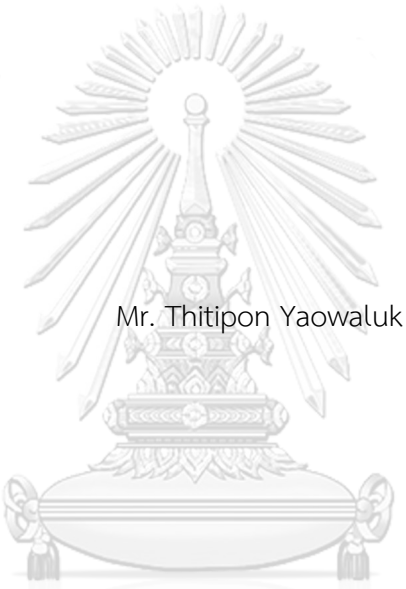
ASSOCIATION OF GENETIC AND NON-GENETIC FACTORS WITH CLINICAL RESPONSES OF DONEPEZIL AND GALANTAMINE IN THAI PATIENTS WITH DEMENTIA

Mr. Thitipon Yaowaluk



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ความสัมพันธ์ของปัจจัยทางพันธุกรรมและปัจจัยที่ไม่เกี่ยวข้องกับพันธุกรรมกับการตอบสนองทางคลินิกของยาโดเนเพซิลและกาแลนทามีนในผู้ป่วยโรคความจำเสื่อมชาวไทย

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
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ฐิติพล เยาวลักษณ์ : ความสัมพันธ์ของปัจจัยทางพันธุกรรมและปัจจัยที่ไม่เกี่ยวข้องกับพันธุกรรมกับการตอบสนองทางคลินิกของยาโดเนเพซิลและกาแลนทามีนในผู้ป่วยโรคความจำเสื่อมชาวไทย. (ASSOCIATION OF GENETIC AND NON-GENETIC FACTORS WITH CLINICAL RESPONSES OF DONEPEZIL AND GALANTAMINE IN THAI PATIENTS WITH DEMENTIA) อ.ที่ปรึกษาหลัก : ผศ. ภญ.ดร.พรพิมล กิจสนาโยธิน, อ.ที่ปรึกษาร่วม : รศ. พญ.วรพรรณ เสนาณรงค์,อ. นพ.ชนินทร์ ลิ่มวงศ์

โดเนเพซิลและกาแลนทามีนเป็นยารักษาภาวะสมองเสื่อมที่สั่งอย่างแพร่หลาย อย่างไรก็ตาม อัตราการตอบสนองต่อ acetylcholinesterase inhibitors มีเพียง 15-35 % ความแตกต่างระหว่างบุคคลในการตอบสนองต่อการรักษาด้วยยาโดเนเพซิลและกาแลนทามีนมีความสัมพันธ์กับปัจจัยทางพันธุกรรมในบางกลุ่มประชากร นอกจากนี้ปัจจัยที่ไม่เกี่ยวข้องกับพันธุกรรม เช่น อายุ เพศ ระดับการศึกษา โรคร่วม และปฏิกิริยาระหว่างยากับยาสามารถส่งผลต่อค่าทางเภสัชจลนศาสตร์และการตอบสนองต่อการรักษา ดังนั้นวัตถุประสงค์ในการศึกษานี้จึงศึกษาความสัมพันธ์ระหว่างความผันแปรทางพันธุกรรมที่เกี่ยวข้องกับผลการรักษาของยาโดเนเพซิลและกาแลนทามีน ได้แก่ยีนที่เกี่ยวข้องกับการเกิดโรค: *APOE*, ยีนที่เกี่ยวข้องการการเปลี่ยนสภาพยา: *CYP2D6*, *CYP3A5*, *UGT1A1*, ยีนที่เกี่ยวข้องกับการนำส่งยา: *ABCB1* และปัจจัยที่ไม่เกี่ยวข้องกับพันธุกรรมกับผลการตอบสนองในการรักษาที่วัดด้วยคะแนน Thai Mental State Examination (TMSE) และระดับยาที่สภาวะคงตัว (Cpss) ในผู้ป่วยความจำเสื่อมชาวไทยที่ได้รับการวินิจฉัยว่าเป็นความจำเสื่อมครั้งแรก ผลการวิเคราะห์ทั้งแบบตัวแปรเดียวและการวิเคราะห์การถดถอยเชิงเส้นพหุคูณแสดงให้เห็นว่าอัลลีล *CYP2D6*10* มีความสัมพันธ์กับระดับยาที่สภาวะคงตัวที่สูงกว่า (p-value = 0.029 และ B = 0.478, p-value = 0.032 ตามลำดับ) และผลการตอบสนองทางคลินิก คือ การเปลี่ยนแปลงของคะแนน TMSE (**Δ**TMSE) ของยาโดเนเพซิลที่ดีกว่า (p-value = 0.023 และ B = 4.107, p-value = 0.002 ตามลำดับ) โดยเฉพาะอย่างยิ่งในโรคอัลซไฮเมอร์ การใช้ยามีแมนทีนเป็นยาร่วมมีความสัมพันธ์กับระดับยาที่สภาวะคงตัวของยาโดเนเพซิลที่สูงขึ้น ในขณะที่การใช้ยาต้านซึมเศร้าเป็นยาร่วมจะลดผลการตอบสนองทางคลินิกของยาโดเนเพซิลในผู้ป่วยโรคอัลซไฮเมอร์ อายุมีความสัมพันธ์ทางลบกับการตอบสนองต่อยาโดเนเพซิลในผู้ป่วยโรคความจำเสื่อมจากภาวะหลอดเลือดสมอง สำหรับยากาแลนทามีน ผลการวิเคราะห์ถดถอยพหุคูณแสดงให้เห็นว่าผู้ป่วยโรคสมองเสื่อมแบบผสมที่มีจำนวนอัลลีลที่ผิดปกติร่วมกันของยีน *CYP2D6*, *CYP3A5*, *UGT1A1* มากกว่ามีความสัมพันธ์กับระดับยาที่สภาวะคงตัวที่ปรับของกาแลนทามีนที่สูงกว่า (B = 34.559, p-value = 0.045) ผลการวิเคราะห์การถดถอยเชิงเส้นพหุคูณและการวิเคราะห์การถดถอยโลจิสติคพหุคูณมีความสอดคล้องกัน คือ ผู้ที่มีอัลลีลของ *CYP2D6*10* มีความสัมพันธ์กับการเปลี่ยนแปลงของคะแนน TMSE ที่สูงกว่า (B = 5.227, p-value = 0.001) อัลลีลที่มีการกลายพันธุ์ของยีน *UGT1A1* และปัจจัยที่ไม่เกี่ยวข้องกับพันธุกรรม ได้แก่ การใช้ยากลุ่ม statin และระดับการศึกษาที่สูงกว่าอาจลดผลการรักษาด้วยยากาแลนทามีน ผลการศึกษานี้เน้นให้เห็นถึงความเป็นไปได้ที่จะนำการตรวจทางพันธุกรรมมาเป็นแนวทางในการรักษาภาวะสมองเสื่อมแบบเฉพาะบุคคลด้วยยาโดเนเพซิลและยากาแลนทามีนในยุคการแพทย์แม่นยำในอนาคตอันใกล้

สาขาวิชา เภสัชวิทยาและพิษวิทยา ลายมือชื่อนิสิต .....

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KEYWORD: Donepezil Galantamine CYP2D6 polymorphisms UGT1A1 polymorphisms Alzheimer's disease (AD) Vascular dementia (VAD) Mixed dementia

Thitipon Yaowaluk : ASSOCIATION OF GENETIC AND NON-GENETIC FACTORS WITH CLINICAL RESPONSES OF DONEPEZIL AND GALANTAMINE IN THAI PATIENTS WITH DEMENTIA. Advisor: Asst. Prof. PORNPIMOL KIJSANAYOTIN, Ph.D. Co-advisor: Assoc. Prof. Vorapun Senanarong, M.D., Chanin Limwongse, M.D.

Donepezil and galantamine are commonly prescribed for the treatment of dementia. However, the response rate of acetylcholinesterase inhibitors is only 15-35 %. Inter-individual variability in donepezil and galantamine response has been associated with genetic factor in some population. Moreover, non-genetic factors such as age, gender, education level, comorbidities and drug-drug interactions can influence pharmacokinetic profiles and drug responses. Therefore, this study aims to investigate the association of genetic variations that involved therapeutic effects of donepezil and galantamine including pathogenic gene; *APOE*, drug metabolizing genes; *CYP2D6, CYP3A5, UGT1A1*, transporter gene; *ABCB1* and non-genetic factors with therapeutic outcomes as measured as Thai Mental State Examination (TMSE) scores and steady-state plasma concentrations (Cpss) of donepezil and galantamine in Thai patients with firstly diagnosed dementia. Both univariate and multiple linear regression analysis indicated that only *CYP2D6*10* allele was associated with higher Cpss (*p*-value = 0.029 and B = 0.478, *p*-value = 0.032, respectively) and a better clinical outcomes of donepezil i.e. ΔTMSE (*p*-value = 0.023 and B = 4.107, *p*-value = 0.002), especially in patients with Alzheimer's disease (AD). Concomitant use of memantine was found to be associated with increased Cpss of donepezil. Whereas, co-medication with antidepressant drugs attenuated clinical responses of donepezil in patients with AD. Age was found to be negative associated with donepezil response in vascular dementia patients. For galantamine, the multivariate regression model revealed that patients with mixed dementia who carried a more detrimental allelic variants in combined *CYP2D6*, *CYP3A5*, and *UGT1A1* were associated with higher galantamine's adjusted Cpss (B = 34.559, *p*-value = 0.045). Both multiple linear and logistic regression analysis consistently revealed that *CYP2D6*10* carriers was significantly associated with higher ΔTMSE (B = 5.227, *p*-value = 0.001). *UGT1A1* mutant alleles and non-genetic factors including concomitant use of statin drugs and higher education level may attenuate the therapeutic outcome of galantamine. The present findings highlight the possibility of using genetic testing to guide personalized dementia therapy with donepezil and galantamine in the forthcoming precision medicine era.

Field of Study:	Pharmacology and Toxicology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Thitipon Yaowaluk

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CHAPTER 1 INTRODUCTION

Background and Rationale

Dementia is a chronic disease caused by various central neurodegenerative and ischemic process. Dementia characterized mainly by progressive cognitive functions decline, loss of initiative thinking, mood and behavioral changes and inability to perform activities of daily living. Dementia is a chronic illness that diminishes quality of life and causes an increased burden on caregivers (1). Moreover, all burdens associated with dementia lead to an increase in family expenses, and ultimately resulting in economic losses to the society as a whole. The prevalence of dementia in elderly is 2-10% and increase 2 times every 5 years after 60 years old.(2) It has become major public health in Thailand.

Alzheimer's disease (AD) is a common neurodegenerative disorder and one of the most common causes of dementia in Thailand and follows by vascular dementia. According to several guidelines such as American Geriatric society 2013 (AGS), European Federation of Neurological Societies 2010 (EFNS), acetylcholinesterase inhibitors are the first line drug for the treatment of dementia. However, the response rate of acetylcholinesterase inhibitors, including donepezil and galantamine that are common acetylcholinesterase inhibitors prescribed in Thailand, is only 15-35 % (3). The previous study on the Thai population concludes that the response rate for cognitive function improvement is quite low (4).

The main goal of pharmacological treatment of dementia is enhancing or modulating neurotransmitters, especially acetylcholine, with the ultimate goal of slowing or halting disease progression. Unfortunately, at the moment, such treatment has varying response, depending on interindividual factors. Donepezil and galantamine are widely used as the first-line drug for treatment of certain dementia-related illnesses including Alzheimer's disease (AD) and vascular dementia (VAD) (3). Donepezil and galantamine's metabolic pathway are through the CYP2D6, an enzyme with genetic polymorphism, which may account for the tremendous interindividual variation in success rate of 20-60% (3, 5-9). In addition, donepezil has

been shown to play a pivotal role in slowing amyloid plaque formation. However, due to elimination via efflux transporter namely P-glycoproteins (P-gp) which is encoded by *ABCB1*, polymorphisms of *ABCB1* might have an influence on the steady-state plasma concentration of donepezil or galantamine (Cpss) and clinical response (8).

CYP2D6 phenotypes of metabolizers can be classified as poor- (PM), intermediate- (IM), extensive- (EM) and ultra-rapid- metabolizers (UM). The metabolic rates in PMs and UMs are distinguished from EMs by 5 to 15 folds (10). Some studies reported the association between CYP2D6 polymorphisms and donepezil response (11, 12). While others reported no such association (13, 14). In Thai population, where *CYP2D6*10* allele frequency is found to be as high as 45% (15), this polymorphism is likely to explain interindividual variability of donepezil response and Cpss.

In addition, studies exploring innate susceptibility in development of AD have suggested the association between apolipoprotein E and the risk of AD. Most of these studies concluded that *APOE* ***ε****4* alleles increase the risk of AD in a gene-dose dependent manner (16). However, effects of *APOE* genotypes on clinical response of donepezil and galantamine are still inconclusive.

Several evidences suggest that approximately 60-70% of therapeutic outcomes of AD treatment depend upon genetic factors (8). Genetic variations may affect safety and efficacy of drug usages. Since dementia has complex pathophysiology and several genes would contribute to variability to drug response, therefore, the association study between single gene on clinical drug response is unlikely to explain therapeutic outcomes being observed (8). Moreover, non-genetic factors such as age, gender, education level, comorbidities and drug-drug interactions can influence pharmacokinetic profiles and drug responses. Therefore, in this study, we investigate the association between genetic variations that involved therapeutic effects of donepezil and galantamine including pathogenic gene; *APOE*, drug metabolizing genes; *CYP2D6, CYP3A5, UGT1A1*, transporter gene; *ABCB1* and non-genetic factors in Thai patients with dementia by using candidate genes approach. The study will enroll patients from the Faculty of Medicine Siriraj Hospital, Mahidol University. Candidate genes approach will be applied by determining the association

of genetic and non-genetic factors with clinical response of donepezil and galantamine by using multivariate linear or logistic regression analysis that could be expected to contribute better prediction of clinical response compared with univariate analysis. The clinical outcomes to be studied in this study were Thai Mental State Examination (TMSE) scores, steady-state plasma concentrations (Cpss) of donepezil and galantamine and adverse drug events.

Objectives

Therefore, the main objectives of this study are:

1. To evaluate the relationships between genetic polymorphisms of genes that involve metabolic pathways including *CYP2D6, CYP3A5, UGT1A1, ABCB1* and steady-state plasma concentrations of donepezil and galantamine in Thai patients with dementia.
2. To investigate the association of genetic factors including *CYP2D6, CYP3A5, UGT1A1, ABCB1* and *APOE* polymorphisms and non-genetic factors including age, gender, drug interaction and education levels with therapeutic response of donepezil and galantamine in Thai patients with dementia.

Scope of study

This study was performed as a retrospective cohort study. The study enrolled Thai patients with firstly diagnosed AD who receive donepezil or galantamine treatment and investigated the association of genetic factors including *CYP2D6, CYP3A5, UGT1A1, ABCB1* and *APOE* polymorphism and non-genetic factors with therapeutic outcomes as measured by Thai Mental State Examination (TMSE) scores and steady-state plasma concentrations (Cpss) of donepezil and galantamine.

Hypothesis

1. Genetic polymorphisms of genes that involve metabolic pathways are significantly correlated with steady-state plasma concentrations of donepezil and galantamine in Thai patients with dementia.

2. *CYP2D6*, *CYP3A5*, *UGT1A1*, *ABCB1*, and *APOE* polymorphisms and non-genetic factors are significantly associated with clinical response to donepezil and galantamine treatment in Thai patients with dementia.

Expected benefits from the study

1. To obtain information about correlations between genetic polymorphisms of genes that involve metabolic pathways and steady-state plasma concentration of donepezil and galantamine.

2. To obtain information about the influence of genetic polymorphisms and non-genetic factors on inter-individual variability of clinical response to donepezil and galantamine treatment in Thai patients with dementia.

Keyword:

Donepezil

Galantamine

CYP2D6 polymorphisms

UGT1A1 polymorphisms

Alzheimer's disease (AD)

Vascular dementia (VAD)

Mixed dementia

CHAPTER II LITERATURE REVIEW

Prevalence and incidence of dementia in Thailand

Dementia is a chronic disease which characterized mainly by cognitive functions declined caused by various central neurodegenerative and ischemic process. Dementia affects the quality of life and caregiver burden. With the aging populations, the prevalence of dementia in the elderly is 2-10% and increase 2 times every 5 years after 60 years old.

The estimated dementia patients in Thailand was about 229,100 persons and will increase to 450,200 and 1,233,200 persons in 2020 and 2050 respectively (2). Dementia is a chronic illness that affects the quality of life and caregiver burden. Moreover, family expenses are increase and loss of pharmacoeconomic outcomes.

Consequently, dementia is a serious healthcare problem in Thailand. Alzheimer's disease (AD) is a common neurodegenerative disorder and one of the most common causes of dementia follow by vascular dementia (VAD) (4).

Pathophysiology

Alois Alzheimer described the key pathological hallmark of AD as Aβ deposition and NFT formation in the cerebral cortex (1). Extracellular amyloid plaques and intracellular neurofibrillary tangles are a key hallmark of pathophysiology (17). The results of this neuropathology of AD lead to apoptosis, inflammation and mitochondria dysfunction of neurons. Eventually, it is the cause of degeneration of cholinergic neurons and depletion of the acetylcholine neurotransmitter (17).

Amyloid precursor protein (APP) is encoded by *the APP* gene on chromosome 21. These proteins are characterized by single-pass transmembrane protein composing large extracellular domain. The functions of APP are promoting nerve growth, cell mobilization, and cell survivability (18). Most of APP is cleaved by alpha-secretase and gamma-secretase. Alpha-secretase cleaves APP at Aβ domain then

gamma-secretase hydrolyzes within hydrophobic transmembrane domain liberating p3 and p7 which show non–toxic effect on the synapse (17). This pathway is called non-amyloidogenic pathways. On the contrary, minor amyloid precursor protein is cleaved by beta-secretase to produce a soluble N-terminal fragment of APP (sAPPβ) and follow by gamma-secretase which hydrolyzes within the hydrophobic transmembrane domain to generate Aβ oligomers (17).

Another neuropathology of Alzheimer's disease is neurofibrillary tangles which result from hyperphosphorylation of tau protein. Tau protein is an abundant soluble protein which responsible for maintaining assembly and stability of microtubules and vesicular transport (17).

Phosphorylation of tau proteins is controlled by various kinases such as glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinase 5 (Cdk5), JNK and microtubule-associated regulatory kinase (MARK) (19) and dephosphorylation is regulated by phosphatases. Hyperphosphorylation of tau results from both an imbalance in tau kinase and phosphatase activities and changes in the conformation of tau. These changes lead to insoluble tau protein and reduce its affinity for microtubules, causing it to detach and spontaneously self-associate into paired helical filament structures. These filaments aggregate into NFTs, disturbing and impairing axonal transport and cause neuron death (17).

The consequence from cholinergic neuron cell death leads to atrophy of gyri and larger sulcus especially the hippocampus, temporal lobe, and frontal lobe. Amyloid plaque and neurofibrillary tangle will be accumulated in these regions causing decrease synapse and glucose metabolism. Choline acetyltransferase; ChAT which produces acetylcholine will be diminished. So, the level of acetylcholinesterase is decreased leading to memory and cognitive impairments. Moreover, noradrenergic cell at locus coeruleus will be destroyed (20).

Generally, stroke is a common cause of dementia so risk factors for stroke are also a risk factor for vascular dementia. Several lines of evidence suggest that the major risk factors for vascular dementia are vascular risk factor which includes increasing age, hypertension, coronary artery disease, atrial fibrillation, diabetes, smoking, elevated total cholesterol levels, lower physical activity, low or high BMI. Most of clinical aspect of pathophysiology of vascular dementia involve brain ischemia and loss of vascular integrity with hemorrhage. The scenario of pathophysiology quite complex and share common neuropathological lesion with AD.

Clinical Presentations and Diagnosis

As mentioned above, the neuropathology of AD leads to neuron cell death particularly cholinergic neurons and therefore acetylcholine is diminished. As a result, the clinical presentations of AD are characterized by cognitive function decline especially loss of recent memory, impairment in activities of daily living (ADL), and change in behavior and personality. Moreover, neuropsychiatric symptoms are dominants symptom in AD especially in the middle and late stage of diseases.

There are two most frequently used clinical guidelines for the diagnosis of AD namely National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and the American Psychiatric Association, in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) as shown in table 1 and table 2, respectively.

Table 1 Diagnosis of AD according to NINCDS-ADRDA criteria (21)

NINCDS-ADRDA
Probable AD • Deficits in two or more domain of cognition • The progressive decline of memory and other cognitive functions • Preserved consciousness • Onset between ages 40 and 90 • Absence of systemic or other brain diseases that could account for symptoms Possible AD • Atypical onset, presentation, or clinical course of dementia • Presence of another illness capable of producing dementia Definite AD • Clinical criteria for probable AD • Tissue diagnosis by autopsy or biopsy

Table 2 Diagnosis of AD according to DSM-IV criteria (22)

DSM-IV
• Insidious onset with a progressive decline of cognitive function resulting in impairment of social or occupational functioning from a previously higher level • Impairment of recent memory and at least one of the following cognitive domains: Aphasia Apraxia Agnosia Executive functioning (planning, organizing, sequencing, abstracting) • Cognitive deficits are not due to other neurological, psychiatric, toxic, metabolic, or systemic diseases • Cognitive deficits do not occur solely in the setting of a delirium

The clinical assessment of AD usually uses neuropsychological testing such as Mini-Mental State Examination (MMSE) or Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS cog score). MMSE is the most widely used and studied worldwide because this method is non- invasive, convenient to routine clinical practice and easy for interpretation. However, neuropsychological testing has some limitation namely MMSE is less sensitive in severe AD (23). Another method for diagnosis of AD is cerebrospinal fluid (CSF) biomarker and neuroimaging. CSF biomarkers for AD are amyloid beta-42 ($A\beta_{42}$), total tau (T-tau) and phosphorylated tau (P-tau). Amyloid beta-42 is a molecular biomarker for amyloid deposition which level will decrease in cerebrospinal fluid whereas total tau and phosphorylated tau, the crucial constituent of neurofibrillary tangles will be increased in AD patients. CSF biomarker is sensitive to early diagnosis of AD. Bob Olsson et al. performed systematic review and meta-analysis and concluded that T-tau, P-tau, and $A\beta_{42}$ in CSF and plasma T-tau are strongly associated with AD and should be used in clinical practice and clinical research (24).

In Thailand, TMSE or Thai Mental State Examination score was modified from MMSE for convenient to use for Thai populations.

Magnetic resonance imaging (MRI) is a widespread neuroimaging used to diagnose AD. The image of brain AD patient manifest by reducing hippocampus volume and medial temporal lobe atrophy. Amyloid PET tracers for examples, F18-florbetapir, F18- flutemetamol were developed to help diagnose of AD particularly in the aspect of rate of progression, and early diagnosis. Amyloid PET tracers binding to amyloid plaque was interpreted as positive scan lead to a measure of amyloid lesion burden in the brain (25). Palmqvist et al. compared head to head the accuracy of regional amyloid PET and CSF biomarkers such as $A\beta_{42/40}$, $A\beta_{42}$, total tau (t-tau). They suggested that PET and CSF biomarkers can identify early AD with high accuracy and there is no difference between the best CSF and PET measures (26).

Vascular dementia is characterized by loss of cognitive function which primarily caused by cerebrovascular disease or impaired cerebral blood flow. Vascular dementia (VAD) is the second most common cause of dementia. Key features of VAD characterize by cognitive impairment as well as cardiovascular event. There are two common clinical scenarios of vascular dementia (27).

- A clinically diagnosed stroke is followed by dementia
- Vascular brain injury is identified on brain imaging in patients with cognitive decline but without a clinical history of stroke

The incidence of vascular dementia is approximately 15-50 % of all types of dementia. Vascular dementia is the second most common type of dementia (28). The estimated prevalence of vascular dementia among individuals older than 65 years is 1.6 % and rises with increasing age (29).Moreover, Senanarong et al. concluded that vascular dementia and AD are the most common causes of demetia in Thailand (30).

The diagnosis of vascular dementia should be differentially diagnosed from other causes of acquired cognitive decline including AD, Parkinson disease and other related dementia (PDD), Dementia with Levy bodies, normal pressure hydrocephalus (NPH), Depression (31-33). The evaluation of vascular dementia should use various modality particularly clinical history (characteristic of cognitive declines, risk factors, underlying disease conditions), neuroimaging and clinical features. In general, the Hachinski ischemic score was used to predict the likelihood of a vascular contribution to dementia (34) .

Table 3 Hachinski ischemic score (34)

+ 2 points	+ 1 point
• Abrupt onset • Fluctuating course • History stroke • Focal neurological symptoms • Focal neurologic signs	• Stepwise deterioration • Nocturnal confusion • Preservation of personality • Depression • Somatic complaints • Emotional incontinence (pseudobulbar affect) • Hypertension • Associated atherosclerosis

A score of 7 or greater indicated that a vascular contribution is likely.

The diagnostic criteria for vascular dementia have been offered by several organization such as DSN-IV, the International Society of Vascular Behavioral and Cognitive Disorders (VAS-COG)(35, 36). The diagnostic criteria are conceptually similar including:

- There is the evidence of stroke based on a sign of neurologic examination or neuroimaging.
- There is a clear relationship in the severity pattern of cognitive impairment and the presence of diffuse, subcortical cerebrovascular disease pathology.
- There should be no history of gradually progressive cognitive deficits before or after the stroke that suggest the presence of a non-vascular cognitive disorder e.g. AD.

Treatment

Because cholinergic neuron at nucleus basalis of Meynert is pronouncedly affected in AD, Moreover, cholinergic neuron regulated memory procedures. Therefore, restore and maintenance of cholinergic neuron are the first goal for AD treatment. At present, there were no disease-modifying drugs for dementia treatment. Because cholinergic neuron at nucleus basalis of Meynert is pronouncedly affected in dementia especially in AD. Moreover, cholinergic neuron regulated memory procedures. Therefore, restore and maintain of a cholinergic neuron are the first goal for dementia treatment.

According to several guidelines such as American Geriatric society 2013 (AGS), European Federation of Neurological Societies 2010 (EFNS), acetylcholinesterase inhibitors (AChEIs) are recommended as the first line therapy for the treatment of mild to moderate AD and plus memantine (NMDA receptor partial agonist) for moderate to severe AD. At present, there are three acetylcholinesterase inhibitors including donepezil, rivastigmine, and galantamine. These drugs have a different precise mechanism of action. Namely, donepezil is a selective reversible noncompetitive inhibitor of acetylcholinesterase. Whereas, rivastigmine is a pseudo-irreversible inhibitor of acetylcholinesterase and butyrylcholinesterase. On the contrary, galantamine is not only the reversible inhibitor of AChE but also a presynaptic modulator of nicotinic ACh receptors which can enhance cholinergic activity.

There are limited data for pharmacological treatment of vascular and mixed dementia. It widely studies that cholinergic dysfunction might be playing a role in the neuropathological condition in vascular dementia as well as AD. Consequently, acetylcholinesterase inhibitors can be used in vascular dementia. Moreover, memantine, the N-methyl-D-aspartates receptor antagonist, has been used for VAD treatment also. Although the evidence of memantine for VAD treatment remains low,

memantine is well tolerated, improve function and reduce caregiver burdens when compared with placebo. In addition, non-pharmacological treatment especially the reduce vascular risk factor can prevent the progression of VAD.

Table 4 and table 5 show some pharmacokinetic and pharmacodynamic properties of acetylcholinesterase inhibitors respectively (3, 37).

Table 4 Some pharmacodynamic properties of acetylcholinesterase drugs (3)

Properties	Donepezil	Rivastigmine	Galantamine
Mode of inhibitions	Non-competitive, rapidly reversible	Non-competitive, very slowly reversible	Competitive, rapidly reversible
AChE/BuChE selectivity	300	1	50
Brain vs peripheral selectivity	Yes	Yes	No
Ach isoform selectivity	No	$G_1 > G_4$	No
nAChR modulation	No	No	Yes
Available dosage form	5,10 mg (tab)	1.5, 3, 4.5,6 mg (cap) 4.6, 9.5 mg/24 hrs. (patch)	8,16 mg (ER form)
Recommended starting dose	5 mg once daily	1.5 mg twice daily 4.6 mg/24 hrs.	8 mg once daily
Max does	10 mg	6 mg twice daily	24 mg once daily
Titration period	4-6 weeks	2-4 weeks	4-6 weeks
Adverse effects	Nausea, vomit, diarrhea	Nausea, vomit, diarrhea	Nausea, vomit, diarrhea
Originator	Eisai	Novartis	Jassen-Cilag
Tradename	Aricept®	Exelon®	Reminyl®

Table 5 Some pharmacokinetic properties of acetylcholinesterase drugs (3)

Properties	Donepezil	Rivastigmine	Galantamine
Bioavailability (%)	100	35 (3 mg), 70 (6 mg)	100
Protein binding (%)	93	40	17
$t_{max,ss}$ (hrs)	4 (IR), 6 (SR)	1 (cap), 8 (patch)	1 (IR), 4-5 (ER)
V_d (L/kg)	12 ± 2	1.8 – 2.7	2.64
Metabolism	Hepatic (CYP2D6, CYP3A4, UGT)	Esterase in liver and intestine	Hepatic (CYP2D6, CYP3A4, UGT1A1)
Kinetics	Linear	Non-linear	Linear
$t_{1/2}$ (hrs)	70	1.5-2 (cap), 3.4 (patch)	17
steady state (days)	14 - 21	1	6
total clearance (L/hr)	10 ± 2.5	120	20 ± 5

Pharmacodynamic characteristic of AChEIs

In humans, cholinesterases are found in two types. The first is acetylcholinesterase which is dominant in CNS. The main function of acetylcholinesterase is to hydrolyze acetylcholine into acetate and choline. Acetylcholinesterase has 3 globular isoforms, G1, G2 and G4 containing 1, 2 or 4 catalytic subunits. In the brain, there are 2 isoforms, i.e., tetrameric (G_4) and monomeric (G_1) isoform with various proportions in different brain regions from up to 15 % in the nucleus basalis of Meynert, to less than 5 % in the caudate nucleus (38). The other cholinesterase is butyrylcholinesterase, which is synthesized in the liver and secreted into the plasma. Butyrylcholinesterase is the predominant form in the peripheral systems such as the gastrointestinal tract and the heart. There is 1 to 10% of the total amount of cholinesterase in the adult CNS, where it is present in glial cells. The physiological effects of butyrylcholinesterase are still unclear, but it accounts for detoxifying of certain chemicals, thus limiting the amounts reaching the CNS. In addition, butyrylcholinesterase plays a role in metabolizing various molecules including neuroactive peptides, butyrylcholine, succinylcholine, organophosphate, and cocaine (3, 38).

