Pitfalls and shortcomings of pharmacogenomic association studies:
The tamoxifen controversy

Hiltrud Brauch

20th International Symposium on Microsomes and Drug Oxidation
Stuttgart, Germany | 18-22, 2014
Tamoxifen in early breast cancer

- Non steroidal anti-estrogen
- Selective estrogen receptor modulator (SERM) first targeted and to date most successful therapy
- Four decades of experience
- Used in > 120 countries for all stages of breast cancer

Recurrence

Mortality

EBCTCG, Lancet 2005
Mechanisms of endocrine therapy in early BC

70% of patients are eligible

Androstenedione → Aromatase → Estrone

Testosterone → Aromatase → Estradiol

Tamoxifen inhibits the binding of ER to EREs, preventing proliferation.

ER +

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Aromatase inhibitor versus tamoxifen outcome

ATAC Trialists’ Group

ATAC: Anastrozole and Tamoxifen alone or in Combination

5 years of tamoxifen versus anastrozole

N=5216

![Graph showing recurrence rates for tamoxifen and anastrozole over 5 years](image)

- **Tamoxifen**
  - 2.5% absolute difference
  - 16.4% at 5 years

- **Anastrozole**
  - 13.9% at 5 years

Relative difference ~ 15%

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Tamoxifen is a prodrug

Tamoxifen (TAM) 4-OH-TAM

2D6 2C19 2B6, 2C9, 3A4

Tamoxifen 4-OH-TAM

Endoxifen

100x more effective

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Sparteine oxidation phenotypes and distribution in a German population

EM
Extensive Metabolizer

UM
Ultrarapid metabolizer

IM
Intermediate Metabolizer

PM 8%
Poor Metabolizer

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Molecular Basis of the \textit{CYP2D6} Polymorphism

>100 genetic variants define phenotypes and their rate of drug metabolism

\textbf{CYP2D gene locus}

\begin{itemize}
  \item \textbf{CYP2D8} (pseudogene)
  \item \textbf{CYP2D7} (pseudogene)
  \item \textbf{CYP2D6}
\end{itemize}

\begin{itemize}
  \item \textbf{UM} ultrarapid
  \item \textbf{EM} extensive
  \item \textbf{IM} intermediate
  \item \textbf{PM} poor metabolizer
\end{itemize}

<table>
<thead>
<tr>
<th>Allele</th>
<th>Function</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1, *2, *35</td>
<td>duplicated alleles</td>
<td>(1.2%)</td>
</tr>
<tr>
<td>*1, *2</td>
<td>functional alleles</td>
<td>(80.7%)</td>
</tr>
</tbody>
</table>

Human CYP Allele Nomenclature Home Page: http://www.cypalleles.ki.se/
Pharmacogenetics / Pharmacogenomics

Personalized cancer treatment and patient stratification

- the right drug
- to the right patient
- at the right time
- in the right dose

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CYP2D6 polymorphism and recurrence probabilities upon tamoxifen treatment

Number of patients: 1325

Brauch H | IKP Stuttgart

Schroth et al. JAMA 302:1429-1436, 2009
CYP2D6 polymorphism and recurrence probabilities upon tamoxifen treatment

Number of patients: 1325

- Unstratified (all)
- EM (full function)
- hetEM/IM (decreased function)
- PM (no function)

Follow-up (years)

Proportion Recurrence

PM 11.5%; P=0.015
EM 2%; P=0.029

Schroth et al. JAMA 302:1429-1436, 2009
The Tamoxifen benefit for patients with fully functional CYP2D6 is similar as that of AI.

Schroth et al. JAMA 302:1429-1436, 2009

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The Stuttgart – Mayo study cohort
Retrospective analysis

Total
- 1580 patients (German and US origin)

Inclusion criteria
- Histologically proven breast cancer
- No previous chemotherapy or endocrine treatment other than adjuvant tamoxifen
- No metastatic disease at diagnosis
- Steroid receptor positivity (ER\(^+\) and/or PgR\(^+\))

Pharmacogenetic analysis with 1361 patients meeting criteria
- median follow-up of 6.3 years
- 95.4% postmenopausal

Schroth et al. JAMA 302:1429-1436, 2009
What level of evidence is needed?

