EFFECTS OF *CYP2D6, CYP3A5* AND *SULT1A1* POLYMORPHISMS ON TAMOXIFEN AND ITS METABOLITES AND ESTROGEN CONCENTRATIONS IN THAI BREAST CANCER PATIENTS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ผลของภาวะพหุสัณฐานของยีน *CYP2D6 CYP3A5* และ *SULT1A1* ต่อยาทาม็อกซิเฟน และเมแทบอไลต์ของยานี้กับความเข้มข้นของเอสโตรเจน ในผู้ป่วยมะเร็งเต้านมชาวไทย

นางสาววรรณพร เจริญโชคทวี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
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วรรณพร เจริญโชคทวี : ผลของภาวะพหุสัณฐานของยีน *CYP2D6 CYP3A5* และ *SULT1A1* ต่อยาทาม็อกซิเฟนและเมแทบอไลต์ของยานี้กับความเข้มข้นของเอสโตรเจนในผู้ป่วยมะเร็งเต้านมชาวไทย (EFFECTS OF *CYP2D6, CYP3A5* AND *SULT1A1* POLYMORPHISMS ON TAMOXIFEN AND ITS METABOLITES AND ESTROGEN CONCENTRATIONS IN THAI BREAST CANCER PATIENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ภญ. ดร.ณัฏฐดา อารีเปี่ยม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นพ. ดร.วิโรจน์ ศรีอุฬารพงศ์, รศ. ภญ. ดร.ดวงจิตต์ พนมวัน ณ อยุธยา, หน้า.

ทาม็อกซิเฟน (TAM) เป็นสมาชิกในกลุ่มสารที่สามารถปรับตัวรับเอสโตนเจนอย่างเฉพาะเจาะจงที่ถูกใช้เพื่อการบำบัดด้วยการต้านฮอร์โมนเอสโตรเจนในผู้ป่วยมะเร็งเต้านมที่มีผลบวกกับตัวรับเอสโตรเจน TAM ต้องการเอ็นไซม์ CYP450 หลายชนิดในการเปลี่ยนจากรูปไม่ออกฤทธิ์เป็นรูปที่ออกฤทธิ์ได้ 2 ชนิด (เอนด็อกซิเฟน (END) และ 4-ไฮดรอกซีทาม็อกซิเฟน (4OHT)) ซึ่งเป็นสารยับยั้งอย่างแข่งขันกับเอสโตรเจนในการออกฤทธิ์ที่ตัวรับเอสโตรเจน ภาวะพหุสัณฐานของยีนที่ถูกถอดรหัสเพื่อสร้างเอ็นไซม์ที่เกี่ยวข้องกับกระบวนการเปลี่ยนสภาพของทาม็อกซิเฟน (*CYP2D6* หรือ *CYP3A5*) มีแนวโน้มที่จะส่งผลต่อความเข้มข้นของ TAM และเมแทบอไลต์ของยานี้ ซึ่งอาจนำไปสู่ความล้มเหลวในการรักษา การกลับเป็นซ้ำหรือการตายต่อไป ความชุกสูงของอัลลีลที่บกพร่องจากยีน *CYP2D6* และ *CYP3A5* ได้ถูกรายงานในชาวไทย แต่ความสัมพันธ์ของอัลลีลเหล่านั้นกับ TAM และเมแทบอไลต์ของยานี้ในผู้ป่วยจำนวนมาก รวมถึงความเข้มข้นของเอสโตรเจนและภาวะพหุสัณฐานของเอ็นไซม์ที่เกี่ยวกับการเปลี่ยนรูปของเอสโตรเจน (จำนวนชุดของยีน *SULT1A1* (*SULT1A1* CNVs)) ในผู้ป่วยมะเร็งเต้านมชาวไทยยังไม่เคยมีการศึกษามาก่อน

งานวิจัยนี้ถูกสร้างขึ้นเพื่อศึกษาผลจาก *CYP2D6*2 CYP2D6*10* และ *CYP3A5*3* ต่อความเข้มข้นของ TAM NDMT END และ 4OHT รวมถึงความเข้มข้นของเอสโตรเจน จำนวนชุดของยีน *SULT1A1* และอาการไม่พึงประสงค์จากการใช้ยาทาม็อกซิเฟน ความชุกของจำนวนชุดของยีน *SULT1A1* รวมถึงความสัมพันธ์ของยีนเหล่านั้นกับความเข้มข้นของเอสโตรเจนและอาการไม่พึงประสงค์ในชาวไทยได้ถูกรายงานเป็นครั้งแรกจากงานวิจัยนี้ ผู้ป่วยมะเร็งเต้านมชาวไทยจำนวน 134 คน ได้ถูกนำเข้ามาในงานวิจัย ตัวอย่างเลือดถูกนำมาวิเคราะห์ด้วยวิธี real-time PCR โดยใช้ Taqman® assay HPLC-FLU และ ELISA ผู้ป่วยส่วนมากเป็นผู้ป่วยมะเร็งเต้านมระยะที่ 1 และระยะที่ 2 มีอายุระหว่าง 27.0-82.0 ปี ดัชนีมวลกายอยู่ในช่วง 15.4-40.0 ค่าเฉลี่ยเวลาในการใช้ยา TAM 21.4 (SD 16.1) เดือน ผู้ป่วยเหล่านั้นเป็น ER+/PR+(71.6%) ER+/PR-(26.9%) ER-/PR+(0.8%) และ ER-/PR-(0.8%) *CYP2D6*10/*10 CYP3A5*1/*3* และ *SULT1A1x2* เป็นจีโนไทป์ที่พบมากที่สุด ในขณะที่ *CYP2D6-IM* และ *CYP3A5-EM* เป็นฟีโนไทป์ที่พบมากที่สุด *CYP2D6* ฟีโนไทป์ส่งผลต่อความเข้มข้นของ NDMT (P=0.013) END (P=0.014) และ 4OHT (P=0.017) อย่างมีนัยสำคัญทางสถิติ แต่กลับไม่พบผลเหล่านั้นจากภาวะพหุสัณฐานของยีน *CYP3A5* จำนวนชุดของยีน *SULT1A1* ไม่มีความสัมพันธ์กับความเข้มข้นของเอสโตรเจนหรืออาการไม่พึงประสงค์จากการใช้ยาจากการวิเคราะห์กลุ่มย่อยในผู้ป่วยที่ยังไม่หมดประจำเดือน อย่างไรก็ตามผู้ป่วยที่เกิดอาการไม่พึงประสงค์จากการใช้ยามีความเข้มข้นของเอสโตนเจนสูงกว่าผู้ป่วยที่ไม่เกิดอาการไม่พึงประสงค์จากการใช้ยาอย่างมีนัยสำคัญทางสถิติ (P=0.014)

ภาควิชา เภสัชกรรมปฏิบัติ
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KEYWORDS: TAMOXIFEN / CYP2D6 / CYP3A5 / SULT1A1 / ESTROGEN / THAI / ENDOXIFEN / ADVERSE DRUG REACTION / BREAST CANCER / ESTRADIOL / PREVALENCE / SERM / POLYMORPHISMS / COPY NUMBERS VARIATIONS

WANAPORN CHAROENCHOKTHAVEE: EFFECTS OF *CYP2D6, CYP3A5* AND *SULT1A1* POLYMORPHISMS ON TAMOXIFEN AND ITS METABOLITES AND ESTROGEN CONCENTRATIONS IN THAI BREAST CANCER PATIENTS. ADVISOR: NUTTHADA AREEPIUM, Ph.D., CO-ADVISOR: ASSOC. PROF. VIROTE SRIURANPONG, M.D, Ph.D., ASSOC. PROF. DUANGCHIT PANOMVANA NA AYUDHYA, Ph.D., pp.

Tamoxifen (TAM) is a member of selective estrogen receptor modulators which has been used as antiestrogen therapy in estrogen receptor positive breast cancer patients. TAM needs several CYP450 enzymes to convert its pro-drug form to the two active metabolites (endoxifen (END) and *4*-hydroxytamoxifen (4OHT)) which are the competitive inhibitors with estrogen for acting on estrogen receptor. The polymorphisms of TAM-metabolizing enzymes encoded genes; *CYP2D6 or CYP3A5*, showed potentially effects on TAM and its metabolites concentrations which might subsequently leaded to treatment failure including recurrent or death. The high prevalence of *CYP2D6* and *CYP3A5* incomplete functional alleles were reported in Thai but the associations of those alleles with TAM and its metabolites in large numbers of patients including estrogen concentration and estrogen-metabolizing enzyme polymorphisms (*SULT1A1* copy numbers variations (CNVs)) in Thai breast cancer patients have never been investigated before.

This research was conducted to determine the effects of *CYP2D6*2, CYP2D6*10 and CYP3A5*3* on TAM, NDMT, END and 4OHT concentrations including estrogen concentrations, *SULT1A1* CNVs and TAM-associated ADRs. The prevalence of *SULT1A1* CNVs including the association of those genes with estrogen concentrations and ADRs in Thai were also firstly reported from this study. The 134 Thai breast cancer patients were recruited to the study. The blood samples were analyzed by real-time PCR with TaqMan® assay, HPLC-FLU and ELISA. Most patients were in stage I and stage II breast cancer ranging in age from 27.0-82.0 years. Their BMI were ranged from 15.4-40.0. Mean of TAM used was 21.4 (SD 16.1) months. They were ER+/PR+(71.6%), ER+/PR-(26.9%), ER-/PR+(0.8%) and ER-/PR-(0.8%). *CYP2D6*10/*10, CYP3A5*1/*3* and *SULT1A1x2* copies were the most common genotypes while *CYP2D6-IM* and *CYP3A5-EM* were the most common phenotypes. *CYP2D6* phenotypes were statistically affected NDMT (P=0.013), END (P=0.014) and 4OHT (P=0.017) concentrations while those effects from *CYP3A5* polymorphisms were not suggested. *SULT1A1* CNVs were not associated with estrogen concentrations or ADRs in premenopausal sub-group analysis; however, patients with ADRs had statistically higher estrogen concentrations than those patients without ADRs (P=0.014).

Department: Pharmacy Practice
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4OHT	4-hydroxy-tamoxifen
ADRs	adverse drug reactions
ALT	alanine aminotransferase
AST	aspartate aminotransferase
AUC	area under the curve
CNVs	copy numbers variations
CV	coefficient of variations
CYP450	cytochrome P450
E1	estrone
E1S	estrone sulfate
E2	estradiol
ELISA	enzyme-linked immunosorbent assay
EM	extensive metabolizer
END	endoxifen (4-hydroxy-*N*-desmethyl-tamoxifen)
ER	estrogen receptor
FLU	fluorescence
HPLC	high performance liquid chromatography
IM	intermediate metabolizer
IS	internal standard
NDMT	*N*-desmethyl-tamoxifen
PBS	phosphate-buffered saline
PM	poor metabolizer
PR	progesterone receptor
qPCR	quantitative polymerase chain reaction
R^2	coefficient of determination
SNPs	single nucleotide polymorphisms
SNVs	single nucleotide variations
TAM	tamoxifen
TamNox	tamoxifen-*N*-oxide
ul	microliters
UNL	upper normal limit
UV	ultra-violet
WT	wild type allele
UNL	upper normal limit

CHAPTER I
INTRODUCTION AND RATIONALE

Tamoxifen (TAM) is a member of selective receptor modulator (SERMs) which has been used as a hormone therapy for prophylaxis (20 mg/day for 5 years), adjuvant (20-40 mg/day for 5-10 years) or neoadjuvant treatment (20 mg/day 3-6 months), including metastasis settings (20-40 mg/day 3-6 months) in breast cancer. TAM is a nonsteroidal antiestrogen which expresses potent estrogenic antagonist effect on estrogen receptor (ER) by competing with estrogen from binding to estrogen receptor (ER) and preventing cell growth[6] in ER-positive breast cancer patients. Despite the fact that TAM provides significant clinical benefit, interindividual variability according to the effects of single nucleotide polymorphisms (SNPs) of Cytochrome P450 (CYP450) enzymes on TAM pharmacokinetics were shown in many studies.[3, 7-9] Investigating of Cytochrome P450 (CYP450) polymorphisms are the key areas involved in relationship exploration between pharmacogenetic effect and breast cancer treatment outcome especially for the polymorphisms of *CYP2D6, CYP2C9, CYP2C19, CYP3A4* and *CYP3A5* which are the main metabolizing enzymes that are responsible for phase I TAM metabolisms including sulfotransferase (SULTs) and UDP-glucuronosyl transferases (UGTs) which are the two metabolizing enzymes involving in TAM phase II metabolism. Many researchers have put their efforts to investigate the association between those enzyme polymorphisms and breast cancer clinical outcomes[7-13] (for example; overall survival (OS), recurrent free survival (RFS) and progression free survival (PFS)); during the time, others have tried to determine the association between those enzymes polymorphisms and TAM and its metabolites concentrations[1, 14, 15] including the associations between TAM and its metabolites levels and their surrogate clinical outcomes[16-21] (for example; mammographic density, endometrial thickness, hot flashes or other TAM-associated adverse drug reactions (ADRs)). Among those CYP450 enzymes, CYP2D6 and CYP3A5 are the main enzymes that implement their roles on TAM transformation from its conventional pro-drug form to its active metabolites including endoxifen (END) and 4-hydroxytamoxifen

(4OHT) through *N*-desmethylation and *4*-hydroxylation pathway.[22-24] Several studies have investigated the association between these two enzymes polymorphisms and breast cancer clinical outcomes[3-9] or TAM and its metabolites concentrations[10-12]; however a role of pharmacogenetic testing on clinical monitoring in breast cancer patient has not been concluded at present. Those inconclusive results might be from various factors; for example, clinical management criteria and pharmacological criteria.[20] Ratain et al suggested that the inconsistent results among those studies were mainly attributable to methodological quality factor and they also claimed that source of DNA, genotyping approach, polymorphisms testing, Hardy-Weinberg Equilibrium (HWE), possible population stratification, phenotyping approach, drug dosing, sample size and data analysis are problematic issues for creating quality metrics of pharmacogenomic studies.[25] Hertz et al. suggested that causes of inconsistent results were from different clinical management criteria (hormone receptor classification, menopausal status, TAM combination therapy) and pharmacological criteria (genotyping comprehensiveness, CYP2D6 inhibitor co-administration and TAM adherence) among those studies. They reported that monotherapy use of TAM, comprehensive test of genotyping and CYP2D6 inhibitor co-administration should be taken in to account to yield clearer results.[26]

The associations between TAM and its metabolites and various TAM metabolizing enzymes polymorphisms have been widely investigated recently. Zefra-Ceres et al. found that *CYP2D6*4/*4* was inversely associated with *4*-hydroxy-*N*-desmethyl-tamoxifen (4OHNDT; END) and 4-hydroxy-tamoxifen (4OHT) levels while the effect from *CYP2C19* polymorphisms on these two metabolites was not presented.[15] Gjerde et al. claimed that *CYP2D6* phenotypes were associated with END, 4OHT and *N*-desmethyltamoxifen (NDMT).[27] Lim et al. found that *CYP2D6*5/*10* and *CYP2D6*10/*10* were associated with low END level while the effect of *CYP3A5, CYP2C9* and *CYP2C19* were not found in this study.[28] Among those TAM and its metabolites, END and 4OHT are the two active metabolites that expected to have pharmacological effect on ER. Murdter et al. found that inhibitory concentration 50 (IC_{50}) of (Z)-END and (Z)-4OHT were 3 and 7 nmol/l (1.23 ng/ml and 2.71 ng/ml), respectively and 93% of poor metabolizer (PM) had (Z)-END levels below inhibitory concentration 90 (IC90) values.[1] Furthermore,

they also claimed that *CYP2D6* genotypes was accounted for 39% and 9% of the variability of steady-state concentrations of (Z)-END and (Z)-4OHT, respectively which suggested the roles of additional factors on TAM metabolic pathways [1]; for example, medication non-adherence or investigating of TAM phase II metabolism enzymes. Irvin et al. suggested that eight patients with extensive metabolizer (EM) (*CYP2D6*1, CYP2D6*2 and CYP2D6*35*) had END levels which were comparable to PM (END level lower than 20 ng/mL) even though six of them reported completely medication adherence.[21] Areepium et al. suggested that *UGT2B7*2* was not associated with END levels but patients with *UGT2B7*2/*2* had higher END levels than patients with *UGT2B7*1/*1* and *UGT2B7*1/*2* among *CYP2D6*10/*10* patients in subgroup analysis.[29]

Even though gene polymorphisms testing before TAM dosing is not a routine standard for TAM treatment at this moment, these backgrounds information might be effective for some patients especially for those carrying one or two decreased or non-functional alleles. Prevalence of *CYP2D6* and *CYP3A5* genotypes were found differently among racial population. *CYP2D6*4* was found higher in Caucasians than in Asian or Thai while *CYP2D6*10* was found more frequent in Asian including Thai than in Caucasian especially for *CYP2D6*10/10* that was accounted for 30-35% in Thai breast cancer patients.[28-30]

Furthermore, It was suggested that *CYP2D6*10/*10* was associated with low END levels[28] and Thai patients with *CYP2D6*10/*10* had lower END levels than those patients with *CYP2D6*1/*10* and *CYP2D6*1/*1* (P=0.045).[29] Kiyotani et al. found that increasing TAM dose to 30 mg/day or 40 mg/day can increase mean plasma END levels to 1.4 and 1.7-fold higher than those patients who were using standard dose of TAM 20 mg/day in the groups of patients with *CYP2D6*1/*10* and *CYP2D6*10/*10*, respectively.[20] Irvin et al. suggested that END levels were rose significantly in intermediate metabolizer (IM) and poor metabolizer (PM) patients who were increased TAM dose from 20 mg/day to 40 mg/day (*CYP2D6*:IM; P=<0.001 and PM; P=0.020)[21] and Walko et al. claimed that TAM dose can be increased to 30 or 40 mg/day to achieve higher END concentration in some patients.[31]

Additionally, Gjerde et al. found that TAM, *N*-dedesmethyltamoxifen (NDDMT), END and tamoxifen-*N*-oxide (TamNox) levels were associated with plasma estrogen

concentrations. TAM level was associated with estradiol (E2) and estrone (E1) concentrations while NDDMT level was associated with both E2 and E1 concentrations and TamNox level was associated with E1 concentration.[4] They also claimed that *SULT1A1* copy numbers variations (CNVs) were associated with E1, E2 and estrone sulfate (E1S) concentrations[4] but not associated with TAM and its metabolites levels.[27] Moyer et al. suggested that *SULT1A1* CNVs were not associated with disease-free survival.[32] Yu et al. claimed that SULT1A1 enzyme activities were strongly associated with *SULT1A1* CNVs (trend test P=0.008) but not associated with *SULT1A1* SNPs (P>0.05).[33] Wegman et al. suggested that *SULT1A1* SNPs were not associated with recurrence[34] while Tengström et al. reported that *SULT1A1* SNPs showed an effect on overall long-term survival in breast cancer patients (HR=0.50, P=0.015).[35]

Previous studies have revealed the relationships between those gene polymorphisms and outcomes in Thai breast cancer patients [11, 12, 29]; however, those studies had small sample size or have not concerned about *CYP2D6*4* according to its low prevalence in Asian or Thai. Absence of *CYP2D6*4* determination might not affect the overall prevalence of *CYP2D6* genotypes but it might affect genotype classification; subsequently, covering true effects of those SNPs on TAM and its metabolites levels. Fernández-Santander et al. suggested that patients with *CYP2D6* variant type/variant type (VT/VT) which included *CYP2D6*3, CYP2D6*4 CYP2D6*5* and *CYP2D6*6* had significant lower endoxifen levels than patients with homozygous wild type alleles (P<0.001).[30]

Regan et al. suggested that *CYP2D6* phenotype was associated with TAM-associated hot flashes (P=0.020)[9] while Dezentjé et al. reported that *CYP2D6* genotypes and phenotypes were not associated with the occurrence of hot flashes.[18] Furthermore, Dieudonne et al. suggested that *CYP2D6* polymorphisms showed a statistically significant association with double endometrial thickness[17] while Ruddy et al. claimed that END levels did not differ significantly between patients with and without hot flashes (P=0.54).[19]

The primary objectives of the present study were to explore the prevalence of incomplete functional alleles for *CYP2D6* and *CYP3A5* by focusing on *CYP2D6*10* and *CYP3A5*3* in larger sample size of Thai breast cancer patients and determine the

association between those polymorphisms of TAM metabolizing enzymes (*CYP2D6 and CYP3A5*) and pharmacokinetic parameters of TAM, NDMT, 4OHT and END including the association between those TAM and its metabolites and estrogen concentrations.

Moreover, this study was firstly explored the prevalence of *SULT1A1* CNVs in Thai breast cancer patients and investigated the association between those *SULT1A1* CNVs and estrogen concentrations. This study also examined the subsequent correlations between TAM and its metabolites levels and estrogen concentrations including the effects of *CYP2D6* and *CYP3A5* polymorphisms on TAM-associated ADRs.

Our findings might indicate the high prevalence of *CYP2D6* and *CYP3A5* incomplete functional alleles in Thai breast cancer patients including the impacts of those alleles on tamoxifen-treating breast cancer patients and lead to create a monitoring protocol by using gene polymorphisms testing for some groups of Thai breast cancer patients that their tamoxifen dose needed to be adjusted to achieve maximum clinical benefits and suitable for each stage of breast cancer in practical setting.

CHAPTER II

LITERATURES REVIEW

The review of related literatures will be sorted in 6 topics:

(1) Tamoxifen (TAM), its pharmacokinetics and pharmacodynamics

(2) TAM-associated adverse drug reactions (ADRs)

(3) Selected single nucleotides polymorphisms (SNPs) of TAM-metabolizing enzymes encoded genes

(4) Estrogens and estrogens metabolizing enzymes polymorphisms

(5) Prevalence of TAM-related single nucleotide variations in different populations

(6) Previous researches of SNVs in Thai TAM-treating breast cancer patients

The review of related literatures:

(1) Tamoxifen (TAM), its pharmacokinetics and pharmacodynamics

Tamoxifen (TAM) is a member of selective estrogen receptor modulators (SERMs) which has a role for the treatment and prevention of estrogen receptor positive (ER+) breast cancer by decreasing breast cancer recurrent (50%) and mortality rate (30%).[22] TAM is used as an estrogen receptor (ER) antagonist, which competitively inhibits cancerous ER+ cells from obtaining the estrogen required for cell growth.[22]

Estradiol (E2) exerts its activity by binding to the ER presented in mammary gland which induces a conformational modification and allows the link to co-activators[36]; in contrast, the binding complex of active metabolites of TAM (END and 4OHT) with ER on estrogen response elements (EREs) which located on DNA increases the association to co-repressor proteins which actively inhibit gene transcription[36] **(Figure 1)**. Additionally, the activity of TAM and other SERMs depends on the balance between co-activators and co-repressor proteins, differently represented in tissues and in breast cancer cells.[36]

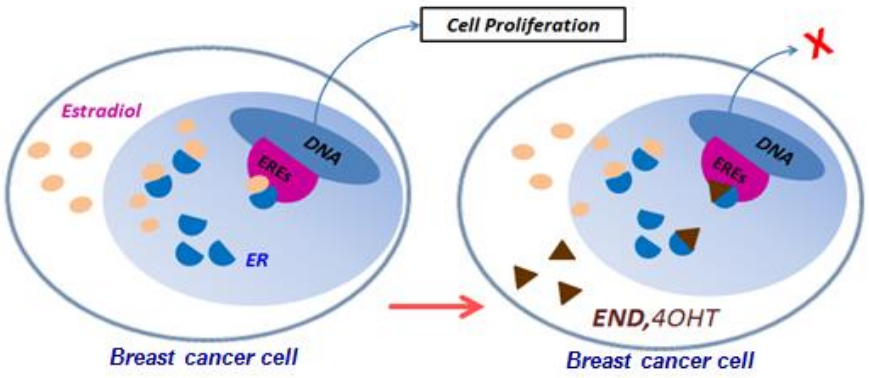


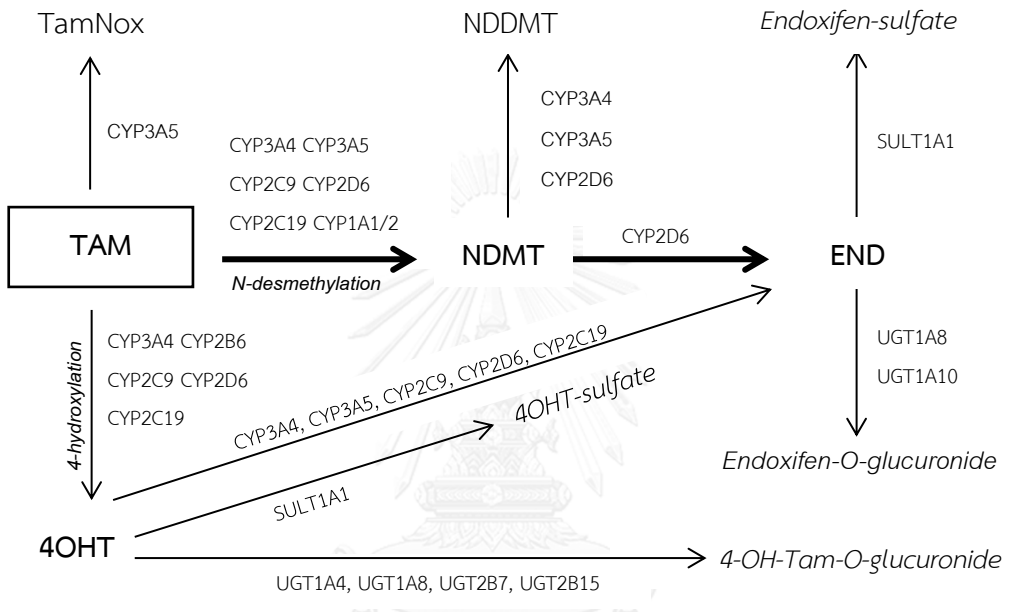
Figure 1: Mechanism of action of TAM on ER in breast cancer cell

Normally it will take 4 weeks to achieve steady state after taking TAM 20 mg/day orally.[6] Many studies have confirmed that TAM is 98% bound to serum albumin which is responsible for its long plasma half-life (7 days).[37] Main CYP450 enzymes which are responsible for TAM phase I metabolism are CYP2D6, CYP2C9, CYP2C19, CYP3A4 and CYP3A5 while sulfotransferase (SULTs) and UDP-glucuronosyl transferases (UGTs) are the two metabolizing enzymes involving in TAM phase II metabolism. TAM is a pro-drug which is mostly metabolized by two metabolic pathways; *N*-desmethylation and *4*-hydroxylation[22] and thereafter converted to at least other 22 metabolites[38] especially for the two major active metabolites; endoxifen (END) and *4*-hydroxytamoxifen (4OHT) [1, 23] including these three transition metabolites; *N*-desmethyltamoxifen (NDMT), *N*-didesmethyltamoxifen (NDDMT) and tamoxifen-*N*-oxide (TamNox)[4] **(Figure 2)**. The END and 4OHT active metabolites shows 30 to 100-fold high potency for suppressing cell proliferation and nearly 100-fold high affinity for ER compared with TAM; however, the 4OHT level is found to be 5-10 times lower than END in plasma.[23]

Although the concentrations of TAM and its metabolites among studies have showed some discrepancies and standard level of END has never been defined, those therapeutic levels of END were drawn from numbers of reports. Jager et al. suggested that predefined therapeutic concentration of (Z)-END was 5.9 ng/ml and

increasing dose of TAM from 20 mg/day to 30 or 40 mg/day leaded to increase (Z)-END level.[39] Ruddy et al. claimed that alternative endocrine therapy should be implemented if baseline END level <6 ng/ml was identified.[19]

Figure 2: TAM metabolic pathways and metabolizing enzymes [1-4]



Note: Tamoxifen (TAM) was metabolized by cytochrome P450 (CYP450) enzyme to its active metabolites; endoxifen (END) and *4*-hydroxy-tamoxifen (4OHT) and eliminated by sulfotransferase (SULTs) and UDP-glucuronosyl transferase (UGTs) enzymes.

