The Role of Molecular Diagnostics in Genetics Testing and Pharmacogenetics

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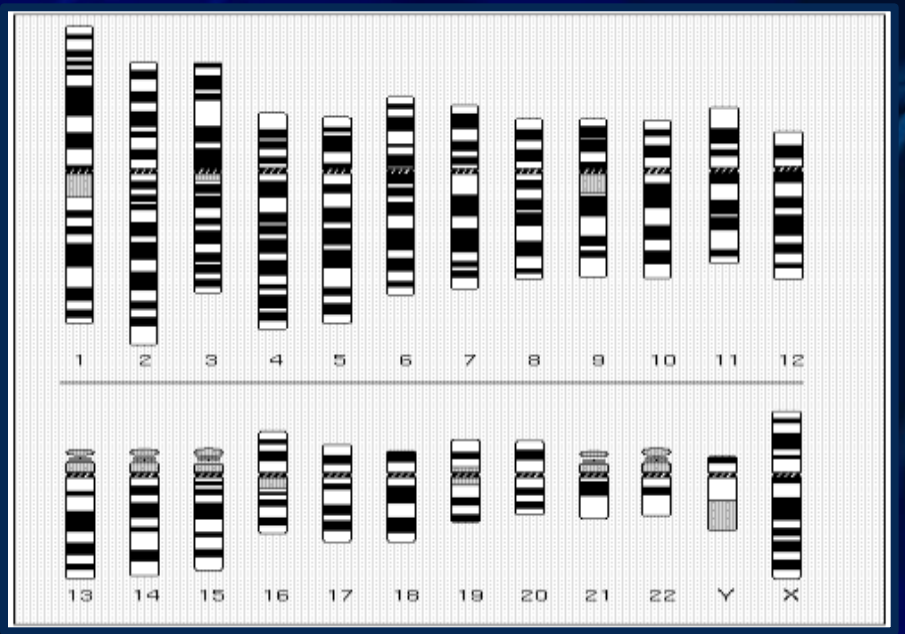
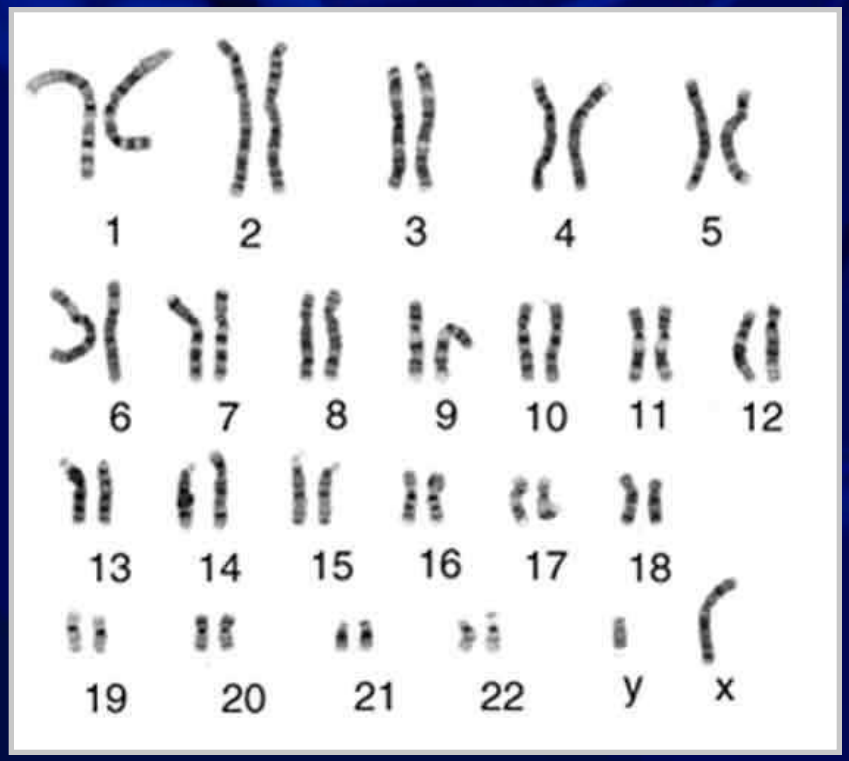
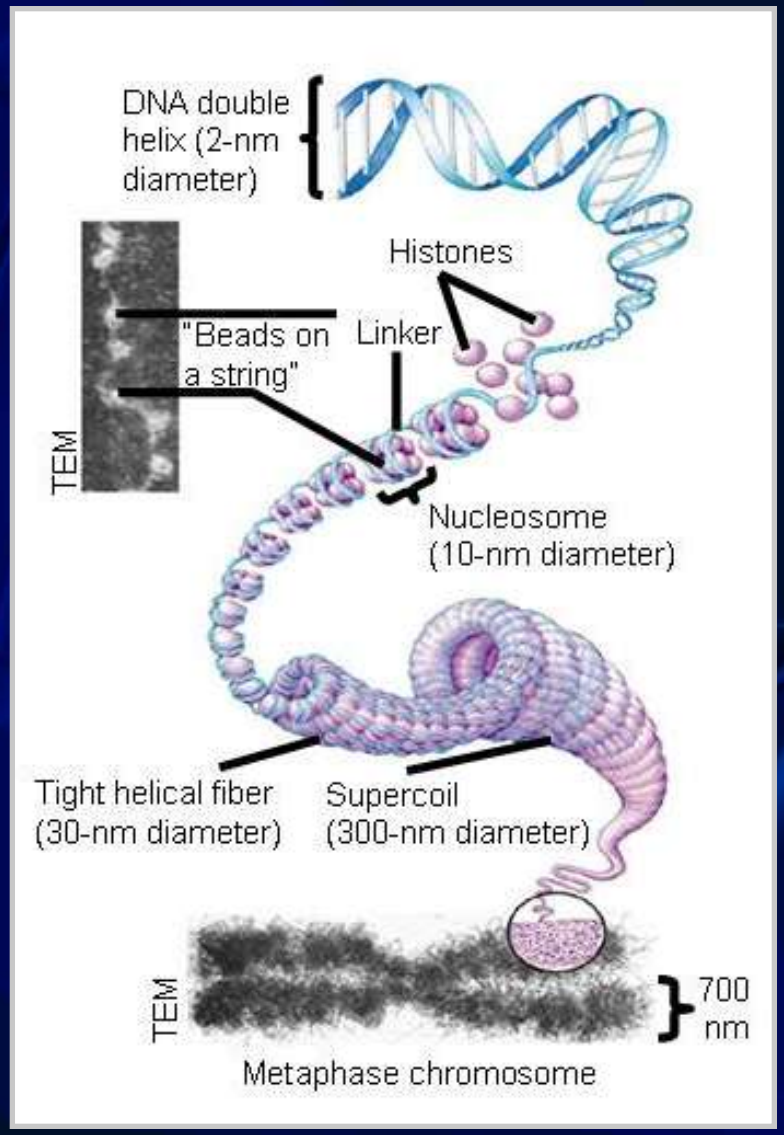
Human DNA

- Diploid genome (two sets of chromosomes)
- Packaged in 23 pairs of chromosomes
- 22 homologous pairs (autosomes)
- 2 sex chromosomes (XX or XY)
- 6 billion bases
- 30,000–60,000 genes



The Structure of DNA





Principles of Genetic Status

- Homozygous—identical alleles at a chromosomal locus
- Heterozygous—two different alleles at a chromosomal locus
- Compound heterozygote—two different alleles at two different loci in the same gene
- Double heterozygote—two different alleles at two different chromosomal loci in different genes

Cystic Fibrosis
(one gene, many mutations)

Autosomal recessive

Carrier frequency 1 in 29 (Northern European)

Ion channel defect leads to irreversible pulmonary damage

Sweat test is the differential diagnosis

Average life span 28 years

CFTR gene cloned in 1989

Over 1000 mutations, majority private

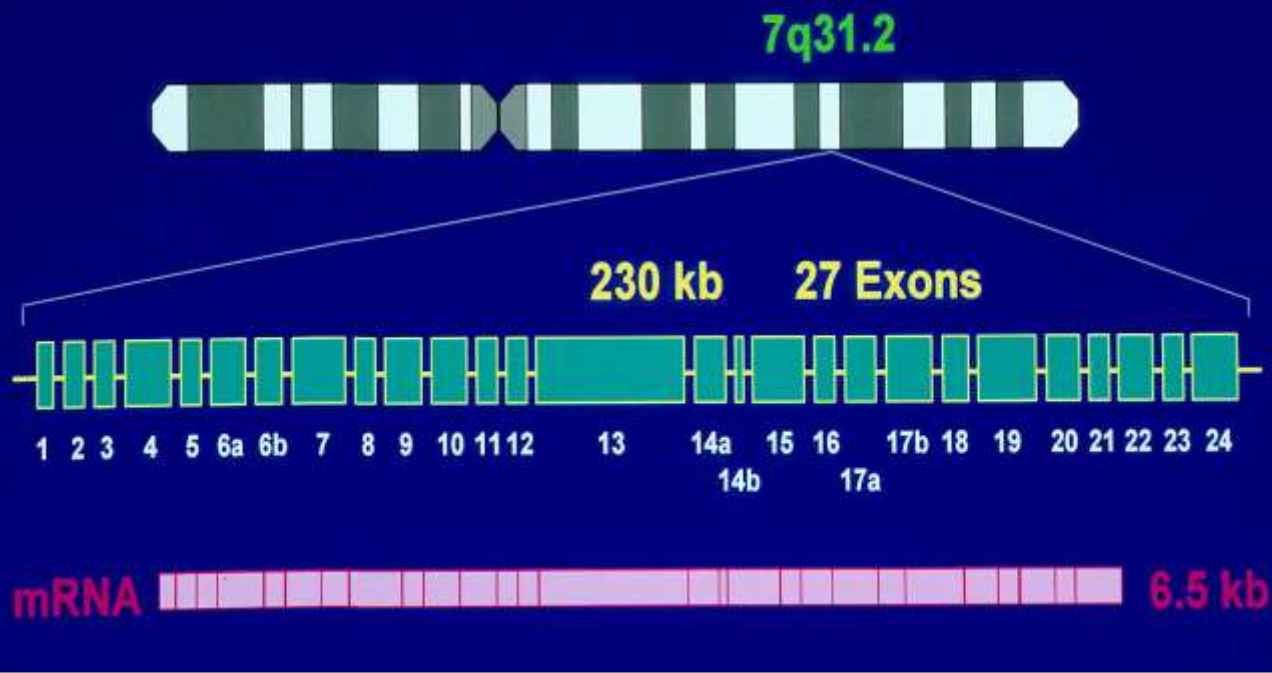
Laboratory Diagnosis and Screening of Cystic Fibrosis

