The Japanese Society for the Study of Xenobiotics Award 2014

Metabolic Activation and Fate of Xenobiotics Determined by Polymorphic Drug-Metabolizing Enzymes

It is really more honor than I deserve.

Hiroshi Yamazaki, PhD

Showa Pharmaceutical University
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- MDO Sapporo 2002
- Joint Meeting Maui 2005
- Fukuoka P450 meeting 2012

Hiroshi Yamazaki is a Professor of Laboratory of Drug Metabolism and Pharmacokinetics in Showa Pharmaceutical University, Tokyo, Japan since 2005. After receiving his BS and MS degrees from Gifu Pharmaceutical University and PhD in Pharmaceutical Sciences from Osaka University in Japan, he trained as a post-doctoral fellow at Vanderbilt University School of Medicine, Nashville, TN, USA in 1994. He was recruited as an Associate Professor of Kanazawa University from Osaka Prefectural Institute of Public Health in 1998 and moved to a post as Associate Professor of Hokkaido University Graduate School of Pharmaceutical Sciences in 2001.

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Hiroshi Yamazaki, PhD

Metabolic activation and fate of xenobiotics determined by polymorphic drug-metabolizing enzymes

- Generalized catalytic P450 enzymes
- Reactive metabolites in humanized-liver mice

  Thalidomide activation and drug interaction
- Monkey and human P450 enzymes
- Physiologically based pharmacokinetic (PBPK) modeling
- Flavin-containing monooxygese (FMO)

Five Decades After the Discovery of Cytochrome P-450

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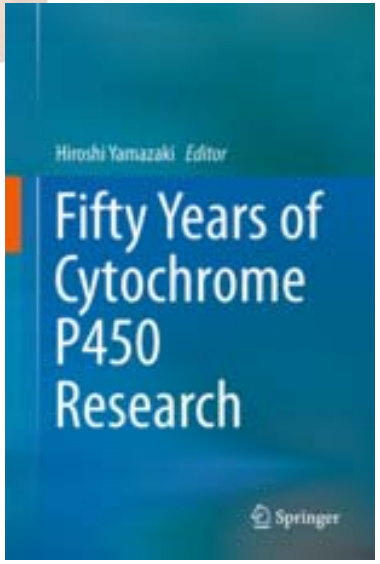
Prof. **Tsuneo Omura** was issued a letter of recognition from JBC Editor at Fukuoka, Japan in 2012 by **F. Peter Guengerich** (an associate editor for JBC) on the 50th anniversary of his seminal P-450 work.



Editor:

Hiroshi Yamazaki

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http://www.springer.com/978-4-431-54991-8

Cytochrome P450 Topics

During the ~30-year history, **human cytochrome P450** (P450) enzymes have been characterized in areas of drug metabolism.

1) Liver microsomal P450 isoform contents, the relatively broad but selective substrate specificities of P450s, P450 induction and inhibition, and genetic polymorphism of P450 enzymes with nomenclature system were shown.
2) P450 involved in oxidation of compounds associated with pharmacological and/or toxicological actions.
3) The knowledge gained so far has enabled researchers (including me) to progress and start challenges.

Interindividual Variations in Human Liver Cytochrome P-450 Enzymes Involved in the Oxidation of Drugs, Carcinogens and Toxic Chemicals: Studies with Liver Microsomes of 30 Japanese and 30 Caucasians

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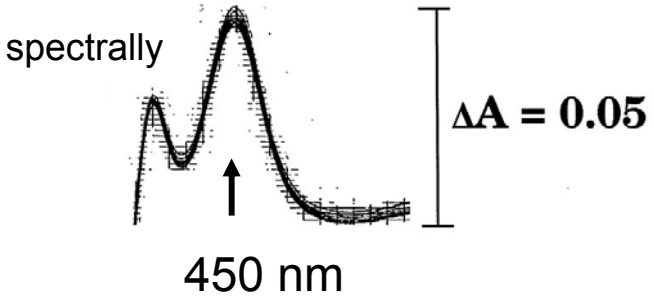
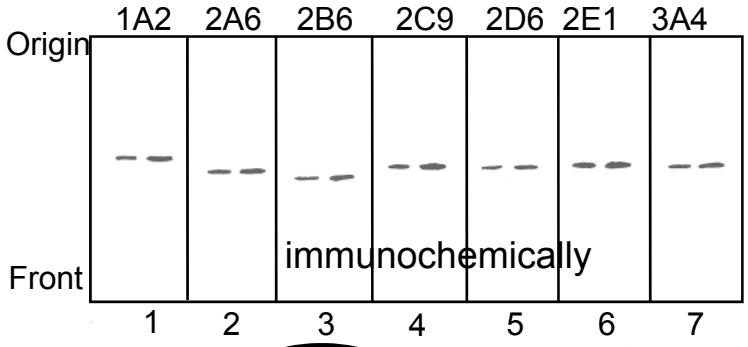
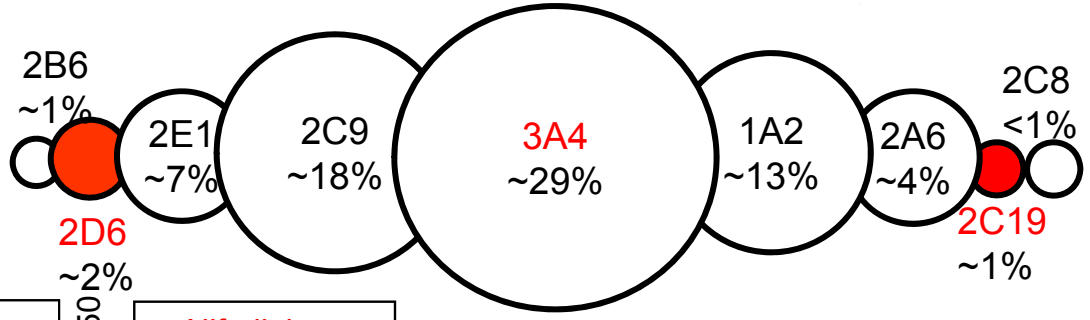
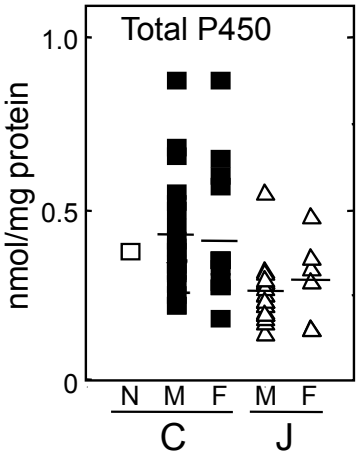
T Shimada, H Yamazaki, M Mimura, Y Inui, and F P Guengerich, J Pharmacol Exp Ther, 270:414-423 (1994). This article has been cited >2,500 times.

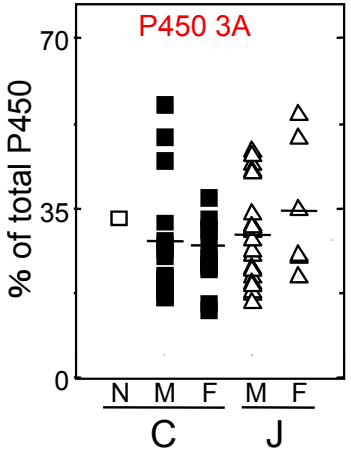


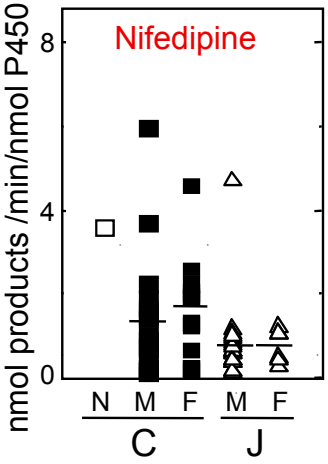


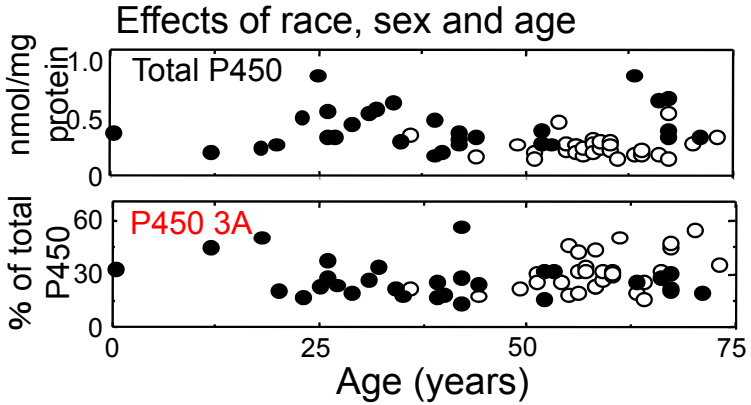
Contents of total P-450 determined spectrally and individual P-450 forms determined immunochemically in liver microsomes from 60 human samples



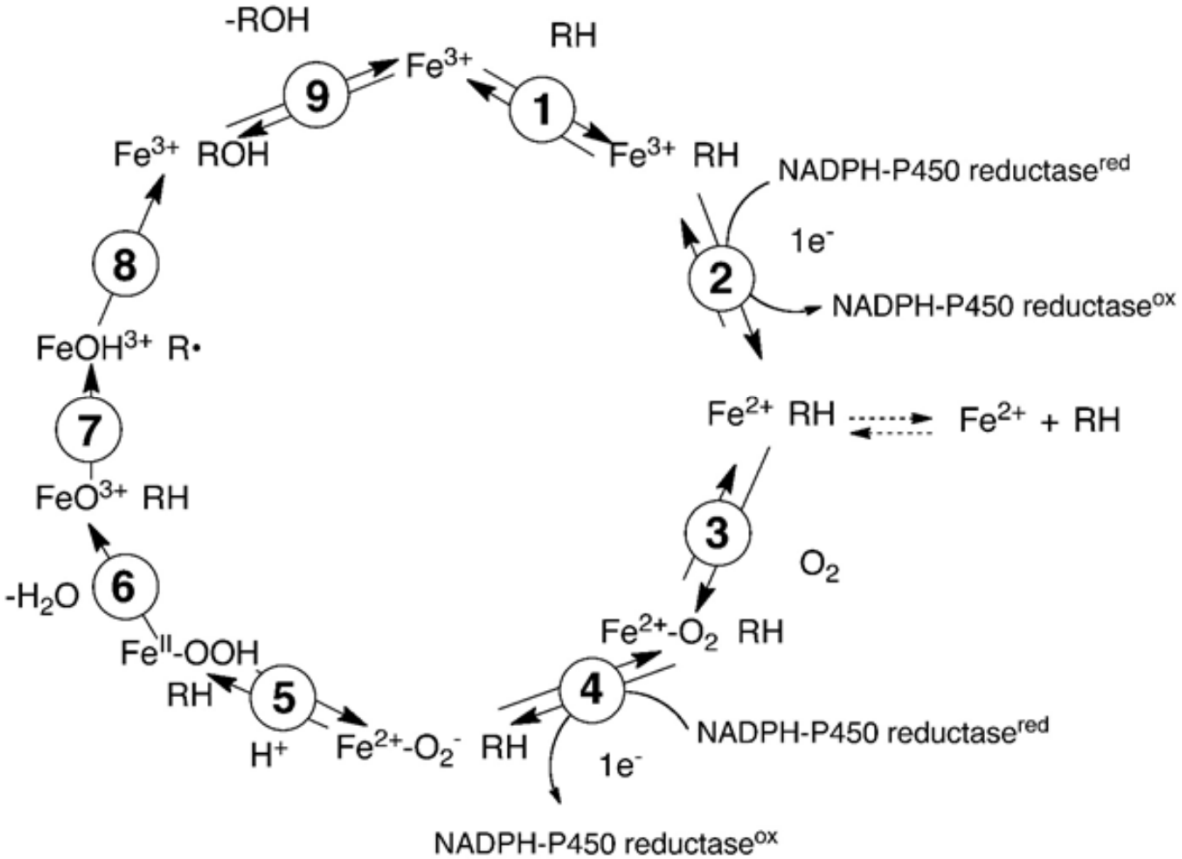


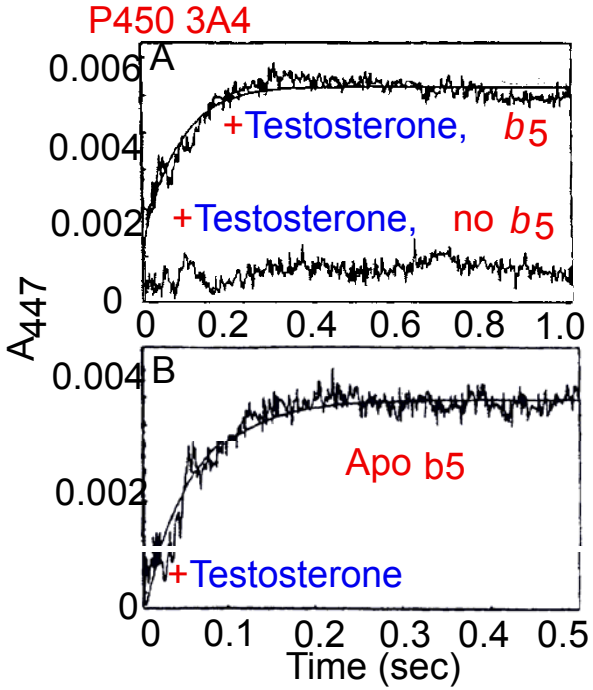






Generalized Catalytic P450 Mechanism





Lack of Electron Transfer from Cytochrome b_5 in Stimulation of Catalytic Activities of Cytochrome P450 3A4
CHARACTERIZATION OF A RECONSTITUTED CYTOCHROME P450 3A4/NADPH-CYTOCHROME P450 REDUCTASE SYSTEM AND STUDIES WITH APO-CYTOCHROME b5
H Yamazaki, WW Johnson, YF Ueng, T Shimada, FP Guengerich
Journal of Biological Chemistry 271, 27438-27444 (1996)
This article has been cited 175 times.

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Hiroshi Yamazaki, PhD

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Non-specific Protein Bindings of Drugs in Livers from Chimeric Mice with Humanized Liver

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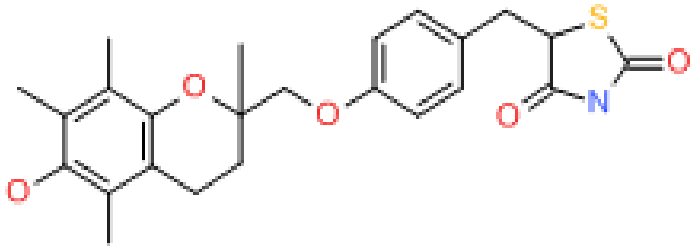
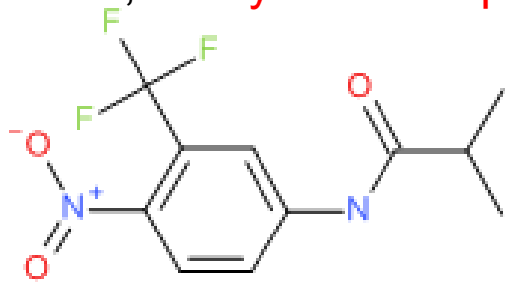
LiverTox
Clinical and Research Information on Drug-Induced Liver Injury

US NIH LiverTox database was developed.

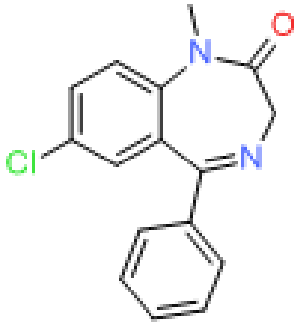
Troglitazone, idiosyncratic hepatotoxicant



Flutamide, idiosyncratic hepatotoxicant



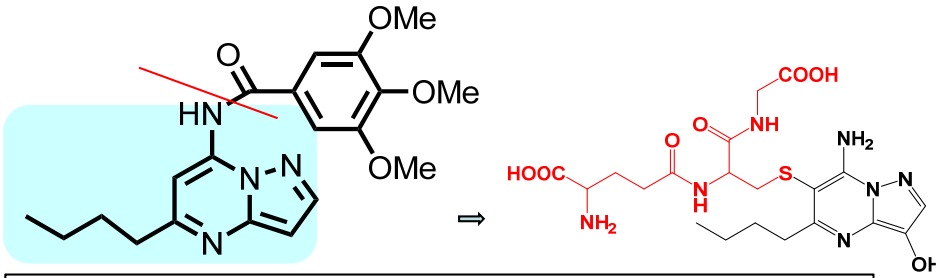
Diazepam, rarely hepatotoxic

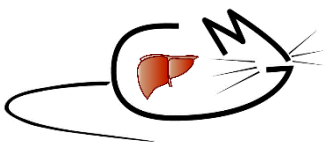


http://livertox.nih.gov/index.html

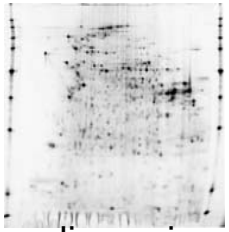
C&EN "Troublesome Drug Metabolites: Diverging Paths"
Toxicologists pool their knowledge to advance safety testing of drug candidates, August 31, 2009, Volume 87, Number 35 pp. 27 – 28.

OT-7100, an experimental analgesic drug, limited hepatotoxic effects in humans



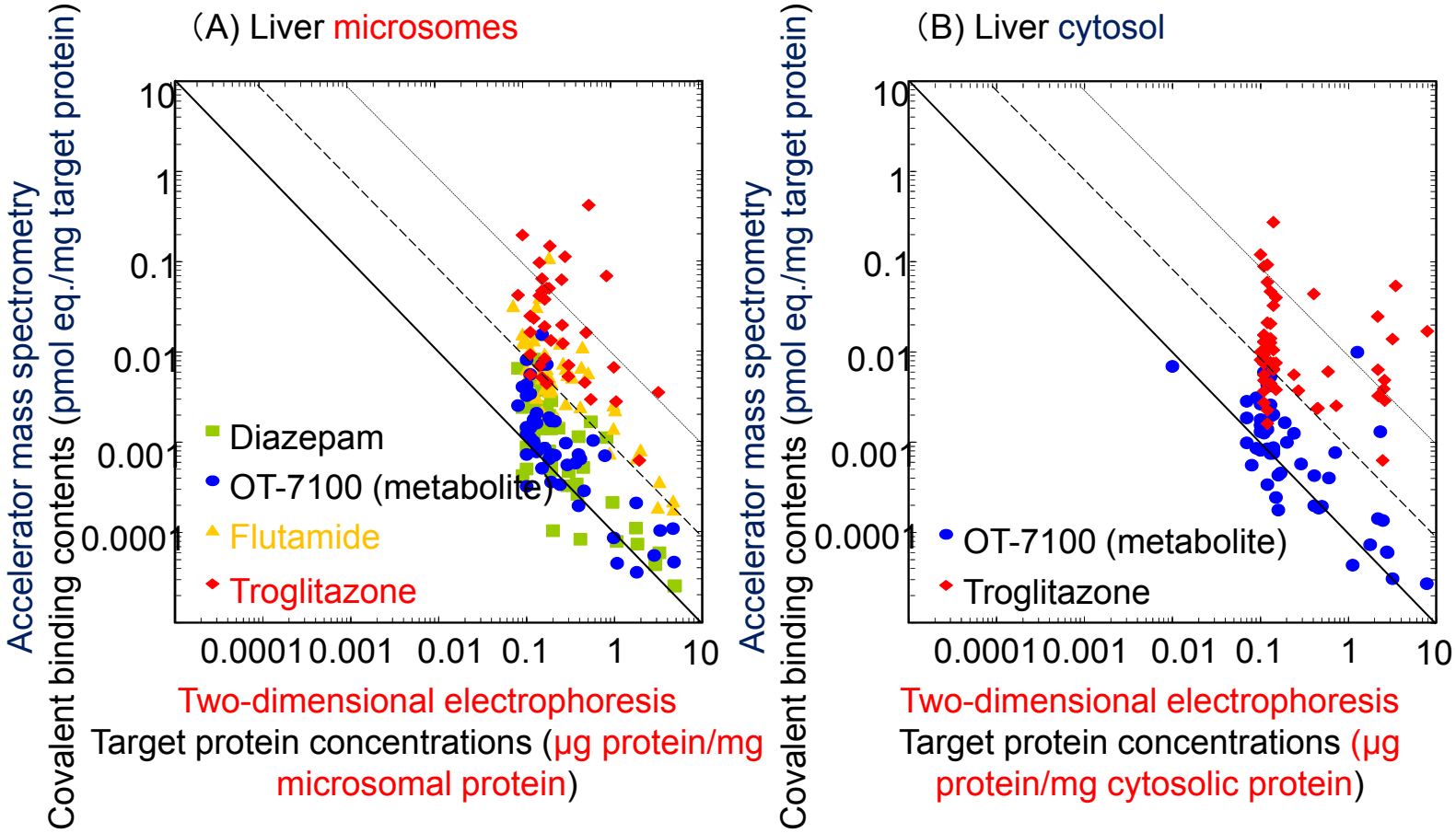


Humanized-liver mice



Two-dimensional electrophoresis associated with accelerator MS

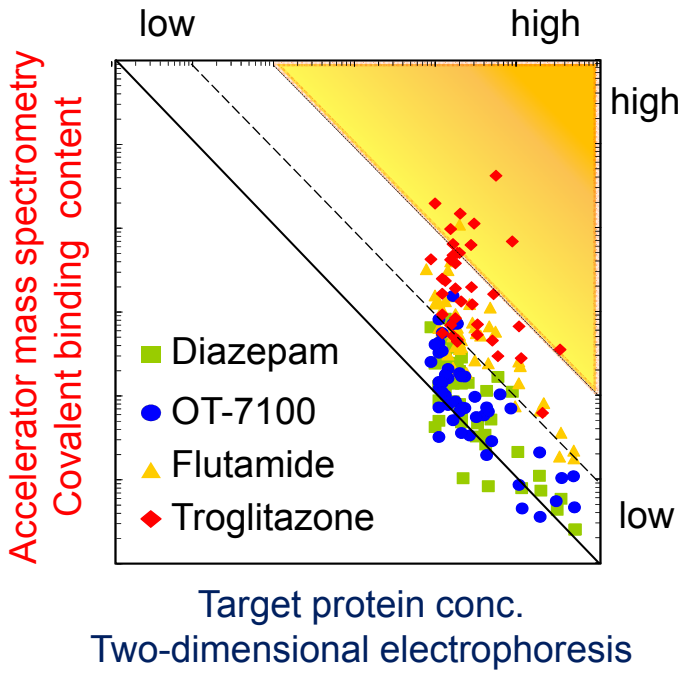
Covalent Binding Profiles of Liver Protein Fractions Separated by Two-dimensional Electrophoresis



- Lines are drawn though convenient axis intersections to indicate an inverse relationship.
- The highest binding level of troglitazone was observed with 17β-hydroxysteroid dehydrogenase in microsomal proteins and with glutathione *S*-transferase M2-2 in cytosolic proteins.
- These values for binding levels and target protein concentrations are considered to be high in **zone analysis.**

Yamazaki et al., *Chem Res Toxicol,* 23, 152-158 (2010); Yamazaki et al., *Toxicol Res,* in press.