TAM-associated adverse drug reactions (ADRs):

TAM is a member of selective estrogen receptor modulators (SERMs) that is not only able to exhibit antiestrogen activity on estrogen receptor (ER) but also able to exhibit estrogen-like activity in some other target organs[40] such as in uterus andcauses endometrial thickness[17] which might potentially be transformed to endometrial carcinoma.[41] Among those TAM-associated ADRs, Westbrook et al. reviewed that hot flashes was reported to be the most common TAM-associated ADRs from the previous study.[23] Lorizio et al. reported that 72.6% of breast cancer patients experienced with some TAM-associated ADRs which hot flashes, sleep

problems and vaginal dryness were the most common ADRs (64.3%, 36.1% and 34.9% , respectively) in 241 mixed-population breast cancer patients (**Table 1**)[42] and Kiyotani et al. reported that hot flashes, hyperhidrosis and vaginal discharge were the most common TAM-associated ADRs in 98 Japanese breast cancer patients for both extensive metabolizers (EM) and intermediate metabolizers (IM) **(Table 2)**.[20]

Table 1: Prevalence of TAM-associated ADRs from previous research [42]

TAM-associated ADRs (Number of patients =241)	Prevalence (%)
No side effects	*27.4*
Hot flashes	64.3
Sleep problems	36.1
Vaginal dryness	34.9
Weight gain	5.8
Irritability and mood swings	3.7
Depression	2.1

Table 2: Prevalence of TAM-associated ADRs related to *CYP2D6* phenotypes [20]

TAM-associated ADRs (Number of patients = 98)	Prevalence (%)		
CYP2D6 genotype	**1/*1*	**1/D and *1/N*	*D/D and D/N*
CYP2D6 phenotype	*EM*	*EM*	*IM*
Hot flashes	90.0	75.0	90.5
Hyperhidrosis	90.0	58.3	61.9
Vaginal discharge	70.0	83.3	57.1
Irregular menstruation	10.0	8.3	9.5
Nausea/Vomiting	10.0	16.7	19.0
Eye disorders	40.0	47.2	38.1
Malaise	70.0	58.3	38.1
Endometrial thickening/Reproductive disorders	0.0	4.4	0.0
Thromboembolic event	4.2	2.2	0.0

Notes: (1) TAM-associated ADRs reported in this table are based from the dosage TAM 20 mg/day.

(2) D=decreased functional allele (*2D6*10, 2D6*41*), N=non-functional allele (*2D6*5, 2D6*21, 2D6*36*)

(3) EM=extensive metabolizer, IM=intermediate metabolizer

Regan et al. found that both *CYP2D6* poor metabolizers (PM) and IM had an increased risk of hot flashes compared with those patients with EM (HR=1.24, 95%CI=1.05-1.43)[9] while Ruddy et al. suggested that hot flashes was not associated with END concentrations <6 ng/ml or genotype polymorphisms.[19] Dezentje et al. reported that *CYP2D6* polymorphisms was not associated with hot flashes (*CYP2D6*3*; P=0.72, *CYP2D6*4*; P=0.31, *CYP2D6*6*; P=0.36, *CYP2D6*14*; P=1.00, *CYP2D6*41*; P=0.94).[43] Additionally, Dieudonne et al. suggested that *CYP2D6* inactive allele was associated with an increased double endometrial thickness in postmenopausal women (P=0.0022).[17]

Selected single nucleotides polymorphisms (SNPs) of TAM-metabolizing enzymes encoded genes

Several genes encoded for CYP450 enzymes are involved in TAM metabolic pathways in terms of their evident genes polymorphisms especially for single nucleotides polymorphisms (SNPs) of those genes. Some genes variations could affect their encoding enzymes functions by increasing or decreasing those enzymes activities while some variations might not affect any enzymes functions depending on their underlined mechanisms of variations **(Table 3)**.

Table 3: Mechanisms of variations of *CYP2D6, CYP3A5* polymorphisms and *SULT1A1* copy numbers variations [44-46]

Gene/Allele	rs numbers	Mechanisms of variations	Enzymes activity
*CYP2D6*1* (wild type)	-	-	Normal
*CYP2D6*2*	rs 16947	Missense	Normal
*CYP2D6*4*	rs 3892097	Splicing defect	None
*CYP2D6*10*	rs 1065852	Missense	Decreased
*CYP3A5*1* (wild type)	-	-	Normal
*CYP3A5*3*	rs rs776746	Intron SNPs, Splicing defect	None
SULT1A1 x 1 copy	-	Copy number variations	Decreased
SUlT1A1 x 2 copies (wild type)	-	-	Normal
SULT1A1 x ≥ 3 copies	-	Copy number variations	Increased

Phase I enzyme metabolism (TAM):

More than 90% of TAM is demethylated by CYP3A4 or CYP3A5 to NDMT, which is subsequently hydroxylated to active metabolites END by CYP2D6.[14] The most common genes variations of TAM metabolizing enzymes are SNPs, duplication or multiplication (for example; *1xN,*2xN,*4xN,*35xN,*36xN), deletion (for example; *5) or tandem gene arrangement (for example; *36+*10).[46] The enzymes activities are depending on their underlined variations. The SNPs of *CYP2D6* and *CYP2C19* may decrease or increase those enzymes activities while polymorphisms of *CYP2C9*, *CYP3A4* and *CYP3A5* cause decreasing or deleting enzymes activities[45] **(Table 4)**. Classification system of those enzymes activities based on their genotypes might be categorized by two methods; conventional phenotype method (extensive metabolizer (EM), intermediate metabolizer (IM) and poor metabolizer (PM)) or genotype activity scores.[14, 47]

The associations between *CYP2D6* polymorphisms and breast cancer outcomes in TAM-treating breast cancer patients have been extensively studied in several reports but those reports have been yielded inconclusive results. Rae et al. found that *CYP2D6* (*CYP2D6*1, CYP2D6*2, CYP2D6*3, CYP2D6*4, CYP2D6*6, CYP2D6*10, CYP2D6*41*) were not associated with recurrence in premenopausal patients (PM vs EM: HR for distance recurrence=1.25, 95%CI=0.55-3.15, P=0.64; HR for any recurrence=0.99, 95%CI=0.48-2.08, P=0.99) [7] which was corresponded with the study from Regan et al. which was investigating *CYP2D6*1, CYP2D6*2, CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6, CYP2D6*10, CYP2D6*41* and reported that PM or IM phenotypes had insignificant reduced risk of recurrence compared with EM (HR of recurrence=0.86, 95%CI=0.60-1.24) in postmenopausal patients.[9] On the contrary, Teh et al. claimed that patients who carried *CYP2D6*10/*10* and heterozygous null allele (IM) showed higher risks of developing recurrence and metastasis than patients with *CYP2D6*1/*1* and *CYP2D6*1/*10* (OR=13.34, 95%CI=0.79-23.2).[48] Karle et al. suggested that patients without any fully functional allele (IM/IM, IM/PM, PM/PM) had a significant shorter progress free survival (PFS) and overall survival (OS) compared with patients with at least one functional allele (EM/EM, EM/IM, EM/PM) (PFS:

HR=2.19, 95%CI=1.15-4.18, P=0.017, OS: HR=2.79, 95%CI=1.12-6.99, P=0.028) in advanced breast cancer setting.[8]

Additionally, two recent meta-analysis researches have shown the conflict results. Lum et al. reported that patients with reduced function of *CYP2D6* had a 11% high risk for all-cause mortality (RR=1.11, 95%CI=0.94-1.31) and a 27% high risk for all-cause mortality with surrogate outcomes compared with those patients with normal function of *CYP2D6* when combining the results from twenty-two treatment-only design studies (association studies) but the association was not found for combining the results from randomized trials (effect modification studies).[49] Province et al. suggested that *CYP2D6* polymorphisms was associated with poorer invasive disease-free survival (IDFS) (HR=1.25, 95%CI=1.06-1.47, P=0.009) when the criterion was specified for postmenopausal women with ER-positive breast cancer who was receiving 20 mg/day TAM for 5 years.[50]

Furthermore, Li et al. suggested that degree of *CYP2D6* enzyme activities was significantly associated with percent mammographic density (PMD) change (P_{trend}=0.021). They also reported that heterozygous EM/IM (het EM/IM) showed 72% (95%CI=0.10-0.79) and PM showed 71% (95%CI=0.03-2.62) less likely to experience a >10% reduction of PMD compared with EM[16] and Karle et al. claimed that clinical benefit (CB) rate of EM group was significantly higher than in IM and PM groups (CB rate 73% vs 38.5%, P=0.019)[8] while Zefra-Ceres et al. suggested that patients with *CYP2D6*4/*4* had 25% lower of END and 4OHT than EM patients (P<0.001) but *CYP2C19*2* did not affect any TAM and its metabolites levels compared with *CYP2C19*1.*[15]

Murdter et al. suggested that 93% of PM/PM patients had (Z)-END levels below the concentration required for 90% ER inhibition (IC_{90}) compared with those patients with fully functional CYP2D6 activity (EM/EM or EM/UM) which had the concentrations above IC_{90} value.[1] Moreover, they also suggested that those patients with *CYP2C9*2* and *CYP2C9*3* had lower plasma concentrations of (Z)-4OHT and (Z)-END (Wilcoxon: P=0.0006 and Wilcoxon: P=0.0032,respectively) while no correlations were found among *CYP2C19*, *CYP3A5,* (Z)-4OHT and (Z)-END concentrations[1] On the contrary, Gjerde et al. found that *CYP2D6* polymorphisms was associated with levels of

4OHT, END and NDMT (P=0.044, 0.003 and 0.001 respectively) and *SULT1A1* polymorphisms was associated with NDMT/TAM ratio and NDDMT/TAM ratio.[27] Fernandez-Santander et al. claimed that *CYP2D6* (WT/WT; *CYP2D6*1, CYP2D6*2, CYP2D6*35*) was associated with higher END level (P<0.001) while *SULT1A2*2* and *SULT1A2*3* were associated with higher 4OHT and END levels (P=0.025 and P=0.006, respectively).[30] Lim et al. reported that *CYP2D6*5* and *CYP2D6*10* were significantly associated with low END and high NDMT concentrations (P<0.001) while *CYP2C9, CYP2C19* and *CYP3A5* polymorphisms were not associated with any of their investigated TAM metabolites.[28]

Moreover, Gjerde et al. suggested that *CYP2D6* (*CYP2D6*2, CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6, CYP2D6*2xN*) were associated with levels of 4OHT, END and NDMT (P=0.019, P=0.009, P=0.044, respectively) while *CYP3A5*3* were associated with TamNox level (P=0.044). It was also reported that *SULT1A1* copy numbers was not associated with any investigated TAM metabolites but was associated with estrone (E1), estradiol (E2) and estrone sulfate (E1S) concentrations (P=0.024, P=0.010 and P=0.005, respectively).[4]

Table 4: Enzymes activities of *CYP2D6, CYP2C9, CYP2C19, CYP3A4, CYP3A5, SULTs and UGTs*

Enzyme activity	Alleles	Enzyme activity	Alleles
CYP2D6[8, 11, 46] (WT=*1,*2)		***CYP3A4***[30] (WT=*1)	
Increased activity	*1xN,*2xN	Non-functional activity	*3,*17
Reduced activity	*10	***CYP3A5***[12] (WT=*1)	
Non-functional activity	*3,*4	Non-functional activity	*3
CYP2C9[45] (WT=*1)		***SULTs***[30, 32, 51] *(WT=1A1*1, 1A2*1)*	
Reduced activity	*2,*3	Increased activity	*1A1xN*
CYP2C19[10, 52] (WT=*1)		Reduced activity	*1A1*2,1A1*3,1A2*2,1A2*3*
Increased activity	*17	***UGTs*** (WT=*2B7*1*)[29]	
Non-functional activity	*2,*3	Reduced activity	*2

Note: WT = wild type

Phase II enzyme metabolism (TAM):

Saladores et al. reported that *UGT2B7*2* was not associated with the concentrations of *trans*-4OHT and *trans*-END while *UGT2B15*2* showed a trend to increase breast cancer recurrence.[3] Areepium et al. found that TAM and END levels were not significantly different among *UGT2B7* genotypes (P=0.613 and P=0.503, respectively).[29] Kiyotani et al. suggested that *SULT1A1*2* did not show any clear association with TAM efficacy and also claimed that copy numbers of *SULT1A1* should be taken into account although the association between *SULT1A1* genotype including the copy numbers variations and TAM and its metabolites levels were not found at the present study.[2] Moreover, Moyer et al. found that *SULT1A1* copy numbers variations were not associated with disease-free survival (DFS) (P=0.667).[32]

Estrogen and estrogen metabolizing enzyme polymorphisms

Sources of estrogens in human body are ovarian and other sources (for example; fat, muscle, skin). The ovarian is a key source for 17β-estradiol (E2) production while estrone (E1) is the estrogen that synthesized from other sources. There are marked differences in E2 concentration during a 28-day menstrual cycle in premenopausal women. The E2 level will be decreased and gradually increased in follicular phase (Day1-Day10 after the first day of menstrual flow (Day0)) then marked increased to peak concentration level in ovulatory phase (Day11- Day15) and gradually decreased again in luteal phase (Day16-Day28) preparing for new menstrual cycle. On the contrary, E2 concentration is quite low and stable during a month in postmenopausal women due to the different source of E2 production.[53] Sources of E2 formation in postmenopausal women is from the transformation process between E1 and E2 which is resulted in lower level of E2 in postmenopausal women than those levels that were observed in premenopausal women.[4]

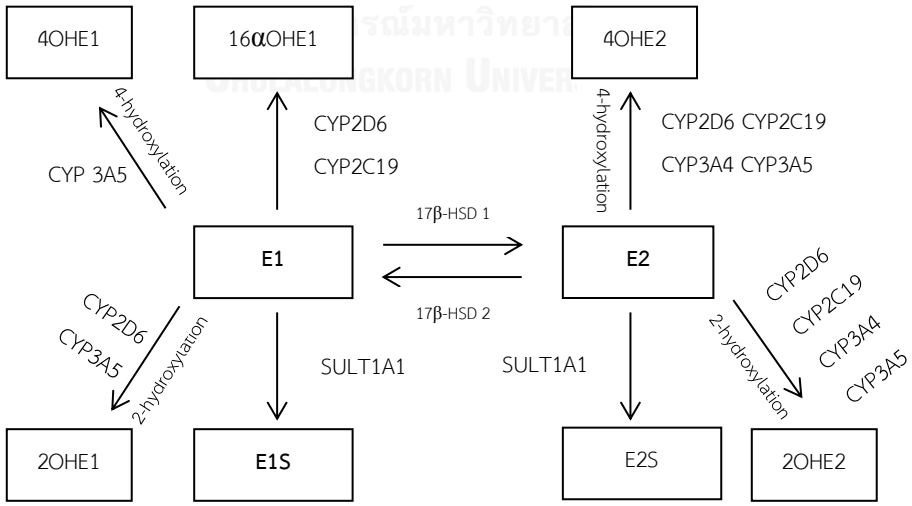
Estrogen metabolisms are also involved in phase I metabolic enzymes (CYP2D6, CYP2C19, CYP3A4 and CYP3A5) and phase II metabolic enzymes (SULT1A1) including two transformed enzymes; 17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD 1) and 17β-Hydroxysteroid dehydrogenase type 2 (17β-HSD 2), which have a role

for reversible converting between E1 and E2. The E2 is a primary ligand that provides estrogenic result that interacts on estrogen receptor (ER)[54] **(Figure 3)**.

Gjerde et al. found that *CYP2D6, CYP3A5* and *SULT1A1* polymorphisms did not affect any estrogen concentrations (P=0.559 (E1), P=0.938 (E2), P=0.804 (E1S), P=0.520 (E1), P=0.248 (E2), P=0.221 (E1S) and P=0.280 (E1), P=0.575, P=734 (E1S) for *CYP2D6, CYP3A5* and *SULT1A1* respectively),whereas *CYP2C19* and *SULT1A1* copy numbers was positively related to the levels of E1, E2 and E1S (P=0.019 (E1), P=0.139 (E2) and P=0.227 (E1S), P=0.024 (E1), P=0.010 (E2), P=0.005 (E1S) for *CYP2C19* and *SULT1A1* copy numbers, respectively).[4]

Lonning et al. found that concentration of E1S (424.5 pM (range 331.3-544.0)) was higher than E1 (74.4 pM (range 64.3-86.1)) and E2 (18.9 pM (15.1-23.7)) in plasma as a result from inhibition of *2*-hydroxylase by TAM in postmenopausal breast cancer patients.[55] They also found that E1S concentration was significantly increased (Level change +18.2%, p=0.031) while E2 concentration was significantly decreased (Level change -18.2%, p=0.004) and E1 concentration level was insignificantly decreased (Level change -6.9%, p=0.093)[55] in plasma when TAM was administered.

Figure 3: Estrogens metabolic pathways and related metabolizing enzymes [4, 5]



Notes: (1) E1S=estrone sulfate, E1=estrone, E2=estradiol

(2) 2OHE1=2-hydroxyl-estrone, 4OHE1=4-hydroxyl-estrone, 16αOHE1=16-α-hydroxyl-estrone

(3) 2OHE2=2-hydroxyl-17β-estradiol, 4OHE2=17-hydroxyl-17β-estradiol

On the contrary, Lum et al. found that mean E2 concentrations significantly increased by 239% from 28 pg/ml to 95 pg/ml ($p<0.05$) and mean E1 concentrations insignificantly increased by 264% from 42 pg/ml to 153 pg/ml ($p=0.06$) in postmenopausal patient while concentrations of E1 and E2 were insignificantly increased in premenopausal patient.[56] Of note, the concentration of E1S was not investigated in that study. Lunardi et al. suggested that measuring of circulating estrogens levels (E1 and E2) might be technically difficult to investigate as a result of very low estrogen concentrations in postmenopausal women, except for E1S that was showed in reliably measurable level in plasma.[57] They also reported that mean circulating E1S was 138 pg/ml (95%CI=117-162) at baseline before starting antiestrogen in postmenopausal patients.[57]

Furthermore, Gjerde et al. suggested that *SULT1A1*2* and *SULT1A1* CNVs were not associated with TAM and its metabolites levels (TAM, NDMT, NDDMT, 4OHT and TamNox) but *SULT1A1* CNVs was associated with levels of E2, E1, and E1S (P= 0.024, 0.010 and 0.005 respectively) in postmenopausal breast cancer women.[4] Yu et al found that *SULT1A1* CNVs was strongly associated with *SULT1A1* enzymatic activity (trend test P=0.008) by investigating of *N*-hydroxy-phenacetin formation in Japanese. Moreover, they also claimed that the subjects who were carrying higher copy numbers (3 or $\geq$4 copy numbers) of *SULT1A1* gene showed higher degree in enzyme activity than those who were carrying only two copies of *SULT1A1* gene.[33]

Prevalence of TAM-related SNVs in different populations

There are marked differences in *CYP2D6* genotypes in population from different racial origins.[46] Prevalence of *CYP2D6*4* has been found higher in Caucasian than in Asian or Thai while *CYP2D6*10* has been found in Asians including Thai than in Caucasian. Early reports have suggested that prevalence of *CYP3A5*1* was the major allele in Thai while *CYP3A5*3* was the major allele in Caucasian and Asian **(Table 5)**.

Table 5: Prevalence of *CYP2D6*, *CYP3A5* and *SULT1A1* polymorphisms in Caucasian, Asian, Chinese and Thai from previous studies [11, 12, 28-30]

Allele/ Genotype	Prevalence (%)				References
	Caucasian	Asian	Chinese	Thai	
CYP2D6					
***1**	76.7[30]	49.0-100.0[28]	14.0-100.0[28]	35.0(11)-47.0[29]	(11, 28-30)
***2**	0.0(30)	19.0[28]	17.0[28]	9.6[11]	(11, 28)
***4**	11.7[30]	<1.0[28]	2.0[28]	0.9[11]	(11, 28, 30)
***10**	0.4[30]	51.0[28]	56.0[28]	53.0[29]	(28-30)
***1/*1**	63.9[30]	30.2-100.0[28]	23.0-100.0[28]	15.7[11]-27.1[29]	(11, 20, 28-30)
***1/*2**	0.0[30]	26.6[28]	35.6[28]	3.5[11]	(11, 28, 30)
***2/*2**	0.0[30]	7.2[28]	1.7[28]	3.5[11]	(11, 28, 30)
***1/*4**	15.0[30]	0.7[28]	2.0[28]	0.0[11]	(11, 28, 30)
***4/*4**	1.5[30]	0.0[28]	0.0[28]	0.0[11]	(11, 28, 30)
***2/*4**	0.0[30]	0.0[28]	0.0[28]	1.8[11]	(11, 28, 30)
***1/*10**	0.0[30]	38.9[28]	47.5[28]	39.0[29]	(20, 28-30)
***2/*10**	0.0[30]	0.0[28]	0.0[28]	7.0[11]	(11, 28, 30)
***4/*10**	0.75[30]	0.0[28]	0.0[28]	0.0[11]	(11, 28, 30)
***10/*10**	0.0[30]	30.9[28]	29.5[28]	33.9[29]	(20, 28-30)
CYP3A5					
***1**	2.2[30]	30.0[28]	25.0[28]	80.0[12]	(12, 28, 30)
***3**	97.8[30]	70.0[28]	76.0[28]	20.0[12]	(12, 28, 30)
***1/*1**	0.0[30]	8.5[28]	8.3[28]	63.0[12]	(12, 28, 30)
***1/*3**	4.4[30]	43.6[28]	32.4[28]	33.0[12]	(12, 28, 30)
***3/*3**	95.6[30]	47.9[28]	59.3[28]	4.0[12]	(12, 28, 30)
SULT1A1 copy number(s)					
1 copy	4.1[32]	0[33]	No data	No data	(32, 33)
2 copies	67.5[32]	65.0[33]	No data	No data	(32, 33)
3 copies	19.5[32]	25.8[33]	No data	No data	(32, 33)
≥ 4 copies	8.9[32]	9.2[33]	No data	No data	(32, 33)

Previous researches of SNVs in Thai TAM-treating breast cancer patients

Sirachainan et al. reported that patients with homozygous *CYP2D6*10/*10 (T/T)* had shorter median DFS than patients with heterozygous *CYP2D6*10 (C/T)* (P=0.036) but it was not different from patients with homozygous *C/C* (P=0.316) while *CYP2D6*4* (1846 G>A; rs3891097) and *CYP2D6*5* (deletion) were not associated with DFS.[58] Sukasem et al. suggested that *CYP2D6* polymorphisms (*CYP2D6*2* and *CYP2D6*3*) were not associated with DFS, except for postmenopausal sub-group analysis and they also found that postmenopausal IM patients showed short DFS compared with other phenotypes (HR=6.85, 95%CI=1.48-31.69, P=0.005) while patients with *CYP2D6*10/*10* had shorter DFS than those patients with heterozygous *CYP2D6*10* and other genotypes (P=0.005).[13]

Chamnanphon et al. claimed that *CYP2D6*10/*10* showed shorter DFS than heterozygous *CYP2D6*10* or others genotypes (log rank test; P=0.046 and P=0.046 respectively) while *CYP2C19*2* and *CYP2C19*3* were not associated with DFS in postmenopausal patients.[11] Sensorn et al reported that *CYP3A5*3* was not associated with DFS but patients with heterozygous *ABCB1 3435 CT* showed shorter DFS than those with homozygous *ABCB1 CC* (P=0.041) and showed higher risk to recurrence than those patients who carried *ABCB1 3435 CC* (HR=5.11, 95%CI=1.05-24.74, P=0.043) while patients with *ABCB1 3435 TT* showed no difference in DFS compared with patients with *ABCB1 3435 CC*.[12]

Areepium et al. suggested that TAM and NDMT concentrations were also significantly different among three different genotypes (*CYP2D6*10/*10, CYP2D6*1/*10, CYP2D6*1/*1*) (TAM; P=0.027, NDMT; P=0.020) and patients with *CYP2D6*10/*10* had lower END concentrations than those patients with *CYP2D6*1/*10* and *CYP2D6*1/*1* (END concentrations 9.62 ng/ml, 15.67 ng/ml and 21.55 ng/ml respectively; P=0.045) while *UGT2B7* polymorphisms did not affect any TAM and its metabolites levels among those genotypes except for the result from subgroup analysis of *CYP2D6*10/*10* which showed that patients with *UGT2B7*2/*2* had higher END concentrations than patients with *UGT2B7*1/*2* or *UGT2B7*1/*1* (P=0.023).[29]

CHAPTER III
RESEARCH METHODOLOGY

Research Questions

1. What are the associations between TAM-metabolizing enzyme polymorphisms and TAM and its metabolites in Thai breast cancer patients?
2. What are the associations between estrogen-metabolizing enzymes polymorphisms and estrogen concentrations in Thai breast cancer patients?
3. What are the correlations between TAM and its metabolites and estrogen concentrations in Thai breast cancer patients?
4. What are the associations between estrogen concentrations and TAM-associated ADRs in Thai breast cancer patients?
5. What are the associations between TAM-metabolizing enzyme polymorphisms and TAM-associated ADRs in Thai breast cancer patients?

Research Hypotheses

1. There is no difference in TAM and its metabolites (END) among different TAM-metabolizing enzyme polymorphisms (*CYP2D6*1, CYP2D6*2* and *CYP2D6*10*) in Thai breast cancer patients.
2. There is no difference in estrogen concentrations (E2) among different metabolizing enzyme polymorphisms (*SULT1A1* copy numbers variations) in Thai breast cancer patients.
3. There is no correlation among TAM and its metabolites (TAM, NDMT, END, and 4OHT) and estrogen concentrations (E2) in Thai breast cancer patients.
4. There is no difference in TAM and its metabolites (TAM, NDMT, END and 4OHT) among other TAM-metabolizing enzyme polymorphisms (*CYP3A5*1* and *CYP3A5*3*) in Thai breast cancer patients.
5. There is no association among estrogen concentrations (E2) and TAM-associated ADRs in Thai breast cancer patients.