In the brain of AD patients, the level of G4 membrane-bound form in a presynaptic neuron is decreased in all stages of diseases. Whereas, the level of G1 form in a postsynaptic neuron is relatively preserved. Conversely, butyrylcholinesterase remains unchanged (38). There are three acetylcholinesterase inhibitors including donepezil, rivastigmine, and galantamine. The main pharmacologic effects of acetylcholinesterases inhibitor are inhibition of acetylcholinesterase so the level of acetylcholine will be increased which can enhance cholinergic activities in AD patients.

However, these drugs have a different precise mechanism. Donepezil is a non-competitive and rapidly reversible inhibitor with a more highly specific for

acetylcholinesterase than butyrylcholinesterase about 300 times. Rivastigmine is a pseudo-irreversible inhibitor of acetylcholinesterase and butyrylcholinesterase. For this reason, the activity of acetylcholinesterase and butyrylcholinesterase in the central nervous system is reduced by the same amount. Because of the progression of AD especially in last stages, glia cell can secrete butyrylcholinesterase to destroy acetylcholine so inhibition of butyrylcholinesterase might be useful for the treatment (39). The duration of central acetylcholinesterase and butyrylcholinesterase that are inhibited by rivastigmine is about 8.5 and 3.5 hours respectively. Therefore, the short elimination half-life of rivastigmine (1-2 hours) is unlikely to impact the duration of inhibitory effects. The unique mechanism of rivastigmine is its preferential inhibition of acetylcholinesterase G1 form. JS Kenedy concluded that G1 isoform plays a role in hydrolyzing acetylcholine but G4 isoform is reduced when disease progression (40). In addition, rivastigmine show inhibition effect in some area of the brain due to G1 form selectivity. G1 form is highly expressed in the cortex and the hippocampus that significantly affected AD when compared with the other areas. Beside therapeutic effect, considering in adverse drug events aspects, rivastigmine shows less incidence of muscle cramp and weakness than other acetylcholinesterase inhibitors. This is because rivastigmine exhibits more selective inhibition of G1 form than G4 form which is the predominant form at the motor-end plate. On the contrary, galantamine is not only the reversible inhibitor of acetylcholinesterase but also allosteric modulation of nicotinic ACh receptors which can enhance cholinergic activity.

Due to differences in precise pharmacologic effects such as selectivity of acetylcholinesterase over butyrylcholinesterase or central over peripheral, these properties can affect their efficacy and adverse drug events profiles. Notwithstanding, several meta-analyses did not reach statistically different to distinguish the efficacy of the three AChEIs, but donepezil shows fewer adverse effects than other AChEIs (3).

Pharmacokinetic characteristic of donepezil and galantamine

Donepezil

Donepezil is the most frequently prescribed acetylcholinesterase inhibitors for the treatment of Alzheimer's disease and vascular dementia. Donepezil exhibits linear pharmacokinetic properties. Bioavailability of donepezil is 100 %. Times to peak concentration of donepezil is 4 hours for immediate release formulation and 6 hours for sustained release formulation. Co-administration with food does not change drug absorption. Donepezil bind with total protein 96 % which consist of albumin and α1-acid glycoprotein approximately 75% and 21%, respectively. Elimination half-life of donepezil is about 70 hours suggesting that once-daily dosing is appropriate. Time to reach steady states is within 14 to 21 days.

Donepezil is metabolized by cytochrome P450 (CYP) 3A4 and 2D6. Renal is the primary route to eliminate the parent drug and metabolites. The metabolic pathways of donepezil comprise of 3 major routes (41, 42).

1. O-demethylation to the M1, M2 metabolites and then glucuronide conjugation to M11 and M12 metabolites
2. N-dealkylation to the M4 metabolites
3. N-oxidation to the M6 metabolites

All of donepezil' s metabolites are inactive due to low plasma concentration and difficult to pass the blood-brain barriers except 6-O-desmethyl-donepezil. The 6-O-desmethyl-donepezil or M1 metabolite is the only one active metabolite and shows AChE inhibition comparable to donepezil. M1 represents approximately 20 % of the parents' drugs in human (43). Study in rat revealed that transportation of M1 into the brain is very low. Therefore, this implies that M1 metabolite cannot significantly influence pharmacological activity of the drug.

Because donepezil and its metabolites are mainly excreted via renal, patients who have renal impairments should be expected to adjust the dose. However, CF Nagy et al concluded that no significant difference pharmacokinetic parameters were observed between the healthy control group and subjects with moderate to severe renal impairments when administering 5 mg of donepezil. Therefore, dosage adjustment does not require for AD patients with moderate renal impairments. In contrast with renal impairment, AD patients with impaired liver function (Child-Pugh grade A and B) have to rise in AUC, $t_{1/2}$, C_{max} and steady-state plasma drug concentration (C_{ss}) when compared with healthy control. These data suggested that patients with mild to moderate hepatic impairment administered 5 mg of donepezil are quite safe and well tolerated.

Galantamine

Galantamine is completely absorbed with a bioavailability of 100% as well as donepezil. Peak plasma concentrations (C_{max}) are achieved at 1 and 4-5 hour after ingestion of immediate and prolonged release formulation respectively. Concomitant with food has no significant effect on total amount of drug absorption but slow t_{max} about 1.5 hours and C_{max} decreased by approximately 25%. Galantamine demonstrates linear pharmacokinetic properties with elimination half-life of 6-8 hours indicating that twice daily dose administration is suitable. A steady state of galantamine is reached approximately 6 days after the first dose of ingestion. The drug has quite low plasma protein binding (17%) and the apparent volume of distribution (V_d) is approximately 2.6 L/kg.

Galantamine is mainly eliminated to clinically inactive metabolites through multiple pathways, primarily by O-demethylation by CYP2D6, O-oxidation by CYP3A4 and glucuronidation. The major pathways of galantamine metabolism are primarily metabolized by cytochrome P450 (CYP) 2D6 and 3A4 with O-demethylation and O-oxidation respectively, follows by glucuronidation. Some studies showed that

UGT1A1 plays a role in glucuronidation. There are two major metabolites of galantamine including O-desmethyl galantamine and galantamine glucuronide. Study in vitro suggested that O-desmethyl galantamine was approximately 3 times more potent than galantamine in AChE inhibition. The rest excrete via kidney approximately 30% via the kidney. Metabolic pathway of galantamine quite complex and no dominant single metabolic pathway.

Dosage adjustment is not required in mild hepatic or renal impairment (creatinine clearance ≥9 ml/min). Maximum dosage of 16 mg/day is recommended for patients with moderate hepatic impairment. Galantamine is contraindicated in patients with severe hepatic (Child-Pugh score >9) and/or renal (creatinine clearance <9 ml/min) impairment (9).

Factor affecting clinical response

Although the effectiveness of AChEI is widely established in several studies, the response is difficult to predict because many factors could be responsible for inter-individual treatment. The factor which influenced the clinical response of AChEI could be divided mainly into 2 groups including genetic and non-genetic factors.

Genetic factors

It is widely accepted that pharmacogenetic is the significant factor that influences drug treatment response. Nowadays, several lines of evidence conclude that genetic variation in pathological, drug-metabolizing, drug transporter genes contribute to the inter-individual clinical response of AChEIs. Cacabelos R. et al. summarized pharmacogenetic genes involve clinical response of AChEIs as shown in table 6.

Table 6 Pharmacogenetic genes associated with therapeutic outcome of AD (8)

Drug	Pharmacogenetic gene
Donepezil	**Pathogenic genes**: *APOE*, *CHAT* **Mechanistic genes**: *CHAT*, *ACHE*, *BCHE* **Drug metabolism-related genes**: - Substrate: *CYP2D6* (major), *CYP3A4* (major), *UGTs*, *ACHE* - Inhibitor: *ACHE*, *BCHE* **Transporter gene**: *ABCB1*
Galantamine	**Pathogenic genes**: *APOE*, *APP* **Mechanistic genes**: *ACHE*, *BCHE*, *CHRNA4*, *CHRNA7*, *CHRNB2* **Drug metabolism-related genes**: - Substrate: *CYP2D6* (major), *CYP3A4* (major), *UGT1A1* - Inhibitor: *ACHE*, *BCHE*
Rivastigmine	**Pathogenic genes**: *APOE*, *APP*, *CHAT* **Mechanistic genes**: *ACHE*, *BCHE*, *CHAT*, *CHRNA4*, *CHRNB2* **Drug metabolism-related genes**: -Inhibitor: *ACHE*, *BCHE* **Pleiotropic genes**: *APOE*, *MAPT*

Note:

***ACHE*:** Acetylcholinesterase, ***APP*:** Amyloid precursor protein, ***BCHE*:** Butyrlcholinesterase, ***CHAT*:** Choline acetyltransferase, ***CHRNA4*:** Cholinergic Receptor Nicotinic Alpha 4 Subunit, ***CHRNA7*:** Cholinergic Receptor Nicotinic Alpha 7 Subunit, ***CHRNB2*:** Cholinergic Receptor Nicotinic Beta 2 Subunit, ***MAPT*:** Microtubule-associated protein tau.

Genetic variations in *CYP2D6*

CYP2D6 is encoded by the *CYP2D6* gene which located on chromosome 22. CYP2D6 enzyme consists of 497 amino acids. Estimated number of commercial drugs about 25 % are metabolized by CYP2D6 enzymes such as antidepressants, beta-blockers, dextromethorphan, codeine, tramadol as shown in table 7(44).

Table 7 Substrates of CYP2D6 (44)

Antidepressants	Amitriptyline, clomipramine, desipramine, doxepin, imipramine, nortriptyline, paroxetine, venlafaxine
Antipsychotics	Aripiprazole, chlorpromazine, haloperidol, olanzapine, perphenazine, paroxetine, risperidone, thioridazine
Antiarrhythmics	Flecainide, mexiletine, propafenone
Antiemetics	Dalasetron, ondansetron, tropisetron
Beta- blocker	Alprenolol, bupranolol, carvedilol, metoprolol, propranolol, timolol
Selective serotonin reuptake inhibitors	Citalopram, fluvoxamine, fluoxetine, paroxetine, venlafaxine
Selectives estrogen receptor modulators	Tamoxifen
Opioids	Codeine, dihydrocodeine, hydrocodone, oxycodone, methadone, tramadol
Others	Atomoxetine, dextromethorphan

The synthesis of CYP2D6 enzyme is regulated by *CYP2D6* gene in chromosome 22q13.1. *CYP2D6* gene consists of 9 exons (4,383 base pairs). *CYP2D7* and *CYP2D8P* are considered as inactivate genes and locate nearby *CYP2D6* gene (45).

Types of *CYP2D6* polymorphisms

Polymorphisms of *CYP2D6* can be divided into 2 major characteristics.

1. Single nucleotide polymorphism; SNP

*CYP2D6*1* is a wild type allele of *CYP2D6* gene that expresses normal function enzyme. Substitution with only single nucleotide base may resulting in phenotype changes. For example, substitution of guanine with adenine at 1846 position named *CYP2D6*4* (46) which is the most common allele found in Caucasians. *CYD2D6*4* expresses defective enzymes resulting in poor metabolizer (PM) phenotype. *CYP2D6*10* results from the substitution of cytosine to guanine at 100 position. *CYP2D6*10* is the most common mutant allele found in Asians which leads to the expression of intermediate metabolizer (IM).

2. Copy number variation; CNV, hybrid or tandem gene

Copy number variation of *CYP2D6* gene resulting from gene duplication or multiduplications of *CYP2D6* gene (*CYP2D6*2xn: n = 2-5 and 13) increase enzyme activity and represent ultra-rapid metabolizer (UM).

Hybrid genes, sometimes called chimeric genes, often composed of fragments from gene deletion of *CYP2D7* on their 5 ´-end and *CYP2D6* on their 3 ´-end. Examples of hybrid genes *are CYP2D6* 13, CYP2D6*16, CYP2D6*66, CYP2D6*67, CYP2D6*79* and *CYP2D6*80*.

Moreover, hybrid gene results from tandem arrangement of variant alleles (*CYP2D6*1, CYP2D6*2*) in the same gene or different gene such as *CYP2D6*36+*10* or *CYP2D6*68 +*4* are commonly found in Asians and Caucasians, respectively (47).

CYP2D6 is a highly polymorphic gene found in population. Different alleles result in the extensive, intermediate, poor and ultrarapid metabolizer phenotypes as described in table 8.

Table 8 Correlation between variant alleles of *CYP2D6* gene and predicted phenotypes (44, 46)

Major variant alleles	Nucleotide changes	Consequence	Enzyme activity	Predict Phenotypes
CYP2D6*1	none	Wild type	Normal	Extensive metabolizer
CYP2D6*2	1661G>C; 2850C>T; 4180G>C	Gene duplication/ multiduplication	Normal	Extensive metabolizer
CYP2D6*4	100C>T; 974C>A; 984A>G; 997C>G; 1661G>C; 1846G>A; 4180G>C	Defective splicing	Inactive enzyme	Poor metabolizer
CYP2D6*5	-	Gene deletion	No enzyme	Poor metabolizer
CYP2D6*10	100C>T; 1039C>T; 1661G>C; 4180G>C	P34S, S486T	Unstable enzyme	Intermediate metabolizer
CYP2D6*17	1023C>T; 1661G>C;2850C>T; 4180G>C	T107I, R296C, S486T	Altered affinity for substrates	Intermediate metabolizer

Correlation of *CYP2D6* polymorphisms and predicted phenotypes

People who carry homozygous or compound heterozygous of normal or increased activity alleles such as *1, *2 were deemed extensive metabolizer (EM) whom optimum drug level and therapeutic response will be achieved. Whereas, those who carry homozygous or compound heterozygous of non-functional alleles such as *3-*8 were classified as poor metabolizer (PM) whom often experience exacerbated blood level and ADR comparing to IM or EM (46).

Table 9 Predicted phenotypes and clinical response of drugs (46)

CYP2D6 allele	Predicted genotype	Effect	
		Parent drug	Pro-drug
homozygous or compound heterozygous of nonfunctional alleles (*3-*8, *11-*16, *18-*21, *31, *36, *38, *42, *44, *47, *51, *56, *62)	Poor metabolizer (PM)	Risk of toxicity	Therapeutic failure
Homozygous or compound heterozygous of reduced activity alleles (*9, *10, *17, *29, *41, *49, *50, *54, *55, *59, *72)	Intermediate metabolizer (IM)	Achieve therapeutic effects	
homozygous or compound heterozygous of normal or increased activity alleles (*1, *2, *33, *35,*53)	Extensive metabolizer (EM)	Achieve therapeutic effects	
homozygous or compound heterozygous of duplication/multiduplication of *CYP2D6* normal alleles (e.g.*1×N, *2×N, *33×N, *35×N, 13>N>2)	Ultra-rapid metabolizer (UM)	Therapeutic failure	Risk of toxicity

CYP2D6 polymorphisms in different ethnicities

*CYP2D6*10* is the most common allele found in the Asian population and thus perhaps the most common *CYP2D6* allele in the world. Whereas, *CYP2D6*4* has the highest frequency in Caucasians and *CYP2D6*17* is found commonly in Africans.

Table 10 Alleles frequencies of *CYP2D6* in various ethnicities (48)

Alleles	*CYP2D6* alleles	Caucasians	African Americans	Asians	Thai
Functional	***1**	33–40	28–50	23–42	21-47
	***2**	22–34	11–78	9–20	9.6-10.8
Reduced function	***9**	0–2.9	0	3.3	-
	***10**	1.9–8	3.1–8.6	38–70	44.6-53.0
	***17**	0.1–0.3	9–34	0.5	-
	***41**	8	-	-	6.5
Nonfunctional	***3**	1–3.9	0–0.5	0.8–1	0.9
	***4**	12–23	1.2–7	0–2.8	0.7-1.3
	***5**	1.6–7.3	0.6–6.1	4.5–6.1	4.3-6.7
	***6**	0.7–1	0	-	-
Duplication	***1 × 2**	0.2–0.5	3.3	0.5	-
	***2 × 2**	0.7–1.6	1.6–2.5	0–1	-
	***4 × 2**	0.1–0.2	0.9	—	-

Polymorphisms of *CYP2D6* in Thai populations

In Thailand, Previous studies reported the allele frequencies of *CYP2D6* variants in Thais individuals by using a variety of techniques. The data showed that *CYP2D6*10* allele was the most common allele found in Thai populations shown in table 11.

Table 11 Distribution of CYP2D6 polymorphisms in Thai population(15, 49-51)

Researchers *CYP2D6* alleles	Suwannasri et.al.	Chamnanphon et.al.	Areepium et.al.	Sukasem et.al.
CYP2D6*1	21	35	72.9	24.6
CYP2D6*2	9.7	9.6	3.2	10.8
CYP2D6*4	0.7	0.9	1.1	1.3
CYP2D6*5	4.3	4.4	-	6.7
CYP2D6*10	44.6	45.6	22.8	49.6
CYP2D6*14	1.04	0.9 (*CYP 2D6*14B*)	-	0.1 (*CYP 2D6*14B*)
CYP2D6*35	-	0.9	-	0.1
CYP2D6*36	16.4	0.9	-	0
CYP2D6*39		-	-	0.2
CYP2D6*41	-	1.8	-	6.5
Functional gene duplication	0.35	-	-	0
Allele coverage	98.09	-	-	100

Genetic variations in *CYP3A5*

CYP3A5 are encoded by *CYP3A5* gene which located on chromosome 22.(52) The *CYP3A5* is a member of *CYP3As* gene. The *CYP3A* composes of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 genes (53, 54). Moreover, there is pseudogene nearby *CYP3As* gene including *CYP3AP1*, *CYP3AP2*, and *CYP3AP3*. The four genes located in the order of *CYP3A43-CYP3A4-CYP3A7-CYP3A5* (55).

In human liver cell, CYP3A4 is the most abundant of drug metabolizing enzyme which plays an important role in drug metabolism process. Contrary to CYP3A4, the CYP3A5 expression is dominant in extrahepatic tissue, especially in the

alimentary canal (54). In general, genetic variation of CYP3A4 might be influenced by interindividual variation in drug level or drug response, especially in Caucasian. In contrast to Caucasians, CYP3A4 is not polymorphic in Thai populations. Consequently, only CYP3A4 may not greatly describe the interindividual variation of drug response because its genetic variants are uncommon and have limited effect on enzyme function. These implied that genetic variation of CYP3A5 responsible for the interindividual variability (56).

Genetic variation of *CYP3A5* is higher compared to *CYP3A4*. There is a large interindividual variation in hepatic and extrahepatic CYP3A5 expression than CYP3A4. In contrast to other CYPs of which the **1* allele is usually common allele, *CYP3A5*3* is the most common defective allele (53) with an allele frequency of about 92%, 73% and 21% in Caucasians, Asians, and Africans, respectively(53). *CYP3A5*3* occurs by splicing variants of intron 3 (g.6986G>A). An allele (*CYP3A5*1*; wild type) encodes a normal splice CYP3A5 whereas, the variants G allele (*CYP3A5*3*) produce stop codon and ultimately exhibit premature termination of translation in CYP3A5 synthesis. Homozygous of *CYP3A5*3* is not express activity of CYP3A5 enzyme (57). The CYP3A5 allele in associate with predicted phenotype is described in table 12.

Table 12 Functionality of CYP3A5 alleles and its related predicted enzyme activity (58)

CYP3A5 alleles	Predicted genotype	Example of diplotypes
homozygous or compound heterozygous of nonfunctional alleles (*3, *6, *7, *8)	Poor metabolizer (PM) (*CYP3A5* non-expresser)	*3/*3, *6/*6, *7/*7, *3/*6, *3/*7, *6/*7
compound heterozygous of functional and non-functional alleles	Intermediate metabolizer (IM) (*CYP3A5* expresser)	*1/*3, *1/*6, *1/*7
homozygous of functional alleles (*1)	Extensive metabolizer (EM) (*CYP3A5* expresser)	*1/*1

CYP3A5 polymorphism in different ethnicities

Previous reported the allele frequencies of *CYP3A5* variants in Thais individuals by using various of techniques. The data showed that *CYP3A5*3* allele was the most common allele as shown in table 13.

Table 13 Alleles frequencies of *CYP3A5* polymorphisms in various ethnicities (59-65)

***CYP3A5* alleles** **Ethnicity**	***CYP3A5*1***	***CYP3A5*3***
Asians		
Thai	38.3	61.7
Chinese	24.0	76.0
Malaysian	39.0	61.0
Japanese	23.0	77.0
Korea	20.0	80.0
Caucasians		
Polish	3.5	96.5
Dutch	8.5	91.5
Bosnia	6.8	93.2
Brazillian	21.0	79.0

Genetic variations in *UGT1A1*

The Uridine diphosphate (UDP)-glucuronosyltransferase or UGT has a crucial role in phase II metabolism, especially in glucuronidation reaction. UGT enzymes are categorized into two subfamilies namely UGT1A and UGT2B. UGT1A1 is encoded from *UGT1* family polypeptide A1, which is located on chromosome 2q37 (66).

The *UGT1A1*1* [$A(TA)_6TAA$] is considered as a wild-type allele which contains six TA repeats in the TATA box of the promoter region. The length of this TA repeat sequence is inversely correlated with the activity of the UGT1A1 enzyme (66). *UGTA1A1*28* [$A(TA)_7TAA$] is the most common mutant allele that comprises of seven TA repeats. *UGT1A1*28* encodes defective enzyme with 25–80% enzymatic activity comparing to the normal allele (66, 67). The allele frequency of *UGT1A1*28* is 33.4–

36.5% in the Caucasian population and 39.0–40.4% in Africans. Whereas, in Asians, the allele frequency is much lower (13.9%) (68). *UGT1A1*6* is the most frequent allele found in Asians (13.0%). The *UGT1A1*6* (211G>A, G71R), encodes enzyme with 50% less activity than the wild-type allele(69). In Thailand, Sukasem et al. developed pyrosequencing techniques to determine *UGT1A1* polymorphism in Thai colorectal cancers. The results showed that allele frequencies of *UGT1A1*1*, *UGT1A1*6*, and *UGT1A1*28* were 74 %, 9 %, and 17% respectively (67). The correlation of variant alleles and phenotypes are shown in table 14.

The decline in the UGT1A1 activity of *UGT1A1*28* variant approximately 25 and 70% depending on the presence of one or two *UGT1A1*28* variant allele respectively (66). At least variant alleles have been reported in UGT Most of them are non-synonymous SNP which resulting in reduced enzyme activity.

Table 14 *UGT1A1* allele naming conventions, locations, and associated phenotypes(69)

Allele	Variant	Location	Enzyme activity	Associated phenotype
*UGT1A1*1*	$(TA)_6TAA$	Promotor	Normal	Wild type
*UGT1A1*28* (rs8175347)	$(TA)_7TAA$	Promotor	Reduced	Gilbert syndrome
*UGT1A1*36*	$(TA)_5TAA$	Promotor	Increased	-
*UGT1A1*37*	$(TA)_8TAA$	Promotor	Reduced	Crigler-Najjar, type II
*UGT1A1*6* (rs4148323)	c.211 211G>A, G71R	Exon1	Reduced	Gilbert syndrome
*UGT1A1*27* (rs35350960)	g. 686 C>A	Exon1	Reduced	Gilbert syndrome

Genetic variations in *ABCB1*

ABCB1 gene (ATP-binding cassette, subfamily B, member 1 also called MDR1; Multidrug resistance 1) encodes P-glycoprotein (P-gp) which located on chromosome 7q21.12. P-gp located on endothelial cell lining BBB, small intestine, liver, and kidney(70). The previous study concludes that *ABCB1* is highly polymorphic. Common three polymorphisms, 1236C>T, 2677G>T/A, and 3435C>T have been studied extensively with respect to their effects on P-gp function and clinical relevance. *ABCB1* 3435C>T is the most common silent SNP in exon 26, associated with a lower expression and function of the protein in human(71) whereas, *ABCB1* 1236C>T SNP, a silent polymorphism occurs in exon 12. *ABCB1* 2677G>T/A is a tri-allelic polymorphism in exon 21. Both variant alleles (A or T) result in an amino acid change, Ala893Thr, and Ala893Ser, respectively, which alter expression and activity of P-gp(72).

In the brain, P-gp is localized on endothelial cell of BBB and brain parenchyma, might attribute to pathogenesis of AD especially clearance of Aβ. Moreover, P-gp plays a role in efflux drug transport pump transporting various drug from the brain back into the blood compartment(8). This implied that genetic variation of *ABCB1* associated with increased donepezil level in CNS due to a decrease efflux of the drug from the central nervous system to the blood compartment (72). The distribution of *ABCB1* 1236 C>T and *ABCB1* 3435 C>T allele frequency are shown in table 15.

Table 15 Alleles frequencies of *ABCB1* polymorphisms in various ethnicities (73, 74)

Ethnicity / **Alleles**	**Caucasian**	**African**	**Asian**	**Thai**
ABCB1 1236 C>T	38.0-45.9	15.0-21.0	43.7-67.2	64.0
ABCB1 3435 C>T	47.0-56.6	10.0-27.0	34.7-63.2	42.6-47.7

Genetic variation in *APOE*

Apolipoprotein E (APOE) is encoded by *APOE* gene which located on chromosome 19q13.32. The structural of apolipoprotein E has two domains. The first domain is the N- terminal domains which responsible for binding with APOE receptor (residues 136-150). The other domain is C-terminal which involves the binding of lipids (residues 244- 272) (75). Apolipoprotein E composes of 299 amino acids and expresses in the liver, the brain, macrophage, and monocyte (76). Apolipoprotein E has three isoforms due to the difference of the amino acid at 112 and 158 positions.

Apolipoprotein E is responsible for transporting cholesterol from the blood to LDL receptor in the liver. In the brain, apolipoprotein is synthesized by glia cell. APOE **ε**2 and APOE **ε**3 facilitate recycling of cholesterol for cell repairment and nerve growth(16).

APOE **ε**4 plays a crucial role in the formation of amyloid plaque and neurofibrillary tangles. It is widely believed that APOE play an important role in Aβ clearance. Moreover, APOE can promote inflammation and apoptosis in neuron(16).

APOE is the important genetic factors that have been elucidated in the late onset of AD. Pharmacogenetic of AD have dominantly involved pharmacodynamic gene especially APOE. *APOE* is the most extensively and consistency studied candidate pharmacogenetic gene in AD treatment. Two of five studies have associate better donepezil response (3).

Several research using both *in vitro* (cell culture) and in vivo (transgenic animal model) methods have explored precise mechanism about APOE **ε**4 that can advocate AD. Most of the studies conclude that APOE **ε**4 play a role in promoting neurofibrillary tangle(77) and amyloid plaque aggregation or reducing amyloid clearance (78).

Mutation in chromosome 19 resulted in increasing production of APOE **ε**4 when comparing with normal chromosome. Hence *APOE **ε**4* increase the risk of AD. Corder EH et al. suggested that homozygous *APOE **ε**4* was associated with the highest frequency of AD (79).

Table 16 Effects of *APOE* ***ε4*** on AD frequency and mean age at onset (16)

***APOE ε4* alleles** **Characteristics**	***APOE ε4*** **non-carrier**	***APOE ε4*** **Heterozygous**	***APOE ε4*** **Homozygous**
AD frequency (%)	20	47	91
Mean age at onset	84	76	68

Several studies have reported the association between apolipoprotein E and the risk of AD. Most of these studies concluded that *APOE* ***ε4*** alleles increase the risk of AD in a gene-dose dependent manner (80). Heterozygous carriers (*APOE* ***ε3ε4***) and homozygous carriers *(APOE* ***ε4ε4***) increase the risk of AD about 3 and 15 folds respectively. Meanwhile, *APOE* ***ε2*** allele may protect from AD and delays the age of onset.(76) Moreover, Mengying Liu et al. performed a meta-analysis and revealed that *APOE* ***ε3*** allele might have a protective effect (81).These results were in concordance with the study of Ping Wu (82).

In Thailand, Senanarong et al. showed that 59.4 percent of AD patients were *APOE* ***ε4*** carriers (positive predictive value of 0.60) and suggested that *APOE* ***ε4*** allele increases the risk of developing dementia. Detection of *APOE* polymorphism may be an adjunct diagnostic for Alzheimer's disease (83). Notwithstanding, there is no report about the association of apolipoprotein E polymorphism and clinical response of donepezil and galantamine in Thailand. Association studies between polymorphism *APOE* and clinical response of donepezil and galantamine are summarized in table 19 and 20, respectively.

APOE polymorphism in different ethnicities

APOE genotypes among the population in the world show in table 17. *APOE*3* is the highest allele frequency in the world and A*POE*2* is lowest in all populations.

Table 17 Alleles frequencies of *APOE* in various ethnicities (84)

***APOE* alleles** / **Ethnicity**	***APOE ε2***	***APOE ε3***	***APOE ε4***
Africa	0.099±0.083	0.690±0.110	0.209±0.090
Europe	0.077±0.033	0.790±0.056	0.127±0.049
Asia	0.063±0.030	0.847±0.054	0.090±0.043
North America	0.049±0.041	0.824±0.060	0.127±0.057
South America	0.046±0.069	0.767±0.129	0.187±0.132
Oceania	0.111±0.052	0.667±0.162	0.221±0.149
India	0.051±0.017	0.881±0.039	0.068±0.030
All populations	0.073±0.047	0.790±0.088	0.133±0.074

Polymorphism of _APOE_ in Thai populations

Polymorphisms of *APOE* in Thai populations are summarized in table 18

Table 18 Allele frequencies of *APOE* polymorphisms in Thai populations(83, 85-87)

Researcher / **Allele**	**Kamruecha (AD)**	**Senanarong (AD)**	**Pulkes et al. (PD)**	**Chaudhary et al. (DM)**
ε2	0.0	3.0	11.0	1.6
ε3	66.7	80.0	79.0	85.8
ε4	33.3	17.0	10.0	12.6
Total	**100**	**100**	**100**	**100**

Table 19 Association study between pharmacogenetic gene and therapeutic outcomes of donepezil

Researcher/Years	Genetic variants	Ethnicity / Number of patients	Main results
		CYP2D6	
Federica Varsaldi et.al. 2006(12)	*CYP2D6*1, CYP2D6*2x2 CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6*	42 Italian	No statistically significant in plasma concentrations between homozygous EM and heterozygous EM. Heterozygous EM showed a better clinical response when compared with homozygous EM.
Albert Pilotto et.al. 2009(11)	rs1080985 C>G	127 Italian	A significantly higher frequency of patients with the G allele of rs1080985 was found in non-responders than in responders.
Davide Seripa et.al. 2011(88)	16 CYP2D6 functional polymorphisms	57 Italian	A significantly higher frequency of gene variants conferring decreased or absent enzyme activity was observed in responder than in non-responder patients.
Diego Albani et.al. 2012(89)	rs1080985	415 Italian	Significant association between rs1080985 and response to donepezil after 6 months of therapy was observed.