Zone Analysis of Protein Bindings in Chimeric Mice with Humanized Liver



- A zone analysis for **imbalance of covalent bindings of substrates** (bioactivated in chimeric mice with humanized liver) **and target proteins** could prove to be a useful tool for predicting hepatotoxic effects.
- Note that the data points in the top right zone (i.e., those with high covalent binding contents and high target protein concentrations) are predominantly those of troglitazone and flutamide, both of which are known to be hepatotoxic.

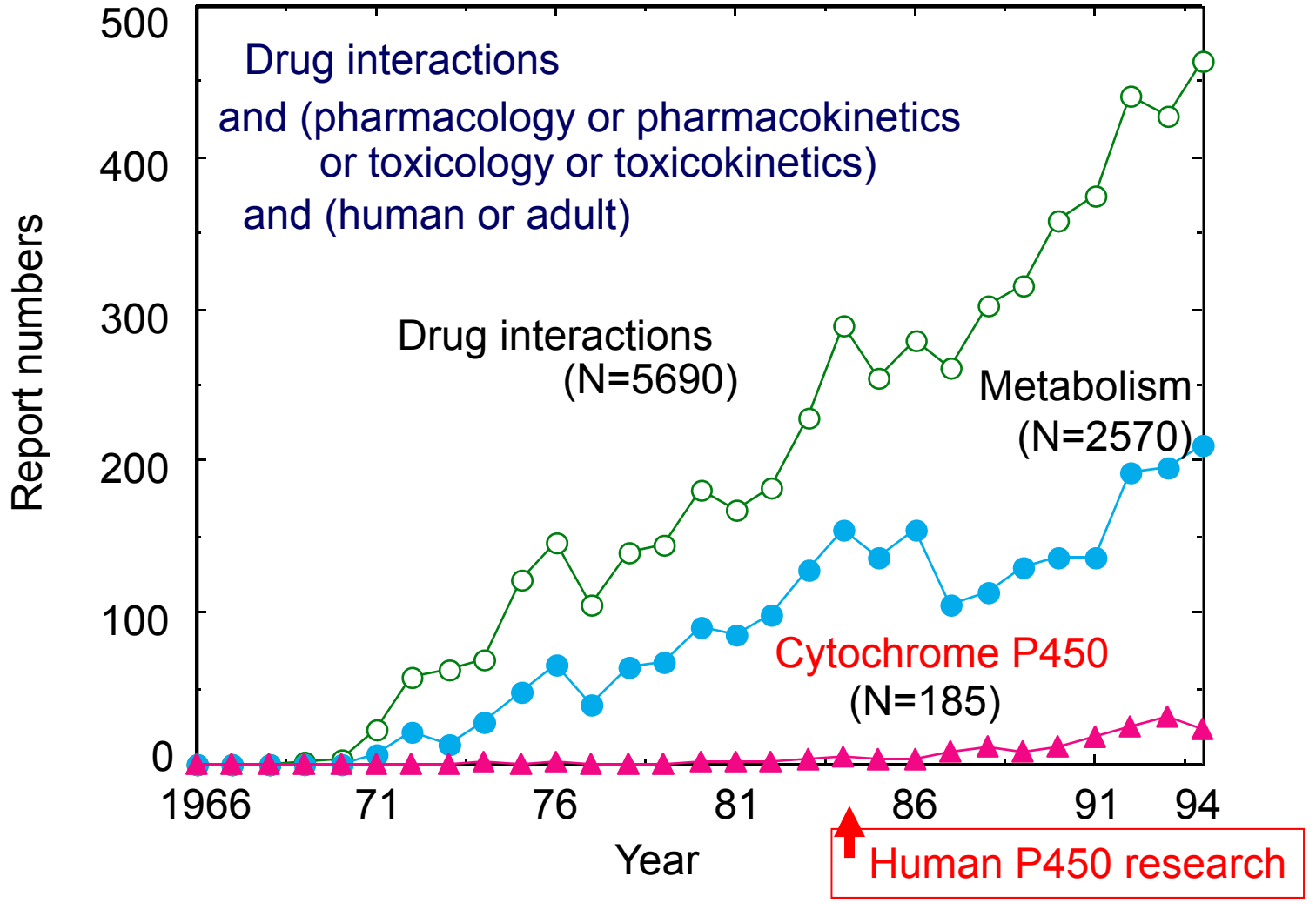
- Drug metabolism mediated by humanized livers (leading to binding *in vivo*) in combination with a zone analysis **of covalent binding contents/target protein concentration** data could be a good tool for evaluating the relationship between the nonspecific protein binding behavior of medicines and potential hepatotoxicity in humans.

Yamazaki et al., *Toxicol Res,* in press.

Numbers of Articles Mentioning Drug Interactions

Yamazaki, *Drug Metab. Pharmacokinet*. 22 (4): 223–224 (2007)

"Drug interactions" in CD-ROM-based Medline database in 1995



Drug interaction studies in the 21st century: research into cytochrome P450s, transporters, and simulations informing their role in drug-drug Interactions

Numbers of Articles Mentioning Drug Interactions in PubMed or Medline

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	03/17/ 1995	06/17/ 2007	04/17/ 2013	06/17/ 2014
Drug interactions*	5,690	53,849	101,369	106,950
+ metabolism	2,570	30,438	62,418	66,611
+ P450 or CYP	185	2,868	3,780	4,122
+ transporter	-	1,611	5,705	6,072

- * Drug interactions and (pharmacology or pharmacokinetics or toxicology or toxicokinetics) and (human or adult) were used for keyword search.
- Metabolic drug interactions are still relevant in the 21st century although papers dealing with “transport" related research are now on the increase.

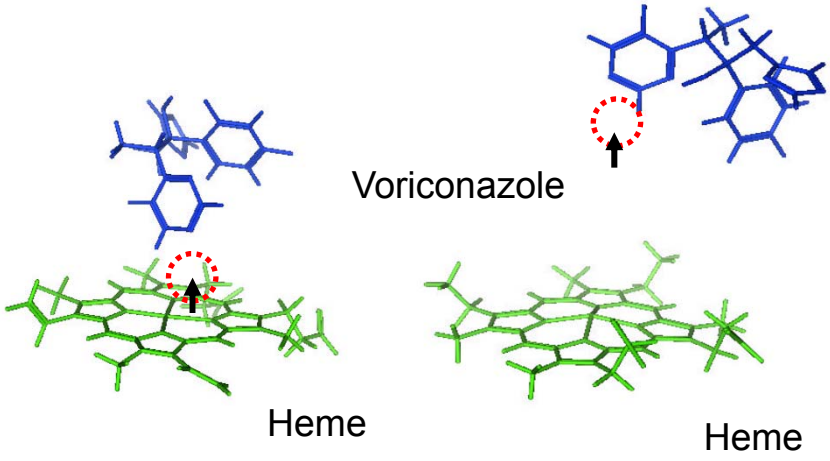
Yamazaki, *Drug Metab Pharmacokinet* 22: 223–224 (2007); Yamazaki, *Fifty Years of Cytochrome P450 Research*, 2014, and updated.

Effects of Voriconazole and Thalidomide on *In Vitro* Midazolam Metabolism

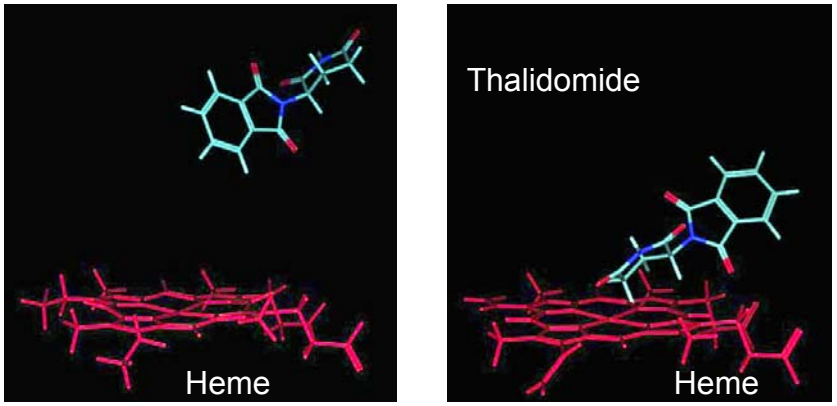
P450	Voriconazole Inhibition, K_i (μM)	Thalidomide Activation, *Ke* (μM)
Recombinantly expressed P450		
3A4	0.15	850
3A5	0.20	40
Liver microsomes		
*3A5*3*/*3	0.20	770
*3A5*1*/*3	0.45	62

Ke, effector constant, where
*K*m'= *K*m / (1+[Thalidomide]/*Ke*).

- Less inhibition of voriconazole on P450 3A5 catalytic function could be derived from far adopting orientation over the heme.

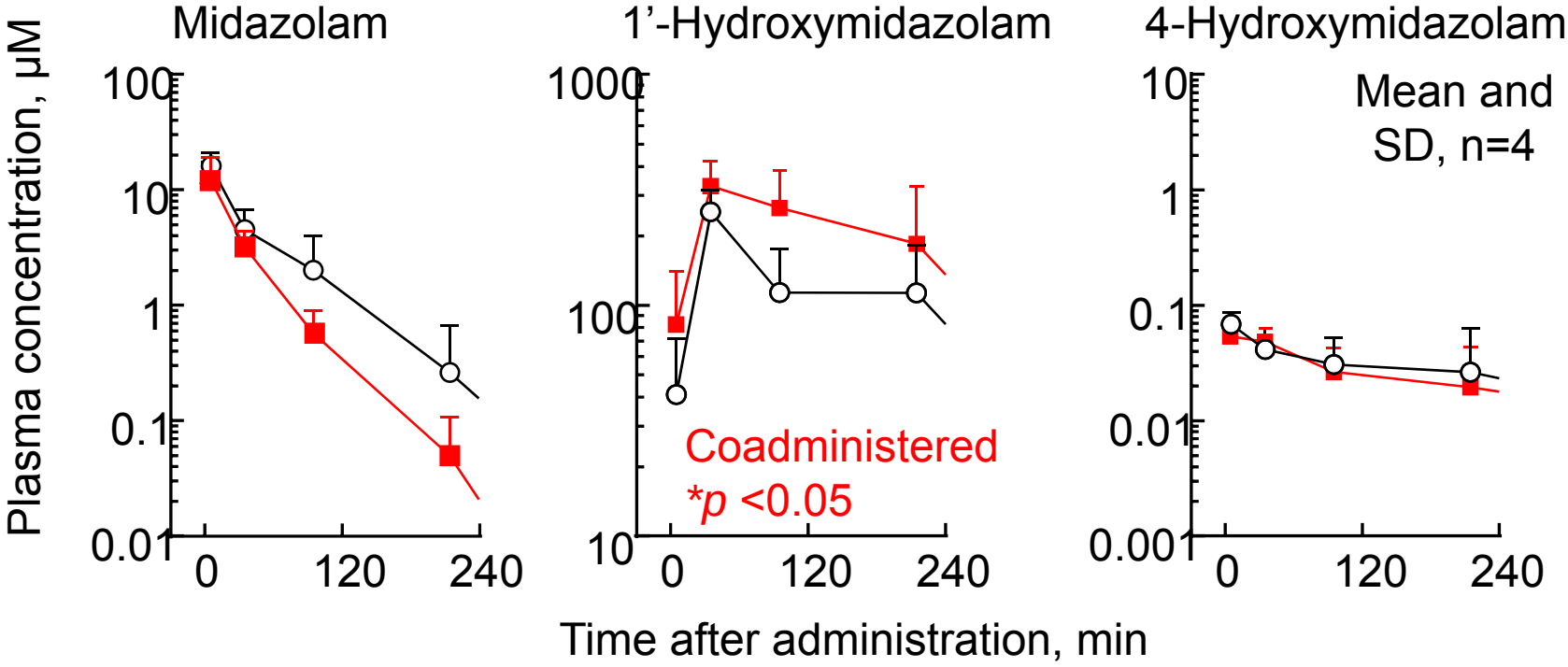


P450 3A4 (PDB 1WOG) P450 3A5



Okada, Yamazaki et al., *Drug Metab Dispos*, 37, 18-23 (2009)
Yamazaki et al., *Brit J Clin Pharmacol,* 69, 593-597 (2010)

Effects of Thalidomide on Midazolam Pharmacokinetics in TK-NOG Mice with Humanized Liver



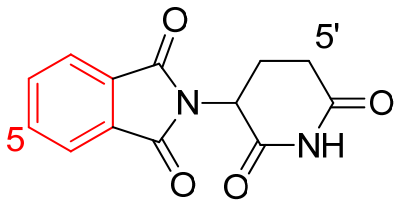
Thalidomide (100 mg/kg) was orally co-administered intravenous midazolam treatment (10 mg/kg) in mice with humanized liver. *p <0.05.

- A higher AUC of 1'- hydroxymidazolam (1.7-fold) was obtained with thalidomide due to heterotropic cooperativity of human P450 3A enzymes.
- Midazolam pharmacokinetics in control mice was not affected by co-administration.

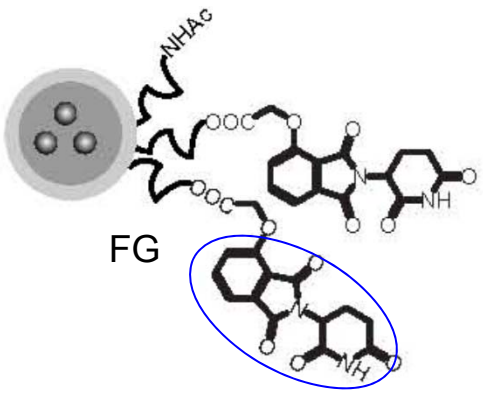
Yamazaki et al., *Chem Res Toxicol,* 26, 486-489 (2013)

Thalidomide: Original Issue Is Birth Defects

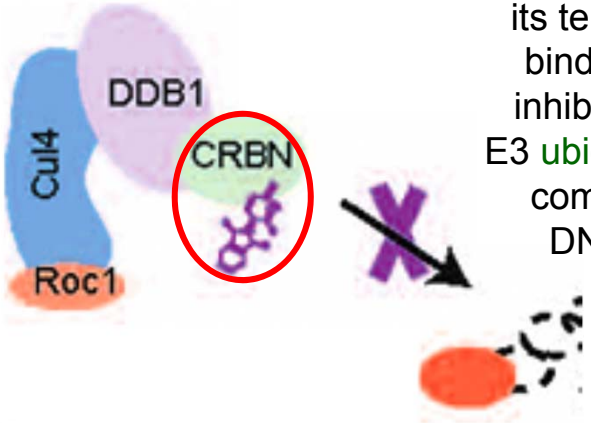
- Cereblon (CRBN) has been reported as a thalidomide-binding protein important for limb outgrowth (Ito et al., Science, 2010) using thalidomide-ferriteglycidyl methacrylate beads (FG, “Handa” beads).



Thalidomide



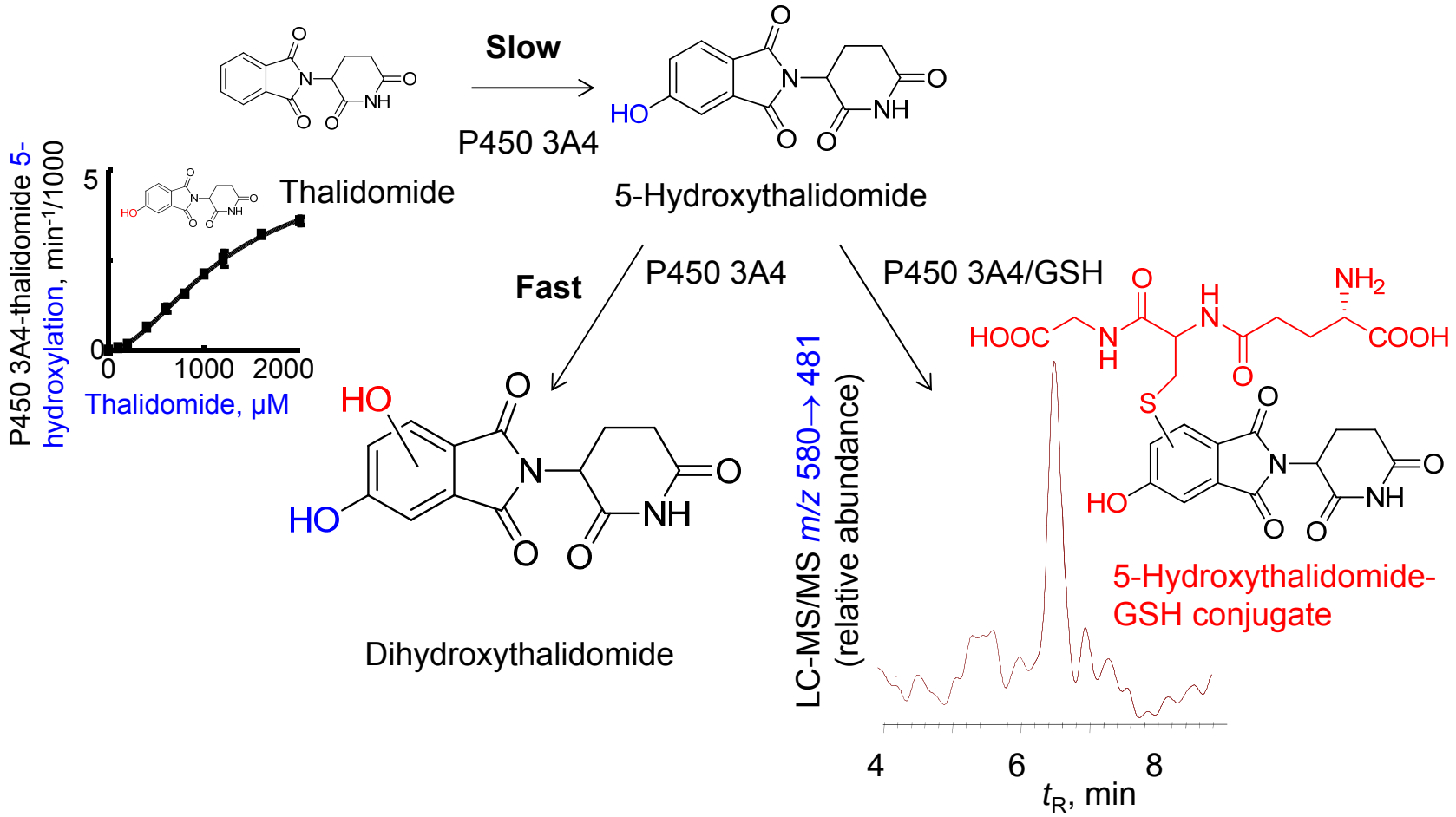
Thal-immobilized beads



Thalidomide would initiate its teratogenic effects by binding to cereblon and inhibiting the associated E3 ubiquitin ligase activity complex with damaged DNA binding protein 1 (DDB1) and Cul4A

- Thalidomide chemically connected to “Handa” beads is unable to be oxidized at its aromatic ring.