6. There is no association among TAM-metabolizing enzyme polymorphisms (*CYP2D6*1, CYP2D6*2, CYP2D6*10, CYP3A5*1* and *CYP3A5*3*) and TAM-associated ADRs in Thai breast cancer patients.

Objectives of the study

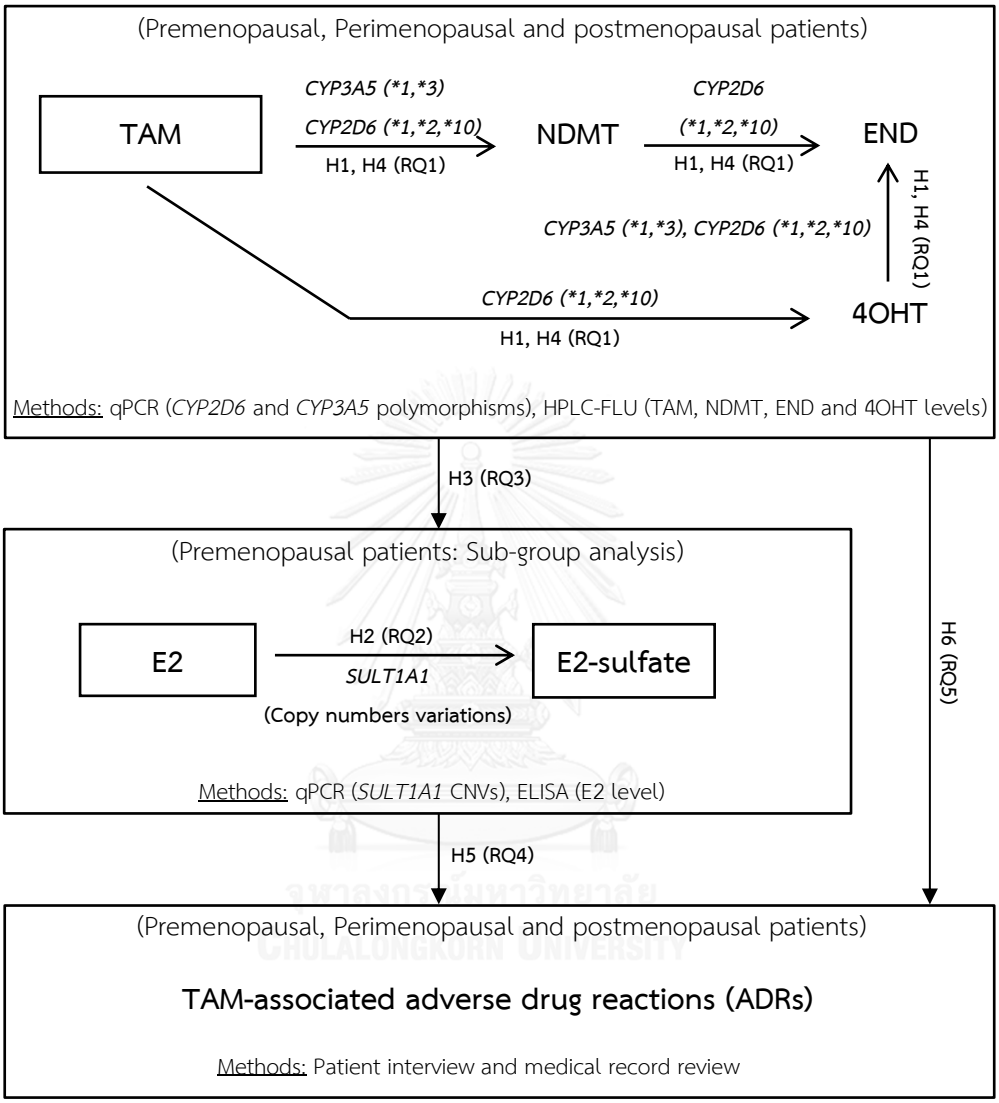
Primary objectives

1. To determine the differences in TAM and its metabolite (END) among different TAM-metabolizing enzyme polymorphisms (*CYP2D6*1, CYP2D6*2* and *CYP2D6*10*) in Thai breast cancer patients.
2. To determine the differences in estrogen concentrations (E2) among different estrogen metabolizing enzyme polymorphisms (*SULT1A1* copy numbers variations) in Thai breast cancer patients.
3. To determine the correlations among TAM and its metabolites (TAM, NDMT, END and 4OHT) and estrogen concentrations (E2) in Thai breast cancer patients.

Secondary objectives

1. To determine the associations among TAM and its metabolites (TAM, NDMT, END and 4OHT) and other TAM-metabolizing enzyme polymorphisms (*CYP3A5*1* and *CYP3A5*3*) in Thai breast cancer patients.
2. To determine the associations among estrogen concentrations (E2) and TAM-associated ADRs in Thai breast cancer patients.
3. To determine the associations among TAM-metabolizing enzyme polymorphisms (*CYP2D6*1, CYP2D6*2, CYP2D6*10, CYP3A5*1* and *CYP3A5*3*) and TAM-associated ADRs in Thai breast cancer patients.

Conceptual framework



Notes: H=research hypothesis, RQ=research question

Figure 4: Conceptual framework

Operational definitions

TAM-metabolizing enzyme polymorphisms were represented by 2 sub-families of TAM-metabolizing CYP450 enzymes; CYP2D6 and CYP3A5 which were referred to all 5 alleles (*CYP2D6*1, CYP2D6*2, CYP2D6*10, CYP3A5*1, CYP3A5*3*) of those enzymes encoded genes. The enzyme activities of the alleles and their rs numbers were shown in **Table 6**.

Table 6: SNPs mechanisms and enzyme activities of *CYP2D6* and *CYP3A5* [45, 59]

Alleles	SNPs mechanism[60]	Global MAF[60]	Prevalence of MAF	Enzyme Activity	rs number
*CYP2D6*1*	SNP was not found	Wild Type	0.47 (Thai)[29]	Normal	-
*CYP2D6*2*	2850C>T(Rev)	A(0.3592)	0.17 (Chinese)[28]	Normal	rs16947
*CYP2D6*10*	100C>T(Rev)	A(0.2380)	0.53 (Thai)[29]	Reduced	rs1065852
*CYP3A5*1*	SNP was not found	Wild Type	0.80 (Thai)[12]	Normal	-
*CYP3A5*3*	6986A>G(Rev)	T(0.3786)	0.20 (Thai)[12]	None	rs776746

These rs numbers were chosen based on their previous publications for global minor allele frequency (global MAF) [60] with higher than 10% of the value including the information of high prevalence in Thai or Chinese population.[29, 45, 58, 61] Even though Gaedigk suggested that 100C>T(Rev) is the SNP position that can be found in both *CYP2D6*4* and *CYP2D6*10* [61], It was believed that detection of 1846G>A position to discriminate *CYP2D6*4* from *CYP2D6*10* was not necessary in this study according to its low prevalence of *CYP2D6*4* in Thai population (*CYP2D6*2/*4*; 2.1%)[13]. Additionally, the 2850C>T (Rev) had also been chosen to determine *CYP2D6*2* in this study which was an additional method to separate *CYP2D6*4* from *CYP2D6*10* by considering their haplotypes. The *CYP2D6*4* haplotypes consisted of 100C>T, 1846G>A and 2850C>T[62] while *CYP2D6*2* and *CYP2D6*10* were determined by 100C>T and 2850C>T positions, respectively.

Estrogen metabolizing enzyme polymorphisms was represented by *SULT1A1* copy number variations (CNVs) which included *SULT1A1*x1 copy, *SULT1A1*x2 copies, and *SULT1A1*x3 copies or more. The activities of those enzymes with copy number variations were shown below **(Table 7)**. The selected *SULT1A1* gene for this study was located on intron 2 of chromosome 16 (NCBI location: 28630011, gene ID 6817, NM_177536.3).[44]

Table 7: *SULT1A1* copy number variations (CNVs) and their enzyme activities[45]

***SULT1A1* CNVs**	**Copy number(s) variations**	**Enzymes activity**
SULT1A1x1 copy	No duplication	Decreased
SULT1A1x2 copies	Duplication	Wild Type
SULT1A1x3 copies or more	Multiplications	Increased

TAM and its metabolites concentrations were represented by tamoxifen (TAM), *N*-desmethyltamoxifen (NDMT), endoxifen (END) and *4*-hydroxytamoxifen (4OHT) concentrations in plasma. All of the concentrations were based on tamoxifen 20 mg/day dosage and were quantified by reverse-phase high performance liquid chromatography (HPLC) technique with fluorescence detector.

Estrogen concentrations *were* represented by plasma concentrations of 17β-estradiol (E2) which were quantified by ELISA technique.

TAM-associated adverse drug reactions (ADRS) were represented by hot flashes, hyperhidrosis, vaginal discharge, irregular menstruation, nausea/vomiting, endometrial thickness, thromboembolic events and other TAM-associated ADRS based on data recorded in medical profile. All TAM-associated ADRs were evaluated by face-to-face interviewing with the patients at the recruitment time or reviewing those patients' medical records or hospital database.

Phenotype was categorized into 3 groups by conventional method; poor metabolizer (PM), intermediate metabolizer (IM) and extensive metabolizer (EM).[45]

Table 8: Phenotype classification by conventional method

Genotypes	Phenotypes	Genotypes	Phenotypes
Functional allele/Functional allele	EM	Reduced allele/Reduced allele	IM
Functional allele/Reduced allele	EM	Reduced allele/Null allele	IM
Functional allele/Null allele	EM	Null allele/Null allele	PM

Menopausal status was categorized into 4 groups; premenopausal, perimenopausal, postmenopausal and unclear status.[63, 64]

Table 9: Menopausal status classification

Menopausal status	Age (years)	Hormonal-related data for classification
Premenopausal	<50	(1) Current use of birth control hormones (2) Reported to have menstrual periods until at least 6 weeks before breast cancer diagnosis
Perimenopausal	50-54	(1) Not sure whether periods have stopped (2) Last menstrual period was reported from 6 weeks to 12 months before breast cancer diagnosis
Postmenopausal	≥55	(1) Report of natural menopause or both ovaries removed or current use of hormone therapy (2) More than 12 months since last menstrual period before breast cancer diagnosis
Unclear status	<55	(1) Hysterectomy without bilateral oophorectomy or not specified surgical menopause (2) The final menstrual period did not reflect the functional endocrine state

Stage of breast cancer was categorized into 5 main groups; stage 0, stage I, stage II, stage III, stage IV including the subcategories of each main stage. The stage of breast cancer was recorded from individual medical record according to the evaluation from physician **(Table 10)**.

Table 10: Stage of breast cancer [65]

Stage of breast cancer	Cancer characteristics
Stage 0	(1) Non-invasive breast cancers (2) Ductal carcinoma in situ (DCIS) (3) There is no evidence of cancer cells or non-cancerous abnormal cells breaking out of the part of the breast, in which they started, or getting through to or invading neighboring normal tissue.
Stage I	
Stage IA	(1) Invasive breast cancer which tumor measures up to 2 cm. AND (2) The cancer has not spread outside the breast; no lymph nodes are involved.
Stage IB	(1) There is no tumor in the breast; instead, small groups of cancer cells (>0.2 mm to ≤2 mm) are found in the lymph nodes. OR (2) There is a tumor in the breast (≤2 cm) and there are small groups of cancer cells (>0.2 mm to ≤2 mm) in the lymph nodes.
Stage II	
Stage IIA	(1) No tumor in the breast but cancer (>2 mm) is found in 1-3 axillary lymph nodes or in the lymph nodes near the breastbone. OR (2) The tumor measures (≤2 cm) has spread to the axillary lymph nodes. OR (3) The tumor (>2 cm-≤5 cm) and has not spread to the axillary lymph nodes.
Stage IIB	(1) The tumor (>2 cm-≤5 cm); small groups of breast cancer cells (>0.2 mm-≤ 2mm) are found in the lymph node. OR (2) The tumor (>2 cm-≤5 cm); cancer has spread to 1-3 axillary lymph nodes or to lymph nodes near the breastbone that were found during a sentinel node biopsy. OR (3) The tumor (>5 cm) but has not spread to the axillary lymph nodes.

Stage of breast cancer	Cancer characteristics
Stage III	
Stage IIIA	(1) No tumor found in the breast or the tumor may be any size; cancer is found in 4-9 axillary lymph nodes or in the lymph nodes near the breastbone. OR (2) The tumor (>5 cm); small groups of breast cancer cells (>0.2 mm-≤2 mm) are found in the lymph nodes. OR (3) The tumor (>5 cm); cancer has spread to 1-3 axillary lymph nodes or to the lymph nodes near the breastbone.
Stage IIIB	(1) The tumor may be any size and has spread to the chest wall and/or skin of the breast and caused swelling or an ulcer. AND (2) May have spread to up to 9 axillary lymph nodes. OR (3) May have spread to lymph nodes near the breastbone. (4) Inflammatory breast cancer is considered at least stage IIIB.
Stage IIIC	(1) The cancer has spread to 10 or more axillary lymph nodes. OR (2) The cancer has spread to lymph nodes above or below the collarbone. OR (3) The cancer has spread to axillary lymph nodes or to lymph nodes near the breastbone.
Stage IV	The cancer has spread beyond the breast and nearby lymph nodes to other organs of the body, such as the lungs, distant lymph nodes or skin, bones, liver or brain.

Research scope

This study was conducted at King Chulalongkorn memorial hospital to investigate the associations among TAM-metabolizing enzyme polymorphisms, TAM and its metabolites, estrogen metabolizing enzyme polymorphisms, estrogen concentrations and TAM-associated adverse drug reactions in Thai breast cancer patient. The study process included patient recruitment, patient interview, medical record review, whole blood collecting, DNA extraction and other laboratories analysis (real time-PCR, HPLC-FLU and ELISA techniques). After whole blood collection, the blood was separated into two layers (buffy coat and plasma) and thereafter analyzed for single nucleotide polymorphisms (DNA from buffy coat), gene copy number variations (DNA from buffy coat), TAM and its metabolites concentrations (plasma) and estrogens concentrations (plasma).

Study place and facilities

(1) King Chulalongkorn memorial hospital
- Patient recruitment and demographic data collection

(2) Department of pharmacy practice (Chulalongkorn University)
- TAM and its metabolites quantification by high performance liquid chromatography (HPLC) with fluorescence detector
- Estrogens concentrations quantification by ELISA technique

(3) Other laboratory settings
- Whole blood separation and DNA extraction
- Single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) determination by real time-PCR (qPCR) with TaqMan assays

Study period 12 months (January 2015-December 2015)

Research design Analytical cross-sectional study

Population and Sample

Target population:

Thai breast cancer patients who were taking tamoxifen (TAM) 20 mg/day

Study population:

Thai breast cancer patients who were taking tamoxifen (TAM) 20 mg/day at King Chulalongkorn memorial hospital during February and March 2015 and eligible to the study

Study sample:

135 Thai breast cancer patients who were taking tamoxifen (TAM) 20 mg/day at King Chulalongkorn memorial hospital during February and March 2015 and eligible to the study (See sample size calculations)

Inclusion and exclusion criteria

***Inclusion criteria*:**

1. Patients who were diagnosed for breast cancer and have filled a prescription for tamoxifen (TAM) 20 mg/day at least for 2 months until the day of recruitment.
2. Patients must be aged at least 18 years.

Exclusion criteria:

1. Patients who were evidenced for abnormal hepatic function (AST or ALT > 2 UNL) or abnormal renal function (Serum creatinine > 1.2 mg/dL) in last four weeks until the recruitment time.
2. Patients who were evidenced for medication non-adherence problem which was evaluated by interviewing with the patients at recruitment time. Patients who reported their missing doses of TAM for more than one time in last four weeks were indicated to have the medication non-adherence problem.
3. Patients who were evidenced for concomitant use of CYP2D6 substrates/ CYP2D6 inhibitors or CYP3A5 substrates/ CYP3A5 inhibitors/ CYP3A5 inducers with

clinically drug-drug interactions problem before the recruitment time (See medication list for drug-drug interaction in **Table 11**).

4. Patients who were diagnosed for psychiatric illness or cognitive impairment.

5. Patients who denied to participate in this study or denied to sign an agreement of participation in this research consent form.

Table 11: Examples of medication list for CYP2D6 and CYP3A5 drug-drug interactions screening[45]

CYP2D6		CYP3A5		
CYP2D6 Substrates	*CYP2D6 Inhibitors*	*CYP3A5 Substrates*	*CYP3A5 Inhibitors*	*CYP3A5 Inducers*
Amitriptyline	Bupropion	Alprazolam	Clarithromycin	Aprepitant
Carvedilol	Celecoxib	Aprepitant	Diltiazem	Carbamazepine
Chlorpromazine	Flecainide	Atorvastatin	Erythromycin	Dexamethasone
Dextromethorphan	Fluoxetine	Carbamazepine	Isoniazid	Ginkgo biloba
Flecainide	Haloperidol	Citalopram	Ketokonazole	Glucocorticoids
Loratadine	Paroxetine	Clarithromycin	Nicardipine	Imatinib
Metoclopramide		Clindamycin	Verapamil	Miconazole
Metoprolol		Dextromethophan	Voriconazole	Phenobarbital
Nortriptyline		Diazepam		Phenylbutazone
Paroxetine		Felodipine		Phenytoin
Propranolol		Fenofibrate		Rifampicin
Tramadol		Haloperidol		Statins
Venlafaxin		Midazolam		Topiramate
		Nifedipine		Troglitazone
		Simvastatin		Valproic acid
		Tramadol		Vinblastine
		Verapamil		
		Zolpidem		

Sample size calculations

Sample size calculations were performed by the G*Power version 3.1 program[66] with statistical power analysis method (Priori method) suggested by Cohen[67] based on their underlining statistical test for the research hypotheses to determine all effect sizes at type-I error = 0.05 (2-tailed) and type-II error = 0.2. The sample size in this research was calculated from the first primary research hypothesis which is there is no association between TAM-metabolizing enzyme polymorphisms (*CYP2D6*1, CYP2D6*2* and *CYP2D6*10*) and TAM metabolite (END) in Thai breast cancer patient. The F-test (3 unequal groups) was employed to test this hypothesis therefore the sample size calculations were calculated based on the effect size of the F-test (one way-analysis of variance (ANOVA) for fixed effect).

The numbers of patients in each *CYP2D6* genotype group (N1, N2, N3, P1, P2 and P3) and the differences of END concentrations among those groups were estimated from a previous study (Areepium et al., 2013).[29] The effect size of the previous study was calculated based on equation derived by Cohen[67] and the standard deviations (SD) within each group were substituted by square root of pool variances as follows **(Table 12)**.

(a) Determine total N and N in each genotype group (N1, N2 and N3) and P1, P2 and P3 from previous study[29]

Number of patients in *CYP2D6*1/*1* (N1) = 16
Number of patients in *CYP2D6*1/*10* (N2) = 23
Number of patients in *CYP2D6*10/*10* (N3) = 20

Therefore, the total number of patients (N) = 16+23+20 = 59

Proportions of patient in each genotype group (P1, P2 and P3) were calculated as follows.

Proportion of patient in *CYP2D6*1/*1* (P1) = N1/N = 16/59 = 0.27
Proportion of patient in *CYP2D6*1/*10* (P2) = N2/N = 23/59 = 0.39
Proportion of patient in *CYP2D6*1/*10 (P3) = N3/N = 20/59 = 0.34

(b) Determine effect size (f) for one-way ANOVA with unequal groups using G*Power program.

$$f = \sqrt{\sum Pi \left(\frac{mi-m}{\sigma}\right)^2}$$

m_i = mean from each group

m = grand mean (computed by G*Power program)

σ = SD within each group calculated from square root of pool variance

Pool variance = $Sp = \sqrt{\frac{\sum_{i=1}^{k}(ni-1)S_i^2}{\sum_{i=1}^{k}(ni-1)}}$

ni = number of sample in i group

S_i^2 = variance of i group

Table 12: Parameters for sample size calculation based on primary hypothesis

TAM Metabolites	N1 *(*1/*1)*	N2 *(*1/*10)*	N3 *(*10/*10)*	Mean N1	Mean N2	Mean N3	σ	ES (f)	N (Total)
Endoxifen (END)[29]	16	23	20	22.4	17.9	14.7	11.93	0.30	111

Note:(1) ES=effect size (standardized mean difference), SD=standard deviation within each group, N=Total sample size

(2) Unit of END concentrations (Mean N1, Mean N2 and Mean N3) is ng/ml.

Total sample size for primary hypothesis was calculated with effect size 0.30 and provided total sample size of 111 patients. Sample size in each group was weighted from P1, P2 and P3.

Sample size for group 1 (*CYP2D6*1/*1*) = P1(N) = 0.27(111) = 29.97 = 30

Sample size for group 2 (*CYP2D6*1/*10*) = P2(N) = 0.39(111) = 43.29 = 44

Sample size for group 3 (*CYP2D6*10/*10*) = P3(N) = 0.34(111) = 37.74 = 38

Therefore, 112 patients were recruited to test the differences of END concentrations among 3 different groups of *CYP2D6* genotypes with effect size 0.30, type-I error 0.05, type-II error 0.2 and the minimum sample size for *CYP2D6*1/*1, CYP2D6*1/*10* and *CYP2D6*10/*10* subgroups were 30, 44 and 38 respectively.

Approximately 20% of patients (23 patients) might be affected from unexpected incomplete data therefore the overall total sample size of 135 patients were drawn to compensate those unexpected effects.

Research Tools

(a) Research documentary tools

(1) Patient's medical profile from breast cancer clinic

(2) Demographics data and TAM-associated ADRs recording form **(Appendix)**

(b) Research laboratory equipment

The research laboratory tools were categorized into 7 categories; whole blood collecting tools, DNA extraction tools, *CYP2D6* polymorphisms determination tools, *CYP3A5* polymorphisms determination tools, *SULT1A1* copy numbers variations determination tools, TAM and its metabolites quantification tools and estrogens concentrations quantification tools **(Table 13)**.

Blood sample

Each participant was interviewed about time of tamoxifen (TAM) ingested and asked to donate their 10 ml of whole blood once at the recruitment time which occurred between 8.30 am and 4.30 pm every Tuesday during February-March 2015. The blood sample was drawn within one hour after finishing patient interview and consent form processes and subsequently collected in BD Vacutainer® plus plastic K_2EDTA tube (BD, USA) by registered nurse. The collected blood was kept at 4 degree Celsius and thereafter separated for its buffy coat and plasma sections. The buffy coat and plasma sections were stored at -20 and -80 degree Celsius, respectively until the next analytical processes.

Table 13: Summary of research laboratory techniques and tools/materials

Research variables	Techniques	Tools/Materials
Blood sample	Whole Blood Collection	BD Vacutainer® plus plastic K_2EDTA tube 10 ml. (BD, USA) (Lavender closure with rubber stopper) Syringe 10 ml, Needle, Cotton ball and Alcohol solution
DNA extraction	DNA Extraction	QIAamp® DNA Mini Kit (Qiagen®, Netherlands) QIAcube Analyzer (Qiagen®, Netherlands)
CYP2D6 polymorphisms *(*1,*2,*10)*	Real time PCR (qPCR)	StepOnePlus realtime® PCR system with ViiA 7 software (Applied Biosystems, USA) TaqMan® Drug Metabolism Genotyping Assay Sets TaqMan® Universal PCR Master Mix
CYP3A5 polymorphisms *(*1,*3)*	Real time PCR (qPCR)	StepOnePlus realtime® PCR system with ViiA 7 software (Applied Biosystems, USA) TaqMan® Drug Metabolism Genotyping Assay Sets TaqMan® Universal PCR Master Mix
SULT1A1 Copy number variations	Real time PCR (qPCR)	StepOnePlus realtime® PCR system with ViiA 7 software (Applied Biosystems, USA) TaqMan® Drug Metabolism Copy Number Assay Sets Reference assay for Copy Number determination TaqMan® Universal PCR Master Mix
TAM and its metabolites Quantification (TAM,NDMT,END,4OHT)	HPLC-FLU	HPLC: Prostar (model 363) with autosampler (model 410) and column oven (model 510) with fluorescence detector and Varian Star software (Varian, USA) Column: Luna 5U C18(2) 100 A, 250 x 4.6 mm (Phenomenex, USA) Mobile phase: 1% TEA and Methanol Standards: TAM and (E/Z)-4OHT (Fluka, Singapore), NDMT and (E/Z)-END (Sigma-Aldrich, Singapore) Internal standard: Mexiletine (Sigma-Aldrich, Singapore)
Estrogen concentrations Quantification (E2)	ELISA	Estradiol (E2) Bioassay™ (Human) ELISA Kit (US Biological, USA) Multilabel counter (VICTOR^3) (PerkinElmer, USA)

DNA extraction process (Spin protocol from buffy coat)

The buffy coat section was drawn for DNA extraction with spin protocol. The extraction process was performed by using QIAamp® DNA Mini kit (Qiagen®, USA) and thereafter quantified for the DNA concentrations by using automated DNA purification machine (QIAcube, Qiagen®, USA). All the DNA samples were stored at -20 degree Celsius until performing next analysis. The content of QIAamp® DNA Mini Kit was described below.

The content of DNA extraction kit (QIAamp® DNA Mini kit)

(1) QIAamp Mini Spin Columns
(2) Collection Tubes (2 ml)
(3) Buffer AL, Buffer AW1, Buffer AW2, Buffer AE, QIAGEN® Pretease
(4) Protease solvent

***Purification of the DNA sample*[68]**

The DNA concentration was determined from the eluate and measured by QIAcube analyzer for absorbance at 260 nm. Purity of the DNA was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm.

Table 14: DNA extraction's quality test criterias by QIAcube[68]

Parameters	QIAcube analyzer criterias for Pure DNA
Absorbance at 260 (A260) nm.	0.1-1.0
Ratio of A260/A280	1.7-1.9

Allelic discrimination assays (SNPs and CNVs determination assays)

The alleles of *CYP2D6 (CYP2D6*1, CYP2D6*2* and *CYP2D6*10), CYP3A5 (CYP3A5*1* and *CYP3A5*3)* and *SULT1A1* copy numbers variations were determined by real time PCR (qPCR) using StepOnePlus® real time PCR with ViiA7 software (Applied Biosystems, USA). All DNA samples were diluted to final concentration of 10 ng/ml with autoclaved Ultra-pure Type I water. The details of TaqMan assays and qPCR conditions were described below **(Table 15 and Table 16)**.

