Cellular, Tissue, and Gene Therapies Advisory Committee Meeting

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Pigs in toxicology:

Differences in metabolism and background findings may be breed dependent

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Breed differences in metabolism and background lesions

- Different breeds
- Drug metabolism
  - Enzymes
  - Locations / organ systems
- Conclusions: drug metabolism
- Background lesions
  - Organ systems
  - Breed differences

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Miniswine lineages: Hanford

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Sinclair

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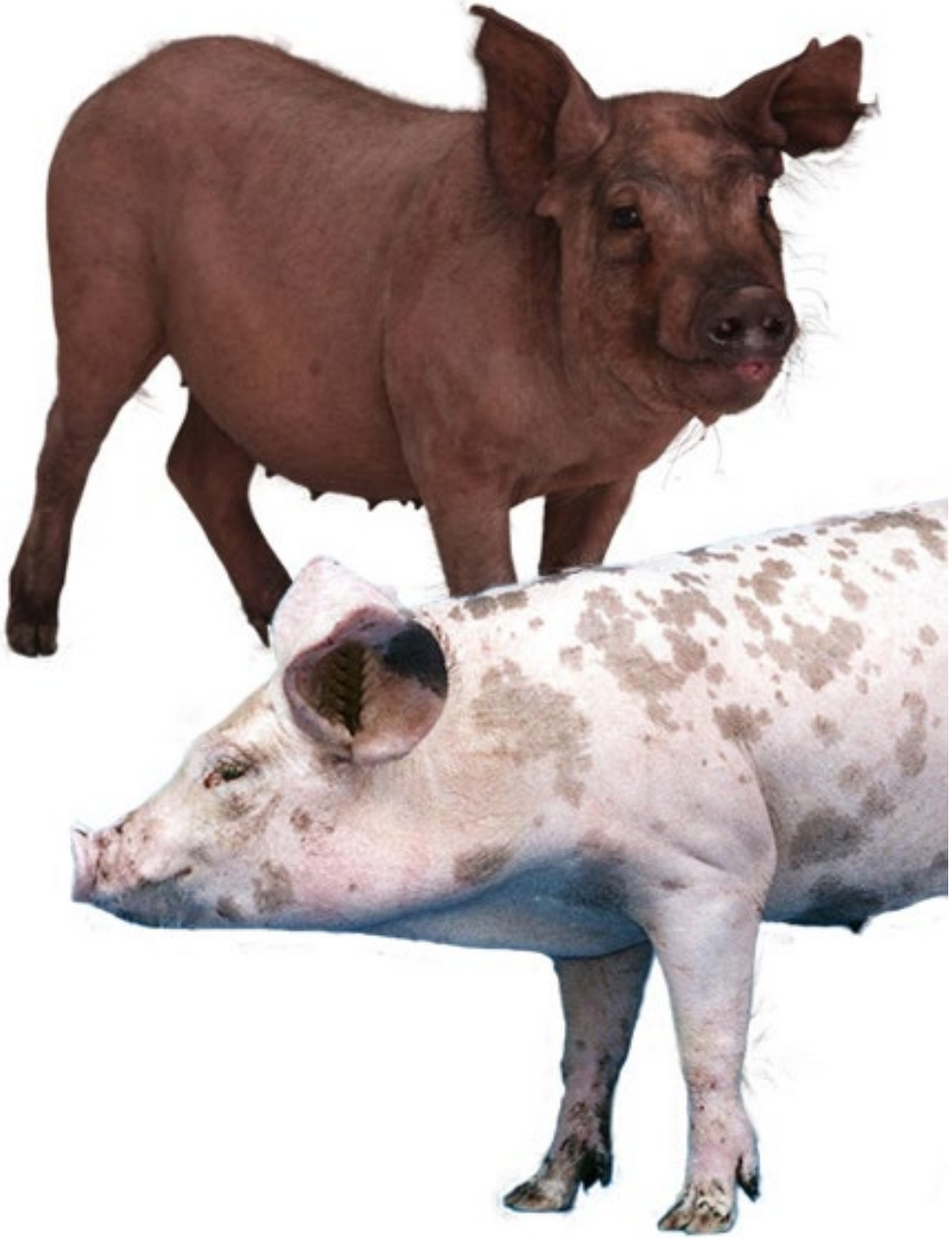
Yucatan



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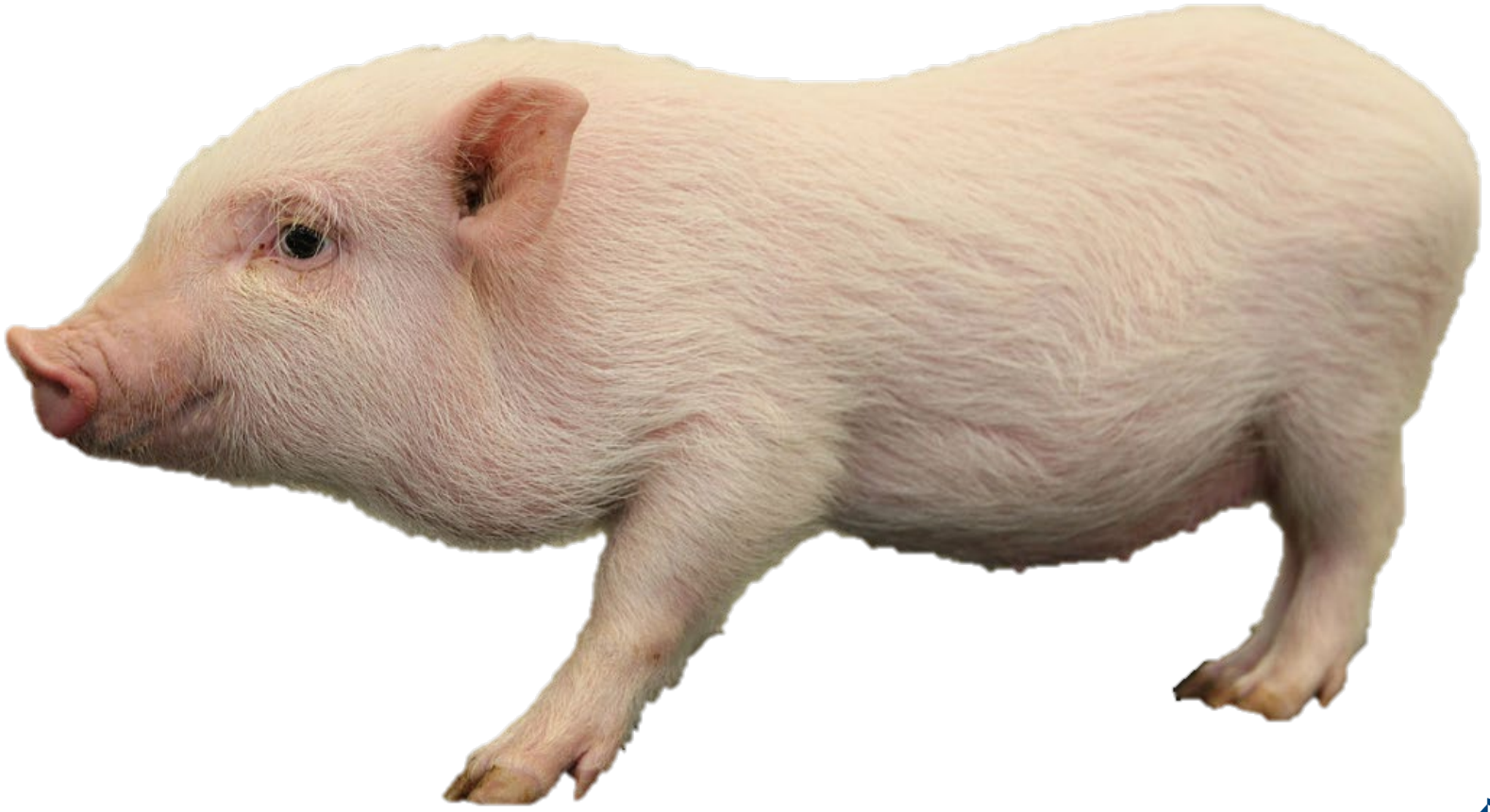
Göttingen…..



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ELLEGAARD

GÖTTINGEN MINIPIGS

EU (1992)

日清製粉グループ

ORIENTAL YEAST CO.,LTD.

Japan (2013)

Czech Republic (Veterinary Medicine Research Institute)

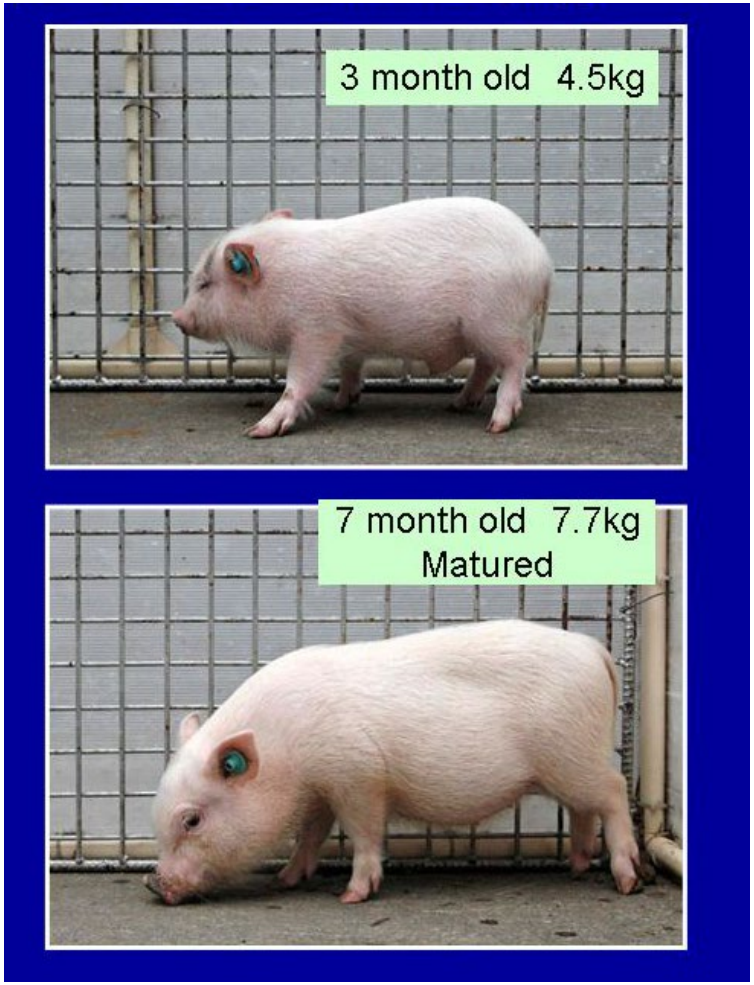
MARSHALL

BIORESOURCES

US (2003)

