ROLE OF CYTOCHROME P450 FATTY ACID METABOLISM IN THE PATHOPHYSIOLOGY OF SUBARACHNOID HEMORRHAGE

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Subarachnoid hemorrhage (SAH) stroke is associated with high rates of morbidity and mortality. One of the major complications of SAH is the development of delayed cerebral ischemia (DCI) due to vasospasm of the cerebrovasculature that typically occurs days after the hemorrhage. Currently, there are no established prognostic indicators of DCI and long-term outcomes in SAH patients. 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) are eicosanoids formed from the oxidation of arachidonic acid by cytochrome P450 (CYP) enzymes. 20-HETE constricts cerebral arteries and contributes to cerebral ischemic injury after SAH. Conversely, EETs dilate the cerebrovasculature and attenuate cerebral ischemic injury. EETs biological action is regulated by its metabolism to inactive DHETs. Human polymorphisms in CYP eicosanoid biosynthesis/metabolism genes are reported to alter enzyme function *in vitro/in vivo*. Thus, we hypothesized that polymorphisms in genes involved in CYP eicosanoid biosynthesis and metabolism will lead to increased 20-HETE or decreased EET concentrations in CSF resulting in the development of ischemic complications and unfavorable long-term functional outcomes in 363 patients with SAH. Patients were genotyped and CYP-eicosanoid CSF levels were measured over 14 days after hemorrhage. Acute outcomes assessed included delayed cerebral ischemia (DCI) and clinical neurological deterioration (CND) over 14 days. Modified Rankin Score (MRS) at 3 and 12 months were obtained for long-term outcome assessment. Multivariate analysis controlled for age, sex, race, and Fisher grade or Hunt & Hess score. Patients with CND and unfavorable 3-month MRS had ~2.2- and 2.7-fold higher mean 20-HETE CSF levels, respectively. Patients in high/moderate 20-HETE trajectory groups (35.7%) were 2.1-, 2.5-, and 2.1-fold more likely to have CND and unfavorable MRS at 3 and 12 months. CYP2C8*4 allele-carriers had 44% and 36% lower mean EET and DHET CSF levels and were 2.2- and 2.5-fold more likely to develop DCI and CND, respectively. Multiple loss-of-function SNPs were associated with lower CYP eicosanoid CSF levels and altered risk for unfavorable outcomes. Several CYP4F2 genotype frequencies differed from Hapmap database indicating putative genetic markers for SAH risk. These are the first clinical data demonstrating an association between genetic polymorphisms, CYP eicosanoid CSF levels, and outcomes in SAH patients.

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PREFACE

First and foremost, I would like to thank my wonderful parents Gail L. Donnelly and Richard C. Donnelly who have provided me with encouragement and support in my educational endeavors and unconditional love throughout the years. I continue to be impressed with their character and feel that they serve as an ideal role model for me to achieve a happy and fulfilling life. I hope this accomplishment makes them proud and that they understand it would not be possible without their support.

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ABBREVIATIONS

20-HETE:	20-hydroxyeicosatetraenoic acid
5-HT:	serotonin
AHA:	American Heart Association
ATP:	adenosine triphosphate
BBB:	blood brain barrier
BOXes:	bilirubin oxidation products
CBF:	cerebral blood flow
cGMP:	cyclic guanosine monophosphate
CND:	clinical neurological deterioration
CO:	carbon monoxide
CPP:	cerebral perfusion pressure
CSF:	cerebrospinal fluid
CT:	computed tomography
CVD:	cardiovascular disease
CVR:	cerebrovascular resistance
CYP:	cytochrome P450
COX:	cyclooxgenase
DCI:	delayed cerebral ischemia
DHA:	docosahexaenoic acid
DHT:	5α-dihydrotestosterone
DIND:	delayed ischemic neurological deficits
DHET:	dihydroxyeicosatrienoic acid
DSA:	digital substraction angiography
EDCF:	endothelium-derived constricting factors
EDRF:	endothelium-derived relaxing factors
EET:	epoxyeicosatrienoic acid
EPA:	eicosapentanoic acid
EpOME:	epoxyoctadecenoic acid
ET-1:	endothelin-1
FDA:	Food and Drug Administration
GCS:	Glasgow Coma Scale
GPCR:	G-protein coupled receptor
GC:	guanylyl cyclase
GWAS:	genome wide association studies
HH:	Hunt and Hess
HPLC:	high performance liquid chromatography
HWE:	Hardy-Weinberg Equilibrium
Hb:	hemoglobin
H_2O_2:	hydrogen peroxide

Hp:	haptoglobin
IA:	intracranial aneurysm
ICP:	intracranial pressure
IL-1α:	interleukin 1-α
K_{Ca+2}:	large-conductance calcium-activated potassium channel
LOX:	lipooxygenase
LLOQ:	lower limit of quantitation
MABP:	mean arterial blood pressure
mGluR:	metabotropic glutamate receptor
MRI:	magnetic resonance image
MRS:	Modified Rankin Scale
MS/MS:	tandem mass spectrometry
NADPH:	nicotinamide adenine dinucleotide phosphate
NIRS:	near-infrared spectroscopy
NO:	nitric oxide
NOS:	nitric oxide synthase
NSAID:	non-steroidal anti-inflammatory drugs
oxyHb:	oxyhemoglobin
PPARα:	peroxisome proliferator-activated receptor alpha
$PGF_{2\alpha}$:	prostaglandin $F_{2\alpha}$
PGI_2:	prostacyclin
PUFA:	polyunsaturated fatty acids
QC:	quality control
ROS:	reactive oxygen species
RBC:	red blood cells
SAH:	subarachnoid hemorrhage
SNP:	single nucleotide polymorphism
SPE:	solid phase extraction
SRM:	selective reaction monitoring
TCD:	transcranial Doppler ultrasonography
TRPC:	transient receptor potential channel
TXA_2:	thromboxane A_2
UPLC:	ultra performance liquid chromatography
VSMC:	vascular smooth muscle cells
WFNSS:	World Federation of Neurological Stroke Scale

1.0 INTRODUCTION

1.1 SUBARACHNOID HEMORRHAGE (SAH)

1.1.1 Background on stroke and SAH

This section provides a brief discussion on the background of stroke, classification of stroke subtypes, brief history of hemorrhagic stroke, etiology of subarachnoid hemorrhage (SAH), and incidence of SAH.

1.1.1.1 Background on stroke: Stroke is a condition caused by an interruption in blood flow to the brain that leads to a lack of nutrients and oxygen resulting in injury to the cells in the affected vascular territory of the brain. When brain cells die, function of the body parts they control is impaired or lost causing speech and sensory problems, memory and reasoning deficits, paralysis, coma, and death.[1] Each year in the United States (US), approximately 795,000 people experience a stroke with 23% of those cases found to be recurrent strokes.[2] Surprisingly, this translates to a stroke occurring on average every 40 seconds in the US.[2] Moreover, the prevalence of stroke in the US from 2007-2010 was 2.8% affecting an estimated 6.8 million Americans ≥20 years of age.[2] When considered separately from cardiovascular disease (CVD), stroke ranks as the fourth leading cause of death in the US behind heart disease, cancer, and chronic lower respiratory diseases.[2, 3] In the United States, stroke accounts for 1 of every 19 deaths and stroke related death are estimated to occur every 4 minutes on average.[2] Also, stroke is a leading cause of serious long-term disability in the US and is associated with direct and indirect costs of $38.6 billion each year.[2]

1.1.1.2 Stroke subtypes: Stroke is typically classified into major subtypes based on its etiology. Strokes caused by occlusion of a blood vessel are referred to as "ischemic" while those caused by the rupture of a blood vessel are referred to as "hemorrhagic". Hemorrhagic strokes are further classified into intracerebral hemorrhage, which is characterized by bleeds in the brain parenchyma, and SAH, which is characterized by bleeds into the subarachnoid cavity between the arachnoid membrane and pia matter. Ischemic strokes are the most common type accounting for 87% of all strokes with the remaining 10% and 3% of strokes accounted for by intracranial hemorrhage and subarachnoid hemorrhage (SAH), respectively.[2, 4] Although hemorrhagic strokes are less common than ischemic strokes, the mortality rates and cost associated with hemorrhagic stroke are much

higher. For example, the 30-day mortality rate was recently reported to be 8-12% in ischemic stroke and 37-44% in hemorrhagic stroke.[2] Furthermore, SAH accounts for 25% of all fatalities related to stroke[5] and 25% of potential years lost to life due to stroke[6]. A study of stroke patients in the US in 1990 reported a lifetime cost of $91,000 for ischemic stroke, $124,000 for intracerebral hemorrhage, and $228,000 for subarachnoid hemorrhage.[7] Based on the high mortality rates and costs associated with SAH, it has been the focus of many clinical studies and reviews in recent years.

1.1.1.3 Brief history of hemorrhagic stroke: A brief description of some key historical events in the diagnosis and treatment of SAH has been previously described.[8] Amazingly, ancient Egyptian writings provide the first known documentation of an intracranial aneurysm (IA).[8] Many years later in 1679, there was a documented association of IAs and cerebral hemorrhages.[9] In the 1760s, the first documented report of an unruptured IA was published[10] and IAs were suggested to be the source of cerebral hemorrhages[11]. The first documented account of an IA rupture was noted in 1814[12] and the first description of neurological deterioration occurring days after a stroke was published in 1859.[13] Autopsy of this patient showed a ruptured middle cerebral artery which was suggested to be the cause of the stroke and subsequent neurological symptoms.[13] In the 1930s, clinical angiograms were developed which aided in the diagnosis and treatment of hemorrhagic stroke.[14] Also, the direct treatment of aneurysms using surgical methods such as clip repair was performed.[15] In the 1940s, ruptured IAs were associated with cerebral infarction.[16]

Since the 1950s, many of the major accomplishments in the diagnosis and treatment of SAH have focused on neurovascular complications and their relationship to outcomes. Prior to 1950, the first description of cerebral vasospasm was made in 1925 after rabbit cortical arties were observed to contract after mechanical stimulation.[17] In the 1940s, blood breakdown products were proposed as vasoactive agents in the brain.[18, 19] In the 1950s, cerebral vasospasm in SAH patients was determined by cerebral angiograms.[20, 21] In the 1960s, angiographic vasospasm was associated with ischemic lesions, worse outcomes, and reduced cerebral blood flow (CBF).[22, 23] Also, it was proposed to delay the timing of surgical treatment in order to reduce the risk of vasospasm that was observed in the first 10 days after SAH.[24] However, larger studies published in 1976 indicated a reduced risk of vasospasm and mortality in patients undergoing surgery within 48 hours.[25] In addition, serial angiograms showed that the onset, peak, and subsidence of vasospasm occurred 3,

6 to 8, and 12 days after SAH, respectively, and that mortality was related to the presence and severity of vasospasm.[26] Katada et al.[27] and Taemae et al.[28] published the first association between the size of the blood clot and the incidence of angiographic vasospasm in the late 1970s. In 1980, the Fisher et al. proposed a formal classification of SAH based on the size and location of blood after noting that severe angiographic vasospasm was almost invariably present in patients with subarachnoid clots larger than 5 × 3 mm or layers of blood ≥1 mm in vertical cisterns and fissures.[29] Subsequent studies confirmed these findings and determined that thick subarachnoid clot completely filling any cistern/fissure was an independent predictor of delayed ischemic neurological deficits (DIND).[30] Thus, many of the major accomplishments in the diagnosis and treatment of SAH in the second half of the 20th century have focused on neurovascular complications and their relationship to outcomes.

1.1.1.4 Etiology of SAH: SAH can be classified into three major groups based on its etiology, which is usually determined by the pattern of blood on a computed tomography (CT) scan or other imaging technology. Previous studies have reported that 75% of SAH cases are due to ruptured IA.[31] The exact means by which IAs form and rupture is unknown, but hypertension and smoking-induced vascular changes are thought to play a major role.[32] IAs commonly occur at the bifurcation of the cerebral arteries at the base of the brain which implies a hemodynamic effect to the vessel wall.[32] More specifically, the incidence of IAs are highest on the anterior communicating artery (30%), posterior communicating artery (25%), middle cerebral artery (20%), internal carotid artery (7.5%), vertebrobasilar tip (7%), and other locations (10.5%).[33] Histological studies of intracranial arteries commonly report structural defects in the vasculature, such as a thin tunica media and internal elastic membrane, which may account for their susceptibility to formation from shear stress.[32] Fortunately, most IA are small and an estimated 50-80% of IA do not rupture during a person's lifetime.[33] The rate of rupture of IAs depends on multiple factors including the location, size, morphology, and previous history of SAH.[34, 35] No identifiable cause is determined in about 20% of SAH cases, which are often classified as idiopathic (spontaneous) or perimesencephalic.[5] Perimesencephalic SAH is characterized by blood which is confined to the cisterns around the midbrain and is rarely due to ruptured aneurysms.[36, 37] Also, perimesencephalic SAH typically follows a relatively benign clinical course with the major complication being hydrocephalus.[37] The remaining 5% of SAH cases are due to

other causes such as arterio-venous malformations of the brain or spine, mycotic aneurysm, intracranial arterial dissection, cerebral trauma, tumors, bleeding diseases, and sympathomimetic drugs.[5, 31] In summary, most SAH cases are due to a ruptured IA in the arteries at the base of the brain. For the purpose of simplicity, the term SAH will refer to aneurysmal SAH in the remainder of this document.

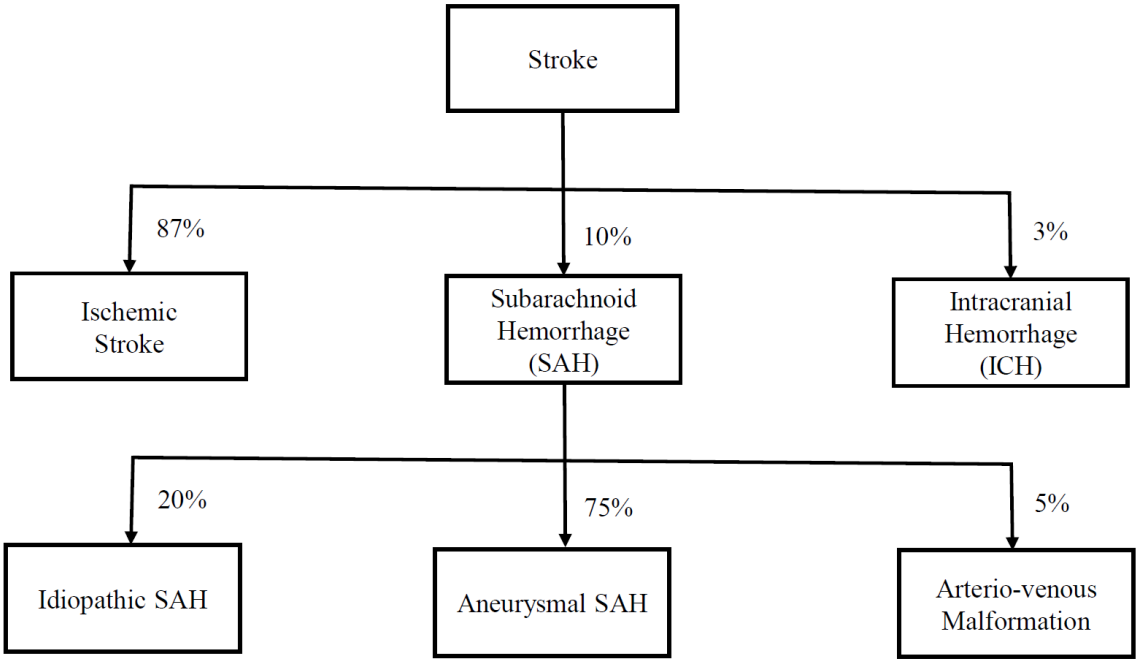


Figure 1-1: SAH etiology

1.1.1.5 Incidence of SAH: In the US, SAH accounts for 3-5% of all strokes and affects approximately 10-15 per 100,000 or approximately 30,000 people each year.[38-41] Because an estimated 12-15% of individuals that suffer from SAH die before hospital admission, the true incidence of SAH may even be higher.[42, 43] A number of studies have reported that the incidence of SAH worldwide has not changed significantly over the past 40 years.[44-48] However, a recent study reports a slight decrease in the incidence of SAH in regions other than Japan, South and Central America, and Finland from 1950 to 2005.[49] Thus, despite medical advances over the past 40 years, the incidence of SAH remains relatively constant and continues to be a major cause of stroke worldwide.

1.1.2 Risk factors for intracranial aneurysms and SAH

This section highlights the risk factors for IAs and SAH including modifiable and non-modifiable risk factors and includes a discussion of genetic disorders and genes associated with the pathogenesis of IAs and SAH. The end of this section includes a table which summarizes these risk factors (Table 1-1: Risk factors for intracranial aneurysm/SAH).

1.1.2.1 Non-modifiable risk factors: There are multiple risk factors associated with SAH, but many are not modifiable. For example, the incidence of SAH is most common in age 40-60 (median ≥50) and increases with age.[50] Also, the risk of SAH is 1.4 to 1.6-fold higher in women than men.[49, 51] This sex difference may be attributed to hormonal factors as some studies have reported a lower risk for SAH with pre-menopausal women[52-55] and older age at onset of menarche or at birth of first child[56]. Interestingly, some studies suggest that the incidence of SAH is influenced by seasonal effects with higher risk of SAH in winter[57] and spring[58], but conflicting results are reported[59]. Furthermore, race and regional differences are suggested to have a major impact on the risk for SAH. In the United States, Black Americans are at higher risk than white Americans.[60] Likewise, Maori and Pacific people are at higher risk than white New Zealanders.[61] Worldwide, the age-adjusted annual incidence of SAH varies 10-fold between different countries.[62] For instance, the incidence of SAH in China and Finland was reported as 2.0 and 22.5 cases per 100,000 individuals, respectively.[62] In addition, the age-adjusted incidence rate of SAH in low- to middle-income countries was found to be almost double that of high-income countries.[44] These data indicate that older age, female sex, seasonal factors, regional differences, race/ethnicity, and economic factors are considered non-modifiable risk factors for SAH.

1.1.2.3 Familial history: Another important risk factor for SAH is previous or familial history of ruptured or unruptured IA. Although the presence of an unruptured IA is relatively common occurring in 1-6% of the adult population[63-65], a greater incidence of IA is reported in families of individuals with ruptured or unruptured IA. To illustrate, four epidemiological studies of familial IAs reveal that 7-20% of SAH patients had 1st or 2nd degree relatives with IA.[66-70] In individuals with ruptured or unruptured IA, the frequency of IA in 1st degree relatives was almost 5-times higher than observed in controls.[69] In addition, multiple IA are observed in about 25% of

individuals with SAH and the formation rate of new aneurysms is reported to be 1-2% per year.[71-77] In addition to presenting an increased risk for IA, previous or familial history of ruptured or unruptured IA increase the risk for SAH as well. For example, SAH patients were almost twice as likely to have a 1st degree relative affected by SAH compared to controls.[78] Likewise, the incidence of SAH in 1st degree relatives of SAH patients is 4-fold higher than the expected incidence of SAH in the same population.[79] Furthermore, studies suggest that 1st degree relatives of SAH patients are at a 3 to 7-fold higher risk for SAH compared to 2nd degree relatives.[79] These studies suggest there is a higher risk of SAH in individuals with previous or familial history of ruptured or unruptured IA, yet screening and surgical treatment of unruptured aneurysms in this population remains controversial.[51, 80]

1.1.2.3 Rare genetic disorders: In additional to familial risk factors, certain rare inherited genetic disorders have been previously associated with SAH. Schievink[81] and others[82, 83] provide excellent reviews on this topic and focus on disorders involved connective tissue and structural support of the vasculature such as Autosomal Dominant Polycystic Kidney Disease (ADPKD)[84], Ehlers-Danlos syndrome Type IV[85], Marfan syndrome[86], Pseudoxantoma Elasticum[87], and Neurofibromatosis[88]. To illustrate, ADPKD results from a mutation in one of the polycystin genes (PKD1 and PKD2) which are integral membrane spanning proteins involved in interactions with the extracellular matrix.[89] The disease is characterized by renal cysts, renal failure and vascular pathology.[90] Moreover, individuals with ADPKD show a 4- and 5-fold greater risk of IAs and SAH, respectively,[91] and the average age of SAH in this group is 41 years, a decade earlier than sporadic cases.[92-94] However, a risk-benefit analysis failed to show any benefit of screening and treatment of unruptured aneurysms in ADPKD patients.[90] These findings suggests that connective tissue disorders and other rare genetic disorders affecting the cerebral vasculature may play an important role in the formation and rupture of IAs, but the identification, screening, and treatment of these patients may have limited clinical utility in reducing the risk for SAH.

1.1.2.4 Candidate genes: Based on the previously established heritable risk factors for IA and SAH, many studies investigated the relationship between genetics and IA. Linkage studies of families with IA have failed to identify any genetic modes of transmission and suggest that the genetics of IA are complex involving multiple genes.[95] Nevertheless, these linkage analyses in combination with genome wide association studies (GWAS) and candidate gene association

studies have identified multiple chromosomal regions that may contain one or more susceptibility genes for IA.[83] Candidate genes identified thus far are involved in diverse biological processes such as inflammation, structural support, remodeling of the extracellular matrix, regulation of blood pressure, and lipid metabolism. For example, collagen and elastin provide much of the content of the vascular extracellular matrix and contribute to the structural integrity and elasticity of the vessels.[96, 97] Endoglin is a glycoprotein highly expressed on endothelial surfaces and serves an important role in the formation of blood vessels.[98] Also, matrix metallopeptidase-9 (MMP-9) is involved in the breakdown of extracellular matrices in various tissues.[99] Together, these proteins play an important role in the formation and maintenance of the cerebral vasculature. By the same token, genetic studies have reported an association between the IA formation and polymorphisms in collagen type I (COL1A2)[100], collagen type III (COL3A1)[101], elastin (ELN)[95, 102], endoglin (ENG)[103], and MMP-9[104] genes. Similarly, endothelial nitric oxide synthase (eNOS) and angiotensin converting enzyme (ACE) play an important role in regulating blood pressure and vascular reactivity and have been associated with the incidence of IA in multiple studies.[105-110] Likewise, polymorphisms in genes involved in inflammation, such as interleukin 1-β (IL-1β)[111], α-1 antichymotrypsin (SERPINA3)[112], and heme oxygenase 1 (HO-1)[113], have been associated with IA. Also, lipid metabolism genes such apolipoprotein A (APOA) have been identified as candidate genes for IA.[114] Although multiple IA candidate genes have been identified, many of the genetic markers were not further studied or the findings could not be replicated in the same or different populations.[83] Furthermore, many of these studies involved patients with unruptured IA and not SAH. Thus, there are currently no genetic markers that identify an increased risk for SAH in diverse populations.

1.1.2.5 Modifiable risk factors: On the other hand, there are multiple potentially modifiable risk factors associated with SAH. CVDs, such as hypertension[53-55, 115, 116] and cerebrovascular disease[53-55], but not diabetes[117], are associated with increased risk for SAH. In addition, SAH has been associated with smoking and heavy alcohol use[115, 116] Some studies suggest that the use of sympathomimetic drugs including phenylpropanolamine[118] and cocaine[119, 120] increase the risk for SAH, especially in younger patients[120]. Furthermore, some dietary factors have been proposed as SAH risk factors. For instance, greater vegetable consumption[121] and yogurt[122] are associated with decreased and increased risk for SAH, respectively, while high consumption of coffee and tea[123]

was not reported to affect SAH risk. Although there are other modifiable risk factors for SAH that have been identified, a recent review suggests that the most important modifiable risk factors for SAH include hypertension, smoking and excessive alcohol intake, each conferring a 2- to 3-fold increase in risk.[124] Furthermore, avoiding tobacco and alcohol misuse and treating high blood pressure with antihypertensive agents has been shown to reduce the risk of SAH.[125] In summary, there are multiple modifiable risk factors for SAH, but avoiding tobacco and alcohol misuse and treating high blood pressure seem to demonstrate the most clinical utility in reducing the risk for SAH.

Table 1-1: Risk factors for intracranial aneurysm/SAH

Category	Risk Factor	High-Risk Group
Demographic factors	Age	Older age[50]
	Sex	Female[49, 51]
	Race/ethnicity	non-Caucasian[60, 61]
Heritable factors	Family history of IA/SAH	1st/2nd Degree Relatives with IA/SAH[66-70, 78, 79]
	Connective tissue disorders	Autosomal Dominant Polycystic Kidney Disease[84]
		Ehlers-Danlos Syndrome Type IV[85]
		Marfan Syndrome[86]
		Pseudoxantoma Elasticum[87]
		Neurofibromatosis[88]
	Variants in genes involved in vascular structure/remodeling	Collagen Type I (COL1A2)[100]
		Collagen Type III (COL3A1)[101]
		Elastin (ELN)[95, 102]
		Endoglin (ENG)[103]
		Matrix Metallopeptidase-9 (MMP-9)[104]
	Variants in genes regulating blood pressure	Endothelial Nitric Oxide Synthase (eNOS)[105-107]
		Angiotensin Converting Enzyme (ACE)[108-110]
	Variants in genes involved in inflammation	Interleukin 1-β (IL-1β)[111]
		α-1 Antichymotrypsin (SERPINA3)[112]
		Heme Oxygenase-1 (HO-1)[113]
Modifiable risk factors	Lifestyle	Smoking / tobacco use[115, 116124]
		Heavy alcohol use[115, 116124]
		Sympathomimetic drugs (cocaine)[118-120]
		Diet (low vegetable consumption/yogurt)[121, 122]
	Medical conditions	Hypertension[53-55, 115, 116, 124]
		Cerebrovascular disease[53-55]
Other factors	Regional differences	Varies per country[62]
	Economic factors	Low income countries[44]
	Seasonal factors	Winter/spring[57-59]

1.1.3 Clinical course and management of SAH

This section highlights the typical symptoms present on admission and the most common intracranial and systemic complications associated with SAH in the clinic. In addition, this section discusses the American Heart Association (AHA) guidelines for the diagnosis, monitoring, and treatment of patients with SAH.

1.1.3.1 Clinical presentation: Clinical symptoms of SAH have been previously described but are of limited use in the diagnosis of SAH. The most common symptom of SAH is a sudden onset of a very severe headache also known as a "thunderclap" headache, which may be the only symptom in up to 1/3 of SAH patients.[126] About 10% of patients with SAH report a sentinel leak that occurs days to weeks before the SAH and results in similar headache symptoms.[127-129] However, others argue that headaches due to sentinel leaks are rare and do not help in SAH diagnosis.[130] Other common symptoms include a disturbance in consciousness, vomiting, or neck stiffness.[126] A smaller percentage of patients show a retinal or vitreous hemorrhage in the eye[131] or other neurological deficits including motor defects, aphasia, seizures, visual troubles, and amnesia.[126] In the first few days after SAH, psychiatric manifestations are common including depression (45%), apathy (42%), denial (21%) and catastrophic reaction (17%).[132] However, no clinical feature is sufficiently reliable to make a diagnosis[126] and SAH is frequently misdiagnosed.[133, 134]

1.1.3.2 Acute intracranial complications: SAH is associated with numerous medical complications that typically develop over different time periods after the hemorrhage. In the first 24 hours after SAH, the primary cause of death is usually attributed to a rise in intracranial pressure (ICP) due to the large volume of bleed in the subarachnoid space and blockage of cerebrospinal fluid (CSF) drainage.[5] A sufficient increase in ICP can lead to decreased cerebral perfusion pressure (CPP), global cerebral ischemia, edema, and ultimately death.[5, 135-137] Extensive cerebral ischemic injury is well documented in the individuals who die within the first 24 hours after SAH.[138-144] For individuals that survive the SAH, the most important immediate concern is the risk of an aneurysm rebleed. Although ruptured IAs tend to heal in survivors of SAH, the risk of rebleed is significant, especially early after SAH, if the IA is not treated surgically. To illustrate, the cumulative incidence of rebleeding in SAH patients with untreated IA is 2-4% within 24

hours[33, 145-147], 15-20% within two weeks[33], and 35-40% within a month[148] After a month, the risk of rebleeding decreases gradually from 1–2%/day to 3%/year[5] Rebleeding is more frequent in patients with poor clinical presentation and large IA.[5, 149] Surgical treatment of IA significantly reduces the risk of IA rebleeds, but also presents additional risks and complications sometimes leading to additional morbidity and mortality.[51, 139, 150, 151] Hydrocephalus occurs in ~20-30% of SAH patients typically within 48 hours after SAH and is highly correlated with poor neurologic grade.[152, 153] When hydrocephalus leads to clinical complications, it can be treated with CSF diversion using external ventricular drains or lumbar drains.[5] In summary, the most common acute intracranial complications after SAH are increased ICP, IA rebleed, and hydrocephalus.

1.1.3.3 Delayed intracranial complications: In the time period following the first day after SAH, the most important complications are the development of cerebral vasospasm and delayed cerebral ischemia (DCI). Cerebral vasospasm describes the abnormal constriction of large cerebral arteries and is often associated with radiographic or CBF evidence of diminished perfusion in the distal territory of the affected artery.[154] The gold standard for assessment of cerebral vasospasm is cerebral angiography.[150] Angiographic vasospasm occurs in 30-70% of SAH patients and typically develops at 3-5 days, reaches maximum constriction at 5-14 days, and gradually resolves over 2-4 weeks after the hemorrhage.[155, 156] Approximately 50% of patients that develop vasospasm also develop DCI.[156-158] In general, DCI describes the delayed onset of a mismatch between CBF and the metabolic oxygen requirement within a given cerebral region resulting in ischemic neuronal damage.[159] The definitions and terms used to describe DCI in the clinic vary greatly, but typically include radiographic evidence of vasospasm with clinical features of cerebral ischemia after exclusion of factors not related to vasospasm.[160] Clinical features of cerebral ischemia that occur in the absence of vasospasm are commonly referred to as clinical neurological deterioration (CND), neurological deficits, or delayed ischemic neurological deficits (DIND) whereas those that occur in the presence of vasospasm are commonly called DCI or symptomatic vasospasm.[160] Conversely, asymptomatic vasospasm is commonly used to refer to the condition when there are no clinical features of cerebral ischemia, but vasospasm is detected.[160] However, it is important to note that these terms are often used interchangeably in the literature.[160] In patients that survive SAH, 50-70% develop angiographic vasospasm, 25-50% develop symptoms of DCI, and 30-50% show radiological signs of infarction attributed to vasospasm.[161] Since cerebral vasospasm and

DCI typically develop days after SAH and may be reversible, these complications serve as a target for aggressive preventive and treatment strategies.[153]

1.1.3.4 Systemic complications: In addition to the commonly observed intracranial complications after SAH, it is common for patients to develop systemic complications, especially in the first few days after the hemorrhage.[153, 162] Patients with SAH commonly develop cardiac dysfunction (75%), fever (54%), anemia (36%), hypertension (27%), hypotension (18%), pneumonia (20%), and pulmonary edema (14%).[153, 163, 164] A recent study reported that 79% patients developed at least one systemic complication after SAH.[165-167] In the Cooperative Aneurysm Study, 23% of deaths were attributed to systemic complications with half of those due to pulmonary complications.[168] Although prevention and treatment of systemic complications might improve outcome after SAH[169], clinicians are often challenged by the potential adverse effects of these interventions on the intracranial complications.[170] Also, therapies aimed at reducing intracranial complications can increase the frequency and severity of systemic complications.[153, 171, 172] Despite the varied and frequent systemic complications that develop after SAH, the prevention and treatment of intracranial complications continues to be the focus in the management of SAH.[150]

1.1.3.5 Diagnosis, monitoring, and treatment: The prevention, diagnosis, and treatment of SAH is the topic of numerous studies and reviews. Recently, the American Heart Association (AHA) performed a comprehensive review of the clinical evidence in this topic and provided guidelines in the management of SAH patients.[125] It should be noted that the evaluation of the clinical data is not fully comprehensive and the guidelines note some of the areas in which there is a lack of quality information. The guidelines are categorized into Class I, II, and III based on the size of the treatment effect and Level A, B, and C as an estimate of certainty of the treatment effect. For instance, Class I guidelines are those in which "there is significant evidence for and/or general agreement that the procedure or treatment is useful and effective". Level A and B guidelines are categorized based on evaluations from multiple populations and limited populations, respectively. The paragraph below summarizes some of the key Class 1 recommendations for the management of SAH. All recommendations are categorized as Class 1B unless specified otherwise.

According to the 2012 AHA guidelines for the management of SAH[125], "a high level of suspicion for SAH should exist in patients with acute onset of severe headache". The acute

diagnosis of SAH should be performed using non-contrast CT, which can be followed by a lumbar puncture if the CT is non-diagnostic. The detection of the aneurysm should be performed using 3-dimensional digital substraction angiography (DSA) and the initial clinical severity of SAH should be determined using of simple validated scales such as Hunt and Hess (Table 1-6: Hunt and Hess Scale) and World Federation of Neurological Surgeons (Table 1-8: World Federation of Neurologic Surgeons Scale). The urgent diagnosis of SAH is emphasized because delays in CT scan allow SAH blood to degrade thus increasing the possibility of a normal CT scan. For example, modern CT scanners failed to detect blood in approximately 2-7% of SAH cases within 12-24 hours.[173, 174] Within 10 days, SAH blood is almost completely dissolved and the sensitivity to detect SAH using CT declines rapidly.[31] Once the SAH is confirmed, urgent treatment of patients is suggested to avoid aneurysm rebleeding. Proper CBF and hemodynamics should be maintained through the control of blood pressure and cerebral perfusion pressure (CPP) and by the maintenance of euvolemia and normal circulating blood volume. Acute hydrocephalus should be prevented by cerebrospinal fluid diversion via external ventricular drainage or lumbar drainage. Chronic SAH-induced hydrocephalus should be treated with permanent CSF diversion (Class IC). Oral nimodipine should be administered to all SAH patients as it has been shown to improve neurological outcomes but not cerebral vasospasm (Class IA). For patients that develop DCI, the induction of hypertension is recommended unless blood pressure is elevated at baseline or cardiac status precludes it. The ruptured aneurysm should be treated by surgical clipping or endovascular coiling followed by cerebrovascular imaging to verify proper obliteration of the aneurysm. If both surgical methods are amenable, endovascular coiling is preferred over surgical clipping. Also, "the determination of aneurysm treatment, as judged by both experienced cerebrovascular surgeons and endovascular specialists, should be a multidisciplinary decision based on characteristics of the patient and the aneurysm" (Class IC). Low-volume hospitals (<10 SAH patients per year should consider transferring SAH patients to high-volume centers (<35 SAH cases per year) with experienced cerebrovascular surgeons, endovascular specialists, and multidisciplinary neuro-intensive care services."[125]

1.1.4 Pathophysiology of SAH

This section describes the mechanisms of acute and delayed injury including oxidative stress, inflammation, acute cerebral ischemia, microvascular dysregulation, microthrombosis, delayed vasospasm, and DCI. Figures at the end of this section summarize the primary mechanisms leading to cellular death and injury (Figure 1-2: Primary mechanisms of injury after SAH) and key regulators of cerebrovascular tone (Figure 1-3: Key regulators of cerebrovascular tone after SAH) after SAH.

1.1.4.1 Mechanisms of acute injury: During SAH, blood is released into the subarachnoid space at arterial pressure and spreads diffusely over the surface of the brain bathing pial arteries with fresh blood.[175] The proposed etiologies of acute mortality include direct neural destruction from the force of the hemorrhage and cerebral ischemia secondary to acute elevations in ICP.[176] Rise in ICP due to blood in subarachnoid space and impeded CSF drainage can lead to cerebrovascular dysfunction and vascular engorgement [177] with subsequent cerebral edema [178]. In additional to the mechanical causes of the rise in ICP, the development of global cerebral edema is a processes that occurs at the cellular level.[179] As ICP rises to levels approximating the mean arterial pressure, the cerebral perfusion pressure approaches zero resulting in a loss of CBF as further described in Section 1.2.2. Changes in CBF can also occur in the absence of increased ICP when cerebral metabolism is altered.[180] This process can lead to cerebral ischemia when the CBF fails to meet the metabolic requirements of the tissues.[159] Also, decreased availability of nitric oxide (NO) [181, 182], acute vasoconstriction[183, 184], and microvascular platelet aggregation[185] have been shown to play an important role in the development of acute cerebral ischemia after SAH. Ultimately, cerebral ischemia results in energy failure in neurons and glia and initiates the cascade of events leading to cytotoxic edema.[186] Cerebral ischemia also leads to apoptosis in the cells that constitute the blood brain barrier (BBB).[187] For example, cell death in astrocytes and endothelial cells leads to the diffusion of serum from the vascular lumen into cerebral tissues (vasogenic

edema). Numerous intracellular second messenger cascades involving caspases, hypoxia-inducible factor 1 (HIF-1), vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs) have been implicated in apoptosis of cells in the cerebrovasculature leading to disrupted BBB.[179, 188] These studies only highlight the complex mechanisms involved in the pathogenesis of acute cerebral ischemia.

SAH also induces acute brain injury through oxidative stress and inflammation. Preclinical[189] and clinical[190, 191] evidence shows that oxidative stress significantly contributes to brain injury after SAH. The largest source of oxidative stress after SAH are superoxide anions released from ischemic mitochondria and free radicals produced from the breakdown of hemoglobin.[179, 189, 192] The free radicals presented by these reactive oxygen species (ROS) directly damage various brain tissues through the promotion of lipid peroxidation, protein breakdown, and DNA damage.[186] There are several antioxidants in the brain that protect tissues against ROS including superoxide dismutases, glutathione peroxidases, and catalase. However, these protective mechanisms are down-regulated or showed reduced antioxidant capabilities after SAH.[189, 190, 193] Also, SAH induces inflammation the secretion of cytokines from leukocytes and erythrocytes in the bleed. These oxidative and inflammatory mechanisms ultimately result in neuronal apoptosis, endothelial injury, and breakdown of the BBB after SAH. Collectively, cerebral ischemia, cerebral edema, oxidative stress, and inflammation following SAH ultimately contribute to cell death and cerebral infarction which have been related to the initial bleed and poor outcomes.[194-196]

1.1.4.2 Mechanisms of delayed injury: The most notable pathogenic mechanisms of DCI are attributed to the development of cerebral vasospasm, altered microvascular regulation of CBF, and thromboembolism after SAH. The pathogenesis of vasospasm has been the subject of numerous reviews.[197-200] The exact cause of vasospasm remains unclear but its development is directly correlated with large bleeds, clots, and the time cerebral arteries are exposed to blood.[201] Kozniewska et al. provides an excellent review on the putative mechanisms involved in the pathophysiology of acute and delayed vasospasm.[200] In the first few hours after SAH, activated platelets and mechanically damaged red blood cells (RBC) release numerous plasmogens including the vasoconstrictors thromboxane A_2 (TXA_2), serotonin, adenosine triphosphate (ATP),

and platelet-derived growth factor (PDGF).[200] Also, hemoglobin (Hb) scavenges the vasodilator NO, which relaxes vascular smooth muscle cells (VSMC) directly by stimulation of cyclic guanosine monophosphate (cGMP) production[202] and indirectly by decreasing the synthesis of the vasoconstrictors endothelin-1 (ET-1)[203] and 20-hydroxyeicosatetraenoic acid (20-HETE)[204]. After a few days, RBCs undergo phagocytosis and lysis releasing oxyhemoglobin (oxyHb) and other break down products.[205] OxyHb scavenges NO to a greater extent than Hb leading to enhanced vasoconstrictive responses[200], especially in the presence of elevated levels of vasoconstrictors such as ET-1, TXA_2, and serotonin (5-HT) levels.[206, 207] Furthermore, vasospastic arteries showed upregulation of the endothelin receptor ETB and serotonin receptor 5-HT1B leading to increased contractile responses to ET-1 and 5-HT.[208, 209] The metabolism of Hb also produces bilirubin oxidation products (BOXes), which contribute to vasoconstriction.[210] Increased levels of BOXes were observed in patients with vasospasm and the timing of the increase correlates well with the onset of vasospasm.[210] The lysis of RBCs leads to the formation of oxidative stress and inflammation, which have been associated with cellular apoptosis, vascular remodeling, and DCI. Other established pathophysiological mechanisms involved in vasospasm focus on endothelial dysfunction and increased contractility of VSMC.[211] Endothelial dysfunction involves damage to the endothelium and reduced production of vasodilators such as NO and increased production of vasoconstrictor such as ET-1.[212, 213] The increased vasoconstrictive response of VSMC has been noted after SAH[200, 212, 213] and is attributed to a decrease in the number of potassium (K^+) channels[214] or activation of signal transduction mechanisms such as protein kinase C (PKC) and Rho kinase that alter calcium (Ca^{+2}) sensitivity.[206] Furthermore, SAH has been shown to induce some degree of vascular remodeling, which has been implicated in the development and maintenance of cerebral vasospasm.[215] Collectively, these studies describe the most notable mechanisms associated with acute and delayed vasospasm and may serve as therapeutic targets to improve outcomes after SAH.

On the other hand, some evidence suggests that cerebral vasospasm plays a limited role in the development of DCI after SAH.[216] Despite the associations between the development of vasospasm and DCI, the incidence, location, severity, and temporal relationship between angiographic vasospasm and DCI does not correlate well.[216, 217] The time course for DCI generally parallels that of cerebral vasospasm, but reductions in CBF often occur before vasospasm can be

visualized.[218] One contributing factor is that cerebral vasospasm is measured large cerebral arteries[219-221], but the microvasculature plays a predominant role in regulating CBF regulation[222, 223], especially after SAH [224]. Direct evidence of microvascular vasospasm in humans after SAH was shown using MRI[225] and orthogonal polarization spectral imaging[226]. Clinical studies investigating the treatment of vasospasm provide additional evidence that vasospasm plays a limited role in the development of DCI and outcomes after SAH. To illustrate, nimodipine improves outcomes without affecting angiographic vasospasm[216] and treatment of angiographic vasospasm often fails to improve outcomes[216]. Together, these studies emphasize the importance of microvascular regulation of CBF on outcomes after SAH and note the limitations of the current monitoring and treatment methods.

Furthermore, growing evidence suggests that microthrombosis in cerebral arteries contributes to the development of cerebral infarction after SAH. First of all, many patients show a diffuse pattern and small size of infarcts after SAH, which indicates pathophysiological mechanisms other than vasospasm.[227] Secondly, clinical studies of SAH have noted that clinical signs of ischemia often do not correlate with pathological evidence of ischemia.[150, 228] In a study of 29 patients that died a mean of 8 days after SAH, clinical ischemia was noted in 48% of patients, but pathological evidence of ischemia was seen in 93% of patients.[228] In this study, thrombi in small cerebral blood vessels were commonly detected in patients with clinical or radiological signs of DCI.[228] Also, transcranial Doppler ultrasound (TCD) commonly shows signals of thromboembolism in SAH patients.[229] Other studies note a relationship between microthrombi and vasospasm in patients who died after SAH.[230] In addition, experimental SAH shows that microcirculatory thrombosis occurs acutely after SAH, which supports the clinical findings.[185] Yet, despite the preclinical and clinical evidence that thromboembolism plays a role in the pathophysiology of SAH, clinical studies report a lack of a beneficial effect of antiplatelet and anticoagulant drugs, such as aspirin and enoxaparin, on outcome in patients with SAH.[231]

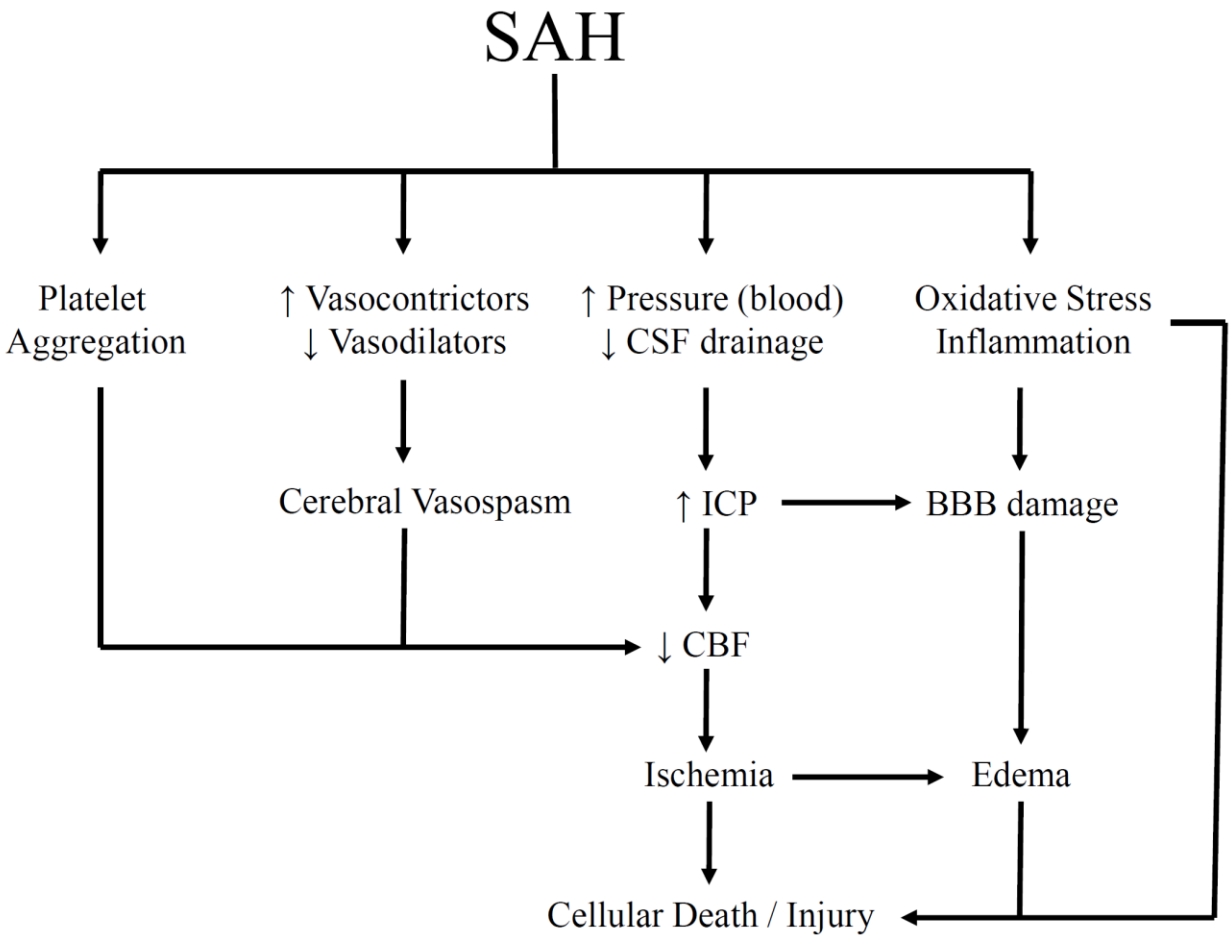


Figure 1-2: Primary mechanisms of injury after SAH

SAH, subarachnoid hemorrhage, CBF, cerebral blood flow; ICP, intracranial pressure; BBB, blood brain barrier

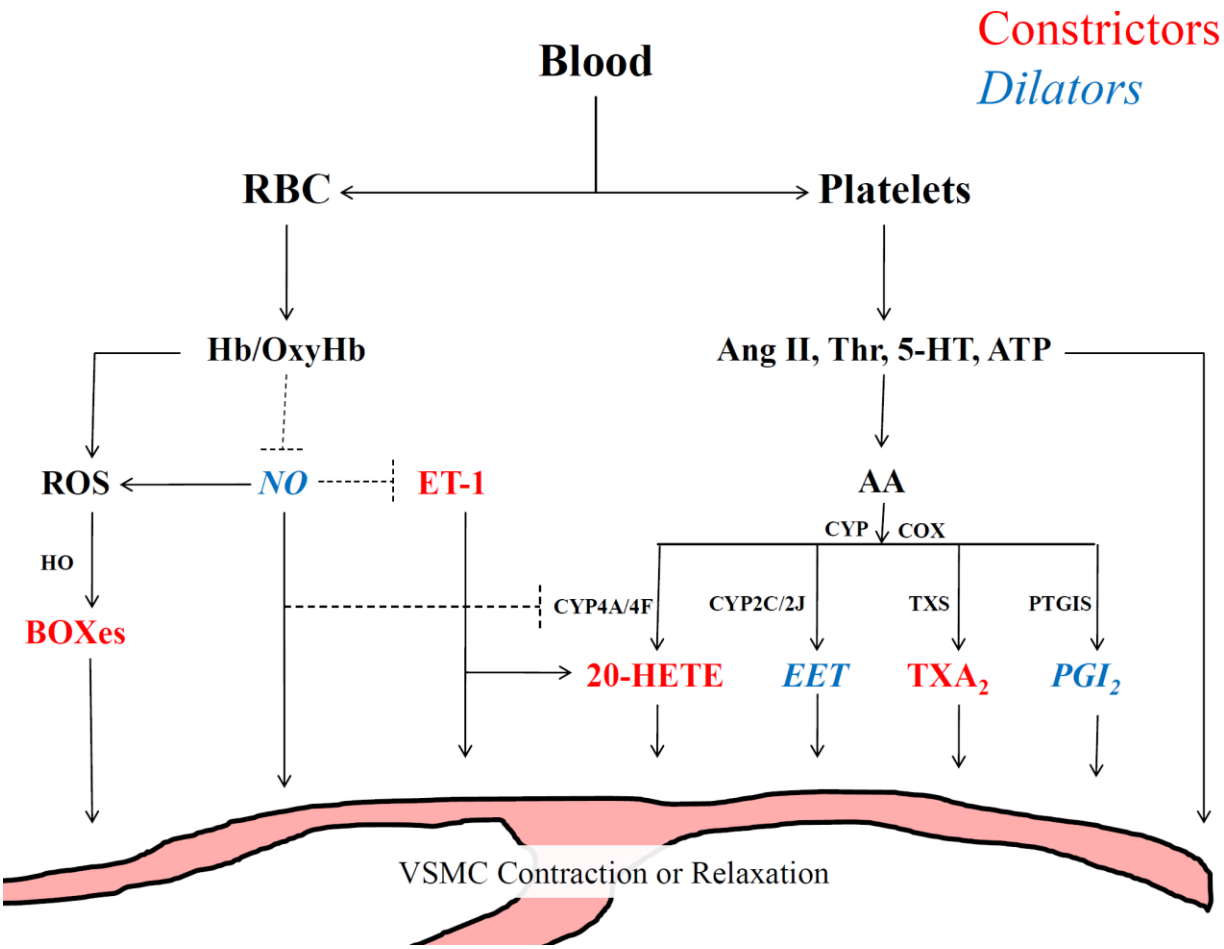


Figure 1-3: Key regulators of cerebrovascular tone after SAH

RBC, red blood cells; Hb/oxyHb, hemoglobin/oxyhemoglobin; AngII, angiotensin II; Thr, thrombin; 5-HT, serotonin; ATP, adenosine triphosphate; AA, arachidonic acid; HO, hemooxygenase; CYP, cytochrome P450; COX, cyclooxygenase; TXS, thromboxane synthase; PTGIS, prostacyclin synthase, ROS, reactive oxygen species; BOXes, bilirubin oxidation products; NO, nitric oxide; TXA2, thromboxane A2; PGI2, prostacyclin; 20-HETE, 20-hydroxyeicosatetraenoic acid, EETs, epoxyeicosatrienoic acids

1.1.5 SAH outcomes

This section notes the morbidity and mortality rates and trends in the US and/or worldwide.

1.1.5.1 Mortality: SAH presents an unusually high risk of death. Approximately 10-20% of individuals that suffer SAH die before reaching the hospital.[42, 43] Among those reaching the hospital, approximately 20% are comatose and need immediate respiratory support[42, 232], 10% die within 24 hours[31], and 25% die within 2 weeks[140]. Population-based studies report 30-day mortality rates of 40-44%.[46, 60, 233] Mortality rates at 6 months are similar to those at 12 months and range from 38-77%.[234] Case fatality rates are estimated to be approximately 50%.[138] Although SAH accounts for only 3-5% of all strokes, SAH accounts for 25% of all fatalities related to stroke.[5] Also, SAH accounts for 25% of potential years lost to stroke due to the relatively young age of onset and the relatively worse outcomes when compared to ischemic stroke.[6] These data demonstrate that the SAH is considered a very serious condition associated with an extremely high mortality.

Despite the relatively high mortality rates of SAH worldwide[44], many studies report a decreasing trend over time.[5] In the US, epidemiological studies of SAH patients have reported 21 to 30-day mortality rates of 57% from 1945 to 1974[46], 42% from 1975 to 1984[46], 45% in 1988[139], and 26-33% from 1993 to 2003[40, 235]. One study estimates that the mortality rate of SAH decreased approximately 1% per year in the US from 1979 to 1994.[6] Worldwide, the mortality rates of SAH vary greatly with values ranging from 8-67%.[236] The large variability may be due in part to inconsistent measurement criteria for some studies, which did not account for deaths that occur before hospital admission.[236] In a meta-analysis of population-based studies, SAH fatality rates decreased worldwide by 17% from 1973 to 2002.[237] High income countries showed lower fatality rates (25-35%) compared to low to medium income countries (40-48%).[44] In addition, regional differences in mortality rates have been noted with median values in the US, Europe, and Japan reported to be 32%, 44%, and 27%, respectively.[236] Taken together, these studies suggest that the mortality rate of SAH is decreasing world-wide, especially in high income countries.

1.1.5.2 Morbidity: For individuals that survive SAH, many suffer from neuropsychological and physical deficits that impact their quality of life. Less than 1/3 of SAH survivors regain their

previous occupation and report no effect on their lifestyle at 18 months.[5, 238, 239] Approximately 1/3 of SAH survivors are left with physical disabilities or cognitive impairments, which leaves them dependent.[138, 240] Recovery to an independent state does not ensure good outcomes because reductions in quality of life are commonly reported in these patients.[239] Although cognitive function tends to improve over the first year after SAH,[241] global cognitive impairment is still present in ~20% of patients at 1 year.[242] In addition, some studies report that SAH patients commonly suffer from neuroendocrinal dysfunction and sleep disorders.[243, 244] Overall, approximately 2/3 of SAH survivors report a reduced quality of life.[240, 245] Many of these individuals do not return to work, retire early, and are unable to function at the same intellectual level as before the rupture.[246-249] When both morbidity and mortality data are considered, death or dependence occurs in almost 70% of patients.[250] In summary, multiple studies have demonstrated that SAH continues to be associated with significant morbidity and mortality.

There is lack of population-based studies investigating long-term outcomes after SAH. One population-based study of 2155 SAH reports very modest improvements in outcome after SAH during the last few decades.[138] In this study, the proportion of patients who remained independent after SAH increased 1.5% per year from 1977 to 1990.[138] Another study of 8739 patients reported that 8-20% suffer from persistent dependence.[236] The International Subarachnoid Aneurysm Trial reports 12% of patients with significant lifestyle restrictions and 6.5% are left functionally dependent at 1 year.[150] A recent study of SAH patients in Australia and New Zealand reported a high proportion of SAH patients with a diminished level of health related quality of life measurements such as incomplete recovery at 1 year (46%), ongoing memory problems (50%), mood abnormalities in (39%), speech problems (14%), and self-care (10%).[240] These data suggest that despite advancements in the treatment of SAH and its subsequent complications, a large percentage of SAH patients continue to suffer from deficits in cognitive, functional, or quality of life indicators.

1.1.5.3 Assessment of Outcomes: Long-term outcomes after SAH can be measured using a wide variety of methods involving cognitive, physical, and quality of life assessments. The Glasgow Outcome Score (GOS) (Table 1-2: Glasgow Outcome Scale) is a commonly used method to measure disability after stroke in the clinic because of its simple scale and history of use.[251] However, several limitations of the GOS scale have been noted including categories that do not

provide information on specific deficits, lack of sensitivity to detect small but clinically relevant changes in outcome, and poor inter-rater reliability.[252] Currently, the Modified Rankin Scale (MRS) and Barthel Index are the most commonly used scales for measuring the degree of disability or dependence in the daily activities of people who have suffered a stroke.[253] Recent studies reported that the MRS is more sensitive method for assessing disability when compared to the Barthel Index.[254] Also, the validation of the MRS scale has been demonstrated through its relationships to physiological indicators such as stroke type, lesion size, perfusion and neurological impairment.[255] Thus, MRS is a validated and clinically useful method to measure the disability or dependence as a long-term outcomes in SAH patients.

Table 1-2: Glasgow Outcome Scale

Grade	Disability	Description
1	Death	Death
2	Persistent vegetative state	Unable to interact with environment; unresponsive
3	Severe disability	Able to follow commands/ unable to live independently
4	Moderate disability	Able to live independently; unable to return to work or school
5	Good recovery	Able to return to work or school

Table 1-3: Modified Rankin Scale

Score	Category	Description
0	No symptoms	NA
1	No significant disability	Able to perform all usual duties and activities despite symptoms
2	Slight disability	Unable to perform all previous activities but able to look after own affairs without assistance
3	Moderate disability	Requires some help, but able to walk without assistance
4	Moderately severe disability	Unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability	Bedridden, incontinent, and requires nursing care and attention

Table 1-4: Barthel Index

Function	Description	Score
Feeding	Unable	0
	Needs help cutting, spreading butter, etc., or requires modified diet	5
	Independent	10
Bathing	Dependent	0
	Independent (or in shower)	5
Grooming	Needs to help with personal care	0
	Independent face/hair/teeth/shaving (implements provided)	5
Dressing	Dependent	0
	Needs help but can do about half unaided	5
	Independent (including buttons, zips, laces, etc.)	10
Bowels	Incontinent (or needs to be given enemas)	0
	Occasional accident	5
	Continent	10
Bladder	Incontinent, or catheterized and unable to manage alone	0
	Occasional accident	5
	Continent	10
Toilet Use	Dependent	0
	Needs some help, but can do something alone	5
	Independent (on and off, dressing, wiping)	10
Transfers (bed to chair and back)	Unable, no sitting balance	0
	Major help (one or two people, physical), can sit	5
	Minor help (verbal or physical)	10
	Independent	15
Mobility (on level surfaces)	Immobile or < 50 yards	0
	Wheelchair independent, including corners, > 50 yards	5
	Walks with help of one person (verbal or physical) > 50 yards	10
	Independent (but may use any aid; for example, stick) > 50 yards	15
Stairs	Unable	0
	Needs help (verbal, physical, carrying aid)	5
	Independent	10

1.1.6 Prognostic factors of long-term outcomes

This section highlights some of the key prognostic factors of long-term outcomes after SAH including factors related to the patient demographics, hospital, hemorrhage, and CBF regulation. This section also notes the inter-relation of these prognostic factors and highlights the most clinically relevant prognostic factors.

1.1.6.1 Patient demographics: Patient demographic factors have been shown to influence outcomes after SAH. Numerous studies report that older patients have worse outcomes than young patients, especially in those over 60 years of age.[46, 250, 256-258] To illustrate, Nieuwkamp et al. reported that only 1 of 6 patients older than 75 years of age leave the hospital alive and independent.[259] Conversely, a study of young SAH patients (<40 years of age) by Ogungbo et al. reported that age does not affect outcomes at discharge and that moderate and severe disability groups continued to improve and achieve good recovery at 6 months after SAH.[260] Likewise, sex and race have been associated with differences in outcome after SAH. For example, higher mortality rates have been reported in women compared to men[6, 43, 46] and in American minorities compared to white Americans.[261] Also, some studies report that socio-economic status can influence outcomes after SAH. For instance, Jakovljevic et al. reported that low socio-economic status was associated with worse outcomes in SAH in a large population-based Finnish stroke incidence study.[262] In summary, demographic factors such as older age, female sex, and low socio-economic status are prognostic factors of SAH outcomes.

1.1.6.2 Hospital/institution: Other factors related to the hospital/institution have been shown to affect outcomes after SAH. These factors include the availability of endovascular services[263], volume of SAH patients treated[235, 263-265] and the type of facility in which the patient is first evaluated[266]. As expected, treatment in these institutions can significantly affect outcome. Many studies report that early treatment can improve outcome.[173, 174, 267, 268] Also, conservative or aggressive treatment in patients with poor clinical grade can reduce mortality rates from ~90% to ~50% and significantly improve favorable outcomes in survivors.[248, 269, 270] Recently, AHA published treatment strategies and guidelines that have been shown to dramatically impact outcomes in SAH patients.[150] These guidelines highlight the importance the hospital/institution and treatment on outcomes.

1.1.6.3 Aneurysm and hemorrhage: Undoubtedly, factors related to the ruptured aneurysm and subsequent severity of bleed are important predictors of outcome after SAH. Aneurysm factors such as size, location, and morphology may affect prognosis by presenting surgical complications or post-operative complications related to the bleed.[271, 272] Surgical treatment of aneurysms usually involves the obliteration of the aneurysm using clips or coils and significantly reduces the risk of aneurysm rebleeds, but surgery can lead to additional morbidity and mortality sometimes without

improvements in the patient's condition.[139, 151] On the other hand, untreated IA are at high risk for rebleeding with a 30-day cumulative risk of 30-60%.[139, 154] Aneurysm rebleeding, especially early after SAH[273], is a major predictor of poor outcome and is associated with mortality rates of ~70-80% and severe functional and neuropsychological deficits.[145, 250, 274-277]. Over the last few decades, the severity and location of the bleed, commonly measured using a CT scan and categorized using clinical grading scales such as the Fisher scale (Table 1-5: Fisher Scale), have shown to be one of the most important prognostic indicators of outcomes after SAH.[234, 278] Larger bleeds have been consistently associated with numerous clinical complications and ultimately worse outcomes.[234] Also, the presence of parenchymal, ventricular and subarachnoid clots results in poor clinical outcome.[250, 274, 279-281] The severity and location of the bleed is also highly associated with the severity of clinical presentation, which is also important prognostic indicator of outcomes.[278] Various grading scales have been used to evaluate the severity of clinical presentation including the Hunt and Hess (HH) Scale (Table 1-6: Hunt and Hess Scale), World Federation of Neurological Surgeons Scale (WFNSS) (Table 1-8: World Federation of Neurologic Surgeons Scale), and Glasgow Coma Scale (GCS) (Table 1-7: Glasgow Coma Scale).[278] Scores on the HH[46, 274, 282, 283], WFNSS[284, 285] and the GCS [246, 274, 280, 285, 286] have predicted outcomes in diverse populations of SAH patients whether assessed on admission or during the clinical course. Collectively, these studies suggest that aneurysm factors, large bleeds, and poor clinical presentation are important prognostic indicators of outcome after SAH.

Table 1-5: Fisher Scale

Grade	Criteria
1	No hemorrhage evident
2	SAH < 1mm thick
3	SAH > 1mm thick
4	SAH of any thickness with IVH or parenchymal extension

IVH, intraventricular hemorrhage

Table 1-6: Hunt and Hess Scale

Grade	Criteria
1	Asymptomatic, mild headache, slight nuchal rigidity
2	Moderate to severe headache, nuchal rigidity, no neurologic deficit other than cranial nerve palsy
3	Drowsiness / confusion, mild focal neurologic deficit
4	Stupor, moderate-severe hemiparesis
5	Coma, decerebrate posturing

Table 1-7: Glasgow Coma Scale

Response	1	2	3	4	5	6
Eye Opening	Spontaneously	To verbal stimuli	To pain	Never	N/A	N/A
Verbal	Oriented and converses	Disoriented and converses	Inappropriate words	Incomprehensible sounds	No response	N/A
Motor	Obeys commands	Localizes pain	Flexion withdrawl	Abnormal flexion (discorticate rigidity)	Extension (decerebrate rigidity)	No response

Table 1-8: World Federation of Neurologic Surgeons Scale

Grade	Glasgow Coma Score	Motor Deficit
1	15	Absent
2	13 - 14	Absent
3	13 - 14	Present
4	7 - 12	Present or Absent
5	3 - 6	Present or Absent

1.1.6.4 CBF dysregulation: Numerous hemodynamic factors are associated with outcomes after SAH. In the first few days after SAH, worse outcomes are expected in patients with increased ICP[135-137], decreased CPP[135-137], vasospasm[183, 184] and cerebral ischemia[137, 180, 182, 184, 287, 288] Likewise, continuous measurement of cerebrovascular autoregulation using the pressure reactivity index were associated with outcomes after SAH.[289] However, Barth et al. reported that pressure-, oxygen-, and flow-related autoregulatory indices were not associated with outcomes.[290] A few days after SAH, hemodynamic factors such as cerebral vasospasm and DCI often lead to cerebral infarction and are leading cause of morbidity and mortality after acute SAH.[171, 291] In a review of 106 studies (3,327 patients), DCI was associated with a mortality rate of 30% and permanent

deficits in 34%.[292] Another study reported that patients with DCI are 6-fold more likely to suffer from moderate to severe neuropsychological deficits when compared to patients that do not develop DCI.[293] Cerebral vasospasm is generally associated with DCI, and to a lesser extent cerebral infarction, but demonstrates poor ability to predict outcomes when compared to DCI or cerebral infarction.[294-298] When multiple secondary insults were evaluated together, the number of these potentially avoidable events after SAH were found to be important predictors of outcome.[299] Other clinical complications associated with worse outcomes include global cerebral edema[150], hydrocephalus[286], cardiac dysfunction[300], hyperglycemia[150], fever[150], anemia[150], pneumonia[150], sepsis[150], pulmonary edema[300], epilepsy[301]. In addition, comorbidities such as untreated and treated hypertension, atrial fibrillation, congestive heart failure, coronary artery disease, and renal disease have been shown to affect outcomes.[235] Collectively, these studies suggest that hemodynamic factors, clinical complications, comorbidities, and secondary insults after SAH are predictors of outcome.

1.1.6.5 Inter-relation and clinical relevance: Although numerous prognostic factors of outcomes after SAH have been identified, studies note the inter-relation of these factors and the limitations of the individual predictors.[245, 302] For example, older patients are more likely to be admitted with poor clinical grade SAH and to develop medical complications that may lead to worse outcomes.[259] Likewise, the aneurysm size and severity of clinical presentation are closely associated with aneurysm rebleeding.[149] Multivariate analysis can be used to address the issue of the inter-relation of these factors and to identify the primary determinants of outcomes. Using multivariate analyses, Hijdra et al. argued that the three most important prognostic indicators for outcomes are age, severity of clinical presentation (Table 1-6: Hunt and Hess Scale), and the severity and location of the bleed (Table 1-5: Fisher Scale).[250, 280] Of these 3 prognostic factors, the severity of clinical presentation, particularly the level of consciousness, was the most important determinant[280], but the mechanisms of this relationship remain unclear.[198] Other studies report additional individual prognostic factors of outcome using multivariate analyses, but the severity of clinical presentation and/or the severity and location of the bleed are consistently noted determinants in these studies as well.[169, 274, 303, 304] Clinical grading scales are commonly used to assess the severity of bleed and clinical presentation and to predict outcomes, but their predictive

ability is limited and none have achieved universal acceptance.[245, 278] Currently, there are no widely accepted prognostic indicators of long-term outcomes after SAH.

1.1.7 Prognostic factors of acute outcomes

This section highlights some of the key methods used for the diagnosis and prediction of acute ischemic complications such as CND and DCI. These methods include clinical grading scales, radiographic monitoring modalities, physiological indicators, biochemical markers, and genetic markers.

1.1.7.1 Diagnosis and management of DCI: The prediction, prevention, diagnosis, and treatment of acute outcomes such as CND and DCI has become a major focus in the management of SAH because it occurs in a high percentage of patients, leads to significant morbidity and mortality, and develops days after SAH potentially providing a time window for therapeutic intervention. As previously mentioned in Section 1.1.3, the development of DCI in SAH patients is a complex process in which the pathological processes involved are not fully understood and the clinical diagnosis is difficult. Vergouwen et al. recently provided a thorough review of the various terms used in the literature to describe DCI in SAH patients and the problems presented by the variety of these terms.[305] One of the primary problems in defining and measuring DCI in the clinic is that the clinical features of cerebral ischemia are difficult to categorize and diagnose, especially in patients who are comatose or sedated. Also, clinical features of DCI require the exclusion of other causes, such as rebleeding, surgery, infection, and hydrocephalus, which may be unrelated to cerebral vasospasm. In order for consistent comparison across studies, many clinicians use clinical grading scales, radiographic methods, and/or physiological indicators to diagnose or predict acute ischemic complications after SAH.[306]

1.1.7.2 Clinical grading scales: The clinical condition of SAH patients is commonly evaluated using the HH (Table 1-6: Hunt and Hess Scale), Fisher (Table 1-5: Fisher Scale), GCS (Table 1-7: Glasgow Coma Scale), and WFNS (Table 1-8: World Federation of Neurologic Surgeons Scale) scales. These scales have been used to categorize the severity of SAH and predict outcomes, but

have been shown to have poor inter-rater reliability and limited success in predicting DCI.[278, 307, 308] The Fisher Scale is the most commonly used clinical grading method used to predict vasospasm after SAH.[278] Disadvantages of the Fisher Scale includes scoring criteria developed using old imaging technology, unclear and subjective classifications, and exclusion of certain characteristics of the bleed such as clot density and clearance rate.[278] As a result of these limitations, clinical grading scales have not approached universal acceptance as predictors of acute ischemic complications after SAH.

1.1.7.3 Radiographic monitoring modalities: Radiographic monitoring modalities such as CT scan and magnetic resonance image (MRI) have been reported as useful tools for monitoring for DCI after SAH. Cerebral angiography is reported to be the gold standard for assessing cerebral vasospasm after SAH.[291] Despite the accurate identification of the presence of vasospasm, angiography has shown limited ability to accurately diagnose or predict DCI.[309-311] In addition, these radiographic monitoring methods are expensive, invasive, and cannot be applied regularly at the patient bed-side. These factors limit the utility of radiographic methods to be used to monitor or predict the onset of acute ischemic complications in SAH patients.

1.1.7.4 Physiological Indicators of DCI: Numerous studies have investigated physiological indicators of DCI using transcranial Doppler ultrasonography (TCD), electroencephalography (EEG), brain tissue oxygen monitoring, and cerebral microdialysis, while a few recent studies have evaluated and near-infrared spectroscopy (NIRS) and thermal diffusion cerebral blood flow (TD-CBF).[306] Limitations of these methods to predict DCI include cost, invasive procedures, inability to continuously monitor patients at bed-side, and studies with small number of patients.[306] The use of TCD to measure the velocities of RBCs in large cerebral arteries and has been previously associated with the development of radiographic evidence of vasospasm.[312-314] Although this method is cheap, non-invasive and bedside technology, significant inter-patient variability is observed because it is highly operator dependent and temporal windows are inadequate in some populations.[314-316] Thus, there are currently no clinically relevant physiological measurements that can be used to predict the onset of acute ischemic complications in SAH patients.