Table 15: TaqMan allelic discrimination assays

Polymorphisms	SNPs position	SNPs mechanism	Enzyme activity	rs number
*CYP2D6*2*	2850C>T (Rev)	Missense	Normal	rs16947
*CYP2D6*10*	100C>T (Rev)	Missense	Reduced	rs1065852
*CYP3A5*3*	6986A>G (Rev)	Intron SNPs, Splicing defect	None	rs776746
*SULT1A1*x1 copy	-	Copy number variations	Decreased	-
*SULT1A1*x>2 copies	-	Copy number variations	Increased	-

Table 16: Real time PCR (qPCR) conditions (TaqMan assays)

Polymorphisms	Pre-PCR	Hold stage	PCR	Post-PCR	Reference assay
*CYP2D6*2*	60 C:30 S	95 C:10 M	95 C:15 S, 60 C: 1 M	60 C:30 S	No
*CYP2D6*10*	60 C:30 S	95 C:10 M	95 C:15 S, 60 C: 1 M	60 C:30 S	No
*CYP3A5*3*	60 C:30 S	95 C:10 M	95 C:15 S, 60 C: 1 M	60 C:30 S	No
SULT1A1 CNVs	None	95 C:10 M	95 C:15 S, 60 C: 1 M	None	Yes

Note: C=degree Celsius, M=minute, S=second

Quantification of TAM and its metabolites

TAM and its metabolites concentrations were quantified from patient's plasma. After the whole blood was collected at the time of recruitment, the plasma was subsequently separated for performing reverse-phase HPLC-FLU analysis. All plasma samples were stored at -80 degree Celsius until next analytical process. The HPLC-FLU method validation and plasma extraction protocol were further modified from the methods developed by Zhu et al.[69] and Areepium et al.[29] The details of HPLC-FLU method in this study was described below.

Instruments:

HPLC-FLU: Prostar (model 363) with autosampler (model 410) and column oven (model 510) with fluorescence detector and Varian Star software (Varian, USA)
Column: Luna 5U C18 (2) 100 A, 250 x 4.6 mm (Phenomenex, USA)
Column temperature: 35 degree Celsius
Mobile phase: 1% TEA and Methanol (19:81 %V/V)
Flow rate: 1.1 ml/min
Volume of injection: 50 ul

Standards and chemicals:

Four of TAM and its metabolites standards: TAM and (E/Z)-4OHT (Fluka, Singapore), NDMT and (E/Z)-END (Sigma-Aldrich, Singapore), and internal standard (IS): Mexiletine (Sigma-Aldrich, Singapore), were purchased to perform the HPLC-FLU method validation and calibration curve.

The methanolic standard stock solutions of TAM, NDMT, END, 4OHT and Mexiletine were prepared by powder dissolution for obtaining 5 mg/ml of Mexiletine, 0.01 mg/ml of END and 4OHT and 0.1 mg/ml and 0.3 mg/ml for TAM and NDMT. The working solution were prepared from each stock solution with proper volume of methanol to obtain 6 non-zero standard solutions containing: TAM (25, 50, 100, 500, 750 and 1,000 ng/ml), NDMT (25, 50, 100, 500, 750 and 1,000 ng/ml), END (5, 10, 50, 75, 100 and 300 ng/ml) and 4OHT (2.5, 5.0, 7.5, 12.5, 25 and 50 ng/ml). All stock solutions were stored at -20 degree Celsius and protected from light.

Triethylamine (TEA) HPLC grade (Sigma-Aldrich, Singapore), methanol HPLC grade (Fisher Scientific, UK), acetonitrile HPLC grade (RCI Labscan, Thailand) and Ultra-pure analytical grade Type I water were used for mobile phase and plasma extraction.

Sample preparation (TAM and its metabolites quantification):

The 10 ul of internal standard (Mexiletine 5 mg/ml) was added to 1,000 ul of plasma sample, followed by 1,000 ul of acetonitrile and 500 ul of methanol into 15 ml of centrifuge tube. The tube was capped and vortex mixed for 10 minutes and subsequently centrifuged 2 times at 3,000 rpm (4 C) for 30 minutes and 10 minutes, respectively. The supernatant was filtered through 0.22 um nylon filter and thereafter derivatized by UV-lamp at wavelength 366 nm for 20 minutes before injected to HPLC column.

Method validation (TAM and its metabolites quantification)

Selectivity (Specificity): Selectivity was the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The chromatogram of analyte from six sources of blank samples (plasma) was compared with the chromatograms of those standards (TAM 750 ng/ml, NDMT750 ng/ml, END 75 ng/ml and 4OHT 25 ng/ml) in blank plasma.

Extraction efficiency (%Recovery): The recovery of an analyte was the measured concentration (standards in methanol) relative to the known amount added to the matrix (standards in plasma). The recovery experiments should be performed for extracted samples at three concentrations.[70] The three concentrations of TAM and its metabolites were prepared as followed: TAM (750, 250 and 50 ng/ml), NDMT (750, 250 and 100 ng/ml), END (100, 75 and 50 ng/ml) and 4OHT (100, 75 and 50 ng/ml).

Linearity: The calibration curves were evaluated by coefficient of determination (R^2). The acceptance criteria of R^2 should not less than 0.999.[70]

Calibration curve (TAM and its metabolites): The calibration curve should consist of a minimum of six non-zero calibrator concentrations covering the entire range of expected concentrations.[70] The standard calibrator concentrations should be within 20% of the nominal concentration at all other concentrations.[70] The

calibration points of standards in this study were prepared as followed: TAM (0, 25, 50, 100, 500, 750 and 1,000 ng/ml), NDMT (0, 25, 50, 100, 500, 750 and 1,000 ng/ml), END (0, 5, 10, 50, 75, 100 and 300 ng/ml) and 4OHT (0, 2.5, 5.0, 7.5, 12.5, 25 and 50 ng/ml). The calibration curves were constructed by calculating the ratios of the peak area of those analyses to the peak area of IS and relating the ratios with known concentrations.

Accuracy: Accuracy was determined by replicate analysis of samples containing known amounts of the analyte.[70] The measure should be performed at least five determinations per concentration. A minimum of three concentrations in the range of expected sample concentrations was recommended.[70] The five replications were prepared from one concentration of TAM and its metabolites as followed: TAM 100 ng/ml, NDMT 750 ng/ml, END 75 ng/ml and 4OHT 25 ng/ml.

Precision: Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected sample concentrations was recommended.[70] The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV).[70] The five replications were prepared from one concentration of TAM and its metabolites as followed: TAM 100 ng/ml, NDMT 750 ng/ml, END 75 ng/ml and 4OHT 25 ng/ml.

Determination of TAM and its metabolites concentrations:

The quantification of TAM and its metabolites was performed by using internal standard technique. The calibration curves of TAM and its metabolites were separately created and interpolated for TAM and its metabolites concentrations in plasma samples.

Estrogen (estradiol, E2) quantification

The Estradiol (E2) Bioassay™ ELISA Kit (Human) (United States Biological, USA) was employed to perform the plasma E2 concentration quantification. The detection range was 250-5,000 pg/ml and the sensitivity of the test was 1 pg/ml. Plasma samples were prepared in 96-wells microtiter strips to perform the quantitative competitive enzyme-linked immunoassay technique. The intensity of color was measured spectrophotometrically at 450 nm in microplate reader. The intensity of the color was inversely proportional to the E2 concentration in plasma sample and the E2 concentration was interpolated from calibration curve. The kit was stored at 4 degree Celsius and used within 6 month after receipt.

Chemicals and reagent preparation (E2 quantification by ELISA):

Wash solution (100X) 10 ml was freshly diluted with 990 ml of deionized water to prepare 1,000 ml of wash solution (1X) before performing the assay. Other kit's components were ready to use.

Sample preparation (E2 quantification):

Plasma samples were stored at -80 degree Celsius and analyzed for E2 concentration within 6 months after sample collection. The plasma samples were brought to room temperature and mixed gently prior to the assay. According to the predicted low E2 concentration in TAM-treating premenopausal breast cancer patients, the sample dilution process was not necessary in this study and blank plasma was subsequently used instead of PBS (dilution buffer). All standards (100 ul/well) and plasma samples (100 ul/well) were added in duplicate to the 96-wells plate.

Calibration curve and estradiol (E2) quantification:

The concentrations of 0, 250, 500, 1,000, 2,500 and 5,000 pg/ml were prepared for E2 calibration curve. The data was created by plotting the known concentration versus the optical density (O.D.) and subsequently constructing the best fit line by regression analysis. The estradiol (E2) concentration was calculated by interpolating from the constructed E2 calibration curve.

Summary of data collections and research procedures

1. Patient's name list, hospital numbers (HN) and patients' medical file were retrieved and sorted by their routine follow-up dates at outpatient cancer clinic.

2. TAM-treating breast cancer patients who met the inclusion criteria were individually invited to join the research by the researcher. All participants were thoroughly explained about the research protocol including risks and benefits of participating the research before deciding to join the research and sign their inform consents.

3. Patient identification (PID) code was generated to represent patient identity in this research to cover individual information and preserve patient's right.

4. Individual demographic data and TAM-associated ADRs were retrieved from medical record, hospital database and face-to-face interview by the researcher. Any inconsistent data were confirmed by an expert opinion to draw a conclusion.

5. The 15 ml. of whole blood was drawn from each patient by a professional nurse between Day1-Day5 of their individual menstrual cycle for premenopausal patients while the blood was drawn on any day of the cycle for postmenopausal patients.

6. The whole blood was separated into 2 sections; buffy coat and plasma. The buffy coat was used for SNVs determinations and the plasma was used for determination of TAM and its metabolites and estrogen.

7. Polymorphisms of *CYP2D6* (*CYP2D6*2* and *CYP2D6*10*) and *CYP3A5* (*CYP3A5*3*) and *SULT1A1* CNVs were determined by real time PCR (qPCR) technique with TaqMan® discrimination assays.

8. TAM and its metabolites (TAM, NDMT, END and 4OHT) were quantified by high performance liquid chromatography (HPLC) with fluorescence (FLU) detector.

9. Estrogen concentration (E2) was quantified by ELISA technique.

10. All collected data (demographics and TAM-associated ADRs recording form and pharmacogenetic data and PK/PD recording form) were summarized and analyzed by SPSS V.22 program (Chulalongkorn University).

Research flow chart

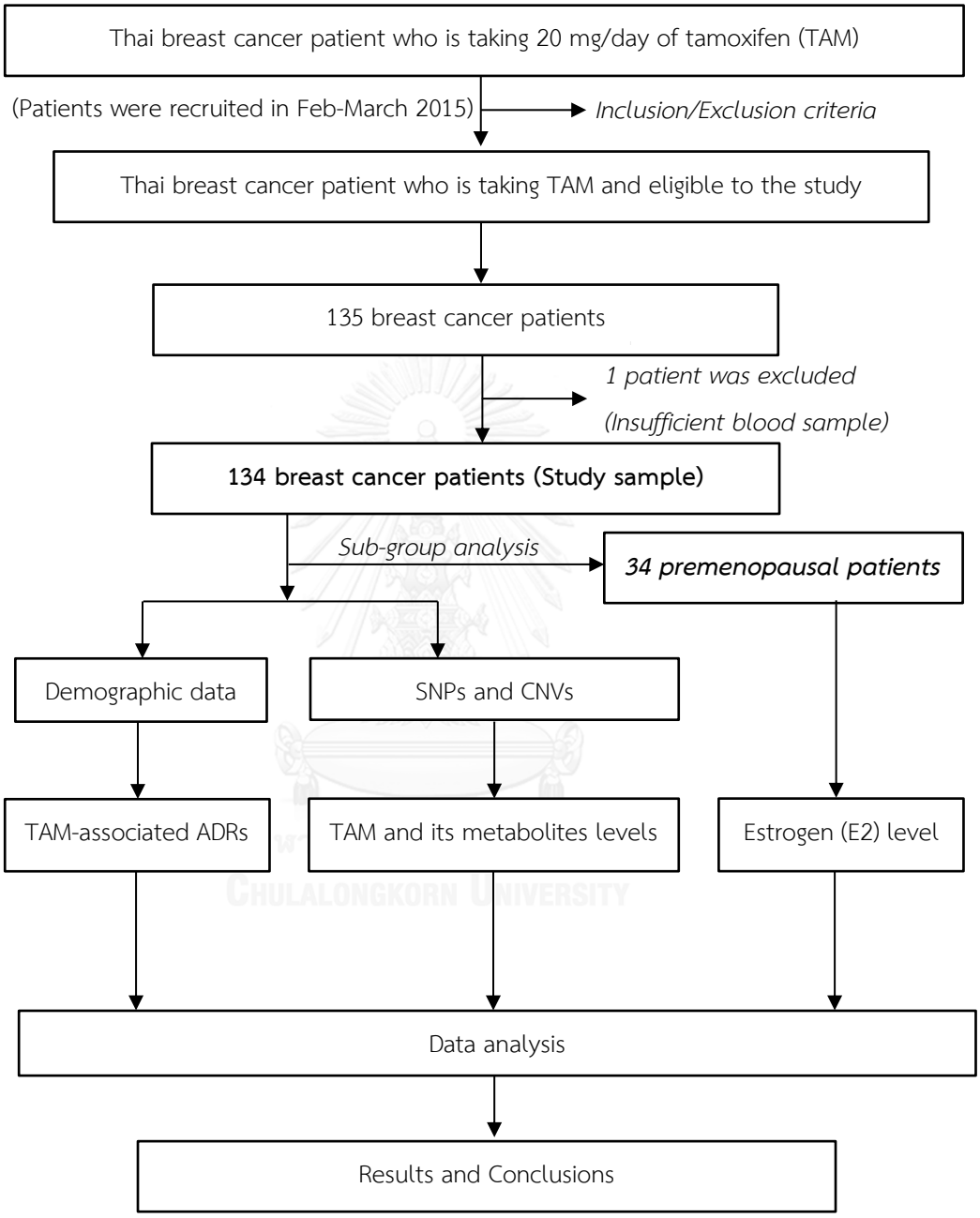


Figure 5: Research flow chart

Data management and statistical analysis

All data were coded and analyzed by the SPSS V.22 (Chulalongkorn University). Descriptive statistics were used to describe the characteristics of the patients including age, weight, BMI, stage of breast cancer, duration of treatment and other demographic data. Continuous variables were summarized by using mean $\pm$ SD for normal distributed data while median (IQR) were used for non-normal distributed data. For categorical variables: frequency (F) and percentage (%) were expressed.

Hypotheses testing

To determine the differences of TAM and its metabolites levels and estrogen concentrations among different genotype/phenotype groups; Kruskal-wallis test or Mann-Whitney U test were employed to compare the median among groups for non-normal distributed data. Relationship between TAM and its metabolites with estrogen concentration were tested by Spearman's rank correlation. For comparison of TAM-associated ADRs among those genotype/phenotype groups; Chi-square test or Fisher's exact test were employed. The significance of all statistical analysis was defined with type I error 0.05 ($P<0.05$) and type II error 0.2 (80% power of analysis) **(Table 17).**

Ethical considerations

All protocols had been approved from the institutional review board of faculty of medicine (Chulalongkorn University) before the research process began.

Table 17: Statistics for hypotheses testing

Research variables	Statistics
Mean differences of END among different *CYP2D6* polymorphisms	Kruskal-Wallis test
Mean difference of END among different *CYP3A5* polymorphisms	Kruskal-Wallis test
Mean differences of TAM among different *CYP2D6* polymorphisms	Kruskal-Wallis test
Mean difference of TAM among different *CYP3A5* polymorphisms	Kruskal-Wallis test
Mean differences of NDMT among different *CYP2D6* polymorphisms	Kruskal-Wallis test
Mean difference of NDMT among different *CYP3A5* polymorphisms	Kruskal-Wallis test
Mean differences of 4OHT among different *CYP2D6* polymorphisms	Kruskal-Wallis test
Mean difference of 4OHT among different *CYP3A5* polymorphisms	Kruskal-Wallis test
Mean difference of E2 between different *SULT1A1* CNVs	Mann-Whitney U test
Correlations between TAM and its metabolites and E2 level	Spearman's rank correlation
Associations between E2 level and TAM-associated ADRs	Mann-Whitney U test
Associations between *CYP2D6* polymorphisms and ADRs	Pearson's Chi square
Associations between *CYP3A5* polymorphisms and ADRs	Pearson's Chi square

Notes: (1) TAM=tamoxifen, NDMT=*N*-desmethyltamoxifen, 4OHT=*4*-hydroxytamoxifen, END=endoxifen
(2) E2=estradiol
(3) ADRs=adverse drug reactions

Obstacles and strategies to solve the problems

1. Identifying the period of Day1-Day 5 from individual premenopausal patients for whole blood collection date was hard to complete according to their individual uncertain menstrual cycle and low feasibility of patient's returning for blood collection; therefore; whole blood collection were performed at the recruitment time for every patients with their individual usual menstrual cycles interview including their medical records review to evaluate their specific menstrual cycle at the time of whole blood collections.

2. The 10 ml. of whole blood was drawn from each patient instead of the 15 ml. of whole blood due to the feasibility of available product for blood collection. Drawing 10 ml. of the whole blood needed only one puncture which should be better for the patients compared with two punctures for 15 ml. of whole blood collection.

Study timeline

The research had been taken for 24 months to complete all the process which included 6 months for sample recruitment, 10 months for the laboratory analysis and two months for summarizing and analyzing all the data **(Table 18)**.

Table 18: Study timeline (Jan 2014-Dec 2015)

Timeline	2014			2015			
	Jan-Jun	Jul-Aug	Sep-Dec	Jan-Mar	Apr-Jun	July-Oct	Nov-Dec
1. Research topic and literature review	→						
2. Research proposal	→						
3. Proposing the proposal to research committee		→					
4. Proposing the proposal to ethic committee			→				
5. Tools preparation		→	→	→			
6. Patient recruitment				→			
7. Demographic and ADRs data collection				→	→		
8. Laboratories analysis				→	→	→	
9. Data analysis							→
10. Research report				→	→	→	→

Budgetary spending for the research

The estimated budget for this research was 501,140.00 THB **(Table 19)**.

Table 19: Budgetary spending for the research

Budget lists	Cost (THB)
Tools/Materials for patient recruitment	
(1) Volunteer expense	42,000.00
(2) Nurse expense	14,000.00
(3) Whole blood collection	16,050.00
(4) DNA extraction	30,000.00
(5) Other expense (For example; standard tips, tubes)	28,000.00
Total (Patient recruitment)	***132,050.00***
Tools/Materials for SNPs and CNVs determinations	
(1) PCR Master mix kit	50,000.00
(2) PCR assays (3 SNPs and 1 CNVs)	72,000.00
(3) Reference assay for CNVs	6,500.00
(4) Other expense (For example; filtered tips)	25,000.00
Total (SNPs and CNVs)	***153,500.00***
Tools/Materials for TAM and its metabolites levels	
(1) Standards for TAM, NDMT, END, 4OHT, IS	38,380.00
(2) Other expense for HPLC analysis	120,000.00
Total (TAM and its metabolites)	***138,380.00***
Tools/Materials for estrogen quantification	
(1) E2 (human) ELISA kits	40,050.00
(2) Other expense for E2 analysis	2,000.00
Total (Estrogen quantification)	***42,050.00***
Additional supporting research expense	***17,160.00***
TOTAL COST (THB)	**501,140.00**

Research funding

This research was funded by the thesis grant for doctoral degree student of the National Research Council of Thailand (NRCT) (2015) and the 90th Anniversary of Chulalongkorn University Scholarship (2015).

CHAPTER IV
RESULTS

Patients and demographic data

The study sample consisted of 134 Thai TAM-treating breast cancer patients. Most of the patients were in stage II and stage I breast cancer (43.3% and 32.8%, respectively) **(Table 20)** ranging in age from 27.0-82.0 years (Mean 51.6±11.6 years). Their mean weight was 60.0±10.7 kg which ranged 36.0-107.5 kg and their BMI were ranged from 15.4-40.0. There was 17.2% of them reported their concomitant diseases which included hypertension (48.4%), dyslipidemia (16.1%) and thyroid disease (9.7%). Median duration of receiving TAM as their treatment was 17.2 (IQR 16.1) months; range 0.8-62.1 months, and 6.7% (9 patients) of them had never received any breast cancer treatment before receiving TAM while the rest of them had experience with breast operation, radiation or chemotherapy treatment. Majority of those patients were positive for estrogen receptor (ER+) and progesterone receptor (PR+) (98.5% and 72.4%, respectively) **(Table 21)** which included 71.6% (96 patients) of ER+/PR+, 26.9% (36 patients) of ER+/PR-, 0.7% (1 patient) of ER-/PR+ and 0.7% (1 patient) of ER-/PR-. Additionally, we found that 4.5% (6 patients) of those patients were recurrent breast cancer patients.

Table 20: Stage of breast cancer in this study

Stage of breast cancer (N=134)	Frequency (%)
Stage 0	1 (0.7)
Stage I (IA and IB)	44 (32.8)
Stage II (IIA and IIB)	58 (43.3)
Stage III (IIIA, IIIB and IIIC)	24 (17.9)
Stage IV	6 (4.5)
Unknown stage	1 (0.7)

Table 21: Demographic data of Thai TAM-treating breast cancer patients

Demographic data (N=134)	Frequencies (%)
Age (years)	Mean 51.6±11.6, Median 49.0 (16.0)
18-30 years	1 (0.7)
30-40 years	17 (12.7)
40-50 years	54 (40.3)
>50 years	62 (46.3)
Weight (kg)	Mean 60.0±10.7, Median 57.0 (11.8)
BMI	Mean 24.0±4.3, Median 23.1 (4.6)
Hormone receptor status	
ER-positive	132 (98.5)
PR-positive	97 (72.4)
Duration of TAM used (months)	Mean 21.4±16.1, Median 17.2 (23.4)
< 6 months	26 (19.4)
6 months-1 year	23 (17.2)
1 year-3 years	57 (42.5)
>3 years	28 (20.9)
Menopausal status	
Premenopausal	71 (53.0)
Perimenopausal	14 (10.4)
Postmenopausal	49 (36.6)
Co-disease	
Hypertension	15 (48.4)
Dyslipidemia	5 (16.1)
Thyroid disease	3 (9.7)
Diabetes mellitus	4 (12.9)
Other diseases	4 (12.9)

Determination of CYP2D6*4 from CYP2D6*2 and CYP2D6*10 assays

*CYP2D6*2* (2850C>T) and *CYP2D6*10* (100C>T) assays were not only used for *CYP2D6*2* and *CYP2D6*10* determination but also used as *CYP2D6*4* (2850C>T and 100C>T) assays. There were five patients were carrying *CYP2D6*1/*2* (*CYP2D6*2* assay) with *CYP2D6*10/*10* (*CYP2D6*10* assay) and two patients carrying *CYP2D6*2/*2* (*CYP2D6*2* assay) with *CYP2D6*10/*10* (*CYP2D6*10* assay) whose genotypes were difficult to define without considering their haplotypes. *CYP2D6**4 haplotype consisted of 100C>T, 1846G>A and 2850C>T [62] while the 2850C>T and 100C>T were detected by *CYP2D6*2* and *CYP2D6*10* assays, respectively; hence, patients who were carrying *CYP2D6*1/*2* with *CYP2D6*10/*10* genotypes and patients who were carrying *CYP2D6*2/*2* with *CYP2D6*10/*10* genotypes would be classified to *CYP2D6*4/*10* and *CYP2D6*4/*4* genotypes, respectively **(Table 22)**.

Table 22: Determination of *CYP2D6*4* from *CYP2D6*2* and *CYP2D6*10* assays

Patient	*CYP2D6* genotype (2850C>T)	CYP2D6 genotype (100C>T)	CYP2D6 genotype (2850C>T and 100C>T)
1	*CYP2D6*1/*2*	*CYP2D6*10/*10*	*CYP2D6*4/*10*
2	*CYP2D6*1/*2*	*CYP2D6*10/*10*	*CYP2D6*4/*10*
3	*CYP2D6*1/*2*	*CYP2D6*10/*10*	*CYP2D6*4/*10*
4	*CYP2D6*1/*2*	*CYP2D6*10/*10*	*CYP2D6*4/*10*
5	*CYP2D6*1/*2*	*CYP2D6*10/*10*	*CYP2D6*4/*10*
6	*CYP2D6*2/*2*	*CYP2D6*10/*10*	*CYP2D6*4/*4*
7	*CYP2D6*2/*2*	*CYP2D6*10/*10*	*CYP2D6*4/*4*

Prevalence of *CYP2D6*, *CYP3A5* and *SULT1A1* polymorphisms

Of those 134 patients, allele frequencies of *CYP2D6*1, CYP2D6*2, CYP2D6*4, CYP2D6*10, CYP3A5*1* and *CYP3A5*3* were 72.9%, 3.2%, 1.1%, 22.8%, 37.3% and 62.7%, respectively. *CYP2D6*10/*10* and *CYP3A5*1/*3* were the most common genotypes that were found in this study (53.7% and 47.8%, respectively) **(Table 23)**.

It was found that 131 patients (97.8%) were carrying at least one incomplete functional allele (*CYP2D6*4, CYP2D6*10* and *CYP3A5*3*) including 28 patients (20.9%) that were carrying only incomplete functional allele for both *CYP2D6* and *CYP3A5*

genes (25 patients with *CYP2D6*10/*10* and *CYP3A5*3/*3*, two patients with *CYP2D6*4/*4* and *CYP3A5*3/*3* and one patient with *CYP2D6*4/*10* and *CYP3A5*3/*3*) which were potentially responsible for their low enzyme activities compared to those patients who were carrying at least one wild type allele (*CYP2D6*1, CYP2D6*2* and *CYP3A5*1*).

Most of Thai breast cancer patients (96.3%) in this study were carrying two copies of *SULT1A1* gene while the rest of them were carrying one copy (1 patient) and three copies (4 patients) of *SULT1A1* genes, respectively **(Table 23)**.

Table 23: Genotypes frequency (%) of *CYP2D6, CYP3A5* and *SULT1A1* (N=134)

Genotypes	Frequency (%)
CYP2D6	
*CYP2D6*1/*1*	13 (9.7)
*CYP2D6*1/*2*	3 (2.2)
*CYP2D6*2/*2*	5 (3.7)
*CYP2D6*4/*4*	2 (1.5)
*CYP2D6*1/*10*	21 (15.7)
*CYP2D6*2/*10*	13 (9.7)
*CYP2D6*4/*10*	5 (3.7)
*CYP2D6*10/*10*	72 (53.7)
CYP3A5	
*CYP3A5*1/*1*	18 (13.4)
*CYP3A5*1/*3*	64 (47.8)
*CYP3A5*3/*3*	52 (38.8)
***SULT1A1* CNVs**	
1 copy	1 (0.7)
2 copies	129 (96.3)
3 copies	4 (3.0)

The *CYP2D6* and *CYP3A5* genotypes were classified to *CYP2D6* and *CYP3A5* phenotypes, respectively by conventional method **(Table 24)**. Prevalence of *CYP2D6* phenotypes were 41.0% (55 patients), 57.5% (77 patients) and 1.5% (2 patients) for extensive metabolizers (EMs), intermediate metabolizer (IMs) and poor metabolizer (PMs), respectively while prevalence of *CYP3A5* phenotypes were 61.2% (82 patients) and 38.8% (52 patients) for EMs and PMs, respectively. It was noticed that *CYP3A5*-IM was not found in this study **(Table 24 and Table 25)**. Furthermore, it was also found

that only 23.1% of them (31 patients) were EMs for both *CYP2D6* and *CYP3A5* while others (75.4%; 101 patients) were IMs or PMs for at least one gene including two patients (1.5%) that were PM for both *CYP2D6* and *CYP3A5* genes **(Table 26)**.