Reactive Metabolites of 5-Hydroxythalidomide by Human P450 3A4



- Reactive metabolite(s) from aromatic 5-hydroxythalidomide was trapped and assigned as a conjugate of 5-hydroxythalidomide formed on the phenyl ring, 5-hydroxythalidomide-GSH conjugate.
- Metabolic activation of thalidomide occurs but details are not known yet.

Okada, Yamazaki et al., *Drug Metab Dispos*, 37, 18-23 (2009); Chowdhury, Yamazaki et al., *Chem Res Toxicol,* 23, 1018-1024 (2010); Yamazaki et al., *Chem Res Toxicol,* 25, 274-279 (2012)

Metabolites in *In Vivo* TK-NOG Mice with Humanized Liver after Oral Administration of Thalidomide

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(A) Thalidomide
m/z 257 → 146

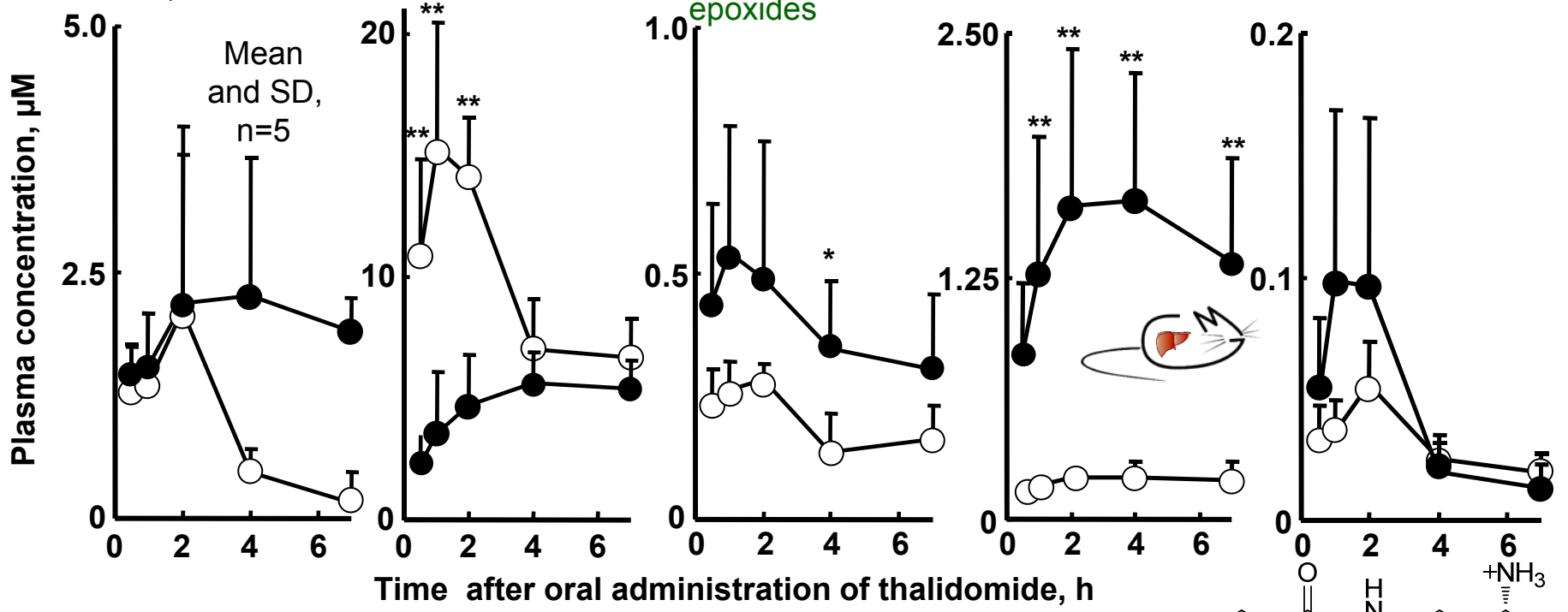
○, control
●, humanized

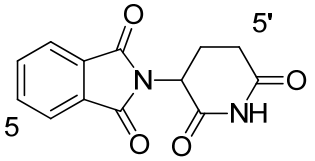
(B) 5'-Hydroxy-thalidomide
m/z 273 → 146

(C) 5-Hydroxy-thalidomide
m/z 273 → 161
activated to reactive epoxides

(D) Dihydroxy-thalidomide
m/z 289 → 177

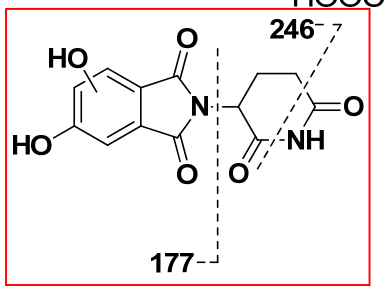
(E) 5-Hydroxy-thalidomide GSH conjugate
m/z 580→451

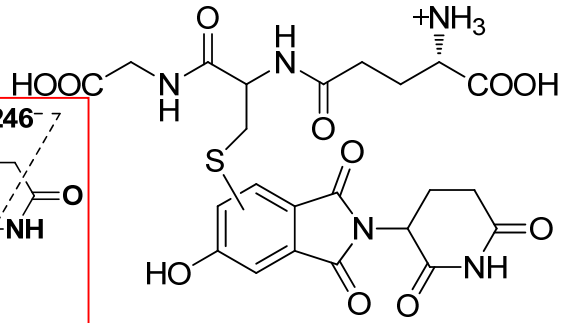




Thalidomide (100 mg/kg)

*p <0.05 two-way ANOVA with Bonferroni post tests.





m/z 580.1

Yamazaki et al., *Chem Res Toxicol,* 25, 274-279 (2012)

Poster-60: Auto-induction of Human P450 3A by Thalidomide via PXR Pathway (confirmed humanized mice)

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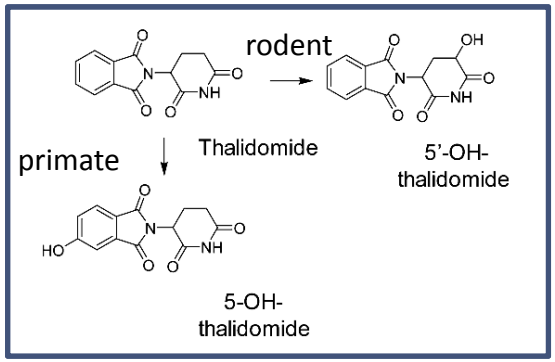
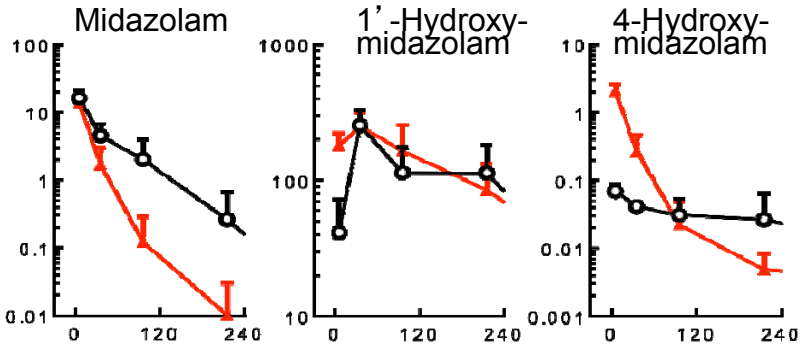


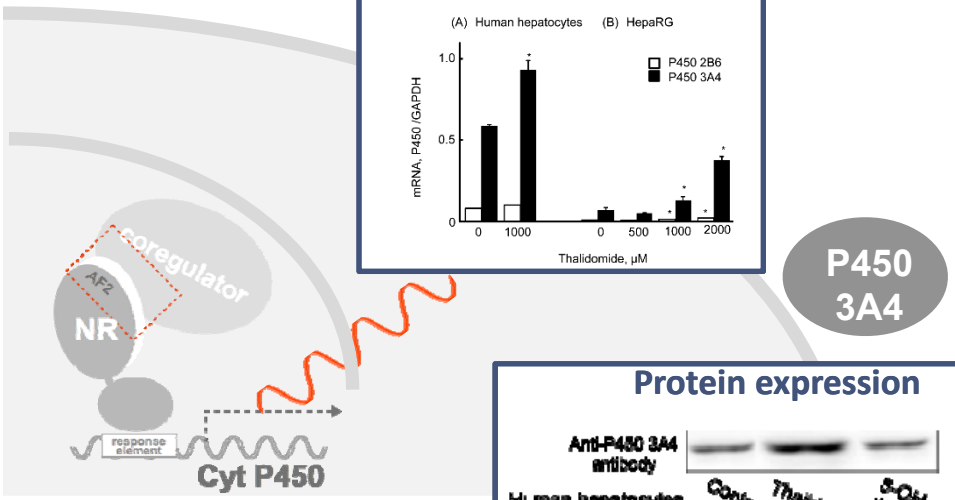
Compound structure

Drug clearance

humanized-liver TK-NOG mice



Effects of pretreatment of thalidomide in TK-NOG mice with humanized liver



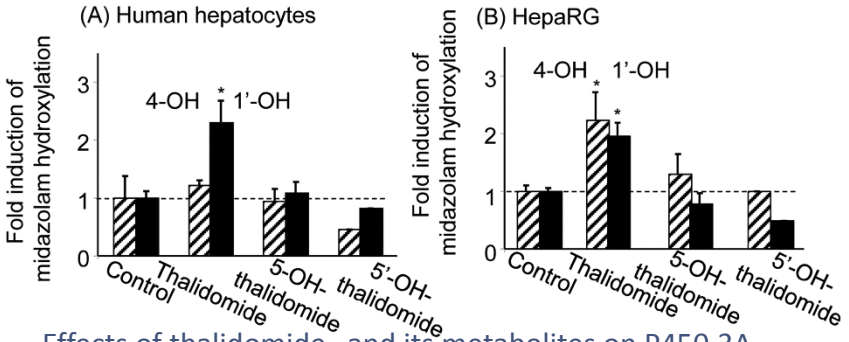
mRNA expression

P450 3A4

Protein expression

liver cells

Enzyme activity



Effects of thalidomide and its metabolites on P450 3A enzymes in HepaRG and human hepatocytes

Micro Array for Realtime Coreglator-nuclear Receptor Interaction

Yamazaki et al., *Chem Res Toxicol,* 26: 486-489 (2013);
Murayama, Yamazaki et al., *Chem.Res.Toxicol.* 27:304-308 (2014).

Showa Pharmaceutical University

Hiroshi Yamazaki, PhD

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Autologous P450 isoforms in Humans, Macaques, and Pigs

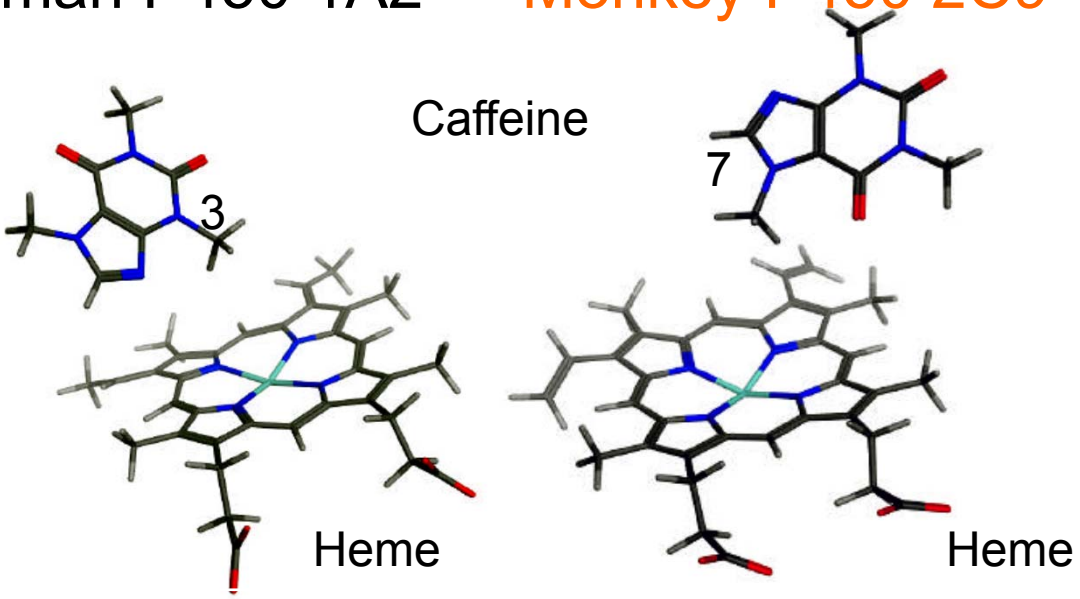
Human	Cynomolgus monkey	Marmo-set	Minipig
1A1	1A1		1A1
1A2	1A2	1A2	1A2
1B1	1B1		
[1D1P]	1D1		
2A6	2A23		2A19
[2A7]	2A24		
2A13	2A26		
2B6	2B6	2B6	2B22
2C8	2C8(20)	2C8	2C33
[2C18]	2C18		2C42
2C9	2C9(43)		2C49
2C19	2C19(75)		
	2C76		
	2C93		

Human	Cynomolgus monkey	Marmo-set	Minipig
2D6	2D17	2D19	2D25
	2D44		
2E1	2E1	2E1	2E1
2G2P	2G2		
2J2	2J2		2J34
3A4	3A4(8)	3A4	3A22
3A5	3A5	3A5	3A29
3A7	(3A7)	3A90	3A39
3A43	3A43		3A46
4A11	4A11		4A21
4A22			4A24

Rendic and Guengerich (2010), Uno et al. (2011), Achour et al (2011), Yamazaki, *Fifty Years of Cytochrome P450 Research*, 2014, and updated.

Caffeine Metabolism by Monkey and Human P450s

- Caffeine 3-*N*-demethylation is major metabolic pathway in humans.
- 7-*N*-demethylation to form pharmacological active theophylline is predominant in monkeys.
- 8-Hydroxylation in mice, rabbits, and rats have been shown to be broadly similar.
- Interspecies differences regarding caffeine oxidation exist.



Reaction :	3-*N*-demethylation	7-*N*-demethylation (**theophylline** formation)
V_{max} / K_m :	1.4 $(mM \cdot min)^{-1}$	1.7 $(mM \cdot min)^{-1}$

Utoh, Yamazaki et al., Xenobiotica 43, 1037-1042 (2013)

Qualitative *De Novo* Analysis of Full Length cDNA and Quantitative Analysis of Gene Expression

Pooled into one sample

Male

Female

Liver Intestine Kidney Brain

[DDBJ Accession number]
SUBMISSION: DRA002207
EXPERIMENT: DRX016291
RUN: DRR018050
http://trace.ddbj.nig.ac.jp/DRASearch

Preparation of random primed normalized cDNA library

GS FLX+ sequencing and *de novo* assembly

cDNA contigs 47,883

Preparation of eight 3'-fragment cDNA libraries

HiSeq 2000 sequencing with eight libraries multiplex

Single read of 3'-end of cDNA

Blastx and GO annotation

Protein A… GO: ……

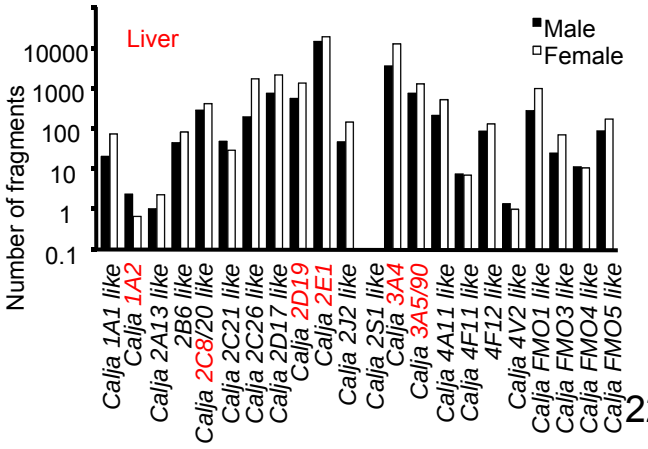
Protein B… GO: ……

Protein C… GO: ……

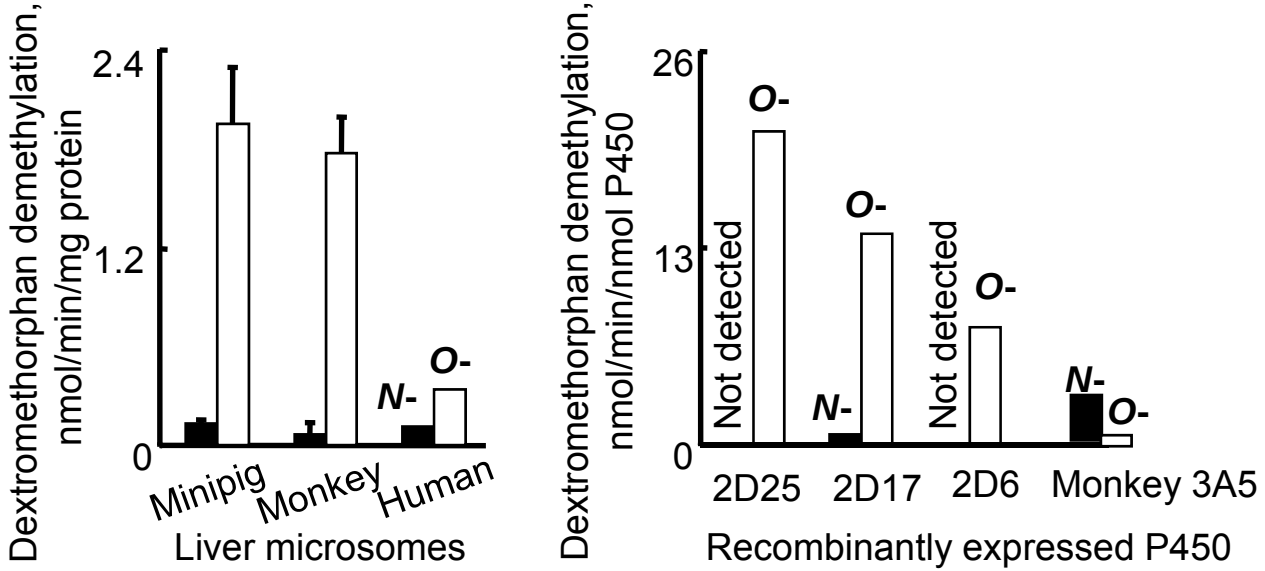
Species	N	%
Callithrix jacchus	13,679	29

Mapping on cDNA contigs

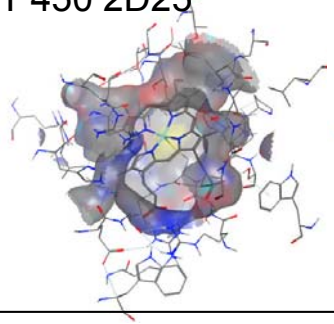
Shimizu, Yamazaki et al, *PLoS One*, 9, e100936 (2014)



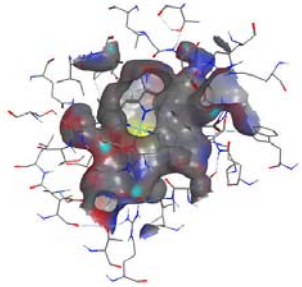
P450 2D-Mediated Dextromethorphan Demethylation



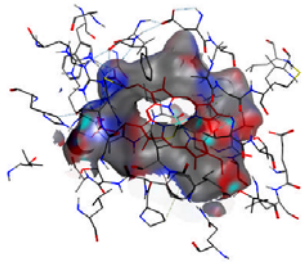
Modeled minipig P450 2D25



Modeled monkey P450 2D17



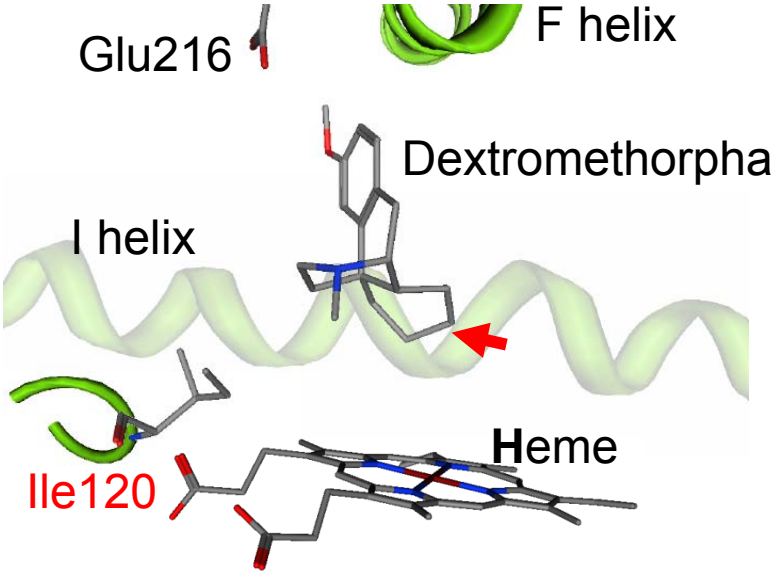
Human P450 2D6

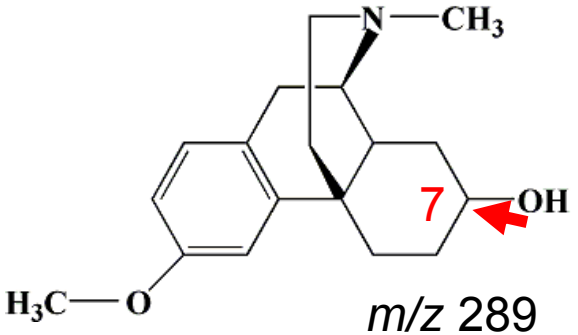


- Monkey P450 2D and 3A enzymes exhibited wider substrate selectivity toward human P450 2D substrates, resulting in high P450 2D-related drug clearance in monkeys.
- Minipig P450 2D-mediated drug oxidation activities were high compared with humans probably because of high P450 2D concentrations in minipig livers