Aleksandra Klimkowicz Mrowiec et.al. 2013(13)	rs1080985 C>G	116 Polish	No association was found between *CYP2D6* rs1080985 SNP and clinical response.
Yuan Zhong et.al. 2013(90)	*CYP2D6*10*	110 Chinese	*CYP2D6*10* carriers may respond better to donepezil when compared with wild allele.
Mengyuan Liu et.al. 2014(14)	rs1080985 C>G	206 Chinese	No significant differences between responders and non-responders to donepezil treatment were observed in the distribution of the *CYP2D6* rs1080985 SNP.
Nirmal Sonali et.al. 2014(91)	*CYP2D6*2*, *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*10*, *CYP2D6*17*	55 Indians	*CYP2D6* polymorphism though not significantly might partially be involved in the plasma concentration of AD drug
Muriel Noetzli et.al. 2014(92)	*CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*	129 Swiss	Significantly decreased and increased CL in CYP2D6 PMs and UMs compared with EMs, respectively.
Jin Lu et.al. 2015(6)	*CYP2D6*10*	77 Han Chinese	Significant association between plasma concentrations of S-donepezil (based on CYP2D6 polymorphisms) and therapeutic responses were found.
Jin Lu et.al.	*CYP2D6*10*	85 Chinese	*CYP2D6*10/*10* showed the best therapeutic

2016(93)			response when compared with other genotypes.
Caterina Chianella 2011(94)	*CYP2D6*1*, *CYP2D6*2*, *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, *CYP2D6*9*, *CYP2D6*10*, *CYP2D6*41*	92 Italian	No significant association was found between CYP2D6 genotype and clinical response.
		CYP3A	
Laura Magliulo et.al. 2011(72)	*CYP3A4*1B, CYP3A4*3, CYP3A4*4 CYP3A5*2, CYP3A5*3, CYP3A5*6*	54 Italian	No association was found between *CYP3A4* or *CYP3A5* polymorphisms and plasma concentration or clinical response.
Muriel Noetzli et.al. 2014(92)	*CYP3A4*1B, CYP3A4*22, CYP3A4* rs4646437 C>T	129 Swiss	The population pharmacokinetic model demonstrated no statistically significantly different in CL was observed between *CYP3A4* or *CYP3A5* genotypes
		ABCB1	
Laura Magliulo et.al. 2011(72)	*ABCB1* 3435C>T, *ABCB1* 1236C>T,	54 Italian	The haplotype 1236T/2677T/3435T showed a tendency towards a better clinical response and

	ABCB1 2677G>T		lower plasma concentration.
		APOE	
Greenberg et al. 2000(95)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	60 American	No significant association of *APOE* genotype and clinical response
Winblad et al. 2001(96)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	286 Caucasian	No significant association of *APOE* genotype and clinical response
Rigaud et al. 2002(97)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	117 French	No significant association of *APOE* genotype and clinical response
Bizarro et al. 2005(98)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	81 Italian	Better efficacy of donepezil was shown in AD patients carrying at least one *APOE* ***ε****4* allele when assessed by MMSE scores
Kanaya et al. 2010(99)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	40 Japanese	*APOE* ***ε****4* groups showed worse clinical response in the third year when evaluated by ADAS-cog score.
Aleksandra Klimkowicz Mrowiec et.al. 2013(13)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	116 Polish	No significant association of *APOE* genotype and clinical response
Yuan Zhong et.al.	*APOE* ***ε****2, APOE* ***ε****3,*	110 Chinese	No significant association of *APOE* genotype and

2013(90)	*APOE* ***ε****4*		clinical response
Mengyuan Liu et.al. 2014(14)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	206 Chinese	No significant association of APOE genotype and clinical response

Table 20 Association study between pharmacogenetic gene and therapeutic outcomes of galantamine

Researcher/Years	Genetic variants	Ethnicity / Number of patients	Main results
		CYP2D6	
Vladimir Piotrovsky et al. 2003(100)	CYP2D6 phenotypes	356 Caucasian	Clearances (CLs) in CYP2D6 PM were found to be lower than EM in a population pharmacokinetic model.
Caterina Chianella 2011(94)	*CYP2D6*1*, *CYP2D6*2*, *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, *CYP2D6*9*, *CYP2D6*10*, *CYP2D6*41*	92 Italian	No significant association was found between CYP2D6 genotype and clinical response.
Muriel Noetzli et.al. 2014(101)	*CYP2D6*3, CYP2D6*4, CYP2D6*41* *CYP3A4*22* *CYP3A5*3* *POR*28* *ABCB1* 3435C>T, *ABCB1* 2677G>T	27 Swiss	Poor metabolizer (PM) was associated with higher plasma concentration compared with extensive metabolizer (EM). No significant association was found between *CYP3A4* rs4646437, *CYP3A4*22*, *CYP3A5*3*, *POR*28*, *ABCB1* 3435C>T, and 2677G>T polymorphisms and dose-

			adjusted galantamine concentrations
		APOE	
Raskind Murray A et. al., 2000(102)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	363 American	No significant association of *APOE* genotype and clinical response
Aerssens Jeroen et al., 2001(103)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	853 Caucasians	No significant association of *APOE* genotype and clinical response
Guk-Hee Suh et al., 2006(104)	*APOE* ***ε****2, APOE* ***ε****3, APOE* ***ε****4*	202 Korean	No significant association of *APOE* genotype and clinical response

Non-genetic factor

1. Age

It is widely accepted that aging might influence the physiological process in the elderly including reducing liver and renal blood flow, declines in liver volume. These physiological processes are predisposed affected pharmacokinetic profile of drugs especially decreases the clearance of drug (105, 106). Consequently, increased age might increase steady state plasma concentration of drugs.

Regarding clinical response, Wattmo et al. perform three-year, nonrandomized, prospective, multicenter study in 843 AD patients who were treated with acetylcholinesterase inhibitors (107). The results suggested that AD patients with older age had better response compared with younger age.

2. Gender

Innate biological and physiological between male and female can contribute to difference pharmacokinetic and pharmacodynamic of drug. The pharmacokinetic processes that affected by gender are distribution and drug elimination process. The difference in volume of distribution between male and female was observed. It is possible that difference in fluid component and body weight among male and female could be attributed to the difference in volume of distribution. In addition, the drug elimination including hepatic metabolism and renal clearance are associated with gender.

Gender has been reported to influence AD susceptibility or treatment. Scacchi et al. observed that female seemed to be more sensitive to acetylcholinesterase inhibitors therapy. Moreover, female have more cognitive score than male when evaluated by MMSE (108). On the contrary, Wattmo et al. reported that male AD patients have better response than female (107). Due to inconsistent results, effect of gender on clinical response of acetylcholinesterase inhibitors are inconclusive. The

studies have explored the influence of gender on response of acetylcholinesterase inhibitor.

3. Drug interaction

Because donepezil and galantamine are metabolized by CYP2D6 and CYP3A4. CYP2D6 or CYP3A4 inhibitors and inducers as shown in table 21 can alter plasma concentration and ultimately affect clinical response. Previous report concludes that co-administration of ketoconazole, a strong CYP3A4 inhibitors, with donepezil showed a significant increased steady-state plasma concentration of donepezil approximately 23-30%. Whereas, plasma concentration of ketoconazole remains stable (109). In case of galantamine, the bioavailability of galantamine is increased about 40, 30 and 10 % when administered with paroxetine (CYP2D6 inhibitor), ketoconazole (CYP3A4 inhibitor) and erythromycin (CYP3A4 inhibitor), respectively (3).

Table 21 Inhibitors of *CYP2D6* and *CYP3A4* enzymes (44)

Enzyme	Strong Inhibitors	Moderate Inhibitors	Weak inhibitors
CYP2D6	bupropion, fluoxetine, paroxetine, quinidine, terbinafine	cinacalcet, cimetidine, duloxetine, fluvoxamine, mirabegron	amiodarone, abiraterone, celecoxib, cimetidine, clobazam, cobicistat, desvenlafaxine, diltiazem, diphenhydramine, Echinacea, escitalopram, febuxostat,

			gefitinib, hydralazine, hydroxychloroquine imatinib, labetalol, locaserin, methadone, oral contraceptives, propafenone, ranitidine, ritonavir, sertraline, telithromycin, verapamil, vemurafenib
CYP3A4	boceprevir, clarithromycin, conivaptan, grapefruit juice, indinavir, itraconazole, ketoconazole, lopinavir/ritonavir, mibefradil, nefazodone, nelfinavir, posaconazole, ritonavir,	amprenavir, aprepitant, atazanavir, ciprofloxacin, darunavir/ritonavir, diltiazem, erythromycin, fluconazole, fosamprenavir, grapefruit juice, imatinib, verapamil	alprazolam, amiodarone, amlodipine, atorvastatin, bicalutamide, cilostazol, cimetidine, cyclosporine, fluoxetine, fluvoxamine, ginkgo, goldenseal, isoniazid,

	saquinavir, telaprevir, telithromycin, voriconazole		nilotinib, oral contraceptives, ranitidine, ranolazine, tipranavir/ritonavir, zileuton

Table 22 Inducers of *CYP2D6* and *CYP3A4* enzymes (44)

Enzyme	**Strong Inducers**	**Moderate Inducers**	**Weak Inducers**
CYP2D6	Not known	Not known	Not known
CYP3A4	avasimibe carbamazepine, phenytoin, rifampin, St. John's wort	bosentan, efavirenz, etravirine, modafinil, nafcillin	amprenavir, aprepitant, armodafinil, echinacea, pioglitazone, prednisone, rufinamide

Considering in pharmacodynamic drug interaction aspects, anticholinergic drug that block muscarinic (M_1) receptor has been reported to disturb cognitive function(110) and counteract the effect of acetylcholinesterase inhibitors.

4. Education levels

Non-genetic factors investigated by several studies is education level. Miranda Lu´ıs F.J.R. and other investigators observed that additional year in the level of

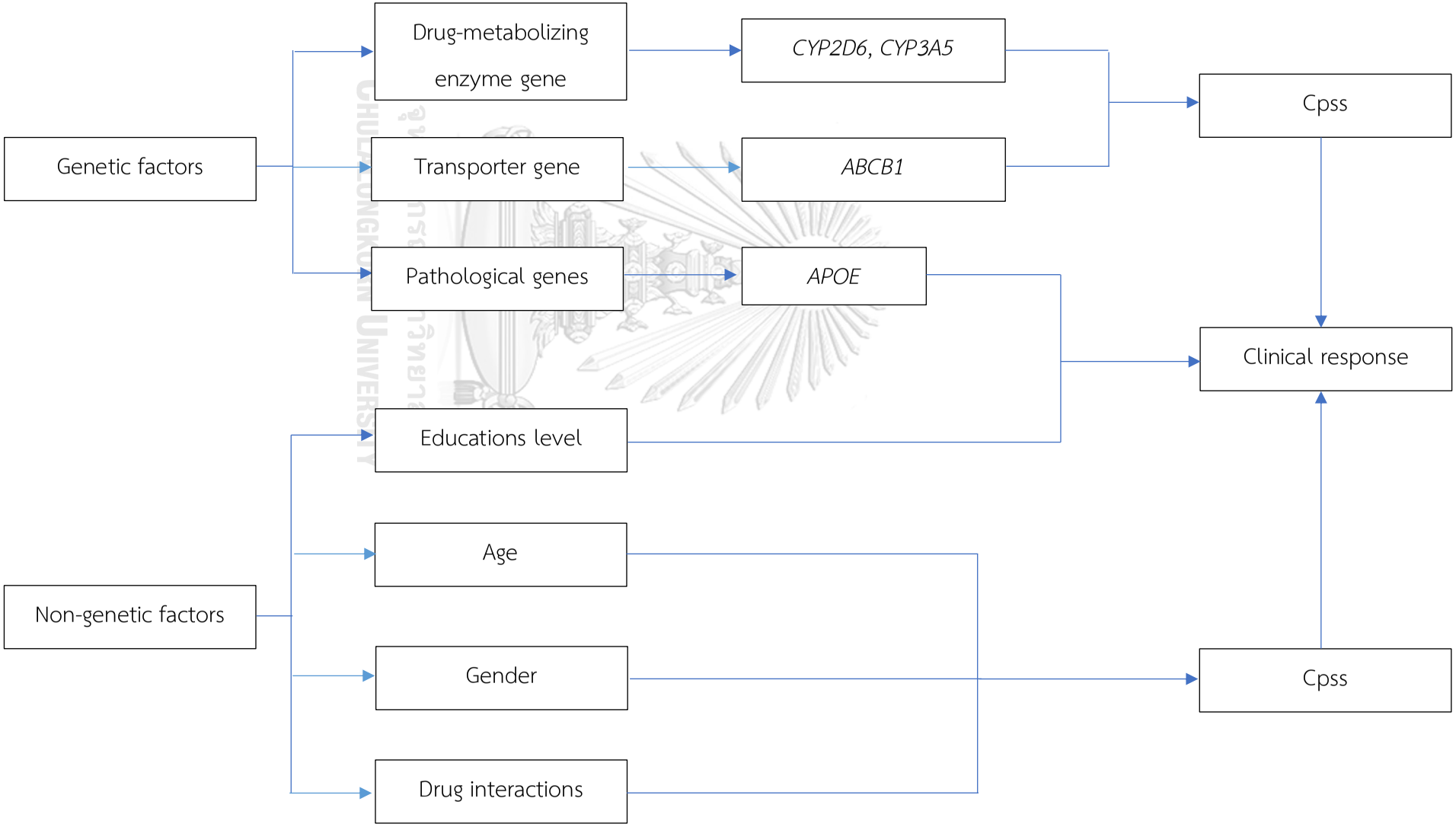
education was associated with worse clinical response (1.14-fold per year). A similar observation was found that AD patients with high education had the worst response to acetylcholinesterase inhibitors (111). Moreover, Wattmo et al. observed that patients with 15 years of education exhibited an average of additional 2.2 points of MMSE and 3.0 points of ADAS-cog deterioration after three years compared with an individual with 9 years of education levels (107).

CHAPTER 3 METHODOLOGY

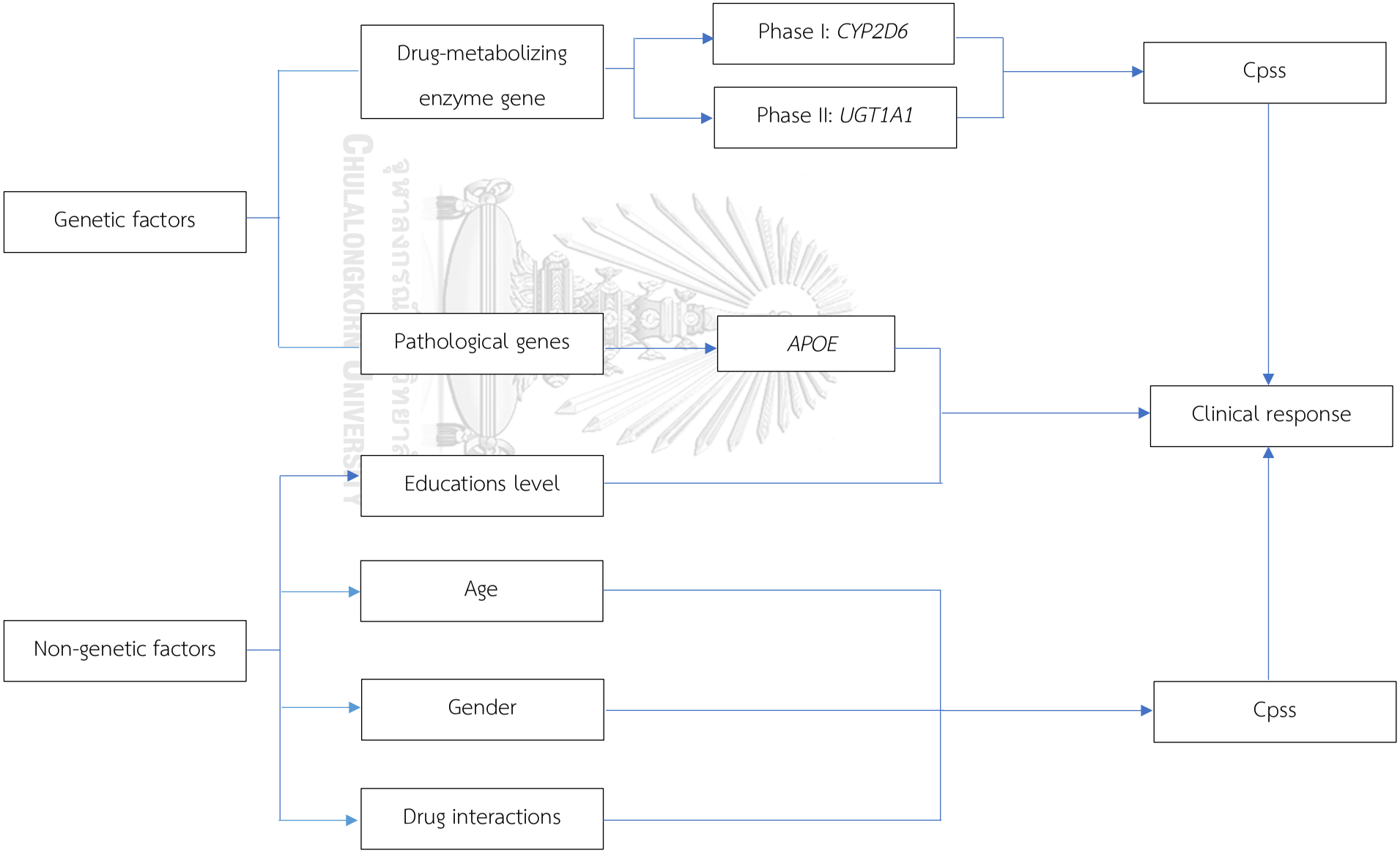
Conceptual framework

As mentioned in literature review, both genetic factor and non-genetic factor, as well as established document and previous clinical studies, guided the conceptual framework of this study. The conceptual framework that might describe interindividual clinical response and Cpss of donepezil and galantamine were illustrated as follow.

Conceptual framework: Donepezil study



Conceptual framework: Galantamine study



Research Methodology

Patients and Protocols

The study was conducted according to the Declaration of Helsinki 1975 and was approved by the Institutional Review Board of the Faculty of Medicine, Siriraj Hospital, Mahidol University (EC: 818/2016). Written informed consents were obtained from participants, from direct relative or legal representative in the case of critical illness and demented patients.

This study was performed as retrospective cohort for donepezil study and prospective cohort for galantamine study. Participants in this study were enrolled from the Neurology outpatient unit in the Department of Internal Medicine, Faculty of Medicine Siriraj Hospital, Mahidol university. The inclusion criteria and exclusion criteria are described in table 23.

Table 23 Inclusion and exclusion criteria

Inclusion criteria
1. Thai patients with AD, VAD, and Mixed AD with CVD who met National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association Work Group (NINCDS-ADRDA) criteria for Alzheimer's disease or NINDS – AIREN criteria for vascular dementia or other criteria as appropriate and first diagnose to Dementia 2. Not early-onset AD and Familial Alzheimer's Disease (FAD) 3. Receive oral donepezil or galantamine daily for the first times and not receive rivastigmine.
Exclusion criteria
1. Patients with Frontotemporal dementia, Dementia with Lewy body 2. Patients who have psychiatric disease i.e. schizophrenia, depression and other neurological disorders such as Parkinson's disease, seizure, and stroke which unstable symptoms 3. Patients or care-givers refusal and reluctant 4. Non- compliance or unable to take donepezil or galantamine for longer than 4 weeks due to side effects or any problems

Non-compliance was defined as being unable to take donepezil or galantamine due to side effects, irregular administration, out of drug supply before the next visit, and loss of drug supply.

The diagnosis was based on Diagnostic and Statistical Manual Mental Disorders-IV (DSM-IV) criteria. Differential diagnosis among AD, VAD, and mixed dementia using Hachiski-Score and neuroimaging evidence which was judged by the neurologist.

Data collection and cognitive evaluation

Participants who met all inclusion criteria were assessed by the same protocols including physical examination, structural interview, and laboratory

screening. Data were obtained from medical records of hospital. All patients' information was recorded in case-record form.

Case-record form contains 4 parts namely demographic data, medical history, treatments data, and genetic data.

Cognitive function was evaluated using TMSE scores which ranged from 0-30 points. This score is positive score, a higher score indicated better cognitive function. Cognitive score was evaluated by psychologist and clinical research nurse using TMSE at baseline every three to six after treatment. These results were reported comparing discrepancy between before and after treatment.

Any adverse drug events that occur will also be recorded. Concomitant drugs data from patients who took concomitant drug for at least three months were collected.

Baseline clinical characteristic and demographic data were obtained from an electronic medical recorded and structural interview.

Definition of responsiveness

According to the NICE (National Institute for Health and Clinical Excellence) criteria, a responder to AChEI treatment was defined as a patient who showed improvement or no deterioration in cognition, as evaluated by means of TMSE score(112).

However, some research defined responder as patients who gets donepezil or galantamine and MMSE increased, remain stable, or the delta MMSE ≥2. Whereas, patients who worsening of more than 3 points in delta MMSE were classified as non-responder (NR) in order to boost statistical power of the study(112).

Sample size determination

In this study, we estimated the number of samples sized which was calculated from univariate analysis. However, due to limited time, resource, and

budget it was not possible for using the number of sample size that was calculated from the univariate analysis.

The numbers of patients were calculated from the rule of thumb suggested for multivariate regression analysis i.e. the number of the less common of the two possible outcomes ("events") divided by the number of predictor variables should be at least 10, and preferably greater (in general 10 to 20) (113).

The number of independent variables considered to be studied in this study was five for donepezil study *(CYP2D6, CYP3A5, ABCB1*, *APOE* genes, and non-genetic factor) and four for galantamine study *(CYP2D6, ABCB1, UGT1A1, APOE* genes, and non-genetic factor).

The probability of poor response will be estimated from previous study of Yuan Zhong et al. who showed that the number of non-responders is 41.7 percent. Therefore, the probability of poor response of donepezil is 0.417. The estimated number of non-responders (less common occurs when compare with responder group) divided by the number of predictor variable should be 10-20, so the number of non-responders is calculated as following this equation:

number of non-responders = 10 x 5 = 50; for donepezil

number of non-responders = 10 x 5 = 50; for galantamine

Because the probability of poor response is 0.417, so

the number of participants 50/0.417 = 120 persons for donepezil

However, in galantamine study, no previous genetic association study was found. Consequently, the number of participants is approximately 50 persons.

Since the expected number of drop out patients is about 5 %, so the total sample size will be 126 and 53 persons for donepezil and galantamine respectively.

Materials and Instruments

Materials

1. DNA extraction
• Gentra Puregene Blood Kit (Qiagen®, Germany) • DNAse free water (AppliChem, Germany) • 70 %, 100 % Ethanol (QRëC, New Zealand)
2. Determination of gene polymorphism by TaqMan® assay
• Universal PCR Master Mix (QIAGEN, U.S.A.) • TaqMan® SNP Genotyping Assays Kit (Applied Biosystems, U.S.A.) ***CYP2D6*** *CYP2D6*2, CYP2D6*10* ***CYP3A5*** *CYP3A5*3* ***ABCB1*** *ABCB1 3435, ABCB1 1236* • DNAse free water (AppliChem, Germany)
3. Determination of *APOE* polymorphism by RFLP techniques.
• 10x buffer • 25 mM magnesium chloride • 10 mM dNTP • DMSO • E1 primer: 5 ' GCA CGG CTG TCC AAG GAG CTG CAG GC 3 ' • E2 primer: 5 ' GGC GCT CGC GGA TGG CGC TGA G 3 ' • Distilled water • Buffer • BSA (Bovine serum albumin) • Restrictive enzyme *Hhal*
4. Determination of UGT polymorphism by direct Sanger sequencing
• 10x buffer • 25 mM magnesium chloride • 10 mM dNTP

- dNTP
- Primer
- Taq Immolase
- 5x buffer
- ExoSAP
- 3M sodium acetate
- 125 mM EDTA
- Milli-Q water
- Absolute ethanol
- 70% Ethanol
- MegaBACE® loading buffer
- BigDye version 3.1

5. Determination of plasma concentration of blood level

- Acetonitrile, HPLC grade (Merck, Darmstadt, Germany)
- Methanol, HPLC grade (Merck, Darmstadt, Germany)
- Trifluoroacetic acid (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany)
- Ammonia solution, Analytical Reagent grade (Merck, Darmstadt, Germany)
- Acetic acid, Analytical Reagent grade (Merck, Darmstadt, Germany)
- Milli Q water Water Purification System (Thermo Scientific, Massachusetts, USA)
- Donepezil hydrochloride monohydrate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) Lot number: 035M4715V, % Assay: 98%, Expiry date: 03/2021
- Diphenhydramine hydrochloride (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) Lot number: 029K8718V, % Assay: 98%, Expiry date: 10/2018
- Drug-Free Human Plasma (Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand)

Instruments

1. Blood samplings
• EDTA tube (Greiner Bio-One, Thailand) • Heparinized tube (Greiner Bio-One, Thailand) • Needle No 22,24,26 • Disposable syringe • Plaster, cotton wool
2. DNA extraction
• Centrifuge (Hettich, Germany) • Refrigerated Centrifuge (Hermle Labortechnik, Germany) • Vortex mixer (Labnet International Inc., USA) • Micropipette (Gilson, USA) • Pipette tips • Centrifugation tube 15 ml (Corning, Mexico) • Microcentrifuge tube 1.5 ml (Hycon, China) • Nanodrop TM 1000 Spectrophotometer (Thermo Scientific, USA)
3. Determination of gene polymorphism by TaqMan® assay
• 9700 Thermal Cycler (Applied Biosystems, USA) • MicroAmp Optical 96-well reaction plate (Applied Biosystems, USA) • MicroAmp Optical Adhesive Film kit (Applied Biosystems, USA) • Applied Biosystems 7500 Real time PCR System; ABI7500
4. Determination of *APOE* polymorphism by RFLP techniques
• Mastercycler PCR machine (Eppendorf, Germany) • gel electrophoresis instruments • UV transilluminator (Gel Doc instruments)
5. Determination of *UGT* polymorphism by direct Sanger sequencing
• Centrifuge (Hettich, Germany) • Refrigerated Centrifuge (Hermle Labortechnik, Germany)

- Vortex mixer (Labnet International Inc., USA)
- Micropipette (Gilson, USA)
- Pipette tips
- Centrifugation tube 15 ml (Corning, Mexico)
- Microcentrifuge tube 1.5 ml (Hycon, China)
- MicroAmp Optical 96-well reaction plate (Applied Biosystems, USA)
- MicroAmp Optical Adhesive Film kit (Applied Biosystems, USA)
- Mastercycler PCR machine (Eppendorf, Germany)
- ABI 3100 automated sequencer

Determination of plasma concentration of blood level

Apparatus	Specification	Manufacturer
Auto pipette	20-200µL, 100-1,000 µL, 500-5,000 µL	Gilson, USA
Micro-centrifuge	1.5 mL clear	Extra gene, California
Volumetric flask	Class A, 5, 10, 20, 25, 50,100 mL	Pyrex Kimble etc., USA Witeg Germany
Vortex	Vortex Genie 2, G5605	Scientific Industries, USA
Centrifuge	Mikro 120	Hettich, USA
Analytical Balance	Libror AEG 320	Shimadzu, Japan
pH meter	Eutech pH 700	Thermo Scientific, USA
Water purification	Barnstead Easy Pure II	Thermo Scientific, USA
Column	ACQUITY UPLC® BEH HSS T3 column (1.8µm, 100 mm x 2.1 mm I.D.)	Waters, USA.
Freezer -20°C	Model 995	Thermo Electron Corporation, USA
Ultra Performance Liquid Chromatography with Photo Diode Array and Data Management System Binary Solvent Manager: Acquity™ Ultra Performance LC, Waters, Co., Ltd. USA. S/N: A10UPB422M Sample Manager: Acquity™ Ultra Performance LC, Waters, Co., Ltd. USA. S/N: A10UPA899M Column Manager: Acquity™ Ultra Performance LC, Waters, Co., Ltd. USA. S/N: D09UPC0100 Photo Diode Array Detector: Acquity™ Ultra Performance LC, Waters, Co., Ltd. USA. S/N: J09UPL101A Data Management system: Empower 2, Waters, Co., Ltd. USA. running on Windows xp on a PC (Dell) S/N: J09UPL101A		

Procedure

Blood samplings

Before starting the study, all participants or caregivers must give written informed consent. Venous blood sample will be collected for 15 milliliters (mL) from all patients by clinical research nurse. Ten milliliters of blood samples will be kept in EDTA tube for genotyping procedure and 5 milliliters will be kept in heparinized tube for determining blood level.

DNA extraction

Genomic DNA will be extracted from whole blood by using Gentra Puregene Blood Kit (QIAGEN®, Germany) and kept at -80 ℃ until genotyping.

DNA extraction procedure: Gentra Puregene Blood Kit

1. Dispense 9 ml RBC Lysis Solution into 15 ml centrifuge tube.

2. Add 3 ml whole blood and mix by inverting 10 times.

3. Incubate 5 minutes at room temperature (15–25°C). Invert at least once during the incubation.

4. Centrifuge for 2 minutes at 2000 g to pellet the white blood cells.

5. Carefully discard the supernatant by pipetting or pouring, leaving approximately 200 µl of the residual liquid and the white blood cell pellet.

6. Vortex the tube vigorously to resuspend the pellet in the residual liquid. Vortexing greatly facilitates cell lysis in the next step. The pellet should be completely dispersed after vortexing.

7. Add 3 ml, Cell Lysis Solution, and pipet up and down to lyse the cells or vortex vigorously for 10 s. Usually no incubation is required; however, if cell clumps are visible, incubate at 37°C until the solution is homogeneous. Samples are stable in Cell Lysis Solution for at least 2 years at room temperature.

8. Add 1 ml Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.

9. Centrifuge for 5 minutes at 2000 x g the precipitated proteins should form a tight, dark brown pellet. If the protein pellet is not tight, incubate on ice for 5 minutes and repeat the centrifugation.

10. Pipet 3 ml isopropanol into a clean 15 ml tube and add the supernatant from the previous step by pouring carefully. Be sure the protein pellet is not dislodged during pouring.

11. Mix by inverting gently 50 times until the DNA is visible as threads or a clump.

12. Centrifuge for 3 minutes at 2000 x g The DNA may be visible as a small white pellet.

13. Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.

