleiser et al, observed myelinoid bodies in the CNS of pigs treated with chloroquine. *Am. J. Pathol.* 53(1):27-45, 1968.
- 1970 Franken et al., reported the presence of huge foam cells in the alveoli of rats fed chlorphentermine for 6 weeks. This study appears to have triggered interest in this condition. *Arzneimittelforschung* 20(3):417.
- 1970 Yamamoto et al., reported a fatty liver condition with lamellar bodies in patients treated with diethylaminoethoxyhexestrol. *Jpn. J. Exper. Med.* 40:127.
- 1975 Lüllmann et al, published the first comprehensive review on drug induced phospholipidosis. *Crit. Rev. Toxicol.* 4:185 -218.

Compounds sharing common chemical features, cause phospholipidosis

DRUG INDUCED PHOSPHOLIPIDOSIS -CATIONIC AMPIPHILIC DRUGS

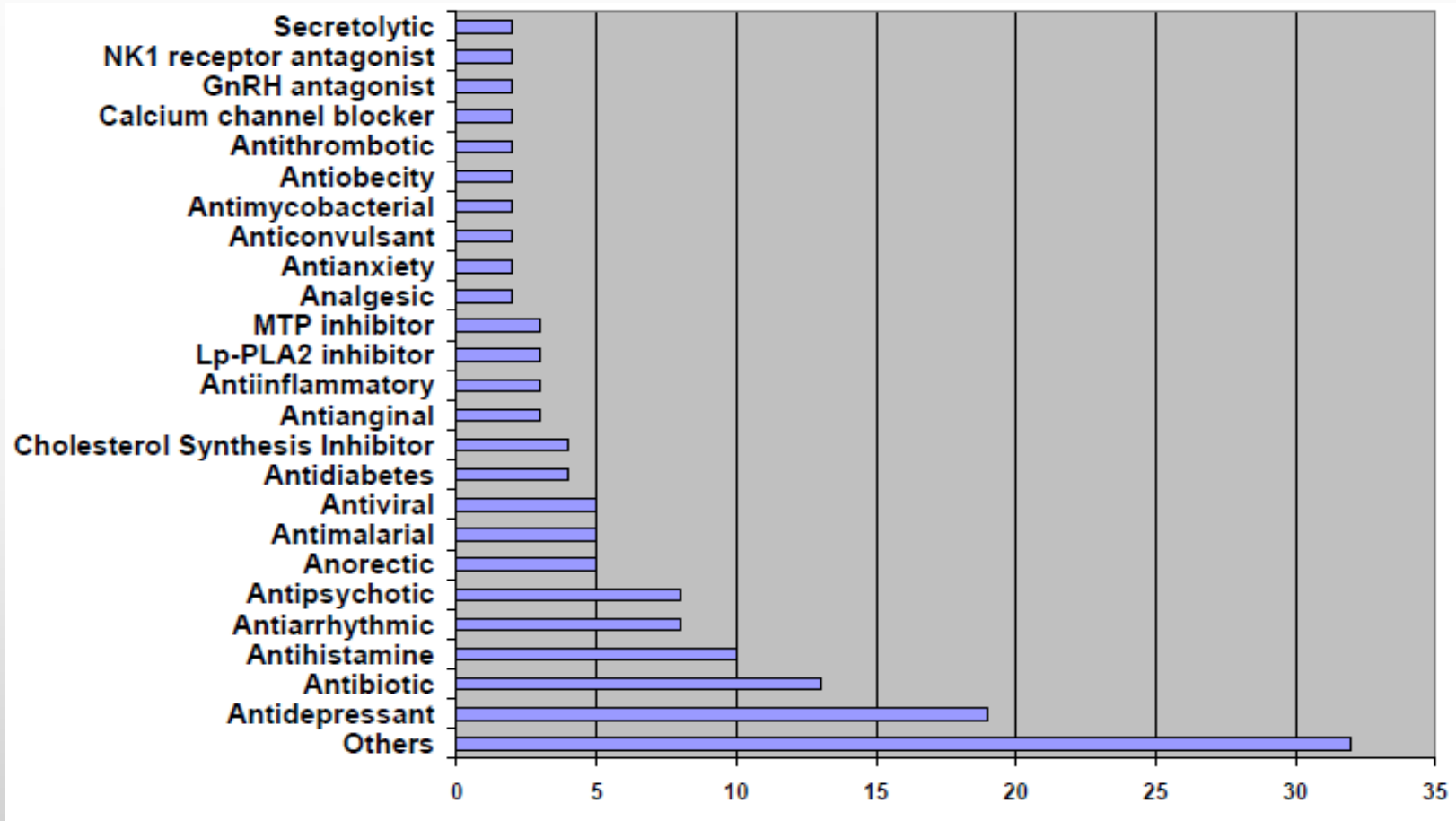
- Wide variety of pharmacologic agents, including antibacterials, antipsychotics, antidepressants, antiarrhythmics, antianginals, antimalarials, anorexic agents, cholesterol-lowering agents, and others induce phospholipidosis.
- These drugs called 'cationic amphiphilic drugs' (CADs), shares several common physiochemical properties. hydrophobic ring structure on the molecule and a hydrophilic side chain with a charged cationic amine group.
- When the molecule are not ionized they can pass through plasma membranes and when in ionized form the molecule tends to remain with the membrane and contribute to membranous changes.
- The membrane phospholipids and their charged ionic groups regulate CAD entry and binding in the cells.



The induction of phospholipidosis is not a function of the pharmacologic action of the drug

PHARMACOLOGICAL CLASSES OF DRUG INDUCING PLD

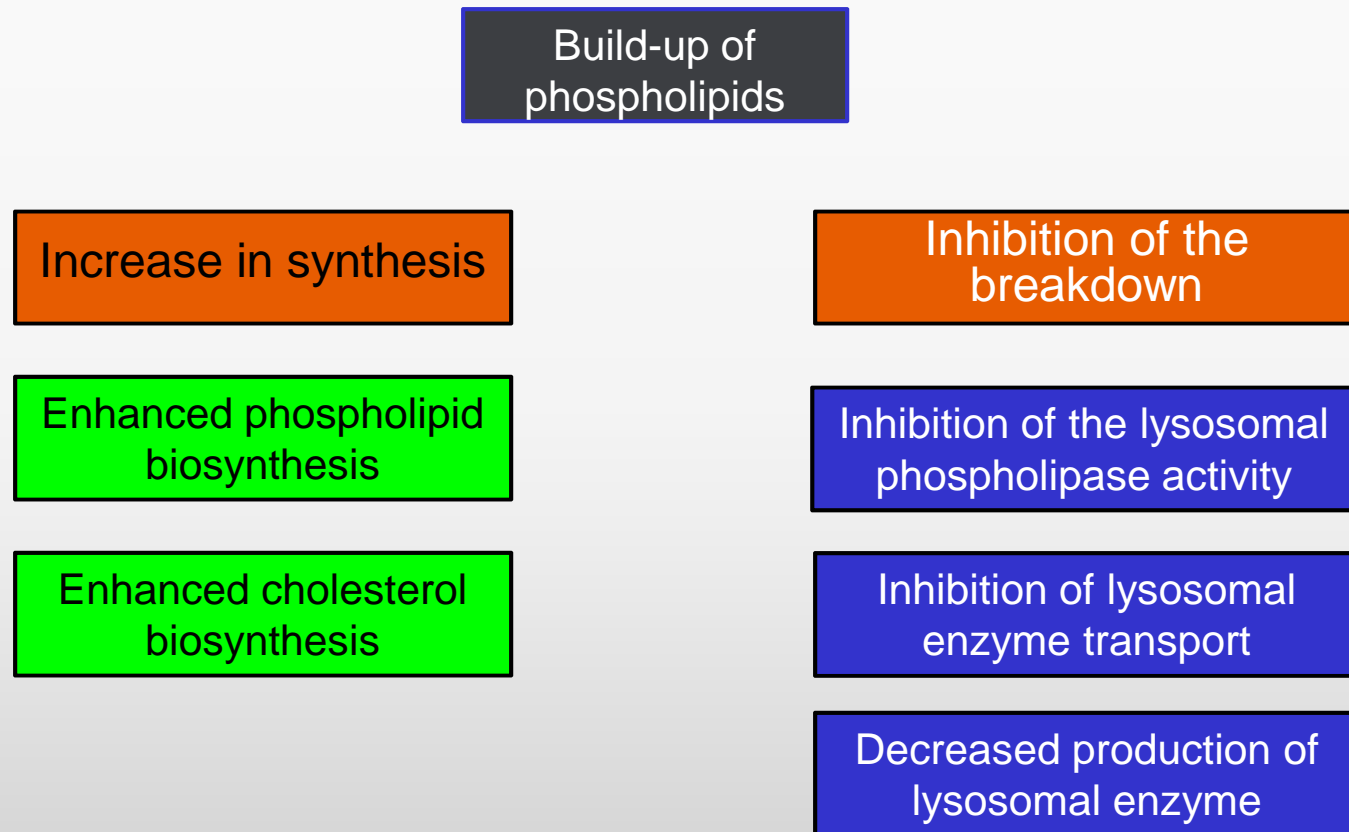
- Over 50 marketed and experimental drugs containing Cationic Amphiphilic Drug (CAD) structure have been reported to induce phospholipidosis.



