UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

INFLUÊNCIA DE FATORES GENÉTICOS E AMBIENTAIS NA ATIVIDADE DA CYP2D6 E CYP3A4 E SUA RELAÇÃO COM A BIOATIVAÇÃO DO TAMOXIFENO EM PACIENTES COM CÂNCER DE MAMA

MARINA VENZON ANTUNES

Porto Alegre, novembro de 2014

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

INFLUÊNCIA DE FATORES GENÉTICOS E AMBIENTAIS NA ATIVIDADE DA CYP2D6 E CYP3A4 E SUA RELAÇÃO COM A BIOATIVAÇÃO DO TAMOXIFENO EM PACIENTES COM CÂNCER DE MAMA

MARINA VENZON ANTUNES

Orientador: Prof. Dr. Gilberto Schwartsmann

Tese apresentada ao Programa de Pós Graduação em Medicina: Ciências Médicas, UFRGS, como requisito para obtenção do título de Doutor.

Porto Alegre, novembro de 2014

CIP - Catalogação na Publicação

Antunes, Marina Venzon
Influência de fatores genéticos e ambientais na atividade da CYP2D6 e CYP3A4 e sua relação com a bioativação do tamoxifeno em pacientes com câncer de mama / Marina Venzon Antunes. -- 2014.
157 f.

Orientador: Gilberto Schwartsmann.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Medicina: Ciências Médicas, Porto Alegre, BR-RS, 2014.

1. tamoxifeno. 2. CYP2D6. 3. CYP3A4. 4. endoxifeno. 5. dried blood spots. I. Schwartsmann, Gilberto, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

AGRADECIMENTOS

Ao Dr. Gilberto Schwartsmann, por oportunizar a realização desta pesquisa, pelo entusiasmo e confiança depositada em meu trabalho.

Ao Dr. Rafael Linden pelas inúmeras contribuições para a execução e qualificação do estudo.

Aos colegas Belgas Dr. Pierre Wallemacq e Dr. Vincent Haufroid da "Université Catholique de Louvain", pela parceria e valiosas contribuições científicas.

A todos os colaboradores do estudo, Dra. Daniela Dornelles Rosa, Dr. José Antônio Cavalheiro, Dr. Jorge Villanova Biazús, bolsistas, funcionários do Centro de Pesquisa Clínica e Laboratório de Análises Toxicológicas Feevale. Este trabalho é resultado da parceria, apoio e dedicação de todos.

À Universidade Feevale e ao Hospital de Clínicas de Porto Alegre (HCPA) por propiciar as condições técnicas necessárias para a execução do estudo.

Ao Conselho Nacional Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) e ao Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE-HCPA), pelo apoio financeiro.

Agradecimento especial os meus pais, irmãs e madrinha, pelo incentivo na busca dos meus objetivos e ao Fabrício Zanella, pelo companheirismo e palavras de apoio e incentivo.

RESUMO

Introdução: a ação antiestrogênica do tamoxifeno (TAM) é dependente da bioativação à endoxifeno (EDF) e 4-hidroxitamoxifeno (HTF). É bastante provável que sua eficácia terapêutica esteja relacionada ao alcance de um limiar nos níveis de EDF (>5,9 ng mL^{-1}). Entretanto, as concentrações plasmáticas de EDF são altamente variáveis, em parte devido a polimorfismos no gene da CYP2D6 e ao uso de inibidores da enzima. A CYP3A4 também contribui para a formação do EDF e pode ser influenciada por interações medicamentosas e exposição solar. Recentemente, o polimorfismo *CYP3A4*22* foi associado à redução da atividade da enzima. Entretanto, pouco se sabe sobre seu impacto na formação do EDF.

Objetivo: Em virtude da alta variabilidade na resposta terapêutica e os múltiplos fatores associados ao metabolismo do TAM, o presente estudo objetivou avaliar o efeito dos polimorfismos da CYP2D6 e CYP3A4, interações medicamentosas e exposição à vitamina D na bioativação do TAM. Adicionalmente, dois métodos analíticos para a otimização do tratamento através da medida das razões metabólicas da CYP2D6 e quantificação do TAM e metabólitos em manchas de sangue seco (*DBS*) foram desenvolvidos.

Patientes & métodos: Cento e dezesseis pacientes em tratamento adjuvante com o TAM forneceram amostras de plasma para dosagens do TAM, metabótilos e $25OHD_3$ no inverno e verão. As concentrações de TAM e metabólitos em plasma e DBS foram medidas por LC-MS/MS. Foram avaliados os genótipos da CYP2D6 e CYP3A4, bem como fenótipos obtidos pelas razões metabólicas determinadas após administração dos fármacos sonda dextrometorfano e omeprazol. As concentrações de vitamina D_3 em plasma foram quantificadas por HPLC-UV. Foram obtidas informações sobre uso de inibidores ou indutores das enzimas e suplementação de vitamina D.

Resultados: Cerca de 20% das pacientes apresentaram atividade metabólica reduzida para a CYP2D6 e 7% para a CYP3A4. Aproximadamente 30% das metabolizadoras lentas (ML), 56% das metabolizadoras intermediárias (MI) e 11.3% das metabolizadoras rápidas (MR) usavam fármaco inibidor da CYP2D6. As concentrações de EDF diminuiram proporcionalmente à redução da atividade metabólica da CYP2D6 (ML 2,79 ng mL^{-1}, MI 5.36 ng mL^{-1} e MR 10,65 ng

mL^{-1}, P<0.01). A mediana das concentrações plasmáticas de TAM e HTF em pacientes CYP2D6 MI com metabolismo reduzido da CYP3A4 (161,50 ng mL^{-1} e 1,32 ng mL^{-1}, respectivamente) foram superiores as encontradas nos pacientes CYP2D6 MI com metabolismo funcional da CYP3A4 (122,07 ng mL^{-1} e 0.61 ng mL^{-1}, respectivamente, P<0.05). Adicionalmente, as concentrações de HTF e TAM foram aproximadamente 50% superiores em pacientes com genótipo *CYP3A4*22* em comparação aos pacientes **1/*1*. A sazonalidade também contribuiu para a variabilidade das concentrações dos metabólitos ativos, os níveis de EDF foram 24% e HTF 42% superiores no verão. Nas análises de DBS, foi possível identificar 96% dos pacientes com concentrações de EDF abaixo do limiar clínico, indicando seu potencial uso no monitoramento terapêutico do TAM.

Conclusão: a CYP3A4 contribui para a bioativação do TAM através da formação de HTF, tornando-se mais importante em condições de atividade diminuída ou ausente da CYP2D6. Os níveis plasmáticos de EDF e HTF demonstraram ser influenciados pela sazonalidade, com aumento significativo no verão. Entretanto o mecanismo relacionado a associação da vitamina D, exposição solar e bioativação do TAM permanecem por ser elucidados.

Palavras-chave: Tamoxifeno; endoxifen; 4-hidroxitamoxifeno; CYP2D6, CYP3A4, DBS, vitamina D.

ABSTRACT

Background: The therapeutic antiestrogenic effect of tamoxifen (TAM) requires metabolic activation to endoxifen (EDF) and 4-hydroxytamoxifen (HTF). Adequate therapeutic outcome seems to be dependent on the achievement of a threshold of EDF concentration (>5.9 ng mL^{-1}). EDF plasma levels are highly variable among patients, which could be partly explained by polymorphisms in the *CYP2D6* gene and the use of enzymes inhibitor drugs. In a lesser extent, CYP3A4 also contributes to EDF formation and can be influenced by drug interactions and sun exposure. From a genetic point of view, a recently described *CYP3A4*22* polymorphism has been associated with reduced enzyme activity. However, there is little knowledge about the impact of *CYP3A4* polymorphisms on EDF formation.

Objective: In view of the large variability on therapeutic response and the multiple factors associated to TAM metabolic activation, the present study aimed to evaluate the effect of CYP2D6 and CYP3A4 polymorphisms, drug interactions and vitamin D exposure on TAM metabolic activation. Additionally, two analytical methods for optimization of TAM treatment by measurement of CYP2D6 metabolic ratios and quantification of TAM and metabolites in dried blood sopts (DBS) were developed.

Patients & methods: One hundred and sixteen patients under TAM therapy provided blood samples for measurement of TAM, NDT, EDF, HTF and $25OHD_3$ at Winter and Summer. TAM and metabolites were measured in plasma and DBS by LC-MS/MS. CYP2D6 and CYP3A4 genotypes and phenotypes, given according to [DMT]/[DTP] and [OME]/[OMS] metabolic ratios after administration of probe drugs, were also evaluated. Vitamine D_3 was measured in plasma by HPLC-UV. Data on use of CYP2D6 and CYP3A4 inhibitor or inducer drugs and vitamin D supplementation were recorded.

Results: About 20% of patients had reduced CYP2D6 metabolic activity and 7% CYP3A4 impaired metabolism. Approximately 30% of CYP2D6 poor metabolizers (PM), 56% of intermediate metabolizers (IM) and 11.3% of extensive metabolizers (EM) were using CYP2D6 inhibitor drugs. EDF levels diminished proportionally to the reduction of CYP2D6 metabolic activity (PM 2.79 ng mL^{-1}, IM 5.36 ng mL^{-1} and EM 10.65 ng mL^{-1}, $P<0.01$). Median plasma

levels of TAM (161.50 ng mL^{-1}) and HTF (1.32 ng mL^{-1}) in CYP2D6 IM patients with redu
CYP3A4 metabolism were higher ($P<0.05$) than those from CYP2D6 IM patients with functio
CYP3A4 metabolism (122.07 ng mL^{-1} and 0.61 ng mL^{-1}, respectively). Indeed, HTF and TAM plasma levels were approximately 50% higher in patients with *CYP3A4*22* genotype compared to patients with alleles **1/*1*. Seasonality also contributed to EDF and HTF variability, summer concentrations were 24% and 42% higher compared to winter. The DBS method was able to identify 96% of patients with plasma EDF concentrations below the clinical threshold and can be used in therapeutic monitoring of TAM.

Conclusion: Our findings suggest that CYP3A4 contributes to the bioactivation of TAM through formation of HTF and becomes increasingly important in conditions of diminished or absent CYP2D6 activity. A significant variability on EDF and HTF exposure related to seasonality was identified, with considerable higher plasma concentrations during summer. The mechanism relating vitamin D status, seasonality and biotransformation of TAM still remains to be elucidated.

Key-words: Tamoxifen; endoxifen; 4-hydroxytamoxifen; CYP2D6, CYP3A4, DBS, vitamin D.

LISTA DE FIGURAS

Revisão da Literatura

Artigo 1.

Artigo 2.

Artigo 3.

Artigo 4.

LISTA DE TABELAS

Revisão da Literatura

Artigo 1.

Artigo 2.

Artigo 3.

Artigo 4.

LISTA DE ABREVIATURAS E SIGLAS

ATLAS – Adjuvant Tamoxifen Longer Against Shorter

ATAC: *Tamoxifen, Alone or in Combination*

BIG: *Breast International Group*

DBS: *Dried Blood Spots* (manchas de sangue seco em papel)

DMT: Dextrometorfano

DTF: Dextrorfano

EDF: Endoxifeno

HTF: 4-Hidroxitamoxifeno

GnRH: *Gonadotropin-Releasing Hormone* (Hormônio liberador de gonadotrofina)

HPLC-FL: *High Performance Liquid Chromatography with Fluorescence Detector* (Cromatografia líquida de alta eficiência com detecção de fluorescência)

IARC: *International Agency for Research on Cancer*

INCA: Instituto Nacional de Câncer

LC-MS/MS: *Liquid Chromatography associated to Mass Espectrometry in Tandem* (Cromatografia Líquida Associada à Espectrometria de Massas Sequencial)

LILACS: Literatura Latino-americana e do Caribe em Ciências da Saúde

ML: Metabolizadores Lentos

MI: Metabolizadores Intermediários

MR: Metabolizador Rápido

MR-R: Metabolizador Rápido com Atividade Rápida

MR-D: Metabolizador Rápido com Atividade Diminuída

NCCN: *National Comprehensive Cancer Network*

NDT: N-Desmetil-tamoxifeno

$25OHD_3$: 25-Hidroxivitamina D_3

$1\alpha,25(OH)_2D_3$: 1α,25-dihidroxivitamina D_3

OME: Omperazol

OMS: Omeprazol sulfona

RE: Receptor de Estrogênio

RP: Receptor de Progesterona

SciELO: *Scientific Eletronic Library Online*

TAM: Tamoxifeno

TGFβ: Fator Transformador de Crescimento Beta

UR: Metabolizador Ultra-rápido

UVB: ultravioleta B

Artigos

BMI: Body Mass Index

DBS: Dried Blood Spots

DMT: Dextrometorphan

DOF: Decrease of Function

DTP: Dextrophan

EDF: Endoxifen

EPC: Estimated Plasma Concentration

HPLC-DAD: High Performance Liquid Chromatography with Diode Array Detection

HPLC-FL: high performance Liquid Chromatography with fluorescence detector

HTF: 4-Hydroxytamoxifen

IM: Intermediate Metabolizer

IQR: interquartile range

LC-MS/MS: Liquid Chromatography associated to Mass Spectrometry in *tanden*

LOF: Loss of Function

EM: Extensive Metabolizer

MPC: Measured Plasma Concentration

NCCN: National Comprehensive Cancer Network

NDT: N-desmethyltamoxifen

NF: Normal Function

$25OHD_3$: 25-Hydroxyvitamin D_3

$1\alpha,25(OH)_2D_3$: 1α,25-dihydroxyvitamin D_3

OME: Omeprazole

OMS: Omeprazole Sulphone

PM: Poor Metabolizer

RSD: Relative Standard Deviation

TAM: Tamoxifen

UM: Ultra Rapid Metabolizer

VDR: Vitamin D Receptor

SUMÁRIO

1. INTRODUÇÃO

O tamoxifeno (TAM) é um fármaco amplamente utilizado no tratamento adjuvante do câncer de mama, com impacto significativo na sobrevida das pacientes [1]. Através do metabolismo de enzimas do sistema citocromo P450, em especial a CYP2D6, o TAM é biotransformado aos metabólitos ativos endoxifeno (EDF) e 4-hidroxitamoxifeno (HTF) [2].

Apesar do benefício clínico do TAM no tratamento do câncer de mama, existe uma substancial variabilidade interpaciente no desenvolvimento de resistência ao tratamento na ocorrência de efeitos adversos [1]. É bastante provável que a eficácia terapêutica do TAM esteja relacionada ao alcance de um limiar nos níveis plasmáticos de seu metabólito ativo, o EDF [3]. A importância da CYP2D6 na biotransformação do TAM à EDF, bem como do impacto dos polimorfismos e do uso de fármacos inibidores da enzima sobre sua atividade tem sido amplamente discutidos na literatura [4]. As pacientes com atividade prejudicada da CYP2D6 apresentam níveis de EDF 3 a 4 vezes inferiores aos encontrados nas pacientes com atividade enzimática completa [5]. Entretanto, algumas pacientes com genótipo funcional da CYP2D6 também apresentam concentrações reduzidas de EDF, possivelmente em virtude de fatores ambientais moduladores da enzima ou, ainda, serem resultados polimorfismos em outros genes [6,7].

Apesar da estreita relação entre o TAM e a atividade da CYP2D6, esta via metabólica é capaz de predizer somente cerca de 40% dos níveis de EDF [8]. Recentemente, sugeriu-se que o papel da enzima CYP3A4 na formação do EDF pode estar sendo subestimado. O polimorfismo no gene da *CYP3A4*22* foi relacionado à redução na atividade hepática da enzima [9]. Entretanto, são escassas as informações acerca do papel da *CYP3A4*22* sobre a ativação metabólica do TAM, especialmente quando associada à CYP2D6. Até o momento, apenas dois estudos avaliaram o papel da enzima na formação do EDF, porém com dados conflitantes [10;11]. Teft *et al*. (2013) identificaram um aumento significativo nas concentrações de EDF nas pacientes com o genótipo *CYP3A4*22* [10], enquanto Heine *et al.* (2014), em uma avaliação fenotípica da via demonstraram níveis reduzidos de EDF em pacientes com metabolismo diminuído da CYP3A4 [11].

Fatores ambientais como a presença de doença hepática, interações medicamentosas [12], bem como a exposição solar e níveis de vitamina D também podem influenciar a atividade da CYP3A4 [13]. Apesar da indução da vitamina D sobre o metabolismo da CYP3A4 intestinal estar relacionada à variação cíclica nas concentrações de alguns substratos da enzima, como o tacrolimus, são escassos os estudos associando a exposição à vitamina D e a sazonalidade ao metabolismo do TAM.

Atualmente, a genotipagem da CYP2D6 buscando otimizar o tratamento hormonal adjuvante não é indicada na prática clínica. Entretanto, é recomendado evitar o uso concomitante de fármacos inibidores da enzima [14]. Não existe recomendação com relação a avaliação genética da CYP3A4, nem tampouco sugestão de limitação do uso concomitante de fármacos moduladores da enzima, possivelmente em virtude do pequeno conhecimento com respeito ao impacto da enzima na bioativação do TAM. O efeito combinado de fatores genéticos e ambientais na atividade da CYP2D6 e CYP3A4 pode ser avaliado através de estratégias de fenotipagem. A estimativa da razão metabólica de um fármaco sonda pode ser uma ferramenta útil para identificar pacientes que provavelmente terão pior resposta ao tratamento [11].

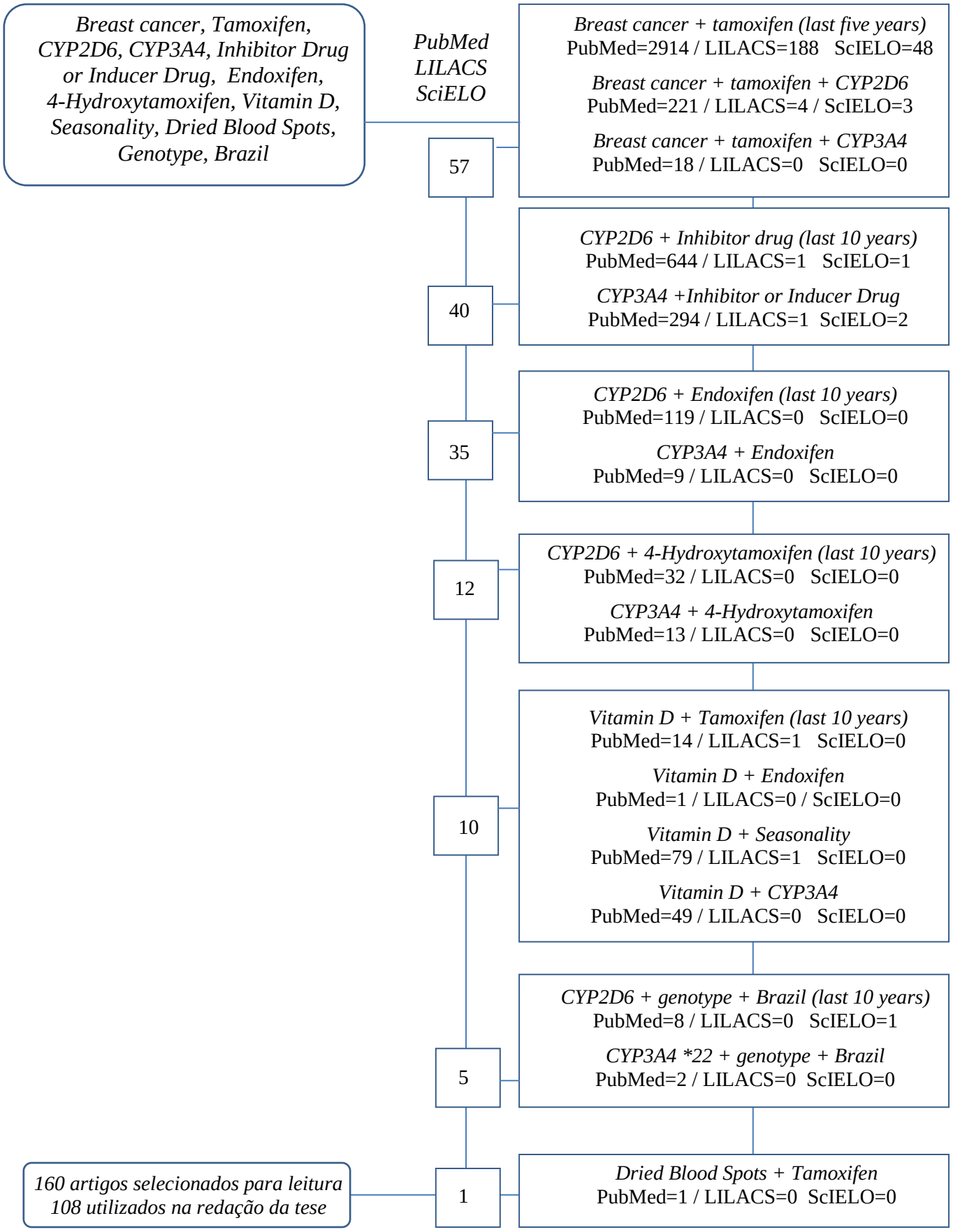
Considerando a larga utilização do tamoxifeno no tratamento do câncer de mama e a ampla variabilidade interindividual na sua bioativação e resposta terapêutica, faz-se de grande relevância clínica a avaliação farmacogenética do seu metabolismo. Desta forma, o propósito deste estudo foi avaliar a influência de fatores genéticos e ambientais na atividade metabólica da CYP2D6 e CYP3A4 e sua relação com a bioativação do tamoxifeno em pacientes com câncer de mama.

Este é o primeiro estudo a avaliar o papel da CYP2D6 e CYP3A4 na bioativação do TAM, através de estratégias de genotipagem e fenotipagem, considerando também o efeito da sazonalidade e vitamina D sobre a formação do EDF e HTF. Na pesquisa foi proposto também o desenvolvimento e validação de um método de amostragem de sangue seco em papel como uma abordagem atrativa, flexível e de fácil coleta para monitorar as concentrações de tamoxifeno e seus principais metabólitos.

2. REVISÃO DA LITERATURA

Para a realização da revisão bibliográfica, buscamos ressaltar os principais aspectos relacionados à terapia com o tamoxifeno e a sua bioativação através do metabolismo da CYP2D6 e CYP3A4. Também foram investigadas as associações do uso de inibidores, sazonalidade e vitamina D sobre o tamoxifeno e seus metabólitos ativos, bem como informação sobre disponibilidade de método para a dosagem do tamoxifeno e metabólitos em amostra de sangue seco em papel (*Dried Blood Spots- DBS*). Foram consultadas as bases de dados do MEDLINE/PubMed (US National Library of Medicine), LILACS (Literatura Latino-americana e do Caribe em Ciências da Saúde) *e* Scientific Eletronic Library Online (ScIELO) com publicações datadas de 2004 a 2014. Foram ainda rastreadas informações nas páginas eletrônicas da Organização Mundial da Saúde e do Instituto Nacional do Câncer (INCA). Além disso, os artigos selecionados serviram de base para localizar outros estudos não contemplados nesta busca. As palavras-chave empregadas foram: *breast cancer, tamoxifen, CYP2D6, CYP3A4, genotype, Brazil, endoxifen, vitamin D, seasonality, inhibitor drug, inductor drug, dried blood spots*, em diversas combinações, buscando a descrição dos tópicos abordados nesta tese. Os artigos que não contemplaram os critérios de cruzamento das palavras-chave ou que não estavam especificamente relacionados com o tema abordado foram excluídos. No total, após a leitura dos títulos e priorizando as publicações mais recentes, foram selecionados 160 artigos para leitura. Foram utilizados 108 artigos para a descrição individual dos temas abordados na revisão da literatura e nos artigos produzidos. A figura 1 mostra o fluxograma do cruzamento das palavras-chave nas bases de dados.

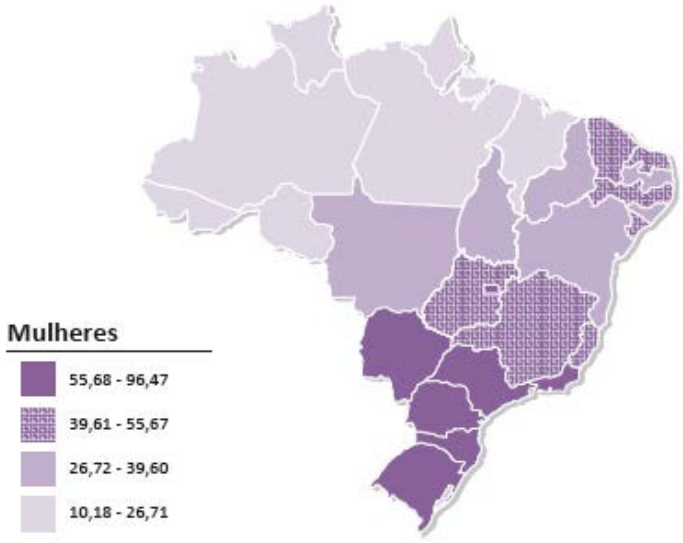
Figura 1. Fluxograma do cruzamento das palavras-chave nas bases de dados



2.1 Câncer de mama

Com exceção do câncer de pele não melanoma, o câncer de mama é a neoplasia mais comum entre as mulheres. Segundo a Agência Internacional de Pesquisa em Câncer (*IARC*), no ano de 2012 foram diagnosticados 1,7 milhões de casos no mundo [15]. No Brasil, estima-se uma incidência de 57 mil casos para o ano de 2014, com taxa bruta prevista para o estado do Rio Grande do Sul de 87,7 casos a cada 100 mil mulheres. A figura 2 apresenta as taxas brutas por 100 mil mulheres estimadas para o ano de 2014 no Brasil [16].

Figura 2. Representação das taxas brutas de incidência de neoplasia maligna da mama por 100 mil mulheres no Brasil, estimadas para 2014.



Fonte: Brasil, Ministério da Saúde, Instituto Nacional do Câncer (2014) [16]

Acredita-se que a maior parte dos casos de câncer de mama (90 a 95%) sejam esporádicos (não familiares) e decorram de mutações somáticas que ocorrem durante a vida. Dentre os fatores reprodutivos/hormonais relacionados a tais mutações incluem-se: idade precoce de menarca, menopausa tardia, nuliparidade, idade avançada na primeira gestação, uso

prolongado de anticoncepcionais orais e uso de terapia de reposição hormonal na pós-menopausa [17]. Entre os fatores de risco para o câncer de mama relacionados ao estilo de vida destacam-se dieta, obesidade, nível de atividade física, consumo de álcool e tabagismo. Com relação ao risco ambiental, são importantes as exposições a compostos químicos sintéticos, radiação ionizante, campos eletromagnéticos e pesticidas organoclorados, entre outros [18].

Apesar de ser considerado um câncer de relativamente bom prognóstico se diagnosticado e tratado oportunamente, as taxas de mortalidade por câncer da mama continuam elevadas no Brasil, muito provavelmente porque a doença ainda é diagnosticada em estádios avançados. A sobrevida média após cinco anos na população de países desenvolvidos tem apresentado um discreto aumento, cerca de 85%. Entretanto, nos países em desenvolvimento, a sobrevida é próxima de 60% [16].

A abordagem terapêutica do câncer de mama envolve tratamento loco-regional com cirurgia e/ou radioterapia e tratamento adjuvante sistêmico, que inclui a quimioterapia, os anticorpos monoclonais, os inibidores de tirosino-quinase e a hormonioterapia. Geralmente, os tratamentos propostos associam duas ou mais abordagens, com estratégia de tratamento dependendo diretamente dos seguintes fatores: estádio da doença e grau do tumor, idade, condição geral da saúde da paciente, presença de mutações conhecidas nos genes para o câncer de mama, *status* pré ou pós-menopáusico e a presença de receptores de estrogênio (RE) ou receptores de progesterona no tumor (RP) [19;20].

A inclusão da terapia endócrina adjuvante é recomendada para as pacientes cujos tumores mostram evidência de resposta endócrina, definida como a presença de qualquer RE ou RP detectável. A maior parte dos tumores malignos invasores de mama apresenta células com receptores de estrogênio (RE 75%), sendo que destes, 65% também possuem receptores de progesterona (RP). Assim como os RE, os RP também são ativados pelo estrogênio e pacientes com câncer de mama com a presença destes receptores podem se beneficiar do tratamento hormonal antiestrogênico [21].

A realização do tratamento hormonal oferece grande benefício terapêutico, reduzindo as taxas de mortalidade e recorrência da doença [19;22;23]. A indicação do tratamento difere entre mulheres na pré e pós-menopausa. Para pacientes na pré-menopausa, o uso de 5 anos de TAM é a

primeira opção de bloqueio hormonal. Pode-se também realizar a supressão da função ovariana com ooforectomia ou bloqueio do eixo hipotálamo-hipofise-gonadas, com inibidores do hormônio liberador de gonadotrofina (GnRH). No entanto, a supressão ou ablação do ovário deve ser considerada somente em circunstâncias extraordinárias [19] e o uso de inibidores GnRh por 2 anos geralmente e uma opção para mulheres que pretendem engravidar logo.

Com relação às pacientes na pós-menopausa, além do TAM pode ser considerada a indicação de fármacos inibidores da aromatase. Apesar da terapia com os inibidores da aromatase estar associada a um percentual de sobrevida livre da doença 3 a 5 % superior em relação ao TAM, este continua sendo um fármaco de alto valor terapêutico. Algumas indicações permanecem exclusivas para ele, como em mulheres na pré-menopausa e no câncer em homens, assim como na terapia profilática [24]. Além disso, o TAM apresenta efeitos secundários benéficos, como a melhora no perfil lipídico e o aumento da densidade óssea em mulheres na pós-menopausa [25], com custo consideravelmente inferior aos inibidores da aromatase.

2.2 Tamoxifeno

Há mais de 30 anos o TAM é considerado a principal terapia endócrina adjuvante para o tratamento do câncer de mama. O tratamento durante 5 anos após cirurgia definitiva reduz as taxas de recorrência e de mortalidade da doença em 41 % e 34 % respectivamente, traduzindo-se em uma redução absoluta de 9,2% na mortalidade em 15 anos pelo câncer de mama [26]. Recentemente, foi publicado o estudo *Longer Against Shorter* (*ATLAS*), que avaliou os resultados alcançados com o tratamento por 5 anos *versus* 10 anos (N=6.848). O uso prolongado do TAM demonstrou ganho aproximado de 3% nos benefícios em relação aos 5 anos de terapia, com taxas inferiores de recorrência (21,4% *versus* 25,1%) e de mortalidade (12% *versus* 15%) [27].

2.2.1 Mecanismo de ação e farmacocinética

O TAM ((Z)-2-[4-(1,2-difenilbut-1-enil)fenoxi]-N,N-dimetil-etanamina) pertence a classe de medicamentos conhecidos como moduladores seletivos dos receptores de estrogênio, apresentando ação antiestrogênica ou pró-estrogênica, dependendo do tecido alvo. No tecido mamário apresenta forte ação antagonista, mediada pela ativação da via de transdução de sinal antiproliferativa do fator de transformação de crescimento beta (TGF β) [28]. Na sua ausência, o

estrogênio estimularia o crescimento das células RE-positivas do tecido mamário maligno, através da ligação com seu sítio no receptor e da interação com o DNA da célula, alterando a atividade do gene e promovendo o crescimento celular [29]. Já no tecido ósseo, o TAM age como pró-estrogênico, protegendo contra a perda óssea em pacientes na pós-menopausa, bem como no epitélio uterino, porém relacionado ao desenvolvimento do câncer de endométrio, o que atualmente gera controvérsia com relação à segurança do uso para a prevenção do câncer de mama [24].

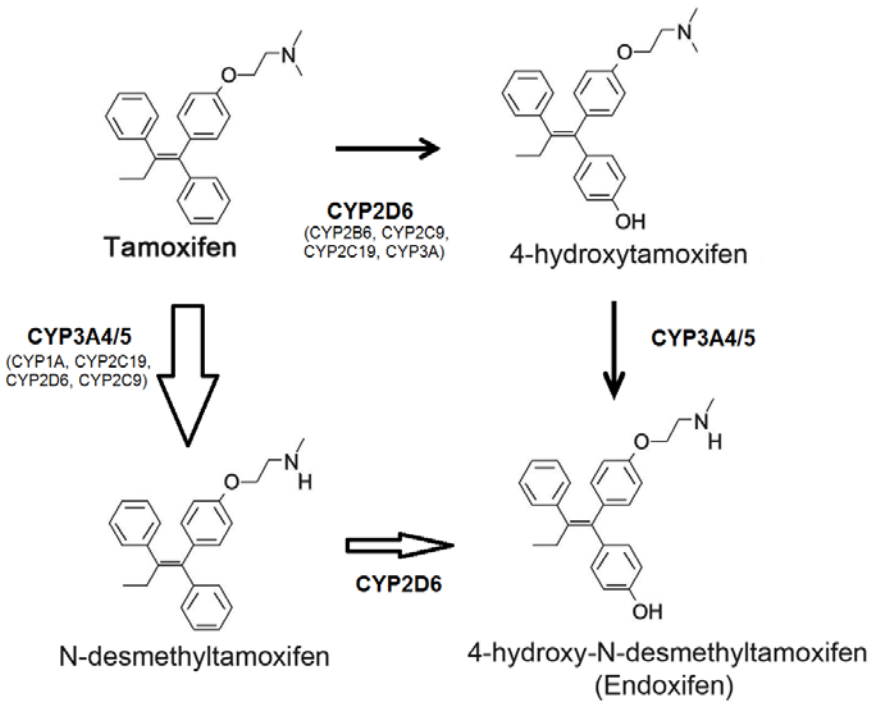
O TAM é apresentado na forma de comprimidos como citrato de tamoxifeno, com dose diária recomendada de 20 mg. Apresenta alta ligação a proteínas plasmáticas (98%) e após a administração oral, atinge concentração sérica máxima em torno de 4 a 7 horas, com meia-vida de sete dias. Devido a longa meia-vida dos metabólitos, que pode chegar a ser de 14 dias, com uma dose típica de 20 mg de TAM o equilíbrio dinâmico é alcançado no período de três a quatro meses. A excreção ocorre predominantemente nas fezes, sendo a excreção urinária mínima [30].

O TAM é extensivamente metabolizado por enzimas hepáticas do sistema citocromo P450 a muitos metabólitos primários e secundários. Alguns metabólitos do TAM formados *in vivo* são mais ativos que o próprio fármaco, motivo pelo qual ele pode ser considerado um pró-fármaco [31]. Na figura 3 são apresentadas as vias de maior relevância para a bioativação do TAM. A formação do metabólito N-desmetiltamoxifeno (NDT) é catalisada pela CYP3A4, com menor contribuição das CYPs 2D6, 1A, 1A2, 2C19 e 2B6 [1]. Apesar das altas concentrações plasmáticas do NDT, aproximadamente o dobro das encontradas para o TAM [5], este metabólito não exerce efeito terapêutico significativo. Posteriormente, via CYP2D6, o NDT sofre 4-hidroxilação e é convertido a 4-hidroxi-N-desmetiltamoxifeno, ou (Z)-endoxifeno (EDF). A CYP2D6 também catalisa o metabolismo do TAM à 4-hidroxitamoxifeno (HTF), com menor contribuição das CYPs 3A4, 2B6, 2C19 e 2C9. O HTF é posteriormente convertido a EDF pela ação da CYP3A4 [2].

A eficácia da terapia hormonal é atribuída aos metabólitos hidroxilados, particularmente ao EDF. Inicialmente, identificou-se a capacidade do HTF em suprimir a proliferação das células estrógeno-dependentes, em cerca de 100 vezes à apresentada pelo TAM [32]. Entretanto, anos depois foi demonstrado que o EDF possui propriedades idênticas com respeito à afinidade pelos

receptores e ação antiproliferativa, com concentrações plasmáticas em equilíbrio dinâmico 6 a 8 vezes maiores que as do HTF [5;7;33].

Figura 3. Principais vias envolvidas na ativação metabólica do tamoxifeno.



2.2.2 Efeitos adversos

O tratamento com o TAM está associado a efeitos secundários benéficos, como a melhora no perfil lipídico e o aumento da densidade óssea em mulheres na pós-menopausa. Entretanto, até 25 % das pacientes apresentam efeitos adversos. Com maior destaque estão náuseas, vômitos e o aumento das “ondas de calor” (“fogachos”), sintoma este relacionado ao climatério, com risco 2 a 3 vezes maior em comparação às mulheres que não usam TAM. Outros efeitos adversos com menor frequência são: irregularidade menstrual, sangramento vaginal, prurido vulvar, dermatite, hipercalcemia, edema periférico, anorexia, depressão, trombocitopenia, leucopenia, cefaléia, doença tromboembólica, retinopatia, carcinoma hepático e carcinoma de endométrio [30].

Para amenizar os “fogachos”, são comumente prescritos antidepressivos inibidores seletivos da recaptação da serotonina (ex. fluoxetina, paroxetina, citalopram e clomipramina) [34;35]. Entretanto, estes fármacos possuem ação inibitória sobre a CYP2D6 e podem promover a redução do metabolismo do TAM, diminuindo a formação dos seus metabolitos ativos [36; 37].

A associação da terapia com TAM a efeitos colaterais indesejáveis em algumas mulheres ainda não está esclarecida, parecendo estar relacionado ao metabolismo do TAM e níveis de EDF, sendo dose/concentração-dependentes [38]. Além disso, é importante ressaltar que a presença constante de efeitos adversos é um fator de resistência à adesão ao tratamento hormonal, podendo levar à ineficácia da terapia [39;40]. Estudos apontam a redução da adesão ao TAM com o passar dos anos, variando de 87 % no primeiro ano para até 50 % no quarto ano de tratamento [41].

2.3 Farmacogenética na terapia com tamoxifeno

Diversos fatores podem afetar a eficácia e segurança de um fármaco, incluindo a idade do paciente, gênero, genética e fatores ambientais. A farmacogenética avalia a influência dos fatores genéticos relacionados à farmacocinética e farmacodinâmica dos fármacos e objetiva, através do estabelecimento de terapia individualizada, o alcance da resposta adequada em um tratamento farmacológico [21].

Apesar do benefício da hormonioterapia ser evidente, até 50% dos pacientes recebendo tratamento adjuvante com o TAM recidivam ou morrem [1]. Em um estudo com 1.380 pacientes, Madlensky *et al.* (2011) exploraram a associação entre o desfecho no tratamento do câncer de mama e as concentrações do TAM e metabólitos. Os autores observaram não haver associação entre os níveis de TAM, HTF, NDT e o desfecho clínico, porém sugerem ser bastante provável que o alcance do resultado terapêutico depende da exposição ao EDF. Neste estudo, as pacientes que apresentam concentrações plasmáticas em vale do EDF acima de 5,9 ng mL^{-1} apresentaram redução de 26% nas taxas de recorrência em comparação ao grupo com níveis inferiores ao proposto limiar [3].

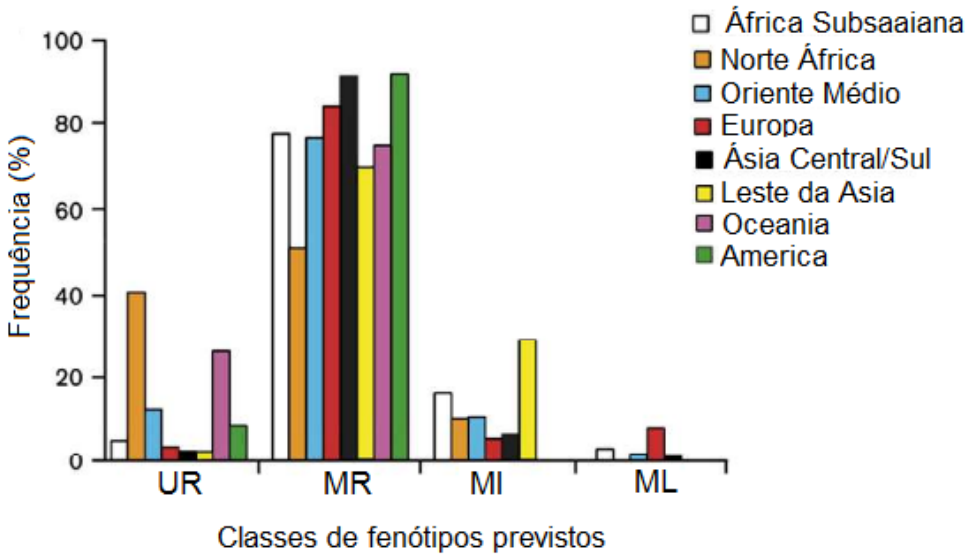
As concentrações plasmáticas de EDF variam amplamente entre os pacientes, o que pode ser em parte explicado por polimorfismos no gene da CYP2D6 e pelas interações da CYP2D6

com agentes moduladores, levando a concentrações plasmáticas insuficientes de EDF em muitos indivíduos [33]. São conhecidos mais de 150 alelos para o gene da *CYP2D6*; o *1 é o alelo selvagem, que codifica a enzima funcional completa, enquanto os alelos **2, *33 e *35* contêm algunas mutações de ponto que não afetam as propriedades catalíticas da enzima. Alguns dos alelos associados à ausência de atividade enzimática são: **3-*8, *11-*16, *18-*20, *38, *40, *42, *44*; os alelos relacionados à atividade enzimática reduzida são: **9, *10, *17, *29, *36, *37, *41* [42].

O genótipo da *CYP2D6* pode ser traduzido em quatro fenótipos distintos: metabolizadores lentos (ML) que não possuem enzima funcional (presença de dois alelos nulos), intermediários (MI) que possuem atividade enzimática residual (presença heterozigótica de alelo com atividade reduzida e nulo ou homozigótica com alelos de atividade reduzida), metabolizador rápido com atividade enzimática normal (presença de pelo o menos 1 alelo funcional) e ultra-rápidos (UR) com elevada atividade enzimática (presença de multiplicação gênica) [24;43]. O fenótipo previsto para o grupo de MR pode ainda ser dividido em dois grupos, os metabolizadores rápidos com atividade rápida (MR-R) (2 alelos funcionais) ou metabolizadores rápidos com atividade diminuída (MR-D) (1 alelo funcional em combinação com alelo de atividade reduzida ou nula) [24]. Os MR-D possuem expressão da enzima pouco prejudicada, porém não é correto afirmar que possam ser classificados como MI [1].

A distribuição dos alelos e genótipos da *CYP2D6* varia de acordo com as etnias. Na figura 4 são apresentadas as frequências dos genótipos da *CYP2D6* em diferentes populações. Estudos indicam que cerca de 7 a 11% dos indivíduos caucasianos (10), 1% dos chineses, coreanos e japoneses, 1 a 19% das populações negras são ML [36]. No Brasil, os poucos trabalhos publicados indicam prevalência de 3 a 5 % de ML e 4 a 12% de MI [44;-46].

Figura 4. Distribuição das frequências dos fenótipos previstos da CYP2D6



Fonte: Adaptado de Ingelman-Sundberg *et al.* (2007)

Schroth *et al.* (2009) em um estudo retrospectivo com 1.325 pacientes, evidenciaram uma forte associação entre os polimorfismos da *CYP2D6* e o desfecho clínico não favorável ao tratamento adjuvante do câncer de mama com TAM. Neste estudo, as pacientes ML apresentaram percentual significativamente reduzido de sobrevida livre da doença em comparação aos MR (29 % contra 14,9 % P=0,005) [4]. Semelhantemente, nosso grupo encontrou em um estudo anterior com mulheres do Sul do Brasil (N=97) níveis reduzidos de EDF em pacientes ML (2,52 ng mL^{-1}) e MI (4,17 ng mL^{-1}) em comparação às MR-R (6,90 ng mL^{-1}) [5].