14. Add 3 ml of 70% ethanol and invert several times to wash the DNA pellet.

15. Centrifuge for 1 minute at 2000 x g.

16. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Air dry the pellet for 5–10 minutes. The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

17. Add 300 µl DNA Hydration Solution and vortex for 5 s at medium speed to mix.

18. Incubate at 65°C for 1 h to dissolve the DNA.

19. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube. Purify DNA must check DNA concentration and purity using Nanodrop as describes in table 24.

Table 24 Interpretation of purifying DNA using Nanodrop

Absorbance 260/280	Results
1.8-2.0	DNA is normal
A ratio lower than 1.8	Presence of proteins and/or other UV absorbers
A ratio higher than 2.0	The samples may be contaminated with chloroform or phenol

Determination of gene polymorphism by TaqMan® assay

Genetic polymorphisms *CYP2D6, CYP3A5* and *ABCB1* were detected by using TaqMan genotyping assay kits. Assay was performed in 96-well plate on a ViiA7 real-time PCR instrument (Applied Biosystems 7500 Real time PCR System; ABI 7500 CA USA) according to the manufacturer's instruction. The process for detecting gene polymorphism by TaqMan® SNP Genotyping Assays Kit using Applied Biosystems 7500 Real time PCR System; ABI 7500 is described as following:

1. Dilute DNA with DNAse free water to get final concentration of 5 ng/µL.
2. Prepare master mixture solution for polymerase chain reaction (PCR) as shown in table 25.

Table 25 Constituent of master mixture solution for TaqMan® assay

Constituent reaction	Volume (µL)
2X Taqman® Genotyping Master Mix	5.0
20X Taqman® SNP Genotyping Assay (Primer-Probe)	0.5
DNAase free water	2.5
DNA sample (5 ng/µL)	2
Total	**10**

3. Pipet 8 µL of master mixture which specific for each SNP into 96-well reaction plate and add DNA sample 2 µL.
4. Spin down at 800 g for 10 seconds by spin down centrifuge.
5. Run PCR with real-time PCR by ViiA™ 7 Real-Time PCR system as condition listed in table 26.

Table 26 Condition in real-time PCR by ViiA™ 7 Real-Time PCR system

Time and Temperature		
Initial Steps	**Denaturation**	**Annealing/Extension**
HOLD	50 Cycles	
10 minutes 95 °C	15 seconds 92 °C	90 seconds 60 °C

6. When reactions are complete, *CYP2D6* genotype will be analyzed by using ViiA™ 7 software version 1.2.4 (Applied Biosystem).

Determination of *APOE* polymorphism by RFLP techniques

Genetic polymorphism of *APOE* determined by Restriction Fragment Length Polymorphism (RFLP) techniques. The procedure of PCR-RFLP techniques was list below.

1. Multiple DNA by PCR techniques for 12 minutes at 95 °C. Polymerase chain reaction (PCR) was performed using a Mastercycler PCR machine (Eppendorf, Germany).

Table 27 Constituent of PCR process for RFLP techniques

Constituent reaction	**Volume (µL)**
10xbuffer	2.5
25 mM $MgCl_2$	1.25
10 mM dNTP	0.5
DMSO	2.5
Primer E1	0.5
Primer E2	0.5
Distilled water	16.05
DNA sample	1
Total	**25**

E1 primer 5 ' GCA CGG CTG TCC AAG GAG CTG CAG GC 3 '

E2 primer 5 ' GGC GCT CGC GGA TGG CGC TGA G 3 '

2. Add Taq DNA polymerase 0.2 µL immediately and run PCR for 110 minutes to activate complete reactions.
3. Restriction DNA by restrictive enzyme *HhaL* and incubate overnight. The components of reaction are listed in table 28

Table 28 Constituent for RFLP techniques

Constituent reaction	**Volume (µL)**
Buffer C	2.0
BSA	0.2
Enzyme *HhaI*	1.0
Distilled water	8.8
PCR product	8.0
Total	**20**

4. Run electrophoresis using 8 % polyacrylamide gel.
5. The DNA fragment will be visualized by UV trans-illuminator (Gel Doc instruments) and *APOE* genotype will be interpreted as shown in table 29.

Table 29 Interpretation of APOE polymorphism from RFLP techniques

DNA fragment (bp)						
	91	91	91	91	91	-
	83	83	83	-	-	-
	-	-	72	-	72	72
	-	48	48	48	48	48
	-	35	35	35	35	35
***APOE* genotype**	*ε2/ε2*	*ε2/ε3*	*ε2/ε4*	*ε3/ε3*	*ε3/ε4*	*ε4/ε4*

Determination of *UGT1A1* polymorphism by direct Sanger sequencing techniques

UGT1A1 genotype (*UGT1A1*6*, *UGT1A1*28*) determined by direct Sanger sequencing techniques. The procedure of Sanger sequencing techniques was list below

1. Multiple DNA by PCR techniques using a Mastercycler PCR machine (Eppendorf, Germany)

Table 30 Constituent of PCR process for detection UGT1A1 genotype

Constituent reaction	**Volume (µL)**
50 mM $MgCl_2$	0.75
dNTP	0.5
Forward or Reverse Primer	1
dd.H_2O	17.125
Taq Immolase	0.125
DNA sample	2
Total	**20**

Table 31 Primers for detection UGT1A1 genotype using direct Sanger sequencing

Variants allele	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
*UGT1A1*6*	GTAGGAGAGGGCGAACCTCT	CTCAGAATGCCTGCTCAGC
*UGT1A1*28*	ATCTCTGAAAGTGAACTCCCTGCTAC	CCTGGGACTCCACAGCCATG

2. Run PCR for 110 minutes to activate complete reactions. PCR was implemented by pre-denaturation at 95°C for 7 minutes, followed by 35

thermal cycles composed of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec for each.

Table 32 Condition in PCR process for detection *UGT1A1* genotype

Time and Temperature			
Pre-denaturation	**Denaturation**	**Annealing**	**Extension**
HOLD	35 Cycles		
10 minutes 95 °C	30 seconds 94 °C	30 seconds 60 °C for *UGT1A1*28* 57 °C for *UGT1A1*6*	45 seconds 72 °C

3. The PCR amplified products were isolated by electrophoresis on a 1% agarose gel and stained with ethidium bromide and visualized under ultraviolet light.
4. Prepare 0.5 µL of PCR amplified products for purifying the sequencing reactions with ethanol/EDTA precipitation as following step
   4.1 Add 14 µL of precipitation solution (2 µL of 125 mM EDTA, 2 µL of 3M sodium acetate and 10 µL of Milli-Q water)
   4.2 Add 55 µL of absolute ethanol
   4.3 Vortex mix for 10 seconds and centrifuge at 13,000 rpm for 15 minutes
   4.4 Discharge supernatant by inverse spin
   4.5 Add 150 µL of 70% ethanol then vortex mix and centrifuge at 13,000 rpm for 15 minutes
   4.6 Discharge supernatant by inverse spin
   4.7 Allow the plate to air dry, face up and protected from light, for 5 to 10 minutes at room temperature

4.8 Add 10 µL of MegaBACE® loading buffer and incubate at room temperature for 5 minutes or keep at - 20 °C overnight for complete dissolve pellet.

4.9 Vortex mix for 10 seconds and load to 96-well plate

5. The nucleotide sequence was determined by direct sequencing using Big Dye Terminator v3.1 Kit (Applied Biosystems, Foster City, USA) on an ABI 3100 automated sequencer according to the manufacturer's instructions (Applied Biosystem, Foster City, CA, USA).

Determination of Cpss

The venous blood sample 5 milliliters will be obtained from each patient and plasma samples will be stored at -20°C before analysis in heparinized tube.

Donepezil

The steady state plasma concentration of donepezil was determined by using reversed-phase Ultra Performance Liquid Chromatography with Photo Diode Array (UPLC-PDA) detection with a minor modification (114). Diphenhydramine was used as an internal standard (115). Method validation had been performed according to US FDA guidance for bioanalytical method validation. The lower limit of quantification (LLOQ) was 10 ng/mL. Average recovery of drug (%) was in a range of 85.14 - 85.57%. QC intra-day precision ranged from 1.22% to 3.90% while inter-day precision range was set at 1.59 - 3.69%.

Samples were prepared by Solid-Phase Extraction (SPE) (OASIS®) and HLB: Hydrophilic-Lipophilic-Balanced reversed-phase sorbent (Waters Corporation, Milford, MA, USA). A 20-µL diphenhydramine solution with a concentration of 10,000 ng/mL was added into 1 mL of the Quality Control Sample (QCs) and Standard Spiked Sample. The mixture’s pH was adjusted with 200 µL orthophosphoric acid. Each 1000-µL sample was loaded in SPE which was pre-conditioned by methanol and equilibrated by deionized water (Milli Q Water). A 1-mL solution of 2% ammonia

solution in 5% methanol and a 1-mL solution of 2% ammonia in 20% methanol were used for washing the samples. The samples were eluted with 500 µL of 2% acetic acid in methanol. The samples were diluted with 200 µL of 0.05% TFA. Each 10-µL final sample solution was injected into the UPLC-PDA which validated parameters and conditions as shown in table 33 and 34.

Table 33 Validated parameters for measuring C_{pss} of donepezil by UPLC- PDA techniques

Parameters	Condition
Extraction type	Solid Phase Extraction (SPE) OASIS® HLB: Hydrophilic-Lipophilic-Balanced reversed-phase sorbent 30 mg 1 mL
Biological Matrix	Drug-Free Human Plasma
Detection method	Photo Diode Array at 230 nm
Column type	ACQUITY UPLC® BEH HSS T3 (1.8µm, 100 mm x 2.1 mm I.D.)
Mobile phase	Gradient program Acetonitrile: 0.05% Trifluoroacetic acid (TFA) (32:68 at 0, 35:65 at 1-2.5 min. and 32:68 at 3 min)
Flow rate	0.48 mL/min
Lower Limit of Quantification	10 ng/mL
Linearity range	10 – 250 ng/mL
Equation type	Y = aX + b, with 1/X weighting
Validated low quality control sample (LQC)	30 ng/mL
Validated medium quality control sample (MQC)	120 ng/mL
Validated high quality control sample (HQC)	220 ng/mL
Auto-sampler stability	10 hours
Freeze-and-thaw stability	3 cycles
Short-term stability	4 hours
Long-term stability	180 Days
Stock stability	90 Days

Table 34 Method validation for measuring Cpss of donepezil by UPLC- PDA techniques

Information from method validation	**Data**
Analyte	Donepezil
Internal standard (IS)	Diphenhydramine
Method description	The plasma was separated and the concentrations of Donepezil acid were determined by using a validated Ultra Performance liquid chromatography with Photodiode Array (UPLC-PDA) method
QC concentrations (µg/mL) for Validation method	30, 120 and 220 ng/mL
Selectivity	No interfering peaks noted in blank plasma samples
Lower Limit of quantitation (µg/mL)	10 ng/mL
Standard curve concentrations (µg/mL)	10 – 250 ng/mL
QC Intraday precision range (%)	Day1: 1.22 – 3.61 Day2: 1.31 – 2.41 Day3: 1.59 – 3.90
QC Intraday accuracy range (%)	Day1: 95.63 – 106.47 Day2: 96.63 – 104.26 Day2: 91.38 – 103.60
QC Interday precision range (%)	1.59- 3.69
QC Interday accuracy range (%)	97.99 – 100.03
Average recovery of drug (%)	85.14 (Low concentration; 30 ng/mL) 84.56 (Middle concentration; 120 ng/mL) 85.57 (High concentration; 220 ng/mL)

Average recovery of IS (%)	74.96 (IS concentration 200 ng/mL)
Freeze and thaw stability (cycles)	3 cycles (1.07%, 4.85% and 7.29% for low middle and high concentration of Donepezil, respectively)
Long-term storage stability (days)	90 days at -20 degree Celsius low middle and high concentration of Donepezil, respectively)
Short-term stability (hrs)	4 hrs at room temperature 25 degree Celsius (0.31%, 5.48% and 2.75% for low middle and high concentration of Donepezil, respectively
Auto sampler or Post-preparative stability (hrs)	10 hrs in Autosampler 8 degree Celsius (2.38%, 2.33% and 1.36% for low middle and high concentration of Donepezil, respectively)
Stock Stability (days)	90 days at -20 degree Celsius (% for Donepezil and-% for Diphenhydramine)

Galantamine

The steady state plasma concentration of galantamine was determined by using reversed-phase Ultra Performance Liquid Chromatography with Photo Diode Array (UPLC-PDA) detection with a minor modification (114). Voriconazole was used as an internal standard (115). Method validation had been performed according to US FDA guidance for bio-analytical method validation (116). The lower limit of quantification (LLOQ) was 10 ng/mL. Average recovery of drug (%) was in a range of 80.03 - 86.88%. QC intra-day precision ranged from 1.00% to 8.15% while inter-day precision range was set at 1.23 – 6.59 %.

Samples were prepared by Solid-Phase Extraction (SPE) (OASIS®) and MCX: Mixed-mode, strong Cation-eXchange reversed-phase sorbent (Waters Corporation, Milford, MA, USA). A 20-µL voriconazole solution with a concentration of 3,000 ng/mL was added into 1 mL of the Quality Control Sample (QCs) and Standard Spiked Sample. The mixture's pH was adjusted with 200 µL orthophosphoric acid. Each 800-µL sample was loaded in SPE which was pre-conditioned by methanol and equilibrated by de-ionized water (Milli Q Water). A 1-mL solution of 2% acetic acid in Milli-Q and a 1-mL solution of 2% ammonia in 20% methanol were used for washing the samples. The samples were eluted with 500 µL of 2% ammonia in methanol. The samples were diluted with 300 µL of Acetonitrile: NH4OAc pH 9, 20:80. Each 10-µL final sample solution was injected into the UPLC-PDA which validated parameters and conditions as shown in table 35 and 36.

Table 35 Validated parameters for measuring C_{pss} of galantamine by UPLC- PDA techniques

Parameter	Condition
Extraction type	Solid Phase Extraction (SPE) OASIS® MCX: Mixed-mode, strong Cation-eXchange reversed-phase sorbent 30 mg 1 mL
Biological Matrix	Drug-Free Human Plasma
Detection method	Photo Diode Array at 289 nm
Column type	ACQUITY UPLC® BEH HSS T3 (1.8µm, 100 mm x 2.1 mm I.D.)
Mobile phase	Gradient program Acetonitrile: Ammonium acetate pH 9 (25:75 at 0, 50:50 at 3-4 min. and 25:75 at 5 min)
Flow rate	0.45 mL/min
Lower Limit of Quantification	10 ng/mL
Linearity range	10 – 250 ng/mL
Equation type	Y = aX + b, with 1/X weighting
Validated low quality control sample (LQC)	30 ng/mL
Validated medium quality control sample (MQC)	120 ng/mL
Validated high quality control sample (HQC)	220 ng/mL
Auto-sampler stability	10 hours
Freeze-and-thaw stability	3 cycles
Short-term stability	4 hours
Long-term stability	180 Days

Table 36 Method validation for measuring Cpss of galantamine by UPLC- PDA techniques

Information from method validation	Data
Analyte	Galantamine
Internal standard (IS)	Voriconazole
Method description	The plasma was separated and the concentrations of Galantamine acid were determined by using a validated Ultra Performance liquid chromatography with Photodiode Array (UPLC-PDA) method
QC concentrations (µg/mL) for Validation method	30, 120 and 220 ng/mL
Selectivity	No interfering peaks noted in blank plasma samples
Lower Limit of quantitation (µg/mL)	10 ng/mL
Standard curve concentrations (µg/mL)	10 – 250 ng/mL
QC Intraday precision range (%)	Day1: 1.00 – 3.34 Day2: 0.85 – 7.63 Day3: 1.17 – 8.15
QC Intraday accuracy range (%)	Day1: 98.88 – 104.27 Day2: 98.05 – 104.37 Day2: 98.54 – 108.19
QC Interday precision range (%)	1.23 - 6.59
QC Interday accuracy range (%)	98.95 – 105.61
Average recovery of drug (%)	86.88 (Low concentration; 30 ng/mL) 86.65 (Middle concentration; 120 ng/mL) 80.03 (High concentration; 220 ng/mL)

Average recovery of IS (%)	76.19 (IS concentration 3,000 ng/mL)
Freeze and thaw stability (cycles)	3 cycles (3.33%, -0.42% and 0.16% for low middle and high concentration of Galantamine, respectively)
Long-term storage stability (days)	90 days at -20 degree Celsius (0.55%,0.30% and -1.83% for low middle and high concentration of Galantamine, respectively)
Short-term stability (hrs)	4 hrs at room temperature 25 degree Celsius (-0.18%, -1.57% and -0.81% for low middle and high concentration of Galantamine, respectively
Auto sampler or Post-preparative stability (hrs)	10 hrs in Autosampler 8 degree Celsius (1.92%, -0.30% and 0.99% for low middle and high concentration of Galantamine, respectively)

Statistical analysis

Baseline demographic and clinical characteristic will be presented as percentage, mean ± SD, and median ± IQR

All genotype and allelic frequency were calculated in percentage. Chi-square is used to test the deviation from Hardy-Weinberg equilibrium.

Normality test of data was performed using Komorgov Smirnov test (N>50).

Univariate analysis was performed to evaluate the association of several common genetic polymorphisms (*CYP2D6*, *CYP3A5*, *UGT1A1*, *ABCB1*, and *APOE*) and non-genetic factor on Cpss or TMSE scores. Student 's t-test and ANOVA (analysis of variance) were performed for parametric continuous data. Mann Whitney U test or Kruskal-Walis test were performed for skewed continuous data.

Multiple comparisons (Post-hoc analysis) was performed by using Scheffer's method.

Correlation’s between adjusted Cpss or TMSE score and continuous variables were tested using Pearson’s correlation coefficients.

For multivariate and logistic regression analysis, this study performed enter and stepwise procedure to select all variable which appeared to be associated with dependent variables by setting significant level for entry (SLE) at *p*-value of 0.25 or lower and was introduced into each multivariate model.

Stepwise multiple linear regression was performed to evaluate the combined association of Cpss or TMSE scores with genetic and non-genetic factors.

If the dependent variable did not pass the assumptions for multiple linear regression analysis, the dependents variable was transformed using appropriate arithmetic function.

Correlation's analysis was tested to examine collinearity. If independent variable that shows correlation coefficient (r) > 0.75 on correlation matrix were excluded from further analysis.

Multicollinearity analysis was performed by using Variance Inflation Factor (VIF) < 10 and Tolerance > 0.1

Residual statistic including histogram and normal P-P plot of regression standardized residual were performed to evaluate deviation from model assumption and identify influential observations.

Differences of genetic polymorphisms between responder and non – responder group will be determined by Chi-square test.

The unstandardized regression coefficients (B) was used in the regression model to estimate the dependent variable, whereas, the standardized regression coefficients (β) was used to compare the strength of association of each covariate.
The determination coefficient (R^2) was presented to indicate the percentage of the variance for dependent variable which was explained by independent variables.

For galantamine study, the prediction of genetic polymorphisms and non- genetic factors with clinical response were determined using multiple logistic regression analysis.

The Hosmer-Lemeshow test was used to examine the fitting and goodness-of-fit from logistic regression model

All data were analyzed using IBM Statistic Package for Social Science (SPSS) statistical software package version 22.

All tests were two-sided. *p*-value less than 0.05 was considered statistically significant.

CHAPTER 4 RESULTS AND DISCUSSION: DONEPEZIL

Association of genetic and non-genetic factors with clinical responses of donepezil in Thai patients with dementia

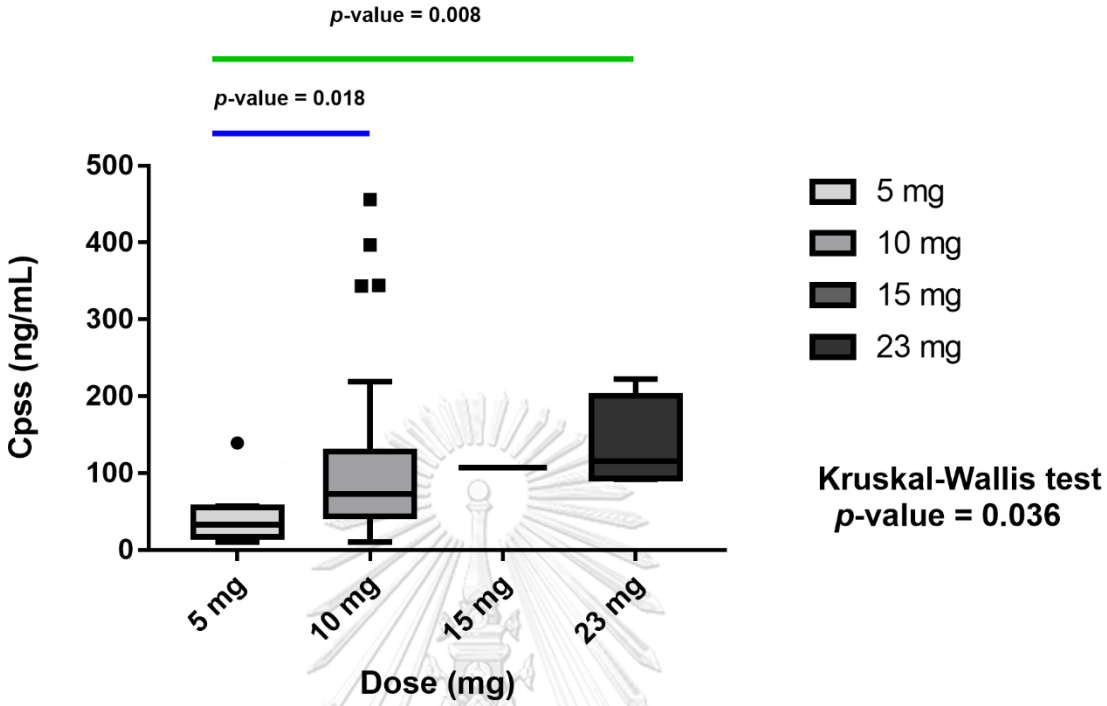
RESULTS

Demographic and clinical characteristic

Among the 105 participants, seven patients were excluded from analysis for the following reasons: irregular administered drug (n=4), poor compliance (n=2) and discontinuation of drug due to adverse drug event (n=1). Eight patients received 5 mg/day of donepezil, 85 patients received 10 mg/day, 1 patient received 15 mg/day, and 4 patients received 23 mg/day.

The results showed that Cpss of donepezil was directly proportional to administered dose, the Cpss levels of 5, 10, 15, and 23 mg donepezil were 44.54, 98.15, 106.86 and 136.37 ng/mL, respectively. Cpss levels corresponding to the four doses were significantly different (*p*-value = 0.036) as show in figure 1.

Figure 1 Association between doses and Cpss of donepezil



Notes:

Each pairwise comparison was calculated from Kruskal-Wallis test.

Each boxplot shows the median as the central line, the extremes of each box are the first and third quartile and the whiskers represent the minimum and maximum values in the sample.

Circles and squares on the top of each boxplot represent outliers.

Because of strong linear association between doses and Cpss was observed, the following studies were used only the data from patients who took 10-mg maintenance dose which were taken by the majority patients, to reduce the effect of doses on Cpss and therapeutic response. Thus, the final analysis included the 85 participants who met eligible criteria. Baseline demographic and clinical characteristics of 85 patients were shown in table 37.

Table 37 Baseline demographic and clinical characteristics of 85 Thai patients with dementia

Demographic and clinical characteristics	Number (%)	Mean ± SD
Age (years)	-	78.42±7.91
Age of onset (years)	-	72.34±8.54
Gender: Male **Female**	38 (44.70) 47 (55.30)	- -
Body weight (Kg)	-	56.69±9.88
Serum creatinine (mg/dL)	-	1.21±1.02
Creatinine clearance (mL/min)	-	60.04±19.71
Years of educations	-	8.56±5.48
Types of dementia: ● **Alzheimer's disease** ● **Vascular dementia** ● **Alzheimer's disease dementia of frontal lobe type** ● **Dementia with Lewy body**	 51 (60.00) 32 (37.64) 1 (1.18) 1 (1.18)	 - - - -
TMSE score at baseline	-	20.01±6.03
TMSE score at steady state	-	18.87±6.92
TMSE score change (ΔTMSE)	-	-0.81±3.09

Of 85 patients who met the eligible criteria, there were slightly more women than men (table 37). The average age was 78.42 years, and the majority of participants were in 75 years or older. The majority were diagnosed with AD (60.00%), followed by VAD (37.64%). Alzheimer's disease dementia of frontal lobe type and dementia with Lewy body were found in negligible proportions. Their initial or baseline TMSE score was 20.01±6.03 points by average. The average years of educations were 8.56±5.48 years.

Genotype distribution

Table 38 Genotype distribution and allele frequencies of the polymorphisms in candidate genes of the study patients

Allele	Allele frequency	Genotype	Number	Genotype frequency	HWE *p*-value	MAF in other Asian populations
ABCB1 c.3435 C>T (rs 1045642)						
C	0.583	CC	32	0.381	0.125	Chinese: 0.40 Japanese: 0.48 (T)
T	0.417	CT	34	0.405		
		TT	18	0.214		
ABCB1 c.1236C>T (rs 1128503)						
C	0.418	CC	16	0.188	0.60	Chinese: 0.34 Japanese: 0.32 (C)
T	0.582	CT	39	0.459		
		TT	30	0.353		
CYP2D6*2 (rs 1135840, g.4180G>C)						
G	0.712	GG (*-/*-)	47	0.553	0.03	Chinese: 0.21 Japanese: 0.41 (C)
C	0.288	GC (*2/*-)	27	0.318		
		CC (*2/*2)	11	0.130		
CYP2D6*10 (rs 1065852, g.100G>A)						
G	0.418	GG (*-/*-)	20	0.235	0.021	Chinese: 0.33 Japanese: 0.50 (G)
A	0.582	AG (*10/*-)	31	0.365		
		AA (*10/*10)	34	0.400		
CYP3A5*3 (rs 776746, g.6986T>C)						
C	0.671	TT (*-/*-)	15	0.176	0.004	Chinese: 0.37 Japanese: 0.26 (T)
T	0.329	CT (*3/*-)	26	0.306		
		CC (*3/*3)	44	0.518		
APOE (rs429358, rs7412)						
APOE ***ε2***	0.055	*APOE* ***ε2/ ε2***	0	0.000	-	Chinese: 0.076(117) Japanese: 0.078(118) (*APOE* ***ε2***)
APOE ***ε3***	0.640	*APOE* ***ε2/ ε3***	7	0.098		
APOE ***ε4***	0.305	*APOE* ***ε2/ ε4***	2	0.019		
		APOE ***ε3/ ε3***	34	0.412		

		APOE ***ε****3/* ***ε****4*	30	0.373		
		APOE ***ε****4/* ***ε****4*	9	0.098		

Note: All MAF data were from Applied Biosystems® except *APOE*.

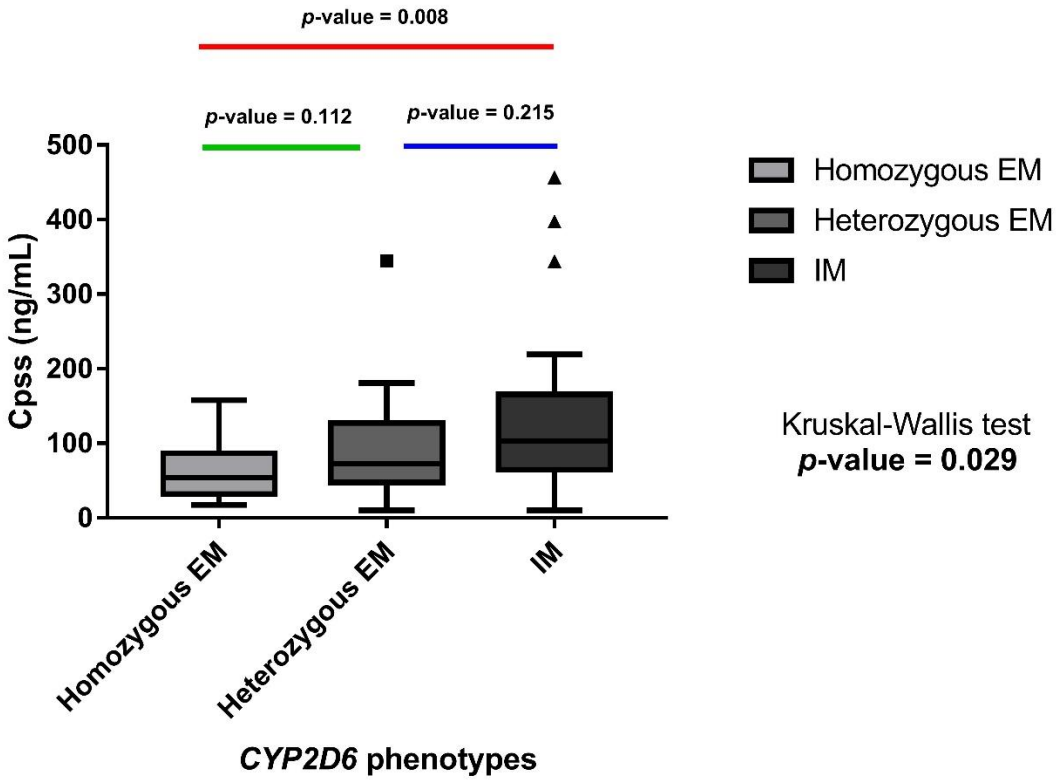
In this study, metabolic phenotypes of *CYP2D6* and *CYP3A5* of the patients were classified using the established common-consensus 'star allele' nomenclature according to CPIC guideline. For *CYP2D6* phenotyping of the 85 patients, 53 of them could be deemed as EM (*CYP2D6*1/*1*, n = 5; *CYP2D6*1/*2*, n = 5; *CYP2D*2/*2*, n =10; *CYP2D*1/*10*, n = 11; *CYP2D*2/*10*, n= 22). Of the 32 patients who carried homozygous *CYP2D6*10* allele, they were all classified as IM (*CYP2D6*10/*10*). Three *CYP3A5* phenotypic groups were identified in this study including EM (*CYP3A5*1/*1*, n = 15), IM (*CYP3A5*1/*3*, n = 26), and PM (*CYP3A5*3/*3*, n = 44). Other genotypes were shown in table 38.

Evaluation of factor affecting Cpss of donepezil

Associations of *CYP2D6*, *CYP3A5*, and *ABCB1* polymorphisms with Cpss of donepezil

At 10-mg maintenance dose of donepezil, homozygous *CYP2D6*10/*10* (i.e., IMs) was found to be associated with the highest Cpss of donepezil. On the other hand, those with heterozygous EMs (*CYP2D6 *1/*10*) and homozygous EMs (*CYP2D6*1/*1/ CYP2D6*1/*2/ CYP2D6*2/*2*) were associated with lower Cpss of donepezil, respectively (Table 39). The Cpss of donepezil among these three phenotypic groups were significantly different (*p*-value = 0.029). Cpss of the IM group was significantly higher than that of the homozygous EM, as shown in figure 2.