- Sweat electrolyte levels
- Immunoreactive trypsin test (IRT)
- DNA analysis

Treatment of Cystic Fibrosis

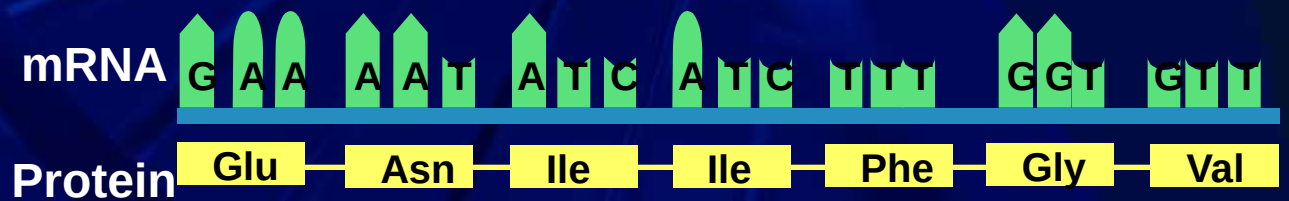
- Pulmonary therapy
  - antibiotics
  - bronchodilators
  - mucolytics (DNases)
  - chest PT
  - lung transplantation
- Digestive therapy
  - pancreatic enzyme replacement
  - dietary supplements
- Reproductive therapy
  - genetic counseling
  - sperm aspiration and ICSI

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)



ΔF508 accounts for ~70% of CFTR mutations among Caucasians

Normal



ΔF508

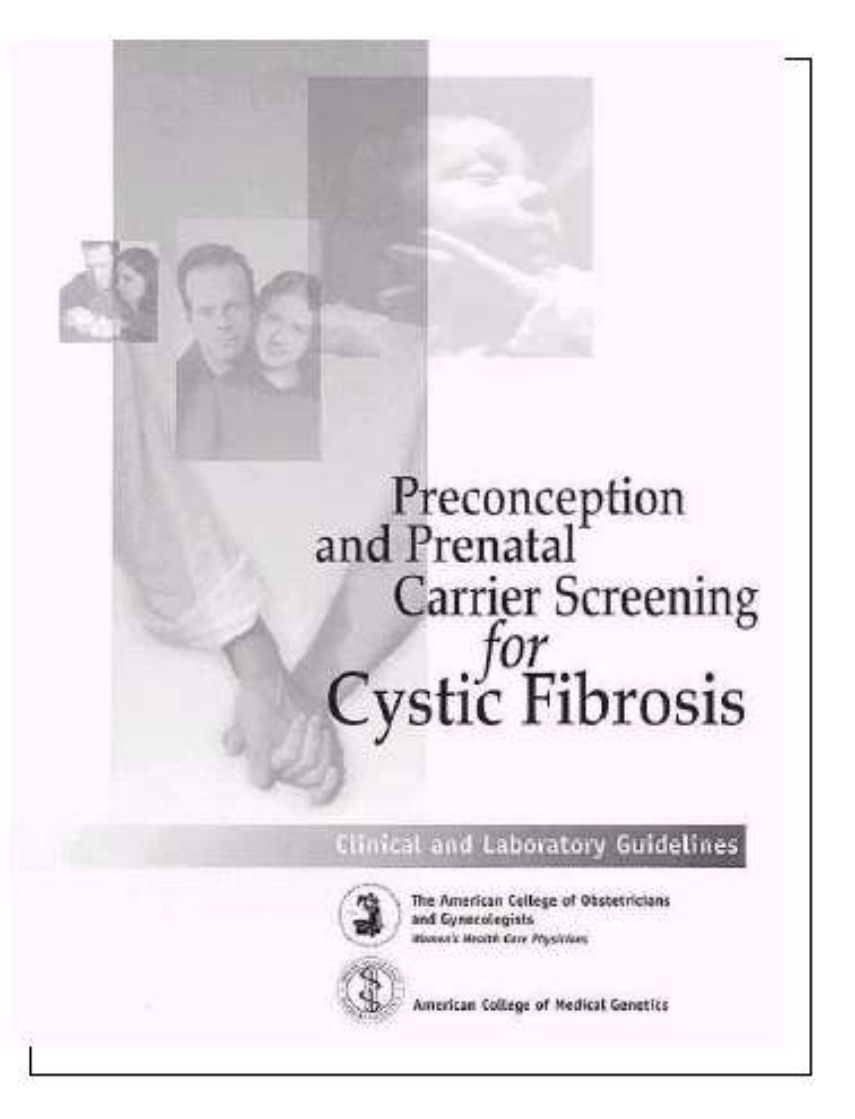
- Loss of one phenylalanine out of 1480 amino acids causes misfolding and degradation of the CFTR protein, and a severe phenotype.

CFTR Gene Mutations

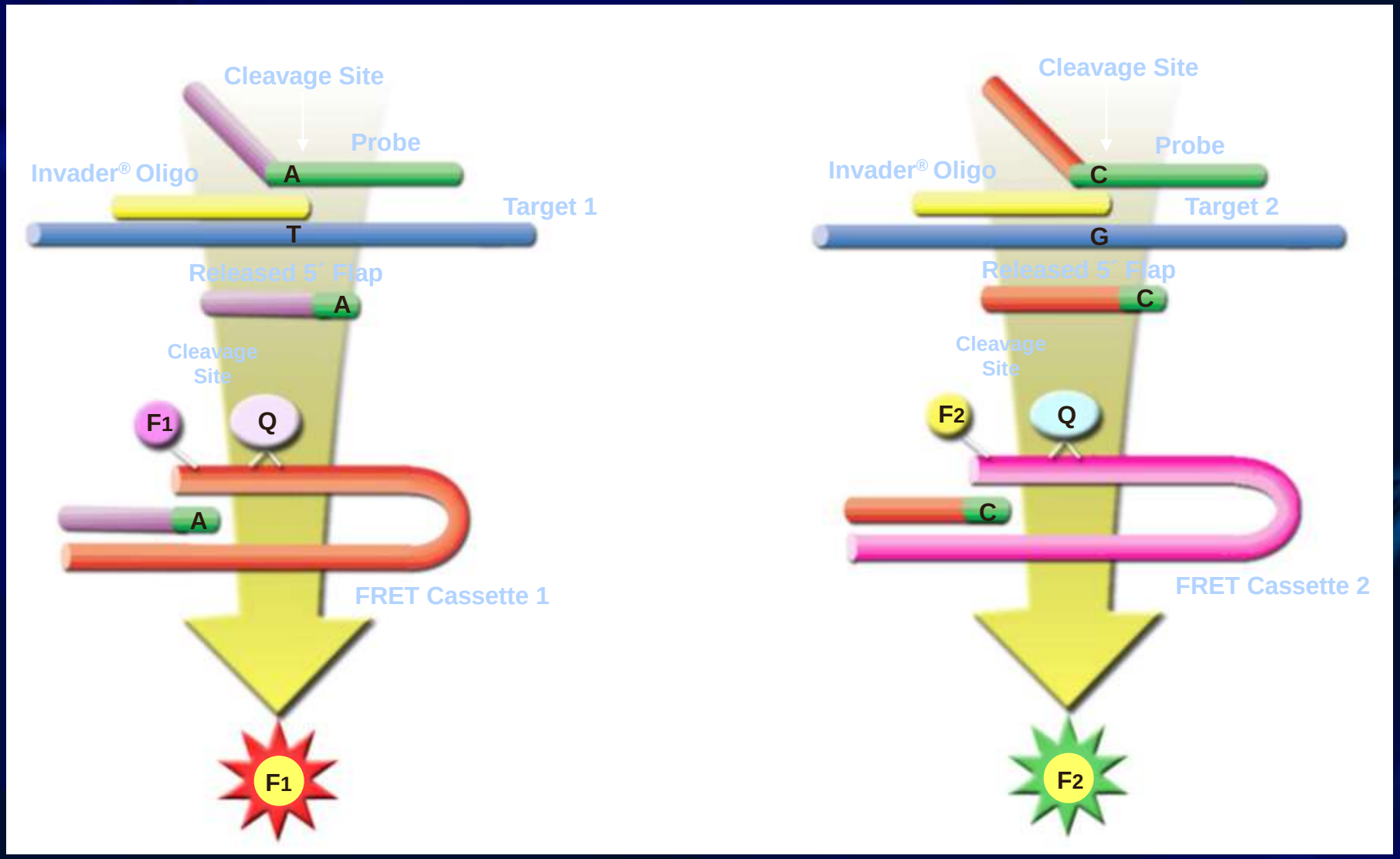
- Class I: defective protein synthesis
- Class II: abnormal or no trafficking of CFTR
- Class III: defective regulation, abnormal CFTR
- Class IV: decreased conductance function
- Class V: reduced trafficking of functional CFTR
- Class VI: decreased stability of CFTR