Use of archived specimens in evaluation of prognostic and predictive markers

Studies with the following characteristics should have the potential to provide valid data:

- prospective clinical trials (gold standard, but costly)
- use of archived specimen (source of genetic material)
- sample size calculation
- determined subject eligibility
- marker cut point specification (phenotype definition)
- analytical plan
- confirmation
Highest level evidence was expected from prospective clinical trials

**ATAC**
Rae *et al.* Lack of correlation between gene variants in tamoxifen metabolizing enzymes with primary endpoints in the ATAC trial.  

**BIG 1-98**
Leyland-Jones *et al.* Outcome according to CYP2D6 genotype among postmenopausal women with endocrine-responsive early invasive breast cancer randomized in the BIG 1-98 Trial.  

No association between CYP2D6 polymorphism and outcome
### Characteristics of Tamoxifen-CYP2D6-pharmacogenetics studies and pitfalls

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<th>Study</th>
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<td>&gt;20 (12%)</td>
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Nakamura et al, JNCI 104:1264; 2012

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Loss of heterozygosity (LOH) is a hallmark of many cancers

The 1980ies

Deletion

LOH

Chromosom 3p

3p Locus

PATIENT genome + TUMOR genome

Tumor tissue contains normal cells (stroma, infiltrating lymphocytes)

Zbar et al Nature 1987
Brauch et al NEJM, 1987
22q13 LOH is common in breast cancer
Concordance Between *CYP2D6* Genotypes Obtained From Tumor-Derived and Germline DNA

James M. Rae, Meredith M. Regan, Jacklyn N. Thibert, Christina Gersch, Dafydd Thomas, Brian Leyland-Jones, Giuseppe Viale, Lajos Pusztai, Daniel F. Hayes, Todd Skaar, Catherine Van Poznak

### Concordance Table

<table>
<thead>
<tr>
<th>CYP2D6 score</th>
<th>From WBCs</th>
<th>From FFPE Ts</th>
<th>From FFPE LNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 4 0 0 0 0 4</td>
<td>0 3 0 0 1 0 4</td>
<td>0 3 0 0 1 0 4</td>
</tr>
<tr>
<td>0.5</td>
<td>0 7 0 0 0 0 7</td>
<td>0 7 0 0 0 0 7</td>
<td>0 7 0 0 0 0 7</td>
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<tr>
<td>1</td>
<td>1 0 1 42 0 0 44</td>
<td>2 0 1 41 0 0 44</td>
<td>2 0 1 41 0 0 44</td>
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<tr>
<td>1.5</td>
<td>0 0 0 15 0 0 15</td>
<td>1 0 0 14 0 0 15</td>
<td>1 0 0 14 0 0 15</td>
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<tr>
<td>2</td>
<td>0 0 0 1 51 51 52</td>
<td>3 0 0 0 49 49 52</td>
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### CYP2D6 phenotype Concordance

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### Kappa Agreement

- **From WBCs:** Concordance 119/121 99.2: [95%; CI 94.2-99.8%], Agreement K = 0.98 (95% CI: 0.94-1.00)
- **From FFPE Ts:** 114/117 97.4: [95% CI 92.7-99.5%], K = 0.96 (95% CI: 0.92-1.00)
- **From FFPE LNs:** 115/117 98.3: [95% CI 94.0-99.8%], K = 0.97 (95% CI: 0.92-1.00)

*white blood cells / FFPE / FFPE of non affected lymphnodes*

Brauch H | IKP Stuttgart
Concordance Between CYP2D6 Genotypes Obtained From Tumor-Derived and Germline DNA

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<th>CYP2D6 score from WBCs</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<td>1</td>
<td>1.5</td>
<td>2</td>
<td></td>
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<tr>
<td>From FFPE-Ts</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>4</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>IM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>7</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>From FFPE-LNs</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>PM</td>
<td>3</td>
<td>0</td>
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<td>4</td>
</tr>
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<td>IM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>3</td>
<td>0</td>
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CYP2D6 score

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CYP2D6 phenotype

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**white blood cells / FFPE / FFPE of non affected lymphnodes**

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The Cancer Genome Atlas (TCGA) Breast Data

CYP2D6 Chr. 22: 42,522,501-42,525,911
Switchplots indicating CYP2D6 LOH in breast tumor
C Perou, personal communication March 20, 2013

All n = 728
ERpos n = 545
ERneg n = 183

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Attention must be paid when genomic DNA is isolated for pharmacogenetic investigations.