1.1.7.5 Biochemical markers: Due to the limitations of the clinical grading scales, radiographic methods, and physiological measurements to predict DCI, many researchers have investigated the ability of certain biomarkers to predict or indicate the onset of acute ischemic complications. A

biomarker can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[263] Potential advantages presented by biomarkers include the ability to elucidate the pathogenic mechanisms, identify early indicators of complications, and evaluate the temporal effects of the biomarker levels over the clinical course of SAH injury. A recent review by Rodriguez-Rodriguez et al. identified numerous biochemicals involved in inflammation, oxidative stress, tissue injury, and vascular pathology as potential biomarkers of vasospasm and outcome after SAH.[317] Lad et al. evaluated proteomic CSF biomarkers after SAH and identified the most promising vasospasm biomarkers to be ET-1, interleukin-6, and thrombin activity markers and outcome biomarkers to be cytokines and neurofilaments.[318] Ducruet et al. summarized the relationships between gene variants with various outcomes after SAH, but noted study limitations such as small cohorts and inconsistent results.[319] Currently, there are no widely accepted genetic or biochemical markers of DCI or long-term outcomes after SAH.

1.1.7.6 Genetic Markers: Other studies have investigated the relationship between genetic markers, DCI, and outcomes in SAH patients. Advantages of using genetic markers to predict DCI include consistent genotyping results, inexpensive methods, and the ability to apply the predictive tool at any time before or after the injury. Most of these genetic studies have focused on candidate genes that have been shown to impact CBF or recovery after SAH.

Nitric Oxide: NO plays a critical role in the regulation of cerebrovascular tone under basal conditions[320] and after SAH[321-324]. The eNOS gene regulates NO synthesis in the endothelium of the cerebral vasculature[325, 326] and thus serves as a candidate gene for genetic studies in SAH patients. Multiple studies investigated the role of the eNOS promoter polymorphism -786T>C (rs2070744) on acute and long-term outcomes in patients with SAH. In SAH patients classified as Fisher grade 3, carriers of the variant C allele were more likely to develop acute complications such as asymptomatic or symptomatic vasospasm when compared to patients with the TT genotype.[327] In this study, vasospasm was determined using TCD or angiography and was considered symptomatic if the vasospasm occurred in the presence of DIND. Likewise, Starke et al. reported that patients with the variant CT genotype showed an approximate 3.5-fold increase in the incidence of cerebrovascular complication, such as angiographic vasospasm, DIND, and angiographic vasospasm with DIND, whereas no significant association was observed with

genotype and infarct on CT or MRI scan.[328] Other studies reported that the -786T>C gene variants were associated with outcomes in SAH patients only after controlling for important clinical covariates in a multivariate analysis. For example, Ko et al. reported that the variant genotype or allele was not associated with angiographic vasospasm or infarct on CT scan in a univariate model.[329] But, the presence of the homozygous variant CC genotype was associated with a 3-fold increase in risk of angiographic vasospasm after adjustment for age, sex, race/ethnicity, Hunt-Hess grade, and Fisher group. In a another study, the presence of the variant C allele was not associated with DIND, but predicted an approximate 4-fold increase in risk for poor outcomes as determined by GOS after controlling for age and sex.[330] Alexander et al. used a tagging SNP approach to characterize the genetic variability of the eNOS gene in SAH patients and reported that angiographic vasospasm was not associated with any of the individual tagging SNP alleles, but was associated with the combination of three tagging SNP variants (rs1799983, rs1800779, rs3918188).[331] In addition, the carriers of the variant allele in rs1799983 was associated with worse long-term functional outcomes as measured by GOS and MRS at 6 months.[331] Taken together, these results suggest that the -786T>C promoter polymorphism and other eNOS variants may be associated with worse acute and long-term outcomes after SAH.

Apolipoprotein E: Apolipoprotein E (APOE) plays an important role in the pathological events after SAH [332] including the impairment of vascular endothelium function [333, 334]. There are multiple studies investigating the relationship between APOE gene variants and acute and long-term outcomes after SAH in humans. The APOE ε4 (rs429358) gene variant has been extensively studies in SAH patients, but results are conflicting. For example, Gallek et al. reported that the APOE ε4 allele was associated with worse functional outcomes, as determined by GOS and MRS scores at 3 and 6 months, after controlling for age, race, HH grade, and Fisher grade.[335] On the other hand, other studies reported that the APOE ε4 was not associated with cerebral infarction[336], functional outcomes as determined by GOS and MRS at 3 months[336] and GOS at 16 months, or neuropsychological outcomes at 16 months.[337] Although there are some conflicting results regarding the relationship of the APOE ε4 polymorphism and outcomes after SAH, a meta-analysis reported that the APOE ε4 allele was associated with a 2-fold increase in incidence of DCI and 2.6-fold increase in incidence of poor outcomes.[338] Another study investigated the relationship of promoter polymorphisms on the APOE gene and reported that patients with the variant -291T

allele were almost 4-times more likely to develop the concomitant presence of TCD vasospasm with DIND in both univariate and multivariate analyses after controlling for age, sex, blood pressure, blood lipid, Hunt and Hess grade, and Fisher grade.[339] These results suggest that the ε4 and -291T promoter variants on APOE gene may be associated with worse acute and long-term outcomes in SAH patients.

Other candidate genes: Other studies have investigated the relationship between certain candidate gene polymorphisms and outcomes in SAH patients, but there are inconsistent results or a lack of studies to verify the findings. For example, Vergouwen et al. reported that the -675 5G>4G (rs1799889) allele on Plasminogen Activator Inhibitor-1 (PAI-1) gene was associated with a 3.3-fold increase in relative risk for DCI and a trend for worse functional outcomes as determined by GOS at 3 months.[340] On the other hand, Ladenvall et al. reported that the -675 5G>4G allele was not associated with functional outcomes as determined by GOS at 1 year.[341] One study reported that the 6178G>T (rs335364374) allele on Ryanodine Receptor 1 (RYR1) was associated with a 6-fold increase in risk for symptomatic vasospasm, which was defined as the combined presence of DIND along with cerebral vasospasm as determined by TCD, angiography, or CT scan.[342] Another study reported that the patient homozygous for the 1080C>T variant on Cystathionine β-synthase (CBS) gene were at increased risk for DCI, but not angiographic vasospasm, after controlling for admission hypertension.[343] In this study, DCI was defined as the presence of DIND or infarct on CT or MR imaging. Also, carriers of the variant Val66Met allele (rs6265) on the Brain Derived Neurotropic Factor (BDNF) gene were 4- and 8-fold more likely to show worse functional outcomes as measured by GOS both before and after controlling for patient age, clinical condition, and radiological severity of the bleeding, respectively.[344] In addition, a Catechol-O-methyltransferase (COMT) gene variant was associated with TCD vasospasm early after SAH both before and after controlling for clinical covariates.[345] In SAH patients classified as Fisher Grade 3, carriers of the variant α2 subunit on the haptoglobin (Hp) gene showed a 23-fold increase in risk for TCD vasospasm.[346] Furthermore, Ruigrok et al. genotyped SAH patients for variants on multiple candidate genes previously associated with outcomes in animal models of cerebral ischemia.[347] In this study, the presence of the -863C>A allele on Tumor Necrosis Factor-α (TNF-α) predicted 2.3-fold increase in risk for worse outcomes as determined by GOS at 3 months after controlling for sex, age, clinical condition on admission, amount of blood on CT scan,

rebleeding, and DCI.[347] In summary, there are a few studies that report an association between genetic polymorphisms in the PAI-1, RYR1, CBS, BDNF, COMT, Hp, and TNF-α genes and altered acute and long-term outcomes in SAH patients, but results need to be confirmed in additional studies. One common element of the genes and biomarkers associated with complications after SAH is that several of these pathways are involved in the regulation of CBF. Therefore, a thorough understanding of the cerebral circulatory system, CBF regulation, and mechanisms of autoregulation is beneficial to understand the impact of these potential prognostic factors on outcomes after SAH.

1.2 CEREBRAL BLOOD FLOW

1.2.1 Cerebral circulatory system

1.2.1.1 Regulation of CBF in microcirculation and macrocirculation: The cerebral circulation can be broadly categorized into two groups based on the size of the arteries: macrocirculation and microcirculation. In the macrocirculation, blood enters the brain through the internal carotid arteries and vertebral arteries and then flows through large arteries in the subarachnoid space. These large arteries include the Circle of Willis at the base of the brain and pial arteries that course the surface of the brain. Blood then flows through arteries that penetrate the cerebral parenchyma into smaller arteries, arterioles, and finally capillaries before returning to the circulatory system through the venous system. There is a notable difference in the relative contribution of the macrocirculation and microcirculation to the regulation of CBF. Large arteries play a major role in the regulation of CBF and contribute significantly to the total cerebrovascular resistance (CVR). For instance, large pial arteries in cats and monkeys comprised of the majority (60%) of the total CVR, but differences in species were noted.[348] Small arteries, arterioles, and capillaries in the

microcirculation also significantly contribute to the regulation of CBF, but primarily through metabolic control and neurovascular coupling.[349] These studies demonstrate that both the macrocirculation and microcirculation are important contributors of CBF regulation.

1.2.1.2 Morphology and function of microcirculation and macrocirculation: There are also notable differences in the morphology and thus function of cerebral arteries in the microcirculation and macrocirculation. The general morphology of cerebral arteries has been reviewed by Lee et al.[96] The artery is comprised of three primary layers: intima, media, and adventitia. These layers are separated by a thin layer of elastic tissue called the elastic lamina. The intima is the innermost (luminal) layer and is comprised of a single layer of endothelial cells that rest on a protein rich layer called the basal lamina. The media contains a thick layer of smooth muscle cells, elastic fibers, and collagen fibers. The outermost layer is the adventitia contains fibroblasts, elastic fibers, collagen fibers, and nerve fibers that innervate VSMC. However, there are significant differences morphology and thus function of the cerebral arteries in the various vessel types. For instance, capillaries contain only endothelial cells and a basement membrane and regulation of CBF through these vessels is controlled by pericytes. Capillary endothelial cells are connected by tight junctions that form the BBB, which controls the exchange of materials between the blood and brain through anatomical, physiochemical, and biochemical mechanisms. Smaller arteries and capillaries are surrounded by astrocytic end feet that couple neuronal and vascular activity.[350] Astrocytes contribute to the integrity of the BBB, provide structural support to neurons, uptake metabolic by-products of nerve activity, and recycle neurotransmitters.[351, 352] Also, large cerebral arteries contain small blood vessels in the adventitia layer that supply nutrients to outer regions of the vessel wall and extrinsic perivascular nerves that can stimulate the VSMC in the media layer. The varied morphology of the cerebral vasculature emphasizes the specialized function of these vessels.

1.2.2 CBF regulation

The adult brain weighs 2% of the total body weight, but consumes 20% of the oxygen produced by the body at resting state. Overall CBF in the brain is ~50ml/100g/min with higher perfusion in

grey matter (70ml/100g/min) and lower perfusion in white matter (20ml/100g/min). [353] The mechanisms involved in the regulation of CBF are numerous and complex. However, the following paragraph will briefly discuss factors involved in the regulation to CBF including myogenic, metabolic, and neurogenic mechanisms.

Cerebral autoregulation is the process in which the cerebrovasculature maintains a constant CBF in the face of changing CPP by altering CVR.[353] CPP is usually not measured directly but is calculated from the difference between mean arterial blood pressure (MABP) and intracranial pressure (ICP).

Equation 1-1: CBF as a function of CPP

$$CBF = CPP / CVR$$
$$CPP = MABP - ICP$$

CBF, cerebral blood flow; CPP, cerebral perfusion pressure; CVR, cerebrovascular resistance; MABP, mean arterial blood pressure; ICP, intracranial pressure

MABP is commonly used as an index of CPP because it is usually higher than ICP. However, in patients with SAH, ICP often rises to levels approaching MABP resulting in loss of perfusion and cerebral ischemia.[354] In normal brain, CBF undergoes autoregulation at MABP from 70 to 120 mmHg to ensure adequate oxygen delivery to the brain.[353] Below 70 mmHg, cerebral vasodilation is maximal and CBF decreases as transmural pressure decreases. Above 120 mmHg, vasoconstriction is maximal and CBF increases as transmural pressure increases.[353] Loss of autoregulation can lead to damage in the structure of the arteries, perfusion insufficient to meet metabolic demands, altered cerebral blood volume and ICP, and tissue damage in the brain.[353] Loss of cerebral autoregulation is seen in SAH and other pathophysiological states.[353] Thus, cerebral autoregulation is a complex process to maintain CBF in the face of dynamic changes in hemodynamics in order to prevent cerebral injury.

1.2.3 Mechanisms of cerebral autoregulation

1.2.3.1 Myogenic, metabolic, and neurogenic processes: The primary paradigms associated with the autoregulation of CBF include myogenic, metabolic, and neurogenic processes.

Myogenic response refers to the constriction that occurs in arteries in the face of increasing arterial pressure. Large cerebral arteries on the Circle of Willis and pial arteries on the surface of the brain are sensitive to myogenic regulation.[355] The cellular mechanisms of pressure-induced myogenic tone have not been clearly elucidated, but the response is associated with activation of mechano-sensitive nonselective cation channels (stretch receptors), activation of phospholipase C (PLC), release of AA from membrane, inhibition of K_{Ca+2} channels, and Ca^{2+} entry through voltage-sensitive channels of VSMC.[351, 356, 357] Metabolic regulation, also referred to as functional hyperemia or neurovascular coupling, refers to the process by which neuronal activity leads to dynamic changes in regional CBF. During neuronal activity, regional CBF is regulated to meet the metabolic demand for glucose and oxygen levels in active regions of the brain and to remove metabolic products.[352] Metabolic regulation of CBF occurs rapidly (within a few seconds) through a complex process involving the neurovascular unit.[352] Astrocytes play a key role in neurovascular coupling through cell signaling events and astrocytic endfeet processes.[351, 352] Neurovascular coupling is controlled by several metabolic factors including potassium, CO_2, O_2, pH, and adenosine and vasoactive substances such as NO and EETs.[352] Regulation of CBF by metabolic processes usually occurs at the level of small precapillary arterioles.[355] Furthermore, CBF in large arteries are subject to neurogenic regulation by extrinsic perivascular nerve.[355] This process appears to protect the brain against large fluctuations in blood pressure, but is reported to play a minor role in cerebral autoregulation compared to myogenic and metabolic regulation.[355, 358] Thus, the primary mechanisms involved in autoregulation of CBF are myogenic response and metabolic regulation.

1.2.3.1 Endothelial derived factors: It is also important to highlight the critical role of the vascular endothelium in the development and maintenance of cerebrovascular tone. Numerous vasodilator and vasoconstrictor substances are synthesized and release in the endothelium of cerebral arteries. Well-known endothelium-derived constricting factors (EDCF) include endothelin 1 (ET-1), thromboxane A2 (TXA_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).[359] The production of ET-1 is catalyzed by endothelin converting enzymes (ECE) and can be stimulated by angiotensin II, vasopressin or thrombin.[359] Well-known constrictors derived from endothelium include NO, prostaglandin I_2 (PGI_2), prostaglandin E_2 (PGE_2), and endothelium-derived relaxing factors (EDRF).[359] EDRF is a general term used to describe factors hyperpolarize VSMC through

activation of K^+ channels and include CO, K^+, hydrogen peroxide (H_2O_2), NO, and EETs.[356, 360] Release of EDRF can be stimulated by numerous plasmogens, hormones, and mechanical stimuli.[359] Endothelial dysfunction is plays a pivotal role in the pathogenesis of many cerebrovascular disease including SAH.[361] Also, the imbalance between vasoconstrictive and vasodilatory factors is thought to contribute to the development of cerebral vasospasm.[159] These findings demonstrate the vascular endothelial factors contribute to regulation of cerebrovascular tone and pathophysiology of cerebrovascular disease.

1.3 EICOSANOIDS AND CYP METABOLISM

1.3.1 Eicosanoids

This section highlights the important role of arachidonic acid metabolites, also known as eicosanoids, in the pathogenesis of numerous disorders. This section notes that arachidonic acid metabolites of the cyclooxgenase and lipooxygenase pathway are implicated in the development of DCI after SAH, but have shown limited clinical utility. Conversely, eicosanoids derived from the cytochrome P450 pathway have not been studied as extensively.

1.3.1.1 Eicosanoid Biosynthesis and Clinical Relevance: Eicosanoids are important signaling molecules derived from the oxidation of either ω-3 or ω-6 essential fatty acids. Arachidonic acid (AA) is a polyunsaturated ω-6 essential fatty acid that is typically stored in the *sn-2* position of phospholipid membranes and comprises approximately 5–15% of total fatty acids in most tissue phospholipids.[362] AA is released primarily through the activation of cytosolic phospholipase A_2 ($cPLA_2$) in response to a variety of physiological, pharmacological, and pathological stimuli.[363] Free AA can be oxidized by several enzymes to produce multiple groups of eicosanoids that play an important role in cell signaling and regulation pathways.[364] The enzymatic oxidation of AA

occurs through three major pathways: cyclooxgenase (COX), lipooxygenase (LOX), and cytochrome P450 (CYP) (Figure 1-4: Eicosanoid synthesis from arachidonic acid).[365] The oxidation of AA by COX and LOX forms multiple families of eicosanoids including prostaglandins (PGs), prostacyclin (PGI_2), thromboxane (TXA_2), leukotrienes (LTs), lipoxins (LPs), and others. These eicosanoids are widely recognized as pathologic mediators of CVD, cancer, pain, fever, inflammation, and other disorders[366-368] and thus serve as targets for numerous pharmacotherapies.

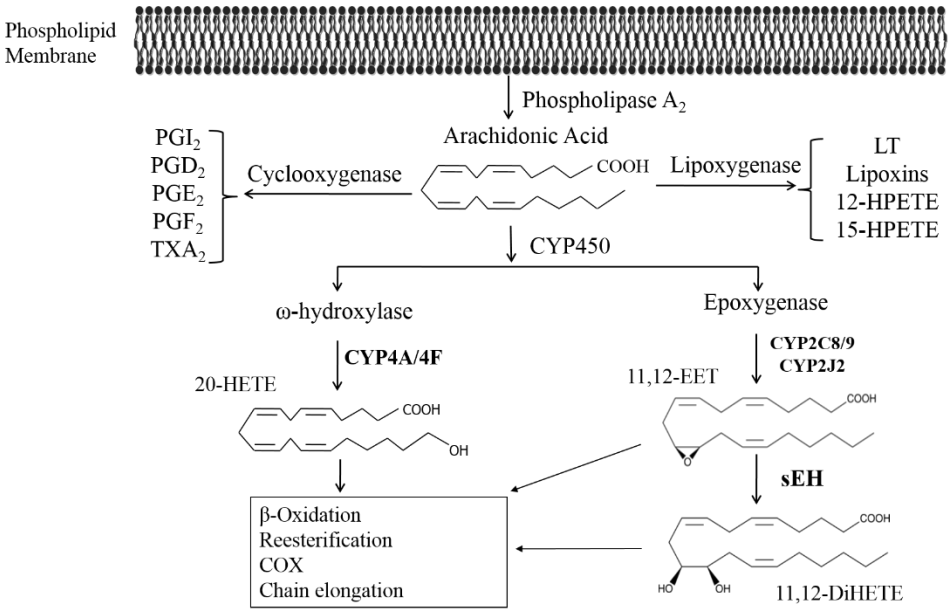


Figure 1-4: Eicosanoid synthesis from arachidonic acid

(Permission for use of figure granted by Jafar Sadik Bhasha Shaik)

1.3.1.2 Role of Eicosanoids in CBF Regulation: It is well established that eicosanoids derived from COX enzymes, and to a lesser extent LOX enzymes, contribute to the regulation of CBF and development of DCI.[369] For example, alterations in the levels of multiple eicosanoids were observed in various experimental models of DCI.[370-374] Studies have shown that the cerebral vasculature constricts *in vitro* and *in vivo* in response to a variety of these eicosanoids.[375, 376] Also, administration of prostacyclin and non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to prevent or reverse vasospasm in experimental models of SAH.[377-381] These studies indicate that eicosanoids are likely to contribute to the development of DCI and pathogenesis of

SAH in humans. However, treatment of SAH patients with agents targeting the COX and LOX pathway has been limited due to either lack of clinical efficacy or unwanted side effects primarily affecting hemostasis.[382-384] On the other hand, eicosanoids formed from the oxidation of AA by CYP enzymes have not been studied as extensively. However, recent evidence demonstrates that eicosanoids of the CYP pathway of AA metabolism are formed in various tissues, exert diverse biological effects, and contribute to the overall pathogenesis of several disease states including SAH.

1.3.2 Cytochrome P450 (CYP) enzymes

This section describes the relevant background on CYP enzymes and their mechanism of action.

1.3.2.1 Background on CYPs: Cytochromes P450 (CYPs) constitute a superfamily of heme-containing proteins that play an important role in the metabolism of a large variety of xenobiotics and endogenous compounds.[385, 386] CYPs are bound to membranes in the endoplasmic reticulum or in the inner mitochondrial membrane.[385] The name for CYPs was derived from the unique spectral properties of these of enzymes that absorb light at 450nm after reduction by carbon monoxide (CO).[385] The oxidation of a substrate by CYPs involves the splitting of molecular oxygen and the transfer of one oxygen atom to the substrate and the other oxygen atom to form a water molecule.[385, 387] Based on their mechanism of action, CYPs are also referred to as monooxygenase, mixed function oxidase, and hydroxylase enzymes.[385] The catalytic activity for CYPs requires the flavoprotein cytochrome P450 reductase for the transfer of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to CYPs.[385, 387] CYPs can also complex with cytochrome b_5 to alter catalytic activity.[385, 387]

1.3.2.2 CYP catalytic cycle: Details of the CYP catalytic cycle are described by Danielson et al. and summarized in Figure 1-5: CYP catalytic cycle.[385] First, the substrate binds to the CYP that is linked to a heme group made of a protoporphyrin IX complex with iron at the center. Before binding of the substrate, the iron is in the Fe^{+3} state and binds water above the plane of the protoporphyrin IX complex. Binding of the substrate releases water bound to Fe^{+3} and facilitates

the transfer of an electron from NADPH-CYP reductase to reduce Fe^{+3} to Fe^{+2}. Next, the CYP/substrate complex binds and reacts with molecular oxygen to produce an unstable ferrous dioxygen-bound complex (Fe+3 -O_2·) that accepts the transfer of a second electron from NADPH-CYP reductase to form Fe^{+3}-O_2-. The reduced complex is protonated twice to form of the reactive ferryl heme ($[Fe\text{-}O]^{+3}$), and the release of a water molecule. Finally, the oxygen atom of the ferryl species is transferred to the substrate and the substrate is released leaving the enzyme in its original Fe^{+3} state bound to water.

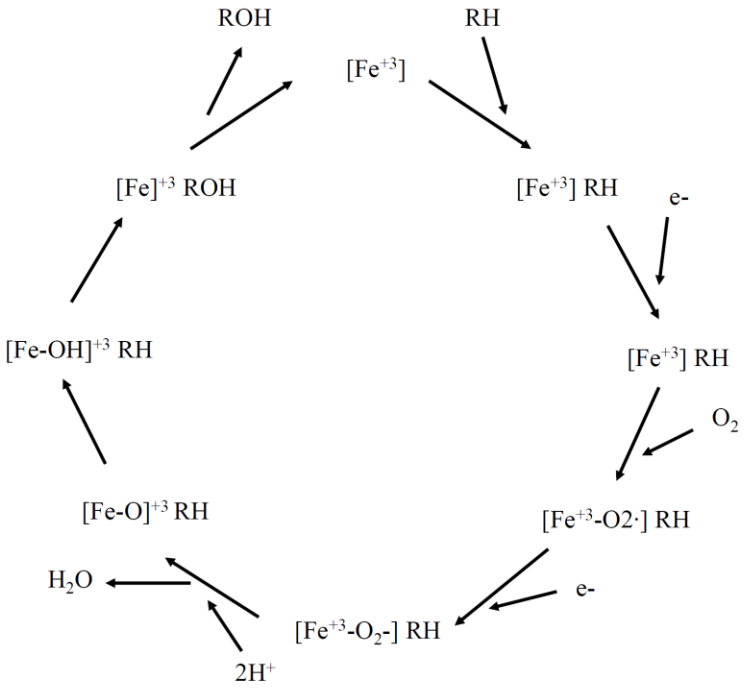


Figure 1-5: CYP catalytic cycle

1.4 CYP EICOSANOID BIOSYNTHESIS AND ELIMINATATION

This section highlights the key enzymes responsible for the formation and elimination of eicosanoids derived from the CYP pathway of AA metabolism. It also discusses the nomenclature for CYP eicosanoids and the regio- and stereo-selectivity of their isomers. Furthermore, the

relevant elimination pathways of these CYP eicosanoids are discussed.

1.4.1 CYP eicosanoid formation

1.4.1.1 CYP eicosanoid regioisomers: CYPs can metabolize AA by hydroxylation, epoxidation, or allylic oxidation reactions. The nomenclature of these CYP eicosanoids is based on the site of oxidation. Hydroxylation or allylic oxidation of AA leads to the formation of hydroxyeicosatetraenoic acids (HETEs) while epoxidation of AA forms epoxyeicosatrienoic acids (EETs). The hydroxylation of AA can occur at the terminal carbon to form 20-hydroxyeicosatetraenoic acid (20-HETE)[388-395] or subterminal positions to form 16-, 17-, 18-, or 19-HETE.[396, 397] Allylic oxidation of AA leads to the formation of 7-, 10-, 11-, 12- 13-, and 15-HETE.[398] Epoxidation of AA can occur at one of the four double bonds of AA leading to the formation of 5,6-, 8,9-, 11,12-, or 14,15-EET.[391, 392, 397, 399-404] EET isomers occur as R- or S-enantiomers and the regio- and stereo-selectivity of EET formation can vary among mammalian species and tissue.[356] Numerous studies have reported that 20-HETE and EETs are the major products produced by the CYP mediated metabolism of AA.[356] Thus, 20-HETE and EETs are the major CYP eicosanoid products.

1.4.1.2 20-HETE and EET synthesis: Numerous human CYP isoforms have been implicated in the formation of 20-HETE including CYP4F2, CYP4A11, CYP2E1, CYP2C18, CYP2C19, CYPF12, CYP1A1, CYP1B2, CYP1B1, and CYP4FB3.[388-395] But, it has been established that CYP4F2 and CYP4A11 are the key human CYP isoforms responsible for the formation of 20-HETE.[356] 20-HETE is formed in tissues various tissues including liver, kidney, heart, lung, brain and vasculature.[356] Human enzymes responsible for the formation of EETs include CYP2C8, CYP2C9, CYP2J2, CYP2C18, CYP2B6, CYP1A2, CYP1A1, and CYP1B2[391, 392, 397, 399-404], but CYP2J2, CYP2C8, and CYP2C9 are primary isoforms responsible for EET synthesis.[356] EETs are formed in various tissues including liver, kidney, heart, lung, brain, pancreas, gastrointestinal tract, and vasculature.[356, 405] In brain, EETs are primarily formed by neurons, astrocytes, and endothelial cells and can be released from the membranes of red blood cells while 20-HETE is primarily synthesized in vascular smooth muscle cells (VSMC) and to a lesser extent white blood

cells.[356, 406, 407] Together, these data demonstrate that CYP eicosanoids are formed in various tissues from the metabolism of AA and the key human CYP enzymes responsible for the formation of 20-HETE are CYP4F2 and CYP4A11 while EETs are primarily formed by CYP2J2, CYP2C8, and CYP2C9.

1.4.2 CYP eicosanoid elimination

Once formed, EETs can be further elimination in a number of pathways. The primary pathway of EET elimination is metabolism by soluble epoxide hydrolase (SEH) to their corresponding diols known as dihydroxyeicosatrienoic acids (DHETs). This process appears to be stereoselective with the preferred substrate of 14,15-EET > 11,12-EET > 8,9-EET and negligible metabolism of 5,6-EET.[408] EETs and to a lesser extent DHETs[409] can also be incorporated into the *sn-2* position of phospholipid membranes through esterification by coenzyme A (CoA)[410, 411] and SEH is proposed to regulate this process.[412] Like AA, these membrane stores of EETs and DHETs can be released through activation of $cPLA_2$.[413] EETs are reported to be highly bound to fatty acid binding proteins thus limiting free concentrations in the plasma.[414, 415] DHETs exhibit protein binding but to a lesser extent than EETs.[415] Other minor pathways of elimination include β-oxidation, glutathione conjugation, and metabolism by CYPs.[414] Thus, can bind to proteins or be incorporated into lipid membranes, but the primary elimination pathway of EETs is metabolism by SEH to form DHETs.

The primary pathways of 20-HETE eliminated have not been clearly elucidated.[416] Porcine coronary endothelial cells have been shown to metabolize 20-HETE to 20-carboxy-aracidonic acid (20-COOH-AA).[417] Likewise, cerebral arteries converted 20-HETE to 20-COOH-AA[418, 419] with catalysis by alcohol dehydrogenase.[419] On the other hand, mouse brain endothelial cells and rat seminal vesicles converted 20-HETE to 20-OH-PGE_2 and 20-hydroxy-$PGF_2\alpha$, respectively.[418, 420] In human tissues, 20-HETE was metabolized by COX and LOX enzymes in platelets to form a series of novel metabolites formed by COX.[421] Also, a glucuronide conjugation of 20-HETE was reported in urine.[422, 423] Like EETs, 20-HETE is highly bound to plasma proteins and is avidly incorporated into lipid membranes through esterification.[356] 20-HETE also reported to undergo β-oxidation.[356] Taken together, these studies report a series of 20-HETE metabolites and elimination pathways, but none have been identified as the primary pathway of elimination.

1.5 CYP EICOSANOID ENZYMES

1.5.1 Expression of CYP eicosanoid enzymes

This section highlights relevant information on the CYP family, gene location, protein expression, tissue localization, and mammalian orthologues for CYP eicosanoid enzymes.

1.5.1.1 CYP4A11 expression: In mammals, six CYP4 gene subfamilies have been identified CYP4A, CYP4B, CYP4F, CYP4V, CYP4X, and CYP4Z [424-427] In humans, the CYP4A family consists of two isoforms CYP4A11 and 4A22, which are located in the CYP4 ABZX gene cluster at 1p33 [425]. CYP4A22 shares 97% nucleotide identity and 94% amino acid identity with CYP4A11 but expression in human tissues is much lower than CYP4A11.[428, 429] CYP4A11 is primarily expressed in human liver and kidney. To illustrate, CYP4A11 is the predominant microsomal CYP4A enzyme in human liver and kidney microsomes[428, 429] and is highly expressed in liver parenchymal cells[390], kidney proximal tubule cells [390, 430] and to a lesser extent in the thick ascending limb and collecting duct of the kidney[430]. The CYP4A isoforms capable of producing 20-HETE in rats include CYP4a1, CYP4a2, CYP4a3, and CYP4a8, which are highly expressed in tissues such as liver, kidney, testes, or muscle.[356, 425] Likewise, the CYP4A isoforms capable of producing 20-HETE in mice include CYP4a10, CYP4a12, and CYP4a14, which are highly expressed in tissues such as liver and kidney.[356, 425] These data suggest that CYP4A enzymes are highly expressed in liver and kidney and to a lesser extent other tissues in mammalian species.

1.5.1.2 CYP4F2 expression: In humans, the CYP4F gene subfamily consists of CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12, and CYP4F22 genes, which are located at chromosome 19p13 as part of a large gene cluster.[396, 431-434] These CYP4F genes have very similar structures and splicing sites.[435] It has been proposed that the CYP4F subfamily is the result of a gene duplication process resulting in multiple CYP4F genes with high amino acid homology and different catalytic specificity.[436] Of the CYP4F enzymes expressed in humans, CYP4F2 is the predominant enzyme responsible for the metabolism of fatty acids (ω-hydrolase).[425] CYP4F2 is

expressed in significant and highly variable levels in liver and kidney[425, 437] CYP4F2 is expressed to a lesser extent in skin, several other tissues and tumors, but not in myeloid cells in humans.[435] The CYP4F isoforms response for 20-HETE formation in rats include CYP4f1, CYP4f4, CYP4f5, and CYP4f6 enzymes, which are highly expressed in liver, kidney, brain, gastrointestinal tract, and lung.[356, 425] Likewise, the CYP4F isoforms response for 20-HETE formation in mice include CYP4f13, CYP4f14, CYP4f15, CYP4f16, CYP4f18, which are highly expressed in liver, kidney, brain, and leukocytes.[356, 425] These data suggest that CYP4F enzymes are highly expressed in liver and kidney and to a lesser extent other tissues in mammalian species.

1.5.1.3 CYP2J2 expression: CYP2J2 is the only isoform of the CYP2J subfamily of enzymes in humans[438]. CYP2J2 gene was mapped on the short arm of human chromosome 1[438], the genomic region spans approximately 40 kb[439]. The gene encodes a protein with a molecular mass of 57.7 kd and consists of 502 amino acids.[401] CYP2J2 is the only human CYP known to be expressed at high levels in heart particularly in cardiac myocytes and endothelial cells in coronary arteries.[401, 440] To illustrate, mRNA levels for CYP2J2 were 10 time higher than CYP2C8/9.[441] CYP2J2 is also expressed to a lesser extent in human liver, intestine, lung, kidney, vasculature, and other tissues [399, 401, 442, 443] In the liver and intestine, CYP2J2 constitutes 1 to 2% of total P450 content.[444] In addition, CYP2J2 showed selective distribution in different brain regions.[445, 446] Although CYP2J2 is widely distributed in human tissues, the pattern of distribution and expression does not seem to correlate with that of CYP2C8/9 and SEH.[447] In other mammalian species, CYP2J isoforms responsible for EET formation include CYP2j3 and CYP2j4 in the rat, CYP2j5 and CYP2j6 in the mouse, and CYP2j1 in the rabbit and sequence homology ranges from 69% to 94%.[448] These CYP2J enzymes are widely expressed in various tissues, but high expression of CYP2j1 is reported in rabbit small intestine, CYP2j3 in the rat heart and liver, CYP2j4 in rat brain, heart, lung, intestine, kidney, and liver, CYP2j5 in mouse kidney, and CYP2j6 in mouse intestine.[356, 448] These data suggest that CYP2J2 plays an important role in the human heart and coronary arteries while CYP2J enzymes in other species are widely distributed among various tissues.

1.5.1.4 CYP2C9 expression: In humans, the CYP2C subfamily consists of CYP2C8, CYP2C9, CYP2C18, and CYP2C19. The CYP2C8 and CYP2C9 genes are in close proximity on chromosome 10q24 located in a CYP2C gene cluster also containing CYP2C18 and CYP2C19

genes[449-451]. Given the close proximity of CYP2C8 and CYP2C9, some degree of linkage disequilibrium has been reported in these genes.[451-453]. The CYP2C enzymes are found predominantly in the liver and comprise 8-35% of the total CYP content.[454, 455] CYP2C8 accounts for 7% of CYP content in the liver, and is expressed to a lesser extent in the kidney, adrenal gland, mammary gland, brain, ovary, uterus, and duodenum.[449, 456-458] CYP2C9 accounts for 19% of CYP content in the liver and, among all CYP isoforms, levels are only exceeded by CYP3A4.[459-461] In extrahepatic tissues, CYP2C8/9 are expressed in a wide variety of tissues such as kidney, lung, vasculature, gastrointestinal tract, skin, and prostate.[447] In one study, tissue-specific patterns of CYP2C9 were very similar to those of SEH.[447] CYP2C enzymes have also been detected in rat brain astrocytes.[462] In other mammalian species, CYP2C isoforms responsible for EET synthesis include CYP2c11, CYP2c12, CYP2c23, and CYP2c24 in the rat and CYP2c29, CYP2c38, CYP2c39, CYP2c40 and CYP2c44 in the mouse and show similar patterns of expression as those described in humans.[356, 391, 463-466] These data suggest that CYP2C8/9 plays an important role in both hepatic and extrahepatic tissues in mammalian species.

1.5.1.5 SEH Expression: Soluble epoxide hydrolase (SEH) is part of the epoxide hydrolase family of enzymes that are found in all living organisms.[413, 467] SEH was first identified in insects while studying the metabolism of a terpenoid epoxide that mimicked the insect juvenile hormone.[468] The term SEH was based on its localization in the soluble and peroxisomal fractions of the cell.[469, 470] In the following 30 years, the gene, protein, and catalytic mechanisms were characterized and studied.[471-474] In humans, SEH is the product of the EPXH2 gene located on chromosome 8p21-p12.[471, 475, 476] The active form of the human SEH protein is a homodimer with each monomer consisting of 555 amino acids and a molecular mass of 62.5 kDa.[475, 477] The SEH enzyme is widely distributed in mammalian tissues and the expression and specific activity of SEH is reported to be highest in the liver, followed by the kidney, with lesser activity in other extrahepatic tissues.[414] For example, studies of various human tissue homogenates have reported that the highest levels of SEH activity are found in liver and kidney.[478] Enayetallah et al. also observed SEH in high levels in liver and kidney but also identified SEH in many other organs including adrenals, pancreatic islets, pituitary gland, lymphoid tissues, muscles, vascular smooth muscle cells, and epithelial cells in the skin, prostatic ducts, and the gastrointestinal tract.[447] In this study, the tissue specific-patterns of SEH expression seemed to correlate well with those of

CYP2C9. In human brain tissues, SEH was found mainly in the neuronal cell bodies, oligodendrocytes, astrocytes, and VSMC and the specific activity of SEH in the various brain regions was approximately 10-fold less than that of the liver.[479] Other studies of Enayetallah et al. identified SEH in different subcellular compartment in an array of human tissues.[480] SEH expression was present in both cytosolic and peroxisomal in human hepatocytes and renal proximal tubules and was found exclusively in the cytosol of pancreatic islet cells, intestinal epithelium, anterior pituitary cells, adrenal gland, endometrium, lymphoid follicles, prostate ductal epithelium, alveolar wall, and blood vessels.[480] Collectively, these data suggest that SEH is found in a wide array of human tissues with the highest levels and activity in the liver and kidney.

1.5.2 Gene regulation of CYP eicosanoid enzymes

This section contains information on activators and repressors of CYP eicosanoid genes and highlights mechanisms involved in this process.

1.5.2.1 CYP411 gene regulation: There are several agents that induce expression of the CYP4A subfamily of enzymes. CYP4A enzymes are induced in several mammalian species by hypolipidemic drugs such as fibrates.[481-483] This response is likely mediated through PPARα, which plays a central role in lipid homeostasis through gene regulation.[484-486] The induction of CYP4A enzymes does not necessarily diminish the activation of PPARα because a number of ω-hydroxylated fatty acid metabolites are also PPARα agonists.[487-489] Caloric restriction and fasting are thought to elevate PPARα responsive genes in the liver in response to the increased release of fatty acids from adipocytes.[490] [491] Also, statins have been shown to induce CYP4A gene expression in rat liver and rat hepatocytes[492] through activation of the transcription factor, sterol regulatory element-binding protein 2 (SREBP-2)[493]. In contrast, statin induction of CYP4A11 mRNA was not seen in primary human hepatocytes.[494] In addition, androgens such as 5α-dihydrotestosterone (DHT) have been shown induce CYP4A enzymes in the renal vasculature of rodents[495], which led to increase blood pressure,[496, 497] especially in males[498]. This effect was accompanied by a down regulation of CYP2C enzymes responsible for the synthesis of EETs[495] and an increase in the 20-HETE to EET ratio in the renal vasculature.[497] Furthermore, a high fat

diet has been shown to down regulate the expression of CYP4A and CYP2C enzymes, reduce the formation of 20-HETE and EETs in renal tubules, and lead to hypertension in rats.[498] These effects can be reversed by administration of fibrates such as clofibrate and fenofibrate.[499, 500] On the other hand, the retinoic acid receptor (RAR) agonist all trans-retinoic acid is reported to suppress CYP4A11 expression in a human hepatoma cell line.[501] Taken together, these studies suggest that CYP4A enzymes in mammals are induced by hypolipidemic drugs, PPARα agonists, statins, androgens, and starvation and suppressed by a high fat diet and RAR agonists with some variable responses among species and sex.

1.5.2.2 CYP4F2 gene regulation: Expression of CYP4F2 is reported to be under the control of several nuclear hormone receptors and transcription factors. For example, CYP4F2 gene expression can be induced by retinoic acid in HepG2 cells through an RAR/retinoid X receptor (RXR) heterodimer response element.[502] Also, CYP4F2 gene expression can be induced in Hep2G cells by saturated fatty acids and repressed by PPARα agonists such as clofibrate and Wy14643.[503] In contrast, CYP4A11 expression in a human hepatoma cell line is repressed by all trans-retinoic acid[501] and induced by hypolipidemic drugs through PPARα[504]. Statins can also induce CYP4F2 expression in human hepatocytes and HepG2 cells through SREBP activation, which are a transcription factors that play an important role in lipid and sterol homeostasis.[494] Several transcription factor binding sites, such as NF-Y, C/EBP, CREB and Sp1, have been shown to contribute to SREBP binding and to efficient target gene transactivation.[494] In addition, direct repeat elements (DR-1 and DR-2) in the 5′ promoter and intron 1 have been shown to cooperate in gene transcription.[503] Taken together, these data suggest that CYP4F2 expression is induced by activators of the nuclear receptors RAR/RXR and PPARα, transcription factors such as SREBP and SP1, and other regulatory elements in the promoter region.

1.5.2.3 CYP2J2 gene regulation: The regulatory mechanisms of CYP2J enzymes have not been well studied. CYP2J2 is alternatively spliced in a tissue-specific fashion that may serve as a mechanism to regulate gene expression or activity.[448] Also, the promoter region contains four binding site consensus sequences for the SP1 transcription factor.[505] Some studies have shown that activator protein-1 (AP-1), a multiprotein transcription factor complex, down regulates CYP2J2 in liver cells.[506] AP-1 activity is activated by stress stimuli such as hypoxia, cytokines, growth factors, carcinogens and UV irradiation and activates signaling pathways regulate cell survival and

apoptosis.[506] In addition, metabolites of CYP2J enzymes are reported to activate the nuclear receptor PPARα *in vitro* and *in vivo*.[507] CYP2J has also been induced with agents such as phenobarbital, β-naphthoflavone, clofibrate, and acetone.[401, 448] CYP2J protein levels do not appear to change in selected animal models of disease, including salt-sensitive hypertension, oxygen-induced lung injury, and cardiac ischemia/reperfusion.[448] Furthermore, CYP2J may be regulated by nutrition[508] and age[448]. These data suggest that CYP2J gene expression is activated by PPARα agonists and repressed by stress stimuli through the activation of AP-1.

1.5.2.4 CYP2C8/9 gene regulation: CYP2C8/9 gene expression is regulated by multiple nuclear receptors and transcription factors.[509, 510] Transcription of CYP2C8/9 is regulated by the nuclear receptors constitutive androstane receptor (CAR)/pregnane X receptor (PXR), glucocorticoid receptor (GR), and hepatocyte nuclear factor-α (HNF-α).[454, 511-513] Recent studies have identified other nuclear receptors and transcriptional factors including HNF4α, HNF3γ, C/EBPα, and RORs that regulate the constitutive expression of CYP2C genes in liver.[454, 514] CYP2C8 protein and mRNA levels are not affected by sex and age.[515] Some studies report correlations in the mRNA expression of 2C8, 2C9, 2C19, and 3A4 due to common regulatory pathways.[515] *In vitro* studies report that CYP2C8 expression is induced strongly by the antibiotic rifampin and to a lesser extent by the glucocorticoid steroid dexamethasone and barbiturate phenobarbital.[512, 516, 517] Clinical studies report that rifampin decreases the plasma exposure of major CYP2C8 substrates such as rosiglitazone[518, 519], pioglitazone[520], and repaglinide[521, 522]. Similarly, CYP2C9 is strongly induced by rifampin but can also be moderately induced by secobarbital.[517, 523] In clinical studies, treatment with rifampicin has been shown consistently to increase the clearance of drugs eliminated by CYP2C9. Also, the clearance of losartan, phenytoin, tolbutamide and S-warfarin is approximately doubled in healthy volunteers or patients treated with rifampicin.[524, 525] Induction of CYP2C8/9 is of concern because it can lead to drug-drug interactions, drug tolerance, or altered drug disposition and response.[454] In summary, CYP2C8/9 gene expression is regulated by multiple nuclear receptors and transcription factors including CAR/PXR, GR, and HNF-α and induction of these enzymes by rifampin or other agents may lead to clinically relevant changes in pharmacotherapy.

1.5.2.5 SEH gene regulation: The expression of SEH is regulated by multiple processes. SEH contains a TATA-less promoter with a GC-rich region and SP-1 binding site that appears to be involved in the basal expression of EPHX2.[526] SP-1 transcription factor has been shown to induce

SEH expression through demethylation in HepG2 cells.[527] In addition, PPARα agonists have been noted to induce SEH expression in rodents.[528-530] Also, SEH expression has been linked to hormonal stimuli. For example, angiotensin II is reported to upregulate SEH expression in human endothelial cells through activation of AP-1[531] and increase SEH protein levels and catalytic activity in rat renal cortical tissue [532] Sex hormones are also suggested to affect SEH expression in rodents. In mice, males show 55% and 28% higher SEH activities in liver and kidney, respectively, in comparison to females[529] and ovariectomy increases SEH activities in liver and kidney[529]. Furthermore, inflammatory stimuli have been shown to affect SEH expression.[413] [531] Cigarette smoke transiently reduces SEH activity in the human lung [533] and exposure to gamma radiation increases the levels of EPHX2 mRNAs through NFkB induction in human cell lines [534] It has also been shown that SEH activity can be affected by post-translational modification through tyrosine nitration of the protein.[535] These data provide evidence that SEH gene expression is induced by SP-1 transcription factor, PPARα agonists, male sex, and reduced by smoking and tyrosine nitration of the protein.

1.5.3 Substrates for CYP eicosanoid enzymes

This section summarizes notable substrates that have been previously reported to be metabolized by CYP eicosanoid enzymes. It includes a discussion of the individual contribution of each enzyme to the formation or elimination of CYP eicosanoids and highlights their role in the metabolism of other endogenous compounds and drugs.

1.5.3.1 CYP4A11 substrates: The CYP4A family of enzymes is generally known for the metabolism of medium chain (C10-C16) and long chain (C16-C26) fatty acids primarily by oxidation of the terminal (ω) carbon and to a lesser extent the ω-1 carbon.[536] CYP4A11 catalyzes the ω-hydroxylation of lauric acid, palmitic acid, and AA in human kidney[537], human liver[538], and in cells expressing the recombinant protein[388, 539]. In these studies, the ω-hydrolase activity of CYP4A11 was greatest toward lauric acid (C12) followed by palmitic acid (C16), and then arachidonic acid (C20). In human liver microsomes, CYP4A11 is responsible for 85% and 13% of the ω-hydrolase activity towards lauric acid[538] and AA[389], respectively. Although CYP4A11

seems to play a minor role in the metabolism of long chain fatty acids in the liver, its effects in the kidney seem more apparent. In human kidneys, CYP4A11 is the predominant isoform responsible for the ω-hydroxylation of arachidonic acid to form 20-HETE.[540] In other mammalian species, the CYP4A subfamily have been shown to catalyze the ω– or ω-1 hydroxylation of lauric acid, palmitic acid, AA, or prostaglandins in liver, kidney, or *in vitro*.[541-543] Taken together, these studies demonstrate that CYP4A enzymes play an important role in the metabolism of fatty acids in the liver and kidney.

1.5.3.2 CYP4F2 substrates: The CYP4F family of enzymes is generally known for the metabolism of long chain (C16-C26) fatty acids and lipids primarily by oxidation of the terminal (ω) carbon and to a lesser extent the ω-1 carbon.[536, 544] CYP4F2 has been reported to be responsible for the metabolism of AA and its metabolites such as prostaglandins E1 and A1, lipotoxins A4 and B1, 5-, 8-, and 12-HETE, prostanoids, leukotriene B4, and anandamide.[426, 463, 481, 545] In human liver, CYP4F2 is the primary ω-hydroxylase enzymes responsible for the metabolism of the pro-inflammatory leukotriene B4 and arachidonic acid.[435, 544] For example, CYP4F2 content in human liver microsomes has been significantly correlated with leukotriene B4 and AA ω–hydroxylase activities ($r>0.63$).[437] Although CYPF2 and CYP4A11 are reported to ω-hydroxylate arachidonic acid in the liver, CYP4F2 has been shown to be the principle enzyme responsible for 20-HETE formation in human liver microsomes.[546] In addition, CYP4F2 is reported to ω-hydroxylate AA in the proximal tubules of the human kidney to form 20-HETE.[390] Likewise, CYP4F2 catalyzes the ω-hydroxylation of phytanic acid (C20), oleic acid (C18), stearic acid (C18), palmitic acid (C16), α- and γ-tocopherols (vitamin E), but not lauric acid (C12).[544, 547, 548] In addition to metabolizing endogenous fatty acids and lipids, CYP4F2 is also responsible for the metabolism of xenobiotics. For example, CYP4F2 is a primary vitamin K1 oxidase in the liver that limits excessive accumulation of vitamin K.[549] Likewise, CYP4F2 is a major human liver microsomal enzymes responsible for the O-demethylation of the anti-parasitic drug pafuramidine (DB289)[550]. Furthermore, CYP4F2 is the predominant enzyme responsible for the hydroxylation of the immunomodulating drug fingolimod.[551, 552] In summary, CYP4F2 is a major enzyme involved in the metabolism of long chain fatty acids, lipids, vitamin K, pafuramidine, and fingolimod.

1.5.3.3 CYP2J2 substrates: Like CYP2C8 and CYP2C9, CYP2J2 is an epoxygenase that catalyzes epoxide formation at the site of a carbon-carbon double bond.[505] There are multiple

xenobiotics that are readily metabolized by CYP2J2.[448] A recent study investigating 139 marketed drugs and compounds reported that CYP2J2 can metabolize numerous structurally diverse compounds such as albendazole, amiodarone, cyclosporine A, danazol, mesoridazine, nabumetone, tamoxifen, and thioridazine.[553] Many of these substrates were also metabolized by CYP3A4, but there were differences in regioselectivity.[553] Also, CYP2J2 metabolism of large compounds was more restricted to a single site.[553] Endogenous substrates of CYP2J include fatty acids such as AA and linoleic acid.[439, 505] CYP2J2 catalyzed the metabolism of AA to four regioisomeric epoxyeicosatrienoic acids (EETs): 14,15-EET; 11,12-EET; 5,6-EET and 8,9-EET.[401, 463] In this study, 14,15-EET was the primary metabolite and the epoxidation was highly stereoselective for 14(*R*),15(*S*)-EET whereas 11,12-EET; 5,6-EET and 8,9-EET seemed to be less selective for specific enantiomers.[401] The regio- and stereo-selective product profile of CYP2J enzymes was different from that of other known AA epoxygenases such as CYP2C enzymes.[397, 402, 554-556] In addition, AA and ebastine strongly inhibited the metabolism of astemizole in microsomes from human small intestines and by recombinant CYP2J2.[557] Furthermore, CYP2J enzymes oxidized other endogenous substrates such as testosterone, but showed low activity toward some fatty acids such as prostaglandins and lauric acid.[448] In summary, CYP2J2 metabolizes numerous structurally diverse drugs and compounds and serves as a primary enzyme involved in the stereo and regio-selective epoxidation of AA and other fatty acids.

1.5.3.4 CYP2C8/9 substrates: The CYP2C8 and CYP2C9 are major phase I metabolizing enzymes that play an integral role in the biotransformation of structurally diverse xenobiotics and endogenous compounds.[558] It has been estimated that CYP2C8 and CYP2C9 are responsible for the metabolic clearance of up to 5% and 15%-20%, respectively, of all drugs undergoing Phase I metabolism [458, 559, 560] Over 100 marketed drugs are metabolized by CYP2C9 including many with a narrow therapeutic index such as warfarin and phenytoin.[460] CYP2C9 metabolizes many structurally diverse drugs such as the NSAIDs diclofenac, hypoglycemic agent tolbutamide, angiotensin II blocker lostartan, and sulphonylurea tolbutamide.[517] Likewise, the CYP2C8 substrate-binding cavity is reported to accommodate large and/or structurally unrelated compounds, such as paclitaxel, amiodarone, cerivastatin, and sorafenib.[517, 561, 562] Although CYP2C8 and CYP2C9 show 70% sequence homology, CYP2C8 shares more common substrates with CYP3A4 than it does with CYP2C9.[458] CYP2C9 also plays a role in the metabolism of

several endogenous compounds such as linoleic acid, AA, serotonin, steroids, melatonin, and retinoids.[460, 563, 564] CYP2C8 also metabolizes endogenous compounds such as retinoids and AA.[510] For example, CYP2C8/9 have been shown to metabolize AA to form various region- and stereo-isomers of EETs in hepatic and extrahepatic tissues.[400, 405, 463, 565] In summary, CYP2C8/9 play an important role in the metabolism of many structurally diverse drugs and endogenous compounds.

1.5.3.5 SEH substrates: SEH has not been reported to be a major metabolizing enzyme for any marketed drugs. However, SEH plays an important role in defense and detoxification in multiple species by converting potentially harmful epoxide-containing compounds into diols, which are less reactive and easier to excrete.[566] SEH of mouse, rat, and human have similar molecular weight and immunoreactivity[567], but SEH of primate and non-primate species differ in substrate specificity.[566] Saturated and unsaturated fatty acid epoxides, including linoleic and AA epoxides, are excellent SEH substrates.[568-573] For example, SEH is the primary metabolizing enzyme of EETs, which have been established as lipid mediators with important biological functions [574] Recently, it has been discovered that SEH is a bi-functional enzyme that possesses an N-terminal phosphatase activity in addition to its C-terminal epoxide hydrolase activity.[575, 576] It has been suggested that endogenous substrates of the N-terminal phosphatase domain include phosphorylated lipid metabolites such as isoprenoid phosphates.[577] Some phosphorylated lipid metabolites are precursors in the biosynthesis of cholesterol[576-578] and isoprenoid phosphates are known to play a role in isoprenylation of small G-proteins and cell signaling[579]. Interestingly, endogenous substrates of the C-terminal domain, such as fatty acid epoxides, are found in the cytosol[488, 580, 581], while endogenous substrates of the N-terminal domain, such as isoprenoid phosphates, are found in the peroxisomes and/or the cytosol[579, 582]. Collectively, these data suggest that SEH plays multiple diverse roles in lipid metabolism and cellular signaling in various tissues and subcellular locations.

1.5.4 Inhibitors of CYP eicosanoid enzymes

This section summarizes some of the key small molecules that have been used to inhibit the enzymes involved in CYP eicosanoid biosynthesis. These inhibitors have been used in preclinical and/or clinical studies investigating the function of these enzymes in various diseases.

1.5.4.1 Inhibitors of 20-HETE Pathway: Numerous ω-hydroxylase inhibitors and 20-HETE antagonists have been developed over the last few decades. Mechanism based inhibitors of CYP4F2 and CYP4A11 include 1-aminobenzotriazole (ABT)[583], 17-octadecynoic acid (17-ODYA)[584, 585], 10-undecynoic acid (10-UDYA)[584, 585], and 10-undecynyl sulfate (10-SUYS)[586]. Competitive and non-competitive inhibitors of 20-HETE synthesis include N-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS)[587], N-hydroxy-N`-(4-butyl-2-methylphenyl)-formamidine (HET0016)[588], and N-(3-Chloro-4-morpholin-4-yl) phenyl-N`-hydroxyimido formamide (TS-011)[589]. There are a few 20-HETE antagonists including the stable structural 20-HETE analogs 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (20-HEDE)[587], and 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (WIT002)[590]. Also, 20-HETE agonists include 4-amino-N-(20-hydroxy-eicosa-5(Z),14(Z)-dienoyl) benzenesulfonamide (ABSA) and 20-hydroxyeicosa-5(Z),14(Z)-dienoic acid (WIT003).[590] These compounds have been used in preclinical studies to investigate the role of 20-HETE in the regulation of cerebral blood flow or cerebrovascular response. Among the ω-hydrolase inhibitors, TS-011 demonstrates superior sensitivity and selectivity and thus appears to be one of the most promising agents for clinical development.[589] In addition, CYP4F2 ω-hydroxylase activity has been shown to be inhibited by some dietary agents. Sesamin has been shown to inhibit CYP4F2 ω-hydroxylase activity towards tocopherols (vitamin E) in rat and human liver microsomes[591] and dietary sesamin has been shown to elevate vitamin E levels *in vivo*[592-594] Likewise, ketaconozole has been shown to inhibit fingolimod metabolism by CYP4F2 in human liver microsomes[595], but showed minimal effects on the pharmacokinetics of fingolimod in humans[551]. In summary, numerous selective ω-hydroxylase inhibitors and 20-HETE antagonists have been used in preclinical studies to help elucidate the mechanisms involved in the pathogenesis of various diseases and demonstrate potential for future clinical studies.

1.5.4.2 Inhibitors of EET Synthesis: A few epoxygenase inhibitors have been used in preclinical studies investigating the role of CYP eicosanoids in various disease models. For example, miconazole[596], 17-ODYA[596], and N-methylsulfonyl-6-(2-propargyloxyphenyl) hexamide (MS-PPOH) [597] have been used to investigate the role of EETs in the regulation of cerebral blood flow *in vivo*. However, some authors have noted that 17-ODYA is not specific and equally inhibits epoxygenase and ω-hydroxylase activity, thus presenting limitations of its use.[598] In addition, there are numerous selective, competitive and mechanism-based inhibitors of epoxygenase enzymes that have been previously reported in other studies.[599] For example, derivatives of ebastine and terfenadine have been shown to be high affinity inhibitors of CYP2J2[600] and the metabolism of astemizole in human small intestinal microsomes and recombinant CYP2J2 microsomes was inhibited by α-naphthoflavone, ketoconazole, troglitazone, tranylcypromine, ebastine and terfenadine[601]. The Department of Medicine at Indiana University provides an excellent table summarizing CYP2C8 and CYP2C9 inhibitors and clinically relevant drug interactions.[517] This table and other studies report that CYP2C8 is strongly inhibited by the hypolipidemic drug gemfibrozil and moderately inhibited by the antibiotic trimethoprim.[509, 517, 602-605] CYP2C9 is strongly and moderately inhibited by fluconazole and amiodarone, respectively.[517, 524] Clinical studies reported altered anticoagulant effect of warfarin in patients coadministered with amiodarone.[606, 607] In summary, numerous inhibitors of epoxygenase enzymes exist, but few have been used in the investigation of CYP eicosanoids in disease.

1.5.4.3 Inhibitors of EET metabolism: Based on the numerous biological functions associated with EETs and their rapid metabolism observed *in vivo*[608], multiple inhibitors of SEH were developed to elevate EETs levels and augment their beneficial biological effects. Shen et al. provides an excellent review that summarizes the history of the development of these inhibitors and their use in various studies and clinical investigations.[609] In this review, it is noted that several pharmaceutical companies and academic institutions were involved in the development of SEH inhibitors as a potential therapeutic treatment for hypertension, atherosclerosis, pulmonary diseases, diabetes, pain, inflammation, immunological disorders and other indications. Pharmaceutical companies involved in the development of these SEH inhibitors were Arête Therapeutics, Astellas, Boehringer Ingelheim, GlaxoSmithKline, Merck, Roche, Taisho,

Dainippon Sumitomo, and Takeda while the primary academic institutions involved included University of California Davis, Columbia University, Chinese Academy of Sciences, and Shanghai Institute of Biological Sciences. Over the years, numerous type of SEH inhibitors were developed in order to improve its potency, solubility, pharmacokinetic properties, and selectivity. The major inhibitor chemotypes include urea, amide, carbamate, thioester, carbonate, ester, thiourea, thioamide, amidine, guanadine, heterocycles, aminoheterocycles, aminoheteroaryls, chalcone oxides, acyl hydrazones, chalcone oxides and trans-3-phenylglycidols.[609] The most common SEH inhibitor used in preclinical models of ischemic and hemorrhagic stroke include 12-(3-Adamantan-1-yl-ureido)-dodecanoic acid (AUDA) and 12-(3-Adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDA-BE). The therapeutic utility of some of these agents have been investigated in clinical trials, however there are currently no approved SEH inhibitors on the market.

1.6 ROLE OF CYP EICOSANOIDS IN THE CEREBROVASCULATURE

1.6.1 Role of CYP eicosanoids in pathogenesis of aneurysms

This section will highlight the key cellular signaling pathways and cellular responses activated by CYP eicosanoids with a focus on those involved in vascular homeostasis and intracranial aneurysm formation or rupture.

1.6.1.1 Cellular action and signaling pathways: 20-HETE and EETs produce diverse array of biological actions in a variety of tissues and cells, but function primarily in the cardiovascular and renal systems. Roman et al. provides an excellent review on the role of CYP eicosanoids in cardiovascular function.[581] EETs are reported to dilate renal and cerebral arteries, promote angiogenesis, mitogenesis, and fibrinolysis, and inhibit inflammation, apoptosis, and platelet aggregation.[581] The cellular actions of EETs are mediated through numerous intracellular signaling mechanisms such as 3,'5'-cyclic monophosphate (cAMP), phosphokinase A (PKA), phosphatidylinositol 3-kinase (PI-3K), protein kinase B (PKB/Akt), mitogen-activated protein kinase (MAPK), IκB kinase (IκK), I-kappa-B-alpha (IκBα), epidermal growth factor receptor

(EGFR), and Src kinase in various cell types.[581] The predominant intracellular signaling pathways appear to be species- and cell-dependent.[356] Evidence suggests that the cellular actions of EETs is mediated through a G-protein coupled receptor (GPCR), but so far only putative receptors have been proposed.[581] The cellular actions of 20-HETE appear to counterbalance those of EETs. 20-HETE constricts renal and cerebral arteries and promotes angiogenesis, inflammation, apoptosis, and platelet aggregation.[356] 20-HETE is reported to activate a wide array of intracellular signaling pathways and 20-HETE receptor has not been identified.[356] Although DHETs have been reported to constrict coronary arteries[610] and activate PPARγ[488], the preponderance of studies report that DHETs do not affect cerebrovascular tone[611] and cellular function[581] and thus are considered inactive metabolites. Based on the biological actions and cellular mechanisms of 20-HETE and EETs, their role in CVD and other disorders has been an intense area of focus in recent years.

1.6.1.2 Hypertension and vascular remodeling: Recent evidence suggests that CYP eicosanoids may play a role in the formation and rupture of IA. Previous studies report a strong link between hypertension and vascular remodeling with the formation and rupture of IA.[124, 612] Growing evidence demonstrates that 20-HETE and EETs are key regulators of blood pressure and play an important role in the development of hypertension.[356, 613] Also, a recent review by Imig et. al. suggests that CYP eicosanoids impact vascular homeostasis and remodeling.[355] *In vitro* studies report that EETs exhibit proliferative, migratory, angiogenic, fibrinolytic, anti-apoptotic, anti-inflammatory, and anti-platelet aggregation effects in vascular endothelial cells and inhibit migration and apoptosis in VSMC.[355] 20-HETE is involved in vascular remodeling and microvessel formation by stimulating proliferation, migration, and mitogenesis in VSMC and proliferation and angiogenesis in microvascular endothelial cells.[355] Collectively, these findings suggest that CYP eicosanoids play an important role in hypertension and vascular homeostasis and may contribute to the pathogenesis of IA and risk of rupture.

1.6.2 CYP eicosanoid regulation of cerebrovascular tone

This section will discuss the responses in cerebrovascular tone to exogenous administration of the CYP eicosanoids to isolated cerebral vessels and some of the key mechanisms involved.

1.6.2.1 Response of isolated vessels: The role of CYP eicosanoids in the regulation of CBF has been a topic of intense area of study in the last two decades. Previous studies demonstrated that CYP eicosanoids regulate cerebrovascular tone *in vitro*. In these studies, cerebral arteries were dissected from the brain, mounted on glass micropipettes with sutures, pressurized in a perfusion chamber, bathed in a physiological saline solution, and exposed to pharmacological agents as previously described.[590] The internal diameters of the vessels were measured with a video system. Harder et al reported that exogenous addition of 20-HETE at 0.1-10nM constricted isolated cat pial arteries in a dose-dependent manner by ~4-13%.[614] Lange et al. studied the effects of higher concentrations of 20-HETE (1-1000nM) on isolated cat pial arteries and reported a dose-dependent constriction of 6-29% that reached a maximum at 2-5 min after application of 20-HETE.[615] In studies of isolated cat microvascular cerebral arteries, exogenous addition of 20-HETE at 10-100nM led to a dose-dependent constriction of ~9-14% that reached a maximum at 2-5 min after application.[616] In rats, isolated pial and basilar arteries were constricted in a dose-dependent manner by 9-26% after exogenous addition of 20-HETE and 20-HETE agonists ABSA and WIT003 at 10-1000nM.[590] In contrast, administration of the 20-HETE antagonist WIT002 at 10-1000nM did not constrict these arteries but it attenuated the vasoconstrictor responses following administration of 20-HETE and 5-hydroxytryptamine.[590] In a similar study by Gebrehedin et al.[587], 20-HETE (10-1000nM) constricted isolated rat pial arteries in a dose-dependent manner by 12-25%, which was attenuated by the administration of 20-HETE antagonist 20-HEDE (1uM). It is important to note that the vasoconstrictive properties of 20-HETE are as potent or greater than those of well-established endogenous regulators such as ET-1.[617] Collectively, these studies demonstrate that 20-HETE constricts cerebral arteries in a dose- and time-dependent manner with a potency comparable to the primary endogenous regulators of cerebrovascular tone.

Similar methods were used to investigate the effect of EETs on the tone of isolated cerebral arteries. Isolated cat cerebral arteries pre-constricted with serotonin were dilated in a dose-dependent manner ~50-110%, 60-109%, and 30-51% by exogenous addition of 11,12-EET, 8,9-EET, and 5,6-EET at 1-50uM, respectively.[611] Half-maximal dilation (EC_{50}) was observed at 2-4uM for all three EET regioisomers.[611] The effects of EETs were transient with a maximum effect observed at 3 min and a returned to baseline at 5 min after EET administration.[611] These results were consistent with previous studies, which report a short half-life of EETs in aqueous

solution.[618, 619] Other studies report similar vasodilator responses in cerebral arteries and other vascular beds in response to exposure to EETs.[356] These findings demonstrate that EETs dilate cerebral arteries in an isomer- and time-dependent manner both *in vitro* and *in vivo*.

1.6.2.2 Mechanisms of vascular response: Numerous studies have elucidated some of the key mechanisms involved in the regulation of cerebrovascular tone by CYP eicosanoids. In isolated cerebral arteries and smooth muscle cells, 20-HETE activates numerous intracellular signaling pathways involved in vasoconstriction including PKC, Ras, tyrosine kinase, and mitogen-activated protein (MAP).[620] Also, 20-HETE activates rho-kinase pathways that increase the sensitivity of cerebral arteries to calcium.[621] In addition, 20-HETE promotes vasoconstriction through the activation of various ion channels. In some studies, 20-HETE constricts cerebral arteries through activation of L-type Ca^{+2} channels leading to a rise in intracellular Ca^{+2} levels.[616] Likewise, 20-HETE activates transient receptor potential channel 6 (TRPC6) promoting vasoconstriction through Ca^{+2}-dependent[622] and Ca^{+2}-independent [623] mechanisms. Also, 20-HETE promotes vasoconstriction by inhibition of large-conductance Ca^{+2}-activated potassium channels (K_{Ca+2} channels) through its phosphorylation by activation of PKC.[615, 624] Conversely, EETs are reported to dilate cerebral arteries by the activation of K_{Ca+2} channels and inhibition of L-type Ca^{+2} channels on smooth muscle cells through a putative GPCR.[625] Other studies suggest that EET mediated dilation of cerebral arteries occurs in an autocrine manner on endothelial cells by the activation of transient receptor potential (TRP) channel V4, C3, or C6.[625] Activation of these receptors promotes Ca^{+2} influx, activation of small conductance (SK) and intermediate (IK) conductance K^+ channels, hyperpolarization, and release of K^+ ions from endothelial cells. K+ ions then stimulate inward rectifying (Kir) K^+ channels and Na^+/K^+ ATPase, thus hyperpolarizing and relaxing the VSMC. These studies are helpful to elucidate the mechanisms involved in the regulation of cerebrovascular tone and CBF by CYP eicosanoids.

1.6.3 20-HETE regulation of CBF

This section will highlight some of the key studies investigating the role of 20-HETE on the regulation of CBF, cerebral ischemic injury, myogenic response, and cerebral vasospasm. This section will also provide evidence that 20-HETE mediates the response to well-known regulators

of CBF and cerebrovascular tone.

1.6.3.1 20-HETE regulation of baseline CBF: Studies have investigated the effects of 20-HETE on baseline CBF and cerebral ischemic injury. Direct injection of 20-HETE (8 mg/kg) into the internal carotid artery in rats reduced baseline CBF for 2 hours and led to a large ischemic infarct.[589] Also, intracisternal administration of the 20-HETE agonist WIT003 (1.5 nmol) reduced baseline CBF by 20% while the 20-HETE antagonist WIT002 (1.5 nmol) and 20-HETE agonist ABSA did not affect baseline CBF.[590] These studies suggest that 20-HETE is a key regulator of CBF and may play a role in cerebral ischemic injury.

1.6.3.2 Role of 20-HETE in myogenic response: As mentioned in Section 1.2.3, the myogenic response plays a key role in the autoregulation of CBF.[358] The similarities between the mechanisms involved in myogenic response and vasoconstriction by 20-HETE led to the hypothesis that 20-HETE mediates the myogenic response of cerebral arteries and autoregulation of CBF. Gebremedhin et al. investigated the role of 20-HETE in the myogenic response of isolated rat pial arteries and reported that 20-HETE concentrations in cerebral arteries increased 6-fold when the intravascular pressure was increased from 20 to 140 mm Hg.[587] The addition of the CYP inhibitor DDMS (10uM) and 20-HETE antagonist 20-HEDE (1uM) eliminated the myogenic response of these cerebral arteries.[587] Extending this research into preclinical animal models, Harder et al. demonstrated that inhibitors and antagonists of 20-HETE attenuated the autoregulation of CBF after elevations of arterial pressure.[356, 587, 614] These studies suggest that 20-HETE significantly contributes to the myogenic response of cerebral arteries in isolated vessels and preclinical animal models.