So many xenobiotics produce phospholipidosis- How? - not well understood

MECHANISM OF ACTION

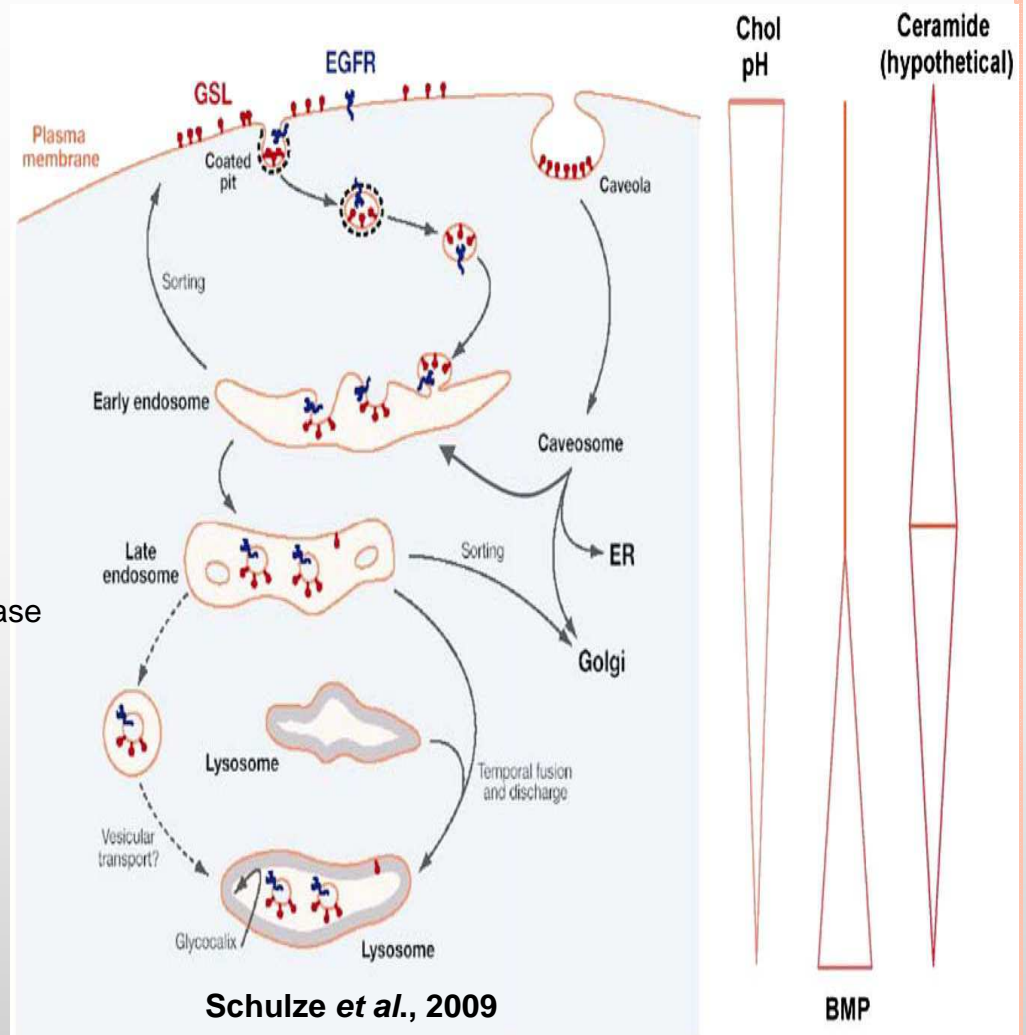
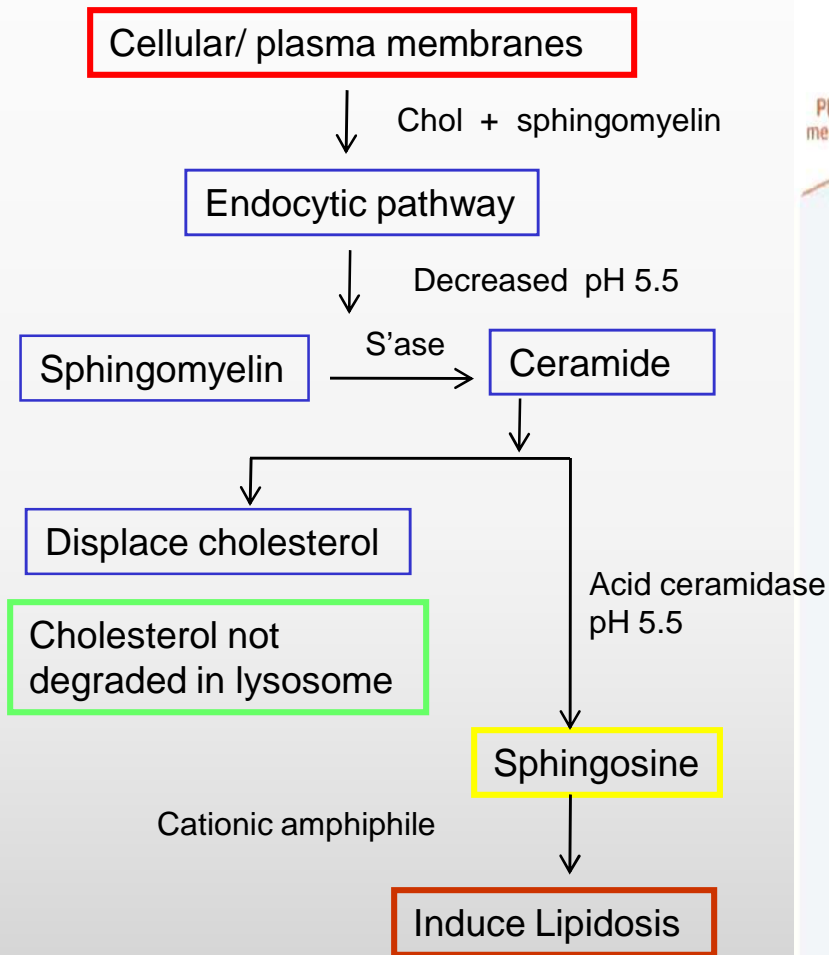
A build-up of phospholipids can be explained by an inhibition of the breakdown or an increase in the synthesis of the phospholipids.



Sawada et al., 2005

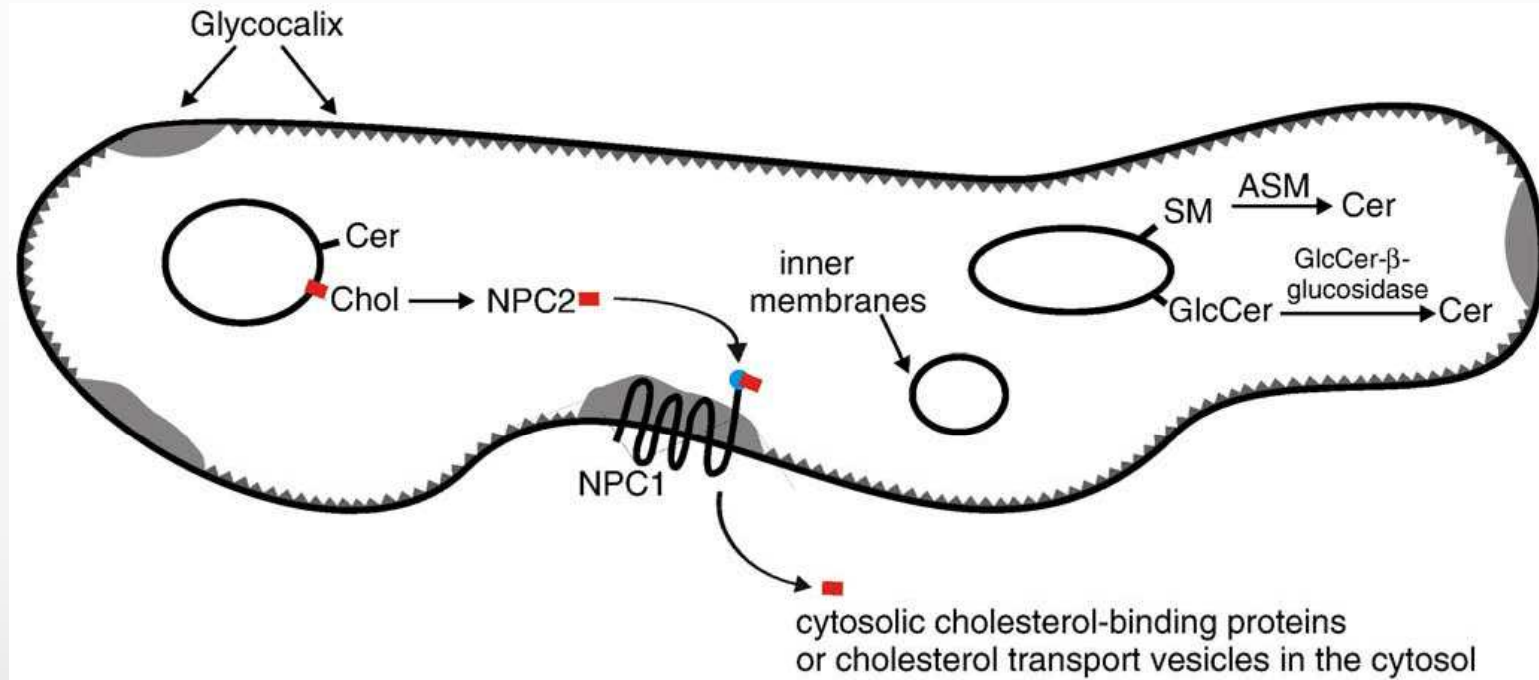
As action of CADs located in the lysosomes – review the lysosomal degradation process

LYSOSOMAL MEMBRANE DEGRADATION

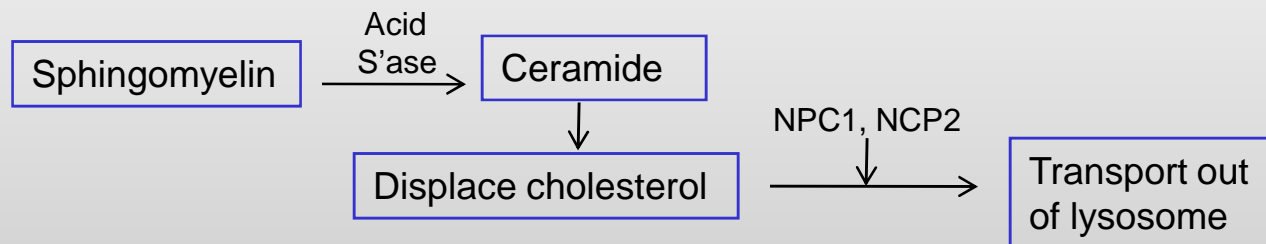


Lysosomal membrane degradation requires presence of phospholipases , low pH, high BMP content and low cholesterol.