Others (Asia)

Micromini (Japan)



Bama (China)

http://microminipig.org/

Zhang 19 cell reports
https://papers.ssrn.com/sol3/papers.cfm?abstract_id=3363854

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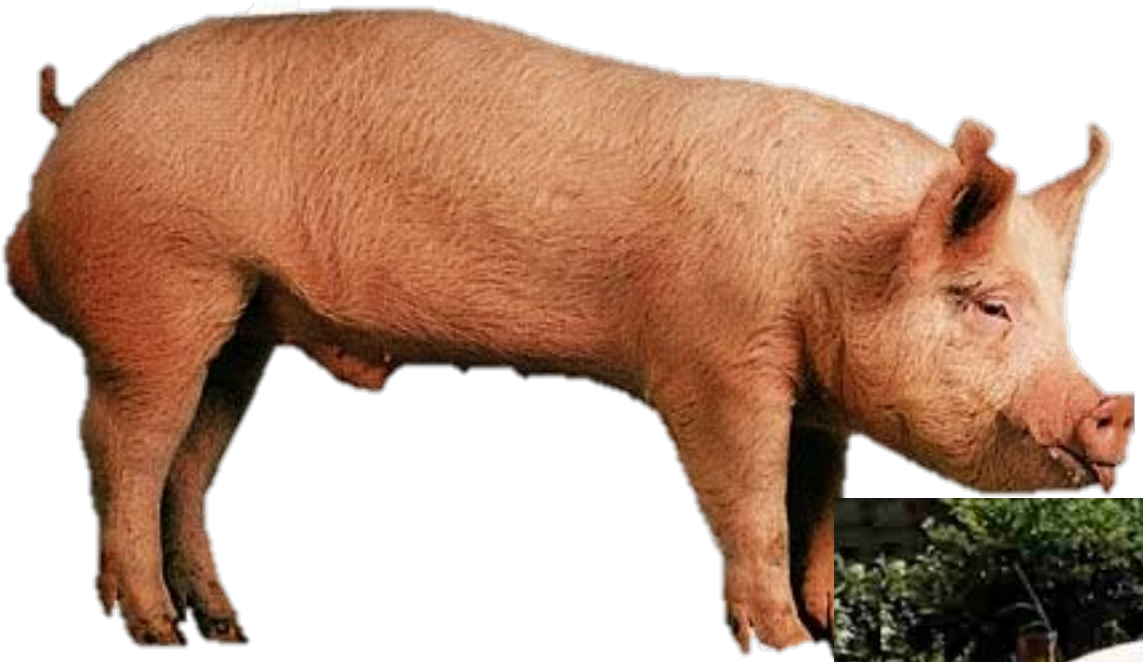
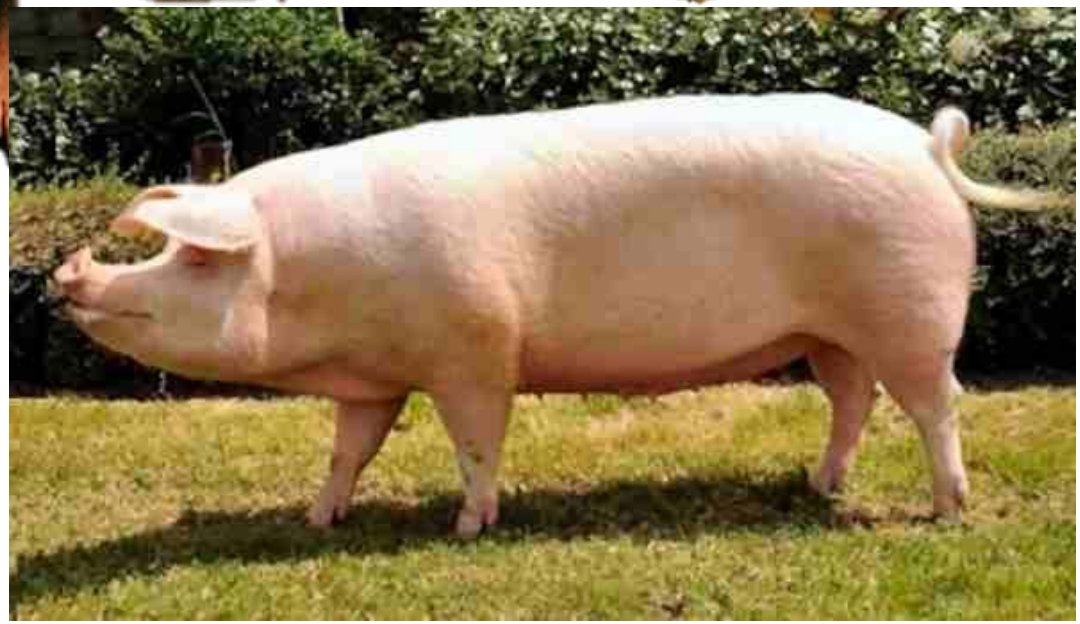
Agricultural Breeds





- Yorkshire
- Duroc
- Landrace

Drug entry pathways

Transporters

- Drug entry –> oral / IV / topical (skin)
- Cross GI tract, renal, or cutaneous epithelium –> passive diffusion or active transporters
- Can undergo transformation in intestinal epithelium, or after in bloodstream, in liver or kidney
  - Phase I reactions
  - Phase II reactions
  - Elimination

Transporters

- All similar (>72%) between pigs and humans
- ABC (ATP- binding cassette: efflux)
  - Pgp1/MDR1 – can be inhibited / induced
  - BCRP
- SLC (Solute carrier: influx)
  - OAT
  - OCT – genetic variations
    - International transporter consortium

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Phase I drug metabolism pathways

Phase I reactions

- Expose functional group of parent compound which may results in increased or loss of drug activity
- Provides functional group for phase II reactions
  - Oxidative
  - Reductive
  - Hydrolytic
  - Dealkylation
- Enzymes Include:
  - CYPs
    - Most frequently involved in drug metabolism
  - Flavin monooxygenases
  - Monoamine oxidases
  - Molybdenum hydroxylases
  - others

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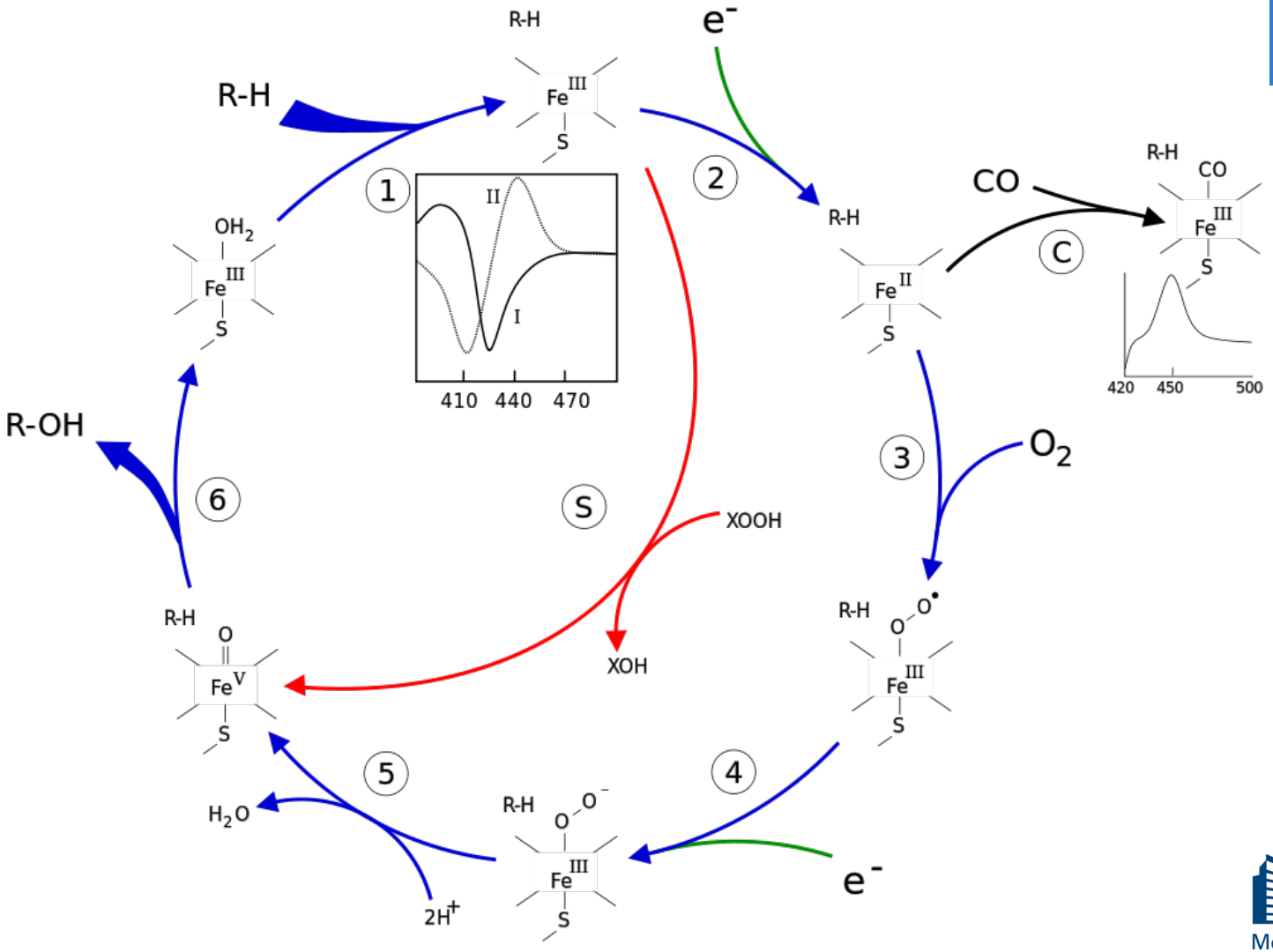
Cytochrome P450 families



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Cytochrome P450 (CYP)

- Family of enzymes that are *functionally* conserved in all mammals
- Most important Phase I biotransformation enzymes in humans are CYP
- In humans, primarily 3 CYP families are involved in majority of all drug biotransformation
  - CYP1
  - CYP2
  - CYP3
- Present in ER and mitochondria of liver, GI tract, kidney, skin, others

Total liver content of CYP enzymes

Species	Total CYP in liver (nmol/mg)
Human	~0.3
Minipig	0.8
Farm pig	0.22-0.46
Dog	0.4
NHP	~1.0

Modified from Dalgaard 15 j pharmacol t

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Total liver content of CYP enzymes

Species	Total CYP in liver (nmol/mg)
Human	~0.3
Minipig (Göttingen)	0.8
Farm pig (Landrace-Yorkshire / Duroc)	0.22-0.46
Dog	0.4
NHP	~1.0

Liver CYP content varies greatly between farm and mini-pigs.