Population Based CF Screening

- October 2001 ACOG recommended offering routine testing to pregnant women and provides educational material for physicians and patients

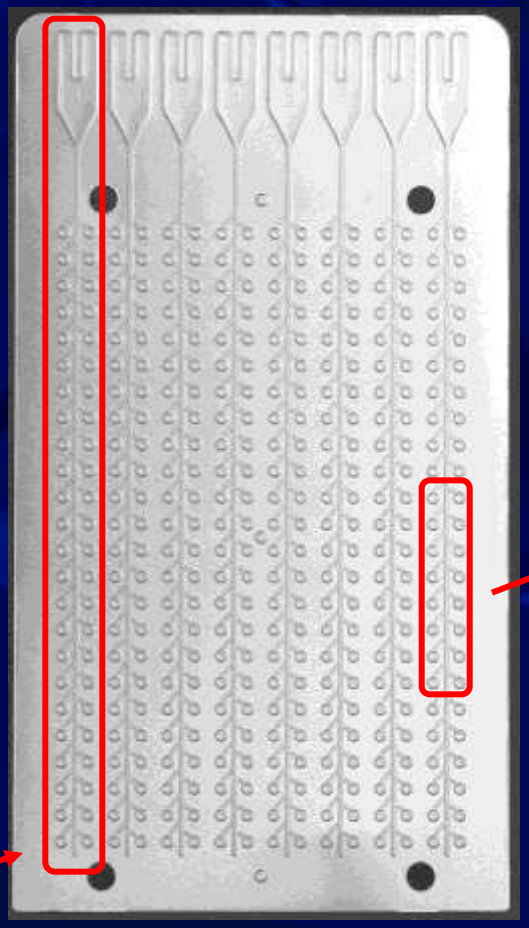


Invader® Biplex Technology



Hologic CFTR InPlex™

- The CFTR InPlex™ card is a lab consumable.
  - The core card consists of thin, heat-stable, molded plastic channels that are backed with heat stable foil.
  - Each card consists of eight raised sample "lanes".
    - Each sample "lane" is further sub-divided into 48 separate reaction chambers.



Enlarged view of reaction chambers

A5 = =SamplePlacement!D$28

Display ALL IVS8-5T/7T/9T Results

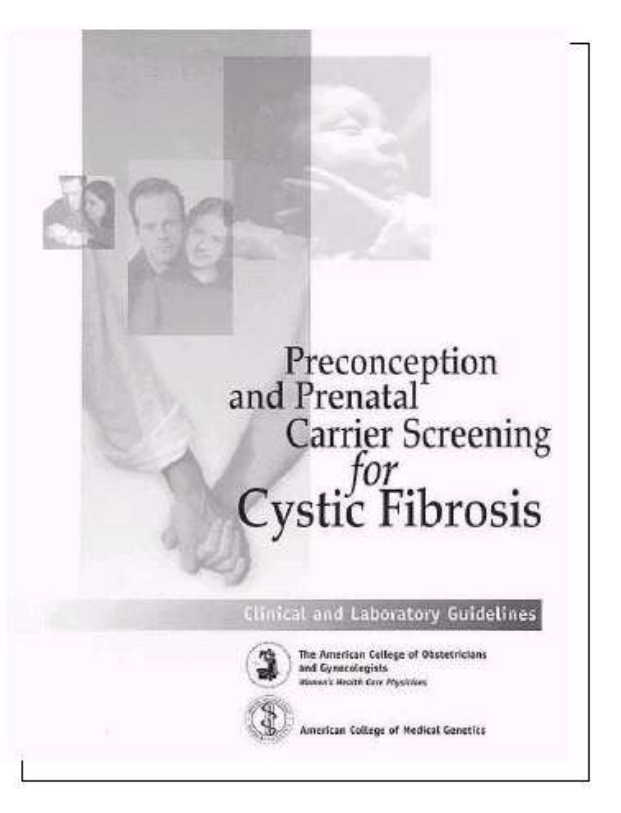
Check controls to ensure plate validity prior to assessing sample results. Check con

	Card 1									
Mutation	101	102	103	104	105	106	107	108	201	202
S549R A>C	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Y122X	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Y1092X C>A	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
R1162X	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
R347H	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2184delA	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3876delA	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
G85E	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
S549R T>G	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
R334W	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	MUT	Normal
R553X	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
621+1G>T	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
deltaI507	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
S549N	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
D1152H	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
394delTT	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
G542X	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
R347P	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2183AA>G	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
R117H	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
IVS8-5T/7T/9T										
G551D	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1078delT	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
deltaF508	Normal	Normal	Normal	HET	Normal	Normal	Normal	Normal	Normal	Normal
3849+4A>G	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
R560T	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
W1282X	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1717-1G>A	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
A455E	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3849+10kbC>T	Normal	Normal	Normal	HET	Normal	Normal	Normal	Normal	Normal	Normal
3659delC	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
V520F	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

MixWorksheet / SamplePlacement / Executive Summary / **Sample Summary** / Card 1 / Card 2 / Card 3 / Card 4 / Card 5 / Card 6

Cystic Fibrosis

- Single gene disorder (CFTR - 1989)
- 27 exons, 23 kb
- ΔF508
- >1,800 mutations
- Clinical heterogeneity
- Screening program – 2001
- Treatment of symptoms
- VX-770
  - small molecule drug
  - G551D specific (4%)
- VX-809 - ΔF508 specific (90%)

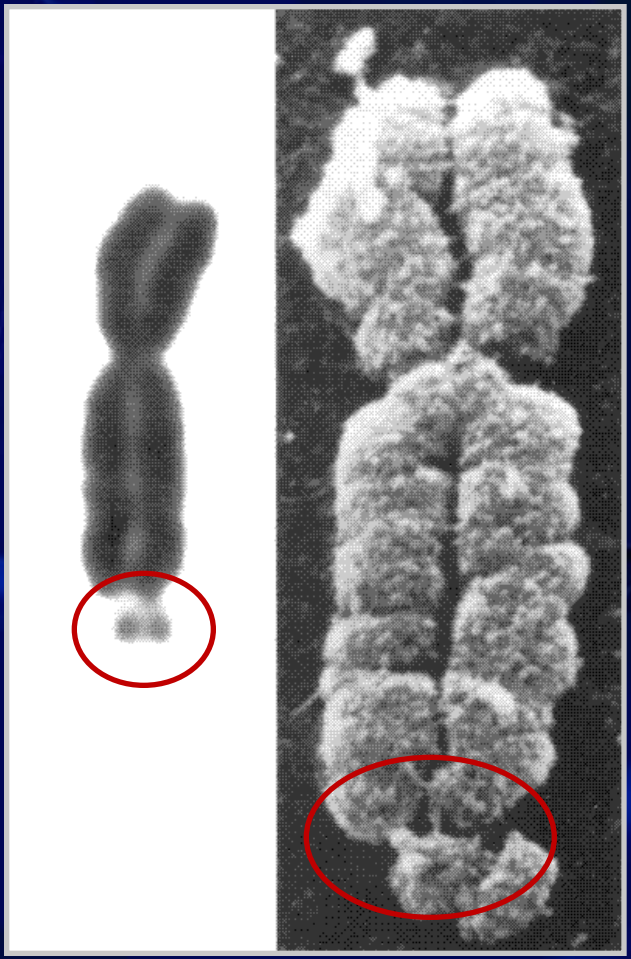


Fragile X Syndrome

- Most common form of inherited mental retardation
- Leading known genetic form of autism
- X-linked dominant with incomplete penetrance and anticipation