1.6.3.3 Role of 20-HETE in cerebral vasospasm: Extending these findings to models of SAH, a few studies investigated the role of 20-HETE in the development of acute and delayed vasospasm. In a rat model of SAH, 20-HETE levels in CSF increased seven-fold and the 20-HETE synthesis inhibitors 17-ODYA and HET0016 attenuated this increase in 20-HETE levels.[626] Pre-treatment of rats with these CYP4A/4F (ω-hydrolase) inhibitors attenuated the acute reduction in CBF that returned to control within 120 minutes after induction of SAH.[626] In similar studies by Yu et al., 20-HETE levels in CSF increased 4-fold after SAH in rats, but levels were not impacted by the administration of the 20-HETE antagonist WIT002 (1.5 nmol) and the 20-HETE agonist

ABSA (1.5 nmol). On the other hand, WIT002 attenuated the acute reduction in CBF while the 20-HETE agonist ABSA exacerbated the acute fall in CBF following SAH.[590] Similar studies using the 20-HETE synthesis inhibitor TS-011 in SAH models revealed similar results.[589] Taken together, these data suggest that 20-HETE levels increase after SAH and contribute to the subsequent acute reduction in CBF.

A few studies investigated the role of 20-HETE in the development of delayed vasospasm after SAH. Roman et al.[620] studied a dual hemorrhage model of SAH in dogs involving an injection of blood into the cisterna magna followed by a second injection of blood 4 days later. At day 7 after the initial injection of blood, 20-HETE levels in CSF increased 8-fold and the basilar arteries constricted to 58% of control. Administration of the 20-HETE synthesis inhibitor TS-011 (0.1 mg/kg i.v.) at day 7 reversed the delayed vasospasm in this model.[620] Using a similar dual hemorrhage model of SAH in rats involving an intracerebroventricular (icv) injection of blood followed by a second injection of blood two days later, Takeuchi et al.[627] reported that the large arteries at the base of the brain constricted by 30% and CBF reduced to 67% after the first injection of blood, but CBF levels returned to control after 24 hours. The second injection of blood led to a sustained constriction of these arteries by 30%. Administration of TS-011 (0.1 mg/kg i.v.) five days after the second injection of blood reversed the vasospasm and the CBF returned to control values. These studies demonstrate that 20-HETE plays a key role in the development of delayed vasospasm after SAH.

Although some mechanisms involved in the regulation of cerebrovascular tone by 20-HETE have been elucidated, it is unclear if these mechanisms are involved in cerebral vasospasm after SAH. Recently, Roman et al. proposed a hypothesis summarizing the role of 20-HETE in mediating these biphasic changes in cerebrovascular tone after SAH.[620] In the acute phase of SAH, 20-HETE levels are increased primarily through scavenging of NO and CO by Hb and release from membranes through the activation of $cPLA_2$ by ischemia or other stimuli. Previous studies have shown that NO and CO inhibit 20-HETE synthesis enzymes.[204, 628] In the secondary phase of SAH, 20-HETE levels are increased through the upregulation of CYP4A/4F enzymes or dissolving of blood clots. However, this area continues to be an area of intense research focus and discussion.

1.6.3.4 20-HETE mediates response to vascular regulators: Growing evidence suggests that

20-HETE affects the vasoconstrictor response of well-known regulators of cerebrovascular tone and CBF (Figure 1-3: Key regulators of cerebrovascular tone after SAH). In cerebral arteries, 20-HETE synthesis is stimulated by angiotensin II, ET-1, 5-HT, and ATP[620], all of which have been implicated in the pathophysiology of cerebral vasospasm after SAH.[200] In addition, administration of 20-HETE synthesis inhibitors HET016 and 17-ODYA attenuated the vasoconstrictor response of 5-HT and was reversed with the co-administration of the 20-HETE agonist WIT003.[590, 629] These findings suggest that the vasoconstrictor effects of 5-HT on cerebral arteries is mediated in part by 20-HETE. It has been well established that NO is a key regulator of CBF and plays an import role in the development of cerebral vasospasm after SAH.[630] NO dilates cerebral arteries through the stimulation of guanylyl cyclase (GC) and subsequent formation of cyclic guanosine monophosphate (cGMP) or through cGMP-independent mechanisms. Other investigators showed that NO binds to CYP4A enzymes and inhibits the formation of 20-HETE thereby affecting the cerebral vasodilator response to NO in a cGMP-independent manner both *in vitro* and *in vivo*.[204] Moreover, recent evidence suggests that 20-HETE mediates CBF regulation by pericytes and 20-HETE inhibition by NO facilitates prostaglandin E_2 mediated dilation.[631] Taken together, these studies suggest that 20-HETE mediates the vasoconstrictor response of certain key regulators of cerebrovascular tone and CBF.

1.6.4 EET regulation of CBF

This section will highlight some of the key studies investigating the role of EETs on the regulation of CBF, cerebral ischemic injury, and neurovascular coupling.

1.6.4.1 Role of EETs in regulation of CBF: Extending the findings from isolated arteries to preclinical animal models, Ellis et al. investigated the effect of EETs on cerebrovascular tone of cat and rabbit pial arteries *in vivo* using a cranial window technique.[632] In this study, administration of 5,6-EET at 15 μg/ml led to a rapid dilation of cat pial arteries by 31% followed by a return to baseline after 5 min.[632] Likewise, rabbit pial arteries were dilated in dose dependent manner by 14,15-EET, 11,12-EET, and 8,9-EET, and 5,6-EET at 15 μg/ml to a maximal dilation of 2%, 5%, 8%, and 23%, respectively.[632] Alkayed et al. reported that that EET synthesis inhibition by

miconazole (20uM for 30min) reduced baseline CBF by 30% in rats.[633]. These studies suggest that EETs play a role in the normal regulation of CBF in preclinical animal models.

1.6.4.2 Role of EETs in neurovascular coupling: Growing evidence suggests that EETs significantly contribute to the regulation of CBF in response to neuronal activity.[352, 634, 635] As discussed in Section 1.4.1, EETs are formed in multiple cell types in the neurovascular unit, including neurons, astrocytes, and endothelial cells.[356] In astrocytes, EETs can be released from the phospholipid membranes of astrocytic foot processes in response to excitatory amino acids such as glutamate.[633] Glutamate has also been shown to upregulate CYP2C enzymes responsible for EET synthesis in rats.[462] Moreover, the functional hyperemic response to whisker stimulation in anesthetized rats is attenuated by inhibition of EETs formation.[351] Conversely, Liu et al. reported that 20-HETE has little impact on the functional hyperemic response to whisker barrel stimulation in rats, unless neuronal nitric oxide synthase (nNOS) was inhibited.[636] More recent studies show that individual administration of an EET synthesis inhibitor, EET antagonist, metabotropic glutamate receptor (mGluR) antagonist, and adenosine A_{2B} receptor antagonist, block the functional hyperemic response to whisker stimulation in rats by ~50%, but co-administration of an EET synthesis inhibitor or EET antagonist with mGluR antagonist or adenosine A_{2B} receptor antagonist had no additional effect on the hyperemic response. These data suggest that the synthesis and release of EETs in models of functional hyperemia may be linked to pathways involving adenosine and glutamate. Furthermore, EETs released from astrocytes promote angiogenesis and through mitogenesis of capillary endothelium, which is blocked by inhibition of EET formation by 17-ODYA.[637] These findings suggest that EETs contribute to angiogenesis, a process related to flow-metabolism coupling. Taken together, these studies suggest that EETs, but not 20-HETE, are key mediators in the regulation of CBF by neurovascular coupling.

1.6.4.3 Role of EETs in cerebral ischemic injury: The role of EETs in preclinical models of SAH has not yet been reported, but investigators report that EETs are an important regulator of CBF and injury resulting from temporary focal ischemia. In a temporary focal ischemia model in mice, administration of the SEH inhibitor AUDA-BE (10mg/kg i.p.) decreased infarct size by 40% when administered 1 hour before reperfusion and by 52% when administered 2 hours after reperfusion.[638] This response was reversed with the administration of the EET synthesis inhibitor

MS-PPOH (0.5 mg/200mL over 24 hr). Likewise, gene deletion of EPHX2 gene, which codes for SEH, or administration of 14,15-EET (1μg over 24 hours) reduced infarct size in mice by 56% and 68%, respectively, in the temporary focal ischemia model.[639] Also, CBF was higher during the occlusion in SEH knockout mice. *In vitro* studies of cerebral ischemia demonstrate that EETs protect astrocytes against ischemic cell death[640]. Also, variants in the EPHX2 gene, which codes for SEH, have been shown to affect SEH activity and neuronal survival after ischemic injury.[641] These studies provide evidence that EETs alter CBF and cerebral ischemic injury *in vitro* and in preclinical animal models and warrant further investigation in humans.

1.7 CLINICAL STUDIES OF CYP EICOSANOID LEVELS IN STROKE

1.7.1 CYP eicosanoid levels SAH patients:

To date, there are only a few studies that measure CYP-eicosanoid levels in plasma or CSF from patients with SAH. Previously, our laboratory measured 20-HETE levels in fresh CSF from four patients with SAH using HPLC-MS and reported values ranging from ~0.1-2.9 ng/ml in two patients with DCI and levels below the detection limit for the majority of samples in two patients without DCI.[642] Then, our lab used UPLC-MS/MS to measure CYP-eicosanoid levels in fresh CSF from seven patients with SAH and reported 20-HETE and 8,9-DHET levels ranging from ~0.2-5.0 ng/ml and ~0.2-2.0 ng/ml, respectively.[643] More recently, our group measured 20-HETE levels in fresh CSF from 108 patients with SAH using HPLC-MS[644] and reported values ranging from 0.1-30.2 ng/ml (mean ~1.4 ng/ml). In this study, detectable 20-HETE levels were associated with the severity of hemorrhage and DCI, but not angiographic vasospasm. Roman et. al measured 20-HETE in CSF from nine SAH patients with DCI and 13 healthy controls using HPLC-MS/MS and reported mean concentrations of ~0.11 ng/ml and non-detectable (<0.02 ng/ml) levels in control samples.[620] Plasma 20-HETE levels (~0.28 ng/ml) were not different than control levels. These data demonstrate the ability to measure CYP eicosanoids in CSF from SAH patients and show associations between 20-HETE levels and DCI.

1.7.2 CYP eicosanoid levels in ischemic stroke patients:

Only one study has measured CYP eicosanoid levels in ischemic stroke (IS) patients. Ward et al. measured CYP eicosanoids in plasma and CSF from 44 patients with acute IS (<96 hours) and 44 healthy age- and sex-matched controls.[645] Stroke patients had elevated plasma 20-HETE (0.62 ± 0.05 ng/ml), EETs (24.96 ± 1.07 ng/ml) compared with and DHET (31.25 ± 1.55 ng/ml) compared with controls (0.36 ± 0.05 ng/ml, 11.33 ± 1.07 ng/ml, and 22.93 ± 1.55 ng/ml, respectively). In a subset of 14 patients, 20-HETE levels (0.22 ± 0.05 ng/ml) and EET levels (16.47 ± 3.2 ng/ml) were decreased at 30 days after the stroke when compared to values at day 0 (0.45 ± 0.07 ng/ml and 25.87 ± 3.4 ng/ml, respectively). In another subset of 24 patients with lesion size and CBF data, there was a significant positive correlation between lesion size and plasma 20-HETE (r=0.44), but not EET and DHET, levels. There were no associations between CBF and CYP eicosanoid levels. Furthermore, 20-HETE levels were also associated with greater neurological impairment, unfavorable functional outcomes, and reduced cognitive function. These data show that CYP eicosanoid levels are increase acutely in stroke patients and decline within a month and that 20-HETE was associated with lesion size and functional indices, but not CBF.

1.8 STUDIES OF HUMAN GENE VARIANTS IN CYP EICOSANOID PATHWAY

1.8.1 *In vitro* studies of substrate metabolism:

This section will identify candidate gene polymorphisms reported to alter transcriptional activity, protein expression, or enzymatic activity *in vitro*. Tables at the end of Section 1.8 summarize key findings from this section.

1.8.1.1 CYP4A11 *in vitro* metabolism: The effect of a few polymorphisms in the promoter and coding regions of CYP4A11 have been evaluated *in vitro*. One such polymorphism is the -825A>G transition found in the promoter region of the CYP4A11 gene. Sugimoto *et al.*

demonstrated that when the variant -825G/G construct was expressed in human renal proximal tubule epithelial cells, the luciferase expression was reduced ~30% compared to the WT construct implying reduced transcriptional expression of CYP4A11.[646] In the same study, probes containing the variant -825G/G construct stimulated DNA binding of nuclear extracts in a gel mobility shift assay. Combined with the results from the luciferase assay, these results suggest that the -825G/G binding factor may serve as a transcriptional repressor of CYP4A11. Conversely, the -296C>T polymorphism did not to affect transcriptional activity of CYP4A11 *in vitro*.[647] Gene polymorphisms in the coding region of CYP4A11 have also been explored. Gainer *et al.* showed that cells expressing the 434Ser (8590T>C) polymorphism in the coding region of CYP4A11 showed reduced arachidonic acid and lauric acid metabolizing activity compared to the 433Phe (WT) protein.[540] The kinetic parameters V_{max} and K_m were reduced to 41% and 68% of WT values for AA metabolism and 35% and 46% of WT values for lauric acid metabolism, respectively. These studies demonstrate that the CYP4A11 -825G and 434Ser polymorphisms are associated with reduced translational activity and enzymatic activity, respectively, resulting a moderate loss-of-function *in vitro*.

1.8.1.2 CYP4F2 *in vitro* metabolism: There are a few polymorphisms in regulatory region of CYP4F2 that have been associated with altered transcriptional activity when expressed *in vitro*. Lui *et al.* evaluated the promoter activity of c.-48G>C (g.421G>C) and showed increased promoter activity of the construct containing the g.-421C polymorphism compared to g.421G (WT) constructs.[648] Also, a gel retardation assay showed Myb binding to the WT protein but not the construct with the variant g.421C allele. Combined with the results from the reporter assay, these results suggest that Myb may repress expression of CYP4F2 and the g.421G polymorphism may result in loss of Myb binding and thus increased expression of CYP4F2. In another study, Lui *et al.* evaluated the transcriptional activity of the CYP4F2 regulatory region in HEK293 cells using reporter constructs of two haplotypes for CYP4F2.[649] In the haplotype analysis, The Haplotype I construct contained the WT alleles c.-91T, c.-48G (g.421G), c.-13T, and c.+34T while the Haplotype II construct contained the variant alleles c.-91C, c.-48C (g.421C), c.-13C, and c.+34G. Haplotype I (WT) construct showed higher basal transcription and LPS-stimulated activity than the variant Haplotype II construct. Other constructs containing the variant -91C allele in the presence and absence of the -48C allele showed lower transcriptional activity than -91T (WT)

construct whereas the variant -48C allele did not affect transcriptional activity in the presence and absence of the variant -91C allele. Also, electrophoretic mobility shift assays with nuclear extracts show that NF-kB binding site existed at position c.-91 and the variant -91C allele altered its binding pattern. Combined with the previous results, these data suggest that NF-kB binding to the c.-91 site on CYP4F2 increases expression and the variant -91C allele attenuates this effect. Collectively, these data suggest that the CYP4F2 regulatory region containing the variant -91C allele and/or the variant alleles present in Haplotype II result in a moderate reduction in basal and LPS-stimulated transcriptional activity whereas the variant -48G allele has been reported to increase or have no effect on transcriptional activity of CYP4F2.

Other CYP4F2 polymorphisms are reported to alter enzymatic activity and protein levels when expressed *in vitro*. Bardowell *et al.* showed that cells expressing the variant 12Gly (*2) and 433Met (*3) alleles increased and decreased enzymatic activity towards both vitamin E and AA when compared to the 12Trp and 433Val (WT) constructs, respectively.[650] Specifically, the variant *2 allele showed a 2.5-fold and 1.9-fold increase in specific activity towards vitamin E and AA, respectively. On the other hand, the variant *3 allele resulted in 34% and 80% lower specific activity towards vitamin E and AA, respectively. Likewise, constructs with both variant *2 and *3 alleles showed resulted in 40% and 78% lower specific activity towards vitamin E and AA, respectively. Conversely, other *in vitro* studies expressing the variant *3 and *2/*3 alleles showed a 34-44% decrease in 20-HETE production whereas the construct with the variant *2 allele did not show any difference when compared to controls.[651] None of these variant constructs affected the ω-hydroxylation of leukotriene B4. Also, McDonald *et al.* reported that human liver microsomes from patients with the *1/*3 and *3/*3 genotypes had 64% and 22% of the CYP4F2 protein content and 52% and 25% of vitamin K1 oxidation compared to samples from patients with the *1/*1 (WT) genotype.[549] No association was observed between transcriptional activity of CYP4F2 in human liver microsomes and the variant *3 allele. Taken together, these data suggest that the variant CYP4F2*3 allele results in decreased ω-hydroxylase enzyme activity towards vitamin E and AA *in vitro* and lower protein levels but no change to mRNA expression of CYP4F2 in human liver microsomes. On the other hand, the variant CYP4F2*2 allele increased ω-hydroxylase enzyme activity towards vitamin E *in vitro*, but the reported effect on ω-hydroxylase enzyme activity towards AA is inconsistent.

1.8.1.3 CYP2J2 *in vitro* metabolism: There are numerous polymorphisms in CYP2J2 that have been reported to alter enzymatic activity, transcriptional activity, or protein levels when expressed *in vitro*. For example, *in vitro* expression of CYP2J2 constructs containing the variant 143Ala (*2), 158Cys (*3), 192Asn (*4), and 404Tyr (*6) alleles showed reduced activity to metabolize fatty acids such as AA or linoleic acid when compared to WT constructs (143Thr, 158Arg, 192Ile, and 404Asp).[439] Specifically, constructs with the variant *2, *3, *4, and *6 alleles metabolized AA to 59%, 41%, 30%, and 5% of WT (*1 allele) values, respectively. Likewise, the variant *2, *3, and *6 alleles metabolized linoleic acid to 58%, 50%, and 10% of WT values, respectively, but the variant *4 allele did not alter CYP2J2 activity toward linoleic acid. Other *in vitro* studies report reduced transcriptional activity of cells expressing the variant c.-50T (*7) polymorphism in the promoter region of CYP2J2.[505, 652, 653] Human cardiac tissue[653] and bovine aortic endothelial cells[505, 652] expressing the *7 polymorphism construct reduced transcriptional activity to 38% and 48-50% of values seen in WT constructs, respectively. Speiker *et al.* proposed that the reduced transcriptional activity is due to the disruption of a Sp1 transcription factor binding site.[505] In addition, human liver microsomes from patients harboring the *7 variant allele showed reduced CYP2J2 protein levels.[654] Another study reported that *in vitro* expression of a construct containing the 186Gly (*8) polymorphism showed almost complete loss of enzyme activity toward astemizole and ebastine metabolism and no carbon monoxide (CO) binding to the heme moiety indicating the lack of a functional heme moiety.[655] In the same study, structural models predicted that the 186 residue is near the heme moiety and the *8 polymorphism may suppress the normal docking of heme into its binding site. Furthermore, *in vitro* expression of a CYP2J2 construct containing the 115Leu (*10) polymorphism showed reduced enzyme activity towards terfenidine, loss of heme binding, and altered protein folding.[656] Collectively, these data suggest that the *2, *3, *4, *7, and *10 polymorphisms result in a moderate loss in enzyme activity while the *6 and *8 polymorphisms demonstrate almost complete loss-of-function *in vitro*.

1.8.1.4 CYP2C8 *in vitro* metabolism: CYP2C8 is one of the most extensively studied CYP isoforms with respect to the effect of polymorphic variants on enzyme activity. Multiple *in vitro* studies showed that constructs expressing the CYP2C8*3 (139Lys/399Arg) polymorphisms did not affect protein expression when compared to WT CYP2C8 protein.[657-659] When the single 399Arg polymorphism was expressed in Hep 2G cells, CYP2C8 protein levels were reduced to

40% of WT values, but introduction of the 139Lys polymorphism to form the *3 construct restored protein levels to WT values.[659] Interestingly, the mRNA levels in cells expressing the 399Arg polymorphism were not significantly different than WT suggesting that the lower protein levels associated with this polymorphism may not be due to altered transduction, but may be due to instability of the protein as previously suggested with CYP2D6*10.[660, 661] In human liver samples, CYP2C8 protein levels and mRNA expression in individuals with the *3 allele were not different than WT.[515, 662] These data suggest that CYP2C8 mRNA expression and protein levels are not affected by the *3 polymorphism.

On the other hand, multiple *in vitro* studies report that *3 allele affects the CYP2C8-mediated metabolism of endogenous substrates such as AA and drugs such as paclitaxel and amiodarone. For example, Smith *et al.* reported that constructs expressing the *3 polymorphism showed 4-fold lower 14,15-EET formation from AA metabolism compared to WT constructs.[663] Kinetic parameters reported showed that K_m, V_{max}, and CL_{int} were 247%, 80%, and 32% of WT values, respectively. A similar study reported that the *3 polymorphism was associated with a 2.2- , 2.7- , and 2.4-fold reduction in activity towards AA metabolism to form 11,12-EET, 14,15-EET, and total EETs, respectively.[664] Furthermore, incubations of human liver microsomes from patients with the *3/*3 and *1/*3 genotype and AA showed a 34% decrease and no difference in the formation of EETs and DHETs when compared to those with the WT genotype. Similarly, constructs expressing the *3, 139Lys and 399Arg polymorphisms are associated with reduced metabolism of several drugs including drugs including paclitaxel[657-659, 662, 664] and amiodarone[657]. On the other hand, a few studies suggest that the *3 polymorphism did not affect amiodarone and paclitaxel metabolism by CYP2C8.[658, 665] Collectively, these studies suggest that the CYP2C8*3 polymorphism may be associated with lower *in vitro* metabolism of AA, paclitaxel, and amiodarone.

Similar studies investigated the effect of the *4 polymorphism on CYP2C8 mRNA and protein expression. One study reported that yeast cells transfected with the *4 polymorphism expressed protein levels ~20% of WT values.[657] Likewise, human liver samples genotyped for a haplotype containing the *4 allele showed reduced protein levels compared to values in WT samples.[665] Conversely, other studies report that human liver microsomes and liver samples from patients with the *4 allele showed no difference in mRNA or protein levels of CYP2C8.[515, 662]

These conflicting results regarding the effect of the CYP2C8*4 variant on CYP2C8 mRNA and protein expression warrants further investigation.

The CYP2C8*4 polymorphism has also been shown to affect the *in vitro* metabolism of AA and several drugs. For example, E. Coli expressing the *4 polymorphism showed 5-fold lower 14,15-EET formation from AA metabolism when compared to WT values.[663] Kinetic parameters reported showed that K_m, V_{max}, and CL_{int} was 67%, 21%, and 30% of WT values, respectively. Similarly, the CYP2C8*4 polymorphism is associated with reduced *in vitro* metabolism of several drugs including drugs including paclitaxel[657, 662, 666], nifedipine[657], and amiodarone[657, 667]. One study reported that paclitaxel metabolism by human liver samples was not affected by the *4 allele, but the haplotype containing the *4 allele was associated with lower paclitaxel metabolism.[665] Although the mechanisms leading to the *in vitro* loss-of-function observed with the variant *4 allele is not fully elucidated, Singh et. al. reported that the constructs expressing the variant *4 allele affected CO binding to the heme moiety and were more sensitive to proteinase K digestion indicating improper heme insertion and protein folding.[667]

Other CYP2C8 gene variants have also been reported to affect CYP2C8 levels, structure, or function. *In vitro* expression studies reported reduced CYP2C8 protein levels associated with the *8[668], *14[668], and 404Ala[659] polymorphisms, but no change in mRNA expression was observed with the *13 polymorphism[668]. The reduced protein levels associated with the *8 polymorphism were restored to WT levels using a proteasome inhibitor MG-132 indicating the decreased levels may be due to enhanced proteasomal degradation.[668] In addition, reduced CO difference spectra observed with the *14 polymorphism suggested a lack of a functional heme group.[669] Likewise, constructs expressing the 404Ala polymorphism showed altered heme and/or substrate binding.[666] Furthermore, there are a few polymorphisms in CYP2C8 that lead to significant loss of the protein structure. The 159Pro (*5) polymorphism results in a frameshift transition at residue 177 and is most likely to be inactive since it lacks 64% of the protein structure including the heme-binding site and 5 of 6 substrate recognition sites.[670] Likewise, the 186Ter (*7) polymorphism leads to an early stop codon that results in a truncated protein that lacks 60% of the C-terminal region including the heme binding site, which was undetectable when expressed *in vitro*.[667] The 274Ter (*11) polymorphism also leads to an early stop codon but has not been evaluated *in vitro*. In regard to samples from humans, liver microsomes from patients with the variant *1B allele showed

increased binding of nuclear proteins and 1.8-fold higher transcription rate than WT.[665] However, another study protein levels in human liver samples from patients with the variant *1B and *1C alleles were not different than WT.[662] Collectively, these studies suggest that CYP2C8 transcription is increased with *1B and unchanged with *8 polymorphisms, CYP2C8 protein expression is reduced with the *2, *8, *14, and 404Ala polymorphisms, and CYP2C8 protein structure and function is significantly altered with the *5, *7, and *11 polymorphisms.

Other studies investigated the effects of CYP2C8 polymorphism on metabolism of some drugs *in vitro*. For example, it has been reported that *in vitro* metabolism of paclitaxel is reduced cell expressing the 269Phe (*2)[657, 664], 186Gly (*8)[668], 238Pro (*14)[669], and 404Ala[659, 666] polymorphism and a haplotype containing the *1B[665] polymorphism whereas complete loss of enzymatic activity is observed with the 186Ter (*7)[668] polymorphism. Similarly, *in vitro* metabolism of amiodarone is reduced with the *2[657] and 404Ala[659] polymorphism. Although the effects of these variants on AA metabolism have not been reported, evaluation of these polymorphism represent an important area of future study due to the significant effects observed on CYP2C8 functional activity. Collectively, these studies suggest that the *2, *8, *14, 404Ala, and possibly *1B polymorphisms lead to moderate loss-of-function while *7 leads to complete loss-of-function of CYP2C8.

1.8.1.5 CYP2C9 *in vitro* metabolism: Zhou *et al.* also provided an extensive and excellent discussion on CYP2C9 functional polymorphisms and their relevance to drug disposition and disease.[671] This review discusses the *in vitro* studies that demonstrate loss of function with the *2, *3, *4, *5, *13, *25, *26, *28, and *30 polymorphisms. Additional studies by Delozier *et al.* report that *in vitro* expression of *15 and *18 polymorphisms resulted in proteins that could not be detected, *17 and *19 polymorphisms exhibited a 30-40% decrease in catalytic activity, and *14 and *16 polymorphisms exhibited 80-90% lower catalytic activity toward tolbutamide, respectively, when compared to WT protein.[672] Likewise, the *in vitro* intrinsic clearance of diclofenac by constructs with the *33 polymorphism was reduced 5-fold when compared to WT enzyme.[673] In addition, Blaisdell *et al.* reported that the *in vitro* metabolism of tolbutamide was altered by genetic polymorphisms.[674] To illustrate, increased metabolism was associated with the *8 polymorphism, decreased metabolism was associated with the *11, and *12 polymorphisms and no change in metabolism was observed in constructs with the *7, *9, and *10 polymorphisms

when compared to WT enzyme.[674] However, another *in vitro* study reported that constructs with the *8 polymorphism led to a 30% reduction in intrinsic clearance of S-warfarin compared to WT enzyme.[675] These data suggest that there are multiple gene variants in CYP2C9 that primarily lead to loss of enzymatic function.

1.8.1.6 EPHX2 *in vitro* metabolism: Currently, there are no published studies investigating the role of gene variants in the EXPH2 regulatory region on mRNA expression or protein levels. However, numerous studies have reported that EPHX2 gene variants can alter SEH activity *in vitro*. For instance, the activity of the 55Arg, 103Cys, 154Tyr, and 287Gln polymorphisms were increased, not changed, increased, and decreased using trans-stilbene oxide (t-SO) and trans-diphenylpropene oxide (t-DPPO) as substrates.[676] Similar results were shown when using 14,15-EET as an SEH substrate.[676] Also, Koerner *et al.* reported that primary cortical neuronal cultures transduced with cDNA containing the 55Arg, 103Cys, 154Tyr, and 287Gln polymorphisms showed increased, no change, no change, and decreased 14,15-DHET formation, respectively, after exogenous administration of 14,15-EET when compared to WT transfected cells.[641] Similarly, Merkel *et al.* reported that cardiomyocytes from SEH knockout and WT mice transduced with the 55Arg and 287Gln polymorphic constructs showed no difference and decreased 14,15-DHET formation.[677] Conversely, Sandberg *et al.* reported that Chinese Hamster Ovary (CHO) and African Green Monkey Kidney (COS-7) cells transfected with the 287Gln polymorphic construct showed no change in SEH activity towards t-SO when compared to WT.[678] Other *in vitro* studies showed that the 287Gln, but not the 55Arg, 103Cys, and 154Tyr polymorphisms, reduce enzyme stability.[678, 679] Although there are some conflicting results, the majority of the studies suggest that SEH activity is increased with the EPHX2 55Arg and 154Tyr polymorphisms, decreased with the 287Gln polymorphism, and not impacted by the 103Cys polymorphism.

A few studies also investigated the effect of the $Arg^{402\text{-}403}$ insertion, 422Ala, and 470Gly polymorphism on the activity of EPHX2. Sandberg *et al.* reported that CHO and COS-7 cells transfected with constructs containing the $Arg^{402\text{-}403}$ insertion polymorphism showed lower SEH activity (~40% of WT) towards t-SO when compared to WT.[678] Another study reported that constructs with the 470Gly polymorphism expressed *in vitro* showed increased SEH activity towards t-SO and t-DPPO, but no difference in activity towards 14,15-EET as compared to WT.[676]

Also, the stability of the $Arg^{402-403}$ insertion enzyme declined more rapidly than the WT enzyme. However, other studies showed that constructs expressing the 470Gly and 422Ala polymorphism did not affect enzyme stability.[679] These studies suggest that $Arg^{402-403}$ insertion leads to decreased SEH activity whereas the 422Ala polymorphism is not likely to affect function of the enzyme. However, the functional effects of the 470Gly polymorphism are unclear and warrant further studies.

1.8.2 *In vivo* studies of substrate metabolism:

This section will identify candidate gene polymorphisms reported to alter endogenous substrate or drug metabolism *in vivo*. Tables at the end of Section 1.8 summarize key findings from this section.

1.8.2.1 CYP4A11 *in vivo* metabolism: As previously discussed, 20-HETE elicits multiple biological effects *in vivo* including a complex role in the regulation of blood pressure.[680] As a result, some studies have investigated the association of CYP4A11 polymorphisms with the urinary excretion of 20-HETE in hypertensive patients. Laffer *et al.* reported that hypertensive patients with the variant 434Ser allele showed reduced 20-HETE in urine in response to salt loading compared to patients with the 433Phe/Phe (WT) genotype after adjusting for the effect of serum insulin on 20-HETE excretion.[681] Another study reported that urinary 20-HETE excretion was 27% higher in hypertensive patients compared to normotensive patients, while no difference in urinary sodium excretion was observed in these groups.[682] In this study, individuals with the variant 434Ser allele showed ~30% reduction in 20-HETE excretion in urine.[682] Also, the variant 434Ser allele showed a trend to predict reduced urinary 20-HETE excretion, but not blood pressure, both before and after adjustment for covariates. These studies demonstrate that the variant 434Ser allele in CYP4A11 is associated with reduced urinary 20-HETE levels in hypertensive and normotensive patients.

1.8.2.2 CYP4F2 *in vivo* metabolism: There are a numerous studies that investigated the association of CYP4F2 polymorphisms with the metabolism of drugs, vitamins, and endogenous compounds in humans. Hu *et al.* reported that urinary concentrations of the AA metabolite 20-

HETE were significantly different across the Val433Met (*3) genotypes in Chinese men, women, and both sexes combined, before and after adjustment for clinical covariates.[683] In men and women combined, the unadjusted geometric mean urinary 20-HETE concentration was 115% and 210% higher in individuals with *1/*3 and *3/*3 genotypes, respectively, compared to the *1/*1 (WT) group. There was no overall association between genotypes describing the -458T>C and -91T>C transitions and urinary 20-HETE concentration. A similar study in an Australian population reported that individuals with variant *3 allele showed increased urinary 20-HETE excretion compared to those with the *1/*1 genotype.[682] The variant *3 allele predicted increased urinary 20-HETE excretion before and after adjustment for age and BMI, but only showed a trend for significance when sex was added to the model. Major *et al.* reported an association between patients with the variant *3 allele and decreased vitamin E metabolism in the α-Tocopherol, β-Carotene Cancer Prevention (ATBC) Study cohort and in a meta-analysis.[684] In a meta-analysis by Denese et al., individuals with the variant *3 allele required an 8.3% higher mean daily warfarin dose to reach a stable international normalized ratio (INR) than those with the *1/*1 (WT) genotype possibly due to reduced vitamin K1 metabolism[549, 685] Collectively, these data suggest that individuals with the variant CYP4F2*3 allele are likely to have reduced 20-HETE in urine, decreased metabolism of vitamin E, and higher dose of warfarin.

1.8.2.3 CYP2J2 *in vivo* metabolism: The effect of the CYP2J2*7 variant allele on the metabolism of endogenous substrates of CYP2J2 in humans was evaluated in multiple studies. In these studies, plasma concentrations of 14,15-DHET were measured as a surrogate marker of AA metabolism by CYP2J2. One study reported that the *7 allele in myocardial infarction patients and controls was associated with a 44% and 54% reduction in plasma 14,15-DHET concentrations, respectively.[652] In a study of German patients with coronary artery disease and controls, individuals with the *7 allele had 29% lower plasma 14,15-DHET concentrations compared to those with the *1/*1 (WT) genotype.[505] Similarly, Chinese patients with type 2 diabetes with the *7 allele showed 67% lower plasma 14,15-DHET levels.[686] On the other hand, Gervasini *et al.* reported that ebastine metabolite levels in urine from Caucasians with the *7 allele were not different than those from individuals with WT genotype.[687] Taken together, these data suggest that the CYP2J2*7 variant is associated with reduced metabolism of AA, but additional studies are needed to determine the effects of CYP2J2*7 and other functional polymorphisms on the

clearance of ebastine and other CYP2J2 substrates in humans.

1.8.2.4 CYP2C8 *in vivo* metabolism: Daily *et al.* provides an excellent review of clinical studies investigating the pharmacogenetics of CYP2C8.[688] One of the most extensively studied CYP2C8 polymorphisms is the loss-of-function *3 variant that has been reported to alter the pharmacokinetics of some drugs and endogenous substrates. For example, most healthy volunteer studies have found variant *3 allele is associated with increased clearance or decreased trough concentrations of pioglitazone and rosiglitazone.[689-694] On the other hand, some studies report that the pharmacokinetics of rosiglitazone and N-desmethylrosiglitizone were not statistically different between *3 allele carriers and *1/*1 groups.[695] Similarly, the *3 polymorphism has been associated with increased clearance and decreased maximum concentration (C_{max}) of repaglinide in most studies[665, 696-698], but some studies report no association between the *3 polymorphism and repaglinide pharmacokinetics or pharmacodynamics at clinically relevant doses[699, 700]. In contrast, multiple studies reported that the variant *3 allele reduces the metabolism of nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen[701-704] and diclofenac[705]. Although some studies have reported an association between *3 polymorphism and an increased risk of neurotoxicity during paclitaxel treatment[706-709], most clinical reports suggest that CYP2C8*3 is not a major determinant of paclitaxel pharmacokinetics[375, 706, 710-712]. The discrepancy between *in vitro* and *in vivo* findings regarding the association between the metabolism of paclitaxel and the *3 polymorphism has been proposed to be the result of the contribution of drug transporters to paclitaxel disposition in humans.[713] There is a lack of studies evaluating the role of CYP2C8 gene variants on the metabolism of endogenous substrates such as AA. However, one study evaluated the relationship between the *3 polymorphism and urinary excretion of DHETs and reported that carriers of the variant *3 allele had paradoxically increased levels of DHETs in urine before and after rosiglitazone treatment.[714] The discrepancy between the *in vitro* studies and *in vivo* studies has not yet been fully elucidated. Taken together, these studies suggest that CYP2C8*3 polymorphism is associated with increased urinary DHET excretion and clearance of structurally diverse drugs with the exception of NSAIDs.

The effect of other CYP2C8 gene variants on drug disposition in humans has not been studied extensively. Studies by Niemi et al. report that the variant *4 allele is not associated with altered clearance of repaglinide in healthy volunteers[697, 698] In addition, there are only a few studies

that have investigated the effect of rare CYP2C8 gene variants and haplotypes on drug disposition in humans. In healthy Asian individuals, the rare *1/*11 genotype was associated with lowered activity towards rosiglitazone compared to the *1/*1 genotype.[376] In healthy Finish Caucasians, individuals with haplotype B containing the *1B polymorphism and haplotype D containing the *3 polymorphism showed increased clearance of repaglinide whereas haplotype C was associated with decreased clearance of repaglinide.[665] Further *in vitro* studies showed *1B polymorphism was the causal variant that led to the increased activity of haplotype B.[665] In another study, individuals with the CYP2C8 haplotype C had lower urinary DHET excretion before and during rosiglitazone, which was shown to decrease urinary DHET excretion by 10%.[714] Collectively, these studies suggest that individual gene variants may not predict drug disposition as well as multiple gene variants or haplotypes.

1.8.2.5 CYP2C9 *in vivo* metabolism: As previously discussed by Zhou *et al.*, CYP2C9*2 and *3 alleles are important determining factors for drug clearance and drug response to multiple NSAIDs, sulfonylurea antidiabetic drugs, and oral anticoagulants [671] Other studies have reported associations of the less frequent CYP2C9 variants and drug disposition. For example, Guo *et al* reported that three patients with the *1/*13 genotype showed a 1.9-fold increase in AUC and 44% decrease in the oral clearance of lornoxicam when compared to patients with *1/*1 genotype.[715] Other studies by Allabi *et al.* showed that the *5, *6, *8 and *11 allele, but not the *9 allele, are associated with a decreased phenytoin metabolism in humans.[716]. On the other hand, patients with the *11 allele did not require different dosing regimens of warfarin.[717] There is a lack of studies investigating the effect of CYP2C9 polymorphic variants on the metabolism of endogenous substrates. However, one study by Lundbad *et al.* reported that the expression of *2 and *3 in yeast shows reduced activity towards AA metabolism with a lower Cl_{int} and V_{max}, and higher K_m.[404] Furthermore, incubations of human liver microsomes from patients with the double homozygous variant *3/*3 and *2/*2 genotype led to a 34% decrease in EET formation.[404] Collectively, these studies suggest that the CYP2C9*2, *3, and *4 variants are the most clinically relevant polymorphisms associated with drug disposition and metabolism of endogenous substrates.

1.8.2.6 EPHX2 *in vivo* metabolism: There are a numerous studies that investigated the association of EPHX2 polymorphisms with the metabolism of drugs and endogenous compounds

in humans. In Korean patients with IgA nephropathy, the 287Gln allele had higher plasma epoxyoctadecenoic acid (EpOME)/dihydroxyoctadecenoic acid (DiHOME) ratios. Specifically, 9,10-EpOME/9,10-DiHOME and 12,13-EpOME/12,13-DiHOME ratios were increased in these patients whereas no association was observed in control patients.[718] Two other EPHX2 gene variants, c.1784A>G (rs1042032) in the promoter region and 55Arg, were not associated with plasma 9,10-EpOME/9,10-DiHOME ratios and 12,13-EpOME/12,13-DiHOME ratios in patients with IgA nephropathy and control patients.[718] Lee *et al.* also investigated the relationship between EPHX2 polymorphisms and plasma 9,10-EpOME/9,10-DiHOME and 12,13-EpOME/12,13-DiHOME ratios in Americans with CVD and control patients in the Atherosclerosis Risk in Communities study (ARIC) and reported that individuals with the variant 55Arg allele had 22% and 30% lower 12,13-EpOME/12,13-DiHOME ratios, respectively, compared to those with the WT genotype.[719] No association of the 55Arg genotype and plasma 9,10-EpOME/9,10-DiHOME ratios was observed. In the race stratified analysis, individuals with the 55Lys/Arg and 55Arg/Arg genotype were associated with 33% and 43% lower plasma 9,10-EpOME/DiHOME ratios and 36% and 40% lower plasma 12,13-EpOME/DiHOME ratios, respectively, in Caucasians but not in African Americans. In addition, Edvardsen *et al.* investigated the relationship between polymorphisms in EPHX2, and other genes involved in reactive oxygen species (ROS) formation, and docetaxel clearance in Norwegian patients with non-small cell lung carcinoma (NSCLC) and reported that the 287Gln polymorphism predicted docetaxel clearance.[720] Sato *et al.* investigated the relationship between the 287Gln polymorphism and plasma cholesterol and triglyceride concentration in individuals with familial hypercholesterolemia.[721] In individuals harboring the IVS14+1G>A polymorphism in the low-density lipoprotein receptor (LDLR), plasma cholesterol and triglyceride levels were 18% and 54% higher, respectively, among those with the variant 287Gln allele compared to those with the WT genotype. No association was observed between genotype and plasma cholesterol and triglyceride levels in non-carriers of the LDLR polymorphism. Chen *et al.* investigated the relationship between the 287Gln polymorphism and concentrations of polycystic aromatic hydrocarbon metabolites in urine from Chinese coke oven workers and controls and reported no association between genotype and urinary 1-hydroxypyrene concentration.[722] Together, these data suggest that individuals with the EPHX2 287Gln polymorphism, and possibly the 55Arg polymorphism, are likely to have reduced metabolism of

various endogenous and xenobiotic substrates.

1.8.3 *In vitro* studies of cerebral ischemic injury

This section will identify candidate gene polymorphisms reported to alter ischemic cell death *in vitro*. Tables at the end of Section 1.8 summarize key findings from this section.

1.8.3.1 EPHX2 *in vitro* ischemia: A few studies investigated the impact of EPHX2 gene variants on cell death using *in vitro* ischemia models. Merkel *et al.* transduced cardiomyocytes from SEH knockout and WT mice with TAT-SEH fusion proteins containing various polymorphic and WT constructs and assessed their tolerance to oxygen and glucose deprivation and reoxygenation and glucose repletion (OGD/RGR).[677] In this study, there was no significant difference in cell death between cardiomyocytes expressing naive, WT, and polymorphic 55Arg, 103Cys, and 154Tyr proteins, but expression of the 287Gln variant showed decreased cell death compared to the WT transfected cells. In a similar study, Koerner *et al.* transduced primary cortical neuronal culture with TAT-hSEH fusion proteins containing various polymorphisms and WT constructs and assessed their tolerance to OGD/RGR.[641] The 55Arg and 154Tyr polymorphisms had no impact on neuronal cell death, but the cells transfected with the 103Cys and 287Gln polymorphisms showed increased and decreased cell death, respectively, compared to WT transfected cells. These data suggest that the 287Gln polymorphism is associated with improved cell survival after ischemia, but the effects of the 103Cys polymorphism on cell survival after ischemia need to be further investigated.

Furthermore, these studies also investigated the effect of SEH inhibitors and 14,15-EET on survival of cells transfected with SEH gene variants after OGD/RGR. Merkel *et al.* reported that inhibition of SEH and addition of excess 14,15-EET improved cardiomyocyte cell survival in WT, 55Arg, 103Cys, and 154Tyr variants, but did not affect cell survival in the 287Gln variant.[677] Also, the 287Gln variant had no effect on cell survival of cardiomyocytes from SEH knockout mice transduced with SEH. Likewise, Koerner *et al.* reported that inhibition of SEH and addition of 14,15-EET improved neuronal cell survival in the 103Cys and 154Tyr variants, but did not affect cell survival in the 55Arg, and 287Gln variants.[641] These data suggest that the 103Cys and 154Tyr

polymorphisms may alter cell survival after ischemia through mechanisms involving SEH.

1.8.4 Candidate gene variants in stroke patients

This section will identify candidate gene polymorphisms associated with various stroke subtypes in clinical studies. Tables at the end of Section 1.8 summarize key findings from this section.

1.8.4.1 CYP4A11 variants in stroke: A few studies have investigated the association between genetic variation in CYP4A11 and the occurrence of stroke. One study by Fava *et al.* showed that the variant 434Ser allele was not associated with the occurrence of ischemic stroke (IS) in a Swedish population.[723] Another study showed that multiple tagging polymorphisms and haplotypes were not associated with IS in a mixed race panel.[724] Similarly, a study by Ding *et al.* investigated the relationship between gene variants of CYP4A11, including tagging polymorphisms and potential functional polymorphisms, and the occurrence of IS and hemorrhagic stroke in a Chinese population and reported that patients with the variant -296T allele had not difference in the occurrence of hemorrhagic stroke (HS), but had higher occurrence of IS when compared to controls.[647] When IS was grouped into large vessel disease and lacunar stroke, the variant -296T allele was associated with an increased occurrence of large vessel disease but not lacunar stroke when compared to controls. Also, patients with the variant 434Ser allele showed a trend for decreased occurrence of IS and HS when compared to controls. Individual haplotypes did not show an association with HS and IS even when IS was broken into lacunar stroke and large vessel disease group. These studies suggest that the variant -296T allele may be associated with a higher risk for IS and worse outcomes in HS.

A study by Fu *et al.* investigated the relationship between tagging polymorphisms of CYP4A11 and the occurrence of cerebral infarction (CI) in Japanese population.[725] When considering individual tagging polymorphisms only in the analysis, it was reported that genotype distribution and frequency of carriers for the variant 434Ser allele and g.10733A allele (rs2269231) was not statistically different between patients with CI and controls in total, men, and women groups in a Japanese population. Another study showed that the presence of the variant g.15254A

allele (rs9333025) was associated with a decreased occurrence of CI when compared to the WT genotype in the total population and in the male group, but not in the female group. Furthermore, a regression model showed that the G/G (WT) genotype was more frequent in patients with cerebral infarction than controls after adjusting for history of hypertension and diabetes mellitus. In the haplotype analysis, seven haplotypes were specified and the overall distribution of haplotypes showed a significant difference between cerebral infarction patients and controls in total group, but not when patients were grouped by sex. Specifically, Haplotype 6 was more frequent in controls compared CI patients in the total, but not in men and women group. These data suggest that the variant g.15254A allele (rs9333025) and Haplotype 6 was associated with a lower occurrence of CI, which may be more prominent in men, thereby implying that future genetic association studies should include sufficient numbers of subjects to evaluate gene polymorphism effects dichotomized by sex.

1.8.4.2 CYP4F2 variants in stroke: A few studies have investigated the association of the loss-of-function CYP4F2*3 (433Met) and the occurrence of stroke. One study showed that that the hazard ratio for IS in Swedish males with the variant *3 allele was significantly higher than those with *1/*1 (WT) genotype after adjustment for major cardiovascular risk factors.[723] Similarly, in a Chinese cohort of stroke patients, the frequency of the genotypes containing the variant *3 allele were higher than the *1/*1 genotype in IS but not in hemorrhagic stroke.[647] When the IS group was further grouped in to large vessel disease and lacunar infarcts, the frequency of the variant *3 allele carriers were higher in the large vessel disease group and showed a trend for significance for lacunar infarcts group in the adjusted model. Also, the individual and combined haplotypes did not show different frequencies in HS, IS, LVD, and lacunar stroke groups when compared to controls. Furthermore, Munshi *et al.* showed that hypertensive and non-hypertensive individuals with the variant *3/*3 genotype and *3 allele have a higher occurrence of IS compared to those with the WT *1/*1 genotype and *1 allele, respectively.[726] On the other hand, Deng at al. reported that the *1/*1 (WT) genotype in men had a higher occurrence of IS compared to those with the *3 allele, but no difference was found in all participants and females.[727] In the multiple logistic regression analysis, the *1/*1 genotype was associated with an increased risk of IS in men compared to the *3/*3 genotype after adjustment for clinical covariates. Although there are some conflicting results, these data suggest that individuals with the CYP4F2*3 variant are at higher

risk for IS. Future meta-analyses of conducted studies or genetic studies in larger populations would likely allow for a more definitive determination of the effects of this variant on stroke risk.

One study investigated the relationship between polymorphisms in CYP4F2 and occurrence of CI. Fu *et al.* reported that the frequency of the WT *1/*1 genotype in males was significantly higher in cerebral infarction patients as compared to *3 allele carriers.[728] In this study, the TCG haplotype was defined by the c.199-105T (WT), c.989C (*3 variant), and c.-698T (WT) alleles. The TCG haplotype containing the variant *3 allele was more frequent in control subjects compared to cerebral infarction patients before and after adjustment for confounding factors. On the other hand, the variant alleles 519Met, *2, c.449A (rs3093135), and c.7256T (rs3093139) were not associated with cerebral infarction. These results suggest that CYP4F2*3 allele is associated with a decreased risk for cerebral infarction.

1.8.4.3 CYP2J2 variants in stroke: The impact of genetic polymorphisms in CYP2J2 on the occurrence and outcomes in stroke has not been studied extensively. One study reported that the frequency of the variant *7 allele and WT genotype in IS patients was not different than in controls.[729] A similar study reported no association with the variant *7/*7 genotype and the cumulative occurrence of IS before and after adjusting for major cardiovascular risk factors.[730] In addition, Marciante *et al.* found no association between ten tagging polymorphisms of CYP2J2 and multiple haplotypes and the occurrence of IS.[724] Currently, there is little data supporting an association between CYP2J2 polymorphisms and the risk for IS.

1.8.4.4 CYP2C8/9 variants in stroke: Few studies have investigated the relationship between CYP2C8/9 polymorphisms on the occurrence of stroke. In a large study involving over 52,000 patients with CVD in Denmark the CYP2C9*2 and CYP2C9*3 polymorphisms were not associated with the risk for IS and ischemic cerebrovascular disease.[731] Similarly, the CYP2C9*2 and CYP2C9*3 genotypes were not associated with IS both before and after adjusting for multiple cardiovascular risk factors when compared to controls in a cohort of 389 patients from the Vienna Stroke Registry.[732] Also, there was no association with the CYP2C9*2 and CYP2C9*3 genotypes and stoke etiology and neurological outcome at one week after the stroke. In a US cohort of 368 patients with IS, multiple tagging polymorphisms and haplotypes of CYP2C8/9 in men, women, and combined sexes were not associated with IS both before and after adjustment for demographic and cardiovascular risk factors when compared to controls.[724] Furthermore, similar results were

observed in subgroups of defined by sex, hypertension, smoking, CVD, and race. These studies suggest that polymorphisms in CYP2C8 and CYP2C9 do not play a major role in the risk for IS.

1.8.4.5 EPHX2 variants in stroke: Multiple studies have investigated the impact of genetic variation in EPHX2 on the occurrence of IS. Fava *et al.* demonstrated that males with the 55Arg/Arg genotype had higher occurrence of ischemic strokes when compared to those with the WT genotype before and after adjustment of cardiovascular risk factors.[733] In contrast, two other studies reported no association with the 55Arg polymorphism and occurrence of IS.[734, 735] Multiple studies reported no association between the 103Cys polymorphism and the occurrence of IS.[733, 735, 736] Gschwendtner *et al.* reported that individuals with 287Gln allele were associated with a higher occurrence of IS compared to those with the WT allele.[734] On the other hand, Zhang *et al.* reported that the 287Gln polymorphism was associated with a lower occurrence of IS after adjustment for sex, age, and multiple cardiovascular risk factors.[729] A few other studies reported no association with the 287Gln polymorphism and occurrence of IS.[733, 735, 736] Also, the variant c.1170+4855C (rs7357432) and c.1291-124A (rs2291635) alleles located in the intronic regions of EPHX2 were associated with increased occurrence of IS.[734] There was no relationship between IS and the Arg$^{402\text{-}403ins}$[735, 736], 470Gly[734, 735], c.-1452C (promoter)[733], c.1784G (near 3'-UTR)[733] polymorphisms[734]. In a haplotype analysis of EPHX2, Fornage *et al.* reported a decreased risk of IS predicted by the ATACGGT Haplotype and increased risk of IS predicted by the ACACAGT Haplotype.[735] Together, these studies that there are no strong relationships with EPHX2 gene variants and IS.

CYP4A11 Gene Variants

SNP (rs#)	*In vitro* Metabolism	*In vivo* metabolism	Stroke
c.-296T>C (NA)	↔ transcription[647]	unknown	↑ IS[647] ↔ IS[723] ↑ LVD[647] ↔ HS[647]
c.-825A>G (rs9332978)	↓ transcription[646]	unknown	unknown
434Phe>Ser [c.8590T>C] (rs1126742)	↓ activity (AA)[540]	↓ 20-HETE (urine)[681, 682]	↔ CI[725]
Haplotype 6	unknown	unknown	↓ CI[725]

IS, ischemic stroke; LVD, stroke from large vessel disease; HS, hemorrhagic stroke; CI, cerebral infarction
AA, arachidonic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid;

CYP4F2 Gene Variants

SNP (rs#)	*In vitro* Metabolism	*In vivo* metabolism	Stroke
12Trp>Gly [*2] (rs3093105)	↑ activity (AA, vit E)[650] ↔ activity (AA)[651]	unknown	↔ CI[728]
433Val>Met [*3] (rs2108622)	↓ activity (AA, vit E, vit K1)[549, 650, 651] ↓ protein levels[549] ↔ translation[549]	↓ metabolism (vit. E)[684] ↑ warfarin dosing[549, 685] ↑ 20-HETE (urine)[682, 683]	↓ CI[728] ↓ IS[727] ↑ IS[647, 723, 726] ↑ LVD[647] ↔ HS [647]
12Trp>Gly / 433Val>Met [*2/*3] (rs3093105/rs2108622)	↓ activity (AA, vit E)[650, 651]	unknown	unknown
c.-48G>C (g.421G>C) (rs3093100)	↑ translation[648] ↔ translation[649]	unknown	unknown
c.-458T>C (rs3093089)	unknown	↔ 20-HETE (urine)[683]	unknown
c.-91T>C (rs3093098)	↓ translation[649]	↔ 20-HETE (urine)[683]	unknown
g.7222002G>A (rs2189784)	unknown	↓ time-to-therapeutic INR[737]	unknown
Haplotype TCG	unknown	unknown	↓ CI[728]
Haplotype II	↓ translation[649]	unknown	unknown

IS, ischemic stroke; LVD, stroke from large vessel disease; HS, hemorrhagic stroke; CI, cerebral infarction
AA, arachidonic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; vit, vitamin; INR, international normalized ratio

CYP2J2 Gene Variants

SNP (rs#)	*In vitro* Metabolism	*In vivo* metabolism	Stroke
143Thr>Ala [*2] (rs55753213)	↓ activity (AA, LA)[439]	unknown	unknown
158Arg>Cys [*3] (rs56307989)	↓ activity (AA, LA)[439]	unknown	unknown
192 Ile>Asn [*4] (rs66515830)	↓ activity (AA)[439]	unknown	unknown
404Tyr>Asn [*6] (rs72547598)	↓ metabolism (AA, LA)[439]	unknown	unknown
c.-50G>T [*7] (rs890293)	↓ transcription[505, 652, 653] ↓ protein levels[654]	↓ DHETs (plasma)[505, 652,686] ↔ metabolism (urinary ebastine)[687]	↔ IS[729, 730]
312Gly>Arg [*8] (rs150461093)	↓ activity (ebastine)[655]	unknown	unknown
115Pro>Leu [*10] (NA)	↓ activity (terfenidine)[656]	unknown	unknown

IS, ischemic stroke; AA, arachidonic acid; LA, linoleic acid; DHET, dihydroxyeicosatetraenoic acid;

CYP2C8 Gene Variants

SNP (rs#)	*In vitro* Metabolism	*In vivo* metabolism	Stroke
-271C>A [*1B] (rs7909236)	↑ translation[665] ↔ protein level[662]	unknown	unknown
-370T>G [*1C] (rs17110453)	↔ protein level[662]	unknown	unknown
269Ile>Phe [*2] (rs11572103)	↓ activity (paclitaxel, amiodarone)[657, 664]	unknown	unknown
139Arg>Lys (rs11572080)	↓ protein level[659] ↓ activity (paclitaxel)[659]	unknown	unknown
399Lys>Arg (rs10509681)	↓ activity (paclitaxel)[659]	unknown	unknown
139Arg>Lys/ 399Lys>Arg [*3] (rs11572080/rs10509681)	↔ translation[515, 662] ↔ protein level[515, 657-659, 662] ↓ activity (paclitaxel, amiodarone, AA)[657-659, 662-664] ↔ activity (paclitaxel, amiodarone)[658, 665]	↑ CL (pioglitazone, rosaglitazone, repaglinide)[665, 689-694, 696-698] ↔ CL (rosiglitazone, repaglinide, paclitaxel)[375, 695, 699, 700, 706, 710-712] ↓ CL (ibuprofen, diclofenac)[701-705] ↑ DHET (urine)[714]	unknown
264Ile>Met [*4] (rs1058930)	↔ translation[515, 662] ↔ protein levels[515, 662] ↓ activity (paclitaxel, amiodarone, nifedipine, AA)[657, 662, 663, 666, 667] ↔ activity (paclitaxel)[665]	↑ CL (repaglinide)[698] ↔ CL (repaglinide)[697]	unknown
159Thr>Pro Frameshift [*5] (rs72558196)	Truncated protein[670]	unknown	unknown
186Arg>Ter [*7] (rs72558195)	Truncated protein[667] Inactive towards paclitaxel[668]	unknown	unknown
186Arg>Gly [*8] (rs72558195)	↔ translation[668] ↓ protein levels[668] ↓ activity (paclitaxel)[668]	unknown	unknown
274Glu>Ter [*11] (NA)	unknown	↓ CL of rosiglitazone[376]	unknown
238Ala>Pro [*14] (rs188934928)	↓ protein levels[668] ↓ activity (paclitaxel)[669]	unknown	unknown
404Pro>Ala (rs66501115)	↓ protein levels[659] ↓ activity (paclitaxel and amiodarone)[659]	unknown	unknown
Haplotype B (containing *1B)	↓ activity (paclitaxel)[665]	↑ CL of repaglinide[665]	unknown

CL; clearance; arachidonic acid; LA, linoleic acid; DHET, dihydroxyeicosatetraenoic acid; tSNPs, tagging single nucleotide polymorphisms

CYP2C9 gene variants

SNP (rs#)	*In vitro* Metabolism	*In vivo* metabolism	Stroke
144Arg>Cys [*2C] (rs1799853)	↓ activity[671]	↓ CL (NSAIDs, anti-coagulants, anti-diabetic drugs)[671]	↔ IS[731, 732] ↔ ICVD[731]
359Ile>Leu [*3A] (rs1057910)	↓ activity[671]	↓ CL (NSAIDs, anti-coagulants, anti-diabetic drugs)[671]	↔ IS[731, 732] ↔ ICVD[731]
359Ile>Thr [*4] (rs56165452)	↓ activity[671]	↓ CL (NSAIDs, anti-coagulants, anti-diabetic drugs)[671]	unknown
360Asp>Glu [*5] (rs28371686)	↓ activity[671]	↓ CL (phenytoin)[716]	unknown
273 Frameshift [*6] (rs9332131)	shortened protein[671]	↓ CL (phenytoin)[716]	unknown
19Ile>Leu (rs67807361)	↔ activity (tolbutamide)[674]	unknown	unknown
150Arg>His [*8] (rs7900194)	↑ activity (tolbutamide)[674] ↓ activity (warfarin)[675]	↓ CL (phenytoin)[716]	unknown
251His>Arg [*9] (rs2256871)	↔ activity (tolbutamide)[674]	unknown	unknown
272Glu>Gly [*10] (rs9332130)	↔ activity (tolbutamide)[674]	unknown	unknown
335Arg>Trp [*11A] (rs28371685)	↓ activity (tolbutamide)[674]	↔ warfarin dosing[717] ↓ CL (phenytoin)[716]	unknown
489Pro>Ser [*12] rs9332239	↓ activity (tolbutamide)[674]	unknown	unknown
90Leu>Pro [*13)] (rs72558187)	↓ activity[671]	↓ CL (lornoxicam)[715]	unknown
125Arg>His [*14] (rs72558189)	↓ activity (tolbutamide)[672]	unknown	unknown
Ser162Ter [*15] (rs72558190)	no protein[672]	unknown	unknown
299Thr>Ala [*16] (rs72558192)	↓ activity (tolbutamide)[672]	unknown	unknown
382Pro>Ser [*17] (C09G1362C)	↓ activity (tolbutamide)[672]	unknown	unknown
359Ile>Leu / 397Asp>Ala [*18] (rs1057910/rs72558193)	no protein[672]	unknown	unknown
454Gln>His [*19] (C09G208C)	↓ activity (tolbutamide)[672]	unknown	unknown
118Lys>Arg (Frameshift) [*25] (rs72558188)	no protein[671]	unknown	unknown
130Thr>Arg [*26] (rs5031019)	↓ activity[671]	unknown	unknown
214Gln>Leu [*28] (C09A641T)	↓ activity[671]	unknown	unknown
477Ala>Thr [*30] (C09G1429A)	↓ activity[671]	unknown	unknown
132Arg>Gln [*33] (rs72558184)	↓ activity (diclofenac)[673]	unknown	unknown

CL, clearance; NSAID, non-steroidal anti-inflammatory; IS, ischemic stroke; ICVD, ischemic cerebrovascular disease

EPHX2 gene variants

SNP (rs#)	*In vitro* Metabolism	*In vivo* metabolism	Stroke	SNP (rs#)
55Lys>Arg (rs41507953)	↑ activity (t-SO, t-DPPO)[676] ↑ activity (EET)[641] ↔ activity (EET)[677]	↔ metabolism (EpOME)[718] ↓ metabolism (EpOME)[719]	↔ death (CM)[677] ↓ death (CM) [+SEHi, +EET][677] ↔ death (NC) [±SEHi, ±EET][641]	↑ IS[733] ↔ IS[734, 735]
103Arg>Cys (rs17057255)	↔ activity (t-SO , t-DPPO, EET)[641, 676]	unknown	↔ death (CM)[677] ↓ death (CM) [+SEHi, +EET][677] ↓ death (NC) [±SEHi, ±EET][641]	↔ IS[733, 735, 736]
154Cys>Tyr (rs57699806)	↑ activity (t-SO , t-DPPO) [676] ↔ activity (EET)[641]	unknown	↔ death (CM) [677] ↔ death (NC)[641] ↓ death (CM) [+SEHi, +EET][677] ↓ death (NC) [±SEHi, ±EET][641]	unknown
287Arg>Gln (rs751141)	↔ activity (t-SO)[678] ↓ activity (t-SO, t-DPPO, EET)[641, 676 738]	↔ metabolism (PAH)[722] ↓ metabolism (EpOME)[718] ↑ cholesterol, triglycerides[721] docetaxel CL[720]	↓ death (CM)[677] ↔ death (NC)[641] ↔ death (CM) [+SEHi, +EET][677] ↓ death (NC) [±SEHi, ±EET] [641]	↑ IS[734] ↓ IS[729] ↔ IS[733, 735, 736]
Arg[402-403ins] (rs2234887)	↓ activity (t-SO)[678]	unknown	unknown	↔ IS[735, 736]
470Glu>Gly (rs68053459)	↑ activity (t-SO, t-DPPO)[676] ↔ activity (EET)[676]	unknown	unknown	↔ IS[734, 735]
g.44201A>C (rs7357432)	unknown	unknown	unknown	↑ IS[734]
g.57498G>A (rs2291635)	unknown	unknown	unknown	↑ IS[734]

EET, epoxyeicosatrienoic acid; t-SO, trans-stilbene oxide, t-DPPO, trans-diphenylpropene oxide; EpOME, epoxyoctadecenoic acid; PAH, polycystic aromatic hydrocarbon; CM, cardiomyocyte; NC, neuronal cell; SEHi, soluble epoxide hydrolase inhibitor; ±, in the presence or absence of; +, in the presence of;

1.9 HYPOTHESIS

Subarachnoid hemorrhage (SAH) due to a ruptured aneurysm is a type of stroke associated with high rates of mortality and significant cost. Survivors of SAH are often left with physical disability and cognitive impairments that can affect their quality of life and ability to return to work. Despite identification of risk factors for SAH and advances in preventative measures, the worldwide incidence of SAH has been relatively constant over the last few decades. Individuals with SAH commonly suffer multiple medical complications that develop over different time periods after the hemorrhage. The most important acute complications after SAH are increased ICP, aneurysm rebleeding, and hydrocephalus whereas development of CND and DCI are important complications that typically occur 3-14 days after the hemorrhage. Mechanisms of acute and delayed injury including oxidative stress, inflammation, cerebrovascular dysregulation, microthrombosis, and cerebral ischemia. Since CND and DCI are potentially reversible and typically develop days after hemorrhage, these conditions serve as a target for monitoring and aggressive preventive and treatment strategies. Key methods used for the diagnosis and prediction of these delayed ischemic complications include clinical grading scales, radiographic monitoring modalities, physiological indicators, biochemical markers, and genetic markers. Yet, despite the time window for therapeutic intervention, strategies to prevent these delayed ischemic complications have had limited success in part due to the lack of understanding of the underlying mechanisms involved.

Growing evidence suggests that AA metabolites of CYP enzymes, also known as CYP eicosanoids, play an important role the pathophysiology of a wide array of diseases including SAH. In humans, AA is metabolized by CYP4A11 and CYP4F2 enzymes to form 20-HETE and by CYP2C8/9 and CYP2J2 enzymes to form four EET regioisomers. In the brain, 20-HETE promotes vasoconstriction, angiogenesis, inflammation, apoptosis, and platelet aggregation while EETs promote vasodilation and angiogenesis and inhibit inflammation, apoptosis, and platelet aggregation. The cellular action of EETs is limited through its metabolism by SEH to form inactive DHETs. Recent evidence suggest that CYP eicosanoids play an important role in hypertension and vascular homeostasis and may contribute to the pathogenesis of IA and risk of

rupture. In addition, CYP eicosanoids have been shown to be key regulators of cerebrovascular tone and CBF and are implicated in the pathophysiology of cerebral injury due to ischemic and hemorrhagic stroke in preclinical studies. Human polymorphisms in genes responsible for CYP eicosanoid biosynthesis and metabolism have been associated with altered function *in vitro* and *in vivo* and have been associated with the risk for SAH and other types of stroke. However, there are few clinical studies that investigate the role of CYP eicosanoids in the pathophysiology of SAH due to the difficulty to measure these compounds in human CSF. Based on these studies, we hypothesized that polymorphisms in genes involved in CYP eicosanoid biosynthesis and metabolism will lead to increased 20-HETE or decreased EET concentrations in CSF resulting in the development of ischemic complications and unfavorable long-term functional outcomes in patients with SAH.

2.0 ANALYTICAL METHOD DEVELOPMENT

[Tricia M. Miller, Mark K. Donnelly, Elizabeth A. Crago, Dana M. Roman, Paula R. Sherwood, Michael B. Horowitz, Samuel M. Poloyac. "Rapid, simultaneous quantitation of mono and dioxygenated metabolites of arachidonic acid in human CSF and rat brain." *J Chromatogr B*; 877 (2009) 3991–4000.]

[Mark K. Donnelly, Elizabeth A. Crago, Tricia M. Miller, Paula R. Sherwood, and Samuel M. Poloyac. "Improved UPLC-MS/MS Quantitation of CYP Eicosanoids in CSF from Patients with Subarachnoid Hemorrhage". Submitted to *J Neurosci Meth.*]

2.1 INTRODUCTION

Based on the preclinical and clinical evidence establishing the importance of CYP eicosanoids in the regulation of CBF and the pathophysiology of SAH, there is a need for sensitive, selective, and reproducible methods for measuring 20-HETE, EET, and DHET metabolites in human biological fluids. Multiple methods have been previously developed for the detection and quantification of CYP Eicosanoids. Gas chromatography-mass spectrometry[739, 740], liquid chromatography (LC) - mass spectrometry (MS)[741, 742], LC-fluorescence detection[743], radioimmunoassay[744], electrophoresis[745], and enzyme immunoassays[746], have all aided in the quantitative analysis of these compounds from many different matrices. Although these assays are useful, limitations of these methods include high cost, limited sensitivity, cross-reactivity, and time-consuming analysis.[747] However, the most significant issue is the specificity for quantification of these highly similar isomeric metabolites in complex biological matrices.

While high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) continues to be in the forefront in the investigation of these compounds from matrices including cell culture[748, 749], tissue[750-752], and biological fluids[620, 753], the limitation of flow rate applied to routine HPLC systems has resulted in extended run times for most reported AA metabolite methods. A more efficient approach has been developed with the use of ultra-performance liquid chromatography (UPLC), which uses high flow rates without the limitations of increased pressure or loss of performance. This new technique, developed in 2004, offers significant advantages over traditional HPLC methods, providing greater separation efficiency, improved sensitivity**,** and shortened run times.[754, 755] UPLC systems, in conjunction with appropriate mobile phases and analytical columns, also offer benefits such as lower sample injection volumes and improved peak resolution.[754, 755] As a result, our lab investigated the utility of ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) to quantify these highly similar isomeric metabolites.

In 2009, our lab developed and validated a rapid, sensitive, and selective UPLC-MS/MS quantitation method to simultaneously measure CYP eicosanoids in CSF from SAH patients, rat cortical tissue, and rat cortical microsomal incubates. Work performed by others in our lab reported the validation of linear calibration curves ranging from 0.208 to 33.3 ng/ml. The inter-

day and intra-day variance was less than 15% at most concentrations with extraction efficiency greater than 73%. My contribution to the method development and validation was to investigate the effects of the matrix on the reproducibility and reliability of the data as recommended by the Food and Drug Administration (FDA) Quantitative Bioanalytical Methods Validation Report and other validation guidance documents.[756, 757] Development of this method is necessary for future evaluation of these metabolites as potential quantitative biomarkers in larger clinical trials.