Modified from Dalgaard 15 j pharmacol t

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CYP sequence

- Polymorphisms result in both **inter**species and **intra**species variations
- Allelic variations
- Some individuals carry multiple copies of CYP genes
- Pseudogenes

Current knowledge: How are CYPs measured?

- Some references measure mRNA (PCR)
- Some measure protein levels (WB, ELISA, MS)
- Some measure activity levels (substrate assays, inhibition assays)
- Some measure both protein and activity

- Need mRNA levels, protein levels and activity levels – evidence for post-transcriptional regulation

How is activity of CYP measured?

- Activity of CYPs are determined by substrate reactions-
  - Does metabolism of a specific substrate or set of substrates happen?
- Substrate reactions are typically specific for a single huCYP
- Not always true in pigs

Cytochrome families: Current knowledge

CYP families / subfamilies

- About 50 cytochromes in humans, of which approximately 6 families metabolize >90% of drugs.
- CYP1
  - CYP1A1, CYP1A2, CYP1B1
- CYP2
  - CYP2A6, CYP2A19, CYP2D6
- CYP3
  - CYP3A4, CYP3A32

CYP1

Hu CYP	pCYP	Similarity (amino acid)	
1A1	1A1	82%	No sex diff, sex diff
1A2	1A2	81.2%	No sex diff

CYP1

- CYP1A
  - Metabolize carcinogens (aromatic and heterocyclic amines), estrogens, mycotoxins, xanthenes, some antidepressants, analgesics
  - Induction by same inducers across species
  - In humans, metabolize about 20% of substances tested
  - In minipigs and humans, CYP1A activity is sex-related
    - Minipig females have 2-4X higher activity level than males
    - Caucasian males have 2-4X higher activity level than females
  - Levels are higher in piglets than adults

CYP1

- CYP1B1
  - In humans is predominant isoform outside the liver
  - Not characterized in minipig

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CYP2

Hu CYP	pCYP	Similarity (amino acid)	
2A6	2A19	87.2%	F>M, Breed diff
2A13	2A19	90.1%	
2B6	2B22	81.1%	F>M
2C9	2C33	64%	M>F, breed diff
	2C44	80%	
	2C49	78%	M>F
2C18	2C33	62%	
	2C42	78%	
	2C49	80%	
2D6	2D25	79%	
2E1	2E1	82.5%	F>M

CYP2

- Metabolize: nicotine, nitrosamines, aflatoxin B1
- cDNA of 2A19 is 99% homologous between Göttingen and conventional breed pigs

CYP2A

- Female Göttingens have 70X higher activity than males, but when males are castrated, activity increases 10x
- Yucatan females have 5X higher activity than males
- No sex difference in activity has been reported in humans

- Marked species, breed, and sex differences

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CYP2B

- Metabolizes diazepam, lidocaine, cyclophosphamide, tamoxifen
- No sex differences in Yucatans
- Levels are increased in conventional pigs relative to humans
- Levels in piglets higher than adults

CYP2B

- Inconsistencies in porcine literature
- 7-pentoxyresorufin dealkylation detected by some groups but not others.
  - Differences in assay used
- May be due to sources of hepatocytes and microsomes
- Or the fact that CYP2B genes can be induced by phenobarbital and others

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CYP2C

- Humans- metabolize 22% of drugs including losartan, propofol, estrogens, testosterone, methadone
- Pigs – different CYP2C enzymes show some cross reactivity toward many of the test substrates, not just those specific for human CYP2C
- Difficult to extrapolate between pigs and humans for CYP2C

CYP2D

- Metabolizes antidepressants, antipsychotics and β-blockers
- High inter-individual variances in humans (polymorphisms)
- Not fully examined in the pig, and many of human CYP2D substrates are metabolized by porcine CYP2B

CYP2E

- Metabolizes alcohols, ketones, anesthetics, nitrosamines
  - Can lead to production of highly reactive toxic or carcinogenic metabolites
- Inducible by alcohol, high fat diet
- Physiologically induced by stress with 8-fold increase in protein and no change in mRNA level
- Female minipigs (Göttingen, Yucatan) have higher activity than males
- No sex differences in conventional pigs or humans
- Many differences in activity between pig and humans

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CYP3

Hu CYP	pCYP	Similarity (amino acid)	
	3A22		
	3A29	76%	F>M
3A4	3A46	77%	Breed diff
3A5		75%	

CYP3

- Most important CYP- metabolizes 27% of compounds and represents 30% of total CYP

- Involved in steroid hydroxylation, converts sex hormones, polycyclic aromatic hydrocarbons, pesticides

CYP3A

- Expressed
  - Liver
  - Intestine
  - Kidney
  - others

CYP3A

- Tissue expression patterns (location, not levels) are similar between pigs and humans
- Transcriptional regulation is different between humans and pigs
- Yucatans have higher activity compared to Göttingen and conventional pigs
- Diet can differentially affect activity between sexes
  - Chicory root decreases activity in males and increases activity in females

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CYP

- No major differences in substrates, inducers, inhibitors, and tissue distribution between humans and pigs in:
  - CYP1A1
  - CYP1A2
  - CYP3A

Review of CYP

- Minipigs (Göttingens) have higher content of CYP overall relative to 3 breeds of conventional pig and 2 races of humans
- CYP content differs between breeds
- CYP activity differs between breeds

- Significant sex differences in pCYP
- Sex differences not observed in all breeds.
  - Meishan vs. Landrace
  - Correlation between CYP levels and testosterone.
    - Dependent upon CYP, may be positive or negative correlation.

- Significant discrepancies in interpretation of CYP levels and substrate specificities are present due to non-comparable data
- Inducibility / magnitude of induction differ across tissues / cell types, even when exposed to same chemical inducer
- CYP protein levels may be affected by androgen levels which are inherited in autosomal dominant manner

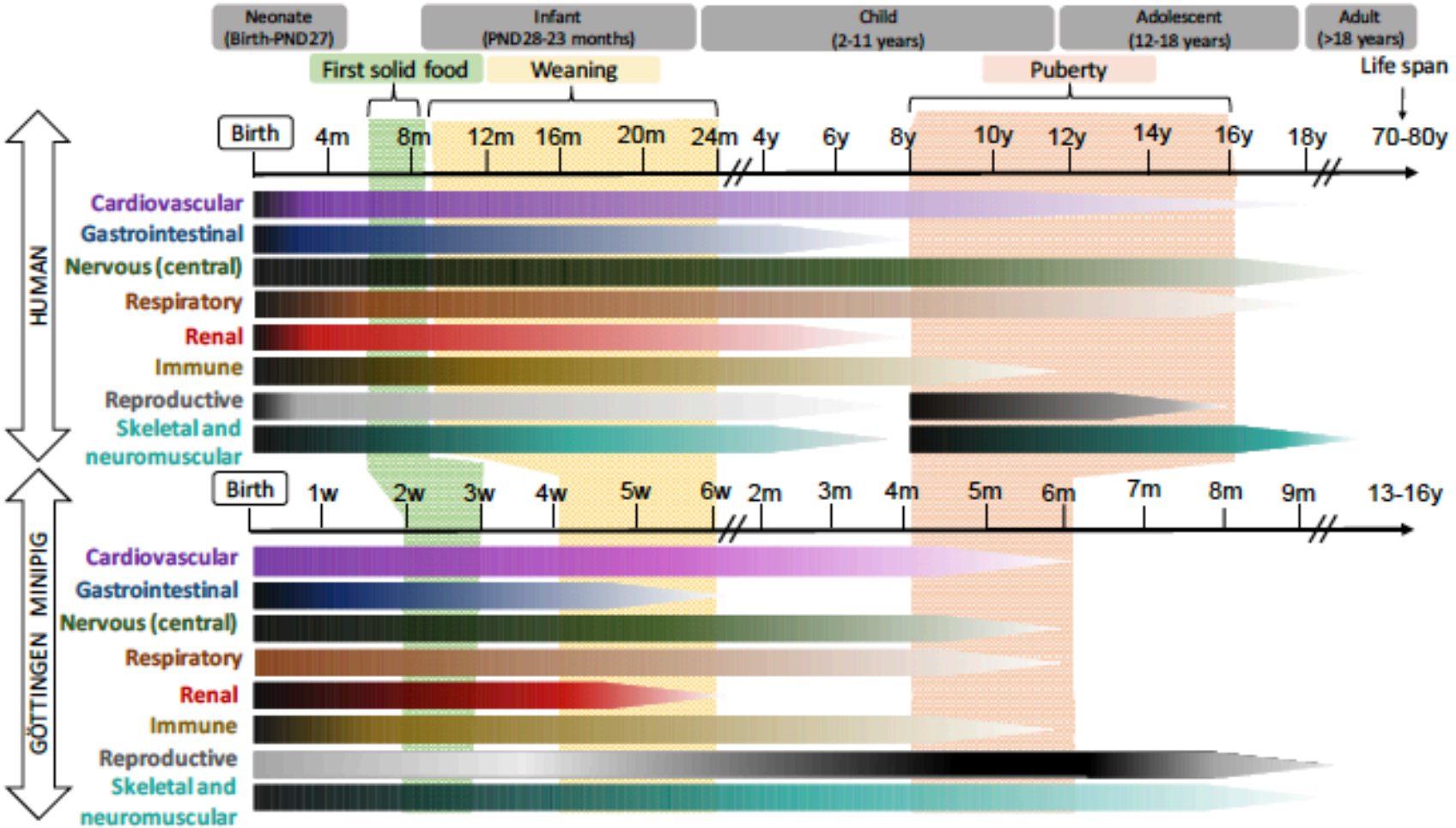
Activity

- Some measure activity as mg/ microsomal protein
- Others measure Activity / mg liver

- These discrepancies account for some of the differences between the sexes.