Table 24: Classification of *CYP2D6* and *CYP3A5* phenotype (N=134)

Genotypes	Phenotypes	Frequency (%)
	CYP2D6	
CYP2D6*1/*1	EM	13 (9.7)
CYP2D6*1/*2	EM	3 (2.2)
CYP2D6*2/*2	EM	5 (3.7)
CYP2D6*4/*4	PM	2 (1.5)
CYP2D6*1/*10	EM	21 (15.7)
CYP2D6*2/*10	EM	13 (9.7)
CYP2D6*4/*10	IM	5 (3.7)
CYP2D6*10/*10	IM	72 (53.7)
	CYP3A5	
CYP3A5*1/*1	EM	18 (13.4)
CYP3A5*1/*3	EM	64 (47.8)
CYP3A5*3/*3	PM	52 (38.8)

Note: EM=extensive metabolizer, IM=intermediate metabolizer, PM=poor metabolizer

Table 25: Phenotype frequency (%) of *CYP2D6* and *CYP3A5* (N=134)

Phenotype	Prevalence (%)
CYP2D6	
EM	55 (41.0)
IM	77 (57.5)
PM	2 (1.5)
CYP3A5	
EM	82 (61.2)
IM	0 (0.0)
PM	52 (38.8)

Note: EM=extensive metabolizer, IM=intermediate metabolizer, PM=poor metabolizer

Table 26: Phenotype frequency (%) of *CYP2D6* with *CYP3A5* (N=134)

Prevalence (%)	*CYP3A5* phenotypes			Total
CYP2D6 phenotypes	EM	IM	PM	
EM	31(23.1)	0(0.0)	24(17.9)	55(41.0)
IM	51(38.1)	0(0.0)	26(19.4)	77(57.5)
PM	0(0.0)	0(0.0)	2(1.5)	2(1.5)
Total	82(61.2)	0(0.0)	52(38.8)	**134(100.0)**

Prevalence of TAM-associated adverse drug reactions (ADRs)

TAM-associated ADRs were examined by interviewing with the patients at recruitment time and reviewing patient's medical record. It was found that most patients (85.1 %, 114 patients) had never experienced with any TAM-associated ADRS while 14.9% of them (20 patients) reported about their TAM-associated ADRS. The most common ADRs were hot flash (3.7%) and myalgia (3.7) **(Table 27)**.

Table 27: TAM-associated adverse drug reactions (ADRs)

TAM-associated ADRs[a]	All stages (N=134)
TAM-associated ADRs were not presented[b]	***114 (85.1)***
TAM-associated ADRs[b]	***20 (14.9)***
Hot flashes	5 (3.7)
Myalgia	5 (3.7)
Endometrial thickness	3 (2.2)
Vaginal discharges/Vaginal dryness	3 (2.2)
Irregular menstruations	2 (1.5)
Palpitations	2 (1.5)

Note: a=numbers of adverse drug reactions (ADRS) events (%), b=prevalence of ADRs (N=134)

TAM and its metabolites quantification

HPLC-FLU method validation:

Selectivity (Specificity)

The chromatogram of blank sample (six sources of plasma) with internal standard (IS) was compared with the chromatograms of TAM 25 ng/ml, NDMT 25 ng/ml, END 5 ng/ml and 4OHT 2.5 ng/ml in blank plasma to perform the selectivity of each metabolite **(Appendix 4)**. The retention times of IS, TAM, NDMT, END and 4OHT were 5.0, 26.1, 19.6, 6.9 and 7.3 minutes, respectively.

Extraction efficiency (% Recovery)

The three concentrations of TAM and its metabolites were prepared to perform the recovery test. Those concentrations were TAM (750 (H), 250 (M) and 50 (L) ng/ml), NDMT (750 (H), 250 (M) and 100 (L) ng/ml), END (100 (H), 75 (M) and 50 (L) ng/ml) and 4OHT (100 (H), 75 (M) and 50 (L) ng/ml). The ranges of recovery were 64.0-152.7%, 84.3-178.1%, 8.9-85.2% and 59.2-270.3% of TAM, NDMT, END and 4OHT, respectively **(Table 28)**.

Table 28: Extraction efficiency (%Recovery) of TAM and its metabolites

% Recovery (N=3)	TAM	NDMT	END	4OHT
High concentration (H)	118.0±12.0	123.7±7.5	130.7±17.5	98.0±13.1
Middle concentration (M)	138.5±33.1	303.4±90.6	89.25±5.2	89.3±5.2
Low concentration (L)	122.3±23.8	109.0±23.8	72.25±13.4	108.4±20.1

Linearity

The coefficient of determination (R^2) of each calibration curve (TAM, NDMT, END and 4OHT) was determined. The R^2 were 0.990-0.995 for TAM **(Table 30 and Figure 6),** NDMT **(Table 31, Figure 7),** 4OHT **(Table 32, Figure 8)** and END **(Table 33, Figure 9).** The regression equations for those calibration curves were provided below **(Table 29).**

Table 29: Regression equation for calibration curves

TAM and its metabolites	Standards concentrations (ng/ml)	Regression equation	R^2
TAM	0, 25, 50, 100, 500, 750, 1,000	y = 0.003(x)+0.064	0.991
NDMT	0, 25, 50, 100, 500, 750, 1,000	y = 0.002(x)-0.007	0.995
END	0, 5, 10, 50, 75, 100, 300	y = 0.003(x)-0.007	0.990
4OHT	0, 2.5, 5.0, 7.5, 12.5, 25, 50	y = 0.023(x)-0.049	0.991

Note: y = AUC (STDs)/AUC (IS), x = STDs concentrations, R^2 = coefficient of determination

Table 30: Calibration curves of TAM (R^2=0.991)

TAM	Standards (ng/ml)	AUC(TAM) / AUC(IS)	Calculated concentration (ng/ml)
Standard 0	0	0	0
Standard 1	25	0.06	21.33
Standard 2	50	0.10	42.72
Standard 3 (1)	100	0.24	100.45
Standard 3 (2)	100	0.26	107.04
Standard 3 (3)	100	0.19	86.31
Standard 3 (4)	100	0.18	80.19
Standard 3 (5)	100	0.21	91.71
Standard 4	500	1.38	481.56
Standard 5	750	2.67	910.87
Standard 6	1,000	3.33	1,129.82

Note: AUC = area under the curve, IS = internal standard (Mexiletine 5 mg/ml)

Table 31: Calibration curve of NDMT (R^2= 0.995)

TAM	Standards (ng/ml)	AUC(NDMT) / AUC(IS)	Calculated concentration (ng/ml)
Standard 0	0	0	0
Standard 1	25	0.06	32.40
Standard 2	50	0.12	62.07
Standard 3	100	0.16	81.66
Standard 4	500	0.79	400.64
Standard 5 (1)	750	1.39	700.12
Standard 5 (2)	750	1.68	843.91
Standard 5 (3)	750	1.73	870.12
Standard 5 (4)	750	1.85	929.73
Standard 5 (5)	750	1.95	918.09
Standard 6	1,000	1.83	983.05

Note: AUC = area under the curve, IS = internal standard (Mexiletine 5 mg/ml)

Table 32: Calibration curve of END (R^2=0.990)

TAM	Standards (ng/ml)	AUC(END) / AUC(IS)	Calculated concentration (ng/ml)
Standard 0	0	0	0
Standard 1	5	0.02	10.60
Standard 2	10	0.07	24.38
Standard 3	50	0.16	55.52
Standard 4 (1)	75	0.23	77.57
Standard 4 (2)	75	0.21	71.38
Standard 4 (3)	75	0.19	64.31
Standard 4 (4)	75	0.19	65.13
Standard 4 (5)	75	0.26	89.26
Standard 5	100	0.26	89.60
Standard 6	300	1.02	343.91

Note: AUC = area under the curve, IS = internal standard (Mexiletine 5 mg/ml)

Table 33: Calibration curve of 4OHT (R^2=0.991)

TAM	Standards (ng/ml)	AUC(4OHT) / AUC(IS)	Calculated concentration (ng/ml)
Standard 0	0	0	0
Standard 1	2.5	0.0475	2.07
Standard 2	5.0	0.0494	2.15
Standard 3	7.5	0.0776	3.37
Standard 4	12.5	0.1956	8.51
Standard 5 (1)	25	0.5275	25.06
Standard 5 (2)	25	0.5113	24.36
Standard 5 (3)	25	0.4038	19.69
Standard 5 (4)	25	0.6573	30.71
Standard 5 (5)	25	0.6613	30.88
Standard 6	50	1.1127	48.38

Note: AUC = area under the curve, IS = internal standard (Mexiletine 5 mg/ml)

Calibration curve

The calibration curve consisted of six non-zero calibrator concentrations ranged 0-1,000 ng/ml, 0-1,000 ng/ml, 0-300 ng/ml and 0-50 ng/ml for TAM **(Figure 6)**, NDMT **(Figure 7)**, END **(Figure 8)** and 4OHT **(Figure 9)**, respectively. The calibration curves were constructed by calculating the ratios of the peak area of those analyses to the peak area of IS and relating the ratios of the known concentrations.

Figure 6: Calibration curve of TAM between concentration and AUC ratio

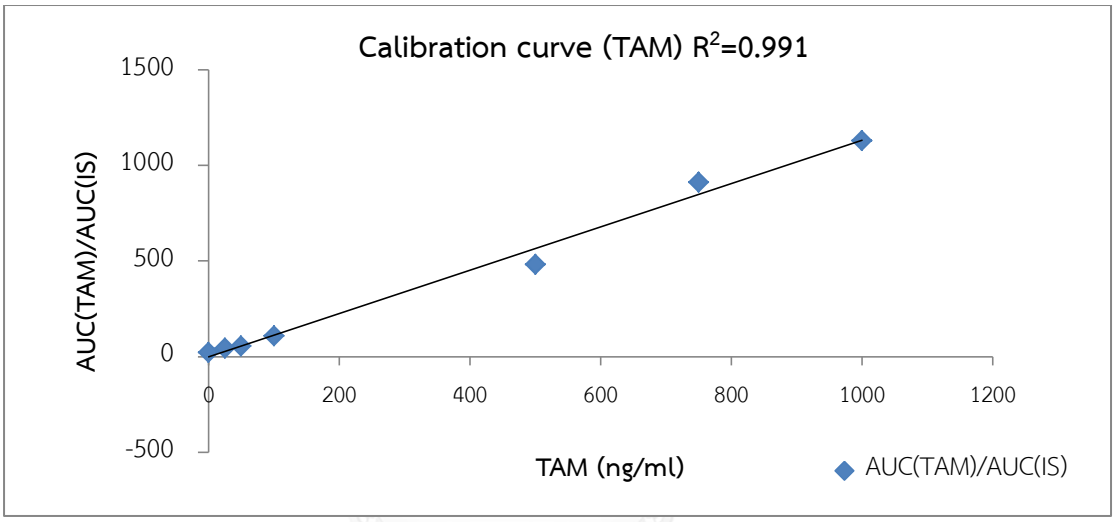


Figure 7: Calibration curve of NDMT between concentration and AUC ratio

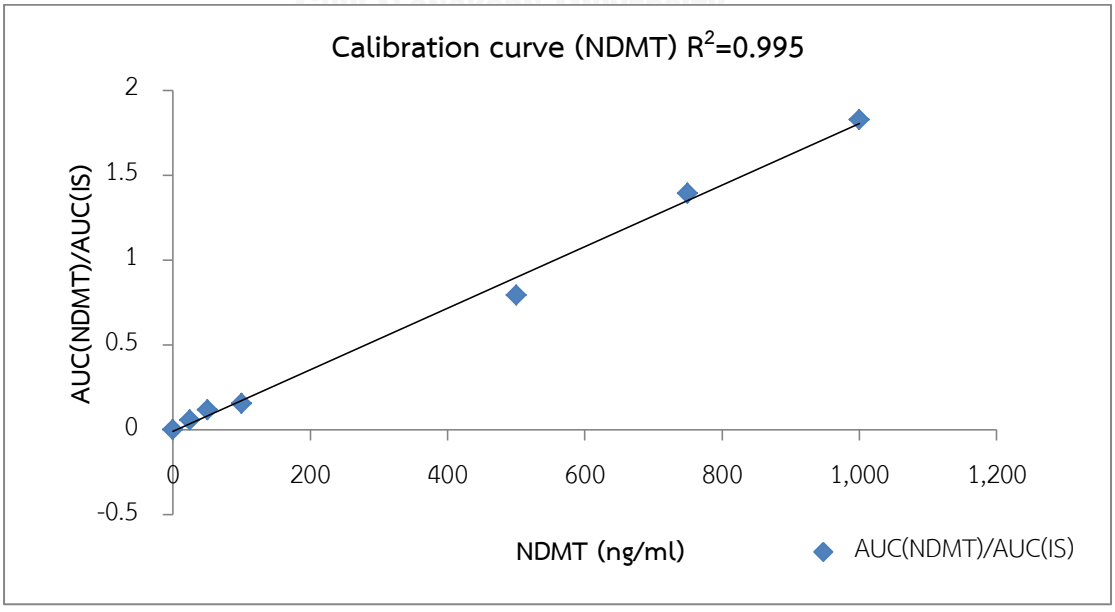


Figure 8: Calibration curve of END between concentration and AUC ratio

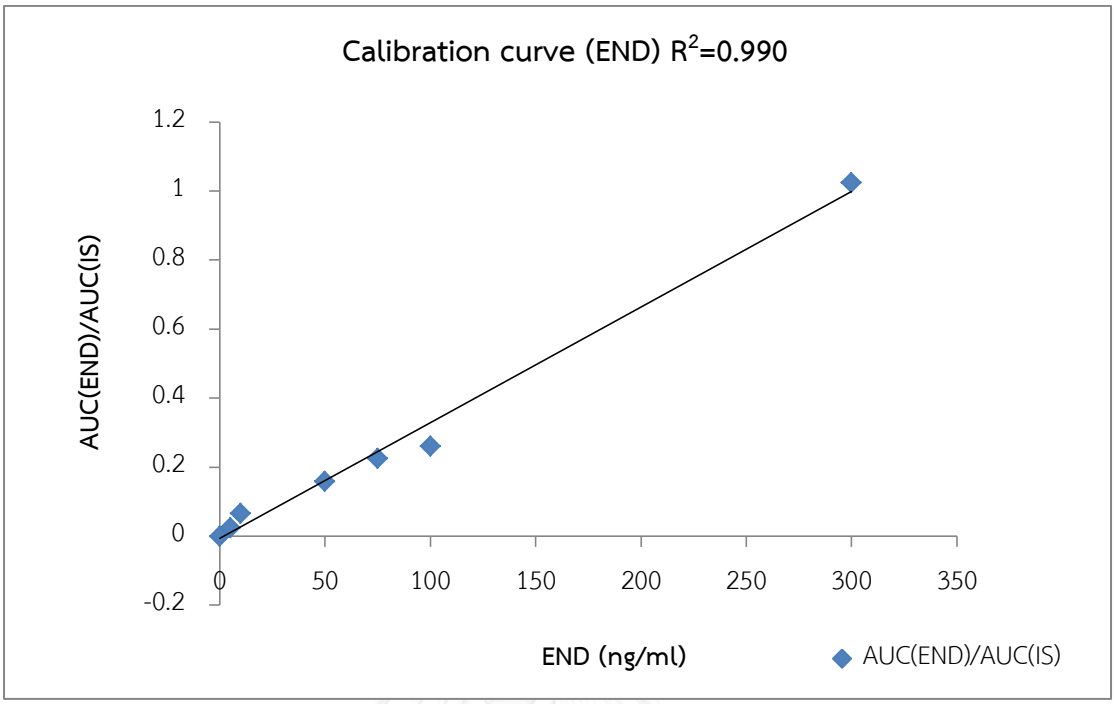
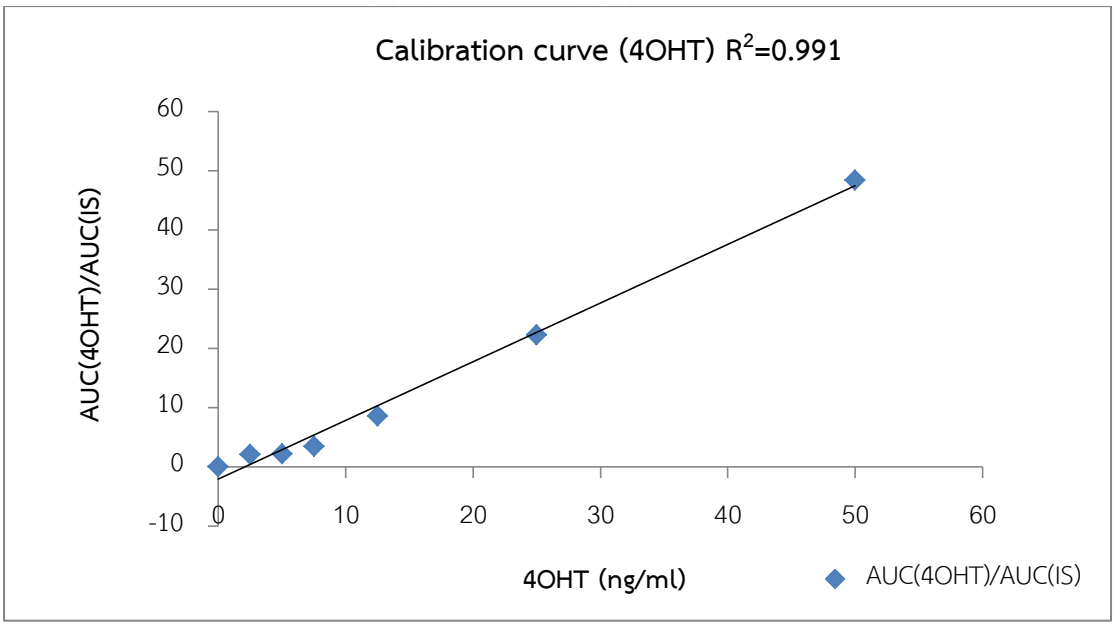


Figure 9: Calibration curve of 4OHT between concentration and AUC ratio



Accuracy

The five replications per concentration of each metabolite were prepared to perform accuracy test; TAM 100 ng/ml, NDMT 750 ng/ml, END 75 ng/ml and 4OHT 25 ng/ml **(Table 30, Table 31, Table 32 and Table 33).** The acceptable range of calculated concentration was calculated from 15% of expected concentrations; therefore, the acceptable ranges were 85-115 ng/ml for TAM, 637.50-862.50 ng/ml for NDMT, 63.75-86.25 ng/ml for END and 21.25-28.75 ng/ml for 4OHT. According to this criterias, four replications of TAM 100 ng/ml and END 75 ng/ml were in acceptable ranges while only two replications of NDMT and 4OHT were in the acceptable ranges; however, the rest of the replications of 4OHT were in borderline of those acceptable ranges **(Table 34).**

Table 34: Accuracy and Precision of standard solutions

Standard solutions	TAM (ng/ml)		NDMT (ng/ml)		END (ng/ml)		4OHT (ng/ml)	
	E.conc	*C.conc*	*E.conc*	*C.conc*	*E.conc*	*C.conc*	*E.conc*	*C.conc*
Standard (1)	100	100.45	750	700.18	75	77.57	25	25.06
Standard (2)	100	107.04	750	843.91	75	71.38	25	24.36
Standard (3)	100	86.31	750	929.73	75	64.31	25	19.69
Standard (4)	100	80.19	750	870.12	75	65.13	25	30.71
Standard (5)	100	91.71	750	983.05	75	89.26	25	30.88
Range (ng/ml)	85.00-115.00		637.50-862.50		63.75-86.25		21.25-28.75	
Mean (ng/ml)	93.14		865.38		72.41		26.28	
SD (ng/ml)	10.76		106.98		10.05		4.99	
% CV	11.55		12.36		13.88		18.99	

Note: E.conc = expected concentration, C.conc = calculated concentration

SD= standard deviation, % CV = % coefficient of variations

Precision

Those five replications were also prepared for precision test **(Table 34)**. The coefficients of variations (%CV) from those replications for TAM, NDMT and END were all less than 15%. The %CV of 4OHT was more than 15% but less than 20%.

Determination of TAM and its metabolites concentrations:

The calibration curves of TAM and its metabolites were interpolated for TAM and its metabolites concentrations in 134 plasma samples. However, one patient was excluded from this analysis according to her extremely low value of TAM (See details in chapter V: discussion).The descriptive data of TAM and its metabolites in this study was provided below **(Table 35)**. The Kolmogorov-Smirnov and Shapiro-Wilk test were used to perform for normality test.

Table 35: Descriptive data of TAM and its metabolites concentrations (ng/ml)

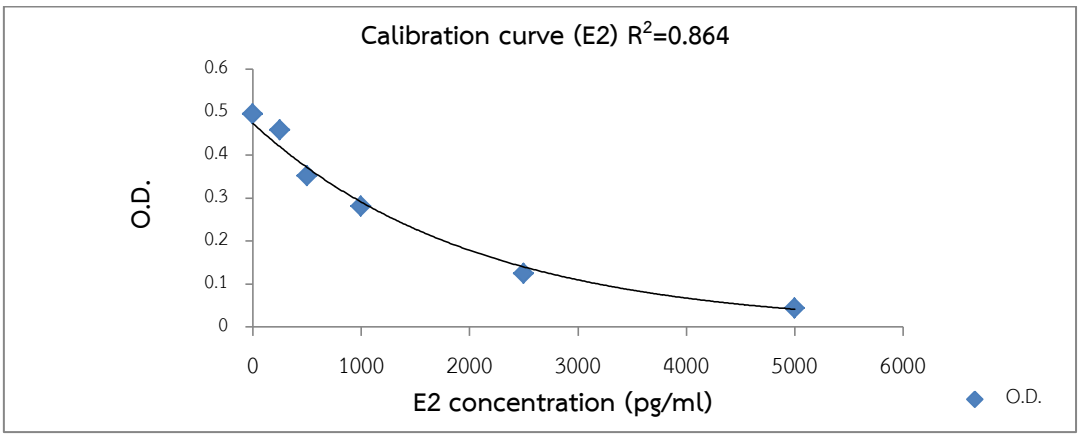
TAM and its metabolites (N=133)	Mean±SD	Median (IQR)	Min-Max
TAM (ng/ml)	406.7 ± 14.9	374.7 (230.2)	82.5 - 984.2
NDMT (ng/ml)	1,149.7 ± 486.7	1,064.9 (599.6)	80.8 - 2,543.8
END (ng/ml)	68.5 ± 4.6	54.5 (52.5)	2.3 - 443.8
4OHT (ng/ml)	5.8 ± 0.3	5.0 (3.1)	2.1 - 21.7

E2 quantification

E2 calibration curve

The standard concentrations of 0, 250, 500, 1,000, 2,500 and 5,000 pg/ml were prepared for E2 calibration curve. The curve was plotted between the known concentrations of standards versus the optical density (O.D.). The regression equation of the E2 calibration curve was $y = -8.76x10^{-5}(x)+0.43$ ($R^2=0.864$) **(Figure 10)**.

Figure 10: Calibration curve (E2)



Quantification of plasma E2 concentration:

The 34 premenopausal patients from the cohort were selected based on their active menstrual cycle data to perform E2 quantification. The premenopausal status was classified according to the pre-defined menopausal status criteria in this study. Mean age of this patient sub-group was 44.3±11.1 years, BMI 23.4±3.6 and were in stage 0, stage I, stage II and stage III of breast cancer (2.9%, 38.2%, 41.2% and 17.6%, respectively). Most patients were ER+/PR+ (82.4%) and ER+/PR- (14.7%) while one patient was reported to be ER-/PR-. Of those, three patients reported about their TAM-associated ADRs. Other demographic and genetic data in this premenopausal sub-group analysis was provided below **(Table 36)**.

Table 36: Demographic data of patients in sub-group analysis (N=34)

Demographic data (N=34)	Frequency (%)
Age (years)	Mean 44.3±11.1, Median 43.0 (12.0)
18-30 years	1 (2.9)
30-40 years	13 (38.2)
40-50 years	17 (50.0)
>50 years	3 (8.8)
TAM used (months)	Mean 23.1±18.1, Median 15.9 (34.1)
< 6 months	6 (17.6)
6 months-1 year	5 (14.7)
1 year-3 years	12 (35.3)
>3 years	11 (32.4)
***CYP2D6* phenotype**	
EM	13 (38.2)
IM	21 (61.8)
***CYP3A5* phenotype**	
EM	23 (67.6)
PM	11 (32.4)
***SULT1A1* CNVs**	
SULT1A1 x 2 copies	33 (97.1)
SULT1A1 x 3 copies	1 (2.9)

TAM and its metabolites concentrations for this sub-group analysis were 334.0 (IQR 162.9) ng/ml, 1,041.1 (IQR 465.8) ng/ml, 42.4 (IQR 47.1) ng/ml and 5.0 (IQR 2.7) ng/ml for TAM, NDMT, END and 4OHT, respectively. The E2 concentration that was found in this study was 1,575.6 (IQR 865.4) pg/ml which ranged from 415.0 pg/ml to 4,186.5 pg/ml **(Table 37)**.

Table 37: TAM and its metabolites and E2 concentrations in sub-group analysis

TAM and its metabolites and E2 levels (N=34)	Mean ± SD	Median (IQR)
Tamoxifen (TAM) (ng/ml)	361.6 ± 151.1	334.0 (162.9)
N-desmethyl-tamoxfien (NDMT) (ng/ml)	1,055.9 ± 455.2	1,041.1 (465.8)
Endoxifen (END) (ng/ml)	60.4 ± 37.0	42.4 (47.1)
4-hydroxy-tamoxfien (4OHT) (ng/ml)	5.8 ± 3.3	5.0 (2.7)
Estradiol (E2) (pg/ml)	1,641.8 ± 789.0	1,575.6 (865.4)

Hypotheses testing

Hypothesis 1: There is no difference in TAM and its metabolites (END) among different TAM-metabolizing enzyme polymorphisms (*CYP2D6*1, CYP2D6*2* and *CYP2D6*10*) in Thai breast cancer patients.

The END concentration among different *CYP2D6* genotypes and phenotypes were compared using Kruskal-Wallis and Mann-Whitney U tests. The END concentrations were not statistically different among different *CYP2D6* genotypes (P=0.226) or *CYP2D6* phenotypes (P=0.128) even though *CYP2D6*-PM had insignificant low END concentration compared with those *CYP2D6*-IM and *CYP2D6*-EM **(Figure 13)**. Moreover, it was found that TAM concentrations were statistically different among *CYP2D6* phenotypes (P=0.031); *CYP2D6*-PM had lower TAM concentration than *CYP2D6*-PM and *CYP2D6*-IM **(Table 38, Figure 13)**. Median concentrations of TAM and its metabolites levels among different *CYP2D6* genotype and *CYP2D6* phenotype were provided below **(Table 38)**.