Figure 2 Association between *CYP2D6* phenotypes and Cpss of donepezil at the 10-mg maintenance dose.



Notes:

Each pairwise comparison was calculated from Kruskal-Wallis test.

Each boxplot shows the median as the central line, the extremes of each box are the first and third quartile and the whiskers represent the minimum and maximum values in the sample.

Triangles and squares on the top of each boxplot represent outliers.

By using univariate analysis, no significant association between *CYP3A5*3*, *ABCB1* 3435 C>T or *ABCB1* 1236C>T polymorphisms and Cpss of donepezil was founded (*p*-value ≥0.05) as show in table 40.

Table 39 Association of the genetic factors and Cpss of donepezil at the 10-mg maintenance dose

Gene	Genotypes/ Phenotypes	N	Cpss (ng/mL)	*p*-value
CYP2D6	Homozygous EM	20	54.08 (32.22, 82.17)	**0.029**
	Heterozygous EM	33	72.85 (52.17, 126.77)	
	IM	32	103.24 (65.63,164.29)	
CYP3A5	*CYP3A5*1/*1* (EM)	15	55.49 (18.9,.101.77)	0.058
	*CYP3A5*1/*3* (IM)	26	100.97 (70.32, 126.77)	
	*CYP3A5*3/*3* (PM)	44	73.04 (41.40, 137.45)	
ABCB1 3435	CC	32	88.96 (57.51, 129.47)	0.563
	CT	34	75.33 (40.06, 137.31)	
	TT	18	72.19 (35.09, 121.00)	
ABCB1 1236	CC	17	71.73 (55.49, 120.60)	0.902
	CT	39	75.50 (39.25, 126.77)	
	TT	29	75.16 (55.27, 136.46)	

Notes: The data were represented as median (IQR).

Association of non-genetic factors with Cpss of donepezil

Non-genetic factors that might have an influence on interindividual variability of Cpss of donepezil were determined. The results demonstrated that there was no statistically significant difference in Cpss of donepezil among gender. However, male patient trend to have lower median (IQR) of Cpss compared with female (71.13 (36.31-110.48) vs 99.16 (52.53-137.31); *p*-value = 0.081). No significant association

between concomitant CYP3A4, CYP2D6, or P-glycoprotein inhibitors and Cpss of donepezil was also observed (Table 39).

Table 40 Association of the non-genetic factors and Cpss of donepezil at the 10-mg maintenance dose

	Categorical variables			Continuous variables		
Factors	**Frequency (%)**	**Cpss (ng/mL)**	***p*-value**	**Factors**	**Correlation Coefficients (r)**	***p*-value**
Gender				**Bodyweight (Kg)**	-0.165	0.131
Male	38	71.31 (36.31,110.48)	0.081	**BMI (Kg/m^2)**	-0.050	0.651
Female	47	99.16 (52.53,137.31)		**Age (year)**	0.178	0.103
Concomitant use of CYP2D6 inhibitors				**TFDI (hour)**	-0.064	0.558
No	60	74.82 (52.71,137.45)	0.401	**CrCL (mL/min)**	-0.057	0.282
Yes	25	72.04 (40.06,121.00)				
Concomitant use of CYP3A4 inhibitors						
No	37	72.04 (37.83,126.77	0.454			
Yes	48	83.14 (52.89,129.39				
Concomitant use of P-glycoprotein inhibitors						
No	39	71.73 (39.25,123.78)	0.232			
Yes	46	87.45 (52.17, 136.92)				
Concomitant use of memantine						
No	66	69.09 (37.83,123.78)	**0.007**			
Yes	19	102.77(75.50,161.27)				

Notes:

The data were represented as median (IQR).

CYP3A4 inhibitors including amlodipine, atorvastatin, diltiazem, and omeprazole.

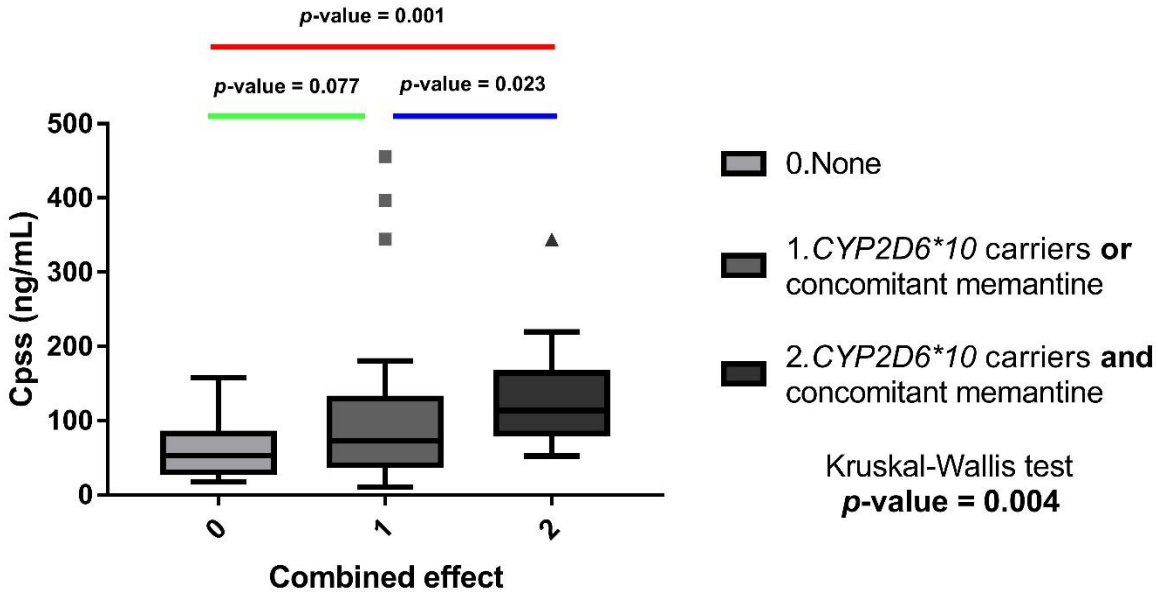
P-glycoprotein inhibitors including atorvastatin, carvedilol, diltiazem, and simvastatin.

There was a strong association between concomitant memantine use and Cpss of donepezil. Patients who received concomitant memantine had higher Cpss of donepezil than those who were memantine non-users (102.77 (75.50-161.27) vs 69.09 (37.83-123.78); *p*-value = 0.007) as shown in Table 40. We further explored the effect of memantine doses on Cpss of donepezil. The results showed that Cpss of donepezil was directly proportional to the administered dose of memantine. The Cpss of donepezil in patients who did not take memantine and who took 10 or 20 mg memantine were 69.09, 93.79, and 173.37 ng/mL, respectively. The Cpss of donepezil corresponding to the three groups were significantly different (*p*-value = 0.012).

The finding also demonstrated a trend toward a combined effect of *CYP2D6*10* carriers and concomitant memantine treatment on Cpss of donepezil. The patients who were *CYP2D6*10* carriers and concurrent memantine users showed the highest Cpss of donepezil when compared with the rest as shown in figure 3.

No significant association between Cpss of donepezil and BMI or body weight was observed.

Figure 3 Association between the combined effect of *CYP2D6*10* carriers and concomitant use of memantine on Cpss of donepezil.



Notes:

Each pairwise comparison was calculated from Kruskal-Wallis test.

Each boxplot shows the median as the central line, the extremes of each box are the first and third quartile and the whiskers represent the minimum and maximum values in the sample.

Triangles and squares on the top of each boxplot represent outliers.

Combined association of genetic and non-genetic factors with adjusted Cpss of donepezil

The regression models were constructed to determine the association of Cpss of donepezil with genetic and non-genetic factors by using stepwise multiple linear regression. The final model was shown in table 41.

Table 41 The final model of stepwise multiple linear regression analysis of explanatory variables for adjusted Cpss of donepezil

Predictive variables	Unstandardized coefficients		Standardized coefficients	95% CI of B	*p*-value
	B	S.E.	β		
Constant	3.420	0.353	-	2.718/4.122	**< 0.001**
***CYP2D6* phenotypes**	0.478	0.220	0.225	0.041/0.916	**0.032**
Concomitant memantine	0.511	0.203	0.261	0.107/0.915	**0.014**
R^2 = 0.133, *p*-value = 0.003					

Notes:

adjusted for *CYP3A5* phenotypes, time from drug intake, age, and gender

CYP2D6 phenotypes: 1.0 = homozygous EM (*CYP2D6*1/*1 or CYP2D6*1/*2 or CYP2D6*2/*2*)

1.5 = heterozygous EM (*CYP2D6*1/*10, CYP2D6*2/*10*)

2.0 = IM (*CYP2D6*10/*10*)

Concomitant memantine use: 0 = non-user, 1 = user

Transformed level by using natural logarithmic function

The results from multivariate analysis were shown in table 41. The stepwise multiple linear regression analysis included *CYP2D6* phenotypes, *CYP3A5* phenotypes, time from drug intake (TFDI), age, and gender as covariates. The final model revealed that *CYP2D6* phenotypes and concomitant memantine use were significantly associated with Cpss of donepezil. These predictive variables could explain approximately 13% of variability in Cpss of donepezil (R^2 = 0.133, *p*-value = 0.003).

Evaluation of factors affecting cognitive function in patients treated with donepezil

In this study, two patients who were frontotemporal lobe dementia and mild cognitive impairment were excluded, because type of dementia might affect

cognitive evaluation. Furthermore, we could not be able to draw any conclusion due to negligible proportions of those patients. We also excluded 1 patient because of missing TMSE score. Therefore, a total of 82 patients were included in the data analysis. The 82 patients were categorized into two groups according to the types of dementia as AD and VAD.

Associations of *CYP2D6*, *CYP3A5*, *ABCB1,* and *APOE* polymorphisms with TMSE score

When cognitive functions of AD patients were tested, IM group of *CYP2D6* showed a tendency toward a better therapeutic outcome with the highest TMSE score (21.10±5.12 points) when compared with those heterozygous EM (20.20±5.30 points) and homozygous EM (14.30±8.10 points) groups (Table 42). In line with that, the decline of cognitive function was the least obvious in the IM group and the most obvious decline was found in the homozygous EM group. There was a statistically significant difference of TMSE score and ΔTMSE between IM and homozygous EM groups as shown in figure 4.

Figure 4 Association between *CYP2D6* phenotypes and TMSE score at steady state (A) or ΔTMSE score (B) in AD patients

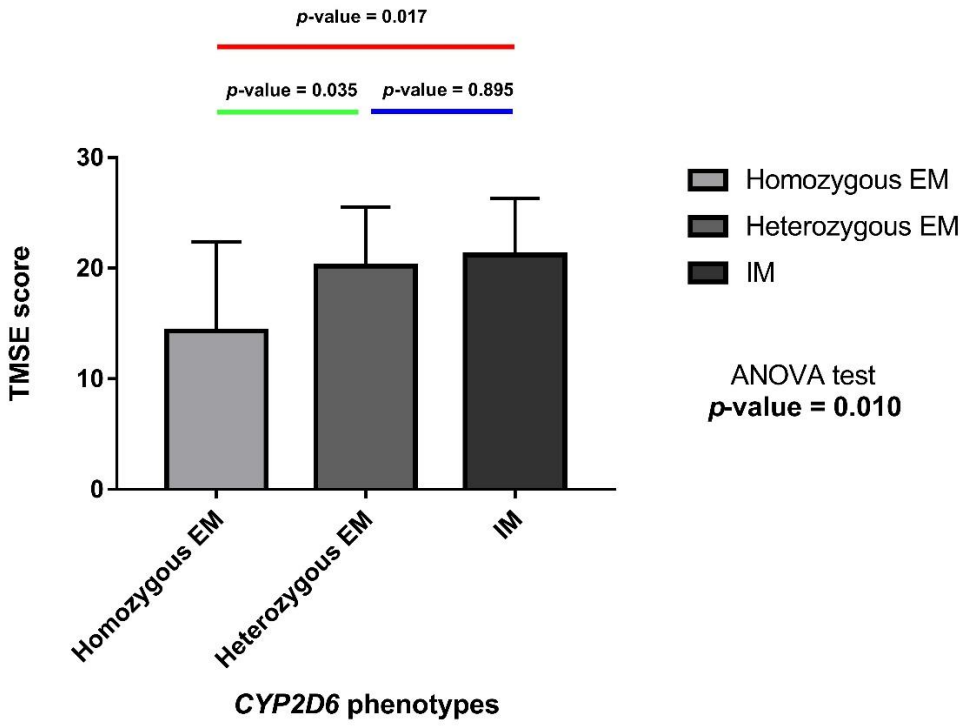


Figure 4A

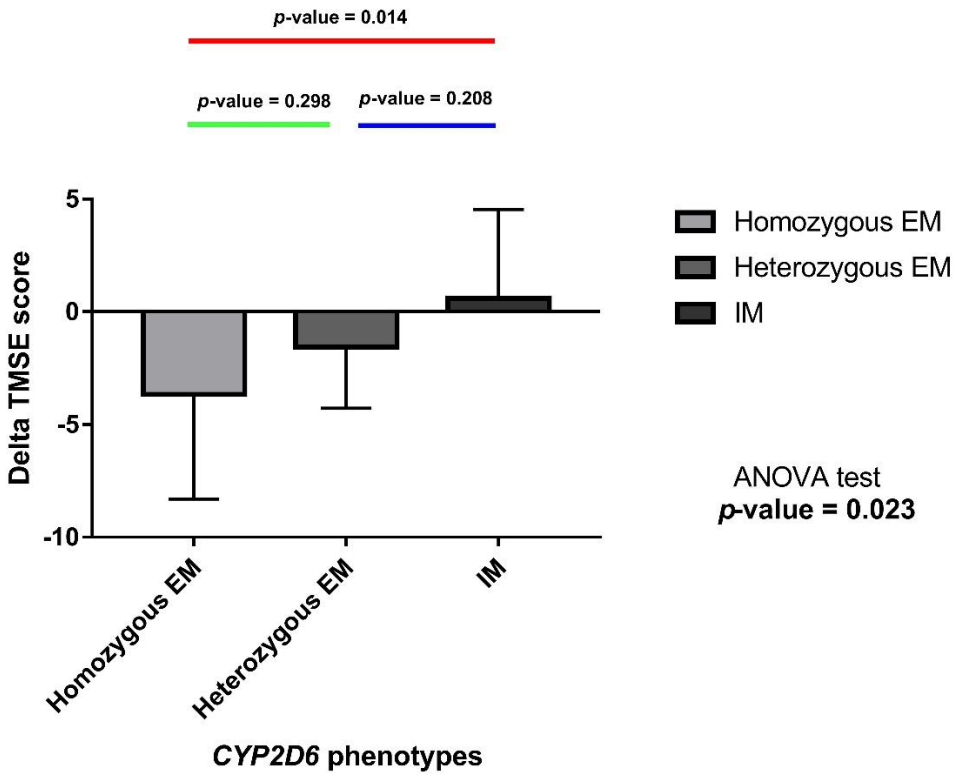


Figure 4B

Notes: Multiple comparisons were performed by Scheffe's method.

Each whisker represents the standard deviation (SD).

In patients with VAD, the decline in cognitive function was high in homozygous EMs, while those who were IMs had some improvement. However, these were not statistically significant.

Regarding univariate analysis, there was no significant association between *CYP3A5, ABCB1*, and *APOE* genetic polymorphisms and TMSE score in both patients with AD and VAD as shown in table 42.

Table 42 TMSE score in association with *CYP2D6*, *CYP3A5*, *ABCB1* and *APOE* genotypes at the 10-mg maintenance dose

Genotypes/ Phenotypes	AD (N=50)			VAD (N=32)		
	N	TMSE score	ΔTMSE score	N	TMSE score	ΔTMSE score
CYP2D6						
Homozygous EM	12	14.30±8.10	-3.67±4.64	8	19.40±4.90	-1.90±2.50
Heterozygous EM	21	20.20±5.30	-1.57±2.71	11	18.30±9.00	-0.50±2.40
IM	17	21.10±5.12	0.59±3.95	13	18.20±8.20	-0.50±4.90
p-value		**0.010**	**0.023**		0.935	0.647
CYP3A5						
CYP3A5*1/*1 **(EM)**	10	18.60±5.80	-1.90±2.50	4	19.50±5.90	-1.80±3.10
CYP3A5*1/*3 **(IM)**	15	19.30±6.20	-0.60±2.90	10	13.40±7.10	-1.80±4.00
CYP3A5*3/*3 **(PM)**	26	19.20±7.20	-0.80±2.70	18	21.10±7.00	-0.10±3.50
p-value		0.962	0.467		0.029	0.421
ABCB1 3435						
CC	19	20.263±5.362	-1.473±3.322	12	19.417±7.668	-2.166±3.459
CT	21	18.095±7.429	-0.904±4.217	11	18.182±7.359	0.727±4.221
TT	9	19.556±6.930	-2.000±5.000	9	17.667±8.5440	-2.000± 6.304
p-value		0.579	0.799		0.868	0.280
ABCB1 1236						
CC	10	19.900±7.766	-1.700±2.311	**7**	19.286±4.572	0.000±3.162
CT	22	17.955±6.425	-1.500±3.776	16	16.625±8.437	-2.687±4.527
TT	18	20.056±6.033	-0.944±4.916	9	21.222±7.661	0.777±5.449
p-value		0.554	0.866		0.343	0.163
APOE						
APOE ε4 **carriers**	28	18.143± 6.392	-1.6071±4.201	11	16.545±9.501	-1.277±5.344
APOE ε4 **non-carriers**	22	20.318±6.614	-1.000±3.664	18	19.222±6.431	-1.000±2.932
p-value		0.245	0.594		0.372	0.876

Notes:

For TMSE and ΔTMSE score, the data were represented as mean ± SD.

ΔTMSE score = change in TMSE score initial treatment to final observation.

CYP2D6 phenotypes: homozygous EM i.e. *CYP2D6*1/*1* or *CYP2D6*1/*2* or *CYP2D6*2/*2*

heterozygous EM i.e. *CYP2D6*1/*10* or *CYP2D6*2/*10*

IM i.e. *CYP2D6*10/*10*

Association of non-genetic factors with TMSE score

Concomitant antidepressant drug was found to be associated with clinical response of donepezil in this study. Both patients with AD and VAD who were receiving antidepressant drugs had poorer cognitive function compared to those who were not receiving the antidepressant drugs, especially in AD as shown in table 43.

There was no significant association between concomitant memantine use, age, gender, education level, and TMSE score in both patients with AD and VAD as shown in table 43.

Table 43 Association of non-genetic factor and TMSE score of donepezil at 10-mg maintenance dose

Non-genetic factor	AD (N=50)			VAD (N=32)		
	N	TMSE score	**Δ**TMSE score	N	TMSE score	**Δ**TMSE score
Gender						
Male	19	20.947±5.317	-1.2105±2.573	17	19.765±7.370	-0.1176±4.226
Female	31	17.968±6.993	-1.4194±4.631	15	17.067±7.851	-2.266±5.091
p-value		0.118	0.839		0.324	0.202
Concomitant use of antidepressant drugs						
No	35	20.343± 6.121	-.5714±3.483	22	18.364±8.144	-0.2727±4.682
Yes	15	16.200± 6.689	-3.133±4.486	10	18.800±6.629	-3.000±4.396
p-value		**0.038**	**0.034**		0.883	0.130
Concomitant use of CYP3A4 inhibitors						
No	22	18.364±7.267	-1.9091± 5.107	12	20.667±7.475	0.166±4.281
Yes	28	19.679± 5.932	-0.8929± 2.739	20	17.200±7.557	-1.900±4.876
p-value		0.484	0.406		0.564	0.235
Concomitant use of P-glycoprotein inhibitors						
No	26	18.731±5.848	-1.5385±3.313	10	15.400±9.045	-2.600±2.547
Yes	24	19.500±7.277	-1.125±4.599	22	19.909±6.596	-0.454±5.324
p-value		0.681	0.714		0.121	0.238
Concomitant use of memantine						
No	36	19.861±6.961	-1.555±4.101	28	19.357±7.592	-0.8929±4.693
Yes	14	17.143±4.881	-0.785±3.598	4	12.500±4.795	-2.750±5.123
p-value		0.188	0.541		0.092	0.469

Table 44 Bivariate analysis: Association of non-genetic continuous variable and TMSE score

	AD				VAD			
Dependent variables	TMSE score		**Δ**TMSE score		TMSE score		**Δ**TMSE score	
Independent variables	Correlation coefficients (r)	*p*-value	Correlation coefficients (r)	*p*-value	Correlation coefficients (r)	*p*-value	Correlation coefficients (r)	*p*-value
Age (year)	0.205	0.153	0.270	0.058	-0.464	**0.008**	-0.458	**0.008**
Baseline TMSE score	0.800	**< 0.001**	-0.143	0.323	0.788	**< 0.001**	-0.107	0.559
Cpss (ng/mL)	0.046	0.749	0.244	0.087	-0.046	0.804	-0.014	0.937
Duration of use (month)	-0.137	0.343	0.286	**0.044**	0.060	0.744	-0.259	0.152
Education levels (year)	0.124	0.391	0.059	0.685	0.199	0.276	0.059	0.748

Combined association of genetic and non-genetic factor with TMSE score

Covariates were selected from the results of univariate analysis (Table 42,43, and 44) by setting significant level for entry (SLE) at *p*-value of 0.25 or lower and were introduced into each multivariate model. The final models are shown in table 45.

Table 45 The final models of stepwise multiple linear regression analysis of explanatory variables for donepezil treatment outcomes as measured by TMSE score at steady state and **Δ**TMSE in patients with AD and VAD

Type of Dementia	Dependent variables	Explanatory variables	Unstandardized coefficients		Standardized coefficients	95% CI of B	*p*-value
			B	S.E.	β		
AD	TMSE score[a]	Constant	-4.113	2.544	-	-9.234/1.008	0.113
		Baseline TMSE score	0.832	0.085	0.738	0.661/1.004	**< 0.001**
		CYP2D6 phenotypes	4.527	1.280	0.265	1.150/5.945	**0.001**
		Concomitant antidepressant use	-2.719	1.052	-0.193	-4.837/-.602	**0.013**
		R^2 = 0.747, *p*-value < 0.001					
	ΔTMSE score[b]	Constant	-8.060	2.092	-	-12.270/-3.85	**< 0.001**
		CYP2D6 phenotypes	4.107	1.259	0.397	1.573/6.641	**0.002**
		Duration of use (year)	0.024	0.011	0.261	0.001/0.047	**0.037**
		Concomitant antidepressant use	-2.348	1.038	-0.275	-4.437/-0.259	**0.028**
		R^2 = 0.321, *p*-value = 0.002					
VAD	TMSE score[c]	Constant	24.816	8.326	-	7.787/41.844	**0.006**
		Baseline TMSE score	0.845	0.119	0.723	0.602/1.089	**< 0.001**
		Age (year)	-0.292	0.095	-0.311	-0.488/-0.097	**0.005**
		R^2 = 0.714, *p*-value < 0.001					
	ΔTMSE score[d]	Constant	19.729	7.433		4.549/34.910	**0.013**
		Age (year)	-0.266	0.094	-0.458	-0.459/-0.073	**0.008**
		R^2 = 0.210, *p*-value = 0.008					

[a]adjusted for concomitant memantine use, age, and gender

[b]adjusted for *CYP3A5* phenotypes, age, and Cpss of donepezil

[c]adjusted for *CYP3A5* phenotypes, concomitant memantine use, and concomitant CYP3A4 inhibitors use

[d]adjusted for *ABCB1* 1236 genotype, concomitant antidepressant use, duration of use and gender

CYP2D6 phenotypes: 1.0 = homozygous EM (*CYP2D6*1/*1 or CYP2D6*1/*2 or CYP2D6*2/*2*)

1.5 = heterozygous EM (*CYP2D6*1/*10, CYP2D6*2/*10*)

2.0 = IM (*CYP2D6*10/*10*)

Concomitant antidepressant use: 0 = non-user, 1 = user

At the 10-mg maintenance dose of donepezil, stepwise multiple linear regression models using TMSE score at steady state or ΔTMSE as the dependent variables were constructed to determine the association of genetic and non-genetic factors associated with donepezil response of AD and VAD patients as shown in Table 45. The results revealed that in AD patients, *CYP2D6* phenotypes was the only genetic factor influencing TMSE score at steady state and ΔTMSE. On the contrary, AD patients who were treated with antidepressant drugs were significantly associated with worsened steady state TMSE score after adjusting for covariates listed in table 45. These two covariates could explain 74% of the variability in TMSE score at steady state (R^2 = 0.747, *p*-value < 0.001). The result also revealed that the only significant predictor of ΔTMSE was *CYP2D6* phenotypes which could explain 32% of the variability (R^2=0.321, *p*-value = 0.002).

In VAD, the final stepwise multiple linear regression model demonstrated that increasing age was significantly associated with a more negative TMSE score at steady state and ΔTMSE. The magnitude of explanation for the variability in the models was 71% for TMSE score (R^2 = 0.714, *p*-value < 0.001) and 21% for ΔTMSE (R^2 = 0.210, *p*-value = 0.008).

Correlation between Cpss and TMSE score

Pearson correlation was performed to illustrate the association between Cpss of donepezil and change in TMSE score at six months (LOCF; last observation carried

forward). A scatter plot of these correlation showed in figure 5. No significant association was found. However, a trend of positive correlation was observed in AD patients (Pearson correlation coefficient (r) = 0.255, *p*-value = 0.074).

Figure 5 Scatter plots show correlation between donepezil Cpss and ΔTMSE score in patients with AD (5A) and VAD (5B)

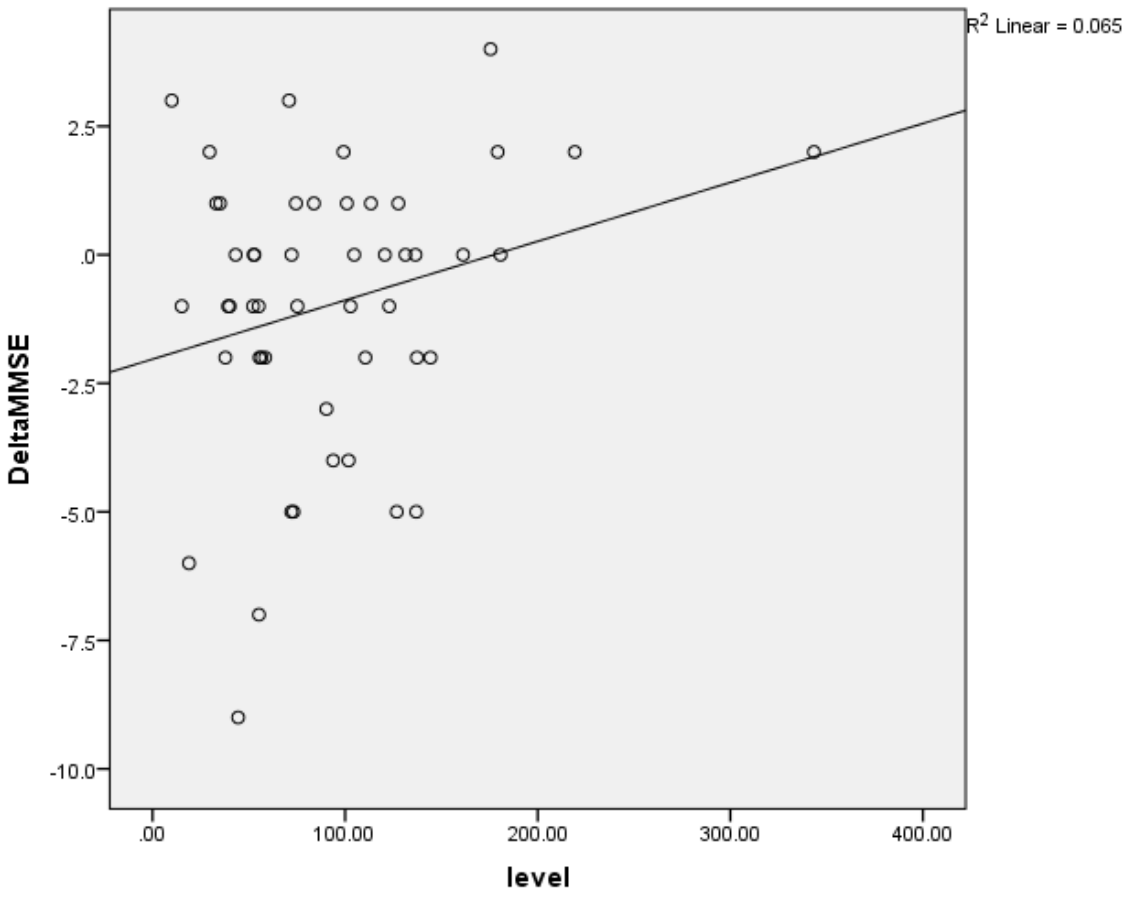


Figure 5A

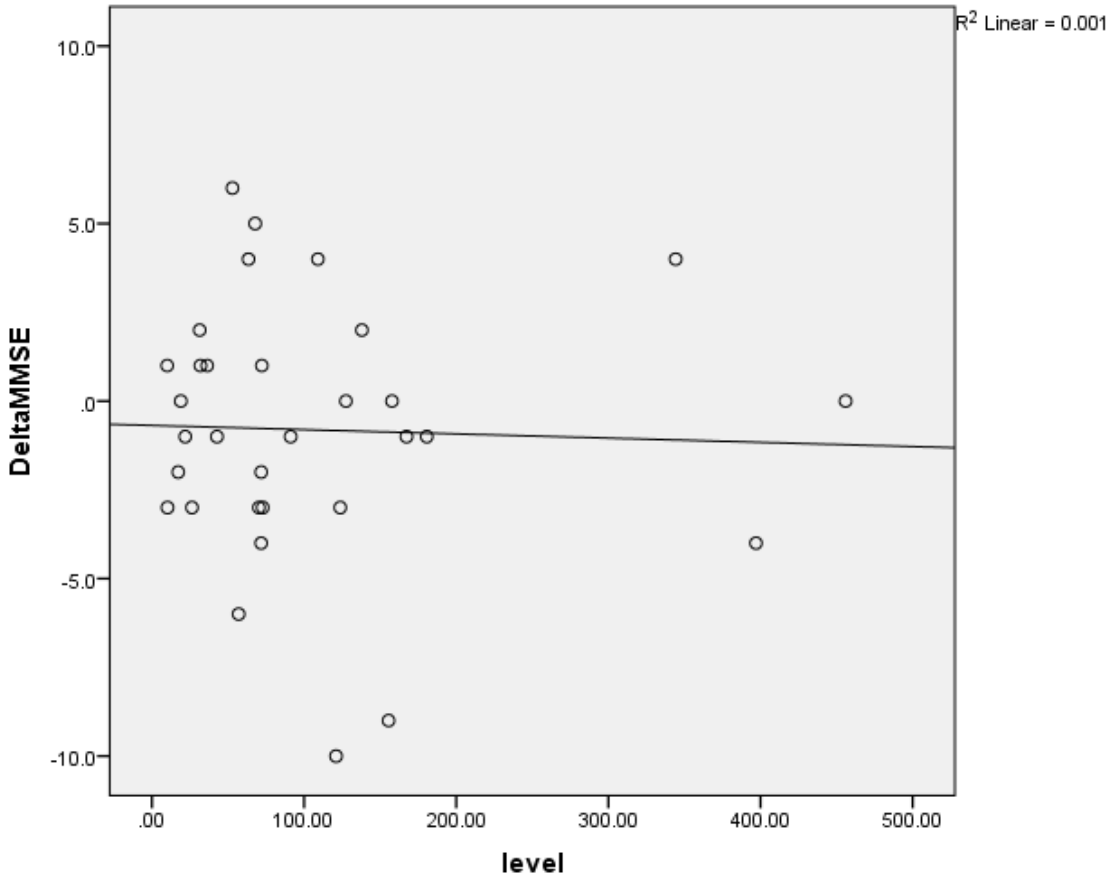


Figure 5B

Evaluation of adverse drug events

The most common presenting adverse drug events of acetylcholinesterase inhibitors is gastrointestinal effects including nausea, vomit, diarrhea followed by bradycardia. Regarding adverse drug events, no significantly association between genetic or non-genetic factor and the existence of ADR was observed. No association was founded between Cpss of donepezil and systolic or diastolic blood pressure or pulse rate in this cohorts. A possible explanation was that this study was a retrospective cohort design, a temporal association was not done in the present study. There were some unrecord data especially in the aspects of adverse drug events.

DISCUSSION

This study aimed to investigate the associations of genetic factors especially genes involved in drug metabolizing enzymes, drug transporter or pathological process (i.e. *CYP2D6*, *CYP3A5*, *ABCB1*, and *APOE*) and certain non-genetic factors simultaneously with plasma concentration and clinical response of donepezil in Thai patients with AD and VAD.

Pharmacokinetic gene of phase I drug metabolizing gene especially polymorphisms of *CYP2D6* are widely studied in various ethnics as showed in table 19. However, most of studies focused only on patients with AD and performed by using univariate analysis. The present study is a study designed to elucidate the influence of several genetic and non-genetic factors simultaneously by using multivariate analysis in both AD and VAD patients.

Genotype distribution

Genotype frequencies of the polymorphisms of the candidate genes in the studied patients were found to be consistent with previous reports in Asian populations. Although, some deviations from Hardy-Weinberg equilibrium were found including *CYP2D6*2* and *CYP2D6*10*. The deviation may be due to the inclusion of undetermined variants of *CYP2D6* gene including *CYP2D6*5* in this study. *CYP2D6*5* was a deleted mutant with an allele frequency of approximately 5% in Thai population. It is possible that *CYP2D6*1/*5* and *CYP2D6*5/*10* may be included in *CYP2D6*1/*1* and *CYP2D6*10/*10* genotype frequencies, respectively. Further analysis of *CYP2D6*5* allele should be investigated. However, if detection of CYP2D6*5 was performed, it is likely not affect the overall phenotype interpretation because recent study from Chamnanphon et al. concluded that *CYP2D6*5/*10* and *CYP2D6*1/*5* were found approximately 4.7% and 4.2 % in Thai population and were classify as IM and EM, respectively. It is possible that *CYP2D6*1/*5* and *CYP2D6*5/*10* may be

included in *CYP2D6*1/*1* (EM) and *CYP2D6*10/*10* (IM) genotype frequencies, respectively.

Previous study does not consider *CYP2D6*2* determination. Identifying of *CYP2D6*2* could provide informative prevalence of the *CYP2D6*2* allele by discriminating between *CYP2D6*1* and *CYP2D6*2*. The latter is another *CYP2D6* allele with normal function frequently found in Thai population. The *CYP2D6*2* determination revealed *CYP2D6*2/*10* genotype which has not been explored in previous studies.

Evaluation of factor affecting Cpss of donepezil

Associations of *CYP2D6*, *CYP3A5*, and *ABCB1* polymorphisms with Cpss of donepezil

Both univariate and multivariate analyses suggest that *CYP2D6* polymorphisms were strongly associated with Cpss of donepezil. Patients carrying loss of function allele of *CYP2D6* (i.e. *CYP2D6*10*) had higher Cpss of donepezil when compared with those non-carriers. The results were concordant with a previous study in Asians population. Yuan Zhong et al. found that patients who were *CYP2D6*10/*10* homozygous had a higher steady state plasma concentration of donepezil and a larger change in MMSE score than those who were *CYP2D6*1/*10* and *CYP2D6*1/*1*, respectively (90). Similar results were found with both racemic donepezil and (S)-donepezil (6).

CYP3A4 gene is not highly polymorphic in Thai population (119). Moreover, previous studies did not find any association between *CYP3A4* variants and Cpss or clinical outcomes of donepezil (12, 72). Therefore, we did not explore the effect of *CYP3A4* polymorphisms in this study. However, in African American ethnicity, Kuehl et al. founded that *CYP3A4*1B* is in linkage disequilibrium with *CYP3A5*1*. Moreover, CYP3A5 was lining through gastrointestinal tract (57). These implied that *CYP3A5*

might play a role in donepezil metabolism but no studies have been done in Asian populations. In the present study, the effect of *CYP3A5* polymorphisms on Cpss of donepezil was investigated but no significant association was found. These results were concordant with studies of Magliulo et. al. (72) and Noetzli et. al.(101). This phenomenon could be possible that donepezil prominently underwent CYP2D6 as its main metabolic pathway when compared with CYP3A. The intrinsic clearance of CYP3A4 is obviously lower than CYP2D6. This suggested that CYP3A4 and CYP3A5 do not play a major role in elimination of donepezil.

Magliulo et al. investigated the association of *ABCB1* polymorphisms on Cpss and therapeutic outcome of donepezil in 54 Italians people (72). The result showed that the most common *ABCB1* haplotypes were 1236C/2677G/3435C (46%) and 236T/2677T/3435T (41%) and TTT haplotype of *ABCB1* showed a tendency towards a better therapeutic outcome and lower plasma concentrations to dose ratio. The latter outcome was also seen when the three SNPs were studied separately. However, the results did not reach statistically significant.

Association of non-genetic factors with Cpss of donepezil

Effect of drug-drug interactions on Cpss of donepezil

A significant association between Cpss of donepezil with drug interactions was identified in this study. Contrary to previous studies which have demonstrated that CYP2D6 inhibitors might increase Cpss of donepezil, the present study found no significant effects of CYP2D6 inhibitors on Cpss of donepezil. This can be due to the disparate strength of CYP2D6 inhibitors in the study including sertraline, venlafaxine, escitalopram, desvenlafaxine which are relatively weak inhibitors compared to other studies that used paroxetine (120). Moreover, evidence has been found that the co-administration with sertraline could decrease Cpss of donepezil. The suggested possible explanation was that sertraline has a slightly stronger affinity for CYP2D6

than donepezil. Thus, at a low plasma level, sertraline could be metabolized competitively with donepezil. Consequently, an increase in donepezil level could be expected. On the contrary, at a higher plasma concentration particularly at steady state, donepezil level was not changed. This can also explain the phenomenon whereby, CYP2D6 exerted less influence at higher plasma concentration due to a shift of donepezil biotransformation to CYP3A4 since the capacity of CYP2D6 was limited by sertraline (121).

We did not find the effect of CYP3A4 inhibitors on Cpss of donepezil. A similar pattern of resulted was obtained in previous study (12, 72, 121-123).

Furthermore, significant higher blood level of donepezil in patients receiving concomitant memantine than non-users was observed. The drug interaction may partially attribute to the effect of *CYP2D6* variants on Cpss of donepezil. This phenomenon could be possible that memantine can inhibit CYP2D6 enzyme as described by Micuda S et.al. (124). This study serves as the first association study to illustrate the effect of concomitant memantine on Cpss of donepezil. The result from the multivariate analysis is concordance with univariate analysis. The result emphasized that concomitant memantine users toward strongly positive associated with Cpss of donepezil.

Effect of gender on Cpss of donepezil

Biological difference among male and female may contribute to difference in both adjusted Cpss in acetylcholinesterase drugs. In general, gender was found to be confounded with body weight. Female gender was associated with lower body weight and ultimately resulted in lower Cpss. In contrast, the result showed that female tend toward higher Cpss when compared with male. This finding should be further determined.

Effect of age on Cpss of **donepezil**

Age tend to be positively correlated with Cpss. It was possible that the decrease in clearance in the elderly could contribute to elevated Cpss of donepezil. Moreover, it is possible that the actual compliance might be associated with age and might be influence on Cpss or therapeutic outcomes.

Combined association of genetic and non-genetic factors with Cpss

The result from the multivariate analysis is concordance with univariate analysis. The result emphasized that *CYP2D6*10* carriers and concomitant memantine users toward strongly positive associated with Cpss of donepezil. These covariates could explain the interindividual variability of Cpss for approximately 13%. The unexplained remaining variability may derive from other contributing factors such as race, gene-environment interaction, nutrition status and some physiological function that cannot assuredly be excluded in this cohorts. Moreover, the comorbid condition in elderly can deteriorating physiological function and may attribute to altered drug concentration in the blood and brain. Consequently, it is difficult to predict precise Cpss. Physiological function especially creatinine clearance may have greater influence in the elderly. However, in the present study no association was found between Cpss of donepezil and creatinine clearance.

Evaluation of factors affecting cognitive function

Associations of *CYP2D6*, *CYP3A5*, *ABCB1,* and *APOE* polymorphisms with TMSE score

In relation to cognitive function, *CYP2D6*10* carriers show a higher TMSE score when compared with non-carriers. Possible association of the genetic polymorphisms of *CYP2D6* in susceptibility to donepezil outcome might be described as the following reasons. Donepezil predominantly metabolized by CYP2D6 and human CYP2D6 in the brain was prominently localized in the pyramidal cell of the cortex and hippocampus which a certain region that account for cognitive function (125, 126). Liam Zaidel et al. showed that donepezil accumulated in the frontal cortex, one of the regions which affected the neuropathology of AD (127). Consequently, *CYP2D6*10* carriers might increase donepezil and greater inhibit acetylcholinesterase in frontal cortex resulting in an improvement in cognitive function as measured by TMSE in AD. Furthermore, Darreh et al. founded that CSF donepezil concentration appears to be approximately tenfold lower compared with plasma levels but

exhibits a similar dose-proportional pattern (128). These implied that *CYP2D6*10* carrier may have higher donepezil level in CSF and could be expected to provide more achievement in clinical responses.

In contrast to AD, in VAD patients *CYP2D6* variants was not found to be associated with the cognitive response of donepezil. This may be reflected of the fact that frontal cortex and hippocampus which abundant of CYP2D6 have a less responsible in the neuropathological process in VAD when compare with AD. In VAD the region of the brain which plays a role in the pathological process is the subcortical area. Jellinger KA found that advance ages may contribute to small vessel disorder (129) and several lines of evidence suggest that advanced age is an additional predisposing factor which aggravates clinical response of acetylcholinesterase inhibitor treatment.

Another possible explanation is that CYP2D6 might play a role in the biotransformation of several endogenous or xenobiotic in the brain. As CYP2D6 is involved in the transformation of several bioactive compounds in the brain (125). It may attribute little effect on a single functional pathway. *CYP2D6* phenotype also influence neurocognition as described by Eva M Peñas-LLedó et al (125). For these reasons, it may imply that genetic variations of *CYP2D6* could mediate the progression of the disease and therapeutic outcomes of donepezil. Furthermore, Kirchheiner J et al. suggested that IM of CYP2D6 has higher brain perfusion in the hippocampus compared with EM (130). This may be one of the reasons to explain the results due to higher brain perfusion in *CYP2D6*10* carriers could restore underlying pathological of disease and provide better response compared to *CYP2D6*10* non-carriers (EM).

In this study, no significant effect of CYP3A5 polymorphisms on Cpss of donepezil and cognitive score was found. This phenomenon could be possible that donepezil prominently underwent CYP2D6 as its main metabolic pathway. Whereas, CYP3A5 might play a minor role in donepezil disposition. Moreover, the distribution

of CYP3A5 in the brain was less than CYP2D6. The present finding was concordant with recent studies of Magliulo et al. and Noetzli et al (72, 92).

The impacts of *ABCB1* polymorphisms on TMSE scores as well as Cpss was elucidated. The results showed that patients with TT genotypes of *ABCB1* 3435 have slightly lower change of TMSE scores compared to the rest. This could be due to the fact that *ABCB1* 3435 C>T and *ABCB1* 1236 C>T are significantly linked with AD risk as indicated by a meta-analysis. Some studies found that T allele of *ABCB1* C1236T, G2677T and C3435T exhibited changes in P-gp activities and promote Aβ aggregation in the brain in a T dose-dependent manner. Chen KD et al. concluded that ABCB1 gene influenced positive correlation with MMSE scores and serves as a novel biomarker of AD. Magliulo et al. also suggested the tendency towards a better therapeutic outcome of patients who were TTT haplotype of *ABCB1* 3435, 1236, and 2677 (72). It might be possible that decreased P-gp activity in *ABCB1* variants may reduce clearance of donepezil from the CNS to the blood compartment and ultimately increased donepezil level in the CNS (72).

To our knowledge, only one study explored the effect of *ABCB1* polymorphisms on therapeutic outcome of donepezil and focused only AD patients as aforementioned. This study is the first study to explore the effect of CYP2D6 genotypes in VAD patients.

Some studies had attempted to explore the association of *APOE* ***ε****4* alleles with acetylcholinesterase response in AD. The rationales whereby APOE **ε**4 plays a role in contributing pathogenesis of AD such as abnormal cholesterol transportation, and the augmentation of amyloid plaque and neurofibrillary tangles might have negative impact on drug treatment. Some observations found that *APOE* ***ε****4* carriers may worsen the TMSE score of donepezil treatment outcome. But no significant association between *APOE* ***ε****4* carriers and TMSE score was found in this study. The effects of *APOE* ***ε****4* on clinical response of donepezil were not homogeneous.

Further investigation with larger and well–designed study should be conducted to illuminate divergent findings.

The inconsistent results from various studies can be attributable to different in cognitive outcome measures, variable in acetylcholinesterase drug and concomitant medication, characteristic of study design including inclusion and exclusion criteria, definition of responses and randomization, interindividual genetic background(131).

Moreover, Jin Lu suggested *APOE* genotype might influence CYP2D6 activity. The probable reason might be as *APOE* correlated with liver enzyme particularly SGOT, SGPT and TG level and these levels may be closely associate with liver steatosis and transaminase activity which mediates the effect APOE on CYP P450 functions. Thus, one of the mechanisms by which *APOE* influence donepezil response may involve CYP2D6 related effects on liver metabolism(93).

A stratify analysis of the two types of dementia suggest that the effect of genetic polymorphisms of the interested genes on clinical response to donepezil is more pronounce in patients AD than VAD.

Association of non-genetic factors with TMSE score

The influence of donepezil doses

Darren and Shori et al. observed that the assessment of cognitive outcomes should be evaluated in association to measurement of acetylcholinesterase inhibition rather than dose of AChEIs (128). Moreover, Wattmo et al. concluded that higher doses of AChEI were associated with a more positive cognitive outcome and this association is regardless effect of type of drug(107). Consequently, the present study included only the patients who received 10-mg of donepezil.

The influence of concomitant use of antidepressants

Concomitant use of CYP2D6 inhibitors was found to be negatively associated with TMSE scores in patients with AD and VAD. This phenomenon was astonishing because one previous study showed that CYP2D6 inhibitors could have increased the Cpss of donepezil and could be expected to provide more achievement in therapeutic responses. The declined TMSE scores were more obvious among patients with moderate AD as indicated by lower baseline TMSE scores compared to those with mild AD. Moreover, patients with moderate AD who used antidepressant drugs which were CYP2D6 inhibitors including sertraline, venlafaxine, escitalopram, desvenlafaxine was found to be associated with lower steady-state TMSE scores than those who did not take CYP2D6 inhibitors. When controlling the degree of dementia severity by introducing baseline TMSE score into multiple linear regression model, the result also showed a significantly negative correlation effect of CYP2D6 inhibitors on TMSE score or **Δ**TMSE scores. These findings suggested the negative impact of antidepressant drugs on cognitive function. It is possible that concomitant antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs) may influence cognitive function (132). These results were in agreement with the finding by Wattmo et al. where responses to acetylcholinesterase therapy were diminished faster in patients with depression treated with antidepressants including SSRIs (133). The possible explanation is that depression condition can deteriorate neurocognitive function which goes beyond the pharmacological effect of antidepressant treatment. Another possible due to anticholinergic effect of some antidepressant drugs may diminish the cognitive function of the patients (109). On the other hand, no significant relationship was found in patients with VAD because depression condition was not commonly found.

The influence of gender

In this study, no significant association of clinical response to donepezil with gender was found. The influence of gender on response was controversial. Previous study reported that female patients seemed to be more sensitive than male patients to treatment with acetylcholinesterase inhibitors and polymorphism of estrogen

receptor gene (ESR1) may contribute to interindividual variability in therapeutic response (108). Other study founded that male patients have better clinical response to acetylcholinesterase treatment when compared with female (107).

The influence of duration of drug exposure

Duration of use is the direct association with clinical response. This finding suggests that long term use of donepezil could be beneficial in improving cognitive function which support by the fact that donepezil might modify underlying mechanism of disease progression in vivo study.

Regarding both genetic and non-genetic factors, the different results observed in previous association studies may be accounted for those differences in assessment scores or definition of response; duration of treatment or follow up period, prediction of CYP2D6 phenotypes from genotypes, inclusion or exclusion criteria. The present study recruited patients in all severity but controlling the effect of severity of dementia on TMSE score by introducing baseline TMSE score into multivariate analysis. Moreover, we evaluate ΔTMSE as well as TMSE at steady state to increase confidently established the results. All patients enrolled in this study were treated for at least 6 months at the same dose. The duration of treatment was also controlled in the multivariate model.

Correlation between Cpss and TMSE score

It remains unclear whether higher plasma concentration of donepezil could improve cognitive outcomes. To address these problems, the correlation between Cpss and change of cognitive function from baseline to final observation as measured by TMSE score were determined. No significant association was found. However, a trend of positive correlation was observed. The finding was consistent with that of Yuan Zhong et al (90). which reported that there was no significant difference in Cpss between responders and non-responders. However, other studies suggested that Cpss of donepezil correlated with therapeutic outcome. Several potential explanations for these divergent results may be as follows:

1. Donepezil consists of two enantiomers. Cpss levels of (S)-donepezil were found to be higher than those of (R)-donepezil which was degraded faster. In clinical setting, the available commercial form of donepezil is in racemic form. It is possible that enantiomers of donepezil might give rise to different Cpss and therapeutic outcome.
2. The differences in assessment scores, inclusion or exclusion criteria, duration of treatment could confound the results.
3. It is possible that other genetic variations besides drug metabolizing enzyme gene such as cholineacetyltransferase(134), butyrylcholinesterase(94) might be associated with clinical response.
4. Levels of donepezil in the brain or cerebrospinal fluid (CSF) may better correlate with cognitive function response of donepezil treatment(135) but could not be included in this cohort. However, determination of drug in CSF is quite invasive and inappropriate in routine clinical practice setting.

CHAPTER 5 RESULTS AND DISCUSSION: GALANATAMINE

Association of genetic and non-genetic factors on clinical responses of galantamine in Thai patients with mixed dementia

RESULTS

Demographic and clinical characteristic

All subjected were born in Thailand. The baseline characteristics of patients in this study was described in table 46.

Table 46 Demographic and clinical characteristics of Thai patients with dementia

Demographic and clinical characteristics	**mean±SD** or **Frequency**
Age (years)	79.16±8.80
Age of onset (years)	72.22±8.28
Gender: Male	21
Female	30
Body weight (kg)	55.87±10.94
Body mass index (BMI) (kg/m^2)	22.05±3.65
Daily galantamine dose (mg/kg)	13.80±4.26
TMSE score at baseline	21.35±5.27
TMSE scores changes (ΔTMSE)	-2.37±5.98
Cpss (ng/mL)	58.60±35.51
Adjusted Cpss (ng/mL per mg/kg)	233.69±125.50

The present analysis showed the result of fifty-one patients who were evaluated after at least 6-month follow-up. Of fifty-one patients who met the eligible criteria, there were slightly more women than men (21 men and 30 women). The average age was 79 years, where the majority were in their 75 years or older because dementia was diagnosed at old age. The average galantamine dose was 13 mg/day.

The average years of education of the patients in this cohort was approximately 9 years. Their initial or baseline TMSE score was 21.35 points by average. TMSE score at steady state after 6 months treatment was about 19.12 points and the average forward TMSE score at least 3 months from the date that measure Cpss is 18.78 points. The delta TMSE which define as forward TMSE minus baseline TMSE was about -2.3 points. Other baseline clinical characteristics and demographics were described in table 47.

Genotype distribution

All allele and genotype frequencies are concordances with previous studies in Thailand. There was no deviation from Hardy-Weinberg equilibrium. For *CYP2D6* phenotyping, of the 51 patients, 18 of them could be deemed as EM. All 33 patients carrying homozygous *CYP2D6*10* allele were classified as IM. For *CYP3A5* phenotyping, 21 patients who carry two alleles of *CYP3A5*3* were classified as *CYP3A5* non-expressers and the rest who carry at least one allele of *CYP3A5*1* were *CYP3A5* expressers. Other genotypes were shown in table 47.

Table 47 Genotype distribution and allele frequencies of the candidate genes in the study patients

Allele	Allele frequency	Genotype	Number	Genotype frequency	HWE *p*-value	MAF (nucleotide)
***ABCB1* 3435** (rs 1045642, c.3435 C>T)						
C	0.570	CC	15	0.300	0.470	Chinese: 0.40 Japanese:0.48 (A)/(T)*
T	0.430	CT	27	0.540		
		TT	8	0.160		
***ABCB1* 1236** (rs 1128503, c.1236C>T)						
C	0.360	CC	8	0.160	0.350	Chinese: 0.34 Japanese:0.32 (G)/(C)*
T	0.640	CT	20	0.392		
		TT	22	0.440		
CYP2D6*2 (rs 1135840, g.4180G>C)						
G	0.706	GG (*-/*-)	27	0.529	0.284	Chinese: 0.21 Japanese: 0.41 (C)
C	0.294	GC (*2/*-)	18	0.353		
		CC (*2/*2)	6	0.118		
CYP2D6*10 (rs 1065852, g.100C>T)						
G	0.559	GG (*-/*-)	18	0.353	0.230	Chinese: 0.33 Japanese:0.50 (G)
A	0.441	AG (*10/*-)	21	0.412		
		AA (*10/*10)	12	0.235		
CYP3A5*3 (rs 776746, g.6986T>C)						
C	0.64	TT (*1/*1)	7	0.412	0.860	Chinese: 0.37 Japanese: 0.26(T)
T	0.36	TC (*1/*3)	23	0.451		
		CC (*3/*3)	21	0.137		
UGT1A1*6 (rs 4148323, c.211G>A)						
G	0.882	GG	40	0.784	0.691	Chinese: 0.11 Japanese: 0.20 (A)
A	0.118	GA	10	0.196		
		AA	1	0.020		
UGT1A1*28[§] (rs8175347, 2-extra-nucleotide insertion (TA))						

TA6	0.863	TA6/TA6	39	0.765	0.219	Chinese: 0.172 Japanese: 0.097 (TA7)
TA7	0.137	TA6/TA7	10	0.196		
		TA7/TA7	2	0.039		
APOE						
APOE ***ε2***	0.098	*APOE* ***ε2/ε2***	0	0		Chinese: 0.076(117) Japanese: 0.078(118) (*APOE* ***ε2***)
APOE ***ε3***	0.676	*APOE* ***ε2/ε3***	7	0.137		
APOE ***ε4***	0.226	*APOE* ***ε2/ε4***	3	0.059		
		APOE ***ε3/ε3***	24	0.470		
		APOE ***ε3/ε4***	14	0.275		
		APOE ***ε4/ε4***	3	0.059		

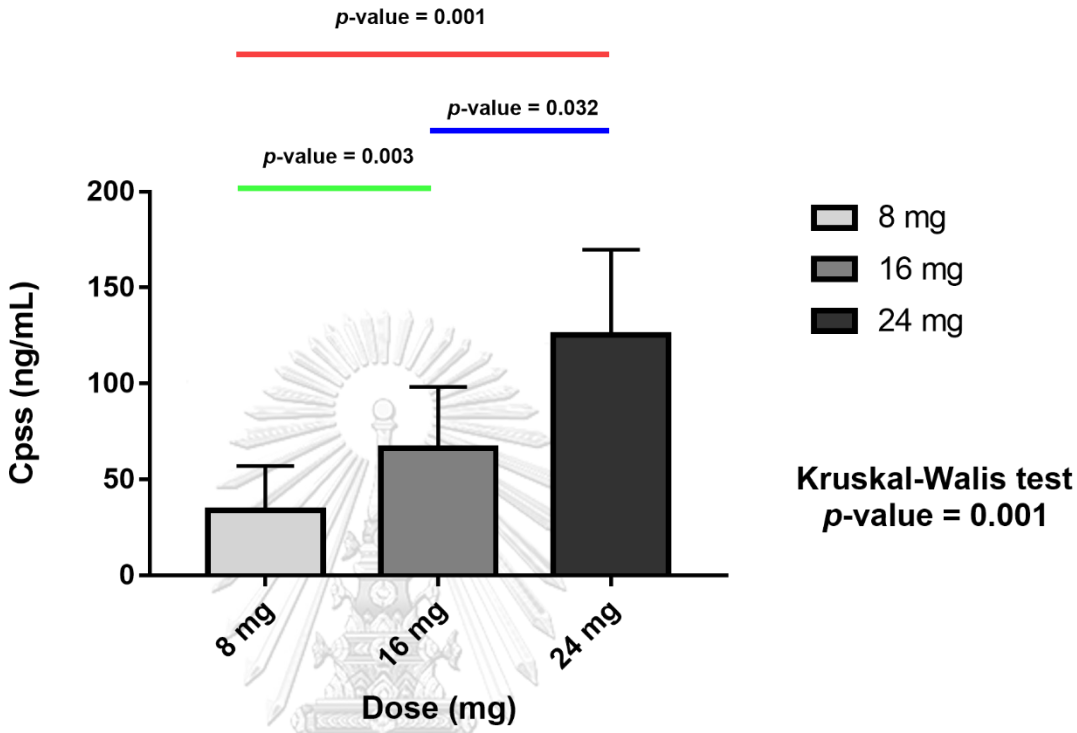
Note: All MAF data from Applied Biosystems® except *APOE*

A and G are polymorphic base on one strand which is complementary to T and C on the other strand.

Evaluation of factors affecting Cpss

The mean dose of galantamine during the study was 13.80±4.25 mg/day. The results showed the Cpss of galantamine was directly proportional to administered dose. The Cpss of 8, 16 and 24 mg galantamine doses were 33.96, 66.49 and 125.39 ng/mL, respectively. Cpss corresponding to the three doses were significantly different (*p*-value = 0.001). There was no significant correlation between time from drug ingestion and Cpss (*p*-value = 0.845).

Figure 6 Association between doses and Cpss of galantamine



Notes: Multiple comparisons were performed by Scheffe's method.

Each whisker represents the standard deviation (SD).

Since galantamine demonstrated linear pharmacokinetic property and a large variation in body weight was observed, the Cpss was adjusted by body weight and daily dose and called adjusted Cpss.

Associations of *CYP2D6*, *CYP3A5*, *UGT1A1*, and *ABCB1* polymorphisms with adjusted Cpss of galantamine

The result from univariate analysis showed that *CYP2D6*10/*10* (i.e. IM) trended to be associated with the higher adjusted Cpss of galantamine but the result did not reach statistical significance. In line with *CYP2D6* genotype, *CYP3A5* non-

expressors showed a trend of higher adjusted Cpss than those of *CYP3A5* expressors. There was no significant association of *ABCB1* with adjusted Cpss.

In relation to *UGT1A1* variants, there was no statistically significant difference among Cpss of galantamine of the wild type group and those of the variant groups. However, a trend of positive correlation of Cpss of galantamine with different genotypic groups was observed. The mean was 224.79, 230.42, 263.89, and 302.13 ng/mL per mg/kg for *UGT1A1*1/*1*, *UGT1A1 *1/*28*, *UGT1A1*1/*6*, and *UGT1A1 *28/*28*, respectively as shown in table 48.

Table 48 Associations of *CYP2D6*, *CYP3A5*, *UGT1A1*, and *ABCB1* polymorphisms with adjusted Cpss

Genotypes	Frequency (%)	Adjusted Cpss (ng/mL per mg/kg)	*p*-value
CYP2D6			
*CYP2D6*10* carriers	33 (64.7)	251.71±139.91	0.167
*CYP2D6*10* non-carriers	18 (35.3)	200.63±87.65	
CYP3A5			
*CYP3A5*3* expressors	30 (58.8)	215.56±99.12	0.221
*CYP3A5*3* non-expressors	21 (41.2)	259.57±154.69	
UGT1A1			
**1/*1*	29 (56.9)	224.79±96.84	0.566[§]
**1/*28*	9 (17.6)	230.42±201.21	
**1/*6*	9 (17.6)	263.89±141.23	
**28/*28*	2 (3.9)	302.13±47.10	
**6/*6*	1 (2.0)	96.20	
**6/*28*	1 (2.0)	249.80	
ABCB1 3435C>T			
CC	15 (29.4)	234.54±173.56	0.842
CT	27 (52.4)	223.88±102.86	
TT	8 (15.7)	253.75±104.50	
ABCB1 1236C>T			
CC	8 (15.7)	189.88±108.10	0.251
CT	20 (39.2)	269.75±156.14	
TT	22 (43.1)	235.81±125.84	

Note: *CYP2D6*10* carriers: *CYP2D6*1/*10*, *CYP2D6*2/*10*, *CYP2D6*10/*10*

*CYP2D6*10* non-carriers: *CYP2D6*1/*1*, *CYP2D6*2/*2*

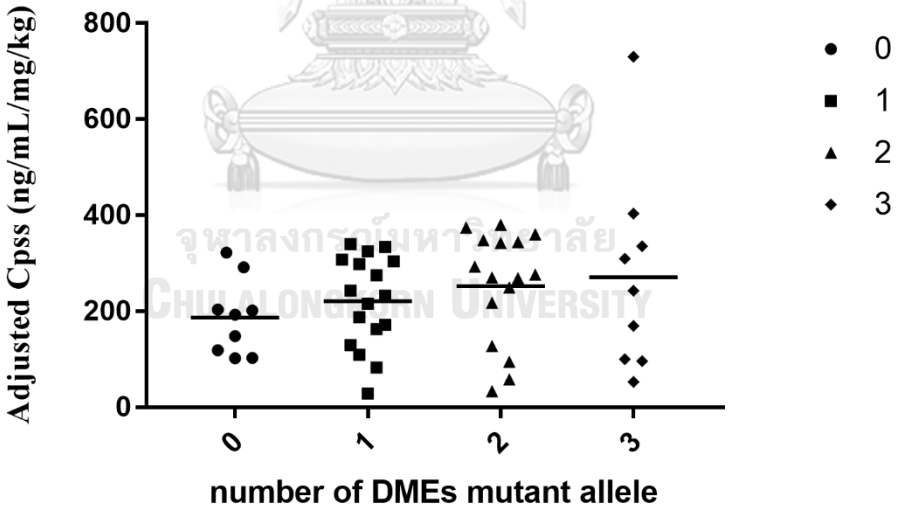
*CYP3A5*3* expressors: *CYP3A5*1/*1*, *CYP3A5*1/*3*

*CYP3A5*3* non-expressors: *CYP3A5*3/*3*

[§]*p*-value calculated by independent t-test; dominant model stratified by the presence of at least one mutant allele versus wild type

When considering the combinations of polymorphisms of the three drug metabolizing enzymes genes including *CYP2D6*, *CYP3A5*, *UGT1A1*, the patients who carry higher numbers of the mutant alleles of drug metabolizing enzyme gene showed the trend of higher adjusted Cpss of galantamine as shown in figure 7.