- Other variables:
  - Genetics
  - Age – young animals may not express or may express at higher than adult levels
  - Sex
  - Sex differences with age
  - Diet
  - Epigenetic factors
  - Circadian variation
  - Transcriptional regulation

Developmental comparison



Drug interactions

- 17/48 drugs tested in pigs had >10% inhibition of specific CYPs

Phase II pathways - conjugation

Phase II reactions

- Result in formation of covalent linkage between functional group and:
  - Glucuronic acid (UGT enzymes)
    - Liver, kidney, intestine, lung, skin
  - Sulfate (SULT)
    - (Liver, kidney, intestine), skin
  - Glutathione
    - Liver, kidney
  - Amino acids
  - Acetate
    - Liver, lung, spleen, stomach, blood cells

- Increases polarity of compound to aid in excretion

Phase II

- Glucuronidation and sulfation are most important in drug biotransformation
- Not as much research done on Phase II to date
- However, it is known that sulfate conjugation in swine is slower
- Pig is more efficient than human at glucuronidation
- Sulfation is major conjugation pathway for phenols and contributes to biotransformation of alcohols, amines, and thiols

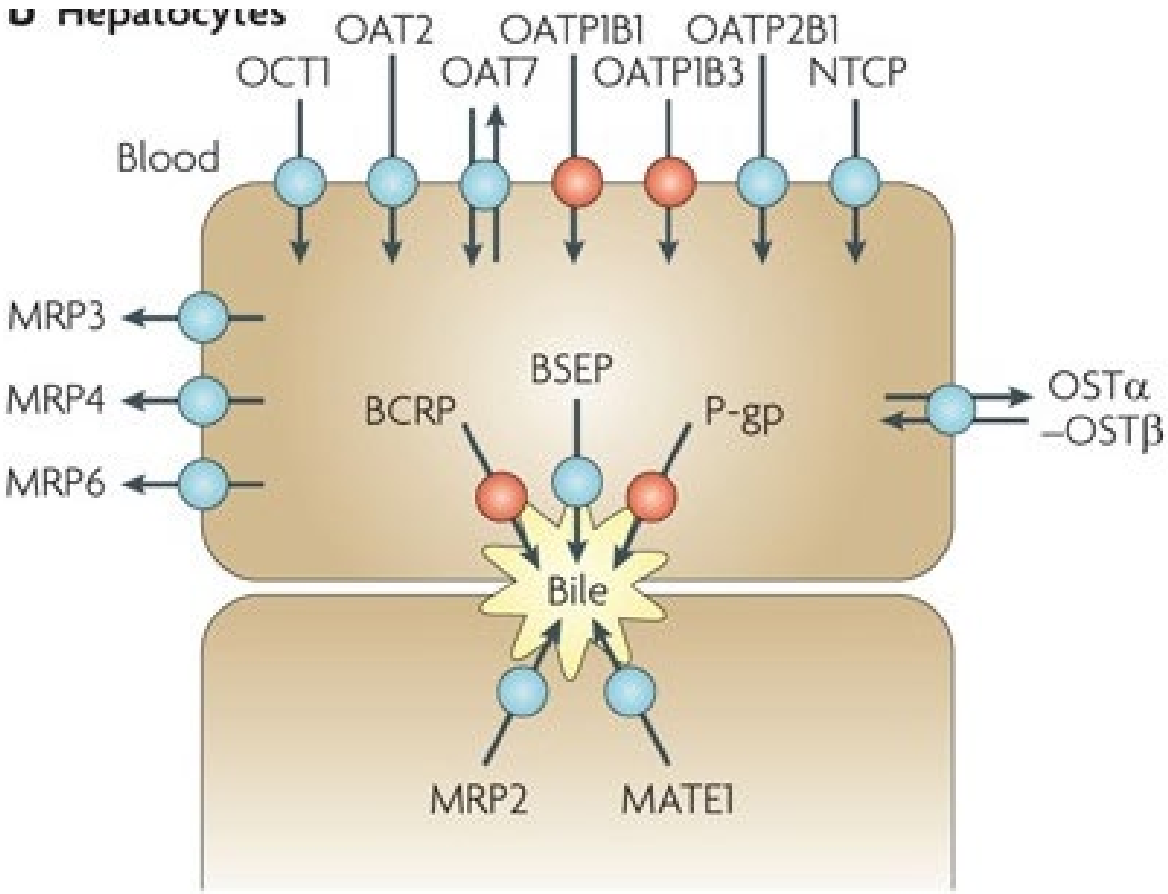
- Pigs compensate by using other phase II pathways in order to metabolize enzymes
- Pigs have high acetylating capability
- Not much is known about UGT (Uridine 5'-diphospho-glucuronosyltransferase) or isoforms in the pig (important in conjugating with glucuronide)

Organ systems

Organs of metabolism

- Liver
- GI
- Kidney

Hepatocytes



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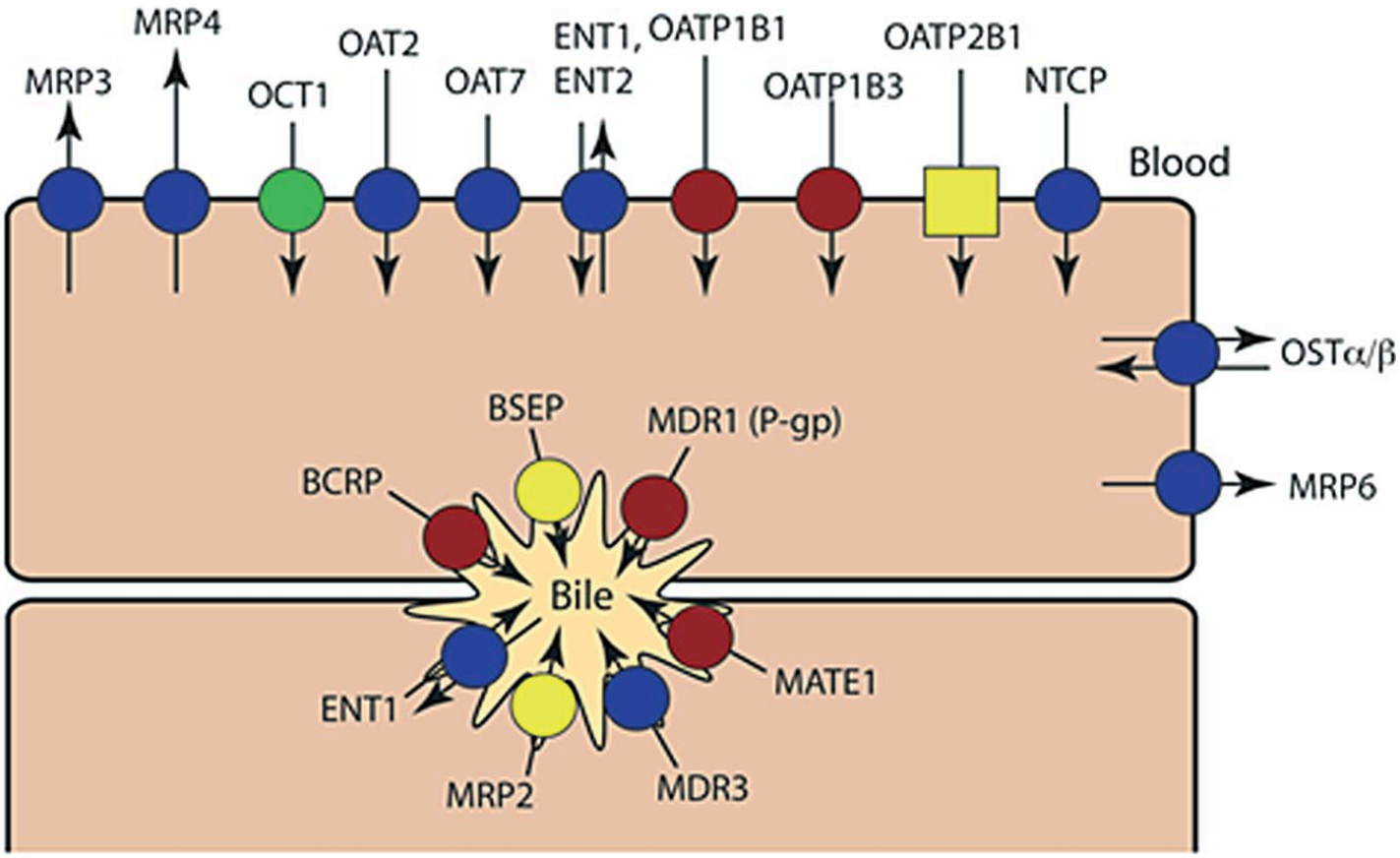
Hepatocytes

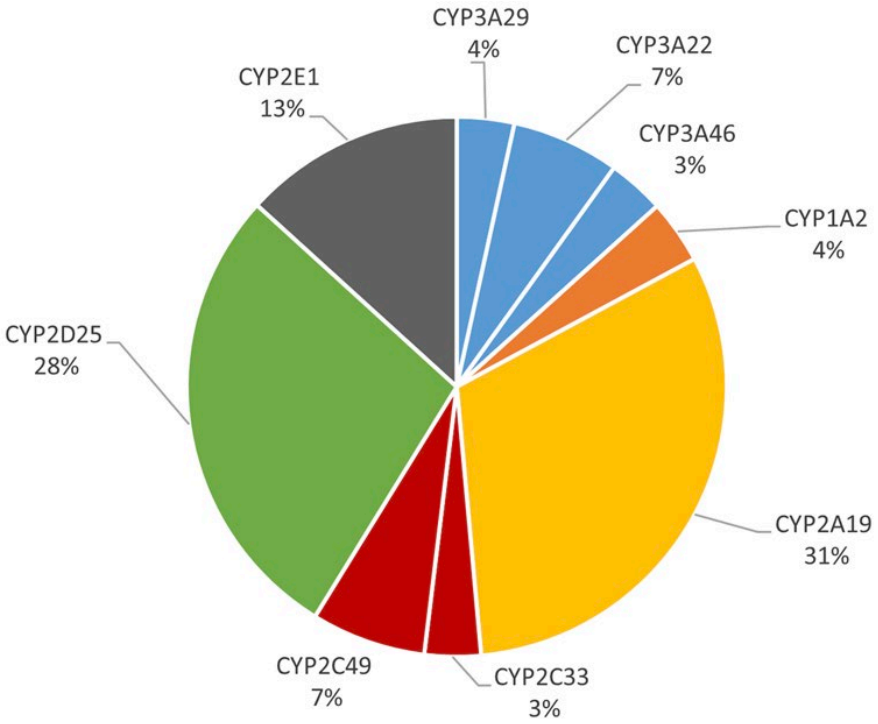


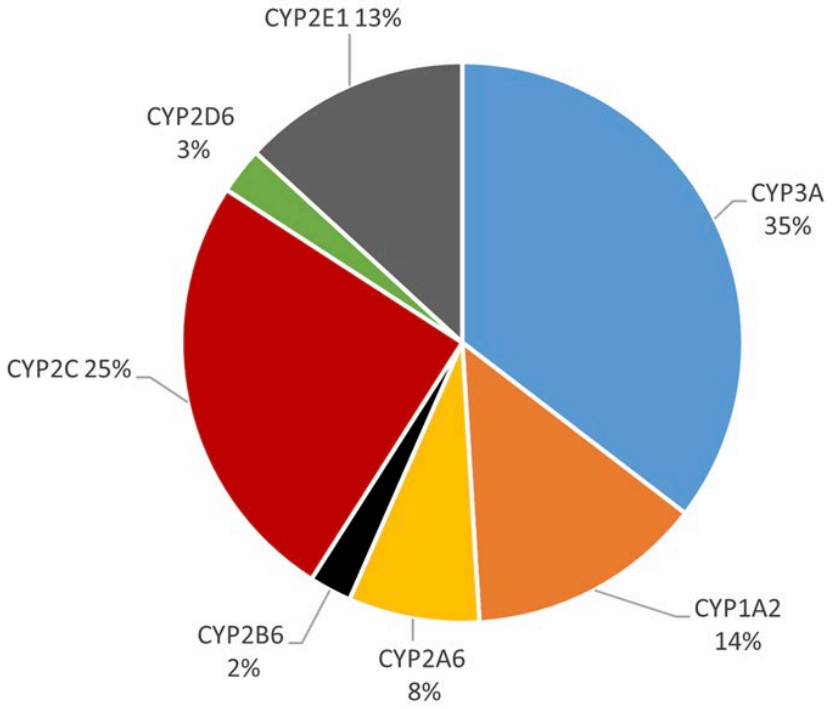
Zamek-Gliszczynski 18 Clin Pharm Therapeut 104:890

Liver

- Primary or pre-systemic extraction / metabolism
- Blood flow: GI → liver → systemic
- Phase I and phase II enzymes present in liver
- Similar levels of GST (glutathione transferase) and UGT (UDP-glucuronosyl transferase) found in humans and pigs (liver)

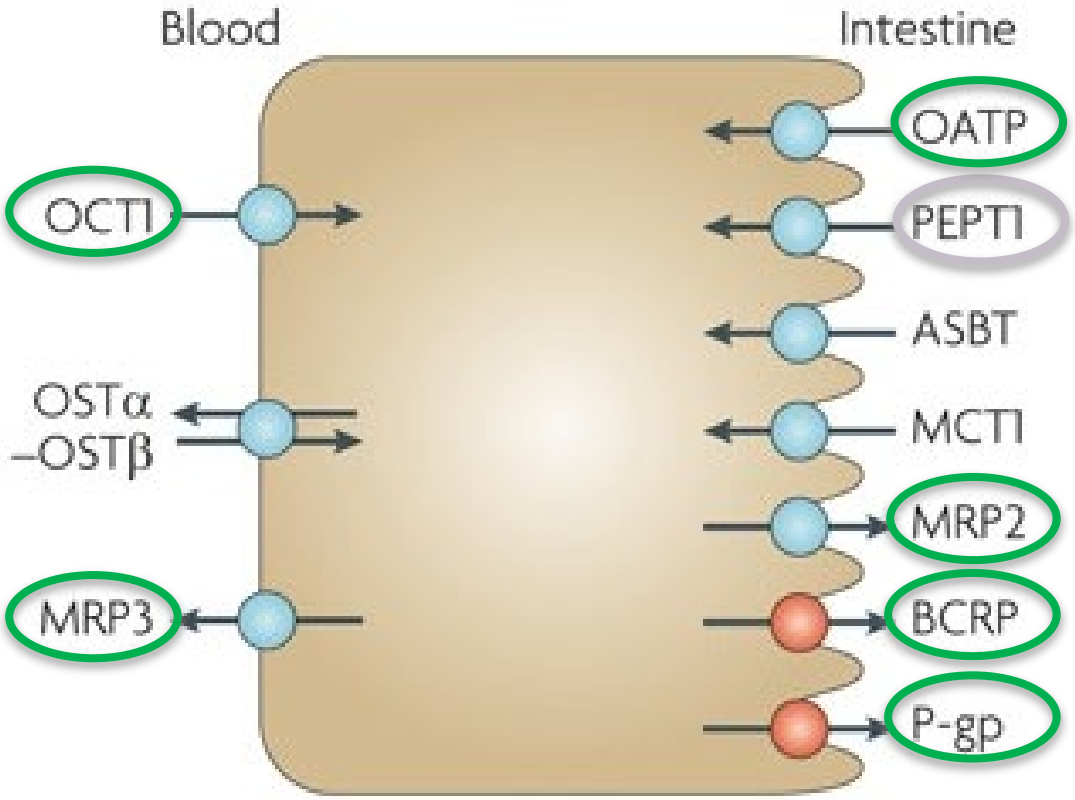
Liver CYP: Pig - Human





N=8M/8F, 12 wk
Hybrid sow X Pietrain Boar

Intestinal epithelia



GLUT1
MCT5
MRP1
OATP4A1

Not detected:
BSEP
OATP1B1
OAT1B3
OAT1C1
OCT3

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Vaessen 17 drug metab disp

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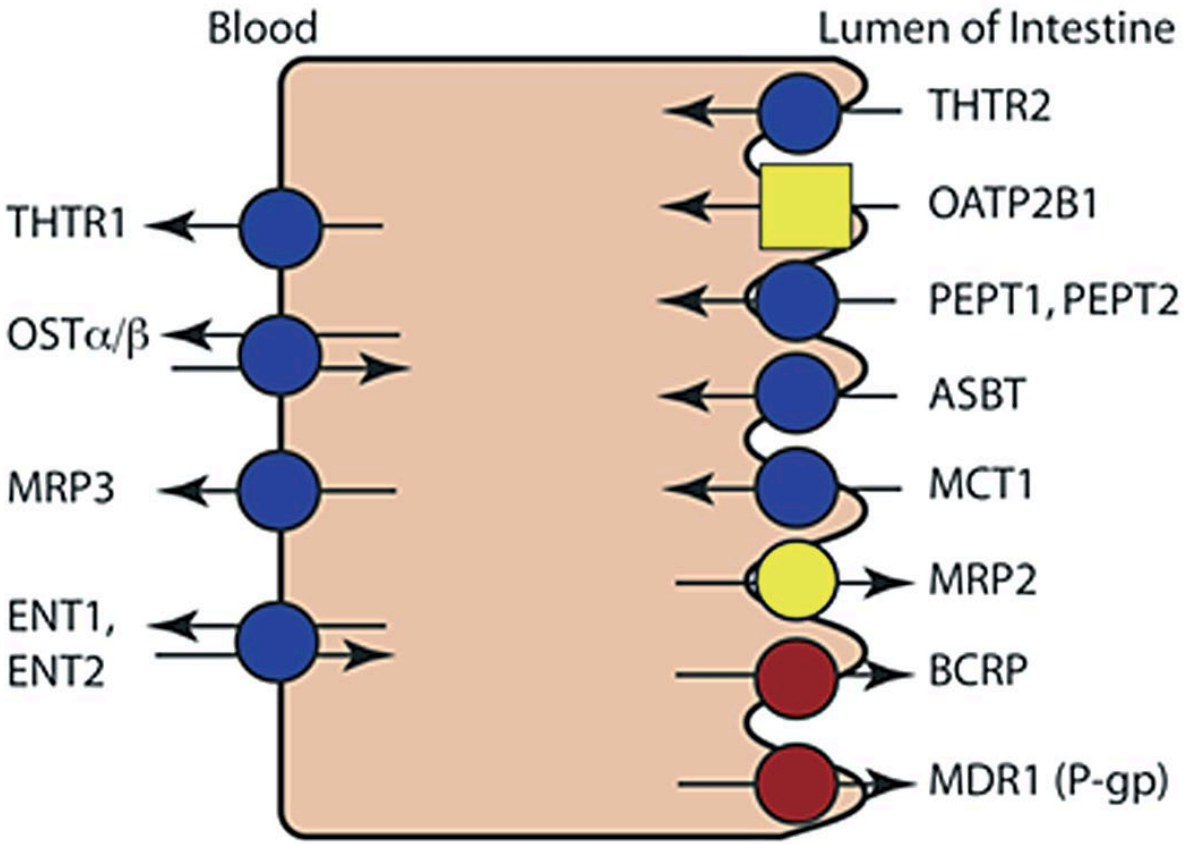
Intestinal epithelia



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GI tract

- Passive cellular diffusion is primary mechanism of intestinal drug absorption
- Profound interspecies differences
  - Salivary amylase
  - pH of stomach, small and large intestines
  - Rate of gastric emptying / GI transit time
  - Age (young pigs more readily absorb large molecules – colostrum)

GI tract

- The GI tract is the most important extrahepatic site of drug biotransformation
- Molecules pass through enterocytes after oral administration
- CYP3A most important
- Overall, pigs have similar gut physiology to humans

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GI tract

- Efflux transporters (permeability glycoprotein, P-gp)
- Bile salts – solubilize lipophilic drugs
- CYP
- CYP2C42 higher expression in jejunum than liver