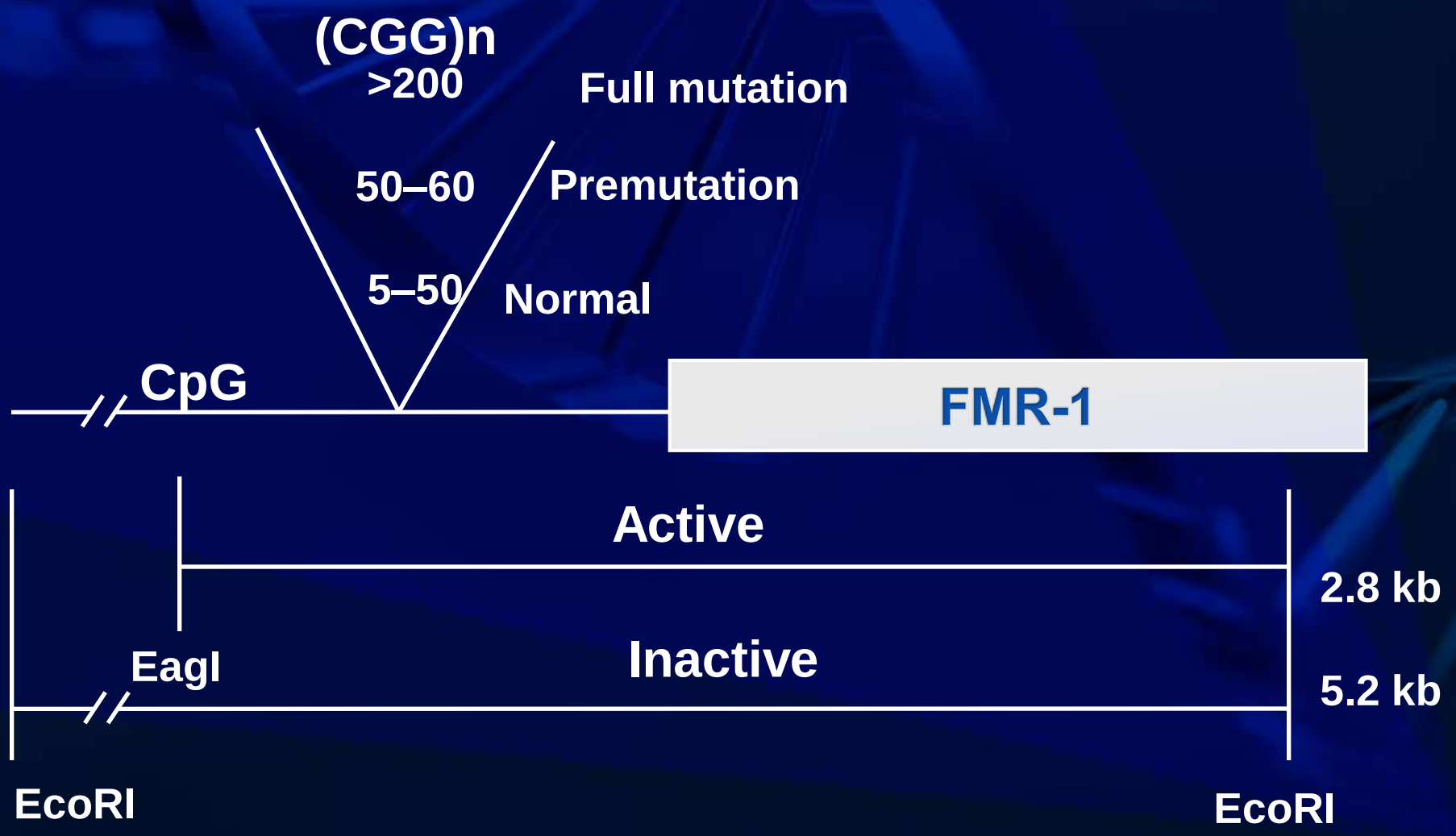
Fragile X Syndrome

- FMR1 gene discovered and cloned in 1991
  - 17 exons, 40 kb
  - 4.4 kb transcript
- First example of trinucleotide repeat (CGG) expansion mutation



http://medicineworld.org/images/blogs/6-2007/fragile-x-12701.gif

FMR1 gene


(CGG)n
>200
Full mutation
50–60
Premutation
5–50
Normal
CpG
FMR-1
Active
2.8 kb
EagI
Inactive
5.2 kb
EcoRI
EcoRI

Fragile X Mutation Expansion

- Slippage of replication fork during replication
  - Cannot account for large expansion and contractions
- Formation of hairpin-like structures not recognized by repair machinery
  - AGG triplets are regularly interspersed throughout the CGG repeat region
  - Expansion occurs at uninterrupted regions
  - Uninterrupted regions more likely to form secondary structures

Fragile X Mutation Expansion

- Expansion from PM to FM only occurs during maternal transmission
  - Only PM found in sperm of FM male patients
  - Timing remains uncertain
    - Probably during maternal meiosis; reductional instability in the fetal testes

Epigenetic Changes in FMR1

Methylation of FMR1 causes features of FXS

- Individuals with expansion, but not methylation, of the CGG repeat do not exhibit the full spectrum of the FXS phenotype
- CGG repeat length is responsible for hypermethylation FMR1
  - Leads to condensation of chromatin
  - Causes inactivation of the FMR1 gene
- First example of trinucleotide repeat (CGG) expansion mutation

FMR1 CGG Repeat Size

Category	(CGG)n	FMR1 Methylation	Female	Male
Stable	6-45	unmethylated	normal	normal
Gray zone	45-55	unmethylated	normal	normal
Premutation	55-200	unmethylated	usually not	usually not
Full mutation	>200	methylated	50% affected	all affected

FMR1 Premutation Phenotypes

- Carriers have increased FMR1 mRNA but decreased FMRP levels
- Accumulation causes intracellular precipitates and neuronal degeneration
- Ovarian dysfunction in females
- Late onset neurodegenerative disorder in males

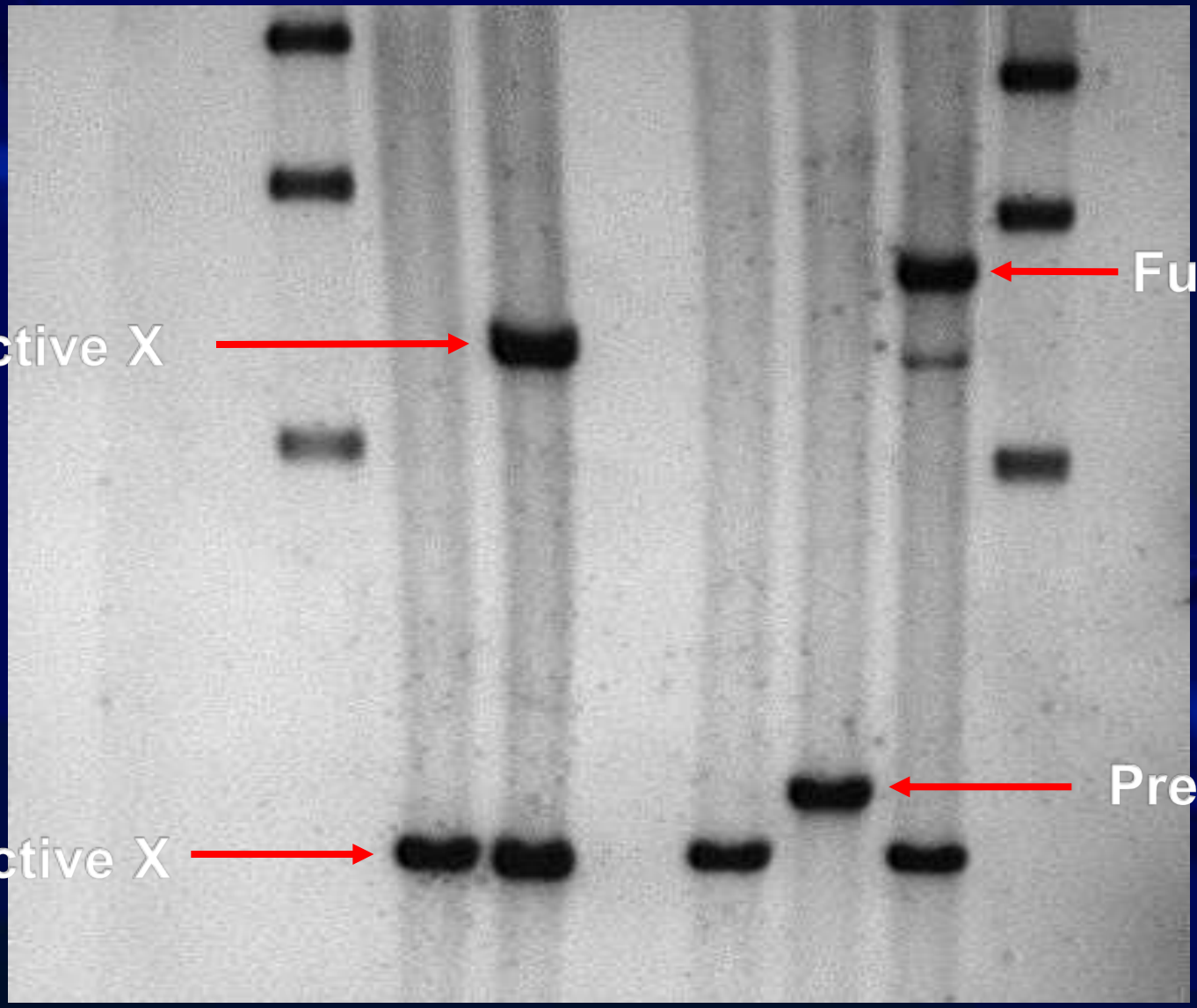
Primary ovarian insufficiency and FMR1 premutation

- Defined as amenorrhea before age 40 and increased FSH levels
- Affects 1% of reproductive age females
- 16%-24% have premutations in FMR1

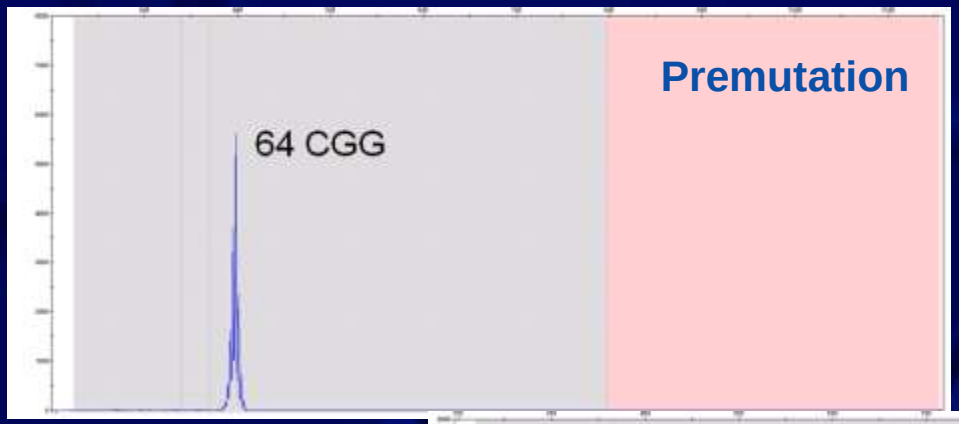
Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS)