LYSOSOMAL MEMBRANE DEGRADATION - CHOLESTEROL TRANSPORT

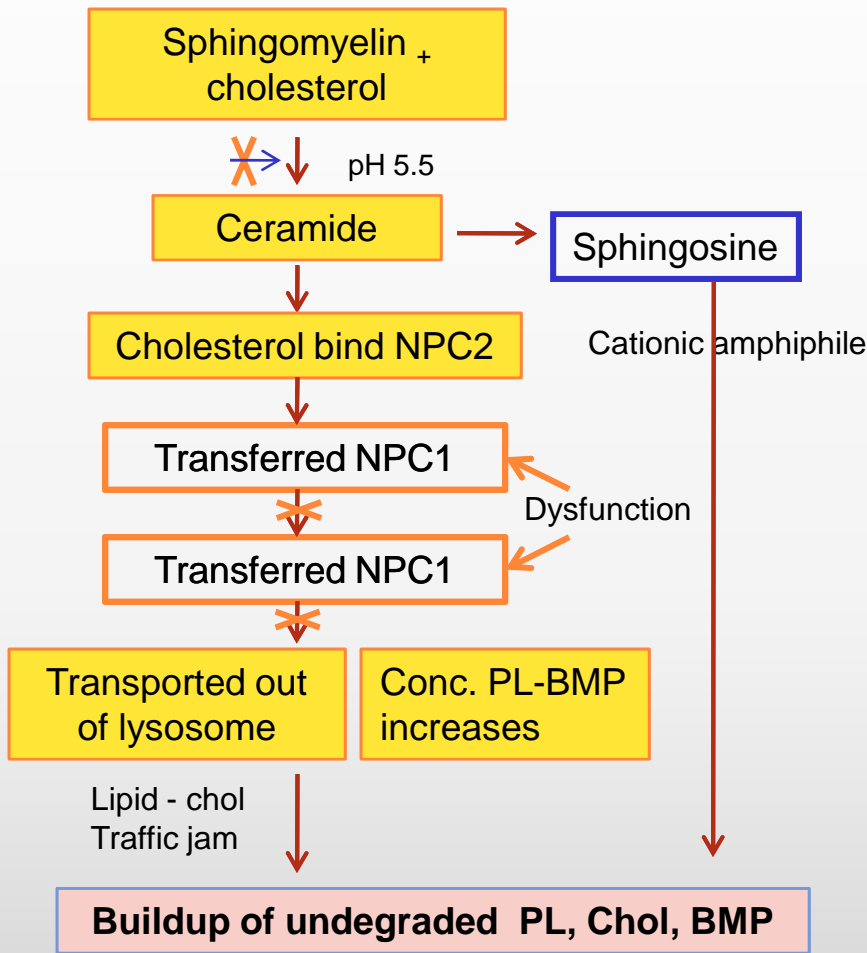


Schulze *et al.*, 2009

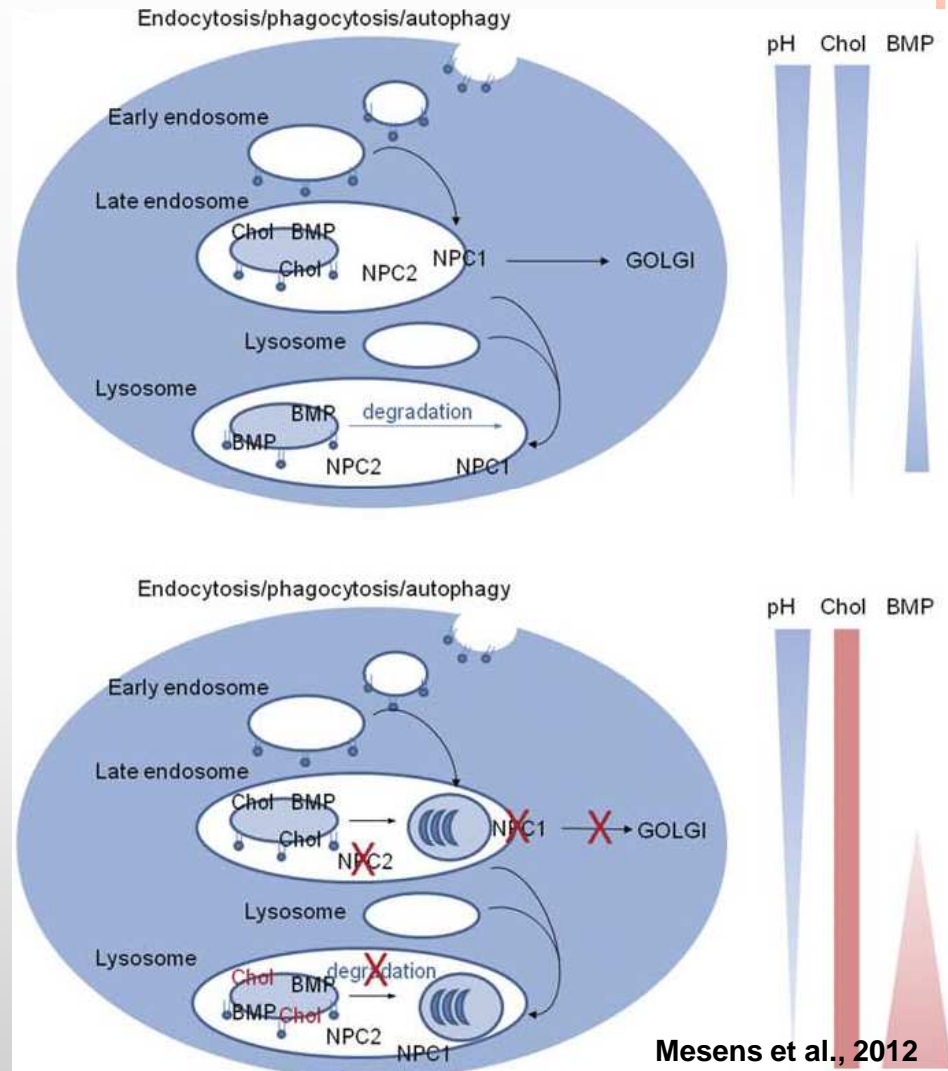


Response of a given species to a particular CAD is unpredictable.