Figure 7 The relationship between adjusted Cpss of galantamine and the combined polymorphisms of drug metabolizing enzymes genes including *CYP2D6*, *CYP3A5*, and *UGT1A1*



Association of non-genetic factor with adjusted Cpss

Regarding the non-genetic factors, there was no significant effect of gender, a concomitant CYP2D6 inhibitor, concomitant use of memantine, time from drug intake and creatinine clearance on adjusted Cpss of galantamine. Correlation analysis revealed significant association of body weight with adjusted Cpss (r = 0.278, *p*-value

= 0.048), as well as BMI. In addition, age trend toward a positive correlation with adjusted Cpss of galantamine as shown in table 49.

Table 49 Association of the non-genetic factors and adjusted Cpss of galantamine

	Categorical variables			Continuous variables		
Factor	**Frequency (%)**	**Adjusted Cpss (ng/mL per mg/kg)**	***p*-value**	**Factor**	**Correlation coefficients (r)**	***p*-value**
Gender				**Bodyweight (kg)**	0.278	0.048
Male	21	237.642 ± 102.747	0.853	**BMI (kg/m^2)**	0.301	0.032
Female	30	230.917 ± 140.897		**Age**	0.178	0.211
Concomitant use of CYP2D6 inhibitors				**TFDI**	-0.028	0.845
No	42	230.388 ± 129.114	0.689	**CrCL**	-0.072	0.625
Yes	9	249.079 ± 112.548				
Concomitant use of memantine						
No	35	227.170 ± 101.916	0.589			
Yes	16	247.940 ± 169.217				

3.3 Combined association of genetic and non-genetic factors with adjusted Cpss of galantamine

By usinging multiple linear regression analysis, genetic and non-genetic factors were selected as covariates base on theirs clinical relevance or biological plausibility according to previous study and were introduced into the multivariate analysis. The final model is shown in table 50.

Table 50 The final model of multiple linear regression analysis of explanatory variables for adjusted Cpss of galantamine

Predictive variables	Unstandardized coefficients		Standardized coefficients	95% CI of B	*p*-value
	B	S.E.	β		
Constant	-550.648	226.68	-	-1007.797/-93.498	**0.019**
***CYP2D6* or *CYP3A5* or *UGT1A1* variants**	34.559	16.769	0.273	0.741/68.377	**0.045**
Body weights (kg)	5.103	1.771	0.447	1.531/8.674	**0.006**
Age (year)	4.751	2.021	0.335	0.675/8.827	**0.023**
***ABCB1* 3435**	-2.473	25.659	-0.013	-54.219/49.273	0.924
Gender	50.143	38.260	0.197	-27.017/127.302	0.197
R^2 = 0.259, *p*-value < 0.036					

Note: Combined *CYP2D6* or *CYP3A5* or *UGT1A1* variants:
no variant=0, one variant=1, two variants =2, three variants =3
ABCB1 3435: CC genotype =0, CT or TT genotype = 1
Gender: male =0, 1 = female

The final model of multiple linear regression analysis revealed that patients who carry a higher number of *CYP2D6*, *CYP3A5*, and *UGT1A1* mutant alleles were associated with higher adjusted Cpss of galantamine. Age and body weights have a positive correlation with adjusted Cpss. These covariates could explain interindividual variability of adjusted Cpss of galantamine for approximately 26% (R^2 = 0.259, *p*-value < 0.036).

Evaluation of factors affecting cognitive function

Associations of *CYP2D6*, *CYP3A5*, *UGT1A1*, *ABCB1,* and *APOE* polymorphisms with TMSE score

The result from univariate analysis showed that *CYP2D6*10* carriers has higher ΔTMSE score when compare with C*YP2D6*10* non-carriers (-0.571 vs -6.231; *p*-value = 0.039). Concomitantly, the trend of better clinical outcome as measured by the change of TMSE scores was found in wildtype of *UGT1A1* as compared with variants. No significant differences between clinical response and *CYP3A5*, *ABCB1*, and *APOE* genotypes was observed as show in table 51.

Table 51 TMSE score in association with *CYP2D6*, *CYP3A5*, *UGT1A1*, *ABCB1* and *APOE* polymorphisms

		TMSE		**Δ**TMSE	
	Frequency (%)	**Score**	***p*-value**	**Score**	***p*-value**
CYP2D6					
*CYP2D6*10* carriers	33	20.030 ± 5.491	0.255	-0.571 ± 2.999	**0.039**
*CYP2D6*10* non-carriers	18	17.444 ± 8.508		-6.2308 ± 8.68	
CYP3A5					
*CYP3A5*3* expressors	30	18.400 ± 7.045	0.369	-2.600 ± 6.205	0.758
*CYP3A5*3* non-expressors	21	20.143 ± 6.311		-2.000 ± 5.797	
ABCB1 3435C>T					
CC	15	18.400 ± 6.185	0.372	-0.4167± 3.343	0.157
CT	27	18.593 ± 7.386		-3.957 ± 7.023	
TT	8	22.250 ± 5.675		-0.1667 ± 4.26	
ABCB1 1236C>T					
CC	8	16.250 ± 8.172	0.351	-1.143 ± 4.705	0.685
CT	20	18.850 ± 5.788		-2.000 ± 5.148	
TT	22	20.318 ± 7.127		-3.375 ± 7.500	
APOE					
APOE ***ε****4* carriers	20	18.950 ± 6.452	0.888	-1.125 ± 4.129	0.294
APOE ***ε****4* non-carriers	31	19.226 ± 7.027		-3.160 ± 6.878	
UGT1A1					
Wild type	29	19.621 ± 7.233	0.546	-1.000 ± 5.234	0.136
Variants	21	18.454 ± 6.139		-3.800 ± 6.502	

Association of non-genetic factor and TMSE scores

Regarding the influence of non-genetic factor on the cognitive function of galantamine as shown in table 52 and 53. Concomitant use of antidepressant and memantine were associated with lower TMSE scores (*p*-value = 0.042 and 0.003 respectively) as well as concomitant statin drugs user showed a tendency toward a worse therapeutic outcome than non-users (-4.000 vs -0.474; *p*-value =0.059). Baseline TMSE was positively correlated with **Δ**TMSE score (r = 0.528, *p*-value **< 0.001**). Whereas, education levels had moderately negative correlated with **Δ**TMSE score (r = -0.413, *p*-value =0.007). There was no significant effect of different doses and **Δ**TMSE.

Table 52 Association of non-genetic factor and TMSE score

		TMSE		ΔTMSE	
	Frequency (%)	**Score**	***p*-value**	**Score**	***p*-value**
Gender					
Male	21	20.143 ± 6.077	0.369	-1.929 ± 4.009	0.741
Female	30	18.400 ± 7.185		-2.593 ± 6.846	
Concomitant use of antidepressant					
No	42	20.000 ± 6.363	**0.042**	-1.647 ± 4.572	0.328
Yes	9	15.000 ± 7.314		-5.857 ± 10.319	
Concomitant memantine					
No	35	20.971 ± 5.874	**0.003**	-1.643 ± 6.273	0.261
Yes	16	15.063 ± 6.913		-3.923 ± 5.188	
Concomitant nicergoline					
No	47	18.936 ± 6.979	0.138	-2.379 ± 6.206	0.968
Yes	4	21.250 ± 2.061		-2.250 ± 3.862	
Concomitant statin drugs					
No	26	20.462 ± 5.673	0.148	-0.474 ± 3.950	0.059
Yes	25	17.720 ± 7.564		-4.000 ± 6.983	

Table 53 Bivariate analysis: Association of non-genetic continuous variable and TMSE score

Dependent variables	TMSE		ΔTMSE	
Independent variables	Correlation coefficients (r)	*p*-value	Correlation coefficients (r)	*p*-value
Age (years)	-0.054	0.705	0.225	0.157
Baseline TMSE	0.528	**< 0.001**	-0.087	0.588
Adjusted level	0.020	0.891	0.001	0.996
Education levels	0.078	0.586	-0.413	**0.007**

4.3 Combined association of genetic and non-genetic factor with TMSE scores

Covariates were selected from the result of univariate analysis by stepwise selection which setting significant level for entry (SLE) as 0.25 and into multivariate analysis. Multivariate regression analysis was performed to evaluate the combined effects of pharmacokinetic related genes and non-genetic factors simultaneously on change of TMSE score as shown in table 54.

Table 54 The models of stepwise multiple linear regression analysis of explanatory variables for ΔTMSE in mixed dementia

Method	Predictative variables	Unstandardized coefficients		Standardized coefficients	95% CI of B	*p*-value
		B	S.E.	β		
TMSE	Constant	9.321	3.472	-	2.329/16.314	**0.010**
	*CYP2D6*10*genotypes	3.444	1.460	0.246	0.503/6.385	**0.023**
	Concomitant use of antidepressant	-5.236	1.817	-0.300	-8.905/-1.587	**0.006**
	Concomitant use of memantine	-4.415	1.643	-0.307	-7.725/-1.106	**0.010**
	Concomitant use of statin drugs	-3.051	1.384	-0.228	-5.838/-0.264	**0.033**
	Baseline TMSE score	0.533	0.144	0.416	0.242/0.823	**0.001**
R^2 = 0.524 *p*-value < 0.001						
ΔTMSE	Constant	5.269	2.753	-	-0.315/10.852	0.064
	*CYP2D6*10* genotype	5.227	1.397	0.412	2.395/8.060	**0.001**
	UGT1A1 variants	-2.794	1.321	-0.236	-5.473/-0.114	**0.041**
	Concomitant use of statin drugs	-5.245	1.349	-0.236	-7.981/-2.508	**< 0.001**
	Education level	-0.478	0.114	-0.474	-0.709/-0.247	**< 0.001**
R^2 = 0.567, *p*-value < 0.001						

Note: TMSE: adjusted for concomitant nicergoline and adjusted Cpss

ΔTMSE: adjusted for age

*CYP2D6*10* carriers: non-carrier =0, carrier = 1

UGT1A1 genotype: wild type (*UGT1A1*) = 0, *UGT1A1*6* or *UGT1A1*28* carrier=1

Concomitant use of memantine: yes = 1, no = 0

Concomitant use of antidepressant: yes = 1, no = 0

Concomitant use of statin drugs: yes = 1, no = 0

In concordance with the univariate analysis, the results of the final model from stepwise multiple linear regression analysis revealed *CYP2D6*10* carriers, baseline TMSE, concomitant memantine, concomitant use of antidepressant were significantly associated with TMSE score at steady state. These covariates could explain interindividual variability of TMSE score at steady state for approximately 52% (R^2 = 0.524, *p*-value < 0.001).

*CYP2D6*10* carriers were positively associated with **Δ**TMSE score (B = 5.227, *p*-value = 0.001). *UGT1A1* variant carriers, concomitant use of statin drugs and education levels were negatively associated with **Δ**TMSE. These covariates could explain overall inter-individual variability of **Δ**TMSE for approximately 57 % (R^2 = 0.567, *p*-value < 0.001).

Prediction of response

A total of 51 patients were enrolled in this study. At the end of follow up (6-month), 21 patients were classified as responder and the rest 20 patients were classified as non-responder.

To investigate the simultaneous effects of genetic and non- genetic factors on the clinical response to galantamine. The logistic regression analysis was further performed. Similar results as described above was found. The final logistic regression model confirmed that clinical responses to galantamine were strongly associated with *CYP2D6*10* carriers (adjusted OR = 19.784, *p*-value = 0.028) as show in table 55.

Table 55 The models of logistic regression analysis of factors associated with galantamine response

Predictive variables	Logistic coefficients (b)	Adjusted OR	95% CI	*p*-value
Genetic factors				
***CYP2D6*10* carriers**	2.985	19.784	1.384/282.849	**0.028**
***UGT1A1* variants**	-1.982	0.138	0.018/1.042	0.055
Non-genetic factors				
Education level (year)	-0.241	0.786	0.641/0.963	**0.020**
Concomitant use of statin drugs	-2.725	0.066	0.006/0.676	**0.022**
Concomitant use of memantine	-3.111	0.045	0.005/0.440	**0.008**
Hosmer and Lemeshow *p*-value = 0.416 (goodness of fit test) **model *p*-valve < 0.001** Cox & Snell R^2 = 0.475, Nagelkerke R^2 = 0.633				

Note: *CYP2D6*10* carriers: non-carrier =0, carrier = 1

UGT1A1 genotype: wild type (*UGT1A1*) = 0, *UGT1A1*6* or *UGT1A1*28* carrier=1

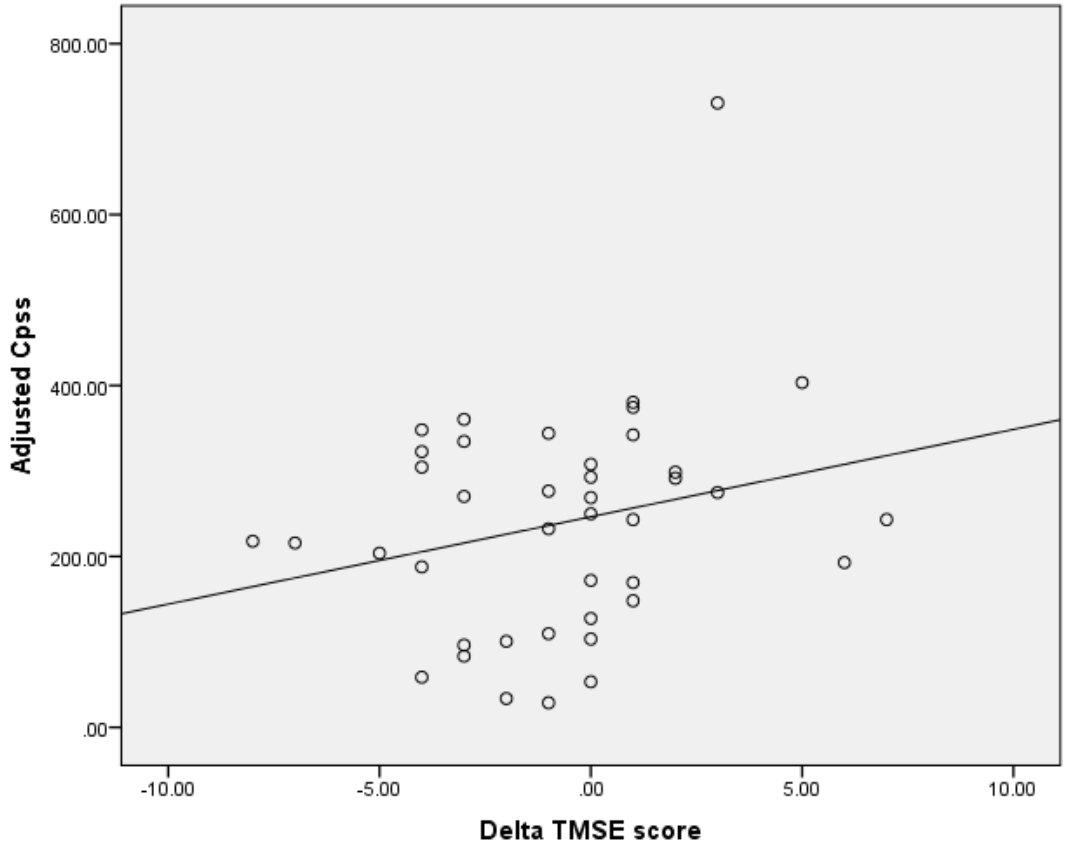
Concomitant use of statin drugs: yes = 1, no = 0

Concomitant use of memantine: yes = 1, no = 0

Correlation between adjusted Cpss and TMSE score

Pearson correlation was performed to illustrate the association between adjusted Cpss of galantamine and change in TMSE score from steady state to final observation. A scatter plot of was showed in figure 18. No significant association was found. However, a trend of positive correlation was observed (Pearson correlation coefficient (r) = 0.246, *p*-value = 0.121).

Figure 8 Scatter plot show correlation between adjusted Cpss and change in TMSE score



Evaluation of adverse drug events

The most common presenting adverse drug events of acetylcholinesterase inhibitors are gastrointestinal effects including nausea, vomiting, diarrhea followed by bradycardia. Regarding adverse drug events, no significantly association between genetic or non-genetic factors and the existence of ADRs was observed. No association was founded between adjusted Cpss of galantamine and systolic or diastolic blood pressure or pulse rate in this cohorts. A possible explanation was limit sample size small sample size and shorter follow- up periods. Moreover, patients had been administered galantamine for long time, so patients can tolerate the adverse drug event.

DISCUSSION

Previous studies elucidated the influence of *CYP2D6*, *CYP3A5* genotyping on Cpss but these studies focused only on phase I drug metabolizing gene. Therefore, the association between genetic polymorphisms of phase II drug metabolizing gene (i.e *UGT1A1*) and Cpss should be investigated. Moreover, some studies showed that *UGT1A1* play a role in xenobiotic biotransformation which involving pathogenesis of neurodegenerative disease such as AD. The allele frequencies of *UGT1A1*6* and *UGT1A1*28* which were common variants that give rise to reduced enzyme activities in Thai population were found to be as high as 9 and 17%, respectively. Consequently, the identification of UGT1A1 genotype may provide further explanation for inter-individual variability in response to galantamine. At present, the influence of SNPs *UGT1A1*6*, *UGT1A1*28* and clinical response of galantamine have not been established.

Genotype distribution

Genotype frequencies are consistency with previous reports in Asian population. No deviation from Hardy-Weinberg equilibrium were found and MAF (minor allele frequency) were consistent with the results reported by ThermoFisher which were studied in Chinese and Japanese ancestry.

Previous study did not consider *CYP2D6*2* determination. Identifying *CYP2D6*2* could provide informative prevalence of the *CYP2D6*2* allele by discriminating between *CYP2D6*1* and *CYP2D6*2*. The latter is another *CYP2D6* allele which encode enzyme with normal function reported in Thai population. Furthermore, the *CYP2D6*2* determination could reveal the *CYP2D6*2/*10* genotypes, which have not been explored in previous study.

Evaluation of factors affecting Cpss of galantamine

Associations of *CYP2D6*, *CYP3A5*, *UGT1A1* and *ABCB1* polymorphisms with adjusted Cpss of galantamine

The results from univariate analysis showed that *CYP2D6*10/*10* (i.e., IM) showed a trend to be associated with the higher adjusted Cpss of galantamine but the result does not reach statistical significance. In line with *CYP2D6* genotype, *CYP3A5*3* non-expressers showed higher adjusted Cpss than that of expressers. Although genetic variations in *CYP2D6* have been discussed to play a significant role in the inter-individual response of galantamine, there were only two studies reported an influence of *CYP2D6* genotypes on the steady-state plasma concentration and not yet study in Asian populations which IM of CYP2D6 (*CYP2D6*10/*10*) is more pronounced. Two recent studies demonstrated that poor metabolizer (PM) of *CYP2D6* has reduced clearance and increased Cpss compared to extensive metabolizer (EM)(100, 101).

Moreover, these studies focused only on phase I drug metabolizing gene. *UGT1A1* may be another gene contributing to inter-individual variability in Cpss of galantamine. This study serves as the first study that explored the effects of phase II drug metabolizing enzymes gene i.e. *UGT1A1* genotype on Cpss of galantamine. The result reveals that no significant association between *UGT1A1* genotypes and adjusted Cpss was observed. This phenomenon could be possible that there is no single dominant metabolic pathway of galantamine.

However, combinations of the three polymorphisms of drug metabolizing enzymes gene including *CYP2D6*, *CYP3A5*, *UGT1A1* trend toward associated with adjusted Cpss of galantamine. The patients who carry higher numbers of the mutant allele of drug metabolizing enzyme gene showed a trend of higher adjusted Cpss suggesting gene-dose dependent manner. In contrast to galantamine, *CYP2D6*10* genotype plays a crucial role in explaining inter-individual variability in adjusted Cpss of donepezil since donepezil prominently underwent CYP2D6 as its main metabolic pathway. These findings emphasized that polymorphisms of phase II drug metabolizing enzymes i.e. *UGT1A1* may provide further explanation in the metabolism of drug that has complicated metabolic pathway such as galantamine (136).

Further investigation of the associations of genetic factors especially genes involved in drug metabolizing enzymes, drug transporter (i.e. *CYP2D6*, *CYP3A5*, and *UGT1A1*) and certain non-genetic factors simultaneously by using multiple linear regression analysis was performed. Covariates were selected base on clinically relevant and biological plausibility and introduced into the multivariate analysis. The results confirmed that the combined effect of *CYP2D6* and *CYP3A5* and *UGT1A1* variants on adjusted Cpss of galantamine. There was no significant difference between adjusted Cpss of galantamine and *ABCB1* genotypes were observed table 51.

Association of non-genetic factors with Cpss of galantamine

The result from multivariate analysis showed that age was positively correlated with adjusted Cpss of galantamine. Itis possible that the decrease in clearance in the elderly could give rise to the elevated Cpss of galantamine. Female has a higher adjusted Cpss when compared with the male. Some studies suggest that CYP2D6 has lower activity in the female when compared with the male (137).

In contrast to the previous study of donepezil, we did not find the effects of concomitant drugs especially antidepressant drugs and memantine which have been considering as CYP2D6 inhibitors on adjusted Cpss of galantamine. This is not surprising since CYP2D6 is not the predominant elimination pathway of galantamine. However, with a relatively small number of patients who take drugs that possess CYP2D6 inhibitory activity, an accurate causative effect of concomitant CYP2D6 inhibitors on adjusted Cpss of galantamine could not be made. Studies with a larger sample size are required to confirm the association.

Combined association of genetic and non-genetic factors with adjusted Cpss

The result from the multivariate analysis is concordance with univariate analysis. The result emphasized that combined effect of *CYP2D6* and *CYP3A5* and *UGT1A1* variants toward positive associated with Cpss of galantamine. These covariates could explain the inter-individual variability of adjusted Cpss for

approximately 25%. The unexplained remaining inter-individual variability may be derived from other contributing factors such as race, concomitant use of P-glycoprotein or CYP3A4 inhibitors, and some physiological function that cannot assuredly be excluded in this cohorts. Physiological function especially creatinine clearance may have greater influence in galantamine' s metabolism since galantamine is excreted as 20% unchanged form via the kidney (3). However, in the present no association was found between adjusted Cpss of galantamine with creatinine clearance.

Evaluation of factors affecting cognitive function

Associations of *CYP2D6*, *CYP3A5*, *UGT1A1*, *ABCB1* and *APOE* polymorphisms with TMSE score

In relation to cognitive function *CYP2D6*10* carriers show a higher TMSE score when compare with non-carriers. The association of the genetic polymorphism of *CYP2D6* in susceptibility to galantamine outcomes might be explained by the following reasons. Galantamine is metabolized by CYP2D6 and human CYP2D6 in the brain was prominently localized in the pyramidal cell of the cortex and hippocampal which a certain region that accounts for cognitive function (138). Keller Connor et al. showed that galantamine increased regional cerebral blood flow in the cortical area of the frontal cortex (139). This finding implied the site of action of galantamine is in the frontal cortex, one of the regions which affected the neuropathology of AD. Consequently, *CYP2D6*10* carriers might increase galantamine level and greater inhibit acetylcholinesterase in frontal cortex resulting in the improvement of cognitive function as measured by TMSE in AD. Furthermore, Kirchheiner J et al. suggested that IM of CYP2D6 has higher brain perfusion in the hippocampus compared to EM (130). This may be one of the reasons to explain the present finding as higher brain perfusion in *CYP2D6*10* carriers could restore underlying pathological of disease and provide better response compared to *CYP2D6*10* non-carriers (EM). Further exploration of the possible explanation is that CYP2D6 might play a role in the biotransformation of several endogenous or xenobiotics in the brain. CYP2D6

phenotype also influencing neurocognition as described by Eva M Peñas-LLedó et al (138). For these reasons, it is likely to further determine whether genetic variants of *CYP2D6* could influence the progression of dementia and therapeutic outcomes of galantamine.

The negative correlation of variants allele of *UGT1A1* and ΔTMSE could be possibly described by the following reason. *UGT1A1* were expressed in the brain and may influenced in the eliminate of endogenous compounds in a region- and age-dependent manner(140). Some endogenous compounds contribute in neuropathological of brain disorder. Thus, *UGT1A1* variants with decreased function in eliminating endogenous substance could deteriorate cognitive function as measured by TMSE score. The UGT mediated neuropathology is a possible indirect effect on cognitive function in AD and might rather be a part of a complicated network of various neuropathological mechanisms.

This study serves as the first study which illustrates the negative relationship between *UGT1A1* variants with clinical response of acetylcholinesterase inhibitors such as galantamine. Further study should be performed.

Association of non-genetic factors with TMSE score

Effect of concomitant use of statin drugs

In contrast to the previous study which showed a significant benefit of statin treatment on vascular dementia, the present study found that co-administered statin drugs for treatment dyslipidemia exhibited a negative correlation with TMSE score. The possible explanation is that dyslipidemia condition can deteriorate vascular pathogenesis of mixed dementia which goes beyond the pharmacological effect of statin treatment in this study. Thus, it seems statin drug could diminish response. However, the effect of statin on dementia treatment or risk still divergence(141, 142). Several factors may contribute to these discrepancies, including the different degree of exposure including duration of use, doses, and types of statins, types of dementia and severity that could confound the outcomes(142). Moreover, *APOE* genotypes might alter the association between use of statins and treatment outcomes of

dementia (142). Nophar Geifman et.al. demonstrated that homozygous *APOE* ***ε****4* genotypes AD patients treated with statins had better cognitive function over the course of 10-year follow-up(143). A well designed randomized clinical trial using multivariate analysis to control confounding variables should be further determined.

Effect of concomitant use of antidepressants

In this study, negative impact of antidepressant drugs on TMSE score at steady state was found. It is possible that concomitant antidepressant drugs such as selective serotonin reuptake inhibitors (SSRIs) may influence cognitive function(132). These results were consistent with the findings of Wattmo et al. who showed that acetylcholinesterase treatment outcomes were diminished faster in patients with depression treated with antidepressants including SSRIs(133). The possible explanation is that depression condition can deteriorate neurocognitive function which goes beyond the pharmacological effect of antidepressant treatment. Another possible may be due to anticholinergic effect of some antidepressant drugs that may diminish the cognitive function of the patients(109).

Effect of concomitant use of memantine

In this study, patients who were taking memantine was founded to be associated with worse clinical response to galantamine. Since patients who used memantine had lower baseline TMSE scores than non-users, so the negative effect of memantine user on TMSE at steady state is reasonable.

Effect of education level

Education level showed negative association with ΔTMSE. This phenomenon was astonishing because some previous studies showed the higher education might increase initial baseline TMSE and could be expected to provide more achievement in therapeutic responses.

The result of the present study is concordance with the study of Wattmo et al. which revealed that high level of education was associated with faster cognitive deterioration and poor response to acetylcholinesterase (107). In concordance with that, the present finding showed that AD patients with higher education level had a lower cognitive score. The negative association between education and clinical response can be described by brain-reserve hypothesis which stated that patients

with higher education have higher cognitive ability, thus requiring a relatively greater burden of pathology when dementia is clinically evident (144, 145).

Moreover, these results concordant with cognitive reserve theory which coined by Stern that patients with high level of schooling may postpone the emergence of clinical manifestation and present milder symptoms and poor response of acetylcholinesterase inhibitors.

Effect of gender

This study did not find any variability in clinical response to galantamine in associate with gender. Previous study concluded that female patients seem to be more sensitive than male patients to treatment with acetylcholinesterase inhibitors and polymorphism of estrogen receptor gene (*ESR1*)(108) may contribute to inter-individual variability in therapeutic response. Other investigators founded that male patients have better clinical response to acetylcholinesterase treatment when compared with female (107).

Regarding both genetic and non-genetic factors, the different results observed in association studies may be accounted for different assessment scores or definition of response, different duration of treatment or follow up period, different in prediction of *CYP2D6* phenotypes from genotypes, different inclusion or exclusion criteria. The present study recruited patients in all severity while using initial severity as determined by baseline TMSE scores as co-variate for multivariate analysis. Moreover, we evaluate **Δ**TMSE as well as TMSE at steady state to confirm the findings.

Correlation between Cpss of galantamine and TMSE score

It remains unclear whether a higher plasma concentration of galantamine can improve cognitive outcomes. To address this question, we determined the correlation between adjusted Cpss and change of cognitive function from baseline to

final observation as measured by TMSE score. No significant association was found. However, a trend of positive correlation was observed. The correlation coefficient was rather low. It is possible that the duration of treatment and level of galantamine in the brain or cerebrospinal fluid (CSF) which would influence cognitive function response of galantamine treatment but could not be included in this cohort. However, determination of CSF drug levels is quite invasive and inappropriate in routinely clinical setting. The impact of Cpss on the efficacy and tolerability of galantamine should be further determined. Moreover, It is possible that other genetic variations besides drug metabolizing enzyme gene such as cholineacetyltransferase (134), butyrylcholinesterase(94) might be associated with clinical response.