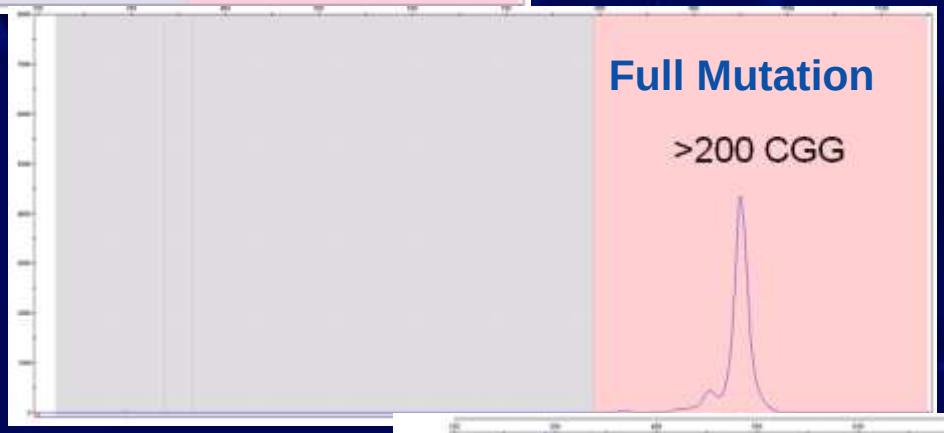
- Mechanism for FXTAS is completely different than FRAX
- Progressive action tremors/ataxia, atypical Parkinson's
- 30% of carrier males have FXTAS
- At autopsy: eosinophilic intranuclear inclusions (neuronal and astrocytic)
- 1 in 3,000 males older than 50

Fragile X Syndrome: Southern Blot Analysis



CE data


Premutation
64 CGG


Full Mutation
>200 CGG

29 CGG
Full Mutation Mosaic
99
131-140
>200

Assay workflow

CGG RP or Gene-Specific PCR

PCR
~30 min setup

13 µL PCR mastermix + 2 µL sample (10-40 ng/mL)

GS PCR → ~3.5 hrs

CGG RP → ~5.5 hrs

PCR
ABI 9700, Veriti,
or
MJ PTC-200

CE
~30 min setup

GS PCR Plate

CGG PCR Plate

Transfer 2 µL of PCR product + ROX Ladder

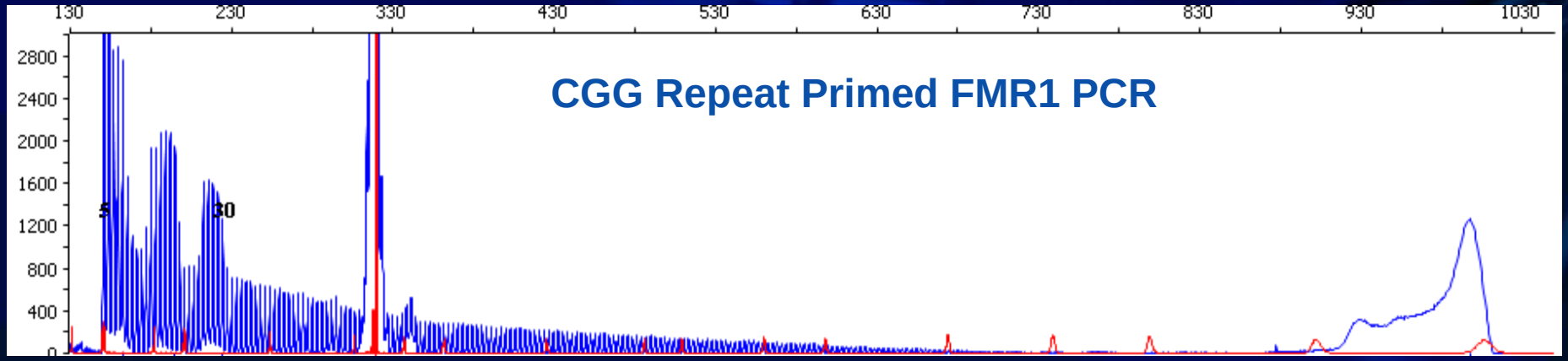
PCR Products
ROX Ladder
+ HiDi formamide

CE Plate

CE
3130, 3130xl, 3730
POP7 (36 cm)

CE data

30 CGG
Gene-specific FMR1 PCR
Full mutation


CGG Repeat Primed FMR1 PCR
5
30

Pharmacogenetics

- Genetic differences resulting in individual variation in response to drugs
  - Side-effects, adverse events, ineffective therapy
- Clinical goals
  - Minimize adverse drug events (ADEs)
  - Maximize drug efficacy

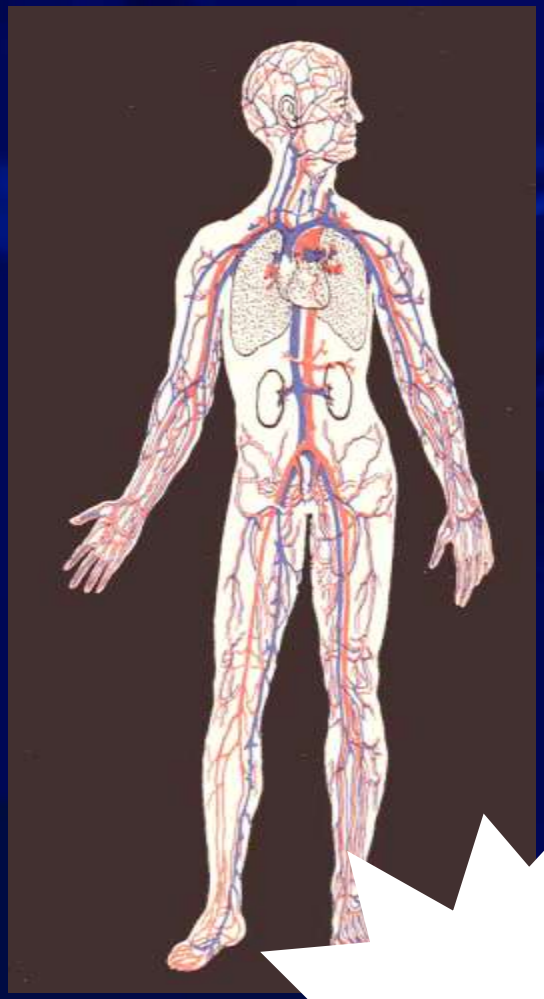
Pharmacogenetics

- Right Drug
- Right Dose
- Right Patient

Current Clinical Applications Of PGx

- Psychiatry
  - SSRIs and other psychotherapeutics
- Oncology
  - Breast cancer: Tamoxifen
  - Solid tumors: Irinotecan
  - ALL: Thiopurines
- Thrombosis and Hemostasis
  - Warfarin
  - Clopidogrel
  - Aspirin
- Anesthesia
  - Succinylcholine
  - Malignant hyperthermia
- Other
  - Anti-retroviral
  - Anti-epileptics/anti-convulsants

PGx: Which Genes?



Drug-metabolizing enzymes
Drug transporters

Pharmacokinetics

Pharmacodynamics

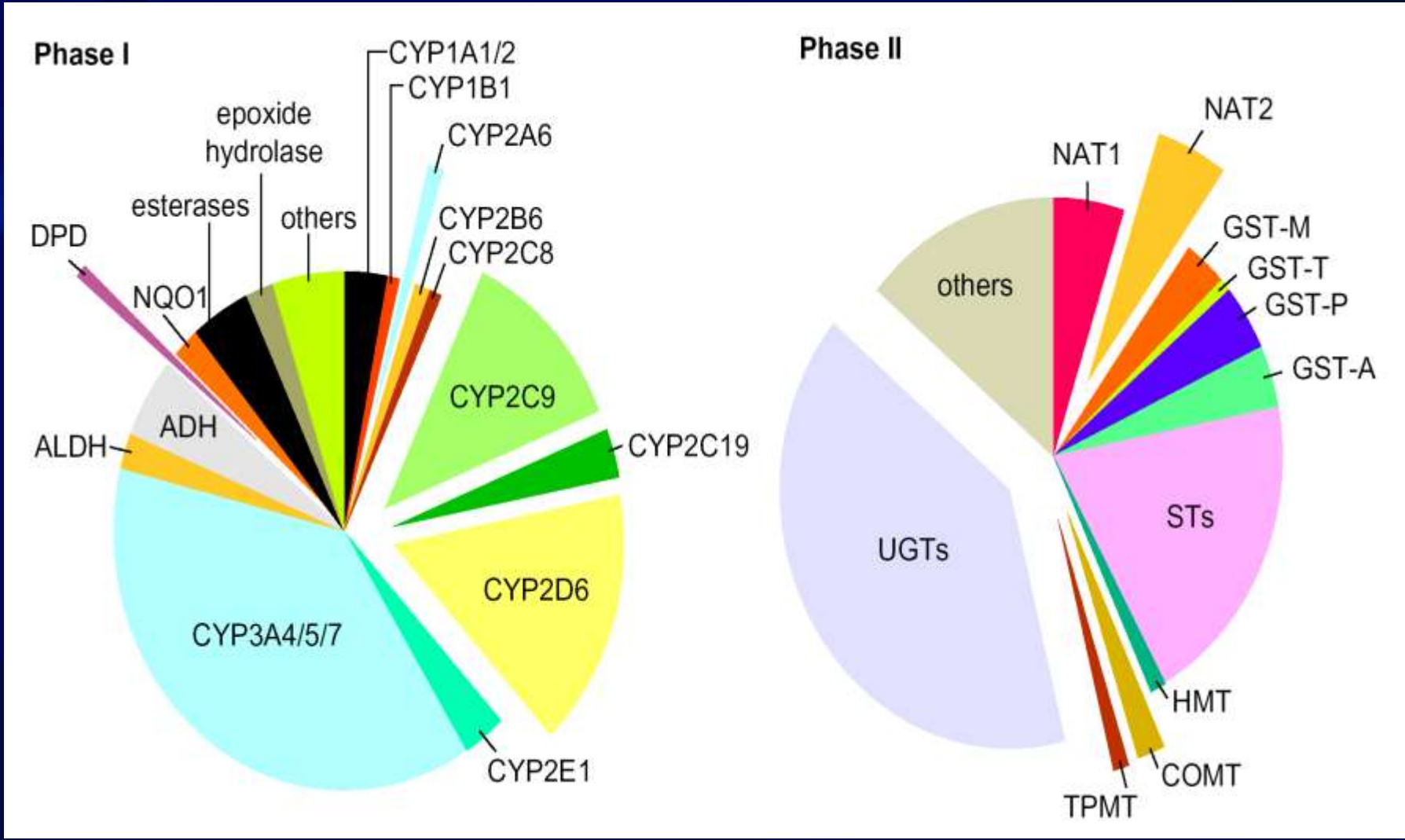
Drug targets

Pharmacokinetics

- “What the body does to the drug”
- ADME
  - **Absorption**
    - Process of substance entering body
  - **Distribution**
    - Dispersion of substances throughout fluids and tissues
  - **Metabolism**
    - Transformation of parent compounds into daughter metabolites
  - **Excretion**
    - Elimination of substances from the body