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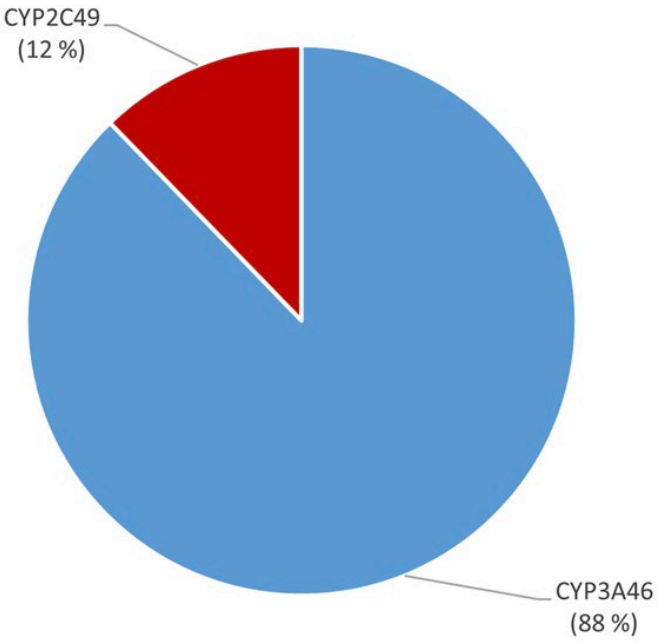
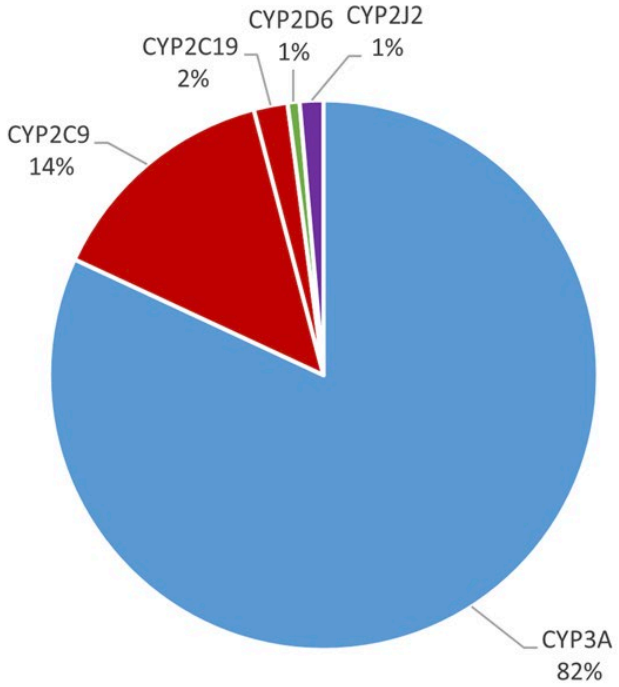
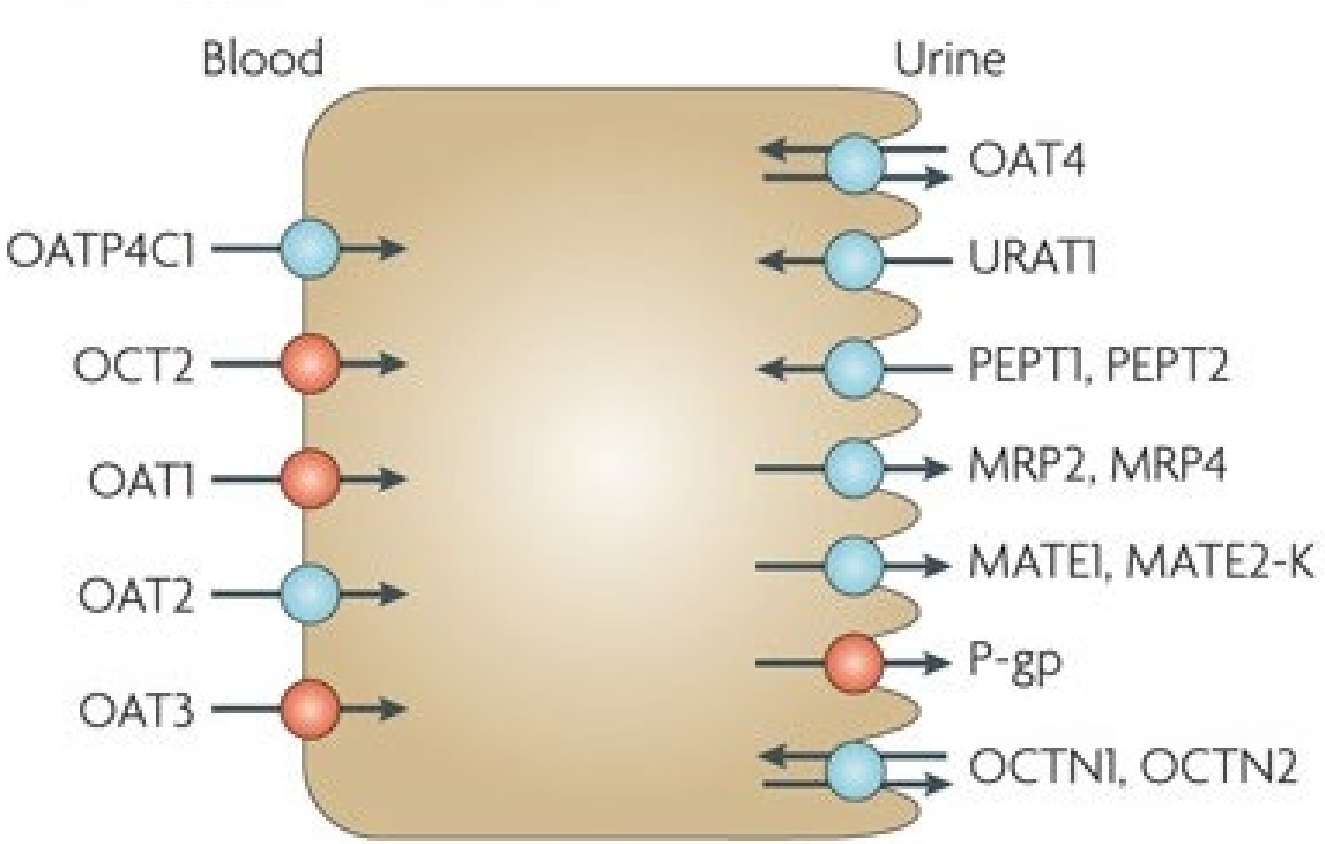
Jejunal CYP: Pig - Human





N=8M/8F, 12 wk
Hybrid sow X Pietrain Boar

Kidney, proximal tubule cells



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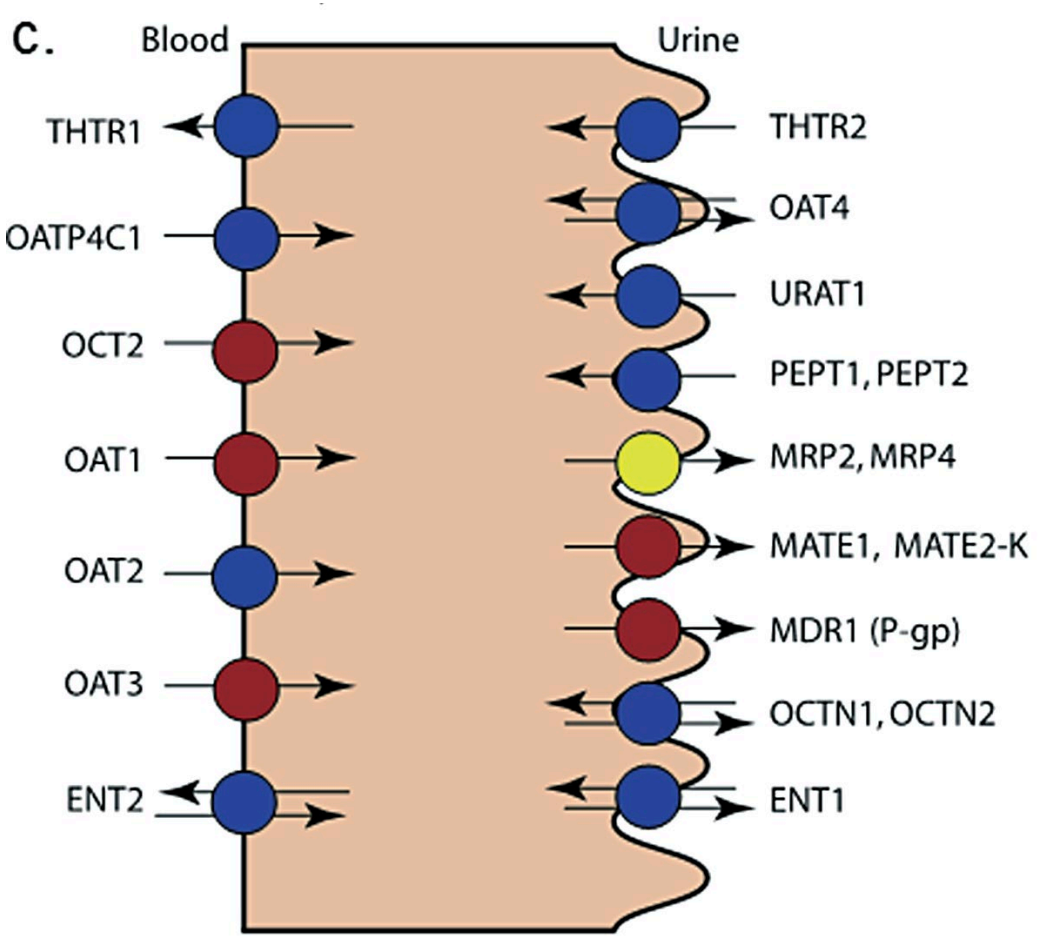
Kidney, proximal tubule cells



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Kidney

- Most important organ for elimination of drugs and their metabolites
- Of top 200 therapeutics, about 1/3 undergo renal eliminaiton
- Kidney expression of CYP is 1/10 of liver expression (all species)
- Metabolically active, and in some cases surpasses the liver
- Regional differences within kidney of enzyme levels
  - Metabolization primarily in proximal tubules

Kidney

- Substrates and inhibitors of renal transporters are well documented
- Porcine renal phase I and II homology remains unknown
- In the rabbit, S2 and S3 segments of proximal tubule have increased levels of CYP
- In the rat, sex differences observed in the liver are not noted in the kidney

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Kidney

- CYPs may be induced in kidney, but by agents different from liver.
  - Barbiturates (liver, not kidney)
  - Polycyclic hydrocarbons (both liver and kidney)
- Difficult to extrapolate without specific pig studies as there are large differences noted between rats and mice
- In Chinese minipigs, results of AKI due to gentamicin inconsistent among groups and individuals
  - Cisplatin reliably induced AKI in same study

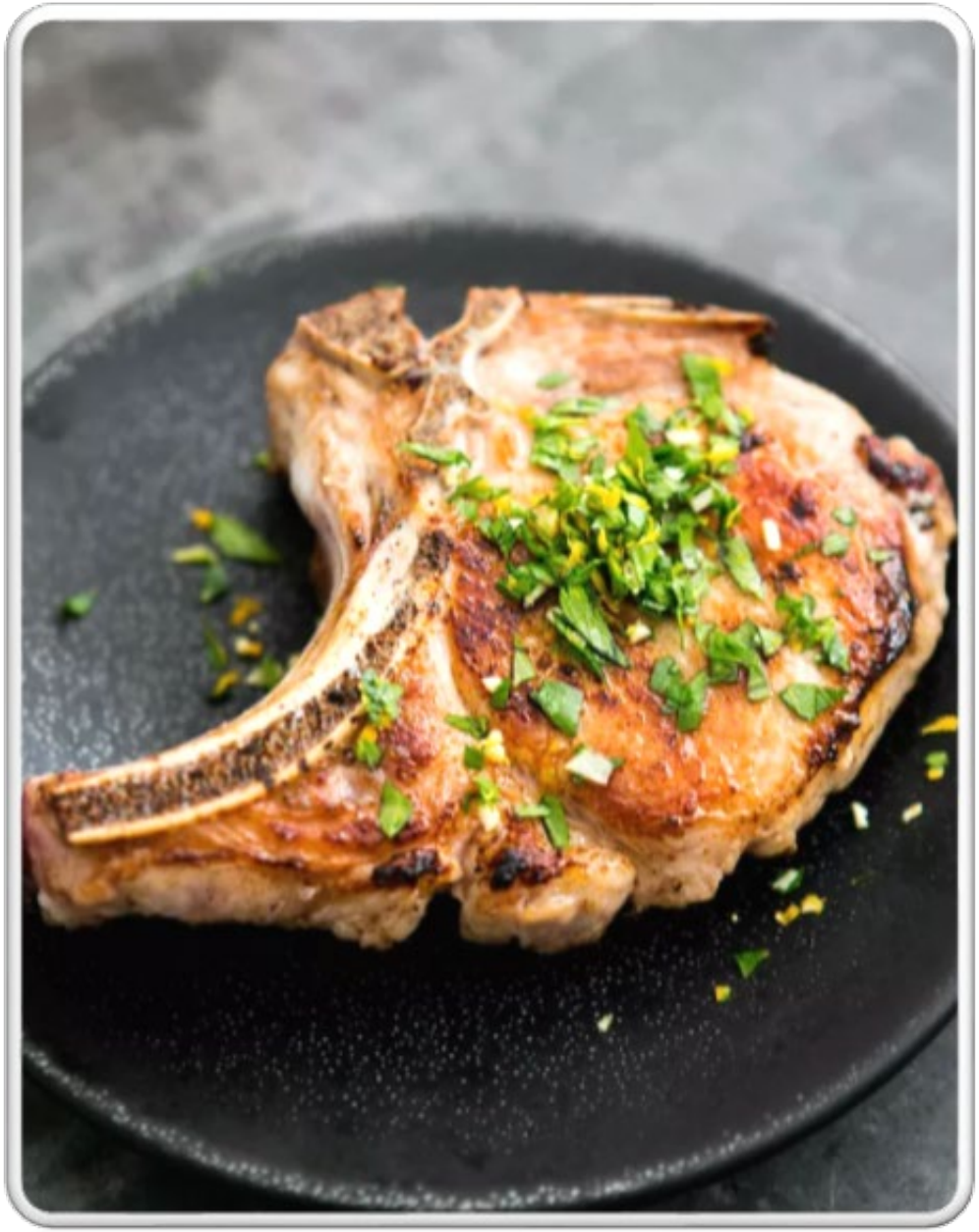
Kidney

- In humans, CYP3A5 is expressed in kidney.
- Levels vary by race.
  - African > Asian > Caucasian
- Cyclosporin and Tacrolimus nephrotoxicity is dependent on CYP3A5 genotype
- Similar processes / pathways exist, but concentration of metabolism / rate may differ between species

Kidney

- Banna minipigs, GFR is ½ of human GFR
- Elimination of cefazolin (excreted unchanged) is 3x faster in Banna pigs
- Drug doses may need to be reassessed if porcine kidneys are transplanted to humans.

Drug metabolism in the Pig: Conclusions



Current knowledge:

- Benefit:
  - Many studies have looked at porcine cytochromes to decrease "boar taint" in pork
  - Studies showed differences among breeds in regard to boar taint
- Drawbacks:
  - These studies may only study CYPs associated with boar taint /3-methyl indole (skatole) metabolism (CYP1A1, 2A19, 2C33v4, 2C49, 2E1, 2D6, 2C49, 3A)
  - Many of the studies were on farm pigs, not minipigs
  - Many studies focused only on males, not females

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However:

- Different species can produce same metabolites as humans in unpredictable ways (Different enzymes)
- Same drug metabolite may be produced by several enzymes within the same species

Problems with current knowledge

- "Because buffer strength, type, and pH can all significantly affect Vmax and Km, standardized assay conditions are recommended."
- Yet-- Specific recommendations are not stated

- Different pig breeds-
  - have different levels (amounts) of CYPs
  - have different activity levels of CYPs
  - CYP mRNA levels change with age
  - Different degree of change between sexes

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Conclusions

- Studies of the cytochromes and xenobiotic metabolizing enzymes are important
- Literature currently uses different isolation and testing techniques
- Interbreed differences are magnified by lack of standardization of testing methods and husbandry (feed, feeding schedule, age, sex, etc.)
- CYPs are inducible
- Also affected by changes in the microbiome
- Many studies only look at a small number of individuals
- Interindividual variation
- Epigenetics play a role in CYP diversity

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Holes in knowledge

- No consistency to selection of breed, age, sex of animals studied
- No consistency as to methods used to test expression, protein amount, or activity of CYPs
- Leads to misinterpretation of results even though there is high sequence homology between pigs and humans
- Other variables in pigs include
  - Dietary fat (both transcriptionally and post-transcriptionally affect levels)
  - Diet (including micronutrients and minerals- Selenium has been shown to affect both activity and expression levels)
  - Microbiome (sex differences in cytochromes were eliminated in germ-free mice)

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Conclusions

- More funding needs to be made available to complete systematic studies such as these in a reproducible manner

- Age, sex, and breed all need to be considered in pharmacological and toxicological testing

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Background lesions and breed differences

Methods

- Contacted several CROs
  - Responses from NA(6), and UK(1)
- Reported as common background if >1%
- Excluded animals from before 2000
  - Genetic drift
- Animals separated into age groups
  - <2mo, 2-6 mo, >6mo

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Systemic diseases

- Thrombocytopenic purpura
- Porcine stress syndrome

- N=1 for each entity
- Only reported at UK site (EU Göttingens)

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+

systems

Cardiovascular

- Göttingen:
  - Inflammatory cell infiltrate
  - Mesothelial hyperplasia and hypertrophy (more common in >6mo)
  - North America:
    - Hemorrhage
    - Myofiber degeneration
- Hanford: (only in 2-6mo)
  - Inflammation, subacute to chronic (14%)
  - Inflammation, endocardial (7%)
- Yucatan:
  - Inflammatory cell infiltrate
  - Epicardium: inflammation, cartilaginous metaplasia

Urinary- kidney

- Göttingen
  - Inflammatory cell infiltrates (19-39%)
  - Inflammation
  - Mineralization
  - Tubular degeneration / regeneration
  - Tubular dilatation
  - Tubular basophilia

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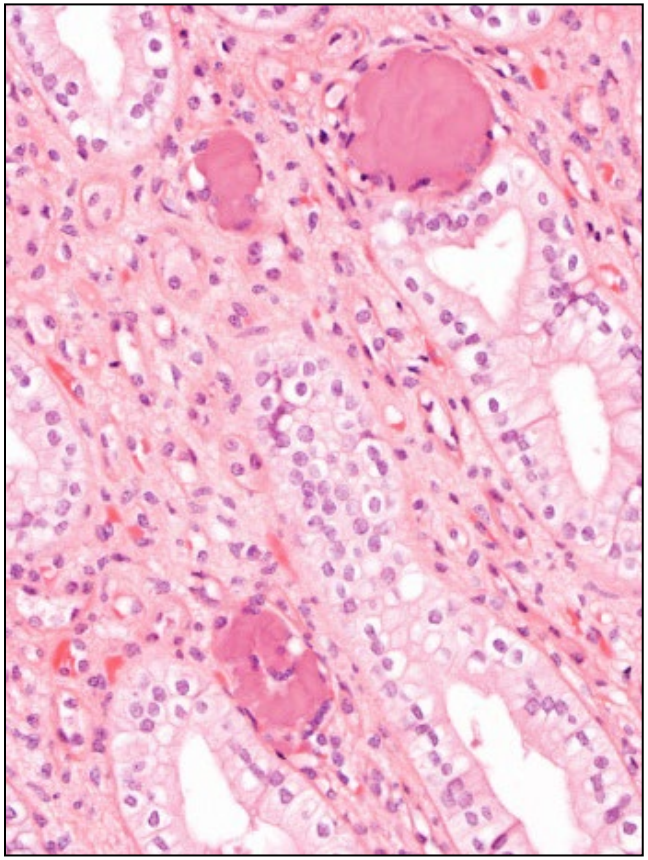
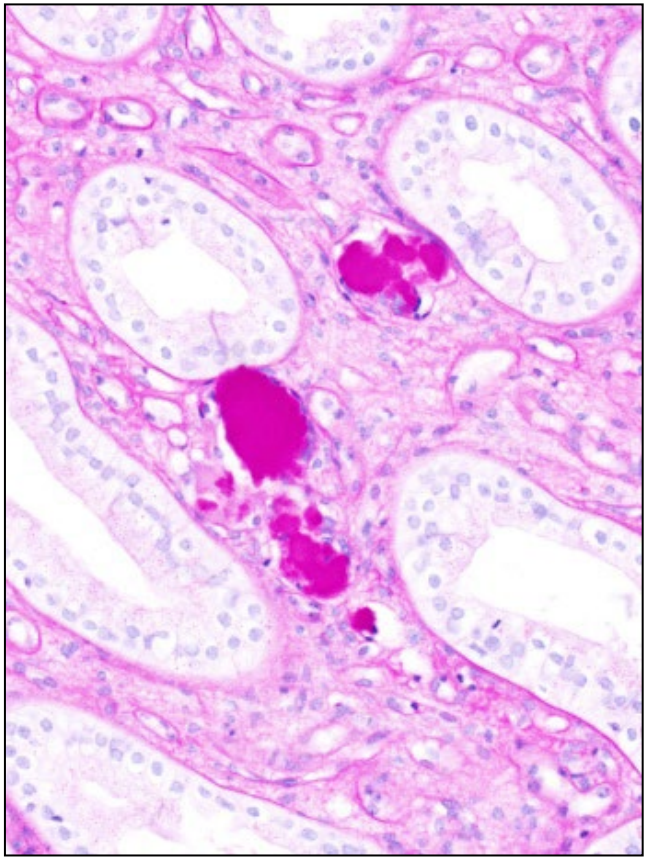
Urinary System – papillary mineralization