Yamazaki, *Fifty Years of Cytochrome P450 Research*, 2014

Novel *CYP2D6*10*-related Haplotypes as Possible Causes of a Poor Metabolic Phenotype





m/z 289
[M+H]+16

7-Hydroxydextromethorphan

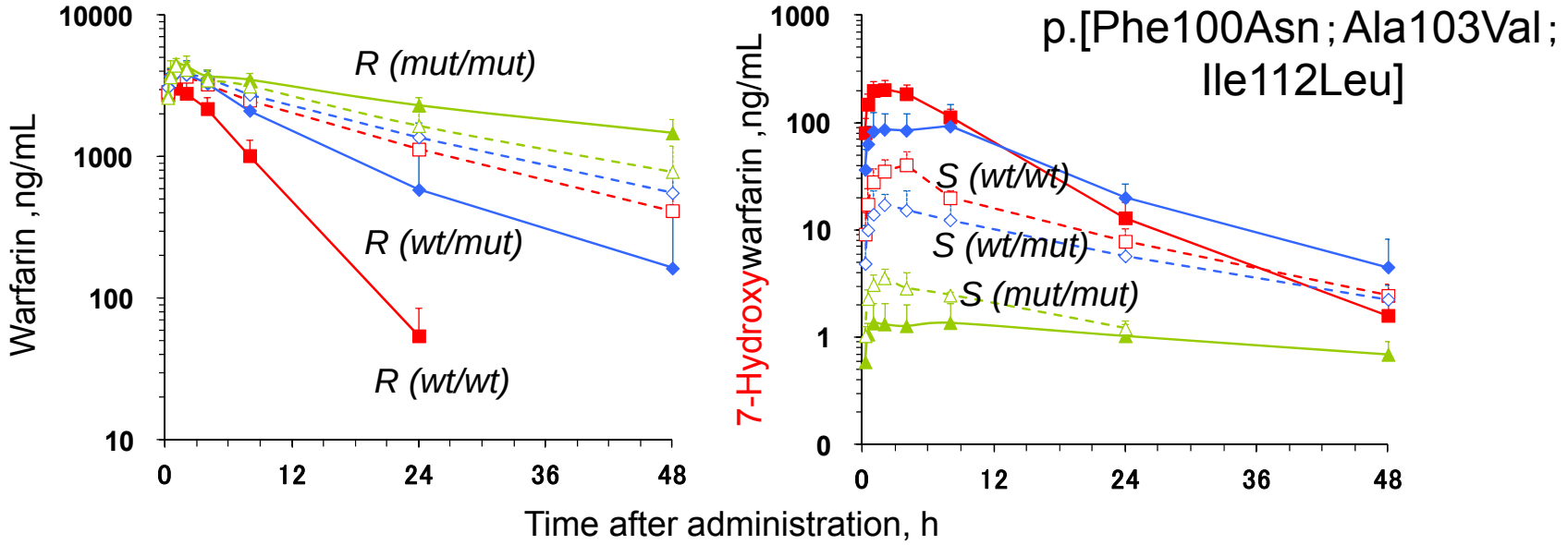
- During the course of sequencing the human *P450 2D6* gene, a novel **F120I variant (*2D6*49*)** was found.
- With dextromethorphan as a substrate, P450 2D6.49 formed a novel 7-hydroxydextromethorphan, with a roughly similar Vmax/Km value to that of *O*-demethylation.
- Variability in P450 2D substrate pockets and contents in livers may result in species and individual differences.

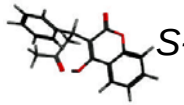
Matsunaga, Yamazaki, *Drug Metab Dispos*, 37, 699-701, 2009; Yamazaki, *Fifty Years of Cytochrome P450 Research*, 2014

Genetic Polymorphism of Cynomolgus Monkey P450s

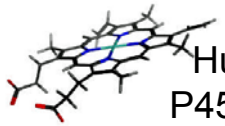
1. Uno, Yamazaki et al. (2014) Polymorphisms of ***CYP2D17*** in cynomolgus and rhesus macaques: an evidence of the genetic basis for the variability of CYP2D-dependent drug metabolism. *Drug Metab. Dispos.*, 42, 1407-1410.
2. Uno, Yamazaki et al. (2014) ***CYP2C19*** polymorphisms account for inter-individual variability of drug metabolism in cynomolgus macaques. *Biochem. Pharmacol.*, 91, 242-248.
3. Uno, Yamazaki et al. (2015) Genetic polymorphism of cynomolgus and rhesus macaque ***CYP2C9***. *Drug Metabolism and Pharmacokinetics*, in press.

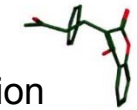
Poster-71: Warfarin Metabolism Mediated by Polymorphic P450 2C19 in Cynomolgus Monkeys

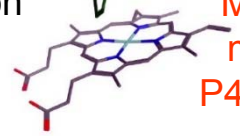



S-Warfarin

7-hydroxylation


Human
P450 2C9


R-Warfarin


Modeled
monkey
P450 2C19

R-/ *S*-	K_m, μM	V_{max}, min^{-1}	*R*-/ *S*-	K_m, μM	V_{max}, min^{-1}
R	16	0.01	*R*	30	7.0
S	10	0.16	*S*	120	0.5

Hosoi, Yamazaki et al., *Biochem Pharmacol* 84, 1691–1695 (2012)

Genetic Polymorphism of Cynomolgus Monkey P450s

1. Uno, Yamazaki et al. (2014) Polymorphisms of *CYP2D17*.. *Drug Metab. Dispos.*, 42, 1407-1410.
2. Uno, Yamazaki et al. (2014) *CYP2C19* polymorphisms.. *Biochem. Pharmacol.*, 91, 242-248.
3. Uno, Yamazaki et al. (2015) Genetic polymorphism of *CYP2C9*... *Drug Metabolism and Pharmacokinetics*, in press.

Showa Pharmaceutical University

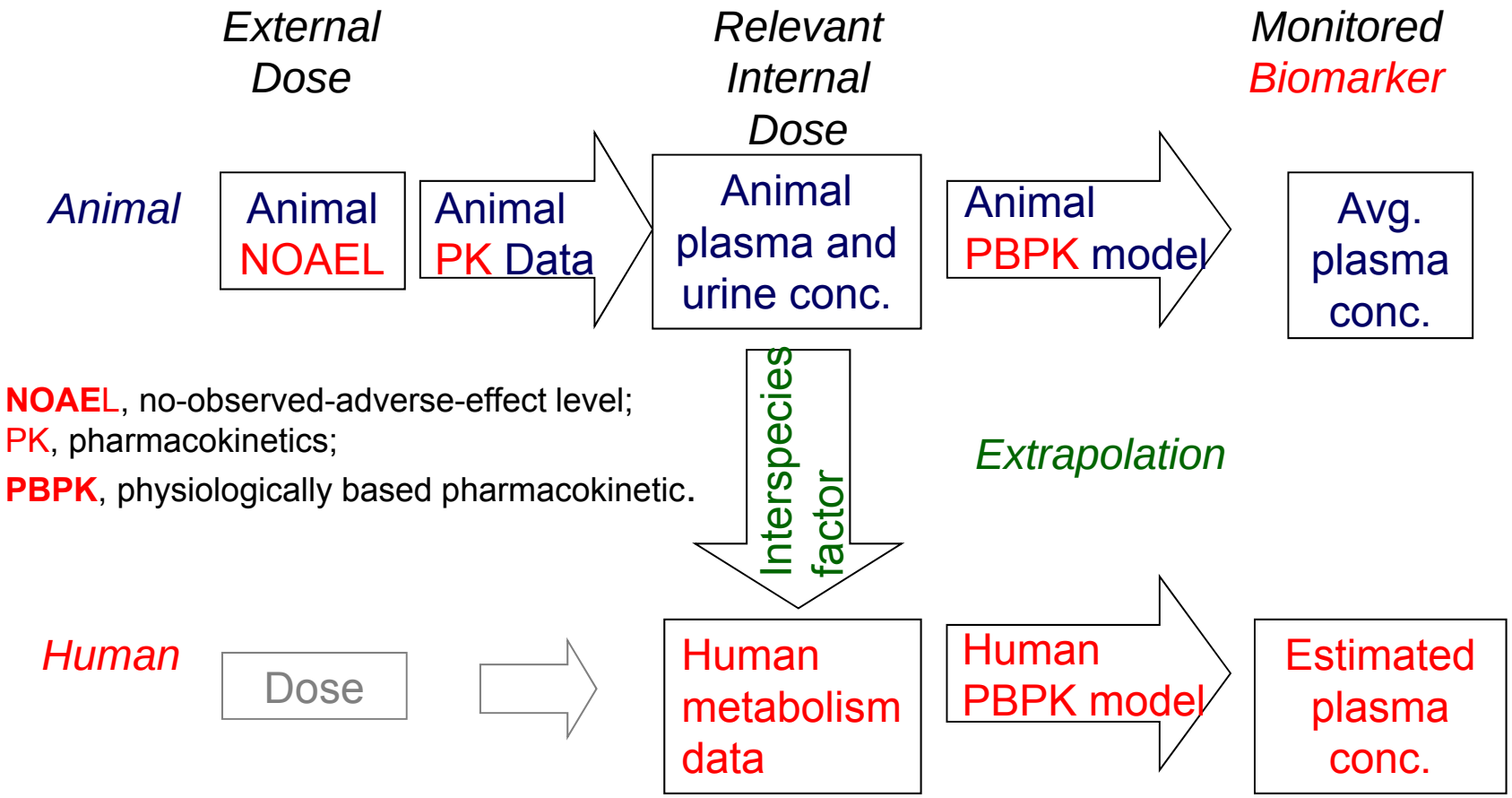
Hiroshi Yamazaki, PhD

Metabolic activation and fate of xenobiotics determined by polymorphic drug-metabolizing enzymes

- Generalized Catalytic P450 Enzymes
- Reactive Metabolites in Humanized-liver Mice

  Thalidomide activation and drug interaction
- Monkey and human P450 enzymes
- Physiologically based pharmacokinetic (PBPK) modeling
- Flavin-containing monooxygese

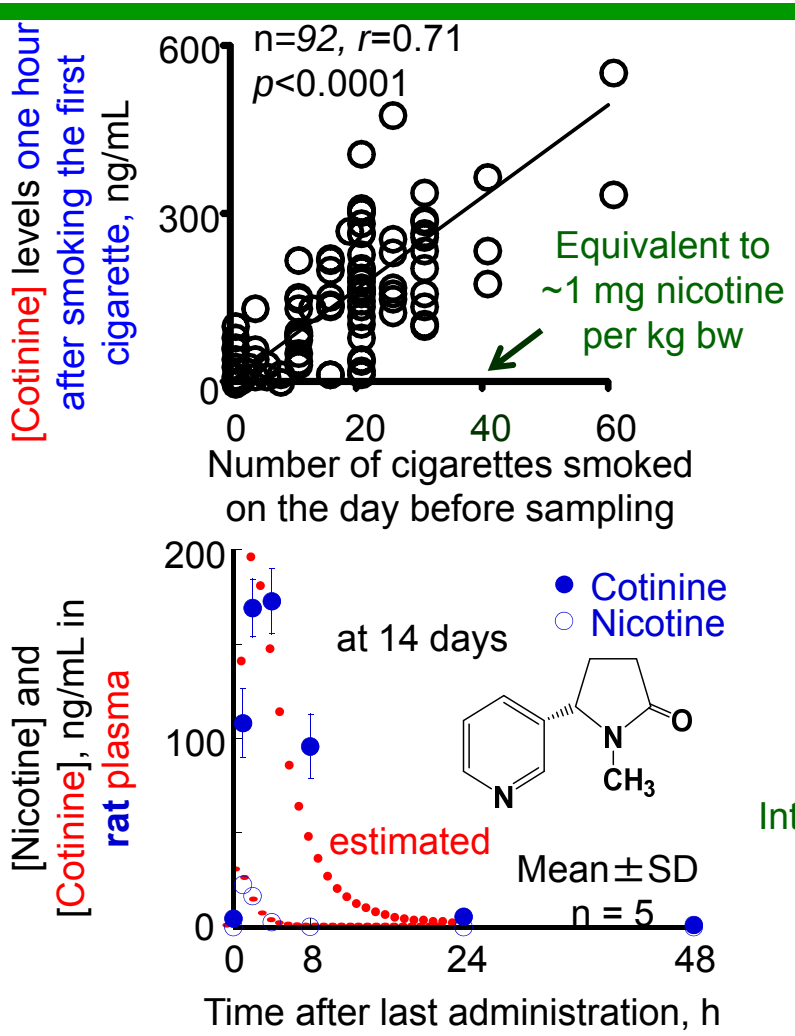
Approach for Calculating Blood-based Biomonitoring Equivalents

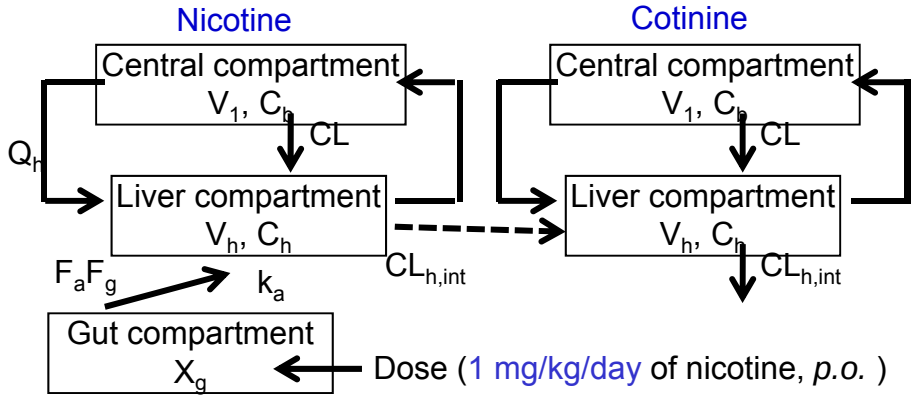


- Adjusted animal biomonitoring equivalents after administered doses in (humanized) animal studies were scaled to human biomonitoring equivalents using known species allometric scaling factors and human metabolic data with a simple PBPK model.

J.Health Sci., 56, 557-565, 2010; *ibid*, 56, 566-575, 2010; *Regul. Toxicol. Pharmaco*l., 58, 252-258, 2010; ; *ibid*, 65, 316-324, 2013, *Int.J.Environ.Res.Public Health*, 7, 3406-3421, 2010

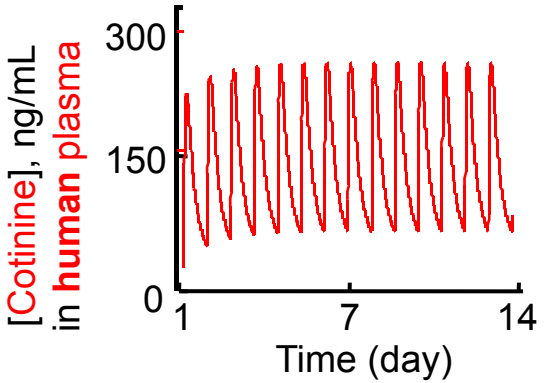
Biomonitoring and Prediction of Human Cotinine Levels





- ✓ PBPK modeling based on rat data
- ✓ *In vitro* nicotine hepatic clearance
- ✓ Possibly simulated in humans with human parameters (bw etc.)

Interspecies factor

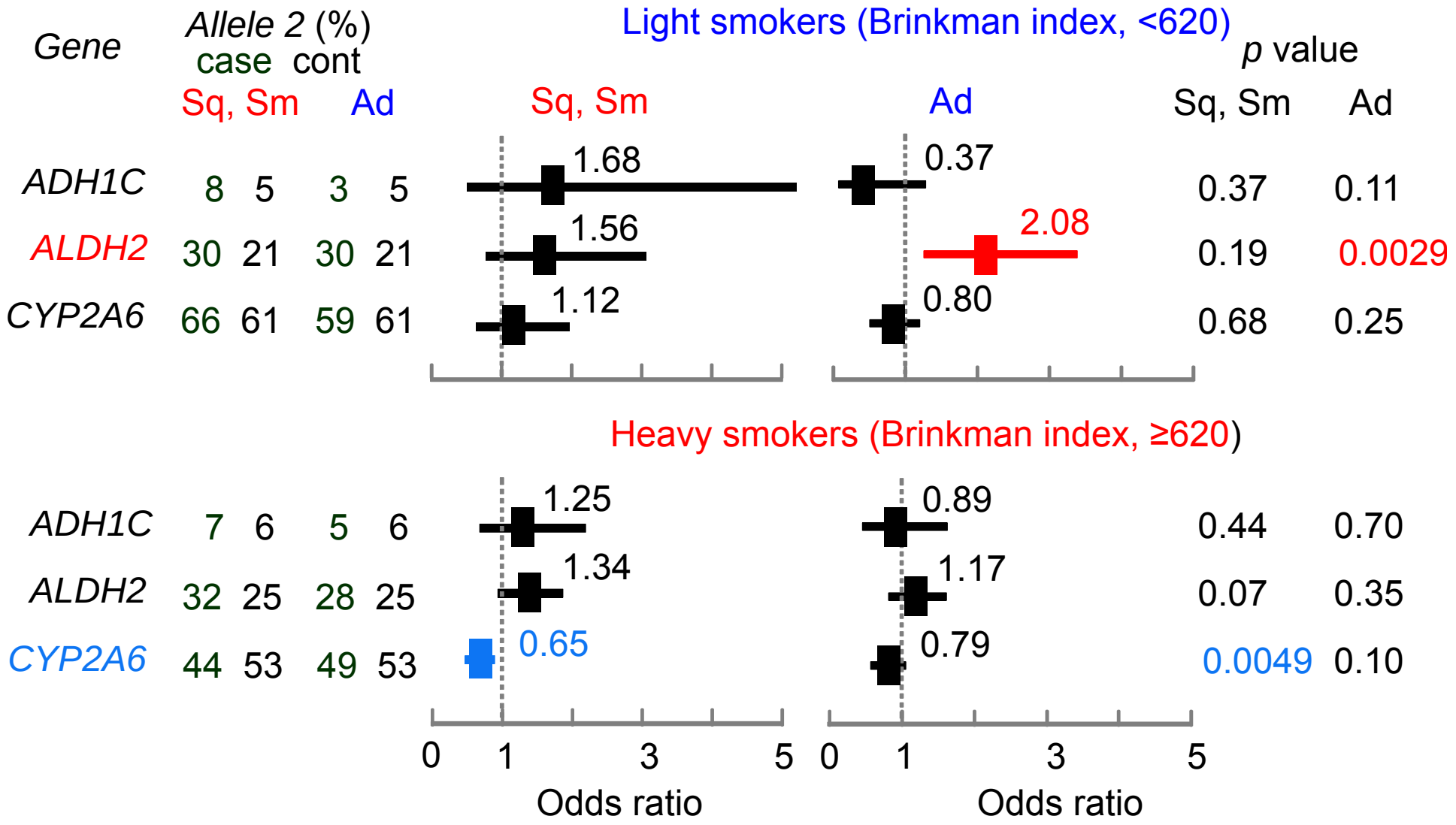


- Plasma cotinine levels were correlated with the number of cigarettes smoked on the day before sampling.
- Simplified PBPK model of nicotine and cotinine was developed and validated with a combination of algorithms, *in vitro* and *in vivo* experiments.