Using our validated UPLC-MS/MS quantitation method, our lab found a low percentage of samples with detectable CYP eicosanoids in 1ml of fresh CSF samples collected directly from external ventricular drains on patients with SAH. This limited our ability to determine temporal concentration profiles of these compounds in CSF from SAH patients. Based on these quantitative limitations, we set out to improve the ability to measure multiple CYP eicosanoids in human CSF using UPLC-MS/MS and determine temporal concentration profiles of these compounds in the CSF of patients with SAH. Specifically, we investigated methods to further concentrate the CSF during sample processing in an effort to improve UPLC-MS/MS peak response and decrease our lower limit of quantitation (LLOQ). In an effort to utilize the larger volumes of CSF (>200ml) available in the drainage bags attached to the ventricular drains, we determined the recovery of these metabolites from the drainage bags and the room temperature stability of these CYP eicosanoids over the 12 hour collection period. In addition, we performed modifications to our solid phase extraction (SPE) method to further concentrate the CSF samples.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Stock standards of 20-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE), (±)14,15-epoxy-5Z, 8Z, 11Z-eicosatrienoic acid (14,15-EET), (±)11,12-epoxy-5Z, 8Z, 14Z-eicosatrienoic acid (11,12-EET), (±)8,9-epoxy-5Z, 11Z, 14Z-eicosatrienoic acid (8,9-EET), (±)14,15-dihydroxy-5Z, 8Z, 11Z, 17Z-eicosatrienoic acid (14,15-DHET), (±)11,12-dihydroxy-5Z, 8Z, 14Z-

eicosatrienoic acid (11,12-DHET), (±)8,9-dihydroxy-5Z, 8Z, 14Z-eicosatrienoic acid (8,9-DHET), (±)5,6-dihydroxy-8Z, 11Z, and 14Z-eicosatrienoic acid (5,6-DHET), 20-hydroxy-5Z, 8Z, 11Z, and 14Z-eicosatetraenoic acid-*d6* (20-HETE-*d6*) were purchased from Cayman Chemical Company (Ann Arbor, MI). In some studies, EETs are also referred to as EpETrE and DHETs are referred to as DiHETrE or DiHETEs. High purity organic solvents were purchased from VWR (West Chester, PA) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Artificial cerebrospinal fluid (aCSF) was purchased from Tocris Bioscience (Ellisville, MO).

2.2.2 Patient population and CSF sample collection

All protocols have been approved by the Institutional Review Board at the University of Pittsburgh and informed consent was obtained from the human subjects or their representatives.

This study included patients with SAH verified by cerebral angiography within 5 days of injury. Also, Fisher grade ≥ 2 were chosen as inclusion criteria to increase the likelihood that subjects will have degree of hemorrhage sufficient for elevated biomarker concentrations. CSF samples used in this study were collected from 38 patients at approximately 12 hour intervals throughout a 14 day period following SAH. In this study, samples were categorized as either fresh CSF or bag CSF based on the method and location of CSF sampling. Fresh CSF samples were collected, stored, and prepared as previously described.[643] Briefly, fresh CSF samples of 0.11 – 1.0 ml were withdrawn directly from the CSF drainage line using a sterile needle and syringe, aliquoted into preservative-free cryostat vials, and immediately stored at -80ºC until analysis. Prior to extraction, fresh CSF samples were diluted using 0.12 M potassium phosphate buffer (pH = 7.4) containing 5 mM magnesium chloride and 0.113 mM butylated hydroxytoluene as an antioxidant to a final sample volume of 2 ml. Bag CSF samples were withdrawn from CSF drainage bags using a needle and syringe. Sample volumes of 2.0-5.0 ml of bag CSF were collected, aliquoted into preservative-free cryostat vials, and immediately stored at -80ºC until analysis.

2.2.3 Preparation of calibration standards and quality control samples

For the validation studies, a stock solution of metabolites was prepared by combining 10µg/ml of each metabolite in methyl acetate. This solution was further diluted with 80:20 methanol: deionized water (MeOH:dH_20) to achieve a series of working solutions at 1µg/ml, 100ng/ml, and 25ng/ml concentrations. A solution of 20-HETE-*d6*, used as the internal standard (IS), was prepared in 80:20 MeOH:dH_20 at a concentration of 1µg/ml. Calibration standards and quality control (QC) samples were prepared from separate stock dilutions by spiking appropriate amounts of the working solutions into buffer. Calibrants were prepared at 0.208, 0.417, 0.833, 1.25, 1.67, 4.17, 8.33, 12.5, 16.67, and 33.33 ng/ml (12.5, 25, 50, 75, 100, 250, 500, 750, 1000, 2000 pg on column, respectively). QC's were prepared at 1.50, 7.50, and 20.83 ng/ml (90, 450, 1250 pg on column, respectively).

For the modified method, stock solutions of CYP eicosanoids were combined and diluted with 80:20 MeOH:dH_20 to achieve a series of working solutions at 0.30, 0.04, and 0.005 ng/ml concentrations. 20-HETE-*d6* was used as IS and was prepared in 80:20 MeOH:dH_20 at a concentration of 0.25 ng/ml. Calibration standards and QCs were prepared from separate stock dilutions by spiking appropriate amounts of the working solutions and IS into buffer. These calibrants and QCs were spiked with IS to a final concentration of 12.5 ng/ml. For quantitation of metabolites using our modified method, calibrants were prepared at 0.014, 0.028, 0.056, 0.11, 0.17, 0.22, 0.56, 1.11, 1.67, 2.22, 4.44 ng/ml (12.5, 25, 50, 75, 100, 250, 500, 750, 1000, 2000 pg on column, respectively) and QCs at 0.20, 1.00, 2.78 ng/ml (90, 450, 1250 pg on column, respectively). These calibrants and QCs were spiked with IS to a final concentration of 1.67 ng/ml. The standard curves for UPLC-MS/MS analysis were constructed the same day as the sample analysis.

2.2.4 Solid phase extraction

The CYP eicosanoid concentrations in the CSF samples, as well as in the calibrants and quality controls, were determined using a solid phase extraction (SPE) procedure. Fresh CSF samples were processed as previously described.[643] Briefly, fresh CSF samples were spiked with 20-HETE-*d6* as the IS to a final concentration of 6.25 ng/ml (750 pg on column). Fresh CSF samples of up to 1ml were loaded onto 1cc Oasis HLB SPE cartridges (30mg sorbent, 30μm particle size) (Waters, Milford, MA) that were conditioned and equilibrated with 1ml of MeOH and 1ml of dH_20, respectively. Columns were washed with three 1 ml volumes of 5% MeOH and were eluted with 100% MeOH. Extracts were dried under nitrogen gas at 37ºC and reconstituted in 125 μl of 80:20 MeOH:dH_20. Bag CSF samples were processed using the method described above with minor modifications. Modifications included spiking the samples with IS to a final concentration of 1.67 ng/ml (750 pg on column), loading 2.0-3.0 ml sample volumes onto 3 cc Oasis HLB SPE cartridges (60mg sorbent, 30μm particle size) (Waters, Milford, MA) that were conditioned and equilibrated with 2 ml of MeOH and 2 ml of dH_20, respectively, washing the column with 3 ml of 5% MeOH, eluting the column with 3 ml of 100% MeOH, and reconstituting the samples in 50 μl of 80:20 MeOH:dH_20.

2.2.5 Chromatographic and mass spectrometric conditions

Liquid chromatography was performed using an Acquity ultra performance LC autosampler (Waters, Milford, MA). Separation of analytes was conducted on a UPLC BEH C18, 1.7μm (2.1 x 100mm) reversed-phase column (Waters, Milford, MA) protected by a guard column (2.1 x 5mm; Waters, Milford, MA,) of the same packing material. Column temperature was maintained at 55º C. Mobile phases, delivered at a flow rate of 0.5ml/min, consisted of 0.005% acetic acid, 5% acetonitrile in deionized water (A) and 0.005% acetic acid in acetonitrile (B) at an initial mixture of 65:35 A and B, respectively. Mobile phase B increased from 35% to 70% in a linear gradient over 4 minutes, and again increased to 95% over 0.5 minutes where it remained for 0.3 minutes. This was followed by a linear return to initial conditions over 0.1 minutes with a 1.5 minute pre-equilibration period prior to the next sample run. Total run time per sample was 6.4 minutes and all injection volumes were 7.5μl.

Mass spectrometric analysis of analyte formation was performed using a TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA) triple quadrupole mass spectrometer coupled with heated electrospray ionization (HESI) operated in negative selective reaction monitoring (SRM) mode with unit resolutions at both Q1 and Q3 set at 0.70 full width at half maximum. Quantitation by SRM analysis on CYP eicosanoids was performed by monitoring their *m/z* transitions. The SRM conditions for these molecules and their retention times are shown in Table 2-1: SRM conditions for quantitation of CYP eicosanoids by UPLC-MS/MS. Parameters were optimized to obtain the highest $[M-H]^-$ ion abundance and were as follows: capillary temperature, 400° C, spray voltage, 3000 kV, and a source collision-induced dissociation set at 0V. Sheath gas, auxiliary gas, and ion sweep gas pressures were set at 65, 55, and 3, respectively. Scan time was set at 0.01s and collision gas pressure was set at 1.3 mTorr. Analytical data was acquired and analyzed using Xcaliber software version 2.0.6 (ThermoFinnigan, San Jose, CA).

Table 2-1: SRM conditions for quantitation of CYP eicosanoids by UPLC-MS/MS

CYP Eicosanoid	Precursor→ Product (m/z)	Collision Energy	Retention Time (min)
20-HETE	319→ 245,289	18	3.69
14,15-EET	319→ 219	14	4.61
11,12-EET	319→ 167	15	4.77
8,9-EET	319→ 127	14	4.81
14,15-DHET	337→ 207	18	3.22
11,12-DHET	337→ 167	19	3.40
8,9-DHET	337→ 127	25	3.57
5,6-DHET	337→ 145	19	3.79
20-HETE-*d6*	319→ 251,295	18	3.69

Selective reaction monitoring (SRM) conditions for UPLC-MS/MS operated in negative heated electrospray ionization (HESI) mode.

2.2.6 Metabolite stability and recovery determination

Stability of CYP eicosanoids at room temperature and recovery from Medtronic Exacta drainage bags (Medtronic Neurosurgery, Goleta, CA) was measured over 12 hours in order to determine the appropriateness of evaluating CSF collection bag samples for these metabolites. The duration of

the experiment was chosen based on the time period between collection of CSF samples. For the evaluation of temperature stability, pooled bag CSF was aliquotted into silanized test tubes. One set of samples was spiked with metabolites at a concentration of 33.33 ng/ml while the other set of vials included control CSF with no metabolites for subtraction of basal CSF concentrations. All samples were kept at room temperature for up to 12 hours. 500µl aliquots were collected at baseline (time 0) and at 1, 6 and 12 hours after start of incubation (n = 3 per time point). Sample aliquots were immediately frozen at -80°C after room temperature incubation.

For evaluation of recovery from collection bags, CSF was pooled from SAH patients and aliquots of 18ml were added into 1L Medtronic Exacta drainage bags constructed of polyvinylchloride (PVC). One bag was spiked with 33.33 ng/ml of metabolites while the other bag included control CSF with no metabolite addition. 500µl aliquots were collected at baseline (time 0) and at 1, 6 and 12 hours after start of incubation (n = 4 per time point). Sample aliquots were immediately frozen at -80°C after room temperature incubation.

At the time of sample processing, frozen samples from the above two experiments were thawed and processed using SPE and concentrations of metabolites were determined by UPLC-MS/MS as described previously. Concentration of metabolites measured in the control CSF was subtracted from the concentration of metabolites measured in the spiked CSF. Results were expressed as a percentage of initial concentration.

It is noteworthy that a direct measurement of 5,6-EET has not been attempted in most studies because, unlike the other EET regioisomers, 5,6-EET has been reported to be chemically unstable in physiological buffer solution and alkaline aqueous solution.[745, 758, 759] Also, 5,6-EET is hydrolyzed to 5,6-DHET and its lactone under neutral and acidic conditions.[745, 758] Therefore, a measurement of 5,6-EET in human CSF was excluded from our analysis.

2.2.7 Evaluation of matrix effects

Matrix effects were evaluated using 2ml volume of the same phosphate buffer used for processing human CSF samples, rat cortical tissue extracts, and rat brain microsomal incubations and also artificial cerebrospinal fluid (aCSF) spiked with 0.113mM BHT. Both matrices were extracted via

SPE using the method described above and the eluate was spiked with low and high concentrations of CYP eicosanoids at 1.5 ng/ml and 20.83 ng/ml, respectively, and 20-HETE-*d6* internal standard at 12.5 ng/ml. Neat samples containing the same concentrations of CYP eicosanoids and 20-HETE-*d6* internal standard were prepared in 80:20 MeOH:dH_20. Samples were analyzed using UPLC-MS/MS as described above. The internal standard normalized matrix factor (IS-normalized MF) for each sample was calculated based on the area ratio (analyte/internal standard) of the post-extraction spiked samples to the neat samples as described by Viswanathan et al.[757] IS-normalized MF results are expressed as average ± standard deviation (SD) with coefficient of variation (CV) (n=6). Values less than 1.00 indicate ion suppression and values greater than 1.00 indicate ion enhancement.[756]

2.2.8 Comparison of published and modified quantitation methods

We investigated the effect of using larger load volumes and SPE cartridges on the recovery of CYP eicosanoids spiked in buffer. The effect of SPE column size on the recovery of CYP eicosanoids was compared by spiking the same amount of analyte and IS (0.83 ng) in 1ml and 3 ml phosphate buffer (pH=7.4), processing the samples using 1cc and 3cc SPE cartridges, respectively, and reconstituting the samples in 125 µl of 80:20 MeOH:dH_20. Next, we investigated the effect of using various reconstitution volumes on the recovery of CYP eicosanoids spiked in buffer. The effect of the reconstitution volume on the recovery of CYP eicosanoids was determined by spiking analyte and IS to 0.276 ng/ml in 3 ml of phosphate buffer, processing samples using 3 cc SPE columns, and reconstituting the samples in 25, 75, 125 and 200 µl of 80:20 MeOH:dH_20. CYP eicosanoids were quantitated using UPLC-MS/MS and recovery was determined using the mean area ratio of analyte to IS.

The combined effect of load volume and reconstitution volume on the peak response of the assay to measure CYP eicosanoids was determined by spiking the analyte and IS to 0.276 ng/ml in phosphate buffer, processing sample load volumes of 1 ml using 1 cc SPE columns and sample load volumes of 3 ml using 3 cc SPE columns, and reconstituting the samples in 50 µl and 125 µl

of 80:20 MeOH:dH_20. CYP eicosanoids were quantitated using UPLC-MS/MS and peak response was compared using the mean area of analytes and IS.

The peak response to measure CYP eicosanoids in bag CSF using our published method[643] was compared to our modified method. Using our published method, the bag CSF samples were processed using a 1 ml sample load volume with 1 cc SPE cartridge and reconstitution volume of 125 µl. Using our modified method, the bag CSF samples were processed using 3ml sample load volume with 3 cc SPE cartridge and reconstitution volume of 50 µl. CYP eicosanoid peak responses were measured using UPLC-MS/MS. The UPLC-MS/MS detector responses were normalized to the maximum value and chromatograms were displayed. In addition, 20-HETE concentrations were measured in fresh and bag CSF from patients with SAH using our published and modified quantitation methods while EET and DHET concentrations in bag CSF were quantified using our modified quantitation method.

2.2.9 Statistical analysis

Statistical analysis was completed using GraphPad Prism software, version 4.03 (GraphPad Software, La Jolla, CA). In the metabolite stability and recovery studies, the percentage of initial concentration at each time point was compared using repeated measures one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test for each metabolite. In the matrix effects studies, IS-normalized MF values were compared using unpaired t-test (2 tailed). In the study investigating the effect of SPE column size on recovery on CYP eicosanoids, data were normalized to the recovery observed using 1cc SPE columns and compared using unpaired t-test (2-tailed). In the study investigating the effect of reconstitution volume on recovery of CYP eicosanoids, data were normalized to the recovery observed using 125µl reconstitution volume and compared using one-way ANOVA with Dunnett *post-hoc* test. For all statistical tests, statistical significance was determined at *$p<0.05$ and **$p<0.0001$

2.3 RESULTS

2.3.1 Development of UPLC-MS/MS method

The chemical structures of CYP eicosanoids and selective reaction monitoring (SRM) conditions for quantitation using UPLC-MS/MS is shown in Figure 2-1: Chemical structures of CYP eicosanoids and internal standard and Table 2-1: SRM conditions for quantitation of CYP eicosanoids by UPLC-MS/MS, respectively. 12-HETE and 15-HETE are AA metabolites that are formed from COX and CYP enzymes. These metabolites are referenced in Figure 2-1: Chemical structures of CYP eicosanoids and internal standard and Figure 2-3: CYP eicosanoid levels in fresh CSF, but will not be discussed in this dissertation.

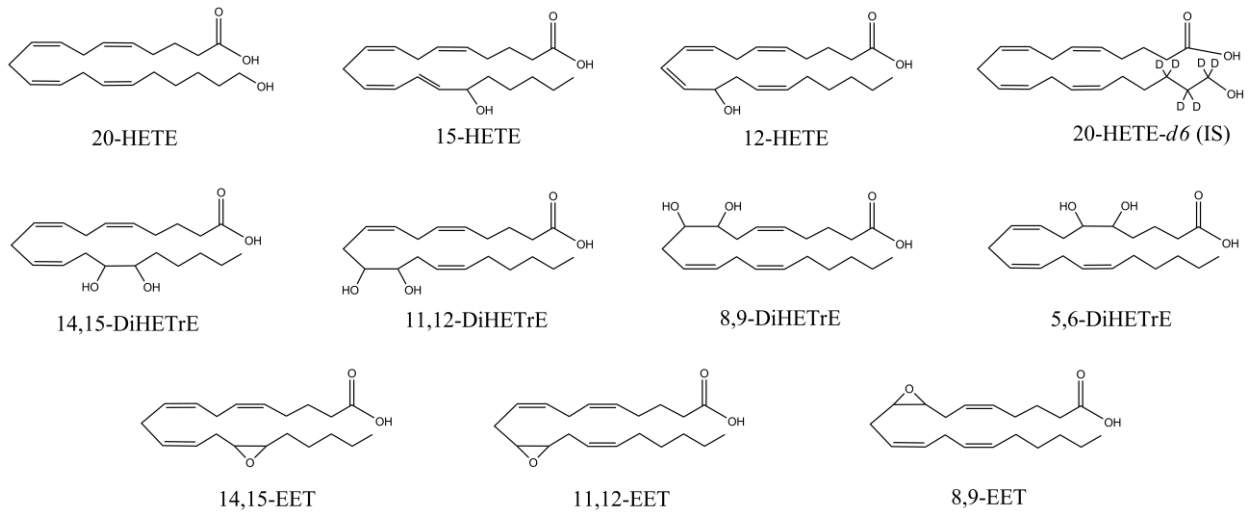


Figure 2-1: Chemical structures of CYP eicosanoids and internal standard

The representative chromatograms of a standard calibrant at 0.208 ng/ml (25pg on column) of CYP eicosanoids are depicted in Figure 2-2: Chromatographic profiles for CYP eicosanoids. The elution sequence was identified as 14,15-DHET, 11,12-DHET, 8,9-DHET, 20-HETE, 5,6-DHET, 14,15-EET, 11,12-EET, and 8,9-EET as determined by comparison to injections of individual compounds and product fragments.

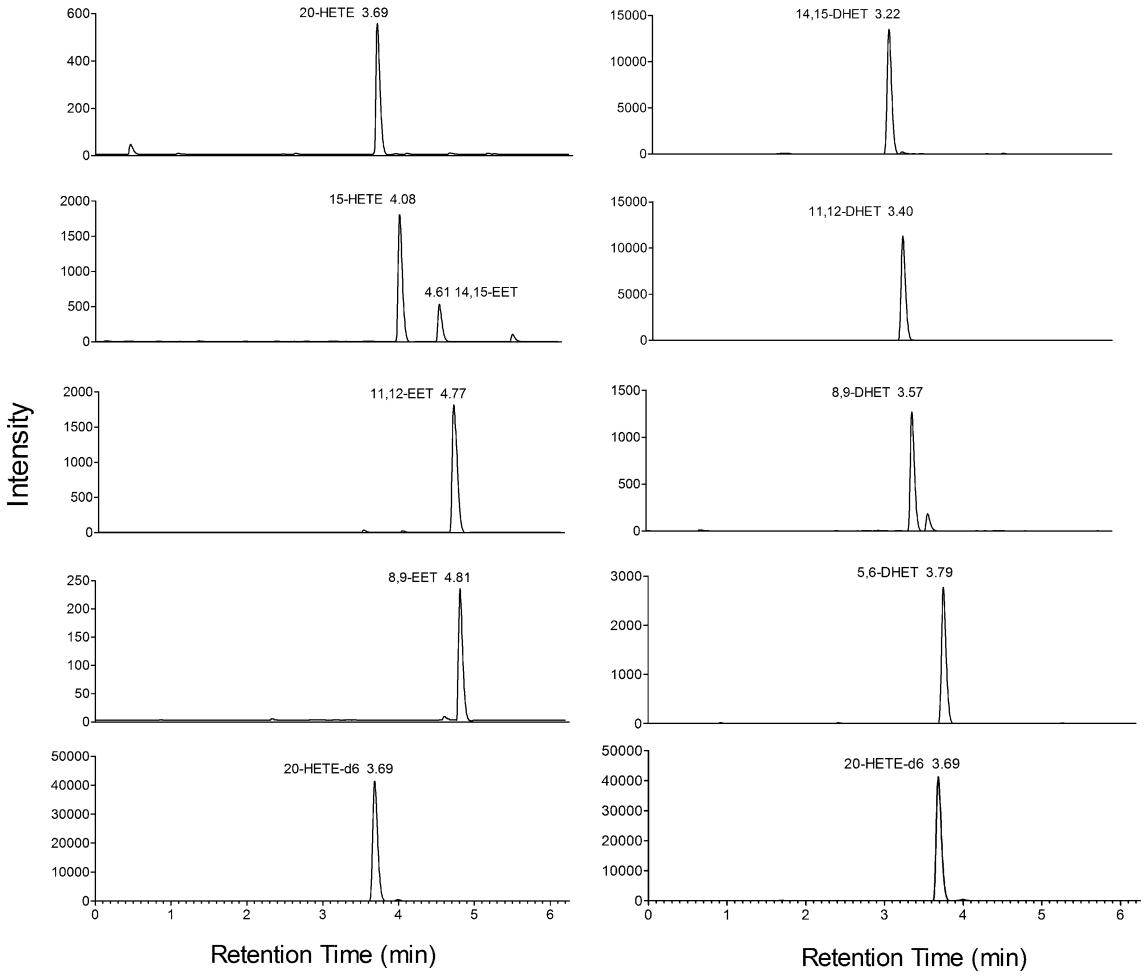


Figure 2-2: Chromatographic profiles for CYP eicosanoids

Chromatographic profiles, corresponding to 25 pg on column, depicting the separation of CYP eicosanoids using a UPLC tandem MS/MS triple quadrupole mass spectrometer. Resolution of the extracted standard mixture was performed on a reversed-phase Acquity BEH (Waters, Milford, MA) C-18 column (2.1mm×100mm; 1.7mm particle size). Metabolites were eluted at a flow rate of 0.5 ml/min over 6.4 min with a gradient from 35% acetonitrile containing 0.005% acetic acid to 95% acetonitrile containing 0.005% acetic acid.

2.3.2 Linearity, accuracy, and precision

Calibration standards over a range of 0.208-33.3 ng/ml were used to construct the curves for all metabolites. The weighting factor 1/Y typically provided the best fit of the plot as determined by visual inspection, correlation coefficient, and analysis of the residuals. Over 93% of the calibration standards fell within 15% deviation of back-calculated amounts from nominal spiked amounts for all levels. The remaining standards fell within 20% RSD and the correlation coefficients (R^2) were >0.99 for each metabolite.

The intra- and inter-day accuracy and precision for the assay were evaluated at three levels: 1.50, 7.50, and 20.83 ng/ml (90, 450, 1250 pg on column, respectively), using the QC samples within the three validation runs. Ten replicates at each concentration within a single day of validation were used to determine the intraday reproducibility. Inter-day reproducibility was determined over three separate days using an n = 5 at these concentrations. Calculated values of the QCs were generated using the equation of linear regression obtained from the calibration curves run within the same sequence. Results are shown in Table 2-2: Quantitation method accuracy and precision. The %RSD for all analytes fell within 15%, indicating favorable reproducibility of the assay. The LLOQ was determined to be 0.208 ng/ml for all metabolites evaluated.

Table 2-2: Quantitation method accuracy and precision

	Spiked Amt (pg on column)	Intra-assay concentration (mean ± std dev) (pg on column)	RE%	RSD%	Inter-assay concentration (mean ± std dev) (pg on column)	RE%	RSD%
20-HETE	90	86.94 ± 12.5	-3.40	14.38	82.81 ± 8.55	-7.99	10.32
	450	479.81 ± 17.76	6.62	3.70	466.49 ± 18.54	3.67	3.98
	1250	1303.41 ± 39.31	4.27	3.02	1311.37 ± 55.07	4.91	4.20
14,15-EET	90	90.90 ± 5.52	1.00	6.08	90.91 ± 5.10	1.01	5.61
	450	464.51 ± 14.59	3.23	3.14	454.51 ± 21.11	1.00	4.64
	1250	1261.26 ± 53.60	0.90	4.25	1258.24 ± 60.54	0.66	4.81
11,12-EET	90	92.07 ± 8.07	2.31	8.77	93.02 ± 6.02	3.35	6.69
	450	470.24 ± 19.81	4.50	4.21	464.44 ± 29.67	3.26	6.39
	1250	1335.41 ± 73.77	6.83	5.52	1271.88 ± 75.94	1.75	5.97
8,9-EET	90	91.54 ± 8.11	1.72	8.86	83.85 ± 8.52	-6.84	10.16
	450	468.64 ± 24.40	4.14	5.21	456.38 ± 39.35	1.42	8.62
	1250	1326.40 ± 65.55	6.11	4.94	1299.05 ± 72.41	3.92	5.57
14,15-DHET	90	91.74 ± 4.23	1.93	4.61	92.17 ± 4.97	2.42	5.39
	450	483.59 ± 18.45	7.46	3.82	472.74 ± 18.05	5.06	3.82
	1250	1281.76 ± 43.98	2.54	3.43	1278.34 ± 55.41	2.27	4.33
11,12-DHET	90	92.03 ± 6.02	2.25	6.54	91.97 ± 4.67	2.20	5.07
	450	477.99 ± 16.92	6.22	3.54	470.92 ± 16.78	4.66	3.56
	1250	1277.72 ± 34.56	2.22	2.7	1271.23 ± 35.35	1.70	2.78
8,9-DHET	90	90.50 ± 6.89	0.56	7.62	90.61 ± 6.14	0.68	6.78
	450	476.17 ± 18.95	5.82	3.98	470.50 ± 17.95	4.55	3.82
	1250	1274.85 ± 34.60	1.99	2.71	1258.13 ± 35.61	0.65	2.83
5,6-DHET	90	89.69 ± 4.99	-0.34	5.56	91.79 ± 2.74	1.98	2.98
	450	476.89 ± 19.79	5.98	4.15	463.42 ± 14.67	2.98	3.17
	1250	1256.58 ± 40.26	0.53	3.20	1268.37 ± 44.23	1.47	3.49

Accuracy and precision of the assay, represented by quality controls, for CYP eicosanoids extracted in buffer. Intra- and inter-assay relative standard deviation (RSD) ranged from 2.70 to 14.38% and 2.78 to 10.32%, respectively. Intra- and inter-assay relative error (RE) ranged from -3.40 to 7.46% and -7.99% to 5.06% and 14.38%, respectively.

2.3.3 Recovery of CYP eicosanoids after extraction

The recovery efficiencies for all metabolites were determined at two different levels, at 1.50 ng/ml and 20.83 ng/ml (90 and 1250 pg on column) as shown in Table 2-3: Recovery efficiencies of CYP eicosanoids after extraction from buffer. The recovery ranged from 73% - 94%, with the EETs

having the lowest recovery, however, all demonstrated favorable reproducibility with a %RSD below 15%.

Table 2-3: Recovery efficiencies of CYP eicosanoids after extraction from buffer

CYP Eicosaniod	Spiked Amnt (pg on column)	Extraction Efficiency %	RSD%
20-HETE	90	83.76	14.86
	1250	85.97	5.12
14,15-EET	90	89.35	12.18
	1250	87.12	6.78
11,12-EET	90	76.55	11.27
	1250	83.80	5.40
8,9-EET	90	72.73	16.75
	1250	80.51	5.51
14,15-DHET	90	89.03	8.34
	1250	92.84	4.66
11,12-DHET	90	90.98	6.04
	1250	94.11	4.27
8,9-DHET	90	87.59	5.72
	1250	92.19	4.81
5,6-DHET	90	86.62	9.18
	1250	89.40	4.67

Recovery efficiencies and relative standard deviation (RSD) of CYP eicosanoids in buffer after solid phase extraction. Recovery efficiencies were calculated from the peak area ratio of the extracted to the unextracted samples.

2.3.4 Analysis of CSF samples from SAH patients

The method was applied to establish CYP eicosanoid concentrations in fresh human CSF samples from eight subarachnoid hemorrhage patients. Samples taken at different time points from each patient were analyzed and concentrations were determined by using the equation of linear regression obtained from the calibration curves. Because these compounds are found endogenously in human CSF, it was not possible to conduct this validation using blank samples of this biological matrix.

Peaks of 8,9-DHET and 20-HETE were found in quantitative amounts in all but one patient. Peaks of 14,15-DHET, 11,12-DHET, and 5,6-DHET were found in amounts above the limit of detection (LOD) but below LOQ. No detectible amounts of 14,15-EET, 11,12-EET, or 8,9-EET were seen in these samples. All peaks were verified by comparing their *m/z* fragment and retention time with that of an authentic standard. The peak concentrations over the 14 day collection period are reported in Figure 2-3: CYP eicosanoid levels in fresh CSF. These results indicate that multiple CYP eicosanoids in CSF from SAH patients can be quantified using this method and the metabolite with the highest concentration measured was 20-HETE.

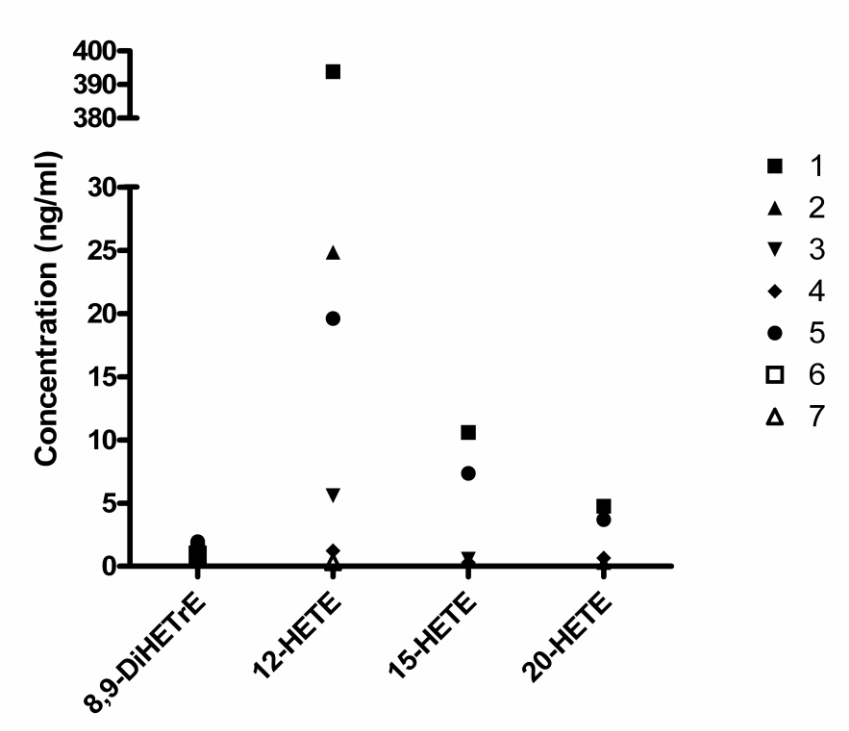


Figure 2-3: CYP eicosanoid levels in fresh CSF

Concentrations of arachidonic acid metabolites detected in fresh human CSF. Concentrations given are the highest concentrations seen in that patient over 14 days. Concentrations from seven patients ranged from 0.293 to 24.9 ng/ml for detected metabolites.

2.3.5 Analysis of matrix effects

The effects of the phosphate buffer and aCSF matrix on the reproducibility of the assay to measure low and high concentrations of metabolites were determined. Table 2-4: Evaluation of matrix effects shows the coefficient of variation (CV) of the peak area of each metabolite and IS and area ratio (analyte/IS) of the post-extraction spike in buffer and aCSF along with the CV of neat samples prepared in 80:20 MeOH:dH_20 (n=6). All of the CV values in Table 2-4: Evaluation of matrix effects were below 15%, which demonstrated favorable precision and reliability of the assay. Most of the CV values (80%) for the area and area ratio of high metabolite concentrations were lower than the CV values for the low metabolite concentration in both aCSF and buffer. This indicates less variability in the assay at high concentrations of metabolites. Also, the CV of the area ratio was larger than the CV of the area of the metabolites and internal standard for most samples (86%) spiked post-extraction. This indicates that there was not a compensating effect of the internal standard on the matrix effects as described by Matusweski et al.[756]

Table 2-4: Evaluation of matrix effects

	Precision (CV %)											
	Low Concentration (1.50 ng/ml)						High Concentration (20.83 ng/ml)					
	Neat		Buffer		aCSF		Neat		Buffer		aCSF	
Metabolite	Area	AR	Area	AR	Area	AR	Area	AR	Area	AR	Area	AR
20-HETE	9.5	10.7	4.3	4.9	6.4	7.3	1.9	2.8	2.8	2.8	2.2	4.5
14,15-EET	6.8	5.5	10.0	10.2	7.9	7.5	4.2	4.2	4.5	5.4	4.6	3.8
11,12-EET	4.0	5.7	4.6	5.1	7.6	5.0	2.6	3.4	4.4	3.9	2.1	3.6
8,9-EET	10.7	8.5	5.9	5.5	14.9	14.8	6.0	6.2	3.4	4.4	4.3	5.2
14,15-DHET	3.7	4.6	1.6	2.1	3.9	3.7	4.6	5.0	4.5	4.5	2.1	4.2
11,12-DHET	3.7	5.5	3.9	4.4	5.4	5.3	5.8	5.2	4.4	5.3	2.9	5.1
8,9-DHET	12.1	9.6	10.4	10.4	11.8	10.3	6.1	5.8	6.9	7.6	4.1	6.3
5,6-DHET	5.2	5.3	5.4	5.7	4.6	4.0	9.2	9.0	5.5	5.7	2.9	5.9
20-HETE-d6	4.0	-	2.4	-	1.5	-	1.9	-	1.9	-	3.7	-

Assessment of matrix effects of the phosphate buffer and artificial cerebrospinal fluid (aCSF) on the reproducibility of the assay to measure low and high concentrations of metabolites. Data is represented as coefficient of variation (CV %) of the peak area of each metabolite and IS and area ratio (analyte/IS) of the post-extraction spike in phosphate buffer and aCSF along with the CV of neat samples prepared in 80:20 MeOH:dH_20 water (n=6). The samples were spiked with low and high concentrations of CYP eicosanoids at 1.5 ng/ml and 20.83 ng/ml, respectively. Results indicate favorable reproducibility and reliability of the assay to measure low and high concentrations of metabolites

in both phosphate buffer and aCSF.

Table 2-5: Evaluation of internal-standard normalized matrix factor shows the average IS-normalized MF values of the samples ± standard deviation (SD) and the CV (n=6). The average IS-normalized MF values ranged from 0.92-1.13 with most of the values (68%) within 5% of 1.00. An IS-normalized MF (or absolute MF) of 1 signifies no matrix effects. A MF value less than 1 signifies ion suppression while a value greater than 1 signifies ion enhancement or analyte loss in the absence of matrix. An IS-normalized MF (or absolute MF) of 1 is not necessary for a reliable bioanalytical assay. However, a highly variable MF would indicate lack of reproducibility in the assay. All of the CV of the IS-normalized MF values were below 15%, which demonstrates minimal effects of the phosphate buffer and aCSF matrix on the precision and reliability of the assay at low and high metabolite concentrations. Also, the matrix effects, measured by IS-normalized MF values, were not significantly different in aCSF as compared to buffer for all metabolites.

Table 2-5: Evaluation of internal-standard normalized matrix factor

	Internal Standard-Normalized Matrix Factor							
	Low Concentration (1.5 ng/ml)				High Concentration (20.83 ng/ml)			
	Buffer		aCSF		Buffer		aCSF	
Metabolite	Value	CV	Value	CV	Value	CV	Value	CV
20-HETE	0.99 ± 0.08	8.6%	0.99 ± 0.14	13.8%	0.98 ± 0.03	3.3%	0.97 ± 0.04	4.6%
14,15-EET	1.13 ± 0.13	11.2%	1.07 ± 0.08	7.8%	1.06 ± 0.07	6.6%	1.09 ± 0.06	5.6%
11,12-EET	1.02 ± 0.09	8.6%	0.92 ± 0.10	11.2%	0.98 ± 0.06	6.0%	0.98 ± 0.04	4.4%
8,9-EET	1.07 ± 0.07	6.6%	0.97 ± 0.14	14.8%	1.01 ± 0.10	10.2%	0.91 ± 0.08	8.8%
14,15-DHET	1.05 ± 0.06	5.5%	1.07 ± 0.08	7.2%	0.98 ± 0.09	9.3%	1.07 ± 0.10	9.1%
11,12-DHET	1.00 ± 0.07	6.9%	0.97 ± 0.06	5.7%	0.98 ± 0.10	10.1%	1.08 ± 0.10	9.3%
8,9-DHET	0.97 ± 0.12	12.2%	0.92 ± 0.10	10.6%	1.00 ± 0.10	10.5%	1.10 ± 0.10	9.1%
5,6-DHET	0.94 ± 0.07	7.4%	1.01 ± 0.07	6.7%	0.99± 0.10	9.9%	1.07 ± 0.12	11.7%

Comparison of the internal standard-normalized matrix factor (IS-normalized MF) of the phosphate buffer and artificial cerebrospinal fluid (aCSF) at low and high concentrations of metabolites. Data is shown as the average IS-normalized MF values ± standard deviation (SD) and the CV for phosphate buffer and aCSF spiked with low and high concentrations of CYP eicosanoids at 1.5 ng/ml and 20.83 ng/ml, respectively (n=6). IS-normalized matrix factor values in phosphate buffer were compared to aCSF. Significant differences are denoted by *$p<0.05$. Results indicate minimal effects of the phosphate buffer and aCSF matrix on the reproducibility and reliability of the assay and no difference in matrix effects of phosphate buffer and aCSF at low and high metabolite concentrations.

2.3.6 Stability and recovery of CYP eicosanoids from CSF drainage bags

Table 2-6: Room temperature stability of CYP eicosanoids demonstrates that CYP eicosanoids were not significantly altered from baseline for up to 12 hours of room temperature incubation in silanized borosilicate glass. Whereas, Table 2-7: Recovery from CSF drainage bags shows some of these metabolites were significantly reduced upon room temperature incubation in PVC bags. Specifically, the recovery of 8,9-EET, 11,12-EET and 14,15-EET was significantly altered by contact with the PVC collection bags over 12 hours ($p<0.05$). Only 8,9-EET, 11,12-EET and 14,15-EET showed a significantly lower recovery as early as 1 hour after addition to the PVC bag and a highly significant recovery loss at 6 and 12 hours after addition to the PVC bag ($p<0.01$). 20-HETE and DHET metabolites were not significantly altered at any of the time points evaluated. These results suggest that the use of CSF collection bag samples for assessment of EET metabolites will be substantially confounded by recovery loss to the PVC material in the tubing and collection bags.

Table 2-6: Room temperature stability of CYP eicosanoids

	t=1hr	t=6hr	t=12hr
Metabolite	**% ± s.d.**	**% ± s.d.**	**% ± s.d.**
20-HETE	97.5 ± 4.0%	93.1 ± 7.8%	95.1 ± 15.5%
14,15-EET	94.5 ± 2.3%	86.1 ± 7.0%	82.4 ± 10.8%
11,12-EET	96.6 ± 2.0%	87.6 ± 4.3%	87.0 ± 10.0%
8,9-EET	100.2 ± 10.3%	93.4 ± 13.7%	88.2 ± 6.4%
14,15-DHET	94.3 ± 7.8%	102.4 ± 3.1%	109.6 ± 11.3%
11,12-DHET	95.9 ± 2.7%	96.7 ± 2.6%	102.3 ± 12.5%
8,9-DHET	95.8 ± 1.9%	93.9 ± 7.6%	94.6 ± 12.6%
5,6-DHET	98.0 ± 5.2%	93.0 ± 4.3%	98.5 ± 10.5%

Comparison of CYP eicosanoid levels during 12-hour incubation in silanized tubes. Data at each time point is presented as mean percentage of initial concentration ± s.d. (n=3). The percentage of initial concentration at each

time point was compared using repeated measures one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. Significant difference was established at *p<0.05. Results indicate that incubation of CSF in silanized test tubes over 12 hours does not significantly affect the measurable amount of the CYP eicosanoids.

Table 2-7: Recovery from CSF drainage bags

	t=1hr	t=6hr	t=12hr
Metabolite	**% ± s.d.**	**% ± s.d.**	**% ± s.d.**
20-HETE	95.3 ± 9.4%	94.8 ± 8.4%	91.3 ± 9.2%
14,15-EET	70.6 ± 20.6%*	71.1 ± 19.4%*	46.1 ± 10.1%**
11,12-EET	60.6 ± 20.1%*	65.9 ± 25.0%*	40.9 ± 19.3%**
8,9-EET	62.1 ± 20.2%*	68.1 ± 26.8%	48.4 ± 25.9%**
14,15-DHET	103.1 ± 8.8%	89.7 ± 11.9%	94.4 ± 18%
11,12-DHET	100.2 ± 3.2%	93.1 ± 7.7%	93.3 ± 5.2%
8,9-DHET	101.1 ± 4.1%	97.9 ± 9.4%	95.6 ± 8.1%
5,6-DHET	94.8 ± 5.8%	90.3 ± 9.1%	85.3 ± 9.4%

Comparison of CYP eicosanoid levels during 12-hour incubation in Medtronic Exacta Drainage Bags. Data at each time point is presented as mean percentage of initial concentration ± SD (n=4). The percentage of initial concentration at each time point was compared using repeated measures one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. Significant differences are denoted by *p<0.05 or **p<0.01. Results indicate that incubation of CSF collected in drainage bags over 12 hours significantly reduces the measurable amount of 14,15-EET, 11,12-EET, and 8,9-EET, while the measurable amount of 20-HETE, 14,15-DHET, 11,12-DHET, 8,9-DHET, and 5,6-DHET did not significantly change.

2.3.7 Effect of method modifications on recovery and peak response

The effect of the SPE column size on the recovery of the CYP eicosanoids in buffer is shown in Figure 2-4: Effect of SPE column size on recovery of CYP eicosanoids. Recovery was represented as the mean area ratio of the analyte to IS ± SD (n=6). Data were normalized to the recovery

observed using 1 cc SPE columns. Results show that using 3 cc SPE columns instead of 1 cc SPE columns did not significantly affect the recovery of each CYP eicosanoid.

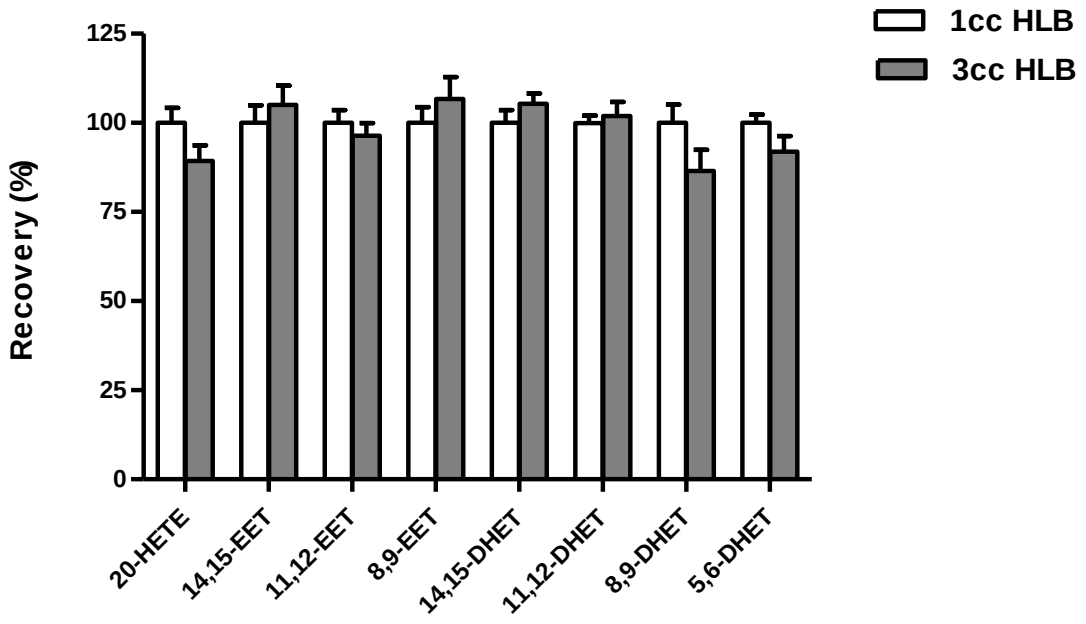


Figure 2-4: Effect of SPE column size on recovery of CYP eicosanoids

CYP eicosanoid analytes and IS (0.83 ng) were spiked in 1 ml and 3 ml phosphate buffer (pH=7.4), processed using 1cc and 3cc SPE cartridges, respectively, and reconstituted in 125 µl. Recovery was represented as mean area ratio of analyte to internal standard ± SD (n=6). Data were normalized to the recovery observed using 1 cc SPE columns and compared using unpaired t-test (2-tailed). Statistical difference was established at *$p<0.05$.

The effect of reconstitution volume on the recovery of CYP eicosanoids in buffer is shown in Figure 2-5: Effect of reconstitution volume on the recovery of CYP eicosanoids. Recovery was represented as the mean area ratio of the analyte to internal standard ± SD (n=6). Recovery data were normalized to account for the different reconstitution volumes and expressed as a percentage recovery of the observed using 125µl reconstitution volume. Results show that using reconstitution volumes of 25, 75 or 200 µl did not significantly affect the recovery of each CYP eicosanoid.

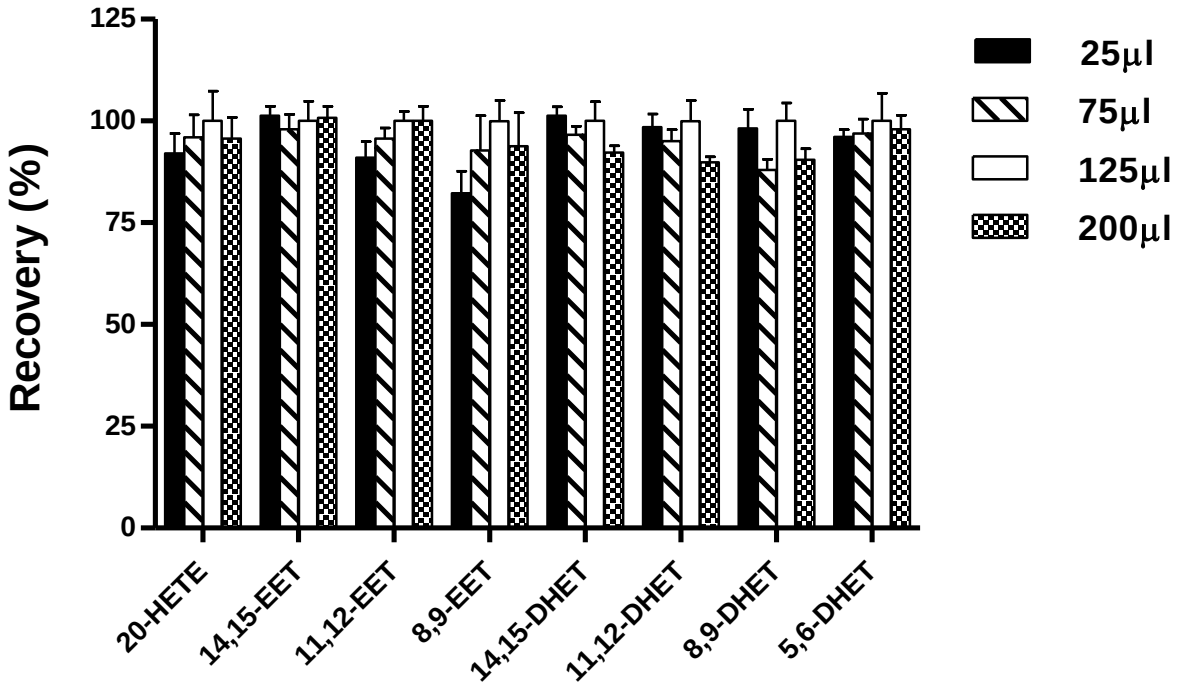


Figure 2-5: Effect of reconstitution volume on the recovery of CYP eicosanoids

CYP eicosanoid analytes and IS was spiked to 0.276 ng/ml in 3ml of phosphate buffer (pH=7.4), processed using 3 cc SPE columns, and reconstituted in 25, 75, 125 and 200 µl. Recovery was represented as mean area ratio of analyte to internal standard ± SD (n=6). Recovery data were normalized to account for the different reconstitution volumes and expressed as a percentage recovery of the observed using 125 µl reconstitution volume. Data were compared using one-way ANOVA with Dunnett *post-hoc* test with a statistical difference established at *p<0.05.

The effect of the load volume and reconstitution volume on the peak response of the UPLC-MS/MS system to measure 20-HETE in buffer solution is shown in Figure 2-6: Improved detection of 20-HETE in buffer with processing modifications. Peak response was expressed as mean peak area ± SD (n=6) and data were normalized to the values observed using the published method (1 ml load volume and 125 µl reconstitution volume). Results show 3.3 ± 0.3 (**p<0.0001), 3.6 ± 0.4 (**p<0.0001), and 10.0 ± 0.6-fold (**p<0.0001) increases in peak response when using 50 µl reconstitution volume, 3 ml load volume, and combination of both modifications, respectively, as compared to the values observed using 1ml load volume and 125 µl reconstitution volume. Data in Appendix A show similar increases in UPLC-MS/MS peak response when using our modified quantitation method to measure 20-HETE-*d6*, EETs, and DHETs. Ultimately, these modifications

led to a decrease in the LLOQ to measure CYP eicosanoids in buffer solution from 0.208 to 0.028 ng/ml when compared to published methods.[643]

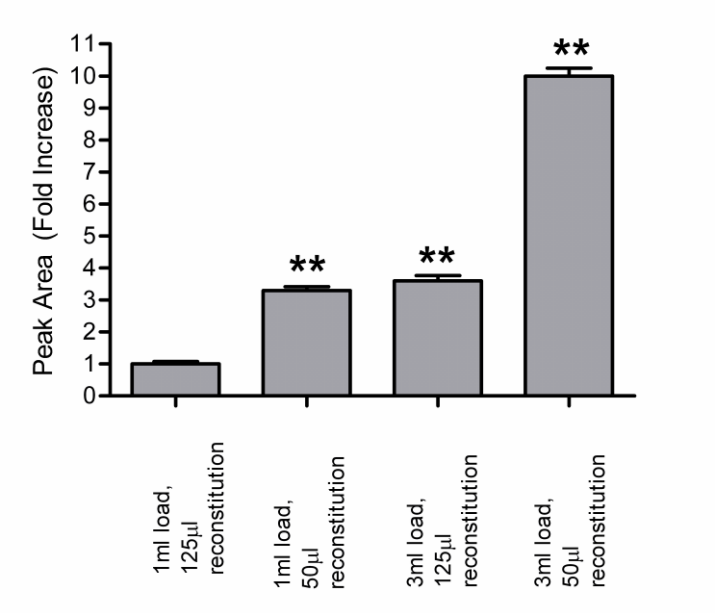


Figure 2-6: Improved detection of 20-HETE in buffer with processing modifications

20-HETE was spiked in buffer to 0.83 ng/ml and measured by UPLC-MS/MS after processing 1 ml sample using 1cc SPE cartridge, 3 ml sample using 3cc SPE cartridge, and reconstituting samples in either 50 µl or 125 µl (n=6). Peak response was expressed as mean peak area ± SD and data were normalized to the values observed using the published method (1 ml load volume and 125 µl reconstitution volume) and compared using unpaired t-test (2-tailed). Statistical difference was established at *$p<0.05$ and **$p<0.0001$.

The effect of processing modifications on the UPLC-MS/MS peak response to measure 20-HETE in bag CSF is shown in Figure 2-7: Improved 20-HETE peak area with method modifications. The UPLC-MS/MS detector responses were normalized to the maximum value and chromatograms were displayed. Results show that the peak response after processing the CSF sample using our published method was approximately 8% of the peak response after processing the CSF sample using our modified.

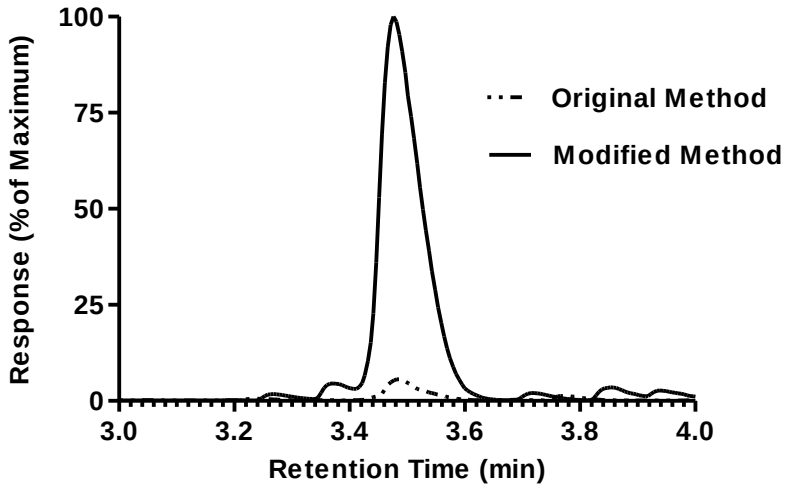


Figure 2-7: Improved 20-HETE peak area with method modifications

A bag CSF sample was simultaneously processed using our published method (1ml sample load volume with 1 cc SPE cartridge and reconstitution volume of 125 μl) and our modified method (3 ml sample load volume with 3cc SPE cartridge and reconstitution volume of 50 μl) and 20-HETE peak responses were measured using UPLC-MS/MS. The UPLC-MS/MS detector responses were normalized to the maximum value and chromatograms were displayed.

2.3.8 Analysis of CSF using method modifications

Next, the metabolite concentrations in bag CSF of 38 patients with SAH were measured using the modified method. Figure 2-8: Samples with detectable CYP eicosanoids in bag CSF shows > 50% of samples had detectable concentrations of each CYP eicosanoid except for 5,6-DHET (13%) and EETs (<6%). Less than 5% of samples were excluded due to sample processing issues such as clogging of the SPE columns or interfering peaks during the UPLC-MS/MS analysis.

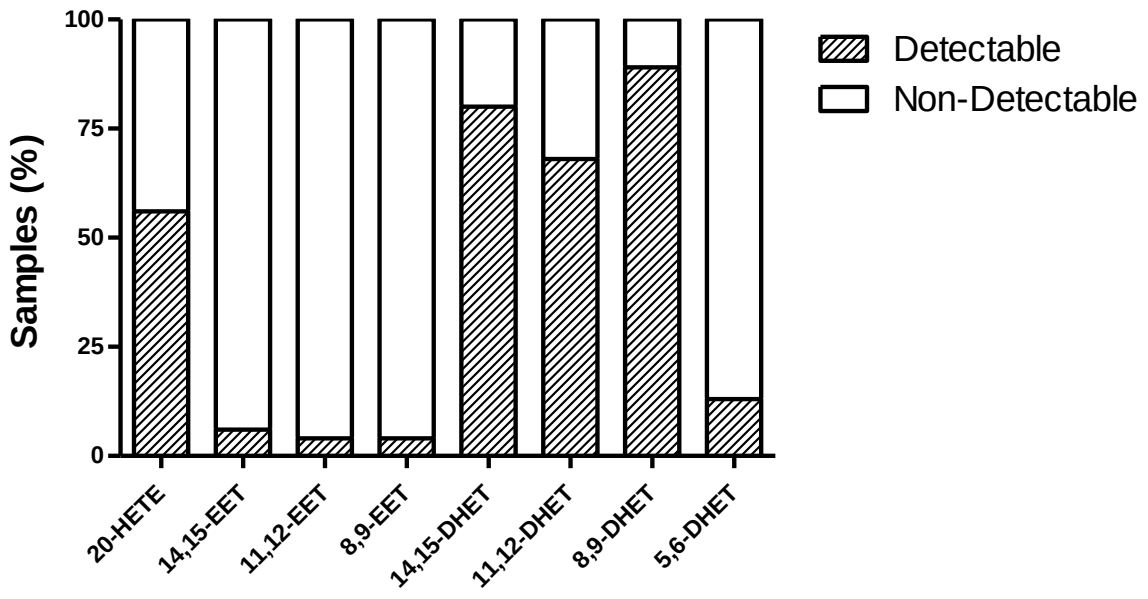


Figure 2-8: Samples with detectable CYP eicosanoids in bag CSF

CYP eicosanoids concentrations were measured in 412 bag CSF samples from 38 patients with SAH using the modified quantitation method (3 ml sample load and 50 μl reconstitution volume) with a lower limit of quantitation (LLOQ) of 0.028 ng/ml. Results were shown as a percentage of samples with detectable metabolite concentrations (above LLOQ) and non-detectable metabolite concentrations (below LLOQ).

In addition, the temporal concentration profiles of CYP eicosanoids from a single patient with SAH were shown. Figure 2-9: Temporal concentration profile of CYP eicosanoids in bag CSF shows a decreasing concentration trend of 20-HETE and increasing concentration trend for DHETs in bag CSF after SAH. The maximum concentration of 20-HETE, 14,15-DHET, 11,12-DHET, and 8,9-DHET in CSF of this patient with SAH was 0.23, 0.14, 0.14, and 0.25 ng/ml at 2.5, 13.5, 13.5, and 6.5 days after SAH, respectively.

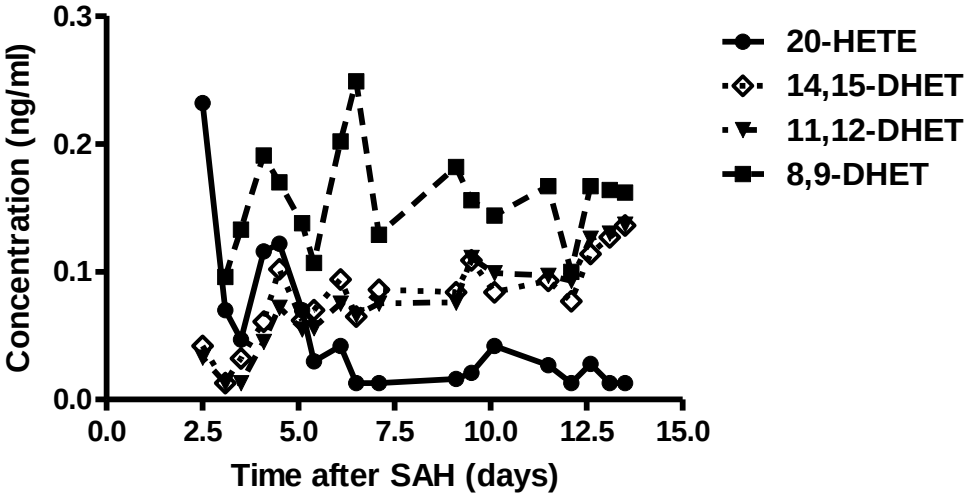


Figure 2-9: Temporal concentration profile of CYP eicosanoids in bag CSF

CYP eicosanoid concentrations were measured in bag CSF of a single patient with SAH over 13 days using the modified quantitation method (3 ml sample load and 50 µl reconstitution volume). The CYP eicosanoid concentration profiles from this patient serve as a representation of concentration profiles observed from the entire SAH patient population.

2.4 DISCUSSION

In this study, we demonstrate that the matrix did not affect the reproducibility and reliability of our UPLC-MS/MS method. Then, we improved our method for detection of CYP eicosanoids by utilizing bag CSF and modifying our sample processing method. In order to utilize bag CSF, we first demonstrated room temperature stability of the CYP eicosanoids over the 12 hour collection period. However, recovery of EETs was significantly altered by contact with the drainage bag, which confounds the quantitation of EET metabolites. Using our modified method, we report a high percentage of samples with detectable 20-HETEs and DHETs in 38 SAH patients and show the temporal concentration profiles of CYP eicosanoids for a single patient. These data demonstrate that our modified quantitation method improves our ability to simultaneously measure

multiple CYP eicosanoids in CSF from SAH patients and demonstrates our ability to establish temporal concentration profiles.

In this section, we describe the development and validation of a rapid, sensitive, and specific UPLC-MS/MS method to measure multiple CYP eicosanoids in CSF of SAH patients. More specifically, we describe the validation of linear calibration curves ranging from 0.208-33.3 ng/ml. The inter-day and intra-day variance was less than 15% at most concentrations with an extraction efficiency of greater than 73%. We demonstrated that the matrix effect of the phosphate buffer and aCSF did not significantly affect the reliability and reproducibility of the assay, the matrix effect of the phosphate buffer is not different than aCSF, and there was no change in UPLC-MS/MS response due to the phosphate buffer and aCSF matrix for all metabolites measured. Therefore, the phosphate buffer can serve as a surrogate matrix for aCSF.

As discussed in Section 1.7.1, there are only a few studies reporting concentrations of CYP eicosanoids in patients with SAH. Previous studies by our group measured 20-HETE concentrations in fresh CSF from 108 patients with SAH using UPLC-MS/MS and found only 32% of patients and 6% of CSF samples had detectable 20-HETE levels.[644] In an effort to improve the quantitative assessment of CYP eicosanoids in human CSF, we modified our method to concentrate our CSF samples and possibly increase MS/MS peak response. We concentrated our samples by using a smaller sample reconstitution volume during sample processing and utilizing the larger sample volumes available from bag CSF compared to fresh CSF. The maximum volume of fresh CSF withdrawn from patients with SAH is limited to approximately 1 ml every 12 hours, while the bag CSF typically accumulates to volumes exceeding 30 ml over the same 12 hour sampling interval. Thus, in order to utilize bag CSF, we first needed to investigate the room temperature stability of CYP eicosanoids and recovery from the collection bags.

We demonstrated room temperature stability of the CYP eicosanoids over the 12 hour collection period. However, significant recovery loss is observed for EETs when incubated in the drainage bags. The largest degree of recovery loss was observed with the EET metabolites, which were reduced below 50% of baseline at 12 hrs. The degree of recovery loss was directly related to the lipophilicity of the individual metabolites. The most lipophilic class of metabolites are the EETs, which demonstrated significant loss of recovery as early as one hour of room temperature incubation in PVC bags. Little to no significant losses were observed with 20-HETE or DHET

metabolites. Collectively, these results suggest that CSF drainage samples from bags kept at room temperature for up to 12 hours are appropriate for quantification of 20-HETE and DHETs. EET metabolites would be expected to undergo significant recovery loss and potentially confound variability if collected from tubing or collection bags made from PVC. These data will be essential in the appropriate design of future clinical studies aimed at determining the utility of these metabolites as therapeutic targets and/or biomarkers for disease progression and complications.

Using our modified method, we demonstrated that the recovery of these compounds during the sample processing step was not affected, but there was increased the peak response of the MS/MS signal without a corresponding increase in the baseline noise. The improved sensitivity of the method can be attributed to increased signal-to-noise ratio. These modifications decrease the LLOQ to measure CYP eicosanoids in CSF from 0.208 ng/ml to 0.028 ng/ml when compared to our published methods.[643] Also, there was sufficient separation of the chromatograms for each metabolite as demonstrated in our published methods [643]. Furthermore, we demonstrated the ability to measure 20-HETE and DHETs, but not EETs, in the majority of bag CSF samples from patients with SAH. We report temporal concentration profiles of CYP eicosanoids in the CSF from a single patient with SAH. Collectively, these data demonstrate the ability to measure multiple CYP eicosanoids in CSF from SAH patients during the inpatient stay of 14 days using a highly sensitive and specific quantitation method.

2.5 CONCLUSIONS

These data are the first to demonstrate the utility of bag CSF for analysis of CYP eicosanoids in SAH patients. The method described in this article showed lower detection limits and a higher percentage of samples with detectable amounts of CYP eicosanoids when compared to published methods thus allowing the analysis of development of temporal concentration profiles. Additional studies are also needed to compare the temporal concentration profiles for CYP eicosanoids in bag and fresh CSF and determine their impact on outcomes in patients with SAH.

3.0 EET RELATIONSHIP WITH OUTCOMES IN SAH PATIENTS

[Mark K. Donnelly, Yvette P. Conley, Elizabeth A. Crago, Dianxu Ren, Paula R. Sherwood, Jeffrey R. Balzer, Samuel M. Poloyac. "Genetic markers in EET metabolic pathway are associated with outcomes in patients with aneurysmal subarachnoid hemorrhage." Submitted to *J Cereb Blood Flow Metab* in June 2014.]

3.1 INTRODUCTION

As discussed in Section 1.1, SAH is devastating condition associated with high rates of mortality and morbidity that can be attributed in large part to the development of ischemic complications such as CND and DCI that typically occur 3-14 days after SAH. Despite this generous time window for therapeutic intervention, strategies to improve outcomes have had limited success in part due to the lack of reliable and accurate methods to identify high-risk patients and a unfavorable understanding of the underlying mechanisms. Currently, there are no established predictors for the development of acute ischemic complications in the first two weeks after SAH and long-term functional outcomes.

Previous studies report that eicosanoids derived from the CYP pathway of AA metabolism regulate cerebrovascular tone and structure.[355] AA is released from phospholipid membranes can be metabolized by CYP enzymes to form EETs that consist of four regioselective isoforms (14,15-, 11,12-, 8,9-, and 5,6-EET). EETs are formed primarily by CYP2J2 and CYP2C8/9 enzymes and metabolized by SEH to their inactive DHETs. In the brain, EETs dilate cerebral arteries, promote angiogenesis, and inhibit inflammation, apoptosis, and platelet aggregation.[355] Collectively, these studies suggest that increasing EET levels in the brain may improve the regulation CBF and vascular homeostasis.

Moreover, preclinical evidence shows that EETs regulate CBF in normal brain and after stroke and affects cerebral ischemic injury. Studies have demonstrated that EET synthesis inhibition reduced baseline CBF in rats.[633] Inhibition or gene deletion of SEH reduces infarct size and increases CBF in animal models of temporary focal ischemia.[638, 639] *In vitro* studies demonstrate that EETs protect astrocytes against ischemic cell death.[640] Also, variants in the EPHX2 gene, which codes for SEH, have been shown to affect SEH activity and neuronal survival after ischemic injury.[641] These studies provide evidence that EETs alter CBF and cerebral ischemic injury in preclinical models and warrant further investigation in humans.

Given the preclinical evidence that EETs are involved in the pathophysiology of stroke, we hypothesized that loss-of-function polymorphisms in EET synthesis genes (CYP2C8, CYP2C9, and CYP2J2) and gain-of-function polymorphisms in the EET metabolism gene EPHX2 will be

associated with decreased EET concentrations in CSF resulting in the development of ischemic complications and unfavorable long-term functional outcomes in patients with SAH. Furthermore, we compared genotype frequencies in our SAH population with those reported in the general population using the Hapmap database to identify potential genetic markers for SAH.

3.2 METHODS

3.2.1 Design and participants

Participants were prospectively recruited from patients admitted to the University of Pittsburgh Medical Center neurovascular intensive care unit (ICU). The protocol was approved by the Institutional Review Board and informed consent was obtained from the patient or their proxy. The study included 363 adult patients (age 18-75) with SAH diagnosed via cerebral angiogram or head computed tomography (CT) and classified as Fisher grade >1. Patients were not enrolled if they had a history of debilitating neurological disease or SAH from trauma, mycotic aneurysm, or arteriovenous malformation. The number of patients was estimated based on the expected variances and differences between CYP eicosanoid levels in outcome groups. CSF from 269 patients was available for analysis of CYP eicosanoid levels. We limited our genetic analyses to 304 Caucasians in an attempt to address population stratification.[760] All patients received standard nursing and medical care in the neurovascular ICU.[760]

3.2.2 Analysis of EET and DHET CSF levels

Chemical standards were purchased from Cayman Chemical Company (Ann Arbor, MI). High purity organic solvents were purchased from VWR (West Chester, PA) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). CSF samples were withdrawn from collection bags on external ventricular drains approximately every 12 hours during the inpatient stay (up to 14 days). Samples were processed using solid phase extraction (SPE). Sample volumes

of 2.0-3.0 ml were loaded onto 3cc Oasis HLB SPE cartridges (Waters, Milford, MA) that were conditioned and equilibrated with 2 ml of MeOH and dH_20, respectively. Columns were washed and eluted with 3 ml of 5% MeOH and 100% MeOH, respectively. Samples were reconstituted in 50 µl of 80:20 MeOH:dH_20. Quantitation of EETs and DHETs was performed using a previously described UPLC-MS/MS method[643] with minor modifications as described in Section 2.0 . Concentrations of EETs and DHETs were determined from the standard curve of the ratio of their peak areas to internal standard peak areas of 14,15-EET-*d11* and 14,15-DHET-*d11*, respectively, over a linear range of 0.014 to 8.88 ng/ml.