Apesar das fortes evidências relacionando o papel da *CYP2D6* na terapia com o TAM, estudos sugerem que outros fatores ainda não identificados podem estar envolvidos com a ativação do TAM à EDF, visto que a CYP2D6 é capaz de predição somente parte da variabilidade dos níveis de EDF (28-38%) [8]. Em menor participação (20 a 30%), a CYP3A4/5 também está relacionada a formação do EDF pela biotransformação do HTF [7]. Assim como a CYP2D6, a atividade da CYP3A4 é caracterizada por alta variabilidade interindividual (10 a 100

vezes), com uma significativa influência de fatores ambientais, como dieta, interações medicamentosas [12] e exposição solar [10], além do ponto de vista genético [9].

Ozdemir *et al.* (2000) sugerem que até 90% da variabilidade no metabolismo da CYP3A4 possa ter uma base genética. Embora diversas mutações no gene da *CYP3A4* tenham sido descritas (*2- *21), não há evidência de que poderiam explicar a ampla variação interindividual na atividade da enzima, possivelmente em virtude de seus efeitos limitados ou ainda em virtude de suas frequências consideravelmente baixas (<0.1%) [47]. Entretanto, no ano de 2011, um promissor biomarcador da atividade hepática da CYP3A4 foi identificado. O polimorfismo no gene *CYP3A4*22* está associado à redução da expressão de mRNA e diminuição da atividade da enzima. A sua frequência e como consequência, de metabolizadores lentos para a CYP3A4 na população caucasiana é de aproximadamente 5 a 7% [9]. Até o presente momento, apenas um estudo retratou a prevalência de indivíduos carreadores do alelo **22* na população brasileira, sendo esta de 6% [48].

Pouco se sabe sobre o impacto dos polimorfismos da *CYP3A4* na formação do EDF. Recentemente, Teft *et al.* (2013), encontraram concentrações superiores de TAM e EDF em indivíduos portadores do alelo *CYP3A4*22*, com risco 2 vezes menor de apresentarem concentrações subterapêuticas de EDF [10]. Heine *et al.* (2014), demonstraram que um modelo baseado na avaliação da conjunta do metabolismo do TAM mediado pela CYP2D6 e CYP3A4 foi capaz de predizer 54% da variabilidade das concentrações plasmáticas de EDF [11]. Contrariamente ao exposto no estudo de Teft *el al*. (2013), os autores indicaram a inibição da CYP3A4 pode resultar em diminuição da eficácia do TAM em virtude de redução na formação do metabólito ativo EDF [10].

2.4 Fatores ambientais na bioativação do tamoxifeno

O metabolismo da CYP2D6 também pode ser afetado por fatores não genéticos, como a presença de disfunção hepática e, sobretudo, a inibição da atividade catalítica devido ao uso simultâneo de medicamentos inibidores da enzima [8]. Na Tabela 1 são apresentados os fármacos administrados com maior frequência durante a terapia com o TAM e a sua influência sobre a atividade da *CYP2D6.* Dentre eles, destacam-se os inibidores da recaptação de serotonina

paroxetina e fluoxetina, usualmente prescritos para reduzir os efeitos adversos resultantes da ação antiestrogênica do TAM e no tratamento da depressão [49].

Tabela 1 - Medicamentos inibidores da *CYP2D6* comumente administrados com TAM, adaptado de Stearns & Rae (2008) [49].

Ação inibitória sobre *CYP2D6*	Fármacos
Forte[a]	Paroxetina, fluoxetina, bupropiona, quinidina
Moderada	Duloxetina, tioridazina, amiodarona, difenidramina, cimetidina
Fraca	Sertralina, venlafaxina, citalopram, escitalopram

[a] Devem ser evitados por mulheres que utilizam o TAM

Tal como acontece com as variações genéticas, as interações medicamentosas também podem levar a importantes consequências clínicas. Nestes casos, é possível que um indivíduo com a presença de dois alelos funcionais expresse a atividade da enzima de forma reduzida, reduzindo a formação do seu metabólito ativo EDF [30;42]. Jin *et al*.(2005) observaram uma redução significativa dos níveis plasmáticos de EDF (n=80) nas pacientes com genótipo ML (20 nM) ou MI (43,1 nM) em comparação aos MR (78 nM) [30]. Dentre os indivíduos com genótipo MR os que utilizavam concomitantemente antidepressivos inibidores da enzima tiveram 58% de redução dos níveis de EDF em comparação aos que não utilizavam (38,6 nM contra 91,4 nM) [8].

Assim como ocorre para a CYP2D6, diversos fármacos podem alterar o metabolismo da CYP3A4. Dentre os principais fármacos inibidores incluem-se a eritromicina, itraconazol, cetoconazol, nefazodona, e troleandomicina, já entre os indutores estão carbamazepina, fenitoína, rifampicina, ritonavir. Além disso, outros fatores ambientais como a presença de doença hepática, o consumo de suco de toranja (*grapefruit*) [12], a exposição solar e níveis de vitamina D já foram citados como moduladores da CYP3A4 [13].

A exposição à vitamina D e o metabolismo da CYP3A4 foram previamente associados. A forma ativa 1α,25-dihidroxivitamina D_3 ($1\alpha,25(OH)_2D_3$) se liga ao receptor de vitamina D e aumenta a transcrição da CYP3A4 [13]. A indução da CYP3A4 intestinal afeta as concentrações e metabolismo de primeira passagem intestinal de alguns substratos. Estudos demontraram que as

concentrações sanguíneas de tacrolimus e sirolimus apresentam variação cíclica ao longo do ano, estando correlacionadas à exposição da luz ultravioleta e às concentrações plasmáticas de 25-hidroxivitamina D_3 ($25OHD_3$) [50]. São escassas as informações a respeito da associação da exposição a vitamina D e o metabolismo do TAM. Recentemente, Kim *et al.* (2014) observaram aumento nas concentrações plasmáticas de $25OHD_3$ durante tratamento com o TAM, o que foi relacionado a ação agonista do TAM nos ossos [51]. Teft *et al.* (2013), exploraram a relação entre vitamina D e a ativação metabólica do TAM através da formação do EDF, os autores observaram concentrações reduzidas de EDF durante o inverno e associaram aos baixos níveis de $25OHD_3$, entretanto sem a explicação de um mecanismo claro [10]. Adicionalmente, uma associação inversa entre níveis séricos de vitamina D e o risco de recorrência também foi demonstrado [52].

Além de sua provável relação com a terapia com o TAM, a vitamina D desempenha um papel importante em outras condições que afetam as pacientes com câncer de mama, incluindo a osteoporose, artralgia e depressão. Desta forma, Kim *et al*. (2014), sugerem que seja imperativo que níveis plasmáticos apropriados de $25OHD_3$ sejam mantidos nesta população de pacientes [51]. A maior fonte de vitamina D circulante é atribuída a produção endógena na pele exposta a radiação (280–320 nm) da luz solar ultravioleta B (UVB), que em circunstâncias usuais, contribui para mais de 90% de suas concentrações plasmáticas [53].

As concentrações séricas de Vitamina D variam conforme a região geográfica, dependendo da latitude. Apesar de não haver um consenso com relação aos níveis ideais de $25OHD_3$ a Sociedade Americana de Endocrinologia (*American Endocrine Society*) estabelece como limítrofes os valores: deficiente <20 ng mL^{-1}, insuficiente 20–29 ng mL^{-1} e suficiente ≥ 30 ng mL^{-1} [54].

A baixa exposição solar e o envelhecimento são fatores de risco para a deficiência de vitamina D [55]. O Brasil, apesar de ser considerado um país ensolarado, apresenta alta prevalência de hipovitaminose D, principalmente em idosos [56;57]. Porto Alegre é uma zona de clima subtropical, com quatro estações bem definidas, localizada a 30º Sul. As baixas temperaturas durante o inverno podem levar a hábitos que diminuem a exposição solar, incluindo o uso de maior quantidade de roupas e a redução do número de atividades realizadas em espaços abertos em contato com o sol [58].

2.5 Abordagens genotípica e fenotípica na avaliação da CYP2D6 e CYP3A4

Diversos estudos apontam para um possível benefício da genotipagem da *CYP2D6* antes do início do tratamento, de forma a auxiliar na decisão da terapia endócrina adjuvante [1;4;59;60]. Irvin *et al.* (2011) avaliaram também a viabilidade do ajuste de dose do TAM de acordo com o genótipo da *CYP2D6*. Os autores avaliaram 190 pacientes utilizando uma dose diária de 20 mg de TAM e obtiveram medianas de concentrações iniciais do EDF superiores em MR (34.3 ng ml^{-1}) em comparação com MI (18.5 ng mL^{-1}) ou ML (4.2 ng mL^{-1}) (P = 0,0045 e P < 0,001, respectivamente). Quando a dose foi aumentada para 40 mg, as concentrações de EDF aumentaram significativamente nos grupos MI e ML (P < 0,001 e P = 0,020), com alteração mediana intrapaciente de + 7,6 ng mL^{-1} (-0,6 a 23,9 ng mL^{-1}) para MIs e + 6,1 ng mL^{-1} (2,6 a 12,5 ng mL^{-1}) para ML. Após 4 meses, os níveis de EDF para pacientes MR (dose 20 mg) e MI (dose 40 mg) não apresentaram mais diferença significativa, entretanto mesmo com o aumento das concentrações de EDF em pacientes ML, ainda eram significativamente inferiores aos MI e MR [61].

Apesar de diversos estudos demonstrarem resultados positivos em relação ao papel da CYP2D6 na formação do EDF [4;5;59;61], alguns trabalhos não encontraram relação entre o status da *CYP2D6* e a resposta ao TAM. Recentemente, dados negativos do *Breast International Group* (BIG), e do *Tamoxifen, Alone or in Combination* (ATAC) sugerem que testar o genótipo da CYP2D6 não apresenta valor clínico [62,63]. Desta forma, considerando as informações conflitantes, a *National Comprehensive Cancer Network* (NCCN) atualmente não recomenda a genotipagem da CYP2D6 como teste para otimizar o tratamento endócrino adjuvante, mas indica evitar o uso concomitante dos inibidores potentes e intermediários da CYP2D6 [14].

Em virtude da escassa informação a respeito do impacto dos polimorfismos da *CYP3A4* e influências de fatores externos sobre a bioativação do TAM. Não há até o momento, indicação clínica para avaliação genotípica da *CYP3A4*, nem tampouco quanto à limitação do uso concomitante de fármacos e demais fatores ambientais relacionados a indução ou inibição da enzima.

É importante destacar que a genotipagem apresenta relevância clínica apenas quando é capaz de predizer o fenótipo [64]. Compreendem-se por fenótipo as características genéticas

observáveis, que se manifestam como resultado de fatores genéticos e ambientais [1]. O efeito combinado destes fatores pode ser medido in vivo através da avaliação da capacidade enzimática com o uso de fármacos sonda [65]. Entretanto, é importante ressaltar que a genotipagem remete a um dado para a vida toda, enquanto a fenotipagem medida através de fármacos sonda reflete um dado momentâneo.

Graan *et al.* (2011) conduziram um estudo considerando a genotipagem e a fenotipagem como preditores dos níveis de EDF em 40 pacientes. Os autores encontraram correlação significativa ($r=-0,72$ $P<0.01$) entre os níveis de EDF e as razões metabólicas do fármaco sonda utilizado na fenotipagem da CYP2D6, enquanto a genotipagem apresentou correlação inferior ($r=0.55$, $P<0.001$). Adicionalmente, 20% das pacientes que apresentaram níveis de EDF no primeiro quartil (25 % mais baixos) eram EM ou UR. Demonstrando a superioridade da fenotipagem em comparação a genotipagem para predizer os níveis de EDF. Segundo os autores, este estudo contribuiu para a personalização e otimização da terapia do câncer de mama com TAM [66].

Uma forma estabelecida para a caracterização do fenótipo da *CYP2D6* é através do seu índice de atividade após a administração de uma dose oral de 30 mg do fármaco sonda dextrometorfano (DMT). Passadas 3 horas é realizada coleta de amostra de sangue periférico e determinações plasmáticas do DMT e metabólito dextrorfano (DTF) [67]. Os fenótipos são classificados considerando os valores das razões metabólicas [DMT] / [DTF] em: ML $\geq$ 0,3; 0,3 > MI $\geq$ 0,03; 0,03 > MR $\geq$ 0,0003; UR <0,0003 [45]. Já a fenotipagem da CYP3A4 pode ser realizada através do fármaco sonda omeprazol (OME). A razão metabólica de biotransformação do OME à omeprazol sulfona (OMS) é determinada em amostra de plasma coletada 3 horas após a administração de dose oral de 20 mg do fármaco sonda. Os fenótipos são determinados de acordo com a razão metabólica log10 [OME]/[OMS] e são classificados em: metabolizador lento (ML) > 0,6; metabolizador rápido (MR) $\leq$ 0,6; metabolizador ultrarrápido (UR) $\leq$ - 0,3 [68].

2.6 Monitoramento das concentrações de EDF e novas estratégias de amostragem

A ampla variação nas concentrações plasmáticas de EDF e a reconhecida relação com os resultados do tratamento [3], além do maior conhecimento a respeito dos agentes moduladores do

metabolismo do TAM, tem despertado o interesse para estudos com ajuste individualizado da dose do TAM em indivíduos com formação insuficiente de EDF (30 ou 40 mg/dia) [69-71]. Desta forma, o monitoramento terapêutico do TAM através de seus principais metabólitos pode ser uma importante ferramenta no acompanhamento clínico e reconhecimento das pacientes elegíveis para terapias alternativas ou doses maiores do TAM.

Diversos métodos para a quantificação do TAM e metabólitos em amostras de soro ou plasma foram descritos [6;33;72;73]. Usualmente, a coleta de amostras convencionais de sangue venoso requer o deslocamento do paciente para um local adequado para a coleta em tempo farmacocineticamente apropriado para permitir a correta avaliação dos resultados [74]. A coleta de sangue capilar em papel filtro tem se destacado como uma nova opção para monitoramento terapêutico de fármacos [74-76], uma vez que os analitos apresentam alta estabilidade nas amostras de sangue seco em papel (*DBS – Dried Blood Spots*). Além disso, as amostras podem ser enviadas ao laboratório através do transporte postal convencional, uma vez que não são consideradas infectantes e possibilitam ainda que os pacientes sejam treinados para conseguirem fazer as próprias coletas, em horários flexíveis, mais adequados a sua posologia [77].

Esta promissora estratégia pode ser considerada para otimizar o tratamento do câncer de mama com o TAM. Entretanto, para a utilização do sangue seco impregnado em papel para determinação de fármacos e metabólitos, em virtude do pequeno volume de amostra é necessária a disponibilidade de métodos analíticos de elevada sensibilidade como a cromatografia líquida associada a espectrometria de massas sequencial (*LC-MS/MS*), que devem ser adequadamente desenvolvidos e validados para serem efetivamente utilizados na prática clínica rotineira. Recentemente, em julho de 2014 foi publicado o primeiro estudo demonstrando a possibilidade do uso de DBS para a dosagem do TAM e EDF [75].

3. OBJETIVOS

3.1 Objetivo geral

Avaliar a influência de fatores genéticos e ambientais na atividade metabólica da CYP2D6 e CYP3A4 e sua relação com a bioativação do tamoxifeno em pacientes com câncer de mama.

3.2 Objetivos Específicos

Artigo 1. Influence of CYP2D6 and CYP3A4 phenotypes, drug interactions and vitamin D status on tamoxifen biotransformation**.**

-Determinar os fenótipos da CYP2D6 das pacientes após dose oral de DMT e quantificação da razão metabólica [DMT]/[DTF]

-Determinar os fenótipos da CYP3A4 das pacientes após dose oral de OME e quantificação da razão metabólica [OME]/[OMS]

-Determinar os níveis plasmáticos de TAM, NDT, HTF, EDF e $25OHD_3$ das pacientes no inverno e no verão.

-Avaliar o efeito modulador da vitamina D e a sazonalidade sobre os níveis dos metabólitos ativos EDF e HTF.

-Avaliar o efeito do uso de inibidores da CYP2D6 sobre os fenótipos da enzima e níveis dos metabólitos ativos EDF e HTF.

-Associar os níveis plasmáticos de TAM, NDT, HTF, EDF e $25OHD_3$ às razões metabólicas [DMT]/[DTF] e [OME]/[OMS] e compará-los entre grupos de fenótipos da CYP3A4 e CYP2D6.

Artigo 2. *CYP3A4*22* is related to increased plasma levels of 4-hydroxytamoxifen and partially compensates for reduced CYP2D6 activation of tamoxifen.

-Determinar as frequências dos alelos da *CYP2D6* com atividade funcional **1, *2, *35*; atividade reduzida **9, *10, *17, *29, *41* e ausência de atividade **3, * 4, *5, *6, *7, *8, *11, *15,* assim como a distribuição do genótipo da enzima na população em estudo.

-Determinar dos alelos da *CYP3A4*, alelos **1* e **22*, assim como a distribuição dos genótipos

-Associar os níveis plasmáticos de TAM, NDT, HTF e EDF aos escores dos genes da *CYP2D6* e *CYP3A4* e compará-los entre os grupos de genótipo das enzimas.

-Avaliar o impacto do polimorfismo do alelo **22* da *CYP3A4* sobre a formação dos metabólitos ativos EDF e HTF, em indivíduos com atividade incompleta da CYP2D6.

Artigo 3. Development, validation and clinical application of a HPLC-FL method for CYP2D6 phenotyping in South Brazilian breast cancer patients.

-Desenvolver e validar um método para a quantificação do DMT e DTF por cromatografia líquida de alta eficiência com detector de fluorescência (CLAE-FL), aplicado à fenotipagem da CYP2D6.

-Determinar os fenótipos da CYP2D6 após a quantificação dos níveis plasmáticos de DMT e DTF em mulheres durante terapia com o TAM, após dose oral de DTF.

-Avaliar o impacto do uso de inibidores da CYP2D6 nas razões metabólicas [DMT]/[DTF].

Artigo 4. Ultra-high performance liquid chromatography tandem mass spectrometric method for the determination of tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in dried blood spots – Development, validation and clinical application during breast cancer adjuvant therapy.

-Desenvolver e validar um método para a quantificação do TAM e seus metabólitos HTF, NDT e EDF em amostras de DBS por cromatografia líquida associada à espectrometria de massas sequencial (LC-MS/MS).

-Monitorar os níveis plasmáticos de TAM, NDT, HTF e EDF nas amostras de DBS e plasma em mulheres durante terapia com o TAM.

4. REFERÊNCIAS DA REVISÃO DA LITERATURA

1. Brauch H, Mürdter TE, Eichelbaum M, Schwab, M. Pharmacogenomics of tamoxifen therapy. Clin Chem. 2009;55:1770-1782.

2. Jung JA, Lim HS. Association between CYP2D6 genotypes and the clinical outocomes of adjuvant tamoxifen for breast cancer: a meta-analysis. *Pharmacogenomics*. 2014;15:49-60.

3. Madlensky L, Natarajan L.,Tchu T, Pu M, Mortimer J, Flatt SW, Nikoloff DM, Hillman G, Fontecha MR, Lawrence HJ, Parker BA, Wu2 AHB, Pierce JP. Tamoxifen Metabolite Concentrations, *CYP2D6* Genotype, and Breast Cancer Outcomes. Clin Pharm & Therap. 2011;89:718-725.

4. Schroth W, Goetz M, Hamma U, Schmidt M, Winter, S, Fritz, P, et al. Association Between *CYP2D6* Polymorphisms and Outcomes Among Women with Early Stage Breast Cancer Treated with Tamoxifen. JAMA, 2009;302:1429-1436.

5. Antunes MV, Linden R, Santos TV, Wallemacq P, Haufroid V, Classen JF, Andreolla H, Costa N, Fontanive TO, Rosa DD. Endoxifen Levels and Its Association With CYP2D6 Genotype and Phenotype: Evaluation of a Southern Brazilian Population Under Tamoxifen Pharmacotherapy. Ther Drug Monit. 2012;34:422-431.

6. Antunes MV, Rosa DD, Viana TS, Andreolla H, Fontanive TO, Linden R. Sensitive HPLC–PDA determination of tamoxifen and its metabolites N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in human plasma, J. Pharm. Biomed. Anal. 2013;76:13– 20.

7. Mürdter, T.E.; Schroth, W.; Bacchus-Gerybadze, L.; Winter, S.; Heinkele, G.; Simon, W.; Fasching, P.A.; Fehm, T.; German Tamoxifen and AI Clinicians Group, Eichelbaum, M.; Schwab, M.; Brauch, H. Activity Levels of Tamoxifen Metabolites at the Estrogen Receptor and the Impact of Genetic Polymorphisms of Phase I and II Enzymes on Their Concentration Levels in Plasma. Clin. Pharm. Therap. 2011:1-10.

8. Borges S, Desta Z, Jin Y, Faouzi A, Robarge JD, Philip S, Nguyen A, Stearns V, Hayes D, Rae JM, Skaar TD, Flockhart DA, Li L. Composite Functional Genetic and Comedication *CYP2D6* Activity Score in Predicting Tamoxifen Drug Exposure Among Breast Cancer Patients. J Clin Pharmacol. 2010;50:450-458.

9. Wang D, Guo Y, Wrighton SA, Cooke GE, Sadee W. Intronic polymorphism in *CYP3A4* affects hepatic expression and response to statin drugs. Pharmacogenomics. 2011;11:274–286.

10. Teft WA et al. CYP3A4 and seasonal variation in vitamin D status in addition to CYP2D6 contribute to therapeutic endoxifen levelduring tamoxifen therapy. Breast Cancer Res Treat. 2013;139:95-105.

11. Heine R, Binkhorst L, de Graan AJM. Population pharmacokinetic modelling to assess the impact of CYP2D6 and CYP3A metabolic phenotypes on the pharmacokinetics of tamoxifen and endoxifen. Br. J Clin. Pharmacol. 2014;78:572–586.

12. Musshoff F, Stamer UM, Madea B. Pharmacogenetics and forensic toxicology. Forensic Sci. Int. 2010;203:53-62.

13. Wang Z, Schuetz EG, Xu Y, Thummel KE. Interplay between vitamin D and the drug metabolizing enzyme CYP3A4. J Steroid Biochem. 2013;136:54– 58.

14. Theuriault RL & Breast Cancer Panel Members. National Comprehensive Cancer Network (NCCN) United States of America, version 3.2013, p.MS-31.

15. International Agency for Research on Cancer (IARC) - World Health Organization. Latest World Cancer Statistics – Press Release N. 233. Dezembro 2013. Disponível em: http://www.iarc.fr/en/media-centre/pr/2013/pdfs/pr223_E.pdf. Acesso em outubro de 2014.

16. Brasil. Ministério da Saúde, Instituto Nacional do Câncer. [Internet]. Rio de Janeiro: INCA. Estimativa 2014: Incidência de Câncer no Brasil. Disponível em: http://www2.inca.gov.br/wps/wcm/connect/inca/portal/home. Acesso em setembro de 2014.

17. Althuis MD, Fergenbaum JH, Garcia-Closas M, Brinton LA, Madigan MP, Mark ES. Etiology of Hormone Receptor–Defined Breast Cancer: A Systematic Review of the Literature. Cancer Epidemiol Biomarkers Prev. 2004;13(10):1558

18. Salehi F, Turner MC, Phillips KP, Wigle DT, Krewski D, Aronson KJ. Review of the Etiology of Breast Cancer with Special Attention to Organochlorines as Potential Endocrine Disruptors. J Toxicol and Environ Health. 2008;11:276–300.

19. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Rlimann BT, Senn HJ. Panel members. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2009. Annals of Oncol. 2009;20:1319–1329.

20. Barros ACSD, Barbosa EM, Gebrim LH, Anelli A, Figueira Filho A, Del Giglio A, Gusmão CB, Pelizon C, Martella E, Motta E, Fernandes JL, Pinotti JA, Ribeiro LCB, Santos MO, Araújo MB, Abreu e Lima MC, Mourão Neto M, Yamaguchi NH, Carmo PAO, Marques R. Diagnóstico e Tratamento do Câncer de Mama. Projeto Diretrizes: Associação Médica Brasileira e Conselho Federal de Medicina. 2001;1:1-15.

21. Hoskins JM, Carey LA, McLeod, HL. *CYP2D6* and tamoxifen: DNA matters in breast cancer. Nature Reviews Cancer. 2009;9:576-586.

22. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet. 2011;378:771–784.

23. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, Buyse M, Baum M, Buzdar A, Colleoni M, Coombes C, Snowdon C, Gnant M, Jakesz R, Kaufmann M, Boccardo F, Godwin J, Davies C, Peto R. Meta-Analysis of Breast Cancer Outcomes in Adjuvant Trials of Aromatase Inhibitors Versus Tamoxifen. J Clin. Oncol. 2010;28:509-518.

24. Ahsen NV, Binder C, Brosckmoller J, Oellerich M. *CYP2D6* and tamoxifen: pharmacogenetic reinvention of an established drug? J Lab Med. 2009;33(5):1-9.

25. Hadji P, Asmar L, van Nes JGH, Menschik T, Hasenburg A, Kuck J, Nortier JWR, van de Velde CJH, Jones SE, Ziller M. The effect of exemestane and tamoxifen on bone health within the Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial: a meta-analysis of the US, German, Netherlands, and Belgium sub-studies. J Cancer Research Clin Oncol. 2011;137:1015-1025.

26. Coombes RC, Kilburn LS, Snowdon CF, Paridaens R, Coleman RE, Jones SE, Jassem J, Van de Velde CJH, Delozier T, Alvarez I, Del Mastro L, Ortmann O, Diedrich K, Coates AS, Bajetta E, Holmberg SB, Dodwell D, Mickiewicz E, Andersen J, Lønning PE, Cocconi G, Forbes J, Castiglione M, Stuart N, Stewart A, Fallowfield LJ, Bertelli G, Hall E, Bogle RG, Carpentieri M, Colajori E, Subar M, Ireland E, Bliss JM. Survival and safety of exemestane versus tamoxifen

after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. Lancet. 2007;369:559–570.

27. Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, Abraham M, Alencar VHM, Badran A, Bonfill X, Bradbury J, Clarke M, Collins R, Davis SR, Delmestri A, Forbes JF, Haddad P, Hou MF, Inbar M, Khaled H, Kielanowska J.Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet.2013;381:805-816.

28. Santana DP, Braga RM, Satrattman R, Albuquerque MM, Bedor DCG, Leal LB. Reversed phase HPLC determination of tamoxifen in dog plasma and its pharmacokinetics after a single oral dose administration. Quim Nova. 2008;31:47-52.

29. Jordan VC. Tamoxifen as the first successful targeted therapy in cancer: the gift that kept on giving. Breast Cancer Manag. 2014;3:321-326.

30. Jin Y, Desta Z, Stearns V, Ward B, Ho H, Lee KH, Skaar T, Storniolo AM, Li L, Araba A, Blanchard R, Nguyen A, Ullmer L, Hayden J, Lemler Z, Weinshilboum RM, Rae JM, Hayes DF, Flockhart DA. *CYP2D6* Genotype, Antidepressant Use, and tamoxifen metabolism during adjuvant breast cancer treatment. J Nac. Cancer Inst. 2005;97:30-39

31. Binkhorst L, Mathijssen RHJ, van Herk-Sukel MPP, Bannink M, Jager A, Wiemer EAC, van Gelder T. Unjustified prescribing of CYP2D6 inhibiting SSRIs in women treated with tamoxifen. Breast Cancer Res. Treat. 2013;139:923-929.

32. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J. Endocrinol.1977;75:305–316.

33. Teunissen SF, Rosing H, Koornstra RHT,Linnb SC, Schellensc JHM, Schinkel, AH, Beijnen JH. Development and validation of a quantitative assay for the analysis of tamoxifen with its four main metabolites and flavonoids daidzein, genistein and glycitein in human serum using liquid chromatography coupled with tandem mass spectrometry. J Chromat B. 2009; 877:2519-2529.

34. Kelly CM, Juurlink DN, Gomes T, Duong-Hua M, Pritchard KI, Austin PC, Paszat LF. Selective serotonin reuptake inhibitors and breast cancer mortality in women receiving tamoxifen: a population based cohort study. BMJ. 2010;340:c693.

35. Orleans RJ, Li L, Kim MJ, Guo J, Sobhan M, Soule L, Joffe HV. FDA Approval of Paroxetine for Menopausal Hot Flushes. N Engl. J Med. 2014; 370:1777-1779.

36. Afshar M, Rouini M, Shahram A. Dextromethorphan metabolic phenotyping in an Iranian population. Eur. J Clin. Pharmacol. 2005;60:849–854.

37. L'Espérance S, Frenette S, Dionne A, Dionne JY, Comité de l'évolution des pratiques en oncologie (CEPO). Pharmacological and non-hormonal treatment of hot flashes in breast cancer survivors: CEPO review and recommendations. Support Care Cancer. 2013;21:1461-1474.

38. Zhu, YB, Zhang Z, Zou JJ, Yu ZX, Xiao DW. Optimizing high-performance liquid chromatography method with fluorescence detection for quantification of tamoxifen and two metabolites in human plasma: Application to a clinical study. J Pharm Biom Anal. 2008; 46:349-355.

39. Grunfeld EA, Hunter MS, Sikka P, Mittal S. Level of adherence was analyzed either as a continuous measure or categorized. Adherence beliefs among breast cancer patients taking tamoxifen. Patient Education and Counseling. 2005;59:97–102.

40. Hershman DL, Shao T, Kushi LH, Buono D, Tsai WY, Fehrenbacher L, Kwan M, Gomes SL, Neugut AI. Early discontinuation and non-adherence to adjuvant hormonal therapy are associated with increased mortality in women with breast cancer. Breast Cancer Res Treat 2011;126:529-537.

41. Dezentjé VO, Van Blijderveen NJC, Gelderblom H, Putter H, Van Herk-Sukel MPP, Casparie MK, Egberts ACG, Nortier JWR, Guchelaar HJ. Effect of Concomitant *CYP2D6* Inhibitor Use and Tamoxifen Adherence on Breast Cancer Recurrence in Early-Stage Breast Cancer. J Clin Oncol. 2010; 28 (14):2423-2429.

42. Borges S, Desta Z, Li L, Skaar TC, Ward BA, Nguyen A, Jin Y, Storniolo AM, Nikoloff M, Wu L, Hillman G, Hayes DF, Stearns V, Flockhart DA. Quantitative effect of *CYP2D6* genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment. Clin Pharmacol Ther. 2006;80(1):61-74.

43. Hinrichs JW, Loovers HM, Scholten B, Van der Weide J. Semi-quatitative *CYP2D6* gene doses in relation to metabolic ratios of pyschotropics. Eur J Clin Pharmacol. 2008;64:979-986.

44. Kohlrausch FB, Gama CS, Lobato MI, Belmonte-de-Abreu P, Gesteira A, Barros F, Carracedo A, Hutz MH. Molecular diversity at the CYP2D6 locus in healthy and schizophrenic Souther Brazilians. Pharmacogenomics. 2009;10:1457-1466.

45. Beverage JN, Sissung T, Sion AM, Danesi R, Figg W. *CYP2D6* polymorphisms and the impact on tamoxifen therapy. J Pharm Scienc. 2007;96(9):2224-2231.

46. Friedrich DC, Genro JP, Sortica VA, Suarez-Kurtz G, de Moraes ME, Pena SDJ, dos Santos AKR, Romano Silva MA, Hutz MA.. Distribution of CYP2D6 alleles and phenotypes in the Brazilian population. Plos One 2014;9:e110691.

47. Elens L, Gelder TV, Hesselink DA, Haufroid V, Schaik RHN. CYP3A4*22: promising newly identified CYP3A4 variant allele for personalizing pharmacotherapy. Pharmacogenomics. 2013;14:47–62.

48. Santoro AB, Struchiner CJ, Felipe CR, Tedesco-Silva H, Medina-Pestana JO, Suarez-Kurtz G. C*YP3A5* Genotype, but Not *CYP3A4*1b*, *CYP3A4*22*, or hematocrit, predicts tacrolimus dose requirements in Brazilian renal transplant patients. Clin. Pharmacol. Ther. 2013;94:201–202.

49. Stearns V, Rae J. Pharmacogenetics and breast cancer endocrine therapy: *CYP2D6* as a predictive factor for tamoxifen metabolism and drug response? Expert Rev Molecular Med. 2008;10:1-13.

50. Lindh JD, Andersson ML, Eliasson E, Bjorkhem-Bergam L. Seasonal variation in blood drug concentrations and a potential relationship to vitamin D. Drug Metab. Disp. 2011;39:933–937.

51. Kim HJ, Koh BS, Yu JH, Lee JW, et al. Changes in serum hydroxyvitamin D levels of breast cancer patients during tamoxifen treatment or chemotherapy in premenopausal breast cancer *Patients.* Eur J Cancer. 2014; 50:1403-1411.

52. Autier P, Gandini S. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. Arch. Intern. Med. 2007;167:1730–1737.

53. Lehmann B, Meurer M. Vitamin D metabolismo. *Dermatol. Ther.* 2010;23:2-12.

54. Holick MF, Binkley NC, Bischoff-Ferrari HA, *et al*. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical practice guideline. J Clin. Endocrinol. Metab. 2011;96:1911-1930.

55. Premaor MO, Furlanetto TW. Hipovitaminose D em adultos: entendendo melhor a apresentação de uma velha doença. Arq. Bras. Endocrinol. Metab. 2006;50:25-37.

56. Araujo MC, Bezerra IN, Barbosa FS, Junger WL, Yokoo EM, Pereira RA, Sichieri R. Consumo de macronutrientes e ingestão inadequada de micronutrientes em adultos. Rev Saúde Púb. 2013;47:177-189.

57. Pedrosa MAC, Castro ML. Papel da vitamina D na função neuro-muscular. Arq. Bras. Endocrinol. Metab. 2005;49:495-502.

58. Benvenuto-Andrade C, Zen B, Fonseca G, de Villa Damiê, Cestari T. Sun Exposure and Sun Protection Habits Among High-school Adolescents in Porto Alegre, Brazil. Photochem. Photobiol. 2005;81:630-635.

59. Schroth W, Hamann U, Fasching PA, Dauser S, Winter S, Wichelbaum M, Schwab M, Brauch H. CYP2D6 polymorphisms as predictors of outcome in breast cancer patients treated with tamoxifen: Expanded polymorphism coverage improves risk stratification. Clin Cancer Res 16:4468-4477, 2010.

60. Brauch H, Schroth W, Goetz MP, Mürdter TE, Winter S, Ingle JN, Schwab M, Eichelbaum M. Tamoxifen Use in Postmenopausal Breast Cancer: CYP2D6 Matters. J Clin Oncol. 2013;31:176-180.

61. Irvin WJ, Walko CM, Weck KE, Ibrahin JG, Chiu WK, Dees EC, Moore SG, Olajide, OA, Grahan ML, Canale ST, Raab, RE, Corso SW, Peppercorn JM, Anderson SM, Friedman KJ, Ogburn ET, Desta Z, Flockhart DA, McLeod HL, Evans JP, Carey LA. Genotype-Guided Tamoxifen Dosing Increases Active Metabolite Exposure in Women With Reduced CYP2D6 Metabolism: A Multicenter Study. J Clin Onc. 2011;29:3232-3239.

62. Regan MM, Leyland-Jones B, Bouzyk M, et al: CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: The Breast International Group 1-98 trial. J Natl Cancer Inst. 2012;104:441-451.

63. Rae JM, Drury S, Hayes DF, et al: CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. J Natl. Cancer Inst. 2012; 104:452-460

64. Hicks, KJ, Swen J; Gaedigk A. Challenges in CYP2D6 Phenotype Assignment from Genotype Data: A Critical Assessment and Call for Standardization. Curr. Drug Metab. 2014;15:218-232.

65. Porcelli S, Fabbri S, Spina E, Serretti A, Ronchi DD. Genetic polymorphisms of cytochrome P450 enzymes and antidepressant metabolism. Expert Opin. Drug Metab. Toxicol. 2011;7:1101-1115.

66. Graan AJM, Teunissen SF, de Vos FYFL, Loos WJ, Van Schaik RHN, Jongh FE, Vos A, Van Alphen, RJ, Van der Holt B, Verweij J, Seynaeve C, Beijnen JH, Mathijssen RHJ. Dextromethorphan As a Phenotyping Test to Predict Endoxifen Exposure in Patients on Tamoxifen Treatment. J Clin Onc. 2011;29:3240-3246.

67. Afshar M, Rouini M, Amini M. Simple chromatography method for simultaneous determination of dextromethorphan and its main metabolites in human plasma with fluorimetric detection. J Chromat B. 2004;802:317-322.

68. González HM, Peregrina ALA, Chávez TJC, Escobar-Islas E, Felipe LK, Hoyo-Vadillo C. CYP2C19 and CYP3A4 Dependent Omeprazole Metabolism in West Mexicans. J. Clin. Pharmacol. 2003, 43:1211-1215.

69. Kiyotani K, Mushiroda T, Imamura CK, Tanigawara Y, Hosono N, Kubo M, Sasa M, Nakamura Y, Zembutsu H. Dose-adjustment study of tamoxifen based on *CYP2D6* genotypes in Japanese breast cancer patients. Breast Cancer Res. Treat. 2012;131:137-145.

70. Irvin WJ, Walko CM, Weck KE, Ibrahin JG, Chiu WK, Dees EC, Moore SG, Olajide, OA, Grahan ML, Canale ST, Raab, RE, Corso SW, Peppercorn JM, Anderson SM, Friedman KJ, Ogburn ET, Desta Z, Flockhart DA, McLeod HL, Evans JP, Carey LA. Genotype-Guided Tamoxifen Dosing Increases Active Metabolite Exposure in Women With Reduced CYP2D6 Metabolism: A Multicenter Study. J Clin Onc. 2011;29(24):3232-3239.

71. Jager NGL, Rosing H, Schellens JHM, Linn SC, Beijnen JH. Tamoxifen dose and serum concentrations of tamoxifen and six of its metabolites in routine clinical outpatient care, Breast Cancer Res. Treat.2014;143:477-483.

72. Lee KH, Ward BA, Desta Z, Flockhart DA, Jones DR. Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection: application to a clinical trial, J Chromatogr. B.2003;791:245-253.

73. Zhu YB, Zhang Z, Zou JJ, Yu ZX, Xiao DW. Optimizing high-performance liquid chromatography method with fluorescence detection for quantification of tamoxifen and two metabolites in human plasma: Application to a clinical study, J. Pharm. Biom. Anal. 2008;46:349-355.

74. Arpini J, Antunes MV, Pacheco LS, Gnatta D, Rodrigues MF, Keitel E, Linden R. Clinical evaluation of a dried blood spot method for determination of mycophenolic acid in renal transplant patients. Clin. Biochem. 2013;46:1905-1908.

75. Jager NGL, Rosing H, Schellens JHM, Beijnen JH, Linn SC. Use of dried blood spots for the determination of serum concentrations of tamoxifen and endoxifen, Breast Cancer Res. Treat. 2014;146:137–144.

76. Rhoden L, Antunes MV, Hidalgo P, da Silva CA, Linden R. Simple procedure for determination of valproic acid in dried bloodspots by gas chromatography–mass spectrometry, J. Pharm. Biom. Anal. 2014;96:207–212.

77. McDade TW, Williams S, Snodgrass J. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography. 2007;4:899-925.

5. ARTIGOS DA TESE

Artigo 1. Submetido em: Therapeutic Drug Monitoring (QUALIS A2)

Influence of CYP2D6 and CYP3A4 phenotypes, drug interactions and vitamin D status on tamoxifen biotransformation

Marina Venzon Antunes[1,2]*, MSc; Tatiana Aparecida da Fontoura Timm[2], BSc; Vanessa de Oliveira[2], BSc; Dilana Elisabeth Staudt[2], Suziane Raymundo[2], PhD; Gustavo Gössling[3], BSc; Jorge Villanova Biazús[3], PhD; José Antônio Cavalheiro[3], PhD; Daniela Dornelles Rosa[4], PhD; Pierre Wallemacq[5], PhD; Vincent Haufroid[5], PhD; Rafael Linden[2], PhD; Gilberto Schwartsmann[1,3], PhD.

[1]Pós-graduação em Ciências Médicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2]Instituto de Ciências da Saúde, Universidade Feevale, Novo Hamburgo, RS, Brazil

[3]Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

[4]Hospital Moinhos de Vento, Porto Alegre, RS, Brazil

[5]Louvain centre for Toxicology and Applied Pharmacology, Institut de recherche expérimentale et clinique, Université catholique de Louvain, Brussels, Belgium.

* Corresponding author:
Universidade Feevale.
ERS 239, n° 2755 - Zip code 93352-000
Novo Hamburgo – RS, Brazil
Phone/fax: (55) 51-358.8800
e-mail: marinaantunes@feevale.br

Abstract

Aim: In view of the large variability on therapeutic response and the multiple factors associated to tamoxifen (TAM) metabolic activation, the present study aimed to evaluate the effect of CYP2D6 and CYP3A4 phenotypes, drug interactions and vitamin D exposure on TAM metabolic activation in a group of breast cancer patients. **Methods:** Trough blood samples were collected from 116 patients. Tamoxifen and metabolites endoxifen (EDF), N-desmethyltamoxifen (NDT) and 4-hydroxy-TAM (HTF) were measured in plasma LC-MS/MS. CYP2D6 and CYP3A4 phenotyping were given according to [DMT]/[DTP] and [OME]/[OMS] metabolic ratios, measured by HPLC in plasma collected 3 hours after oral administration of 33 mg of dextromethorphan and 20 mg of omeprazole. Vitamin D_3 was measured in plasma by HPLC-UV. Data on concomitant use of drug considered as CYP2D6 and CYP3A4 inhibitor or inducer and vitamin D supplementation were recorded. **Results:** about 20% of patients had reduced CYP2D6 metabolic activity and 7% CYP3A4 impaired metabolism. EDF levels diminished proportionally to the reduction of CYP2D6 metabolic activity (PM 2.79 ng mL^{-1}, IM 5.36 ng mL^{-1} and EM 10.65 ng mL^{-1}, $P<0.01$). Median plasma levels of TAM (161.50 ng mL^{-1}) and HTF (1.32 ng mL^{-1}) in CYP2D6 IM/CYP3A4 PM patients were higher ($P<0.05$) than those from CYP2D6 IM/CYP3A4 EM patients (122.07 ng mL^{-1} and 0.61 ng mL^{-1}, respectively). Seasonality contributed to the interpatient variability of EDF and HTF levels, summer concentrations were 24% and 42% higher compared to winter. VitaminD_3 was not associated to CYP3A4 metabolic activity, indicating that other mechanism might be involved on the relation between TAM metabolism and vitamin D exposure. **Conclusions**: CYP3A4 contributes to the bioactivation of TAM through formation of HTF and becomes increasingly important in conditions of diminished or absent CYP2D6 activity. EDF and HTF exposure were associated to seasonality, with considerable higher plasma concentrations during summer.

Key-words: tamoxifen; *CYP3A4*; *CYP2D6;* vitamin D.

INTRODUCTION

Tamoxifen (TAM) is the main adjuvant endocrine therapy for pre- and postmenopausal estrogen receptor (ER)-positive breast cancer. It has both estrogenic and antiestrogenic effects, depending on the target tissue. It is strongly antiestrogenic on mammary epithelium and thereby arresting the cell cycle; it is proestrogenic on bone tissue given significant protection against postmenopausal bone loss [1].