DNA source:
- Germline genome
- Periperal blood mononuclear cells
- Tumor genome
- FFPE tissue
- Core punch

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Austrian ABCSG 8 Trial: Reduced CYP2D6 metabolism and higher risk for recurrence


<table>
<thead>
<tr>
<th></th>
<th>Tamoxifen only (Arm A)</th>
<th>Tamoxifen followed by anastrozole (Arm B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>112 cases</td>
<td>102 cases</td>
</tr>
<tr>
<td><strong>OR (95% CI)</strong></td>
<td><strong>P</strong></td>
<td><strong>OR (95% CI)</strong></td>
</tr>
<tr>
<td>PM/PM relative to EM/EM</td>
<td>2.45 (1.05 - 5.73)</td>
<td>0.04</td>
</tr>
<tr>
<td>EM/PM and PM/IM relative to EM/EM</td>
<td>1.67 (0.95 - 2.93)</td>
<td>0.07</td>
</tr>
<tr>
<td>EM/IM and IM/IM relative to EM/EM</td>
<td>1.23 (0.58 - 2.61)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Case-control study: 5 year treatment

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CYP2D6 genotype is associated with a higher risk of recurrence

International Tamoxifen Pharmacogenetics Consortium (ITPC)

Metaanalysis of 1996 patients
Invasive Disease-free Survival

HR 1.25 (95% CI 1.06-1.47)

Univ Yonsai / Korea
Univ Dundee / UK
Univ Salzburg / Austria
UCSF / USA
IKP Stuttgart / Germany
Univ Orebrö / Sweden
Univ Manchester / UK
Mayo Clinic / USA
Univ Indiana / USA
RIKEN Center / Japan
Univ Leuven / Belgium
Roswell Park / USA
Variability of the (Z)-endoxifen plasma levels

N=236

IC$_{90}$ (ER) 20 mg/day

IC$_{90}$ Endoxifen

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CYP2D6 and (Z)-endoxifen formation (N=236)

(Z)-Endoxifen plasma level

Intrinsic formation rate

Gene-Dose-Effect

P < 10^{-16}

~40% of variability explained by CYP2D6 genotype

IM: *9, *10, *41 : 0.5
EM: *1, *2 : 1
UM: 2x*1, 2x*2 : 2

Breast cancer outcomes in the WHEL study


Q1: Women with low endoxifen concentrations are at higher risk to develop recurrences

<table>
<thead>
<tr>
<th>Quartile</th>
<th>Median (ng/ml)</th>
<th>Range</th>
<th>Recurrence rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>4.2</td>
<td>0-5.9</td>
<td>16.0</td>
</tr>
<tr>
<td>Q2</td>
<td>8.2</td>
<td>5.9-10.2</td>
<td>11.7</td>
</tr>
<tr>
<td>Q3</td>
<td>12.4</td>
<td>10.2-14.6</td>
<td>14.7</td>
</tr>
<tr>
<td>Q4</td>
<td>17.4</td>
<td>14.6-20.4</td>
<td>10.1</td>
</tr>
<tr>
<td>Q5</td>
<td>26.2</td>
<td>20.4-73.7</td>
<td>12.4</td>
</tr>
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HR 0.74
95% CI 0.55-1.00
Tamoxifen Use in Postmenopausal Breast Cancer: CYP2D6 Matters

Hiltrud Brauch and Werner Schroth, Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart; and University Tübingen, Tübingen, Germany
Matthew P. Goetz, Mayo Clinic, Rochester, MN
Thomas E. Müldter and Stefan Winter, Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart; and University Tübingen, Tübingen, Germany
James N. Ingle, Mayo Clinic, Rochester, MN
Matthias Schwab, Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart; University Tübingen; and Institute of Experimental and Clinical Pharmacology and Toxicology, University Hospital Tübingen, Tübingen, Germany
Michel Eichelbaum, Dr Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart; and University Tübingen, Tübingen, Germany
Modeling the pharmacological importance of endoxifen for the treatment of ER positive breast cancer in premenopausal women
Maximov et al. AACR 2013, Washington DC

Long term adjuvant tamoxifen therapy for five years is the antiestrogenic standard of care for ER positive breast cancer in premenopausal patients. The metabolic activation of tamoxifen by CYP2D6 to endoxifen remains controversial to plan the treatment of patients with breast cancer. However, all retrospective studies focus entirely on postmenopausal patients and no studies have been undertaken in the relevant premenopausal treatment population. We have addressed the issue of the pharmacological importance of endoxifen to control the estrogen-stimulated growth of four ER positive breast cancer cell lines. We have modeled the actual estrogen environment in the laboratory (estradiol plus estrone) based on previous data from premenopausal patients taking tamoxifen [1]. Our strategy was to evaluate the anti-proliferative actions of actual concentrations of tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen combined, based on the actual measurements reported for those metabolites in patients who were extensive metabolizers (EM), intermediate metabolizers (IM) or poor metabolizers (PM) [2]. The results demonstrate the essential requirement with the appropriate concentration of endoxifen necessary to block estrogen-mediated cell replication.
The premenopausal situation

Tamoxifen metabolite levels are similar between ethnic groups

Network for the investigation of Tamoxifen Outcome in Premenopausal Breast Cancer

LC-MS/MS; N=587

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The premenopausal situation

Endoxifen concentration depends on CYP2D6 activity score

N=548
median age: 39.1 yrs [range 22-59 yrs]

$R^2=53\%$; $P < 10^{-77}$

Network for the investigation of Tamoxifen Outcome in Premenopausal Breast Cancer

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women diagnosed with invasive breast cancer aged 40 years or younger at diagnosis

women aged 41-50 years with BRCA1 or BRCA2 mutation diagnosed with invasive cancer

N = 306
Endoxifen predicts outcome in POSH
Prospective Study of Outcomes in Sporadic versus Hereditary Breast Cancer

Patients aged 40 years or younger at diagnosis & BRCA carriers

The Premenopausal Breast Cancer Study Group

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Impact of CYP2C19 and CYP2C9 on tamoxifen metabolite ratios

CYP2C19 phenotype (loss of function alleles *2/*3)

CYP2C9 phenotype (reduced activity alleles *2/*3)
Tamoxifen Pharmacogenetics

- **Postmenopausal patients**: CYP2D6 predicts outcome

- The mechanism of tamoxifen metabolism is the same in pre- and postmenopausal women

- **Premenopausal patients**: first evidence that endoxifen concentrations and CYP2D6 activity predict outcome

- Regular and continuous drug intake is necessary for treatment success (no pain no gain)

- CYP2D6 inhibitors must be avoided

ASCO guidelines July 2010 (JCO)
Cancer Pharmacogenomic Study Design

Choosing patient cohort
- Clinical trial
- Prospective study
- Retrospective study
- Has consent for genetic studies been given?
- Has germline DNA been collected

Optimizing sample size
- Any previous estimates of effect size
- Are at least 300 patients available for a discovery GWAS
- Are there similar trials or data sets that could be compiled

Phenotypes to consider
- Adverse events or toxicities
- Tumor response
- Progression free survival
- Overall survival

Endophenotypes to consider
- Drug or metabolite clearance
- RNA expression
- Methylation patterns
- Serum protein levels

Statistical analysis
- Traditional GWAS or meta-analysis?
- Sequencing or rare variant analysis
- Polygenic modelling?
- Pathway-based analysis?

Replication and validation
- Is a suitable replication patient cohort available?
- Resequencing study for functional variants?
- Cell or animal model functional studies?

Wheeler H et al
Nat Rev Genet 14:23-34; 2013
Lessons learned from pitfalls in tamoxifen pharmacogenomics

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CYP2D6 allele coverage sufficient

Brauch H | IKP Stuttgart
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Lessons learned from pitfalls in tamoxifen pharmacogenomics

Brauch H | IKP Stuttgart
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Lessons learned from pitfalls in tamoxifen pharmacogenomics

Oncology
DNA source: tumor

Cave: tumor DNA and germline DNA are not the same

Lack of quality control

Invalid analysis
### Choosing patient cohort
- Clinical trial
- Prospective study
- Retrospective study
- Has consent for genetic studies been given?
- Has germline DNA been collected

### Phenotypes to consider
- Adverse events or toxicities
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- Overall survival

### Endophenotypes to consider
- Drug or metabolite clearance
- RNA expression
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- Serum protein levels

### Statistical analysis

#### Optimizing sample size
- Any previous estimates of effect size
- Are at least 300 patients available for a discovery GWAS
- Are there similar trials or data sets that could be compiled

#### Quality Control:

- **Phenotyping**
  - Has sequencing or rare variant analysis been performed?
  - Is a replication patient cohort available?
- **Genotyping**
  - Traditional GWAS or meta-analysis?
  - Polygenic modelling?
  - Pathway-based analysis?
- **Statistical analysis**
  - N/A

### Oncology
**DNA source:** tumor

**Cave:** tumor DNA and germline DNA are not the same

### Lessons learned from pitfalls in tamoxifen pharmacogenomics