3.2.3 Analysis of gene variants in EET pathway

Candidate genes in the EET metabolic pathway included CYP2C8, CYP2C9, CYP2J2, and EPHX2. Tagging single nucleotide polymorphisms (tSNPs) were selected using the CEU population from Hapmap database (Release 27; www.hapmap.org) and criteria included $r^2 > 0.8$ and minor allele frequency ≥20% while functional SNPs (fSNPs) were defined as those previously reported to affect mRNA transcription, protein expression, or enzyme activity *in vitro*. Our genetic analysis excluded SNPs with variant genotype frequencies <1% in our SAH population. Also, rs11572080 and rs71220599 were excluded from the analysis because these SNPs were in 100% linkage disequilibrium with rs10509681 and rs71553864, respectively. Our genetic analysis included 14 tSNPs (rs11572133, rs11572139, rs1934952, rs1934953, rs12772884, rs1934967, rs2253635, rs4086116, rs4918766, rs9332104, rs1155002, rs7515289, rs2071575, and rs7816586), eight fSNPs (rs10509681, rs1058930, rs1799853, rs1057910, rs890293, rs41507953, rs751141, and rs71553864) and one fSNP/tSNP (rs7909236) as shown in Table 3-1: Candidate gene variants in EET pathway included in the analysis. Genotyping was performed for using Taqman allele discrimination assay with ABI Prism 7000 Sequence Detection System (Applied Bioscience, Carlsbad, CA, USA) for rs1057910, Affymetrix Human Genome-wide SNP Array 6.0 (Affymetrix, Santa Clara, CA) for rs1934952, and iPLEX MassArray (Sequenom, San Diego, CA) for all other SNPs. Consistency and integrity of genotyping data was checked by inclusion of duplicate CEPH controls on each plate for internal as well as plate-to-plate consistency, using

genotype call rate criteria of >85%, comparing the observed and Hapmap Caucasian (CEU) frequencies, and performing checks for Hardy-Weinberg Equilibrium (HWE) consistency. Analyses involving genetic data included both genotype groups (codominant model) and presence or absence of the variant allele (dominant model) groups. SNPstats software was used to assign haplotypes for candidate genes and combinations of fSNPs on different candidate genes.[761] Haplotypes with frequencies < 5% were not evaluated.

Table 3-1: Candidate gene variants in EET pathway included in the analysis

Gene	rs# (fSNP/tSNP)	Genomic	cDNA	Protein	CYP Nomenclature
CYP2C8	rs10509681 (†)	g.35506T>C	c.1196T>C	399 Lys>Arg	CYP2C8*3
	rs1058930 (†)	g.16136C>G	c.792C>G	264 Ile>Met	CYP2C8*4
	rs7909236 (†‡)	g.4825G>T	c.-271G>T		CYP2C8*1B
	rs11572133 (‡)	g.24879A>T	c.610-3668A>T		
	rs11572139 (‡)	g.25369C>T	c.610-3178C>T		
	rs1934952 (‡)	g.36755G>A	c.1082-434G>A		
	rs1934953 (‡)	g.36785A>G	c.1082-404A>G		
CYP2C9	rs1799853 (†)	g.8633C>T	c.430C>T	144Arg>Cys	CYP29*2C
	rs1057910 (†)	g.47639A>C	c.1075A>C	359Ile>Leu	CYP2C9*3A
	rs12772884 (‡)	g.723T>A	c.169-985T>A		
	rs1934967 (‡)	g.48012C>T	c.1149+299C>T		
	rs2253635 (‡)	g.816A>G	c.169-1078A>G		
	rs4086116 (‡)	g.13788C>T	c.482-334C>T		
	rs4918766 (‡)	g.18470G>A	c.819+2843G>A		
	rs9332104 (‡)	g.5276T>C	c.168+83T>C		
CYP2J2	rs890293 (†)	g.4930G>T	c.-76G>T		CYP2J2*7
	rs1155002 (‡)	g.30345693C>T	c.862-176C>T		
	rs7515289 (‡)	g.14742T>G	c.211-910T>G		
EPHX2	rs41507953 (†)	g.14861A>G	c.5A>G	55Lys>Arg	
	rs751141 (†)	g.30221C>T	c.860C>T	287Arg>Gln	
	rs71553864 (†)	g.50690-50691insGTC	c.1204-1205insGTC	404Thr>del	
	rs2071575 (‡)	g.54788T>C	c.1379+259T>C		
	rs7816586 (‡)	g.25690G>A/C	c.661-19G>A/C		

† Functional SNP; ‡ Tagging SNP

3.2.4 Outcomes assessment

Acute outcomes included the presence or absence of CND and/or DCI during the inpatient stay (up to 14 days).[762] CND was determined by a decline in neurological exam for >1 hour evidenced by a documented global or focal deficit, decrease in NIH Stroke Scale or Glasgow Coma Scale score in the absence of medication administration, fever, seizure, rebleed, increased intracranial pressure, hydrocephalus. DCI was defined as the presence of CND accompanied by evidence of impaired CBF (simultaneously or within 12 hours pre- or post-determination of CND). Impaired CBF was determined using surrogate markers of blood flow including angiography (≥25% cerebral vessel narrowing) and/or elevated transcranial Doppler flow velocities (≥200cm/s or Lindegaard ratio ≥3) or CT/magnetic resonance (MR) perfusion scans (impaired perfusion or new cerebral infarction). Long-term outcomes were determined by global functional recovery at 3 and 12 months using the Modified Rankin Scale (MRS) obtained during face-to-face interview or phone call with the patient or their surrogate.

3.2.5 Statistical analysis

For the purpose of analysis, HH scores were dichotomized into high (3-5) and low (1-2) groups and MRS scores were dichotomized into favorable (MRS 0-2) and unfavorable (MRS 3-6) groups. CYP eicosanoid CSF concentrations below the lower limit of quantitation (LLQ) were reported as LLQ/2. The mean and maximum CYP eicosanoid levels for each patient were calculated and were used to compare the mean ± standard error of the mean (SEM) in the genotype and outcome groups using t-test (with Welch's correction as appropriate) or ANOVA with Bonferroni's *post-hoc* test. To examine homogeneous latent trajectory classes of DHET CSF levels following SAH, group-based trajectory analysis was performed with the PROC TRAJ macro in SAS version 9.4 as previously described.[763] The time range of 2-11 days was selected to minimize missing data and the time from hemorrhage was rounded up to the nearest day. Log transformation was applied to

reduce sample variation and skewness for better model fitting (See Appendix D and E). The Bayesian Information Criterion (BIC) and the substantive utility of the classes (e.g., distinctiveness of the trajectories, proportion assigned to a given class) were used to determine the optimal solution for the number of trajectory groups. Mean concentration values from the trajectory analysis were reported as the geometric mean ± 95% confidence interval (CI). DHET trajectory groups and genotype/allele frequencies were compared to acute outcomes, long-term outcomes, and covariate groups using chi-square analysis or t-test. Also, the relationship between genotype, CYP eicosanoid concentrations/trajectory groups, and outcomes was determined using logistic regression after controlling for covariates such as age, sex, race (for comparison of CYP eicosanoid levels and outcomes), and either Fisher grade (for analyses involving acute outcomes) or HH score (for analyses involving long-term outcomes). Previous studies report that Fisher grade and HH score are strongly associated with acute and long-term outcomes, respectively.[278] The cumulative incidence of acute outcomes during the inpatient stay in each genotype group was compared using Kaplan-Meier log-rank analysis and the model was adjusted for covariates using Cox regression. In addition, CYP eicosanoid levels and outcomes were compared in genetic haplotype groups before and after adjustment for covariates using SNPstats software.[761] All other statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was determined at p-values<0.05.

3.3 RESULTS

3.3.1 Acute and long-term outcomes comparison

A comparison of acute and long-term outcomes is shown in Table 3-2: Acute and long-term outcomes comparison. The presence of CND was significantly associated with DCI and unfavorable MRS at 3 and 12 months (p<0.001). In addition, the presence of DCI was significantly associated with unfavorable MRS at 3 and 12 months (p<0.001). Also, unfavorable MRS scores

at 3 months were significantly associated with unfavorable MRS scores at 12 months (p<0.001).

Table 3-2: Acute and long-term outcomes comparison

Outcome	Group	DCI		Chi-square Association	
		(+)	(-)	OR (95% CI)	P-value
CND	(+)	124 (67.8)	59 (32.2)	3.10 (2.51 - 3.83)	p<0.001
	(-)	0 (0)	149 (100)		
Outcome	Group	MRS3		Chi-square Association	
		Unfavorable (3-6)	Favorable (0-2)	OR (95% CI)	P-value
CND	(+)	54 (40)	81 (60)	6.07 (2.90 - 12.69)	p<0.001
	(-)	10 (9.9)	91 (90.1)		
Outcome	Group	MRS12		Chi-square Association	
		Unfavorable (3-6)	Favorable (0-2)	OR (95% CI)	P-value
CND	(+)	45 (33.3)	90 (66.7)	5.38 (2.40 - 12.06)	p<0.001
	(-)	8 (8.5)	86 (91.5)		
Outcome	Group	MRS3		Chi-square Association	
		Unfavorable (3-6)	Favorable (0-2)	OR (95% CI)	P-value
DCI	(+)	38 (39.2)	59 (60.8)	2.70 (1.50 - 4.84)	p<0.001
	(-)	27 (19.3)	113 (80.7)		
Outcome	Group	MRS12		Chi-square Association	
		Unfavorable (3-6)	Favorable (0-2)	OR (95% CI)	P-value
DCI	(+)	33 (35.1)	61 (64.9)	2.96 (1.58 - 5.56)	p<0.001
	(-)	21 (15.4)	115 (84.6)		
Outcome	Group	MRS12		Chi-square Association	
		Unfavorable (3-6)	Favorable (0-2)	OR (95% CI)	P-value
MRS3	Unfavorable (3-6)	53 (72.6)	20 (27.4)	57.42 (21.8 – 151.0)	p<0.001
	Favorable (0-2)	6 (4.4)	130 (95.6)		

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Unfavorable (3-6), Favorable (0-2); EET, epoxyeicosatrienoic acids; DHET, dihydroxyeicosatetraenoic acid (DHET); SEM, standard error of the mean. § Multivariate analysis included correction for age, sex, and either Fisher Grade (for acute outcomes) or Hunt & Hess Score (for long-term outcomes)

*Statistical significance established at p<0.05

3.3.2 Covariates and outcomes comparison

Results comparing covariates with acute and long-term outcomes are shown in Table 3-3: Covariate and outcome comparison. As expected, the severity of injury measured by Fisher grade and HH scores were associated with worse acute and long-term outcomes ($p<0.001$), respectively. Increased age was associated with the presence of CND ($p=0.001$) but not DCI and long-term outcomes. Race was associated with better outcomes at 3 months, but not at 12 months, in Caucasians compared to Non-Caucasians ($p=0.003$). Sex was not associated with acute and long-term outcomes.

Table 3-3: Covariate and outcome comparison

Covariate	Group	CND (+) N (%)	CND (-) N (%)	OR (95% CI)	P-value	DCI (+) N (%)	DCI (-) N (%)	OR (95% CI)	P-value
Race	non-Caucasian	21 (58.3)	15 (41.7)	1.16	0.681	16 (44.4)	20 (55.6)	1.40	0.344
	Caucasian	162 (54.7)	134 (45.3)	(0.57 - 2.33)		108 (36.4)	189 (63.6)	(0.70 - 2.82)	
Sex	Male	53 (57)	40 (43)	1.11	0.669	37 (39.8)	56 (60.2)	1.16	0.549
	Female	130 (54.4)	109 (45.6)	(0.69 - 1.80)		87 (36.3)	153 (63.8)	(0.71 - 1.90)	
Age	Years	54.4 (11.2)	50.5 (10.2)	NA	0.001*	52.3 (10.9)	53.3 (11.1)	NA	0.434
Fisher grade	2	39 (36.4)	68 (63.6)	NA	<0.001*	26 (24.3)	81 (75.7)	NA	<0.001*
	3	98 (58)	71 (42)			67 (39.6)	102 (60.4)		
	4	46 (83.6)	9 (16.4)			31 (55.4)	25 (44.6)		
Covariate	Group	MRS3 (3-6) N (%)	MRS3 (0-2) N (%)	OR (95% CI)	P-value	MRS12 (3-6) N (%)	MRS12 (0-2) N (%)	OR (95% CI)	P-value
Race	non-Caucasian	14 (58.3)	10 (41.7)	3.51	0.003*	8 (40)	12 (60)	1.83	0.202
	Caucasian	65 (28.5)	163 (71.5)	(1.48 - 8.30)		60 (26.7)	165 (73.3)	(0.71 - 4.70)	
Sex	Male	24 (33.8)	47 (66.2)	1.17	0.599	16 (24.2)	50 (75.8)	0.78	0.456
	Female	55 (30.4)	126 (69.6)	(0.65 - 2.10)		52 (29.1)	127 (70.9)	(0.41 - 1.50)	
Age	Years	53.0 (10.9)	55.0 (10.9)	NA	0.175	53.9 (11.1)	55.5 (11.6)	NA	0.326
Hunt & Hess	Favorable (0-2)	17 (15.3)	94 (84.7)	4.34	<0.001*	13 (11.9)	96 (88.1)	5.01	<0.001*
	Unfavorable (3-5)	62 (44)	79 (56)	(2.35 - 8.02)		55 (40.4)	81 (59.6)	(2.56 - 9.83)	

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Unfavorable (3-6), Favorable (0-2); OR, odds ratio; CI, confidence interval

3.3.3 CYP eicosanoid quantitation

Results showing the percentage patients and samples with detectable CYP eicosanoid levels and the mean and maximum CYP eicosanoid levels in all patients are shown in Table 3-4: Quantitation of EETs and DHETs in CSF samples. Detectable EET and DHET levels were measured in 64.3% and 98.9% of patients (n=269) patients and 13.1% and 97.9% of samples (n=3151), respectively. Mean and maximum EET levels were 0.073 ± 0.007 ng/ml and 0.153 ± 0.018 ng/ml, respectively. Mean and maximum DHET levels were 1.271 ± 0.069 ng/ml and 2.462 ± 0.140 ng/ml, respectively.

Table 3-4: Quantitation of EETs and DHETs in CSF samples

CYP-Eicosanoid	Mean Conc. Mean ± SEM (ng/ml)	Maximum Conc. Mean ± SEM (ng/ml)	Detectable Samples N (%) [Total=3151]	Detectable Patients N (%) [Total=269]
EET	0.073 ± 0.007	0.153 ± 0.018	414 (13.1)	173 (64.3)
14,15-EET	0.016 ± 0.001	0.037 ± 0.006	140 (4.4)	123 (45.7)
11,12-EET	0.013 ± 0.001	0.023 ± 0.004	166 (5.3)	87 (32.3)
8,9-EET	0.044 ± 0.006	0.101 ± 0.015	271 (8.6)	149 (55.4)
DHET	1.271 ± 0.069	2.462 ± 0.140	3084 (97.9)	266 (98.9)
14,15-DHET	0.053 ± 0.003	0.114 ± 0.008	2152 (68.3)	241 (89.6)
11,12-DHET	0.031 ± 0.002	0.078 ± 0.007	1948 (61.8)	189 (70.3)
8,9-DHET	1.171 ± 0.069	2.329 ± 0.138	3025 (96.0)	264 (98.1)
5,6-DHET	0.016 ± 0.001	0.048 ± 0.007	382 (12.1)	113 (42.0)

Detectable, Concentration > 0.014 ng/ml

3.3.4 HWE and Hapmap comparison of genetic data

Results of the HWE test and the observed vs. Hapmap frequency comparison are shown in Table 3-5: HWE and Hapmap comparison of genetic data. The genotype frequencies for CYP2C9 g.18470G>A were not in HWE (p=0.003). Also, our SAH population showed different genotype frequencies than those reported in the Hapmap CEU population for CYP2C8 g.24879A>T (p=0.041), CYP2C9 g.18470G>A (p=0.009), and EPHX2 g.54788T>C (p=0.031) and a trend for a difference for CYP2J2 g.14742T>G (p=0.053).

Table 3-5: HWE and Hapmap comparison of genetic data

Gene SNP (rs#)	Genotype	Observed N (%)	Hapmap N (%)	P-value	
				HWE	Obs-Hap
CYP2C8 g.24879A>T‡ (rs11572133)	A/A	148 (50.5)	135 (46.0)	0.067	0.041*
	T/A	111 (37.9)	137 (46.9)		
	T/T	34 (11.6)	21 (7.1)		
CYP2C9 g.18470G>A‡ (rs4918766)	G/G	157 (52.9)	122 (41.1)	0.003*	0.009*
	G/A	103 (34.7)	138 (46.4)		
	A/A	37 (12.5)	37 (12.5)		
EPHX2 g.54788T>C‡ (rs2071575)	T/T	149 (49.3)	150 (49.6)	0.666	0.031*
	C/T	124 (41.1)	139 (46)		
	C/C	29 (9.6)	13 (4.4)		
CYP2J2 g.30354600T>G‡ (rs7515289)	T/T	160 (53.5)	183 (61.1)	0.466	0.053
	G/T	114 (38.1)	103 (34.5)		
	G/G	25 (8.4)	13 (4.4)		

HWE, Hardy-Weinberg Equilibrium; Obs-Hap, observed vs. Hapmap frequency comparison; † Functional SNP; ‡ Tagging SNP; *Statistical significance established at $p<0.05$

3.3.5 CYP eicosanoid levels in genetic groups

Results comparing CYP eicosanoid CSF levels in genetic groups are shown in Figure 3-1: EET CSF levels in genetic groups, Figure 3-2: DHET CSF levels in genetic groups. CYP2C8*4 (g.16136G) allele-carriers (n=24) had 44% and 36% lower mean EET and DHET levels when compared to CYP2C8*1/*1-carriers (n=197) ($p=0.002$ and $p<0.001$), respectively. Similar results were observed when comparing maximum EET and DHET levels in these genotype groups. EPHX2 55Arg (g.14861G) allele-carriers (n=38) had 42% and 41% lower mean and maximum EET levels ($p=0.003$ and $p=0.049$, respectively) and showed a trend for increased maximum DHET levels ($p=0.094$) when compared to EPHX2 55Lys/Lys carriers (n=180). CYP2C8*1B (g.4825A) allele-carriers (n=95) had 23% reduction in maximum DHET levels when compared to CYP2C8*1/*1-carriers (n=123) ($p=0.044$). Also, CYP2J2*7 (g.4930T) allele-carriers (n=28) had 36% lower mean DHET levels when compared to CYP2J2*1/*1 carriers (n=193) ($p=0.004$). In the tSNP analysis, CYP2C8 g.25369T allele-carriers (n=119) had 60% and 93% higher mean and maximum EET levels when compared to CYP2C8 g.25369C/C carriers (n=98) ($p=0.024$ and $p=0.012$), respectively. CYP2C8 g.36755A allele-carriers (n=104) had 49% and 84% higher mean

and maximum EET levels when compared to CYP2C8 g.36755G/G carriers (n=71) (p=0.033 and p=0.028), respectively. CYP2C8 g.24879T allele-carriers (n=101) showed a trend for lower mean and maximum EET levels when compared to CYP2C8 g.24879A/A carriers (n=114) (p=091 and p=0.094), respectively. CYP2C8 g.36785G allele-carriers had 22% lower maximum DHET levels compared to CYP2C8 g.36785A/A carriers (p=0.044).

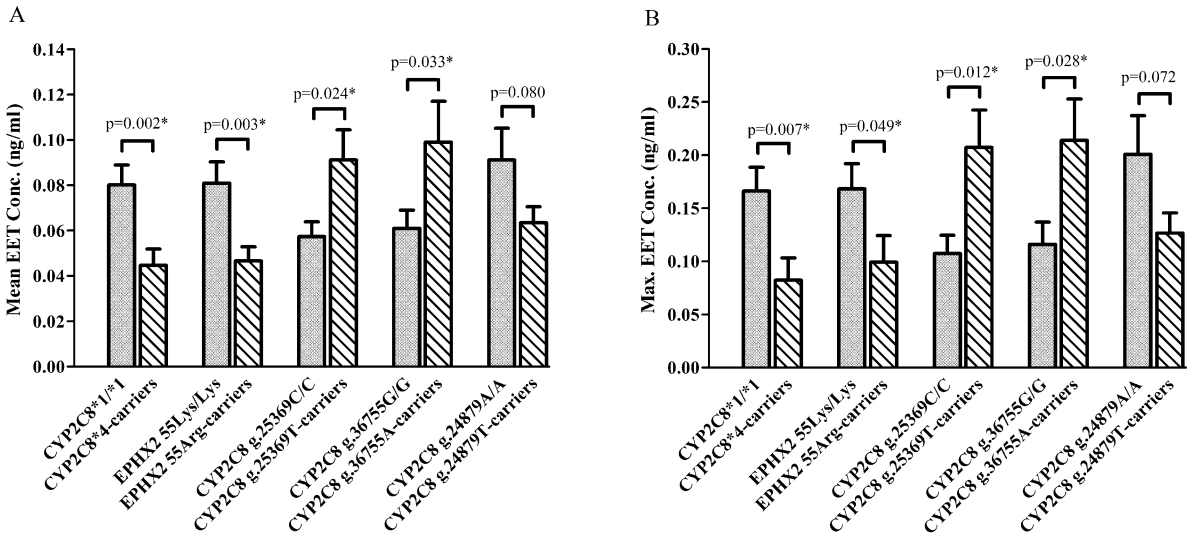


Figure 3-1: EET CSF levels in genetic groups

Mean (A) and maximum (B) EET levels in CSF from patients with SAH are compared in genotype groups. EET CSF levels for each patient were calculated and were used to compare the population mean ± SEM of EETs in the variant allele-carrier (striped bars) and WT-genotype (solid bars) groups using t-test with Welch's correction as appropriate. Statistical significance established at *p<0.05.

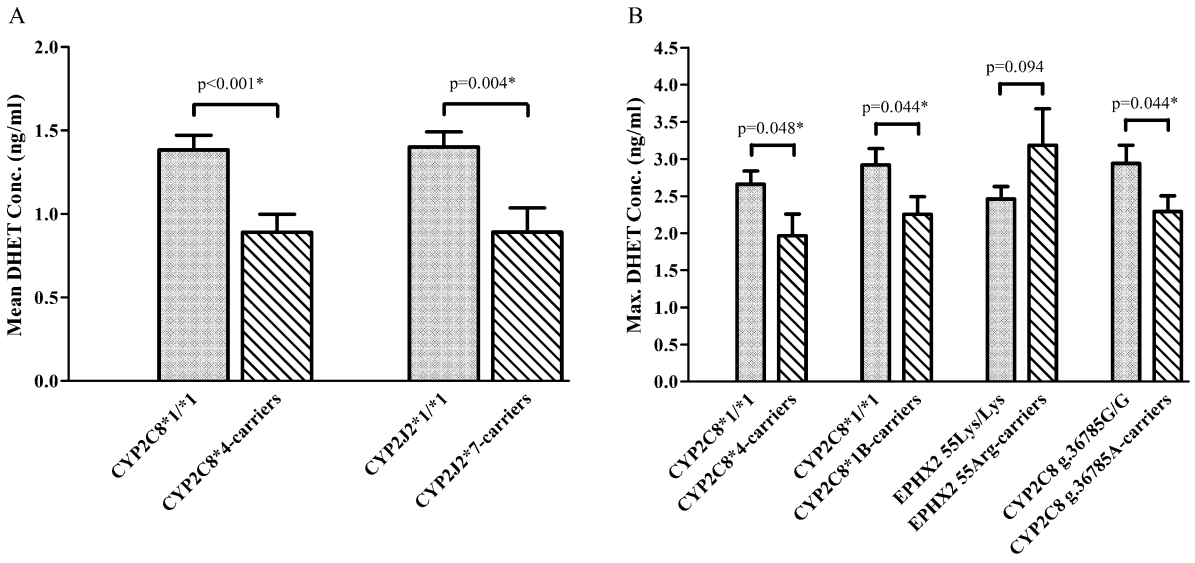


Figure 3-2: DHET CSF levels in genetic groups

Mean (A) and maximum (B) DHET levels in CSF from patients with SAH are compared in genotype groups. DHET CSF levels for each patient were calculated and were used to compare the population mean ± SEM of DHETs in the variant allele-carrier (striped bars) and WT-genotype (solid bars) groups using t-test with Welch's correction as appropriate. Statistical significance established at *p<0.05.

EET and DHET CSF levels in candidate gene haplotypes are shown in Table 3-6: DHET CSF levels in CYP2C9 haplotypes, Table 3-7: EET and DHET CSF levels in CYP2C8 haplotypes. The most common haplotype (Haplotype 1) for CYP2C8, CYP2C9, CYP2J2 and EPHX2 was GTCAG (rs7909236, rs11572133, rs11572139, rs1934953, rs1934952), ATGCGT (rs12772884, rs1934967, rs2253635, rs4086116, rs4918766, rs9332104), CT (rs1155002 and rs7515289), and TA (rs2071575 and rs7816586) seen in 30.2%, 22.7%, 47.0%, and 39.0% of patients, respectively. CYP2C9 Haplotype 4 (ACGCGT) was present in 14.8% of patients and was associated with 0.44 ng/ml and 1.06 ng/ml higher mean and maximum DHET levels (p=0.028 and p=0.008), respectively. CYP2C8 Haplotype 2 (GATAA) was present 29.1% of patients and was associated with 0.03 ng/ml and 0.1 ng/ml higher mean and maximum EET levels (p=0.019 and p=0.006), respectively. Haplotype 2 also showed a trend for 0.25 ng/ml higher mean DHET levels (p=0.072). CYP2C8 Haplotype 4 (GACGG) was present 8.3% of patients and was associated with 0.54 ng/ml and 1.0 ng/ml higher mean and maximum DHET levels (p=0.010 and p=0.018), respectively.

CYP2C8 Haplotype 4 also showed a trend for 0.04 ng/ml higher mean EET levels (p=0.058). EPHX2 Haplotype 4 (CA) present in 2.1% of patients was associated with 2.09 ng/ml and 3.84 ng/ml higher mean and maximum DHET levels (p<0.001). Similar relationships between CYP eicosanoids and haplotypes were observed after controlling for age, sex, and Fisher grade. There were no differences in CYP eicosanoid levels in haplotype groups when compared to the most common haplotype for CYP2J2 and EPHX2.

Table 3-6: DHET CSF levels in CYP2C9 haplotypes

Haplotype	CYP2C9 Haplotypes						Frequency	Mean DHET (ng/ml)	P-value	P-value§
Group	rs12772884	rs1934967	rs2253635	rs4086116	rs4918766	rs9332104	(n=222)	Difference (95% CI)		
1	A	T	G	C	G	T	22.7%	0	---	---
2	T	C	A	C	G	C	22.5%	0.24 (-0.11 - 0.6)	0.190	0.110
3	T	C	A	C	A	T	15.6%	0.02 (-0.35 - 0.38)	0.930	0.820
4	A	C	G	C	G	T	14.8%	0.44 (0.05 - 0.84)	0.028*	0.031*
5	T	C	A	T	A	T	13.8%	0.29 (-0.1 - 0.67)	0.150	0.082
6	T	C	A	T	G	T	5.4%	0.13 (-0.44 - 0.7)	0.660	0.550
7	A	C	A	C	G	T	5.3%	0.09 (-0.46 - 0.64)	0.750	0.710
Global									0.75	0.71
Haplotype	CYP2C9 Haplotypes						Frequency	Maximum DHET	P-value	P-value§
Group	rs12772884	rs1934967	rs2253635	rs4086116	rs4918766	rs9332104	(n=222)	Difference (95% CI)		
1	A	T	G	C	G	T	22.7%	0	---	---
2	T	C	A	C	G	C	22.5%	0.55 (-0.17 - 1.26)	0.130	0.088
3	T	C	A	C	A	T	15.6%	0.03 (-0.7 - 0.76)	0.930	0.890
4	A	C	G	C	G	T	14.8%	1.06 (0.28 - 1.85)	0.008*	0.012*
5	T	C	A	T	A	T	13.8%	0.56 (-0.21 - 1.33)	0.160	0.100
6	T	C	A	T	G	T	5.4%	0.5 (-0.65 - 1.64)	0.400	0.350
7	A	C	A	C	G	T	5.3%	0.84 (-0.26 - 1.95)	0.140	0.120
Global									0.14	0.12

§ Multivariate analysis included correction for age, sex, and either Fisher Grade (for acute outcomes) or Hunt & Hess Score (for long-term outcomes); *Statistical significance established at p<0.05

Table 3-7: EET and DHET CSF levels in CYP2C8 haplotypes

Haplotype	CYP2C8 Haplotypes					Frequency	Mean DHET (ng/ml)	P-value	P-value§
Group	rs7909236	rs11572133	rs11572139	rs1934953	rs1934952	(n=220)	Difference (95% CI)		
1	G	T	C	A	G	30.2%	0	---	---
2	G	A	T	A	A	29.1%	0.25 (-0.02 - 0.53)	0.072	0.063
3	T	A	C	G	G	22.7%	-0.06 (-0.36 - 0.24)	0.690	0.730
4	G	A	C	G	G	8.3%	0.54 (0.13 - 0.94)	0.010*	0.018*
5	G	A	C	A	A	4.5%	-0.28 (-0.83 - 0.26)	0.310	0.380
Global								0.33	0.430
Haplotype	CYP2C8 Haplotypes					Frequency	Maximum DHET (ng/ml)	P-value	P-value§
Group	rs7909236	rs11572133	rs11572139	rs1934953	rs1934952	(n=220)	Difference (95% CI)		
1	G	T	C	A	G	30.2%	0	---	---
2	G	A	T	A	A	29.0%	0.47 (-0.09 - 1.02)	0.100	0.100
3	T	A	C	G	G	22.7%	-0.3 (-0.91 - 0.31)	0.330	0.360
4	G	A	C	G	G	8.3%	1 (0.17 - 1.82)	0.018*	0.038*
5	G	A	C	A	A	4.5%	0.03 (-1.06 - 1.13)	0.950	0.860
Global								0.430	0.570
Haplotype	CYP2C8 Haplotypes					Frequency	Mean EET (ng/ml)	P-value	P-value§
Group	rs7909236	rs11572133	rs11572139	rs1934953	rs1934952	(n=220)	Difference (95% CI)		
1	G	T	C	A	G	30.3%	0	---	---
2	G	A	T	A	A	29.1%	0.03 (0.01 - 0.06)	0.019*	0.040*
3	T	A	C	G	G	22.6%	0.01 (-0.02 - 0.04)	0.340	0.340
4	G	A	C	G	G	8.4%	0.04 (0 - 0.08)	0.058	0.180
5	G	A	C	A	A	4.5%	-0.01 (-0.07 - 0.04)	0.670	0.640
Global								0.630	0.760
Haplotype	CYP2C8 Haplotypes					Frequency	Maximum EET (ng/ml)	P-value	P-value§
Group	rs7909236	rs11572133	rs11572139	rs1934953	rs1934952	(n=220)	Difference (95% CI)		
1	G	T	C	A	G	30.3%	0	---	---
2	G	A	T	A	A	29.1%	0.1 (0.03 - 0.17)	0.006*	0.012*
3	T	A	C	G	G	22.6%	0.05 (-0.03 - 0.12)	0.250	0.250
4	G	A	C	G	G	8.4%	0.06 (-0.05 - 0.16)	0.290	0.590
5	G	A	C	A	A	4.5%	-0.03 (-0.17 - 0.11)	0.700	0.670
Global								0.660	0.810

§ Multivariate analysis included correction for age, sex, and either Fisher Grade (for acute outcomes) or Hunt & Hess Score (for long-term outcomes); *Statistical significance established at $p<0.05$

3.3.6 Outcomes in genetic groups

Results comparing the genotype and allele frequencies with acute outcomes are shown in Table 3-8: Acute outcomes in genetic groups. Carriers of the CYP2C8*4 (g.16136G) allele were ~2.2- and 2.5-fold more likely to develop DCI (p=0.041) and CND (p=0.039), respectively. Conversely, patients with CYP2J2 g.30345693C/T genotype were ~1.9-fold less likely to develop CND (p=0.025).

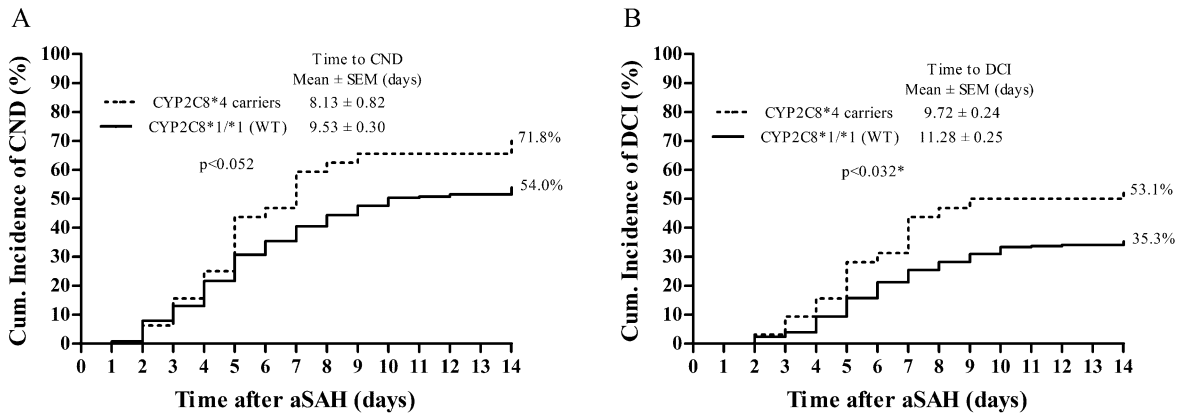
Table 3-8: Acute outcomes in genetic groups

Gene SNP (rs#)	Genotype	CND (+) N (%)	CND (-) N (%)	Unadjusted P-value	Adjusted§	
					OR (95% CI)	P-value
CYP2C8 g.16136C>G† Ile264Met [*4] (rs1058930)	C/C	137 (53.9)	117 (46.1)	0.054	Reference	-
	C/G	23 (71.9)	9 (28.1)		2.48 (1.05-5.89)	0.039*
	G/G	0 (0)	0 (0)		-	-
	G-carriers	17 (53.1)	15 (46.9)	0.054	2.48 (1.05-5.89)	0.039*
CYP2J2 g.30345693C>T‡ (rs1155002)	Overall					0.059
	C/C	68 (62.4)	41 (37.6)	0.135	Reference	-
	C/T	69 (50.0)	69 (50.0)		0.53 (0.31-0.92)	0.025*
	T/T	22 (59.5)	15 (40.5)		0.96 (0.42-2.19)	0.922
	T-carriers	91 (52.0)	84 (48.0)	0.086	0.60 (0.36-1.02)	0.057
Gene SNP (rs#)	Genotype	DCI (+) N (%)	DCI (-) N (%)	Unadjusted P-value	Adjusted§	
					OR (95% CI)	P-value
CYP2C8 g.16136C>G† Ile264Met [*4] (rs1058930)	C/C	90 (35.3)	165 (64.7)	0.049*	Reference	-
	C/G	17 (53.1)	15 (46.9)		2.21 (1.03-4.73)	0.041*
	G/G	0 (0)	0 (0)		-	-
	G-carriers	17 (53.1)	15 (46.9)	0.049*	2.21 (1.03-4.73)	0.041*

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; † Functional SNP; ‡ Tagging SNP; § Multivariate analysis included correction for age, sex, and Fisher grade; *Statistical significance established at $p<0.05$.

Figure 3-3: Cumulative incidence of CND in CYP2C8*4 carriers shows patients with the variant CYP2C8*4 (g.16136G) allele had a greater cumulative incidence of DCI (53.1%) and a trend for greater cumulative incidence of CND (71.8%) over 14 days compared to those with CYP2C8*1/*1 genotype (35.3% and 54%, p=0.032 and p=0.052, respectively). These relationships did not change after controlling for clinical covariates.

Figure 3-3: Cumulative incidence of CND in CYP2C8*4 carriers



The cumulative incidence of CND and DCI during the inpatient stay is compared in CYP2C8*4-carrier (11.1%) and CYP2C8*1/*1 (WT) genotype (88.9%) groups using Kaplan-Meier log rank analysis (n=287). Statistical significance was established at *p<0.05.

Table 3-9: Long-term outcomes in genetic groups shows the results comparing the genotype and allele frequencies with long-term outcomes. Results show that patients with the EPHX2 Thr404del (g.50690-50691insGTC) and CYP2C8*1/*2C (g.8633C/T) genotype were ~2.6-, ~2.6-, and 2.0-fold more likely to have unfavorable outcomes at 3 months (p=0.040, p=0.037, and p=0.045), respectively. Conversely, patients with CYP2C9 g.816G/A, CYP2J2*1/*7 (g.4930G/T) and EPHX2 287Arg/Gln (g.30221G/A) genotypes were ~2.1, 3.5, and 2.6-fold less likely develop unfavorable outcomes at 3 or 12 months (p=0.048, p=0.048, p=0.027), respectively. Our haplotype analysis showed no differences in outcomes among haplotype groups when compared to the most common haplotype for each candidate gene.

Table 3-9: Long-term outcomes in genetic groups

Gene SNP (rs#)	Genotype	MRS3 (3-6) N (%)	MRS3 (0-2) N (%)	Unadjusted P-value	Adjusted§ OR (95% CI)	Adjusted§ P-value
CYP2C9	Overall					0.096
g.8633C>T†	C/C	39 (25)	117 (75)		Reference	-
Arg144Cys [*2C]	C/T	22 (37)	38 (63)	0.186	2.01 (1.01-3.97)	0.045*
(rs1799853)	T/T	1 (50)	1 (50)		4.46 (0.23-84.97)	0.32
	T-carriers	23 (37)	39 (63)	0.074	2.06 (1.05-4.04)	0.036*
EXPH2	Overall					0.089
g. 50690-50691insGTC†	DEL/DEL	51 (26)	145 (74)		Reference	-
Thr404del	DEL/GTC	12 (52)	11 (48)	0.021*	2.81 (1.12-7.05)	0.028*
(rs71553864)	GTC/GTC	0 (0)	2 (100)		-	0.999
	GTC-carriers	12 (48)	13 (52)	0.022*	2.55 (1.04-6.24)	0.040*
Gene SNP (rs#)	Genotype	MRS12 (3-6) N (%)	MRS12 (0-2) N (%)	Unadjusted P-value	Adjusted§ OR (95% CI)	Adjusted§ P-value
CYP2C9	Overall					0.085
g.816A>G‡	A/A	28 (33)	58 (67)	0.034*	Reference	-
(rs2253635)	A/G	17 (18)	78 (82)		0.48 (0.23-0.99)	0.048*
	G/G	10 (37)	17 (63)		1.15 (0.44-2.97)	0.78
	G-carriers	27 (22)	95 (78)	0.093	0.61 (0.32-1.18)	0.14
CYP2J2	Overall					0.141
g.4930G>T†	G/G	55 (29)	133 (71)		Reference	-
c.-76G>T[*7]	G/T	3 (11)	24 (89)	0.037*	0.28 (0.08-0.99)	0.048*
(rs890293)	T/T	1 (100)	0 (0)		-	1
	T-carriers	4 (14)	24 (86)	0.115	0.36 (0.12-1.14)	0.082
EXPH2	Overall					0.086
g.30221G>A†	C/C	51 (32)	109 (68)		Reference	-
Arg287Gln	T/C	8 (15.4)	44 (84.6)	0.047*	0.38 (0.16-0.89)	0.027*
(rs751141)	T/T	0 (0)	2 (100)		-	0.999
	T-carriers	8 (15)	46 (85)	0.015*	0.36 (0.15-0.84)	0.019*

MRS, Modified Rankin Scale score at 3 and 12 months: Poor (3-6), Good (0-2); † Functional SNP; ‡ Tagging SNP. § Multivariate analysis included correction for age, sex, and Hunt & Hess Score. *Statistical significance established at $p<0.05$.

3.3.7 CYP eicosanoid levels in outcome groups

Results comparing CYP eicosanoid CSF levels in outcome groups are shown in Figure 3-4: EET and DHET CSF levels in outcome groups and Table 3-10: EET and DHET CSF levels in outcome groups. Mean and maximum EET CSF levels were ~2-fold higher in patients with unfavorable outcomes at 3 months (n=67) when compared to those with favorable outcomes (n=115) (p=0.030

and p=0.025), respectively. Similar relationships were observed in the multivariate analysis. Also, there was a trend for increased maximum DHET CSF levels in patients with CND compared (n=153) compared to those without CND (n=88) (p=0.056). No relationship was observed between mean and maximum CSF levels of individual DHET isomers and outcomes.

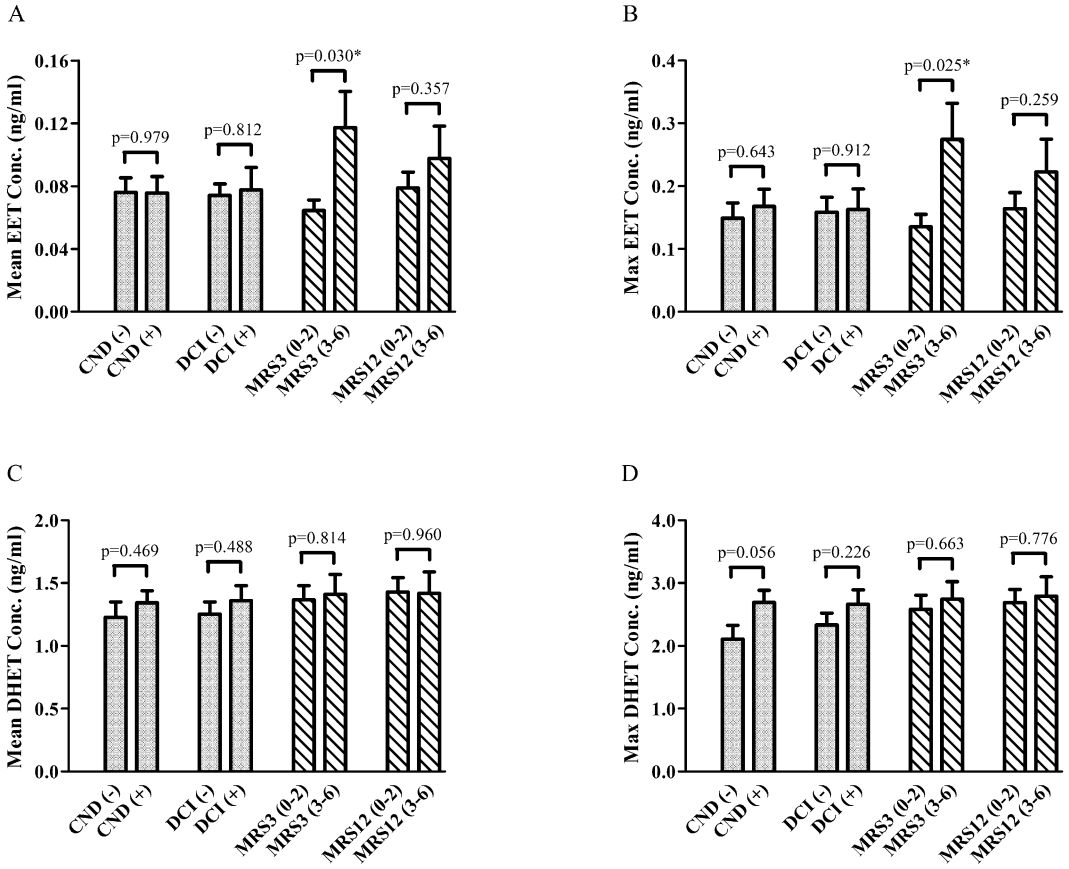


Figure 3-4: EET and DHET CSF levels in outcome groups

Mean EET (A), maximum EET (B), mean DHET (C) and maximum DHET (D) levels in CSF from patients with SAH are compared in outcomes groups. Acute outcomes (solid bars) included the presence or absence of delayed cerebral ischemia (DCI) and/or clinical neurological deterioration (CND) up to 14 days after the hemorrhage. Long-term outcomes (stripped bars) were determined by global functional recovery at 3 and 12 months using the Modified Rankin Scale (MRS) and were dichotomized into favorable (MRS 0-2) and unfavorable (MRS 3-6) groups. The mean and maximum EET and DHET CSF levels for each patient were calculated and were used to compare the population mean ± SEM of EET and DHET CSF levels in outcome groups using t-test with Welch's correction as appropriate.

Statistical significance established at *p<0.05.

Table 3-10: EET and DHET CSF levels in outcome groups

Outcome	Group	N	Mean EET Mean ± SEM (ng/ml)	P-value Unadjusted	P-value Adjusted§	Maximum EET Mean ± SEM (ng/ml)	P-value Unadjusted	P-value Adjusted§
CND	(-)	88	0.076 ± 0.009	0.979	0.935	0.149 ± 0.024	0.643	0.839
	(+)	153	0.076 ± 0.010			0.168 ± 0.027		
DCI	(-)	139	0.074 ± 0.007	0.812	0.285	0.158 ± 0.024	0.912	0.634
	(+)	103	0.078 ± 0.014			0.163 ± 0.033		
MRS3	Low (0-2)	115	0.065 ± 0.007	0.030*	0.007*	0.135 ± 0.020	0.025*	0.004*
	High (3-6)	67	0.117 ± 0.023			0.274 ± 0.058		
MRS12	Low (0-2)	117	0.079 ± 0.010	0.357	0.207	0.164 ± 0.025	0.259	0.096
	High (3-6)	61	0.098 ± 0.021			0.222 ± 0.052		
Outcome	Group	N	Mean DHET Mean ± SEM (ng/ml)	P-value Unadjusted	P-value Adjusted§	Maximum DHET Mean ± SEM (ng/ml)	P-value Unadjusted	P-value Adjusted§
CND	(-)	88	1.228 ± 0.122	0.469	0.434	2.109 ± 0.217	0.056	0.257
	(+)	153	1.342 ± 0.097			2.692 ± 0.194		
DCI	(-)	139	1.252 ± 0.097	0.488	0.788	2.333 ± 0.190	0.266	0.137
	(+)	103	1.359 ± 0.120			2.663 ± 0.230		
MRS3	Favorable (0-2)	115	1.365 ± 0.115	0.814	0.317	2.582 ± 0.225	0.663	0.469
	Unfavorable (3-6)	67	1.410 ± 0.157			2.741 ± 0.280		
MRS12	Favorable (0-2)	117	1.430 ± 0.112	0.96	0.545	2.688 ± 0.212	0.776	0.636
	Unfavorable (3-6)	61	1.420 ± 0.170			2.793 ± 0.305		

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Unfavorable (3-6), Favorable (0-2); § Multivariate analysis included correction for age, sex, and either Fisher Grade (for acute outcomes) or Hunt & Hess Score (for long-term outcomes); *Statistical significance established at p<0.05.

CYP eicosanoid levels in Fisher grade groups are shown in Figure 3-5: EET and DHET CSF levels by Fisher grade. Fisher grade was associated with mean EET levels (p=0.014), maximum EET levels (p=0.012), and showed a trend for an association with maximum DHET levels (p=0.084). Patients classified as Fisher grade 4 had higher mean and maximum EET levels (0.104 ± 0.021 ng/ml and 0.227 ± 0.047 ng/ml) compared with those in Fisher grade 2 (0.043 ± 0.003 ng/ml and 0.071 ± 0.010 ng/ml, p=0.010 and p=0.012, respectively). Patients classified as Fisher grade 3 showed a trend for higher maximum DHET levels (2.661 ± 0.196 ng/ml) compared to those in the Fisher grade 2 group (1.969 ± 0.221 ng/ml) (p=0.090).

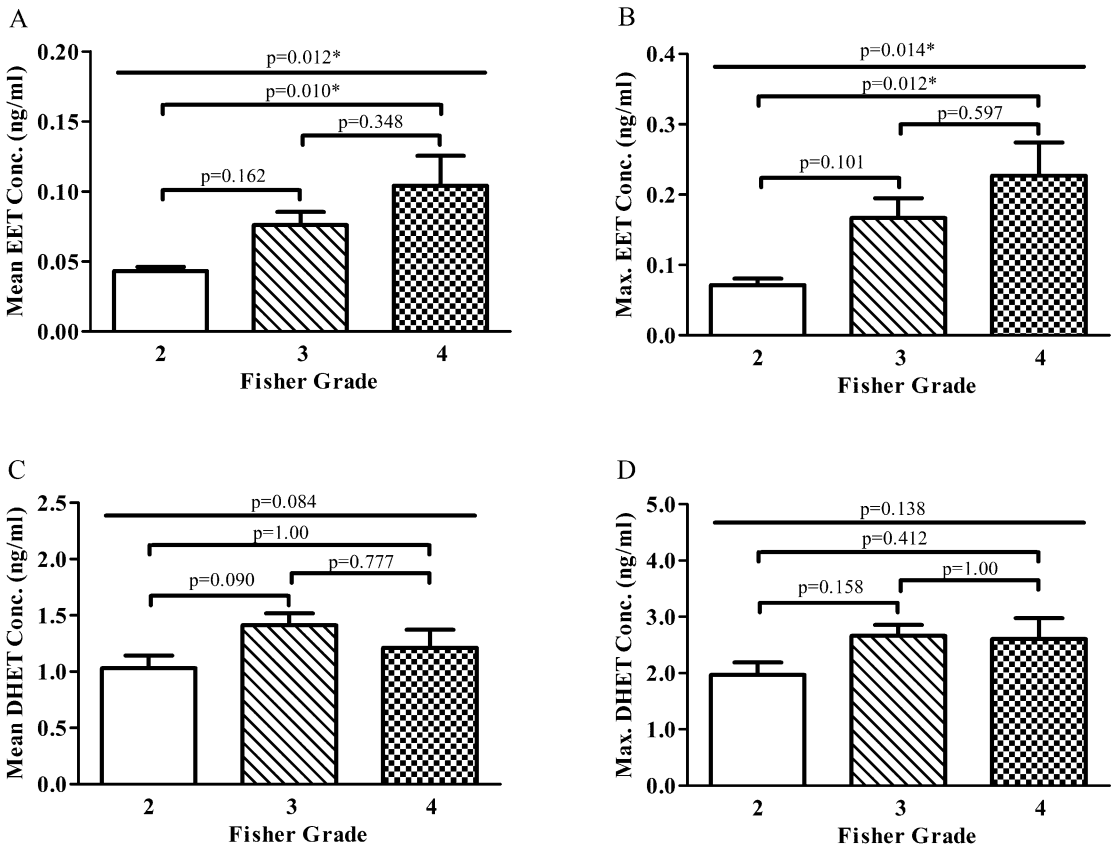


Figure 3-5: EET and DHET CSF levels by Fisher grade

The mean and maximum EET and DHET CSF levels for each patient were calculated and were used to compare the mean ± standard error of the mean (SEM) in Fisher Grade 2 (open bars, 24.1%), Fisher Grade 3 (stripped bars, 53.3%), and Fisher Grade 4 (checkerboard bars, 22.6%) using ANOVA with Bonferroni's *post-hoc* test (n=257). Statistical significance established at *p<0.05.

3.3.8 Trajectory analysis of CYP eicosanoid levels

DHET temporal concentration profiles and trajectory patterns are shown in Figure 3-6: DHET temporal concentration profiles and trajectory patterns. The trajectory model evaluating DHET CSF levels show three groups of patients with significantly different concentration profiles from days 2-11 after SAH (p<0.001). DHET trajectory groups were not associated with acute or long-term outcomes. Concentration values in trajectory groups are presented as geometric mean with

95% confidence interval. Patients in the "low" group (n = 45, 16.7%) have relatively low DHET levels that slightly increase over time (day 2: 0.27 ± 1.30 ng/ml; day 11: 0.39 ± 1.26 g/ml). Patients in the "moderate" group (n = 159, 59.1%) have relatively moderate DHET levels that slightly decrease over time (day 2: 0.98 ± 1.12 ng/ml; day 11: 0.75 ± 1.09 ng/ml). Patients in the "high" group (n = 65, 24.2%) have relatively high DHET levels that decrease over time (day 2: 3.30 ± 1.16 ng/ml; day 11: 1.89 ± 1.21 ng/ml). DHET trajectory groups were not associated with acute or long-term outcomes before and after adjusting for clinical covariates (Table 3-11: Outcomes in trajectory groups of DHET CSF levels).

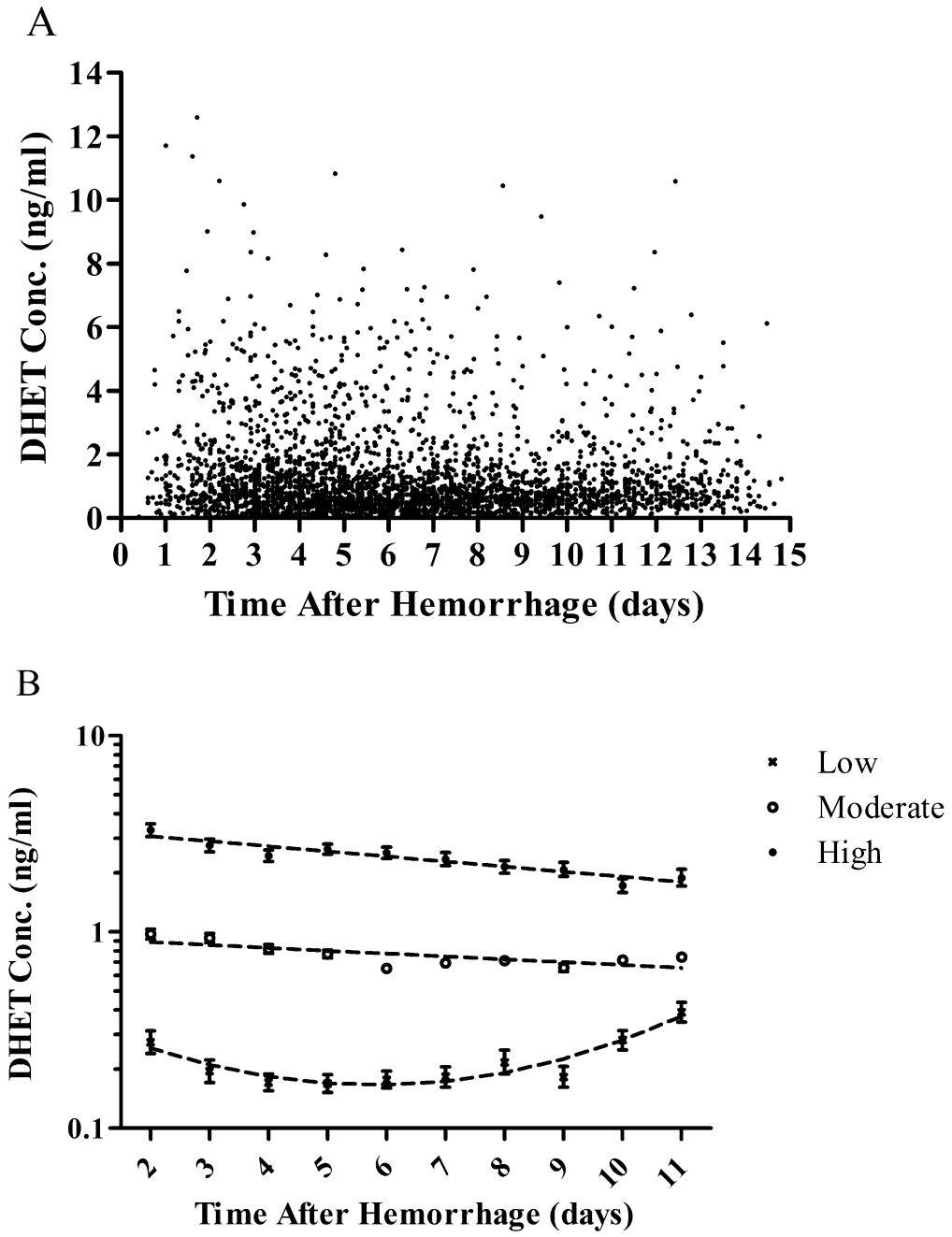


Figure 3-6: DHET temporal concentration profiles and trajectory patterns

Panel (A) shows raw population values of DHET CSF concentrations (ng/ml) from 269 patients up to 14 days after hemorrhage. Panel (B) shows DHET CSF concentration versus time from hemorrhage (days) in high [filled circles, n=65 (24.2%)], moderate [open circles, n=159 (59.1%)], and low [X, n=45 (16.7%)] concentration groups as identified by trajectory analysis. Concentration data are presented as geometric mean with 95% confidence interval.

Table 3-11: Outcomes in trajectory groups of DHET CSF levels

DHET Trajectory Group	CND (-) N (%)	CND (+) N (%)	P-value Unadjusted	P-value Adjusted§
Low	13 (31.7)	28 (68.3)	0.567	0.583
Moderate	55 (39.3)	85 (60.7)		
High	20 (33.3)	40 (66.7)		
DHET Trajectory Group	DCI (-) N (%)	DCI (+) N (%)	P-value Unadjusted	P-value Adjusted§
Low	22 (53.7)	19 (46.3)	0.409	0.465
Moderate	86 (61)	55 (39)		
High	31 (51.7)	29 (48.3)		
DHET Trajectory Group	MRS3 (0-2) N (%)	MRS3 (3-6) N (%)	P-value Unadjusted	P-value Adjusted§
Low	16 (57.1)	12 (42.9)	0.759	0.363
Moderate	68 (64.8)	37 (35.2)		
High	31 (63.3)	18 (36.7)		
DHET Trajectory Group	MRS12 (0-2) N (%)	MRS12 (3-6) N (%)	P-value Unadjusted	P-value Adjusted§
Low	14 (58.3)	10 (41.7)	0.699	0.727
Moderate	69 (66.3)	35 (33.7)		
High	34 (68)	16 (32)		

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Unfavorable (3-6), Favorable (0-2); § Multivariate analysis included correction for age, sex, and either Fisher Grade (for acute outcomes) or Hunt & Hess Score (for long-term outcomes); *Statistical significance established at $p<0.05$.

3.4 DISCUSSION

This clinical study is the first to investigate the impact of gene variants in the EET metabolic pathway on CYP eicosanoid CSF levels, risk for SAH, and subsequent acute/long-term outcomes in SAH patients. We report that patients with CYP2C8*4 showed lowered EET and DHET levels in CSF and had a greater likelihood of DCI and CND during the inpatient stay. Also, patients with

the loss-of-function variants CYP2J2*7 and CYP2C8*1B showed lower EET and DHET levels, respectively, while those with the CYP2C8 g.25369T allele had higher EET levels. Patients with the gain-of-function EPHX2 55Arg variant had lower EET levels and a trend for increased DHET levels. Patients classified as Fisher grade 4 (ventricular bleed) had increased EET levels compared to those in the Fisher grade 2 group (bleed <1mm thick). DHET CSF levels and trajectory patterns were not associated with outcomes. Patients with EPHX2 404del and CYP2C8*2C variants were more likely to have unfavorable long-term outcomes. Conversely, patients with CYP2J2*7, EPHX2 287Gln, and CYP2C9 g.816G>A variants were less likely to develop unfavorable long-term outcomes. Furthermore, we identified tSNPs that were not in HWE and showed genotype frequencies in our SAH population that were different than those in Hapmap CEU database, which may indicate putative genetic markers for SAH risk.

We observed previously reported relationships between CND, DCI, and MRS scores.[764] Likewise, we observed previously established relationships between HH score, Fisher grade, race, and increasing age with acute and/or long-term outcomes.[765] Caucasians had better 3-month outcomes compared to non-Caucasians as previously reported in patients with SAH and other stroke subtypes.[261] Collectively, these data suggest that the impact of race and age on EET formation/metabolism and outcomes after SAH may warrant further investigation.

Our genetic analysis showed that that multiple gene variants were associated with altered EET and DHET CSF levels. Patients with the CYP2C8*4 allele (11%) have reduced EET and DHET levels in CSF and are ~2.1- to 2.5-fold more likely to develop DCI and CND. Previous studies report that expression of CYP2C8*4 *in vitro* results in a ~5-fold reduction in protein levels and enzymatic activity towards AA to form 14,15-EET.[657, 663] CYP2C8*4 enzymes showed reduced carbon monoxide binding to the heme moiety and were more sensitive to proteinase K digestion indicating improper heme insertion and protein folding.[667] Furthermore, human liver samples and microsomes from Caucasians harboring CYP2C8*4 or a haplotype containing CYP2C8*4 showed reduced protein expression.[515, 665] Collectively, these studies suggest that CYP2C8*4 is a loss-of-function SNP associated with reduced EET and DHET levels and worse outcomes, which is consistent with our results.

Patients with at least one copy of the variant EPHX2 55Arg had lower mean and maximum EET levels and a trend for increased maximum DHET levels. This reduction in EET concentration

is consistent with the findings of Przybyla-Zawislak et al. who demonstrated that this variant increases SEH enzyme activity *in vitro*.[676] Similarly, studies report that CYP2J2*7 results in lower mRNA transcription *in vitro*.[505] In this study, we did not observe lower EET levels, but we report lower mean DHET levels in patients with the variant CYP2J2*7 allele. These results are expected since DHETs are considered surrogate marker for EETs. We expect worse outcomes in patients with CYP2J2*7, but we observed the opposite trend suggesting that other mediators may be involved. Since *in vitro* expression of CYP2C8*1B results in increased mRNA transcription compared to CYP2C8*1, we expect that patients with CYP2C8*1B may have higher EET and DHET levels.[665] However, CYP2C8*1B was associated with reduced maximum DHET levels in our study possibly due to differences in gene regulation in various species.[665] Also, CYP2C8 tSNPs and CYP2C8, CYP2C9, and EPHX2 haplotypes were associated with altered CYP eicosanoid levels. These data suggest that there are multiple SNPs on our candidate genes, potentially including SNPs that were not investigated in this study, which may affect EET synthesis and metabolism.

Results revealed that patients heterozygous for CYP2C9*2 (21%) are 2-fold more likely to develop unfavorable outcomes at 3 months. Lundbad et al. reported that expression of CYP2C9*4 *in vitro* results in 33% reduced activity towards AA to form EETs and that incubations of human liver microsomes from patients homozygous for CYP2C9*2 and CYP2C8*3 led to a 34% decrease in EET formation.[404] Moreover, human liver samples from two patients heterozygous for CYP2C9*2 showed 5- to 10-fold greater mRNA expression of 144Cys over 144Arg.[766] Collectively, these studies suggest that CYP2C9*2 is a loss-of-function SNP associated with reduced EET and DHET levels and worse outcomes. This hypothesis is consistent with our results regarding long-term outcomes.

We report that patients with the EPXH2 287Gln variant (25%) are ~2.6-fold less likely to develop unfavorable outcomes at 12 months. Previous studies report that *in vitro* expression of the EPHX2 (SEH) 287Gln enzyme resulted in ~5-fold reduction in 14,15-EET hydrolysis, higher SEH monomer:dimer ratio, and reduced enzyme stability.[676, 678, 679] Rat neuronal cell cultures and cardiomyocytes from SEH knockout mice transduced with the EPHX2 287Gln enzyme reduced 14,15-DHET levels to 34% and ~40% of WT values after administration of excess 14,15-EET and reduced ischemic cell death to 80% and 78% of values for untreated cells, respectively.[641, 677]

Moreover, clinical studies report that the EPHX2 287Gln variant was associated with reduced epoxyoctadecenoic acid (EpOME) metabolism and higher cholesterol and triglyceride levels in plasma.[718, 721] These findings suggest that the EPHX2 287Gln variant is a loss-of-function SNP associated with reduced EET metabolism and ischemic cell death. Therefore, it is expected that patients with EPHX2 287Gln would have increased EET levels, reduced DHET levels, and favorable outcomes. This hypothesis is consistent with our results regarding long-term outcomes.

We report that the EPHX2 404del variant was present in 11% of patients and was associated with a 2.6- to 3-fold increase in unfavorable outcomes at 3 months. Although the loss-of-function EPHX2 402-403ins/del variant (rs2234887)[678] was not genotyped, our genetic analysis includes rs71553864, which captures the variability in this gene region. The functional effects of this SNPs have not been previously investigated. Therefore interpretation of these results are difficult without further studies. Although multiple fSNPs and tSNPs were associated with acute and long-term outcomes, candidate gene haplotypes were not associated with outcomes. This discrepancy may be due to reduced power when using haplotypes or because the causative variant shows different linkage disequilibrium with haplotypes compared to tSNPs. These data suggest that additional studies are needed using a more focused genotype approach and improved characterization of candidate gene haplotypes.

HWE tests show that CYP2C9 g.18470G>A is not in HWE and, similar to other tSNPs in our candidate genes, showed genotype frequencies in our SAH population that were different than those in the Hapmap CEU population. Given that our population is enriched for individuals with SAH, these results suggest that genetic variation in the EET metabolic pathway may contribute to the formation and rupture of intracranial aneurysms. Support for these results includes numerous *in vivo* studies demonstrate that demonstrate EETs exhibit diverse physiologic functions including vasodilation of the cerebrovasculature and anti-hypertensive effects.[613, 767] *In vitro* studies report that EETs exhibit proliferative, migratory, angiogenic, fibrinolytic, anti-apoptotic, anti-inflammatory, and anti-platelet aggregation effects in vascular endothelial cells and inhibit migration and apoptosis in vascular smooth muscle cells.[355] Based on these reported mechanisms of action, it is expected that EETs play an important role in vascular homeostasis and remodeling and may impact intracranial aneurysm formation, rupture, or recovery after SAH.

We demonstrated the ability to measure DHET, and to a lesser extent EET, levels in CSF from patients with SAH. The low percentage of samples with detectable EET levels in our study may be due to rapid EET metabolism or binding to the collection bag. As expected, we measured a large percentage of CSF samples with 14,15-DHET and 11,12-DHET. *In vitro* studies suggest that CYP2C8/9 and CYP2J2 produce high, moderate, and low amounts of 14,15-EET, 11,12-EET, and 8,9-EET, respectively.[768] Based on these data, we expect high, moderate, and low amounts of 14,15-DHET, 11,12- DHET, and 8,9- DHET in CSF from SAH patients. As expected, we reported a large percentage of samples with 14,15-DHET and 11,12- DHET and low percentage of samples with detectable 5,6-DHET. But, we also reported a large percentage of samples with high concentrations of 8,9-DHET. This may be due to differences in the metabolism of 8,9-EET or storage and release of 8,9-DHET compared to other EET regioisomers in brain tissues.[769] Thus, future studies are needed to identify the factors responsible for the differences in EET and DHET regioisomer formation and release in humans and patients with SAH.

Although EET CSF levels in patients with SAH have not been previously reported in clinical studies, our DHET levels were consistent with our previous studies.[642, 643] EET and DHET CSF levels measured in this study are comparable to EET levels reported to demonstrate physiological effects on cerebrovascular tissues. For example, EETs have been shown to relax isolated cerebral arteries and increase the activity of K_{Ca+2} channels in cerebral VSMC at 1 nM (0.32 ng/ml).[625] It is expected that the CYP eicosanoid levels in the brain will be higher than those reported in this study because these compounds act locally in an autocrine/paracrine fashion[355] and then are diluted in CSF. In addition, any spikes in concentration will be diluted in the CSF drainage bags during the collection period.

Our analysis of CYP eicosanoids showed that DHET CSF levels and trajectory patterns were not associated with acute or long-term outcomes, but increased EET CSF levels were associated with unfavorable 3-month outcomes. This relationship was not expected due to the anti-inflammatory, vasodilatory, and neuroprotective properties of EETs.[355] On the other hand, increased EET levels were associated with Fisher grade 4, which is reported to be strongly associated with worse acute and long-term outcomes.[765] These data suggest that the degree and location of the hemorrhage may affect EET formation and/or release in the brain and possibly long-term outcomes. Red blood cells can release EETs directly from phospholipid membranes or

synthesize EETs through the release and subsequent metabolism of AA from phospholipid membranes.[406] However, it is unknown whether an increase in EET CSF levels are due to EET release or synthesis directly from the blood in the CSF or from brain tissues. Also, it is important to note that this relationship was not observed in acute outcomes and 12-month outcomes and that the analysis did not include the measurement of vasoconstrictors that may counteract the effects of EETs in the brain. The role of the EET metabolic pathway in SAH has not been previously investigated in clinical studies and therefore warrants further study.

In spite of these novel findings, several limitations of this study should be noted. The analysis of CYP eicosanoid CSF levels were not compared control values due to lack of access to control CSF. Since the CSF samples were taken from drainage bags, which collected CSF over a 12 hour time period, reported CYP eicosanoid levels represent time-averaged values that may be lower than those at the site of action. Also, there was a significantly lower number of CSF samples with EET levels above the quantitation limit when compared to samples with detectable DHET levels possibly due EET conversion to DHETs or reduced recovery from the collection bag. In our genetic analysis, some functional genetic variants in the EET metabolic pathway were not genotyped. Many SNPs had allele frequencies that were too low for informative analysis. Our analyses were limited to single fSNP analyses, thus the effects of multiple fSNPs simultaneously were not assessed. It is not possible to determine if our putative genetic markers were causative or were in linkage disequilibrium with the causative SNP. In addition, p-values from significant findings did not pass correction criteria for multiple testing. Furthermore, this study represents one of the first reports of a large population of SAH patients for genotype/CYP eicosanoid analysis, therefore, a validation cohort is not readily available for confirmation of these genotype findings. Future studies that include a focused genotype approach, validation cohort, and/or extensive CYP eicosanoid analysis including other vasoconstrictors, such as 20-HETE, are needed.

3.5 CONCLUSIONS

In summary, EETs have been shown to play an important role in the regulation of cerebrovascular

tone and vessel remodeling *in vitro* and significantly affect CBF and cerebral ischemic injury *in vivo*. Other studies report that polymorphisms in the genes responsible for the formation or metabolism of EETs alter enzyme expression or activity. This study, involving one of the largest SAH cohorts to date, suggests that gene variants involved in EET formation/metabolism are associated with the risk for complications after SAH and subsequent acute and long-term outcomes. These results are important to help elucidate the mechanisms involved in the pathogenesis and pathophysiology of SAH and possibly identify patients at high risk for unfavorable outcomes so that intervention strategies may be implemented earlier or more aggressively in these patients.

4.0 20-HETE RELATIONSHIP WITH OUTCOMES AFTER SAH

[Mark K. Donnelly, Yvette P. Conley, Elizabeth A. Crago, Paula R. Sherwood, Dianxu Ren, Jeffrey R. Balzer, Patrick M. Kochanek, Samuel M. Poloyac. "20-HETE is associated with ischemic complications and unfavorable outcomes in patients with aneurysmal subarachnoid hemorrhage." Submitted to *Lancet Neurol* in June 2014.]

4.1 INTRODUCTION

As discussed in Section 1.1, SAH is devastating condition associated with high rates of mortality and morbidity that can be attributed in large part to the development of ischemic complications such as CND and DCI that typically occur 3-14 days after SAH. Despite this generous time window for therapeutic intervention, strategies to improve outcomes have had limited success in part due to the lack of reliable and accurate methods to identify high-risk patients and a poor understanding of the underlying mechanisms. Currently, there are no established predictors for the development of acute ischemic complications in the first two weeks after SAH and long-term functional outcomes.