Nagano, Yamazaki et al., *Int. J. Environ. Res. Public Health* 2010, 7, 2953-2964; Yamazaki et al, *ibid*, 2010, 7, 3406-3421

Association of *Alcohol Dehydrogenase 1C* (*ADH1C*), Aldehyde Dehydrogenase 2 (*ALDH2*), and *CYP2A6* with Lung Cancer Risk in Male Japanese Smokers

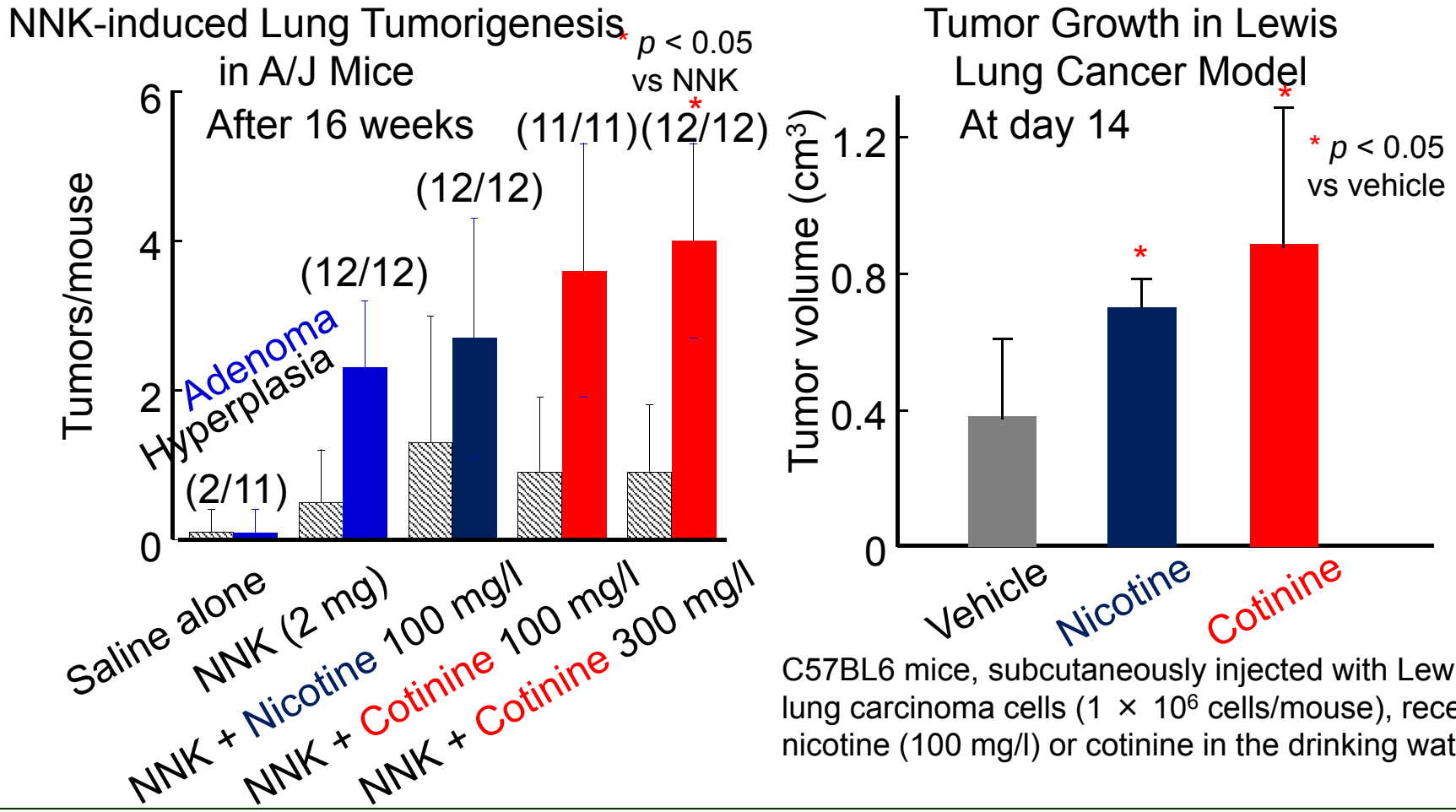
Showa Pharmaceutical University



Sq, squamous cell carcinoma; Sm, small cell carcinoma; Ad, adenocarcinoma.

Shimizu, Yamazaki *et al.*, *Journal of Cancer Therapy* 4, 29-35 (2013)

Lung Tumogigenesis Promoted by Cotinine, A Nicotine Metabolite by P450 2A Enzymes



C57BL6 mice, subcutaneously injected with Lewis lung carcinoma cells (1 × 10^6 cells/mouse), received nicotine (100 mg/l) or cotinine in the drinking water.

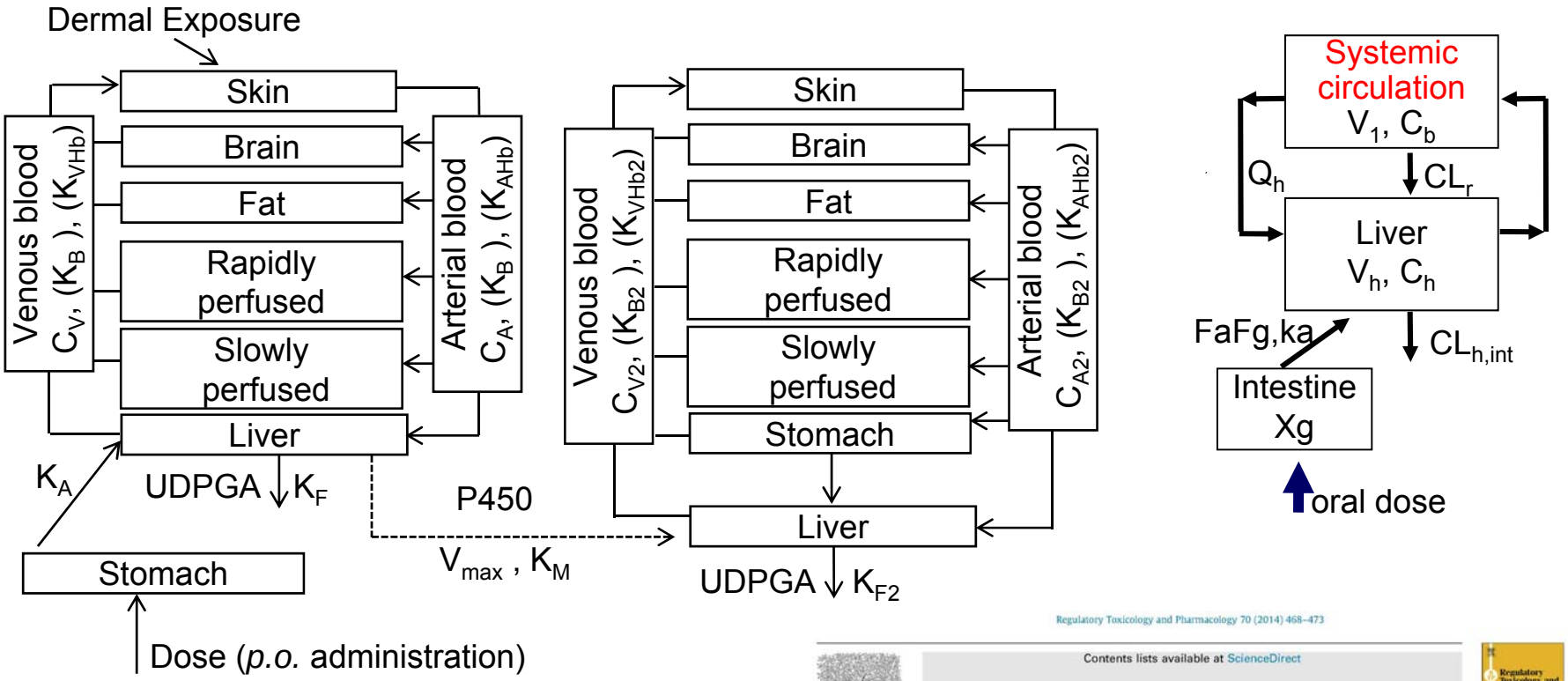
- Plasma concentrations of nicotine and cotinine in smokers are 0.12 and 0.85 μM.
- Cotinine could stimulate anti-apoptotic effects and tumor proliferative effects more than nicotine.

Nakada, Yamazaki *et al.*, *J Toxcol Sci,* 37, 555-563 (2012).

Organophosphorus Pesticide Chlorpyrifos Concentrations Hypothetically Modeled in Humans

(A) Timchalk model (2002)

(B) Suemizu model (2014)



TOXICOLOGICAL SCIENCES 66, 34–53 (2002)
Copyright © 2002 by the Society of Toxicology

A Physiologically Based Pharmacokinetic and Pharmacodynamic (PBPK/PD) Model for the Organophosphate Insecticide Chlorpyrifos in Rats and Humans

C. Timchalk,*,1 R. J. Nolan,† A. L. Mendrala,‡ D. A. Dittenber,‡ K. A. Brzak,‡ and J. L. Mattsson†

*Battelle Pacific Northwest Division, Chemical Dosimetry, PO Box 999, Richland, Washington 99352; †Dow AgroSciences, 9330 Zionsville, Indianapolis, Indiana 46268; and ‡The Dow Chemical Co., Midland, Michigan 48674

Regulatory Toxicology and Pharmacology 70 (2014) 468–473

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

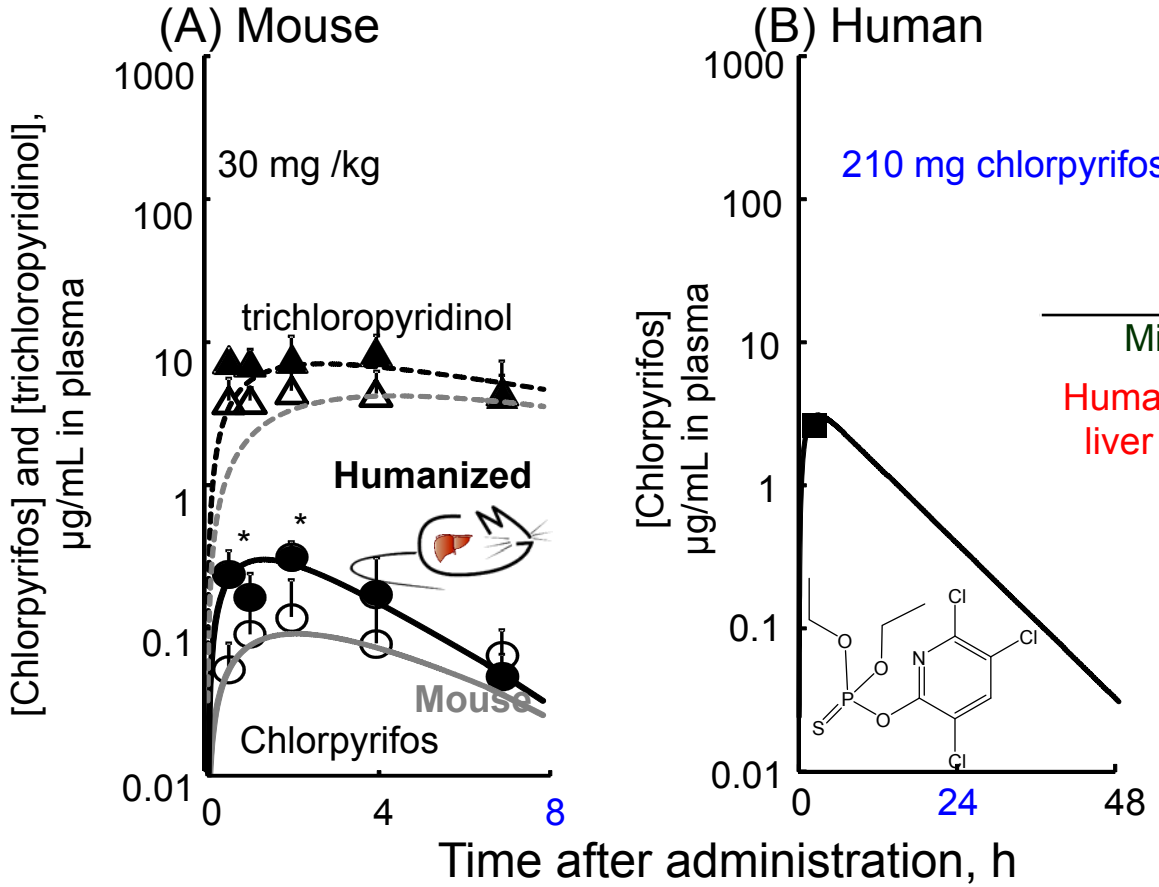
journal homepage: www.elsevier.com/locate/yrtph

Pharmacokinetics and effects on serum cholinesterase activities of organophosphorus pesticides acephate and chlorpyrifos in chimeric mice transplanted with human hepatocytes

Hiroshi Suemizu[a], Shigeto Sota[b], Miyuki Kuronuma[a], Makiko Shimizu[b], Hiroshi Yamazaki[b,*]

[a] Central Institute for Experimental Animals, Kawasaki-ku, Kawasaki 210-0821, Japan
[b] Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan

Plasma Concentrations of Chlorpyrifos in Mice with and without Humanized Liver and Humans after Oral Doses



	Butyrylcholinesterase (U/L)	
	Before treatment	A day after treatment
Mice	20	10
Humanized-liver mice	163	48

Serum Butyrylcholinesterase (U/L) in the range 5–500 U/L could be detected

*p< 0.05, two-way ANOVA. Reported values for chlorpyrifos in humans are taken from the literature (Drevenkar et al., 1993).

- Incidental overdose levels of chlorpyrifos cleared more slowly from plasma in humans than it did in mice because of liver metabolism dependent clearance.
- Chimeric mice with humanized liver in combination with a simple PBPK model can assist evaluations of toxicological potential of organophosphorus pesticides

Suemizu, Yamazaki et al., Regul Toxicol Pharmacol, 70, 468-473 (2014)

Showa Pharmaceutical University

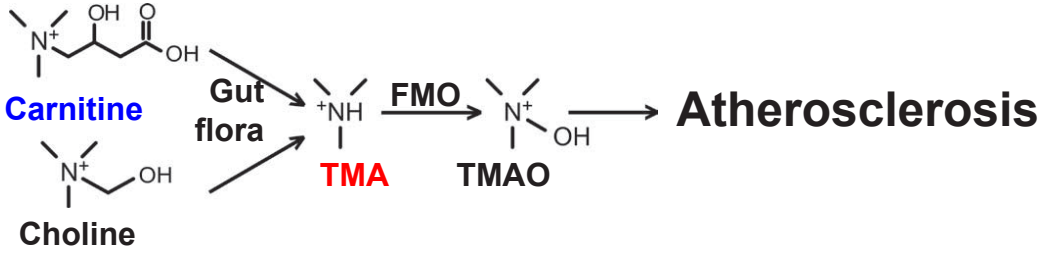
Hiroshi Yamazaki, PhD

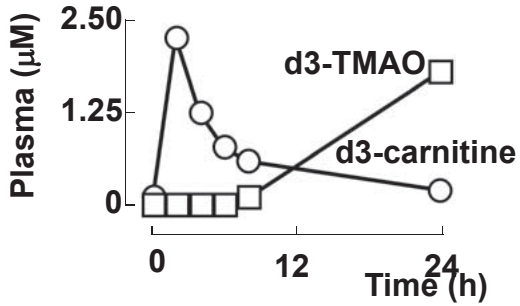
Metabolic activation and fate of xenobiotics determined by polymorphic drug-metabolizing enzymes

- Generalized Catalytic P450 Enzymes
- Reactive Metabolites in Humanized-liver Mice

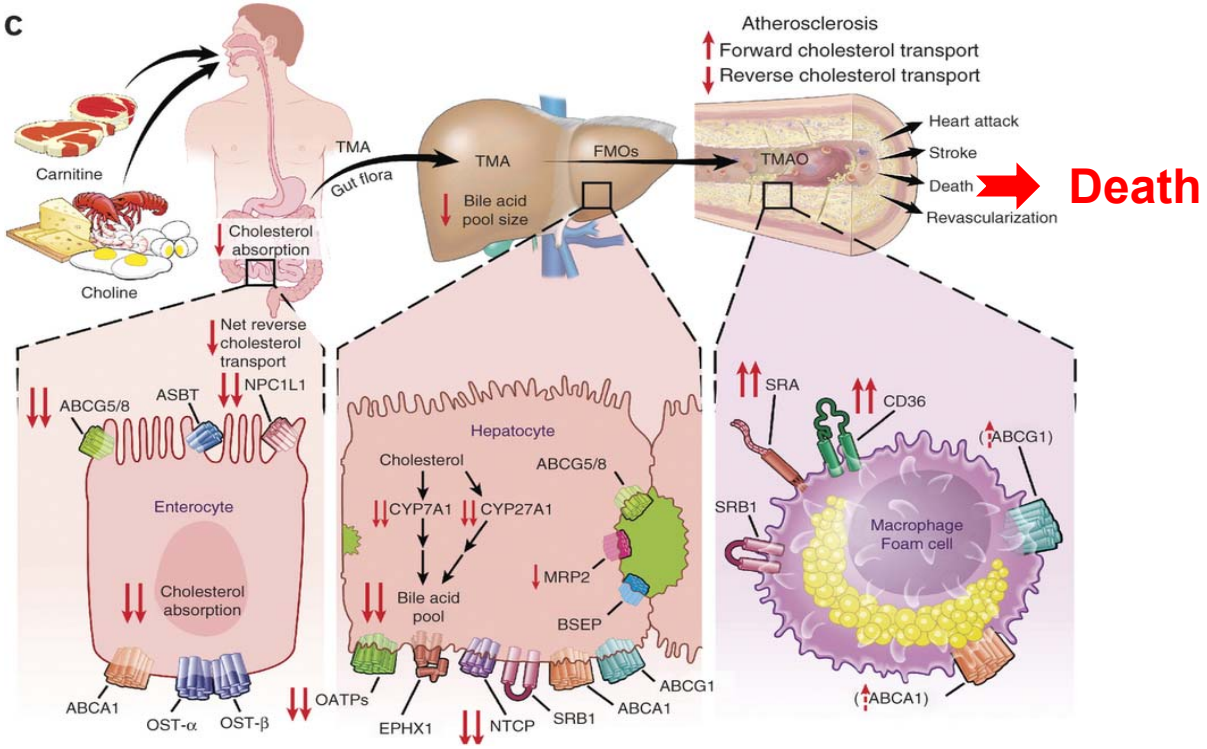
  Thalidomide activation and drug interaction
- Monkey and human P450 enzymes
- Physiologically based pharmacokinetic (PBPK) modeling
- Flavin-containing monooxygese

Intestinal Microbiota Metabolism of L-Carnitine, a Nutrient in Red Meat, Promotes Atherosclerosis



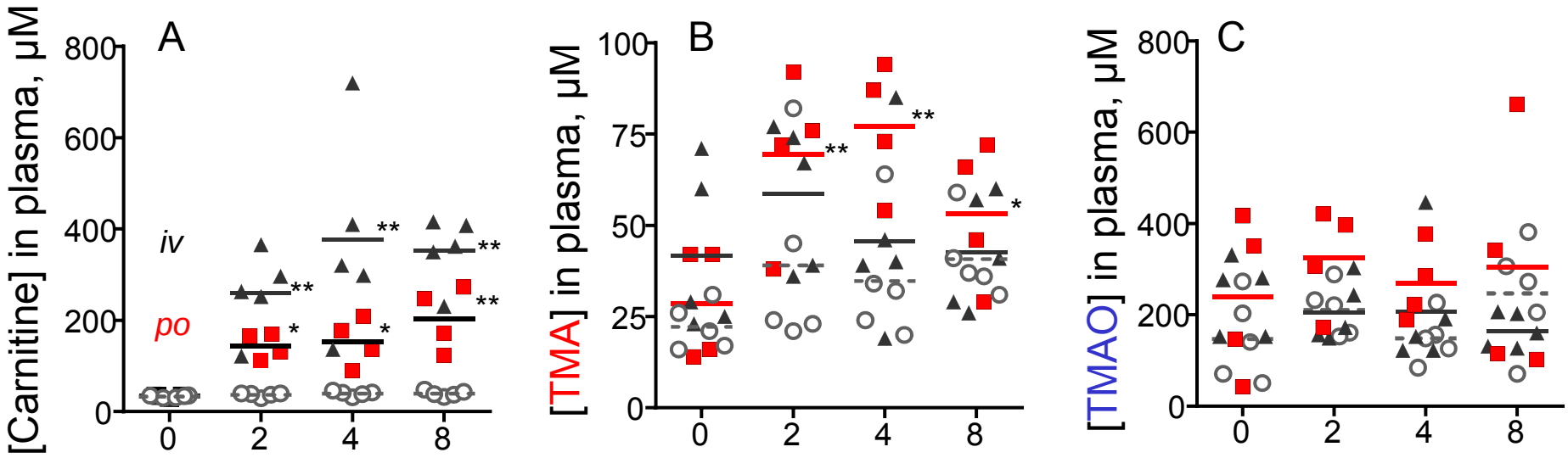


In the presence of carnitine (an important medical compound and a nutrient in red meat), the intestinal microbiota produces the waste product trimethylamine, TMA.

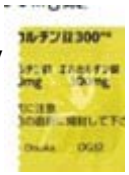


Koeth et al. Nature Medicine 2013; 19: 576–585

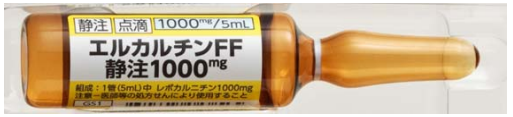
Trimethylamine Generation in Patients Receiving Hemodialysis Treated with L-Carnitine



po 300 mg x 3 x 7 day per week (6.3 g)



iv 1,000 mg x 3 days per week (3.0 g)



Plasma concentrations of total L-carnitine (A), TMA (B) and TMAO (C) in untreated patients (circles) and in patients treated with L-carnitine intravenously (triangles) and orally (squares). The three bars are averages of the three groups (n = 4 or 5). *$P < 0.05$ and **$P < 0.01$ compared with untreated patients, two-way ANOVA with Dunnett's post test.

- Plasma TMA concentrations after oral treatment with L-carnitine were higher than those of the untreated group. TMAO concentrations did not increase significantly after treatments and were in the range of daily interindividual variations.

Ozasa, Yamazaki et al., Clin Kidney J, 7, 329 (2014).

Urinary Excretions of Total TMA from Food and Plasma Levels of TMA in Japanese

	Ingested (μmol/200g)	Urinary excretion (μmol/8h)	Corrected excretion (μmol/8h, %)
Control	8	71	0
Bonito	224 (100)	266	195 (87)
Tuna	2,500 (100)	1,140	1,070 (43)
Cod	14,000 (100)	5,430	5,350 (38)

Shimizu, Yamazaki et al., Drug Metab Pharmacokinets 24, 549-552 (2009)

Japanese	[TMA] in plasma, μM	[TMAO] in plasma, μM
Pre-dialysis, n=22	34±20	189±94
Post-dialysis, n=22	25± 8*	140±77*
Volunteers, n=10	26±17	197±100

- Plasma TMA/TMAO concentrations in hemodialysis patients may be in the same range as those of healthy subjects consuming a typical diet in Japan

Ozasa, Yamazaki et al., Clin Kidney J, 7, 329 (2014)

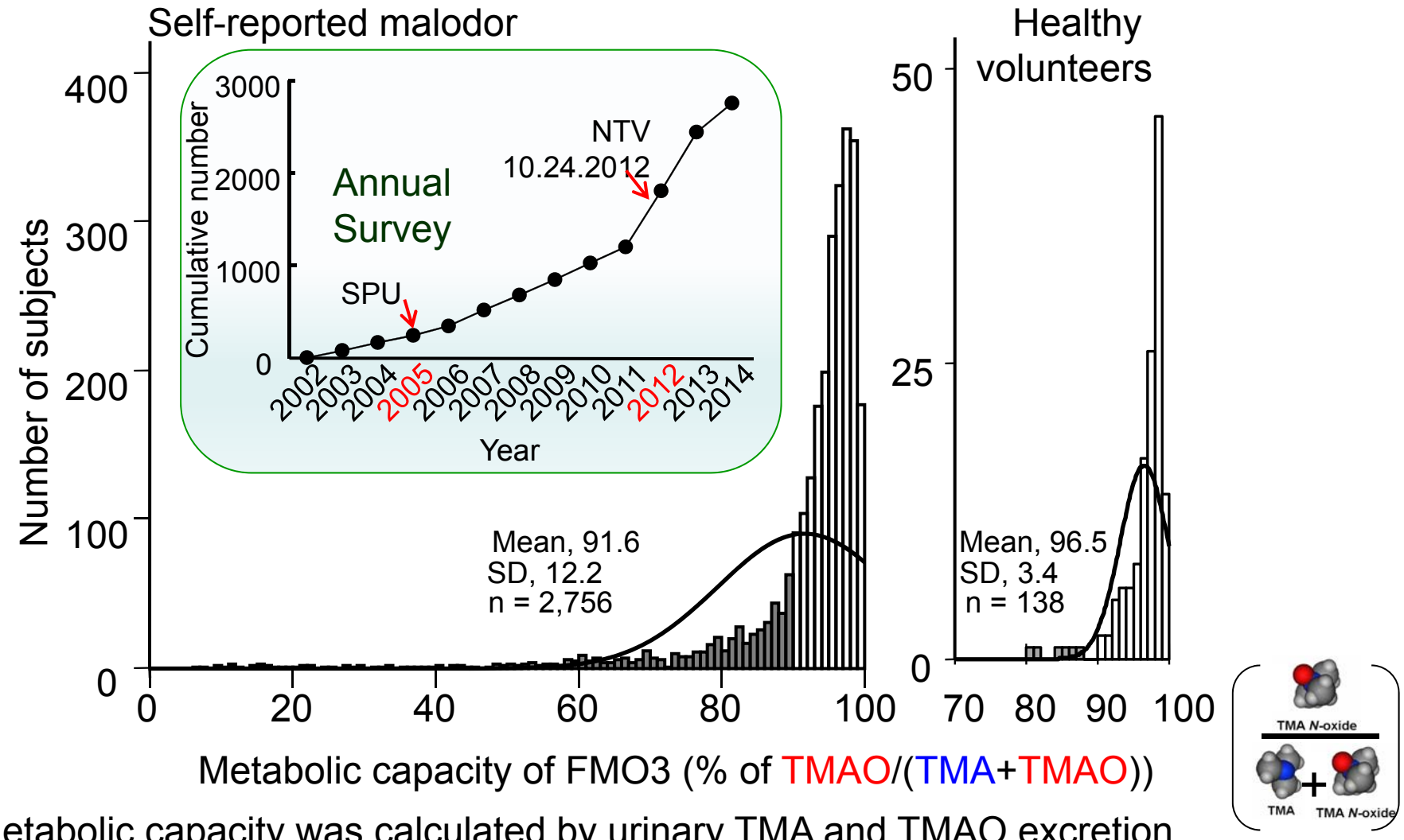
Trimethylaminuria

- Trimethylaminuria, also known as fish-like odor syndrome, is a metabolic disorder characterized by excretion of dietary-derived trimethylamine (TMA).
- Unpleasant malodor from urine, sweat or breath caused by excess TMA may lead to social problems.
- This causal factor is a decreased capacity to oxidize free malodorous TMA to non-odorous TMA *N*-oxide (TMAO) by the flavin-containing monooxygenease (FMO).

TMA TMA *N*-oxide

Trimethylaminuria: Fish-odor Syndrome

Urinary Trimethylamine Excretion by Daily Food Intake

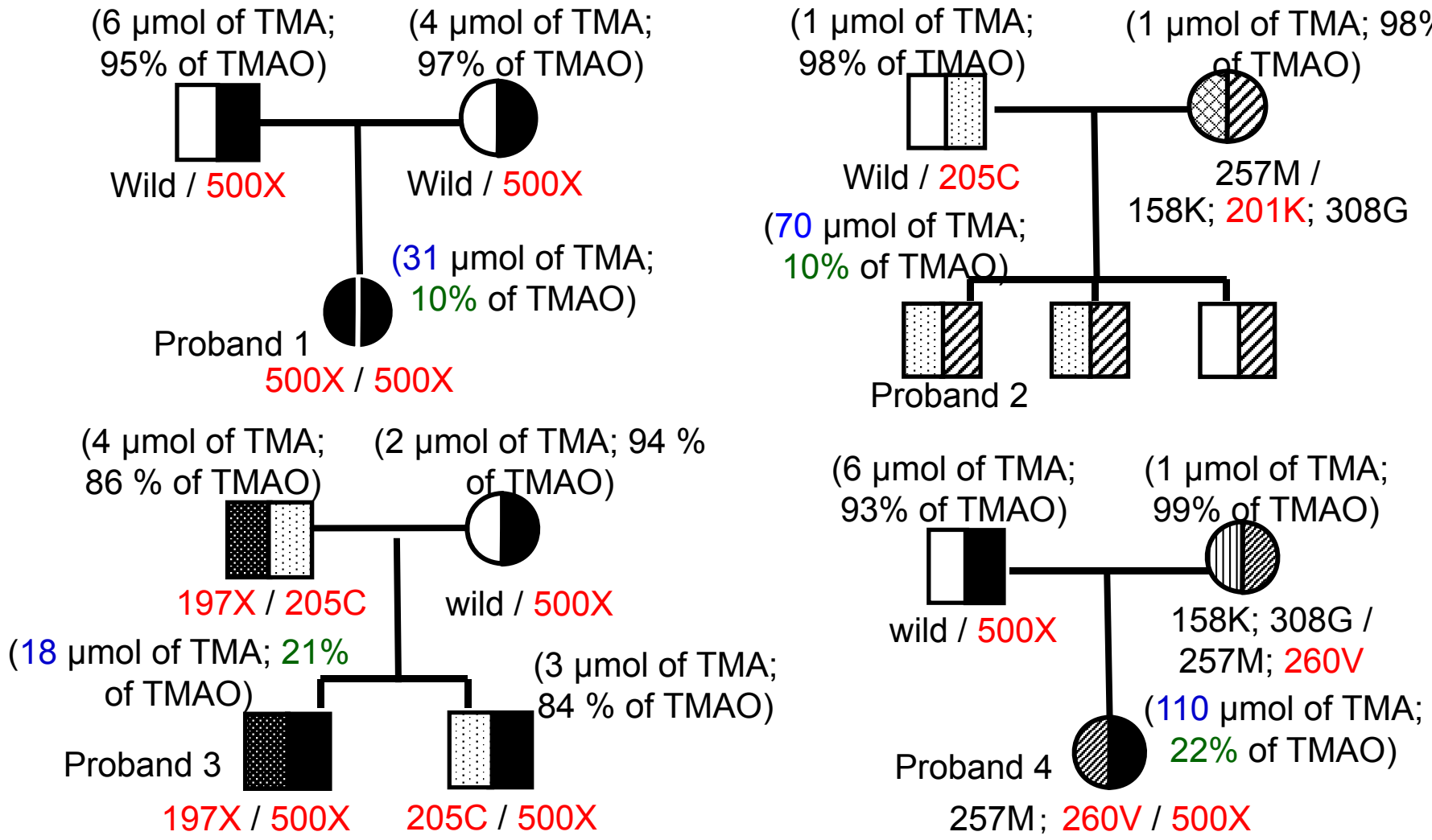


Metabolic capacity was calculated by urinary TMA and TMAO excretion.

Approximately 1.3% of subjects showed less than 40% of FMO3 metabolic capacity in urine tests.

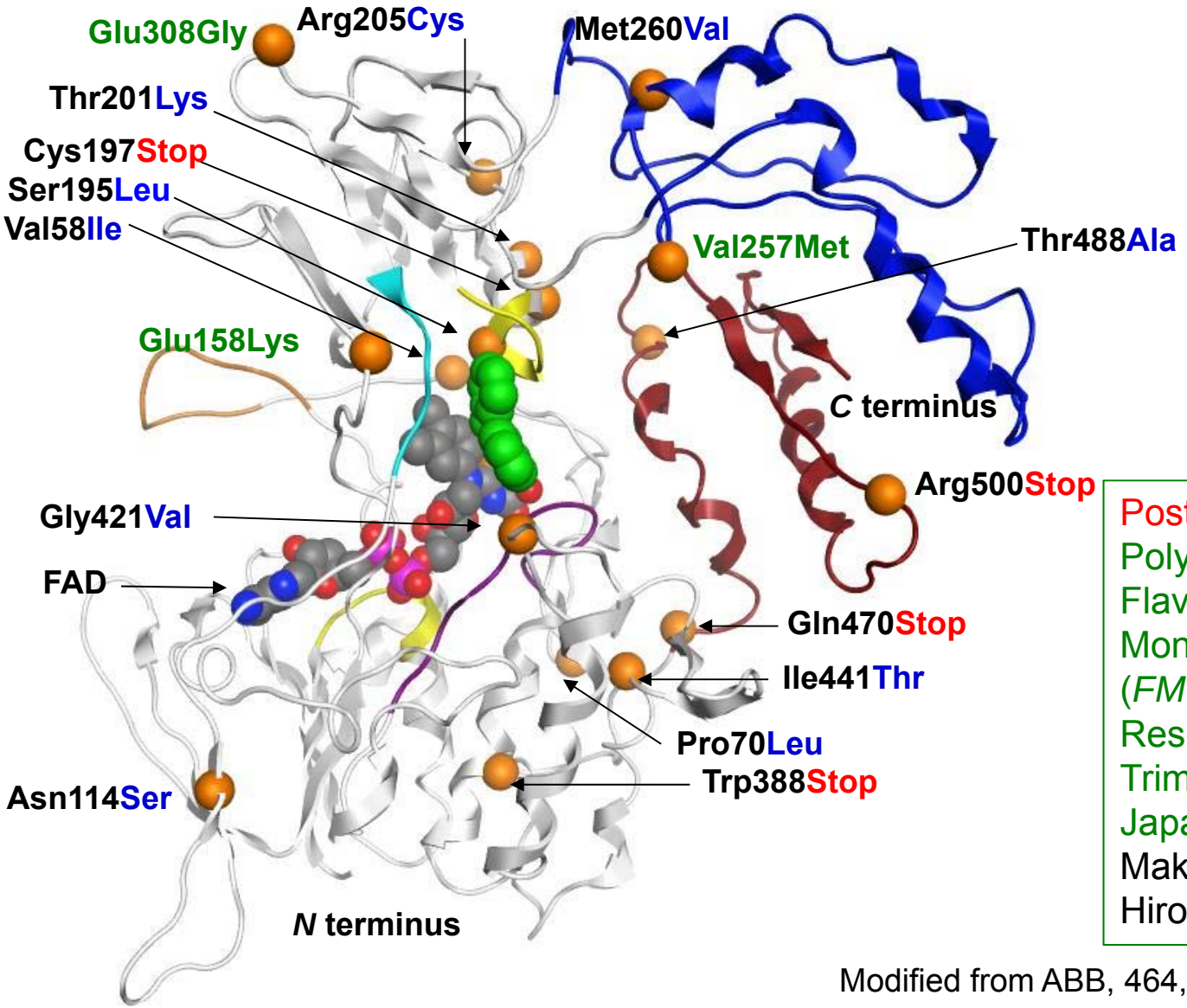
Yamazaki and Shimizu, Curr Drug Metab, 8, 487-491, 2007 and updated

Representative Pedigree Analyses in *FMO3* Gene



Shimizu,Yamazaki et al., *Drug Metab Pharmacokinet*, 21, 245-247 (2006); *ibid* 22, 61-64 (2007); Yamazaki, Shimizu et al., *Mol Genet Metab*, 90, 58-63 (2007)

Amino Acid Substitutions of FMO3 Found in Japanese



Poster 369 Genetic Polymorphisms in Flavin-containing Monooxygenase 3 (*FMO3*) Gene Responsible for Trimethylaminuria in a Japanese Population

Makiko Shimizu and Hiroshi Yamazaki

Modified from ABB, 464, 251 (2007)

PCR-RFLP Analysis of *FMO3* Gene Found in a Japanese Cohort

Showa Pharmaceutical University

Mutation	Position at Accession number AL021026	rs number in NCBI *	Exon	Restriction enzyme for PCR products	Length (bp) of RFLP products, uncut / cut (fragment)
Val58Ile	g.16882 G>A		3	Allele Specific (-cag / -caa)	276
Pro70Leu	g.16919 C>T		3	*Xsp* I	272 / 219 + 59 (mutant)
Asn114Ser	g.20752 A>G		4	*Hpy*8 I	699 / 609 + 90 (mutant)
Glu158Lys	g.20883 G>A	rs2266782	4	*Hinf* I	283 / 217 + 66 (wild)
Ser195Leu	g.21236 C>T		5	EcoRI	699/567+132 (wild)
Cys197Stop	g.21243_21244 TG deletion	rs3832024	5	*Mwo* I	196 / 116 + 78 (mutant)
Thr201Lys	g.21254 C>A		5	Allele specific (- cac / - caa)	124
Arg205Cys	g.21265 C>T	rs28363549	5	*Pvu* II	699 / 599 + 100 (mutant)
Val257Met	g.23997 G>A	rs1736557	6	BsaA I	378 / 243 + 135 (wild)
Met260Val	g.24006 A>G		6	*Bfu*A I	378 / 240 + 138 (mutant)
Glu308Gly	g.27159 A>G	rs2266780	7	*EcoO109* I	464 / 318 + 146 (mutant)
Trp388Stop	g.27400 G>A		7	*Blp* I	464 / 384 + 80 (mutant)
Gly421Val	g.30162 G>T	rs61757397	9	Allele Specific (-tgg / -tgt)	409
Ile441Thr	g.30222 T>C		9	*Bmr* I	251 / 127 + 124 (mutant)
Gln470Stop	g.30308 C>T		9	*Spe* I	443 / 244 + 199 (mutant)
Thr488Ala	g.30362 A>G		9	*Bgl* I	443 / 252 + 194 (mutant)
Arg500Stop	g.30398 C>T		9	*BssS* I	443 / 289 + 154 (wild)

* URL: http://www.ncbi.nlm.nih.gov/SNP/.

Yamazaki and Shimizu, Curr Drug Metab, 8, 487-491 (2007) and updated

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Commentary

Biochemical Pharmacology 85 (2013) 1588–1593

Survey of variants of human flavin-containing monooxygenase 3 (FMO3) and their drug oxidation activities

Hiroshi Yamazaki *, Makiko Shimizu

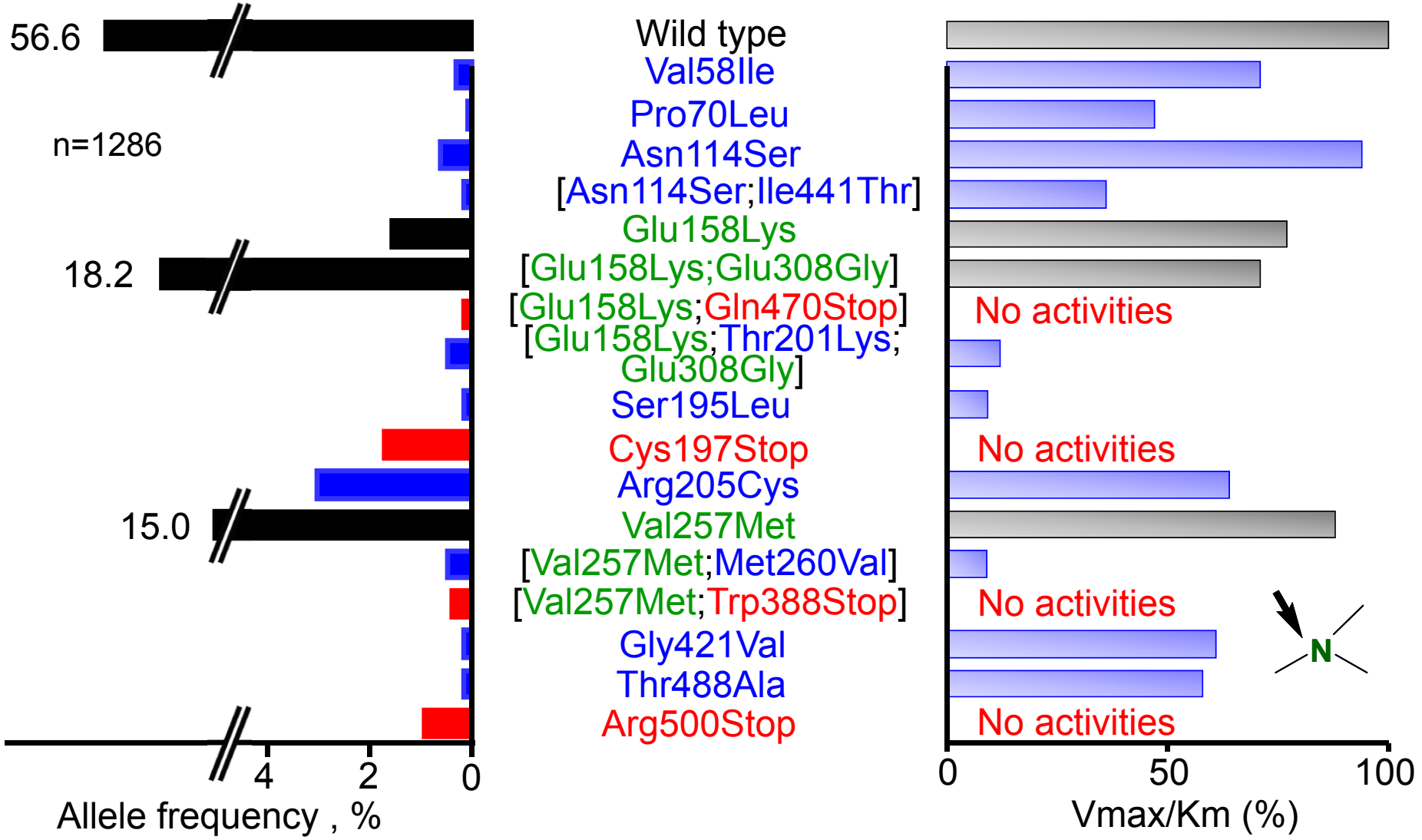
Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan

Table 2
Reported frequency of common *FMO3* mutations in three ethnic groups.