Cytochrome P450 plays an essential role on TAM metabolic activation. The formation of the major metabolite N-desmethyl-tamoxifen (NDT) is catalyzed through CYP3A4/5, with minor contributions by CYPs 2D6, 1A, 1A2, 2C19 e 2B6 [2]. Besides the high plasma steady state concentrations of NDT, about twice as TAM [3], this metabolite exerts no significant therapeutic effect. NDT undergoes further 4-hydroxylation by CYP2D6 being converted to 4-hydroxy-N-desmethyltamoxifen or (Z)-endoxifen (EDF). CYP2D6 also catalyzes the metabolism of TAM to 4-hydroxy-tamoxifen (HTF), with minor contribution of CYPs 3A4, 2B6, 2C19 and 2C9. Moreover, HTF is further converted to EDF by CYP3A4/5 [4]. The efficacy of hormonal therapy is attributed to the hydroxylated metabolites, particularly EDF, once its plasma concentrations are about 6 times higher than the equipotent HTF [5; 6]. Recently, Madlensky *et al.* (2011) proposed a clinical threshold of EDF plasma levels of 5.9 ng mL-1, which was related to approximately 30% reduction on recurrence rate [7].

Besides the clinical benefits of TAM in breast cancer treatment, there is substantial inter-patient variability in both the development of resistance to TAM and the occurrence of adverse reactions [2]. Hot flashes are the commonest symptom reported by women receiving adjuvant therapy with TAM and may hinder adherence [8]. Indeed, selective serotonin reuptake inhibitor (SSRI) antidepressants, some of which are known to inhibit CYP2D6, are commonly prescribed to treat hot flashes in women who take TAM, reducing systemic exposure to EDF [9].

The high variability on EDF plasma concentrations has also been attributed to polymorphisms in the gene encoding CYP2D6 [5; 10]. Frequencies and distributions of *CYP2D6* alleles and genotypes vary depending on the origin of the population; 7-11% of Caucasians are poor metabolizers (PM) and about 10% are intermediate metabolizers (IM) [11]. Similar frequencies were found in a South Brazilian population with 5.6% PM and 7.8% IM, both related to lower exposure to EDF [12]. CYP3A4 contributes in some extend to EDF formation from the

metabolism of HTF [6]. Like CYP2D6, CYP3A4 metabolic indices are highly variable among people (10 to 100 fold) due to genetic and/or environmental factors, such as diet, drug interactions [13] and sun exposure [14]. The presence of the polymorphic allele *CYP3A4*22*, with a frequency of 5-7% in Caucasians, is associated to reduced enzyme activity [15]. Conflicting data regarding the impact of impaired CYP3A4 activity on TAM biotransformation have been reported. While Heine *et al.* (2014) indicated, through a phenotype-based evaluation, that CYP3A4 inhibition might result in decreased TAM efficacy due to reduced EDF levels [16], Teft *et al.* (2013) and Antunes *et al.* (submitted) found higher levels of active metabolites EDF and HTF within patients carriers of the mutant allele *CYP3A4*22* [12; 17].

Vitamin D and CYP3A4 metabolism have been previously associated. The active form 1α,25-dihydroxyvitamin D3 ($1\alpha,25(OH)_2D_3$) binds to vitamin D receptor and up regulate the transcription of CYP3A4 [14]. The impact of intestinal CYP3A4 induction on several substrates have been demonstrated. For instance, blood levels of tacrolimus and sirolimus showed cyclic variation throughout the year that correlated with ultraviolet light exposure and plasma levels of 25-hydroxyvitamin D ($25OHD_3$) [18]. Sparse information associating vitamin D exposure and TAM metabolism is available. Recently, Kim *et al*. (2014) observed increased serum $25OHD_3$ concentrations during TAM treatment, which was related to estrogen antagonist action of TAM on bone [19]. Teft *et al.* (2013) exploited the relation of between $25OHD_3$ and TAM metabolic activation, and reported that reduced EDF plasma concentrations during the winter months was associated to low plasma $25OHD_3$ levels, but without a clear mechanistic explanation [17]. Indeed, an inverse association between serum $25OHD_3$ levels and risk of breast cancer recurrence has also been demonstrated [20].

CYP2D6 genotyping is not currently suggested in clinical practice for optimizing adjuvant endocrinal treatment with TAM, but avoiding concurrent use of CYP2D6 inhibitor drugs is recommended [21]. There is no clinical indication regarding CYP3A4 genetic evaluation, neither on limitation of concurrent use of enzyme inducer or inhibitor drugs. The combined effect of genetic and environmental factors on CYP2D6 and CYP3A4 activity can be evaluated by phenotyping approaches. The estimation of the metabolic ratio of a probe drug might be a useful tool to identify patients with possibly poor treatment response [3;16].

In view of the large variability on therapeutic response and the multiple factors associated to TAM metabolic activation, the present study aimed to evaluate the effect of CYP2D6 and CYP3A4 phenotypes, drug interactions and vitamin D exposure on TAM metabolic activation in a group of breast cancer patients.

METHODS

Study Population and Data Collection

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Hospital de Clínicas de Porto Alegre. A total of 116 patients on adjuvant hormonal treatment with TAM (20 mg/day) for at least 4 months were enrolled in the study, after informed consent. Exclusion criteria were use of omeprazole (OME) on a daily basis or single dose administration of dextromethorphan (DMT) or OME within 7 days before the phenotyping procedure. Data on age, body weight, body mass index (BMI), race, menopause status and presence of adverse effects, such as hot flashes, were recorded. In addition, the duration of TAM therapy and use of vitamin D supplementation, as well as any drug considered as a CYP2D6 and CYP3A4 inhibitor or inducer was also recorded. Treatment adherence was evaluated from monthly TAM pill counts.

Biological sample collection

Participants provided blood samples at two different times, at winter and summer at the South Hemisphere (July and January, respectively). Phenotypic analysis of CYP2D6 and CYP3A4 metabolism was performed only at the first evaluation, at winter. Blood samples for measurement of TAM metabolites and $25OHD_3$ were taken both at Winter and Summer, 16-24 h after the last TAM intake, with patients being fasten for 4 hours and abstain from alcohol for 48 hours prior to the tests. An 8 mL sample of venous blood was drown into a tube containing EDTA as anticoagulant. Tubes were centrifuged immediately and plasma was transferred to 2 mL polypropylene tubes, which were stored at -70 °C until analysis.

CYP2D6 Phenotyping

CYP2D6 activity index was assessed by calculating the metabolic ratio between the probe drug DMT and its metabolite DTP [22]. Each volunteer received a 30 mg oral dose of DMT and

3 hours later a 4 mL sample of venous blood was drown into a tube containing EDTA as anticoagulant. Tubes were centrifuged immediately and plasma was transferred to 2 mL polypropylene tubes, which were stored at -70 °C until analysis. DMT and DTP plasma concentrations were measured by high performance liquid chromatography with fluorescence detection (HPLC-FL), as described in Antunes *et al.* (2014). Briefly, plasma samples were enzymatically hydrolyzed and the compounds were extracted by liquid–liquid extraction. Separation was conducted in a Hypersil Gold® C18 column (150 x 4.6 mm) with a mobile phase composed of phosphate buffer 0.1 M pH 6.0 and acetonitrile (76:24, v/v), with 0.1 % triethylamine, eluated at a flow rate of 1.0 mL min^{-1}. Chromatograms were acquired at an excitation wavelength of 280 nm and an emission wavelength of 320 nm. Method was linear through 1-250 ng mL^{-1} for DTP and 10-2,500 ng mL^{-1} for DMT, within and between assay RSD were <9.7 % and accuracy in the range of 92.1-106.9 % [23]. Concentrations of DMT and DTP were estimated from their calibration curves and then [DMT]/[DTP] metabolic ratios were calculated. CYP2D6 phenotypes were classified into four categories on the basis of the resultant ratios [DMT]/[DTP]: PM ≥ 0.3; 0.3 > IM ≥ 0.03; 0.03 > EM ≥ 0.0003; UM > 0.0003 [24].

CYP3A4 phenotyping

CYP3A4 phenotypes were assessed by calculating the metabolic ratio between the probe drug OME and its metabolite omeprazole sulphone (OMS) [25]. Each volunteer received a 20 mg oral dose of OME and 3 hours later a 4 mL sample of venous blood was drown into a tube containing EDTA as anticoagulant. Tubes were centrifuged immediately and plasma was transferred to 2 mL polypropylene tubes, which were stored at -70 °C until analysis. OME and OMS plasma concentrations were measured by high performance liquid chromatography with diode array detector (HPLC-DAD), according to Linden et al. (2007), with minor modifications [26]. Plasma samples were prepared by liquid-liquid extraction. The compounds and internal standard were separated in a Lichrospher® RP18 reversed phase column (250 x 4 mm, 5 µm), eluted with a mobile phase composed from phosphate buffer 0.1 M pH 7.6 and acetonitrile (72:28, v/v). Chromatograms were monitored at 302 nm. Method was linear through 20-1,500 ng mL-1, within and between assay RSD were <11.0 % and accuracy in the range of 98-105 %. Concentrations of OME and OMS were estimated from their calibration curves and then log10

[OME]/[OMS] metabolic ratios were calculated. CYP3A4 phenotypes were classified into three categories on the basis of the resultant ratios $\log_{10}$ [OME]/[OMS]: PM > 0.6; 0.6 ≥ EM > -0.3; UM ≤ -0.3 [25].

Determination of TAM and metabolites concentrations in plasma

Concentrations of TAM, NDT, HTF and EDF were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after a simple liquid-liquid extraction of 200 µL plasma sample, as described in Antunes *et al.* (2015) [27]. The method was linear from 0.5 to 600.0 ng mL^{-1} with lower limit of quantification of 7.5 ng mL^{-1} for TAM, 15.0 ng mL^{-1} for NDT, 1.0 ng mL^{-1} for EDF and 0.5 ng mL^{-1} for HTF. Accuracy was in the range 90.7-107.5 %, and precision RSD were in the range of 4.8-13.4% [27].

Determination of 25-hydroxyvitamin D3 concentrations in plasma

Plasma concentrations of $25OHD_3$ were estimated by HPLC-DAD, after solid-phase extraction. Separation was performed in a Hypersil Gold® C18 column (150 x 4.6 mm, 3 µm), eluted with a mixture of water and acetonitrile (69:31, v/v). Chromatograms were recorded at 264 nm. The method was linear in the range of 5-100 ng mL^{-1}. Precision RSD were <15.0 % and accuracy was in the range of 98-105 %. Although no consensus exists regarding the optimal level of $25OHD_3$, we categorized it according to the American Endocrine Society as: deficient <20 ng mL^{-1}, insufficient 20–29 ng mL-1 and sufficient ≥ 30 ng mL^{-1} [28].

Analysis of data

Descriptive analysis was performed for all variables. The frequency distributions for the CYP2D6 and CYP3A4 metabolic ratios, expressed as log [DMT]/[DTP] and log [OME]/[OMS], respectively, were used to plot histograms. The normality of the metabolic distributions curves were tested using the Shapiro-Wilk test. TAM and metabolites and $25OHD_3$ plasma concentrations, as well as the metabolic ratios [NDT]/[EDF] and [TAM]/[NDT] were compared between CYP3A4 phenotype groups with Mann-Whitney test and among groups classified according to CYP2D6 phenotype, race, menopause status and use of CYP2D6 inhibitor drugs

with Kruskal-Wallis test, followed by Mann-Whitney test. Quantitative variables were associated in Spearman correlation analysis. The predictive power of EDF and HTF plasma levels from CYP2D6 and CYP3A4 phenotypes was evaluated using multiple linear regression, including demographic variables at the model. The prevalence of patients with reduced EDF (<5.9 ng mL^{-1}) and deficient $25OHD_3$ plasma (<20 ng mL^{-1}) concentrations were evaluated within groups classified according to CYP2D6 and CYP3A4 phenotypes, menopause status, race, season and use of CYP2D6 inhibitor drug, using Chi-square or Fisher exact test. Likewise, the presence of hot flashes was compared among groups the same groups. Plasma concentrations of $25OHD_3$ and TAM and metabolites were also compared between winter and summer months by Wilcoxon test. Ten patients were excluded from this analysis due to the reported use of supplementation of vitamin D. Analyses were conducted using SPSS version 17.0 and results with $P<0.05$ were considered statistically significant.

RESULTS

Patient Characteristics

A total of 116 women using TAM on steady-state participated in the study. The demographic and clinical characteristics of the patients are listed in Table 1. The mean age was 56 years (28 to 81) and BMI was 27.3 Kg/m^2 (18.4 to 47.6). Most of the patients were white (82.8 %), with a small representation of other ethnic groups (brown or black). As expected due to TAM anti-estrogenic effects, hot flashes were the most prevalent reported side effect, occurring in 55% of patients. Ten patients reported the use of vitamin D supplementation, 7 women reported the use of strong CYP2D6 inhibitor drug (fluoxetine or paroxetine) and 15 reported the use of weak inhibitors (sertraline, venlafaxine, citalopram). There was no report of concomitant use of any CYP3A4 inhibitor or inducer drugs. All patients were classified as adherent to TAM, at the first data collection, 2 patients reported the use of 27 tablets (90% adherence), 4 patients of 29 tablets (96.6% adherence) and 110 patients of 30 tablets (100% adherence) monthly. Similar observation was found at second data collection, when 4 patients reported the use of 29 tablets (96.6%) and 112 patients of 30 tablets (100% adherence).

Table 1. Patients demographic characteristics according to CYP2D6 phenotypes (N=116).

Characteristic	(N=116)	CYP2D6 PM (n=9)	CYP2D6 IM (n=16)	CYP2D6 EM (n=88)	CYP2D6 UM (n=3)
Age (years)					
Mean (SD)	56 (10)	57 (10)	59 (11.7)	55 (10)	69 (9)
Range	28–81	42-74	42-81	28-81	56-74
Race/Ethnicity (n)					
White	96	9	12	73	2
Others	20	0	4	15	1
BMI (Kg/m^2)					
Mean (SD)	27.3 (5.3)	28.3 (4.6)	26.9 (5.8)	27.1 (5.2)	35.4 (7.5)
Range	(18.4-47.6)	(19.3-34.8)	(18.9-43.7)	(18.4-47.6)	(23.4-37.2)
Marital status (n)					
Single	20	1	2	17	0
Married	55	6	5	43	1
Separared/divorced	15	1	3	11	0
Widowed	26	1	6	17	2
Menopause status (n)					
Pre menopause	57	5	5	46	1
Post menopause	59	4	11	42	2
Vitamin D supplementation (n)	10	2	2	5	1
CYP3A4 phenotypes (n)					
PM	8	0	4	4	0
EM	107	9	11	84	3
UM	1	0	1	0	0
Strong CYP2D6 inhibitor (n)					
Fluoxetine/paroxetine	7	1	4	2	0
Weak CYP2D6 inhibitor (n)					
Sertraline/venlafaxine/citalopram	15	2	5	8	0
Adverse effects (n) no/yes					
Hot flashes	52/64	3/6	7/9	41/47	1/2
Vaginal bleeding	111/5	9/0	15/1	84/4	3/0
Vaginal discharge	78/38	5/4	10/6	60/28	3/0
Vulvar Itching	93/23	8/1	10/6	73/15	2/1
Nausea or vomiting	96/20	7/2	13/3	73/15	3/0
Dizzeness	95/21	8/1	13/3	71/17	3/0
Cutaneous rash	104/12	8/1	13/3	80/8	3/0
Alopecia	89/27	8/1	10/6	68/20	3/0
Thrombosis	110/6	9/0	15/1	83/5	3/0

BMI: body mass index

CYP2D6 and CYP3A4 Phenotypes

DMT and DTP plasma concentrations were highly variable among patients, ranging from 1.0 to 251.1 ng mL^{-1} and from 28.4 to 4,995.50 ng mL^{-1} for DMT and DTP, respectively. Thus, a a marked difference of 1,103 times was found between the lowest (0.003) and the highest (3.31) [DMT]/[DTP] metabolic ratio. The [DMT]/[DTP] metabolic ratios were not normally distributed according to the Shapiro-Wilk test (P<0.001). Figure 1.A shows a histogram illustrating the frequency distribution of CYP2D6 metabolic ratios, expressed as $\log_{10}$ [DMT]/[DTP], in which the groups PM (n=9, 7.7%), IM (n=16, 13.8%), EM (n=88, 75.9%) and UM (n=3, 2.6%) can be clearly identified.

Regarding CYP3A4 phenotypes, OME and OMS plasma concentrations were also highly variable, ranging from 34.6 to 1,392.9 ng mL^{-1} and from 20.10 to 790.1 ng mL^{-1} for OME and OMS, respectively. The amplitude between the lowest (0.40) and the highest (10.21) [OME]/[OMS] metabolic ratio was of 26 times. Figure 1.B shows the $\log_{10}$ [OME]/[OMS] metabolic ratio distribution. Besides Shapiro-Wilk test indicates a normal distribution (P=0.200), probably due to the small number of patients with reduced OME to OMS metabolism, 8 patients were classified as PM (6.9%), 1 as UM (0.9%) and 107 as EM (92.2%), according to classes previously proposed by Gonzalez *et al.* (2003) [25].

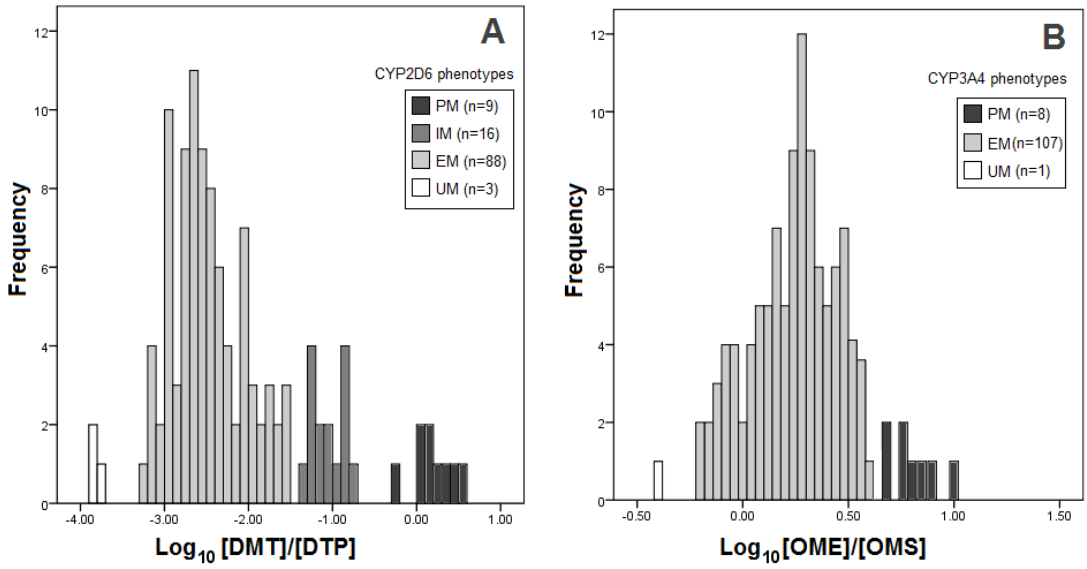


Figure 1. Frequency histogram distribution of CYP2D6 and CYP3A4 metabolic ratios, described as Log_{10} [DMT]/[DTF] and Log_{10} [OME]/[OMS] (N=116).

Approximately 20% of the study population exhibited incomplete CYP2D6 metabolism, whereas about 7% of patients demonstrated poor CYP3A4 metabolic activity. All poor metabolizers for both enzymes were white. Regarding the effect of inhibitor drugs on CYP2D6 metabolism, 33.3% of PM patients, 56% of IM patients and 11.3% of EM patients informed the concomitant use of either weak or strong CYP2D6 inhibitor drugs.

Tamoxifen metabolism in association to CYP2D6 and CYP3A4 phenotypes and drug interaction

CYP2D6 phenotype, measured as [DMT]/[DTP] metabolic ratio, was inversely correlated to the plasma concentration of the active metabolites EDF and HTF (r_s=-0.524 and r_s=-0.319, P<0.01). CYP2D6 phenotype presented a strong association to [NDT]/[EDF] metabolic ratio (r_s=0.603, P<0.01). CYP3A4 phenotype, described as [OME]/[OMS] metabolic ratio, showed significant association to TAM plasma levels (r_s=0.405, P<0.01) and [TAM]/[NDT] metabolic ratio (r_s=0.344, P<0.05). However, CYP3A4 phenotype was not correlated to EDF or HTF (r_s=0.052, P=0.578 and r_s=0.120, P=0.20).

As TAM metabolism is complex and CYP2D6 has a substantial impact on EDF and HTF formation, we investigated whether the CYP3A4 metabolism would have a greater contribution on the formation of active metabolites within patients with CYP2D6 impaired metabolism (PM and IM phenotypes). Even with a small number of observations (n=25), we identified a significant association of CYP3A4 phenotype with HTF concentrations (r_s=0.374 P<0.05). However, EDF concentrations were not significantly associated to CYP3A4 phenotype (r_s=0.284, P=0.169).

TAM and metabolites plasma concentrations were compared among CYP2D6 and CYP3A4 phenotype groups (Table 2). Box plot graphs are shown in supplementary material. Excepting for TAM, all measured metabolites plasma concentrations, as well as [TAM]/[NDT] and [NDT]/[EDF] metabolic ratios, were significantly different among CYP2D6 phenotype groups (P<0.01).

Table 2. Tamoxifen, endoxifen, 4-hydroxytamoxifen, N-desmethyltamoxifen and 25-hydroxyvitamin D_3 plasma concentrations and metabolic ratios according to *CYP2D6* and *CYP3A4* (n=116). Data are expressed as median and percentiles 25% and 75%.

	N		TAM (ng mL^{-1})	EDF (ng mL^{-1})	HTF (ng mL^{-1})	NDT (ng mL^{-1})	[TAM]/[NDT]	[NDT]/[EDF]	25OHD$_3$ (ng mL^{-1})*
CYP2D6 PHENOTYPES					**Median (P25–P75)**				
PM	9		122.05 [a] (98.78-129.50)	2.79 [a] (2.52-2.96)	0.57 [a] (0.47-0.68)	241.05 [b] (230.13-284.09)	0.48 [a] (0.44-0.50)	99.50 [c] (80.95-106.35)	13.20 [a,b] (9.95-24.90)
IM	16		130.26 [a] (103.74-177.66)	5.36 [b] (3,32-9.02)	0.86 [b,] (0.58-1.92)	241.17 [b] (165.49-356.55)	0.55 [a,b] (0.49-0.63)	51.16 [b] (24.27-72.05)	11.85 [a] (7.95-13.80)
EM	88		115.67 [a] (87.29-134.79)	10.65 [c] (8.11-14.44)	1.12 [b] (0.84-1.82)	192.90 [a] (130.82-225.46)	0.63 [b] (0.49-0.74)	16.86 [a] (10.93-25.28)	14.55 [b] (9.70-23.20)
UM	3		138.65 [a] (94.87-161.21)	20.46 [d] (17.73-20.77)	1.88 [c] (1.63-3.46)	309.51 [b] (178.22-323.07)	0.50 [a,b] (0.45-0.53)	15.78 [a] (8.58-17.46)	21.70 [c] (17.0-33.20)
	116	**P**	0.251	0.000	0.001	0.004	0.009	0.000	0.029
CYP3A4 PHENOTYPES					**Median (P25–P75)**				
PM	8		141.93 (123.71-211.52)	8.84 (4.45-17.76)	1.38 (0.92-1.96)	238.98 (150.19-365.04)	0.58 (0.43-0.66)	19.43 (12.74-71.62)	12.95 (10.68-17.43)
EM/UM	108		115.82 (92.82-134.94)	9.96 (5.39-14.39)	1.01 (0.75-1.72)	201.72 (146.26-256.88)	0.58 (0.49-0.70)	18.67 (12.11-37.35)	13.80 (9.60-22.70)
	116	**P**	0.034	0.878	0.482	0.227	0.656	0.644	0.672
CYP2D6 IM & CYP3A4 PHENOTYPES					**Median (P25–P75)**				
CYP2D6 IM & CYP3A4 PM	4		161.50 (140.42-250.01)	6.74 (3.63-10.81)	1.32 (0.92-1.94)	317.70 (228.26-590.97)	0.52 (0.43-0.62)	67.23 (32.26-85.32)	12.95 (8.68-15.20)
CYP2D6 IM & CYP3A4 EM	12		122.07 (103.09-130.82)	4.36 (3.01-8.31)	0.61 (0.50-1.16)	240.82 (190.72-292.61)	0.69 (0.32-0.76)	55.18 (28.1-79.76)	10.55 (8.53-14.45)
	16	**P**	0.030	0.421	0.048	0.245	0.907	0.588	0.627

* n=106 for 25-hydroxyvitamin D_3 analysis, as patients supplementing vitamin D were not included in statistical analysis.
P value as Mann-Whitney test ($\alpha = 0.05$) for CYP3A4 phenotypes and as Kruskal-Wallis test for CYP2D6 phenotypes ([a.b.c,d] same letter in the column do not differ in at 5 % of significance for CYP2D6 phenotypes).

PM: poor metabolizer, IM: intermediate metabolizer, EM: extensive metabolizer, UM: ultra-rapid, TAM: tamoxifen, EDF: endoxifen, HTF: 4-hydroxytamoxifen, NDT: N-desmethyltamoxifen, 25OHD$_3$: 25-hydroxyvitamin D[3]

NDT hydroxylation to EDF, mediated by CYP2D6, was responsible for most of the variance on EDF plasma concentrations (r_s=-0.789, P<0.001). Moreover, EDF levels decreased proportionally to the reduction of CYP2D6 metabolic activity. Median concentrations in PM (2.79 ng mL^{-1}) were approximately 4 times and IM (5.36 ng mL^{-1}) 2 times lower than that from EM (10.65 ng mL^{-1}). Similarly was observed for HTF, in which PM (0.57 ng mL^{-1}) had lower median levels compared to EM-F (1.12 ng mL^{-1} P<0.01).

Regarding CYP3A4 phenotypes, median TAM plasma concentrations were 22% higher in PM (141.93 ng mL^{-1}) compared to EM/UM patients (115.82 ng mL^{-1}, P<0.05). Differently, EDF, NDT and HTF plasma concentrations in the whole cohort were not different among groups of CYP3A4 phenotypes. Since a significant correlation was found between HTF and CYP3A4 phenotype within patients with reduced CYP2D6 metabolism, we compared TAM and metabolites concentrations among CYP2D6 IM patients (n=16), according to their CYP3A4 phenotypes. Median plasma concentrations of TAM (161.50 ng mL^{-1}) and HTF (1.32 ng mL^{-1}) in CYP2D6 IM/CYP3A4 PM patients (n=4) were significantly higher (P<0.05) than those from CYP2D6 IM CYP3A4 EM patients (122.07 ng mL^{-1} and 0.61 ng mL^{-1}, respectively, n=12). Differences on EDF plasma concentrations were not detected, and might be further investigated in studies including a higher number of patients.

We also examined the prevalence of patients with EDF plasma levels below 5.9 ng mL^{-1} according to CYP2D6 and CYP3A4 phenotypes and the use of CYP2D6 inhibitor drugs. Concerning CYP2D6 phenotypes, there was a significant reduction on the frequency of patients with EDF levels < 5.9 ng mL^{-1} with the increase of the CYP2D6 metabolic ratio (P<0.01). In fact, 100% of PM, and 56.3 % of IM subjects had EDF levels below the suggested threshold. In contrast, only 18.2 % of EM and none of UM subjects were below this value. No difference was found within CYP3A4 PM (37.5%) and EM phenotypes (28.7%) (P=0.691). Additionally the prevalence of patients with EDF concentrations below the proposed therapeutic threshold increased with the use and potency of the CYP2D6 inhibitor drug. Frequencies during winter were 20.2% for non-users, 60.0% for users of weak inhibitor drug and 85.7% for users of strong inhibitor drug (P<0.001). Same trend was observed during summer with 14.9% for non-users, 46.7% for users of weak inhibitor drug and 66.7% for users of strong inhibitor drugs (P<0.001).

25-Hydroxyvitamin D_3 plasma concentrations

Ten patients reported taking vitamin D supplementation and were excluded from statistical evaluation. We found no significant correlation between $25OHD_3$ plasma levels and age or BMI (rs=0.067, P=0.706 and rs=0.019 P=0.932). Median $25OHD_3$ plasma concentrations and interquartiles (IQR_1 and IQR_3) were lower in the winter 13.80 ng mL^{-1} (9.60-23.05 ng mL^{-1}), compared to summer 23.02 ng mL^{-1} (17.93-27.67 ng mL^{-1}) (P<0.001, n=106) as showed in Figure 2. Indeed, the frequency of patients with deficient [28] $25OHD_3$ plasma levels was lower during summer time (35.8% *versus* 66%, P<0.001).

Pre-menopausal patients were less likely to have deficient $25OHD_3$ plasma levels (54.7%) during winter, compared to post-menopausal women (73.6%) (P<0.05). There was a significant reduction on the number of patients with deficient $25OHD_3$ plasma concentrations in the summer (p<0.05), with a frequency of 35.8% for both menopausal status groups.

The impact of CYP3A4 and CYP2D6 metabolism on $25OHD_3$ plasma levels was also evaluated. CYP3A4 phenotype, assessed as [OME]/[OMS] metabolic ratio, was not associated to $25OHD_3$ plasma concentrations (r_s=-0.038, P=0.705). Additionally, we found no differences in $25OHD_3$ concentrations between groups of CYP3A4 phenotypes (P=0.672) (Table 2.). Although no significant association was found between $25OHD_3$ concentrations and [DMT]/[DTP] metabolic ratios (r_s=-0.124, P=145), patients with reduced CYP2D6 metabolic activity had median plasma $25OHD_3$ concentrations lower (PM of 13.20 ng mL^{-1} and IM of 11.85 ng mL^{-1}), than those with functional enzyme (EM of 14.55 ng mL^{-1} and UM of 21.70 ng mL^{-1}, P<0.05) (Table 2).

Tamoxifen metabolism, seasonality and inhibitor drugs

TAM and NDT plasma concentrations were strong correlated to age (r_s=0.636 and r_s=0.435, respectively, P<0.01), while EDF and HTF were inversely correlated to BMI (r_s=-0.207 and r_s=-0.220, P<0.05 respectively). Plasma concentrations of the equipotent active metabolites EDF and HTF had a very strong relationship (r_s=0.828, P<0.001), with median EDF concentration being 9.6-fold higher than that of HTF in the winter and 8.4-fold in the summer. EDF levels were also positively correlated to $25OHD_3$ plasma concentrations (r_s=0.204, P<0.05) as did [TAM]/[NDT] metabolic ratio (r_s=0.233, P<0.05).

Median plasma concentrations and range of TAM and metabolites in the winter were: TAM 117.84 ng mL^{-1} (23.50 to 277.45 ng mL^{1}); HTF 1.02 ng mL^{-1} (0.49 to 3.64 ng mL^{-1}); EDF 9.81 ng mL^{-1} (1.58 to 32.45 ng mL^{1}); NDT 202.71 ng mL^{-1} (71.30 to 662.96 ng mL^{1}). Except for TAM, metabolites plasma concentrations were significantly higher in the summer compared to winter (P<0.01), as follows: TAM 119.70 ng mL^{-1} (27.60 to 417.70 ng mL^{1}); HTF 1.45 ng mL^{-1} (0.65 to 4.52 ng mL^{-1}); EDF 12.15 ng mL^{-1} (2.22 to 35.36 ng mL^{1}); NDT 216.81 ng mL^{-1} (79.58 to 493.96 ng mL^{1}) (Figure 2). The increase on metabolites plasma concentrations was prominent for the active metabolites HTF and EDF, of 42% and 24%, respectively, while NDT levels were 6% higher in the summer. Besides the increase of EDF plasma concentrations at summer time, a considerable number of patients still had plasma levels lower than the proposed clinical threshold (21%) [7], but without significant difference from winter (28%, P=0.226).

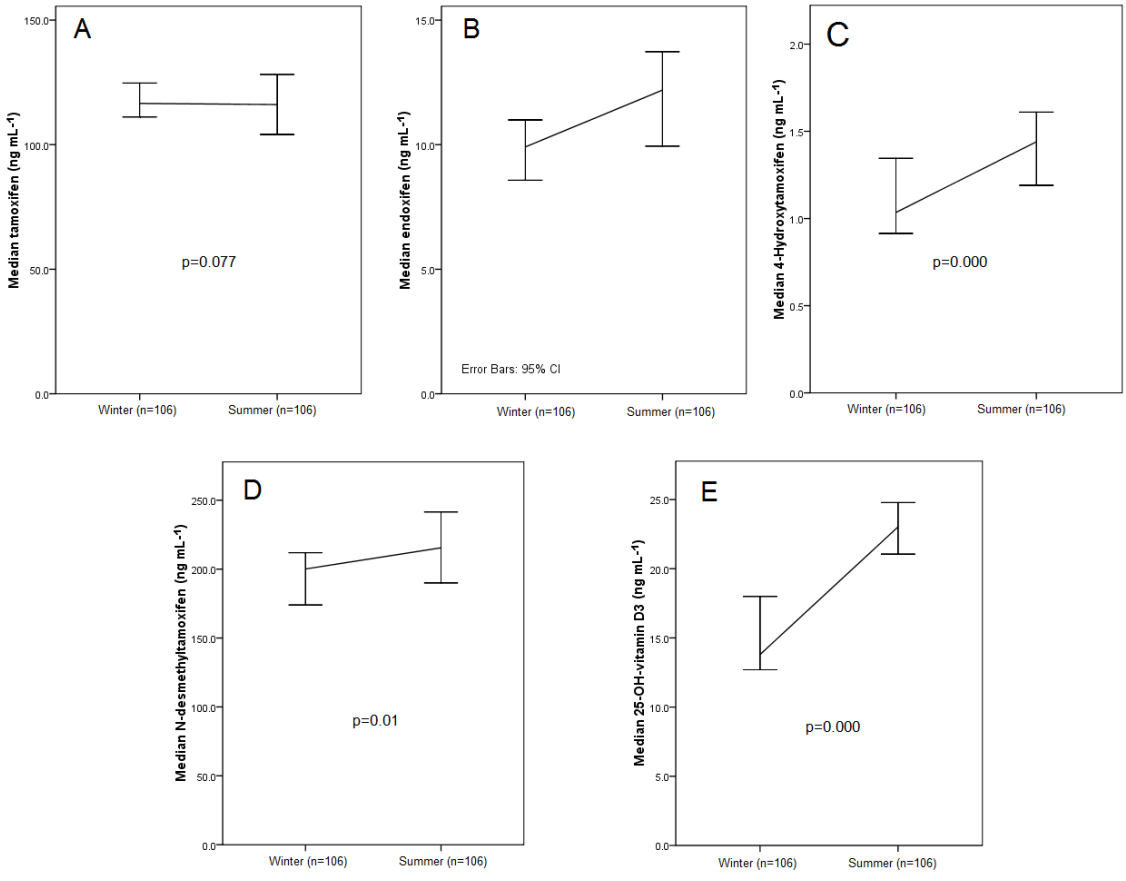


Figure 2. Plasma concentrations of tamoxifen (A), endoxifen (B), 4-hydroxytamoxifen (C), N-desmethyltamoxifen (D) and 25-hydroxyvitamin D_3 (E) according to the season (winter/summer). Data are represented as median and 95% confidence interval (n=106). P value as Wilcoxon test.

We also investigated the effect of concomitant use of CYP2D6 inhibitor drugs on EDF, HTF and $25OHD_3$ plasma concentrations during summer and winter (Figure 3). Lower exposure to EDF was found within patients using strong or weak inhibitor drugs, compared those not using these drugs, with median concentrations in the winter of 3.42 ng mL^{-1} and 4.84 ng mL^{-1} *versus* 10.59 ng mL^{-1} ($P<0.001$) and in the summer of 4.15 ng mL^{-1} and 6.72 ng mL^{-1} *versus* 13.07 ng mL^{-1} ($P<0.01$). Same trend was found for HTF in the winter of 0.63 ng mL^{-1} and 0.82 ng mL^{-1} *versus* 1.15 ng mL^{-1} ($P<0.05$) but not in the summer of 1.18 ng mL^{-1} and 1.10 ng mL^{-1} *versus* 1.48 ng mL^{-1} ($P=0.114$). With respect to vitamin D, the impact of the use of CYP2D6 inhibitor drugs was higher during the winter, comparing with results obtained from summer. Median $25OHD_3$ plasma concentrations in the winter were lower in patients using strong or weak CYP2D6 inhibitor drugs, of 12.70 ng mL^{-1} and 10.70 ng mL^{-1} respectively *versus* 15.25 ng mL^{-1} ($p<0.05$). However, no differences were found during summer, with $25OHD_3$ plasma levels of 18.0 ng mL^{-1}, 21.06 ng mL^{-1} and 23.10 ng mL^{-1} for strong, weak and no use of CYP2D6 inhibitor drug, respectively ($p=0.233$).

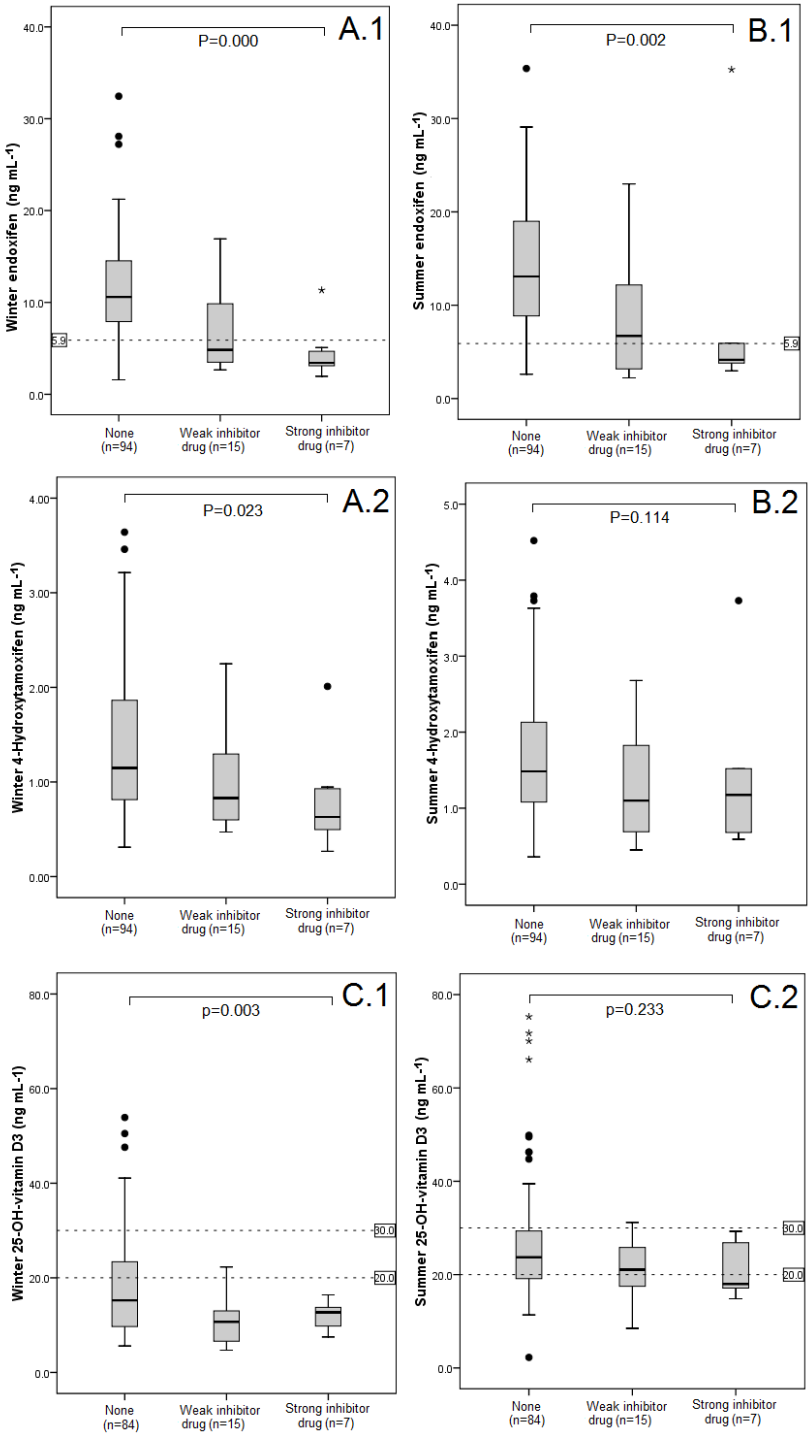


Figure 3. Endoxifen, 4-hydroxytamoxifen (N=116) and 25-hydroxyvitamin D_3 plasma concentrations (n=106) according to the concomitant use of CYP2D6 inhibitor drugs during winter (A.1, B.1, C.1) and summer (A.2, B.2, C.2). Data are represented as median, 25% and 75% percentiles, and range. P value as Kruskal Wallis test.

Prediction of EDF and HTF plasma concentrations

Multiple linear regression revealed that age, BMI, $25OHD_3$ plasma concentrations and phenotypes, assessed as [DMT]/[DTP] and [OME]/[OMS] metabolic ratios, can predict 30% of EDF plasma levels ($P<0.001$). Nevertheless, CYP3A4 metabolic ratio, $25OHD_3$ and BMI showed no statistically significance in the regression model ($P=0.915$, $P=0.465$ and $P=0.721$), in contrast to CYP2D6 phenotype and age ($P<0.001$, $P<0.05$). Same evaluation was performed for HTF, but with lower prediction power of 13% ($P<0.05$), in which [DMT]/[DTP] metabolic ratio was the only statistically significant variable in the multiple linear regression ($P<0.05$). As the correlation between HTF and [OME]/[OMS] metabolic ratio showed to be significant only within patients with CYP2D6 impaired metabolism, we repeated the multiple regression evaluation with CYP2D6 PM/IM (n=25) women and found a considerable higher prediction power of 48% ($P<0.05$). Age, [DMT]/[DTP] and [OME]/[OMS] metabolic ratios were statistically significant in the regression model ($P<0.05$).

DISCUSSION

Although the link between CYP2D6 status and TAM metabolic activation and treatment efficacy have already been recognized [7;29;30] the current work incorporates the evaluation of the impact of CYP3A4 metabolism associated to vitamin D status on the formation of TAM active metabolites.

Previously studies within Brazilian population indicated the prevalence of 3 to 5% *CYP2D6* PM and 4 to 12% *CYP2D6* IM genotypes [3;31;32]. In our study, about 20% of participants had incomplete CYP2D6 metabolism, probably as a result of *CYP2D6* polymorphisms, combined to an expressive number of patients concomitantly using CYP2D6 inhibitor drugs. More than one third of CYP2D6 PM and half of IM patients reported taken some CYP2D6 inhibitor drug in the evaluated period.

CYP2D6 phenotypes, assessed as [DMT]/[DTP] metabolic ratios, were moderately correlated to EDF and HTF plasma concentrations. Median EDF concentrations differed among groups of CYP2D6 phenotypes and diminished proportionally to the reduction of enzyme metabolic activity. In fact, all CYP2D6 PM and about half of IM patients had EDF plasma levels below the proposed clinical threshold [7]. However, a significant variability in EDF exposure still

exists within patients with complete enzyme activity, since a considerable number of CYP2D6 EM patients had low EDF plasma concentrations. In addition, the formation of EDF through NDT hydroxylation, which is mediated by CYP2D6, responded for most but not all of the variance on EDF plasma concentrations. Indeed, even in the absence of CYP2D6 activity EDF is still formed. These observations confirmed previous findings on the participation of other enzymes on EDF formation [3;6], suggesting that additional variables remain to be elucidated. Lower median HTF plasma concentration was also seen in patients with CYP2D6 incomplete metabolism compared to extensive metabolizers, but in a lower range compared to EDF. Since HTF formation is also attributed to *CYP3A4, 2C9, 2B6,* and *2C19* [4], this metabolite is not as closely dependent on CYP2D6.