Figure 11: TAM concentrations (ng/ml) among different *CYP2D6* genotypes

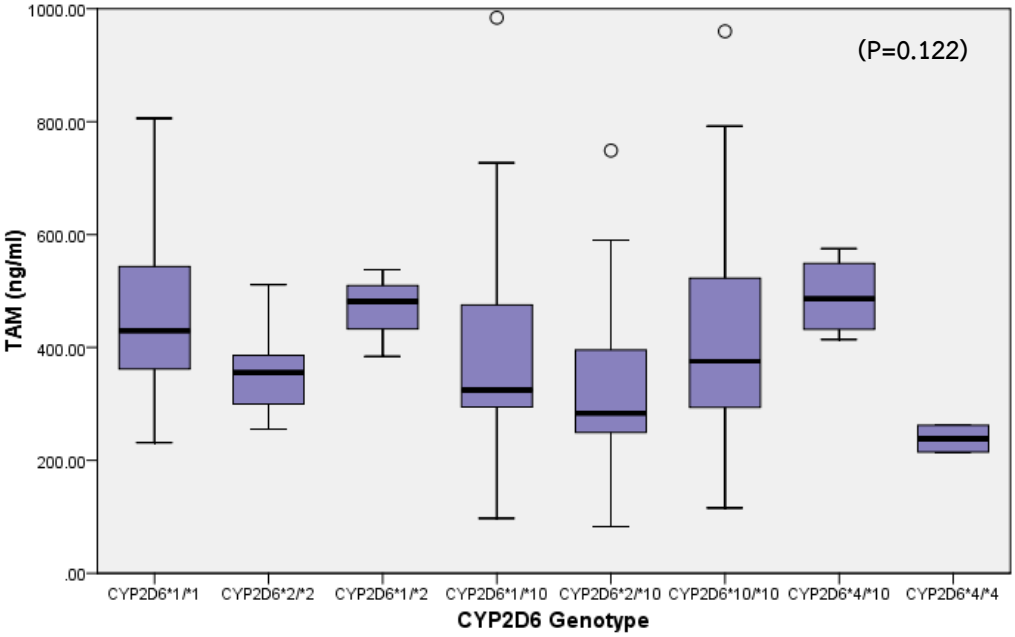


Figure 12: END concentrations (ng/ml) among different *CYP2D6* genotypes

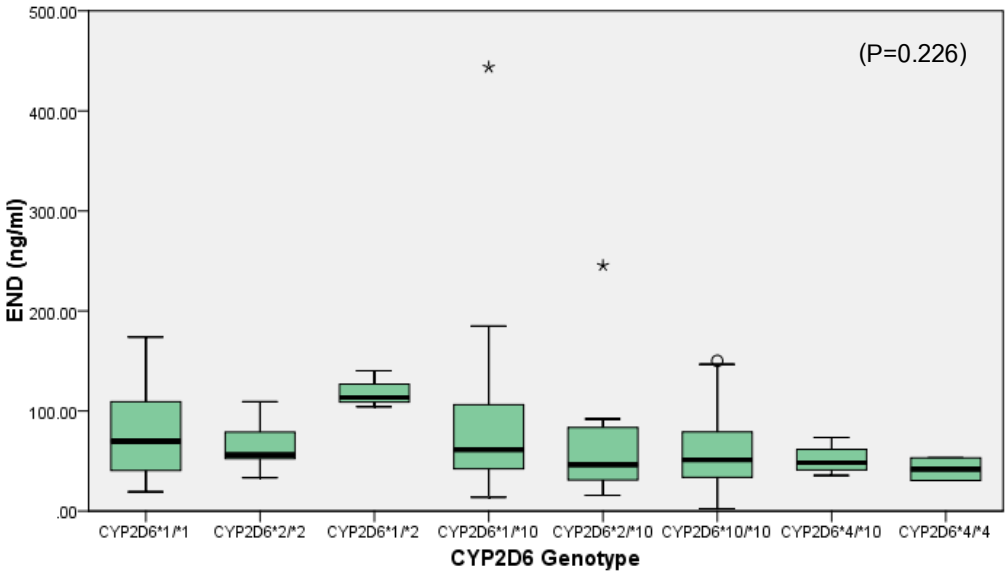
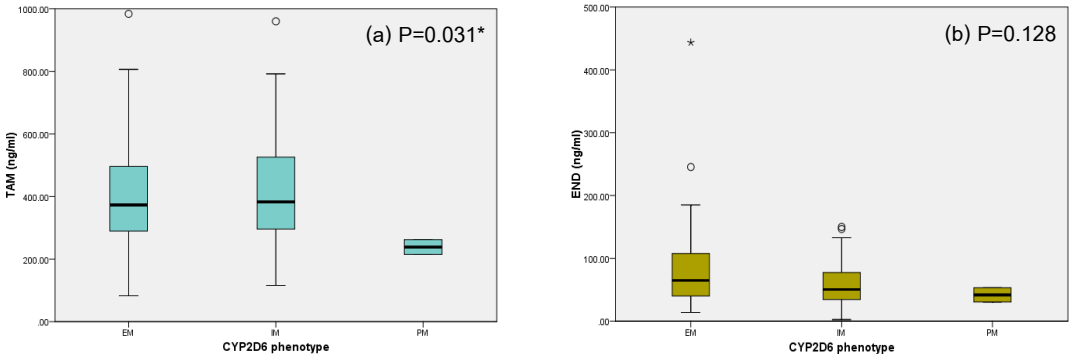


Figure 13: TAM and END concentrations among different *CYP2D6* phenotypes



Note: Figure 13 (a) TAM concentration (ng/ml) among *CYP2D6* phenotype

Figure 13 (b) END concentration (ng/ml) among *CYP2D6* phenotype

Table 38: TAM and its metabolites concentrations among *CYP2D6* polymorphisms

CYP2D6 Polymorphisms	N	TAM (ng/ml)	NDMT (ng/ml)	END (ng/ml)	4OHT (ng/ml)
CYP2D6 genotype	133	P=0.122	P=0.078	P=0.226	P=0.491
*CYP2D6*1/*1*	13	429.6 (266.5)	948.5 (466.0)	69.9 (71.8)	5.9 (9.0)
*CYP2D6*1/*10*	21	324.7 (239.5)	997.8 (741.6)	61.2 (79.9)	6.5 (3.4)
*CYP2D6*10/*10*	72	375.5 (231.9)	1,095.8 (597.4)	51.1 (46.0)	4.5 (2.5)
*CYP2D6*2/*2*	5	355.7 (171.2)	1,085.9 (838.9)	56.2 (51.2)	4.6 (2.5)
*CYP2D6*2/*10*	13	283.3 (174.9)	994.6 (462.0)	46.3 (57.3)	5.4 (3.1)
*CYP2D6*1/*2*	3	481.7 (0.0)	1,170.9 (0.0)	113.5 (0.0)	5.1 (0.0)
*CYP2D6*4/*4*	2	238.4 (0.0)	803.2 (0.0)	41.9 (0.0)	6.2 (0.0)
*CYP2D6*4/*10*	4	486.5 (138.7)	1,924.6 (730.5)	48.2 (29.4)	7.0 (9.6)
CYP2D6 phenotype	133	P=0.031*	P=0.052	P=0.128	P=0.156
EM	55	373.1 (224.7)	1,031.0 (503.6)	64.8 (69.3)	5.8 (3.3)
IM	76	382.9 (232.5)	1,113.1 (612.0)	50.4 (43.5)	4.6 (3.2)
PM	2	238.4 (0.0)	803.2 (0.0)	41.9 (0.0)	6.2 (0.0)

Note: P = P-value from Kruskal-Wallis test

It was noticed that *CYP2D6*-PM had low TAM, NDMT and END concentrations compared with *CYP2D6*-EM and *CYP2D6*-IM while *CYP2D6*-IM had lower END and 4OHT concentrations than *CYP2D6*-EM but had higher TAM and NDMT concentrations than *CYP2D6*-EM **(Table 38)**. These inconclusive findings suggested that considering the original values of TAM and its metabolites concentrations might not reveal the effects of *CYP2D6* polymorphisms on those TAM and its metabolites levels according to the other several TAM-metabolizing enzymes that involved in END transformation including the two sub-pathways for END formation; through NDMT and 4OHT **(Figure 2)**. Therefore, the TAM and its metabolites concentrations were converted to metabolic ratio (MR)[1] to adjust the baseline concentration of input metabolite compared with output metabolite in each sub-pathway of TAM-metabolizing process. The method of MR calculation of each metabolite was provided below **(Table 39)**.

Table 39: Metabolic Ratio (MR) calculation method (TAM and its metabolites)

Metabolic Ratio (MR)	MR calculation method
MR (TAM-NDMT)	TAM (ng/ml) / NDMT (ng/ml)
MR (NDMT-END)	NDMT (ng/ml) / END (ng/ml)
MR (TAM-4OHT)	TAM (ng/ml) / 4OHT (ng/ml)
MR (4OHT-END)	4OHT (ng/ml) / END (ng/ml)

MR (TAM-NDMT) and MR (TAM-4OHT) were represented for NDMT and 4OHT concentrations, respectively while MR (NDMT-END) was represented for END concentrations through NDMT sub-pathway and MR (4OHT-END) was represented for END concentrations through 4OHT sub-pathway **(Figure 2)**. It was noticed that MR analysis could be used to discriminate END concentration with NDMT sub-pathway which was the main pathway for END formation from another minor sub-pathway through 4OHT **(Figure 2)**. According to this analytical concept, MR of all metabolites should be low in *CYP2D6*-EM compared with *CYP2D6*-IM and *CYP2D6*-PM if the effects

of *CYP2D6* polymorphisms on TAM and its metabolites in each sub-pathway were proved to be existed

In this study, it was suggested that the MR (TAM-NDMT) was statistically different among different *CYP2D6* genotypes (P=0.000) and *CYP2D6* phenotypes (P=0.013). The MR (NDMT-END) and MR (TAM-4OHT) were statistically different among different *CYP2D6* phenotypes (P=0.014 and P=0.017, respectively) but were not statistically different among *CYP2D6* genotypes (P=0.078 and P=0.094, respectively) while MR (4OHT-END) was not statistically different among *CYP2D6* phenotypes and *CYP2D6* genotypes (P=0.594 and P=0.470, respectively) **(Table 40, Figure 14)**.

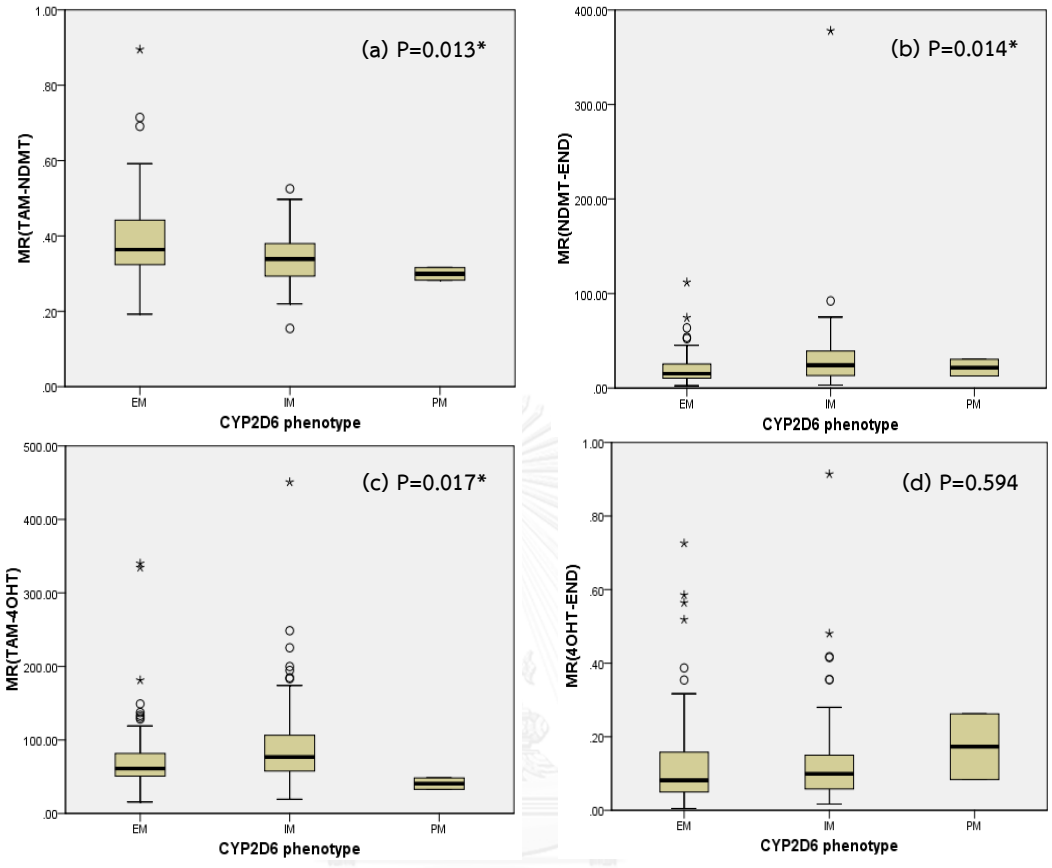
CYP2D6-IM had higher MR (NDMT-END) and MR (TAM-4OHT) than *CYP2D6*-EM but lower than *CYP2D6*-PM. *CYP2D6*-PM had higher MR (4OHT-END) than *CYP2D6*-IM and *CYP2D6*-EM, respectively but had lower MR (TAM-NDMT) than *CYP2D6*-IM and *CYP2D6*-EM, respectively **(Table 40, Figure 14)**.

Table 40: MR of TAM and its metabolites among *CYP2D6* polymorphisms

CYP2D6 Polymorphisms	N	MR (TAM-NDMT)	MR (NDMT-END)	MR (TAM-4OHT)	MR (4OHT-END)
***CYP2D6* genotype**	**133**	**P=0.000***	**P=0.078**	**P=0.094**	**P=0.470**
***CYP2D6*1/*1**	13	0.51 (0.23)	11.72 (22.57)	61.05 (52.86)	0.08 (0.33)
***CYP2D6*1/*10**	21	0.38 (0.11)	15.19 (20.04)	56.18 (60.29)	0.08 (0.16)
***CYP2D6*10/*10**	72	0.34 (0.09)	23.57 (25.79)	77.07 (49.80)	0.10 (0.09)
***CYP2D6*2/*2**	5	0.32 (0.09)	16.84 (13.87)	63.50 (52.33)	0.08 (0.10)
***CYP2D6*2/*10**	13	0.34 (0.10)	19.53 (20.39)	60.66 (18.32)	0.11 (0.06)
***CYP2D6*1/*2**	3	0.40 (0.00)	10.96 (0.00)	92.99 (0.00)	0.04 (0.00)
***CYP2D6*4/*4**	2	0.30 (0.00)	21.56 (0.00)	40.48 (0.00)	0.17 (0.00)
***CYP2D6*4/*10**	4	0.27 (0.10)	39.96 (33.71)	69.34 (119.84)	0.15 (0.30)
***CYP2D6* phenotype**	**133**	**P=0.013***	**P=0.014***	**P=0.017***	**P=0.594**
EM	55	0.10 (0.03)	15.19 (15.55)	61.05 (34.92)	0.08 (0.13)
IM	76	0.09 (0.02)	24.06 (26.15)	76.80 (49.8)	0.10 (0.09)
PM	2	0.08 (0.00)	21.56 (0.00)	40.48 (0.00)	0.17 (0.00)

Note: P = P-value from Kruskal-Wallis test

Figure 14: MR of TAM and its metabolites among different *CYP2D6* phenotypes



Note: Figure 14 (a): MR (TAM-NDMT), Figure 14 (b): MR (NDMT-END) among *CYP2D6* phenotype
Figure 14 (c): MR (TAM-4OHT), Figure 14 (d): MR (4OHT-END) among *CYP2D6* phenotype

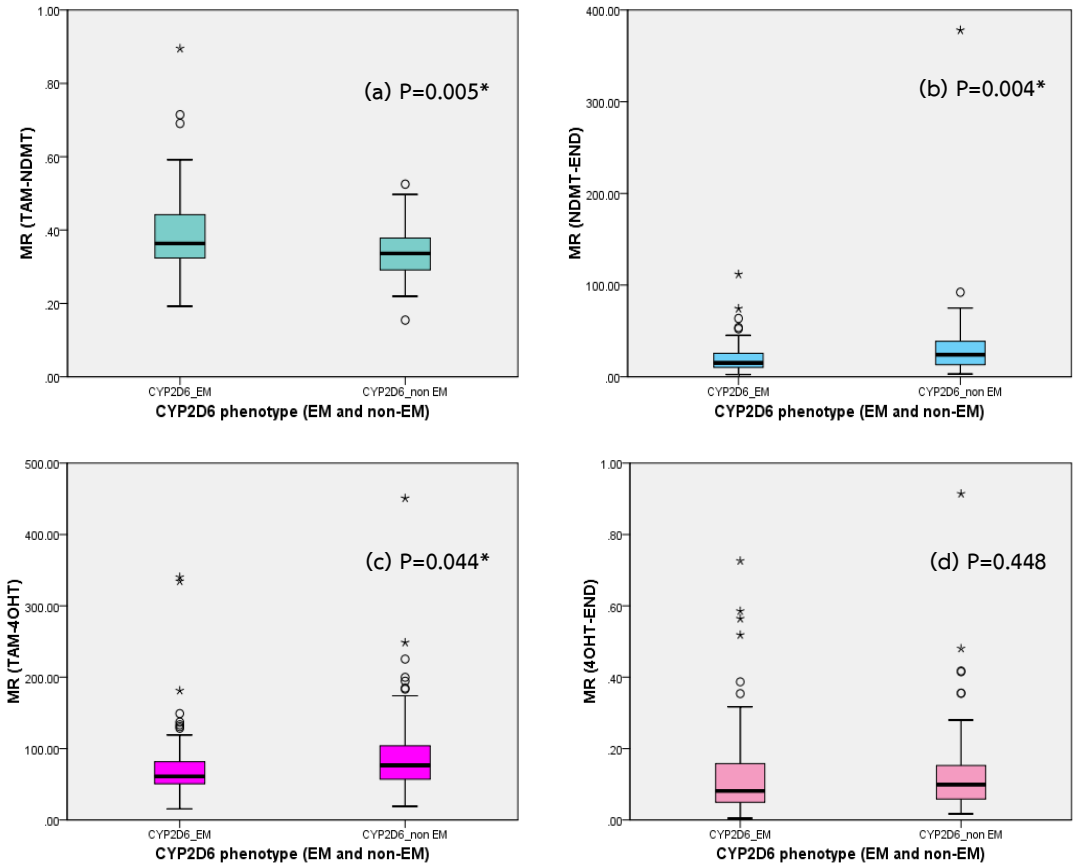
Moreover, It was suggested that MR (TAM-NDMT), MR (NDMT-END) and MR (TAM-4OHT) were also statistically different between *CYP2D6*-EM and *CYP2D6*-non-EM (*CYP2D6*-IM and *CYP2D6*-PM) (P=0.005, P=0.004 and P=0.044, respectively) while MR (4OHT-END) was not statistically different between those groups (P=0.448) **(Table 41, Figure 15)**. *CYP2D6*-EM had lower MR (NDMT-END), MR (TAM-4OHT) and MR (4OHT-END) than *CYP2D6*-non-EM but had higher MR (TAM-NDMT) than *CYP2D6*-non-EM **(Table 41, Figure 15)**.

Table 41: MR of TAM and its metabolites (*CYP2D6*-EM and *CYP2D6*-non-EM)

CYP2D6 Polymorphisms	N	MR (TAM-NDMT)	MR (NDMT-END)	MR (TAM-4OHT)	MR (4OHT-END)
CYP2D6 phenotype	133	P=0.005*	P=0.004*	P=0.044*	P=0.448
EM	55	0.36 (0.12)	15.19 (15.55)	61.05 (34.92)	0.08 (0.13)
Non-EM	78	0.34 (0.09)	24.06 (25.89)	76.47 (48.12)	0.10 (0.09)

Note: P = P-value from Mann-Whitney U test, *CYP2D6*-non-EM = *CYP2D6*-IM and *CYP2D6*-PM

Figure 15: MR of TAM and its metabolites (*CYP2D6*-EM and non-EM)



Note: Figure 14 (a): MR (TAM-NDMT), Figure 14 (b): MR (NDMT-END) among *CYP2D6*-EM and non-EM
Figure 14 (c): MR (TAM-4OHT), Figure 14 (d): MR (4OHT-END) among *CYP2D6*-EM and non-EM

Hypothesis 2: There is no difference in estrogen concentration (E2) among different metabolizing enzyme polymorphisms (*SULT1A1* copy numbers variations) in Thai breast cancer patients.

Since 96.3% (36 patients) of these 134 patients had two copies of *SULT1A1* genes, the sample size of each *SULT1A1* copy numbers group in this sub-group analysis was subsequently not enough to determine the difference of E2 concentration among those different *SULT1A1* copy numbers variations. The E2 concentration showed a trend to increase with more *SULT1A1* copy numbers; however, the E2 concentration was not statistically different among those groups (P=0.241). The E2 concentrations of different *SULT1A1* copy numbers variations in this sub-group analysis were provided below **(Table 42)**.

Table 42: Estrogen concentration among different *SULT1A1* CNVs

SULT1A1 CNVs	N (34)	E2 (pg/ml): Mean ± SD	E2 (pg/ml): Median (IQR)	P
***SULT1A1* x 2 copies**	33	1,622.4 ± 793.0	1,567.9 (829.1)	0.353
***SULT1A1* x 3 copies**	1	2,280.8 ± 0.0	2,280.8 (0.0)	

Note: P = P-value (Mann-Whitney U test)

Hypothesis 3: There is no correlation among TAM and its metabolites (TAM, NDMT, END, and 4OHT) and estrogen concentration (E2) in Thai breast cancer patients.

Spearman's rank correlation was performed to test the correlation between TAM and its metabolites and E2 concentrations but the correlations between those variables were not found in this study **(Table 43).** Additionally, the correlation test between MR of TAM and its metabolites and E2 concentration were also performed but the significant correlations of those variables were not found in this study **(Table 44)**.

Table 43: Correlation between TAM and its metabolites and E2 concentrations

TAM and its metabolites	Spearman's rho (with E2)	P
TAM (ng/ml)	0.062	0.728
NDMT (ng/ml)	-0.044	0.805
END (ng/ml)	0.055	0.758
4OHT (ng/ml)	-0.062	0.728

Table 44: Correlation between MR and E2 concentrations

MR of TAM and its metabolites	Spearman's rho (with E2)	P
MR (TAM-NDMT)	0.105	0.556
MR (NDMT-END)	-0.118	0.505
MR (TAM-4OHT)	0.105	0.555
MR (4OHT-END)	-0.208	0.238

Hypothesis 4: There is no difference in TAM and its metabolites (TAM, NDMT, END and 4OHT) among other TAM-metabolizing enzyme polymorphisms (*CYP3A5*1* and *CYP3A5*3*) in Thai breast cancer patients.

TAM and its metabolites concentrations among different *CYP3A5* genotypes and phenotypes were compared using Kruskal-Wallis and Mann-Whitney U tests. The TAM and its metabolites concentrations were not statistically different among different *CYP3A5* genotypes or *CYP3A5* phenotypes **(Table 45)**. The MR of those metabolites were not also statistically different among *CYP3A5* genotypes (P= 0.307, 0.786, 0.742 and 0.642, respectively) or *CYP3A5* phenotypes (P=0.831, 0.657, 0.508 and 0.400, respectively); for those MR (TAM/NDMT), MR (NDMT/END), MR (TAM/4OHT) and MR (4OHT/END), respectively **(Table 46)**.

Table 45: TAM and its metabolites concentrations in *CYP3A5* polymorphisms

CYP3A5 Polymorphisms	N	TAM (ng/ml)	NDMT (ng/ml)	END (ng/ml)	4OHT (ng/ml)
***CYP3A5* genotype**	**133**	**P=0.771**	**P=0.680**	**P=0.844**	**P=0.223**
CYP3A5*1/*1	18	363.8 (199.2)	3,903.9 (2,729.8)	51.1 (67.1)	4.4 (2.4)
CYP3A5*1/*3	64	364.2 (249.3)	3,870.9 (2,004.5)	53.4 (54.2)	5.0 (2.9)
CYP3A5*3/*3	51	384.0 (235.1)	4,017.6 (2,898.5)	56.2 (49.4)	5.4 (3.2)
***CYP3A5* phenotype**	**133**	**P=0.493**	**P=0.451**	**P=0.813**	**P=0.100**
EM	82	363.8 (234.9)	3,870.9 (2,215.7)	53.4 (57.3)	4.8 (2.9)
PM	51	384.0 (235.1)	4,017.6 (2,898.5)	56.2 (49.4)	5.4 (3.2)

Note: P = P-value from Mann-Whitney U and Kruskal-Wallis tests

Table 46: MR of TAM and its metabolites in *CYP3A5* polymorphisms

CYP3A5 Polymorphisms	N	MR (TAM-NDMT)	MR (NDMT-END)	MR (TAM-4OHT)	MR (4OHT-END)
***CYP3A5* genotype**	**133**	**P=0.307**	**P=0.786**	**P=0.742**	**P=0.642**
CYP3A5*1/*1	18	0.32 (0.10)	21.72 (30.33)	79.49 (51.14)	0.10 (0.15)
CYP3A5*1/*3	64	0.36 (0.10)	15.45 (23.37)	70.34 (41.05)	0.08 (0.09)
CYP3A5*3/*3	51	0.34 (0.09)	23.26 (19.97)	63.23 (47.45)	0.10 (0.08)
***CYP3A5* phenotype**	**133**	**P=0.831**	**P=0.657**	**P=0.508**	**P=0.400**
EM	82	0.36 (0.10)	15.89 (25.49)	71.04 (42.44)	0.08 (0.12)
PM	51	0.34 (0.09)	23.26 (19.97)	63.23 (47.45)	0.10 (0.08)

Note: P = P-value from Mann-Whitney U and Kruskal-Wallis tests

Hypothesis 5: There is no association among estrogen concentration (E2) and TAM-associated ADRs in Thai breast cancer patients.

Kruskal-Wallis and Mann-Whitney U tests and were performed to test this hypothesis. Those premenopausal patients who reported their TAM-associated ADRs had significantly higher E2 concentration than those patients without ADRs report (P=0.014) but there was not any statistically difference of E2 concentration among those different TAM-associated ADRs symptoms (P=0.065) **(Table 47)**.

Table 47: E2 concentrations among different TAM-associated ADRs

TAM-associated ADRs (N=34)	N (%)	E2 (pg/ml) Mean ± SD	E2 (pg/ml) Median (IQR)	P
No ADRs	31 (92.5)	1,521.8 (652.5)	1,557.6 (740.9)	0.014[a*]
ADRs presented	3 (7.5)	2,882.1 (1,164.2)	2,511.2 (0.0)	*0.065*[b]
Irregular menstruation	*2*	*3,067.5 (1,582.6)*	*3,067.5 (0.0)*	
Vaginal discharge/dryness	*1*	*2,511.2 (0.0)*	*2,511.2 (0.0)*	

Note: a=Mann-Whitney U test (No ADRs versus ADRs presented), b=Kruskal-Wallis test, * = P-value < 0.05

Hypothesis 6: There is no association among TAM-metabolizing enzyme polymorphisms (*CYP2D6*1, CYP2D6*2, CYP2D6*10, CYP3A5*1* and *CYP3A5*3*) and TAM-associated ADRs in Thai breast cancer patients.

Pearson's chi-square test was performed to test the associations among *CYP2D6* or *CYP3A5* phenotypes and TAM-associated ADRs. There was not any association among those TAM-associated ADRs and *CYP2D6* (P=0.910) or *CYP3A5* (P=0.361) phenotypes were found in this study **(Table 48)**.

Table 48: *CYP2D6, CYP3A5* polymorphisms and TAM-associated ADRs

Polymorphisms	ADRs Presented	No ADRs	Total	P-value
***CYP2D6* phenotype**				0.910
EM	9 (45.0)	46 (40.7)	55 (41.4)	
Non-EM (IM and PM)	11 (55.0)	67 (59.3)	78 (58.6)	
Total	20 (100.0)	113 (100.0)	133 (100.0)	
***CYP3A5* phenotype**				0.361
EM	10 (50.0)	72 (63.7)	82 (61.7)	
PM	10 (50.0)	41 (36.3)	51 (38.3)	
Total	20 (100.0)	113 (100.0)	133 (100.0)	

CHAPTER V
DISCUSSION

Patients and demographic data

The patients consisted of 134 Thai breast cancer women which included all stages of breast cancers and all stages of menopausal status. Majority of those patients were positive for estrogen receptor (ER+) and progesterone receptor (PR+) (98.5% and 72.4% respectively). Their ages were ranged from 27.0 to 82.0 years and their BMI were ranged from 15.4 to 40.0.

According to previous studies, Saladores et al. suggested that combined genetic factors (*CYP2C9, CYP2C19* and *CYP3A5*) and non-genetic factors (age and BMI) had 2.8% contribution on MR (NDMT-END)[71] and Lien et al. reported that age was positively correlated to TAM and its metabolites concentrations (TAM, NDMT and END)[72] therefore those correlations between the demographic factors (age, BMI, duration of TAM used) and TAM and its metabolites concentrations were explored to ensure the other possible effects in the analysis. It was found that age showed significant correlation to MR (TAM-4OHT) (P=0.026) and BMI showed significant correlation to MR (TAM-NDMT) (P=0.020) and MR (TAM-4OHT) (P=0.023) **(Table 49)** while duration of TAM used did not show any significant correlation to TAM and its metabolites concentrations. However, the distributions of those variables (BMI and age) were not statistically different among *CYP2D6* and *CYP3A5* phenotypes **(Table 50)** which indicated the same baseline of age and BMI among those phenotype groups.

Table 49: Correlations between BMI, age and MR of TAM and its metabolites

Demographic data	P-value			
	MR (TAM-NDMT)	*MR (NDMT-END)*	*MR (TAM-4OHT)*	*MR (4OHT-END)*
Age	0.201	0.335	0.026*	0.452
BMI	0.020*	0.603	0.023*	0.162

Note: P-value (Spearman's rank correlation)

Table 50: Associations among BMI, Age and *CYP2D6*, *CYP3A5* polymorphisms

Polymorphisms	P-value (BMI)	P-value (Age)
***CYP2D6* phenotype**	0.791	0.200
***CYP3A5* phenotype**	0.865	0.154
Combined *CYP2D6* with *CYP3A5* phenotypes	0.892	0.185

Note: P-value (Kruskal-Wallis test and Man-Whitney U test)

Moreover, the correlations between the demographic variables (age, BMI and duration of TAM used) and TAMs and its metabolite concentrations in sub-group analysis (34 premenopausal breast cancer patients) were also explored. It was found that BMI was correlated to 4OHT concentration (P=0.026) while age was correlated to TAM and NDMT concentrations (P=0.008 and P=0.006, respectively) but was not correlated to END concentration (P=0.251) which was corresponded with previous report from Lien et al.[72] However, the distribution of age and BMI were not statistically different among *SULT1A1* CNVs (P=0.471 and P=0.941, respectively). Additionally, It was found that age, BMI and duration of TAM used were not statistically correlated to those E2 concentrations (P=0.056, P=0.928 and P=0.709, respectively) which could be implied that these demographic variables did not affect the scope of this sub-group analysis.

Determination of *CYP2D6*4* from *CYP2D6*2* and *CYP2D6*10* assays:

There were 7 patients that their genotypes were classified to *CYP2D6*4* instead of *CYP2D6*10* according to their haplotypes **(Table 22)**. Gaedigk et al. and Lyon et al. suggested that the 100C>T was the SNPs that occurs on both *CYP2D6*4* and *CYP2D6*10* and these two alleles were assigned by ruling out of 1846G>A to confirm the *CYP2D6*4* allele.[61, 62] The 1846G>A SNPs was not used in this study according to the low prevalence of *CYP2D6*4* in Thai; however, the *CYP2D6*4* was noticed in this study by considering the haplotypes of 100C>T with 2850C>T[62] which

were detected by *CYP2D6*10* and *CYP2D6*2* assays, respectively. The TAM and its metabolites concentrations from those 7 patients were provided below **(Table 51)**.

Table 51: TAM and its metabolites concentrations of patients with *CYP2D6*4*

CYP2D6 polymorphisms	TAM (ng/ml)	NDMT (ng/ml)	END (ng/ml)	4OHT (ng/ml)
CYP2D6*4/*10 **(IM)**	44.9	80.8	17.2	2.5
CYP2D6*4/*10 **(IM)**	414.3	1,135.0	73.5	2.1
CYP2D6*4/*10 **(IM)**	450.0	1,926.6	46.6	7.1
CYP2D6*4/*10 **(IM)**	523.0	1,922.7	49.9	6.9
CYP2D6*4/*10 **(IM)**	574.9	2,107.7	35.5	14.8
CYP2D6*4/*4 **(PM)**	214.8	679.1	53.2	4.5
CYP2D6*4/*4 **(PM)**	262.0	927.0	30.6	8.0

Additionally, since *CYP2D6*4* was determined by using *CYP2D6*2* (2850C>T) and *CYP2D6*10* (100C>T) assays without using *CYP2D6*4* (1846G>A) assay, there were 13 patients (9.7%) who expressed *CYP2D6*1/*2* (*CYP2D6*2* assay) with *CYP2D6*1/*10* (*CYP2D6*10* assay) which could be classified to *CYP2D6*2/*10* genotype (2850C>T and 100C>T were located on different alleles) or *CYP2D6*1/*4* genotype (2850C>T and 100C>T were located on same alleles). However, it was suggested that those alleles should be classified to *CYP2D6*2/*10* according to the low prevalence of *CYP2D6*4* in Thai population.[11] Other investigations might be performed to explore other possible allele patterns; for example, exploring genotypes of their parents[62] or considering TAM and its metabolites levels[29] to confirm those genotype classification results. Nevertheless, both *CYP2D6*2/*10* and *CYP2D6*1/*4* genotypes were classified to EM phenotype which should provide the same enzyme activities.

Prevalence of *CYP2D6*, *CYP3A5* and *SULT1A1* polymorphisms

The 9.7% of *CYP2D6*1/*1* in Thai breast cancer patients which was suggested in this research was lower than the prevalence that was reported from previous study[29] as a result of simultaneous *CYP2D6*2* determination. Areepium et al. used only *CYP2D6*10* (100C>T) assay to discriminate *CYP2D6*10* (SNPs) from *CYP2D6*1* (WT) based on qPCR technique with TaqMan® assay[29] while Chamnanphon et al. used Amplichip™ CYP450 test[11] which could detect several SNPs simultaneously including *CYP2D6*2* (2549delA), *CYP2D6*4* (1846G>A) and *CYP2D6*10* (100C>T)[62] Therefore, the prevalence of *CYP2D6*1/*1* that was reported in those studies depending on the numbers of SNPs position that included in each platform which were different among those three studies **(Table 52)**. Moreover, the prevalence of *CYP2D6*10/*10* was high in this study compared with previous studies in Thai breast cancer patients **(Table 52)** which might be influenced from the larger numbers of patients that were recruited in this study.

Table 52: Comparison of *CYP2D6* genotype in this study with previous studies

CYP2D6 genotype	Prevalence (%)		
	TaqMan® assay	TaqMan® assay	Amplichip™ CYP450
	This study (N=134)	Areepium et al. (N=59)[29]	Chamnanphon et al. (N=57)[11]
CYP2D6*1/*1	9.7	27.1	15.7
CYP2D6*2/*2	3.7	No detection	3.5
CYP2D6*1/*2	2.2	No detection	3.5
CYP2D6*1/*10	15.7	39.0	28.1
CYP2D6*2/*10	9.7	No detection	7.0
CYP2D6*10/*10	53.7	33.9	22.8
CYP2D6*4/*4	1.5	No detection	0.0
CYP2D6*2/*4	0.0	No detection	1.8
CYP2D6*4/*10	3.7	No detection	0.0

Note: Chamnanphon et al. also reported *CYP2D6*5, CYP2D6*14B, CYP2D6*35, CYP2D6*36* and *CYP2D6*41.*

However, summation of those genotype frequencies with fully functional alleles (*CYP2D6*1/*1, CYP2D6*1/*2* and *CYP2D6*2/*2*) was 15.6% which was agreed with previous studies **(Table 52)**.[11, 29] Determination of *CYP2D6*2* allele was useful for separating *CYP2D6*2/*10* (EM), *CYP2D6*4/*4* (PM) and *CYP2D6*4/*10* (IM) from *CYP2D6*1/*10* (EM) that was responsible for low prevalence of *CYP2D6*1/*10* (15.7%) in this study compared with previous research **(Table 52)**. The *CYP2D6*4/*4* and *CYP2D6*4/*10* would potentially contributed to low enzyme activities compared with *CYP2D6*10/*10* and might affect TAM and its metabolite concentrations differently.

Additionally, it was suggested that the prevalence of *CYP3A5*1/*3* and *CYP3A5*3/*3* were high in this study (47.8% and 38.8%, respectively) compared with *CYP3A5*1/*1* (13.4%) which was corresponded with previous studies in Caucasian[30] and Asian[28] but disagreed with another early report in Thai which suggested that *CYP3A5*1/*1* was the most common *CYP3A5* genotype in Thai breast cancer patients **(Table 53)**.[12] Those different results might be affected from its limited numbers of patients recruited in that report (30 patients) compared with the other studies **(Table 53)**. Furthermore, it was noticed that low prevalence of *CYP3A5*1/*1* was reported in Thai from this study and previous report in Asian but was not found in Caucasian from previous research **(Table 53)** which implied the high prevalence of *CYP3A5* polymorphisms in both Caucasian and Asian including Thai population.

Table 53: Comparison of *CYP3A5* genotype in this study with previous study

CYP3A genotype	Prevalence (%)			
	TaqMan® assay	TaqMan® assay	Amplichip™ CYP450	INFINITI™ CYP450
	This study (N=134)	Thai (N=30)[12]	Caucasian (N=135)[30]	Asian (N=165)[28]
CYP3A5*1/*1	13.4	63.0	0.0	8.5
CYP3A5*1/*3	47.8	33.0	4.4	43.6
CYP3A5*3/*3	38.8	4.0	95.6	47.9

The prevalence of *SULT1A1* copy numbers variations (CNVs) in Thai breast cancer patients was firstly reported in this study. It was suggested that two copies of *SULT1A1* gene was the most common variations in Thai breast cancer patients which was corresponded with early reports in healthy Japanese[33] and Caucasian breast cancer patients[32] **(Table 54)**. It was noticed that most Thai breast cancer patients were carrying wild type of *SULT1A1* copy number variations which was accounted for 96.3% while only 3.0% and 0.7% of those patients that were carrying three copies and one copy of *SULT1A1* gene, respectively **(Table 54)**.

Table 54: Comparison of *SULT1A1* CNVs in this study with previous study

SULT1A1 Copy numbers variations	Prevalence (%)		
	TaqMan® assay	TaqMan® assay	qPCR with GeneMarker®
	This study Thai (N=134)	Yu et al.[33] Japanese (N=101)	Moyer et al.[32] Caucasian (N=190)
SULT1A1x1	0.7	0.0	4.1
SULT1A1x2	96.3	65.0	67.5
SULT1A1x3	3.0	25.8	19.5
SULT1A1x4 or more	0.0	9.2	8.9

Note: Yu et al. reported the prevalence of SULT1A1 CNVs in healthy Japanese.

Prevalence of TAM-associated adverse drug reactions (ADRs)

It was suggested that 14.9% (20 patients) of patients in this study reported about their TAM-associated ADRs which hot flashes was ranked to be the most common TAM-associated ADRs (25.0%). This finding was corresponded with previous reports from Kiyotani et al.[20] and Lorizio et al.[42] **(Table 55)**.

Table 55: Comparison of TAM-associated ADRs events with previous studies

TAM-associated ADRs events	Prevalence (%)		
	This study Thai (N=134)	Kiyotani et al.[20] Japanese (N=98)	Lorizio et al.[42] Caucasian (N=241)
No ADRs reported	***85.1***	***No report***	***27.4***
Hot flashes	25.0	42.6	64.3
Sleep problems	0.0	0.0	36.1
Myalgia	25.0	27.9	0.0
Vaginal discharge/dryness	15.0	40.0	34.9
Endometrial thickness	15.0	1.6	0.0
Irregular menstruations	10.0	4.7	0.0
Palpitations	10.0	0.0	0.0
Weight gain	0.0	0.0	5.8

The overall report of ADRs was accounted for 14.9% in this study which was lower than the report from Lorizio et al. which suggested 72.6% of TAM-associated ADRs.[42] This underestimated data might be affected from the ADRs collection process in this study which was medical record review and patient interview. Those data collecting method might be influenced by the limitation of retrospective data collection, such as missing data and recall bias. On the contrary, Lorizio et al. collected those ADRs data by using self-report questionnaire [42] which might lead to overestimation of TAM-associated ADRs report especially for some non-specific ADRs such as sleep problems or weight gain.

Moreover, it was mentioned that those reported TAM-associated ADRs could be classified into two groups; the specific ADRs and the non-specific ADRs. The specific ADRs were resulted from cessation of estrogen effect (estrogen antagonist) which included hot flashes, vaginal dryness and irregular menstruation or over expression of estrogen effect (estrogen agonist) which included endometrial thickness. The non-specific ADRs were those common symptoms that could be indirectly related to or unrelated to the estrogen effect which included myalgia,

sleep problems, palpitations or weight gain. It was noticed that those specific ADRs were consistently reported among studies which implied that those specific ADRs (Hot flashes, vaginal dryness, irregular menstruation and endometrial thickness) were TAM-associated ADRs which could be found from both medical record review (This study) and self-report questionnaire (Lorizio et al.[42]) On the contrary, those non-specific ADRs (Myalgia, sleep problems, palpitations and weight gain) showed some discrepancies among studies which implied to the overestimation of those ADRs reports from tamoxfien **(Table 55)**. Further investigations or evaluation process might need to perform to confirm whether those non-specific ADRs were resulted from tamoxifen used. In conclusions, the prevalence of TAM-associated ADRs reported in this study might be underestimated or overestimated depending on the types of those ADRs symptoms.

TAM and its metabolites quantification

HPLC-FLU method validation:

It was found that some TAM and its metabolites were yielded more than 100% of the extraction efficiency (TAM (H, M and L), NDMT (H M and L), END (H) and 4OHT (L)) **(Table 28)** which was the practical problem that could be occurred in sample extraction method by protein precipitation technique as a result of the volume displacement error.[73] This circumstance was raised from the volume that was occupied by the protein precipitate itself and causing a somewhat increased concentration of solutes in the filtrate.[73] Nevertheless, the extraction efficiency can be analyzed from the consolidating trend result of those three concentrations of each metabolite instead of their individual recovery values **(Table 28)**.

The calibration curve of NDMT and END were constructed in range of 0-1,000 ng/ml and 0-300 ng/ml, respectively **(Table 29)**. However, there were 79 patients whose NDMT or END concentrations were greater than their expected theoretical concentrations (78 patients for NDMT and one patient for END) therefore their

metabolites concentrations were extrapolated from NDMT and END calibration curves, respectively. The range of NDMT and END concentrations of those patients were 1,002.23-2,543.78 ng/ml and 443.76 ng/ml, respectively.

TAM and its metabolite concentrations in plasma sample:

The TAM and its metabolites concentrations were tested for normality by Kolmogorov-Smirnov and Shapiro-Wilk test (P=0.000 for TAM, NDMT, END and 4OHT) therefore median and IQR were used to represent those data. It was found that the TAM concentration in this research was corresponded with previous studies [28, 29] which suggested the same baseline concentration of TAM from patients who taking tamoxifen 20 mg/day among those different studies **(Table 56)**; however, the NDMT and END concentrations in this study were higher than those concentrations from previous reports.[28, 29] Those differences might be resulted from the several factors. Firstly, there were marked differences of sample preparation methods among those studies. Zhu et al. performed their research by liquid-liquid extraction technique with methanol and trimethylamine[69], Lim et al. extracted their samples with hexane and butanol[28] and Areepium et al. retrieved them by using protein precipitation technique with acetonitrile[29] which was the technique that was also used in this study with the modification by adding methanol in precipitation process to increase amount of those TAM and its metabolites in extracted solution. It was noticed that those different sample preparation methods might affect the metabolites concentrations among studies except TAM which level was found to be consistent among different research **(Table 56)**. Secondly, the types of column that were used in those HPLC-FLU techniques were also different among studies. The C8 HPLC column was used in the study from Lim et al.[28], short length of C18 column (150 mm x 4.6 mm) was used in the studies from Zhu et al.[69] and Areepium et al.[29] while long length of C18 column (250 mm x 4.6 mm) was used in this study. Thirdly, the wavelength of UV lamp and time of UV exposure that were used in each study were also different. Zhu et al. prepared their samples under UV lamp 254 nm for 10

minutes[69] while Areepium et al. performed the experiment by using UV lamp 375 nm for 20 minutes[29] compared with the UV lamp 366 nm for 20 minutes that was used in this study. The UV lamp was essential equipment for transforming TAM and its metabolites in extracted solvent to become fluorescence-detectable form. The over-exposure time might lead to degradation of those TAM and its metabolites[69] while incomplete exposure might be responsible for low detectable concentrations of those TAM and its metabolites by fluorescence detector. Finally, the differences of numbers of patients and race of those patients that were recruited in each study needed to be taken into account. The larger sample size of 134 patients was recruited in this research which included those 78 patients who had outstanding high NDMT concentrations and leaded to higher median of NDMT concentrations compared with previous report in Thai.[29] Nevertheless, the sub-group analysis showed that the rest of them (56 patients) provided similar result of NDMT concentration (768.7 (382.9) ng/ml) compared with the previous study in Thai (59 patients)[29] **(Table 56)**. Lim et al. determined those TAM and its metabolites levels in Asian breast cancer patients which included Chinese, Malays and Indians that was different from this study which collected data in Thai breast cancer patients.

Table 56: Comparison of TAM and its metabolites levels with previous studies

TAM and its metabolites	This study: Thai Median (N=133)	Areepium et al.[29]: Thai Median (N=59)	Lim et al.[28]: Asian Median (N=111)
TAM (ng/ml)	374.7 (230.2)	336.5	205.2 (39.3-599.9)
NDMT (ng/ml)	1,064.9 (599.6)	532.7	304.1 (40.8-803.0)
END (ng/ml)	54.5 (52.5)	15.3	13.7 (1.7-42.8)
4OHT (ng/ml)	5.0 (3.1)	Undetectable	2.0 (0.5-5.3)

Additionally, one patient was excluded from the gene polymorphisms-concentration association analysis according to her extremely low concentration of TAM (44.9 ng/ml) which was lower than 20% of the median concentration. This low

concentration might be a consequence from non-adherent problem or any unknown factors. It was implied that the non-adherence screening method by patient interview showed some over-estimation of medication adherence. Saladores et al. reported the use of TAM level as a criterion for medication adherence screening in TAM-treating breast cancer patients and they found that 39/587 of patients were excluded from the analysis.[71] The genotype data and TAM and its metabolites concentrations of the excluded patient were provided below **(Table 57)**.

Table 57: Genotype and TAM and its metabolites data of the excluded patient

Genotype data		TAM and its metabolites concentrations	
CYP2D6	*CYP2D6*4/*10* (IM)	**TAM (ng/ml)**	44.9
CYP3A5	*CYP3A5*3/*3* (PM)	**NDMT (ng/ml)**	80.8
SULT1A1	*SULT1A1x2*	**END (ng/ml)**	17.2
Copy numbers variations		**4OHT (ng/ml)**	2.5

E2 quantification

E2 concentrations in plasma samples:

The E2 concentrations from those 34 premenopausal patients were ranged from 415.0-4,186.5 pg/ml which was higher than E2 concentration reported from previous studies.[27, 56] Those differences might come from several factors. Firstly, the premenopausal breast patients were recruited in this study which had naturally higher E2 concentration compared with those postmenopausal patients included in study from Gjerde et al.[4] **(Table 58)**. Secondly, the E2 quantitative technique that was used in this study was ELISA which was different from the radioimmunoassay technique that was used in 1997 by Lum et al.[56] Thirdly, the patients were using tamoxifen 20 mg once daily for at least two months in this study while the patients were using tamoxifen 10 mg twice a day in previous study but the duration of TAM used was not mentioned in that report[56] **(Table 58)**. Finally, the larger numbers of

patient was recruited in this study (N=34) compared with small number of patients in previous study (N=6)[56] **(Table 58)**. Additionally, the different phase of individual menstrual cycle provided different E2 concentration. The E2 was increased in follicular phase until reached the peak of E2 concentration then was decreased in ovulatory phase and increase again in luteal phase then started to decreased again to begin the new menstrual cycle. Even though the phase in menstrual cycle could not be controlled in this study, the menstrual phase during individual menstrual cycle was predicted by interviewing with the patient at patient recruitment time. It was found that the E2 concentration was not statistically different among those different menstrual phase in this study (P=0.195) **(Table 59)**.

Table 58: Comparison of E2 concentration in this study with previous studies

Estrogen concentration	This study (N=34) Premenopausal	Lum et al.[56] (N=6) Premenopausal	Gjerde et al.[4] (N=90) Postmenopausal
E2 (pg/ml)	1,575.6 (865.4)	493.0 ± 574.0	2.56 (0.35-14.9)

Table 59: E2 concentration during menstrual cycle (N=25)

Menstrual cycle	N (25)	Median (IQR): E2 (pg/ml)	P
Follicular phase (Day1-Day10)	10	1,608.5 (887.0)	0.195
Ovulatory phase (Day11-Day15)	6	1,057.7 (1,052.2)	
Luteal phase (Day16-Day28)	9	1,567.9 (1,138.3)	

Note: Day0 = First day of menstrual flow

Hypotheses testing

Hypothesis 1 and Hypothesis 4:

When these two hypotheses were combined, it was found that TAM and its metabolites were not statistically different among *CYP2D6* or *CYP3A5* genotypes **(Table 60)**. Even though TAM and its metabolites concentration was statistically different among *CYP2D6* phenotypes, those differences were not existed when both *CYP2D6* and *CYP3A5* were combined in the analysis (P=0.265) **(Table 60)**. This result indicated the same baseline concentration of TAM before entering to the gene polymorphisms-concentrations association analysis; however, since there are several enzymes involved in TAM-metabolic pathway **(Figure 2)**. The metabolic ratio (MR) was used to adjust the baseline concentration of each metabolite in each TAM-metabolic sub-pathway. It was found that MR (TAM-NDMT) and MR (NDMT-END) were statistically different among combined *CYP2D6* and *CYP3A5* phenotypes (P=0.032 and P=0.026, respectively) **(Table 61)**. This result indicated that combined *CYP2D6* and *CYP3A5* phenotypes affected to MR (TAM-NDMT) and MR (NDMT-END) but did not affect to MR (TAM-4OHT) and MR (4OHT-END) which were implied to the effects of *CYP2D6* and *CYP3A5* phenotypes on NDMT and END.

Moreover, the result was showed that MR (TAM-NDMT), MR (NDMT-END) and MR (TAM-4OHT) were statistically different among *CYP2D6* phenotypes (P=0.013, P=0.014 and P=0.017, respectively) but were not statistically different among different *CYP3A5* phenotype (P=0.967 and P=0.595, respectively). These findings indicated that *CYP2D6* phenotype was responsible for NDMT, END and 4OHT concentrations while *CYP3A5* did not show that effect. Moreover, it was suggested that MR (TAM-4OHT) was significantly different among *CYP2D6* phenotypes (P=0.017) **(**Table 40**)** but this association was not existed when *CYP2D6* and *CYP3A5* were combined in the analysis (P=0.079) **(Table 61)** which was confirmed that the effects of gene polymorphisms on MR (TAM-4OHT) were from *CYP2D6* polymorphisms regardless of *CYP3A5* polymorphisms.

Table 60: TAM and its metabolites concentrations among combined *CYP2D6* and *CYP3A5* phenotypes

Combined phenotype	N (133)	TAM (P=0.265)	NDMT (P=0.114)	END (0.244)	4OHT (0.224)
CYP2D6(EM)-*CYP3A5*(EM)	31	373.1	1,031.0	72.9	5.3
CYP2D6(EM)-*CYP3A5*(PM)	24	365.2	1,030.4	58.7	6.3
CYP2D6(IM)-*CYP3A5*(EM)	51	358.3	1,094.7	46.6	4.7
CYP2D6(IM)-*CYP3A5*(PM)	25	425.6	1,193.6	57.5	4.4
CYP2D6(PM)-*CYP3A5*(PM)	2	238.4	803.2	41.9	6.2

Table 61: MR of TAM and its metabolites among combined phenotype

Combined phenotype	N (133)	MR TAM-NDMT (P=0.032*)	MR NDMT-END (P=0.026*)	MR TAM-4OHT (P=0.079)	MR 4OHT-END (P=0.622)
CYP2D6(EM)-*CYP3A5*(EM)	31	0.38 (0.19)	11.83 (7.06)	65.09 (42.45)	0.07 (0.08)
CYP2D6(EM)-*CYP3A5*(PM)	24	0.34 (0.11)	18.06 (27.87)	60.29 (28.26)	0.11 (0.17)
CYP2D6(IM)-*CYP3A5*(EM)	51	0.34 (0.09)	23.84 (27.62)	75.47 (46.76)	0.10 (0.14)
CYP2D6(IM)-*CYP3A5*(PM)	25	0.35 (0.08)	24.28 (17.3)	78.98 (68.10)	0.10 (0.07)
CYP2D6(PM)-*CYP3A5*(PM)	2	0.30 (0.00)	21.56 (0.00)	40.48 (0.00)	0.17 (0.00)

Note: *=P-value<0.05

These results were corresponded with the study from Mürdter et al which found that *CYP2D6* phenotype was associated with MR (NDMT/END) ($P<10^{-16}$) [1] and the study from Saladores et al which suggested that *CYP2D6* showed 53% of contribution on MR (NDMT/END)[71] and the studies from Lim et al [28] and Fernández-Santander[30] which suggested no association between *CYP3A5* polymorphisms and those TAM and its metabolites concentrations.