Previous studies report that 20-HETE is formed in multiple brain regions and regulates cerebrovascular tone and structure.[355] In humans, CYP4F2 and CYP4A11 are the primary enzymes responsible for 20-HETE synthesis from AA.[356] In the brain, 20-HETE promotes vasoconstriction, angiogenesis, inflammation, apoptosis, and platelet aggregation.[356] Recent evidence suggests that 20-HETE mediates CBF regulation by pericytes and 20-HETE inhibition by NO facilitates prostaglandin E_2 mediated dilation.[631] Collectively, these studies suggest that 20-HETE is a critical mediator of CBF by regulating microvascular tone and vascular remodeling.

Recent evidence implicates 20-HETE in the pathophysiology of cerebral injury due to ischemic and hemorrhagic stroke. Inhibitors of 20-HETE synthesis improve CBF after SAH and reduce infarct size after temporary focal ischemia while 20-HETE agonists reduce baseline CBF in rats.[589, 590] Also, 20-HETE formation is reduced by NO, a known mediator of SAH pathophysiology.[317, 356] Currently, no studies have evaluated the role of 20-HETE in a large SAH cohort. Therefore, we hypothesized that loss-of-function polymorphisms in 20-HETE synthesis genes (CYP4A11 and CYP4F2) will be associated with decreased 20-HETE concentrations in CSF resulting and decreased risk for ischemic complications and unfavorable long-term functional outcomes in patients with SAH. Furthermore, we compared genotype frequencies in our SAH population with those reported in the general population using the Hapmap database to identify potential genetic markers for SAH.

4.2 METHODS

4.2.1 Design and participants

The study design and participants for this section are identical to those described in Section 3.2.1. Briefly, the study included 363 adult patients with SAH classified as Fisher grade >1. CSF was available from 269 patients for 20-HETE quantitation and blood samples were taken from 304 Caucasians for genotyping. All patients received standard medical care.[760]

4.2.2 Analysis of 20-HETE CSF levels

Analysis of 20-HETE levels in CSF from SAH patients was performed in the manner described in Section 2.2. Briefly, samples were withdrawn from collection bags on external ventricular drains approximately every 12 hours for up to 14 days. We previously demonstrated stability of 20-HETE assessment from sample collection bags.[643] CSF samples of 2-3 ml were processed using SPE and reconstituted in 50 μl of 80:20 MeOH:dH_20. Quantitation of CYP-eicosanoids was performed using a published UPLC-MS/MS method with minor modifications.[643] 20-HETE concentrations were determined from the standard curve of the ratio of their peak area to IS peak areas of 20-HETE-*d6* over a linear range of 0.014-8.88 ng/ml. EET and DHET concentrations were determined as described in section 3.2.2.

4.2.3 Analysis of gene variants in 20-HETE synthesis pathway

Candidate genes associated with 20-HETE synthesis include CYP4A11 and CYP4F2. Tagging single nucleotide polymorphisms (tSNPs) were selected using the CEU population from Hapmap database (Release 27; www.hapmap.org) and criteria included $r^2 > 0.8$ and minor allele frequency $\geq 20\%$ while functional SNPs (fSNPs) were defined as those previously reported to affect mRNA transcription, protein expression, or enzyme activity *in vitro*. Our genetic analysis excluded SNPs with variant genotype frequencies <1% in our SAH population. Our genetic analysis included five tSNPs (CYP4A11-g.13414C>G, CYP4F2-g.4593T>C, CYP4F2-g.4211A>T, CYP4F2-g.8575T>C, CYP4F2-g.16162A>G), six fSNPs (CYP4A11-g.4207A>G, CYP4A11-g.13661G>A, CYP4F2-g.7222002G>A, CYP4F2-g.5416G>C, CYP4F2-g.5373T>C CYP4F2-g.5497T>C), and one fSNP/tSNP (CYP4F2-g.14389C>T) as shown in Table 4-1: Candidate gene variants in 20-HETE pathway included in the analysis. Genotyping was performed using Taqman allele discrimination assay with ABI Prism 7000 Sequence Detection System (Applied Bioscience, Carlsbad, CA, USA) for rs3093105, sequencing with BigDye and ABI 3730xl (Applied Bioscience, Carlsbad, CA, USA) for rs1126742, and iPLEX MassArray (Sequenom, San Diego, CA) for all other SNPs. Consistency and integrity of genotyping data was checked by inclusion of duplicate CEPH controls on each plate for internal as well as plate-to-plate consistency, using genotype call rate criteria of >85%, comparing the observed and Hapmap Caucasian (CEU) frequencies, and performing checks for Hardy-Weinberg Equilibrium (HWE) consistency. Analyses of genetic data included both genotype groups (codominant) and presence or absence of the variant allele (dominant) groups. SNPstats software was used to assign haplotypes for candidate genes and combinations of fSNPs on different candidate genes.[761]

Table 4-1: Candidate gene variants in 20-HETE pathway included in the analysis

Gene	rs# (fSNP/tSNP)	Genomic	cDNA	Protein	CYP Nomenclature
CYP4A11	rs9332978 (†)	g.4207A>G	c.-825A>G	NA	NA
	rs1126742 (†)	g.13661G>A	c.1301G>A	434Phe>Ser	NA
	rs3890011 (‡)	g.13414C>G	c.1223-24C>G	NA	NA
CYP4F2	rs2108622 (†‡)	g.14389C>T	c.1297C>T	433Val>Met	CYP4F2*3
	rs2189784 (†)	g.7222002G>A	NA	NA	NA
	rs3093100 (†)	g.5416G>C	c.-48G>C	NA	NA
	rs3093098 (†)	g.5373T>C	c.-91T>C	NA	NA
	rs3093105 (†)	g.5497T>C	c.34T>G	12Trp>Arg	CYP4F2*2
	rs3093089 (‡)	g.4593T>C	c.-458T>C	NA	NA
	rs3093156 (‡)	g.4211A>T	c.648-106A>T	NA	NA
	rs3093168 (‡)	g.8575T>C	c.1115+489T>C	NA	NA
	rs3093207 (‡)	g.16162A>G	c.*923A>G	NA	NA

4.2.4 Outcomes assessment

Outcomes were assessed as described in Section 3.2.4. Briefly, acute outcomes included the presence or absence of CND and/or DCI during the inpatient stay (up to 14 days).[762] Long-term outcomes were determined by global functional recovery at 3 and 12 months using the Modified Rankin score (MRS) obtained during face-to-face interview or phone call with the patient or their surrogate.

4.2.5 Statistical analysis

Statistical analysis was performed as described in Section 3.2.5. Statistical significance was determined at *p-values<0.05. Bonferroni's multiple comparison correction was used in analyses involving genetic and biomarker concentration data (**p<0.0042 and **p<0.0167, respectively).

4.3 RESULTS

4.3.1 Quantitation of 20-HETE CSF samples

Table 4-1: Candidate gene variants in 20-HETE pathway included in the analysis shows the concentrations of CYP eicosanoids in CSF from SAH patients along with the percentage of samples and patients with detectable levels. Detectable 20-HETE, EET, and DHET levels were found in 71.6%, 13.1%, and 97.9% of samples (n=3151) and 98.5%, 64.3%, and 98.9% of patients (n=269), respectively.

CYP-Eicosanoid	Mean Conc. Mean ± SEM (ng/ml)	Maximum Conc. Mean ± SEM (ng/ml)	Detectable Samples N (%) [Total=3151]	Detectable Patients N (%) [Total=269]
20-HETE	0.099 ± 0.012	0.307 ± 0.038	2255 (71.6)	265 (98.5)
EET	0.073 ± 0.007	0.153 ± 0.018	414 (13.1)	173 (64.3)
14,15-EET	0.016 ± 0.001	0.037 ± 0.006	140 (4.4)	123 (45.7)
11,12-EET	0.013 ± 0.001	0.023 ± 0.004	166 (5.3)	87 (32.3)
8,9-EET	0.044 ± 0.006	0.101 ± 0.015	271 (8.6)	149 (55.4)
DHET	1.271 ± 0.069	2.462 ± 0.140	3084 (97.9)	266 (98.9)
14,15-DHET	0.053 ± 0.003	0.114 ± 0.008	2152 (68.3)	241 (89.6)
11,12-DHET	0.031 ± 0.002	0.078 ± 0.007	1948 (61.8)	189 (70.3)
8,9-DHET	1.171 ± 0.069	2.329 ± 0.138	3025 (96.0)	264 (98.1)
5,6-DHET	0.016 ± 0.001	0.048 ± 0.007	382 (12.1)	113 (42.0)

Figure 4-1: Quantitation of CYP eicosanoids in CSF samples

Detectable Concentration ≥ 0.014 ng/ml

4.3.2 20-HETE CSF levels in Fisher grade groups

A comparison of 20-HETE CSF levels SAH patients by Fisher grade is shown in Figure 4-2: 20-HETE CSF levels by Fisher grade. 20-HETE was higher in patients classified as Fisher grade 4 when compared to Fisher grade 2/3.

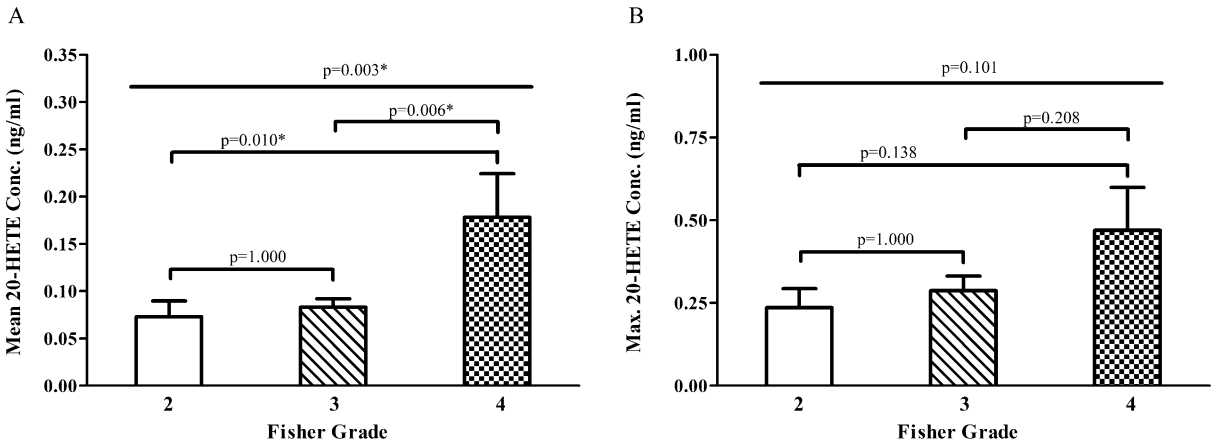


Figure 4-2: 20-HETE CSF levels by Fisher grade

The mean and maximum 20-HETE CSF levels for each patient were calculated and were used to compare the mean ± standard error of the mean (SEM) in Fisher grade 2 (open bars, 24.1%), Fisher grade 3 (stripped bars, 53.3%), and Fisher grade 4 (checkerboard bars, 22.6%) using ANOVA with Bonferroni's *post-hoc* test (n=257). Statistical significance established at *p<0.05.

4.3.3 20-HETE CSF levels in outcome groups

20-HETE CSF levels in acute and long-term outcomes groups is shown in Figure 4-3: 20-HETE CSF levels in outcome groups. Patients with CND had ~2.2- and 2.6-fold higher mean and maximum 20-HETE levels, respectively, when compared to those without CND (p=0.001). Patients with unfavorable 3-month outcomes had ~2.7- and 2.6-fold higher mean and maximum 20-HETE levels when compared to those with favorable outcomes (p=0.009 and p=0.010), respectively. Patients with unfavorable 12-month outcomes showed a trend for a 2.2- and 1.6-fold increase in mean and maximum 20-HETE levels when compared to those with favorable outcomes (p=0.052 and p=0.063), respectively. Similar relationships were observed between 20-HETE:EET and 20-HETE:DHET CSF levels and outcomes (Figure 4-3: 20-HETE CSF levels in outcome groups and Table 4-2: 20-HETE CSF levels and ratios in outcome groups). Also, adjusting for covariates did not significantly change the relationship between 20-HETE, 20-HETE:EET, and 20-HETE:DHET CSF levels and outcomes in the multivariate analysis.

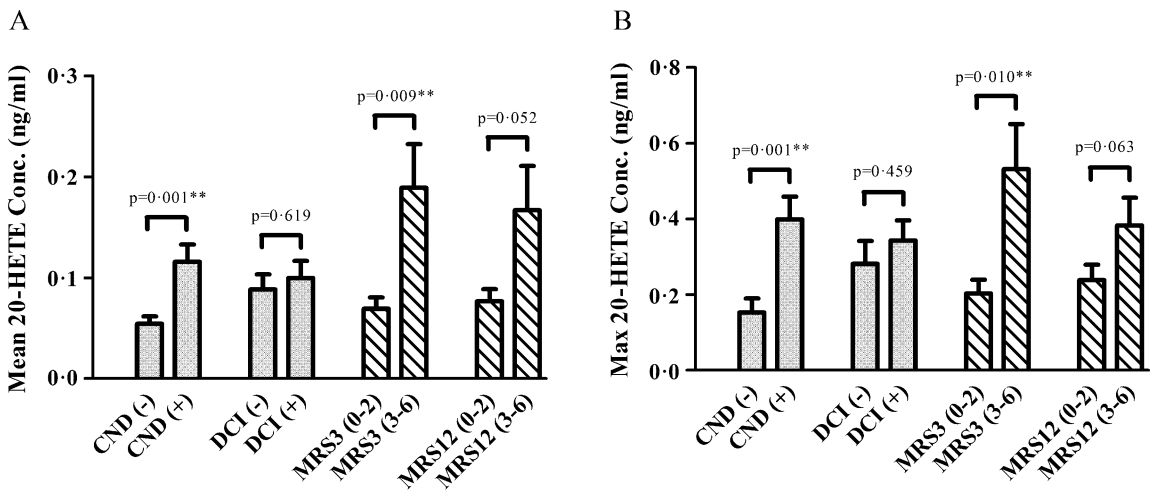


Figure 4-3: 20-HETE CSF levels in outcome groups

Mean (panel A) and maximum (panel B) 20-HETE levels in CSF from patients with SAH are compared in outcomes groups. Acute outcomes (solid bars) included the presence or absence of clinical neurological deterioration (CND) and/or delayed cerebral ischemia (DCI) up to 14 days after the hemorrhage. Long-term outcomes (stripped bars) were determined by global functional recovery at 3 and 12 months using the Modified Rankin Scale (MRS) and were dichotomized into favorable (MRS 0-2) and unfavorable (MRS 3-6) groups. The mean and maximum 20-HETE CSF levels for each patient (n=269) were calculated and were used to compare the mean ± SEM of the CYP eicosanoid levels in outcome groups using t-test with Welch's correction as appropriate. Statistical significance established at *$p<0.05$ and **$p<0.0167$ (correction for multiple comparisons).

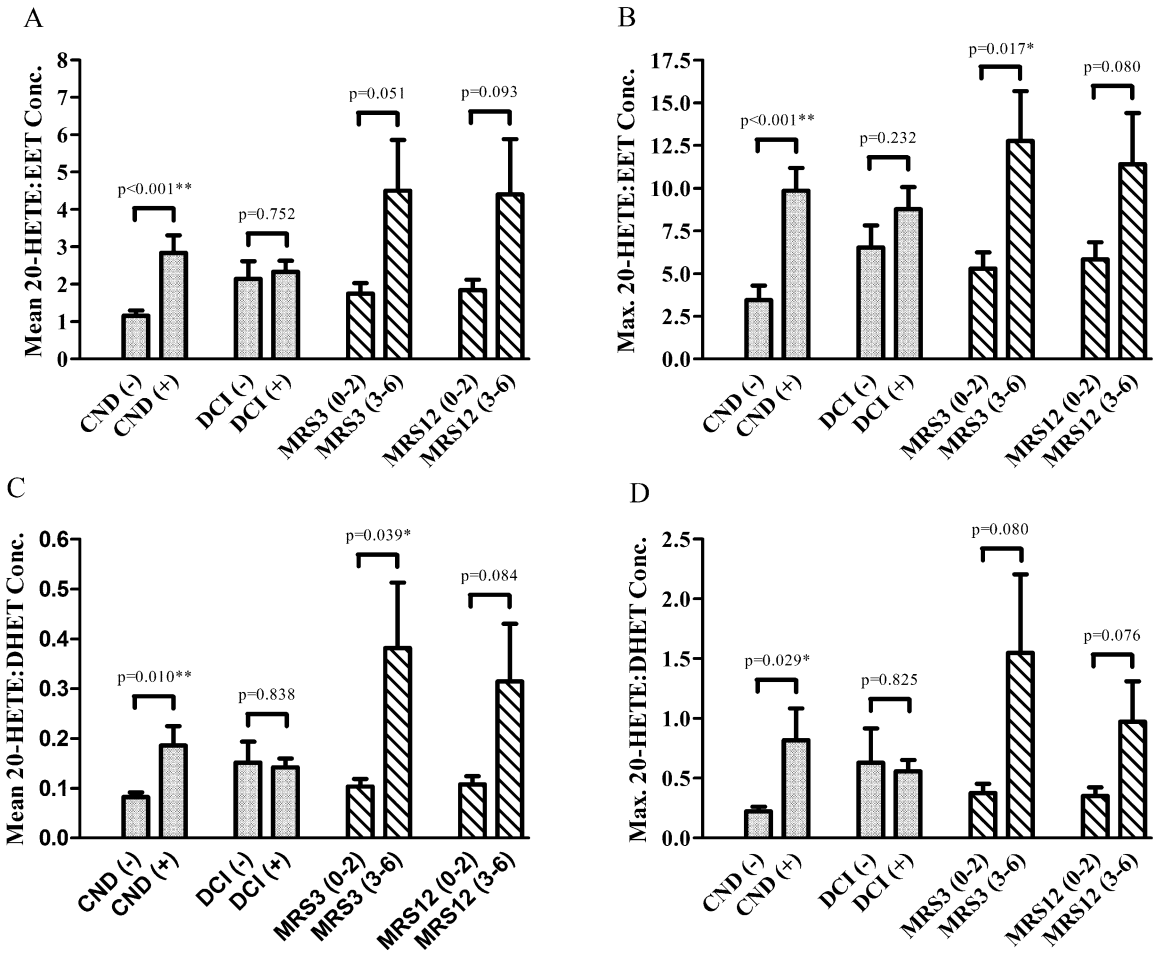


Figure 4-4: 20-HETE:EET and 20-HETE:DHET CSF levels in outcome groups

Mean 20-HETE:EET (panel A), maximum 20-HETE:EET (panel B), mean 20-HETE:DHET (panel C), and maximum 20-HETE:DHET (panel D) levels CSF in patients with SAH are compared in outcomes groups. Acute outcomes (solid bars) included the presence or absence of delayed cerebral ischemia (DCI) and/or clinical neurological deterioration (CND) up to 14 days after the hemorrhage. Long-term outcomes (stripped bars) were determined by global functional recovery at 3 and 12 months using the Modified Rankin Scale (MRS) and were dichotomized into good (MRS 0-2) and poor (MRS 3-6) groups. The mean and maximum 20-HETE CSF levels for each patient (n=269) were calculated and were used to compare the mean ± SEM of the CYP eicosanoid levels in outcome groups using t-test with Welch's correction as appropriate. Statistical significance established at *p<0.05 and **p<0.0167 (correction for multiple comparisons).

Table 4-2: 20-HETE CSF levels and ratios in outcome groups

Outcome	Group	N	Mean 20-HETE (ng/ml)	Unadjusted P-value	Adjusted§ P-value	Max. 20-HETE (ng/ml)	Unadjusted P-value	Adjusted§ P-value
CND	(-)	88	0.054 ± 0.007	0.001**	0.019*	0.153 ± 0.037	0.001**	0.009**
	(+)	153	0.116 ± 0.017			0.399 ± 0.061		
DCI	(-)	139	0.088 ± 0.015	0.619	0.817	0.281 ± 0.060	0.459	0.587
	(+)	103	0.100 ± 0.017			0.343 ± 0.053		
MRS3	Low (0-2)	115	0.069 ± 0.011	0.009**	0.033*	0.203 ± 0.036	0.010**	0.012**
	High (3-6)	67	0.189 ± 0.043			0.532 ± 0.119		
MRS12	Low (0-2)	117	0.077 ± 0.012	0.052	0.087	0.239 ± 0.040	0.063	0.152
	High (3-6)	61	0.167 ± 0.044			0.383 ± 0.074		
Outcome	Group	N	Mean 20-HETE:DHET	Unadjusted P-value	Adjusted§ P-value	Max. 20-HETE:DHET	Unadjusted P-value	Adjusted§ P-value
CND	(-)	88	0.082 ± 0.010	0.010**	0.008**	0.225 ± 0.037	0.029*	0.002**
	(+)	153	0.186 ± 0.039			0.818 ± 0.267		
DCI	(-)	139	0.152 ± 0.041	0.838	0.565	0.630 ± 0.288	0.835	0.614
	(+)	103	0.142 ± 0.018			0.558 ± 0.096		
MRS3	Low (0-2)	115	0.103 ± 0.016	0.039*	0.045*	0.375 ± 0.078	0.080	0.062
	High (3-6)	67	0.381 ± 0.131			1.550 ± 0.656		
MRS12	Low (0-2)	117	0.108 ± 0.016	0.084	0.074	0.352 ± 0.072	0.076	0.085
	High (3-6)	61	0.314 ± 0.116			0.974 ± 0.337		
Outcome	Group	N	Mean 20-HETE:EET	Unadjusted P-value	Adjusted§ P-value	Max. 20-HETE:EET	Unadjusted P-value	Adjusted§ P-value
CND	(-)	88	1.155 ± 0.140	0.001**	0.003**	3.449 ± 0.849	<0.001**	0.005**
	(+)	153	2.844 ± 0.465			9.848 ± 1.348		
DCI	(-)	139	2.140 ± 0.479	0.752	0.915	6.532 ± 1.292	0.232	0.291
	(+)	103	2.334 ± 0.297			8.775 ± 1.301		
MRS3	Low (0-2)	115	1.753 ± 0.275	0.051	0.165	5.303 ± 0.948	0.017*	0.029*
	High (3-6)	67	4.505 ± 1.357			12.762 ± 2.919		
MRS12	Low (0-2)	117	1.841 ± 0.282	0.093	0.125	5.844 ± 0.984	0.080	0.115
	High (3-6)	61	4.405 ± 1.476			11.418 ± 2.987		

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Poor (3-6), Good (0-2); § Multivariate analysis included correction for age, sex, race, and either Fisher Grade (for acute outcomes) or Hunt & Hess score (for long-term outcomes); *Statistical significance established at *p<0.05 or **p<0.0167 (for multiple comparison correction)

4.3.4 20-HETE temporal concentration profiles and trajectory patterns

20-HETE temporal concentration profiles and trajectory patterns are shown in Figure 4-5: 20-HETE temporal concentration profiles and trajectory patterns. The trajectory model evaluating 20-HETE CSF levels identified three groups of patients with concentration profiles categorized "low", "moderate", and "high" ($p<0.001$). A comparison of 20-HETE trajectory patterns with acute and long-term outcomes is shown in Table 4-3: Outcomes in trajectory groups of 20-HETE CSF levels. 20-HETE trajectory groups showed an association with CND, 3-month MRS, and 12-

month MRS (p=0.012, p=0.001, and p=0.046, respectively). The cumulative incidence of CND over 14 days in 20-HETE trajectory groups are shown in Figure 4-6: Cumulative incidence of CND and DCI in 20-HETE trajectory groups. Patients in moderate/high 20-HETE trajectory groups were 2.1-, 2.5-, and 2.1-fold more likely to have CND and unfavorable 3- and 12-month MRS (p=0.004, p=0.002, and p=0.014, respectively) and had lower median time to develop CND (p=0.002).

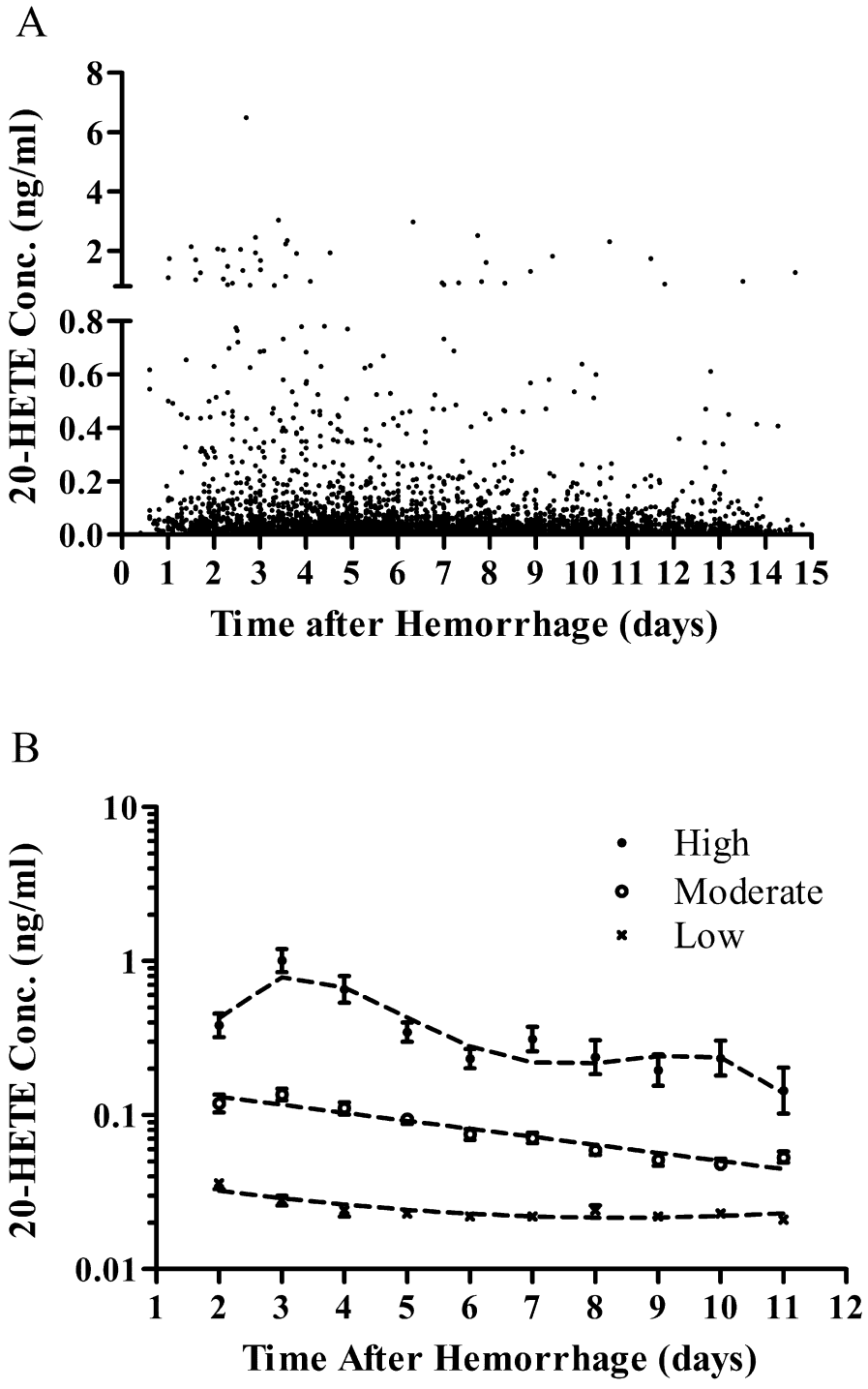


Figure 4-5: 20-HETE temporal concentration profiles and trajectory patterns

Panel (A) shows raw population values of 20-HETE CSF concentrations from 269 patients up to 14 days after hemorrhage. Panel (B) shows 20-HETE CSF concentration versus time from hemorrhage (days) in high [filled circles, n=21 (7.8%)], moderate [open circles, n=75 (27.9%), and low [X, n=173 (64.3%)] concentration groups as identified by trajectory analysis. Concentration data are presented as geometric mean with 95% confidence interval (CI).

4.3.5 Outcomes in 20-HETE trajectory groups

Table 4-3: Outcomes in trajectory groups of 20-HETE CSF levels

20-HETE Trajectory	**CND (-) N (%)**	**CND (+) N (%)**	**Unadjusted P-value**	**Adjusted§**	
				OR (95% CI)	**P-value**
Overall					0.047*
Low	68 (43)	90 (57)	0.012**	Reference	-
Moderate	17 (25.8)	49 (74.2)		1.98 (1.02 - 3.82)	0.043*
High	3 (17.6)	14 (82.4)		3.07 (0.82 - 11.47)	0.096
Moderate/High	20 (24.1)	63 (75.9)	0.004**	2.14 (1.16 - 3.96)	0.015**
20-HETE Trajectory	**DCI (-) N (%)**	**DCI (+) N (%)**	**Unadjusted P-value**	**Adjusted§**	
				OR (95% CI)	**P-value**
Overall					0.342
Low	97 (61)	62 (39)	0.283	Reference	-
Moderate	34 (51.5)	32 (48.5)		1.46 (0.81 - 2.66)	0.208
High	8 (47.1)	9 (52.9)		1.66 (0.59 - 4.66)	0.334
Moderate/High	42 (50.6)	41 (49.4)	0.120	1.50 (0·87 - 2.61)	0.148
20-HETE Trajectory	**MRS3 (0-2) N (%)**	**MRS3 (3-6) N (%)**	**Unadjusted P-value**	**Adjusted§**	
				OR (95% CI)	**P-value**
Overall					0.011**
Low	85 (71.4)	34 (28.6)	0.001**	Reference	-
Moderate	25 (55.6)	20 (44.4)		1.88 (0.88 - 4.03)	0.105
High	5 (27.8)	13 (72.2)		5.33 (1.65 - 17.22)	0.005**
Moderate/High	30 (47.6)	33 (52.4)	0.002**	2.49 (1.16 - 4.93)	0.009**
20-HETE Trajectory	**MRS12 (0-2) N (%)**	**MRS12 (3-6) N (%)**	**Unadjusted P-value**	**Adjusted§**	
				OR (95% CI)	**P-value**
Overall					0.111
Low	83 (72.2)	32 (27.8)	0.046*	Reference	-
Moderate	26 (55.3)	21 (44.7)		2.08 (0.99 - 4.38)	0.053
High	8 (50)	8 (50)		2.00 (0.65 - 6.15)	0.224
Moderate/High	34 (54)	29 (46)	0.014**	2.06 (1.05 - 4.06)	0.036*

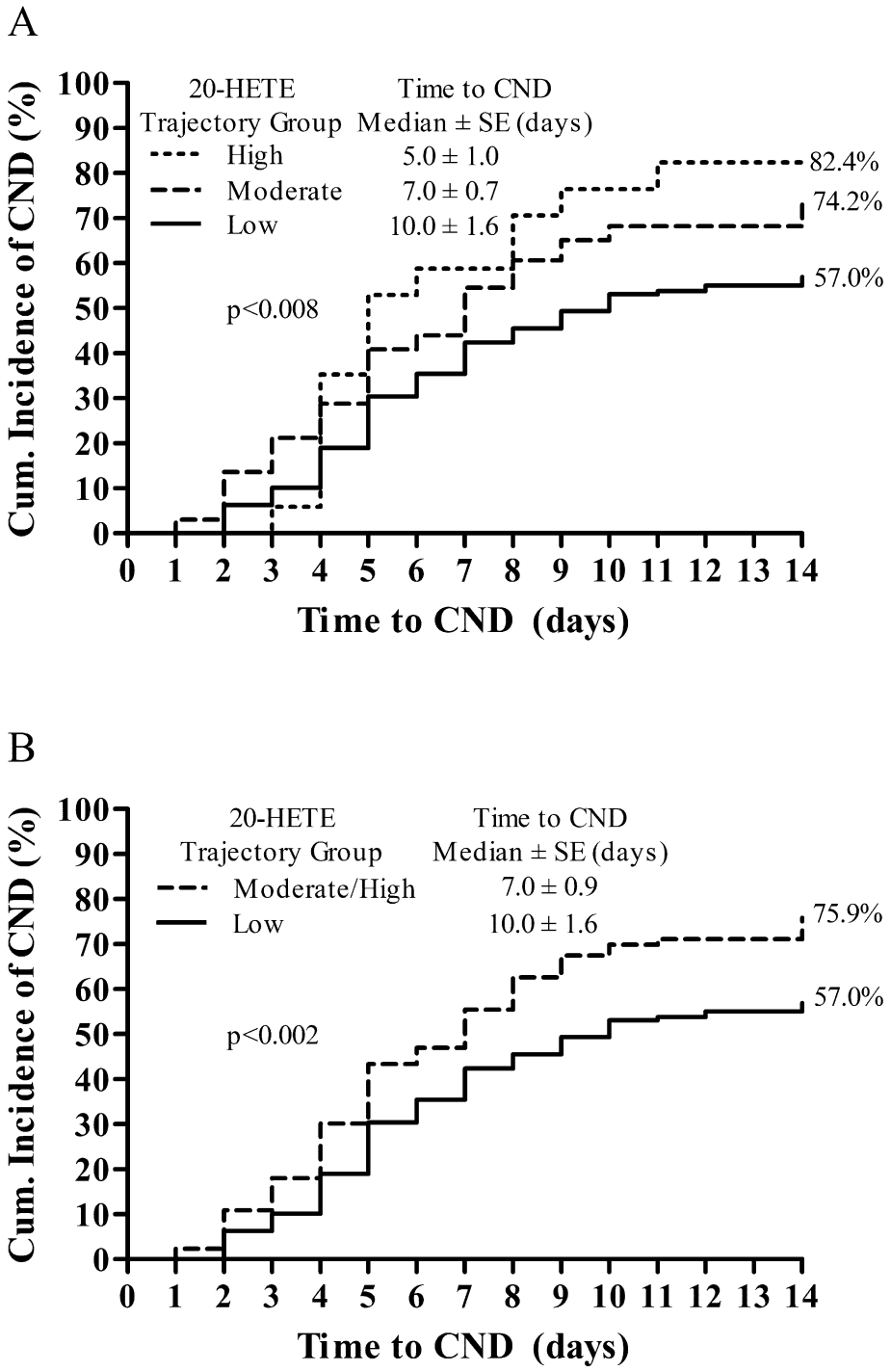


Figure 4-6: Cumulative incidence of CND and DCI in 20-HETE trajectory groups

The cumulative incidence of clinical neurological deterioration (CND) in 153 patients (panel A) and delayed cerebral ischemia (DCI) in 103 patients (panel B) was compared in trajectory groups using Kaplan-Meier log-rank analysis.

For the analysis, the time to develop CND and DCI was rounded up to the nearest day and the trajectory groups were defined in Figure 4-5: 20-HETE temporal concentration profiles and trajectory patterns. Cox regression was used to control for age, sex, race, and Fisher grade or Hunt and Hess (HH) score.

4.3.6 20-HETE:EET and 20-HETE:DHET trajectory patterns and relation to outcomes

Temporal concentration profiles for 20-HETE:EET and 20-HETE:DHET ratios are shown in Figure 4-7: Temporal concentration profiles and trajectory patterns for 20-HETE:EET and 20-HETE:DHET. Similar relationships were observed when comparing 20-HETE:EET and 20-HETE:DHET CSF trajectory groups showed similar relationships to outcomes as the 20-HETE trajectory groups (Table 4-4: Outcomes in 20-HETE:EET CSF level trajectory groups and Table 4-5: Outcomes in 20-HETE:DHET CSF level trajectory groups).

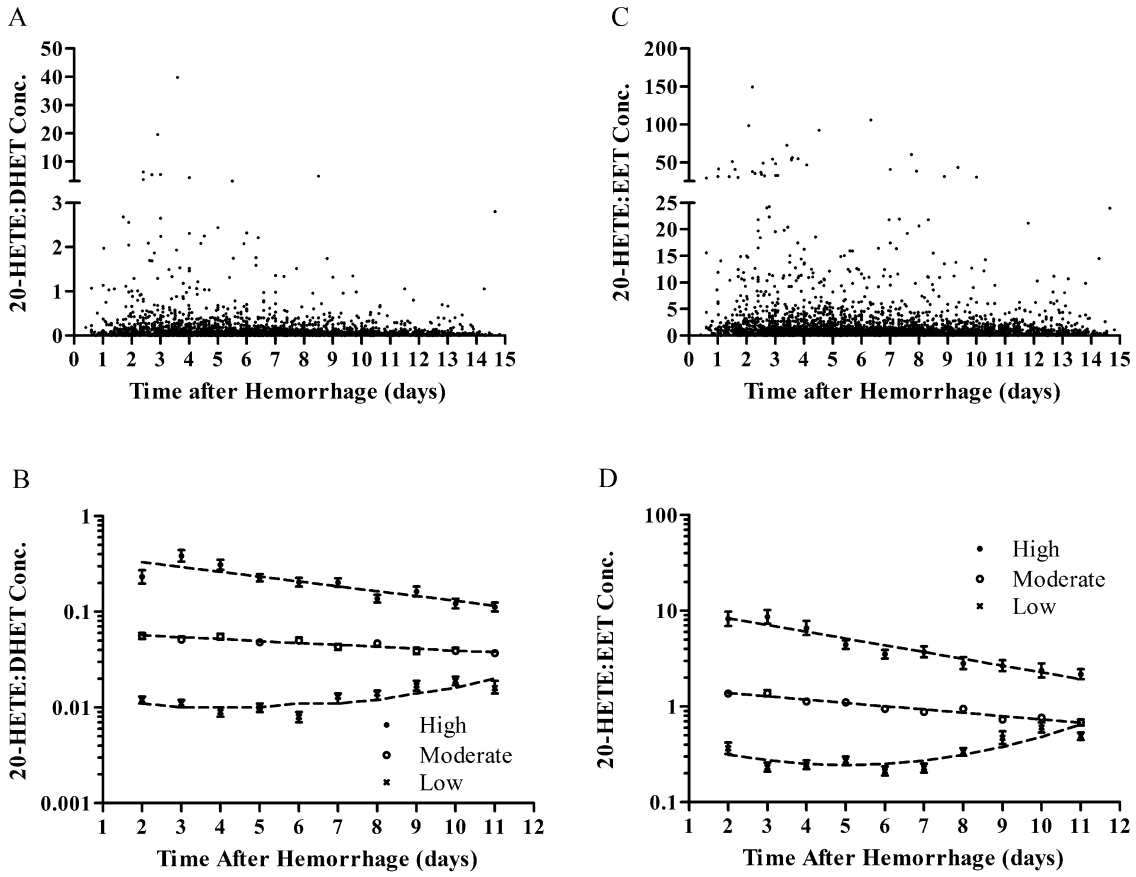


Figure 4-7: Temporal concentration profiles and trajectory patterns for 20-HETE:EET and 20-HETE:DHET

Panels (A) and (C) show the raw population values of 20-HETE:EET and 20-HETE:DHET CSF concentrations from 269 patients up to 14 days after hemorrhage, respectively. Panels (B) and (D) shows 20-HETE:EET and 20-HETE:DHET CSF concentration versus time from hemorrhage (days) in high (filled circles), moderate (open circles), and low (X) concentration groups as identified by trajectory analysis, respectively. Concentration data are presented as geometric mean with 95% confidence interval.

Table 4-4: Outcomes in 20-HETE:EET CSF level trajectory groups

20-HETE:EET Trajectory Group	CND (-) N (%)	CND (+) N (%)	Unadjusted P-value	Adjusted	
				OR (95% CI)	P-value
Overall					0.008**
Low	34 (51.5)	32 (48.5)		Reference	-
Moderate	45 (33.1)	91 (66.9)	0.006**	2.21 (1.18 - 4.13)	0.013**
High	9 (23.1)	30 (76.9)		3.66 (1.46 - 9.16)	0.006**
Moderate/High	54 (30.9)	121 (69.1)	0.003**	2.45 (1.34 - 4.48)	0.004**
20-HETE:EET Trajectory Group	**DCI (-) N (%)**	**DCI (+) N (%)**	**Unadjusted P-value**	**Adjusted**	
				OR (95% CI)	**P-value**
Overall					0.006**
Low	49 (74.2)	17 (25.8)		Reference	-
Moderate	71 (51.8)	66 (48.2)	0.005**	2.73 (1.42 - 5.30)	0.009**
High	19 (48.7)	20 (51.3)		3.13 (1.34 - 7.32)	0.003**
Moderate/High	90 (51.1)	86 (48.9)	0.001**	2.82 (1.49 - 5.32)	0.001**
20-HETE:EET Trajectory Group	**MRS3 (0-2) N (%)**	**MRS3 (3-6) N (%)**	**Unadjusted P-value**	**Adjusted**	
				OR (95% CI)	**P-value**
Overall					0.092
Low	40 (76.9)	12 (23.1)		Reference	-
Moderate	59 (57.8)	43 (42.2)	0.052	2.53 (1.1 - 5.83)	0.029*
High	16 (57.1)	12 (42.9)		2.14 (0.72 - 6.4)	0.173
Moderate/High	75 (57.7)	55 (42.3)	0.015**	2.44 (1.08 - 5.51)	0.031*
20-HETE:EET Trajectory Group	**MRS12 (0-2) N (%)**	**MRS12 (3-6) N (%)**	**Unadjusted P-value**	**Adjusted**	
				OR (95% CI)	**P-value**
Overall					0.028*
Low	43 (81.1)	10 (18.9)		Reference	-
Moderate	59 (59)	41 (41)	0.019*	3.12 (1.35 - 7.21)	0.008**
High	15 (60)	10 (40)		2.47 (0.82 - 7.48)	0.109
Moderate/High	74 (59.2)	51 (40.8)	0.005**	2.98 (1.32 - 6.73)	0.009

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Poor (3-6), Good (0-2); § Multivariate analysis included correction for age, sex, race, and either Fisher Grade (for acute outcomes) or Hunt & Hess score (for long-term outcomes); *Statistical significance established at *$p<0.05$ or **$p<0.0167$ (for multiple comparison correction)

Table 4-5: Outcomes in 20-HETE:DHET CSF level trajectory groups

20-HETE:DHET Trajectory Group	CND (-) N (%)	CND (+) N (%)	Unadjusted P-value	Adjusted	
				OR (95% CI)	P-value
Overall					0.046*
Low	25 (50)	25 (50)		Reference	-
Moderate	48 (37.5)	80 (62.5)	0.015**	1.62 (0.81 - 3.24)	0.173
High	15 (23.8)	48 (76.2)		2.92 (1.25 - 6.83)	0.013**
Moderate/High	63 (33)	128 (67)	0.026*	1.92 (0.98 - 3.74)	0.056
20-HETE:DHET Trajectory Group	**DCI (-) N (%)**	**DCI (+) N (%)**	**Unadjusted P-value**	**Adjusted**	
				OR (95% CI)	**P-value**
Overall					0.037*
Low	37 (74)	13 (26)		Reference	-
Moderate	67 (51.9)	62 (48.1)	0.026*	2.62 (1.25 - 5.48)	0.011**
High	35 (55.6)	28 (44.4)		1.96 (0.85 - 4.53)	0.114
Moderate/High	102 (53.1)	90 (46.9)	0.008**	2.4 (1.18 - 4.88)	0.016**
20-HETE:DHET Trajectory Group	**MRS3 (0-2) N (%)**	**MRS3 (3-6) N (%)**	**Unadjusted P-value**	**Adjusted**	
				OR (95% CI)	**P-value**
Overall					0.207
Low	30 (78.9)	8 (21.1)		Reference	-
Moderate	63 (62.4)	38 (37.6)	0.034*	1.76 (0.69 - 4.5)	0.234
High	22 (51.2)	21 (48.8)		2.6 (0.9 - 7.48)	0.076
Moderate/High	85 (59)	59 (41)	0.024*	1.98 (0.8 - 4.9)	0.139
20-HETE:DHET Trajectory Group	**MRS12 (0-2) N (%)**	**MRS12 (3-6) N (%)**	**Unadjusted P-value**	**Adjusted**	
				OR (95% CI)	**P-value**
Overall					0.168
Low	31 (81.6)	7 (18.4)		Reference	-
Moderate	64 (64)	36 (36)	0.040*	2.33 (0.9 - 6.05)	0.082
High	22 (55)	18 (45)		2.63 (0.89 - 7.75)	0.080
Moderate/High	86 (61.4)	54 (38.6)	0.020*	2.41 (0.96 - 6.09)	0.062

DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Poor (3-6), Good (0-2); § Multivariate analysis included correction for age, sex, race, and either Fisher Grade (for acute outcomes) or Hunt & Hess score (for long-term outcomes); *Statistical significance established at *$p<0.05$ or **$p<0.0167$ (for multiple comparison correction)

The time-to-maximum 20-HETE concentration was compared to the time-to-CND in Figure 4-8: Time to maximum 20-HETE CSF levels vs. time to CND. There was a weak and moderate correlation between time-to-maximum 20-HETE concentrations and time-to-CND in all patients ($r=0.274$, $p<0.001$) and those with moderate/high trajectory groups, respectively ($r=0.439$, $p<0.001$).

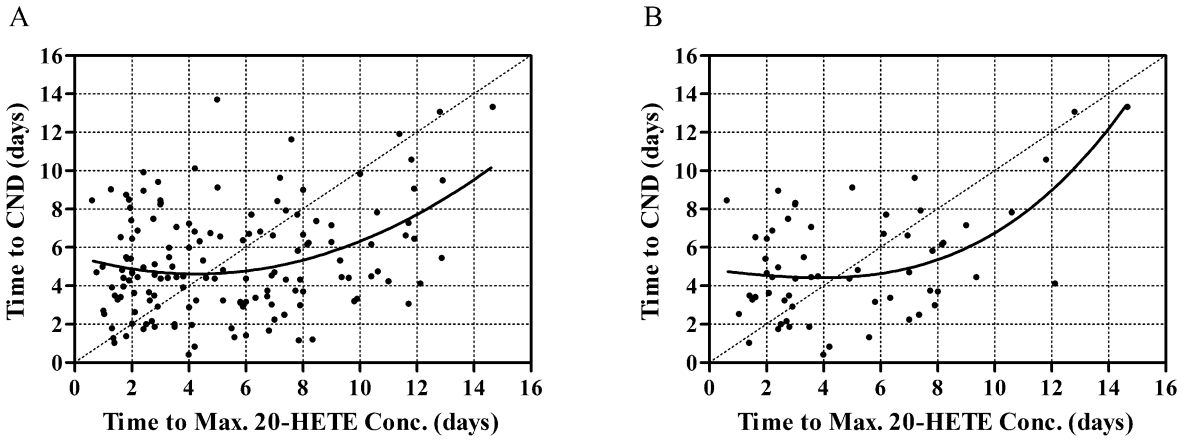


Figure 4-8: Time to maximum 20-HETE CSF levels vs. time to CND

The time to reach maximum 20-HETE levels in CSF was compared to the time to reach CND in all patients (panel A, n=148) and in patients with high/moderate trajectory patterns (panel B, n=60) as identified in Figure 4-5: 20-HETE temporal concentration profiles and trajectory patterns. Comparison was performed using Pearson correlation coefficient (r) and statistical significance established at *p<0.05.

4.3.7 Evaluation of genotype frequency data

Table 4-6: HWE and observed vs. Hapmap comparison shows a comparison of the observed genotype frequencies in this study with the expected frequencies based on the Hapmap CEU population data. Multiple CYP4F2 gene variants were not in HWE including g.5416G>C (p<0.001), g.5373T>C (p=0.023), g.14389C>T(*3) (p<0.001), g.7222002G>A (p=0.001), g.4593T>C (p=0.013) and g.16162A>G (p=0.002). Also, our SAH population showed different allele frequencies than those reported in the Hapmap CEU population for CYP4F2 gene variants including g.14389C>T(*3), g.7222002G>A, g.16162A>G (p<0.001) and a trend for a difference for g.5416G>C (p=0.074) and g.5373T>C (p=0.083).

Table 4-6: HWE and observed vs. Hapmap comparison

Gene SNP (rs#)	Genotype	Observed N (%)	Hapmap N (%)	HWE P-value	Obs-Hap P-value
CYP4F2	GG	194 (65.8)	244 (82.6)		
g.5416G>C	CG	96 (32.5)	51 (17.4)	<0.001**	0.074
(rs3093100)†	CC	5 (1.7)	0 (0)		
CYP4F2	TT	199 (66.3)	228 (76.1)		
g.5373T>C	CT	96 (32)	66 (22.1)	0.023*	0.083
(rs3093098)†	CC	5 (1.7)	5 (1.8)		
CYP4F2	CC	147 (51.4)	174 (60.7)		
g.14389C>T [*3]	CT	137 (47.9)	92 (32.1)	<0.001**	<0.001**
(rs2108622)†‡	TT	2 (0.7)	20 (7.1)		
CYP4F2	GG	90 (30.1)	111 (37.2)		
g.7222002G>A	GA	178 (59.5)	135 (45.1)	0.001**	<0.001**
(rs2189784)‡	AA	31 (10.4)	53 (17.7)		
CYP4F2	TT	134 (47.5)	117 (41.6)		
g.4593T>C	TC	107 (37.9)	127 (45.1)	0.013*	0.226
(rs3093089)‡	CC	41 (14.5)	38 (13.3)		
CYP4F2	AA	125 (41.7)	151 (50.4)		
g.16162A>G	AG	155 (51.7)	112 (37.2)	0.002**	<0.001**
(rs3093207)‡	GG	20 (6.7)	37 (12.4)		

HWE, Hardy-Weinberg Equilibrium; Obs, observed frequency in SAH cohort; Hap, expected frequency based on Hapmap CEU population.

4.3.8 Acute and long-term outcomes in genetic groups

A comparison of the acute outcomes in genetic groups is shown in Table 4-7: Acute and Long-term outcomes in genetic groups. In the multivariate analysis, patients with CYP4F2-g.4593C/C genotype were 2.3-fold less likely to develop CND compared to CYP4F2-g.4593T/T carriers (p=0.047). CYP4A11-g.13414C allele-carriers were ~2-fold more likely to have unfavorable 3- and 12-month MRS (p=0.019 and p=0.042), respectively. Also, patients with the variant CYP4F2-g.4211T allele or CYP4F2-g.8575T/T genotype were ~2.4-fold less likely and ~3.3-fold more likely to have unfavorable outcomes at 3 months (p=0.016 and p=0.017), respectively.

Table 4-7: Acute and Long-term outcomes in genetic groups

Gene SNP (rs#)	Genotype	CND (+) N (%)	CND (-) N (%)	Unadjusted P-value	Adjusted§ OR (95% CI)	Adjusted§ P-value
CYP4F2 g.4593T>C (rs3093089)‡	Overall					0.138
	TT	77 (60.6)	50 (39.4)		Reference	-
	TC	59 (56.7)	45 (43.3)	0.223	0.82 (0.46-1.44)	0.482
	CC	16 (44.4)	20 (55.6)		0.44 (0.20-0.99)	0.047*
	C-carriers	75 (53.6)	65 (46.4)	0.245	0.70 (0.41-1.17)	0.175
Gene SNP (rs#)	**Genotype**	**MRS3 (3-6) N (%)**	**MRS3 (0-2) N (%)**	**Unadjusted P-value**	**Adjusted§ OR (95% CI)**	**Adjusted§ P-value**
CYP4A11 g.13414G>C (rs3890011)‡	Overall					0.025*
	GG	31 (23)	106 (77)		Reference	-
	GC	31 (42)	43 (58)	0.004*	2.43 (1.28-4.62)	0.007*
	CC	0 (0)	6 (100)		-	0.999
	C-carriers	31 (39)	49 (61)	0.011*	2.12 (1.13-3.99)	0.019*
CYP4F2 g.4211A>T (rs3093156)‡	Overall					0.055
	AA	22 (40)	33 (60)		Reference	-
	TA	25 (23)	82 (77)	0.08	0.42 (0.2-0.89)	0.023*
	TT	15 (27)	41 (73)		0.44 (0.19-1.03)	0.059
	T-carriers	40 (25)	123 (75)	0.028*	0.43 (0.21-0.85)	0.016*
CYP4F2 g.8575C>T (rs3093168)‡	Overall					0.055
	CC	23 (25)	69 (75)		Reference	-
	CT	26 (26)	74 (74)	0.088	1.21 (0.61-2.41)	0.579
	TT	12 (46)	14 (54)		3.30 (1.23-8.83)	0.017*
	T-carriers	38 (30)	88 (70)	0.402	1.51 (0.8-2.88)	0.206
Gene SNP (rs#)	**Genotype**	**MRS12 (3-6) N (%)**	**MRS12 (0-2) N (%)**	**Unadjusted P-value**	**Adjusted§ OR (95% CI)**	**Adjusted§ P-value**
CYP4A11 g.13414G>C (rs3890011)‡	Overall					0.067
	GG	29 (21)	108 (79)		Reference	-
	GC	27 (40)	41 (61)	0.007*	2.22 (1.13-4.34)	0.020*
	CC	0 (0)	5 (100)		-	0.999
	C-carriers	27 (37)	46 (63)	0.014*	1.99 (1.03-3.86)	0.042*

† Functional SNP; ‡ Tagging SNP, DCI, delayed cerebral ischemia; CND, clinical neurological deterioration; MRS, Modified Rankin Scale score at 3 and 12 months: Unfavorable (3-6), Favorable (0-2); § Multivariate analysis included correction for age, sex, race, and either Fisher grade (for acute outcomes) or Hunt & Hess score (for long-term outcomes); *Statistical significance established at *$p<0.05$ or **$p<0.0042$ (for multiple comparison correction)

4.3.9 20-HETE levels in genetic groups

A comparison of 20-HETE CSF levels in acute and long-term outcome groups is shown in Figure

4-9: 20-HETE CSF levels in acute and long-term outcome groups. CYP4A11-g.4207G allele-carriers had 43% lower mean 20-HETE levels when compared to CYP4A11-g.4207A/A carriers (p=0.005). 20-HETE levels were not associated with genotype in the codominant model. The most common CYP4F2 haplotype (Haplotype 1) was CTCAC (31.9%) for rs3093089, rs3093156, rs3093168, rs3093207, and rs2108622, respectively. Compared to Haplotype 1, haplotype 4 (TTCGT, 9.5%) was associated with 0.26 ng/ml lower 20-HETE (p=0.034), but there were no differences in other haplotype groups.

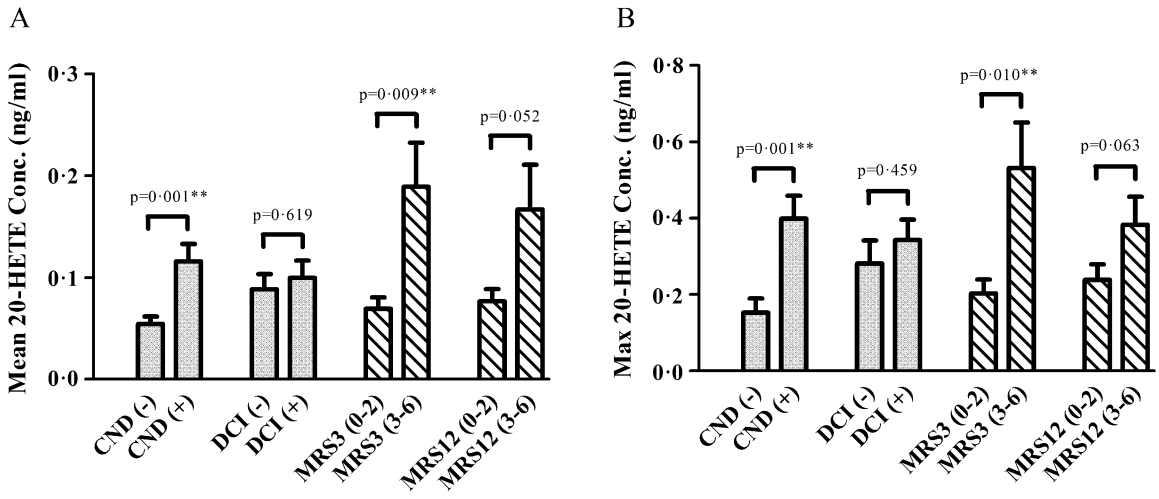


Figure 4-9: 20-HETE CSF levels in acute and long-term outcome groups

Mean (panel A) and maximum (panel B) 20-HETE levels in CSF from patients with SAH are compared in outcomes groups. Acute outcomes (solid bars) included the presence or absence of clinical neurological deterioration (CND) and/or delayed cerebral ischemia (DCI) up to 14 days after the hemorrhage. Long-term outcomes (stripped bars) were determined by global functional recovery at 3 and 12 months using the Modified Rankin Scale (MRS) and were dichotomized into favorable (MRS 0-2) and unfavorable (MRS 3-6) groups. The mean and maximum 20-HETE CSF levels for each patient (n=269) were calculated and were used to compare the mean ± SEM of the CYP eicosanoid levels in outcome groups using t-test with Welch's correction as appropriate. Statistical significance established at *p<0.05 and **p<0.0167 (correction for multiple comparisons).

4.4 DISCUSSION

This clinical study is the first to investigate the relationship between SNPs in 20-HETE synthesis genes, 20-HETE CSF levels, and acute/long-term outcomes in a large SAH cohort. We report that higher 20-HETE CSF levels and trajectory patterns were associated with unfavorable acute and long-term outcomes before and after controlling for age, sex, race, and Fisher grade or HH score. Also, SNPs in 20-HETE synthesis genes were associated with long-term outcomes and showed different frequencies compared to control populations in Hapmap. Collectively, these results demonstrate the translational importance of 20-HETE in pathophysiology of SAH.

Growing evidence suggests that 20-HETE contributes to the pathophysiology of SAH along with other biomarkers.[317] We observed that CYP-eicosanoids are present in SAH patients at concentrations reported to affect cerebrovascular function. 20-HETE constricts isolated cat and rat cerebral arteries at 0.1 nM (0.032 ng/ml) and 10 nM (3.2 ng/ml) concentrations, respectively.[590, 614] A dog model of SAH-induced DCI was associated with 20-HETE CSF levels of ~1.2 nM.[620] Our clinical study reports maximum 20-HETE, EET, and DHET CSF concentration of ~1.0, 0.5, and 7.3 nM, respectively, which are comparable to levels reported to alter cerebrovascular tone. Moreover, it is expected that CYP-eicosanoid levels in the cerebrovasculature are higher than those in CSF.[356] These results suggest that CYP-eicosanoids are present at physiologically relevant levels in the brain after SAH in humans.

We observed that elevated 20-HETE CSF levels and temporal concentration profiles were associated with CND, but not DCI, both before and after adjustment for covariates. Patients with high/moderate 20-HETE trajectory patterns developed CND earlier and to a greater extent than those in the low group. 20-HETE generally reached maximum levels before the onset of CND in first few days after SAH, suggesting that 20-HETE may contribute to secondary ischemic insults. The terms used to describe the delayed onset of cerebral ischemia and neurological deterioration after SAH are inconsistent and often used interchangeably in clinical studies.[160] Roman et al. reported that nine SAH patients with angiographic vasospasm and neurological deficits had elevated 20-HETE CSF levels compared to controls.[620] Similarly, our lab reported that detectable 20-HETE in CSF was associated with DCI in a smaller SAH cohort.[642, 762] In these studies,

multivariate analyses were not performed and 20-HETE was measured in fresh CSF using a less sensitive quantitation method with dichotomization of patients into detectible and non-detectible groups. Also, growing evidence suggests that cerebral vasospasm in large arteries plays a limited role in DCI after SAH.[216] 20-HETE is a primary regulator of cerebral microvasculature tone[356] and therefore may affect CND to a greater extent than DCI after SAH.[224] Future studies are needed to delineate the temporal relationships between 20-HETE CSF levels, microvascular/macrovascular tone, and regional CBF after SAH.

We observed that increased 20-HETE CSF levels were associated with unfavorable long-term outcomes. Since CND is a strong prognostic factor for unfavorable long-term outcomes after SAH[150], it is possible that 20-HETE is a causative factor in this relationship. Conversely, the effects of 20-HETE on long-term outcomes may be mediated through inflammation, vascular remodeling, or other mechanisms that occur at various time periods after SAH.[355] Future studies are needed to elucidate the mechanisms involved in the relationship between 20-HETE and long-term outcomes after SAH.

We observed previously established relationships between Fisher grade, HH score, and increasing age with acute and/or long-term outcomes.[765, 770] 20-HETE was associated with ventricular hemorrhage but not the severity of hemorrhage. Since 20-HETE is released from white blood cells and vascular tissue[356], it is difficult to determine the source of 20-HETE in CSF. Nevertheless, the multivariate analysis showed that 20-HETE CSF levels/trajectory groups remained strong predictors of outcomes with comparable prognostic ability as Fisher grade and HH score, respectively. Collectively, these studies suggest that 20-HETE may serve as a potential biomarker of acute and long-term outcomes in patients with SAH.

Our genetic integrity tests show that multiple gene variants on CYP4F2 violated HWE conditions possibly due to enrichment in the SAH population. Many of the SNPs that violated HWE conditions also showed genotype frequencies in our SAH population that were different than those in the Hapmap CEU population, suggesting putative genetic markers for the formation and rupture of intracranial aneurysms. Previous studies report a strong link between hypertension and vascular remodeling with the incidence of SAH.[124] 20-HETE elicits a wide array of effects including regulation of blood pressure, CBF, and vascular remodeling.[355, 356] Thus, it is expected that CYP4F2 variants that lead to alterations in 20-HETE may impact the occurrence of SAH

possibly through its effects on blood pressure, cerebrovascular tone, and vascular homeostasis.

The CYP4F2 putative genetic markers for SAH identified in this study were not associated with altered 20-HETE CSF levels, but previous *in vitro* studies associated the CYP4F2 variants g.14389C>T [*3], g.5373T>C, and g.5416G>C with reduced enzymatic activity, reduced transcriptional activity, and increased transcriptional activity, respectively.[648, 649, 651] Interpretation of our results is difficult because of the discrepant *in vitro/in vivo* findings on the effect of CYP4F3*3 on 20-HETE levels and 20-HETE levels on vascular remodeling.[355, 682] Also, it is possible that the genotype frequencies observed in our SAH population were different than those in the Hapmap CEU population due to differences in genotyping methods. Thus, the effects of CYP4F2 gene variants and 20-HETE on the formation and rupture of intracranial aneurysms warrants further investigation.

Our genetic analysis showed that patients with the variant g.4207G-allele located in the promoter region of CYP4A11 showed 43% lower mean 20-HETE CSF levels. In-silico models predicted that this SNP will lower CYP4A11 transcriptional activity.[771] When the CYP4A11 g.4207G/G (c.-825G/G) mutant construct was expressed *in vitro*, CYP4A11 transcriptional activity was reduced by ~30% compared to the WT-construct.[646] Based on these studies, we expect that patients with the variant CYP4A11 g.4207G-allele would have decreased 20-HETE CSF levels, which is consistent with our results.

Multiple tSNPs in CYP4F2 and CYP4A11 were associated with acute and long-term outcomes. Patients with the variant CYP4F2 g.4593C/C genotype were 2.3-fold less likely to develop CND compared to those with g.4593T/T genotype in the multivariate analysis. In a study of Chinese hypertensive patients, no association was observed between the CYP4F2 g.4593T>C polymorphism and both urinary 20-HETE levels and hypertension.[683] Thus factors other than 20-HETE may mediate the lower risk to develop CND in patients with the variant CYP4F2 g.4593C/C genotype. We report that CYP4A11 g.13414C allele-carriers were ~2-fold more likely to develop unfavorable outcomes at 3 and 12 months. Also, patients with the variant CYP4F2 g.4211T allele or CYP4F2 g.8575T/T genotype were ~2.4-fold less likely and ~3.3-fold more likely to have unfavorable outcomes at 3 months, respectively. These tSNPs are located at the intron regions of CYP4A11 or CYP4A11 and are not likely to alter enzyme expression or activity. However, these

tSNPs may be linkage disequilibrium with fSNPs or contribute to haplotype constructs and thus may serve as surrogate genetic markers for clinical phenotypes.

We also note some limitations of our results. First, our biomarker assessment was performed only in patients with ventricular drains that commonly have more significant hemorrhage than those without ventricular drains. 20-HETE CSF levels represent time-averaged values during the 12hr collection period. 20-HETE levels in the cerebrovasculature were not measured but are expected to be higher than CSF levels. Also, our findings implicate regions of association and not necessarily causative genetic variation.

4.5 CONCLUSIONS

In summary, 20-HETE has been shown to play an important role in the regulation of cerebrovascular tone and vessel remodeling *in vitro* and significantly affect CBF and cerebral ischemic injury *in vivo*. Our study, involving one of the largest SAH cohorts to date, implicates 20-HETE and CYP4F2 gene variants in the risk for SAH and subsequent acute and long-term outcomes. These results are important to help elucidate the mechanisms involved in the pathophysiology of SAH and possibly identify patients at high risk for unfavorable outcomes; thereby, lending insight into future interventional strategies.

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

5.1.1 Summary of research goals

The primary goal of this research was to investigate the role of CYP eicosanoids in the pathophysiology of SAH. This was achieved by investigating key polymorphisms in the genes responsible for CYP eicosanoid synthesis and metabolism and their relationship to CYP eicosanoid levels in CSF, ischemic complications during the inpatient stay, and long-term functional outcomes at 3 and 12 months in a large SAH cohort. Similarly, the relationship between CYP eicosanoid CSF levels and acute/long-term outcomes was also determined. In order to accomplish these goals, we first set out to develop and validate a sensitive analytical method to measure CYP eicosanoid levels in CSF and then modified this assay in order to improve our ability to evaluate temporal concentration profiles. Key findings from this research are summarized below.

5.1.2 Key research findings

In the first part of this research, we developed and validated a rapid, sensitive, and specific UPLC-MS/MS method for measuring CYP eicosanoids in CSF from SAH patients. Linear calibration curves ranged from 0.208-33.3 ng/ml and the matrix did not affect the accuracy or precision of the assay. Then, we modified this method to lower detection limits from 0.208 ng/ml to 0.028 ng/ml, thus significantly improving our ability to develop temporal concentration profiles. Modifications included utilizing larger volumes of CSF available in the drainage bags and modifications to our

sample processing methods. We also established room temperature stability of the CYP eicosanoids over the 12 hour collection period and measured the recovery from the collection bags. These findings were critical to accomplish the other goals of our research.

Next, we investigated the relationships between key polymorphisms in the genes responsible for EET synthesis and metabolism, EET and DHET CSF levels, acute ischemic complications (CND and DCI), and long-term functional outcomes as determined by MRS at 3 and 12 months. We report that patients with the CYP2C8*4 allele had 44% and 36% lower EET and DHET CSF levels and were 2.2- and 2.5-fold more likely to develop DCI and CND, respectively. We identified a few candidate gene variants that were associated with altered EET and/or DHET CSF levels and favorable or unfavorable outcomes. Many of these polymorphisms were previously reported to affect gene expression, protein levels or activity *in vitro* and our results generally agreed with the expected impact of these gene variants on CYP eicosanoid CSF levels and outcomes in our SAH cohort. Also, EET levels were associated with the severity/location of the hemorrhage and poor 3-month outcomes, but DHET levels and trajectory patterns were not associated with outcomes. These are the first clinical data demonstrating the association between the EET biosynthesis/metabolic pathway and the pathophysiology of SAH.

Finally, we investigated the relationships between key polymorphisms in the genes responsible for 20-HETE synthesis, 20-HETE CSF levels, acute ischemic complications (CND and DCI), and long-term functional outcomes as determined by MRS at 3 and 12 months. Patients with CND and unfavorable 3-month MRS had ~2.2- and 2.7-fold higher mean 20-HETE CSF levels, respectively. 20-HETE CSF levels were described by three trajectory groups with decreasing concentrations over time. Patients in high/moderate 20-HETE trajectory groups were 2.1-, 2.5-, and 2.1-fold more likely to have CND and unfavorable MRS at 3 and 12 months after controlling for age, sex, race, and Fisher grade or Hunt & Hess score. We identified a few candidate gene variants that were associated with altered 20-HETE CSF levels and favorable or unfavorable outcomes. Many of these polymorphisms were previously reported to affect gene expression, protein levels or activity *in vitro* and our results generally agreed with the expected impact of these gene variants on CYP eicosanoid CSF levels and outcomes in our SAH cohort. Furthermore, several CYP4F2 genotype frequencies in our SAH population differed from the Hapmap CEU database indicating putative genetic markers for SAH risk. These findings in

SAH patients support the emerging role of 20-HETE in cerebral microvascular dysregulation after a neurovascular hemorrhage and implicates this pathway as a target for future intervention.

5.2 FUTURE DIRECTIONS

5.2.1 Future Studies and Potential Areas for Improvement

The source of CYP eicosanoids in CSF after SAH has not been studied extensively. Recently, Alkayed et al. reported that SAH patients with DCI had low EET CSF levels that increased over 14 days.[772] These results suggest that EETs may be produced and secreted into CSF following SAH in humans. Conversely, the same study reported that 20-HETE CSF levels were high early after SAH and decreased over 14 days in SAH patients with DCI.[772] Our study reported a similar trends with 20-HETE CSF levels over time. These temporal concentration profiles suggest that 20-HETE could be released directly from the vascular tissue at aneurysm rupture, extravasated blood, or the breakdown of blood after SAH. In addition, preclinical studies have shown that 20-HETE is released from white blood cells and vascular tissue.[356] On the other hand, evidence from this study shows that 20-HETE CSF levels were not associated with the severity of hemorrhage as determined by Fisher grade. Although the Fisher Scale is the most commonly used clinical grading method used to predict vasospasm after SAH, disadvantages of the Fisher Scale includes scoring criteria developed using old imaging technology, unclear and subjective classifications, and exclusion of certain characteristics of the bleed such as clot density and clearance rate.[278] Thus, Fisher grade has limited ability to accurately measure the amount and location of blood over time. These limitations make it difficult to determine the relationship of CYP eicosanoid levels with hemorrhagic factors and limit our ability to determine source of CYP eicosanoids in CSF after SAH.

In order to further investigate the source of CYP eicosanoids in CSF after SAH in humans, it may be valuable to measure the concentration of blood constituents and blood breakdown products to assess the degree of bleed more accurately than Fisher grade. Levels of blood

constituents such as red blood cells, white blood cells, and platelets can be measured using standard blood count panels. Blood breakdown products such as bilirubin, oxyhemoglobin, and methemoglobin can be measured using spectrophotometry. These spectrophotometry methods are commonly used to diagnose SAH by quantify the level of xanthochromia (yellow appearance) in CSF due to the presence of blood breakdown products. If these methods were employed in this clinical study, the relationship between the temporal concentration profiles of blood constituents and blood breakdown products in CSF from SAH patients and CYP eicosanoid levels could be investigated. These investigations may help elucidate the primary source of CYP eicosanoid release in CSF after SAH in humans.

Until recently, there were no published clinical studies investigating the relationship of CYP eicosanoid levels over time with outcomes after SAH. A few months ago, Alkayed et al. published a study that showed similar trends in 20-HETE CSF levels over time when compared to our study.[772] Although our study showed limited ability to measure EETs, the stable EET metabolites 11,12-DHET and 14,15-DHET, but not 8,9-DHET, appeared to show similar temporal concentration profiles to those reported by Alkayed et al. Since the 8,9-DHET levels were significantly higher than those of the other DHET regioisomers, the overall DHET temporal concentration profiles appeared to parallel those of 8,9-DHET. However, our study did not perform trajectory analysis of the individual CYP eicosanoid regioisomers. Thus, the analysis of our study should be extended to include an evaluation of the CYP eicosanoid regioisomer concentration over time and their relationship to clinical outcomes.

Despite our significant efforts to develop robust experimental methods and analyses, numerous areas of our research project can be modified and evaluated for potential improvements. For instance, other studies have reported alternative methods of CSF collection and preparation. Alkayed et al. collected 3ml of CSF directly from the ventricles instead of the drainage bags and measured CYP eicosanoids using our method with minor modifications to the sample preparation.[772] An evaluation of these methods may be beneficial since a significant number of CSF samples from SAH patients showed quantifiable EET levels in this study.[772] In order to further address our limited ability to measure EETs in CSF from SAH patients, we could add an SEH inhibitor to the CSF sample immediately after collection in an effort to limit the conversion of EETs to DHETs. Currently, it was unknown whether increased DHET concentrations are the

result of increased EET availability or increased SEH expression after SAH. In order to address this issue, previous studies have measured 9,10-epoxyoctadecenoic acid (9,10-EpOME) and 9,10-Dihydroxyoctadecenoate (9,10-DiHOME) as surrogate markers of EETs and DHETs and evaluated the changes in 9,10-DiHOME/9,10-EpOME ratio after SAH.[773] This study also measured 9,10-EpOME and 9,10-DiHOME in a subset of our SAH cohort, but further analysis has not yet been completed. Since we did not have access to control CSF, we were not able to compare control levels of CYP eicosanoids to those of SAH patients. However, others reported that CYP eicosanoids were below LLQ for all control CSF samples.[620, 772] In a similar fashion, we could genotype control patients with similar demographic factors (age, sex, race, etc.) to our SAH patients instead of using genetic data from the Caucasian (CEU) population in the Hapmap database for our analyses. Furthermore, we could perform full sequencing of our candidate genes instead of limiting our study to include the analysis of only functional and tagging SNPs. Finally, we can improve the clinical utility of our studies by expanding our analysis to include an assessment and evaluation of concomitant medications and medical conditions and their relationship with CYP eicosanoid levels and outcomes in SAH patients. In summary, limitations of this clinical study can be potentially be addressed or improved with modifications to our experimental methods and additional analyses.

5.2.2 Pharmacological agents targeting the CYP eicosanoid pathway

Drug candidates targeting the EET metabolic pathway show promise for the treatment of SAH and CVD in general. Hammock et al. provides an excellent review discussing the history, rationale, and status of the development of pharmaceutical agents targeting the EET metabolic pathway.[609] The beneficial actions of EETs can be enhanced or increased by utilizing EET agonists or SEH inhibitors to inhibit of EET metabolism. The potential benefits of an EET agonist includes the initiation of biological action in the absence of endogenous EETs, extended half-life when compared to endogenous EETs, and the flexibility to attain supraphysiological levels and responses if desired. Advantages of SEH inhibitors include the extended half-life of endogenous EETs present in the stereo- and regio-specific EET isomers unique to each tissue. Also, the biological effects of SEH inhibitors are limited by the level of endogenous EETs thus reducing the

risk for potential side effects. On the other hand, disadvantages of using EET agonists include differences between endogenous stereo- and regio-specific EET isomer formation and differences in tissue specific levels when compared to endogenous EET formation. Disadvantages of SEH inhibitors include a limited biological response and increase in alternative metabolic pathways such as β-oxidation. Currently, there are no FDA approved SEH inhibitors or EET agonists, however numerous compounds are in various stages of development. Based on the wide therapeutic index presented by SEH inhibitors, it is likely that one of these compounds will be approved for the prevention or treatment of chronic vascular conditions such as hypertension or ischemic complications after SAH.

Candidates targeting 20-HETE synthesis appear less promising compared to SEH inhibitors. For instance, many inhibitors of 20-HETE synthesis, such as DDMS and 17-ODYA, have limited potency and selectivity. These compounds along with the 20-HETE antagonist WIT002 are highly protein bound and have minimal bioavailability when delivered systemically. HET0016 is a potent and selective 20-HETE synthesis inhibitor, but has a short half-life with limited solubility. Among the ω-hydrolase inhibitors, TS-011 demonstrates superior potency and selectivity along with sufficient bioavailability and half-life in preclinical studies.[589] Therefore, TS-011 appears to be one of the most promising 20-HETE synthesis inhibitors for clinical development. Currently, phase I clinical trials for the use of TS-011 in acute ischemic stroke patients is underway.

Numerous factors must be considered when targeting CYP eicosanoid pathways for pharmacological intervention. Since AA is metabolized by multiple enzymatic pathways, it is possible that inhibition of one enzymatic pathway may lead to shunting to another pathway through increased substrate availability. To illustrate, a few studies reported that inhibition of COX enzymes leads to shunting of AA metabolism to CYP and LOX pathways. Ren et al. reported that administration of the COX inhibitor indomethacin to isolated rabbit afferent arterioles led to an increase in 20-HETE levels.[774] Similarly, Hammock et al. reported that oral administration of the selective COX-2 inhibitor rofecoxib (Vioxx) to mice for 3 months resulted in a 120-fold increase in 20-HETE levels in plasma and shorter bleeding times.[775] Based on these data, it is also expected that inhibition of CYP enzymes responsible for 20-HETE and EET formation may lead to increased COX and LOX metabolites through shunting of AA metabolism. Therefore, the impact

of CYP eicosanoid inhibition on the formation of COX and LOX metabolites warrants further clinical investigation.

Similarly, a wide variety of endogenous substrates and drugs are metabolized by CYP eicosanoid enzymes and thus inhibition of these enzymes may lead to considerable side effects. As discussed in Section 1.5.3, CYP eicosanoid enzymes are responsible for the metabolism of medium chain (C10-C16) and long chain (C16-C26) fatty acids leading a wide array of metabolites that exhibit diverse biological actions and play important roles in numerous diseases.[776] For example, one of the primary endogenous substrates of CYP4F2 is leukotriene B_4 (LTB_4), which is a chemotactic agent involved in inflammation.[777] Thus, inhibition of CYP4F2 would be expected to alter the termination of the inflammatory response mediated by CYP4F2. In addition, numerous FDA approved drugs are metabolized by CYP eicosanoid enzymes. CYP2C8 and CYP2C9 are responsible for the metabolic clearance of up to 5% and 15%-20%, respectively, of all drugs undergoing Phase I metabolism.[458, 559, 560] Therefore, it is possible that pre- or post-admission use of medications may impact endogenous CYP eicosanoid formation. Alternatively, inhibition of the CYP eicosanoid pathway may also lead to potentially important drug-drug interactions. As a result, the benefit of pharmacological agents targeting the CYP eicosanoid pathway should be compared to the potential side effects or drug-drug interactions associated with these target enzymes.

Alternatively, altered CYP eicosanoid levels may be achieved through dietary ω-3 supplementation. Enzymes involved in the synthesis of CYP eicosanoids have recently been reported to preferentially metabolize ω-3 polyunsaturated fatty acids (PUFAs) such as eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) over AA.[778] EPA and DHA promote vasodilation and reduce inflammation, thrombosis, and triglycerides and diets rich in ω-3 PUFAs have been associated with protection against CVD.[779] The mechanisms of ω-3 PUFA action on CVD are not fully understood, but some have argued that the reduction in ω-6 eicosanoid biosynthesis due to competitive inhibition of COX, LOX, and CYP enzymes by ω-3 PUFAs substrates plays an important role.[778, 780, 781] The effect of ω-3 PUFAs on cerebrovascular tone and CBF regulation have not been studied extensively, but it is reported that 17(R),18(S)-EEQ, an epoxide metabolite of EPA, is a more potent vasodilator of rat cerebral arteries than 11(R),12(S)-

EET.[782] Therefore, it is expected that administration of ω-3 supplements may reduce the risk for SAH and improve outcomes in SAH patients by reducing the formation of ω-6 PUFAs.

5.2.3 Expected trends and research focused on SAH

It is expected that advances in the treatment of SAH will continue to focus on CND and DCI due to their strong associations with outcomes, the time window for therapeutic intervention, the reversible nature of these conditions, the marginal efficacy of existing pharmacotherapies, and the high risk presented by aggressive treatments. As explained in Section 1.1.3, AHA recommends the administration of oral nimodipine to all SAH patients and induction of hypertension in patients that develop DCI. These therapies can significantly affect cerebral hemodynamics and are effective in preventing or treating the development of the delayed onset of ischemic complications. However, recent evidence suggests that the efficacy of induced hypertension in SAH patients with DCI is closely linked to their autoregulatory status.[783] Therefore, future studies are needed to individualize treatment modalities based on autoregulatory status and other factors.

It is also expected that future experimental and clinical studies of SAH will focus on the cerebral microcirculation. As explained in section 1.1.4, growing evidence suggests that vasospasm in large cerebral arteries plays a limited role in the development of DCI after SAH.[216] Some studies suggest that microvasculature plays a predominant role in regulating CBF regulation[222, 223], especially after SAH[224], and imaging technologies provide direct evidence of microvascular vasospasm in humans after SAH[225, 226]. Moreover, the definition of DCI should account for clinically relevant alterations in both macrocirculation and microcirculation that lead to ischemia.[160] Thus, the relative contribution of the microcirculation and macrocirculation to the regulation of CBF after SAH has not been well studied and thus presents a promising area of future research.

Moreover, CBF regulation after SAH is a complex process involving the interaction between mechanical factors presented by the hemorrhage, altered metabolic needs of brain tissue,

loss of cerebral autoregulation due to altered myogenic response and neurovascular coupling, structural alterations of cells by edema, inflammation, and oxidative stress, and cell death by necrosis and apoptotic mechanisms. However, few clinical studies have investigated the interaction of these factors. Therefore, it is likely that future studies will combine multiple structural and physiological measurements with clinical findings.

It is expected that clinical grading scales will continue to be the most clinically relevant prognostic indicators of acute and long-term outcomes after SAH. As discussed in Section 1.1.7, advantages of clinical grading scales include history of use, simple grading criteria, and moderate sensitivity and specificity as prognostic indicators. Disadvantages presented by clinical grading scales includes poor inter-rater reliability due to subjective and broad grading criteria and inconsistent timing of use. A genetic marker would be an ideal prognostic factor based on consistent genotyping results, inexpensive methods, and the ability to apply the predictive tool at any time before or after the injury. However, no genetic markers have demonstrated sensitive and specific prediction of DCI in large and/or diverse SAH cohorts. Potential advantages presented by biomarkers include the ability to elucidate the pathogenic mechanisms, identify early indicators of complications, and evaluate the temporal effects of the biomarker levels over the clinical course of SAH injury. However, biochemical markers would not be expected to be a clinically relevant prognostic indicator because the cost and time required for the assessment and the difficulty in determining if the biomarker is a causal factor or indicator of injury. In order for any of these potential prognostic factors to be widely accepted in the clinic, their predictive ability would need to be classified and validated according to criteria previously described in studies evaluating genetic[784, 785], biomarker[318, 786, 787], clinical grading scales[788], and combinations of prognostic factors and other clinical criteria[789-791].

Currently, there is a lack of understanding of the underlying mechanisms by which CYP eicosanoids are synthesized/metabolized and affect CBF and injury after SAH. For instance, the source of CYP eicosanoids in CSF after SAH is currently unknown. Likewise, there is a lack of understanding of the mechanisms leading to the altered CYP eicosanoid synthesis by the release of plasmogens from blood in the CSF after SAH. The interaction of CYP eicosanoids with other vasoactive compounds and their relative contribution to the pathophysiology of SAH should be further investigated. In addition, the predominant metabolite of 20-HETE has not been identified

and it is unknown if 20-HETE elicits its vasoconstrictive effects directly or through further metabolism to COX metabolites.[621, 792] More importantly, the 20-HETE and EET receptors should be identified and targeted for pharmacological intervention after SAH. Further investigation in these areas would greatly enhance our understanding of the biological mechanisms of CYP eicosanoids in SAH.

5.2.4 Focus on Translational Research

The development of a drug is a costly and high-risk process with high failure rates. The average length of time from target discovery to approval of a new drug currently averages about 13 years, the failure rate exceeds 95%, and the cost per successful drug exceeds $1 billion, after adjusting for all of the failures.[793] Recently, the National Institute of Health (NIH) formed the National Center for Advancing Translational Sciences (NCATS) in order to address common barriers to clinical and translational research, develop innovative and effective therapies, and transform healthcare. One of the key elements of the NCATS approach is to develop and support innovative collaborations across traditionally separate scientific disciplines and convene teams with diverse expertise to reduce, remove or bypass significant bottlenecks in translational sciences.[794] The clinical project discussed in this dissertation utilized this key NCATS element of diverse collaboration by including a team of researchers with expertise in biomarker analysis, genetics, statistics, neuroscience and neurosurgery, nursing and critical care medicine, and stroke research and outcomes assessments. On the other hand, the NCATS approach would most likely identify the limited number of promising drug candidates for the treatment of DCI after SAH as a barrier to translational sciences. In addition, the NCATS approach would likely identify the modifiable risk factors for SAH as a key target for the prevention SAH. In summary, the strategies and methods presented by NCATS can be used to identify barriers to clinical research focused on SAH and develop innovative therapies for conditions with unmet medical needs such as DCI.

APPENDIX A: EFFECT OF PROCESSING MODIFICATIONS ON UPLC-MS/MS PEAK RESPONSE TO MEASURE CYP EICOSANOIDS

Load Volume	1 ml	1 ml	3 ml	3 ml
Reconstitution Volume	125 ml	50 ml	125 ml	50 ml
Theoretical Fold Increase	-	2.5	3	7.5
20-HETE-d6	1.00 ± 0.04	2.46 ± 0.15	2.81 ± 0.20	7.08 ± 0.47
20-HETE	1.00 ± 0.11	3.30 ± 0.27	3.60 ± 0.39	10.00 ± 0.59
14,15-EET	1.00 ± 0.06	3.01 ± 0.31	3.66 ± 0.33	9.35 ± 1.06
11,12-EET	1.00 ± 0.12	2.67 ± 0.50	3.42 ± 0.51	9.06 ± 1.02
8,9-EET	1.00 ± 0.19	3.46 ± 0.83	3.96 ± 0.75	11.62 ± 2.52
14,15-DHET	1.00 ± 0.05	2.69 ± 0.22	3.07 ± 0.23	8.96 ± 0.75
11,12-DHET	1.00 ± 0.07	2.70 ± 0.14	2.91 ± 0.29	8.07 ± 0.80
8,9-DHET	1.00 ± 0.14	2.86 ± 0.30	3.12 ± 0.38	8.41 ± 1.02
5,6-DHET	1.00 ± 0.04	2.52 ± 0.18	2.85 ± 0.12	7.54 ± 0.51

Peak response was expressed as mean peak area ± SD and data were normalized to the values observed using our published method (1 ml load volume and 125 µl reconstitution volume).

APPENDIX B: 20-HETE CSF LEVELS IN GENETIC GROUPS

Gene	rs#	Genotype	N	Mean 20-HETE (ng/ml)	P-value	Max. 20-HETE (ng/ml)	P-value
CYP4A11	rs9332978 (†)	g.4207AA	185	0.117 ± 0.018	0.005*	0.346 ± 0.053	0.347
		g.4207G-carriers	35	0.061 ± 0.008		0.228 ± 0.060	
	rs1126742 (†)	g.13661G>A	5	0.080 ± 0.042	0.747	0.418 ± 0.322	0.772
		g.13661A-carriers	172	0.114 ± 0.018		0.3273 ± 0.052	
	rs3890011 (‡)	g.13414CC	132	0.117 ± 0.019	0.561	0.361 ± 0.066	0.364
		g.13414G-carriers	84	0.099 ± 0.026		0.275 ± 0.058	
CYP4F2	rs2108622 (†‡)	g.14389CC	113	0.110 ± 0.022	0.455	0.352 ± 0.073	0.482
		g.14389T-carriers	95	0.090 ± 0.014		0.286 ± 0.054	
	rs2189784 (†)	g.7222002GG	68	0.105 ± 0.023	0.841	0.319 ± 0.061	0.85
		g.7222002A-carriers	150	0.112 ± 0.019		0.338 ± 0.061	
	rs3093100 (†)	g.5416GG	149	0.112 ± 0.02	0.713	0.330 ± 0.060	0.842
		g.5416C-carriers	67	0.099 ± 0.022		0.311 ± 0.066	
	rs3093098 (†)	g.5373TT	152	0.112 ± 0.019	0.712	0.338 ± 0.060	0.774
		g.5373C-carriers	68	0.100 ± 0.022		0.31 ± 0.065	
	rs3093105 (†)	g.5497T>C	159	0.110 ± 0.017	0.581	0.346 ± 0.057	0.348
		g.5497C-carriers	60	0.093 ± 0.023		0.252 ± 0.050	
	rs3093089 (‡)	g.4593TT	94	0.103 ± 0.022	0.554	0.293 ± 0.053	0.37
		g.4593C-carriers	113	0.122 ± 0.023		0.380 ± 0.077	
	rs3093156 (‡)	g.4211AA	58	0.129 ± 0.034	0.462	0.390 ± 0.081	0.46
		g.4211T-carriers	160	0.103 ± 0.017		0.313 ± 0.056	
	rs3093168 (‡)	g.8575TT	90	0.102 ± 0.020	0.721	0.332 ± 0.087	0.968
		g.8575C-carriers	128	0.113 ± 0.022		0.328 ± 0.049	
	rs3093207 (‡)	g.16162AA	100	0.126 ± 0.028	0.307	0.383 ± 0.083	0.293
		g.16162G-carriers	120	0.095 ± 0.015		0.286 ± 0.047	

*Statistical significance established at *p<0.05

APPENDIX C: EET CSF LEVELS IN GENETIC GROUPS

Gene	rs#	Genotype	N	Max. EET Mean ± SEM (ng/ml)	P-value	Mean EET Mean ± SEM (ng/ml)	P-value
CYP2C8	rs10509681	g.35506T/T	175	0.155 ± 0.024	0.823	0.076 ± 0.009	0.945
		g.35506C-carriers	46	0.166 ± 0.036		0.077 ± 0.013	
	rs1058930	g.16136C/C	197	0.166 ± 0.022	0.007$^{\chi}$*	0.080 ± 0.009	0.002$^{\chi}$*
		g.16136G-carriers	24	0.082 ± 0.021		0.045 ± 0.007	
	rs7909236	g.4825G/G	123	0.155 ± 0.023	0.626	0.077 ± 0.011	0.923
		g.4825T-carriers	95	0.176 ± 0.038		0.078 ± 0.012	
	rs11572133	g.24879A/A	114	0.201 ± 0.036	0.072$^{\chi}$	0.091 ± 0.014	0.080$^{\chi}$
		g.24879T-carriers	101	0.127 ± 0.019		0.063 ± 0.007	
	rs11572139	g.25369C/C	98	0.107 ± 0.017	0.012$^{\chi}$*	0.057 ± 0.006	0.024$^{\chi}$*
		g.25369T-carriers	119	0.207 ± 0.035		0.091 ± 0.013	
	rs1934952	g.36755G/G	71	0.116 ± 0.021	0.028$^{\chi}$*	0.061 ± 0.008	0.033$^{\chi}$*
		g.36755A-carriers	104	0.214 ± 0.039		0.099 ± 0.018	
	rs1934953	g.36785A/A	100	0.149 ± 0.024	0.494	0.069 ± 0.008	0.33
		g.36785G-carriers	117	0.178 ± 0.034		0.085 ± 0.013	
CYP2C9	rs1799853	g.8633C/C	166	0.155 ± 0.025	0.422	0.076 ± 0.010	0.78
		g.8633T-carriers	52	0.195 ± 0.038		0.082 ± 0.012	
	rs1057910	g.47639A/A	190	0.158 ± 0.022	0.464	0.076 ± 0.009	0.584
		g.47639C-carriers	24	0.208 ± 0.069		0.090 ± 0.024	
	rs12772884	g.723T/T	72	0.149 ± 0.026	0.598	0.070 ± 0.009	0.493
		g.723A-carriers	145	0.173 ± 0.029		0.082 ± 0.011	
	rs1934967	g.48012C/C	128	0.136 ± 0.019	0.214$^{\chi}$	0.067 ± 0.007	0.209$^{\chi}$
		g.48012T-carriers	92	0.193 ± 0.042		0.090 ± 0.016	
	rs2253635	g.816A/A	85	0.136 ± 0.022	0.287	0.066 ± 0.008	0.297
		g.816G-carriers	128	0.183 ± 0.032		0.083 ± 0.012	
	rs4086116	g.13788C/C	140	0.150 ± 0.027	0.421	0.076 ± 0.011	0.962
		g.13788T-carriers	77	0.185 ± 0.032		0.076 ± 0.009	
	rs4918766	g.18470G/G	117	0.180 ± 0.034	0.429	0.086 ± 0.013	0.234$^{\chi}$
		g.18470A-carriers	101	0.146 ± 0.022		0.068 ± 0.008	
	rs9332104	g.5276T/T	127	0.177 ± 0.029	0.421	0.080 ± 0.011	0.644
		g.5276C-carriers	88	0.142 ± 0.031		0.072 ± 0.012	
CYP2J2	rs890293	g.4930G/G	193	0.167 ± 0.023	0.661	0.078 ± 0.009	0.899
		g.4930T-carriers	28	0.139 ± 0.038		0.075 ± 0.018	
	rs1155002	g.30345693C/C	87	0.179 ± 0.034	0.554	0.093 ± 0.017	0.176$^{\chi}$
		g.30345693T-carriers	132	0.154 ± 0.027		0.067 ± 0.007	
	rs7515289	g.14742T/T	121	0.177 ± 0.032	0.41	0.078 ± 0.010	0.725
		g.14742G-carriers	98	0.142 ± 0.024		0.073 ± 0.012	
EPHX2	rs41507953	g.14861A/A	180	0.168 ± 0.024	0.049$^{\chi}$*	0.081 ± 0.009	0.003$^{\chi}$*
		g.14861G-carriers	38	0.099 ± 0.025		0.047 ± 0.006	
	rs751141	g.30221C/C	169	0.163 ± 0.024	0.609	0.076 ± 0.009	0.928
		g.30221T-carriers	51	0.139 ± 0.039		0.078 ± 0.019	
	rs71553864	404Thr/Thr	202	0.164 ± 0.022	0.793	0.077 ± 0.008	0.973
		404del-carriers	20	0.145 ± 0.048		0.076 ± 0.020	
	rs2071575	g.54788T/T	111	0.164 ± 0.028	0.967	0.079 ± 0.012	0.828
		g.54788C-carriers	110	0.162 ± 0.031		0.075 ± 0.011	
	rs7816586	g.25690G/G	72	0.164 ± 0.036	0.928	0.080 ± 0.015	0.904
		g.25690A/C-carriers	143	0.168 ± 0.027		0.078 ± 0.010	

EET, epoxyeicosatrienoic acids; SEM, standard error of the mean; Max, maximum;

$^{\chi}$ Unequal variances per Levene's test; *Statistical significance established at $p<0.05$

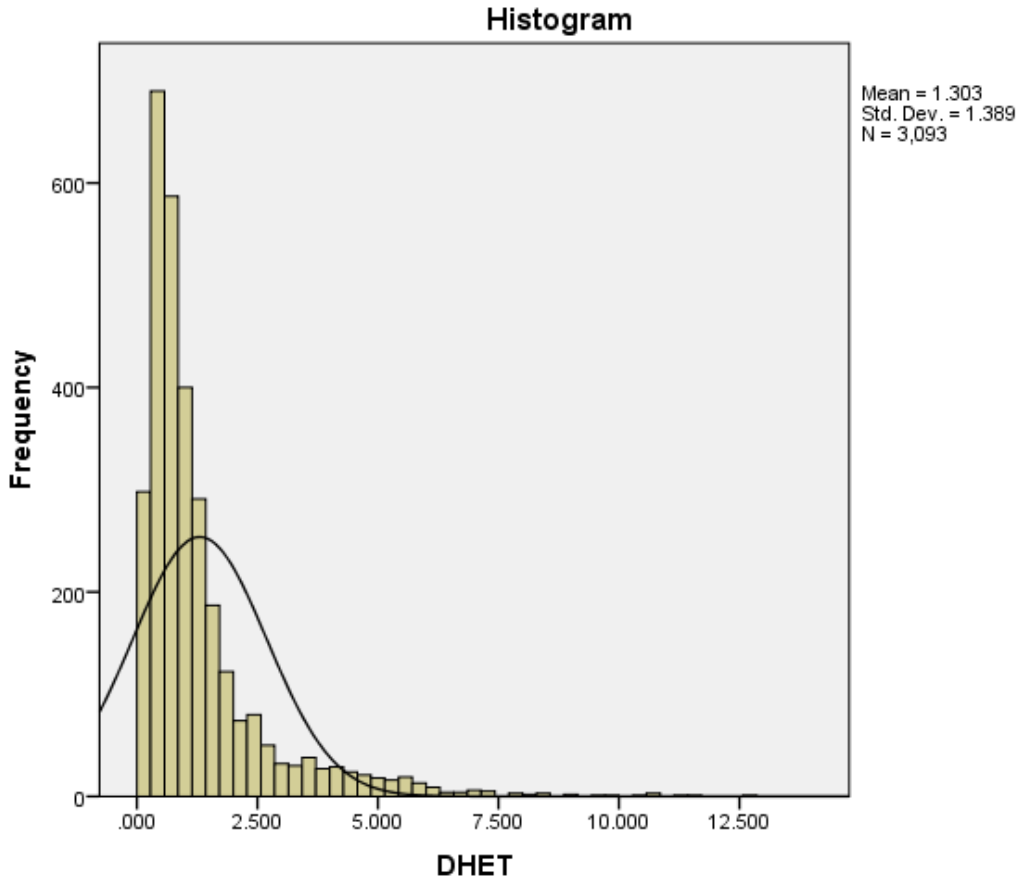
APPENDIX D: DHET CSF LEVELS IN GENETIC GROUPS

Gene	rs#	Genotype	N	Max. DHET Mean ± SEM (ng/ml)	P-value	Mean DHET Mean ± SEM (ng/ml)	P-value
CYP2C8	rs10509681	g.35506T/T	175	2.601 ± 0.188	0.837	1.331 ± 0.090	0.979
		g.35506C-carriers	46	2.518 ± 0.316		1.326 ± 0.180	
	rs1058930	g.16136C/C	197	2.658 ± 0.178	0.048*	1.384 ± 0.089	<0.001$^{\chi}$*
		g.16136G-carriers	24	1.968 ± 0.289		0.891 ± 0.107	
	rs7909236	g.4825G/G	123	2.920 ± 0.221	0.044*	1.451 ± 0.113	0.154
		g.4825T-carriers	95	2.253 ± 0.241		1.217 ± 0.116	
	rs11572133	g.24879A/A	114	2.736 ± 0.241	0.506	1.424 ± 0.117	0.408
		g.24879T-carriers	101	2.514 ± 0.226		1.287 ± 0.115	
	rs11572139	g.25369C/C	98	2.429 ± 0.244	0.291	1.213 ± 0.119	0.159
		g.25369T-carriers	119	2.779 ± 0.223		1.443 ± 0.111	
	rs1934952	g.36755G/G	71	2.448 ± 0.281	0.272	1.330 ± 0.150	0.521
		g.36755A-carriers	104	2.862 ± 0.244		1.455 ± 0.124	
	rs1934953	g.36785A/A	100	2.942 ± 0.244	0.044*	1.430 ± 0.124	0.252
		g.36785G-carriers	117	2.294 ± 0.210		1.246 ± 0.103	
CYP2C9	rs1799853	g.8633C/C	166	2.616 ± 0.194	0.882	1.334 ± 0.093	0.743
		g.8633T-carriers	52	2.673 ± 0.301		1.397 ± 0.167	
	rs1057910	g.47639A/A	190	2.610 ± 0.177	0.609	1.344 ± 0.088	0.661
		g.47639C-carriers	24	2.881 ± 0.510		1.460 ± 0.245	
	rs12772884	g.723T/T	72	2.478 ± 0.261	0.615	1.359 ± 0.149	0.805
		g.723A-carriers	145	2.650 ± 0.203		1.317 ± 0.094	
	rs1934967	g.48012C/C	128	2.803 ± 0.213	0.122	1.420 ± 0.108	0.204
		g.48012T-carriers	92	2.292 ± 0.251		1.212 ± 0.120	
	rs2253635	g.816A/A	85	2.759 ± 0.262	0.377	1.412 ± 0.137	0.399
		g.816G-carriers	128	2.464 ± 0.209		1.273 ± 0.098	
	rs4086116	g.13788C/C	140	2.626 ± 0.216	0.967	1.339 ± 0.106	0.997
		g.13788T-carriers	77	2.612 ± 0.250		1.339 ± 0.125	
	rs4918766	g.18470G/G	117	2.751 ± 0.246	0.426	1.383 ± 0.116	0.659
		g.18470A-carriers	101	2.488 ± 0.212		1.310 ± 0.114	
	rs9332104	g.5276T/T	127	2.603 ± 0.220	0.758	1.327 ± 0.106	0.644
		g.5276C-carriers	88	2.708 ± 0.253		1.405 ± 0.131	
CYP2J2	rs890293	g.4930G/G	193	2.680 ± 0.175	0.222	1.402 ± 0.089	0.004$^{\chi}$*
		g.4930T-carriers	28	2.083 ± 0.438		0.892 ± 0.145	
	rs1155002	g.30345693C/C	87	2.558 ± 0.237	0.754	1.308 ± 0.120	0.701
		g.30345693T-carriers	132	2.663 ± 0.223		1.372 ± 0.109	
	rs7515289	g.14742T/T	121	2.722 ± 0.226	0.373	1.400 ± 0.111	0.354
		g.14742G-carriers	98	2.428 ± 0.237		1.249 ± 0.117	
EPHX2	rs41507953	g.14861A/A	180	2.460 ± 0.168	0.094	1.293 ± 0.089	0.374
		g.14861G-carriers	38	3.184 ± 0.493		1.483 ± 0.198	
	rs751141	g.30221C/C	169	2.631 ± 0.187	0.639	1.377 ± 0.098	0.319
		g.30221T-carriers	51	2.450 ± 0.332		1.186 ± 0.128	
	rs71553864	404Thr/Thr	202	2.653 ± 0.170	0.278	1.371 ± 0.086	0.168
		404del-carriers	20	2.038 ± 0.523		0.984 ± 0.189	
	rs2071575	g.54788T/T	111	2.486 ± 0.209	0.496	1.307 ± 0.115	0.734
		g.54788C-carriers	110	2.708 ± 0.251		1.362 ± 0.114	
	rs7816586	g.25690G/G	72	2.587 ± 0.290	1	1.256 ± 0.129	0.559
		g.25690A/C-carriers	143	2.587 ± 0.195		1.354 ± 0.100	

DHET, dihydroxyeicosatetraenoic acid (DHET); SEM, standard error of the mean; Max, maximum;

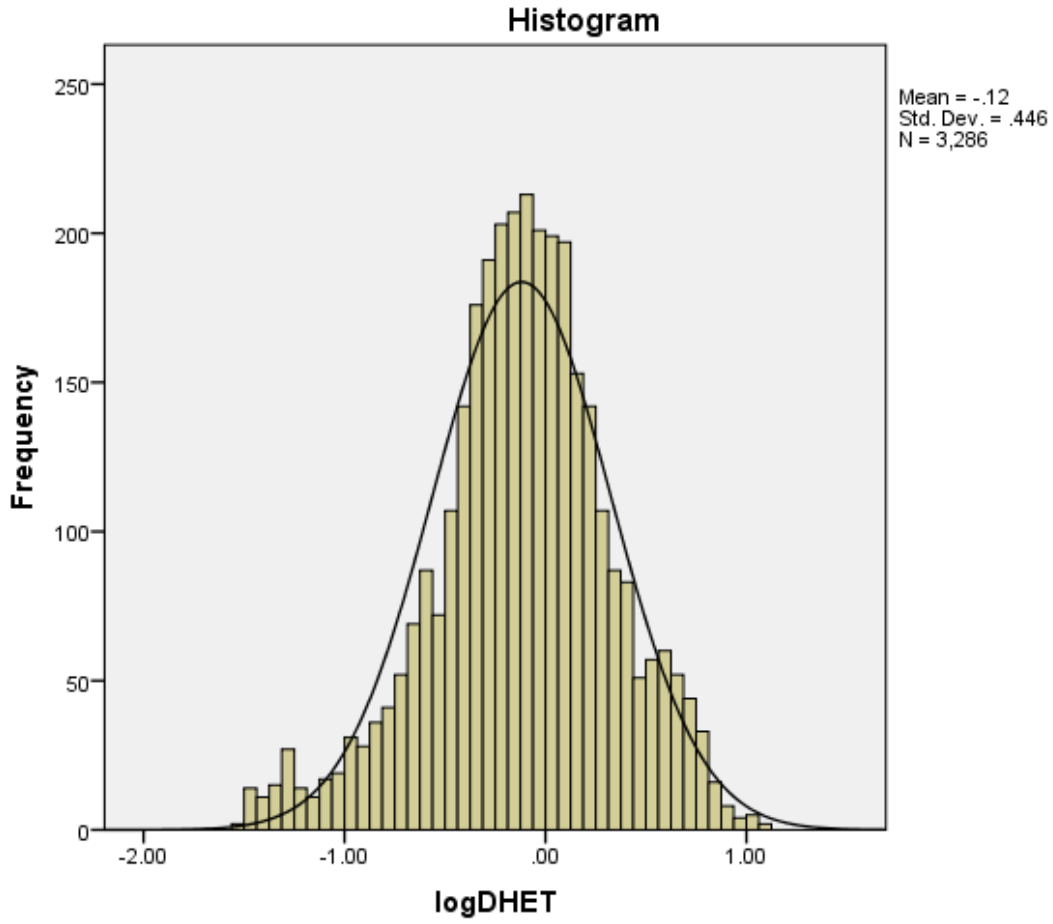
$^{\chi}$ Unequal variances per Levene's test; *Statistical significance established at $p<0.05$

APPENDIX E: DHET CONCENTRATION FREQUENCY DISTRIBUTION



x-axis: DHET levels (ng/ml); y-axis: frequency of CSF samples

APPENDIX F: LOG-TRANSFORMED DHET CONCENTRATION FREQUENCY DISTRIBUTION



x-axis: log-transformed DHET levels (ng/ml); y-axis: frequency of CSF samples

BIBLIOGRAPHY

1. Mohr JP, Caplan LR, Melski JW, Goldstein RJ, Duncan GW, Kistler JP, et al. The harvard cooperative stroke registry: A prospective registry. *Neurology* 1978; **28**: 754-762.
2. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics--2013 update: A report from the american heart association. *Circulation* 2013; **127**: e6-e245.
3. Murphy S. Deaths: Final data for 2010. *National Vital Statistics Reports*. 2013;61
4. Bederson JB, Awad IA, Wiebers DO, Piepgras D, Haley EC, Jr., Brott T, et al. Recommendations for the management of patients with unruptured intracranial aneurysms: A statement for healthcare professionals from the stroke council of the american heart association. *Stroke; a journal of cerebral circulation* 2000; **31**: 2742-2750.
5. Ferro JM, Canhao P, Peralta R. Update on subarachnoid haemorrhage. *Journal of neurology* 2008; **255**: 465-479.
6. Johnston SC, Selvin S, Gress DR. The burden, trends, and demographics of mortality from subarachnoid hemorrhage. *Neurology* 1998; **50**: 1413-1418.
7. Taylor TN. The medical economics of stroke. *Drugs* 1997; **54 Suppl 3**: 51-57; discussion 57-58.
8. Al-Tamimi YZ, Orsi NM, Quinn AC, Homer-Vanniasinkam S, Ross SA. A review of delayed ischemic neurologic deficit following aneurysmal subarachnoid hemorrhage: Historical overview, current treatment, and pathophysiology. *World neurosurgery* 2010; **73**: 654-667.
9. Bonet T. *Sepulcretum anatomicum*. Geneva; 1679.
10. Biumi F. *Observationes anatomicae, scholiis illustrati*. Milan: S & J Luchtmans; 1765.
11. Morgagni J. De sedibus et causis morborum per anatomen indagatis. 1769; **Book 1, Letters 3 and 4**:
12. Blackall J. *Observations on the nature and cure ofdropsies*. London; 1814.
13. Gull W. Cases of aneurism of the cerebral vessels*Guys Hosp Rep* 1859; **5**: 281-304.
14. Moniz E. L'angiographie cérébrale, ses applications et résultats en anatomic, physiologie te clinique [cerebral angiography, its applications and results in anatomy, physiology, and clinic]. 1934:
15. Dandy W. Intracranial aneurysms of the internal carotid artery. Cured by operation. . *Ann Surg* 1938; **107**: 654-659.
16. Robertson E. Cerebral lesions due to intracranial aneurysms. *Brain* 1949; **72**: 150-185.
17. Florey H. Microscopical observations on the circulation of the blood in the cerebral cortex *Brain* 1925; **48**: 43-64.
18. Zucker M. A study of substances in blood serum and platelets which stimulate smooth muscle. *AmJ Physiol* 1944; **142** 12-26.
19. Jackson IJ. Aseptic hemogenic meningitis; an experimental study of aseptic meningeal reactions due to blood and its breakdown products. *Archives of neurology and psychiatry* 1949; **62**: 572-589.
20. Reid BJ, RT. . Proceedings of the sixth international congress of radiology in london. 1950:

21. Ecker A, Riemenschneider PA. Arteriographic demonstration of spasm of the intracranial arteries, with special reference to saccular arterial aneurysms. *Journal of neurosurgery* 1951; **8**: 660-667.
22. Stornelli SA, French JD. Subarachnoid hemorrhage--factors in prognosis and management. *Journal of neurosurgery* 1964; **21**: 769-780.
23. Kak VK, Taylor AR. Cerebral blood flow in subarachnoid hemorrhage. *Lancet* 1967; **1**: 875-877.
24. Allcock JM, Drake CG. Ruptured intracranial aneurysms--the role of arterial spasm. *Journal of neurosurgery* 1965; **22**: 21-29.
25. Suzuki J, Yoshimoto T. [early operation for the ruptured intracranial aneurysms--especially the cases operated within 48 hours after the last subarachnoid hemorrhage (author's transl)]. *No shinkei geka* 1976; **4**: 135-141.
26. Weir B, Grace M, Hansen J, Rothberg C. Time course of vasospasm in man. *Journal of neurosurgery* 1978; **48**: 173-178.
27. Katada K, Kanno T, Sano H, Shibata T, Shah MY. [computed tomography of ruptured intracranial aneurysm in acute stage (author's transl)]. *No shinkei geka* 1977; **5**: 955-963.
28. Takemae T, Mizukami M, Kin H, Kawase T, Araki G. [computed tomography of ruptured intracranial aneurysms in acute stage--relationship between vasospasm and high density on ct scan (author's transl)]. *No to shinkei = Brain and nerve* 1978; **30**: 861-866.
29. Fisher CM, Kistler JP, Davis JM. Relation of cerebral vasospasm to subarachnoid hemorrhage visualized by computerized tomographic scanning. *Neurosurgery* 1980; **6**: 1-9.
30. Claassen J, Bernardini GL, Kreiter K, Bates J, Du YE, Copeland D, et al. Effect of cisternal and ventricular blood on risk of delayed cerebral ischemia after subarachnoid hemorrhage: The fisher scale revisited. *Stroke; a journal of cerebral circulation* 2001; **32**: 2012-2020.
31. Al-Shahi R, White PM, Davenport RJ, Lindsay KW. Subarachnoid haemorrhage. *Bmj* 2006; **333**: 235-240.
32. Schievink WI. Intracranial aneurysms. *The New England journal of medicine* 1997; **336**: 28-40.
33. Brisman JL, Song JK, Newell DW. Cerebral aneurysms. *The New England journal of medicine* 2006; **355**: 928-939.
34. Juvela S, Porras M, Poussa K. Natural history of unruptured intracranial aneurysms: Probability and risk factors for aneurysm rupture. *Neurosurgical focus* 2000; **8**: Preview 1.
35. Unruptured intracranial aneurysms--risk of rupture and risks of surgical intervention. International study of unruptured intracranial aneurysms investigators. *The New England journal of medicine* 1998; **339**: 1725-1733.
36. Schwartz TH, Solomon RA. Perimesencephalic nonaneurysmal subarachnoid hemorrhage: Review of the literature. *Neurosurgery* 1996; **39**: 433-440; discussion 440.
37. van Gijn J, van Dongen KJ, Vermeulen M, Hijdra A. Perimesencephalic hemorrhage: A nonaneurysmal and benign form of subarachnoid hemorrhage. *Neurology* 1985; **35**: 493-497.
38. King JT, Jr. Epidemiology of aneurysmal subarachnoid hemorrhage. *Neuroimaging clinics of North America* 1997; **7**: 659-668.

39. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: A report from the american heart association. *Circulation* 2011; **123**: e18-e209.
40. Labovitz DL, Halim AX, Brent B, Boden-Albala B, Hauser WA, Sacco RL. Subarachnoid hemorrhage incidence among whites, blacks and caribbean hispanics: The northern manhattan study. *Neuroepidemiology* 2006; **26**: 147-150.
41. Shea AM, Reed SD, Curtis LH, Alexander MJ, Villani JJ, Schulman KA. Characteristics of nontraumatic subarachnoid hemorrhage in the united states in 2003. *Neurosurgery* 2007; **61**: 1131-1137; discussion 1137-1138.
42. Schievink WI, Wijdicks EF, Parisi JE, Piepgras DG, Whisnant JP. Sudden death from aneurysmal subarachnoid hemorrhage. *Neurology* 1995; **45**: 871-874.
43. Truelsen T, Bonita R, Duncan J, Anderson NE, Mee E. Changes in subarachnoid hemorrhage mortality, incidence, and case fatality in new zealand between 1981-1983 and 1991-1993. *Stroke; a journal of cerebral circulation* 1998; **29**: 2298-2303.
44. Feigin VL, Lawes CM, Bennett DA, Barker-Collo SL, Parag V. Worldwide stroke incidence and early case fatality reported in 56 population-based studies: A systematic review. *Lancet neurology* 2009; **8**: 355-369.
45. Harmsen P, Tsipogianni A, Wilhelmsen L. Stroke incidence rates were unchanged, while fatality rates declined, during 1971-1987 in goteborg, sweden. *Stroke; a journal of cerebral circulation* 1992; **23**: 1410-1415.
46. Ingall TJ, Whisnant JP, Wiebers DO, O'Fallon WM. Has there been a decline in subarachnoid hemorrhage mortality? *Stroke; a journal of cerebral circulation* 1989; **20**: 718-724.
47. Sacco S, Totaro R, Toni D, Marini C, Cerone D, Carolei A. Incidence, case-fatalities and 10-year survival of subarachnoid hemorrhage in a population-based registry. *European neurology* 2009; **62**: 155-160.
48. Lovelock CE, Rinkel GJ, Rothwell PM. Time trends in outcome of subarachnoid hemorrhage: Population-based study and systematic review. *Neurology* 2010; **74**: 1494-1501.
49. de Rooij NK, Linn FH, van der Plas JA, Algra A, Rinkel GJ. Incidence of subarachnoid haemorrhage: A systematic review with emphasis on region, age, gender and time trends. *Journal of neurology, neurosurgery, and psychiatry* 2007; **78**: 1365-1372.
50. Rinkel GJ, Djibuti M, Algra A, van Gijn J. Prevalence and risk of rupture of intracranial aneurysms: A systematic review. *Stroke; a journal of cerebral circulation* 1998; **29**: 251-256.
51. Wardlaw JM, White PM. The detection and management of unruptured intracranial aneurysms. *Brain* 2000; **123 (Pt 2)**: 205-221.
52. Longstreth WT, Nelson LM, Koepsell TD, van Belle G. Subarachnoid hemorrhage and hormonal factors in women. A population-based case-control study. *Annals of internal medicine* 1994; **121**: 168-173.
53. Ellamushi HE, Grieve JP, Jager HR, Kitchen ND. Risk factors for the formation of multiple intracranial aneurysms. *Journal of neurosurgery* 2001; **94**: 728-732.
54. Juvela S. Risk factors for multiple intracranial aneurysms. *Stroke; a journal of cerebral circulation* 2000; **31**: 392-397.

55. Qureshi AI, Suarez JI, Parekh PD, Sung G, Geocadin R, Bhardwaj A, et al. Risk factors for multiple intracranial aneurysms. *Neurosurgery* 1998; **43**: 22-26; discussion 26-27.
56. Okamoto K, Horisawa R, Kawamura T, Asai A, Ogino M, Takagi T, et al. Menstrual and reproductive factors for subarachnoid hemorrhage risk in women: A case-control study in nagoya, japan. *Stroke; a journal of cerebral circulation* 2001; **32**: 2841-2844.
57. Inagawa T. Seasonal variation in the incidence of aneurysmal subarachnoid hemorrhage in hospital- and community-based studies. *Journal of neurosurgery* 2002; **96**: 497-509.
58. Gallerani M, Portaluppi F, Maida G, Chieregato A, Calzolari F, Trapella G, et al. Circadian and circannual rhythmicity in the occurrence of subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 1996; **27**: 1793-1797.
59. Oyoshi T, Nakayama M, Kuratsu J. Relationship between aneurysmal subarachnoid hemorrhage and climatic conditions in the subtropical region, amami-oshima, in japan. *Neurologia medico-chirurgica* 1999; **39**: 585-590; discussion 590-581.
60. Broderick JP, Brott T, Tomsick T, Huster G, Miller R. The risk of subarachnoid and intracerebral hemorrhages in blacks as compared with whites. *The New England journal of medicine* 1992; **326**: 733-736.
61. Anderson C, Anderson, N., Bonita, R. Epidemiology of aneurysmal subarachnoid hemorrhage in australia and new zealand: Incidence and case fatality from the australasian cooperative research on subarachnoid hemorrhage study (across). *Stroke; a journal of cerebral circulation* 2000; **31**: 1843-1850.
62. Ingall T, Asplund K, Mahonen M, Bonita R. A multinational comparison of subarachnoid hemorrhage epidemiology in the who monica stroke study. *Stroke; a journal of cerebral circulation* 2000; **31**: 1054-1061.
63. Atkinson JL, Sundt TM, Jr., Houser OW, Whisnant JP. Angiographic frequency of anterior circulation intracranial aneurysms. *Journal of neurosurgery* 1989; **70**: 551-555.
64. Nakagawa T, Hashi K. The incidence and treatment of asymptomatic, unruptured cerebral aneurysms. *Journal of neurosurgery* 1994; **80**: 217-223.
65. Wiebers DO, Whisnant JP, Huston J, 3rd, Meissner I, Brown RD, Jr., Piepgras DG, et al. Unruptured intracranial aneurysms: Natural history, clinical outcome, and risks of surgical and endovascular treatment. *Lancet* 2003; **362**: 103-110.
66. Norrgard O, Beckman G, Beckman L, Cedergren B, Fodstad H, Angquist KA. Genetic markers in patients with intracranial aneurysms. *Human heredity* 1987; **37**: 255-259.
67. Schievink WI, Schaid DJ, Michels VV, Piepgras DG. Familial aneurysmal subarachnoid hemorrhage: A community-based study. *Journal of neurosurgery* 1995; **83**: 426-429.
68. Ronkainen A, Hernesniemi J, Ryynanen M. Familial subarachnoid hemorrhage in east finland, 1977-1990. *Neurosurgery* 1993; **33**: 787-796; discussion 796-797.
69. De Braekeleer M, Perusse L, Cantin L, Bouchard JM, Mathieu J. A study of inbreeding and kinship in intracranial aneurysms in the saguenay lac-saint-jean region (quebec, canada). *Annals of human genetics* 1996; **60**: 99-104.
70. Norrgard O, Angquist KA, Fodstad H, Forsell A, Lindberg M. Intracranial aneurysms and heredity. *Neurosurgery* 1987; **20**: 236-239.
71. Peterson EW, Cardoso ER. The blood-brain barrier following experimental subarachnoid hemorrhage. Part 2: Response to mercuric chloride infusion. *Journal of neurosurgery* 1983; **58**: 345-351.

72. David CA, Vishteh AG, Spetzler RF, Lemole M, Lawton MT, Partovi S. Late angiographic follow-up review of surgically treated aneurysms. *Journal of neurosurgery* 1999; **91**: 396-401.
73. Zacks DJ, Russell DB, Miller JD. Fortuitously discovered intracranial aneurysms. *Archives of neurology* 1980; **37**: 39-41.
74. Inagawa T, Hirano A. Autopsy study of unruptured incidental intracranial aneurysms. *Surgical neurology* 1990; **34**: 361-365.
75. Rinne JK, Hernesniemi JA. De novo aneurysms: Special multiple intracranial aneurysms. *Neurosurgery* 1993; **33**: 981-985.
76. King JT, Jr., Berlin JA, Flamm ES. Morbidity and mortality from elective surgery for asymptomatic, unruptured, intracranial aneurysms: A meta-analysis. *Journal of neurosurgery* 1994; **81**: 837-842.
77. Winn HR, Almaani WS, Berga SL, Jane JA, Richardson AE. The long-term outcome in patients with multiple aneurysms. Incidence of late hemorrhage and implications for treatment of incidental aneurysms. *Journal of neurosurgery* 1983; **59**: 642-651.
78. Wang PS, Longstreth WT, Jr., Koepsell TD. Subarachnoid hemorrhage and family history. A population-based case-control study. *Archives of neurology* 1995; **52**: 202-204.
79. Bromberg JE, Rinkel GJ, Algra A, Greebe P, van Duyn CM, Hasan D, et al. Subarachnoid haemorrhage in first and second degree relatives of patients with subarachnoid haemorrhage. *Bmj* 1995; **311**: 288-289.
80. Mitchell P, Gholkar A, Vindlacheruvu RR, Mendelow AD. Unruptured intracranial aneurysms: Benign curiosity or ticking bomb? *Lancet neurology* 2004; **3**: 85-92.
81. Schievink WI. Genetics of intracranial aneurysms. *Neurosurgery* 1997; **40**: 651-662; discussion 662-653.
82. Nahed BV, Bydon M, Ozturk AK, Bilguvar K, Bayrakli F, Gunel M. Genetics of intracranial aneurysms. *Neurosurgery* 2007; **60**: 213-225; discussion 225-216.
83. Krischek B, Inoue I. The genetics of intracranial aneurysms. *Journal of human genetics* 2006; **51**: 587-594.
84. Chapman AB, Rubinstein D, Hughes R, Stears JC, Earnest MP, Johnson AM, et al. Intracranial aneurysms in autosomal dominant polycystic kidney disease. *The New England journal of medicine* 1992; **327**: 916-920.
85. de Paepe A, van Landegem W, de Keyser F, de Reuck J. Association of multiple intracranial aneurysms and collagen type iii deficiency. *Clinical neurology and neurosurgery* 1988; **90**: 53-56.
86. ter Berg HW, Bijlsma JB, Veiga Pires JA, Ludwig JW, van der Heiden C, Tulleken CA, et al. Familial association of intracranial aneurysms and multiple congenital anomalies. *Archives of neurology* 1986; **43**: 30-33.
87. Leftheriotis G, Omarjee L, Le Saux O, Henrion D, Abraham P, Prunier F, et al. The vascular phenotype in pseudoxanthoma elasticum and related disorders: Contribution of a genetic disease to the understanding of vascular calcification. *Frontiers in genetics* 2013; **4**: 4.
88. Schievink WI, Piepgras DG. Cervical vertebral artery aneurysms and arteriovenous fistulae in neurofibromatosis type 1: Case reports. *Neurosurgery* 1991; **29**: 760-765.
89. Bastos AP, Onuchic LF. Molecular and cellular pathogenesis of autosomal dominant polycystic kidney disease. *Brazilian journal of medical and biological research = Revista*

brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al 2011; **44**: 606-617.
90. Gieteling EW, Rinkel GJ. Characteristics of intracranial aneurysms and subarachnoid haemorrhage in patients with polycystic kidney disease. *Journal of neurology* 2003; **250**: 418-423.
91. Hughes PD, Becker GJ. Screening for intracranial aneurysms in autosomal dominant polycystic kidney disease. *Nephrology* 2003; **8**: 163-170.
92. Chauveau D, Pirson Y, Verellen-Dumoulin C, Macnicol A, Gonzalo A, Grunfeld JP. Intracranial aneurysms in autosomal dominant polycystic kidney disease. *Kidney international* 1994; **45**: 1140-1146.
93. Pirson Y, Chauveau D. Adpkd-associated intracranial aneurysm: New insights and unanswered questions. *Contributions to nephrology* 1995; **115**: 53-58.
94. Schievink WI, Torres VE, Piepgras DG, Wiebers DO. Saccular intracranial aneurysms in autosomal dominant polycystic kidney disease. *J Am Soc Nephrol* 1992; **3**: 88-95.
95. Onda H, Kasuya H, Yoneyama T, Takakura K, Hori T, Takeda J, et al. Genomewide-linkage and haplotype-association studies map intracranial aneurysm to chromosome 7q11. *American journal of human genetics* 2001; **69**: 804-819.
96. Lee RM. Morphology of cerebral arteries. *Pharmacology & therapeutics* 1995; **66**: 149-173.
97. Watton PN, Ventikos Y, Holzapfel GA. Modelling the growth and stabilization of cerebral aneurysms. *Mathematical medicine and biology : a journal of the IMA* 2009; **26**: 133-164.
98. Lopez-Novoa JM, Bernabeu C. The physiological role of endoglin in the cardiovascular system. *American journal of physiology. Heart and circulatory physiology* 2010; **299**: H959-974.
99. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. *The Journal of biological chemistry* 1999; **274**: 21491-21494.
100. Yoneyama T, Kasuya H, Onda H, Akagawa H, Hashiguchi K, Nakajima T, et al. Collagen type i alpha2 (col1a2) is the susceptible gene for intracranial aneurysms. *Stroke; a journal of cerebral circulation* 2004; **35**: 443-448.
101. Brega KE, Seltzer WK, Munro LG, Breeze RE. Genotypic variations of type iii collagen in patients with cerebral aneurysms. *Surgical neurology* 1996; **46**: 253-256; discussion 256-257.
102. Ruigrok YM, Seitz U, Wolterink S, Rinkel GJ, Wijmenga C, Urban Z. Association of polymorphisms and haplotypes in the elastin gene in dutch patients with sporadic aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2004; **35**: 2064-2068.
103. Takenaka K, Sakai H, Yamakawa H, Yoshimura S, Kumagai M, Yamakawa H, et al. Polymorphism of the endoglin gene in patients with intracranial saccular aneurysms. *Journal of neurosurgery* 1999; **90**: 935-938.
104. Peters DG, Kassam A, St Jean PL, Yonas H, Ferrell RE. Functional polymorphism in the matrix metalloproteinase-9 promoter as a potential risk factor for intracranial aneurysm. *Stroke; a journal of cerebral circulation* 1999; **30**: 2612-2616.
105. Khurana VG, Sohni YR, Mangrum WI, McClelland RL, O'Kane DJ, Meyer FB, et al. Endothelial nitric oxide synthase t-786c single nucleotide polymorphism: A putative

genetic marker differentiating small versus large ruptured intracranial aneurysms. *Stroke; a journal of cerebral circulation* 2003; **34**: 2555-2559.
106. Khurana VG, Meissner I, Sohni YR, Bamlet WR, McClelland RL, Cunningham JM, et al. The presence of tandem endothelial nitric oxide synthase gene polymorphisms identifying brain aneurysms more prone to rupture. *Journal of neurosurgery* 2005; **102**: 526-531.
107. Khurana VG, Meissner I, Meyer FB. Update on genetic evidence for rupture-prone compared with rupture-resistant intracranial saccular aneurysms. *Neurosurgical focus* 2004; **17**: E7.
108. Slowik A, Borratynska A, Pera J, Betlej M, Dziedzic T, Krzyszkowski T, et al. Ii genotype of the angiotensin-converting enzyme gene increases the risk for subarachnoid hemorrhage from ruptured aneurysm. *Stroke; a journal of cerebral circulation* 2004; **35**: 1594-1597.
109. Keramatipour M, McConnell RS, Kirkpatrick P, Tebbs S, Furlong RA, Rubinsztein DC. The ace i allele is associated with increased risk for ruptured intracranial aneurysms. *Journal of medical genetics* 2000; **37**: 498-500.
110. Takenaka K, Yamakawa H, Sakai H, Yoshimura S, Murase S, Okumura A, et al. Angiotensin i-converting enzyme gene polymorphism in intracranial saccular aneurysm individuals. *Neurological research* 1998; **20**: 607-611.
111. Slowik A, Borratynska A, Turaj W, Pera J, Dziedzic T, Wloch D, et al. Interleukin 1beta-511 c/t polymorphism and risk of aneurysmal subarachnoid haemorrhage. *Journal of neurology, neurosurgery, and psychiatry* 2006; **77**: 279-280.
112. Slowik A, Borratynska A, Turaj W, Pera J, Dziedzic T, Figlewicz DA, et al. Alpha1-antichymotrypsin gene (serpina3) a/t polymorphism as a risk factor for aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2005; **36**: 737-740.
113. Morgan L, Hawe E, Palmen J, Montgomery H, Humphries SE, Kitchen N. Polymorphism of the heme oxygenase-1 gene and cerebral aneurysms. *British journal of neurosurgery* 2005; **19**: 317-321.
114. Roberts GA, Corcoran BT, Pfouts LL, Phillips JP, Farrell MA, Bouchier-Hayes DJ, et al. Genetic evaluation of lipoprotein(a) in intracranial aneurysm disease. *Neurosurgery* 2001; **49**: 133-140; discussion 141-132.
115. Taylor CL, Yuan Z, Selman WR, Ratcheson RA, Rimm AA. Cerebral arterial aneurysm formation and rupture in 20,767 elderly patients: Hypertension and other risk factors. *Journal of neurosurgery* 1995; **83**: 812-819.
116. Qureshi AI, Suri MF, Yahia AM, Suarez JI, Guterman LR, Hopkins LN, et al. Risk factors for subarachnoid hemorrhage. *Neurosurgery* 2001; **49**: 607-612; discussion 612-603.
117. Adams HP, Jr., Putman SF, Kassell NF, Torner JC. Prevalence of diabetes mellitus among patients with subarachnoid hemorrhage. *Archives of neurology* 1984; **41**: 1033-1035.
118. Kernan WN, Viscoli CM, Brass LM, Broderick JP, Brott T, Feldmann E, et al. Phenylpropanolamine and the risk of hemorrhagic stroke. *The New England journal of medicine* 2000; **343**: 1826-1832.
119. Oyesiku NM, Colohan AR, Barrow DL, Reisner A. Cocaine-induced aneurysmal rupture: An emergent negative factor in the natural history of intracranial aneurysms? *Neurosurgery* 1993; **32**: 518-525; discussion 525-516.
120. Nanda A, Vannemreddy PS, Polin RS, Willis BK. Intracranial aneurysms and cocaine abuse: Analysis of prognostic indicators. *Neurosurgery* 2000; **46**: 1063-1067; discussion 1067-1069.

121. Larsson SC, Mannisto S, Virtanen MJ, Kontto J, Albanes D, Virtamo J. Dietary fiber and fiber-rich food intake in relation to risk of stroke in male smokers. *European journal of clinical nutrition* 2009; **63**: 1016-1024.
122. Larsson SC, Mannisto S, Virtanen MJ, Kontto J, Albanes D, Virtamo J. Dairy foods and risk of stroke. *Epidemiology* 2009; **20**: 355-360.
123. Larsson SC, Mannisto S, Virtanen MJ, Kontto J, Albanes D, Virtamo J. Coffee and tea consumption and risk of stroke subtypes in male smokers. *Stroke; a journal of cerebral circulation* 2008; **39**: 1681-1687.
124. Feigin VL, Rinkel GJ, Lawes CM, Algra A, Bennett DA, van Gijn J, et al. Risk factors for subarachnoid hemorrhage: An updated systematic review of epidemiological studies. *Stroke; a journal of cerebral circulation* 2005; **36**: 2773-2780.
125. Connolly ES, Jr., Rabinstein AA, Carhuapoma JR, Derdeyn CP, Dion J, Higashida RT, et al. Guidelines for the management of aneurysmal subarachnoid hemorrhage: A guideline for healthcare professionals from the american heart association/american stroke association. *Stroke; a journal of cerebral circulation* 2012; **43**: 1711-1737.
126. Linn FH, Rinkel GJ, Algra A, van Gijn J. Headache characteristics in subarachnoid haemorrhage and benign thunderclap headache. *Journal of neurology, neurosurgery, and psychiatry* 1998; **65**: 791-793.
127. Fox JL. Warning headache in aneurysmal subarachnoid hemorrhage. *Archives of neurology* 1989; **46**: 839.
128. Jakobsson KE, Saveland H, Hillman J, Edner G, Zygmunt S, Brandt L, et al. Warning leak and management outcome in aneurysmal subarachnoid hemorrhage. *Journal of neurosurgery* 1996; **85**: 995-999.
129. Verweij RD, Wijdicks EF, van Gijn J. Warning headache in aneurysmal subarachnoid hemorrhage. A case-control study. *Archives of neurology* 1988; **45**: 1019-1020.
130. Polmear A. Sentinel headaches in aneurysmal subarachnoid haemorrhage: What is the true incidence? A systematic review. *Cephalalgia* 2003; **23**: 935-941.
131. McCarron MO, Alberts MJ, McCarron P. A systematic review of terson's syndrome: Frequency and prognosis after subarachnoid haemorrhage. *Journal of neurology, neurosurgery, and psychiatry* 2004; **75**: 491-493.
132. Caeiro L, Santos CO, Ferro JM, Figueira ML. Neuropsychiatric disturbances in acute subarachnoid haemorrhage. *Eur J Neurol* 2011; **18**: 857-864.
133. Edlow JA. Diagnosis of subarachnoid hemorrhage. *Neurocritical care* 2005; **2**: 99-109.
134. Liebenberg WA, Worth R, Firth GB, Olney J, Norris JS. Aneurysmal subarachnoid haemorrhage: Guidance in making the correct diagnosis. *Postgraduate medical journal* 2005; **81**: 470-473.
135. Nornes H. Cerebral arterial flow dynamics during aneurysm haemorrhage. *Acta neurochirurgica* 1978; **41**: 39-48.
136. Nornes H. The role of intracranial pressure in the arrest of hemorrhage in patients with ruptured intracranial aneurysm. *Journal of neurosurgery* 1973; **39**: 226-234.
137. Bederson JB, Germano IM, Guarino L. Cortical blood flow and cerebral perfusion pressure in a new noncraniotomy model of subarachnoid hemorrhage in the rat. *Stroke; a journal of cerebral circulation* 1995; **26**: 1086-1091; discussion 1091-1082.

138. Hop JW, Rinkel GJ, Algra A, van Gijn J. Case-fatality rates and functional outcome after subarachnoid hemorrhage: A systematic review. *Stroke; a journal of cerebral circulation* 1997; **28**: 660-664.
139. Broderick JP, Brott TG, Duldner JE, Tomsick T, Leach A. Initial and recurrent bleeding are the major causes of death following subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 1994; **25**: 1342-1347.
140. Adams HP, Jr., Kassell NF, Torner JC, Nibbelink DW, Sahs AL. Early management of aneurysmal subarachnoid hemorrhage. A report of the cooperative aneurysm study. *Journal of neurosurgery* 1981; **54**: 141-145.
141. Cook DA. Mechanisms of cerebral vasospasm in subarachnoid haemorrhage. *Pharmacology & therapeutics* 1995; **66**: 259-284.
142. Crompton MR. The pathogenesis of cerebral infarction following the rupture of cerebral berry aneurysms. *Brain* 1964; **87**: 491-510.
143. Sundt TM, Jr., Whisnant JP. Subarachnoid hemorrhage from intracranial aneurysms. Surgical management and natural history of disease. *The New England journal of medicine* 1978; **299**: 116-122.
144. Weir B. The pathophysiology of cerebral vasospasm. *British journal of neurosurgery* 1995; **9**: 375-390.
145. Larsen CC, Astrup J. Rebleeding after aneurysmal subarachnoid hemorrhage: A literature review. *World neurosurgery* 2013; **79**: 307-312.
146. Inagawa T, Kamiya K, Ogasawara H, Yano T. Rebleeding of ruptured intracranial aneurysms in the acute stage. *Surgical neurology* 1987; **28**: 93-99.
147. Rosenorn J, Eskesen V, Schmidt K, Ronde F. The risk of rebleeding from ruptured intracranial aneurysms. *Journal of neurosurgery* 1987; **67**: 329-332.
148. Hijdra A, Braakman R, van Gijn J, Vermeulen M, van Crevel H. Aneurysmal subarachnoid hemorrhage. Complications and outcome in a hospital population. *Stroke; a journal of cerebral circulation* 1987; **18**: 1061-1067.
149. Naidech AM, Janjua N, Kreiter KT, Ostapkovich ND, Fitzsimmons BF, Parra A, et al. Predictors and impact of aneurysm rebleeding after subarachnoid hemorrhage. *Archives of neurology* 2005; **62**: 410-416.
150. Connolly ES, Jr., Rabinstein AA, Carhuapoma JR, Derdeyn CP, Dion J, Higashida RT, et al. Guidelines for the management of aneurysmal subarachnoid hemorrhage: A guideline for healthcare professionals from the american heart association/american stroke association. *Stroke; a journal of cerebral circulation* 2012; **43**: 1711-1737.
151. Kissela BM, Sauerbeck L, Woo D, Khoury J, Carrozzella J, Pancioli A, et al. Subarachnoid hemorrhage: A preventable disease with a heritable component. *Stroke; a journal of cerebral circulation* 2002; **33**: 1321-1326.
152. Germanwala AV, Huang J, Tamargo RJ. Hydrocephalus after aneurysmal subarachnoid hemorrhage. *Neurosurgery clinics of North America* 2010; **21**: 263-270.
153. Smith M. Intensive care management of patients with subarachnoid haemorrhage. *Current opinion in anaesthesiology* 2007; **20**: 400-407.
154. Bederson JB, Connolly ES, Batjer HH, Dacey RG, Dion JE, Diringer MN, et al. Guidelines for the management of aneurysmal subarachnoid hemorrhage, a statement for healthcare professionals from a special writing group of the stroke council, american heart association. *Stroke; a journal of cerebral circulation* 2009; **40**: 994-1025.

155. Fisher CM, Roberson GH, Ojemann RG. Cerebral vasospasm with ruptured saccular aneurysm--the clinical manifestations. *Neurosurgery* 1977; **1**: 245-248.
156. Heros RC, Zervas NT, Varsos V. Cerebral vasospasm after subarachnoid hemorrhage: An update. *Annals of neurology* 1983; **14**: 599-608.
157. Sundt TM, Jr., Kobayashi S, Fode NC, Whisnant JP. Results and complications of surgical management of 809 intracranial aneurysms in 722 cases. Related and unrelated to grade of patient, type of aneurysm, and timing of surgery. *Journal of neurosurgery* 1982; **56**: 753-765.
158. Kassell NF, Kongable GL, Torner JC, Adams HP, Jr., Mazuz H. Delay in referral of patients with ruptured aneurysms to neurosurgical attention. *Stroke; a journal of cerebral circulation* 1985; **16**: 587-590.
159. Sakowitz OW, Unterberg AW. Detecting and treating microvascular ischemia after subarachnoid hemorrhage. *Current opinion in critical care* 2006; **12**: 103-111.
160. Vergouwen MD, Vermeulen M, van Gijn J, Rinkel GJ, Wijdicks EF, Muizelaar JP, et al. Definition of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage as an outcome event in clinical trials and observational studies: Proposal of a multidisciplinary research group. *Stroke; a journal of cerebral circulation* 2010; **41**: 2391-2395.
161. Weir B. *Subarachnoid hemorrhage: Causes and cures*. Oxford University Press; 1998.
162. Stevens RD, Nyquist PA. The systemic implications of aneurysmal subarachnoid hemorrhage. *Journal of the neurological sciences* 2007; **261**: 143-156.
163. Khechinashvili G, Asplund K. Electrocardiographic changes in patients with acute stroke: A systematic review. *Cerebrovascular diseases (Basel, Switzerland)* 2002; **14**: 67-76.
164. Macrea LM, Tramer MR, Walder B. Spontaneous subarachnoid hemorrhage and serious cardiopulmonary dysfunction--a systematic review. *Resuscitation* 2005; **65**: 139-148.
165. Wartenberg KE, Mayer SA. Medical complications after subarachnoid hemorrhage: New strategies for prevention and management. *Current opinion in critical care* 2006; **12**: 78-84.
166. Wartenberg KE, Schmidt JM, Claassen J, Temes RE, Frontera JA, Ostapkovich N, et al. Impact of medical complications on outcome after subarachnoid hemorrhage. *Critical care medicine* 2006; **34**: 617-623; quiz 624.
167. Suarez JI, Tarr RW, Selman WR. Aneurysmal subarachnoid hemorrhage. *The New England journal of medicine* 2006; **354**: 387-396.
168. Solenski NJ, Haley EC, Jr., Kassell NF, Kongable G, Germanson T, Truskowski L, et al. Medical complications of aneurysmal subarachnoid hemorrhage: A report of the multicenter, cooperative aneurysm study. Participants of the multicenter cooperative aneurysm study. *Critical care medicine* 1995; **23**: 1007-1017.
169. Claassen J, Vu A, Kreiter KT, Kowalski RG, Du EY, Ostapkovich N, et al. Effect of acute physiologic derangements on outcome after subarachnoid hemorrhage. *Critical care medicine* 2004; **32**: 832-838.
170. Lim HB, Smith M. Systemic complications after head injury: A clinical review. *Anaesthesia* 2007; **62**: 474-482.
171. Lee KH, Lukovits T, Friedman JA. "Triple-h" therapy for cerebral vasospasm following subarachnoid hemorrhage. *Neurocritical care* 2006; **4**: 68-76.
172. Egge A, Waterloo K, Sjoholm H, Solberg T, Ingebrigtsen T, Romner B. Prophylactic hyperdynamic postoperative fluid therapy after aneurysmal subarachnoid hemorrhage: A

clinical, prospective, randomized, controlled study. *Neurosurgery* 2001; **49**: 593-605; discussion 605-596.
173. Rinkel GJ, Feigin VL, Algra A, van den Bergh WM, Vermeulen M, van Gijn J. Calcium antagonists for aneurysmal subarachnoid haemorrhage. *Cochrane database of systematic reviews (Online)* 2005: CD000277.
174. van der Schaaf I, Algra A, Wermer M, Molyneux A, Clarke M, van Gijn J, et al. Endovascular coiling versus neurosurgical clipping for patients with aneurysmal subarachnoid haemorrhage. *Cochrane database of systematic reviews (Online)* 2005: CD003085.
175. Zhang B, Fugleholm K, Day LB, Ye S, Weller RO, Day IN. Molecular pathogenesis of subarachnoid haemorrhage. *The international journal of biochemistry & cell biology* 2003; **35**: 1341-1360.
176. Manno EM. Subarachnoid hemorrhage. *Neurologic clinics* 2004; **22**: 347-366.
177. Grubb RL, Jr., Raichle ME, Eichling JO, Gado MH. Effects of subarachnoid hemorrhage on cerebral blood volume, blood flow, and oxygen utilization in humans. *Journal of neurosurgery* 1977; **46**: 446-453.
178. Rabinstein AA, Weigand S, Atkinson JL, Wijdicks EF. Patterns of cerebral infarction in aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2005; **36**: 992-997.
179. Ayer R, Zhang J. Connecting the early brain injury of aneurysmal subarachnoid hemorrhage to clinical practice. *Turkish neurosurgery* 2010; **20**: 159-166.
180. Prunell GF, Mathiesen T, Svendgaard NA. Experimental subarachnoid hemorrhage: Cerebral blood flow and brain metabolism during the acute phase in three different models in the rat. *Neurosurgery* 2004; **54**: 426-436; discussion 436-427.
181. Sehba FA, Schwartz AY, Chereshnev I, Bederson JB. Acute decrease in cerebral nitric oxide levels after subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 2000; **20**: 604-611.
182. Sehba FA, Ding WH, Chereshnev I, Bederson JB. Effects of s-nitrosoglutathione on acute vasoconstriction and glutamate release after subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 1999; **30**: 1955-1961.
183. Jackowski A, Crockard A, Burnstock G, Russell RR, Kristek F. The time course of intracranial pathophysiological changes following experimental subarachnoid haemorrhage in the rat. *J Cereb Blood Flow Metab* 1990; **10**: 835-849.
184. Bederson JB, Levy AL, Ding WH, Kahn R, DiPerna CA, Jenkins AL, 3rd, et al. Acute vasoconstriction after subarachnoid hemorrhage. *Neurosurgery* 1998; **42**: 352-360; discussion 360-352.
185. Sehba FA, Mostafa G, Friedrich V, Jr., Bederson JB. Acute microvascular platelet aggregation after subarachnoid hemorrhage. *Journal of neurosurgery* 2005; **102**: 1094-1100.
186. Ostrowski RP, Colohan AR, Zhang JH. Molecular mechanisms of early brain injury after subarachnoid hemorrhage. *Neurological research* 2006; **28**: 399-414.
187. Keep RF, Andjelkovic AV, Stamatovic SM, Shakui P, Ennis SR. Ischemia-induced endothelial cell dysfunction. *Acta neurochirurgica* 2005; **95**: 399-402.

188. Park S, Yamaguchi M, Zhou C, Calvert JW, Tang J, Zhang JH. Neurovascular protection reduces early brain injury after subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2004; **35**: 2412-2417.
189. Marzatico F, Gaetani P, Cafe C, Spanu G, Rodriguez y Baena R. Antioxidant enzymatic activities after experimental subarachnoid hemorrhage in rats. *Acta neurologica Scandinavica* 1993; **87**: 62-66.
190. Gaetani P, Pasqualin A, Rodriguez y Baena R, Borasio E, Marzatico F. Oxidative stress in the human brain after subarachnoid hemorrhage. *Journal of neurosurgery* 1998; **89**: 748-754.
191. Marzatico F, Gaetani P, Tartara F, Bertorelli L, Feletti F, Adinolfi D, et al. Antioxidant status and alpha1-antiproteinase activity in subarachnoid hemorrhage patients. *Life sciences* 1998; **63**: 821-826.
192. Arai T, Takeyama N, Tanaka T. Glutathione monoethyl ester and inhibition of the oxyhemoglobin-induced increase in cytosolic calcium in cultured smooth-muscle cells. *Journal of neurosurgery* 1999; **90**: 527-532.
193. Gaetani P, Lombardi D. Brain damage following subarachnoid hemorrhage: The imbalance between anti-oxidant systems and lipid peroxidative processes. *Journal of neurosurgical sciences* 1992; **36**: 1-10.
194. Kreiter KT, Copeland D, Bernardini GL, Bates JE, Peery S, Claassen J, et al. Predictors of cognitive dysfunction after subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2002; **33**: 200-208.
195. Pickard JD, Murray GD, Illingworth R, Shaw MD, Teasdale GM, Foy PM, et al. Effect of oral nimodipine on cerebral infarction and outcome after subarachnoid haemorrhage: British aneurysm nimodipine trial. *Bmj* 1989; **298**: 636-642.
196. Schmidt JM, Rincon F, Fernandez A, Resor C, Kowalski RG, Claassen J, et al. Cerebral infarction associated with acute subarachnoid hemorrhage. *Neurocritical care* 2007; **7**: 10-17.
197. Dumont AS, Dumont RJ, Chow MM, Lin CL, Calisaneller T, Ley KF, et al. Cerebral vasospasm after subarachnoid hemorrhage: Putative role of inflammation. *Neurosurgery* 2003; **53**: 123-133; discussion 133-125.
198. Macdonald RL, Pluta RM, Zhang JH. Cerebral vasospasm after subarachnoid hemorrhage: The emerging revolution. *Nature clinical practice. Neurology* 2007; **3**: 256-263.
199. Weir B, Macdonald RL, Stoodley M. Etiology of cerebral vasospasm. *Acta neurochirurgica* 1999; **72**: 27-46.
200. Kozniewska E, Michalik R, Rafalowska J, Gadamski R, Walski M, Frontczak-Baniewicz M, et al. Mechanisms of vascular dysfunction after subarachnoid hemorrhage. *J Physiol Pharmacol* 2006; **57 Suppl 11**: 145-160.
201. Reilly C, Amidei C, Tolentino J, Jahromi BS, Macdonald RL. Clot volume and clearance rate as independent predictors of vasospasm after aneurysmal subarachnoid hemorrhage. *Journal of neurosurgery* 2004; **101**: 255-261.
202. Ignarro LJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. *Annual review of pharmacology and toxicology* 1990; **30**: 535-560.
203. Clozel M, Watanabe H. Bq-123, a peptidic endothelin eta receptor antagonist, prevents the early cerebral vasospasm following subarachnoid hemorrhage after intracisternal but not intravenous injection. *Life sciences* 1993; **52**: 825-834.

204. Alonso-Galicia M, Hudetz AG, Shen H, Harder DR, Roman RJ. Contribution of 20-hete to vasodilator actions of nitric oxide in the cerebral microcirculation. *Stroke; a journal of cerebral circulation* 1999; **30**: 2727-2734; discussion 2734.
205. Macdonald RL, Weir BK. A review of hemoglobin and the pathogenesis of cerebral vasospasm. *Stroke; a journal of cerebral circulation* 1991; **22**: 971-982.
206. Nishizawa S, Laher I. Signaling mechanisms in cerebral vasospasm. *Trends in cardiovascular medicine* 2005; **15**: 24-34.
207. Kessler IM, Pacheco YG, Lozzi SP, de Araujo AS, Jr., Onishi FJ, de Mello PA. Endothelin-1 levels in plasma and cerebrospinal fluid of patients with cerebral vasospasm after aneurysmal subarachnoid hemorrhage. *Surgical neurology* 2005; **64 Suppl 1**: S1:2-5; discussion S1:5.
208. Beg SA, Hansen-Schwartz JA, Vikman PJ, Xu CB, Edvinsson LI. Erk1/2 inhibition attenuates cerebral blood flow reduction and abolishes et(b) and 5-ht(1b) receptor upregulation after subarachnoid hemorrhage in rat. *J Cereb Blood Flow Metab* 2006; **26**: 846-856.
209. Vikman P, Beg S, Khurana TS, Hansen-Schwartz J, Edvinsson L. Gene expression and molecular changes in cerebral arteries following subarachnoid hemorrhage in the rat. *Journal of neurosurgery* 2006; **105**: 438-444.
210. Clark JF, Sharp FR. Bilirubin oxidation products (boxes) and their role in cerebral vasospasm after subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 2006; **26**: 1223-1233.
211. Sasaki T, Kikkawa Y. Proposed mechanism of cerebral vasospasm: Our hypothesis and current topics. *Acta neurochirurgica* 2013; **115**: 53-56.
212. Clozel M, Breu V, Burri K, Cassal JM, Fischli W, Gray GA, et al. Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. *Nature* 1993; **365**: 759-761.
213. Hansen-Schwartz J, Hoel NL, Zhou M, Xu CB, Svendgaard NA, Edvinsson L. Subarachnoid hemorrhage enhances endothelin receptor expression and function in rat cerebral arteries. *Neurosurgery* 2003; **52**: 1188-1194; 1194-1185.
214. Weyer GW, Jahromi BS, Aihara Y, Agbaje-Williams M, Nikitina E, Zhang ZD, et al. Expression and function of inwardly rectifying potassium channels after experimental subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 2006; **26**: 382-391.
215. Zhang ZD, Macdonald RL. Contribution of the remodeling response to cerebral vasospasm. *Neurological research* 2006; **28**: 713-720.
216. Rowland MJ, Hadjipavlou G, Kelly M, Westbrook J, Pattinson KT. Delayed cerebral ischaemia after subarachnoid haemorrhage: Looking beyond vasospasm. *British journal of anaesthesia* 2012; **109**: 315-329.
217. Geraud G, Tremoulet M, Guell A, Bes A. The prognostic value of noninvasive cbf measurement in subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 1984; **15**: 301-305.
218. Weinberger J, Frishman WH, Terashita D. Drug therapy of neurovascular disease. *Cardiology in review* 2003; **11**: 122-146.
219. Macdonald RL, Marton LS, Andrus PK, Hall ED, Johns L, Sajdak M. Time course of production of hydroxyl free radical after subarachnoid hemorrhage in dogs. *Life sciences* 2004; **75**: 979-989.

220. Macdonald RL, Curry DJ, Aihara Y, Zhang ZD, Jahromi BS, Yassari R. Magnesium and experimental vasospasm. *Journal of neurosurgery* 2004; **100**: 106-110.
221. Lan C, Das D, Wloskowicz A, Vollrath B. Endothelin-1 modulates hemoglobin-mediated signaling in cerebrovascular smooth muscle via rhoa/rho kinase and protein kinase c. *American journal of physiology. Heart and circulatory physiology* 2004; **286**: H165-173.
222. Kontos HA, Wei EP, Raper AJ, Rosenblum WI, Navari RM, Patterson JL, Jr. Role of tissue hypoxia in local regulation of cerebral microcirculation. *The American journal of physiology* 1978; **234**: H582-591.
223. Kontos HA, Wei EP, Navari RM, Levasseur JE, Rosenblum WI, Patterson JL, Jr. Responses of cerebral arteries and arterioles to acute hypotension and hypertension. *The American journal of physiology* 1978; **234**: H371-383.
224. Naraoka M, Matsuda N, Shimamura N, Asano K, Ohkuma H. The role of arterioles and the microcirculation in the development of vasospasm after aneurysmal sah. *BioMed research international* 2014; **2014**: 253746.
225. Shimoda M, Takeuchi M, Tominaga J, Oda S, Kumasaka A, Tsugane R. Asymptomatic versus symptomatic infarcts from vasospasm in patients with subarachnoid hemorrhage: Serial magnetic resonance imaging. *Neurosurgery* 2001; **49**: 1341-1348; discussion 1348-1350.
226. Uhl E, Lehmberg J, Steiger HJ, Messmer K. Intraoperative detection of early microvasospasm in patients with subarachnoid hemorrhage by using orthogonal polarization spectral imaging. *Neurosurgery* 2003; **52**: 1307-1315; disacussion 1315-1307.
227. Griffiths PD, Wilkinson ID, Mitchell P, Patel MC, Paley MN, Romanowski CA, et al. Multimodality mr imaging depiction of hemodynamic changes and cerebral ischemia in subarachnoid hemorrhage. *Ajnr* 2001; **22**: 1690-1697.
228. Stein SC, Browne KD, Chen XH, Smith DH, Graham DI. Thromboembolism and delayed cerebral ischemia after subarachnoid hemorrhage: An autopsy study. *Neurosurgery* 2006; **59**: 781-787; discussion 787-788.
229. Giller CA, Giller AM, Landreneau F. Detection of emboli after surgery for intracerebral aneurysms. *Neurosurgery* 1998; **42**: 490-493; discussion 493-494.
230. Suzuki S, Kimura M, Souma M, Ohkima H, Shimizu T, Iwabuchi T. Cerebral microthrombosis in symptomatic cerebral vasospasm--a quantitative histological study in autopsy cases. *Neurologia medico-chirurgica* 1990; **30**: 309-316.
231. van den Bergh WM, Algra A, Dorhout Mees SM, van Kooten F, Dirven CM, van Gijn J, et al. Randomized controlled trial of acetylsalicylic acid in aneurysmal subarachnoid hemorrhage: The mash study. *Stroke; a journal of cerebral circulation* 2006; **37**: 2326-2330.
232. Huang J, van Gelder JM. The probability of sudden death from rupture of intracranial aneurysms: A meta-analysis. *Neurosurgery* 2002; **51**: 1101-1105; discussion 1105-1107.
233. Broderick JP, Brott T, Tomsick T, Miller R, Huster G. Intracerebral hemorrhage more than twice as common as subarachnoid hemorrhage. *Journal of neurosurgery* 1993; **78**: 188-191.
234. Hanel RA, Xavier AR, Mohammad Y, Kirmani JF, Yahia AM, Qureshi AI. Outcome following intracerebral hemorrhage and subarachnoid hemorrhage. *Neurological research* 2002; **24 Suppl 1**: S58-62.

235. Cross DT, 3rd, Tirschwell DL, Clark MA, Tuden D, Derdeyn CP, Moran CJ, et al. Mortality rates after subarachnoid hemorrhage: Variations according to hospital case volume in 18 states. *Journal of neurosurgery* 2003; **99**: 810-817.
236. Nieuwkamp DJ, Setz LE, Algra A, Linn FH, de Rooij NK, Rinkel GJ. Changes in case fatality of aneurysmal subarachnoid haemorrhage over time, according to age, sex, and region: A meta-analysis. *Lancet neurology* 2009; **8**: 635-642.
237. Nieuwkamp DJ, Vaartjes I, Algra A, Bots ML, Rinkel GJ. Age- and gender-specific time trend in risk of death of patients admitted with aneurysmal subarachnoid hemorrhage in the netherlands. *International journal of stroke : official journal of the International Stroke Society* 2013:
238. Rosenorn J, Eskesen V, Schmidt K, Espersen JO, Haase J, Harmsen A, et al. Clinical features and outcome in 1076 patients with ruptured intracranial saccular aneurysms: A prospective consecutive study. *British journal of neurosurgery* 1987; **1**: 33-45.
239. Hop JW, Rinkel GJ, Algra A, van Gijn J. Quality of life in patients and partners after aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 1998; **29**: 798-804.
240. Hackett ML, Anderson CS. Health outcomes 1 year after subarachnoid hemorrhage: An international population-based study. The australian cooperative research on subarachnoid hemorrhage study group. *Neurology* 2000; **55**: 658-662.
241. Samra SK, Giordani B, Caveney AF, Clarke WR, Scott PA, Anderson S, et al. Recovery of cognitive function after surgery for aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2007; **38**: 1864-1872.
242. Springer MV, Schmidt JM, Wartenberg KE, Frontera JA, Badjatia N, Mayer SA. Predictors of global cognitive impairment 1 year after subarachnoid hemorrhage. *Neurosurgery* 2009; **65**: 1043-1050; discussion 1050-1041.
243. Kreitschmann-Andermahr I, Hoff C, Saller B, Niggemeier S, Pruemper S, Hutter BO, et al. Prevalence of pituitary deficiency in patients after aneurysmal subarachnoid hemorrhage. *The Journal of clinical endocrinology and metabolism* 2004; **89**: 4986-4992.
244. Schuiling WJ, Rinkel GJ, Walchenbach R, de Weerd AW. Disorders of sleep and wake in patients after subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2005; **36**: 578-582.
245. van Gijn J, Rinkel GJ. Subarachnoid haemorrhage: Diagnosis, causes and management. *Brain* 2001; **124**: 249-278.
246. Longstreth WT, Jr., Nelson LM, Koepsell TD, van Belle G. Clinical course of spontaneous subarachnoid hemorrhage: A population-based study in king county, washington. *Neurology* 1993; **43**: 712-718.
247. Saveland H, Brandt L. Which are the major determinants for outcome in aneurysmal subarachnoid hemorrhage? A prospective total management study from a strictly unselected series. *Acta neurologica Scandinavica* 1994; **90**: 245-250.
248. Bailes JE, Spetzler RF, Hadley MN, Baldwin HZ. Management morbidity and mortality of poor-grade aneurysm patients. *Journal of neurosurgery* 1990; **72**: 559-566.
249. Mayer SA, Kreiter KT, Copeland D, Bernardini GL, Bates JE, Peery S, et al. Global and domain-specific cognitive impairment and outcome after subarachnoid hemorrhage. *Neurology* 2002; **59**: 1750-1758.

250. Kassell NF, Torner JC, Haley EC, Jr., Jane JA, Adams HP, Kongable GL. The international cooperative study on the timing of aneurysm surgery. Part 1: Overall management results. *Journal of neurosurgery* 1990; **73**: 18-36.
251. Jennett B, Bond M. Assessment of outcome after severe brain damage. *Lancet* 1975; **1**: 480-484.
252. Weir J, Steyerberg EW, Butcher I, Lu J, Lingsma HF, McHugh GS, et al. Does the extended glasgow outcome scale add value to the conventional glasgow outcome scale? *Journal of neurotrauma* 2012; **29**: 53-58.
253. Ghandehari K. Challenging comparison of stroke scales. *Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences* 2013; **18**: 906-910.
254. Balu S. Differences in psychometric properties, cut-off scores, and outcomes between the barthel index and modified rankin scale in pharmacotherapy-based stroke trials: Systematic literature review. *Current medical research and opinion* 2009; **25**: 1329-1341.
255. Banks JL, Marotta CA. Outcomes validity and reliability of the modified rankin scale: Implications for stroke clinical trials: A literature review and synthesis. *Stroke; a journal of cerebral circulation* 2007; **38**: 1091-1096.
256. Sarti C, Tuomilehto J, Salomaa V, Sivenius J, Kaarsalo E, Narva EV, et al. Epidemiology of subarachnoid hemorrhage in finland from 1983 to 1985. *Stroke; a journal of cerebral circulation* 1991; **22**: 848-853.
257. Inagawa T, Shibukawa M, Inokuchi F, Tokuda Y, Okada Y, Okada K. Primary intracerebral and aneurysmal subarachnoid hemorrhage in izumo city, japan. Part ii: Management and surgical outcome. *Journal of neurosurgery* 2000; **93**: 967-975.
258. Lanzino G, Kassell NF, Germanson TP, Kongable GL, Truskowski LL, Torner JC, et al. Age and outcome after aneurysmal subarachnoid hemorrhage: Why do older patients fare worse? *Journal of neurosurgery* 1996; **85**: 410-418.
259. Nieuwkamp DJ, Rinkel GJ, Silva R, Greebe P, Schokking DA, Ferro JM. Subarachnoid haemorrhage in patients > or = 75 years: Clinical course, treatment and outcome. *Journal of neurology, neurosurgery, and psychiatry* 2006; **77**: 933-937.
260. Ogungbo B, Gregson B, Blackburn A, Barnes J, Vivar R, Sengupta R, et al. Aneurysmal subarachnoid hemorrhage in young adults. *Journal of neurosurgery* 2003; **98**: 43-49.
261. Ayala C, Greenlund KJ, Croft JB, Keenan NL, Donehoo RS, Giles WH, et al. Racial/ethnic disparities in mortality by stroke subtype in the united states, 1995-1998. *American journal of epidemiology* 2001; **154**: 1057-1063.
262. Jakovljevic D, Sivenius J, Sarti C, Torppa J, Mahonen M, Immonen-Raiha P, et al. Socioeconomic inequalities in the incidence, mortality and prognosis of subarachnoid hemorrhage: The finmonica stroke register. *Cerebrovascular diseases (Basel, Switzerland)* 2001; **12**: 7-13.
263. Johnston SC. Effect of endovascular services and hospital volume on cerebral aneurysm treatment outcomes. *Stroke; a journal of cerebral circulation* 2000; **31**: 111-117.
264. Berman MF, Solomon RA, Mayer SA, Johnston SC, Yung PP. Impact of hospital-related factors on outcome after treatment of cerebral aneurysms. *Stroke; a journal of cerebral circulation* 2003; **34**: 2200-2207.
265. Johnston SC, Dudley RA, Gress DR, Ono L. Surgical and endovascular treatment of unruptured cerebral aneurysms at university hospitals. *Neurology* 1999; **52**: 1799-1805.

266. Johnston SC, Wilson CB, Halbach VV, Higashida RT, Dowd CF, McDermott MW, et al. Endovascular and surgical treatment of unruptured cerebral aneurysms: Comparison of risks. *Annals of neurology* 2000; **48**: 11-19.
267. Whitfield PC, Kirkpatrick PJ. Timing of surgery for aneurysmal subarachnoid haemorrhage. *Cochrane database of systematic reviews (Online)* 2001: CD001697.
268. de Gans K, Nieuwkamp DJ, Rinkel GJ, Algra A. Timing of aneurysm surgery in subarachnoid hemorrhage: A systematic review of the literature. *Neurosurgery* 2002; **50**: 336-340; discussion 340-332.
269. Murayama Y, Nien YL, Duckwiler G, Gobin YP, Jahan R, Frazee J, et al. Guglielmi detachable coil embolization of cerebral aneurysms: 11 years' experience. *Journal of neurosurgery* 2003; **98**: 959-966.
270. Le Roux PD, Elliott JP, Newell DW, Grady MS, Winn HR. Predicting outcome in poor-grade patients with subarachnoid hemorrhage: A retrospective review of 159 aggressively managed cases. *Journal of neurosurgery* 1996; **85**: 39-49.
271. Pierot L, Aggour M, Moret J. Vasospasm after aneurysmal subarachnoid hemorrhage: Recent advances in endovascular management. *Current opinion in critical care* 2010; **16**: 110-116.
272. Schievink WI, Wijdicks EF, Piepgras DG, Chu CP, O'Fallon WM, Whisnant JP. The poor prognosis of ruptured intracranial aneurysms of the posterior circulation. *Journal of neurosurgery* 1995; **82**: 791-795.
273. Cha KC, Kim JH, Kang HI, Moon BG, Lee SJ, Kim JS. Aneurysmal rebleeding : Factors associated with clinical outcome in the rebleeding patients. *Journal of Korean Neurosurgical Society* 2010; **47**: 119-123.
274. Inagawa T, Tokuda Y, Ohbayashi N, Takaya M, Moritake K. Study of aneurysmal subarachnoid hemorrhage in izumo city, japan. *Stroke; a journal of cerebral circulation* 1995; **26**: 761-766.
275. Ohkuma H, Tsurutani H, Suzuki S. Incidence and significance of early aneurysmal rebleeding before neurosurgical or neurological management. *Stroke; a journal of cerebral circulation* 2001; **32**: 1176-1180.
276. Juvela S. Rebleeding from ruptured intracranial aneurysms. *Surgical neurology* 1989; **32**: 323-326.
277. Hijdra A, Vermeulen M, van Gijn J, van Crevel H. Rerupture of intracranial aneurysms: A clinicoanatomic study. *Journal of neurosurgery* 1987; **67**: 29-33.
278. Rosen DS, Macdonald RL. Subarachnoid hemorrhage grading scales: A systematic review. *Neurocritical care* 2005; **2**: 110-118.
279. Phillips LH, 2nd, Whisnant JP, O'Fallon WM, Sundt TM, Jr. The unchanging pattern of subarachnoid hemorrhage in a community. *Neurology* 1980; **30**: 1034-1040.
280. Hijdra A, van Gijn J, Nagelkerke NJ, Vermeulen M, van Crevel H. Prediction of delayed cerebral ischemia, rebleeding, and outcome after aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 1988; **19**: 1250-1256.
281. Qureshi AI, Sung GY, Razumovsky AY, Lane K, Straw RN, Ulatowski JA. Early identification of patients at risk for symptomatic vasospasm after aneurysmal subarachnoid hemorrhage. *Critical care medicine* 2000; **28**: 984-990.

282. Schutz H, Krack P, Buchinger B, Bodeker RH, Laun A, Dorndorf W, et al. Outcome of patients with aneurysmal and presumed aneurysmal bleeding. A hospital study based on 100 consecutive cases in a neurological clinic. *Neurosurgical review* 1993; **16**: 15-25.
283. Bonilha L, Marques EL, Carelli EF, Fernandes YB, Cardoso AC, Maldaum MV, et al. Risk factors and outcome in 100 patients with aneurysmal subarachnoid hemorrhage. *Arquivos de neuro-psiquiatria* 2001; **59**: 676-680.
284. Lagares A, Gomez PA, Lobato RD, Alen JF, Alday R, Campollo J. Prognostic factors on hospital admission after spontaneous subarachnoid haemorrhage. *Acta neurochirurgica* 2001; **143**: 665-672.
285. Chiang VL, Claus EB, Awad IA. Toward more rational prediction of outcome in patients with high-grade subarachnoid hemorrhage. *Neurosurgery* 2000; **46**: 28-35; discussion 35-26.
286. Qureshi AI, Sung GY, Suri MA, Straw RN, Guterman LR, Hopkins LN. Prognostic value and determinants of ultraearly angiographic vasospasm after aneurysmal subarachnoid hemorrhage. *Neurosurgery* 1999; **44**: 967-973; discussion 973-964.
287. Kamiya K, Kuyama H, Symon L. An experimental study of the acute stage of subarachnoid hemorrhage. *Journal of neurosurgery* 1983; **59**: 917-924.
288. Frontera JA, Ahmed W, Zach V, Jovine M, Tanenbaum L, Sehba F, et al. Acute ischaemia after subarachnoid haemorrhage, relationship with early brain injury and impact on outcome: A prospective quantitative mri study. *Journal of neurology, neurosurgery, and psychiatry* 2014:
289. Rasulo FA, Girardini A, Lavinio A, De Peri E, Stefini R, Cenzato M, et al. Are optimal cerebral perfusion pressure and cerebrovascular autoregulation related to long-term outcome in patients with aneurysmal subarachnoid hemorrhage? *Journal of neurosurgical anesthesiology* 2012; **24**: 3-8.
290. Barth M, Woitzik J, Weiss C, Muench E, Diepers M, Schmiedek P, et al. Correlation of clinical outcome with pressure-, oxygen-, and flow-related indices of cerebrovascular reactivity in patients following aneurysmal sah. *Neurocritical care* 2010; **12**: 234-243.
291. Nolan CP, Macdonald RL. Can angiographic vasospasm be used as a surrogate marker in evaluating therapeutic interventions for cerebral vasospasm? *Neurosurgical focus* 2006; **21**: E1.
292. Dorsch NW. Cerebral arterial spasm--a clinical review. *British journal of neurosurgery* 1995; **9**: 403-412.
293. Stienen MN, Smoll NR, Weisshaupt R, Fandino J, Hildebrandt G, Studerus-Germann A, et al. Delayed cerebral ischemia predicts neurocognitive impairment following aneurysmal subarachnoid hemorrhage. *World neurosurgery* 2014:
294. Etminan N, Vergouwen MD, Macdonald RL. Angiographic vasospasm versus cerebral infarction as outcome measures after aneurysmal subarachnoid hemorrhage. *Acta neurochirurgica* 2013; **115**: 33-40.
295. Lee Y, Zuckerman SL, Mocco J. Current controversies in the prediction, diagnosis, and management of cerebral vasospasm: Where do we stand? *Neurology research international* 2013; **2013**: 373458.
296. Charpentier C, Audibert G, Guillemin F, Civit T, Ducrocq X, Bracard S, et al. Multivariate analysis of predictors of cerebral vasospasm occurrence after aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 1999; **30**: 1402-1408.

297. Hoh BL, Topcuoglu MA, Singhal AB, Pryor JC, Rabinov JD, Rordorf GA, et al. Effect of clipping, craniotomy, or intravascular coiling on cerebral vasospasm and patient outcome after aneurysmal subarachnoid hemorrhage. *Neurosurgery* 2004; **55**: 779-786; discussion 786-779.
298. Niskanen MM, Hernesniemi JA, Vapalahti MP, Kari A. One-year outcome in early aneurysm surgery: Prediction of outcome. *Acta neurochirurgica* 1993; **123**: 25-32.
299. Enblad P, Persson L. Impact on clinical outcome of secondary brain insults during the neurointensive care of patients with subarachnoid haemorrhage: A pilot study. *Journal of neurology, neurosurgery, and psychiatry* 1997; **62**: 512-516.
300. McLaughlin N, Bojanowski MW, Girard F, Denault A. Pulmonary edema and cardiac dysfunction following subarachnoid hemorrhage. *The Canadian journal of neurological sciences* 2005; **32**: 178-185.
301. Claassen J, Peery S, Kreiter KT, Hirsch LJ, Du EY, Connolly ES, et al. Predictors and clinical impact of epilepsy after subarachnoid hemorrhage. *Neurology* 2003; **60**: 208-214.
302. Harrod CG, Bendok BR, Batjer HH. Prediction of cerebral vasospasm in patients presenting with aneurysmal subarachnoid hemorrhage: A review. *Neurosurgery* 2005; **56**: 633-654; discussion 633-654.
303. Artiola L, Fortuny I, Adams CB. Predicting outcome in patients with intracranial aneurysms with the help of microsurgery. *Journal of neurology, neurosurgery, and psychiatry* 1981; **44**: 615-620.
304. Jaja BN, Cusimano MD, Etminan N, Hanggi D, Hasan D, Ilodigwe D, et al. Clinical prediction models for aneurysmal subarachnoid hemorrhage: A systematic review. *Neurocritical care* 2013; **18**: 143-153.
305. Vergouwen MD, Vermeulen M, van Gijn J, Rinkel GJ, Wijdicks EF, Muizelaar JP, et al. Definition of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage as an outcome event in clinical trials and observational studies: Proposal of a multidisciplinary research group. *Stroke; a journal of cerebral circulation*; **41**: 2391-2395.
306. Diringer MN, Bleck TP, Claude Hemphill J, 3rd, Menon D, Shutter L, Vespa P, et al. Critical care management of patients following aneurysmal subarachnoid hemorrhage: Recommendations from the neurocritical care society's multidisciplinary consensus conference. *Neurocritical care* 2011; **15**: 211-240.
307. Kramer AH, Hehir M, Nathan B, Gress D, Dumont AS, Kassell NF, et al. A comparison of 3 radiographic scales for the prediction of delayed ischemia and prognosis following subarachnoid hemorrhage. *Journal of neurosurgery* 2008; **109**: 199-207.
308. Woertgen C, Ullrich OW, Rothoerl RD, Brawanski A. Comparison of the claassen and fisher ct classification scale to predict ischemia after aneurysmatic sah? *Zentralblatt fur Neurochirurgie* 2003; **64**: 104-108.
309. Edlow JA, Rothman RE, Barsan WG. What do we really know about neurological misdiagnosis in the emergency department? *Mayo Clinic proceedings* 2008; **83**: 253-254; author reply 255.
310. Lysakowski C, Walder B, Costanza MC, Tramer MR. Transcranial doppler versus angiography in patients with vasospasm due to a ruptured cerebral aneurysm: A systematic review. *Stroke; a journal of cerebral circulation* 2001; **32**: 2292-2298.
311. Suarez JI, Qureshi AI, Yahia AB, Parekh PD, Tamargo RJ, Williams MA, et al. Symptomatic vasospasm diagnosis after subarachnoid hemorrhage: Evaluation of

transcranial doppler ultrasound and cerebral angiography as related to compromised vascular distribution. *Critical care medicine* 2002; **30**: 1348-1355.
312. Rigamonti A, Ackery A, Baker AJ. Transcranial doppler monitoring in subarachnoid hemorrhage: A critical tool in critical care. *Canadian journal of anaesthesia = Journal canadien d'anesthesie* 2008; **55**: 112-123.
313. Tsivgoulis G, Alexandrov AV, Sloan MA. Advances in transcranial doppler ultrasonography. *Current neurology and neuroscience reports* 2009; **9**: 46-54.
314. White H, Venkatesh B. Applications of transcranial doppler in the icu: A review. *Intensive care medicine* 2006; **32**: 981-994.
315. Bazzocchi M, Quaia E, Zuiani C, Moroldo M. Transcranial doppler: State of the art. *European journal of radiology* 1998; **27 Suppl 2**: S141-148.
316. Masdeu JC, Irimia P, Asenbaum S, Bogousslavsky J, Brainin M, Chabriat H, et al. Efns guideline on neuroimaging in acute stroke. Report of an efns task force. *Eur J Neurol* 2006; **13**: 1271-1283.
317. Rodriguez-Rodriguez A, Egea-Guerrero JJ, Ruiz de Azua-Lopez Z, Murillo-Cabezas F. Biomarkers of vasospasm development and outcome in aneurysmal subarachnoid hemorrhage. *Journal of the neurological sciences* 2014; **341**: 119-127.
318. Lad SP, Hegen H, Gupta G, Deisenhammer F, Steinberg GK. Proteomic biomarker discovery in cerebrospinal fluid for cerebral vasospasm following subarachnoid hemorrhage. *J Stroke Cerebrovasc Dis* 2012; **21**: 30-41.
319. Ducruet AF, Gigante PR, Hickman ZL, Zacharia BE, Arias EJ, Grobelny BT, et al. Genetic determinants of cerebral vasospasm, delayed cerebral ischemia, and outcome after aneurysmal subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 2010; **30**: 676-688.
320. Toda N, Ayajiki K, Okamura T. Cerebral blood flow regulation by nitric oxide: Recent advances. *Pharmacological reviews* 2009; **61**: 62-97.
321. Khurana VG, Smith LA, Baker TA, Eguchi D, O'Brien T, Katusic ZS. Protective vasomotor effects of in vivo recombinant endothelial nitric oxide synthase gene expression in a canine model of cerebral vasospasm. *Stroke; a journal of cerebral circulation* 2002; **33**: 782-789.
322. Weir B, MacDonald L. Cerebral vasospasm. *Clinical neurosurgery* 1993; **40**: 40-55.
323. Sadamitsu D, Kuroda Y, Nagamitsu T, Tsuruta R, Inoue T, Ueda T, et al. Cerebrospinal fluid and plasma concentrations of nitric oxide metabolites in postoperative patients with subarachnoid hemorrhage. *Critical care medicine* 2001; **29**: 77-79.
324. Woszczyk A, Deinsberger W, Boker DK. Nitric oxide metabolites in cisternal csf correlate with cerebral vasospasm in patients with a subarachnoid haemorrhage. *Acta neurochirurgica* 2003; **145**: 257-263; discussion 263-254.
325. Knowles RG, Palacios M, Palmer RM, Moncada S. Formation of nitric oxide from l-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. *Proceedings of the National Academy of Sciences of the United States of America* 1989; **86**: 5159-5162.
326. Robinson LJ, Weremowicz S, Morton CC, Michel T. Isolation and chromosomal localization of the human endothelial nitric oxide synthase (nos3) gene. *Genomics* 1994; **19**: 350-357.
327. Khurana VG, Sohni YR, Mangrum WI, McClelland RL, O'Kane DJ, Meyer FB, et al. Endothelial nitric oxide synthase gene polymorphisms predict susceptibility to aneurysmal

subarachnoid hemorrhage and cerebral vasospasm. *J Cereb Blood Flow Metab* 2004; **24**: 291-297.
328. Starke RM, Kim GH, Komotar RJ, Hickman ZL, Black EM, Rosales MB, et al. Endothelial nitric oxide synthase gene single-nucleotide polymorphism predicts cerebral vasospasm after aneurysmal subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 2008; **28**: 1204-1211.
329. Ko NU, Rajendran P, Kim H, Rutkowski M, Pawlikowska L, Kwok PY, et al. Endothelial nitric oxide synthase polymorphism (-786t->c) and increased risk of angiographic vasospasm after aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2008; **39**: 1103-1108.
330. Song MK, Kim MK, Kim TS, Joo SP, Park MS, Kim BC, et al. Endothelial nitric oxide gene t-786c polymorphism and subarachnoid hemorrhage in korean population. *Journal of Korean medical science* 2006; **21**: 922-926.
331. Alexander S, Poloyac S, Hoffman L, Gallek M, Dianxu R, Balzer J, et al. Endothelial nitric oxide synthase tagging single nucleotide polymorphisms and recovery from aneurysmal subarachnoid hemorrhage. *Biol Res Nurs* 2009; **11**: 42-52.
332. Guo ZD, Sun XC, Zhang JH. The role of apolipoprotein e in the pathological events following subarachnoid hemorrhage: A review. *Acta neurochirurgica* 2011; **110**: 5-7.
333. d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck GC, et al. Mechanism of endothelial dysfunction in apolipoprotein e-deficient mice. *Arteriosclerosis, thrombosis, and vascular biology* 2001; **21**: 1017-1022.
334. Mesis RG, Wang H, Lombard FW, Yates R, Vitek MP, Borel CO, et al. Dissociation between vasospasm and functional improvement in a murine model of subarachnoid hemorrhage. *Neurosurgical focus* 2006; **21**: E4.
335. Gallek MJ, Conley YP, Sherwood PR, Horowitz MB, Kassam A, Alexander SA. Apoe genotype and functional outcome following aneurysmal subarachnoid hemorrhage. *Biol Res Nurs* 2009; **10**: 205-212.
336. Juvela S, Siironen J, Lappalainen J. Apolipoprotein e genotype and outcome after aneurysmal subarachnoid hemorrhage. *Journal of neurosurgery* 2009; **110**: 989-995.
337. Morris PG, Wilson JT, Dunn LT, Nicoll JA. Apolipoprotein e polymorphism and neuropsychological outcome following subarachnoid haemorrhage. *Acta neurologica Scandinavica* 2004; **109**: 205-209.
338. Lanterna LA, Ruigrok Y, Alexander S, Tang J, Biroli F, Dunn LT, et al. Meta-analysis of apoe genotype and subarachnoid hemorrhage: Clinical outcome and delayed ischemia. *Neurology* 2007; **69**: 766-775.
339. Wu HT, Ruan J, Zhang XD, Xia HJ, Jiang Y, Sun XC. Association of promoter polymorphism of apolipoprotein e gene with cerebral vasospasm after spontaneous sah. *Brain research* 2010; **1362**: 112-116.
340. Vergouwen MD, Frijns CJ, Roos YB, Rinkel GJ, Baas F, Vermeulen M. Plasminogen activator inhibitor-1 4g allele in the 4g/5g promoter polymorphism increases the occurrence of cerebral ischemia after aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2004; **35**: 1280-1283.
341. Ladenvall C, Csajbok L, Nylen K, Jood K, Nellgard B, Jern C. Association between factor xiii single nucleotide polymorphisms and aneurysmal subarachnoid hemorrhage. *Journal of neurosurgery* 2009; **110**: 475-481.

342. Rueffert H, Gumplinger A, Renner C, Dengl M, Reske A, Kaisers UX, et al. Search for genetic variants in the ryanodine receptor 1 gene in patients with symptomatic cerebral vasospasm after aneurysmal subarachnoid hemorrhage. *Neurocritical care* 2011; **15**: 410-415.
343. Grobelny BT, Ducruet AF, DeRosa PA, Kotchetkov IS, Zacharia BE, Hickman ZL, et al. Gain-of-function polymorphisms of cystathionine beta-synthase and delayed cerebral ischemia following aneurysmal subarachnoid hemorrhage. *Journal of neurosurgery* 2011; **115**: 101-107.
344. Siironen J, Juvela S, Kanarek K, Vilkki J, Hernesniemi J, Lappalainen J. The met allele of the bdnf val66met polymorphism predicts poor outcome among survivors of aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2007; **38**: 2858-2860.
345. He Z, Sun X, Guo Z, Zhang JH. The correlation between comt gene polymorphism and early cerebral vasospasm after subarachnoid hemorrhage. *Acta neurochirurgica* 2011; **110**: 233-238.
346. Borsody M, Burke A, Coplin W, Miller-Lotan R, Levy A. Haptoglobin and the development of cerebral artery vasospasm after subarachnoid hemorrhage. *Neurology* 2006; **66**: 634-640.
347. Ruigrok YM, Slooter AJ, Bardoel A, Frijns CJ, Rinkel GJ, Wijmenga C. Genes and outcome after aneurysmal subarachnoid haemorrhage. *Journal of neurology* 2005; **252**: 417-422.
348. Faraci FM, Heistad DD. Regulation of large cerebral arteries and cerebral microvascular pressure. *Circulation research* 1990; **66**: 8-17.
349. Kulik T, Kusano Y, Aronhime S, Sandler AL, Winn HR. Regulation of cerebral vasculature in normal and ischemic brain. *Neuropharmacology* 2008; **55**: 281-288.
350. Petzold GC, Murthy VN. Role of astrocytes in neurovascular coupling. *Neuron* 2011; **71**: 782-797.
351. Harder DR, Roman RJ, Gebremedhin D. Molecular mechanisms controlling nutritive blood flow: Role of cytochrome p450 enzymes. *Acta physiologica Scandinavica* 2000; **168**: 543-549.
352. Filosa JA, Blanco VM. Neurovascular coupling in the mammalian brain. *Experimental physiology* 2007; **92**: 641-646.
353. Strandgaard S, Paulson OB. Cerebral autoregulation. *Stroke; a journal of cerebral circulation* 1984; **15**: 413-416.
354. Mak CH, Lu YY, Wong GK. Review and recommendations on management of refractory raised intracranial pressure in aneurysmal subarachnoid hemorrhage. *Vascular health and risk management* 2013; **9**: 353-359.
355. Imig JD, Simpkins AN, Renic M, Harder DR. Cytochrome p450 eicosanoids and cerebral vascular function. *Expert reviews in molecular medicine* 2011; **13**: e7.
356. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiological reviews* 2002; **82**: 131-185.
357. Schubert R, Brayden JE. Stretch-activated cation channels and the myogenic response of small arteries. In: Kamkin A, Kiseleva I, eds. *Mechanosensitivity in cells and tissues*. Moscow; 2005.
358. Faraci FM, Heistad DD. Regulation of the cerebral circulation: Role of endothelium and potassium channels. *Physiological reviews* 1998; **78**: 53-97.

359. Vanhoutte PM. How to assess endothelial function in human blood vessels. *Journal of hypertension* 1999; **17**: 1047-1058.
360. Qi M, Hang C, Zhu L, Shi J. Involvement of endothelial-derived relaxing factors in the regulation of cerebral blood flow. *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology* 2011; **32**: 551-557.
361. Miller AA, Budzyn K, Sobey CG. Vascular dysfunction in cerebrovascular disease: Mechanisms and therapeutic intervention. *Clin Sci (Lond)* 2010; **119**: 1-17.
362. Rosa AO, Rapoport SI. Intracellular- and extracellular-derived ca(2+) influence phospholipase a(2)-mediated fatty acid release from brain phospholipids. *Biochimica et biophysica acta* 2009; **1791**: 697-705.
363. Ghosh M, Tucker DE, Burchett SA, Leslie CC. Properties of the group iv phospholipase a2 family. *Progress in lipid research* 2006; **45**: 487-510.
364. Kniepeiss D, Wagner D, Wasler A, Tscheliessnigg KH, Renner W. The role of cyp2c8 genotypes in dose requirement and levels of everolimus after heart transplantation. *Wiener klinische Wochenschrift* 2013:
365. Iliff JJ, Jia J, Nelson J, Goyagi T, Klaus J, Alkayed NJ. Epoxyeicosanoid signaling in cns function and disease. *Prostaglandins & other lipid mediators* 2010; **91**: 68-84.
366. de Gaetano G, Donati MB, Cerletti C. Prevention of thrombosis and vascular inflammation: Benefits and limitations of selective or combined cox-1, cox-2 and 5-lox inhibitors. *Trends in pharmacological sciences* 2003; **24**: 245-252.
367. Funk CD. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science (New York, N.Y* 2001; **294**: 1871-1875.
368. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer* 2010; **10**: 181-193.
369. Needleman P, Turk J, Jakschik BA, Morrison AR, Lefkowith JB. Arachidonic acid metabolism. *Annual review of biochemistry* 1986; **55**: 69-102.
370. Karonen T, Filppula A, Laitila J, Niemi M, Neuvonen PJ, Backman JT. Gemfibrozil markedly increases the plasma concentrations of montelukast: A previously unrecognized role for cyp2c8 in the metabolism of montelukast. *Clinical pharmacology and therapeutics* 2010; **88**: 223-230.
371. Maeda Y, Tani E, Miyamoto T. Prostaglandin metabolism in experimental cerebral vasospasm. *Journal of neurosurgery* 1981; **55**: 779-785.
372. Nosko M, Schulz R, Weir B, Cook DA, Grace M. Effects of vasospasm on levels of prostacyclin and thromboxane a2 in cerebral arteries of the monkey. *Neurosurgery* 1988; **22**: 45-50.
373. English BC, Baum CE, Adelberg DE, Sissung TM, Kluetz PG, Dahut WL, et al. A snp in cyp2c8 is not associated with the development of bisphosphonate-related osteonecrosis of the jaw in men with castrate-resistant prostate cancer. *Therapeutics and clinical risk management* 2010; **6**: 579-583.
374. Walker V, Pickard JD, Smythe P, Eastwood S, Perry S. Effects of subarachnoid haemorrhage on intracranial prostaglandins. *Journal of neurology, neurosurgery, and psychiatry* 1983; **46**: 119-125.
375. Bergmann TK, Brasch-Andersen C, Green H, Mirza M, Pedersen RS, Nielsen F, et al. Impact of cyp2c8*3 on paclitaxel clearance: A population pharmacokinetic and

pharmacogenomic study in 93 patients with ovarian cancer. *The pharmacogenomics journal* 2011; **11**: 113-120.
376. Yeo CW, Lee SJ, Lee SS, Bae SK, Kim EY, Shon JH, et al. Discovery of a novel allelic variant of cyp2c8, cyp2c8*11, in asian populations and its clinical effect on the rosiglitazone disposition in vivo. *Drug metabolism and disposition: the biological fate of chemicals* 2011; **39**: 711-716.
377. Teh LK, Zahri MK, Zakaria ZA, Ismail R, Salleh MZ. Mutational analysis of cyp2c8 in hypertensive patients using denaturing high performance liquid chromatography. *Journal of clinical pharmacy and therapeutics* 2010; **35**: 723-728.
378. Haschke-Becher E, Kirchheiner J, Trummer O, Grunbacher G, Kainz A, Boehm BO, et al. Impact of cyp2c8 and 2c9 polymorphisms on coronary artery disease and myocardial infarction in the luric cohort. *Pharmacogenomics* 2010; **11**: 1359-1365.
379. Nebot N, Crettol S, d'Esposito F, Tattam B, Hibbs DE, Murray M. Participation of cyp2c8 and cyp3a4 in the n-demethylation of imatinib in human hepatic microsomes. *British journal of pharmacology* 2010; **161**: 1059-1069.
380. Karonen T, Neuvonen PJ, Backman JT. The cyp2c8 inhibitor gemfibrozil does not affect the pharmacokinetics of zafirlukast. *European journal of clinical pharmacology* 2011; **67**: 151-155.
381. Chyatte D, Rusch N, Sundt TM, Jr. Prevention of chronic experimental cerebral vasospasm with ibuprofen and high-dose methylprednisolone. *Journal of neurosurgery* 1983; **59**: 925-932.
382. Chyatte D. Anti-inflammatory agents and cerebral vasospasm. *Neurosurgery clinics of North America* 1990; **1**: 433-450.
383. Young AM, Karri SK, Ogilvy CS. Non-steroidal anti-inflammatory drugs used as a treatment modality in subarachnoid hemorrhage. *Current drug safety* 2012; **7**: 197-201.
384. Niemi T, Tanskanen P, Taxell C, Juvela S, Randell T, Rosenberg P. Effects of nonsteroidal anti-inflammatory drugs on hemostasis in patients with aneurysmal subarachnoid hemorrhage. *Journal of neurosurgical anesthesiology* 1999; **11**: 188-194.
385. Danielson PB. The cytochrome p450 superfamily: Biochemistry, evolution and drug metabolism in humans. *Current drug metabolism* 2002; **3**: 561-597.
386. Guengerich FP. Cytochrome p450s and other enzymes in drug metabolism and toxicity. *The AAPS journal* 2006; **8**: E101-111.
387. Hedlund E, Gustafsson JA, Warner M. Cytochrome p450 in the brain; a review. *Current drug metabolism* 2001; **2**: 245-263.
388. Hoch U, Zhang Z, Kroetz DL, Ortiz de Montellano PR. Structural determination of the substrate specificities and regioselectivities of the rat and human fatty acid omega-hydroxylases. *Archives of biochemistry and biophysics* 2000; **373**: 63-71.
389. Powell PK, Wolf I, Jin R, Lasker JM. Metabolism of arachidonic acid to 20-hydroxy-5,8,11, 14-eicosatetraenoic acid by p450 enzymes in human liver: Involvement of cyp4f2 and cyp4a11. *The Journal of pharmacology and experimental therapeutics* 1998; **285**: 1327-1336.
390. Lasker JM, Chen WB, Wolf I, Bloswick BP, Wilson PD, Powell PK. Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney. Role of cyp4f2 and cyp4a11. *The Journal of biological chemistry* 2000; **275**: 4118-4126.

391. Imaoka S, Hashizume T, Funae Y. Localization of rat cytochrome p450 in various tissues and comparison of arachidonic acid metabolism by rat p450 with that by human p450 orthologs. *Drug metabolism and pharmacokinetics* 2005; **20**: 478-484.
392. Choudhary D, Jansson I, Stoilov I, Sarfarazi M, Schenkman JB. Metabolism of retinoids and arachidonic acid by human and mouse cytochrome p450 1b1. *Drug metabolism and disposition: the biological fate of chemicals* 2004; **32**: 840-847.
393. Hashizume T, Imaoka S, Hiroi T, Terauchi Y, Fujii T, Miyazaki H, et al. Cdna cloning and expression of a novel cytochrome p450 (cyp4f12) from human small intestine. *Biochemical and biophysical research communications* 2001; **280**: 1135-1141.
394. Christmas P, Jones JP, Patten CJ, Rock DA, Zheng Y, Cheng SM, et al. Alternative splicing determines the function of cyp4f3 by switching substrate specificity. *The Journal of biological chemistry* 2001; **276**: 38166-38172.
395. Wu JH, Hodgson JM, Clarke MW, Indrawan AP, Barden AE, Puddey IB, et al. Inhibition of 20-hydroxyeicosatetraenoic acid synthesis using specific plant lignans: In vitro and human studies. *Hypertension* 2009; **54**: 1151-1158.
396. Bylund J, Bylund M, Oliw EH. Cdna cloning and expression of cyp4f12, a novel human cytochrome p450. *Biochemical and biophysical research communications* 2001; **280**: 892-897.
397. Rifkind AB, Lee C, Chang TK, Waxman DJ. Arachidonic acid metabolism by human cytochrome p450s 2c8, 2c9, 2e1, and 1a2: Regioselective oxygenation and evidence for a role for cyp2c enzymes in arachidonic acid epoxygenation in human liver microsomes. *Archives of biochemistry and biophysics* 1995; **320**: 380-389.
398. Bylund J, Kunz T, Valmsen K, Oliw EH. Cytochromes p450 with bisallylic hydroxylation activity on arachidonic and linoleic acids studied with human recombinant enzymes and with human and rat liver microsomes. *The Journal of pharmacology and experimental therapeutics* 1998; **284**: 51-60.
399. Zeldin DC, Foley J, Goldsworthy SM, Cook ME, Boyle JE, Ma J, et al. Cyp2j subfamily cytochrome p450s in the gastrointestinal tract: Expression, localization, and potential functional significance. *Molecular pharmacology* 1997; **51**: 931-943.
400. Zeldin DC, Moomaw CR, Jesse N, Tomer KB, Beetham J, Hammock BD, et al. Biochemical characterization of the human liver cytochrome p450 arachidonic acid epoxygenase pathway. *Archives of biochemistry and biophysics* 1996; **330**: 87-96.
401. Wu S, Moomaw CR, Tomer KB, Falck JR, Zeldin DC. Molecular cloning and expression of cyp2j2, a human cytochrome p450 arachidonic acid epoxygenase highly expressed in heart. *The Journal of biological chemistry* 1996; **271**: 3460-3468.
402. Daikh BE, Lasker JM, Raucy JL, Koop DR. Regio- and stereoselective epoxidation of arachidonic acid by human cytochromes p450 2c8 and 2c9. *The Journal of pharmacology and experimental therapeutics* 1994; **271**: 1427-1433.
403. Daikh BE, Laethem RM, Koop DR. Stereoselective epoxidation of arachidonic acid by cytochrome p-450s 2caa and 2c2. *The Journal of pharmacology and experimental therapeutics* 1994; **269**: 1130-1135.
404. Lundblad MS, Stark K, Eliasson E, Oliw E, Rane A. Biosynthesis of epoxyeicosatrienoic acids varies between polymorphic cyp2c enzymes. *Biochemical and biophysical research communications* 2005; **327**: 1052-1057.

405. Kaspera R, Totah RA. Epoxyeicosatrienoic acids: Formation, metabolism and potential role in tissue physiology and pathophysiology. *Expert opinion on drug metabolism & toxicology* 2009; **5**: 757-771.
406. Jiang H. Erythrocyte-derived epoxyeicosatrienoic acids. *Prostaglandins & other lipid mediators* 2007; **82**: 4-10.
407. Hill E, Murphy RC. Quantitation of 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-hete) produced by human polymorphonuclear leukocytes using electron capture ionization gas chromatography/mass spectrometry. *Biological mass spectrometry* 1992; **21**: 249-253.
408. Zeldin DC, Kobayashi J, Falck JR, Winder BS, Hammock BD, Snapper JR, et al. Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. *The Journal of biological chemistry* 1993; **268**: 6402-6407.
409. Bernstrom K, Kayganich K, Murphy RC, Fitzpatrick FA. Incorporation and distribution of epoxyeicosatrienoic acids into cellular phospholipids. *The Journal of biological chemistry* 1992; **267**: 3686-3690.
410. Karara A, Dishman E, Blair I, Falck JR, Capdevila JH. Endogenous epoxyeicosatrienoic acids. Cytochrome p-450 controlled stereoselectivity of the hepatic arachidonic acid epoxygenase. *The Journal of biological chemistry* 1989; **264**: 19822-19827.
411. Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, Spector AA. Potentiation of endothelium-dependent relaxation by epoxyeicosatrienoic acids. *Circulation research* 1997; **81**: 258-267.
412. Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, Spector AA. Epoxide hydrolases regulate epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids. *The American journal of physiology* 1999; **277**: H2098-2108.
413. Newman JW, Morisseau C, Hammock BD. Epoxide hydrolases: Their roles and interactions with lipid metabolism. *Progress in lipid research* 2005; **44**: 1-51.
414. Zeldin DC. Epoxygenase pathways of arachidonic acid metabolism. *The Journal of biological chemistry* 2001; **276**: 36059-36062.
415. Widstrom RL, Norris AW, Spector AA. Binding of cytochrome p450 monooxygenase and lipoxygenase pathway products by heart fatty acid-binding protein. *Biochemistry* 2001; **40**: 1070-1076.
416. Kroetz DL, Xu F. Regulation and inhibition of arachidonic acid omega-hydroxylases and 20-hete formation. *Annual review of pharmacology and toxicology* 2005; **45**: 413-438.
417. Kaduce TL, Fang X, Harmon SD, Oltman CL, Dellsperger KC, Teesch LM, et al. 20-hydroxyeicosatetraenoic acid (20-hete) metabolism in coronary endothelial cells. *The Journal of biological chemistry* 2004; **279**: 2648-2656.
418. Fang X, Faraci FM, Kaduce TL, Harmon S, Modrick ML, Hu S, et al. 20-hydroxyeicosatetraenoic acid is a potent dilator of mouse basilar artery: Role of cyclooxygenase. *American journal of physiology. Heart and circulatory physiology* 2006; **291**: H2301-2307.
419. Collins XH, Harmon SD, Kaduce TL, Berst KB, Fang X, Moore SA, et al. Omega-oxidation of 20-hydroxyeicosatetraenoic acid (20-hete) in cerebral microvascular smooth muscle and endothelium by alcohol dehydrogenase 4. *The Journal of biological chemistry* 2005; **280**: 33157-33164.
420. Schwartzman ML, Falck JR, Yadagiri P, Escalante B. Metabolism of 20-hydroxyeicosatetraenoic acid by cyclooxygenase. Formation and identification of novel

endothelium-dependent vasoconstrictor metabolites. *The Journal of biological chemistry* 1989; **264**: 11658-11662.
421. Hill E, Fitzpatrick F, Murphy RC. Biological activity and metabolism of 20-hydroxyeicosatetraenoic acid in the human platelet. *British journal of pharmacology* 1992; **106**: 267-274.
422. Watzer B, Reinalter S, Seyberth HW, Schweer H. Determination of free and glucuronide conjugated 20-hydroxyarachidonic acid (20-hete) in urine by gas chromatography/negative ion chemical ionization mass spectrometry. *Prostaglandins, leukotrienes, and essential fatty acids* 2000; **62**: 175-181.
423. Prakash C, Zhang JY, Falck JR, Chauhan K, Blair IA. 20-hydroxyeicosatetraenoic acid is excreted as a glucuronide conjugate in human urine. *Biochemical and biophysical research communications* 1992; **185**: 728-733.
424. Baer BR, Rettie AE. Cyp4b1: An enigmatic p450 at the interface between xenobiotic and endobiotic metabolism. *Drug metabolism reviews* 2006; **38**: 451-476.
425. Hsu MH, Savas U, Griffin KJ, Johnson EF. Human cytochrome p450 family 4 enzymes: Function, genetic variation and regulation. *Drug metabolism reviews* 2007; **39**: 515-538.
426. Kalsotra A, Strobel HW. Cytochrome p450 4f subfamily: At the crossroads of eicosanoid and drug metabolism. *Pharmacology & therapeutics* 2006; **112**: 589-611.
427. Stark K, Guengerich FP. Characterization of orphan human cytochromes p450. *Drug metabolism reviews* 2007; **39**: 627-637.
428. Bellamine A, Wang Y, Waterman MR, Gainer JV, 3rd, Dawson EP, Brown NJ, et al. Characterization of the cyp4a11 gene, a second cyp4a gene in humans. *Archives of biochemistry and biophysics* 2003; **409**: 221-227.
429. Savas U, Hsu MH, Griffin KJ, Bell DR, Johnson EF. Conditional regulation of the human cyp4x1 and cyp4z1 genes. *Archives of biochemistry and biophysics* 2005; **436**: 377-385.
430. Ito O, Nakamura Y, Tan L, Ishizuka T, Sasaki Y, Minami N, et al. Expression of cytochrome p-450 4 enzymes in the kidney and liver: Regulation by ppar and species-difference between rat and human. *Molecular and cellular biochemistry* 2006; **284**: 141-148.
431. Kikuta Y, Kato M, Yamashita Y, Miyauchi Y, Tanaka K, Kamada N, et al. Human leukotriene b4 omega-hydroxylase (cyp4f3) gene: Molecular cloning and chromosomal localization. *DNA and cell biology* 1998; **17**: 221-230.
432. Christmas P, Ursino SR, Fox JW, Soberman RJ. Expression of the cyp4f3 gene. Tissue-specific splicing and alternative promoters generate high and low k(m) forms of leukotriene b(4) omega-hydroxylase. *The Journal of biological chemistry* 1999; **274**: 21191-21199.
433. Bylund J, Hidestrand M, Ingelman-Sundberg M, Oliw EH. Identification of cyp4f8 in human seminal vesicles as a prominent 19-hydroxylase of prostaglandin endoperoxides. *The Journal of biological chemistry* 2000; **275**: 21844-21849.
434. Cui X, Nelson DR, Strobel HW. A novel human cytochrome p450 4f isoform (cyp4f11): Cdna cloning, expression, and genomic structural characterization. *Genomics* 2000; **68**: 161-166.
435. Kikuta Y, Kusunose E, Kusunose M. Prostaglandin and leukotriene omega-hydroxylases. *Prostaglandins & other lipid mediators* 2002; **68-69**: 345-362.
436. Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW. Comparison of cytochrome p450 (cyp) genes from the mouse and human genomes, including

nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* 2004; **14**: 1-18.
437. Hirani V, Yarovoy A, Kozeska A, Magnusson RP, Lasker JM. Expression of cyp4f2 in human liver and kidney: Assessment using targeted peptide antibodies. *Archives of biochemistry and biophysics* 2008; **478**: 59-68.
438. Ma J, Ramachandran S, Fiedorek FT, Jr., Zeldin DC. Mapping of the cyp2j cytochrome p450 genes to human chromosome 1 and mouse chromosome 4. *Genomics* 1998; **49**: 152-155.
439. King LM, Ma J, Srettabunjong S, Graves J, Bradbury JA, Li L, et al. Cloning of cyp2j2 gene and identification of functional polymorphisms. *Molecular pharmacology* 2002; **61**: 840-852.
440. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, et al. Anti-inflammatory properties of cytochrome p450 epoxygenase-derived eicosanoids. *Science (New York, N.Y* 1999; **285**: 1276-1279.
441. Delozier TC, Kissling GE, Coulter SJ, Dai D, Foley JF, Bradbury JA, et al. Detection of human cyp2c8, cyp2c9, and cyp2j2 in cardiovascular tissues. *Drug metabolism and disposition: the biological fate of chemicals* 2007; **35**: 682-688.
442. Zeldin DC, Foley J, Ma J, Boyle JE, Pascual JM, Moomaw CR, et al. Cyp2j subfamily p450s in the lung: Expression, localization, and potential functional significance. *Molecular pharmacology* 1996; **50**: 1111-1117.
443. Zeldin DC, Foley J, Boyle JE, Moomaw CR, Tomer KB, Parker C, et al. Predominant expression of an arachidonate epoxygenase in islets of langerhans cells in human and rat pancreas. *Endocrinology* 1997; **138**: 1338-1346.
444. Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC. The human intestinal cytochrome p450 "pie". *Drug metabolism and disposition: the biological fate of chemicals* 2006; **34**: 880-886.
445. Nishimura M, Yaguti H, Yoshitsugu H, Naito S, Satoh T. Tissue distribution of mrna expression of human cytochrome p450 isoforms assessed by high-sensitivity real-time reverse transcription pcr. *Yakugaku Zasshi* 2003; **123**: 369-375.
446. Dutheil F, Dauchy S, Diry M, Sazdovitch V, Cloarec O, Mellottee L, et al. Xenobiotic-metabolizing enzymes and transporters in the normal human brain: Regional and cellular mapping as a basis for putative roles in cerebral function. *Drug metabolism and disposition: the biological fate of chemicals* 2009; **37**: 1528-1538.
447. Enayetallah AE, French RA, Thibodeau MS, Grant DF. Distribution of soluble epoxide hydrolase and of cytochrome p450 2c8, 2c9, and 2j2 in human tissues. *J Histochem Cytochem* 2004; **52**: 447-454.
448. Scarborough PE, Ma J, Qu W, Zeldin DC. P450 subfamily cyp2j and their role in the bioactivation of arachidonic acid in extrahepatic tissues. *Drug metabolism reviews* 1999; **31**: 205-234.
449. Klose TS, Blaisdell JA, Goldstein JA. Gene structure of cyp2c8 and extrahepatic distribution of the human cyp2cs. *Journal of biochemical and molecular toxicology* 1999; **13**: 289-295.
450. Inoue K, Inazawa J, Suzuki Y, Shimada T, Yamazaki H, Guengerich FP, et al. Fluorescence in situ hybridization analysis of chromosomal localization of three human cytochrome

p450 2c genes (cyp2c8, 2c9, and 2c10) at 10q24.1. *The Japanese journal of human genetics* 1994; **39**: 337-343.
451. Gray IC, Nobile C, Muresu R, Ford S, Spurr NK. A 2.4-megabase physical map spanning the cyp2c gene cluster on chromosome 10q24. *Genomics* 1995; **28**: 328-332.
452. Yasar U, Lundgren S, Eliasson E, Bennet A, Wiman B, de Faire U, et al. Linkage between the cyp2c8 and cyp2c9 genetic polymorphisms. *Biochemical and biophysical research communications* 2002; **299**: 25-28.
453. Speed WC, Kang SP, Tuck DP, Harris LN, Kidd KK. Global variation in cyp2c8-cyp2c9 functional haplotypes. *The pharmacogenomics journal* 2009; **9**: 283-290.
454. Chen Y, Goldstein JA. The transcriptional regulation of the human cyp2c genes. *Current drug metabolism* 2009; **10**: 567-578.
455. Guengerich FP. Human cytochrome p-450 enzymes. *Life sciences* 1992; **50**: 1471-1478.
456. Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome p-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: Studies with liver microsomes of 30 japanese and 30 caucasians. *The Journal of pharmacology and experimental therapeutics* 1994; **270**: 414-423.
457. Rendic S, Di Carlo FJ. Human cytochrome p450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug metabolism reviews* 1997; **29**: 413-580.
458. Totah RA, Rettie AE. Cytochrome p450 2c8: Substrates, inhibitors, pharmacogenetics, and clinical relevance. *Clinical pharmacology and therapeutics* 2005; **77**: 341-352.
459. Soars MG, Gelboin HV, Krausz KW, Riley RJ. A comparison of relative abundance, activity factor and inhibitory monoclonal antibody approaches in the characterization of human cyp enzymology. *British journal of clinical pharmacology* 2003; **55**: 175-181.
460. Rettie AE, Jones JP. Clinical and toxicological relevance of cyp2c9: Drug-drug interactions and pharmacogenetics. *Annual review of pharmacology and toxicology* 2005; **45**: 477-494.
461. Shu Y, Cheng ZN, Liu ZQ, Wang LS, Zhu B, Huang SL, et al. Interindividual variations in levels and activities of cytochrome p-450 in liver microsomes of chinese subjects. *Acta pharmacologica Sinica* 2001; **22**: 283-288.
462. Alkayed NJ, Narayanan J, Gebremedhin D, Medhora M, Roman RJ, Harder DR. Molecular characterization of an arachidonic acid epoxygenase in rat brain astrocytes. *Stroke; a journal of cerebral circulation* 1996; **27**: 971-979.
463. Capdevila JH, Falck JR, Harris RC. Cytochrome p450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. *Journal of lipid research* 2000; **41**: 163-181.
464. Karara A, Makita K, Jacobson HR, Falck JR, Guengerich FP, DuBois RN, et al. Molecular cloning, expression, and enzymatic characterization of the rat kidney cytochrome p-450 arachidonic acid epoxygenase. *The Journal of biological chemistry* 1993; **268**: 13565-13570.
465. Tsao CC, Foley J, Coulter SJ, Maronpot R, Zeldin DC, Goldstein JA. Cyp2c40, a unique arachidonic acid 16-hydroxylase, is the major cyp2c in murine intestinal tract. *Molecular pharmacology* 2000; **58**: 279-287.
466. DeLozier TC, Tsao CC, Coulter SJ, Foley J, Bradbury JA, Zeldin DC, et al. Cyp2c44, a new murine cyp2c that metabolizes arachidonic acid to unique stereospecific products. *The Journal of pharmacology and experimental therapeutics* 2004; **310**: 845-854.

467. Fretland AJ, Omiecinski CJ. Epoxide hydrolases: Biochemistry and molecular biology. *Chemico-biological interactions* 2000; **129**: 41-59.
468. Gill SS, Hammock BD, Casida JE. Mammalian metabolism and environmental degradation of the juvenoid 1-(4'-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-trans-2-octene and related compounds. *Journal of agricultural and food chemistry* 1974; **22**: 386-395.
469. Gill SS, Hammock BD. Distribution and properties of a mammalian soluble epoxide hydrase. *Biochemical pharmacology* 1980; **29**: 389-395.
470. Arand M, Knehr M, Thomas H, Zeller HD, Oesch F. An impaired peroxisomal targeting sequence leading to an unusual bicompartmental distribution of cytosolic epoxide hydrolase. *FEBS letters* 1991; **294**: 19-22.
471. Beetham JK, Tian T, Hammock BD. Cdna cloning and expression of a soluble epoxide hydrolase from human liver. *Archives of biochemistry and biophysics* 1993; **305**: 197-201.
472. Borhan B, Jones AD, Pinot F, Grant DF, Kurth MJ, Hammock BD. Mechanism of soluble epoxide hydrolase. Formation of an alpha-hydroxy ester-enzyme intermediate through asp-333. *The Journal of biological chemistry* 1995; **270**: 26923-26930.
473. Pinot F, Grant DF, Beetham JK, Parker AG, Borhan B, Landt S, et al. Molecular and biochemical evidence for the involvement of the asp-333-his-523 pair in the catalytic mechanism of soluble epoxide hydrolase. *The Journal of biological chemistry* 1995; **270**: 7968-7974.
474. Morisseau C, Hammock BD. Epoxide hydrolases: Mechanisms, inhibitor designs, and biological roles. *Annual review of pharmacology and toxicology* 2005; **45**: 311-333.
475. Sandberg M, Meijer J. Structural characterization of the human soluble epoxide hydrolase gene (ephx2). *Biochemical and biophysical research communications* 1996; **221**: 333-339.
476. Larsson C, White I, Johansson C, Stark A, