Current Considerations in Toxicologic Pathology Research

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Outline

- **Introduction**
- Exposome and One Health
- Microbiome and Gut-Brain Axis
- Drug Development
- Biomedical Research and the Toxicologic Pathologist
  - Study Design Considerations
  - Considerations in Pathology Evaluation
- Resources

Leading Causes of Death in Brazil in 2020 (April, Ministerio da Saude)

- 1. Diseases of circulatory system – heart disease, stroke
- 2. Some infectious and parasitic diseases – COVID-19
- 3. Cancer – lung, prostate/breast, colon
- 4. Respiratory system diseases – COVID-19, COPD, bronchitis, emphysema, asthma, pneumonia
- 5. Accidents and violence (external causes)
- 6. Endocrine, nutritional and metabolic diseases (e.g., diabetes)
- 7. Digestive system diseases
- 8. Genitourinary system diseases e.g., kidney disease
- 9. Nervous system diseases e.g., Alzheimer's disease,
- NOTE: **urban air pollution** is major environmental issue – highest cause of premature mortality by 2050

Toxicologic Pathology Research

Medical therapies

- Antibiotics, vaccines, chemotherapy, dialysis, organ transplant, joint replacement
- New areas – stem and other cell therapies, gene and immune therapies
- Safety/risk assessment
- Efficacy of therapy
- Virtually all developed with help of animals

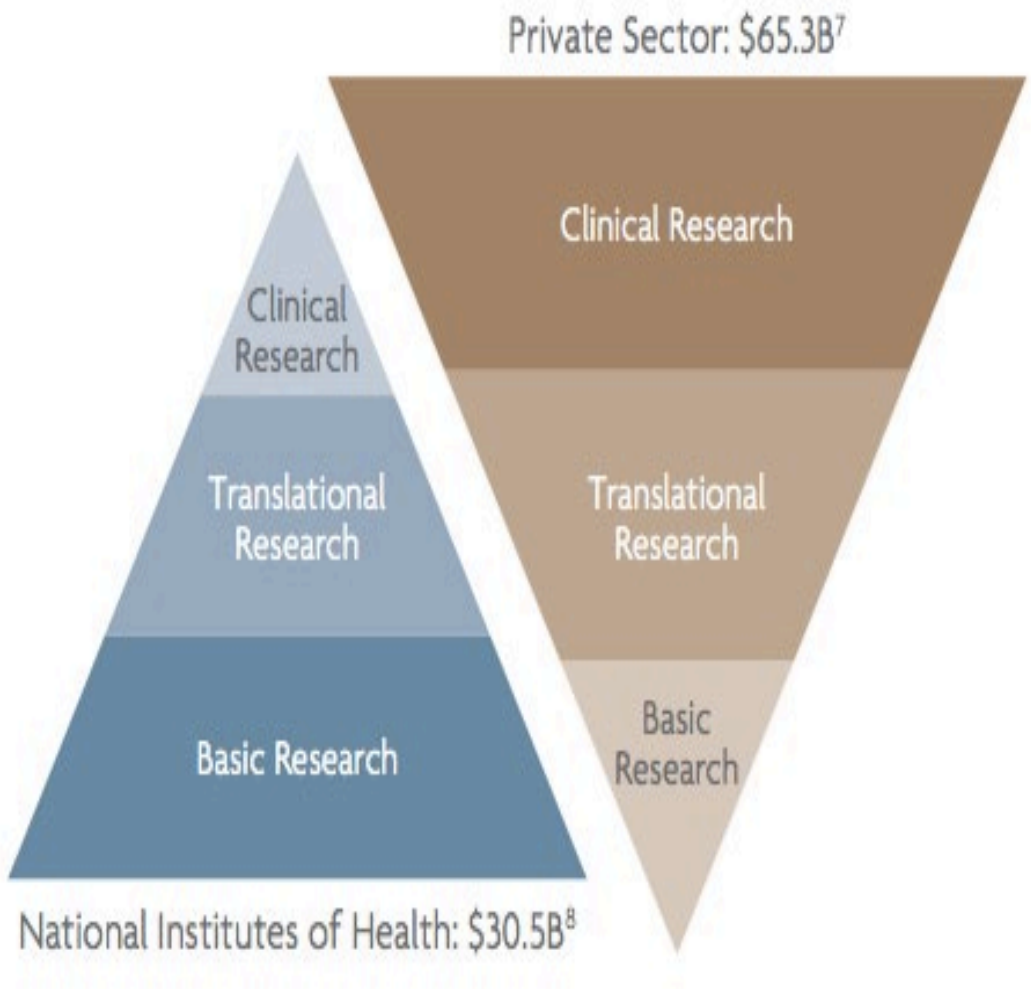
Environmental research

- Toxicity of exogenous agents (hazard identification)
- Man made chemicals or naturally occurring agents
- Includes radiation and physical agents.
- End point is risk assessment

Toxicologic Pathology Research

- Basic research – emphasis on in silico, in vitro, gene based studies
- Translational research – importance of animal studies – e.g., safety assessment, efficacy studies, animal models, risk assessment
- Clinical research e.g., target species, generally in humans, drug trials, epidemiology

Complimentary Roles of Government/ Academia and Industry in Research and Drug Development

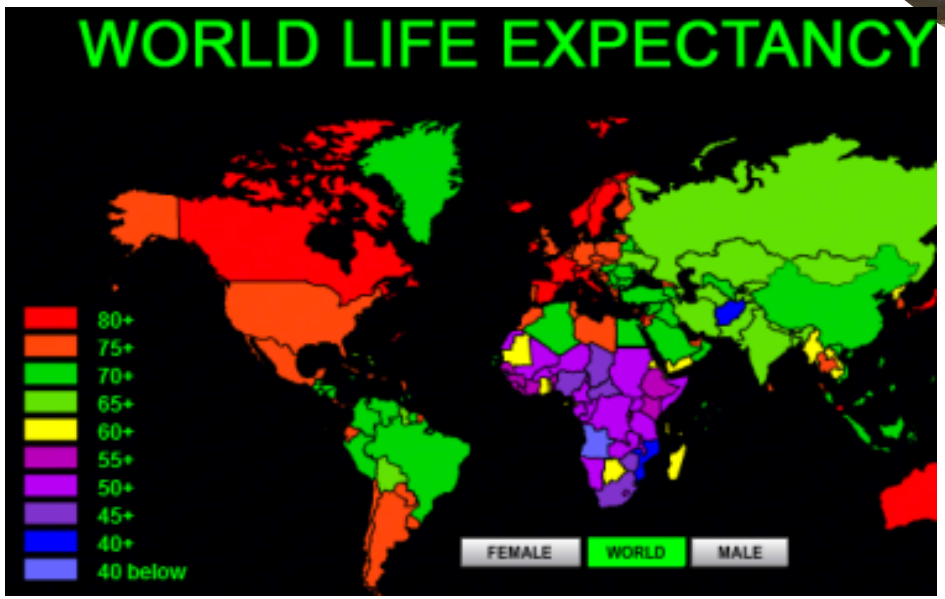


Spending is for 2009. Private Sector is estimated.

Source: Adapted from E. Z

Benefits of Biomedical Research

- Brazilian human life span - increased from 46 yrs in 1945 to 77 yrs in 2020
  - But COVID-19 cut off almost 2 yrs by 2021
- Quality of life improved
- Similar benefits for animals



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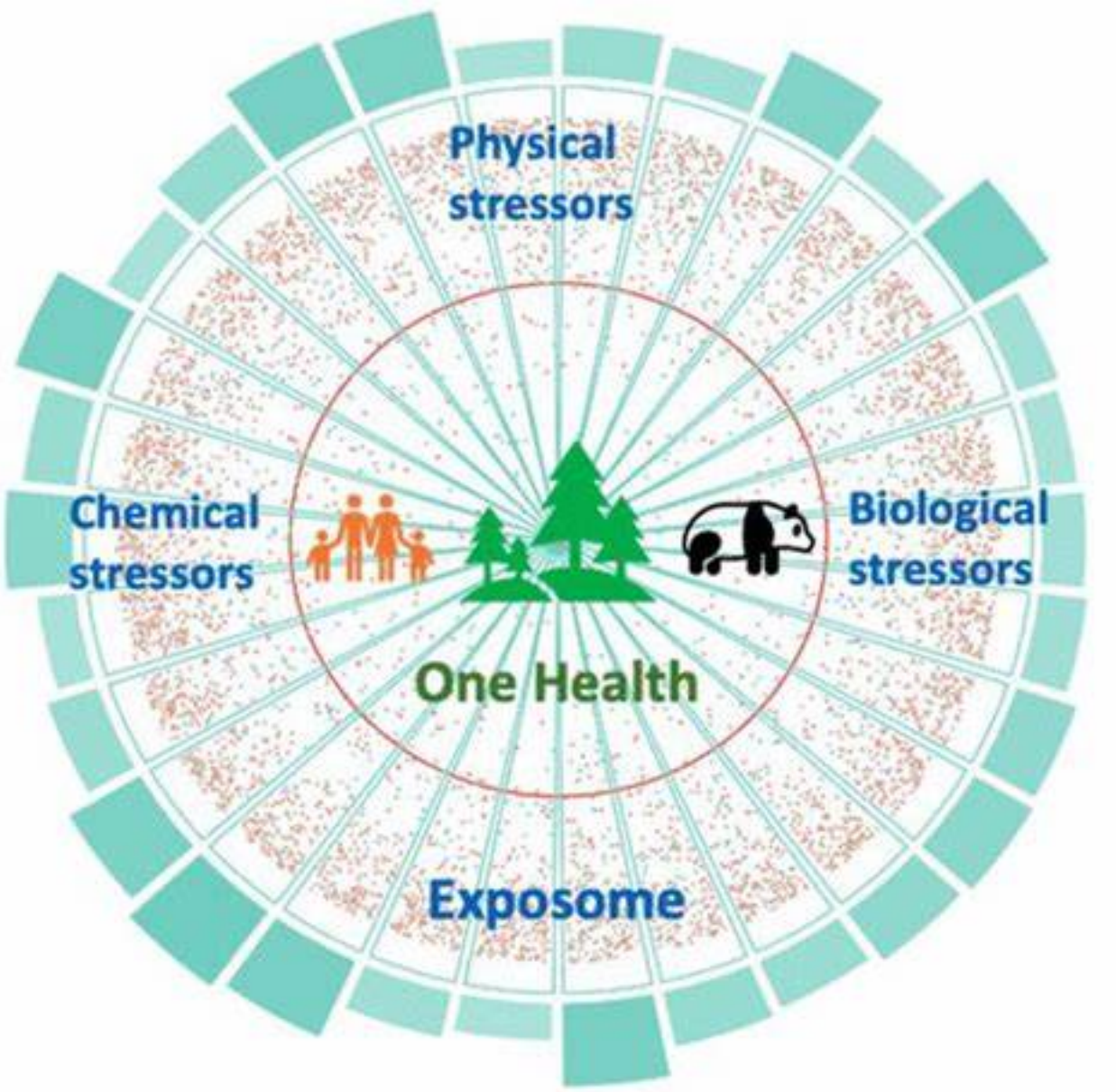
One Health

- Interconnectedness of people, animals and environment (ecosystem) in health arena
- Concept existed for centuries - Hippocrates 400 BC
- Recent momentum
  - Zoonotic (spread between humans and animals) and vector borne (infected animals to humans via insects) diseases e.g., COVID-19
  - Environmental contamination e.g., Rio Deuce iron ore spill in Brasil
- Use of animals in human and animal drug development

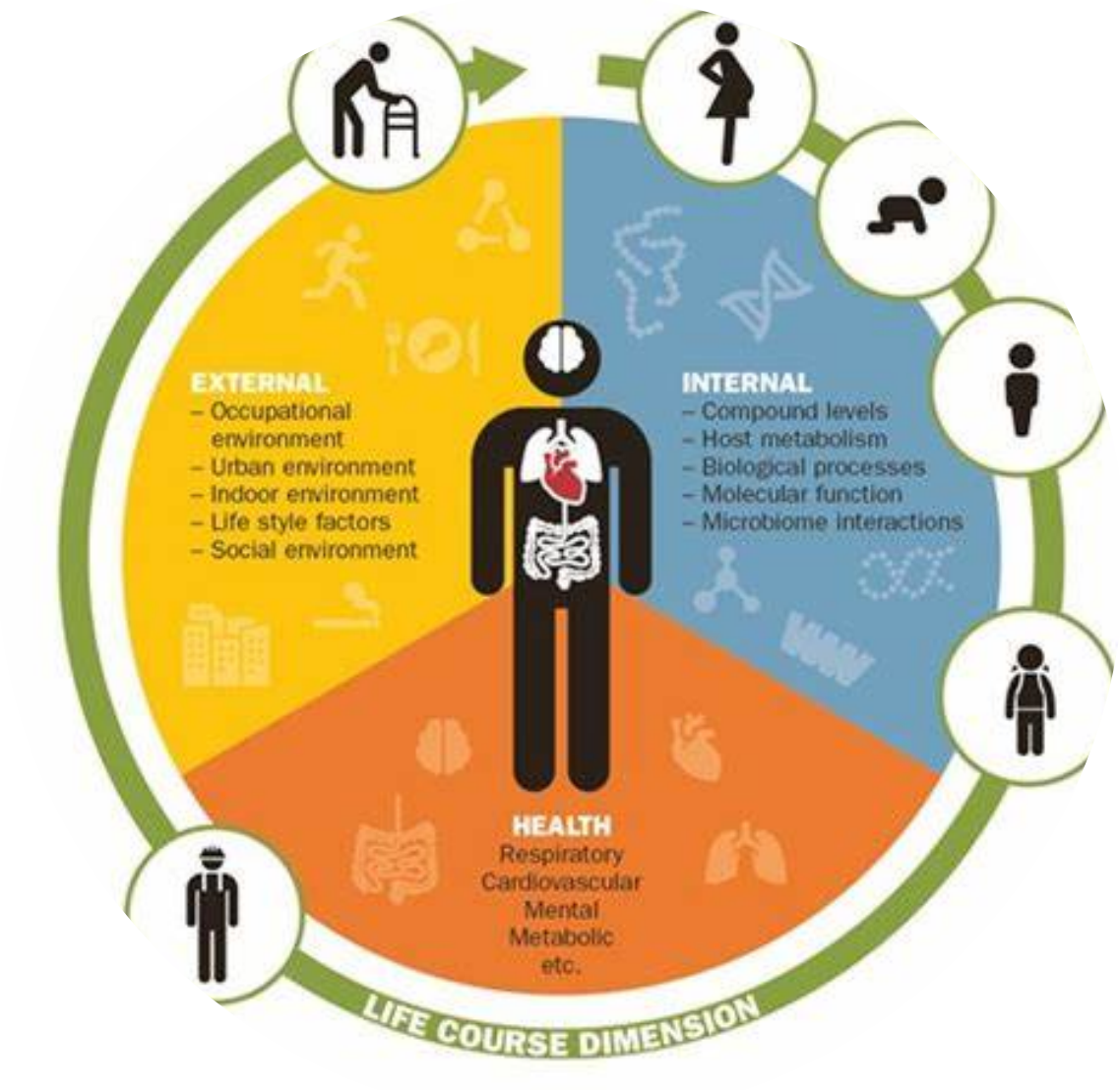


- Risk assessment of stressors
- Medical therapies

Need for Toxicologic Pathology

Exposome

- Personalized exposure menu of chemicals, drugs, microbes and other stressors over lifetime, beginning in utero
- Environment and gene-environment interaction
- Exposotype reason for individual response to xenobiotics
- Biomarkers of exposure – “omics” –use in epidemiology and chemical risk assessment – adverse outcome pathways (AOP)
- Leads to need for personalized medicine
- Same considerations for laboratory animals in research setting



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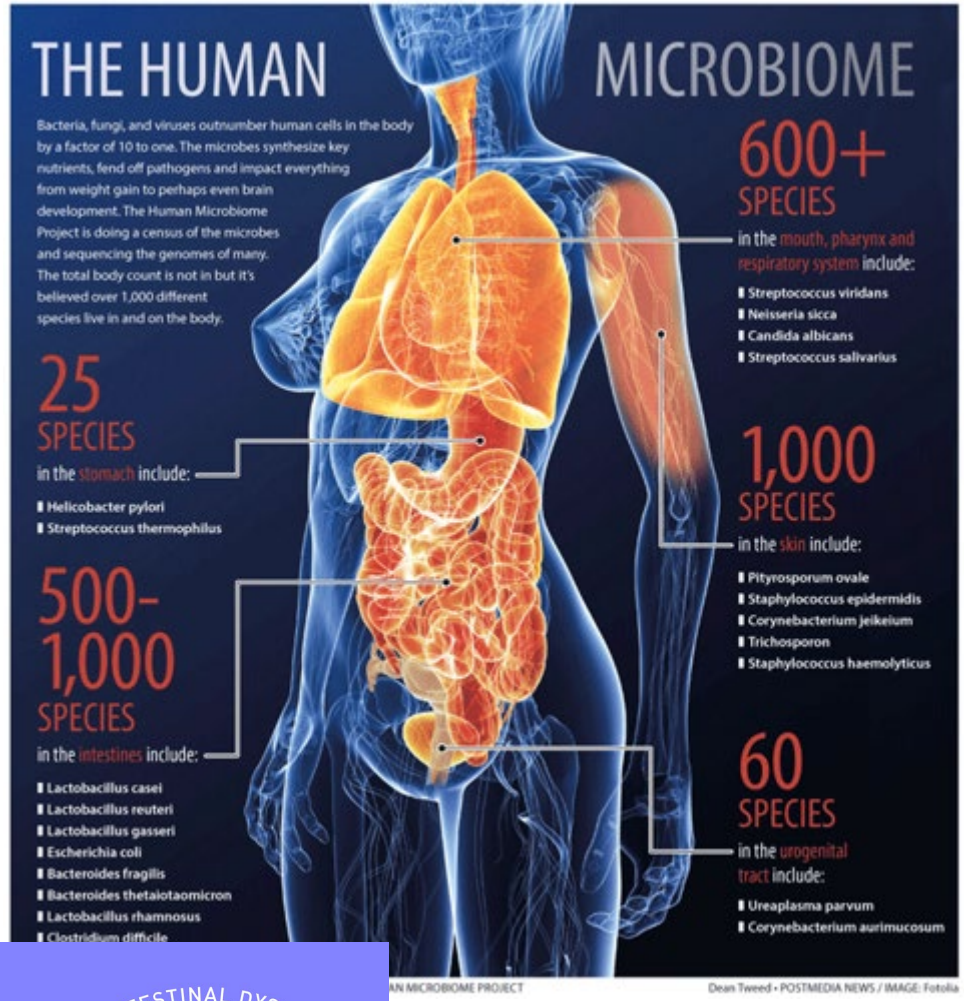
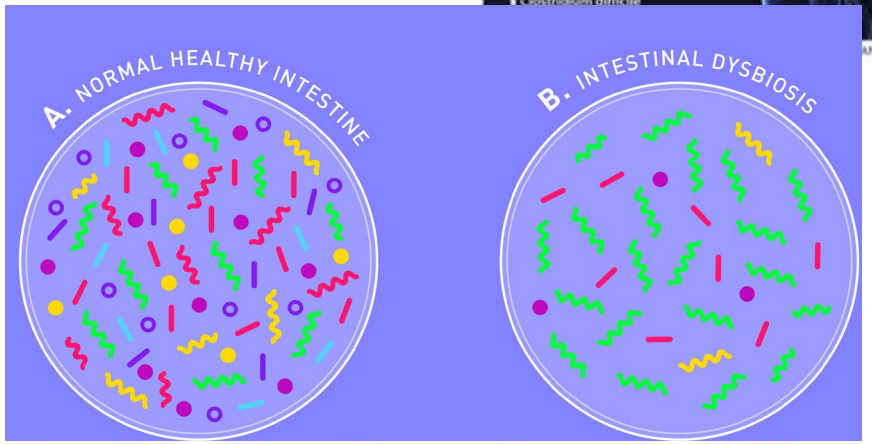
Microbiome (another organ?)

- Eukaryotic, bacterial, viral -up to 10x more microbial cells than human
- Collective genome >100 times human genome
- Metagenomics for research
- Dysbiosis= dysregulated (abnormal) microbiota. Common to many diseases including obesity, diabetes, colon cancer, and IBD



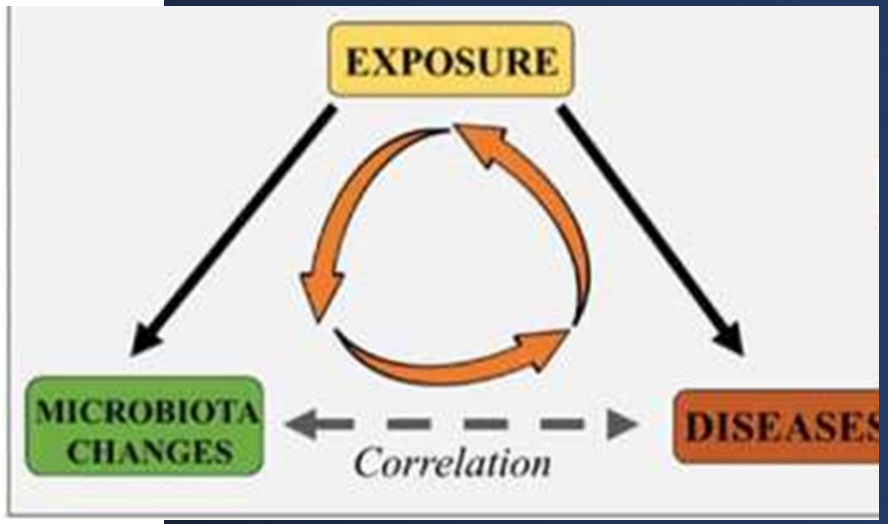


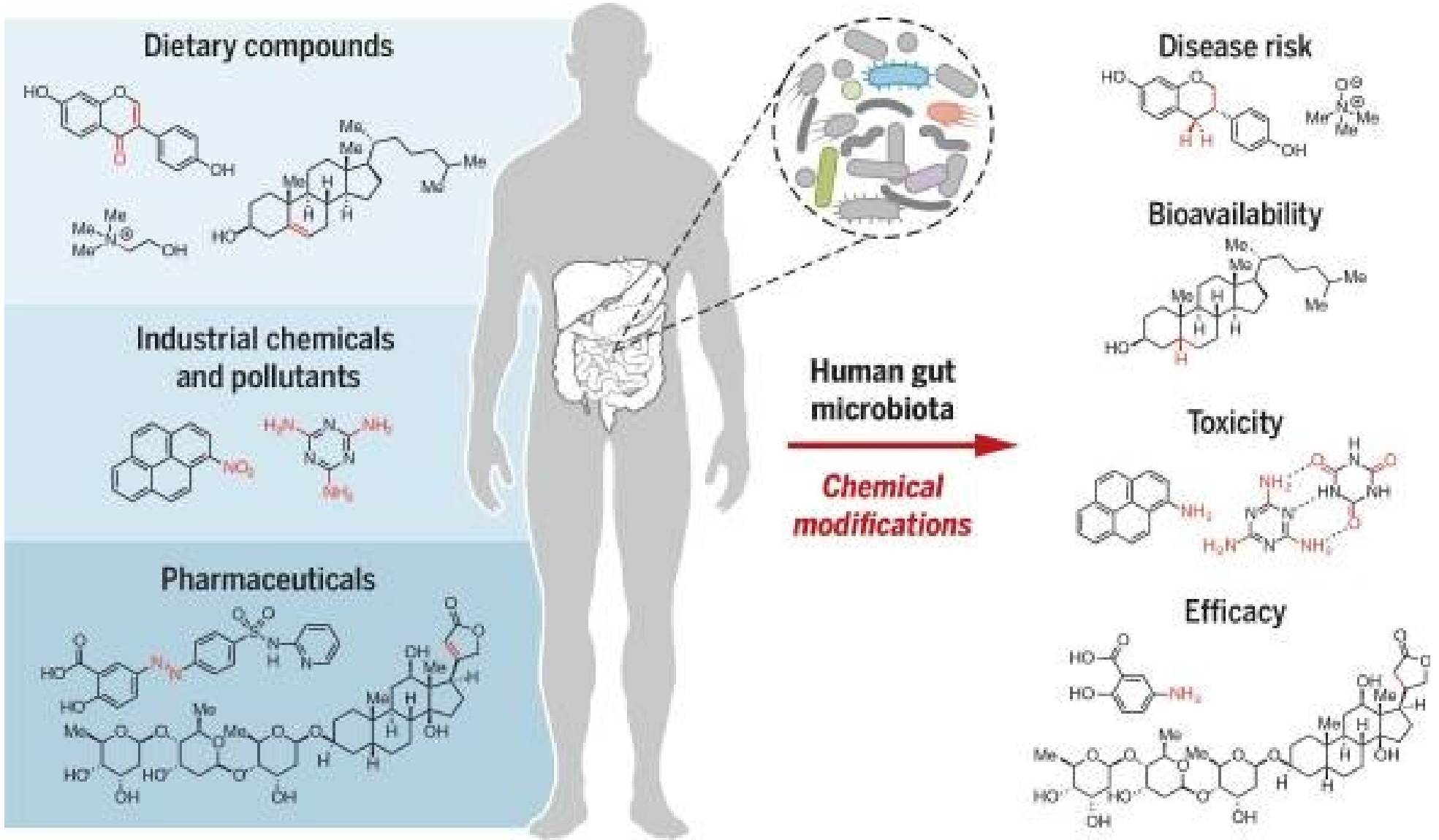
What Do Microbes Do For Us?

- Provide ability to harvest nutrients
- Produce additional energy otherwise inaccessible to the host
- Produce vitamins
- Metabolize carcinogens and other xenobiotics
- Prevent colonization by pathogens
- Assist in the development of a mature immune system
- Properties of both microbiome and host important - microbial composition & multi ‘omics (i.e., transcripts, proteins & metabolites) from microbiome and host studied

Microbiome-Associated Conditions

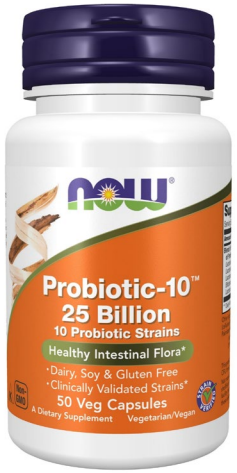
- Skin: eczema, psoriasis, acne
- GI/oral: esophageal adenocarcinoma, necrotizing enterocolitis, IBS/IBD, ulcerative colitis, Crohn's Disease
- Urogenital: bacterial vaginosis
- Cardiovascular disease (Hazen Curr. Opin. Lipidol. 25: 48, 2014)
- Obesity (Ley et al. PNAS 444:1022-1023, 2006)
- Diabetes
- Immune disorders
- Mental health conditions





Koppel et al, Science. 2018; 356(6344): eaag2770.

Diet/Drugs/Xenobiotics Can Alter Microbiome



- Food sources influence growth of different bacteria – health and affect disease susceptibility
- Artificial sweeteners – e.g., saccharin induced health effects mediated via dysbiosis
- Antibiotics e.g., vancomycin, ampicillin, streptomycin, metronidazole
  - Increased susceptibility to *C. difficile*, autism?
- Heavy metals e.g, inorganic arsenic, lead
- Pesticides – e.g., glyphosate (bees), organophosphates (mice)

Chi et al, Gut Microbes 2021, 13(1):1921912

Gut Microbiome Can Influence Drug/Xenobiotic Biotransformation

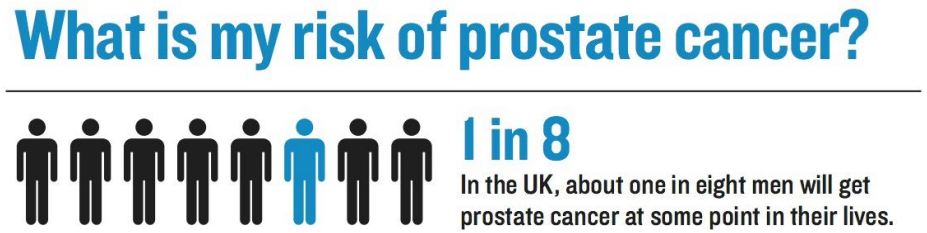
- Drugs
  - Activation: lovastatin, sulfasalazine
  - Inactivation: digoxin
  - Toxicity: irinotecan (chemotherapy) – reactivation by bacterial beta-glucuronidases in gut
- Xenobiotics
  - Arsenic, etc
- Aryl Hydrocarbon (Ah) receptor activated by metabolites from microorganism metabolism

Gut Microbiome Can Influence Drug Bioavailability

- Gut bacteria accumulate many common medications decreasing effectiveness and changing bacterial composition and metabolism (Klunemann et al, 2021, Nature)
  - *In vitro* and *C. elegans* studies
  - Examples: antidepressants (duloxetine), antidiabetic (rosiglitazone), asthma (montelusk)
- Further evidence for need for personalized medicine
- Possible new targets to modulate disease, treatment resistance and side effects

Gut Microbiome and Prostate Cancer (PC)

- Prostate cancer is second most common cancer in men, approx. 1 in 8 men will develop prostate cancer
- Role of short-chain fatty acids (SCFAs) (Matsushita et al, Cancer Sci 2021; 112 (8), 3125)
  - PC (mouse model) promoted by bacterial short-chain fatty acids (SCFAs)
  - Japanese men with high risk PC had significantly increased SCFA producing bacteria than low risk/negative PC
  - Potential for biomarker

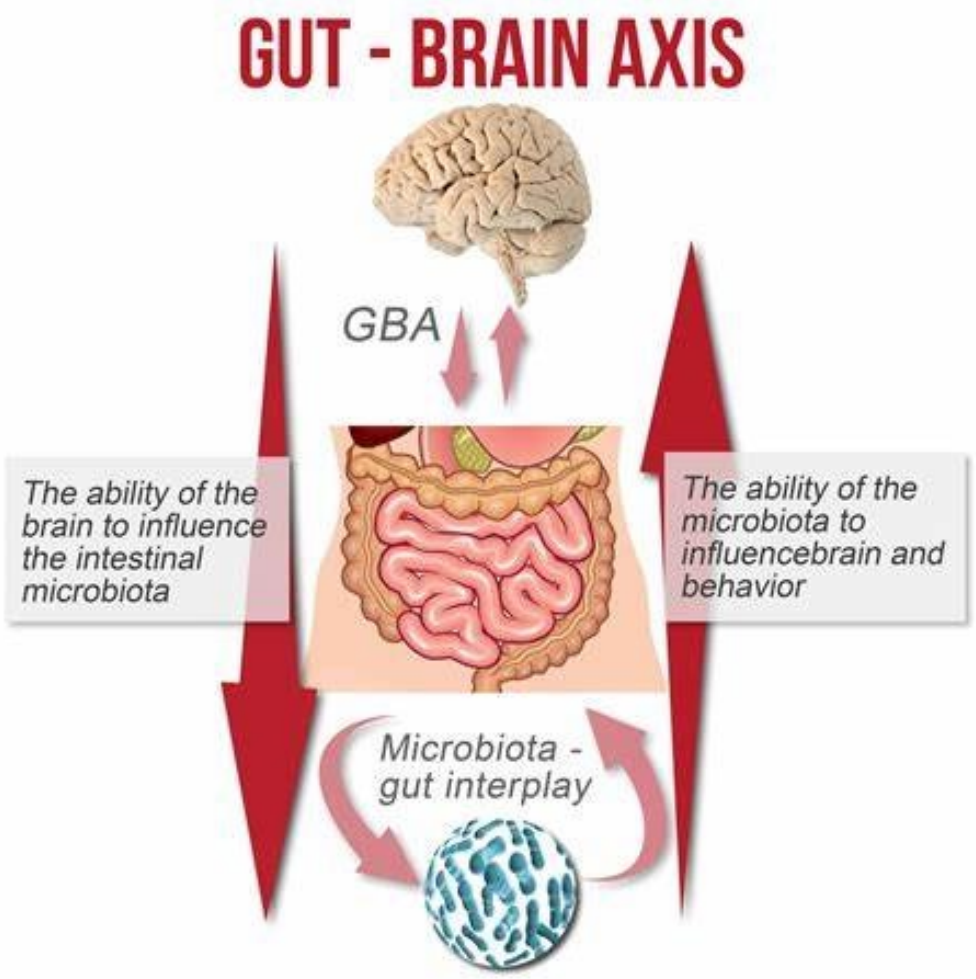


Gut Microbiome and Prostate Cancer (PC) continued

- Androgens (testosterone) drive progression of PC
- Men with PC receive androgen deprivation therapy (ADT) (chemical castration) as first line treatment
  - become resistant to ADT after about 2 years
- Potential mechanism of ADT resistance (Pernigoni et al, Science, 2021, 374: 216)
  - Mice PC model + castration (ADT equivalent) led to microbiome shift
  - Altered microbiome converted precursors to active androgens (drug resistance)
  - Fecal microbiota transplantation (FMT) from resistant men/mice to PC mouse model led to PC growth