MECHANISM OF PLD - INCREASE IN BIOSYNTHESIS

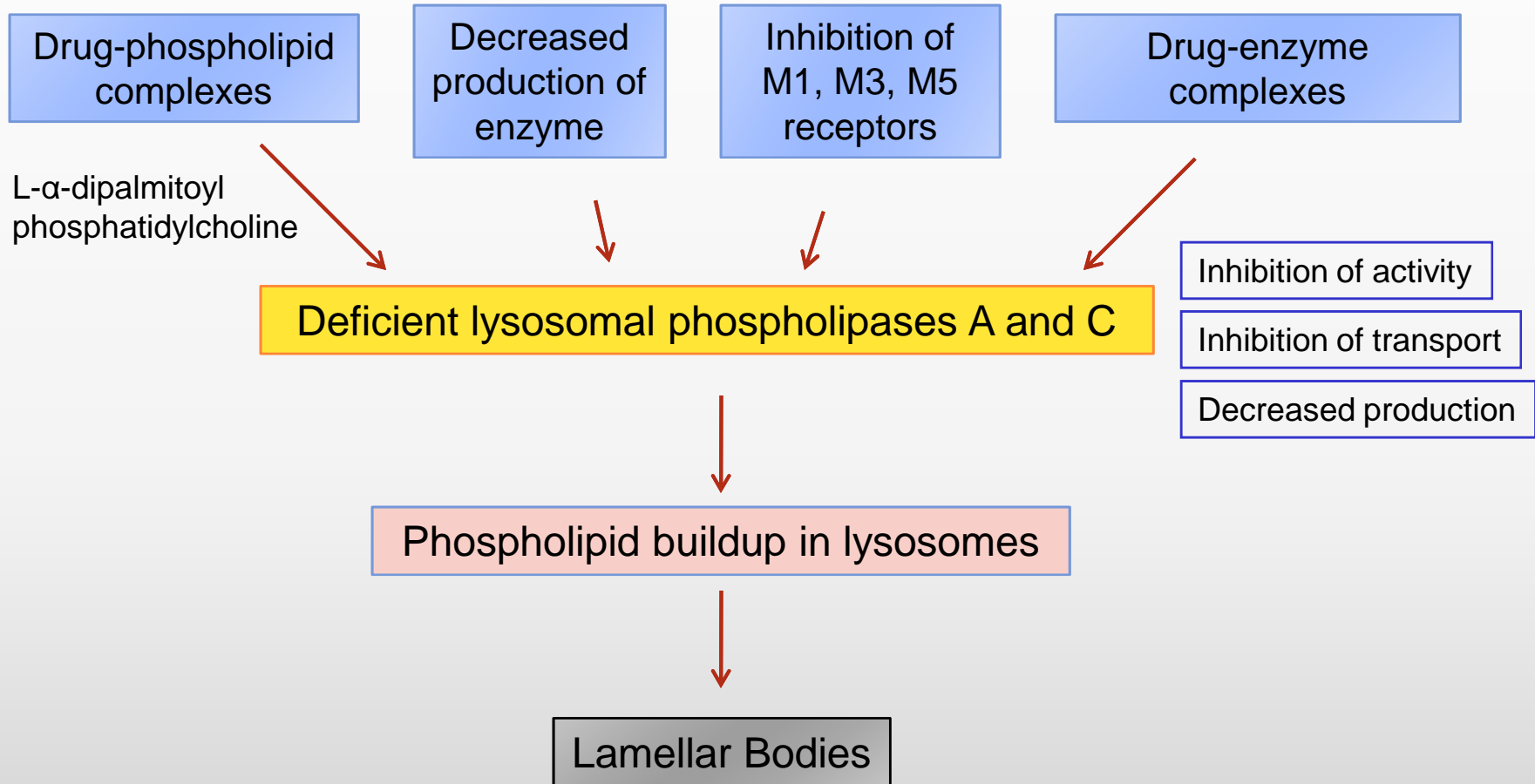


ASM – Acid Sphingomyelinase; BMP- bis(monoacylglycerol) phosphate; NCP- Niemann-Pick proteins



Other theory of inhibition of the breakdown of phospholipidosis

MECHANISM OF PLD - INHIBITION OF BREAKDOWN



CAD change the susceptibility of membrane to breakdown

MECHANISM OF PLD – SUGGESTED BY SAWADA

Sr. no	Mechanism	Targets
1	Inhibition of lysosomal phospholipase activity	Sphingomyelin phosphodiesterase (SMPD) (h)
		Lysosomal Phospholipase A1 (LYPLA1) (r)
		Phospholipase A2 (PLA2) (h)
2	Inhibition of lysosomal enzyme transport	down-regulation of genes involved in lysosomal enzyme transport; adaptor-related protein complex 1 sigma 1 subunit (AP1S1) -transports newly synthesized lysosomal enzymes between the transgolgi network and lysosomes
3	Enhanced phospholipid biosynthesis	Up-regulation of fatty acid biosynthesis-related genes (ELOVL6 and stearyl-CoA desaturase) – unlikely
4	Enhanced cholesterol biosynthesis	up-regulation of cholesterol biosynthesis-related genes (HMGCS1, HMGCR, lanosterol synthase)

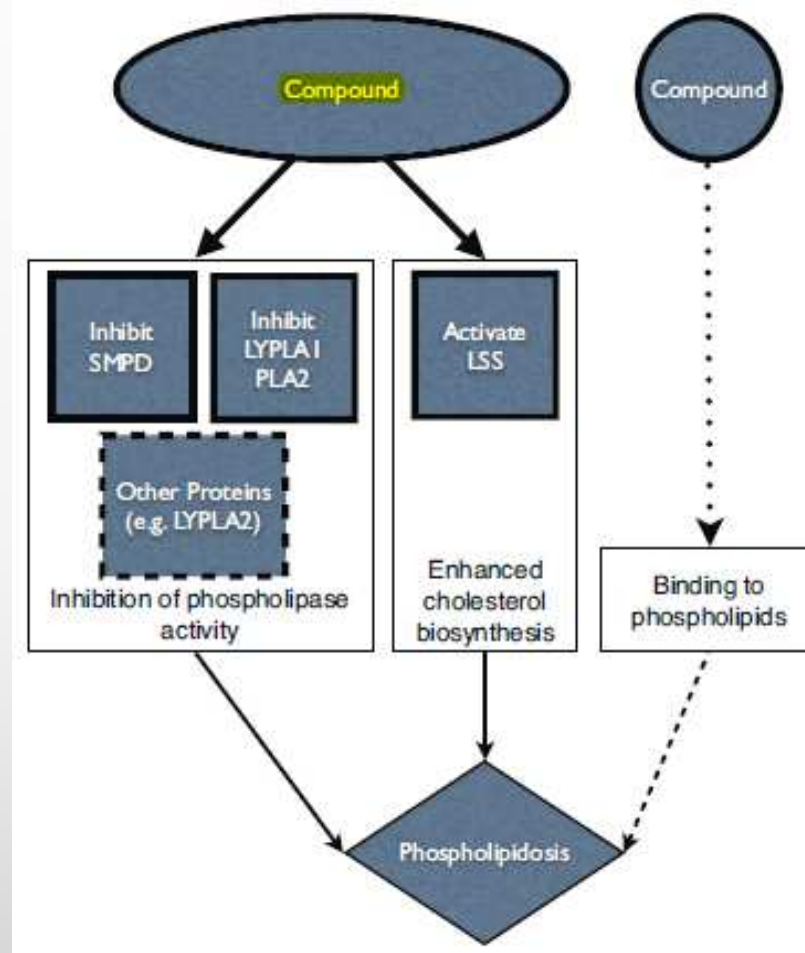
Sawada et al., 2005

Response of a given species to a particular CAD is unpredictable.

MECHANISM OF PLD – SUMMARY

Important mechanisms responsible for PLD

- Inhibition of phospholipase activity- likely targets
 - Sphingomyelin phosphodiesterase (SMPD),
 - Phospholipase A2 (PLA2) and
 - Lysosomal phospholipase A1 (LYPLA1)
- Enhanced Cholesterol Biosynthesis
 - Lanosterol synthase (LSS)



Lowe et al 2012

No single target or even mechanism is responsible for phospholipidosis

MECHANISM OF PLD – IN NUTSHELL

- There is no single mechanism for induction of PLD, as diverse drugs induce it in different ways.
- Multiple causative mechanisms may be involved.
- Response of a given species to a particular CAD is unpredictable.
- Lysosome rich cells are prominent responders such as macrophages.



CHARACTERISTICS OF PLD

- PLD induced in many tissues in the body, and in cell culture.
- No species, gender or age group specificity.
- The time of induction varies based on affinity of CAD for susceptible cell.
- In cell cultures, the LB formed in few hrs.
- In *in vivo* it depends upon dose, exposure duration and animal species used.
- Site of induction is not predictable based upon structure of a drug and may vary among species and cells or tissues.



There are various factors that determine the incidence and severity of phospholipidosis.

CHARACTERISTICS OF PLD – SPECIES, AGE, STRAIN SPECIFICITY

Specificity	Characteristics	Example
Species	<ul style="list-style-type: none"> - Differences in capacity for metabolic elimination of CADs. - Status of metabolic enzymes in target or non-target tissues. - Overall turnover of CADs by tissues. 	<ol style="list-style-type: none"> 1. Chlorphentermine-induced PLD in lung & adrenal gland. 2. Chlorphentermine or gentamicin induce PLD in rat embryo not in chick embryo.
Age	<ul style="list-style-type: none"> - Young age more susceptible. - Influence of enzyme development pattern at different stages. - Differences in the enzyme profile. - Differences in structural components of the cell 	<ol style="list-style-type: none"> 1. Gentamicin induced increase in p-inositol in newborn rats, unlike in adults.
Strain	<ul style="list-style-type: none"> - Strain differences may be related to the dispositional location of the drug. - Not to be related to biochemical or cellular features of the AM. 	<ol style="list-style-type: none"> 1. Amiodarone more phospholipogenic in male Fischer-344 rats than in male SD rats
Metabolism	<ul style="list-style-type: none"> - Rapidly metabolized Drugs fail to induce PLD. - Enhancement of metabolism by prior administration of inducing agents does not cause PL. - inhibition of metabolism - increased potential for PLD. 	<ol style="list-style-type: none"> 1. Reduced incidence & severity of Chlorphentermine induced PLD by concurrent administration of phenobarbital.

It is difficult to predict in what species, tissues or cells CAD will induce phospholipidosis

CHARACTERISTICS OF PL - TISSUE SPECIFICITY

Tissue	Characteristics	PL type increased	Example
Lung	<ul style="list-style-type: none"> - High content in lung surfactant - CAD reacts - polar ionic moiety vigorously 	phosphatidylcholine	Chlorphentermine
Macrophages	<ul style="list-style-type: none"> - Accumulation of neutral di saturated phosphatidylcholine, with cholesterol, cholesterol esters, free fatty acids. 	Phosphatidylcholine, cholesterol and FA	Fluoxetine, chlorcyclizine
Liver	<ul style="list-style-type: none"> -induce cytoplasmic vacuoles, but not lamellar bodies. - binds only to hydrophobic moiety of the phospholipids. 	Phosphatidylserine , phosphatidylethanolamine	amiodarone
	<ul style="list-style-type: none"> -has a divalent cationic group. -accumulation of ganglioside. 	Phosphatidylglycerol, phosphatidylinositol	Chloroquine
Kidney	<ul style="list-style-type: none"> - Excreted by glomerular filtration - binds to brush-border of cells of PCT –adsorbed – accumulate in lysosomes. 	Phosphatidylinositol, phosphatidylserine, phosphatidylcholine	Gentamicin, amantadine
Brain	<ul style="list-style-type: none"> -PL rich organ. -PLD is unusual in brain due to BBB -Affected areas have incomplete BBB. 	P-ethanolamine, p-cholin, sphingo myelin, p-serine, p-inositol	Chloroquine- and amiodarone, anti fungal

Ionic and hydrophobic interactions of CADs with phospholipids determines tissue specificity of phospholipidosis.

MORPHOLOGICAL PATTERNS OF PLD

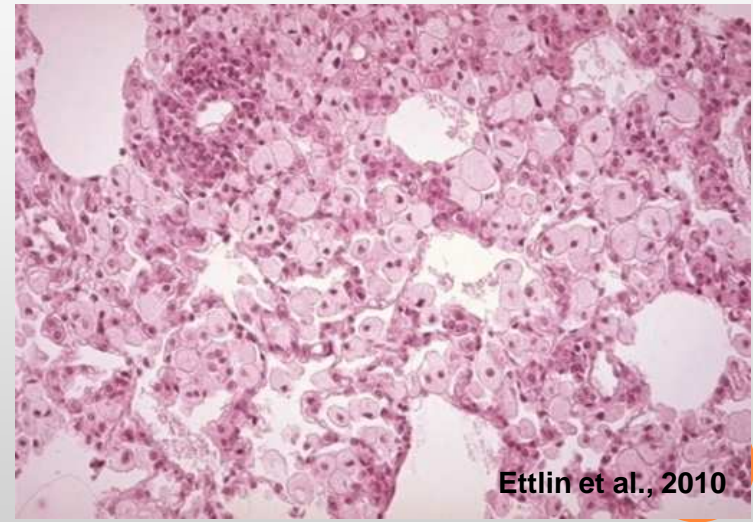
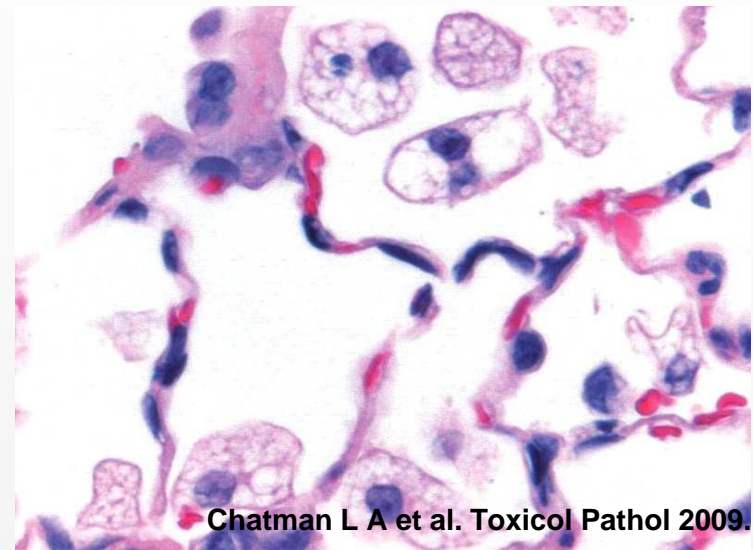
1. Macrophage dominant
 - Resident macrophages of the tissues demonstrate phospholipidosis
2. Parenchymal cell-dominant
 - PLD is also evident in the parenchymal cells of the tissues
3. Localized
 - Ductal cells of the bile are affected.



CAD administration induces dramatic morphologic changes

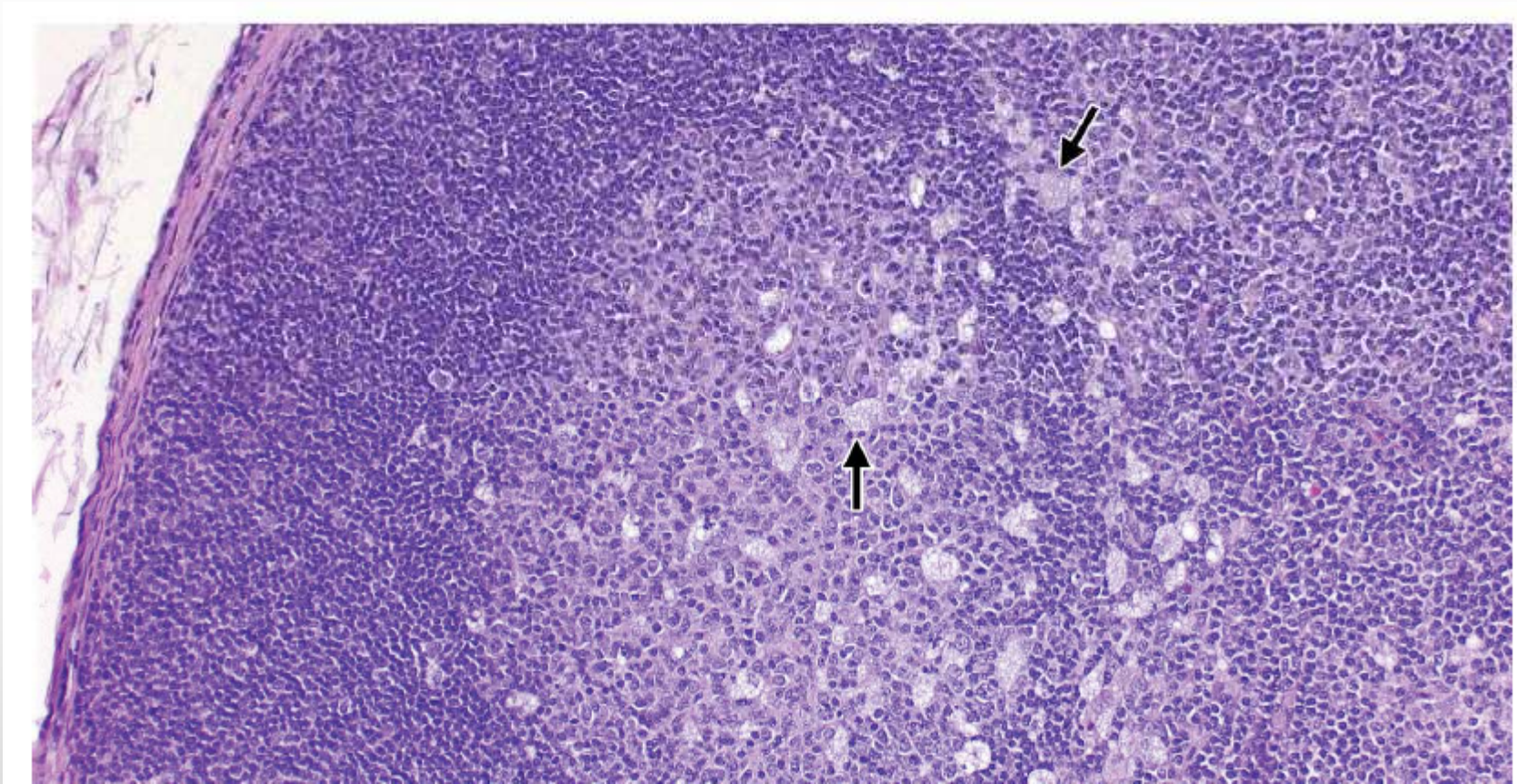
MORPHOLOGICAL PATTERNS - MACROPHAGE DOMINANT

- Initial manifestation - infiltration of foamy macrophages in lung and lymph nodes.
- Multi-focal /diffuse infiltrate in alveolar space or lymphatic sinuses of LN (MLN).
- Lung and LN findings correlate .
- No of foamy cells in lymphatic sinuses correlate with increase in doses.
- Tissue macrophages in liver, spleen, thymus, BM also show foamy appearances.



Macrophages show foamy appearance, enlarged and increased in number.