Pharmacokinetic Metabolism

- Parent compounds are usually converted to metabolites that are more water soluble (easier excretion)
- Prodrugs can also be converted into therapeutically active compounds (bioactivation)
- Pathways of drug metabolism are:
  - **Phase I reactions**
    - Oxidation, reduction, hydrolysis, dealkylation, deamination, desulfuration, dehydrogenation
  - **Phase II reactions (Conjugation)**
    - Acetylation, glucuronidation, methylation

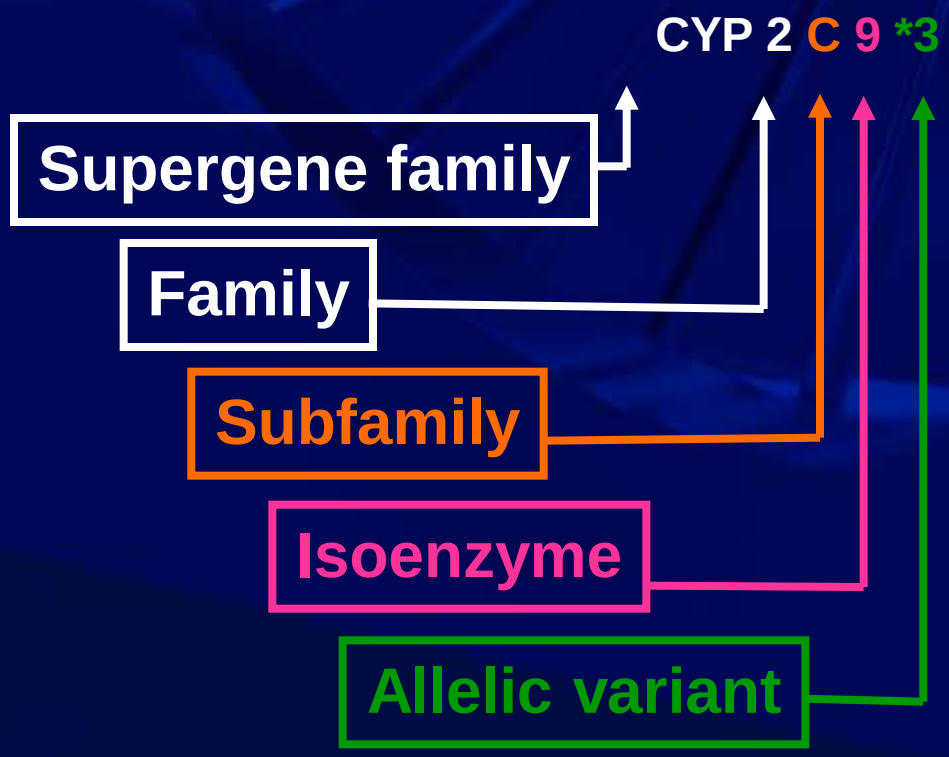


Evans E et al, Science 286:487-491 (1999). Reprinted with permission from the American Association for the Advancement of Science.

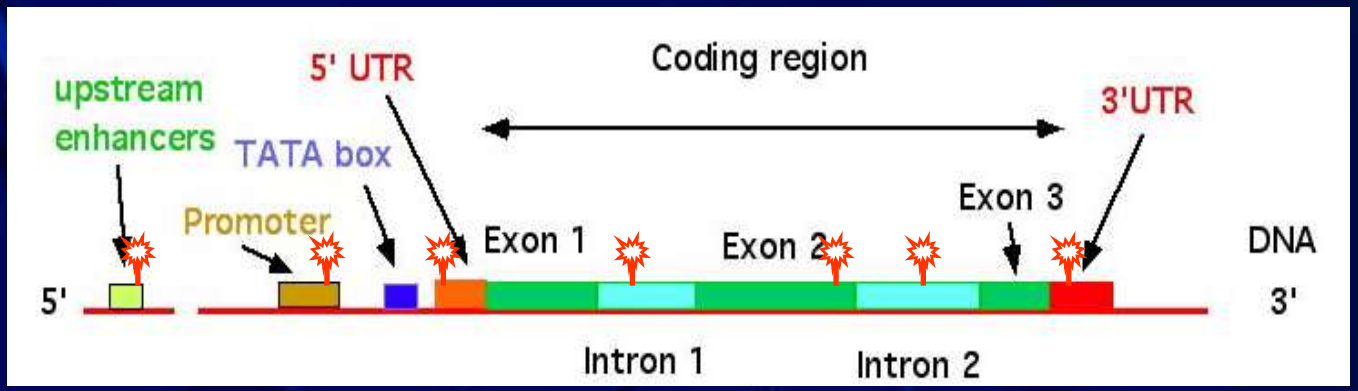
Cytochrome P450 Enzymes

- **Supergene family of heme-thiolate proteins**
- **Found in liver and small intestine**
  - Most located in ER, some mitochondrial
    - Mostly membrane-associated
- **Characteristic absorption spectra of the protein products**
  - peak at 450 nm
- **57 CYP genes in the human genome**
  - **15 involved in the metabolism of xenobiotics**
    - Account for 75% of total metabolism of drugs
  - 14 involved in metabolism of sterols
  - 4 oxidize fat-soluble vitamins
  - 9 involved in metabolism of fatty acids and eicosanoids
  - 15 – substrates essentially unknown

CYP Nomenclature



Polymorphisms and Enzyme Activity



Spectrum of Drug Metabolism

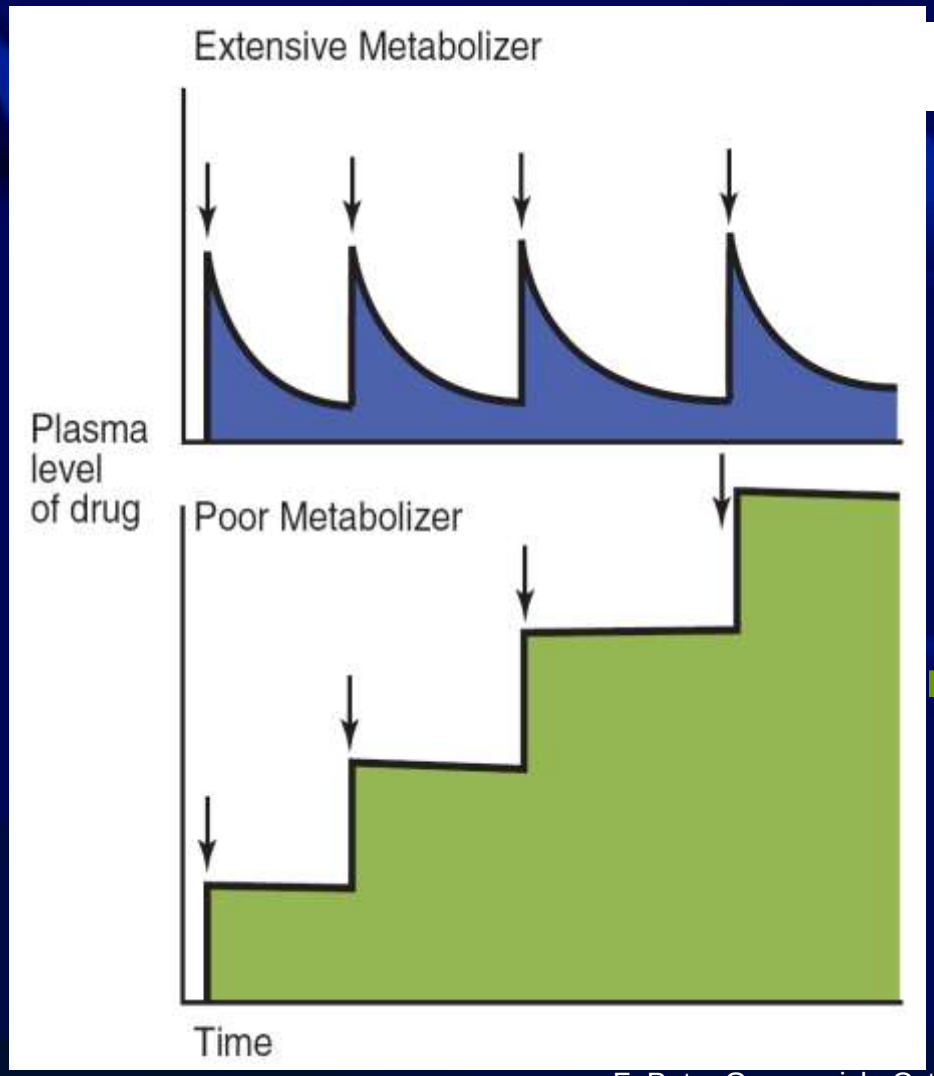
Poor (PM)

Intermediate (IM)

Extensive (EM)

Ultra-Rapid (UM)

Significance of low drug metabolism by P450s



- ADEs
- Ineffective therapy

F. Peter Guengerich, Cytochromes P450, Drugs, and Diseases, Mol Interv June 2003 3:194-201. © by the American Society for Pharmacology and Experimental Therapeutics.