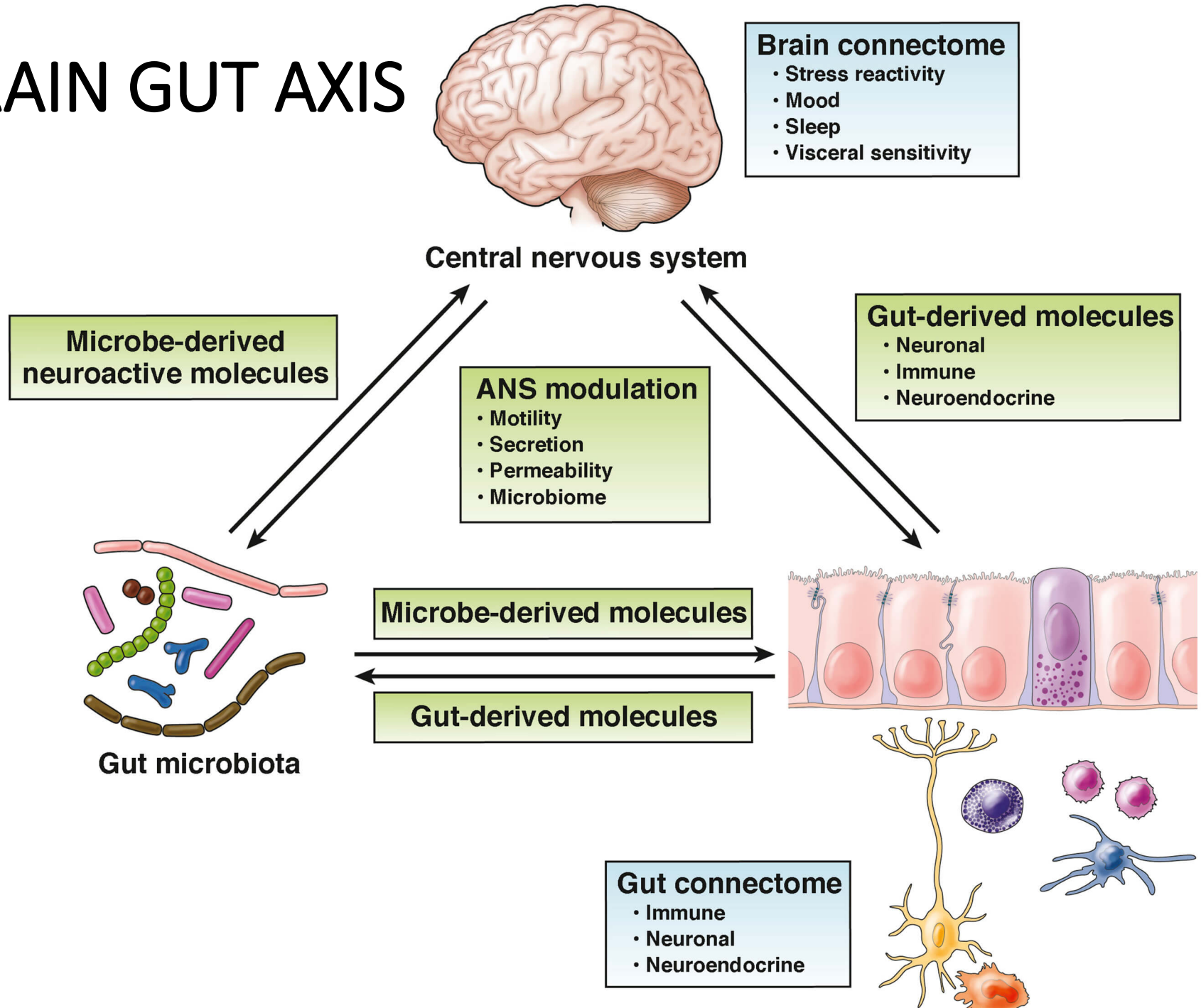
Microbiota-Gut/Organ Axes

- Small molecules produced by microbes are absorbed and influence host physiology (e.g., serotonin or 5HT)
- Gut/brain
- Gut/intestine
- Gut/liver
- Gut/lung



MICROBIOTA BRAIN GUT AXIS

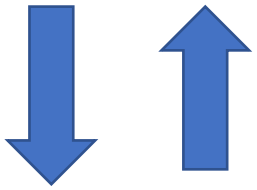
Vital.ly hub



Microbiota-Gut-Brain Axis

Healthy Status

Healthy CNS function

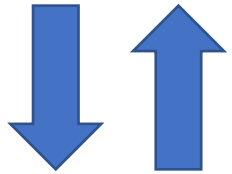


Normal gut physiology

- Physiological levels of inflammatory cells/mediators
- Normal gut microbiota

Stress/disease

Alterations in behavior, cognition, emotion, nociception



Abnormal gut function

- Increased levels of inflammatory cells/mediators
- Intestinal dysbiosis

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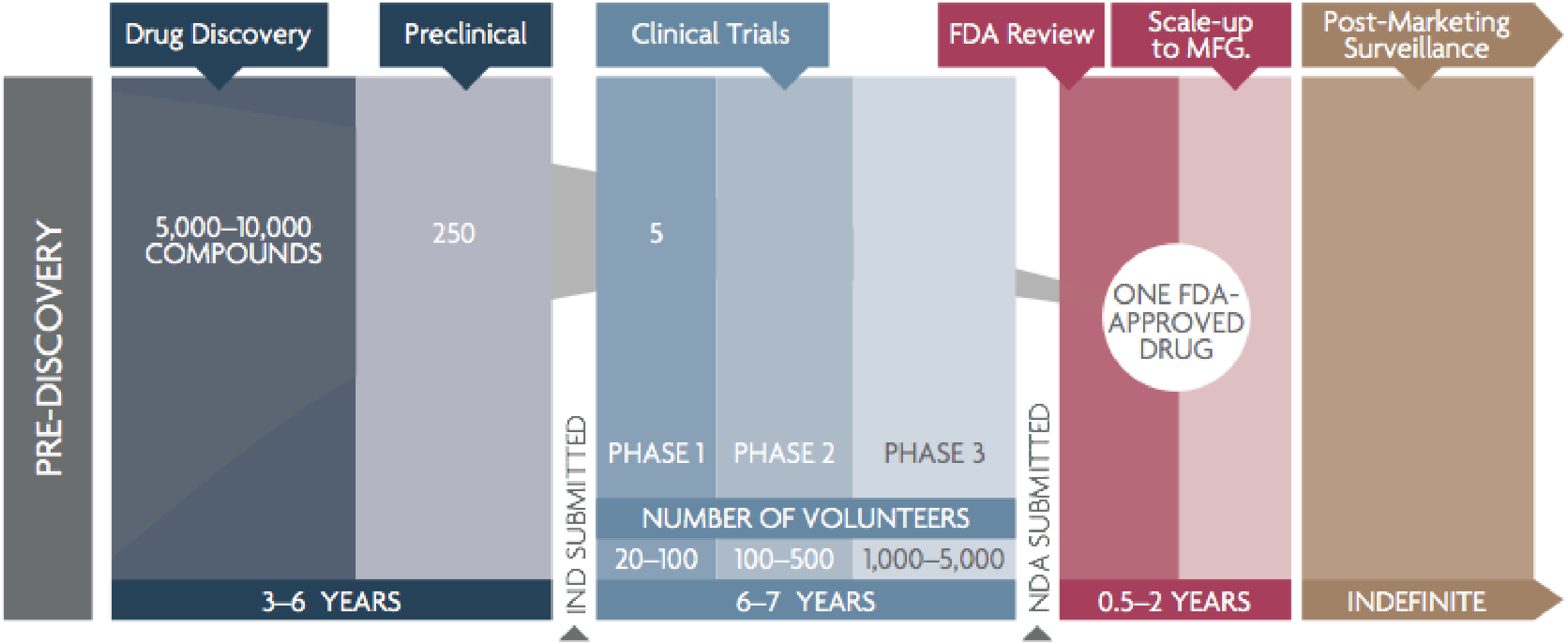
Drug Discovery and Development One Health: Finding the Cure

- Inventors
- Investors/shareholders
- Business persons
- Chemists
- Biochemists
- Pharmacologists
- Pharmacokineticists
- Toxicologists
- Toxicologic pathologists
- Lawyers
- Immunologists
- Microbiologists
- Clinicians
- Biostatisticians
- Molecular biologists
- Veterinarians

Drug Discovery and Development

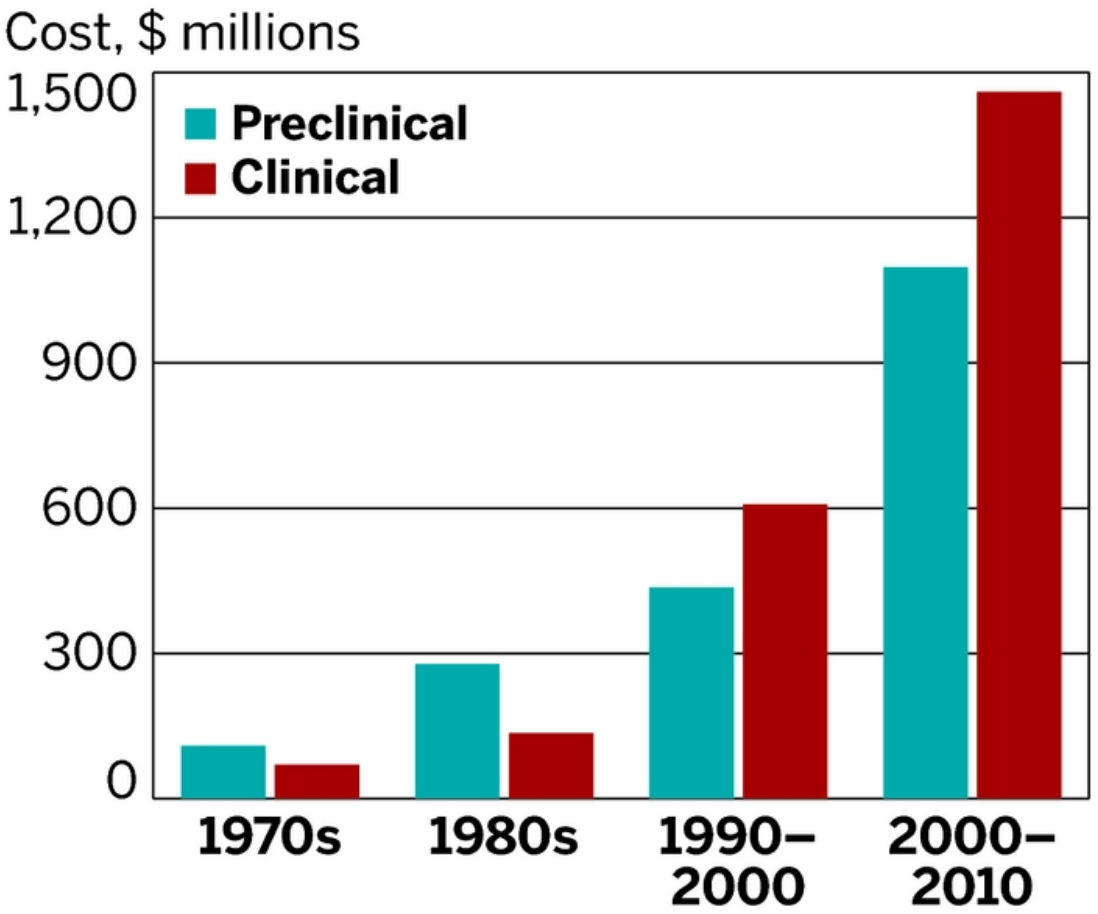
- Discovery – basic research – not GLP (Good Laboratory Practice)
- Safety and Efficacy Assessment (animal testing) – GLP, regulatory constraints
  - Efficacy – **does it work?**
  - Safety – **is it safe?** In animal model? In people?
- Clinical trials – people - phase I, II and III
- In US, Food and Drug Administration (FDA) reviews and approves drugs
- Post marketing surveillance

Finding the Cure: Drug Development
10 + Year Window, $3 - 6 billion



Source: PhRMA[5]

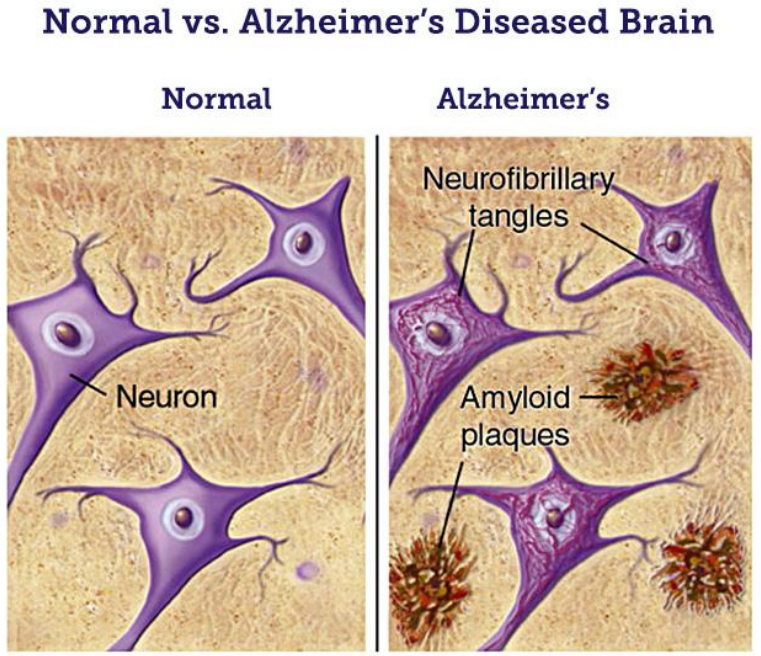
Cost to Develop New Pharmaceutical Drug in US Now Exceeds $2.6B (up to $6B)