H&E



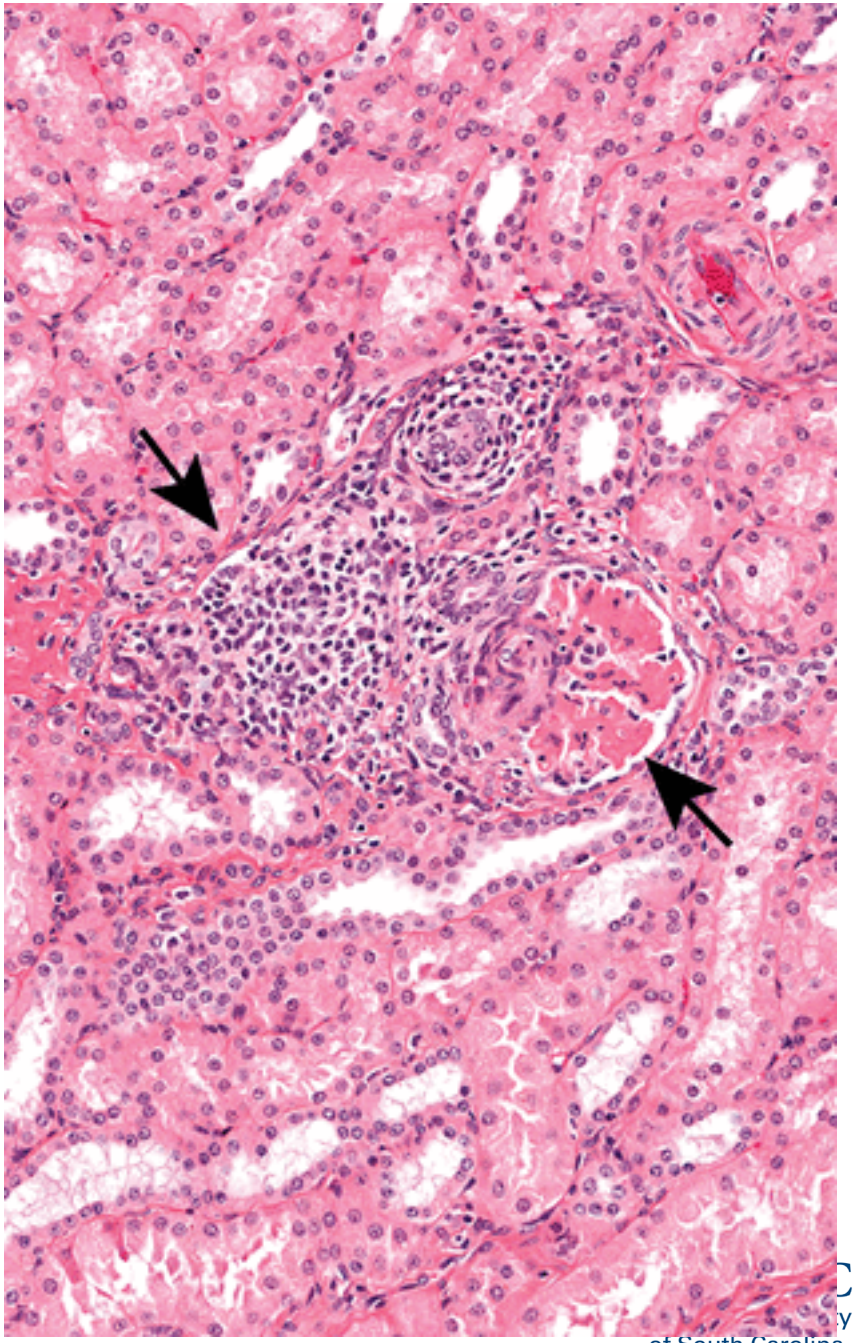
PAS

Urinary - kidney

- Hanford
  - Inflammatory cell infiltrates (20%)
- Yucatan
  - Inflammatory cell infiltrates (36%)
  - Hemorrhage
  - Fibrosis
  - Vacuolation of renal tubular epithelium (3% <2mo)

Overview- urinary

- Among all organs, kidney had highest prevalence of inflammatory cell infiltrate (19-50%)
- All breeds, females overrepresented for inflammatory cell infiltrate,
- Females overrepresented:
  - Tubular dilatation, and fibrosis- Gottingen
  - Tubular degeneration and regeneration - Yucatan



McInnis 16 tox path DOI: 10.1177/0192623315622423

Musculoskeletal

- Göttingen
  - Degeneration, degeneration / regeneration
  - Inflammatory cell infiltrate
- No changes noted in Hanford or Yucatan

Integument

- Göttingen
  - Hyperkeratosis
  - Hyperplasia
  - Exudate
  - Serocellular crust / scab
  - Abscesses or pustules
  - Inflammation
  - Inflammatory cell infiltrates
- Hanford
  - Inflammatory cell infiltrates
- Yucatan
  - Hyperplasia
  - Exudate
  - Inflammation

Male Reproductive- Testis

- Göttingen
  - Testicular hypoplasia, degeneration, atrophy, tubular (25%)
    - minimal to severe
    - Most common finding in male reproductive tract
    - Does not decrease with age
- Hanford (59 animals)
  - >6mo = sexual maturity in 100%
- Yucatan (37 animals)
  - <2mo: immature, hematopoiesis (47%)

Overview male reproductive:

- Göttingen
  - Hypoplasia, degeneration, atrophy of testicle common (25%) - all age groups
  - Vacuolation of tubular cells of testes
  - Aspermia / oligospermia- common in epididymis, may be related to testicular lesions
  - Prostate mineralization
  - Inflammatory cell infiltrate / inflammation both common in seminal vesicle
- Hanford
  - All male repro organs examined in animals >6 mo are mature
- Yucatan
  - Most changes in animals <2 mo and associated with immaturity
  - Hematopoiesis reported in young animals (<2mo)

Nervous system

- Göttingen
  - Inflammatory cell infiltrates
  - Perivascular inflammation
  - Mineralization of meninges (NA only)
- Hanford
  - Inflammatory cell infiltrate
- Yucatan
  - Inflammatory cell infiltrate

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Endocrine

- Thyroid – hemorrhage
  - Göttingen, Hanford, Yucatan
- Pituitary – mineralization
  - Göttingen, Yucatan
  - Increase with age
- Adrenal gland
  - Inflammatory cell infiltrate
  - Göttingen, Hanford, Yucatan

Conclusions

- Göttingen more likely to mineralize tissues than Hanford or Yucatan.
  - Mineralization / concretions more common in NA than EU (male repro, CNS, ovary)
  - EU more common than NA (urinary, lung)
  - Genetics or husbandry?
- Iron supplementation
- Urinary lesions more common in females

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Future directions

- Need to continue to look at breed differences
- No studies in farm / agricultural pigs
- No aging studies – If an organ from a young animal is placed into a middle-aged human, will the organ age at a rate higher than the recipient?
- Sex differences

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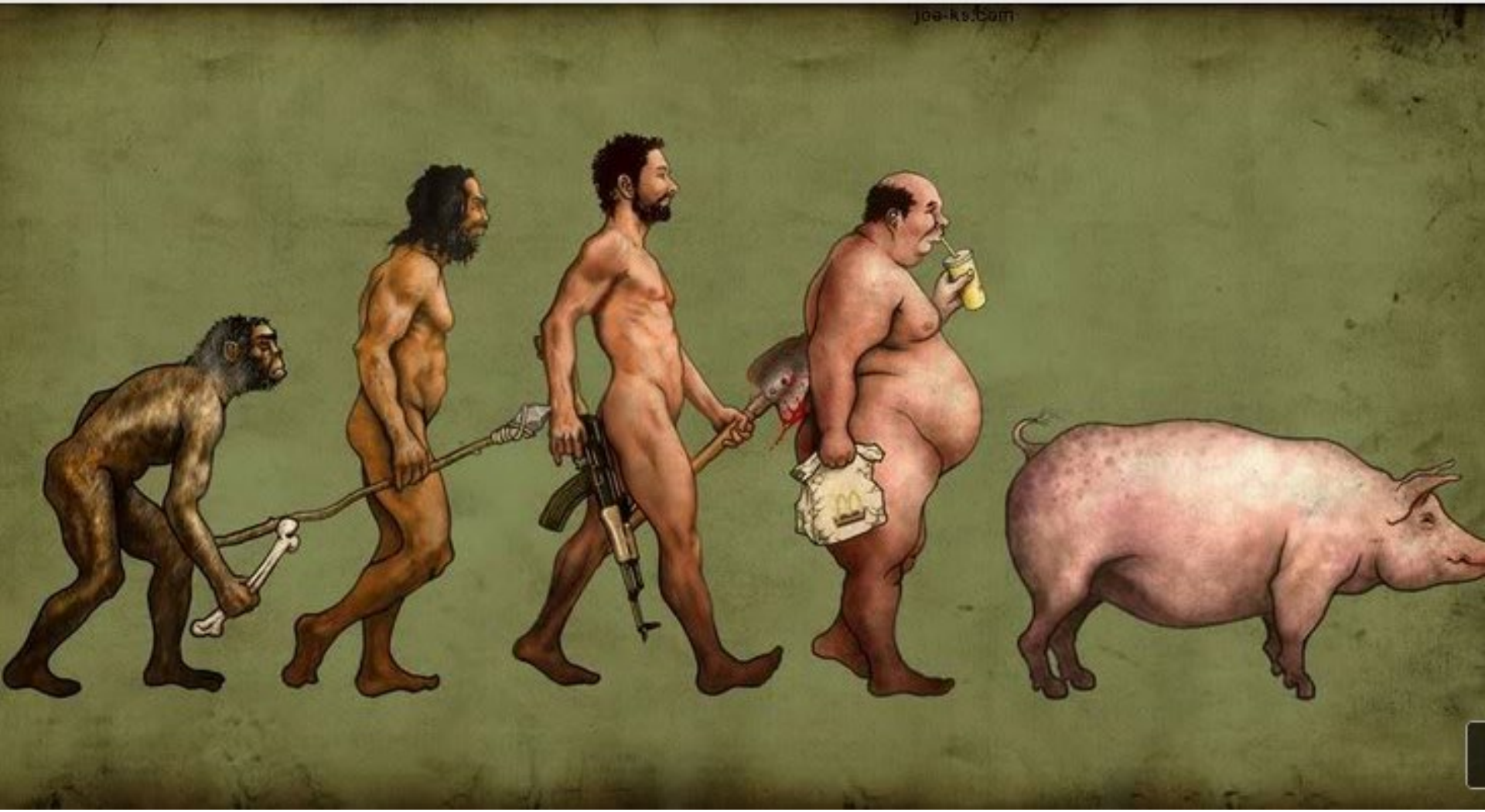
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