CHAPTER 6 CONCLUSIONS

Pharmacogenetic association study of donepezil

Patients with AD or VAD carrying *CYP2D6*10* allele were associated with higher Cpss of donepezil and better therapeutic outcomes, in AD. Non-genetic factors including concomitant memantine use was also significantly associated with increased Cpss of donepezil. Whereas, concomitant antidepressant treatment and age may attenuate clinical responses in AD and VAD, respectively. The negative impact of concomitant antidepressant treatment on donepezil outcomes should be further investigated. There was no statistically significant association of *CYP3A5* and *ABCB1* genetic polymorphisms with Cpss or cognitive response as measured by TMSE score. In overall, the findings suggest no significant effect of the *APOE* genotypes on clinical outcome of donepezil. Determination of genetic factors i.e. *CYP2D6*10* genotypes together with non-genetic factors including individual demographics and concomitant drug exposure could be useful for tailoring of donepezil treatment in the forthcoming personalized medicine.

Pharmacogenetic association study of galantamine

Genetic variations in genes participating metabolic pathways (*CYP2D6*, *CYP3A5*, and *UGT1A1*) are likely to synergistically influence the interindividual Cpss of galantamine because of it complicates metabolic pathways. In addition to *CYP2D6*10,* polymorphism of *UGT1A1* gene which encode phase II drug metabolizing enzyme, might partially be associated with clinical response of galantamine. However, additional study with larger sample size is required to confirm these association. Non-genetic factors including age and gender might be influenced on adjusted Cpss. These findings provide additional evidence that concomitant statin and higher education level could attenuate clinical response. This is the first findings to illustrate the influence of genetic and non-genetic factors on Cpss and therapeutic outcomes of galantamine in mixed dementia. Determination of drug metabolizing

genetic polymorphisms together with non-genetic factors including individual demographics and concomitant could be useful for tailoring the therapeutic outcome of galantamine in patients with mixed dementia in forthcoming aging societies.

By identifying of certain candidate genetic variants that responsible for drug metabolism or transporter genes and pathogenic gene together with non-genetic factors could provide more information to understand the inter-individual clinical response of donepezil and galantamine treatment. The present findings highlight the possibility of using genetic testing to guide personalized dementia therapy with donepezil and galantamine in the forthcoming personalized medicine era.

Strength

To our knowledge, these are the very first pharmacogenetic studies of donepezil and galantamine conducted in Thai populations. The findings gain information from clinical practice.

The studies examined several genes including phase I and phase II drug metabolizing enzymes gene (*CYP2D6*, *CYP3A5*, *UGT1A1*), transporter gene (*ABCB1*) pathological gene (*APOE*) and certain non-genetic factors simultaneously that could have an influence on Cpss as well as the therapeutic outcome of donepezil and galantamine by using multivariate analysis. The use of multivariate analysis could identify covariables that could better explain inter-individual variability in clinical response than the univariate analysis.

By using clinical setting, several non-genetic factors especially age, gender, and concomitant drugs were not limited and tested as non-genetic covariates in the multivariate analysis.

Limitation

The studies were performed in tertiary medical school, it remains to be warranted whether the findings are applicable to other cohorts especially in rural

community setting which different healthcare policy. Although, dementia management including diagnosis and treatment was appropriately performed in tertiary medical school but a large number of demented patients live in rural communities. Therefore, prospective study in multicenter larger cohort of patients should be conducted.

The evaluation of cognitive performance using only TMSE scores instead of full set of measurement might have some limitation. TMSE score is less sensitivity to identify mild cognitive impairment especially in patients with high educational level. Moreover, TMSE cannot distinguish a small clinical change in severe AD patients (Ceiling and Flooring effect). However, it has been suggested that TMSE is sensitive and specific enough for examination the therapeutic outcome and appropriate in routine clinical setting which have limited specialist and time. Evaluation of treatment outcomes by TMSE can be finished in approximately 10 minutes whereas, Alzheimer's Disease Assessment Scale - Cognitive section (ADAS-Cog), a well-established scale for evaluate cognitive function could take around 1 hours which is impractical for routine clinical practices.

The assessment of Behavioral and Psychological Symptoms of Dementia (BPSD) symptoms which may influence cognitive function score was not performed in this study. However, this limitation was apparent in most of previous studies. However, to compensate somewhat for this limitation, the concomitant antidepressant drugs were introduced into multivariate model and significant influence on clinical outcome was founded.

Future prospective

To confirm the association of Cpss with genetic or non-genetic factors. Population pharmacokinetic study can be subjected of the further investigations. Population pharmacokinetic analysis is a suitable method for sparse data. Moreover,

it can eliminate the effect of the time differences of drug ingestion and blood sampling for each individual person.

In addition, to provide better understanding of the underlying mechanism of variability in clinical response. Future investigation should be performed by using neuroimaging particularly the use of amyloid PET scan for evaluation clinical response in addition to TMSE score. Moreover, the neurophysiological and neuropathological in associated with cognitive function can be drawn from neuroimaging such as MRI. Neuroimaging might also serve as a novel surrogate outcome for evaluating therapeutic effect of acetylcholinesterase inhibitors in dementia patients.

Prospective study, especially randomized controlled trials with stratification on doses of galantamine or donepezil according to individual genotypes, should be conducted.

The findings cannot fully elaborate all of factors which affect association study. The weakness attributable to residual confounding from unknown or unmeasured co-variate. To overcome confounding bias, the introduction of appropriate covariates base on clinical relevance and biological plausibility that may confound the association study. However, the study cannot rule out the residual un-explain confounders. Others non-genetic factors including smoking, foods and behavior can affect therapeutic outcomes. Notably, the genetic variations in pharmacodynamic gene such as acetylcholinesterase, butyrylcholinesterase, choline acetyltransferase, and nicotine acetylcholine receptors which might have an influence on clinical response of acetylcholinesterase inhibitors were not identified in the present study.

It should be acknowledged that the association study provided plausible clues for possibly describing association but not prove a causal relationship. Only

statistical procedure alone cannot demonstrate a relationship between an associated factors and outcome is causal. Causality is established on the basis of biological plausibility and well-designed study which minimize sources of potential bias. Therefore, any findings from pharmacogenetic studies should be replicated in a well study designed

The molecular mechanism of such relationship especially the role of ABCB1, CYP2D6 and UGT1A1 for xenobiotic disposition in CNS should be further investigate.

APPENDIX

APPENDIX I

Abbrevations

AD	Alzheimer's disease
CrCL	creatinine clearance
Cpss	Steady state plasma concentration
MAF	Minor allele frequency
MMSE score	Mini-Mental State Examination score
MCI	Mild cognitive impairment
TMSE score	Thai Mental State Examination score
VAD	Vascular dementia
R^2	determination coefficient
B	unstandardized regression coefficients
β	standardized regression coefficients
VIF	Variance Inflation Factor
rs	reference SNP
Kg	Kilogram
95% CI	95 % confidence intervals

APPENDIX II

Full method validation of determination Cpss

Donepezil

Table 1 Lower limit of quantification (LLOQ) of unextracted donepezil

LLOQ	Concentration of Donepezil (ng/mL)						Mean	CV	RV
(ng/mL)	N1	N2	N3	N4	N5	N6	(ng/mL)	(%)	(%)
10	10.70059	10.58035	10.12097	10.39648	10.56771	10.47826	10.4732	1.92	104.74

Table 2 Linearity data of donepezil in human plasma for 3 days

Nominal concentration	Experimental concentration (ng/mL)			Mean	S.D.	%CV	%Recovery
(ug/mL)	Day 1	Day 2	Day 3				
10.00	10.7006	10.1210	10.5677	10.1407	0.7887	7.78	101.41
	8.5804	10.3965	10.4783				
50.00	54.4585	51.4151	47.7557	49.2767	3.0281	6.15	98.55
	48.1152	46.8902	47.0259				
100.00	94.4978	105.0947	99.7747	99.1584	3.9952	4.03	99.16
	100.2738	94.7649	100.5445				
150.00	151.3672	153.3597	153.7386	152.6063	2.6737	1.75	101.74
	154.9332	147.7637	154.4755				
200.00	197.0325	204.5235	198.9904	199.5297	2.6107	1.31	99.76
	199.2088	197.9460	199.4769				
250.00	254.5002	244.9874	246.6819	249.2881	3.8609	1.55	99.72
	246.3320	252.7373	250.4900				
r^2	0.998483	0.998324	0.999259				

Table 3 Average data for linearity of donepezil in human plasma

Nominal concentration (ng/mL)	Black calculate concentration (ng/mL)	% Nominal value
10.00	10.1407	101.41
50.00	49.2767	98.55
100.00	99.1584	99.16
150.00	152.6063	101.74
200.00	199.5297	99.76
250.00	249.2881	99.72

Table 4 Accuracy and precision of LLOQ, LQC, MQC, and HQC

(10, 30, 120, and 220 ng/mL) of within-batch human plasma donepezil

Sample Number	Within-batch Accuracy & Precision (Day 1)							
	LLOQ (10 ng/mL)		LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
1	9.32	93.20	28.93	96.43	114.98	95.82	223.30	101.50
2	10.05	100.50	29.80	99.33	117.66	98.05	222.26	101.03
3	10.07	100.70	30.04	100.13	115.70	96.42	221.05	100.48
4	10.65	106.50	31.94	106.47	116.77	97.31	227.01	103.19
5	9.25	92.50	29.04	96.80	121.45	101.21	224.08	101.85
6	10.67	106.70	29.95	99.83	121.28	101.07	228.10	103.68
Mean	10.0017	100.02	29.9500	99.83	117.9733	98.31	224.3000	101.95
SD	0.6170		1.0825		2.7819		2.7407	
% CV	6.17		3.61		2.36		1.22	
Sample Number	**Within-batch Accuracy & Precision (Day 2)**							
	LLOQ (10 ng/mL)		**LQC (30 ng/mL)**		**MQC (120 ng/mL)**		**HQC (220 ng/mL)**	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
1	9.74	97.40	30.07	100.23	119.49	99.58	222.85	101.30
2	10.24	102.40	30.63	102.10	123.27	102.73	226.31	102.87
3	10.03	100.30	28.99	96.63	122.12	101.77	221.43	100.65
4	9.94	99.40	30.62	102.07	122.46	102.05	229.38	104.26
5	10.63	106.30	29.23	97.43	118.12	98.43	225.10	102.32
6	9.77	97.70	30.89	102.97	121.04	100.87	227.62	103.46
Mean	10.0583	100.58	30.0717	100.24	121.0833	100.90	225.4483	102.48
SD	0.3344		0.7258		1.9531		2.9643	
% CV	3.32		2.41		1.61		1.31	

Sample Number	Within-batch Accuracy & Precision (Day 3)							
	LLOQ (10 ng/mL)		LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
1	9.81	98.10	28.79	95.97	112.40	93.67	220.73	100.33
2	9.70	97.00	30.98	103.27	121.17	100.98	215.27	97.85
3	9.67	96.70	30.29	100.97	116.90	97.42	225.16	102.35
4	9.93	99.30	31.08	103.60	109.66	91.38	219.18	99.63
5	9.56	95.60	29.85	99.50	110.84	92.37	220.50	100.23
6	10.44	104.40	29.06	96.87	111.25	92.71	223.85	101.75
Mean	9.8517	98.52	30.0083	100.03	113.7033	94.75	220.7817	100.36
SD	0.3147		0.9572		4.4325		3.5103	
% CV	3.19		3.19		3.90		1.59	

Table 5 Accuracy and precision of LLOQ, LQC, MQC, and HQC

(10, 30, 120, and 220 ng/mL) of between-batch human plasma donepezil

Sample / Batch	Between-batch Accuracy & Precision							
	LLOQ (10 ng/mL)		LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
Day 1	9.32	93.20	28.93	96.43	114.98	95.82	223.30	1488.67
	10.05	100.50	29.80	99.33	117.66	98.05	222.26	1481.73
	10.07	100.70	30.04	100.13	115.70	96.42	221.05	1473.67
	10.65	106.50	31.94	106.47	116.77	97.31	227.01	1513.40
	9.25	92.50	29.04	96.80	121.45	101.21	224.08	1493.87
	10.67	106.70	29.95	99.83	121.28	101.07	228.10	1520.67
Day 2	9.74	97.40	30.07	100.23	119.49	99.58	222.85	1485.67
	10.24	102.40	30.63	102.10	123.27	102.73	226.31	1508.73
	10.03	100.30	28.99	96.63	122.12	101.77	221.43	1476.20
	9.94	99.40	30.62	102.07	122.46	102.05	229.38	1529.20
	10.63	106.30	29.23	97.43	118.12	98.43	225.10	1500.67
	9.77	97.70	30.89	102.97	121.04	100.87	227.62	1517.47
Day 3	9.81	98.10	28.79	95.97	112.40	93.67	220.73	1471.53
	9.70	97.00	30.98	103.27	121.17	100.98	215.27	1435.13
	9.67	96.70	30.29	100.97	116.90	97.42	225.16	1501.07
	9.93	99.30	31.08	103.60	109.66	91.38	219.18	1461.20
	9.56	95.60	29.85	99.50	110.84	92.37	220.50	1470.00
	10.44	104.40	29.06	96.87	111.25	92.71	223.85	1492.33
Mean	9.9706	99.71	30.0100	100.03	117.5867	97.99	223.5100	101.60
SD	0.4267		0.8959		4.3436		3.5484	
% CV	4.28		2.99		3.69		1.59	

Table 6 Recovery of extraction of donepezil in human plasma

Assay no.	Concentration of Donepezil (ng/mL)					
	LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Unextract	Extract	Unextract	Extract	Unextract	Extract
1	2923	2279	7128	6163	13873	11611
2	2849	2483	8046	6835	13917	11580
3	2714	2344	8039	6580	14446	11650
4	2890	2530	7829	6344	13705	11982
5	2790	2490	7746	6466	13566	12170
6	2840	2353	7236	6532	14105	12551
Mean	2834.33	2413.17	7670.67	6486.67	13935.33	11924.00
SD	74.37	100.83	397.67	227.00	310.89	386.88
%CV	2.62	4.18	5.18	3.50	2.23	3.24
% Absolute Recovery	85.14		84.56		85.57	

Table 7 Recovery of extraction of Internal standard (Diphenhydramine)

Assay no.	Concentration of Diphenhydramine (200 ng/mL)	
	Un-extracted	Extract
1	13546	10155
2	12784	10368
3	14572	10000
4	14463	10549
5	14435	10654
6	13655	10833
Mean	13909.1667	10426.5000
SD	704.9923	313.3820
%CV	5.07	3.01
% Absolute Recovery	74.96	

Table 8 Recovery of extraction Donepezil and Diphenhydramine

Analyte	Concentration (ug/mL)	Absolute Recovery (% Mean)
Donepezil	30.00	85.14
(n=6)	120	84.56
	220	85.57
Diphenhydramine	200	74.96
(n=6)		
N = number of replicates		

Table 9 Human Donepezil concentration in spiked human plasma samples at 30, 120, and 220 ng/mL before and after freeze and thaw condition 3 cycles

	Concentration of Donepezil(ng/mL)					
	LQC (30 ng/mL)		**MQC (120 ng/mL)**		**HQC (220 ng/mL)**	
	Fresh	After 3 cycles	Fresh	After 3 cycles	Fresh	After 3 cycles
	30.29	29.06	112.40	122.61	220.73	240.15
	29.85	31.14	121.17	119.08	219.18	234.98
	29.06	29.95	116.90	125.79	220.50	233.44
Mean	29.7333	30.0500	116.8233	122.4933	220.1367	236.1900
SD	0.6232	1.0436	4.3855	3.3565	0.8364	3.5148
% CV	2.10	3.47	3.75	2.74	0.38	1.49
% Recovery	99.11	100.17	97.35	102.08	100.06	107.36
% Variation	1.07		4.85		7.29	

Table 10 Long term stability of Galantamine in spiked human plasma samples at 30, 120 and 220 ng/mL (6-months)

	Concentration of Donepezil(ng/mL)		
	LQC (30 ng/mL)	**MQC (120 ng/mL)**	**HQC (220 ng/mL)**
	Fresh	Fresh	Fresh
	30.29	112.40	220.73
	29.85	121.17	219.18
	29.06	116.90	220.50
Mean	29.733	116.823	220.136
SD	0.6232	4.3855	0.8364
% CV	2.10	3.75	0.38
% Recovery	99.11	97.35	100.06

Table 11 Short term stability of Galantamine in spiked human plasma samples at 30, 120, and 220 ng/mL

	Concentration of Donepezil (ng/mL)					
	LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Fresh	After Thawed for 4 hr	Fresh	After Thawed for 4 hr	Fresh	After Thawed for 4 hr
	30.29	29.37	112.40	121.75	220.73	235.59
	29.85	30.01	121.17	123.44	219.18	222.47
	29.06	30.10	116.90	124.50	220.50	220.50
Mean	29.7333	29.8267	116.8233	123.2300	220.1367	226.1867
SD	0.6232	0.3980	4.3855	1.3870	0.8364	8.2029
% CV	2.10	1.33	3.75	1.13	0.38	3.63
% Recovery	99.11	99.42	97.35	102.69	100.06	102.81
% Variation	0.31		5.48		2.75	

Table 12 Auto-sampler stability of donepezil in spiked human plasma samples at 30, 120, and 220 ng/mL

	Concentration of Donepezil (ng/mL)					
	LQC (30 ng/mL)		**MQC (120 ng/mL)**		**HQC (220 ng/mL)**	
	Fresh	After 10 hr	Fresh	After 10 hr	Fresh	After 10 hr
	30.29	30.07	112.40	119.49	220.73	222.85
	29.85	30.63	121.17	118.12	219.18	221.43
	29.06	30.62	116.90	121.04	220.50	225.10
Mean	29.7333	30.4400	116.8233	119.5500	220.1367	223.1267
SD	0.6232	0.3205	4.3855	1.4609	0.8364	1.8506
% CV	2.10	1.05	3.75	1.22	0.38	0.83
% Recovery	99.11	101.47	97.35	99.63	100.06	101.42
% Variation	2.38		2.33		1.36	

Galantamine

Table 1 Lower limit of quantification (LLOQ) of unextracted galantamine

LLOQ	Concentration of Galantamine (ng/mL)						Mean	CV	RV
(ng/mL)	N1	N2	N3	N4	N5	N6	(ng/mL)	(%)	(%)
10	10.2791	10.0048	9.0886	10.4278	11.8372	11.8966	10.3275	10.33	105.89

Table 2 Linearity data of galantamine in human plasma for 3 days

Nominal concentration	Experimental concentration (ng/mL)			Mean	S.D.	%CV	%Recovery
(ug/mL)	Day 1	Day 2	Day 3				
10.00	8.2791	9.0886	11.8372	9.9223	1.7258	17.39	99.22
	8.0048	10.4278	11.8966				
50.00	53.5476	49.4934	49.5685	49.2922	2.6782	5.43	98.58
	45.6458	50.1188	47.3795				
100.00	106.4026	100.8664	102.6449	102.0349	3.0287	2.97	102.03
	103.1692	101.9661	97.1605				
150.00	147.3053	151.3373	144.8405	149.4470	2.7336	1.83	99.63
	151.3445	150.9234	150.9309				
200.00	204.7763	198.3000	201.1153	198.2050	5.7022	2.88	99.10
	190.2725	192.5539	202.2118				
250.00	256.7871	250.8708	252.8636	251.0985	4.4910	1.79	100.44
	244.4652	254.0535	247.5508				
r^2	**0.996471**	**0.998984**	**0.999071**				

Table 3 Average data for linearity of galantamine in human plasma

Nominal concentration (ng/mL)	Black calculate concentration (ng/mL)	% Nominal value
10.00	9.9223	99.22
50.00	49.2922	98.58
100.00	102.0349	102.03
150.00	149.4470	99.63
200.00	198.2050	99.10
250.00	251.0985	100.44

Table 4 Accuracy and precision of LLOQ, LQC, MQC, and HQC (10, 30, 120, and 220 ng/mL) of within-batch human plasma galantamine

Sample Number	Within-batch Accuracy & Precision (Day 1)							
	LLOQ (10 ng/mL)		LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
1	10.28	102.79	29.26	97.53	118.39	98.66	220.75	100.34
2	10.00	100.05	29.93	99.77	119.85	99.88	219.71	99.87
3	10.73	107.31	32.06	106.87	117.09	97.58	217.31	98.78
4	10.46	104.56	29.61	98.70	118.52	98.77	221.72	100.78
5	10.18	101.82	30.84	102.80	117.65	98.04	219.21	99.64
6	10.91	109.08	30.11	100.37	120.43	100.36	223.72	101.69
Mean	10.4267	104.27	30.3017	101.01	118.6550	98.88	220.4033	100.18
SD	0.3418		1.0119		1.2749		2.2058	
% CV	3.28		3.34		1.07		1.00	
Sample Number	Within-batch Accuracy & Precision (Day 2)							
	LLOQ (10 ng/mL)		LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
1	9.09	90.89	30.09	100.30	116.74	97.28	221.04	100.47
2	10.43	104.28	30.28	100.93	117.62	98.02	221.11	100.50
3	10.77	107.67	32.02	106.73	118.48	98.73	218.77	99.44
4	10.11	101.06	29.97	99.90	119.75	99.79	221.91	100.87
5	11.45	114.45	29.51	98.37	117.27	97.73	216.88	98.58
6	10.78	107.85	30.37	101.23	116.12	96.77	220.72	100.33
Mean	10.4366	104.37	30.3733	101.24	117.6633	98.05	220.0717	100.03
SD	0.7968		0.7862		1.2971		1.8813	
% CV	7.63		2.59		1.10		0.85	
Sample	Within-batch Accuracy & Precision (Day 3)							

Number	LLOQ (10 ng/mL)		LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
1	11.84	118.37	29.84	99.47	119.58	99.65	216.73	98.51
2	11.90	118.97	30.12	100.40	122.32	101.93	219.21	99.64
3	10.96	109.56	29.64	98.80	120.29	100.24	225.50	102.50
4	10.02	100.15	30.50	101.67	118.10	98.42	221.26	100.57
5	10.07	100.75	28.24	94.13	119.87	99.89	214.27	97.40
6	10.13	101.34	29.04	96.80	119.22	99.35	222.36	101.07
Mean	10.8189	108.19	29.5633	98.54	119.8967	99.91	219.8883	99.95
SD	0.8816		0.8118		1.4010		4.0369	
% CV	8.15		2.75		1.17		1.84	

Table 5 Accuracy and precision of LLOQ, LQC, MQC, and HQC (10, 30, 120, and 220 ng/mL) of between-batch human plasma galantamine

Sample / Batch	Between-batch Accuracy & Precision							
	LLOQ (10 ng/mL)		LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy	Measured Value	% Accuracy
Day 1	10.28	102.79	29.26	97.53	118.39	98.66	220.75	100.34
	10.00	100.05	29.93	99.77	119.85	99.88	219.71	99.87
	10.73	107.31	32.06	106.87	117.09	97.58	217.31	98.78
	10.46	104.56	29.61	98.70	118.52	98.77	221.72	100.78
	10.18	101.82	30.84	102.80	117.65	98.04	219.21	99.64
	10.91	109.08	30.11	100.37	120.43	100.36	223.72	101.69
Day 2	9.09	90.89	30.09	100.30	116.74	97.28	221.04	100.47
	10.43	104.28	30.28	100.93	117.62	98.02	221.11	100.50
	10.77	107.67	32.02	106.73	118.48	98.73	218.77	99.44
	10.11	101.06	29.97	99.90	119.75	99.79	221.91	100.87
	11.45	114.45	29.51	98.37	117.27	97.73	216.88	98.58
	10.78	107.85	30.37	101.23	116.12	96.77	220.72	100.33
Day 3	11.84	118.37	29.84	99.47	119.58	99.65	216.73	98.51
	11.90	118.97	30.12	100.40	122.32	101.93	219.21	99.64
	10.96	109.56	29.64	98.80	120.29	100.24	225.50	102.50
	10.02	100.15	30.50	101.67	118.10	98.42	221.26	100.57
	10.07	100.75	28.24	94.13	119.87	99.89	214.27	97.40
	10.13	101.34	29.04	96.80	119.22	99.35	222.36	101.07
Mean	10.5607	105.61	30.0794	100.26	118.7383	98.95	220.1211	100.06
SD	0.6964		0.9247		1.5601		2.7043	
% CV	6.59		3.07		1.31		1.23	

Table 6 Recovery of extraction of Galantamine in human plasma

Assay no.	Concentration of Galantamine (ng/mL)					
	LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Unextract	Extract	Unextract	Extract	Unextract	Extract
1	692	568	4012	3526	7551	6095
2	627	576	4018	3504	7408	5965
3	613	563	4032	3546	7604	5945
4	605	587	4008	3484	7585	6024
5	694	523	4079	3534	7515	6025
6	640	546	4097	3416	7456	6055
Mean	645.17	560.50	4041.00	3501.67	7519.83	6018.17
SD	38.94	22.90	37.74	47.45	76.04	55.72
%CV	6.04	4.09	0.93	1.36	1.01	0.93
% Absolute Recovery	86.88		86.65		80.03	

Table 7 Recovery of extraction of Internal standard (Voriconazole)

Assay no.	Concentration of Voriconazole (3,000 ng/mL)	
	Un-extracted	Extract
1	4599	3360
2	4528	3411
3	4616	3949
4	4559	3404
5	4637	3233
6	4737	3729
Mean	4612.6667	3514.3333
SD	72.5222	268.6393
%CV	1.57	7.64
% Absolute Recovery	76.19	

Table 8 Recovery of extraction Galantamine and Voriconazole

Analyte	Concentration (ug/mL)	Absolute Recovery (% Mean)
Galantamine	30.00	86.88
(n=6)	120	86.65
	220	80.03
Voriconazole	3,000	76.19
(n=6)		
n = number of replicates		

Table 9 Human Galantamine concentration in spiked human plasma samples at 30, 120, and 220 ng/mL before and after freeze and thaw condition 3 cycles

	Concentration of Galantamine (ng/mL)					
	LQC (30 ng/mL)		**MQC (120 ng/mL)**		**HQC (220 ng/mL)**	
	Fresh	After 3 cycles	Fresh	After 3 cycles	Fresh	After 3 cycles
	29.84	31.45	119.58	120.88	219.21	223.79
	30.12	30.00	120.29	118.57	221.26	218.04
	29.64	31.13	119.87	118.79	222.36	222.03
Mean	29.8667	30.8600	119.9133	119.4133	220.9433	221.2867
SD	0.2411	0.7618	0.3570	1.2749	1.5987	2.9462
% CV	0.81	2.47	0.30	1.07	0.72	1.33
% Recovery	99.56	102.87	99.93	99.51	100.43	100.58
% Variation	3.33		-0.42		0.16	

Table 10 Long term stability of Galantamine in spiked human plasma samples at 30, 120 and 220 ng/mL (6-months)

	Concentration of Galantamine(ng/mL)					
	LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Fresh	After 6 months	Fresh	After 6 months	Fresh	After 6 months
	29.84	30.25	119.58	119.37	219.21	216.67
	30.12	30.18	120.29	119.13	221.26	217.91
	29.64	29.66	119.87	122.31	222.36	216.13
Mean	29.8667	30.0300	119.9133	120.2700	220.9433	216.9033
SD	0.2411	0.3223	0.3570	1.7708	1.5987	0.9127
% CV	0.81	1.07	0.30	1.47	0.72	0.42
% Recovery	99.56	100.10	99.93	100.23	100.43	98.59
% Variation	0.55		0.30		-1.83	

Table 11 Short term stability of Galantamine in spiked human plasma samples at 30, 120, and 220 ng/mL

	Concentration of Galantamine(ng/mL)					
	LQC (30 ng/mL)		MQC (120 ng/mL)		HQC (220 ng/mL)	
	Fresh	After Thawed for 4 hr	Fresh	After Thawed for 4 hr	Fresh	After Thawed for 4 hr
	29.84	30.30	119.58	116.36	219.21	217.58
	30.12	29.85	120.29	117.81	221.26	219.12
	29.64	29.29	119.87	119.93	222.36	220.77
Mean	29.8667	29.8133	119.9133	118.0333	220.9433	219.1567
SD	0.2411	0.5060	0.3570	1.7954	1.5987	1.5953
% CV	0.81	1.70	0.30	1.52	0.72	0.73
% Recovery	99.56	99.38	99.93	98.36	100.43	99.62
% Variation	-0.18		-1.57		-0.81	

Table 12 Auto-sampler stability of Galantamine in spiked human plasma samples at 30, 120, and 220 ng/mL

	Concentration of Galantamine(ng/mL)					
	LQC (30 ng/mL)		**MQC (120 ng/mL)**		**HQC (220 ng/mL)**	
	Fresh	After 10 h	Fresh	After 10 h	Fresh	After 10 h
	29.84	30.07	119.58	119.49	219.21	222.85
	30.12	30.63	120.29	118.12	221.26	221.43
	29.64	30.62	119.87	121.04	222.36	225.10
Mean	29.8667	30.4400	119.9133	119.5500	220.9433	223.1267
SD	0.2411	0.3205	0.3570	1.4609	1.5987	1.8506
% CV	0.81	1.05	0.30	1.22	0.72	0.83
% Recovery	99.56	101.47	99.93	99.63	100.43	101.42
% Variation	1.92		-0.30		0.99	

APPENDIX III

Ethic Document

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Siriraj Institutional Review Board
Certificate of Approval (Renewal)

COA no. Si 818/2016

Protocol Title(English) : Association of genetic factors and non-genetic factors with clinical response of Donepezil and Galantamine in Thai patients with Dementia

Protocol Title(Thai) : ความสัมพันธ์ระหว่างปัจจัยทางพันธุกรรมและปัจจัยที่ไม่เกี่ยวข้องกับพันธุกรรม กับผลการตอบสนองทางคลินิกของยาโดนเพซิลหรือกาแลนทามีนในผู้ป่วยโรคความจำเสื่อมชาวไทย

Protocol number : 539/2559(EC1)

Principal Investigator/Affiliation : Mr.Thitipon Yaowaluk / Faculty Of Pharmaceutical Sciences Chulalongkorn University

Research site : Faculty of Medicine Siriraj Hospital

Renewal date (2nd) : December 27, 2018

Expired date : December 26, 2019

This is to certify that Siriraj Institutional Review Board is in full compliance with International Guidelines For Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonizationin Good Clinical Practice (ICH-GCP)

(Prof. Chairat Shayakul, M.D.)
Chairperson

- 4 JAN 2019
date

(Prof.Dr. Prasit Watanapa, M.D., Ph.D.)
Dean of Faculty of Medicine Siriraj Hospital

-7 JAN 2019
date

Approval Includes :

1. SIRB submission form amendment 1, dated 19 Sep 2017
2. Protocol
3. Participant information sheet, dated 17 Jan 2018
4. Informed consent form, dated 17 Jan 2018
5. Telephone script
6. Case record form
7. Curriculum vitae

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