Pharmacodynamics

- **“What the drug does to the body”**
- **Biochemical and physiological effects of drugs on the body**
- **Five main drug actions:**
  - Depression
  - Stimulation
  - Cytotoxic
  - Irritation
  - Replacement

The FDA and PGx-related Black Box Warnings

January 2010
Updated Warfarin label to include effect of VKORC1 and PGx-guided dosing ranges

March 2010
Clopidogrel resistance in CYP2C19 poor metabolizers

July 2005
Irinotecan toxicity due to UGT1A1 deficiency

August 2007
Warfarin sensitivity in CYP2C9 poor metabolizers

July 2008
Abacavir hypersensitivity due to HLA-B*5701

October 2006
CYP2D6 poor metabolizers have increased risk of breast cancer recurrence on Tamoxifen

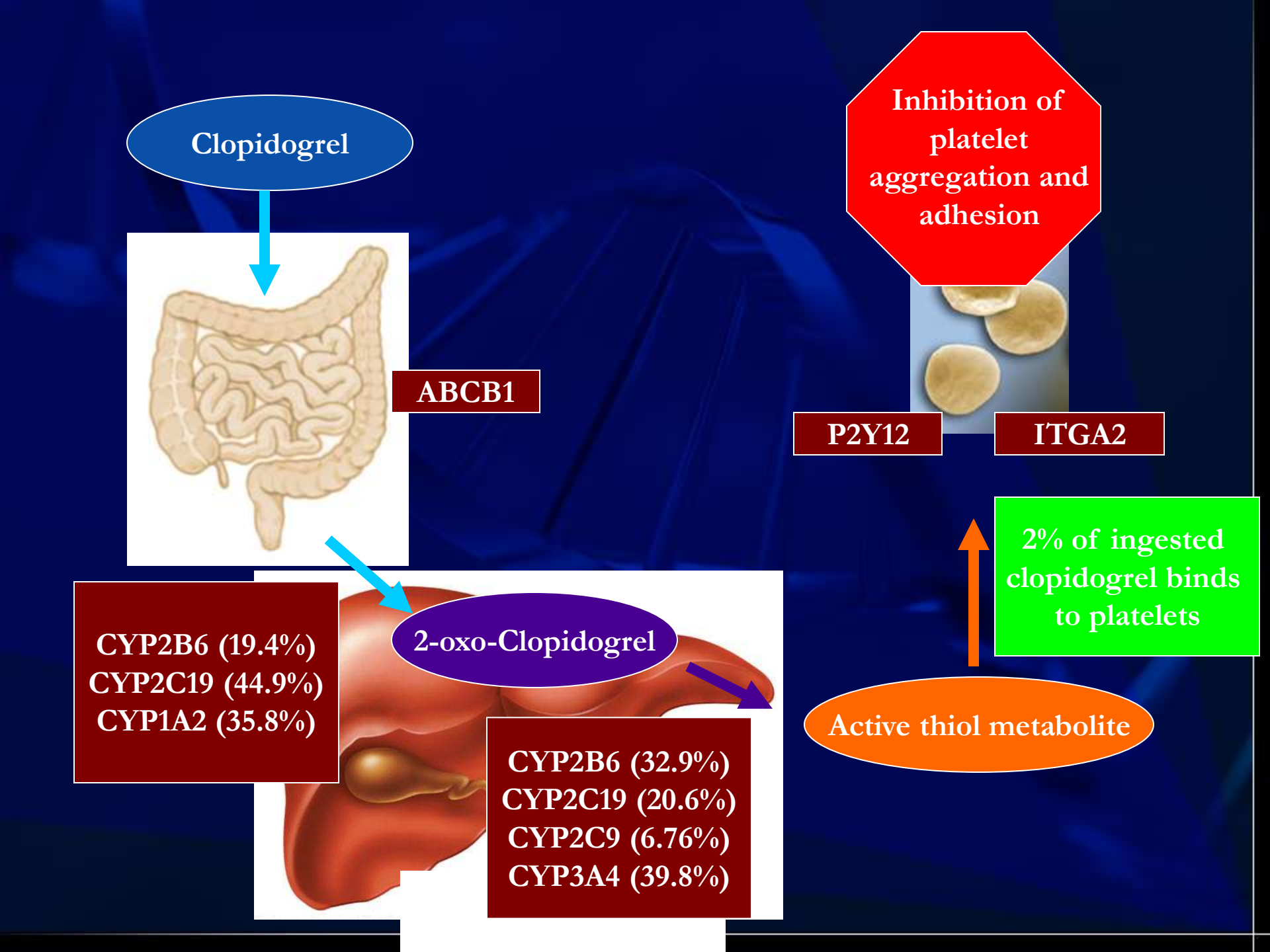
December 2007
Carbamazepine-associated SJS or TEN due to HLA-B*1502

March 2010

Clopidogrel resistance in CYP2C19 poor metabolizers

Clopidogrel

- Pro-drug
  - Requires activation by CYP enzymes
    - CYP3A4/5, CYP2C19, CYP2C9, CYP2B6, and CYP1A2
- Clopidogrel's active metabolite blocks the platelet P2Y12 receptor
  - ~50% reduction in ADP-mediated platelet aggregation
- High rate of response variability and non-response to clopidogrel
  - Related to variable and suboptimal active metabolite generation
  - Due to many variables
    - Underdosing
    - Interactions with CYP inhibitors and substrates
      - e.g. lipophilic statins, calcium antagonists, PPIs
    - Genetics
- Metabolism of clopidogrel has been only recently elucidated


Clopidogrel
ABCB1
CYP2B6 (19.4%)
CYP2C19 (44.9%)
CYP1A2 (35.8%)
2-oxo-Clopidogrel
CYP2B6 (32.9%)
CYP2C19 (20.6%)
CYP2C9 (6.76%)
CYP3A4 (39.8%)
Active thiol metabolite
2% of ingested clopidogrel binds to platelets
Inhibition of platelet aggregation and adhesion
P2Y12
ITGA2

CYP2C19 variants

- At least 25 polymorphic variants in CYP2C19
  - Most common is *2
    - Loss of function allele in exon 5
    - Caucasians, 25-35%; African Americans, 33-34%; Chinese, 51%; Mexican Americans, 18-19%
  - *3, *4, *5, and *8 are also associated with clopidogrel resistance, but occur at a much lower frequency
- Overall, CYP2C19 polymorphisms that result in inadequate platelet inhibition are found in approximately 30% of N. Am. population
- CYP2C19*17 promoter variant leads to increased platelet inhibition
  - 41- 46% of Caucasians; 4% of Chinese; 30 -46% African

CYP2C19 loss of function alleles

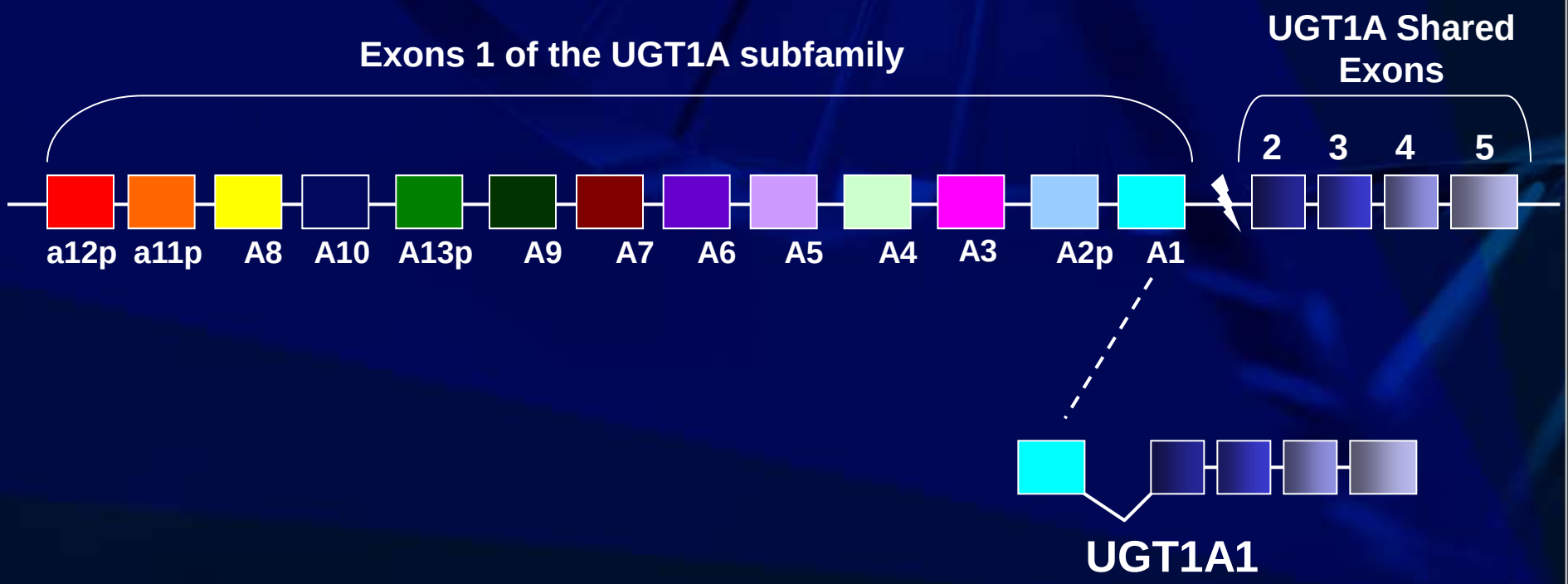
- Associated with marked decrease in platelet responsiveness to clopidogrel
- Associated with increased rate of subsequent cardiovascular events and death
- Mega et al, NEJM, 2009, 360: 354-62 (TRITON-TIMI 38)
  - n=1477, patients with ACS and planned PCI, randomly assigned to treatment with clopidogrel
  - Relative 53% increased risk of death from CV causes, MI, or stroke
  - 3-fold increased risk of stent thrombosis
- Simon et al, NEJM, 2009, 360: 363-75 (FAST-MI)
  - n=2208, patients with acute ST-elevation and non-ST-elevation MI
  - Higher event rate in carriers of two CYP2C19 loss of function alleles
    - 21.5% vs. 13.3%, adjusted HR, 1.98; 95% CI, 1.10 – 3.58
  - Adjusted risk of death, MI, or stroke in patients who underwent PCI (n=1535) was 3.58 times higher in patients with 2 CYP2C19 LOF alleles (95% CI, 1.71-7.51, p=0.005)
  - Presence of two CYP2C19 LOF alleles and one or two ABCB1 variants had highest risk of events (adjusted HR, 5.31, 95% CI, 2.13 – 13.20, p=0.009)