COVID-19 VACCINE

- SARS-CoV-2 discovered December 2019 in Wuhan, China
- WHO – Public Health Emergency - 30 January 2020; Pandemic - 1 March 2020
- Deaths - global 5m, USA 800,000, Brazil 650,000
- Vaccines developed/approved in record time, first by end 2020
  - New technology - mRNA e.g., Moderna, Pfizer-BioNTech
  - Traditional - adenovirus vector e.g., Janssen, AstraZenica, Sputnik
- Animal research – mice, hamsters, nonhuman primates, rats, rabbits
- Vaccines also for mink, zoo animals

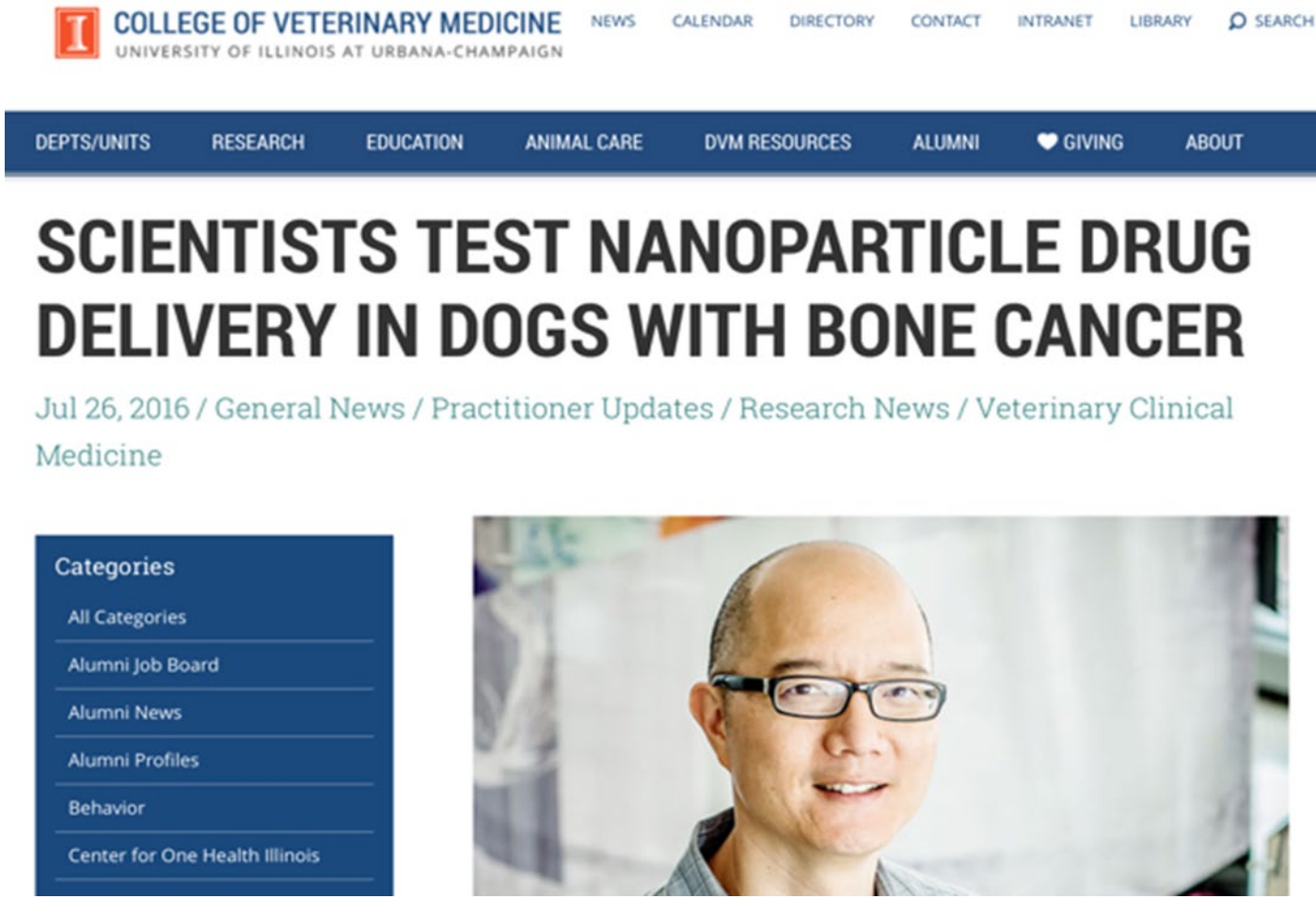
Alzheimers Disease (AD)



- Progressive neurodegenerative disease leading to memory loss, dementia and ultimately, death
- Marked increase in Brazil
- Beta amyloid containing plaques in brain correlate with AD
- New therapies developed using rodents, tested in non-human primate models

CANCER

- Development of more effective treatments, gene and immunotherapy, with help of dogs, rodents and other species
- At UI, an engineer and veterinarian tested a bone cancer drug delivery system in dogs (standard animal model is mouse).
- Dogs get naturally occurring bone cancers similar to humans... – so dog as animal model and target species as well



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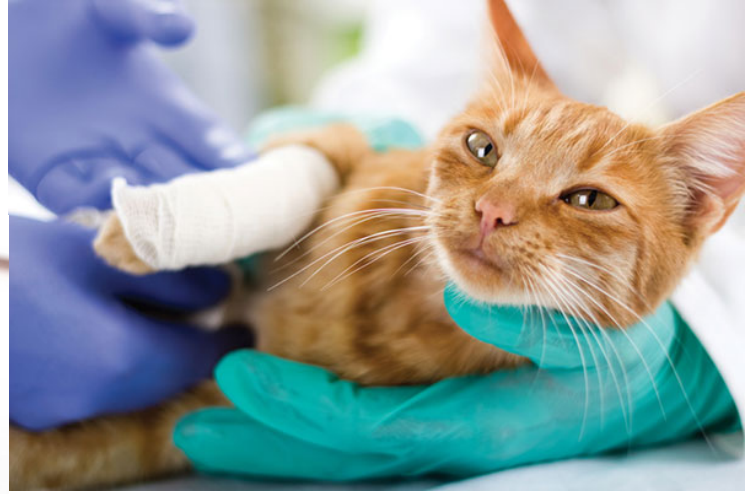
  - Animal Use
  - Study Design Considerations
  - Considerations in Pathology Evaluation
    - INHAND – nomenclature
    - Quantification
- Resources

Biomedical Research and the Contribution of Toxicologic Pathologists

- Integrative (whole organism) biology expertise – trained to evaluate functional, biomarker and morphologic data
- Comparative expertise – from mice to men
- Art and science of observation - pattern recognition – interpretation of histopathology and other data points such as omics
- Comparative biology expertise - translation of animal data to identify hazards, assess safety and manage potential risks
- Work and communicate well in team setting – appreciate collaborative nature of research

Biomedical Research and Use of Animals



- To understand a disease, need living system with a genetic makeup similar to humans
- Computer models or cells grown in a dish cannot provide accurate picture of e.g., infection, in a whole living system
- People share 95 percent of genes with mice and 98 percent with non-human primates, like rhesus macaque monkeys
- Carried out in an ethical and humane way
- 3Rs – replacement, reduction and refinement
- Animal research advances veterinary and human medicine and helps pets and wildlife live longer, happier, and healthier lives

Toxicology Study Design Considerations

- Pathologist needs to be involved during planning stage
- Hypothesis important in decision making
- Species selection
  - Toxicity/safety assessment or efficacy
  - “Best” model - comparative anatomy, physiology, pharmacokinetics, etc
  - Source of animal – pathogen status of animal
- Animal number – consult with statistician, consider 3Rs (replace, reduce, refine)
- Route of administration – potential for treatment induced lesions
- Duration of administration and time to termination
- Housing – potential for lesion induction
- Evaluation end points

Toxicology Study Design Considerations (cont.)

- Dose/time response
- Acute vs chronic
- Reversible vs nonreversible
- No observable effect level – NOEL
- No observable adverse effect level – NOAEL
- Adverse or adaptive effect - definition - clinical effect? Reversibility?
- Biological vs statistical differences
- Risk assessment - extrapolation to human/other target species

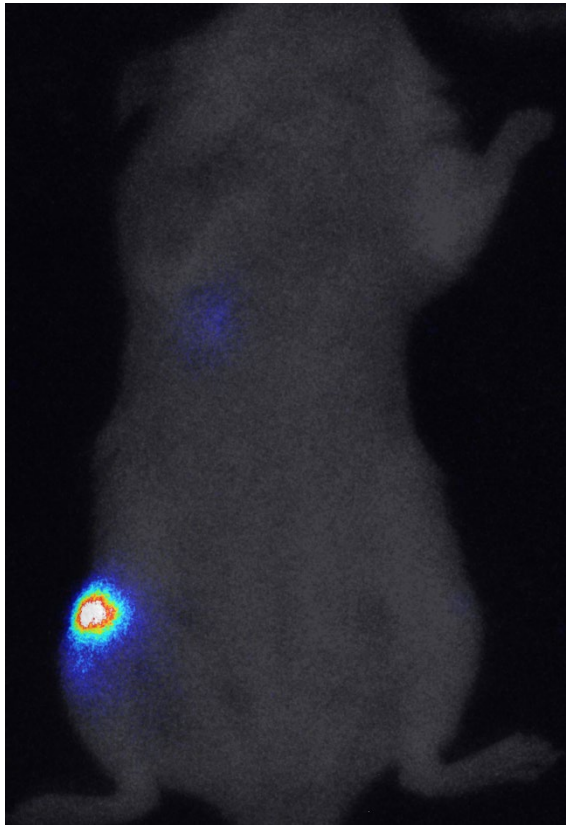
“In Life” Toxicity Evaluation

- Behavioral changes
- Weight changes
- Ophthalmologic examination
- Clinical Pathology (biomarkers) – “in life” or terminal procedure
  - Hematology
  - Clinical chemistry
  - Urinalysis and urine chemistry
- Imaging - a relatively new and powerful tool

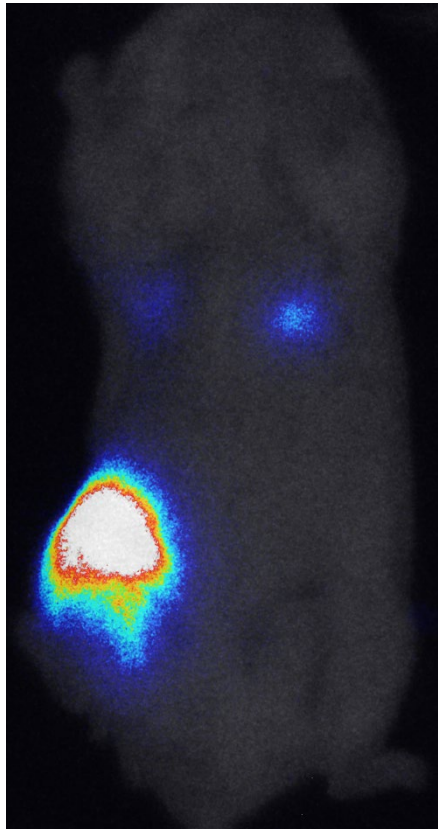
Bioluminescent Imaging: Day 20 Post-Injection of Mammary Tumor Cells in Tibia

(Cam and Helferich, 2015)

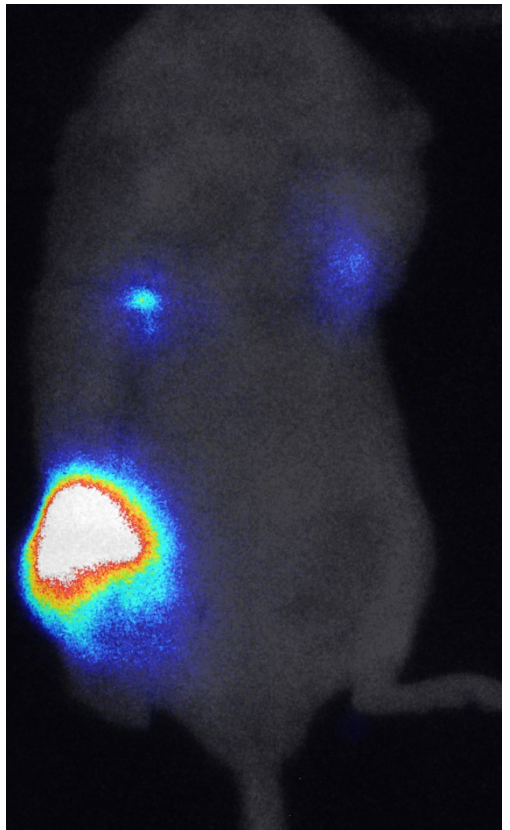
Low fat control



Low-fat Refried Fish Oil



High fat control



End of Life Toxicity Evaluation (STP Best Practices documents at toxpath.org)