Polymorphisms on *CYP3A4* gene [15] and environmental factors, such as diet, drug interactions [13] and sun exposure have been associated to the high variability on CYP3A4 metabolism [14]. The occurrence of patients with reduced enzyme metabolism was in agreement to the frequency of *CYP3A4*22* polymorphism identified in Brazilian individuals (6%) [33]. Besides the Brazilian population being highly heterogeneous [31], all CYP3A4 PM patients were white, being in accordance to reported by Elens *et al.* (2013) of the absence of allele **22* within African and Asian individuals [34]. TAM was significantly correlated to CYP3A4 phenotype, as did [TAM]/[NDT] metabolic ratio, as a result of reduction on the abundant primary TAM biotransformation to NDT, mediated by CP3A4/5 [4]. In addition, TAM plasma concentrations were higher in CYP3A4 PM compared to CYP3A4 EM/UM, demonstrating the lower rate of TAM conversion to NDT on individuals with CYP3A4 reduced metabolism.

CYP3A4 has been related in some extent (20-30%) to the formation of EDF after HTF metabolism [6]. Besides HTF and EDF plasma concentrations being highly correlated, none of metabolites were significantly associated to CYP3A4 phenotype, assessed as [OME]/[OMS] metabolic ratios. Further, median EDF and HTF were not different between groups of CYP3A4 phenotypes. As CYP2D6 plays a main role in EDF and HTF formation, we investigated whether CYP3A4 metabolism would have greater contribution on active metabolites formation within patients with CYP2D6 impaired metabolism (PM and IM phenotypes). At this evaluation, we identified a significant association between CYP3A4 metabolic activity and HTF concentrations, in which patients with lower metabolism tend to have higher HTF plasma levels. In addition, median HTF concentrations in CYP2D6 IM patients with CYP3A4 PM phenotype was 2.2 times

higher than CYP2D6 IM patients with CYP3A4 EM phenotype. These findings could partly be attributed to the reduction of CYP3A4 mediated pathway (TAM to NDT), with higher CYP2D6 mediated transformation of TAM to HTF, as an alternative route. In addition, further metabolization of HTF to EDF requires CYP3A4 activity and low enzyme activity explains a lower degradation/higher accumulation, in addition to higher formation rate of HTF. Thus, the decrease on CYP3A4 metabolic activity might compensate reduction of the formation of EDF related to CYP2D6 inactivity, due to increased HTF concentrations.

Our data demonstrated that seasonal variation contributes to the interpatient variability of the levels of the active metabolites EDF and HTF and $25OHD_3$ concentrations. Median EDF and HTF plasma concentrations were markedly increased (24% and 42 %, respectively) during summer compared to winter. Similarly, Teft *et al.* (2013) found 20% lower EDF levels during winter in comparison to mean level across seasons, no evaluation regarding HTF was set [17]. Even though multiple factors interferers on TAM biotranformation, a weak positive correlation of EDF and $25OHD_3$ plasma concentrations was seen, whereas no significant relation was identified between $25OHD_3$ concentrations and HTF. Additionally, vitamin D plays an important role in other conditions that affect breast cancer patients, including osteoporosis, arthralgia and depression. Thus, Kim *et al*. (2014) suggested that it is imperative that proper $25OHD_3$ levels are maintained in this population [19].

A large number of patients had deficient $25OHD_3$ plasma concentrations [28] during winter, particularly post-menopausal women (>70%). Median $25OHD_3$ plasma levels increased about 60% during summer and the prevalence of patients with vitamin D deficiency was about twofold lower. Major source of circulation vitamin D is the endogenous production in the skin exposed to sunlight ultraviolet B (UVB) radiation (280–320 nm), which, under usual circumstances, contributes to more than 90% of its plasma concentrations [35]. Poor sunlight exposure and aging are risk factors for vitamin D deficiency. Porto Alegre (Brazil) is a zone of subtropical climate with four well-defined seasons, located at 30º South. Low temperatures during winter might lead to habits that diminish sunlight exposure, including use of more clothing and minor number of outdoor activities [36].

Vitamin D status have been associated to increased CYP3A4 metabolism, mediated by $1\alpha,25(OH)_2D_3$ binding to vitamin D receptor (VDR), with reported impact in the blood

concentrations of tacrolimus and other substrates [18]. However, we found no association between $25OHD_3$ plasma levels and CYP3A4 activity. The primary sites of CYP3A4 expression are the liver and mucosa of the small intestine. There is considerable data supporting the role of VDR in regulating intestinal CYP3A4. However, the role of VDR in regulating hepatic CYP3A4 is more controversial. Early reports found that VDR was only expressed in non-parenchymal and biliary epithelial cells and not in hepatocytes [37]. Therefore, considering the cellular composition of the liver, induction of hepatic CYP3A4 expression by VDR in non-parenchymal cells may not be that relevant to overall hepatic drug clearance [14]. Additionally, previously studies evaluated other markers of CYP3A4 phenotype and also had negative results while associating to $25OHD_3$. Nylen *et al.* (2014) found no association between $25OHD_3$ and CYP3A4 activity assessed as 4 different phenotyping strategies (quinine metabolic ratio, 4β-hydroxycholesterol to cholesterol ratio, midazolam clearance and as well as 6β-hydroxycortisol to cortisol ratio urinary ratio) [38]. Indeed, Teft *et al.* (2013) found no correlation between $25OHD_3$ levels to 4β-hydroxycholesterol to total free cholesterol [17].

The lack of association between CYP3A4 metabolic ratios and $25OHD_3$, combined to the weak relevance of VDR to hepatic drug clearance, indicates that an alternative mechanism rather than CYP3A4 metabolism is enrolled on the association among TAM metabolic activation, vitamin D and sun exposure. Recently, *Kim et al.* (2014) suggested that TAM increases serum vitamin D and may also influence VDR status, by antagonistic action on oestrogen and alteration of bone calcium metabolism [19]. However, seasonality differences on TAM and vitamin D were not evaluated.

Even with a substantial increase on EDF plasma levels during summer, about 20% of patients still had plasma concentrations below the proposed clinical threshold, mostly as a result of CYP2D6 impaired metabolism and concomitant CYP2D6 inhibitor drug use. During summer, about 50% of patients using weak inhibitor drug and more than 60% of women using strong CYP2D6 inhibitor drug had EDF plasma concentrations <5.9 ng mL^{-1}. HTF levels were lower in patients using CYP2D6 inhibitor drugs during winter, but not during summer, highlighting the greater impact of seasonality on increase of HTF, which is not as closely dependent on CYP2D6 metabolism as EDF.

The multiple linear regression model indicated that age, BMI, $25OHD_3$ plasma concentrations and CYP2D6 and CYP3A4 phenotypes could predict only 30% of EDF and 13%

of HTF concentrations variability. Thus, even with multiple variables, perfect prediction of the metabolic behavior is an unachievable goal. Although the contribution of CYP3A4 activity to HTF concentrations appears to be small and not statistically significant in the regression model, this route may become apparent when CYP2D6 activity is reduced. Indeed, when the same regression model was applied only to CYP2D6 PM/IM patients, a higher predicting power of HTF levels (48%) was achieved, highlighting the significant contribution of CYP3A4..

CONCLUSION

Our findings demonstrate the complexity of TAM metabolism and suggest that CYP3A4 contributes to the bioactivation of TAM through formation of HTF and becomes increasingly important in conditions of diminished or absent CYP2D6 activity. We identified a significant variability on EDF and HTF exposure related to seasonality, with considerable higher plasma concentrations during summer. The mechanism relating vitamin D status, seasonality and biotransformation of TAM still remains to be elucidated.

ACKNOWLEDGMENTS

This work was supported by Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE-HCPA), CNPq/FNRS (CNPq registry number 485013/2013-4).

SUPPLEMENTARY MATERIAL

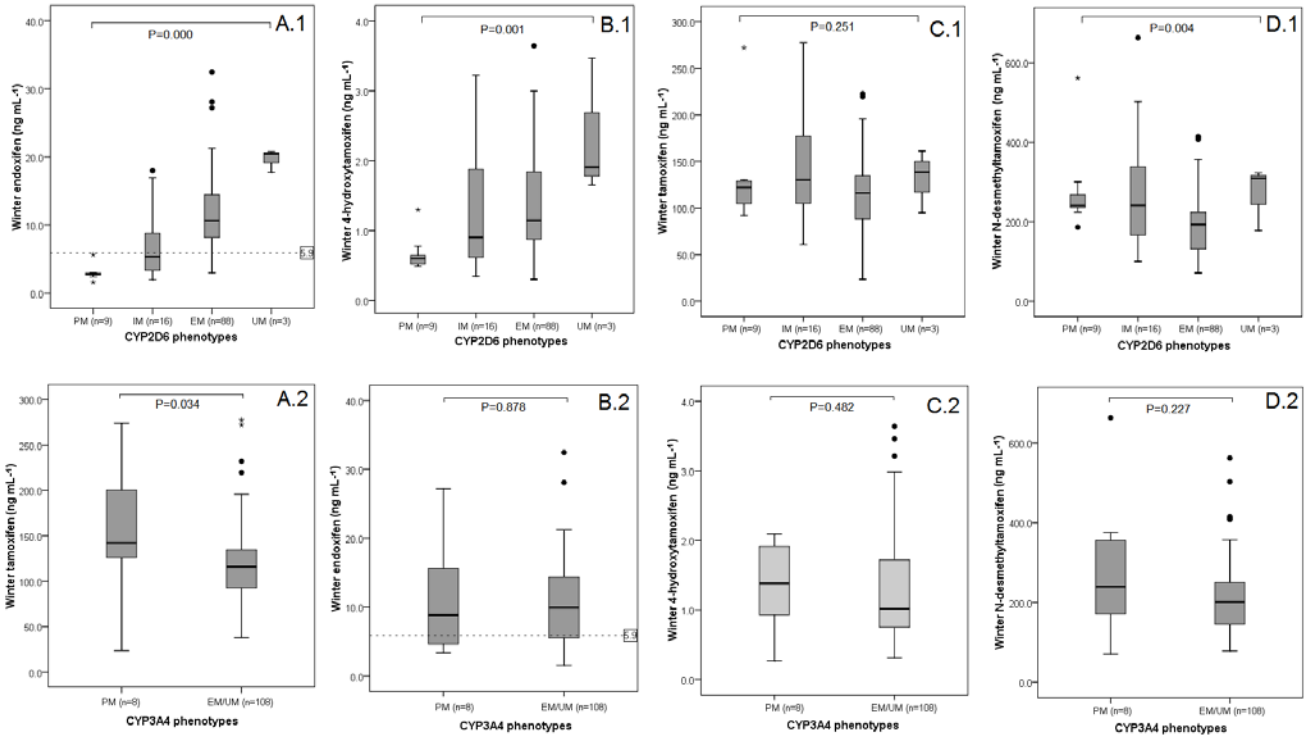


Figure Tamoxifen, endoxifen, 4-hydroxytamoxifen and N-desmethyltamoxifen plasma concentrations (n=116) in the winter according to CYP2D6 phenotypes (A.1, B.1, C.1, D.1, E.1) and CYP3A4 phenotypes (A.2, B.2, C.2, D.2). Data are represented as median, 25% and 75% percentiles, and range. P value as Kruskal Wallis test for CYP2D6 phenotypes and Mann-Whitney test for CYP3A4 phenotypes.

REFERENCES

[1] Ahsen NV, Binder C, Brosckmoller J, et al. CYP2D6 and tamoxifen: pharmacogenetic reinvention of an established drug? *J Lab. Med*. 2009;33:1-9.

[2] Brauch H, Murdter TE, Eichelbaum M, *et al.* Pharmacogenomics of tamoxifen therapy. *Clin Chem.* 2009; 55:1770-1782.

[3] Antunes MV, Linden R, Santos TV, *et al.* Endoxifen levels and its association With CYP2D6 genotype and phenotype: evaluation of a southern Brazilian population under tamoxifen pharmacotherapy. *Ther. Drug Monit.* 2012;34:422-431.

[4] Jung JA, Lim HS. Association between CYP2D6 genotypes and the clinical outocomes of adjuvant tamoxifen for breast cancer: a meta-analysis. *Pharmacogenomics*. 2014;15:49-60.

[5] Teunissen SF, Rosing H, Koornstra RHT, *et al.* Development and validation of a quantitative assay for the analysis of tamoxifen with its four main metabolites and flavonoids daidzein, genistein and glycitein in human serum using liquid chromatography coupled with tandem mass spectrometry. *J Chromatogr. B.* 2009; 877:2519-2529.

[6] Mürdter TE, Schroth W, Bacchus-Gerybadze L, *et al.* Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma, *Clin. Pharmacol. & Therap*. 2011;89:708-717.

[7] Madlensky L, Natarajan L, Tchu T, *et al.* Tamoxifen metabolite concentrations, *CYP2D6* genotype, and breast cancer outcomes. *Clin. Pharm. & Therap*. 2011;89:718-725.

[8] Baxter S, Teft WA, Yun-Hee C, *et al.* Tamoxifen-associated hot flash severity is inversely correlated with endoxifen concentration and CYP3A4*22. *Breast Cancer Res. Treat.* 2014;145:419-428.

[9] Jin Y, Desta Z, Stearns V, *et al. CYP2D6* Genotype, Antidepressant Use, and tamoxifen metabolism during adjuvant breast cancer treatment. *J Nac Cancer Inst*. 2005;97:30-39.

[10] Lotsch J, Rohrbacher M, Schmidt H, *et al.* Can extremely low or high morphine formation from codeine be predicted prior to therapy initiation? *Pain.* 2009;144:119-124.

[11] Teh LK, Bertilsson L. Pharmacogenomics of CYP2D6: Molecular genetics, interethnic differences and clinical importance. *Drug Metab. Pharmacokinet.* 2012;17:55-67.

[12] Antunes MV, Oliveira V, Raymundo S, *et al. CYP3A4*22* is related to increased plasma levels of 4-hydroxytamoxifen and partially compensates for reduced CYP2D6 activation of tamoxifen. Submitted to Pharmacogenomics.

[13] Musshoff F, Stamer UM, Madea B. Pharmacogenetics and forensic toxicology. *Forensic Sci. Int*. 2010;203:53-62.

[14] Wang Z, Schuetz EG, Xu Y, *et al.* Interplay between vitamin D and the drug metabolizing enzyme CYP3A4. *J Steroid Biochem*. 2013;136:54– 58.

[15] Wang D, Guo Y, Wrighton SA, *et al*. Intronic polymorphism in *CYP3A4* affects hepatic expression and response to statin drugs. *Pharmacogenomics J*. 2011;11:274–286.

[16] Heine R, Binkhorst L, de Graan AJM. Population pharmacokinetic modelling to assess the impact of CYP2D6 and CYP3A metabolic phenotypes on the pharmacokinetics of tamoxifen and endoxifen. *Br. J Clin. Pharmacol.* 2014;78:572–586.

[17] Teft WA, Gong IY, Dingle B, *et al*. CYP3A4 and seasonal variation in vitamin D status in addition to CYP2D6 contribute to therapeutic endoxifen level during tamoxifen therapy. *Breast Cancer Res. Treat*. 2013;139:95-105.

[18] Lindh JD, Andersson ML, Eliasson E. *et al.* Seasonal variation in blood drug concentrations and a potential relationship to vitamin D. *Drug Metab. Disp*. 2011;39:933–937.

[19] Kim HJ, Koh BS, Yu JH, Lee JW, et al. Changes in serum hydroxyvitamin D levels of breast cancer patients during tamoxifen treatment or chemotherapy in premenopausal breast cancer *Patients. Eur J Cancer.* 2014; 50:1403-1411.

[20] Autier P, Gandini S. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. *Arch. Intern. Med.* 2007;167:1730–1737.

[21] Theuriault RL & Breast Cancer Panel Members. National Comprehensive Cancer Network (NCCN) United States of America, version 3.2013, p.MS-31.

[22] Afshar M, Rouini M-R, Amini M. Simple chromatography method for simultaneous determination of dextromethorphan and its main metabolites in human plasma with fluorimetric detection *J. Chromatogr. B.* 2004;802:317–322.

[23] Antunes MV, Staudt DE, Raymundo S, *et al.* Development, validation and clinical application of a HPLC-FL method for CYP2D6 phenotyping in South Brazilian breast cancer patients. *Clin. Biochem.* 2014;47:1084-1090.

[24] Beverage JN, Sissung T, Sion AM, *et al*. *CYP2D6* polymorphisms and the impact on tamoxifen therapy. *J Pharm Scienc*. 2007;96:2224-2231.

[25] González HM, Romero EMT, Aaron AL. *et al.* CYP2C19- and CYP3A4-Dependent Omeprazole Metabolism in West Mexicans. *J.Clin. Pharmacol.* 2003;43:1211-1215.

[26] Linden R, Ziulkoski AL, Wingert M, *et al.* Simultaneous determination of omeprazole, hydroxyomeprazole and omeprazole sulphone in human plasma by isocratic HPLC-DAD: application to the phenotyping of CYP2C19 and CYP3A4 in Brazilian Volunteers. *J. Braz. Chem. Soc.* 2007;18:733-740.

[27] Antunes MV, Raymundo S, Oliveira V, *et al*. Ultra-high performance liquid chromatography tandem mass spectrometric method for the determination of tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in dried blood spots – Development, validation and clinical application during breast cancer adjuvant therapy. *Talanta*. 2015;132:775-784.

[28] Holick MF, Binkley NC, Bischoff-Ferrari HA, *et al*. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical practice guideline. J Clin. Endocrinol. Metab. 2011;96:1911-1930.

[29] Bijl MJ, van Schaik RHN, Lammers LA, *et al*. The CYP2D6*4 polymorphism affects breast cancer survival in tamoxifen users. *Breast Cancer Res. Treat*. 2009;118:125-130.

[30] Schroth W, Goetz M, Hamma U, *et al.* Association between *CYP2D6* polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. *JAMA*. 2009;302:1429-1436.

[31] Kohlrausch FB, Gama CS, Lobato MI, *et al.* Molecular diversity at the *CYP2D6* locus in healthy and schizophrenic souther Brazilians. *Pharmacogenomics*. 2009;10:1457-1466.

[32] Friedrich DC, Genro JP, Sortica VA, *et al.* Distribution of *CYP2D6* alleles and phenotypes in the Brazilian population. *Plos One* 2014;9:e110691.

[33] Santoro AB, Struchiner CJ, Felipe CR, *et al.* C*YP3A5* Genotype, but Not *CYP3A4***1b*, *CYP3A4***22*, or hematocrit, predicts tacrolimus dose requirements in Brazilian renal transplant patients. *Clin. Pharmacol. Ther*. 2013;94:201–202.

[34] Elens L, Gelder TV, Hesselink DA, *et al. CYP3A4***22*: promising newly identified *CYP3A4* variant allele for personalizing pharmacotherapy. *Pharmacogenomics*. 2013;14:47-62.

[35] Lehmann B, Meurer M. Vitamin D metabolismo. *Dermatol. Ther*. 2010;23:2-12.

[36] Benvenuto-Andrade C, Zen B, Fonseca G, *et al.* Sun Exposure and Sun Protection Habits Among High-school Adolescents in Porto Alegre, Brazil. *Photochem. Photobiol.* 2005;81:630-635.

[37] Gascon-Barre M, Demers C, Mirshahi A, *et al.* The normal liver harbors the vitamin D nuclear receptor in nonparenchymal and biliary epithelial cells. *Hepatology*. 2003;37:1034–1042.

[38] Nylen H, Björkhem-Bergman L, Ekström L, *et al*. Plasma levels of 25-hydroxyvitamin D3 and in vivo markers of cytochrome P450 3A activity in Swedes and Koreans: effects of a genetic polymorphism and oral contraceptives. *Basic Clin. Pharmacol. Toxicol*. 2014;115:366–371.

Artigo 2. Submetido em: Pharmacogenomics (QUALIS A1)

*CYP3A4*22* is related to increased plasma levels of 4-hydroxytamoxifen and partially compensates for reduced CYP2D6 activation of tamoxifen

Marina Venzon Antunes[1,2]*, Vanessa de Oliveira[2], Suziane Raymundo[2], Dilana Elisabeth Staudt[2], Gustavo Gössling[3], Jorge Villanova Biazús[3], José Antônio Cavalheiro[3], Daniela Dornelles Rosa[4], Geneviève Mathy[5], Pierre Wallemacq[5], Rafael Linden[2], Gilberto Schwartsmann[1,3], Vincent Haufroid[5]

[1]Pós-graduação em Ciências Médicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[2]Instituto de Ciências da Saúde, Universidade Feevale, Novo Hamburgo, RS, Brazil
[3]Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[4]Hospital Moinhos de Vento, Porto Alegre, RS, Brazil
[5]Louvain centre for Toxicology and Applied Pharmacology, Institut de recherche expérimentale et clinique, Université catholique de Louvain, Brussels, Belgium;

* Corresponding author:
Universidade Feevale.
ERS 239, n° 2755
Zip code 93352-000
Novo Hamburgo – RS, Brazil
Phone/fax: (55) 51-358.8800
e-mail: marinaantunes@feevale.br

Abstract

Aim: To evaluate the impact of *CYP3A4**22 in the formation of endoxifen (EDF) and hydroxytamoxifen (HTF), under different *CYP2D6* genotypic backgrounds. Materials & Materials & methods: 178 patients were enrolled in the study. Blood samples were collected for *CYP2D6* and *CYP3A4* genotyping and tamoxifen (TAM) and metabolites quantification. Results: Regarding *CYP2D6* genotype, EDF concentrations were lower in poor (2.77 ng mL^{-1}) and intermediate metabolizers (5.84 ng mL^{-1}), comparing to functional group (EM-F) (10.67 ng mL^{-1}, $p<0.001$). Hydroxytamoxifen levels were 47% and TAM 53% higher in *CYP3A4*22* compared to **1/*1* patients in the whole group. Patients with impaired CYP2D6 metabolism and carriers of *CYP3A4*22* had EDF levels comparable to *CYP2D6 EM-F* group (9.06 and 10.67 ng mL^{-1}, $p=0.247$). Conclusion: The presence of *CYP3A4*22* might compensate the reduction of EDF concentrations related to CYP2D6 inactivity, especially due to increased HTF concentrations.

Key-words: tamoxifen; endoxifen; 4-hydroxytamoxifen; genotype; *CYP3A4*; *CYP2D6*.

INTRODUCTION

The therapeutic antiestrogenic effect of tamoxifen (TAM) [(*Z*)-1-(4-2-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene], a mainstay in the endocrine treatment of pre- and postmenopausal estrogen receptor (ER)-positive breast cancer, requires metabolic activation by cytochrome P450 (CYP) enzymes [1;2]. In the largest primary metabolic route, CYP3A4 converts TAM to N-desmethyl-tamoxifen (NDT). Via CYP2D6, NDT undergoes 4-hydroxylation and is converted to 4-hydroxy-N-desmethyltamoxifen or (Z)-endoxifen (EDF). CYP2D6 also catalyzes the metabolism of TAM to 4-hydroxy-tamoxifen (HTF), with minor contribution of CYP3A4, 2B6, 2C19 and 2C9 HTF is further converted to EDF by CYP3A4/5 [2]. The metabolic pathway of TAM is demonstrated in figure 1.

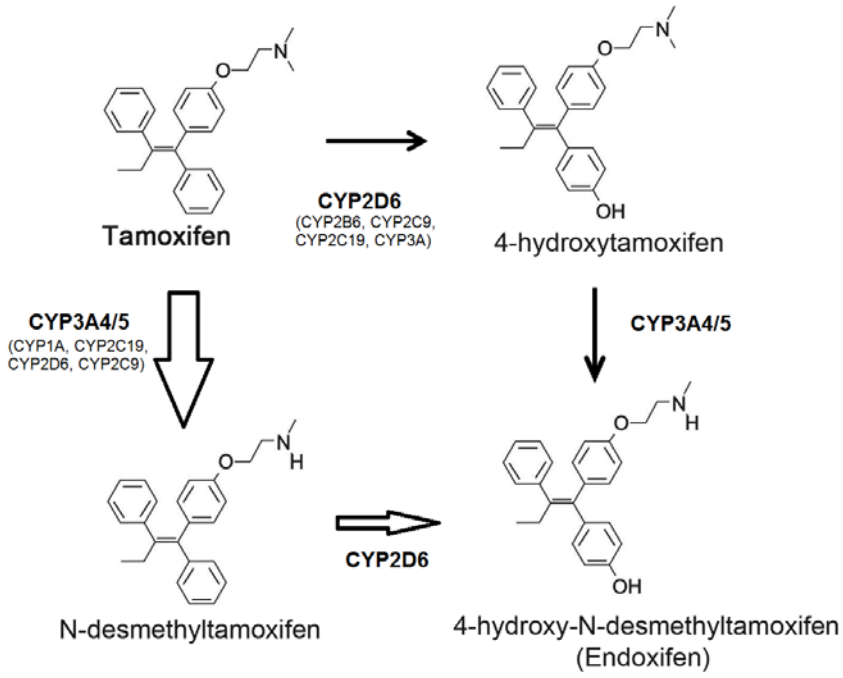


Figure 1. Metabolic pathway of tamoxifen

Tamoxifen's efficacy had initially been attributed to HTF, which is about 100 times more potent than TAM in suppressing the proliferation of estrogen-dependent cells [3]. However, EDF has been recognized to have identical properties with respect to receptor affinity and antiproliferative action, with plasma steady-state concentrations approximately 6 times higher than HTF [4,5].

The clinical benefit of TAM has been evident for more than 3 decades reducing mortality by approximately 31% and recurrences by 50% [6]. However, up to 50% of patients who receive adjuvant TAM relapse or die from tumor-specific resistance or host genome-associated factors [1]. Recently, Madlensky *et al.* (2011) exploited the relation between the clinical outcome of hormonal therapy and plasma concentrations of TAM and its metabolites, suggesting that adequate therapeutic outcome is dependent to the achievement of a threshold EDF concentration. In this study, patients presenting trough EDF plasma levels above 5.9 ng mL^{-1} had a 26 % reduction of recurrences comparing with patients with EDF concentrations below this threshold [7].

EDF plasma concentrations are highly variable among patients, what could be partly explained by polymorphisms in the *CYP2D6* gene and by interactions with CYP2D6 modulating agents, leading to insufficient plasma EDF levels in many individuals [4]. Over 150 alleles of the *CYP2D6* gene are known [8], including alleles encoding complete functional enzyme *(*1, *2, *33 ,*35*), decrease of function (DOF) enzyme *(*9, *10, *17, *29, *36, *37, *41*) and loss of function (LOF) enzymes *(*3-*8, *11-*16, *18-*20, *38, *40, *42, *44*) [9].

C*YP2D6* genotype may be translated into distinct phenotypes: poor metabolizer (PM), with no enzyme activity; intermediate metabolizer (IM), with residual enzyme activity; extensive metabolizer (EM), with normal enzyme activity, and ultra-rapid metabolizer (UM), with increased enzymatic activity. Depending on whether the EM carries one or two functional alleles, the phenotype for this group can be sub-divided into EM with homozygous fast activity (EM-F) or heterozygous slow activity (EM-S) [6]. The distribution of *CYP2D6* genotypes varies among different ethnicities. Studies demonstrate that about 7-11% of Caucasians are PM [10]. Among Brazilians, 3-5% are PM and 4-12% are IM [11-13]. Several studies systematically indicated lower EDF exposure in individuals with reduced CYP2D6 activity; average EDF plasma concentrations in EM are 3.9-8.2 fold higher than in PM and 1.9 than in IM [12,14].

In a lesser extent (20-30%), CYP3A was also related to the formation of the EDF from the biotransformation of HTF [5]. Like CYP2D6, the metabolic activity of CYP3A4 is characterized by high interindividual variability (10 to 100-fold) with a significant influence of environmental factors, such as diet, drug interactions [15] and sun exposure [16]. From a genetic point of view, a recently described *CYP3A4*22* polymorphism , within intron 6 (rs35599367; C>T), and with a

frequency of 5-7% in Caucasians, has been associated with reduced mRNA expression and CYP3A4 enzyme activity [17].

There is little knowledge about the impact of *CYP3A4* polymorphisms on EDF formation. Recently, Teft *et al*. (2013) showed higher concentrations of TAM and EDF in patients carriers of the *CYP3A4*22* allele, with two fold lower risk of sub therapeutic EDF concentrations [18]. However, Heine et al. (2014) recently reported that a model based on CYP2D6 and CYP3A4 phenotypes could predict 54% of EDF plasma concentration variability. The authors indicated that both enzymes influence exposure to EDF and CYP3A4 inhibition might result in decreased TAM efficacy due to reduced EDF levels [19].

In view of the impact of *CYP2D6* polymorphisms in EDF formation and the possible underestimation of CYP3A4 role in the metabolic activation of TAM, the present study evaluates the impact of *CYP3A4**22 polymorphism in the formation of the active equipotent TAM metabolites, EDF and HTF, under different *CYP2D6* genotypic backgrounds.

MATERIALS & METHODS

Study Population and Data Collection

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Hospital Fêmina and Hospital de Clínicas de Porto Alegre. A total of 178 patients on adjuvant hormonal treatment with tamoxifen (20 mg/day) for at least 4 months were enrolled in the study, after informed consent. Data on age, weight, body mass index (BMI), race, treatment adherence (obtained from the number of TAM tablets taken monthly), menopause status and presence of adverse effects, such as hot flashes, were recorded. In addition, the duration of TAM therapy and the reported use of any concomitant drugs considered as CYP2D6 and CYP3A4 inhibitors or inducers were also recorded. Blood samples were taken 16-24 h after the last TAM intake, with patients being fasten for 4 hours.

CYP2D6 Genotyping

DNA was extracted from EDTA-whole blood samples using the automated extraction system Magna Pure Compact® (Roche Diagnostics). Genotyping was performed using the Luminex xTAG ® *CYP2D6* Kit v3 (Luminex Corporation), which identifies the following

alleles: normal function (NF) **1, *2, *35*; decrease of function (DOF) **9, *10, *17, *29, *41*; loss of function (LOF) **3, * 4, *5, *6, *7, *8, *11, *15* and duplications, according to the manufacturer instructions. The Luminex xTAG ® technology, based on the flow cytometry principles, is a multiplex microsphere-based suspension array capable of analyzing 19 *CYP2D6* SNPs as well as the presence of gene deletion or duplication in a single well (*Duplication, deletion, -1584C>G, 31G>A, 100C>T, 124G>A, 138insT, 883G>C, 1023C>T, 1659G>A, 1661G>C, 1707T>del, 1758G>T/A, 1846G>A, 2549A>del, 2613delAGA, 2850C>T, 2935A>C, 2988G>A, 3183G>A and 4180G>C*). The amount of DNA engaged in the PCR reaction was standardized at 10 ng/mL. Briefly, the first step of the reaction is a long range multiplex PCR, followed by a multiplex Allele Specific Primer Extension (ASPE). ASPE products are then hybridized with a bead mix and incubated with a reporter solution. A TPersonal Thermocycler (Westburg) was used for the PCR and hybridization reactions. Data were acquired on the Luminex® 200™ Instrument. The data analysis software is the xTAG® Data Analysis Software (TDAS) that proposes automatically the final CYP2D6 genotype. Genotypes were classified according to the scoring system proposed by Gaedigk *et al.* (2008) in which NF alleles receive 1.0, DOF alleles 0.5 and LOF alleles 0 points. According to the sum of the alleles, patients were classified as PM (0 LOF/LOF), IM (0.5 or 1.0, LOF/DOF, DOF/DOF), EM-S (1.0 or 1.5, NF/LOF, NF/DOF), EM-F (2.0 NF/NF); UM (>2.0 NF/NF xN) [6].

CYP3A4 genotyping

Allelic discrimination analysis was performed for the determination of *CYP3A4* intron 6 C>T genotype (rs35599367). These analyses were realized using TaqMan® (Applied Biosystems, CA, USA) genotyping assays (C__59013445_10) on the ABI PRISM 7000® Sequence Detection Systems (Applied Biosystems). The final volume for each reaction was 25 µl, consisting of 12.5 µL TaqMan Universal PCR Master Mix ® (Applied Biosystems), 900 nmol/L of each primer, 200 nmol/L of each TaqMan probe, and 50 ng genomic DNA. The PCR profile consisted of an initial denaturation step at 95°C for 10 min and 50 cycles with 92°C for 15 s and 60°C for 1 min. The fluorescence level was measured with the ABI PRISM 7000 sequence detector. Genotypes were determined by Applied Biosystems Sequence Detection Systems (Elens et al Pharmacogenomics (2011) 12: 1383-1396). CYP3A4 activity scores (or gene scores) were set at

1 for patients with the *CYP3A4*1/*1* genotype and at 0 for patients possessing at least one *CYP3A4*22* allele.

Determination of TAM and metabolites concentrations in plasma

Plasma concentrations of TAM, NDT, HTF and EDF were measured by LC-MS/MS after a simple liquid-liquid extraction, as described by Antunes *et al.* (2015). Briefly, a 0.2 mL aliquot of plasma was transferred to screw cap glass tube added with 0.1 mL of internal standard solution (IS, clomiphene 0.01 μg mL^{-1}), 0.1 mL of Tris buffer pH 10 and 2 mL of extraction solvent (hexane:n-propanol 95:5, v/v). After homogenization and centrifugation, the supernatant was dried under an air stream. The extract was suspended mobile phase and a 25 μL aliquot was injected into an Ultimate 3000 XRS UHPLC system (Thermo Scientific, San Jose, USA). Separation was performed in an Acquity C18 column (150 × 2.6 mm, p.d. 1.7 μm), from Waters (Milford, USA) and mobile phase consisted of formic acid 0.1% pH 2.7 and acetonitrile plus 0.1% formic acid in gradient mode (60:40 to 50:50, v/v). Detection was performed in a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Scientific, San Jose, USA). The following transitions were used for quantification: TAM *m/z* 372→72; NDT *m/z* 358→58; EDF *m/z* 374→58; HTF *m/z* 388→72; IS (clomifene) *m/z* 406→100. The method was linear from 0.5 to 600 ng mL^{-1} with LLOQ of 7.5 ng mL^{-1} for TAM, 15 ng mL^{-1} for NDT, 1 ng mL^{-1} for EDF and 0.5 ng mL^{-1} for HTF. Accuracy was in the range 90.7-107.5 %, and within and between assay RSD were in the range of 4.8-13.4% [20].

Statistical analysis

Statistical analyses were performed with SPSS version 17.0. Descriptive analysis was initially performed for all variables. The hydroxylation ratio [NDT]/[EDF], demethylation ratios [TAM]/[NDT] and [HTF]/[EDF], TAM, NDT, HTF and EDF and the sum of EDF and HTF (active metabolites) concentrations were compared among *CYP2D6* and *CYP3A4* genotype groups and between individuals with functional and impaired CYP2D6 metabolism carriers (or not) of *CYP3A4*22* with Kruskal-Wallis test, followed by Mann-Whitney test and variables were associated in Spearman correlation analysis. The prevalence of patients with reduced EDF levels (<5.9 ng mL^{-1}) was evaluated within *CYP2D6* and *CYP3A4* genotypes through Chi-square or

Fisher exact test. The predictive power of EDF levels from *CYP2D6* and *CYP3A4* genotypes was evaluated using multiple linear regression. In all tests $p < 0.05$ was considered as statistically significant.

RESULTS

Patient Characteristics

A total of 178 women using TAM on steady-state were recruited between April 2010 and July 2013. All patients received adjuvant TAM at a dose of 20 mg daily, and were pathologically diagnosed with ER and/or PR-positive cancer. The demographic and clinical characteristics of the patients are listed in Table 1. The mean age was 57 years (range 28 to 90 years). Most of the patients were white (85.4 %), with a small representation of other ethnic groups. As expected due to TAM anti-estrogenic effects, hot flashes were the most prevalent reported side effect, occurring in 56 % of patients. Patients who indicated the use of drugs that could modulate CYP2D6 or CYP3A4 activities were excluded from further statistical evaluation. Eleven women reported the use of strong CYP2D6 inhibitor drug (fluoxetine, paroxetine or bupropione), ten of weak inhibitors (sertraline, venlafaxine, citalopram, chlorpromazine) and two of both strong and weak inhibitors (haloperidol together with bupropione or fluoxetine) on a daily basis. Additionally, one patient was using a CYP3A4 inductor (carbamazepine) and other was using a CYP3A4 inhibitor drug (ritonavir). All patients were classified as adherent to TAM, reporting the use of at least 29 tablets (96.7% of adherence) monthly.

Table 1. Patients demographic characteristics according to CYP2D6 predicted phenotypes (N=178).

Characteristic	(N=178)	CYP2D6 PM (n=10)	CYP2D6 IM (n=14)	CYP2D6 EM-S (n=65)	CYP2D6 EM-F (n=75)	CYP2D6 UM (n=8)
Age (years)						
Mean (SD)	57 (10.8)	54 (13.2)	58 (12.2)	57 (10.1)	57 (11.1)	59 (9.7)
Range	28–90	30-74	44-90	40-81	28-81	46-73)
Race/Ethnicity (n)						
White	152	10	13	52	67	7
Others	26	0	1	14	8	1
Tamoxifen duration (months)						
Median (Percentiles 25-75)	24.5 (11–40)	17 (6-25)	15 (8-29)	16 (8-30)	22 (12-32)	10 (7-23)
Range	(4–82)	(4-32)	(4-40)	(4-82)	(4-65)	(5-47)
Menopause status (n)						
Pre menopause	57	4	3	8	23	3
Post menopause	121	2	5	14	14	2
CYP3A4 genotypes (n)						
*1/*1	161	10	11	59	69	6
*1/*22	16	-	3	5	6	2
*22/*22	1	-	-	1	-	-
Strong CYP2D6 inhibitor* (n)						
Fluoxetine/paroxetine/bupropione	13	0	0	5	6	0
Weak CYP2D6 inhibitor (n)						
Sertraline/venlafaxine/citalopram/ Chlorpromazine/haloperidol**	12	1	2	4	5	0
CYP3A4 inductor/inhibitor (n)						
Carbamazepine	1	0	0	1	0	0
Ritonavir	1	0	0	1	0	0
Adverse effects (n) no/yes						
Hot flashes	78/100	3/7	8/6	25/41	37/37	2/6
Vaginal bleeding	163/15	2/8	1/13	5/61	6/68	0/8
Vaginal discharge	118/60	5/5	10/4	40/26	56/18	3/5
Vulvar Itching	139/39	8/2	12/2	48/18	61/13	6/2
Nausea or vomiting	134/43	8/2	10/4	46/20	59/15	7/1
Dizzeness	131/47	10/0	11/3	46/20	52/22	8/0
Cutaneous rash	164/14	9/1	14/0	59/7	68/6	8/0
Alopecia	133/45	8/2	12/2	47/19	53/21	8/0
Thrombosis	165/12	9/1	12/2	64/2	67/7	8/0

ER +: estrogen receptor positive, PR +: progesterone receptor positive. * two patients with heterozygote allelic duplication for CYP2D6 (unknown gene scores of 1 or 2) were taking CYP2D6 strong inhibitor drugs **patients taking haloperidol together with bupropione or fluoxetine.

CYP2D6 and CYP3A4 Genotypes

CYP2D6 and *CYP3A4* alleles and frequencies are presented in Table 2. *CYP3A4*22* allele, associated to reduced enzyme activity, was present with an allelic frequency of 5.1% while functional *CYP3A4*1* allele was present with an allelic frequency of 94.9%. *CYP3A4*22* allele was present only in white women. We screened 16 different alleles for CYP2D6, including multiple copies of the gene and gene deletion. Gene scores were given according to Gaedigk *et al.* (2008) CYP2D6 activity scoring system. The most frequent *CYP2D6* alleles were **1* (39.3%), **2* (21.6%) and **4* (16.6%). Considering all tested alleles, 69.6 % were considered as functional, 22.3 % as null and 11.5 % as dysfunctional.

Table 2. *CYP2D6* and *CYP3A4* alleles and frequencies (N=178).

	Allele	**n**	**Allele frequency (%)**
CYP2D6	**1*	**131**	36.8
	**1 x N*	**9**	2.5
	**2*	**63**	17.7
	**2 x N*	**14**	3.9
	**35*	**19**	5.3
	**9*	**12**	3.4
	**10*	**3**	0.8
	**17*	**4**	1.1
	**29*	**4**	1.1
	**41*	**17**	4.8
	**41 x N*	**1**	0.3
	**3*	**6**	1.7
	**4*	**49**	13.8
	**4 x N*	**10**	2.8
	**5*	**12**	3.4
	**6*	**2**	0.6
N		**356**	100
CYP3A4	**22*	**18**	5.1
	**1*	**338**	94.9
N		**356**	100

The frequencies of CYP3A4 and CYP2D6 genotype-based predicted phenotypes are shown in Table 3. Carriers of *CYP3A4*22* with reduced enzyme activity (**1/*22* and **22/*22*), corresponded to 9.5% of patients, whereas 91.5% of the study population had a functional genotype (**1/*1*). Regarding CYP2D6, the prevalence of patients with impaired enzyme activity was 5.6% (PM, n=10) and patients with deficient enzyme activity were 7.8% of the whole group (IM, n=14). Considering the patients possessing at least one functional allele, 36.5% were classified as EM-S (n=65) with *CYP2D6* activity scores of 1 or 1.5, related to moderate/rapid activity, and 42.1 % as EM-F (n=75) with activity scores of 2, corresponding to rapid activity. The UM group (NF alleles with duplication, score >2) represented 4.5% of the patients (n=8). Patients with heterozygous alleles duplication (n=6, 3.4%), which can have gene score of 1 or 2 were not included in CYP2D6 genotype statistics.

The distribution of *CYP3A4* reduced activity genotypes (*1/*22 or *22/*22) through CYP2D6 genotype-based predicted phenotype groups was: 21.4% of IM patients (**1/*22*, n=3), 9.2% of EM-S *(*1/*22*, n=5 and **22/*22*, n=1), 8% of EM-F (**1/*1*, n=6) and 25% of UM (**1/*1*, n=2). There was no impaired *CYP3A4* genotype in the *CYP2D6* PM group (Table 1).

Table 3. *CYP2D6* and *CYP3A4* genotypes, predicted phenotypes and frequencies (N=178).

(Genotype) n	Alleles phenotype	Score	Activity	Predicted phenotype	n	Frequency (%)
CYP2D6 Genotypes						
6 (**4/*4*) 2 *(*4/*4 DUP)* 2 (**4/*5*)	LOF/LOF	0	Deficient	PM	10	5.6
1 *(*4*17)* 3 *(*4/*41)* 1 (**4/*9*) 2 *(*5/*41, DEL)* 1 (**6/*41*)	LOF/DOF	0,5	Slow	IM	14	7.8
2 (*41/*41) 1 (*9/*9) 1 (**17/*29*) 2 (**9/*29*)	DOF/DOF	1				
19 (**1/*4*) 7 (**1/*5, DEL*) 10 (*2/*4) 2 (**1/*3*) 1 (**4/*35*) 1 (**5/*35, DEL*) 2 (**2/*3*) 2 (**3/*35*) 1 (**1/*6*)	NF/LOF	1	Moderate			
2 (**2/*9*) 2 (**2/*41*) 5 (**1/*41*) 4 (**1/*9*) 2 (**1/*10*) 1 (**9/*35*) 1 (**2/*17*) 1 (**1/*17*) 1 (**10/*35*) 1 (**1/*29*)	NF/DOF	1,5	Moderate/ Rapid	EM-S	65	36.5
28 (**1/*1*) 5 (**2/*35*) 25 (**1/*2*) 8 (**1/*35*) 8 (**2/*2*) 1 (**2/*41*,DUP)	NF/NF NF/DOF, xN	2	Rapid	EM-F	75	42.1
4 (*2/*4 DUP)* 2 (*1/*4 DUP)*	NF/LOF, xN		Moderate to Rapid	EM-S/EM-F	6	3.4
7 (**1/*2*,DUP) 1 (**2/*2*, DUP)	NF/NF xN	3	Ultra-rapid	UM	8	4.5
CYP3A4 Genotypes						
1 (*22/*22) 16 (*1/*22)	TT CT	.0..	Deficient	PM	17	9.5
161 (*1/*1)	CC	1	Rapid	EM	161	90.5

DUP: duplication; DEL: deletion; LOF: loss of function; DOF: decrease of function; NF: normal function; PM: poor metabolizer; IM: intermediate metabolizer; EM-S: extensive metabolizer slow activity; EM-F: extensive metabolizer fast activity; UM: ultra-rapid metabolizer.

Tamoxifen metabolism in association to CYP2D6 and CYP3A4 genotypes

TAM and metabolite concentrations were highly variable among patients. Median plasma concentrations and 25-75% percentiles were: TAM 92.42 ng mL^{-1} (57.16-125.37 ng mL^{-1}); NDT 178.62 ng mL^{-1} (117.92-256.52 ng mL^{-1}); EDF 7.94 ng mL^{-1} (4.62-11.91 ng mL^{-1}) and HTF 1.10 ng mL^{-1} (0.79-1.81 ng mL^{-1}). EDF levels ranged from 1.48 ng mL^{-1} to 28.08 ng mL^{-1} and were in average 7.2-fold higher than HTF concentrations. The sum of both equipotent active metabolites (EDF+HTF) was in the range of 1.56 to 31.06 ng mL^{-1}, with median of 9.11 ng mL^{-1}.

Table 4 shows the correlations among TAM, NDT, EDF and HTF plasma concentrations, metabolic ratios and genotypic activity scores for *CYP2D6* and *CYP3A4*. Patients under concomitant use of drugs that could interfere on TAM metabolism were not included at this evaluation. EDF concentrations were highly correlated to HTF levels (rs=0.626, P<0.01) and to the metabolic ratio [NDT]/[EDF] (r_s=-0.541, P<0.01), as well as to the ratio [HTF]/[EDF] (r_s=-0.510, P<0.01).

CYP2D6 gene score was moderately correlated to EDF concentrations (r_s=0.434, P<0.01), whereas presented a higher correlation to the metabolic ratio [NDT]/[EDF] (r_s=-0.567, P<0.01). CYP3A4 gene score was inversely correlated to TAM (r_s=-0.184, P<0.05) and HTF concentrations (r_s=-0.196, P<0.05), association even stronger when excluding *CYP2D6* EM-F/UM patients and users of CYP2D6/CYP3A4 inhibitors/inducers drugs (n=81) (r_s=-0.289, P<0.01 and r_s=-0.269, P<0.01, respectively). However, *CYP3A4* gene score was not associated to EDF plasma concentrations (r_s=-0.064, P=0.43).

Multiple linear regression revealed that *CYP2D6* and *CYP3A4* gene scores together can predict only 16% of EDF plasma levels. Nevertheless, *CYP3A4* gene scores showed no statistically significance in the regression model (P=0.144), in contrast to CYP2D6 gene scores (P<0.001). Similar situation was found for HTF with 17% of prediction power, where both gene scores were statistically significant in the multiple linear regression (CYP3A4, p<0.01; CYP2D6, P<0.05).

Table 4. Correlations of TAM, metabolites, metabolic ratios and CYP2D6 and CYP3A4 gene scores among patients (n=153, $^{+}$CYP2D6 scores n=149)

	EDF	**HTF**	**NDT**	**EDF+HTF**	**[NDT]/[EDF]**	**[TAM]/[NDT]**	**[HTF]/[EDF]**	***CYP3A4* gene score**	***CYP2D6* gene score^{+}**
TAM	0.436**	0.362**	0.714**	0.449**	0.220**	0.249**	-0.127	-0.184*	-0.114
EDF		0.626**	0.202*	0.991**	-0.541**	0.264**	-0.510**	-0.064	0.434**
HTF			0.257**	0.711**	-0.246**	0.129	0.270**	-0.196*	0.252**
NDT				0.221**	0.629**	-0.464**	0.075	-0.085	-0.367**
EDF + HTF					-0.467**	0.258**	-0.418**	-0.073	0.428**
[NDT]/[EDF]						-0.626**	0.419**	-0.007	-0.567**
[TAM]/[NDT]							-.210**	-0.108	0.338**
[HTF]/[EDF]								-0.153	-0.299**
***CYP3A4* gene score**									0.050

Spearman correlation coefficient, *p<0,05, **p<0,01.
+patients with heterozygote allelic duplication for CYP2D6 (unknown gene scores of 1 or 2) were not included in CYP2D6 correlation analysis. Additionally, two of the six patients using concomitant CYP2D6 inhibitor drugs were also excluded.

Tamoxifen and metabolites concentrations according to CYP2D6 genotypes

Medians and percentiles of TAM and metabolites plasma concentrations among *CYP2D6* and *CYP3A4* genotypes are summarized in Table 5. Evaluations were performed excluding users of CYP2D6 or CYP3A4 inhibitor/inductor drugs. Besides TAM (P=0.173), all metabolites concentrations and metabolic ratios were significantly different among *CYP2D6* genotypes groups (P<0.01). Median plasma EDF concentrations increased according to the number of functional *CYP2D6* alleles. Median EDF plasma concentrations were 2.77, 5.84 and 7.83 ng mL^{-1} in PM, IM and EM-S patients, respectively. These patients had lower EDF levels than EM-F (10.67 ng mL^{-1}, P<0.001), with median concentrations representing 26.0%, 54.7% and 73.4 %, respectively, of those measured in the functional group EM-F. EDF concentrations in PM were also statistically significantly different from IM and EM-S groups (P<0.05). The same trend was observed for HTF, in which PM (0.57 ng mL^{-1}) presented lower median levels compared to IM, EM-S, EM-F and UM (P<0.01). The impact of *CYP2D6* genotypes on the sum of EDF and HTF plasma concentrations reflected those obtained for the metabolites alone, increasing

proportionally to enzyme functionality (P<0.01). Median NDT was superior in PM and IM compared to those with functional alleles (P<0.001), resulting from a lower hydroxylation ratio in subjects with reduced *CYP2D6* activity.

The metabolic ratio [NDT]/[EDF], which is dependent on CYP2D6 metabolism, was 7.7-fold higher in PM (108.8) compared to EM-F (14.12), as well as IM and EM-S (54.77 and 23.06, respectively) compared to functional genotype (P<0.01). Despite the demethylation process is not dependent on CYP2D6 metabolism, [TAM]/[NDT] metabolic ratios were different among *CYP2D6* genotypes (P<0.001), probably due to equivalent TAM levels within the different *CYP2D6* genotypes groups and the reduction of NDT concentrations with the increase of enzyme functionality. Similar observation was noted with [HTF]/[EDF] metabolic ratio, as PM had higher ratios compared to other groups (P<0.01). Since biotransformation of HTF to EDF is not dependent on CYP2D6, this finding can be attributed to significant lower EDF concentrations found in PM, compared to other *CYP2D6* genotypes.

Tamoxifen and metabolites concentrations according to CYP3A4 genotypes

TAM metabolism mediated by CYP3A4 is responsible for demethylation of TAM to NDT and HTF to EDF. Impaired CYP3A4 activity due to the presence of the **22* allele led to a reduction of the biotransformation of TAM to NDT, resulting on an increase of TAM concentrations in **22* carriers (n=13), with median level of 130.82 *versus* 85.05 ng mL^{-1} in the **1/*1* group (n=140) (P<0.05). Also, a higher [TAM]/[NDT] metabolic ratio of 0.72 *versus* 0.52 for **22* carriers compared to **1/*1* patients was observed (P<0.05). The same trend was observed concerning HTF level, which is converted to EDF by CYP3A, where *CYP3A4*22* carriers had a 47% increase of median concentrations of the active metabolite compared to **1/*1* group (1.71 *versus* 1.16 ng mL^{-1}, P<0.05). Box plot charts of TAM and metabolites concentrations according to *CYP2D6* and *CYP3A4* genotypes are shown in figure 2.

Table 5. Median tamoxifen, metabolites concentrations and metabolic ratios according to *CYP2D6* (n=149) and *CYP3A4* predicted phenotypes (n=153).

	n	TAM (ng mL^{-1}) MD (P25 – P75)	EDF (ng mL^{-1}) MD (P25–P75)	HTF (ng mL^{-1}) MD (P25–P75)	EDF+HTF (ng mL^{-1}) MD (P25 – P75)	NDT (ng mL^{-1}) MD (P25 – P75)	[TAM]/[NDT] MD (P25 – P75)	[NDT]/[EDF] MD (P25–P75)	[HTF]/[EDF MD (P25–P75)
CYP2D6 Genotypes									
PM	9	122.04 [a] (69.18-142.82)	2.77 [a] (2.38-3.40)	0.57 [a] (0.47-0.96)	3.35 [a] (2.85-4.35)	300.46 [b] (232.38-442.89)	0.33 [a] (0.21-0.50)	108.8 [d] (92.90-125-60)	0.22 [b] (0.20-0.29)
IM	12	102.43 [a] (85.03-161.58)	5.84 [b] (3.61-10.47)	1.10 [b,c] (0.86-2.08)	6.98 [b] (4.48-11.96)	257.32 [b] (164.99-419.36)	0.43 [a] (0.32-0.69)	54.77 [c] (18.58-65.47)	0.19 [a] (0.12-0.22)
EM-S	56	79.29 [a] (52.81-114.73)	7.83 [b] (5.00-9.82)	1.04 [b] (0.81-1.72)	8.90 [b] (5.82-11.34)	170.06 [a] (119.57-255.42)	0.44 [a] (0.32-0.64)	23.06 [b] (15.40-28.97)	0.14 [a] (0.11-0.21)
EM-F	64	89.68 [a] (59.13-124.65)	10.67 [c] (6.85-14.51)	1.51 [c] (0.93-2.06)	12.14 [c] (8.31-16.49)	145.34 [a] (110.53-211.51)	0.59 [b] (0.45-0.71)	14.12 [a] (10.18-19.54)	0.12 [a] (0.09-0.19)
UM	8	82.70 [a] (52.50-112.14)	10.06 [c] (8.81-17.21)	1.41 [c] (0.99-1.87)	11.47 [c] (10.39-18.64)	104.26 [a] (97.07-195.53)	0.81 [b] (0.44-1.01)	10.35 [a] (7.07-11.19)	0.11 [a] (0.08-0.17)
	149	**p** 0.173	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	<0.01
CYP3A4 Genotypes									
***22/*22 *1/*22**	13	130.82 (65.93-166.33)	9.25 (7.31 – 11.40)	1.71 (1.35-2.05)	10.97 (9.03-13.5)	191.71 (115.50-253.59)	0.72 (0.48-0.78)	16.37 (11.46-23.43)	0.18 (0.12-0.22)
***1/*1**	140	85,05 (54.75-119.89)	8.21 (5.21-13.04)	1.16 (0.81- 1.84)	9.49 (6.36-14.03)	165.69 (116.92-243.73)	0.52 (0.35-0.66)	18.54 (10.86-27.94)	0.14 (0.10-0.20)
	15	**p** <0.05	0.357	<0.05		0.531	<0.05	0.700	0.06
CYP2D6 and CY3A4 Genotypes									
CYP2D6 EM-F & CYP3A4*22 or *1/*1	64	89.68 (59.13-124.65)	10.67 (6.85-14.51)	1.51 (0.93-2.06)	12.14 (8.31-16.49)	145.34 (110.53-211.51)	0.59 (0.45-0.71)	14.12 (10.18-19.54)	0.12 (0.09-0.19)
CYP2D6 IM/EM-S & CYP3A4*22	8	150.72 (99.96-188.54)	9.08 (5.97-13.13)	1.94 (1.72-2.04)	10.94 (7.34-15.16)	244.72 (214.72-440-41)	0.69 (0.32-0.76)	23.33 (13.87-65.47)	0.20 (0.15-0.23)
	72	**p** <0.01	0.274	0.124	0.351	<0.05	0.641	<0.05	<0.05
CYP2D6 EM-F & CYP3A4*22 or *1/*1	64	89.68 (59.13-124.65)	10.67 (6.85-14.51)	1.51 (0.93-2.06)	12.14 (8.31-16.49)	145.34 (110.53-211.51)	0.59 (0.45-0.71)	14.12 (10.18-19.54)	0.12 (0.09-0.19)
CYP2D6 IM/EM-S & *CYP3A4 *1/*1*	60	81.97 (54.37-114.73)	7.36 (4.77-9.92)	1.02 (0.81-1.70)	8.84 (5.66-11.92)	176.08 (120.55-265.37)	0.49 (0.32-0.62)	24.85 (15.39-36.50)	0.14 (0.11-0.20)
	72	**p** 0.642	<0.01	<0.05	<0.01	0.09	0.134	<0.01	0.14

P value as Mann-Whitney test for *CYP3A4* genotypes and Kruskal-Wallis test for *CYP2D6* genotypes($\alpha = 0.05$) [a,b,c,d] same letter in the column do not differ in at 5 % of significance

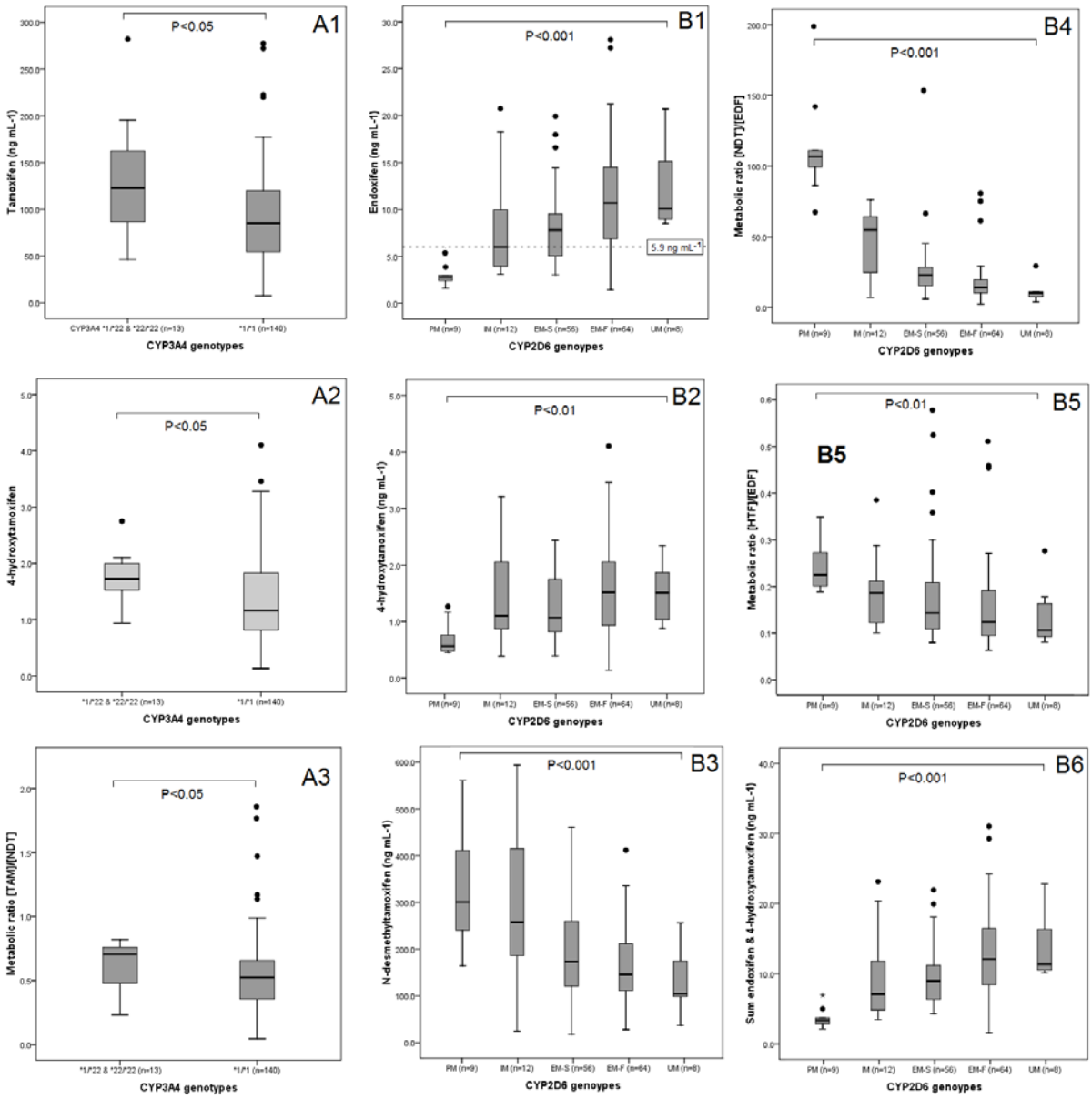


Figure 2. Tamoxifen and metabolites plasma concentrations and metabolic ratios according to *CYP3A4* and *CYP2D6* genotypes. Statistically significant differences between *CYP3A4* genotypes groups: (A1) tamoxifen; (A2) 4-hydroxytamoxifen; (A3) Metabolic ratio [TAM]/[NDT]; and among groups of *CYP2D6* genotypes: (B1) endoxifen; (B2)4-hydroxytamoxifen; (B3) N-desmethyltamoxifen; (B4) metabolic ratio [NDT]/[EDF]; (B5) metabolic ratio [HTF]/[EDF] and (B6) sum of endoxifen and 4-hydroxytamoxifen. Data are represented as median, 25% and 75% percentiles, and range.

CYP3A4*22 and TAM metabolic activation within individuals with CYP2D6 impaired metabolism

Based on the findings that *CYP3A4* genotype was associated to TAM and HTF plasma concentrations, with higher levels found in **22* carriers, we evaluated if the presence of *CYP3A4*22* allele could compensate for impaired CYP2D6 metabolism by increasing the concentrations of the active metabolites EDF and HTF. Thus, we compared TAM and metabolites concentrations, as well as metabolic ratios, at two groups, according to the combination of *CYP2D6* and *CYP3A4* genotypes. The first evaluation was performed comparing patients with *CYP2D6* gene score < 2 (IM and EM-S, n=8) carriers of *CYP3A4*22* with patients possessing *CYP2D6* rapid gene score of 2, independently of CYP3A4 genotype (EM-F, n=64). For the second evaluation, the group with *CYP2D6* gene score < 2 (IM and EM-S) was associated to *CYP3A4*1/*1* patients (n=60) and compared to the group of patients with *CYP2D6* gene score of 2 (EM-F, n=64). Participants under concomitant use of enzyme inhibitor/inductor drugs were not included in this evaluation. TAM plasma concentrations were higher in the presence of *CYP3A4*22* allele at CYP2D6 IM/EM-F group comparing to functional CYP2D6 group ($P<0.01$). While no difference was found between CYP3A4*1/*1 patients with impaired CYP2D6 metabolism and CYP2D6 score 2 patients, confirming our findings. *CYP2D6* IM/EM-S patients with *CYP3A4*1/*1* had lower median concentrations of EDF, HTF and the sum of both (7.36, 1.02 and 8.84 ng mL^{-1}, respectively), when compared to *CYP2D6* EM-F group (10.67, 1.51 and 12.84 ng mL^{-1}, respectively; $P<0.01$ for EDF and sum EDF+HTF, $P<0.05$ for HTF), reinforcing the significant impact of CYP2D6 activity on TAM metabolic activation. When the evaluation was performed with *CYP3A4*22* carriers at *CYP2D6* IM/EM-S group, the concentrations of the active metabolites (EDF 9.06 ng mL^{-1}, HTF 1.94 ng mL^{-1}, EDF + HTF 10.94 ng mL^{-1}) were not different from EM-F patients (EDF 10.67 ng mL^{-1}, HTF 1.51 ng mL^{-1}, EDF + HTF 12.14 ng mL^{-1}; P=0.247, 0.124 and 0.351, respectively), indicating that the presence of the **22* allele could , at least partly, compensate for impaired CYP2D6 metabolism. Box-plot charts are presented in Figure 3. Median EDF, HTF and sum of both concentrations in *CYP2D6* IM/EM-S *CYP3A4*22* group were 23, 91 and 24%, respectively, superior to median concentrations in *CYP3A4*1/*1* individuals.

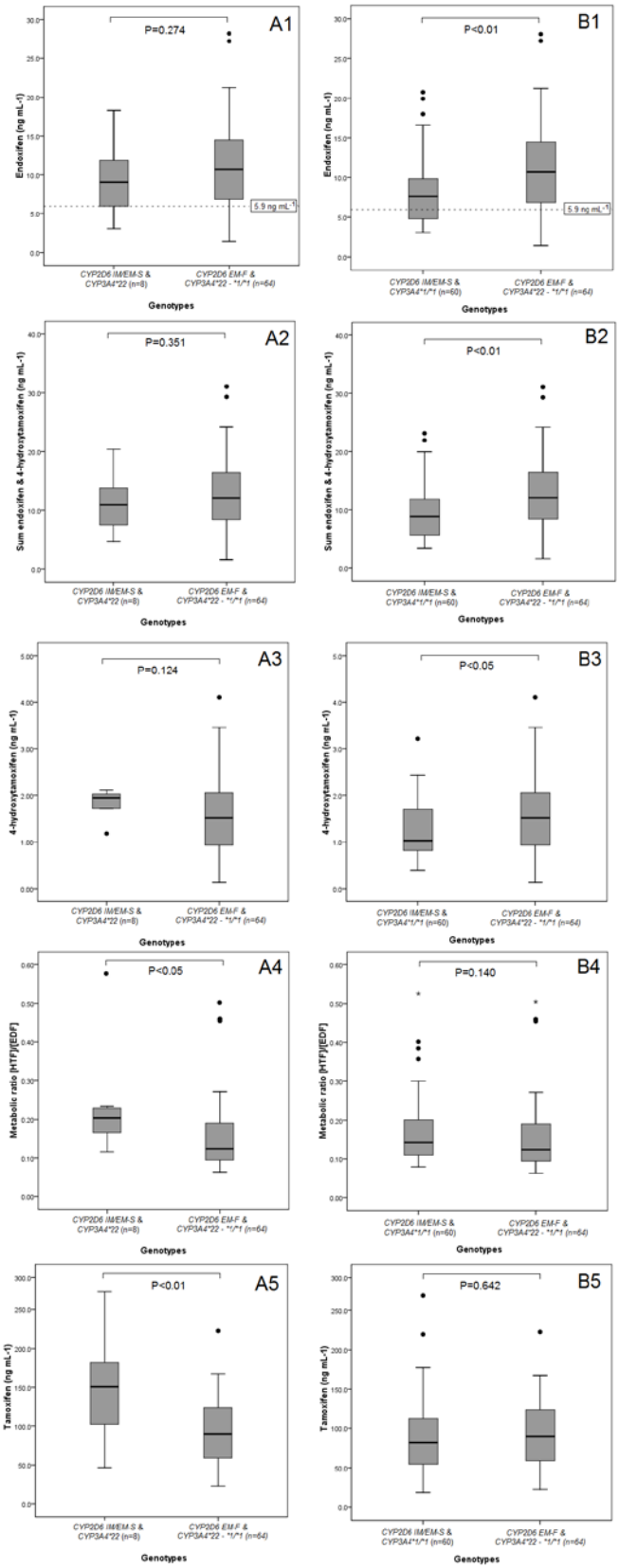


Figure 3 Tamoxifen and metabolites plasma concentrations and metabolic ratios according to *CYP2D6 EM-F* (*CYP3A4*22* or **1/*1*) genotype and *CYP2D6 IM/EM-S* genotype (*CYP3A4*22*). (A1 and B1) endoxifen; (A2 and B2) sum of endoxifen and 4-hydroxytamoxifen; (A3 and B3) 4-hydroxytamoxifen; (A4 and B4) metabolic ratio [HTF]/[EDF]; (A5 and B5) tamoxifen. Data are represented as median, 25% and 75% percentiles, and range.

We also examined the prevalence of patients with EDF plasma levels below 5.9 ng mL^{-1}, the concentration previously related to better clinical outcomes [7], according to *CYP2D6* and *CYP3A4* genotypes, excluding patients with use of CYP2D6 or CYP3A4 inhibitor/inducer drugs. Concerning *CYP2D6* genotypes, there was a significant reduction on the frequency of patients with EDF levels < 5.9 ng mL^{-1} with the increase of genotypic activity score ($P<0.01$ in Chi-square test). In fact, 100% of PM, 50 % of IM and 36 % of EM-S subjects had EDF below levels the suggested threshold. In contrast, only 19 % of EM-F and none of UM subjects were below this value. Considering the CYP3A4 genotype, the reduction of enzyme activity was related to lower prevalence of EDF concentrations below the proposed concentration threshold [7] ($p<0.01$ in Fisher exact test), which was observed in 15.3% of carriers of the **22* allele and 31.4% of *1/*1 carriers (Figure 4).

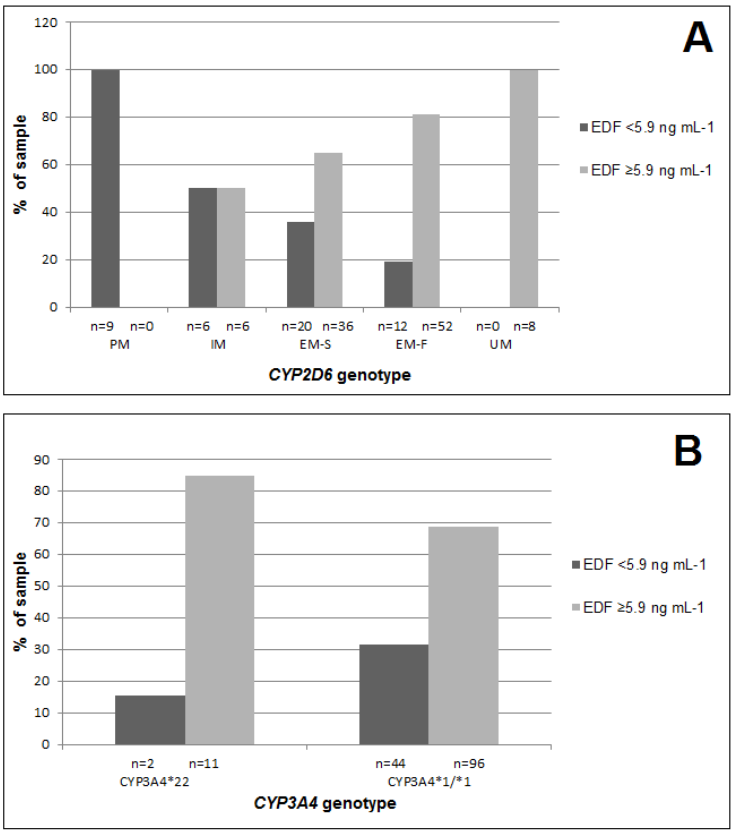


Figure 4. Frequency of patients with endoxifen levels below clinical threshold [7], according to *CYP2D6* and *CYP3A4* genotypes.

DISCUSSION

CYP2D6 genetic polymorphisms and their implication on TAM efficacy have been intensively studied; worse outcomes are related to the presence of reduced or non-functional *CYP2D6* alleles and low EDF exposure [7;21;22]. Although the link between *CYP2D6* status and TAM metabolism was established in our previous study [12], the current work incorporates an evaluation of the impact of *CYP3A4*22* polymorphism on the formation of TAM active metabolites in a larger population of patients. We identified five CYP2D6 predicted phenotypes, based on gene scores: PM (5.6%), IM (7.8 %), EM-S (36.5%), EM-F (42.1%) and UM (4.5%). The frequencies of PM and IM predicted phenotypes are similar to those observed in Caucasians (5.9% PM and 10.6% IM), possible due to Brazilian Southern population being characterized by a higher density of citizens from European origin than any other Brazilian regions [11].

EDF plasma concentrations were consistent with those previously reported in the literature [12;23;24] and showed a *CYP2D6* gene-dose effect. *CYP2D6* PM patients had the lowest levels, confirming CYP2D6 as the major enzyme in the formation of this metabolite. Lower HTF concentrations were also found in PM, which was expected based on the biotransformation pathway of TAM to HTF, involving mainly CYP2D6. However, the difference was not observed within other genotype groups, as CYPs 3A4, 2B6, 2C19 and 2C9 participate with minor contribution to its formation [5] and could compensate the impaired CYP2D6 activity. EDF and HTF concentrations were highly correlated ($r=0.626$, $P<0.01$). Also, the sum of the equipotent metabolites EDF and HTF presented a higher contribution from EDF ($r=0.991$, $P<0.01$) over HTF ($r=0.711$, $P<0.01$), due to higher EDF concentrations. The magnitude of EDT to HTF difference (7.2-fold) was in accordance to previously reported by Teunissen *et al.* (2009) and Murdet *et al.* (2011), of approximately 6-fold [4,5].

NDT conversion to EDF, assessed as the metabolic ratio [NDT]/[EDF], can be used as an index to measure CYP2D6 activity. As expected, we found a significant inverse correlation between [NDT]/[EDF] and *CYP2D6* genotype score ($r_s=-0.567$, $P<0.01$). Similar trend was found for the metabolic ratio [TAM]/[NDT], as a result of NDT reduced biotransformation. In addition, the metabolic ratio [HTF]/[EDF] was inversely proportional to CYP2D6 functionality, due to reduced EDF plasma concentrations.

CYP2D6 gene scores were moderately correlated to EDF plasma concentrations (r_s=0.434, p<0.01), indicating that *CYP2D6* polymorphisms do not fully predict the variability of plasma EDF concentrations. Additionally, even in the absence of CYP2D6 activity, there is still some formation of EDF, with median plasma concentrations about 4 times lower than in individuals with complete enzyme activity. Importantly, all CYP2D6 PM, as well as half of IM patients, had EDF plasma concentrations bellow the proposed clinical threshold (5.9 ng mL^{-1}) [7]. However, we identified a considerable number of patients with functional *CYP2D6* alleles presenting EDF plasma concentrations below this threshold as well (35% EM-S and 19% EM-F).

The above data suggest that other enzymes and/or environmental factors must contribute to plasma EDF exposure and remain to be elucidated. Recently, Heine *et al*. (2014) indicated that CYP3A inhibition might result in decreased TAM efficacy due to reduced EDF levels [19]. In contrast, Teft *et al.* (2013) demonstrated an increase on TAM and metabolites concentrations in *CYP3A4*22* carriers [18]. Thus, the role of CYP3A4 on metabolic activation of TAM still needs to be clarified.

*CYP3A4*22* polymorphism has been previously related to enzyme reduced expression and activity [17]. Our findings suggest a significant inverse correlation of TAM plasma concentrations with *CYP3A4* gene scores, as well as higher metabolic ratios [TAM]/[NDT], within *CYP3A4*22* carriers. The increase of TAM plasma concentrations might be a result of its reduced biotransformation to NDT, the most abundant primary metabolic route, mediated through CYP3A4 [5]. Interestingly, NDT concentrations were not lower in the *CYP3A4*22* group, probably due to NDT further conversion to EDF, mediated through CYP2D6 and affected by its polymorphisms, as PM and IM metabolizers had higher levels compared to others with at least one functional CYP2D6 allele. In a recent study, Teft *et al.* (2013), observed higher levels of all TAM measured metabolites, including NDT, while retaining the predicted higher TAM levels, in accordance with our results. The authors suggested that a reduction in intestinal CYP3A4 activity in *CYP3A4*22* carriers may reduce first pass metabolism resulting in enhanced TAM bioavailability and consequently to increased levels of its metabolites in *CYP3A4*22* carriers [18]. However, Wang *et al.* (2011) described no reduction on CYP3A4 activity in the intestine but only in the liver, with first pass metabolism mainly due to the liver [17]. Additionally, it is important to highlight that CYP3A4 also mediates other important biotransformation pathway of

TAM, therefore being unlikely that impaired enzyme activity would not also reduce the metabolism of TAM to NDT.

Given that the *CYP2D6* gene-dose effect on HTF concentrations was found to be less pronounced (r_s=0.252, P<0.01), enzyme activity seems not to be a rate-limiting factor in the formation of this active metabolite. Rather, plasma HTF concentrations were inversely associated to *CYP3A4* gene score. HTF plasma concentrations were higher in *CYP3A4*22* carriers, and this difference could partly be attributed to the reduction of CYP3A4 mediated pathway (TAM to NDT), with higher CYP2D6 mediated transformation of TAM to HTF, as an alternative route. In addition, further metabolization of HTF to EDF requires CYP3A4 activity and low enzyme activity explains a lower degradation/higher accumulation, in addition to higher formation rate of HTF.

As TAM metabolism is complex, it is difficult to determine the effect of single CYP isoform polymorphisms [18]. The association between *CYP3A4* gene scores and TAM plasma concentrations, as well as HTF, was significantly higher within CYP2D6 impaired metabolizers (PM/IM/EM-S), when comparing to the entire cohort (r_s=-0.289 *versus* r_s=-0.184 and r_s=-0.269 *versus* r_s=-0.196, respectively). The increase on TAM and HTF plasma concentrations, already observed in the presence of *CYP3A4*22* allele, was confirmed with an increase on TAM and HTF median levels of 68 and 28%, respectively (even not statistically significant), in *CYP2D6* IM/EM-S *CYP3A4*22* carriers, when compared to *CYP2D6 EM-F* (independently of CYP3A4 genotype). At the contrary, HTF was significantly lower for *CYP2D6* IM/EM-S *CYP3A4*1/*1* compared to *CYP2D6* EM-F, while TAM was comparable between the groups.

Although the contribution of CYP3A4 to the EDF concentrations appears to be small and not statistically significant, this route may become apparent when CYP2D6 activity is reduced. While *CYP3A4* gene scores had no statistical significant correlation to EDF levels (rs=-0.064, P=0.416), a smaller number of patients carriers of *CYP3A4*22* (2.2%) had EDF plasma concentrations below the proposed clinical threshold [7], compared to CYP3A4*1/*1 patients (11%). EDF concentrations were comparable between *CYP2D6 IM/EM-S* patients carrying also at least one *CYP3A4*22* allele and *CYP2D6 EM-F* patients (independently of CYP3A4 genotype), representing 85% of the concentrations found in patients with two *CYP2D6* functional alleles. A similar evaluation was performed within the same impaired *CYP2D6* genotype group,

but with the presence of *CYP3A4*1/*1*. In this case, we found lower EDF levels, corresponding to 69% of those found in CYP2D6-EM patients. Since the metabolic ratio [NDT]/[EDF] was higher for *CYP2D6 IM/EM-S*, even in the presence of *CYP3A4*22*, when compared to *CYP2D6 EM-F*, we excluded a direct role of CYP3A4 in the formation of EDF from NDT, but confirmed the influence of CYP3A4 activity in EDF formation from HTF. Same trend was observed for sum of EDF and HTF concentrations, which was comparable between *CYP2D6* IM/EM-S carriers of *CYP3A4*22* and *CYP2D6* EM-F patients, but lower in CYP2D6 IM/EM-S carriers of *CYP3A4 *1/*1*. Patients with impaired CYP2D6 metabolism and concurrent presence of *CYP3A4*22* had EDF + HTF concentrations 24% higher than patients at the same groups of *CYP2D6* genotype, but carrying *CYP3A4*1/*1*.

CONCLUSION

Our data suggest that CYP3A4 contributes to the metabolic activation of TAM through formation of HTF and EDF. CYP2D6 is the major enzyme responsible for EDF formation, but in conditions of reduced or absent CYP2D6 activity, it seems that CYP3A4 becomes increasingly important. The reduction of CYP3A4 enzyme activity due to the presence of the polymorphic allele *CYP3A4*22* might compensate the reduction of the formation of EDF related to CYP2D6 inactivity, especially due to increased HTF concentrations. We demonstrated that patients with impaired *CYP2D6* metabolism (IM/EM-S) carriers of *CYP3A4*22* had EDF and HTF plasma concentrations comparable to CYP2D6 EM-F patients. Additionally, the sum of equipotent metabolites was considerably higher in the group with *CYP3A4*22* than in the group with *CYP3A4 *1/*1*, under the same *CYP2D6* activity score.

FUTURE PERSPECTIVE

While vast information is available regarding the impact of *CYP2D6* polymorphisms on the metabolic activation of TAM to EDF, little is known about the influence of the variation of other *CYP* genes on the formation of TAM active metabolites. A better understanding of the role of multiple genetic factors on TAM metabolism, like polymorphisms on the *CYP3A4* gene, will give bases for the introduction of pharmacogenetic-based dose selection for specific groups of patients. The combined approach of initial TAM dose selection based on the patient's genetic

profile, followed by periodic monitoring of the plasma concentrations of the active metabolites EDF and HTF seems to be a promising alternative to obtain optimal efficacy in hormonal therapy of breast cancer with TAM.

EXECUTIVE SUMMARY

Background

- The therapeutic antiestrogenic effect of tamoxifen requires metabolic activation by cytochrome P450 (CYP) enzymes.
- CYP2D6 and CYP3A4 are involved on tamoxifen metabolism and are characterized by high interindividual variability, with a significant influence of genetic and environmental factors such as drug interactions.
- 4-Hydroxytamoxifen and endoxifen have identical properties with respect to receptor affinity and antiproliferative action. However plasma steady-state concentrations of endoxifen are higher than 4-hydroxytamoxifen.
- Few studies had evaluated *CYP3A4* polymorphisms impact on endoxifen formation. Recently enzyme inhibition was associated to reduced endoxifen plasma level. In contrast, others found an increase on endoxifen concentrations in *CYP3A4*22* carriers. Thus, the role of CYP3A4 on metabolic activation of TAM still needs to be clarified.

Results

- *CYP2D6* gene scores were moderately correlated to EDF plasma concentrations, indicating that *CYP2D6* polymorphisms do not fully predict the variability of plasma endoxifen concentrations.
- Endoxifen levels were lower in patients with impaired CYP2D6 metabolism, (*CY2D6 PM/IM/EM-S*) compared to functional metabolism (*EM-F*).
- Tamoxifen and 4-hydroxytamoxifen plasma concentrations were higher in the presence of *CYP3A4*22* allele. Additionally, the prevalence of patients with endoxifen concentrations below the clinical threshold was lower in this group.
- Our data indicate that the presence of the **22* allele of CYP3A4 could, at least partly, compensate for impaired CYP2D6 metabolism

Conclusion

- Our data suggest that CYP3A4 contributes to the metabolic activation of tamoxifen through formation of 4-hydroxytamoxifen and endoxifen.
- CYP2D6 is the major enzyme responsible for endoxifen formation, but in conditions of reduced or absent CYP2D6 activity, it seems that CYP3A4 becomes increasingly important.

REFERENCES

1. Brauch H, Murdter TE, Eichelbaum M, Schwab M. Pharmacogenomics of tamoxifen therapy. *Clin Chem*. 55, 1770-1782 (2009).
2. Jung JA, Lim HS. Association between CYP2D6 genotypes and the clinical outocomes of adjuvant tamoxifen for breast cancer: a meta-analysis. *Pharmacogenomics*. 15(1), 49-60 (2014).*
3. Jordan VC, Collins MM, Rowsby L, Prestwich G. A monohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. *J. Endocrinol.* 75, 305–316 (1977).
4. Teunissen SF, Rosing H, Koornstra RHT.; *et al.* Development and validation of a quantitative assay for the analysis of tamoxifen with its four main metabolites and flavonoids daidzein, genistein and glycitein in human serum using liquid chromatography coupled with tandem mass spectrometry. *J Chromatogr. B*. 877, 2519-2529 (2009).
5. Mürdter TE, Schroth W, Bacchus-Gerybadze L, *et al.* Activity levels of tamoxifen metabolites at the estrogen receptor and the impact of genetic polymorphisms of phase I and II enzymes on their concentration levels in plasma, *Clin. Pharmacol. & Therap*. 89, 708-717 (2011).
6. Ahsen NV, Binder C, Brosckmoller J, Oellerich M. *CYP2D6* and tamoxifen: pharmacogenetic reinvention of an established drug? *J Lab. Med.* 33(5), 1-9 (2009).
7. Madlensky L, Natarajan L, Tchu T, *et al.* Tamoxifen metabolite concentrations, *CYP2D6* genotype, and breast cancer outcomes. *Clin. Pharm. & Therap*. 89(5), 718-725 (2011).**
8. Gaedigk A, Simon SD, Pearce RE, Bradford LD, Kennedy MJ, Leeder JS. The *CYP2D6* activity score: translating genotype information into a qualitative measure of phenotype. *Clin. Pharmacol. Ter.* 83, 234-242 (2008).

9. Lotsch J, Rohrbacher M, Schmidt H, *et al.* Can extremely low or high morphine formation from codeine be predicted prior to therapy initiation? *Pain.* 144, 119-124 (2009).

10. Teh LK, Bertilsson L. Pharmacogenomics of CYP2D6: Molecular genetics, interethnic differences and clinical importance. *Drug Metab. Pharmacokinet.* 17(1), 55-67 (2012).

11. Kohlrausch FB, Gama CS, Lobato MI, *et al.* Molecular diversity at the *CYP2D6* locus in healthy and schizophrenic souther Brazilians. *Pharmacogenomics*. 10(9), 1457-1466 (2009).

12. Antunes MV, Linden R, Santos TV, *et al.* Endoxifen levels and its association With CYP2D6 genotype and phenotype: evaluation of a southern Brazilian population under tamoxifen pharmacotherapy. *Ther. Drug Monit.* 34, 422-431 (2012).

13. Friedrich DC, Genro JP, Sortica VA, *et al.* Distribution of CYP2D6 alleles and phenotypes in the Brazilian population. *Plos One* 9(10), e110691 (2014).

14. Irvin WJ, Walko CM, Weck KE, *et al.* Genotype-guided tamoxifen dosing increases active metabolite exposure in women with reduced *CYP2D6* metabolism: a multicenter study. *J Clin Onc.* 29(24), 3232-3239 (2011).

15. Musshoff F, Stamer UM, Madea B. Pharmacogenetics and forensic toxicology. *Forensic Sci. Int*. 203, 53-62 (2010).

16. Wang Z, Schuetz EG, Xu Y, Thummel KE. Interplay between vitamin D and the drug metabolizing enzyme CYP3A4. *J Steroid Biochem*. *136, 54– 58* (2013).**

17. Wang D, Guo Y, Wrighton SA, Cooke GE, Sadee W. Intronic polymorphism in *CYP3A4* affects hepatic expression and response to statin drugs. *Pharmacogenomics J*. 11, 274–286 (2011).**

18. Teft WA, Gong IY, Dingle B, et al, CYP3A4 and seasonal variation in vitamin D status in addition to CYP2D6 contribute to therapeutic endoxifen level during tamoxifen therapy. *Breast Cancer Res Treat.* 139, 95-105 (2013).**

19. Heine R, Binkhorst L, de Graan AJM. Population pharmacokinetic modelling to assess the impact of CYP2D6 and CYP3A metabolic phenotypes on the pharmacokinetics of tamoxifen and endoxifen. *Br. J Clin. Pharmacol.* 78(3), 572–586 (2014).

20. Antunes MV, Raymundo S, Oliveira, V, *et al*. Ultra-high performance liquid chromatography tandem mass spectrometric method for the determination of tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in dried blood spots – Development, validation and clinical application during breast cancer adjuvant therapy. *Talanta*. 132, 775-784 (2015).*

21. Bijl MJ, van Schaik RHN, Lammers LA, *et al*. The CYP2D6*4 polymorphism affects breast cancer survival in tamoxifen users. *Breast Cancer Res. Treat*. 118(1), 125-130 (2009).

22. Schroth W, Goetz M, Hamma U, *et al.* Association between *CYP2D6* polymorphisms and outcomes among women with early stage breast cancer treated with tamoxifen. *JAMA*, 302(13), 1429-1436 (2009).

23. Jager NGL, Rosing H, Schellens JHM, Linn SC, Beijnen JH. Tamoxifen dose and serum concentrations of tamoxifen and six of its metabolites in routine clinical outpatient care. *Breast Cancer Res. Treat.* 143, 477-483 (2014).

24. Heath DD, Flat SW, Wu AH, Pruitt MA, Rock CL. Evaluation of tamoxifen and metabolites by LC-MS/MS and HPLC methods. *Br. J. Biomed. Sci*. 71, 33-39. (2014).

REFERENCE ANNOTATIONS

*Jung & Lin (2014). Meta-analysis evaluating the association between *CYP2D6* genotype and hazard ratios for the recurrence risk of breast cancer after tamoxifen treatment.

**Madlenski *et al.* (2011). Authors associated endoxifen serum concentrations to therapeutic outcomes and proposed a clinical threshold associated to lower recurrence rates.

**Wang *et al.* (2013). Review on regulation of CYP3A4 expression by vitamin D and metabolism of vitamin D by CYP3A4.

**Wang *et al.* (2011). Describes that *CYP3A4*22* could serve as a biomarker for predicting response to CYP3A4 metabolized drugs.

**Teft *et al.* (2013). Describes an endoxifen concentration prediction algorithm, based on *CYP2D6* and *CYP3A4*22* genotypes, seasonal variation, and concomitant use of CYP2D6 inhibiting antidepressants.

*Antunes *et al*. (2015) Describes an analytical method for tamoxifen and metabolites dried blood spots samples (DBS) quantification, as a tool to optimize adjuvant breast cancer treatment.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

This work was supported by Fundo de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE-HCPA), CNPq/FNRS (CNPq registry number 485013/2013-4) and FAPERGS (registry number 1255-2551/13-2) grants. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Artigo 3. Publicado em Clinical Biochemistry. 2014, 47:1084-1090.
doi: dx.doi.org/10.1016/j.clinbiochem.2014.04.008; QUALIS B1.

Development, validation and clinical application of a HPLC-FL method for CYP2D6 phenotyping in South Brazilian breast cancer patients

Marina Venzon Antunes[1,2]*, Dilana Elisabeth Staudt [2], Suziane Raymundo [2], Vanessa de Oliveira [2], Gustavo Gössling[3], Rafaela Pirolli[3], Jorge Villanova Biazús[3], José Antônio Cavalheiro[3], Daniela Dornelles Rosa[4], Gilberto Schwartsmann[1,3], Rafael Linden[2]

[1] Pós-graduação em Ciências Médicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil

[2] Instituto de Ciências da Saúde, Universidade Feevale, Novo Hamburgo, RS, Brasil

[3] Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brasil

[4] Hospital Moinhos de Vento, Porto Alegre, RS, Brasil

* Corresponding author:
Universidade Feevale.
ERS 239, n° 2755
Zip code 93352-000
Novo Hamburgo – RS, Brazil
Telephone: (55) 51-358.8800 (9039)
e-mail: marinaantunes@feevale.br

ABSTRACT:

Objective: To develop and validate a method for determination of dextromethorphan (DMT) and dextrorphan (DTP) in plasma samples using HPLC-FL and to apply it to CYP2D6 phenotyping of a population from the south of Brazil. **Methods:** Samples were prepared by hydrolysis and liquid-liquid extraction. Analysis was conducted in a reversed phase column, with isocratic elution and fluorescence detection. One hundred and forty patients being treated with tamoxifen were given 30 mg of dextromethorphan and their CYP2D6 phenotypes were determined on the basis of [DMT]/[DTP] metabolic ratios in plasma samples collected after 3 hours. **Results:** total chromatography running time was 12 min. Precision (CV %) was below 9.7 % and accuracy was between 92.1 and 106.9 %. The lower limits of quantification were 1 ng mL^{-1} for DMT and 10 ng mL^{-1} for DTP. Mean extraction yield of analytes was 86.6 %. Mean age of patients was 55.7 years. Phenotype frequencies were as follows: 7.1% poor metabolizers, 13.6% intermediate metabolizers, 77.1 % extensive metabolizers and 2.1 ultra-rapid metabolizers. Metabolic ratios for patients on strong (n=11) and weak (n=16) CYP2D6 activity inhibitors were different from each other and also different from ratios for patients not taking enzyme inhibitors (n=113). **Conclusions:** A sensitive method for determination of dextromethorphan and its metabolite in plasma samples was developed and successfully applied, providing evidence of the impact of that CYP2D6 inhibitors have on the enzyme's metabolic capacity.

Keywords: CYP2D6; phenotyping; dextromethorphan; dextrorphan; HPLC-FL; tamoxifen

INTRODUCTION

Cytochrome P450 2D6 (CYP2D6) is responsible for oxidative metabolism of approximately 25 % of commonly-prescribed drugs. There is a high degree of interindividual variability in the level of activity of this enzyme, primarily caused by genetic polymorphisms [1]. More than 150 different alleles of the CYP2D6 gene are known [2]. The wild type allele is CYP2D6*1, which codes a complete and functional enzyme, while alleles *2, *33 and *35 have point mutations that do not affect the enzyme's catalytic properties. Some alleles are associated with absent enzyme activity, such as *3-*8, *11-*16, *18-*20, *38, *40, *42, and *44, or with reduced activity, such as *9, *10, *17, *29, *36, *37, and *41 [3].

CYP2D6 genotype can be classified into four distinct phenotypes, depending on the resulting enzyme's metabolic activity. An extensive metabolizer (EM) has at least one functional wild allele. An intermediate metabolizer (IM) will have one allele coding for an enzyme with reduced activity and one allele with null activity, while a poor metabolizer (PM) will be homozygous for two alleles with null activity. Finally, an ultra-rapid metabolizer (UM) will be dominant autosomal with a duplicated functional allele [4]. Frequencies and distributions of CYP2D6 alleles and genotypes vary depending on the origin of the population. There is a lack of studies that have investigated the frequency of CYP2D6 polymorphisms in Brazilian population, which is a highly admixed population originating in three distinct populations; slaves brought from Africa, the indigenous population and the European colonists [5-7].

The importance of CYP2D6 in breast cancer treatment has become a focus of interest in the literature. For more than 30 years, tamoxifen (TAM) has been considered the primary adjuvant endocrine treatment and it has a significant impact on patient survival. Treatment with TAM for 5 years after definitive surgery reduces the disease's recurrence rate by 41 % and the mortality rate by 34 %, and is linked with a 9.2% absolute reduction in 15-year breast cancer mortality [8]. Tamoxifen's antiestrogenic activity is especially attributed to its active metabolite endoxifen, which is metabolically formed mainly by CYP2D6 [9]. Additionally, environmental factors, such as co-administration of drugs that inhibit the enzyme (e.g. fluoxetine and paroxetine) and alterations of liver and/or kidneys functions can cause a given genotype to be expressed as phenotype from a different group [10].

The relationship between CYP2D6 genotype and clinical outcomes of TAM treatment has been discussed in the literature. Studies have shown a robust association between polymorphisms of the enzyme and use of inhibitors with unfavorable clinical outcomes, including increase in recurrence rates [11] and reduction in survival rates [12]. Recently, studies conducted by the Breast International Group (BIG) and the Tamoxifen, Alone or in Combination (ATAC) group suggested that there is no clinical value in testing for CYP2D6 genotype [13,14]. In view of the conflicting information, the National Comprehensive Cancer Network (NCCN 2013) does not currently recommend genotyping CYP2D6 as a test for optimizing adjuvant endocrinal treatment, but does recommend avoiding concurrent use of strong and intermediate CYP2D6 inhibitors [15].

It is important to point out that genotyping is only clinically relevant if it is capable of predicting the phenotype. Employing a combination of genetic and environmental factors when evaluating CYP2D6 activity during treatment with TAM is very valuable since it is common for patients to be given drugs concurrently that inhibit the enzyme's catalytic activity [16]. Phenotype analysis is less expensive than genetic assessment, which demands that the correct alleles be chosen for screening, on the basis of the profile of the population.

One established method for determining the metabolic phenotype of CYP2D6 in terms of an activity index is by calculating the metabolic ratio between a probe drug, dextromethorphan (DMT), and its metabolite o-demethylated dextrorphan (DTP), in plasma samples taken 3 hours after oral administration of 30 mg of DMT [17]. There are other substrates of CYP2D6 such as debrisoquine, sparteine, tramadol and metoprolol that can be employed instead of DMT [1]. DMT is usually preferred due its availability and safety. This drug is an opioid receptor agonist that is normally administered as an antitussive agent, it rarely causes adverse effects and when these are present they are tolerable and include phenomena such as dizziness or gastrointestinal discomfort [18]. Additionally, relatively low concentrations of the substrate can saturate CYP2D6 with high specificity, meaning that the probe drug dosage can be small, further reducing the risk of adverse effects [19].

The concentrations of DMT and DTP found in plasma samples for CYP2D6 phenotyping are very low, in the order of 1 ng mL^{-1} [6,20,21], meaning that very sensitive methods are needed for quantification of analytes. High Performance Liquid Chromatography combined with mass spectrometry (LC-MS/MS) is the first choice method in terms of sensitivity, but these

sophisticated detection systems are expensive and rarely available in clinical laboratories, especially in Developing Countries. An alternative is to use the more affordable liquid chromatography systems with fluorescence detection (HPLC-FL), taking advantage of the native fluorescence of DMT and DTP [17,21].

The objective of this study was to develop a sensitive method for determination of DMT and DTP levels in plasma samples using HPLC-FL to phenotype CYP2D6. The proposed method was tested for pharmacogenetic assessment in 140 Southern Brazilian breast cancer patients under tamoxifen pharmacotherapy.

EXPERIMENTAL COMPONENT

Reagents and materials

Analytical standards for dextrorphan (DTP) and propranolol were acquired from Sigma (Saint Louis, United States), while the standard for dextromethorphan (DMT) was obtained from Sanofi-Aventis (Paris, France). The reagents monopotassium phosphate, dipotassium phosphate, sodium acetate, sodium hydroxide and tris(hydroxymethyl)aminomethane were obtained from Nuclear (Diadema, Brazil). HPLC grade methyl *tert*-butyl ether, 85% orthophosphoric acid, methanol and acetonitrile were all obtained from Merck (Darmstadt, Germany). Both 98% triethylamine and β-glucuronidase Helix Pomatia (HP-2) 122,700 units mL^{-1} were acquired from Sigma (Saint Louis, United States). The water used was purified in a Pure Lab Ultra system, from Elga Lab Water do Brasil (Cotia, Brazil).

Preparation of reagents and standard solutions

Stock solutions of DMT 0.1 $mg.mL^{-1}$ and DTP 1 $mg.mL^{-1}$ were made by dissolving the analytical standards in methanol. The working solution was made up from a mixture of the standards, at concentrations of 10 $\mu g\ mL^{-1}$ for DMT and 100 $\mu g\ mL^{-1}$ for DTP, by diluting stock solutions with methanol. The stock solution of propranolol 1 $mg\ mL^{-1}$ was prepared by dissolving the analytical standard in methanol. The internal standard propranolol 50 $ng\ mL^{-1}$ solution was prepared by diluting the stock solution with methanol.

Phosphoric acid 0.1 % (v/v) was prepared by diluting 85 % orthophosphoric acid with ultrapure water. Tris 0.2 M pH 10 buffer was prepared by dissolving 2.43 g of tris(hydroxymethyl)aminomethane in 100 mL of ultrapure water and duly adjusting pH using 85 % orthophosphoric acid. Preparation of β-glucuronidase 1,000 units mL^{-1} was by dilution of 815 μL β-glucuronidase Helix Pomatia (HP-2) 122,700 units mL^{-1} in 100 mL of 0.2 M acetate buffer pH 5.

The mobile phase was prepared by mixing phosphate buffer 0.1 M pH 6.0 and acetonitrile (76:24, v/v) with 0.1 % triethylamine. The pH 6.0 phosphate buffer was prepared by dissolving 13.8 g of monosodium phosphate and 14.198 g of disodium phosphate in 900 mL of ultrapure water. The pH was adjusted by adding phosphoric acid 85% or 1.5 M sodium hydroxide and then volume was made up to 1000 mL with ultrapure water. The buffer was filtered through a cellulose acetate membrane filter with 0.45 μm pores (Sartorius, Germany). After mixing the acetonitrile, the mobile phase was gasified in an ultrasonic bath for 5 min.

Chromatography equipment and conditions

A Shimadzu Class VP high performance liquid chromatograph (Kyoto, Japan) comprising a quaternary LC-10AT pump system, an SCL-10A system controller, a DGU-14A degasser, a CTO-10AS column oven, an SIL-10AF autosampler and a fluorescence detector was used. The chromatography system was controlled by Class VP 6.13 SP2 software, also provided by Shimadzu. Separation was conducted in a Hypersil Gold C18 reversed phase column (150 x 4.6 mm, 3 μm) from Thermo Scientific (Waltham, United States), kept at 35 ºC throughout analysis. The mobile phase was a mixture of phosphate buffer 0.1 M pH 6.0 and acetonitrile (76:24, v/v) 0.1 % triethylamine, with a flow rate of 1 mL min^{-1}. Chromatographic analysis lasted 12 minutes. Chromatograms were acquired at an excitation wavelength of 280 nm and an emission wavelength of 320 nm. Detector sensitivity was set at an initial gain of 32 times and increased to 512 times at 6 minutes.

Preparation of calibration solutions

Calibration solutions were prepared by diluting the DMT and DTP working solutions in varying volumes of methanol to achieve concentrations of 10 and 100; 50 and 500; 100 and 1,000; 200 and 2,000; 500 and 5,000; 750 and 7,500; 1,000 and 10,000; 2,500 and 25,000 ng mL^{-1} of DMT and DTP respectively. Plasma calibration solutions were prepared by diluting the calibration solutions 1:10 in plasma free from analytes, in order to obtain samples with concentrations of 1 and 10; 5 and 50; 10 and 100; 20 and 200; 50 and 500; 75 and 750; 100 and 1,000; 250 and 2,500 ng mL^{-1}, for DMT and DTP respectively

Sample preparation

Plasma samples were prepared by enzymatic hydrolysis of the conjugated forms of DTP and DMT with glucuronic acid, followed by liquid–liquid extraction. Test tubes with threaded lids were filled with a 500 µL plasma sample, followed by 500 µL β-glucuronidase 1,000 U/mL, and put in a water bath at 37 ºC. After 18 hours' incubation, 100 µL of propranolol internal standard 50 ng mL^{-1}, 400 µL of buffer tris pH 10 and 3,000 µL of methyl *tert*-butyl ether were added to the samples. Tubes were mixed for 10 minutes and centrifuged at 3,000 g for 10 minutes. The organic phase was then transferred to a new test tube with a threaded lid and analytes re-extracted by adding 200 µL of 0.1 % phosphoric acid. After mixing for 15 minutes, tubes were centrifuged at 3,000 g for 15 minutes. After separation, 150 µL of the aqueous phase was transferred to a microvial and 50 µL injected into the HPLC-FL system.

Validation of the analytical method

The method was validated as described by Shah *et al.* (2000) [22]. The parameters evaluated were specificity, linearity, sensitivity, precision, accuracy, extraction yield and stability.

Specificity

Six plasma samples free from DMT and DTP and prepared as described under "sample preparation" were analyzed, finding that interferents with the same retention time as analytes were present [22].

Linearity

The linearity of the method was assessed by constructing calibration curves. Calibration solutions with concentrations in the range of 1 to 250 ng mL^{-1} for DMT and 10 to 2500 ng mL^{-1} for DTP were analyzed in sextuplicate. Calibration curves were constructed by calculating the correlations between the calibration solutions' nominal concentrations and the ratios between the areas for the analytes and the areas for the internal standard. The homogeneity of variance of the calibration data was assessed using the F test, with a 95 % confidence interval. Curves were fitted using least squares linear regression with a range of weighting factors ($1/x$, $1/x^{0.5}$, $1/x^2$, $1/y$, $1/y^{0.5}$, $1/y^2$). Calibration models were evaluated on the basis of their coefficients of correlation (r) and sum percentage relative error ($\sum$%RE), as described by Almeida et al. (2002) [23]. Calibration was conducted daily with one replicate of each concentration throughout all experiments of validation and application of the method.

Precision and accuracy

Precision and accuracy were evaluated over 5 days by analyses in triplicate of samples quality control low (QCL) 3 and 30 ng mL^{-1}, quality control medium (CQM) 30 and 300 ng mL^{-1} and quality control high (CQH) 90 and 900 ng mL^{-1} for DMT and DTP respectively. Within-assay and between-assay precision were calculated by analysis of variance (single-factor ANOVA), with the grouping variable "day" and were expressed as coefficient of variation CV %. Accuracy was defined as the percentage concentration estimated by the calibration curve in relation to nominal concentration. The criterion for acceptability of precision was a maximum CV of 15 % (for both within and between-assay) and acceptable accuracy values were from 85 to 115 % of nominal concentrations [22].

Extraction yield

Extraction efficiency was determined over 3 days by comparing the areas of analytes obtained in triplicate analyses of the QCL, CQM and QCH samples with areas for direct injection of metabolic solutions of DMT and DTP at concentrations equivalent to complete extraction. Percentage extraction yield was calculated by assuming that the mean areas for each DMT and DTP solution represented 100%, for the purposes of comparison with the mean areas for the same concentrations for the control plasma samples extracted.

Sensitivity

The method's lower limit of quantification (LLOQ) was assessed over 3 days by triplicate analyses of the lowest concentration calibration solutions, at concentrations 1 and 10 ng mL^{-1}, for DMT and DTP respectively. The CV % and accuracy of measurements were calculated. The criterion for acceptability was a maximum CV of 20 % and accuracy values in the range of 80 to 120 % [22].

Stability

In order to assess bench stability of the samples processed under the analytical conditions described, six control samples of DMT and DTP at concentrations of 8.0 and 400 ng mL^{-1} were extracted as described in "sample preparation". Extracts at each concentration were mixed and injected under conventional conditions analytical at 1-hour intervals, over a 12-hour period. The extracted analytes were defined as stable if variation between the areas measured over the 12 hours was less than or equal to 10 %.

Stability after freezing and thawing cycles was tested with quality control DMT and DTP samples at concentrations of 8; 40 and 400 ng mL^{-1}, analyzed before (n=3) and after 3 freeze-thaw cycles (n=9). For each freeze-thaw cycle, samples were frozen at – 20 °C for 24 hours, then thawed and kept at room temperature for 2 hours before being prepared. The concentrations of the analytes from each experiment were calculated using the daily calibration curves and variance analyzed using ANOVA. P values < 0.05 were considered statistically significant.

CYP2D6 phenotyping of patients on tamoxifen

This study was approved by the Research Ethics Committee at the Hospital de Clínicas de Porto Alegre under hearing number 240.253. A total of 140 patients on adjuvant hormonal treatment with tamoxifen were enrolled on the study, were provided with a full explanation and signed free and informed consent forms. Exclusion criteria for this study were underlying liver or kidney diseases.

Participants were instructed to fast for 4 hours and abstain from alcohol for 48 hours prior to the tests. Data on age, weight, body mass index (BMI) and race were recorded, in addition to the time each patient had been on tamoxifen and any drugs they were taking that are CYP2D6 inhibitors. For the phenotyping procedure, each volunteer received a 30 mg oral dose of DMT and 3 hours later an 8 mL sample of venous blood was drown into a tube containing EDTA as anticoagulant. Tubes were centrifuged immediately and plasma was transferred to 2 mL polypropylene tubes, which were stored at -70 °C until analysis.

Concentrations of DMT and DTP were estimated from their calibration curves and then [DMT]/[DTP] metabolic ratios were calculated. CYP2D6 phenotypes were classified into four categories on the basis of the resultant ratios [DMT]/[DTP]: PM $\geq$ 0.3; 0.3 > IM $\geq$ 0.03; 0.03 > EM $\geq$ 0.0003; UM > 0.0003 [24]. Although these limits were originally defined on the basis of urine sample testing, a high correlation (r^2=0.8736, p<0.0001) with minor deviation (0.023) has been observed between metabolic ratios from urine and plasma samples [25] and so the same classification is also applicable to the context of this study.

Analysis of data

Initially, a descriptive analysis of the study variables was conducted. The precision and stability validation parameters for the analytes were evaluated using ANOVA. Medians and 25% and 75% percentiles for DTP and DMT levels were determined. The frequency distributions for the CYP2D6 metabolic ratios, expressed as log [DMT]/[DTP], were used to plot a histogram. The normality of the metabolic distribution curve was tested using the Dagostino-Pearson test. Median DMT and DTP concentrations and median metabolic ratios were compared across races, age groups, BMI groups and level of CYP2D6 inhibitors use using the Mann-Whitney test.

Analyses were conducted using SPSS version 17.0, and results with $P<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Chromatography, preparation of samples and validation

The chromatography running time was 12 minutes, and retention times for analytes were 3.4, 7.4 and 10.2 min for DTP, internal standard and DMT, respectively. Figure 1 shows chromatograms for a control sample and for patients with poor and extensive CYP2D6 metabolic phenotypes. The chromatographic conditions employing a reversed phase column and isocratic elution with fluorescence detection proved capable of selectively separating analytes and endogenous compounds.

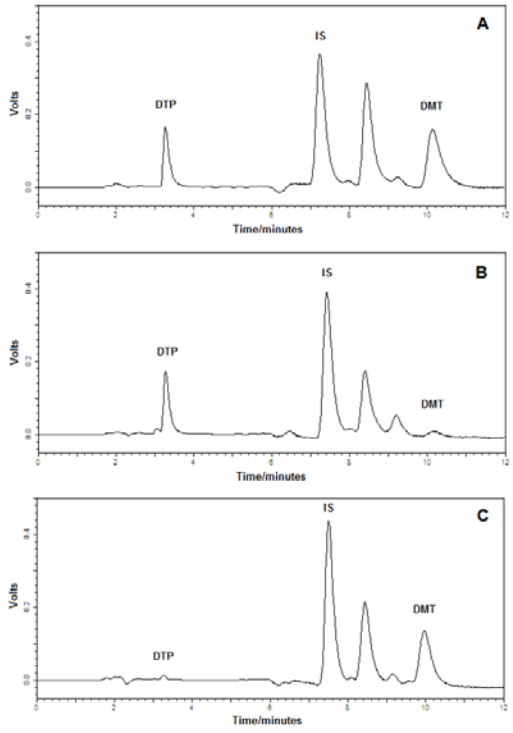


Figure 1 CYP2D6 phenotyping chromatograms **A:** Control samples**:** DTP 750 $ng.mL^{-1}$ and DMT 75 $ng.mL^{-1}$. **B:** Extensive metabolizer patient sample: DTP 810.4 $ng.mL^{-1}$ and DMT 6.81 $ng.mL^{-1}$, [DMT]/[DTP] = 0.0084; **C:** Poor metabolizer patient sample: DTP 59.61 $ng.mL^{-1}$ and DMT 50.7 $ng.mL^{-1}$, [DMT]/[DTP] = 1.176

Several different methods for detection of DMT and DTP by HPLC-FL have already been described in the literature [17,21,26]. In those studies, chromatography running times ranged from 15 to 33 minutes, using isocratic elution or mobile phase gradients.

The method developed here was linear for the interval 1 to 250 ng mL^{-1} DMT and 10 to 2,500 ng mL^{-1} DTP. The calibration data exhibited significant heteroscedasticity and so several weighted regression models were tested, leading to selection of the reciprocal of concentration (1/x) as the best weighting factor for both analytes, with ∑R values of 4.47 for DMT and 7.69 for DTP and ∑%RE values of 1.5 x 10^{-15} for DMT and 7.5 x 10^{-15} for DTP, contrasting with the values obtained when unweighted regression was used, giving ∑R of -24.52 for DMT and -40 for DTP and ∑%RE values of 119.59 for DMT and 176.59 for DTP. The calibration curves exhibited acceptable linearity, all with correlation coefficients of $r > 0.99$.

The parameters sensitivity, precision and accuracy were all within their limits of acceptability for validation of bioanalytical methods (Table 1). The coefficients of variation (CV%) ranged from 3.4 to 9.7 % intra-day and 4.0 to 8.8 % inter-day. The method's accuracy was within the range of 92.1 to 106.9%.

The sample preparation technique based on liquid–liquid extraction and including a re-extraction step provided extracts with a high degree of purity and offered a high yield, greater than 85 %. The limits of quantification for analytes were satisfactory for application of the method to clinical samples, at 10 ng mL^{-1} DTP and 1 ng.mL^{-1} DMT. Afshar *et al.* (2004) proposed a method in which preparation of samples was based on simple precipitation of proteins, but their limit of DMT quantification was 10 ng mL^{-1}, with the result that their method was only able to quantify DMT in 4 of 39 EM patients and could not distinguish between UM, EM and IM phenotypes [17].

Using a modified version of their previously published method, Afshar *et al.* (2005) were able to reduce the limit of quantification to 5 ng mL^{-1} [20]. However, these conditions were still unable to quantify DMT in any samples from EM patients (n=195). Since the lack of normality in the distribution of metabolic ratios within the EM group suggested that IM patients may overlap into this group, these authors reanalyzed 80 samples chosen at random using a method with a LLOQ of 2 ng mL^{-1}. Despite the improved sensitivity, it was only possible to quantify DMT in four of the 80 samples and for the remainder the metabolic ratio was calculated taking the limit of detection of 1 ng mL^{-1} as the DMT concentration. As a result, concentrations between the LOD

and the LLOQ were disregarded in the calculations and the possibility that this range may had been determinant in the classification and differentiation of IM, EM and UM phenotypes in their study could not therefore be ruled out.

A study by Lin *et al.* (2007) reported a limit of quantification of 0.27 ng mL^{-1} for DMT and 0.26 ng mL^{-1} for DTP utilizing 1 mL of plasma, without a hydrolysis stage, resulting in an upper limit to the calibration curve of 54.3 ng mL^{-1} for DMT and 51.5 ng mL^{-1} for DTP [26]. In the present study, with a hydrolysis step, the range of values detectable in samples was considerably wider, with a DMT interval of <1.0 to 251.1 ng mL^{-1} and a DTP interval of 28.4 to 2,349.0 ng mL^{-1}.

Table 1: Sensitivity (n=9), precision and accuracy (n=45) and extraction yield (n=27).

Analyte		**Nominal concentration ($ng.mL^{-1}$)**	**Precision (CV%)**		**Accuracy (%)**	**Extraction yield (%)**
			Intra-assay	Inter-assay		
Dextrorphan	LLOQ	10	8.7	9.3	105.9	-
	QCL	30	4.4	5.8	95.5	89.2
	QCM	300	5.6	7.9	100.3	86.3
	QCH	900	3.4	4.0	98.9	87.4
Dextromethorphan	LLOQ	1	15.0	12.1	108.4	-
	QCL	3	9.7	8.8	92.1	85.8
	QCM	30	8.1	7.0	97.1	84.2
	QCH	90	6.3	6.6	106.9	86.6

LLOQ: lower limit of quantification, QCL: quality control low, QCM: quality control medium, QCH: quality control high.

The results of the analyte stability tests are shown in Table 2. In the bench stability tests the analytes remained stable for a period of 12 hours for both concentrations tested with a maximum variation of 6% in area (Figure S1, supplementary material). It is therefore possible to conduct simultaneous extraction from a number of samples, storing them at room temperature

before proceeding to injection. Signs of analyte instability were also not detected during the freezing and thawing experiments ($P > 0.05$).

Table 2: Analysis of bench stability of DTP and DMT over time and stability after freeze-thaw cycles (n=27).

Analyte	(ng mL^{-1})	Bench stability	Freeze-thaw stability			
			Control concentration after three cycles (n=3)			
		Variance of concentration after 12 h (%)	First	Second	Third	P (ANOVA)
DTP	8	4.3	7.6	7.2	8.1	0.25
	40	-	41.7	40.2	40.5	0.48
	400	3.9	404.3	403.5	403.0	0.59
DMT	8	6.0	8.0	8.2	8.5	0.56
	40	-	38.7	38.0	39.4	0.54
	400	4.8	409.4	407.2	409.8	0.37

DTP: dextrorphan, DMT: dextrometorphan

Phenotyping of CYP2D6 in patients on tamoxifen

A total of 140 patients on adjuvant hormonal treatment with tamoxifen were assessed. The sample had mean age of 55.7 ± 10.35 years (28 to 81), mean weight of 69.38 ± 13.63 kg (51 to 116 Kg) and mean BMI of 27.0 ± 5.33 kg/m^2 (18.4 to 46.5 kg/m^2), while 114 patients were white, 17 were brown (*parda* – a Brazilian national census classification) and 9 were black. Twenty-seven patients reported concurrently taking drugs that inhibits CYP2D6, 16 of whom were on weak inhibitors such as venlafaxine, citalopram, chlorpromazine and haloperidol and 11 of whom were on strong inhibitors, such as fluoxetine and bupropion.

The [DMT]/[DTP] metabolic ratios were not distributed normally according to the Dagostino-Pearson test ($P<0.001$). Figure 2 shows a histogram illustrating the frequency

distribution of CYP2D6 metabolic ratios, expressed as $\log_{10}$ [DMT]/[DTP], in which the groups PM, IM, EM and UM can be identified.

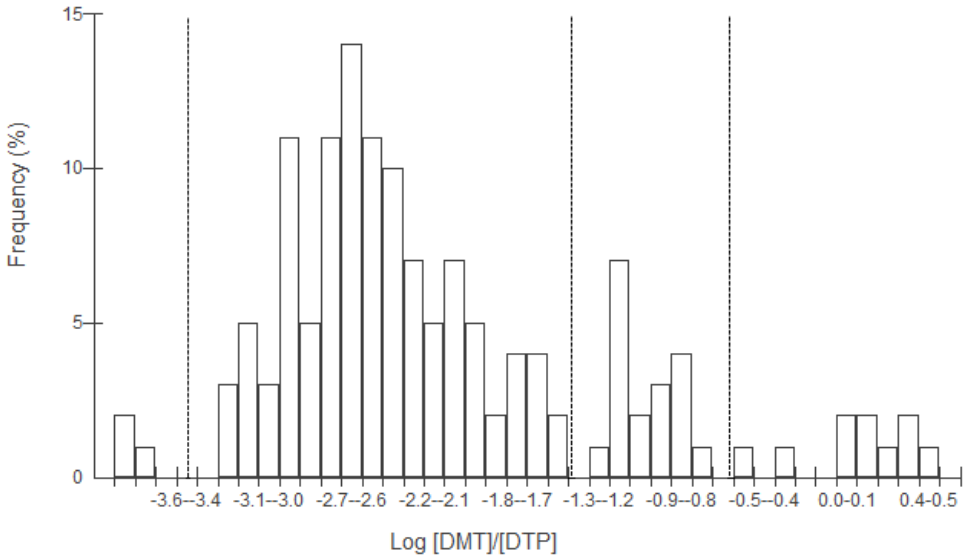


Figure 2. Histogram showing distribution of frequencies of CYP2D6 metabolic ratios, expressed as $\log_{10}$[DMT]/[DTP] N=140.

In samples from three patients, DMT was detected, but at concentrations below the lower limit of quantification and in these cases the metabolic ratio was calculated using a DMT concentration of 1 ng mL^{-1}. Approximately 20 % of the study population exhibited incomplete CYP2D6 metabolism. The participants were classified into phenotype groups by [DMT]/[DTP] metabolic ratios as follows: 10 (7.1 %) PM, 19 (13.6%) IM, 108 (77.1 %) EM and 3 (2.1%) UM. Medians and ranges (P25 – 75) for DMT and DTP are given in Table 3. Both DMT and DTP levels and metabolic ratios were statistically different between phenotype groups. The median metabolic ratio for PM (1.490) was 497 times greater than that for EM (0.003), and IM (0.091) was 30 times EM. The magnitude of the difference between PM and EM was similar to that reported by Afshar *et al.* (2005), at 520 times difference between the lowest and highest metabolic ratios (N=200) [20].

Table 3. CYP2D6 phenotypes, DMT and DTP concentrations and [DMT] / [DTP] ratios (N=140)

Phenotype	n	Frequency (%)	Median DTP ng mL^{-1} (P25 – P75)	Median DMT ng mL^{-1} (P25 – P75)	Median [DMT] / [DTP] ratios and ranges
PM	10	7.1	60.1 [a] (49.3-87.4)	93.4 [a] (63.2–113.0)	1.4905 [a] 0.302 - 3.313
IM	19	13.6	552.0 [b] (354.3–852.3)	48.1 [b] (31.0–66.9)	0.0905 [b] 0.031 - 0.177
EM	108	77.1	1290.7 [c] (1020.1–1597.6)	3.8 [c] (2.2–7.1)	0.003 [c] 0.0005 - 0.0278
UM	3	2.1	1990.1 [d] (1522.5–1995.3)	< 1.0	0.001 [d] 0.0001 - 0.0002

DMT: dextromethorphan; DTP: dextrorphan; PM: poor metabolizers; IM: intermediate metabolizers; EM: extensive metabolizers; UM: ultra-rapid metabolizers
The same letter (a,b,c,d) in the column does not differ in Mann–Whitney at 5% significance.

It is known that there is wide variability in the prevalence of non-functional alleles across different races. This study investigated patients from the South of Brazil, which is a region with a history of European immigration and the frequencies of CYP2D6 phenotypes found were similar to the distributions of genotypes found in Caucasian populations at 7 to 11% PM; 10 to 15 % IM; 70 to 80 % EM and 3 to 5 % UM [1].

The percentages of PM reported in previous studies of Brazilian populations have varied in the order of 2.3 to 6.2 % [5,6,7]. In a previous study, we found prevalence rates of CYP2D6 genotypes of 4.1 % PM, 4.1 % IM, 88.7 % EM and 3.1 % UM (N=97) [6]. Jardim *et al*. (2010) found genotype frequencies of 3% PM, 27 % IM and 70% EM (N=30) [5]. Neves *et al.* (2013) assessed CYP2D6 phenotypes using metoprolol metabolic conversion to α-hydroxymetoprolol as enzyme activity surrogate (N=130) and did not identified any IM or UM, only PM (n=3, 2.3%) and EM (n=127, 97.7%) [7]. The marked differences between the prevalence rates of CYP2D6 phenotypes found in different studies can be attributed to the genetic heterogeneity of the studies populations and the contribution of non-genetic factors to enzyme activity, in particular to taking enzyme inhibiting substances.

In this study, [DMT]/[DTP] metabolic ratios were compared across racial groups, BMI groups, age groups and across CYP2D6 inhibiting drug use groups. There was no statistically

significant difference in median metabolic ratios between Caucasian individuals (n=116) and people of other races (n=24), nor between people with BMI < 27 (n=78) or ≥ 27 kg/m^2 (n=62), or between people aged < 55 (n=71) or ≥ 55 years (n=69). In contrast, the results highlight the effect of taking inhibitors on enzyme activity, with statistically significant differences between median metabolic ratios for all three groups: weak inhibitors (n=16), strong inhibitors (n=11) and no inhibitors (n=113) (P<0.05). Furthermore, 3 (30%) of the PM patients (n=10) and 9 (47 %) of the IM patients (n=19) reported taking CYP2D6 inhibitors, in contrast with the EM patients (n=108), fewer than 13 of whom were taking them (12 %).

Table 4. Concentrations of DMT and DTP and [DMT] / [DTP] ratios by age, BMI, race and CYP2D6 inhibitors (N=140)

Groups		n	[DMT]/[DTP] ratio	P
Age (years)	< 55	71	0.0042 (0.0021-0.0280)	0.684
	≥ 55	69	0.0049 (0.0018-0.0222)	
BMI (kg/m^2)	< 27	78	0.0036 (0.0018-0.019)	0.826
	≥ 27	62	0.0036 (0.0018-0.019)	
Race	White	114	0.0039 (0.0019-0.0230)	0.547
	Others	26	0.0039 (0.0018-0.0084)	
CYP2D6 inhibitors	No	113	0.0031 (0.0017-0.0110) [a]	<0.05
	Weak	16	0.0092 (0.0027-0.0593) [b]	
	Strong	11	0.0746 (0.0048-0.1024) [c]	

DMT: dextromethorphan; DTP: dextrorphan; MD: median; PM: poor metabolizers; IM: intermediate metabolizers; EM: extensive metabolizers; UM: ultra-rapid metabolizers
P values according to Mann-Whitney (age, BMI, race) or Kruskall Wallis (inhibitors) tests.
The same letter (a,b,c,d) in the column does not differ in Mann–Whitney at 5% significance. test.

Our results offer insight into the effects of CYP2D6 strong and weak inhibitor drugs on its activity and may have implications for tamoxifen and other drugs that interact with the enzyme, of which there are many . Moreover, dextromethorphan has shown to be an efficient tool in the classification of CYP2D6 phenotypes, of whom breast cancer patients under tamoxifen

pharmacotherapy are candidates for evaluation due to the formation of the active metabolite endoxifen.

CONCLUSIONS

A sensitive method for determination of dextromethorphan and its metabolite samples of plasma was developed and validated. The sample preparation technique employed allowed high recovery of the analytes. The linearity range of the method makes it appropriate for CYP2D6 phenotyping. The method was applied to the pharmacogenetic evaluation of CYP2D6 metabolism in 140 patients on adjuvant therapy with tamoxifen, and 20.7% of these patients exhibited reduced enzyme activity. Additionally, evidence of the impact of CYP2D6 inhibitors on their metabolic capacity was clearly identified.

ETHICAL CONSIDERATIONS

The authors state they have no conflicts of interests related to this study. Experiments were all conducted in compliance with Brazilian legislation and with prior approval from the Research Ethics Committee.

ACKNOWLEDGEMENTS

The authors would like to thank Universidade Feevale, the Brazilian National Council for Scientific and Technological Development (CNPq, grant n° 479225/2011-7) and the Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS) for financial support.

SUPPLEMENTARY MATERIAL

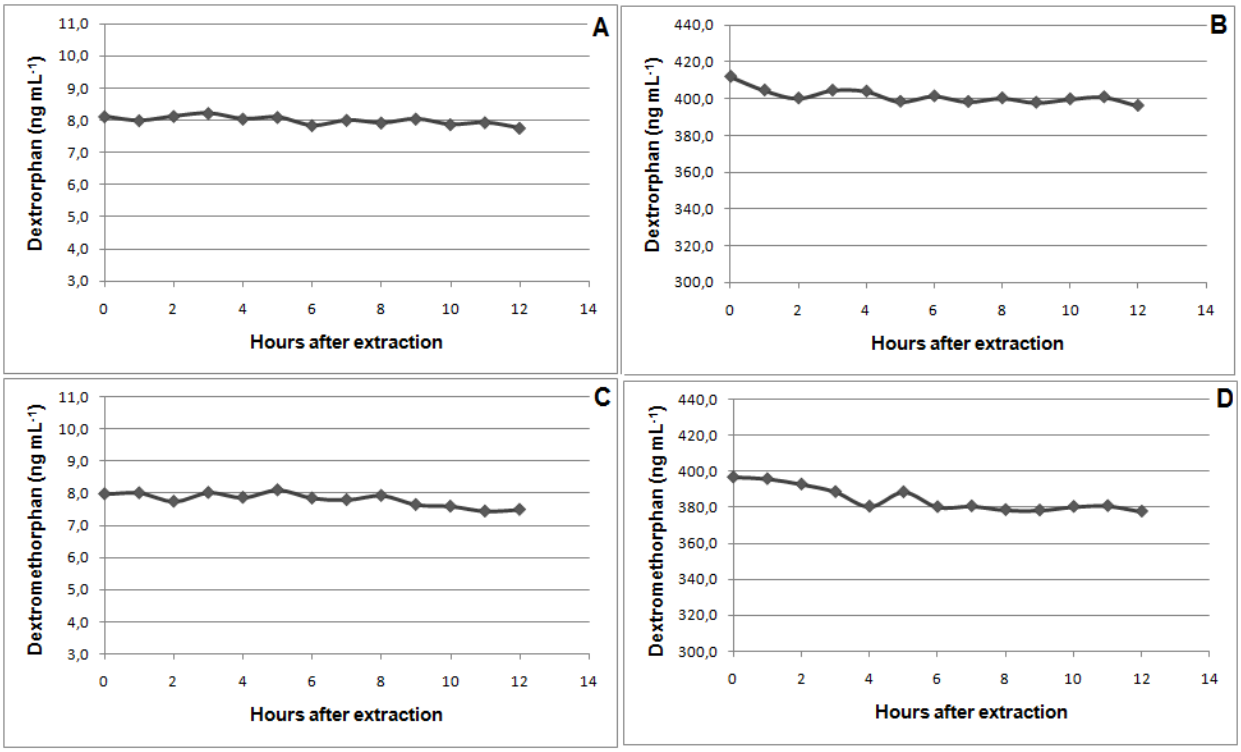


Figure evaluation DMT and DTP stability.

REFERENCES

1. D. Frank, U. Jaehde, U. Fuhr, Evaluation of probe drugs and pharmacokinetic metrics for CYP2D6 phenotyping, Eur J Clin Pharmacol., 63 (2007) 321-333.

2. B. Arneth, M. Shams, C. Hiemke, S. Härtter, Rapid and reliable genotyping procedure for detection of alleles with mutations, deletion, or/and duplication of the CYP2D gene, Clin Biochem., 42 (2009) 1282-1290.

3. S. Borges, Z. Desta, L.Li, T.C. Skaar, B.A. Ward, A. Nguyen, Y. Jin, A.M. Storniolo,

D.M. Nikoloff, L. Wu, G. Hillman, D.F. Hayes, V. Stearns, D.A. Flockhart, Quantitative effect of CYP2D6 genotype and inhibitors on tamoxifen metabolism: implication for optimization of breast cancer treatment, Clin Pharmacol Ther., 80 (2006) 61-74.

4. J.W. Hinrichs, H.M. Loovers, B. Scholten, J.V. Weide, Semi-quantitative CYP2D6 gene doses in relation to metabolic ratios of pyschotropics, Eur J Clin Pharmacol., 64 (2008) 979-986.

5. D.L.F Jardim, A.F.C Calabrich, A. Katz, Determinação da frequência de polimorfismos da CYP2D6 em mulheres brasileiras e revisão de literatura, Rev. Bras. Mastologia, 20 (2010) 15-21.

6. M.V. Antunes, R. Linden, T.V. Santos, P. Wallemacq, V. Haufroid, J.F. Classen, H. Andreolla, N. Costa, T.O. Fontanive, D.D. Rosa, Endoxifen levels and its association with CYP2D6 genotype and phenotype: evaluation of a southern Brazilian population under tamoxifen pharmacotherapy, Ther. Drug Monit., 34 (2012) 422–431.

7. D.V. Neves, V.L. Lanchote, L. Souza, M. Hayashida, M.S. Nogueira, N.V. Moraes, E.J. Cesarino, Metoprolol oxidation polymorphism in Brazilian elderly cardiac patients, J Pharm. Pharmacol., (2013) 1347-1353.

8. R.C. Coombes, L.S. Kilburn, C.F. Snowdon, R. Paridaens, R.E. Coleman, S.E. Jones, J. Jassem, C.J.H. Van de Velde, T. Delozier, I. Alvarez, L. Del Mastro, O. Ortmann, K. Diedrich, A.S. Coates, E. Bajetta, S.B. Holmberg, D. Dodwell, E. Mickiewicz, J. Andersen, P.E. Lønning, G. Cocconi, J. Forbes, M. Castiglione, N. Stuart, A. Stewart, L.I. Fallowfield, G. Bertelli, E. Hall, R.G. Bogle, M. Carpentieri, E. Colajori, M. Subar, E. Ireland, J.M. Bliss. Survival and safety of exemestane versus tamoxifen after 2–3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial, Lancet, 369 (2007) 559–570.

9. Y. Jin, Z. Desta, V. Stearns, B. Ward, H. Ho, K.H. Lee, T. Skaar, A.M. Storniolo, L. Li, A. Araba, R. Blanchard, A. Nguyen, L. Ullmer, J. Hayden, Z. Lemler, R.M. Weinshilboum, J.M. Rae, D.F. Hayes, D.A. Flockhart, *CYP2D6* Genotype, Antidepressant Use, and tamoxifen metabolism during adjuvant breast cancer treatment, J Nac Cancer Inst., 97 (2005) 30-39.

10. K.A. Bachmann, Genotyping and Phenotyping the cytochrome p-450 Enzymes. American J Therapeut, 9 (2002) 309-316.

11. W. Schroth, M. Goetz, U. Hamma, M. Schmidt, S. Winter, P. Fritz, et al., Association Between *CYP2D6* Polymorphisms and Outcomes Among Women with Early Stage Breast Cancer Treated with Tamoxifen, JAMA, 302 (2009) 1429-1436.

12. L.A. Lammers, R.H.J. Mathijssen, T. Gelder, M.J. Bijl, A.J.M. Graan, C. Seynaeve, M.A. Fessem, E.M.A Berns, G. Vulto, R.H.N. Schaik, The impact of *CYP2D6*-predicted phenotype on tamoxifen treatment outcome in patients with metastatic breast cancer, Brit J of Cancer, 103 (2010) 765–771.

13. M.M. Regan, B. Leyland-Jones, M. Bouzyk, et al, CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: The Breast International Group 1-98 trial, J Natl Cancer Inst., 104 (2012) 441-451.

14. J.M. Rae, S. Drury, D.F. Hayes, et al: CYP2D6 and UGT2B7 genotype and risk of recurrence in tamoxifen-treated breast cancer patients. J Natl Cancer Inst 104:452-460, 2012

15. R.L.Theuriault e Breast Cancer Panel Members. National Comprehensive Cancer Network (NCCN) dos EUA, versão 3.2013, p.MS-31.

16. JF Rogers, AN Nafziger , JS Bertino Jr., Pharmacogenetics affects dosing, efficacy, and toxicity of cytochrome P450-metabolized drugs, Am J Med., 113 (2002) 746-50.

17. M. Afshar, M. Rouini, M. Amini, Simple chromatography method for simultaneous determination of dextromethorphan and its main metabolites in human plasma with fluorimetric detection, J Chromat B., 802 (2004) 317-322.

18. A.A. Weinbroum, V. Rudick, G. Paret, R. Ben-Abraham, The role of dextromethorphan in pain control, Can J Anesth., 47 (2000) 585-596.

19. D.S. Streetman, J.S. Bertino Jr, A.N. Nafziger, Phenotyping of drug-metabolizing enzymes in adults: a review of in-vivo cytochrome P450 phenotyping probes, Pharmacogenetics, 10 (2000) 187-216.

20. M. Afshar, M. Rouini, A. Shahram, Dextromethorphan metabolic phenotyping in an Iranian population, Eur J Clin Pharmacol., 60 (2005) 849–854.

21. S.Y. Lin, C. Chien-Ho, H. Hsiu-O, C. Hsueh-Hui, S. Ming-Thau, Simultaneous analysis of dextromethorphan and its three metabolites in human plasma using an improved HPLC method with fluorometric detection, J Chromat B., 859 (2007) 141-146.

22. V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Bioanalytical method validation - A revisit with a decade of progress, Pharm. Res., 17 (2000) 1551-1557.

23. A.M. Almeida, M.M. Castel-Branco, A.C. Falcão, Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci., 774 (2002) 215-222.

24. N. Beverage, T. Sissung, A.M. Sion, R. Danesi, W. Figg, *CYP2D6* polymorphisms and the impact on tamoxifen therapy, J Pharm Scienc., 96 (2007) 2224-2231.

25. S. Raimundo, C.Toscano, K.Klein, J.Fischer, E.U. Griese, M. Eichelbaum, M. Schwab, U.M. Zanger, A novel intronic mutation, 2988G>A, with high predictivity for impaired function of cytochrome P450 2D6 in white subjects, Clin Pharmacol Ther., 76 (2004) 128–38.

26. H.P. Hendrickson, B.J. Gurley, W.D. Wessinger, Determination of dextromethorphan and its metabolites in rat serum by liquid-liquid extraction and liquid chromatography with fluorescence detection, J. Chromatogr. B, 788 (2003) 261-268.

Artigo 4. Publicado em Talanta. 2015, 132:775-784.
doi:10.1016/j.talanta.2014.10.040; QUALIS A2

Ultra-high performance liquid chromatography tandem mass spectrometric method for the determination of tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in dried blood spots – Development, validation and clinical application during breast cancer adjuvant therapy

Marina Venzon Antunes[1,2]*, Suziane Raymundo [2], Vanessa de Oliveira [2], Dilana Elisabeth Staudt [2], Gustavo Gössling[3], Giovana Piva Peteffi[2], Jorge Villanova Biazús[3], José Antônio Cavalheiro[3], Marie Tre-Hardy[4], Arnaud Capron[4], Vincent Haufroid[4], Pierre Wallemacq[4], Gilberto Schwartsmann[1,3], Rafael Linden[2]

[1] Pós-graduação em Ciências Médicas, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

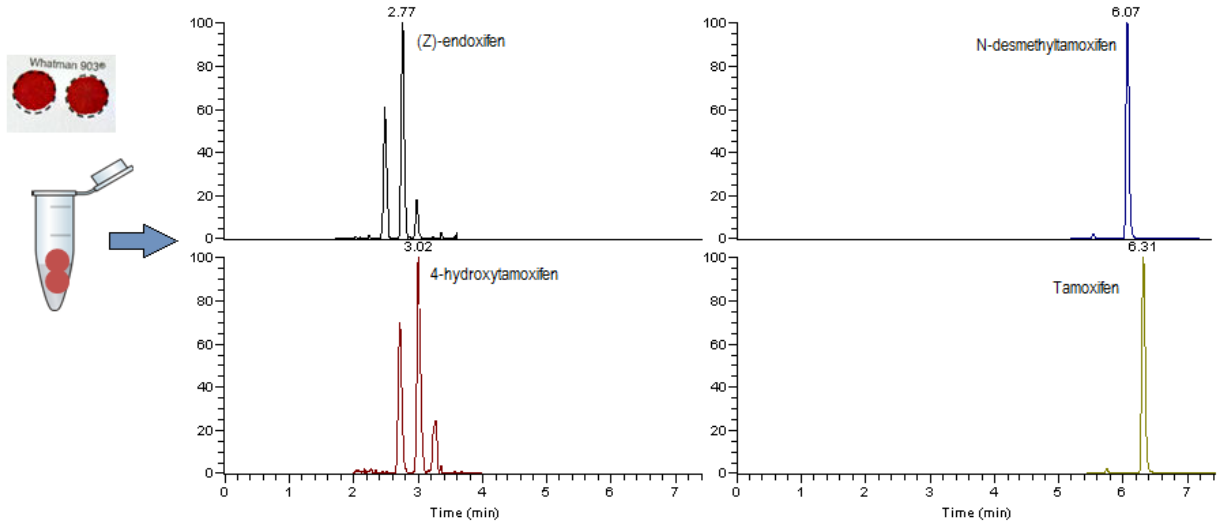
[2] Instituto de Ciências da Saúde, Universidade Feevale, Novo Hamburgo, RS, Brazil

[3] Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

[4] Louvain Center for Toxicology and Applied Pharmacology, Université Catholique de Louvain, Brussels, Belgium;

* Corresponding author:
Universidade Feevale.
ERS 239, n° 2755
Zip code 93352-000
Novo Hamburgo – RS, Brazil
Telephone: (55) 51-358.8800 (9039)
e-mail: marinaantunes@feevale.br

GRAPHICAL ABSTRACT



ABSTRACT

A LC-MSMS method for the simultaneous determination of tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in Dried Blood Spots samples was developed and validated. The method employs an ultrasound-assisted liquid extraction and a reversed phase separation in an Acquity® C18 column (150 x 2.1 mm, 1.7 µm). Mobile phase was a mixture of formic acid 0.1% (v/v) pH 2.7 and acetonitrile (gradient from 60:40 to 50:50, v/v). Total analytical run time was 8 min. Precision assays showed CV % lower than 10.75% and accuracy in the range 94.5 to 110.3%. Mean analytes recoveries from DBS ranged from 40% to 92%. The method was successfully applied to 91 paired clinical DBS and plasma samples. Dried blood spots concentrations were highly correlated to plasma, with rs>0.83 (P<0.01). Median estimated plasma concentrations after hematocrit and partition factor adjustment were: TAM 123.3 ng mL^{-1}; NDT 267.9 ng mL^{-1}, EDF 10.0 ng mL^{-1} and HTF 1.3 ng mL^{-1}, representing in average 98 to 104% of the actually measured concentrations. The DBS method was able to identify 96% of patients with plasma EDF concentrations below the clinical threshold related to better prognosis (5.9 ng mL^{-1}). The procedure has adequate analytical performance and can be an efficient tool to optimize adjuvant breast cancer treatment, especially in resource limited settings.

Key-words: Dried blood spot; tamoxifen; endoxifen; 4-hydroxytamoxifen; N-desmethyltamoxifen; LC-MS/MS

INTRODUCTION

Tamoxifen (TAM) [*trans*-1-(4-*β*-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-butene] has been the mainstay hormonal treatment for breast cancer for more than 30 years, with significant impact on the survival rates of patients. However, besides the therapeutic benefits of TAM in the general population, 30 to 50% of patients under TAM pharmacotherapy have tumor recidives or present resistance to the treatment [1].

The usual presentation of TAM is TAM citrate tablets, containing 10 or 20 mg of the pure *trans* isomer, with the recommended posology of 20 mg per day. TAM pharmacologic activity is dependent of its bioativation by cytochrome P 450 enzymes [2;3]. CYP3A4 and 3A5 convert TAM to N-desmethyltamoxifen (NDT), this being the largest primary metabolic route. NDT undergoes 4-hydroxylation via CYP2D6, being biotransformed into 4-hydroxy-N-desmethyltamoxifen or (Z)-endoxifen (EDF). CYP2D6 also catalyzes the metabolism of TAM to 4-hydroxy-TAM (HTF) [4]. The antiestrogenic activity of TAM is mainly due to EDF, which, as HTF, is up to 100 times more potent to inhibit the proliferation of estrogen-dependent cells, and usually have plasma concentrations up to 6 times higher than HTF [5].

EDF plasma concentrations were widely variable among patients, what could be explained by polymorphisms in the gene encoding CYP2D6 and by interactions with enzyme modulating agents, leading to insufficient plasma EDF levels in many individuals [6]. Along CYP2D6, CYP3A and CYP2C9 enzymes were related to EDF formation, but to a lesser extend [2]. Recently, Teft *et al*. (2013) described reduced EDF plasma concentrations during the winter months, which was associated to low plasma 25-hydroxyvitamin D levels. These authors suggested that CYP3A4 activity together with sunlight exposure is an underscored factor in the evaluation of the therapeutic efficacy of TAM [7].

In a recent clinical study, Madlensky *et al.* (2011) exploited the relation between the clinical outcome of hormonal therapy and serum concentrations of TAM and its metabolites, suggesting that adequate therapeutic outcome is dependent to the achievement of a threshold EDF concentration. Patients presenting trough EDF plasma levels above 5.9 ng mL^{-1} had a 26 % reduction of recurrences comparing with patients with EDF concentrations below this threshold. [8]. These findings, along with the increasing knowledge about TAM metabolism modulating agents, encouraged studies with individualized dose adjustments, with increased doses for

patients with impaired EDF formation (30 or 40 mg/day) [9;10;11]. Moreover, the metabolic ratio [NDF]/[EDF] has been described as an appropriate surrogate of CYP2D6 activity, rendering useful clinical information about TAM metabolism [5]. Considering the available evidence, therapeutic drug monitoring of TAM and its main metabolites during hormonal therapy of breast cancer could be an important tool to obtain optimal pharmacological response, recognizing patients eligible to higher TAM doses or alternative pharmacotherapy.

Several methods for the measurement of TAM and metabolites in serum or plasma samples were described [5;6;12;13]. To obtain these conventional samples, it is necessary to perform a phlebotomy in a proper facility, also considering pharmacokinetically appropriated collection times. A novel option for therapeutic drug monitoring that has been emerging recently is testing in dried blood spots on paper (DBS), collected by finger prick [15-17], mainly due to the stabilization of the analytes by drying and the possibility of postal transportation, once DBS samples are usually non-bio hazardous. Also, this novel matrix allows the possibility of training patients to take their own samples and flexible collection times, more adequate to their personal posology [18].

The use of DBS for TAM and metabolites measurements could be a very useful strategy to optimize the use of this drug in breast cancer, potentially allowing continuous monitoring of EDF levels during a long-time treatment, without requiring patients to come to a specialized center. However, considering the small blood volume present in a DBS obtained after a finger prick and the low concentrations of TAM and metabolites, especially EDF, this application requires highly sensitive analytical methods. In view of the above, the objective of this study was to develop and validate a method for determination of TAM and its metabolites NDT, EDF e HTF in DBS using ultra performance liquid chromatography-positive electrospray ionization tandem mass spectrometry and evaluate its performance in clinical samples of breast cancer patients.

EXPERIMENTAL

Reagents, materials and reference standard samples

Tamoxifen, 4-hydroxy tamoxifen e clomifen citrate (E/Z) were acquired from Sigma (Saint Louis, USA). N-desmethyl tamoxifen chloridrate and N-desmethyl-4-hidroxy tamoxifen (E/Z) were obtained from Toronto Research Chemicals (North York, Canada).

Tris(hidroxymethyl)aminomethane was obtained from Nuclear (Diadema, Brazil). Formic acid, metanol, acetonitrile, n-propanol and hexane (60% n-hexane) were bought from Merck (Darmstadt, Germany). Purified water was obtained from an Elga Purelab Ultra® system from Veolia Labwater (High Wycombe, UK). Whatman 903 paper was obtained from GE Healthcare (Westborough, USA).Finger prick blood samples were collected using Medlance® Plus Special lancets (0.8 mm blade) from Medlance Plus Special (Marietta, USA).

Preparation of solutions and standards

TAM, NDT and EDF (E/Z) stock solutions were prepared in methanol to obtain a concentration of 1 mg mL^{-1}. The stock solution of HTF was prepared in methanol to obtain a concentration of 0.1 mg mL^{-1}. Stock was diluted with methanol to obtain intermediate solutions of EDF and HTF at 20 µg mL^{-1} and intermediate solutions of TAM, NDT e EDF at at 40 µg mL^{-1} (20 µg mL^{-1} of the Z-fraction of EDF). Working solutions were prepared by dilution of the intermediate solution with methanol. Calibration samples were prepared in whole blood diluting working solutions at 1:20 with whole blood free from the analytes, as described in the *linearity* section. Clomifene citrate stock (internal standard, IS) solution was prepared in methanol at 1 mg mL^{-1} concentration. Intermediate clomifene solutions were prepared by dilution of the stock with methanol (10.0 µg mL^{-1}) and further diluted to 0.1 µg mL^{-1}. DBS extraction solution was prepared by dilution of the clomifene intermediate (0.1 µg mL^{-1}) with methanol to obtain a concentration of 0.1 ng mL^{-1}.

Sample preparation

Quality control and calibration samples were prepared by pipetting 60 µL aliquots of spiked blood onto Whatman 903 paper and leaving them to dry at room temperature for 3 h before processing. DBS discs were obtained by perforation, using a 10 mm punch cutter, and two discs were used for extraction. Discs were cut in half, transferred to polypropylene microtubes and 1,000 µL of extraction solution was added (clomifene 0.1 ng mL^{-1} in methanol), followed by 1 min vortexing. After 45 min h in an ultrasonic bath, 850 µL of extract was transferred to a clean polypropylene microtube and dried at 55 °C under a gentle stream of air. The dried extract was

recovered with 100 µL of mobile phase, transferred to an autosampler vial and a 25 µL aliquot was injected into the LC-MS/MS system.

UHPLC–MS-MS equipment and conditions

Samples were analyzed using a TSQ Quantum Access triple quadrupole mass spectrometer with an electrospray source, coupled to an Ultimate 3000 XRS UHPLC system, controlled by the Xcalibur software, all from Thermo Scientific (San Jose, USA). Separation was performed in an Acquity C18 column (150 × 2.6 mm, p.d. 1.7 µm) from Waters (Milford, USA), maintained at 50 °C and eluted at a mobile phase flow rate of 0.4 mL min^{-1}. The mobile phase consisted of formic acid 0.1% pH 2.7 (eluent A) and acetonitrile plus 0.1% formic acid (eluent B). Initial eluent composition was 60% A, maintained for 3 min, and followed by a linear 1 min ramp to 50%, which was maintained for until 5.5 min. The mobile composition returned to 60% A at 6 min. Equilibration time was 2 min. Injection volume was 25 µL. The MS conditions were as follows: electrospray ionization, positive mode, capillary voltage of 4 kV; sheath gas, nitrogen at a flow rate of 50 arbitrary units; auxiliary gas, nitrogen at flow rate of ten arbitrary units; collision gas, argon; vaporizer temperature, 400 °C; and ion transfer capillary temperature, 220 °C. The scan time was set at 0.3 s per transition. The following transitions were used for MRM acquisition: TAM *m/z* 372→72 (quantitation) and *m/z* 372→70 and 372→129 (qualification); NDT *m/z* 358→58 (quantitation) and *m/z* 358→129 and 358→178 (qualification); EDF *m/z* 374→58 (quantitation), *m/z* 374→152 and 374→223 (qualification); HTF *m/z* 388→72 (quantitation) and m/z 388→129 and 388→223 (qualification); IS (clomifene) *m/z* 406→100 (quantitation) and *m/z* 406→72 and 406→58 (qualification). Collision energies were 23, 19, 19, 24 and 24 eV for TAM, NDT, EDF, HTF and IS, respectively.

Selectivity

Blank DBS samples obtained from 6 different human sources were prepared as described above to check for the presence of chromatographic peaks that might interfere with detection of analytes or IS.

Benchtop stability

For estimation of stability of processed samples under the conditions of analysis, control DBS samples were extracted as described above, in sextuplicate. Quality control low (QCL) samples had TAM, NDT, EDF e HTF at 21.0; 45.0; 3.0 and 1.8 ng mL^{-1}, respectively. Quality control high (QCL) samples had TAM, NDT, EDF and HTF at 210.0; 450.0; 30.0 and 10.8 ng mL-1, respectively. The extracts obtained at each concentration were pooled. Aliquots of these pooled extracts at each concentration were transferred to autosampler vials and injected under the conditions of a regular analytical run at time intervals of 1 h, during 12 h. Stability of analytes was tested by regression analysis plotting absolute peak areas corresponding to each compound at each concentration vs. injection time. Using the obtained linear regression, the concentration after 12 h was calculated. A decrease or increase of up to 10% in the measured peak areas was considered as acceptable.

Stability of analytes in DBS at varying temperatures

For evaluation of thermal stability of TAM and metabolites in DBS samples, quality control DBS samples (QCL and QCH), previously described, were maintained at -20 °C, 25 °C e 45 °C and analyzed (triplicate) on days 2, 6, 8, 10, 13, 15, 17 e 20 after spotting in the paper. Stability was considered acceptable if all results were within the range of 85–115% of the concentrations measured at the beginning of the series.

Linearity

Aliquots of 950 µL blank blood (Hct 35%), were enriched with 50 µL of the corresponding stock solutions to obtain seven calibration levels of samples containing TAM (300, 225, 150, 75, 30, 15, 7.5 ng mL^{-1}), NDT (600, 450, 300, 150, 60, 30, 15 ng mL^{-1}), EDF (40, 30, 20, 10, 4, 2, 1 ng mL^{-1}) and HTF (50, 15, 10, 5, 2, 1, 0.5 ng mL^{-1}). Calibration samples were applied to Whatman 903 paper (12 x 60 µL for each level), dried at room temperature and analyzed within 24 h. Replicates (n = 6) at each concentration were analyzed as described at item 2.5. Calibration curves were calculated relating the area ratios from TAM, NDT, EDF and HTF peaks to the IS peak and with the nominal concentrations of the calibration samples. Homoscedasticity of calibration data was evaluated with F-test at the confidence level of 95%. Curves were fitted using least-squares linear regression using several weighting factors (1/x, $1/x^2$,

$1/y, 1/y^2$). Calibration models were assessed using coefficients of correlation (r) and cumulative percentage relative error ($\sum$%RE) [19]. Daily calibration curves using the same concentrations (single measurement at each concentration level) were analyzed with each batch of validation and clinical samples.

Precision and accuracy

Aliquots of blank blood were enriched with methanolic solutions and applied to paper to obtain quality control DBS samples containing TAM, NDT, EDF and HTF at concentrations of 21.0; 45.0; 3.0 and 1.8 ng mL^{-1}, respectively (quality control low, QCL), 70.0; 150.0; 10.0 and 6.0 ng mL^{-1}, respectively (quality control medium, QCM) and 210.0; 450.0; 30.0 and 18.0 ng mL^{-1}, respectively (quality control high, QCH). Control samples were maintained at room temperature and analyzed within 24 h as item 2.5. The quality control samples were analyzed as described above in triplicate on each of 5 days. Within-assay precision and between-day precision were calculated by one-way ANOVA with the grouping variable “day” and were expressed as CV %. Accuracy was defined as the percentage of the nominal concentration represented by the concentration estimated with the calibration curve. The acceptance criteria for accuracy were mean values within ±15% of the theoretical value and for precision a maximum CV of 15% was accepted [20].

Lower limit of quantification

An independent DBS quality control sample at the lowest point of the calibration curve at concentrations of 7.5; 15.0; 1.0; 0.5 ng mL^{-1} for TAM, NDT, EDF and HTF, respectively, was included in the accuracy and precision experiments (quality control at the limit of quantitation, QCLOQ) and was tested in triplicate in three different days. The acceptance criteria established for the limit of quantification was accuracy within 100 ± 20% of the nominal value and an imprecision with of maximum 20% [20].

Influence of hematocrit on analytes concentrations assayed in DBS samples

Aliquots of blood containing different Hct % (25, 30, 35, 40, 45 and 50 %) were prepared by centrifuging EDTA whole blood and then combined with appropriate volumes of cells and plasma. TAM and metabolites were added to these aliquots of blood to achieve concentrations of QCL: 21.0; 45.0; 3.0 and 1.8 ng mL^{-1} and QCH: 210.0; 450.0; 30.0 and 18.0 ng mL^{-1} for TAM, NDT, EDF and HTF, respectively, which were then pipetted onto Whatman 903 paper, followed by drying at room temperature for 3 h. The DBS obtained were analyzed in triplicate for each concentration and Hct% as described in item 2.5. The influence of the Hct% on TAM and metabolites measurements was determined as the percentages of nominal concentrations that were actually measured in the DBS.

Matrix effect and extraction yield

Matrix effect was evaluated by a post-extraction spike method. Three series of quality control samples (QCL, QCM and QCH) were prepared in order to assess extraction yield and matrix effect on ionization as follows: (A) solutions of TAM, metabolites and IS prepared in mobile phase solutions in order to obtain final concentrations equivalent to 100% extraction yield and directly injected onto column. (B) DBS extracts samples from 5 different sources (mixed before application on paper), post-extraction spiked with TAM, metabolites in mobile phase containing IS. (C) DBS extracts samples from 5 different sources (mixed before application on paper) enriched with TAM and metabolites before extraction. Each quality control sample was analyzed in quintuplicate. Matrix effect (ME) on ionization was estimated as the percentages of reduction or increase of TAM, NDT, EDF, HTF and IS areas on post-extraction spiked (B), comparing to directly injection of solutions (B), calculated as ME = [100%-(B/A%)]. Extraction yields (EY) were calculated comparing the analyte/IS area ratio before extraction (C) and after extraction (B), using the formula EY= C/B%.

Application of the method

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee at the Hospital de Clínicas de Porto Alegre. A total of 91 patients on adjuvant hormonal treatment with tamoxifen (20 mg/day) for at least 4 months were

enrolled in the study with informed consent. Data on age, weight, body mass index (BMI), race, compliance (obtained from the number of TAM tablets taken monthly) and menopause status were recorded, in addition to the duration of TAM therapy and any concomitant therapies considered as CYP2D6 inhibitors. Blood samples were taken 18-24 h after the last TAM intake, patients being fasten for 4 hours. Capillary blood was collected after finger prick using a 2.0 mm penetration, 0.8 mm blade contact-activating lancet (Medlance®). Blood spots were collected on 2 circles of the Whatman 903 DBS card and allowed to dry for at least 3 h at room temperature. Analyses were performed within 3 days. Venous blood was simultaneously collected within an interval of ± 15 min by venipuncture into two EDTA tubes. One tube was used for Hct% determination, the other was immediately centrifuged and plasma was transferred to polypropylene tubes, stored at -70 °C until analysis within one month. Concentrations of TAM, NDT, EDF and HTF were measured through daily calibration curves and estimated plasma concentrations (EPC) were obtained after adjustment of DBS concentrations by patients Hct% and use of a correction factor, as follows:

$$EPC = [(DBS\ concentration)/ (1 – Hct)] \times correction\ factor$$

Correction factors were estimated so that the ratio of mean DBS to mean plasma levels would be equal to 1.

Determination of TAM and metabolites in plasma

A 0.2 mL aliquot of plasma was transferred to screw cap glass tube and 0.1 mL of the IS solution (clomiphene 0.01 µg mL^{-1}), 0.1 mL of Tris buffer pH 10 and 2 mL of extraction solvent (hexane:n-propanol 95:5, v/v). After homogenization for 30 min and centrifugation at 3,000 g for 10 min, a 1.6 mL aliquot of the supernatant was transferred to a polypropylene microtube and dried at 55 °C for 15 min under air stream. The extract was resuspended with 0.15 mL of mobile phase and a 25 µL aliquot was injected into the LC-M/MS system. The chromatographic conditions and mass detector parameters were the same as those used for the DBS samples. Concentrations were calculated with daily calibration curves. The method was linear from 0.5 to 600 ng mL^{-1} with LLOQ of 7.5 ng mL^{-1} for TAM, 15 ng mL^{-1} for NDT, 1 ng mL^{-1} for EDF and

0.5 ng mL^{-1} for HTF. Accuracy and imprecision were acceptable with accuracy of 90.7%–107.5 %, and percent coefficient of variation within and between assay in the range of 4.8–13.4.

Statistical analysis

Initially, a descriptive analysis of the study variables was conducted. The precision validation parameters were evaluated using ANOVA. Medians and 25% and 75% percentiles for TAM, NDT, EDF, HTF levels and metabolic ratio [NDT]/[EDF] were determined. Analytes in DBS (y) versus measured plasma concentrations (x), as well as estimated plasma (y) versus measured plasma concentrations (x) were assessed by Passing-Bablok regression (Passing and Bablok, 1983) and Spearman correlation. The presence of outliers and tendencies were evaluated using Bland Altman plots (Bland and Altman, 1986) of estimated plasma concentrations versus measured plasma concentrations as well as DBS versus measured plasma concentrations. Acceptation criteria for the agreement between estimated and measured plasma concentrations were based on the guideline on Bioanalytical Method Validation of the EMA, the difference in concentration should be within ± 20 % of their mean for at least 67 % of the samples (EMA, 2011).

The effect of concomitant CYP2D6 inhibitors was tested for TAM, NDT, EDF, HTF and metabolic ratio [NDT]/[EDF] from measured and estimated plasma data obtained from patients stratified in 2 cohorts based on the use or not of CYP2D6 inhibitors by Mann-Whitney test. DBS levels of EDF and the DBS metabolic ratio [NDT]/[EDF] were further compared between these 2 cohorts with plasma EDF above or below the threshold of efficacy of 5.9 ng mL^{-1} by Mann-Whitney test. Analyses were conducted using SPSS version 17.0 and MedCalc version 12.3, $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Chromatography and preparation of samples

The LC-MS/MS system proved to be an appropriate system for determination of TAM and its main metabolites in DBS samples. TAM, NDT, HTF and EDF are structurally very similar, with demethylation and/or hydroxylation, resulting in slight differences of polarity. Reversed phase C18 columns appeared appropriate to separate TAM metabolites (Teunissen et

al., 2010). In this study an Acquity® C18 column (150 x 2.1 mm, 1.7 µm) was used, separating TAM and the 3 metabolites in a 8 min run, with reduced mobile phase consumption of 3.2 mL per analysis. Retention times were 2.77 min for EDF, 3.02 min for HTF, 5.85 min for IS, 6.07 min for NDT and 6.31 min for TAM. Figure 1 illustrates chromatograms from DBS and plasma samples.

No interfering compounds were identified in the 6 DBS samples processed free from TAM and analytes. Additionally, the tandem mass spectrometry results in inherent specificity analysis, in which chromatograms are monitored through the transition of molecular ion to three products (1 quantifying and 2 confirmatory ions).

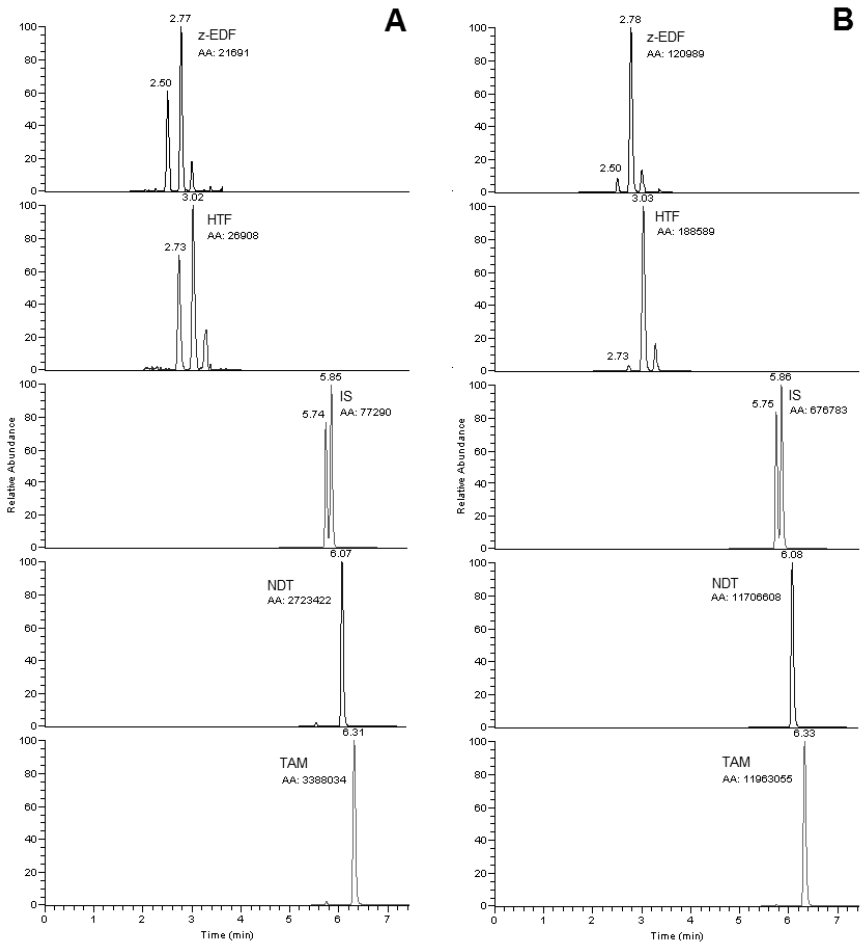


Figure 1. Chromatograms obtained from DBS and plasma analyses. A: plasma EDF 9.9, HTF 1.5, NDT, 188.3, TAM 111.8 (ng mL^{-1}); B: dried blood spot EDF 4.8, HTF 1.2, NDT, 166.0, TAM 100.0 (ng mL^{-1}). Transitions: EDF 374 →58 (m/z), HTF 388→72 (m/z), IS 406→100 (m/z), NDT 358→58 (m/z)TAM 372→72 (m/z).

Sample preparation was simple, based on ultrasound-assisted liquid extraction of analytes from paper punches containing approximately 90 µL of blood. Extraction yield was higher for the least polar compounds, with average of 91.5% for TAM and 84.8% for NDT, comparing to 47.5% for (Z)-(Z)-EDF and 39.6% for HTF. Besides the lower extraction recoveries of EDF and HTF, the method presented satisfactory sensitivity considering the extent of concentrations found in clinical samples, with LLOQ of 1.0 ng mL^{-1} for EDF and 0.5 ng mL^{-1} for HTF [11;14;25;26].

Method validation

Extracts from DBS containing TAM and metabolites at QCL and QCH concentrations were stable during the 12 h bench stability test, with maximum variation in analytical response of 9.2 %, indicating that large batches can be analyzed without taking special measures to preserve extracts. Calibration data exhibited significant heteroscedasticity, with F_{exp} ranging from 495.1 to 1237.0 ($F_{tab\ (6;5;0.95)}$=4.95). The regression using a weighting factor of 1/x offered the best ∑%RE of the models tested, with values below 1 x 10^{-9} and this weighting factor was used for all subsequent validation tests and for analysis of the clinical samples. The coefficients of correlation were above 0.99 for all weighting factors. The results of the precision and accuracy experiments, assessed by analyzing low, medium and high concentration quality control samples, were satisfactory, as shown in Table 1. Intra-assay imprecision were in the range of 5.13 to 12.30% and inter-assay imprecision was 5.10 to 11.54%, demonstrating that the method is adequately repeatable. Accuracy was estimated at 94.6 to 110.3%, which is also within the limits of acceptance for bioanalytical methods recommended by Shah *et al.* (2000). The quality control samples at the lower limit of quantification (LLOQ) of 7.5; 15; 1.0; 0.5 ng mL^{-1} for TAM, NDT, EDF and HTF presented acceptable imprecision (from 7.01 to 12.07%) and accuracy (90.6 to 97.5%).

Due to its high specificity and sensitivity LC-MS/MS is considered as the method of choice for quantitative analysis of compounds in biological matrices. However molecules originating from the sample matrix that coelute with the compounds of interest can interfere with the ionization process, causing ion suppression or enhancement [27]. Post-extraction spiked method analysis showed (Z)-EDF and HTF ionization suppression within 12% and IS ionization enhancement effect of 8 %, indicating that co-eluting matrix components appear to have a

minimal effect on the considered analytes. TAM and NDT demonstrated to be more affected by matrix effect with average suppression of 22.9 and 34.7 % respectively. Similar observation was pointed out on a previously described LC-MS/MS method [28], where matrix ionization suppression (plasma samples) was larger for NDT (38% area reduction) comparing to other TAM metabolites.

As reported above, matrix components do influence to some extent TAM and NDT ionization and consequently the overall process efficiency, requiring therefore the preparation of calibration and control samples in a DBS matrix reflecting at best the composition of the samples to be analyzed. More important that the absolute matrix effect, no significant variability among concentrations and samples was observed in our method. Additionally, TAM and NDT presented high extraction yields (>81 %), allowing analysis with satisfactory sensitivity and linearity range comprising clinical samples concentrations after a 20 mg/daily intake of TAM [11;14;25;26].

Thermal stability was also tested in DBS samples at the concentrations of QCL and QCH after storage at -20 °C, 25 °C and 45 °C up to 20 days. These temperatures were selected to simulate conditions that samples sent by regular mail could potentially be exposed. No changes were observed neither for TAM nor for NDT on DBS stored over a period of 20 days at the tested temperatures (maximum variance of 14%). However, (Z)-EDF and HTF were stable at -20 and 25°C (maximum variance of 15 %) but not at 45 °C, with an increase on concentrations, which ranged from 38% at day 2, to 47% after 20 days. In absence of any data in the literature regarding TAM and metabolites stability on DBS samples, we assumed that increase of metabolite concentrations at higher temperature could be the consequence of TAM and NDT degradation. Tested EDF and HTF levels were approximately 10 fold lower than TAM and NDT, therefore more likely affected by any minor changes in TAM and NDT concentrations. Another possible explanation for this finding could be the production of isobaric interfering compounds on DBS samples under high temperatures. Both hypotheses should be further tested.

Table 1. Method validation parameters, linearity, precision, accuracy and extraction yield and benchtop stability

Analyte	**Linear regression** ***Weighting factor 1/x***	**Quality Control**	**Nominal concentration (ng ml^{-1})**	**Precision (CV %)**		**Accuracy (%)**	**Extraction yield (%)**	**Ion suppression/ enhancement (%)**	**Concentration change after 12 h (%)**
				Intra-assay	**Inter-assay**				
Tamoxifen	y = 0.613x - 0.403 r= 0.996	LLOQ	7.5	9.09	7.01	93.9	-	-	
		QCL	21.0	8.95	6.31	100,2	89.8	-20.4	-3.9
		QCM	70.0	5.13	8.58	98.5	91.3	-24.2	-
		QCH	210.0	8.18	5.10	102.8	93.4	-24.0	-8.1
N-desmethyltamoxifen	y = 0.222 x - 0.1913 r= 0.997	LLOQ	15.0	7.93	10.04	92.0	-	-	
		QCL	45.0	6.73	7.61	99.5	81.5	-34.4	-0.8
		QCM	150.0	6.75	8.51	99,4	88.5	-34.5	-
		QCH	450.0	9.10	5.17	94.5	84.5	-35.3	-3.2
(Z)-Endoxifen	y = 0.117x - 0.0701 r= 0.991	LLOQ	1.0	7.46	11.48	97.5	-	-	-
		QCL	3.0	6.59	7.68	96.7	47.9	-11.1	9.2
		QCM	10.0	8.72	10.77	105.2	48.5	-11.3	-
		QCH	30.0	12.30	6.53	98.0	46.1	-12.0	-2.7
4-hydroxytamoxifen	y = 0.533 x - 0.0912 r= 0.991	LLOQ	0.5	12.07	7.93	90.6	-	-	-
		QCL	1.8	8.98	11.54	94.6	38.1	-5.9	5.2
		QCM	6.0	10.42	5.75	110.3	39.4	-6.6	-
		QCH	18.0	8.53	7.13	98.9	41.2	-2.7	-5.8
Clomiphene (IS)		LLOQ						-	
		QCL						+ 9.1	
		QCM						+ 6.7	
		QCH						+ 8.7	

LLOQ: lower limit of quantification, QCL: quality control low, QCM: quality control medium, QCH: quality control high. (linearity n=42, precision and accuracy n=45, extraction yield n=30, ion suppression n=30)

Information about the thermal, light, and chemical stability of the analytes in DBS matrix must be available in order to explore its logistic advantages. When using transport by regular mail it is important to be aware that the inner temperature of mailboxes in full sun exposure can reach about 60 °C in summer months [29]. Up to now there is no data in the literature regarding the stability of EDF and HTF in DBS samples. Considering our findings on EDF and HTF stability and the high temperatures during summer time, it is reasonable to recommend that temperature during transport and storage of DBS cards for TAM and metabolites measurement should be controlled and not exceed 25 C°.

Impact of hematocrit on determination of TAM and metabolites

The impact of different Hct% levels on calculated concentrations of TAM and metabolites during analysis of DBS samples was tested at concentrations of QCL and QCH prepared in blank blood with Hct% ranging from 25 to 50 and quantified by analytical curves prepared using blood with Hct% of 35. Variation from nominal concentrations found in control samples were within ± 13% for Hct 25%, ± 10% for Hct 30%, ± 10% for Hct 35%, ± 8% for Hct 40%, ± 16% for Hct 45% and ± 20% for Hct 50%. Highest variations from target were found at Hct 45% and 50% and were above 15% which was the cutoff for acceptability. This is probably due to increased viscosity of the blood and hence reduced dispersion through the paper. In contrast, DBS prepared with blood with Hct percentages ranging from 25 to 40 presented results within the range of acceptability (87-108%). On this basis, analytical curves prepared using blood with Hct% of 35 are applicable to samples from patients with Hct% within 25-40, covering most of patients Hct% range (30.6 to 43.6, mean 36). Seven patients had Hct% above 40, where calculated concentrations have to be corrected accordingly.

Besides DBS samples being obtained from capillary blood, venous blood was used to determine the patient Hct%, considering the absence of difference between capillary and venous Hct% [29].

Clinical application and method comparison

A total of 91 patients on adjuvant hormonal treatment with tamoxifen (20 mg/day) were enrolled in this study. Patient's demographic and clinical data are presented in table 2. All patients were classified as adherent to TAM, with reported use of 30 tablets monthly. The patient population displayed a mean age of 56.3 years, mean BMI of 27.0 kg/m^2 and mean Hct% of 36.0

(range 30.6-43.5), 75 patients were Caucasian and 16 were brown or black. Hot flashes were the major adverse effect (51.6%). Eighteen patients (19.8%) reported concomitant use of CYP2D6 inhibitors drugs: 11 on weak inhibitors (e.g. venlafaxine, citalopram and haloperidol) and 7 on strong inhibitors (e.g. fluoxetine and bupropion).

Table 2. Patients demographic characteristics (N=91)

Characteristic	(N=91)
Age (years)	
Mean (± SD)	56.3 (10.1)
Range	28–78
Hematocrit (%)	
Mean (± SD)	36.0 (2.4)
Range	30.6 - 43.5
Race/Ethnicity (n)	
Caucasian	75
Others	16
Menopause status (n)	
Pre menopause	41
Post menopause	50
Tamoxifen duration (months)	
Median (Percentiles 25-75)	15 (9-23)
Range	(4–45)
Marital status (n)	
Single	14
Married	45
Divorced	12
Widowed	20
CYP2D6 inhibitor drug (n)	
Weak/moderately	11
Strong	7
Adverse effects (n) no/yes	
Hot flashes	44/47
Vaginal bleeding	88/3
Vaginal discharge	60/31
Vulvar Itching	73/18
Nausea or vomiting	76/15
Dizzeness	75/16
Cutaneous rash	82/9
Alopecia	68/23
Thrombosis	87/4

The developed method was used to analyze DBS concentrations of TAM and its metabolites, paired with plasma analyses. Analytes concentrations were highly variable in both matrices, as presented in table 3. Median DBS levels in the whole cohort were: TAM 100.0 ng

mL^{-1}; NDT 202.0 ng mL^{-1}, (Z)-EDF 5.9 ng mL^{-1} and HTF 0.9 ng mL^{-1} and median plasma concentrations were: TAM 123.2 ng mL^{-1}; NDT 263.5 ng mL^{-1}, EDF 9.1 ng mL^{-1} and HTF 1.4 ng mL^{-1}. Plasma trough concentrations were consistent with those previously reported in the literature [11;14;25]. EDF levels were 6.5-fold higher than HTF concentrations, with the 5.7-fold [14] and 6.8-fold [30] previously reported, confirming the predominance of EDF over HTF.

Tamoxifen, NDT, EDF and HTF concentrations in DBS were in average 80%, 77%, 59% and 72% of those measured in plasma. EPC were calculated from DBS concentrations after adjustment by patients Hct% and using the correction factors of 0.84; 0.78; 1.12; 0.87 for TAM, NDT, EDF and HTF respectively. After Hct% and factor correction, median estimated plasma levels were: TAM 123.3 ng mL^{-1}; NDT 267.9 ng mL^{-1}, EDF 10.0 ng mL^{-1} and HTF 1.3 ng mL^{-1}, thus estimated levels represented in average 98 to 104% of the actually measured plasma concentrations. The differences between EPC and measured plasma concentrations were within ±20 % for most samples, 87 (97%) TAM, 86 (95%) NDT, 73 (80%) EDF and 80 (88%) HTF, being in accordance with the EMA acceptation criteria for the agreement between methods [23].

DBS concentrations and EPC were highly correlated to measured plasma concentrations with $rs>0.83$ ($P<0.01$) for all associations. Passing-Bablok regressions were performed in order to evaluate the existence of constant and/or proportional errors. Comparison of DBS (y) and measured plasma (x) concentrations returned in intercept 95% confidence intervals of -0.6472 to 10.0400 for TAM, -9.1216 to 28.0722 for NDT, -0.5097 to 0.4635 for EDF, -0.09409 to 0.09373 for HTF and -5.9314 to 0.3608 for the ratio [NDT]/[EDF] indicating no constant error. However, slope 95% confidence intervals showed the presence of proportional difference between methods: 0.6086 to 0.7879 for TAM, 0.6577 to 0.8289 for NDT, 0.4979 to 0.6402 for EDF, 0.6598 to 0.8512 for HTF and 1.3389 to 1.6789 for the ratio [NDT]/[EDF]. When EPC (y) and measured plasma levels (x) were compared, intercept 95% confidence intervals were -11.8954 to 17.8583 for TAM, -30.1816 to 26.4940 for NDT, -1.0641 to 0.7439 for EDF and 0.1460 to 0.1349 for HTF, indicating no constant error. At this evaluation, slope 95% confidence intervals showed to have no proportional differences: 0.8343 to 1.1120 for TAM, 0.9113 to 1.1465 for NDT, 0.9205 to 1.1744 for EDF, and 0.9289 to 1.1778 for HTF, indicating that when the effect of Hct% and multiplication factor are taken into account, methods are comparable. Cusum test P values were all >0.05, indicating no significant difference from linearity.

Table 3. Patients median, percentiles, range and correlations of TAM, NDT, EDF, HTF, and metabolic ratio [NDT]/[EDF] concentrations in plasma, dried blood spot and estimated plasma (N=91).

Analyte	Sample	Median (P25 - P75) (ng mL^{-1})	Range (ng mL^{-1})	Mean ±SD relation to measured plasma (%)	Passing bablok regression to measured plasma	Spearman correlation to measured plasma (rs)
Tamoxifen	Measured plasma	123.2 (101.5 - 165.5)	52.6 - 307.8	-	-	-
	Dried Blood Spot	100.0 (82.58 - 123.0)	40.0 - 290.0	80 ± 11	y = 0.688 x + 0.872 (P=0.65)	0.917**
	Estimated plasma	123.3 (101.4 - 154.1)	49.9 - 364.3	98 ± 14	y = 0.982 x + 0.804 (P=0.69)	0.911**
N-desmethyltamoxifen	Measured plasma	263.5 (188.3 - 353.0)	85.6 - 575.9	-	-	-
	Dried Blood Spot	202.0 (140.0 - 262.1)	76.3 -504.0	77 ± 14	y = 0.739 x + 9.592 (P=0.20)	0.883**
	Estimated plasma	267.9 (177.4 - 350.0)	104.4 - 664.3	102 ± 19	y = 1.018 x + 3.854 (P=0.39)	0.877**
(Z)-Endoxifen	Measured plasma	9.1 (5.8 - 15.1)	2.4 - 40.3	-	-	-
	Dried Blood Spot	5.9 (3.1 - 9.0)	1.0 - 24.2	59 ± 17	y = 0.566 x - 0.041 (P=0.31)	0.895**
	Estimated plasma	10.0 (5.2 - 15.4)	1.5 - 41.3	104 ± 30	y = 1,053 x -0,4376 (P=0.35)	0.897**
4-Hydroxytamoxifen	Measured plasma	1.4 (0.9 - 1.8)	0.7 - 3.9	-	-	-
	Dried Blood Spot	0.9 (0.6 - 1.3)	0.5 - 3.2	72 ± 18	y = 0.761 x + 0.008 (P=0.31)	0.842**
	Estimated plasma	1.3 (0.8 - 1.8)	0.5 - 4.4	99 ± 25	y = 1.061 x - 0.012 (P=0.33)	0.839**
Metabolic ratio [NDT]/[EDF]	Measured plasma	25.8 (16.3 - 45.9)	5.1 - 175.4	-	-	-
	Dried Blood Spot	37.5 (19.6 - 67.9)	7.4 - 230.7	139 ± 41	y = 1.508 x - 3.308 (P=0.63)	0.895**
	Estimated plasma	28.1 (14.7 - 50.9)	5.5 - 173.0	105 ± 31	y = 1.120 x -2.134 (P=0.71)	0.897**

NDT: N-desmethyltamoxifen; EDF: Endoxifen; P25: percentile 25; P75: percentile 75, r_s: spearman correlation coefficient. P value as Cusum test for linearity, ** P<0.01 Spearman correlation

Estimated plasma concentrations (EPC) of TAM and metabolites were also compared to measured plasma concentrations (MPC) by Bland–Altman plots (Figure 2). The mean difference of the concentrations in plasma and estimated plasma concentrations using individual Hct% were 4.0 ng mL^{-1} for TAM, -0.7 ng mL^{-1} for NDT, 0.0 for EDF and 0.06 ng mL^{-1} for HTF, with only 4, 5 and 3 values (out of 91) outside the ± 1.96 standard deviation range for TAM and NDT, EDF and HTF respectively. Moreover, differences were also distributed randomly around the mean, indicating the absence of systematic errors which were proportional to TAM and metabolites levels.

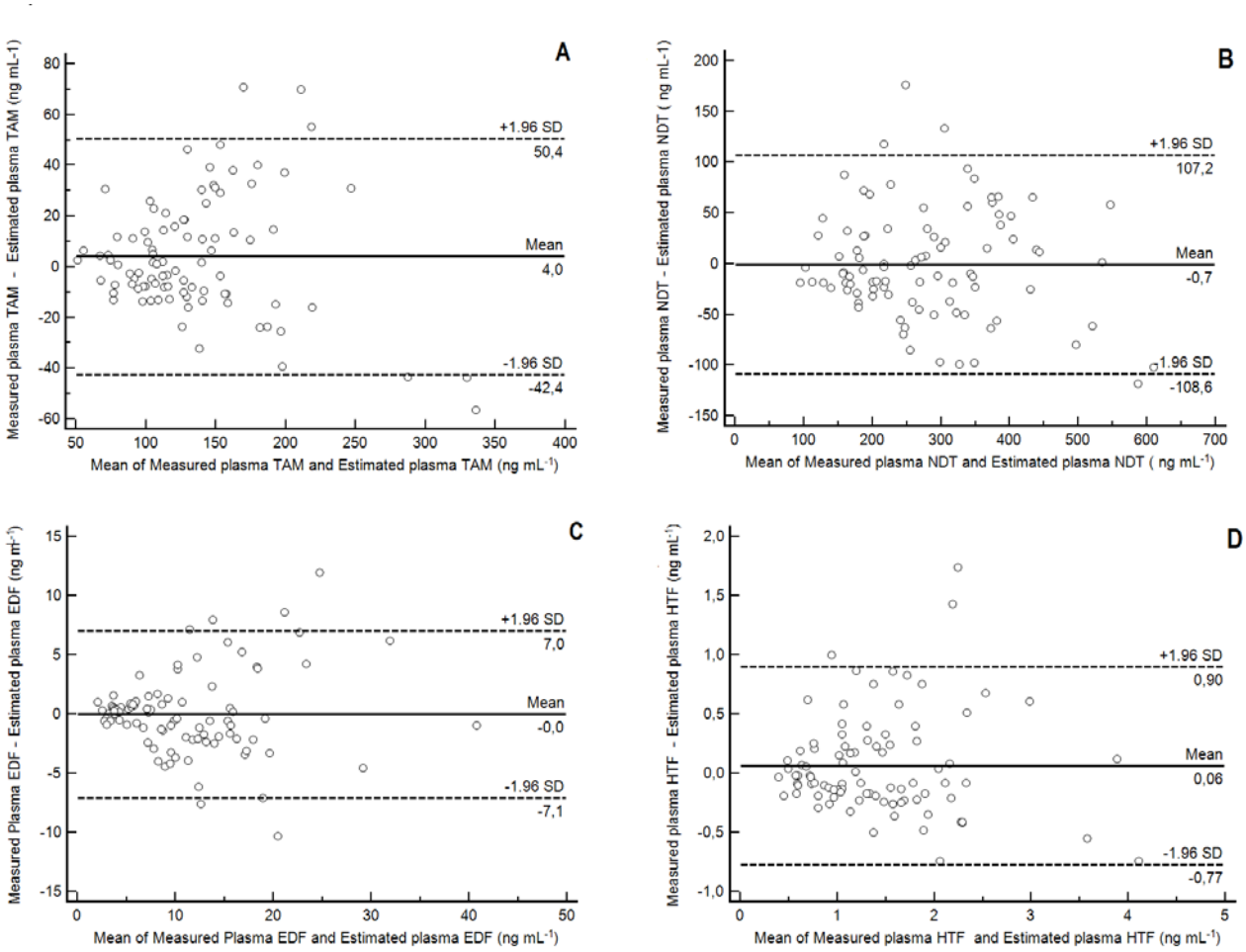


Figure 2. Method comparison, Bland Altman plot of estimated plasma *vs* measured plasma concentrations of TAM (A), NDT (B), EDF (C) and HTF (D) in clinical samples (N=91).

Our data showed a wide range of EDF plasma concentrations among patients, with a 16.8 fold difference between the lowest (2.4 ng ml^{-1}) and the highest (40.3 ng ml^{-1}) measurement. The amplitude of concentrations may be attributed to the interindividual pharmacokinetic variability, including polymorphisms of CYP2D6 and CYP3A4 and drugs interactions [1-7]. Thus, we evaluated the impact of CYP2D6 inhibitors on EDF levels and [NDT]/[EDF] metabolic ratio in both matrices and found reduced metabolites concentrations in patients under concomitant use of CYP2D6 inhibitors drug inhibitors (Table 4.) as compared to the group without use of drug inhibitors, as follows: median DBS 3.5 ng mL^{-1} *vs* 5.8 ng mL^{-1}, EPC 6.1 ng mL^{-1} *vs* 10.2 ng mL^{-1} and measured plasma 5.5 ng mL^{-1} *vs* 10.1 ng mL^{-1} ($P<0.05$ in DBS and EPC, and $P<0.01$ in measured plasma). Moreover, the frequency of patients with EDF levels below the threshold of optimal clinical activity (5.9 ng mL^{-1}) was higher in patients taking CYP2D6 inhibitor drug, 50% (9 of 18), compared to patients without CYP2D6 inhibitors, 20.5% (15 of 73) (Fisher Exact test, $P<0.05$).

The metabolic ratio [NDT]/[EDF], depending on CYP2D6 metabolism, was also affected by the use of drug inhibitors, as observed in both matrices: median DBS 40.5 *vs* 32.0, EPC 24.0 *vs* 40.5 and measured plasma 20.8 *vs* 53.3 for patients taking or not inhibitors respectively ($P<0.05$ in DBS and EPC, and $P<0.01$ in measured plasma). Thus, DBS based monitoring method showed to have equivalent capacity to identify differences on EDF levels and metabolic ratio between CYP2D6 metabolism affected or not by drug interactions as does the usual plasma measurement.

Based on these findings, it was interesting to evaluate if it was possible to identify, through DBS samples, patients with EDF plasma levels below 5.9 ng mL^{-1}. Overall, 24 patients (26.4%) had EDF measured plasma concentrations <5.9 ng mL^{-1}, of these 23 (96%) also presented estimated plasma concentrations below the threshold. Only 4 patients with estimated plasma concentrations <5.9 ng mL^{-1} had measured plasma concentrations above it (6.07 ng mL^{-1}, 6.18 ng mL^{-1}, 6.48 ng mL^{-1} and 7.94 ng mL^{-1}), but still close to the threshold. Thereafter, using Passing-Bablok regression equation, we extrapolated the plasma EDF threshold to DBS concentration and found a proposed DBS threshold of 3.3 ng mL^{-1} (Figure 3). Using the DBS proposed threshold, as previously observed on estimated plasma results, we were able to identify 23 of 24 samples with measured plasma concentrations < 5.9 ng mL^{-1}. The high prevalence of patients with low EDF levels could be related to the high frequency of use of inhibitor drugs

(20%) and other factors such as genetic polymorphisms of CYP2D6 and/or other enzymes to be further evaluated.

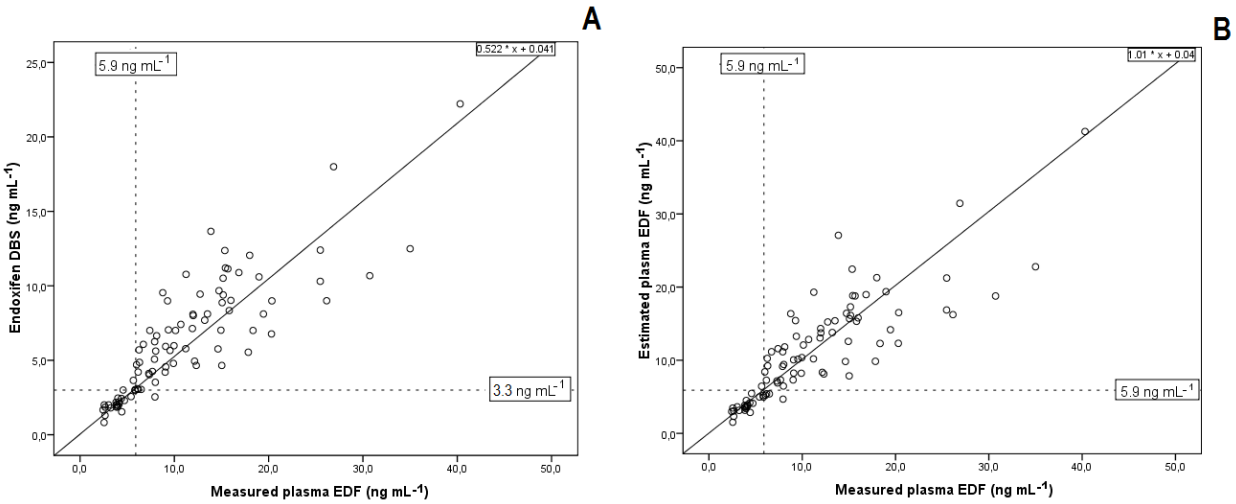


Figure 3. Correlation of EDF concentrations in DBS and plasma (A); Correlation of estimated plasma EDF concentration and EDF measured plasma concentration (B) (N=91). Dashed lines indicate EDF minimum therapeutic threshold concentration in plasma (5.9 ng mL^{-1}) [8] and proposed EDF concentration threshold in DBS, extrapolated from plasma concentrations (3.3 ng mL^{-1}).

Additionally we compared DBS and estimated plasma EDF concentrations between groups of measured EDF above or below 5.9 ng mL^{-1} and found reduced levels at the last group as well: median EDF DBS 2.0 ng mL^{-1} *vs* 7.0 ng mL^{-1} and 3.6 ng mL^{-1} *vs* 12.5 ng mL^{-1} ($P<0.01$), for DBS and estimated plasma respectively. Similarly DBS and estimated plasma [NDT]/[EDF] metabolic ratio were also significantly different between groups of EDF plasma levels (Table 4). In summary, the DBS method was able to identify with high accuracy (96%) patients with plasma EDF levels below the clinical threshold related to better prognosis as well as impaired CYP2D6 metabolism through [NDT]/[EDF] metabolic ratio.

Table 4. Endoxifen levels and metabolic ratio of [NDT]/[EDF] in patients taking or not CYP2D6 inhibitor drugs and according to EDF plasma threshold 5.9 ng mL^{-1}(N=91).

Patients	N	EDF DBS (ng mL^{-1})	Estimated plasma EDF (ng mL^{-1})	Measured plasma EDF (ng mL^{-1})	DBS [NDT]/[EDF]	Measured plasma [NDT]/[EDF]	Estimated plasma [NDT]/[EDF]
		Median (P25 - P75)					
No CYP2D6 inhibitor Drug	73	5.8 (3.3 - 9.2)	10.2 (5.9 - 16.2)	10.1 (6.2- 15.5)	32.0 (12.9 - 42.8)	20.8 (14.6 - 41.7)	24.0 (13.0 - 42.8)
Taking CYP2D6 inhibitor drug	18	3.5 (2.1 - 6.7)	6.1 (3.7 - 11.6)	5.5 (4.0 - 8.4)	40.5 (27.8 - 73.8)	53.3 (34.1 - 69.5)	40.5 (27.8 - 73.8)
	P	<0.05	<0.05	<0.01	<0.05	<0.01	<0.05
Plasma EDF < 5.9 ng mL^{-1}	24	2.0 (1.9-2.5)	3.6 (3.2 - 4.4)	-	90.2 (61.3 - 144.1)	67.5 (42.7- 91.5)	67.3 (45.9 - 108.0)
Plasma EDF ≥ 5.9 ng mL^{-1}	67	7.0 (4.9-9.5)	12.5 (9.2 - 16.4)	-	28.6 (16.6 - 39.7)	19.7 (13.6 - 31.4)	21.4 (12.5 - 29.7)
	P	<0.01	<0.01	-	<0.01	<0.01	<0.01

P value as Mann-Whitney test.

CONCLUSIONS

A method for determination of TAM and its major metabolites in DBS using LC-MS/MS was developed and validated. Transport and storage of DBS samples require special attention, since EDF and HTF concentrations in control samples increased after storage at 45 °C. TAM, NDT, EDF, HTF concentrations in DBS and estimated plasma were highly correlated to measured plasma concentrations. We found a high prevalence of patients with EDF level below the clinical threshold of 5.9 ng mL^{-1} which were effectively identified through DBS samples. These findings suggest that DBS based therapeutic monitoring of TAM can be an efficient tool to optimize adjuvant breast cancer treatment, allowing patients to collect blood samples at their home within the appropriate time to obtain trough samples, facility transport conditions and handling safety. In addition, our results also offer insight into the effects of CYP2D6 inhibitors on TAM metabolism to EDF.

ACKNOWLEDGMENTS

This work was supported by CNPq/FNRS (CNPq registry number 490408/2011-7) and FAPERGS (registry number 1255-2551/13-2) grants.

REFERENCES

1. W. Schroth, L. Antoniadou, P. Fritz, M. Schwab, T. Muerdter, U.M. Zanger, W. Simon, M. Eichelbaum, H. Brauch, Breast cancer treatment outcome with adjuvant tamoxifen relative to patient CYP2D6 and CYP2C19 genotypes, J Clin Oncol. 33 (2007) 5187-5193.
2. T.E. Mürdter, W. Schroth, L. Bacchus-Gerybadze, S. Winter, G. Heinkele, W. Simon, P.A. Fasching, T. Fehm, German Tamoxifen and AI Clinicians Group, M. Eichelbaum, M. Schwab, H. Brauch, Activity Levels of Tamoxifen Metabolites at the Estrogen Receptor and the Impact of Genetic Polymorphisms of Phase I and II Enzymes on Their Concentration Levels in Plasma, Clin. Pharmacol. & Therap. 89 (2011) 708-717.
3. V.C. Jordan, New insights into the metabolism of tamoxifen and its role in the treatment and prevention of breast cancer, Steroids 72 (2007) 829-842.
4. M.E. Irarrázaval, Tamoxifeno y antidepresivos: ¿Antagonistas en la prevención del cáncer de mama?, Rev. Med. Chile 139 (2011) 89-99.
5. M.V. Antunes, R. Linden, T.V. Santos, P. Wallemacq, V. Haufroid, J.F. Classen, H. Andreolla, N. Costa, T.O. Fontanive, D.D. Rosa, Endoxifen Levels and Its Association With CYP2D6 Genotype and Phenotype: Evaluation of a Southern Brazilian Population Under Tamoxifen Pharmacotherapy, Ther. Drug Monit. 34 (2012) 422-431.
6. S.F. Teunissen, H. Rosing, R.H.T. Koornstra, S.C. Linnb, J.H.M. Schellensc, A.H. Schinkel, J.H. Beijnen, Development and validation of a quantitative assay for the analysis of tamoxifen with its four main metabolites and flavonoids daidzein, genistein and glycitein in human serum using liquid chromatography coupled with tandem mass spectrometry, J. Chromatgr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 2519-2529.

7. W.A. Teft, I.Y. Gong, B. Dingle, K. Potvin, J. Younus, T.A. Vandenberg, M. Brackstone, F.E. Perera, Y.H. Choi, G. Zou, R.M. Legan, R.G. Tirona, R.B. Kim, CYP3A4 and seasonal variation in vitamin D status in addition to CYP2D6 contribute to therapeutic endoxifen level during tamoxifen therapy, Breast Cancer Res Treat. 139 (2013) 95-105.

8. L. Madlensky, L. Natarajan, T. Tchu, M. Pu, J. Mortimer, S.W. Flatt, D.M. Nikoloff, G. Hillman, M.R. Fontecha, H.J. Lawrence, B.A. Parker, A.H.B. Wu, J.P. Pierce. Tamoxifen Metabolite Concentrations, CYP2D6 Genotype, and Breast Cancer Outcomes. Clin Pharm & Therap. 89 (2011) 718-725.

9. K. Kiyotani, T. Mushiroda, C.K. Imamura, Y. Tanigawara, N. Hosono, M. Kubo, M. Sasa, Y. Nakamura, H. Zembutsu, Dose-adjustment study of tamoxifen based on CYP2D6 genotypes in Japanese breast cancer patients, Breast Cancer Res Treat. 131 (2012) 137–145.

10. W.J. Irvin Jr, C.M. Walko, K.E Weck., J.G. Ibrahim, W. K. Chiu, E. C. Dees, S.G. Moore, O.A. Olajide, M. L. Graham, S. T. Canale, R..E. Raab, S.W. Corso, J. M. Peppercorn, S. M. Anderson, K. J. Friedman, E. T. Ogburn, Z. Desta, D. A. Flockhart, H.L. McLeod, J. P. Evan, L. A. Carey, Dees, Genotype-guided tamoxifen dosing increases active metabolite exposure in women with reduced CYP2D6 metabolism: a multicenter study, J Clin Oncol. 29 (2011) 3232–3239.

11. N.G.L. Jager, H. Rosing, J.H.M. Schellens, S.C. Linn, J.H. Beijnen, Tamoxifen dose and serum concentrations of tamoxifen and six of its metabolites in routine clinical outpatient care, Breast Cancer Res. Treat. 143 (2014) 477-483.

12. K.H. Lee, B.A. Ward, Z. Desta, D.A. Flockhart, D.R. Jones, Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection: application to a clinical trial, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 791 (2003) 245-253.

13. Y.B. Zhu, Z. Zhang, J.J Zou, Z.X Yu, D.W. Xiao, Optimizing high-performance liquid chromatography method with fluorescence detection for quantification of tamoxifen and two metabolites in human plasma: Application to a clinical study, J. Pharm. Biom. Anal. 46 (2008) 349-355.

14. M.V. Antunes, D.D. Rosa, T.S Viana, H. Andreolla, T.O. Fontanive, R. Linden, Sensitive HPLC–PDA determination of tamoxifen and its metabolites N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in human plasma, J. Pharm. Biomed. Anal. 76 (2013) 13–20.

15. J. Arpini, M.V. Antunes, L.S. Pacheco, D. Gnatta, M.F. Rodrigues, E. Keitel, R. Linden, Clinical evaluation of a dried blood spot method for determination of mycophenolic acid in renal transplant patients, Clin. Biochem. 46 (2013) 1905-1908.

16. N.G.L. Jager, H. Rosing, J.H.M. Schellens, J.H. Beijnen, S.C. Linn, Use of dried blood spots for the determination of serum concentrations of tamoxifen and endoxifen, Breast Cancer Res. Treat. 146 (2014) 137–144.

17. L. Rhoden, M.V. Antunes, P. Hidalgo, C.A. da Silva, R. Linden, Simple procedure for determination of valproic acid in dried bloodspots by gas chromatography–mass spectrometry, J. Pharm. Biom. Anal. 96 (2014) 207–212.

18. T.W. McDade, S. Williams, J. Snodgrass, What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research, Demography 4 (2007) 899-925.

19. A.M. Almeida, M.M. Castel-Branco, A.C. Falcão, Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods, J. Chromatgr. B: Anal. Technol. Biomed. Life Sci. 774 (2002) 215-222.

20. V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hil, J.D. Hulse, J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Bioanalytical method validation—a revisit with a decade of progress, Pharm. Res. 17 (2000) 1551-1557.

21. H. Passing, W. Bablok, A New Biometrical Procedure for Testing the Equality of Measurements from Two Different Analytical Methods, J. Clin. Chem. Clin. Biochem. 21 (1983) 709-720.

22. J.M. Bland, D.G. Altman, Statistical methods for assessing agreement between two methods of clinical measurement, Lancet, i (1986) 307-310.

23. European Medicines Agency, Guideline on Bioanalytical Method Validation, European Medicines Agency (2011) 4–10.

24. S.F. Teunissen, H. Rosing, A.H. Schinkel, J.H.M Schellens, J.H. Beijnen, Bioanalytical methods for determination of tamoxifen and its phase I metabolites: A review. Anal. Chim. Acta. 683 (2010) 21-37.

25. J. Gjerde, M. Hauglid, H. Breilid, S. Lundgren, J.E. Varhaug, E.R. Kisanga, G. Mellgren, V.M. Steen, E.A. Lien, Effects of CYP2D6 and SULT1A1 genotypes including SULT1A1 gene copy number on tamoxifen metabolism, Ann Oncol. 19 (2008) 56–61.

26. D.D. Heath, S.W. Flat, A.H. Wu, M.A. Pruitt, C.L. Rock, Evaluation of tamoxifen and metabolites by LC-MS/MS and HPLC methods, Br. J. Biomed. Sci. 71 (2014) 33-39.

27. A.V. Eeckhaut, K. Lanckmans, S. Sarre, I, Smolders, Y. Michott, Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects, J. Chromatgr. B: Anal. Technol. Biomed. Life Sci. 23 (2009) 2198-2207.

28. E. Dahmane, T. Mercier, B. Zanolari, S. Cruchona, N. Guignarda, T. Buclina, S. Leyvrazc, K. Zamanc, C. Csajkaa,b, L.A. Decosterd, An ultra performance liquid chromatography–tandem MS assay for tamoxifen metabolites profiling in plasma: First evidence of 4 – hydroxylated metabolites in breast cancer patients, J. Chromatgr. B: Anal. Technol. Biomed. Life Sci. 878 (2010) 3402–3414.

29. P. Timmerman, S. White, S. Globig, S. Lüdtke, L. Brunet, J. Smeraglia, EBF recommendation on the validation of bioanalytical methods for dried blood spots, Bioanalysis 14 (2011) 1567–1575.

30. Y. Jin, Z. Desta, V. Stearns, B. Ward, H. Ho, K.H. Lee, T. Skaar, A.M. Storniolo, L. Li, A. Araba, R. Blanchard, A. Nguyen, L. Ullmer, J. Hayden, Z. Lemler, R.M. Weinshilboum, J.M. Rae, D.F. Hayes, D.A. Flockhart, CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment, J. Natl. Cancer Inst. 97 (2005) 30–39.

6. CONSIDERAÇÕES FINAIS

A CYP2D6 é a maior responsável pela bioativação do TAM, seus genótipos e fenótipos estão associados significativamente com as concentrações plasmáticas do EDF, sendo estas proporcionais à capacidade metabólica da enzima. Os resultados desta pesquisa demonstram a complexidade do metabolismo do TAM e sugerem que a CYP3A4 contribui para a sua bioativação, através da formação do metabólito HTF. Ambas as enzimas são caracterizadas por alta variação interindividual, com influência significativa de fatores genéticos e ambientais. No presente estudo, cerca de 20% das pacientes apresentaram diminuição no metabolismo da CYP2D6 e 7% da CYP3A4. A inibição da CYP3A4 parece compensar, pelo o menos em parte, a redução na formação do EDF relacionada à inatividade da CYP2D6, especialmente devido ao aumento significativo das concentrações de HTF. Estes achados podem ser atribuídos à redução na biotransformação do TAM à NDT, com maior transformação do TAM a HTF mediada pela CYP2D6 ou outras enzimas envolvidas na formação do metabólito.

Neste estudo, também foi identificada uma variabilidade significativa na exposição ao EDF e HTF, relacionada a sazonalidade, com concentrações consideravelmente superiores no verão. Como esperado, as concentrações de $25OHD_3$ também foram maiores no verão e tiveram correlação positiva com os níveis de EDF. O mecanismo envolvido na relação entre status de vitamina D, sazonalidade e biotransformação do TAM permanece por ser elucidado, uma vez que a hipótese de ação através da indução da CYP3A4 foi descartada, uma vez que os níveis de $25OHD_3$ não tiveram correlação significativa com as razões metabólicas. Adicionalmente, as concentrações de $25OHD_3$ não foram diferentes entre os grupos ML e MR da CYP3A4. Adicionalmente, nossos achados indicam que a inibição da CYP3A4 pelo alelo **22* aumenta os níveis do HTF, sendo pouco provável que a indução da enzima pela vitamina D tivesse ação semelhante.

Foram produzidos dois trabalhos importantes na área tecnológica envolvendo o desenvolvimento de métodos bioanalíticos. Inicialmente foi desenvolvido e validado um método sensível para fenotipagem da CYP2D6 através da quantificação do fármaco sonda DMT e seu metabólico DTF através de cromatografia líquida. O método se mostrou eficaz na identificação dos diferentes fenótipos da CYP2D6, incluindo os efeitos dos fármacos inibidores da enzima

sobre as razões metabólicas. Esta ferramenta poderá ser utilizada em futuros estudos que envolvam a avaliação desta via metabólica, inclusive para predizer o metabolismo de outros fármacos biotransformados pela CYP2D6.

Foi desenvolvido também um método para a quantificação do TAM e metabólitos em amostras de DBS. Os achados demonstram a aplicabilidade do sangue capilar coletado por meio de DBS no monitoramento terapêutico do TAM. As concentrações plasmáticas estimadas a partir das medidas em DBS foram comparáveis às quantificadas em amostras pareadas de plasma. Além disso, foi possível identificar com precisão as pacientes com níveis de EDF inferiores ao limiar clínico, utilizando o corte em DBS proposto no presente estudo, de 3,3 ng mL^{-1}. Este é o primeiro estudo a determinar simultaneamente o TAM e metabólitos NDT, EDF e HTF em amostras DBS. O método foi extensivamente validado e foram disponibilizadas informações a respeito da estabilidade dos analitos nas amostras de DBS.

7. PERSPECTIVAS

A aplicação da farmacogenética de forma a predizer o prognóstico terapêutico do câncer de mama ou orientar a conduta terapêutica individual ainda está longe da rotina clínica. Apesar do amplo conhecimento a respeito do impacto dos polimorfismos da *CYP2D6* na ativação metabólica do TAM à EDF, pouco se sabe sobre a influência das variações nos genes de outras ezimas envolvidas em seu metabolismo. O desafio atual é ampliar o entendimento sobre o papel de fatores genéticos múltiplos no metabolismo do TAM, como os polimorfismos no gene da *CYP3A4*, além do efeito dos fatores ambientais, em especial o mecanismo envolvido entre o efeito da sazonalidade e exposição à vitamina D sobre a formação dos metabólitos ativos. Estes conhecimentos servirão de base para a realização de estudos bem controlados, com seguimento clínico das pacientes, de forma a possibilitar a introdução do ajuste de dose baseado na farmacogenética para grupos específicos de pacientes ou ainda o uso de terapia alternativa. A abordagem combinada compreendendo a escolha inicial da dose do TAM baseada no genótipo do paciente e o monitoramento periódico das concentrações plasmáticas ou em DBS dos metabólitos ativos EDF e HTF, parece ser uma estratégia promissora na busca da eficácia no tratamento hormonal adjuvante do câncer de mama com o TAM.

ANEXOS

Anexo 1. Termo de Consentimento Livre e Esclarecido

PESQUISA: **Correlação dos níveis plasmáticos de endoxifeno e TGF β2 associados à atividade da CYP2D6 durante terapia com o tamoxifeno**

Código de identificação da paciente # _____
PACIENTE _____

Prezada Sra.:

Estamos conduzindo um estudo para identificar variações genéticas ou ocasionadas pelo uso de medicamentos que interferem no metabolismo do tamoxifeno, o que pode levar à ineficiência do tratamento com este medicamento.

Como a Sra. teve diagnóstico de câncer de mama e realiza tratamento com o tamoxifeno, gostaríamos de convidá-la a participar do estudo. Caso você concorde em participar, realizaremos uma entrevista com questões pertinentes ao tratamento com o tamoxifeno e registraremos suas informações médicas. Você receberá uma dose oral de 33 mg de dextrometorfano e passadas três horas de sua administração coletaremos uma amostra de sangue venoso (16 mL de sangue) e sangue capilar (três gotas de sangue do dedo médio ou indicador). A Sra será solicitada a comparecer ao Hospital para outra avaliação daqui a 6 meses. Somente neste primeiro encontro a Sra receberá o dextrometorfano, no seguinte responderá às perguntas relacionadas ao tratamento com o tamoxifeno, como por exemplo a presença de efeitos adversos e fornecerá novas amostras de sangue venoso (12 mL) e capilar (três gotas de sangue do dedo médio ou indicador).

Com as amostras de sangue, verificaremos se há a presença de uma alteração genética na enzima que metaboliza o tamoxifeno, que implique na diminuição da quantidade de seu composto ativo (o endoxifeno) no sangue e também na quantidade do marcador de efeito TGF beta 2. A dosagem do dextrometorfano também fornecerá informações sobre o comportamento do metabolismo do tamoxifeno. Após 6 meses serão avaliados os níveis do composto ativo (endoxifeno) e do marcador de efeito TGF beta 2 no sangue.

Os materiais biológicos não serão utilizados para outras finalidades que não aquelas expostas neste termo. Durante a realização do estudo as amostras permanecerão armazenados a -20 °C no laboratório em que serão executadas as análises. Após o término do estudo as amostras serão descartadas.

A assinatura desse consentimento informado dará autorização aos pesquisadores do estudo de utilizarem os dados obtidos somente para fins científicos, incluindo a divulgação dos mesmos, sempre preservando a identidade dos pacientes.

Eu,_____fui informado (a) dos objetivos e da justificativa desta pesquisa, de forma clara e detalhada. Recebi informações específicas sobre os procedimentos diagnósticos e tratamento aos quais serei submetido(a), ressaltando que fui suficientemente informado de que minha participação neste estudo não ocasionará qualquer alteração em meu tratamento quimioterápico. Declaro que fui suficientemente esclarecido que: A) Para a realização dos testes laboratoriais serão coletadas amostras de sangue.

B) Que não haverá riscos à minha saúde, podendo, entretanto, haver desconforto em função da punção venosa e transcutânea e devido a ingesta do dextrometrorfano, em casos raros, presença de efeitos adversos brandos, como vertigem ou indisposição gastrointestinal. C) Que poderei consultar os pesquisadores responsáveis em qualquer época pessoalmente ou por telefone, para esclarecimento de qualquer dúvida. D) Que estou livre para, a qualquer momento, deixar de participar dessa pesquisa, que não precisarei apresentar quaisquer justificativas para isso, e caso deixe o estudo, não terei prejuízos no atendimento por esta instituição. E) Que todas as informações por mim fornecidas e/ou resultados obtidos serão mantidos em sigilo e que, estes somente serão utilizados para divulgações em reuniões e revistas sem a minha identificação. F) Que serei informado de todos os resultados obtidos, independentemente do fato de mudar o meu consentimento em participar da pesquisa. G) Que não terei quaisquer benefícios e/ou direitos financeiros sobre os eventuais resultados decorrentes da pesquisa. H) Todos os custos relacionados a exames diagnósticos serão cobertos por verbas próprias do Projeto de Pesquisa.

Todas as minhas dúvidas foram respondidas com clareza e sei que poderei solicitar novos esclarecimentos a qualquer momento. Além disso, terei liberdade de retirar meu consentimento de participação na pesquisa durante o andamento da mesma.

O profissional Drª._____certificou-me de que as informações por mim fornecidas terão caráter confidencial. Em caso de dúvidas, poderei contatar a Sra. *Marina Venzon Antunes*, no telefone *(51) 3586.8800 ramal 9039*, ou através do e-mail *marinaantunes@feevale.br* para esclarecimentos.

Para eventuais esclarecimentos éticos poderá ser contatado o Comitê de Ética em Pesquisa (CEP) no telefone (51) 3359 8304.

Declaro ser de livre vontade minha participação nesta pesquisa.

Assinatura do paciente:_____

Assinatura do Pesquisador Responsável:_____

Porto Alegre, _____de _____de 20____.

OBS: Este termo apresenta duas vias, uma destinada ao usuário (a) ou seu representante legal e a outra, ao pesquisador responsável.

Anexo 2. Adendo ao Termo de Consentimento Livre e Esclarecido

PESQUISA: **Correlação dos níveis plasmáticos de endoxifeno e TGF β2 associados à atividade da CYP2D6 durante terapia com o tamoxifeno**

Código de identificação da paciente # _____
PACIENTE _____

Prezada Sra.:

A Sra. está participando deste estudo sobre a avaliação do metabolismo do tamoxifeno e forneceu amostras de sangue para verificar se possui alteração no gene da enzima CYP2D6, que implique na diminuição da quantidade de seu composto ativo (o endoxifeno) e também na quantidade do marcador de efeito TGF beta 2. Recentemente, foi sugerido em outras pesquisas que uma segunda enzima, chamada CYP3A4 também contribui para a metabolização do tamoxifeno a seu produto ativo. A CYP3A4 é uma enzima produzida no fígado e intestino, cuja atividade pode ser afetada por alterações genéticas e também depende dos níveis de vitamina D. Desta forma, gostaríamos de solicitar sua autorização para a utilizarmos as amostras de sangue que já foram coletadas anteriormente e encontram-se armazenadas, na realização de novas análises incluindo a genotipagem da CYP3A4 e dosagem da vitamina D. Estas análises fornecerão informações adicionais sobre o comportamento do metabolismo do tamoxifeno e permitirão identificar se os níveis de vitamina D são suficientes. Não são conhecidos riscos para este procedimento.

A assinatura desse consentimento informado dará autorização aos pesquisadores do estudo de utilizarem os dados obtidos somente para fins científicos, incluindo a divulgação dos mesmos em reuniões e revistas científicas, sempre preservando a identidade dos pacientes e de forma agrupada.

A sua participação neste estudo não ocasionará qualquer alteração em seu tratamento quimioterápico, sendo que você está livre para, a qualquer momento, deixar de participar dessa pesquisa, sem a necessidade de quaisquer justificativas para isso, e caso deixe o estudo, não terá prejuízos no atendimento por esta instituição. Você será informada de todos os resultados obtidos, independentemente do fato de mudar o seu consentimento em participar da pesquisa. Informamos também que todos os custos relacionados a exames diagnósticos serão cobertos por verbas próprias do Projeto de Pesquisa e você não recebera nenhuma remuneração pela participação.
Os pesquisadores poderão ser consultados em qualquer época pessoalmente ou por telefone, para esclarecimento de qualquer dúvida, sendo o pesquisador responsável Dr. Gilberto Schwartsmann, médico do serviço de Oncologia, telefone 3359.8012. Você também poderá contatar a pesquisadora Marina Venzon Antunes, no telefone (51) 3586.8800 ramal 9039, ou através do e-mail marinaantunes@feevale.br para esclarecimentos.

Para eventuais esclarecimentos éticos poderá ser contatado o Comitê de Ética em Pesquisa (CEP) no 2 ° andar do HCPA, sala 2227, ou através do telefone 33597640, das 8 às 17 h, de segunda a sexta.

Declaro ser de livre vontade minha participação nesta pesquisa.

Nome do participante:_____Assinatura do paciente:_____

Nome do pesquisador:_____ Assinatura do pesquisador:_____

Porto Alegre, _____de _____de 20____.

OBS: Este termo apresenta duas vias, uma destinada ao usuário (a) l e a outra, ao pesquisador responsável.

Anexo 3. Ficha de Avaliação

Código de identificação da paciente:_____

Data de nascimento: ___/___/___

Cor:_____

Estado civil:_____

Escolaridade:_____

Idade da menarca:____ anos

Idade da menopausa:____ anos

Número de anos em menopausa:____ anos

Paridade: ( ) sim número de filhos: ___ ( ) não

Idade do primeiro parto:____ anos

Uso prévio de anticoncepcional: ( ) sim Número de anos: ____ ( ) não

Uso prévio de reposição hormonal: ( ) sim Número de anos: ____ ( ) não

História familiar de câncer: ( ) sim _____ ( ) não

Hormonioterapia com tamoxifeno (data de início):____/____/____

Dados prontuário médico:

Patologia do tumor:

- tamanho:
- tipo histológico (ductal invasor, lobular invasor, carcinoma não especificado, doença de Paget ou outro)
- grau histológico (1, 2 ou 3):
- receptores de estrogênio:
- receptores de progesterona:
- c-erbB2
- linfonodos axilares:
- metástases à distância:

Estadiamento clínico: T N M

Cirurgia:

- Mastectomia radical modificada:
- Setorectomia:
- Linfonodo sentinela:
- Esvaziamento axilar:

Quimioterapia:

- Neoadjuvante (esquema com doses e datas)
- Adjuvante (esquema com doses e datas)
- Paliativa (esquemas com doses e datas)

Anexo 4. Ficha de acompanhamento da paciente

Código de identificação da paciente:_____

Data Avaliação: ___/___/___ Data próxima avaliação: ___/___/___

Peso:____ Kg Altura: _____ m IMC:____

Número comprimidos último mês:_____
Tabagismo: ( ) sim número cigarros dia:_____ ( ) não
Uso de bebida alcoólica ( ) sim freqüência:_____ ( ) não
Nível de atividade física: ( ) sim freqüência:_____ ( ) não

Presença de co-morbidades:

Doença cardíaca ( ) sim ( ) não
Diabete mélito ( ) sim ( ) não
Doença vascular cerebral ( ) sim ( ) não
Doença respiratória ( ) sim ( ) não
Outra (s):_____

Uso de inibidores da CYP2D6:

( ) citalopram Posologia:_____
( ) fluoxetina Posologia:_____
( ) paroxetina Posologia:_____
( ) venlafaxina Posologia:_____
( ) bupropiona Posologia:_____
( ) duloxetina Posologia:_____
( ) terbinafrina Posologia:_____
( ) quinidina Posologia:_____
( ) amiodarona Posologia:_____
( ) difenidramina Posologia:_____
( ) clorpromazina Posologia:_____
( ) haloperidol Posologia:_____
( ) celecoxib Posologia:_____
( ) cimetidina Posologia:_____
( ) clomipramina Posologia:_____
( ) cloranfenicol Posologia:_____
( ) metoclopramida Posologia:_____
( )levomepromazina Posologia:_____

Presença de efeitos adversos do tamoxifeno:

- ondas de calor (fogachos) ( ) sim ( ) não
- sangramento vaginal ( ) sim ( ) não
- corrimento vaginal ( ) sim ( ) não
- prurido vulvar ( ) sim ( ) não
- náuseas ( ) sim ( ) não
- vômitos ( ) sim ( ) não
- tonturas ( ) sim ( ) não
- erupção cutânea ( ) sim ( ) não
- alopecia (queda de cabelo) ( ) sim ( ) não
- eventos tromboembólicos ( ) sim ( ) não