Hypothesis 2:

This is the first research that explored the prevalence of *SULT1A1* CNVs in Thai breast cancer patients. The prevalence of those variations was 0.7%, 96.3% and 3.0% for one, two and three copy numbers of *SULT1A1* genes, respectively. Of those 134 patients, the reported prevalence of *SULT1A1* CNVs was corresponded with the prevalence from other population except for the 4 copies of *SULT1A1* genes which was not found in Thai patients but was reported in Caucasian and Japanese [28, 33]. However, the association between *SULT1A1* CNVs and E2 concentration was not observed in this study which was inconsistent with previous study that reported statistically positive association between *SULT1A1* CNVs and E2 concentration (P=0.010).[4] The negative result might be affected from very low prevalence of *SULT1A1x1* copy and *SULT1A1x3* copies in this study in terms of power of analysis. Further study might need to recruit more patients to confirm the result.

Hypothesis 3:

TAM and its metabolites levels were not correlated to E2 concentration in this study **(Table 43)**. This result was not corresponded with previous study. Gjerde et al. claimed that E2 concentration was statistically correlated to TAM concentration ($P<0.05$). It was also found that E2 had positive correlation with TAM ($r=0.295$).[4] The different result might be a consequence of three major factors. Firstly, the patients recruited in this study were premenopausal patients which was different from the previous study[4] that included postmenopausal patients. Lum et al. reported that E2 concentration was increased from 94 ± 60 pg/ml to 493 ± 574 pg/ml in premenopausal patients while increased from 18 ± 6 to 37 ± 43 pg/ml in postmenopausal patients after receiving tamoxifen.[56] Secondly, the E2 level in postmenopausal patient was quite stable during a month compared with premenopausal patient whose E2 levels were changing during individual menstrual cycle. Finally, a limited numbers of patients in this sub-group analysis might affect power of the test **(Table 58)**.

Hypothesis 5 and Hypothesis 6:

The association between E2 concentration and TAM-associated ADRs was found in this premenopausal sub-group analysis (P=0.017) **(Table 47)**. Those TAM-associated ADRs in this sub-group analysis included irregular menstruation and vaginal discharge/dryness which might be a consequence of estrogen antagonist effect from tamoxifen mechanism of action. However, several factors needed to be considered before performing the conclusive result. Firstly, the patient interview and medical record review were used to collect those ADRs data in this study without using other ADRs evaluation instruments; therefore, the limitations from retrospective data or subjective data collection were existed and might lead to over-estimation or under-estimation of those ADRs data which might subsequently affect the validity of those ADRs records. Secondly, the exact menstrual phase in individual patient could not be confirmed in this study and might affect the results. Other factors might need to be explored for controlling these possible confounders (for example; follicle stimulating hormone (FSH), sex hormone-binding globulin (SHBG) or their individual E2 baseline). Thirdly, the limited numbers of patients in this premenopausal sub-group analysis could be problematic, especially for those three patients who reported their TAM-associated ADRs. More patients needed to be explored for this relationship before generalizing the result to the target population. Finally, those TAM-associated ADRs in this study did not include hot flashes which was the most common TAM-associated ADRs in breast cancer patients.

Furthermore, several factors were reported to affect those ADRs. Lorizio et al. suggested that duration of TAM used, age, END concentration and menopausal status can predict TAM-associated ADRs[42] ;however, those factors were not associated with TAM-associated ADRs in this study (Age; P=0.273, duration of TAM use; P=0.847 and END concentration; P=0.591).

Additionally, it was found that *CYP2D6* and *CYP3A5* polymorphisms were not associated with those TAM-associated ADRs **(Table 48)** which were corresponded with previous study that reported that *CYP2D6* and *CYP3A5* polymorphisms were not associated with hot flashes.[18]

Limitations

Even though the research was prudently constructed, some expected or unexpected limitations were existed in practical setting. The details of those limitations were summarized below.

1. TAM-associated ADRs data in this study was collected from medical record and patient interview; therefore, those ADRs data might be interfered with limitations of retrospective data which included missing data and recall bias that might lead to under-estimation or over-estimation of TAM-associated ADRs events.

2. Co-medications list was recorded from hospital database and medical record for drug-drug interaction problems screening without plasma drug level determination.

3. Medication adherence for TAM was assessed by face-to-face interview with the patients without other medication adherent assessment tools which might lead to over-estimation of medication adherence.

4. *CYP2D6*4* was determined by considering *CYP2D6*2* (2850C>T) and *CYP2D6*10* (100C>T) haplotypes without using *CYP2D6*4* (1846G>A) assay. According to the low prevalence of *CYP2D6*4* in Thai breast cancer patients [11], those genotypes were classified to *CYP2D6*2/*10*; however, other investigations needed to be performed to explore other possible allele patterns and confirm the genotype classification result.

5. The extrapolation of NDMT concentration in 78 patients and END concentration in one patient needed to be taken into account for the accuracy of those metabolites quantification reported in this study. However, the relevant hypotheses results should not be affected from those limitations according to the non-parametric analyses which were based on the rank sum test rather than their actual values.

6. The limited numbers of patients in those premenopausal sub-group analyses was a critical issue to provide the conclusive results and was an obstacle to generalize those results to the target population. Further research need to recruit more patients to determine the conclusive results.

CHAPTER VI
CONCLUSION

This is the first study that determined the associations among *CYP2D6* with *CYP3A5* polymorphisms and TAM and its metabolites in Thai breast cancer patients. The 134 Thai breast cancer cpatients who were taking TAM 20 mg/day was recruited in the study and their genotypes were determined by real-time PCR with TaqMan® assays. Those patients included premenopausal (53.0%), perimenopausal (10.4%) and postmenopausal patients (36.6%) with covering all stages of breast cancer. The mean age of patients was 51.6±11.6 years which BMI ranged from 15.4-40.0 and mean time of TAM used was 21.4±16.1 months which was ranged from 0.8-62.1 months.

*CYP2D6*10/*10* (53.7%) and *CYP3A5*1/*3* (47.8%) were the most common genotypes while *CYP2D6*-IM (57.5%) and *CYP3A5*-EM (61.2%) were the most common phenotypes that were found in this research. The wild type of *CYP2D6* (*CYP2D6*1/*1, CYP2D6*1/*2* and *CYP2D6*2/*2*) and *CYP3A5* (*CYP3A5*1/*1*) genes were accounted for 15.6% and 13.4%, respectively. There were only 23.1% of those patients carrying EM phenotypes for both *CYP2D6* and *CYP3A5* genes. The rest of them were carrying at least one decreased functional or non-functional allele. Additionally, *CYP2D6*4/*4* (PM) (1.5%) and *CYP2D6*4/*10* (IM) (3.7%) were determined from this study by simultaneous considering *CYP2D6*2* with *CYP2D6*10* haplotypes.

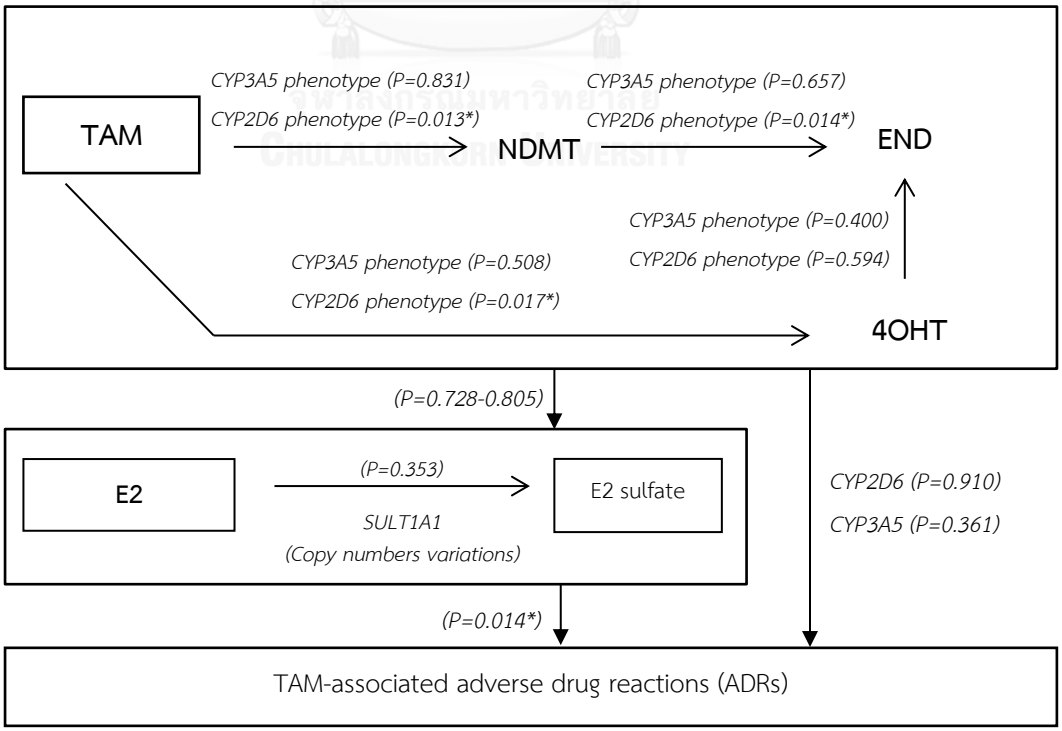
TAM and its metabolites concentrations from patients were quantified by HPLC-FLU technique which included TAM (82.5-984.2 ng/ml), NDMT (80.8-2,543.8 ng/ml), END (2.3-443.8 ng/ml) and 4OHT (2.1-21.7 ng/ml). The effects of *CYP2D6* phenotypes on NDMT, END and 4OHT concentrations were found in this study (P=0.013, P=0.014 and P=0.017, respectively) while those effects were not showed among different *CYP2D6* genotypes. However, the effects of those *CYP3A5* genotypes and phenotypes on TAM and its metabolites concentrations were not suggested from this research.

The prevalence of *SULT1A1* copy numbers variations in Thai breast cancer patients was also firstly reported from this study. Most of Thai breast cancer patients were carrying 2 copies of *SULT1A1* genes (96.3%) which exhibited normal enzyme

activity. Among those patients, 14.9% of them had experiences with TAM-associated ADRs which included hot flashes (3.7%), myalgia (3.7%), endometrial thickness (2.2%), vaginal discharge (2.2%), irregular menstruations (1.5%) and palpitations (1.5%).

The sub-group analysis of 34 premenopausal breast cancer patients provided the range of estradiol (E2) concentration between 415.0 and 4,186.5 pg/ml by ELISA technique but the association between those E2 concentrations and *SULT1A1* copy number variations were not found in this study (P=0.353). Nevertheless, the association between those E2 concentrations and TAM-associated adverse drug reactions (ADRs) was observed in this research (P=0.014). Those patients with TAM-associated ADRs experiences had high E2 concentrations compared with those patients without TAM-associated ADRs. However, several factors needed to be taken into account before drawing the conclusive result. Furthermore, The associations among those TAM-associated ADRS and *CYP2D6* or *CYP3A5* polymorphisms were not suggested from this study (P=0.910 and P=0.361, respectively). The overall findings of this research were provided below **(Figure 16)**.

Figure 16: Overall findings of this study presented in conceptual framework



In conclusions, the effects of *CYP2D6* polymorphisms on NDMT, END and 4OHT concentrations in Thai breast cancer patients were suggested from this research. The *CYP2D6* polymorphisms is a potential biomarker for END concentrations in TAM-treating breast cancer patients. Plasma estradiol concentrations were not correlated to any TAM and its metabolites concentrations but statistically associated with TAM-associated ADRs in premenopausal breast cancer patients.

The high prevalence of *SULT1A1x2* genes was firstly suggested from this study which indicated that most of Thai breast cancer patients are carrying *SULT1A1* genes with normal enzyme activities.

Implementation of the research result

To simplify the research results to clinical practice, the phenotype groups were categorized to *CYP2D6*-EM and other phenotypes (*CYP2D6*-IM and *CYP2D6*-PM). It was suggested that MR (TAM-NDMT), MR (NDMT-END) and MR (TAM-4OHT) were statistically different between those two phenotype groups (P=0.005, P=0.004 and P=0.044, respectively) which implied that those patients who are not carrying at least one full-functional allele (*CYP2D6*10/*10, CYP2D6*4/*4* and *CYP2D6*4/*10*) are in the high risk group to have low concentrations of NDMT, END and 4OHT compared with those patients who are carrying at least one full-functional allele (*CYP2D6*1/*1, CYP2D6*2/*2, CYP2D6*1/*2, CYP2D6*1/*10* and *CYP2D6*2/*10*).

This result can be further applied to identify the high risk group for ineffective tamoxifen treatment in terms of the low concentrations of its active metabolites or improve medication plan by adjusting tamoxifen dosage based on individual genetic data. However, the associations among those TAM and its metabolites concentrations and true clinical outcomes should be confirmed before these results are applied in practical settings.

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APPENDIX

APPENDIX I

Demographics data and Tamoxifen-associated ADRs recording form

Demographics data

Age | Height (m) | BW (Kg) | BSA | PID

E | T | Stage | Ki-67 | Day

P | N | Allergies: | Date

HER-2 | M | Time

Tamoxifen started date: | *Recurrence:*

History/Co-treatment:

Chemotherapy | Operations

Radiation | Her-2 specific

Current Medication list:

Underlying disease:

HTN | CVD

DM (I, II) | Dyslipidemias

Tamoxifen adherence:

Menopausal status:

Premenopausal | Hysterectomy

Perimenopausal | Oophorectomy

Postmenopausal

Last menstrual period (……..)

ADRs Events:

None

Hot flashes | Hyperhidrosis

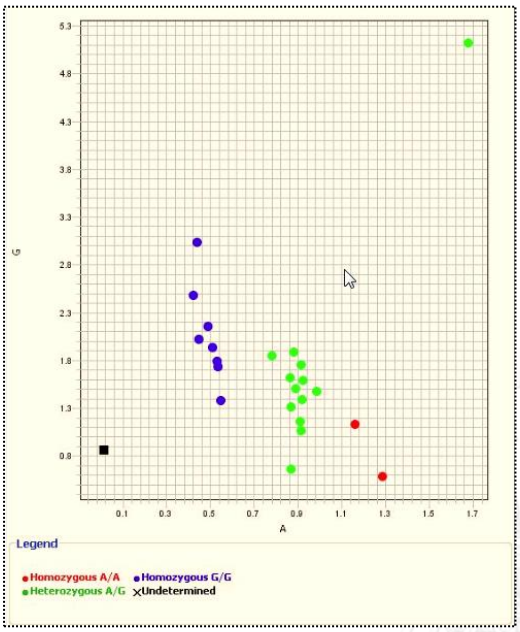
Irregular period | Discharge

Bleeding | N/V

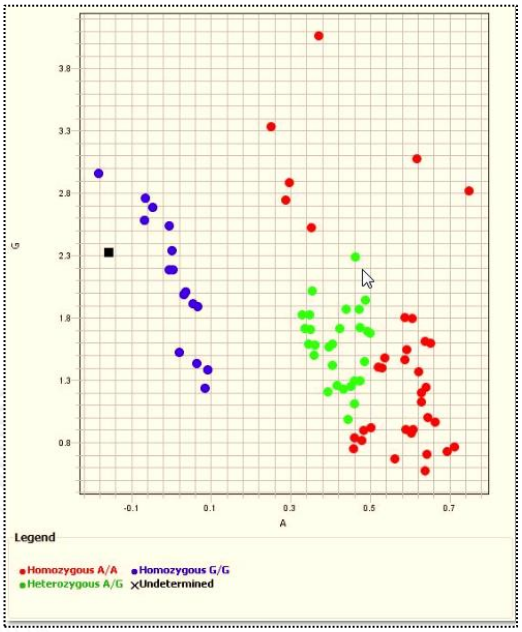
ENM thickness | TE event

APPENDIX II

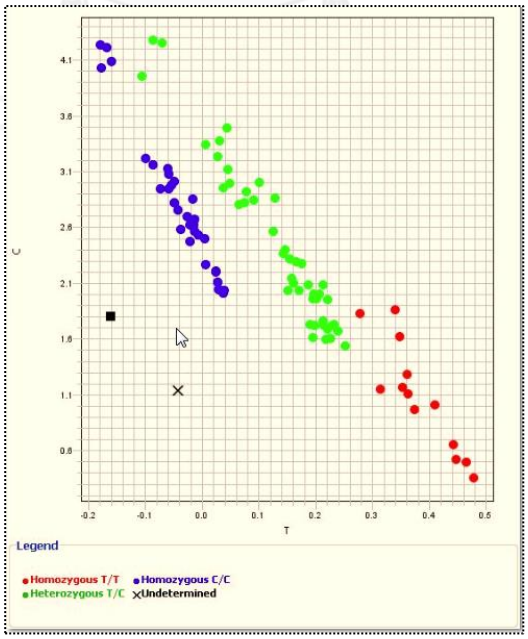
Allelic discrimination plot (*CYP2D6* and *CYP3A5* polymorphisms)



(a) Allelic discrimination plot (*CYP2D6*2* assay)



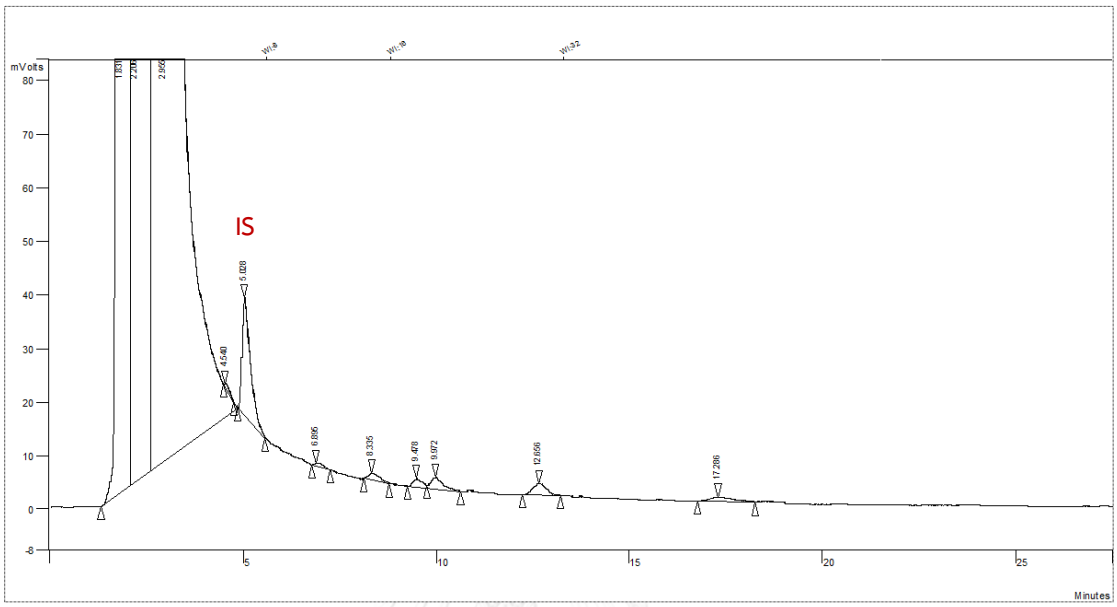
(b) Allelic discrimination plot (*CYP2D6*10* assay)



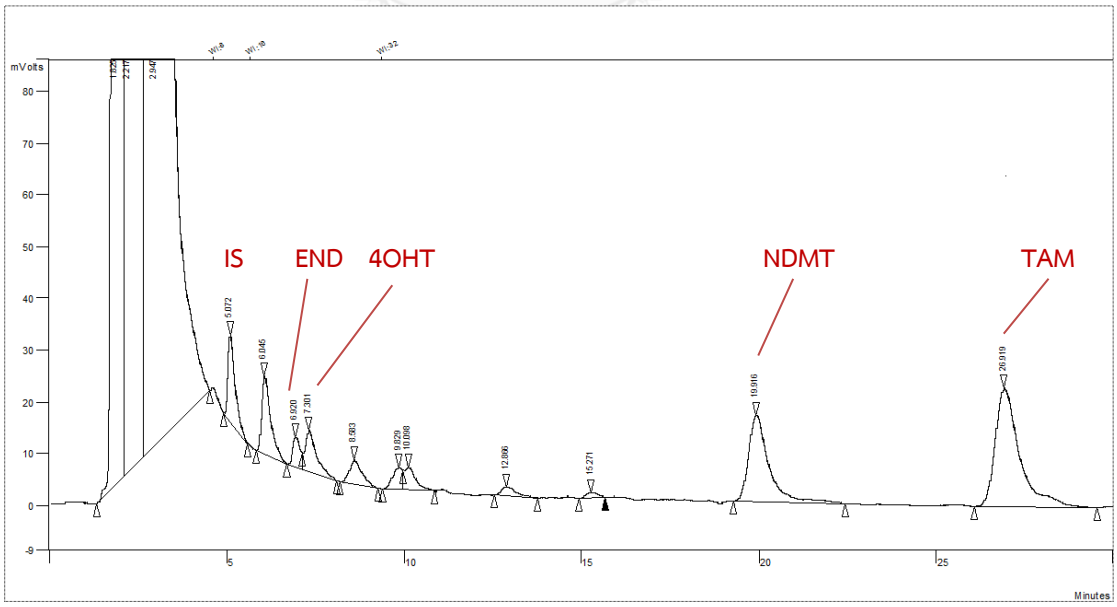
(c) Allelic discrimination plot (*CYP3A5*3* assay)

APPENDIX III

Chromatogram of TAM and its metabolites and IS in blank plasma



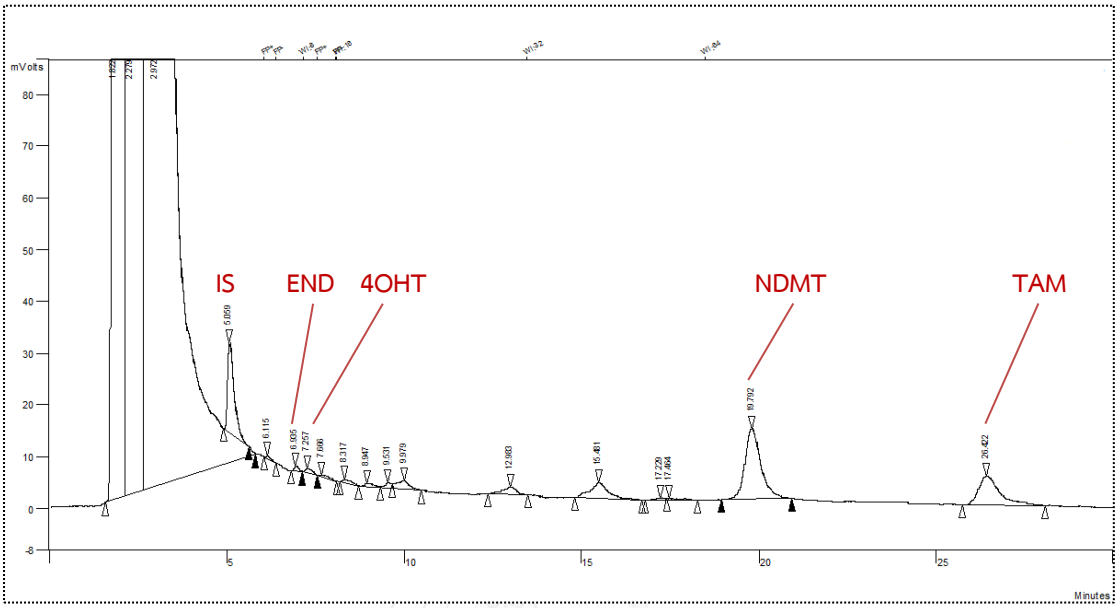
(a) Chromatogram of blank plasma with internal standard (IS) (RT 5.0 min)



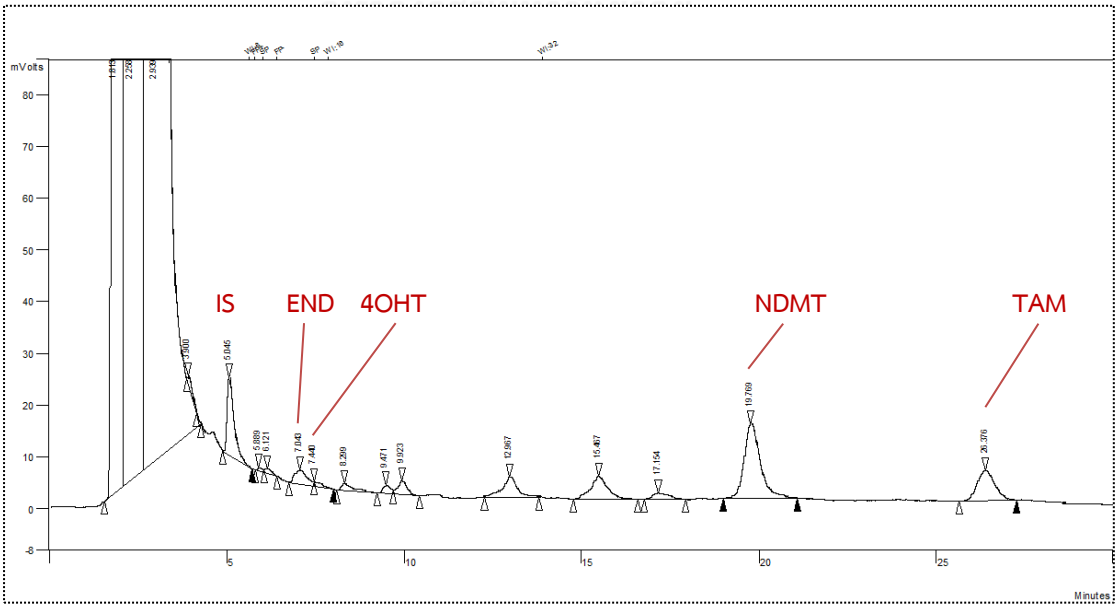
(b) Chromatogram of TAM (RT 26.1 min), NDMT (RT 19.6 min), END (RT 6.9 min)
4OHT (RT 7.3 min) with IS (RT 5.0 min) in blank plasma

APPENDIX IV

Chromatogram of TAM and its metabolites and IS in plasma sample



(a) Chromatogram of TAM (RT 26.1 min), NDMT (RT 19.6 min), END (RT 6.9 min) 4OHT (RT 7.3 min) with IS (RT 5.0 min) in plasma sample



(b) Chromatogram of TAM (RT 26.1 min), NDMT (RT 19.6 min), END (RT 6.9 min) 4OHT (RT 7.3 min) with IS (RT 5.0 min) in plasma sample

VITA

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