- Organ weight changes
  - Absolute
  - Ratio of tissue/organ to body weight or brain
- Clinical pathology
- Anatomic pathology
  - Gross observations
  - Microscopic evaluation - histopathology
  - Special studies
    - Ultrastructural
    - IHC
    - ISH
    - Special techniques
- ‘Omics
- Other

Considerations in Pathology Evaluation

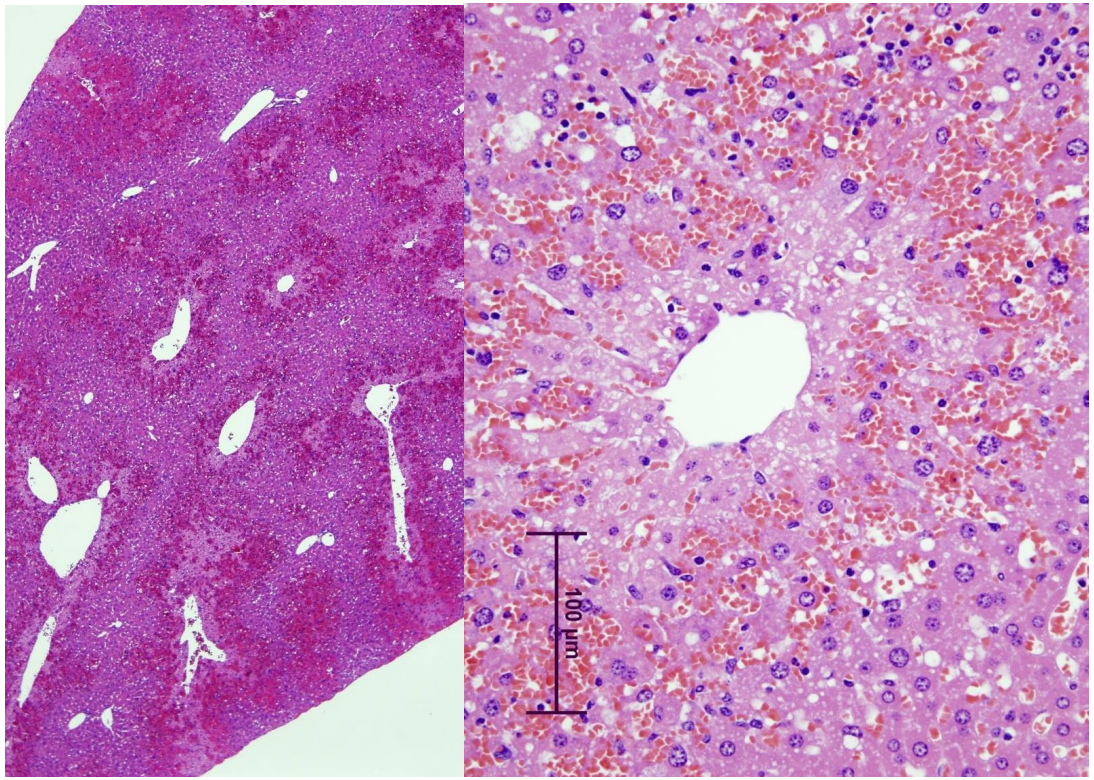
- Method of euthanasia – can induce alterations
- Tissues/fluids to be collected and for what procedures
  - Clinical pathology
  - Organ weights
  - Histopathology
  - Biochemistry
  - Molecular biology
- Morphological evaluation
  - Gross changes
  - Tissue collection/trimming – site, size, thickness -depends on endpoint - consistency
  - Fixation – organ specific, special study specific
  - Processed or frozen sections
  - Staining (at same time)
  - Special studies

Considerations in Pathology Evaluation (cont)

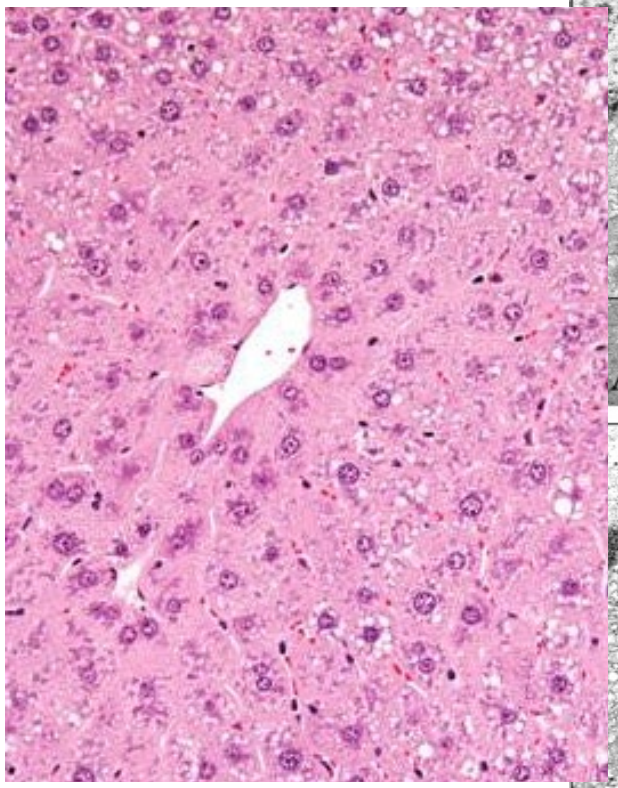
- Informed vs masked (blinded) evaluation. Knowledge of control and treated groups important.
- Standardized nomenclature for altered morphology
- Background changes - importance of control group – concurrent, source, etc (microbiome differences)
- Treatment-induced changes
  - Dose-response relationships including NOEL (no observed effect level) and NOAEL (no observed **adverse** effect level)
  - Grading/scoring of lesions for more quantitative data
  - Determination of adverse (harm to the affected individual) vs non-adverse treatment induced changes or background lesions

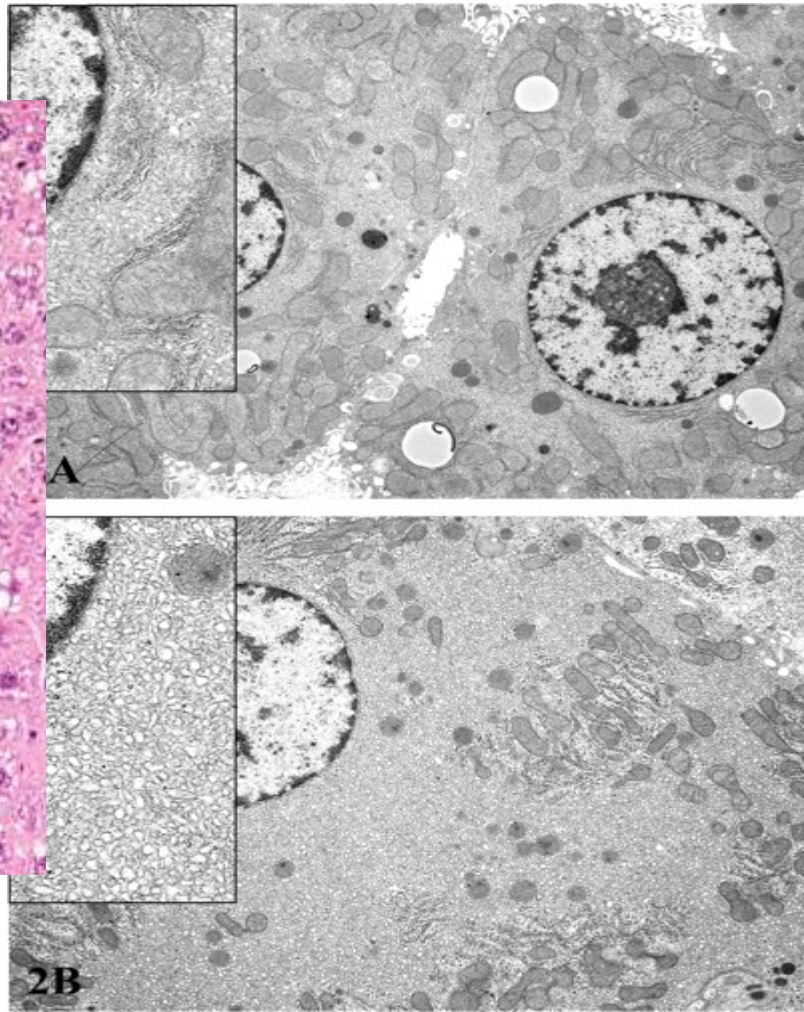
Adversity: Liver

Adverse: Necrosis



Non Adverse: Hypertrophy (SER proliferation)



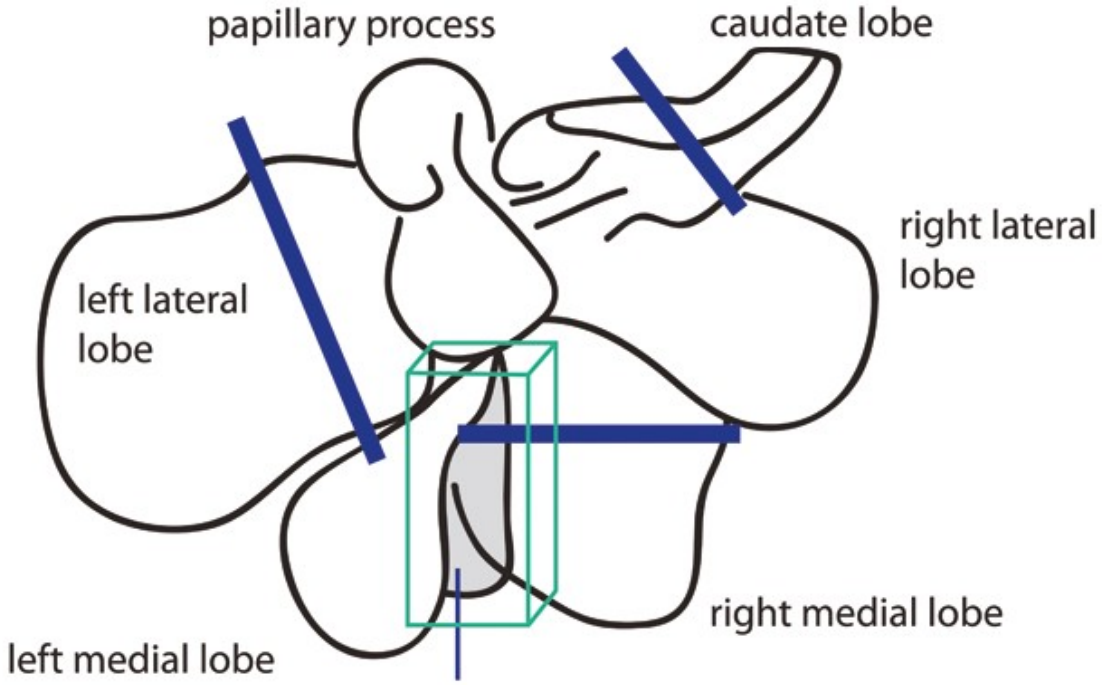


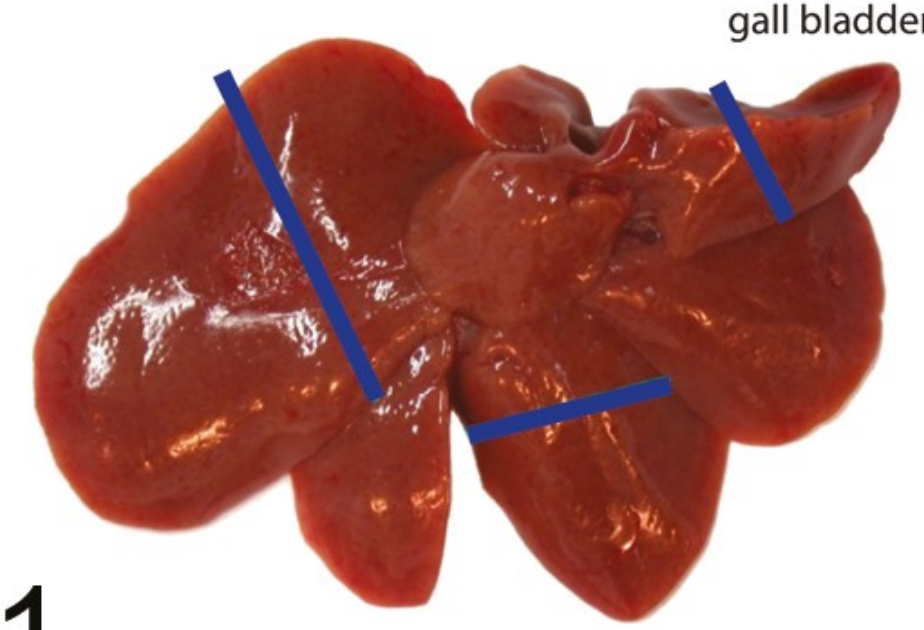
Standardized Terminology in Pathology Evaluation

INHAND – International Harmonization of Nomenclature and Diagnostic Criteria

- Background and induced lesions, also normal anatomy and histology
  - Rodent – organ based
  - Non-rodent – dog, rabbit, minipig, nonhuman primate
  - Fish – in progress
- Information on trimming and scoring in some publications
- All publications freely available at toxpath.org
- More info for STP members at GoReni.org
- Also see NTP Nonneoplastic Lesion Atlas at ntp.niehs.nih.gov/nnl
- Useful for all experimental pathology

GoReni: Gross appearance and tissue trimming recommendations for a normal rodent liver.





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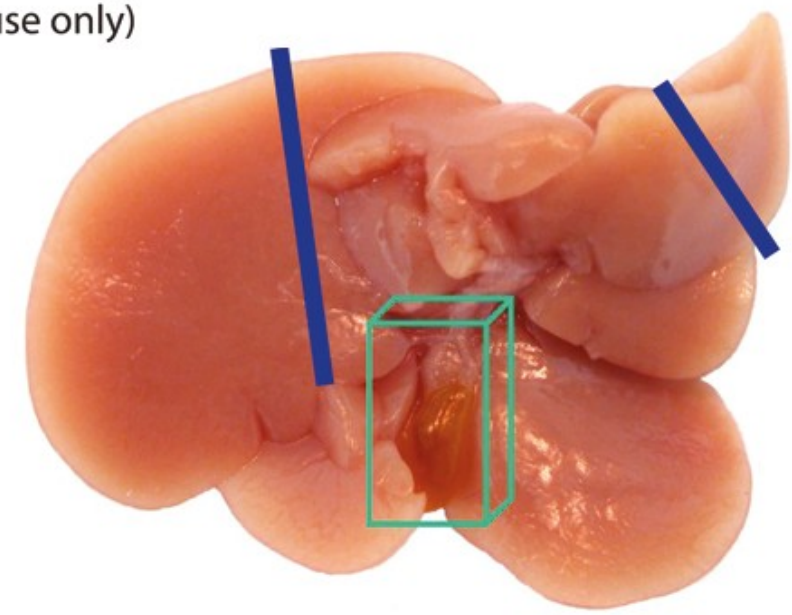
Rat liver, visceral aspect



Mouse liver, visceral aspect, with gall bladder

GoReni.org
A descriptive approach for classifying inflammatory responses in the liver.

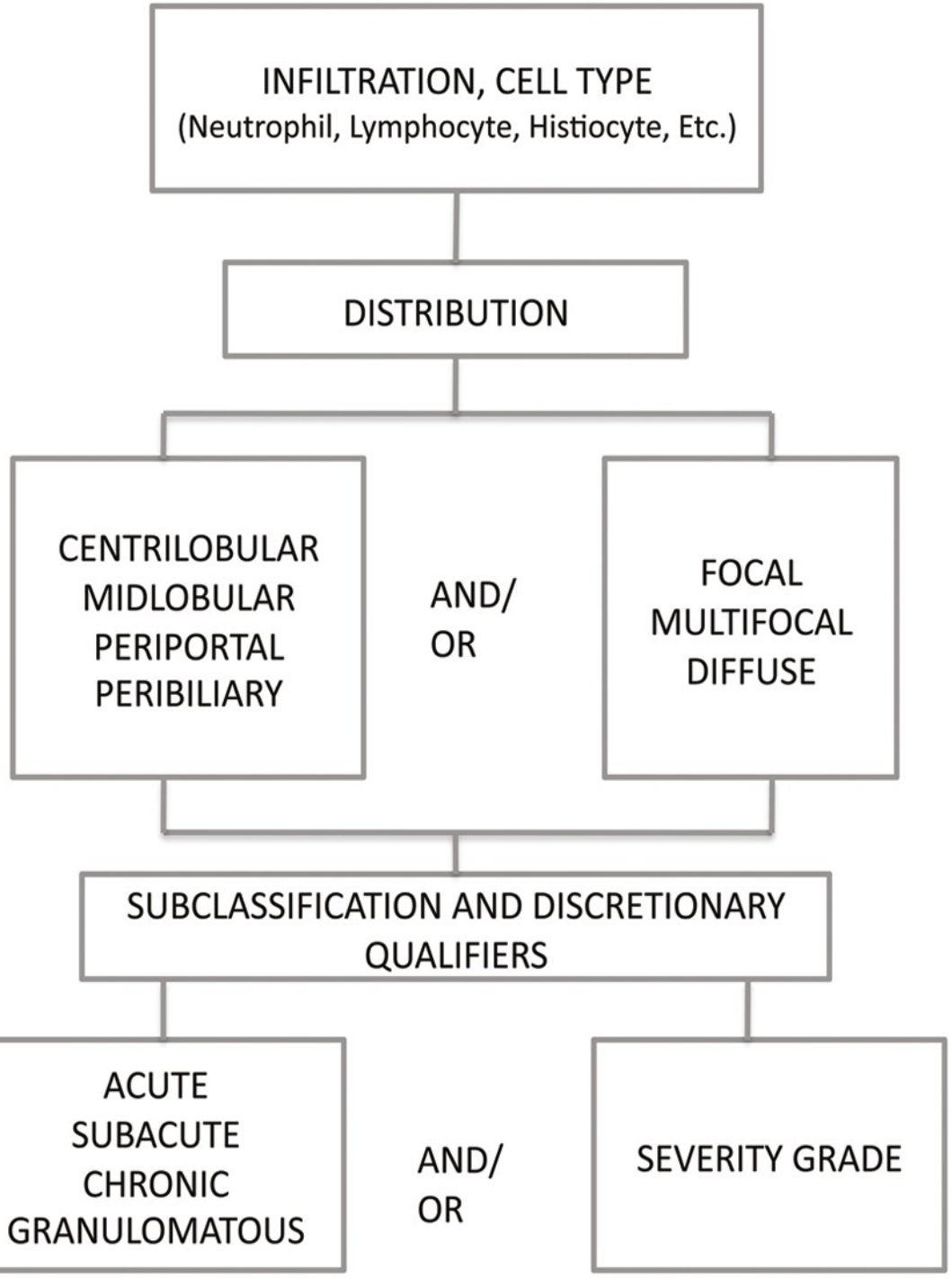
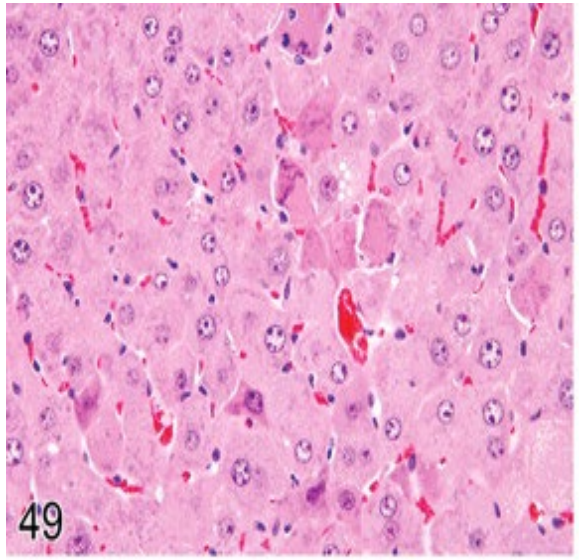
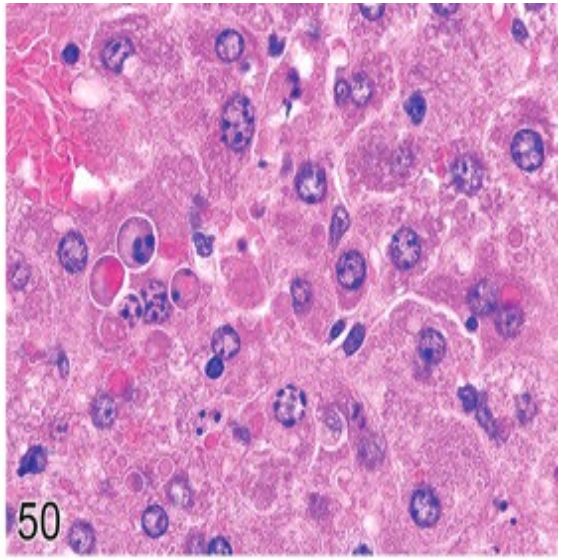


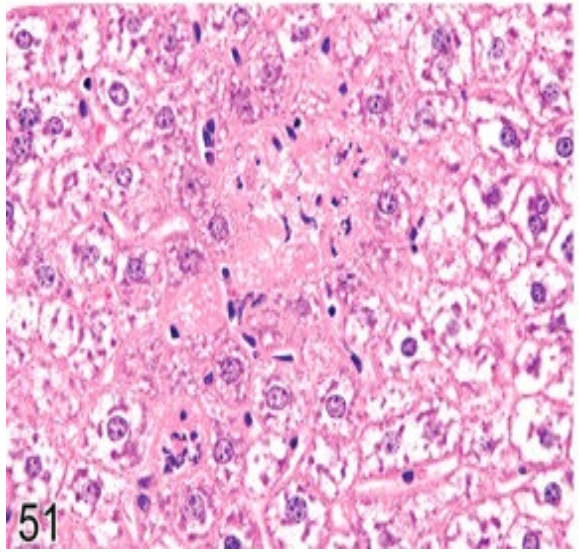
Figure 49. Mouse liver.

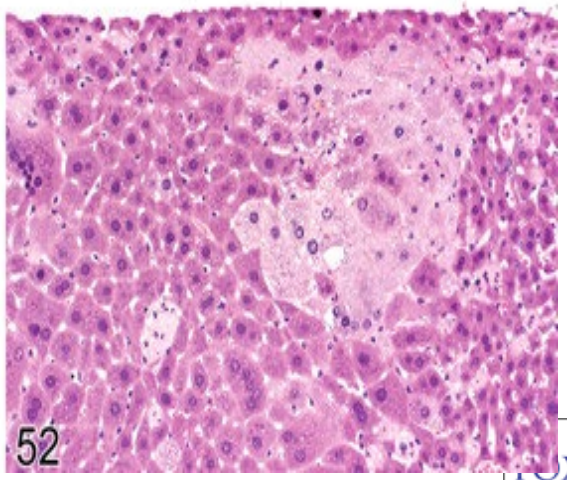
Apoptosis





Focal Necrosis



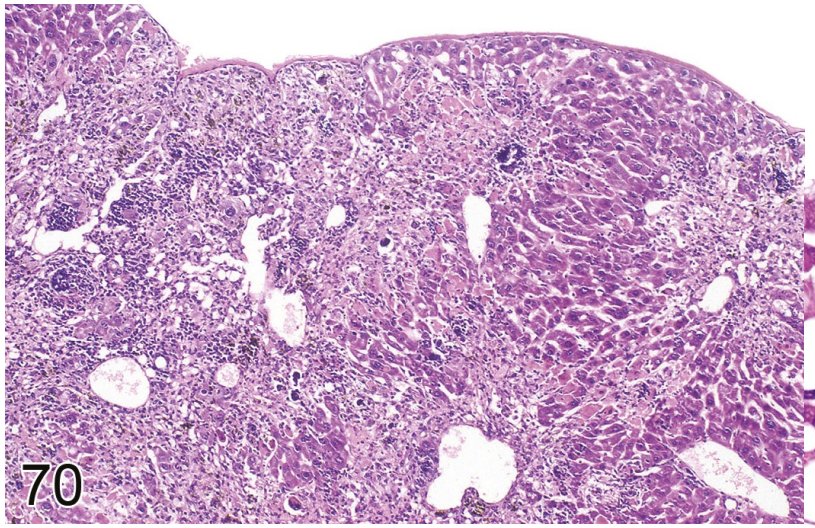


TOXICOLOGIC PATHOLOGY

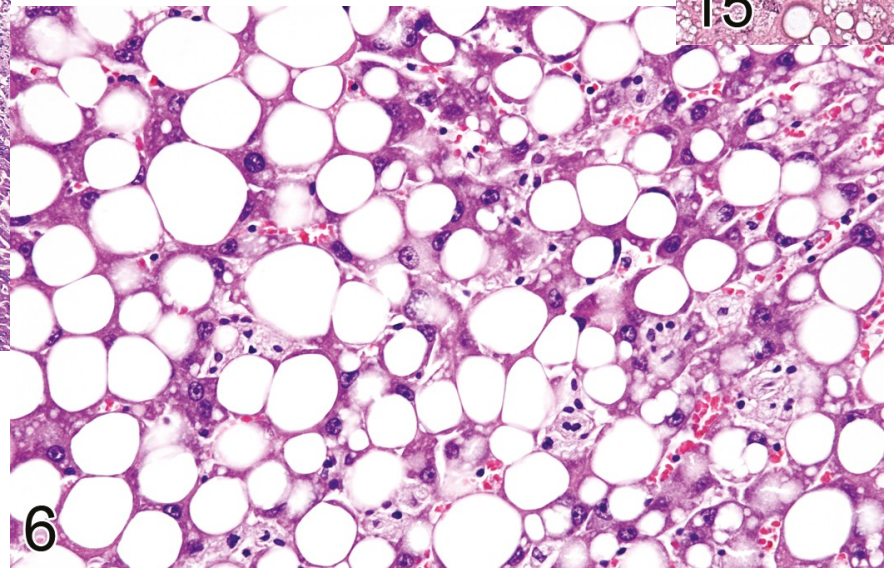
Thoolen B et al. Toxicol Pathol 2010;38:5S-81S

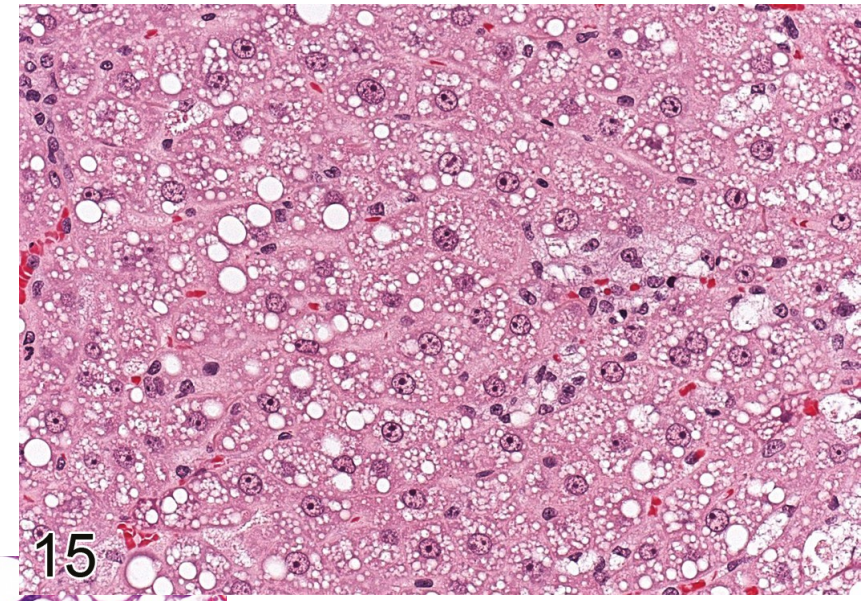
GoReni Liver Images

Generalized inflammation, postnecrotic mild fibrosis. Mouse hepatitis virus infection.



Macrovesicular fatty change.





Phospholipidosis

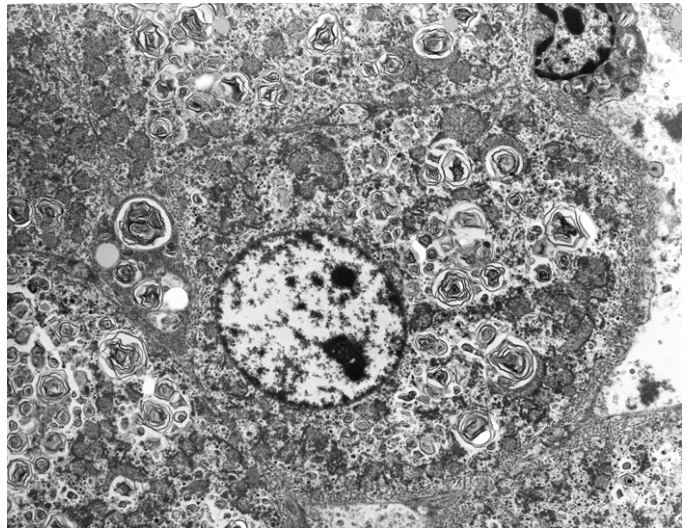


Table 2: Selected immunohistochemical stains that have been used to identify different cell types in liver sections.

Immunohistochemical stains of liver cells	
Cell type	**Antibody**
Hepatocytes	CK8, CK18
Bile canaliculi	Polyclonal CEA
Bile duct epithelium	CK7, CK19, AE1/AE3
Endothelial cell	Factor VIII, CD31, CD34
Exudate macrophages (monocytes)	ED1
Kupffer cells	CD68, F4/80, ED2, SRA-E5
Hepatic stellate cells (activated), myofibroblasts and smooth muscle cells	α-SMA
Dendritic cells	NLDC-145, OX-6
Oval cells	α-fetoprotein (AFP), CK20
Apoptosis	Bcl-2, Caspase 3 and 7
Proliferation markers	Ki67/MIB-1, PCNA

Geller, Dhall, and Alsabeh (2008); Malhotra, Sakhuja, and Gondal (2004); Hurlimann and Gardiol (1991);Davenport et al. (2001); Kashiwagi, Kaidoh, and Inoué (2001); Faa et al. (1998).

Quantitative Pathology Data

- Advantages
  - Group/treatment effect delineation
  - Statistical analysis
  - Publication
  - Communication to non-pathologists
- Semiquantitative Data - Scoring
  - Criteria definition e.g., extent, severity, number of cells, length/area affected
  - Literature can provide scoring suggestions e.g., INHAND publications
  - Whole slide digital imaging can aid quantitation
- Quantitative Data - Stereology/morphometry
- Artificial intelligence (AI)/deep learning technology under development

GoReni: Liver Grading Scheme

Table 3: A sample grading scheme for focal and multifocal liver lesions (modified from Hardisty and Eustis 1990; World Health Organization 1978; Derelanko 2000).

Severity	Proportion of liver affected	Grade	Quantifiable finding
Marginal or minimal	Very small amount	1	1-2 foci
Slight or few	Small amount	2	3-6 foci
Moderate or several	Medium amount	3	7-12 foci
Marked or many	Large amount	4	> 12 foci
Severe	Very large amount	5	Diffuse

Some Common Issues without Trained Pathologist

Species specific	Tissue/slide preparation	Pathology findings mis-interpretated
• Normal anatomical structures/processes called lesions • Background changes misinterpreted as treatment induced lesions • Sex specific morphology in some species	• Trimming • Improper fixation • Staining issues	• Adversity • Reversibility • Risk assessment

Integration of Study Findings

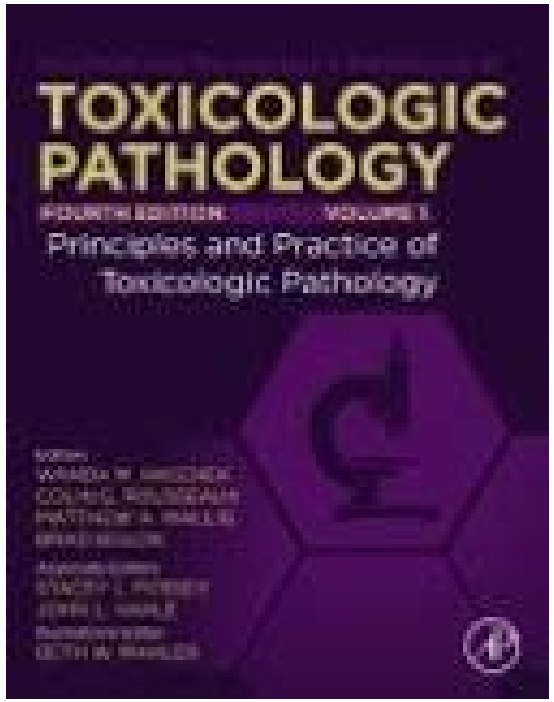
- Pathologist may
  - Issue pathology report only together with images if appropriate
  - Be responsible for integration of all study findings
  - Determine/suggest possible mechanism
  - Review report/manuscript to make sure integration and interpretation appropriate
- Pathologist should be included on authorship list or at least be acknowledged (no treatment related effect is still an important finding) – this should be decided upfront

Weight of Evidence in Risk Assessment

- Pathologic alterations
  - Are they adverse?
  - Progression
  - Reversibility
  - Organ’s functional reserve
- Kinetics
- Metabolism
- Mechanism of toxicity
- Variation in species sensitivity
- Other

Online Resources

- Society of Toxicologic Pathology (STP)
  - www.toxpath.org
    - INHAND publications (also Go RENI www.goreni.org if member STPs)
    - Best Practice documents
    - Annual Meeting - Student Travel and Other Awards
- National Toxicology Program (NTP) Nonneoplastic Lesion Atlas at ntp.niehs.nih.gov/nnl



Resources (continued)

- Greaves P (2011) Histopathology of Preclinical Toxicity Studies, Interpretation and Relevance in Drug Safety Evaluation, 4th Edition Elsevier Inc., Academic Press.
- Gopinath, C and V Mowat, (2014). Atlas of Toxicological Pathology. 2nd Ed, Humana Press.
- Haschek WM et al., eds. (2013) “Haschek and Rousseaux’s Handbook of Toxicologic Pathology”, 3rd Edition. Elsevier Inc., Academic Press. 4th Ed in progress, Volume 1 (2021) available online
- Wallig MA et al., eds. (2018) "Fundamentals of Toxicologic Pathology", 3rd Ed, Elsevier
- Sahota PS et al., eds (2018) “Toxicologic Pathology: Nonclinical Safety Assessment”. CRC Press
- Sahota PS et al., eds (2019) “The Illustrated Dictionary of Toxicologic Pathology and Safety Science”. CRC Press