Amino acid change	dbSNP rs number	Frequency		
		European (HapMap-CEU)	Asian (HapMap-JPT)	Sub-Saharan African (HapMap-YRI)
p.Asp132His	rs12072582	0 (130 alleles)	0 (88 alleles)	0.137 (124 alleles)
p.Glu158Lys	rs2266782	0.420 (224 alleles)	0.208	0.483
p.Arg205Cys	rs28363549	n/a	0.018	n/a
p.Val257Met	rs1736557	0.071	0.142	0.020
p.Val277Ala	rs2066530	0 (130 alleles)	0 (88 alleles)	0.055 (290 alleles)
p.Glu308Gly	rs2266780	0.217	0.181	0.014
p.Glu362Gln	rs2066532	0 (130 alleles)	0 (88 alleles)	0.008 (126 alleles)
p.Ser147Ser	rs1800822	0.066	0.235	0.037
p.Asn285Asn	rs909530	0.279	0.385	0.544

HapMap-CEU (Utah residents with ancestry from northern and western Europe, 226 chromosomal samples), HapMap-JPT (Japanese residents of Tokyo, Japan, 226 samples), and HapMap-YRI (Yoruba in Ibadan, Nigeria, 294 samples) are representative populations studied in the International HapMap project in HapMap Data release #28, August 2010, on NCBI B36 assembly, dbSNP b126 (http://www.hapmap.org).

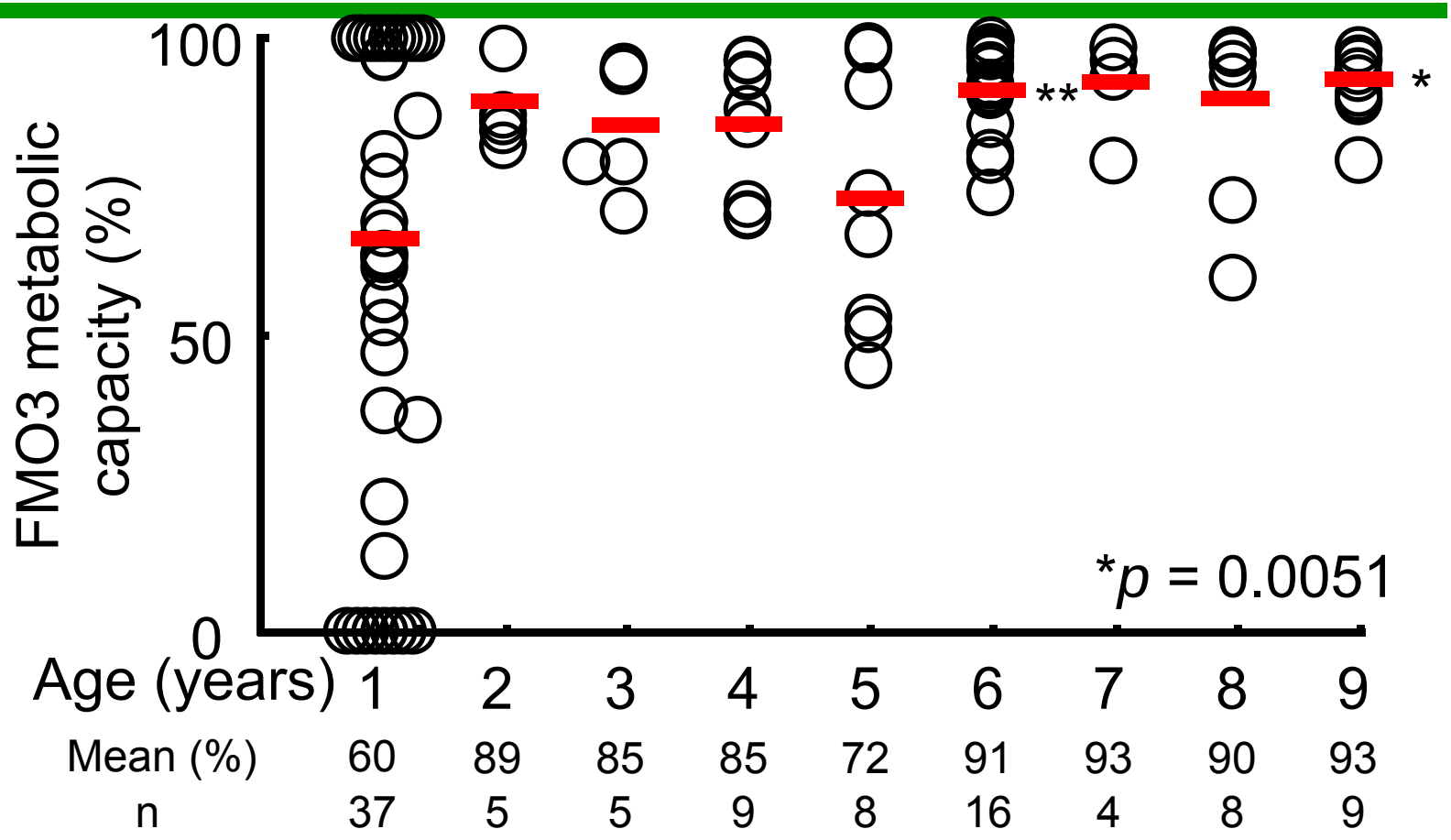
Allele Frequency of *FMO3* and Trimethylamine *N*-Oxygenation Activities of Recombinant FMO3



Yamazaki and Shimizu, Curr Drug Metab, 8, 487-491 (2007) and updated

Wild-type (532 amino acids) and variant FMO3 proteins were expressed in *E. coli* membranes.

FMO3 Metabolic Capacity of Children in Urine Tests



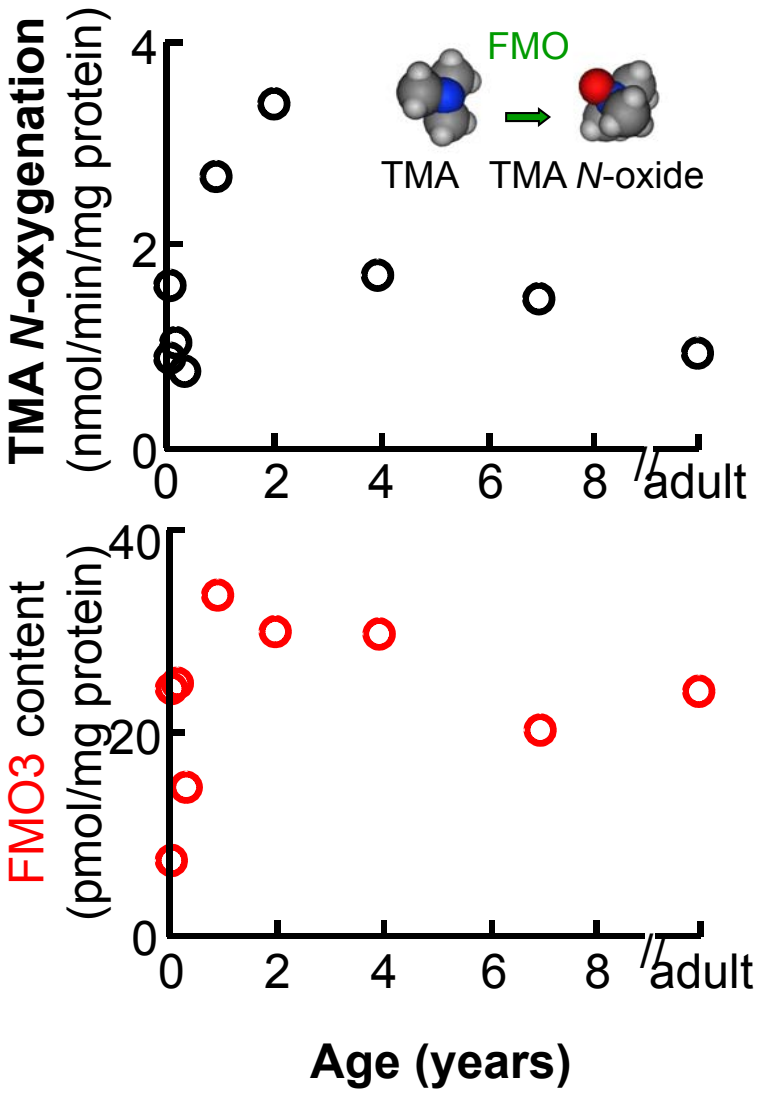
- When FMO3 metabolic capacity was evaluated as a function of age by one-way ANOVA, a significant increase in FMO3 metabolic capacity was observed ($p < 0.01$).
- Mean metabolic capacity at ages of 6 and 9 years were significantly higher than at age 1 year by post-Dunnett's test (comparing all *vs.* the youngest group).

Shimizu, Yamazaki et al., *Brit J Clinical Pharmacol*, 71, 585-591 (2011).

Trimethylamine *N*-Oxygenation by Recombinatly Expressed FMO and in Human Liver Microsomes

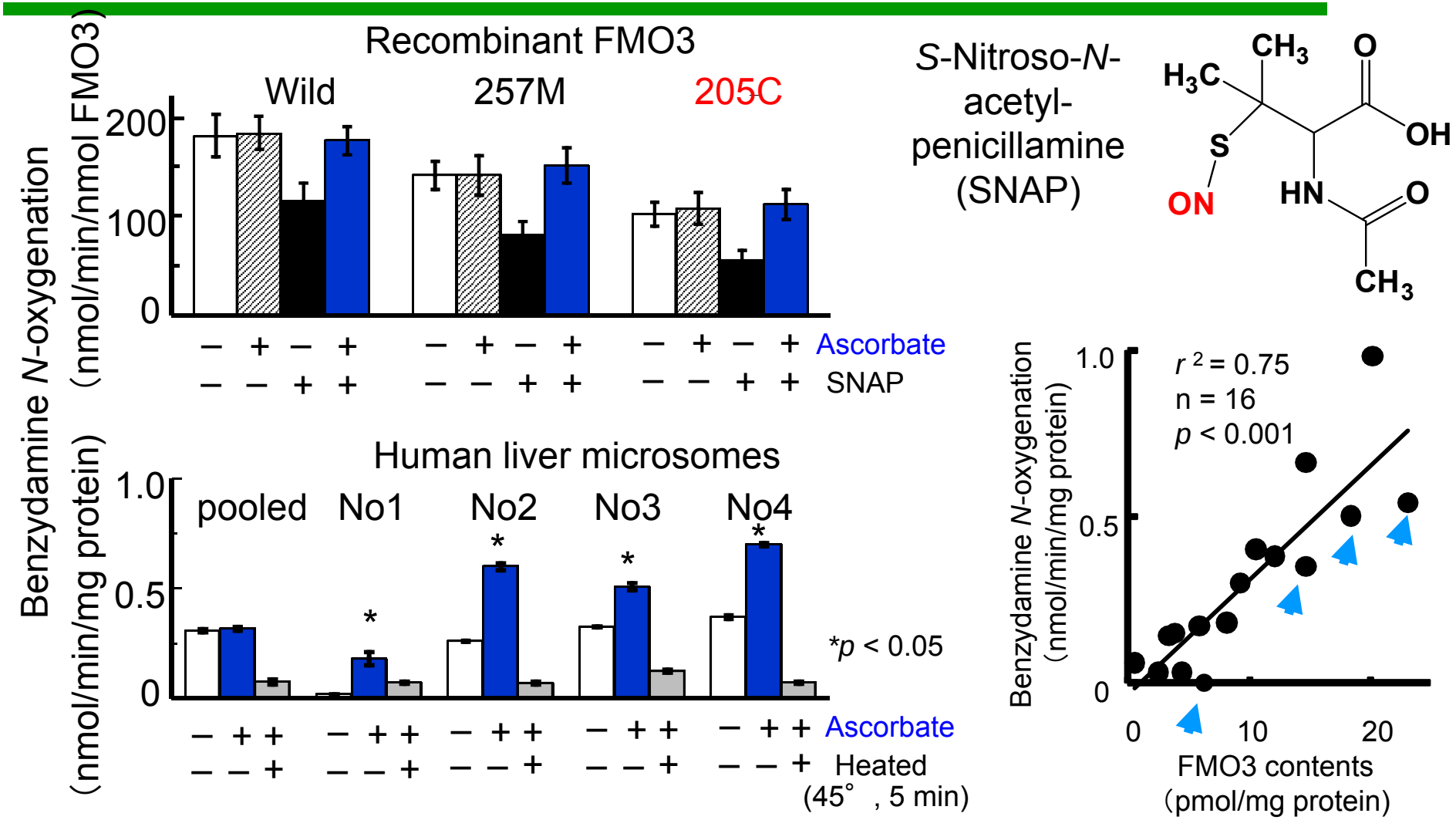
N-Oxygenation by recombinatly expressed FMO

	TMA		Benzydamine	
	V_{max} (min^{-1})	K_m (μM)	V_{max} (min^{-1})	K_m (μM)
FMO1	< 1	-	110	54
FMO3	91	110	250	40
FMO5	< 1	-	7	330



Developmental variations in functional metabolic capacity of FMO3 in childhood were shown both on the basis of *in vivo* phenotyping tests and in *in vitro* liver microsomal determinations. Shimizu, Yamazaki et al., *Brit J Clinical Pharmacol*, 71, 585-591 (2011).

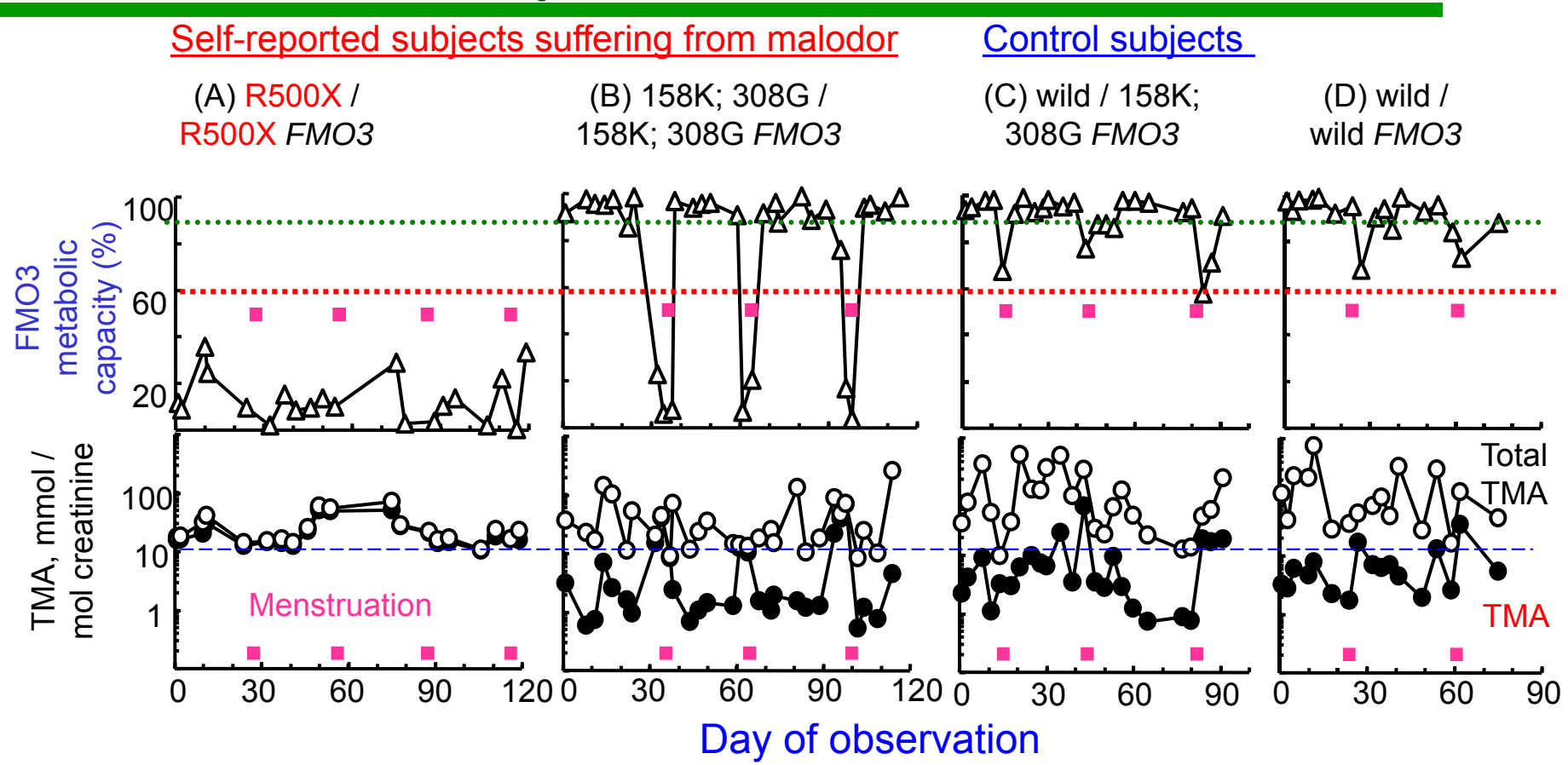
Recovery of Nitrosylation-modulated FMO3 Protein with Ascorbate



- Preincubation with the reducing agent ascorbate revealed that FMO3 activities in some liver samples were suppressed by nitrosylation.

Nagashima, Yamazaki et al., *Drug Metab Pharmacokinet*, 24, 218-225 (2009).

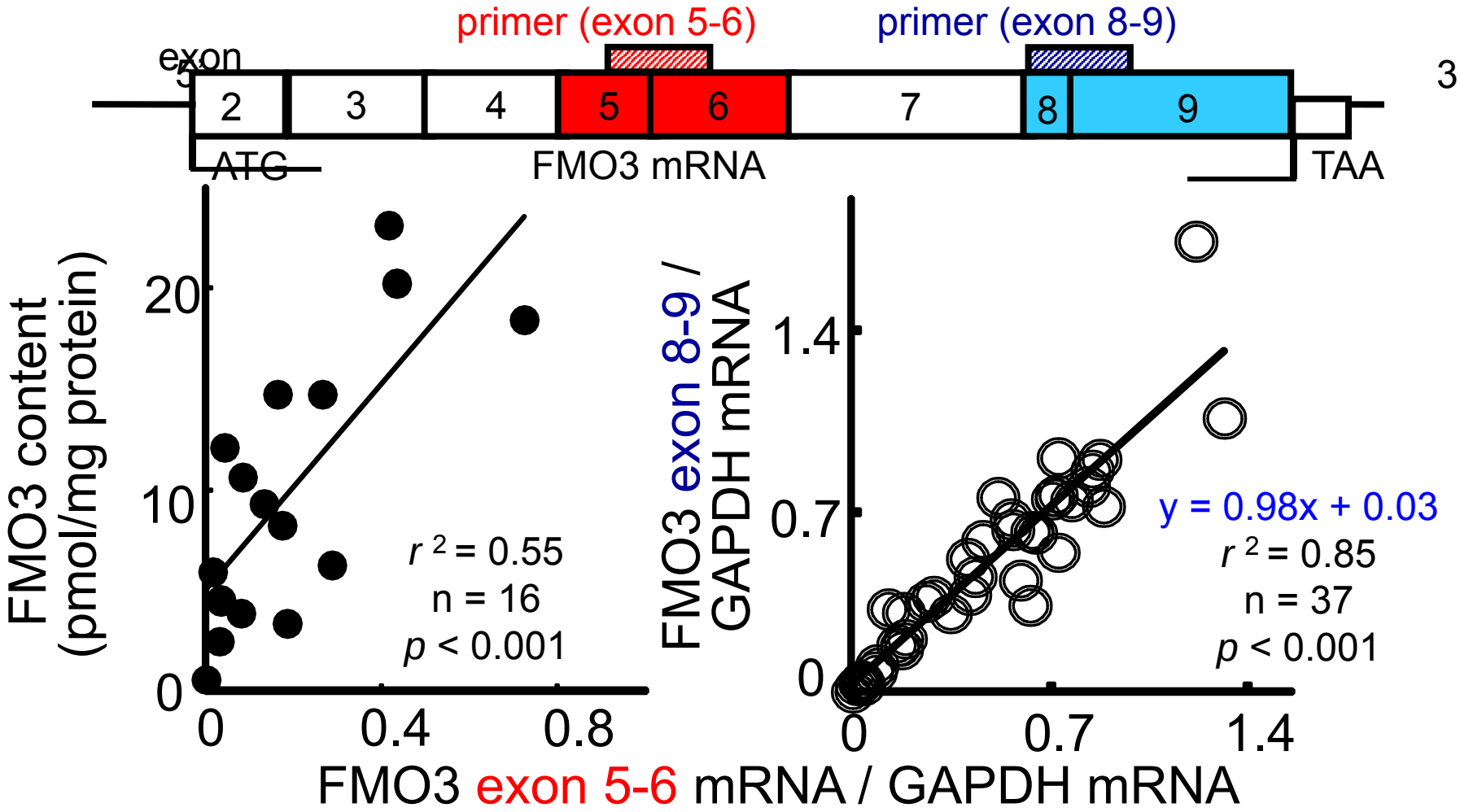
Transient trimethylaminuria Related to Menstruation



- Abnormal FMO3 metabolic capacity is caused by menstruation particularly in the presence of mild genetic variants such as [Glu158Lys; Glu308Gly].
- This would further suggest that sex hormones play a role in the variable regulation of FMO3 to cause intra-individual variations.

Shimizu, Yamazaki et al., *BMC Med Genet*, 8, 2 (2007)

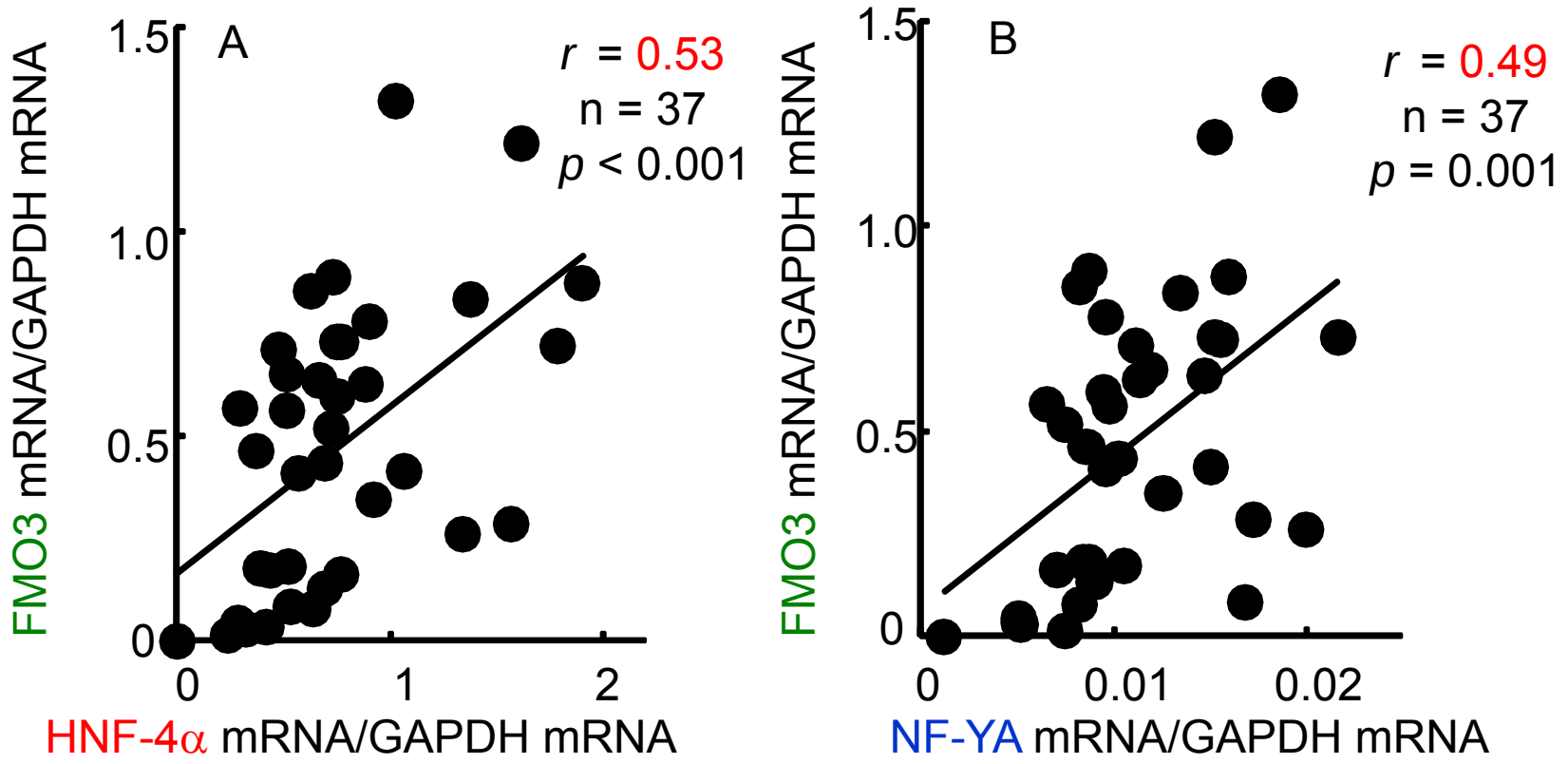
Expression Levels of FMO3 in Human Livers



- Microsomal FMO3 protein contents were correlated with FMO3 mRNA levels.
- Apparently no alternative processing events were suggested.

Nagashima, Yamazaki et al., *Drug Metab Pharmacokinet*, 24, 218-225 (2009)

Correlations between Expression Levels of FMO3 mRNA and HNF-4α or NF-YA mRNA in Human Livers



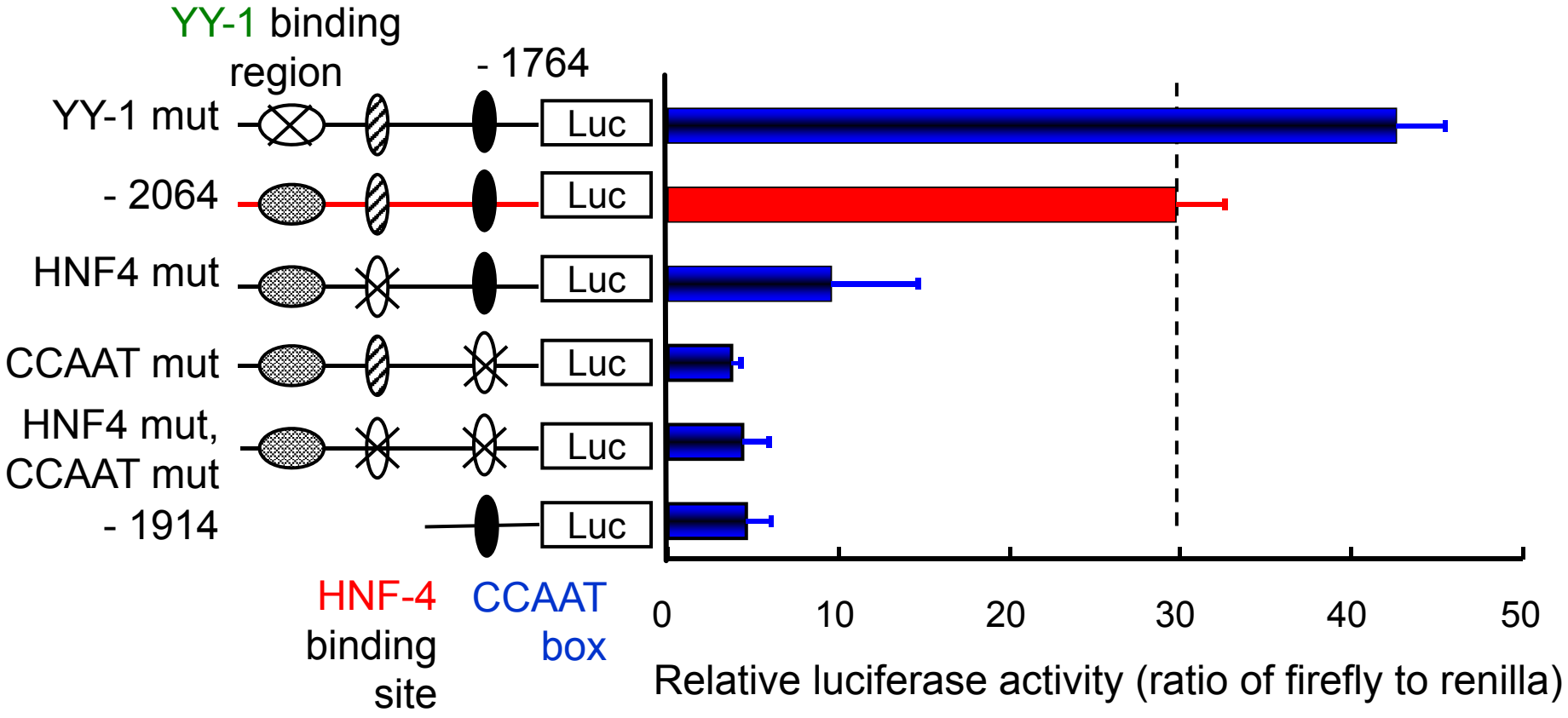
Multivariate linear regression analysis, r = 0.56, p =0.0017, n = 37.

[FMO3] = 0.28 x [HNF-4α] + 19 x [NF-YA] + 0.055

FMO3 mRNA levels were multivariately correlated with *trans*-acting factors, i.e. hepatic nuclear factor 4 (HNF-4) mRNA and nuclear factor Y box-binding protein (NF-Y) mRNA.

Nagashima, Yamazaki et al., *Drug Metab Pharmacokinet*, 24, 218-225 (2009)

Mutational Analysis of Binding Sites for HNF-4 and CCAAT Box in the Promoter Activities of the *FMO3* Gene



- Putative hepatocyte nuclear factor-4 (HNF-4) binding site and CCAAT box, but not Yin Yang 1 element, could be responsible *cis*-acting elements of the *FMO3* gene, by site-directed mutagenesis analysis.

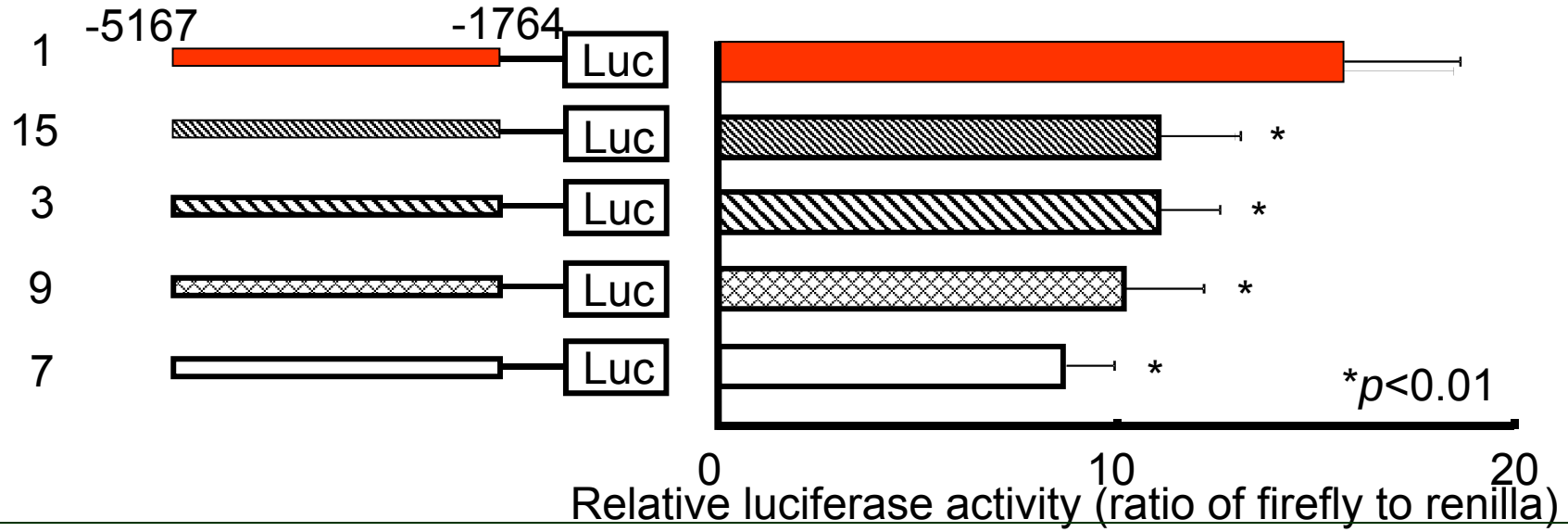
Shimizu, Yamazaki et al., *Drug Metab Pharmacokinet*, 23, 54-58 (2008)

Transcriptional Activities of the 5'-Flanking Region of *FMO3* Haplotypes

Showa Pharmaceutical University

Haplo-type	Nucleotide position											
	-5167	-4608	-3784	-3606	-3549	-3548	-3544	-2962	-2854	-2650	-2543	-2137
1	G	T	T	G	C	A	C	T	T	C	T	G
15	C	-	-	A	-	G	-	-	-	G	-	-
3	C	C	C	-	-	G	T	C	C	G	A	C
9	C	-	-	-	T	G	-	-	-	G	-	-
7	C	-	-	-	-	-	-	-	-	-	-	-

FMO3 mutations segregate into 19 haplotypes, including four different combinations of upstream mutations, each of which reduces transcriptional activity in comparison with the ancestral upstream sequence of *FMO3*.



- Comparisons of genotype and phenotype reveal that severe trimethylaminuria is caused by loss-of-function mutations in *FMO3*. For moderate and mild cases the situation is more complex, with most resulting from factors other than *FMO3* genotype.

Shimizu, Yamazaki et al., Brit J Clini Pharmacol 77, 839-851 (2014)

Kinetic Parameters for *N*- and *S*-Oxygenations by Liver Microsomes and Recombinant FMO Enzymes

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		N-Oxygenation						*S*-Oxygenation					
		Benzydamine			Tozasertib			Methimazole			Sulindac sulfide		
		K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
	Human	42	0.7	0.02	25	0.8	0.03	40	0.5	0.01	28	0.5	0.02
	Monkey	120	1.7	0.01	28	1.0	0.04	120	0.6	0.01	130	0.8	0.01
	Rat	63	18	0.29	40	14	0.35	45	12	0.27	58	5.4	0.09
	Minipig	34	39	1.1	37	36	0.97	32	24	0.75	11	32	2.9
Human	FMO1	55	11	0.2	30	4	0.1	19	2	0.08	280	3	0.01
	FMO3	53	60	1.1	24	33	1.4	39	69	1.8	46	55	1.2
Monkey	FMO1	94	22	0.23	8	4	0.57	130	4	0.03	250	9	0.04
	FMO3	230	39	0.17	26	25	0.96	110	12	0.11	48	19	0.40
Rat	FMO1	8	103	13	13	42	3.2	3	72	21	4	23	6.6
	FMO3	850	95	0.1	34	42	1.2	47	34	0.7	130	28	0.2
Minipig	FMO1	35	33	0.9	15	16	1.1	50	9	0.18	28	5.9	0.21
	FMO3	>2000	8	<0.1	28	1	0.03	87	2	0.03	180	3.1	0.02

Yamazaki et al., *Biochem Pharmacol*, 90, 159-165 (2014).

Effects of Methimazole on Sulindac Sulfide *S*-Oxygenation Activities in Liver Microsomes and FMO3

Enzyme	Sulindac sulfide *S*-oxygenation		Methimazole
	V_{max},	K_m	K_i
Liver microsomes genotyped for p.[Glu158Lys; Glu308Gly] *FMO3*			
	nmol/min/mg protein	μM	μM
Wild homozygotes (n=3)	3.5 (3.0, 3.6, 4.0)	53 (50, 58, 59)	22 (18, 24, 25)
Heterozygotes (n=2)	2.9 (2.7, 3.0)	45 (35, 54)	23 (23, 24)
Mutant homozygote	1.9	50	12
Recombinatly expressed FMO3 in *E. coli* membranes			
	min^{-1}	μM	μM
Wild-type FMO3	230 ± 54	54 ± 20	22 ± 5
158Lys; 308Gly FMO3	160 ± 43	56 ± 25	11 ± 2

Competitive inhibition by methimazole (0-50 μM) on sulindac sulfide *S*-oxygenation was observed in six individual human liver microsomes or recombinatly expressed FMO3 proteins after co-incubation. Inhibition constants were calculated by non-linear regression analysis and are represented as mean ± SE values for recombinant FMO3.

Yamazaki, Shimizu, Biochem Pharmacol, 85, 1588-1893 (2013).

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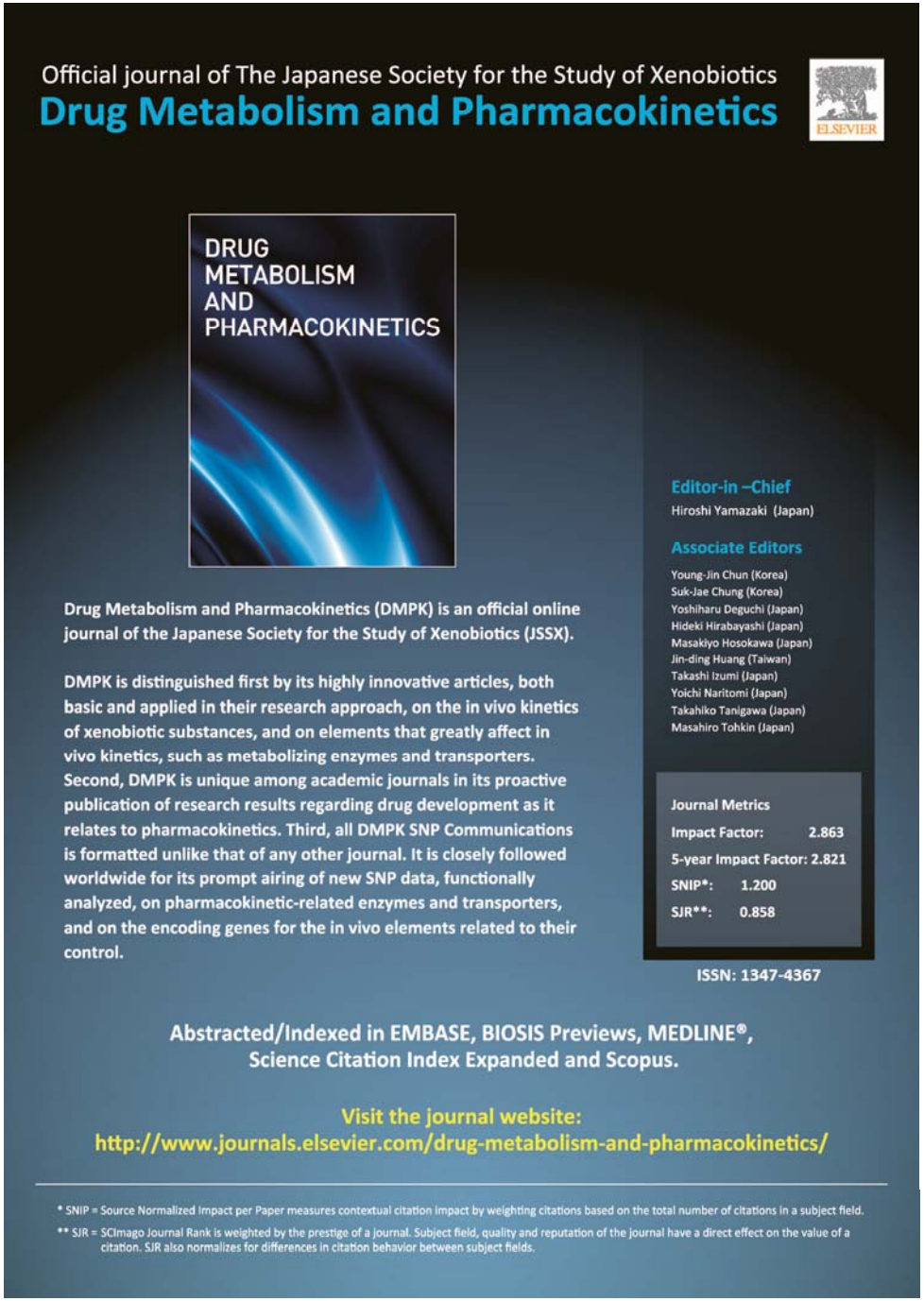
Take Home Messages

- **Imbalance of covalent bindings and target protein concentrations** could be predicting hepatotoxicity.
- **Reactive metabolite** formation and drug interactions via P450 3A4/5 with **thalidomide** would be suggested.
- Monkey PK could be influenced by polymorphic P450 2C and 2D enzymes.
- Chimeric mice with humanized liver in combination with a simple **PBPK** model can assist evaluations of toxicological potential of chemicals.
- Importance of human FMO3 in individual xenobiotic oxygenations could be indicated, including those of **new medicines** and dietary derived **trimethylamine**.



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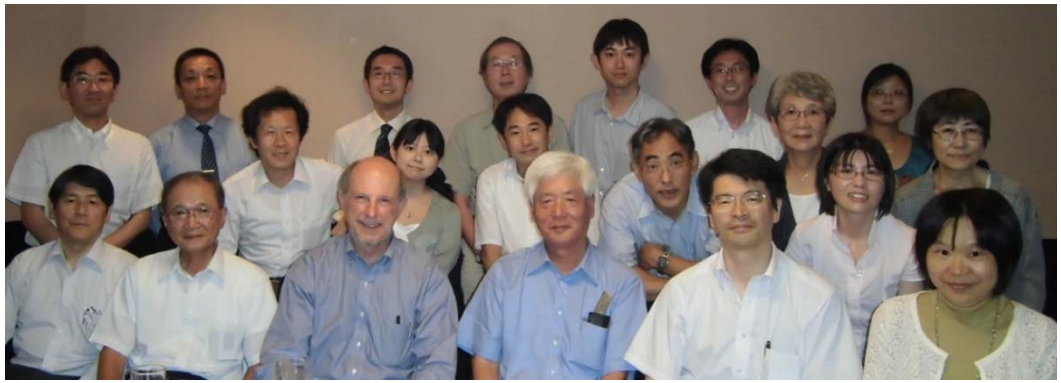

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