July 2005

Irinotecan toxicity due to UGT1A1 deficiency

UGT1A1

- UDP-glucuronosyltransferase
- UGT1A subfamily has 13 different gene isoforms

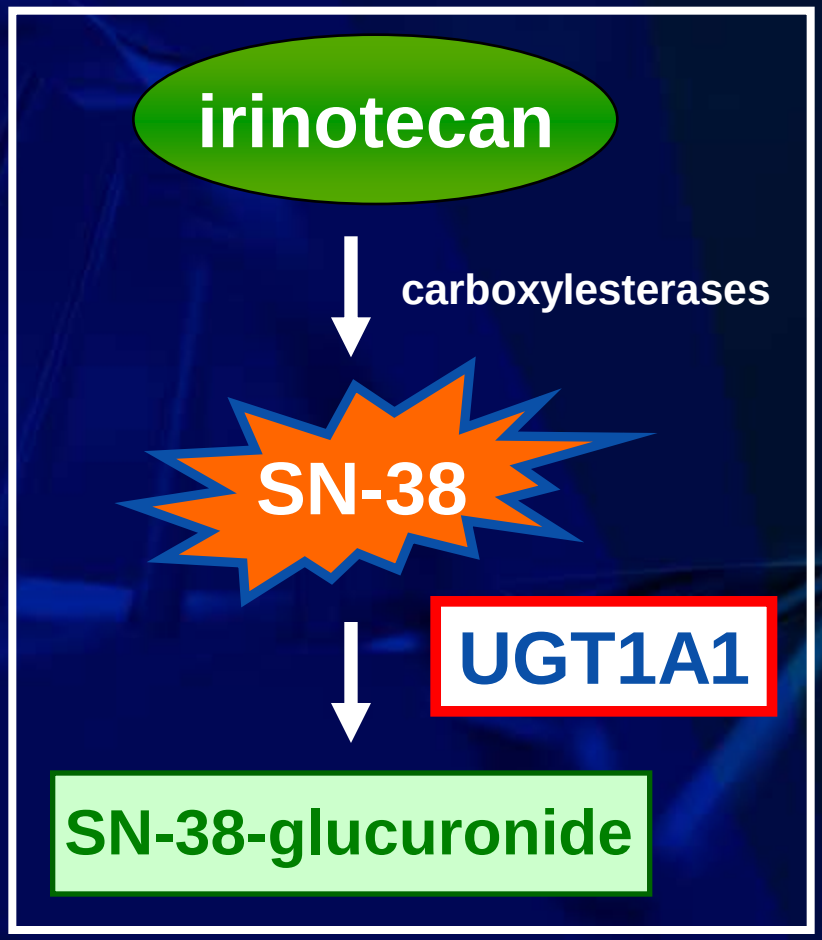


UGT1A1

- **UGT1A1 is primary enzyme involved in glucuronidation of irinotecan**
- **Irinotecan is a camptothecin analogue widely used for treatment of solid tumors**
  - Severe, occasionally fatal, leukopenia/neutropenia or diarrhea
    - Linked to *UGT1A1* polymorphisms
- **FDA approved revisions to safety labeling on irinotecan to recommend reduced dosing in patients with specific *UGT1A1* genotypes**

Irinotecan metabolism

- Irinotecan activated by carboxylesterases
- SN-38 is active drug
  - Blocks DNA replication
- SN-38 inactivated by glucuronidation (UGT1A1)
- SN-38 conjugate secreted into bile and the intestines



UGT1A1 Promoter Repeats

U.S. FDA-Cleared PGx Tests

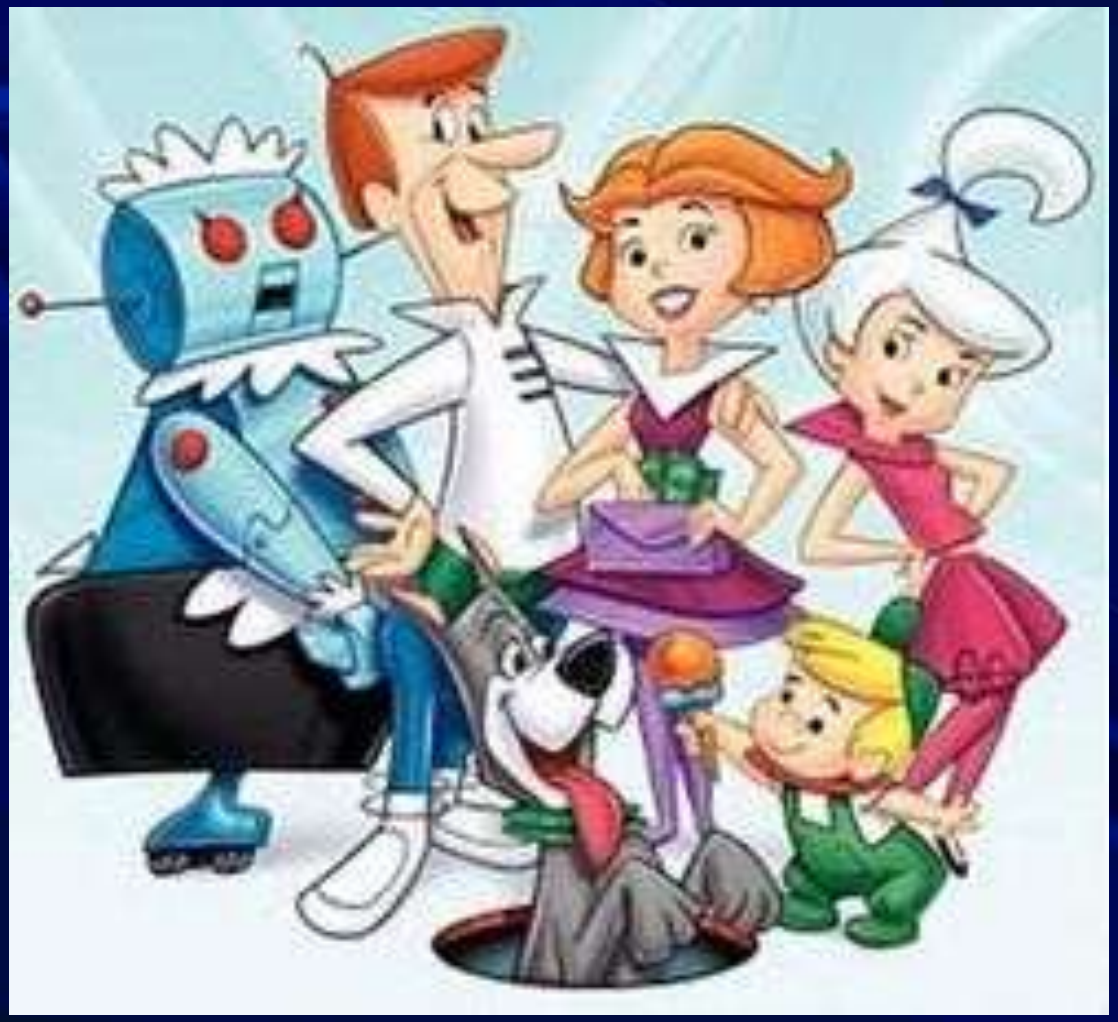
Gene(s)	Test Platform	Manufacturer	Approval Date	Target Drug
CYP2D6	AmpliChip® CYP450	Roche	Jan 2005	Not specified
CYP2C19	AmpliChip® CYP450	Roche	Jan 2005	Not specified
UGT1A1	Invader®	Hologic	Sept 2005	Irinotecan
CYP2C9/VKORC1	Verigene®	Nanosphere	Sept 2007	Warfarin
CYP2C9/VKORC1	INFINITI™	Autogenomics	Jan 2008	Warfarin
CYP2C9/VKORC1	RT-PCR	Paragon	May 2008	Warfarin
CYP2C9/VKORC1	eSensor®	GenMark	July 2008	Warfarin
CYP2C9/VKORC1	eQ-PCR LightCycler	TrimGen	Feb 2009	Warfarin
CYP2D6	xTAG®	Luminex	Nov 2010	Not specified

http://www.amp.org/FDATable/FDATable.doc

Summary

- CF has become a model for molecular genetic testing
- FRAX testing highlights the importance of gene associations with other disease entities
- PGX represents a new opportunity to apply genetics in patient management

What if…….



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