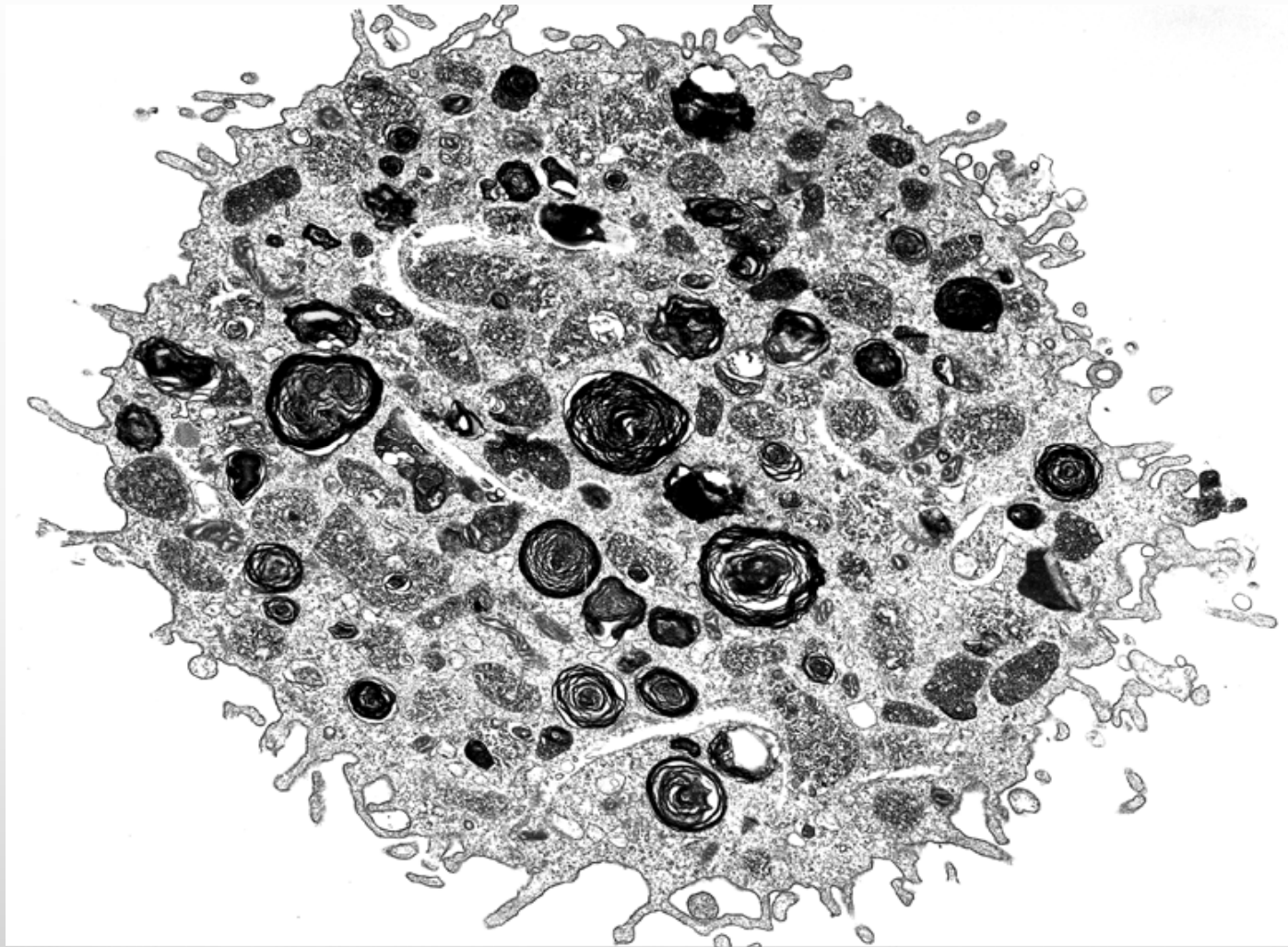
MORPHOLOGICAL PATTERNS - MACROPHAGE DOMINANT



Dog treated with posaconazole (30 mg/kg) for twelve months

MLN- cortex contains many swollen macrophages that have abundant eosinophilic, foamy to clear cytoplasmic vacuoles

MORPHOLOGICAL PATTERNS - MACROPHAGE DOMINANT



TEM of alveolar macrophage. Electron dense lamellar inclusion

MORPHOLOGICAL PATTERNS – PARANCHYMAL CELL DOMINANT

- Also called Generalized PLD.
- Hepatocytes, renal tubular epithelial cells, bile ductal cells, endocrine cells, nerve cells, vascular endothelial cells, muscle cells are affected.
- PL accumulation in cells is associated with physiological lysosome distribution.
- Vacuoles are fine to small and may be associated with cytotoxic changes including degeneration/necrosis and infiltration of inflammatory cells.



Consequences of parenchymal cell dominant PLD are imp in safety evaluation

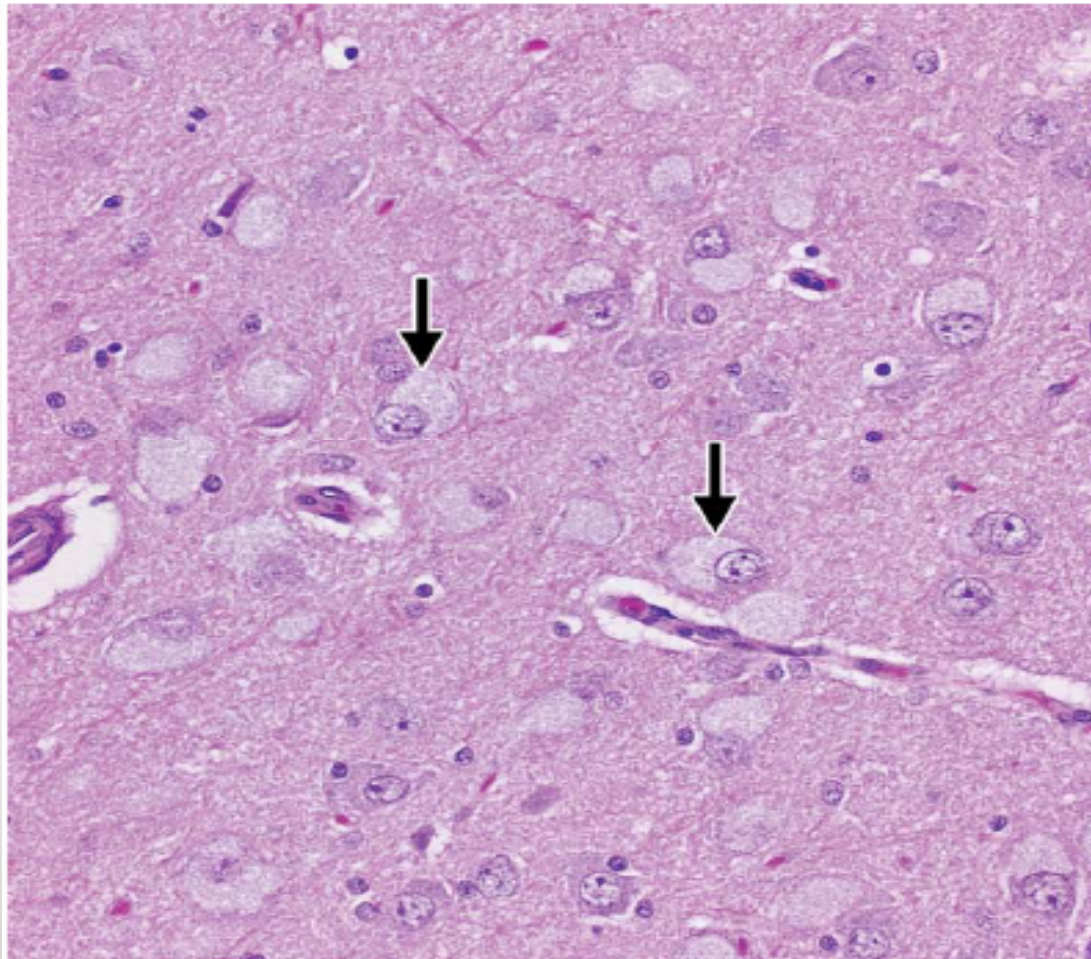
MORPHOLOGICAL PATTERNS – LOCALIZED

- This pattern is not common – special type parenchymal cell dominant PLD.
- Certain compounds induce PLD only in one type of parenchymal cells.
- Cells are exposed to higher concentration than other tissues.
- Ex: renal PLD, bile duct or Nerve cell PLD.



Consequences of parenchymal cell dominant PLD are imp in safety evaluation

MORPHOLOGICAL PATTERNS – LOCALIZED

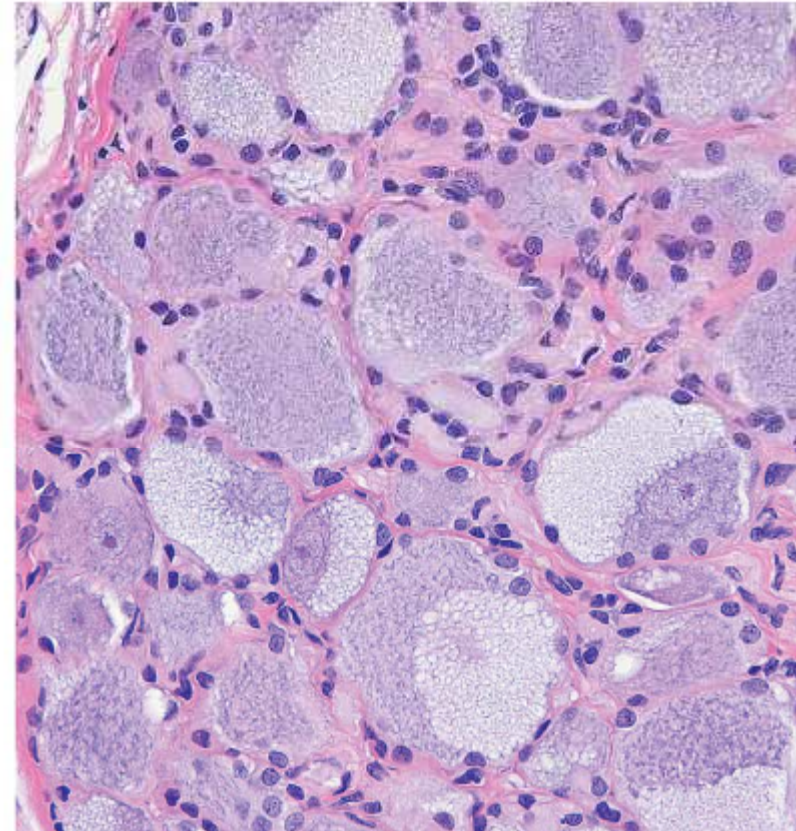
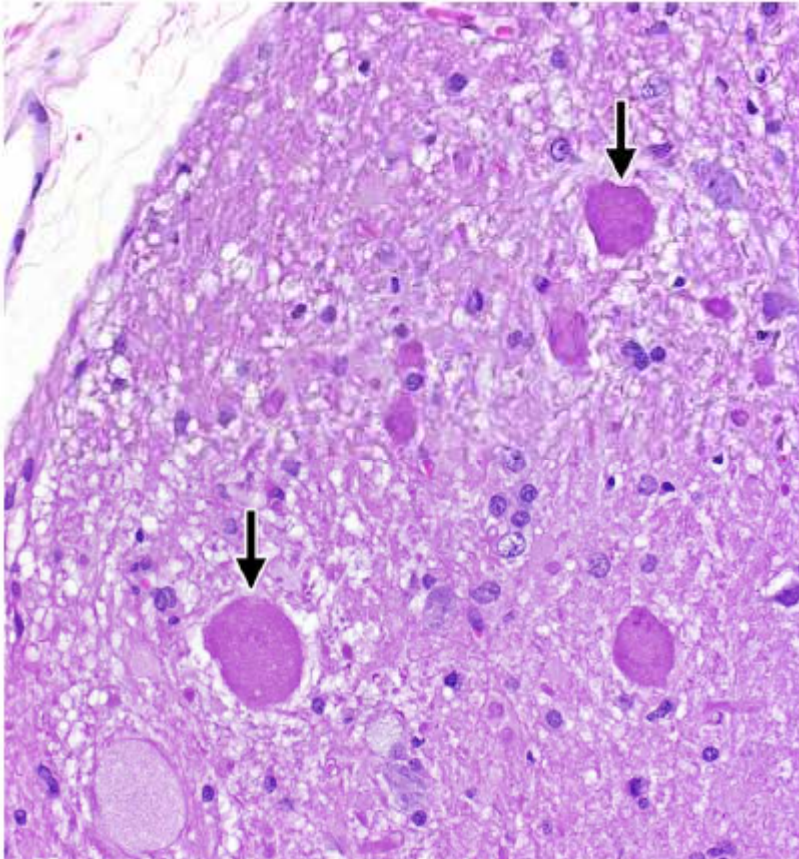


Cartwright, et al., 2009

Dog treated with posaconazole (30 mg/kg) for twelve months

Thalamus- multiple, enlarged neurons with abundant eosinophilic foamy cytoplasm

MORPHOLOGICAL PATTERNS – LOCALIZED

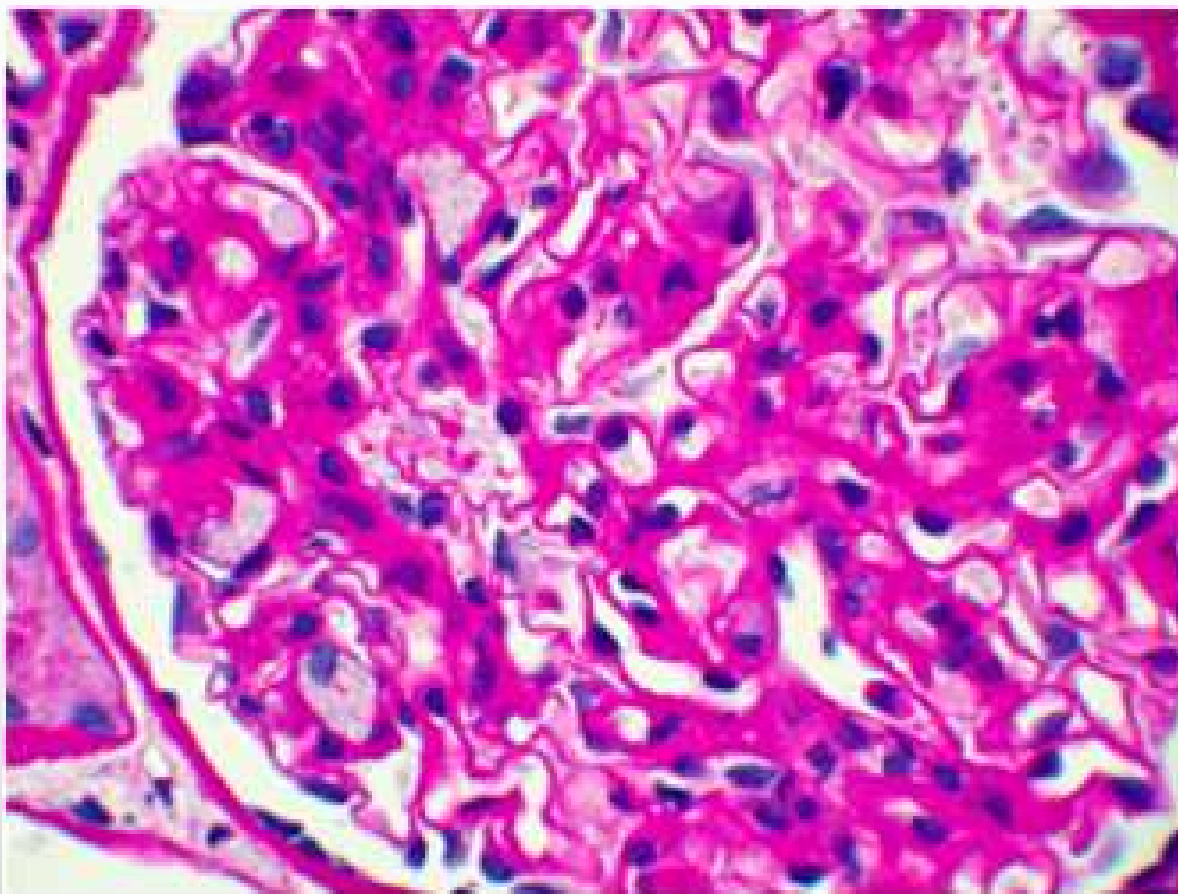


Cartwright, et al., 2009

Dog treated with posaconazole (30 mg/kg) for twelve months

**Multiple enlarged axons with abundant foamy to dense eosinophilic material;
Swollen neurons with abundant eosinophilic, foamy to clear vacuoles**

MORPHOLOGICAL PATTERNS – LOCALIZED



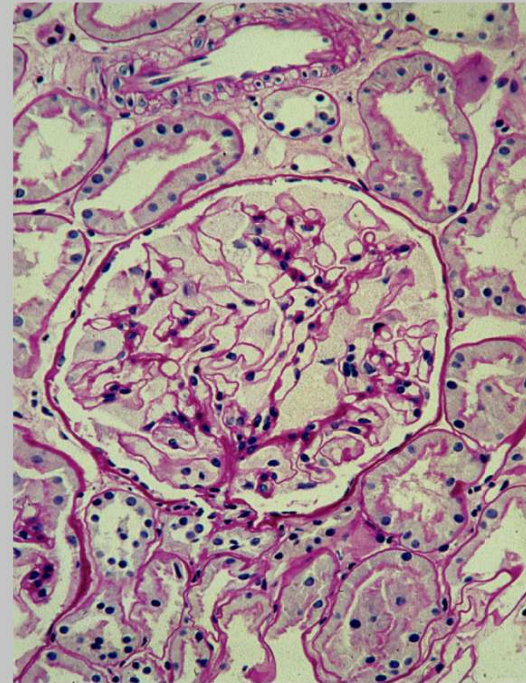
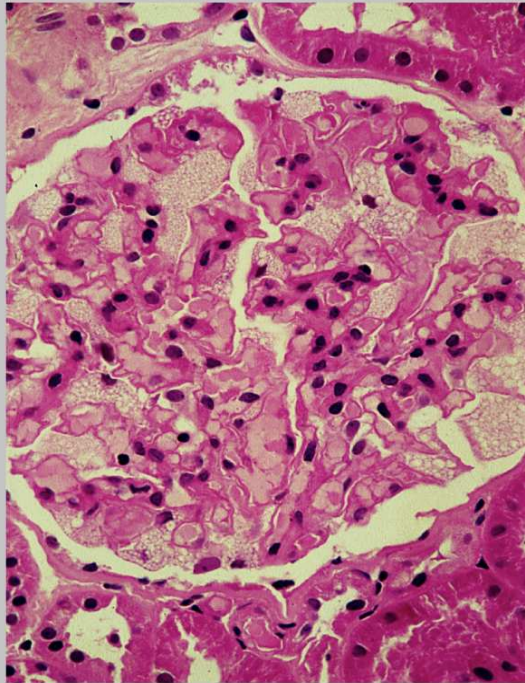
Pintavorn and Cook, 2008

Amiodaron induced- Glomerulus with dilated capillary lumina containing vacuolated cells

MORPHOLOGICAL PATTERNS — LOCALIZED

Phospholipidosis

Example: Fabry`s disease



Podocytic foam cells (HE stain, left; PAS, right)



PHOSPHOLIPIDOSIS – TOXICOLOGICAL IMPLICATIONS

	Non-adverse	Adverse
Doses	Significantly higher (>10 folds) than clinical doses	
Reversibility	Generally considered reversible	Rate varies with drugs, tissues and species. Little information in humans.
Functional deficit	Is adaptive response	Toxicity may result from higher drug concentration.
	Pulmonary phospholipidosis offered partial protection against inhaled nitrogen dioxide.	Some times associated with toxicities both pre-clinically and clinically. Physico-chemical properties of CAD may predispose them to cause indirect toxicities.
Incidence	Only few CADs reported to induce PLD in humans	Variation in species, tissue, age specificity – response is unpredictable.

Studies not adequate to draw any definitive conclusion

PHOSPHOLIPIDOSIS – TOXICOLOGICAL IMPLICATIONS

- ❑ Tissue damage has been observed in association with the induction of phospholipidosis by some drugs.
- ❑ Results of *in vivo and ex vivo* studies are variable - some studies showing impaired function, some no change in function and some enhanced.
- ❑ These changes have not definitively linked to presence of PLD.
- ❑ Some studies are suggestive of toxicity –ex; gentamycin induced nephrotoxicity.
- ❑ From a regulatory perspective and consistent with the task of determining drug safety, PLD has been considered as an adverse finding, whether justified or not.

When phospholipidosis is observed in cells or tissues, - assess the function of the affected tissue and establish whether any changes in function, if present, are causally related to the phospholipidosis.

It is essential to evaluate phospholipidosis-inducing potential of compounds at an early stage in order to reduce the risk of attrition in drug development.

DIAGNOSIS / BIOLOGICAL MARKERS

- **In Silico techniques QSAR models** -
 - Correlation between structural features of molecules and PLD activity. Amphiphilicity of drug molecules is linked to their ability to induce PLD.
 - CDER constructed 3 QSAR software programs; MC4PC, MDL-QSAR, and Leadscope
- **Physicochemical properties** – CAD would be phospholipidosis inducing provided that it has $pK_a > 8$ and $ClogP > 1$ and also the eq is satisfied i.e. $(ClogP)^2 + (pK_a)^2 > 90$.
- **Histopathological analyses** - presence of foamy cells - only suggests that PLD may have occurred.
- **Electron microscopy** – gold standard - confirmatory method - expensive, time consuming, not for high-throughput screenings.



Each Model has specific strengths and weaknesses

DIAGNOSIS / BIOLOGICAL MARKERS

- **In vitro screen**
 - Measurement of binding of dyes to the phospholipids.
 - Use of various cell lines (primary hepatocytes, HepG2, U-937, CHO-K1, and CHL/IU cell lines, spleen macrophages).
 - Fluorescently labeled surrogate phospholipid -
 - PL - phosphatidylcholine (PC) and fluorescent moiety-nitrobenzoxadiazole (NBD) – NBD-PC- fluorescent spectrophotometer.
 - Phosphoethanolamine –NBD-PE – laser scanning confocal microscopy.
 - understanding the mechanisms.
- **Toxicogenomic assays – Microarray technology**
 - Marker genes which could be used as signatures to predict the potential for PLD.
 - Contribute to understanding the mechanisms.
 - High cost and low throughput.



Significance of PL findings in these models is not translatable to the clinical setting

DIAGNOSIS / BIOLOGICAL MARKERS

○ **Non-invasive biomarker – BMP**

- Bis(monoacylglycerol)phosphate – a phospholipid.
- Is present in the membranes of late endosomal vesicles and in the multilamellar membranes of myeloid bodies.
- Is present in high levels in plasma from patients with lysosomal storage disorders, especially Niemann-Pick disease.
- LC-MS/MS method used to extract BMP from urine samples.
- Also detected in human samples.
- Can be used to monitor the onset and time-course of phospholipidosis with drug-induced toxicities.



WHAT HAVE WE LEARNED ABOUT PLD-INDUCING DRUGS?

- ❑ PLD is Associated with every pharmacological class.
- ❑ Signals a change in cell membrane integrity and accumulation of intracellular drug or metabolite in tissues.
- ❑ Most of PLD inducing drugs are cationic amphiphilic drugs (CADs).
- ❑ No direct association with clinical outcome.
- ❑ The sensitivity of preclinical models to detect PL vary with therapeutic agents
- ❑ PL is expected to be reversible after discontinuation of drug treatment .
- ❑ There is no clear understanding about the considering PLD as adverse or non-adverse effect.



REGULATORY PERSPECTIVE

- ❑ Currently no regulatory guidelines. FDA created PLD WG (2004) to address PLD concerns.

- ❑ PLDWG goals.
 - To assess clinical concern of PLD findings in animals.
 - To develop better tools to predict if drugs could induce PLD.
 - To develop non-invasive biomarker to detect PLD in patients.
 - To help industry by developing a guidance document.
 - To understand potential relationship between PLD and QT Prolongation.

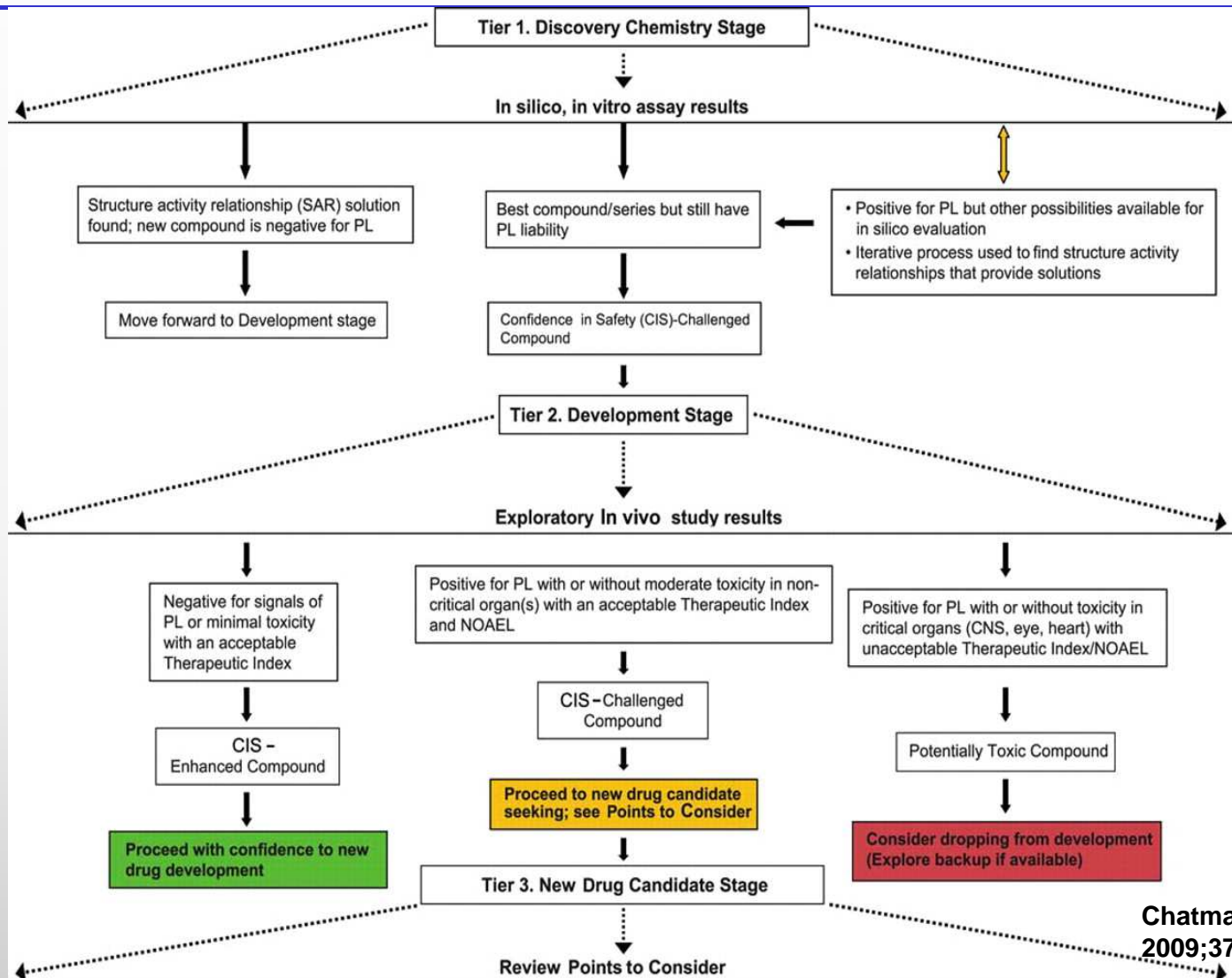


A STRATEGY FOR RISK MANAGEMENT

It has been stated in an expert opinion paper that ,”from a regulatory perspective, and consistent with the task of determining drug safety, PLD has been considered an adverse finding, whether justified or not” (Reasor, Hastings, and Ulrich 2006).

- PL findings may have an adverse impact on the success of the project. potential for development success through the use of three major decision-making stages
- Rapidly identify and screen out those compounds early in the discovery process.
- Pharmaceutical companies apply appropriate strategies to select, develop, and market safe compounds that benefit the health of patients .

RISK MANAGEMENT STRATEGY FOR PLD



Chatman et al. Toxicol Pathol
2009;37:997-1005

A well-thought-out risk management strategy improve selection and development of new drugs

CONCLUSION

- ❑ Drug-induced PL refers to an excessive, reversible accumulation of PL and associated drug in lysosomes.
- ❑ CADs are capable of inducing a phospholipidosis in various tissues. No species, gender or age group specificity.
- ❑ Mechanism is complex. 3 concepts
 - ❑ CAD bind with PL and render them more resistant to phospholipase activity.
 - ❑ CADs interact with phospholipases and limit their ability
 - ❑ CADs influence the synthesis of phospholipids.
- ❑ Morphological patterns – macrophage dominant, parenchymal dominant and localized.



CONCLUSION

- ❑ Linkage between toxicological implications and clinical responses of PLD is unclear.
- ❑ For diagnosis of PLD – various *in silico*, *in vitro* models, histopathology, electron microscopy, Microarray technology.
- ❑ Noninvasive biomarkers – BMP can be used during clinical development.
- ❑ FDA created PLD WG (2004) to address PLD concerns.
- ❑ 3- tier risk management strategy for selection and development of compounds with potential to produce PL



Thank you

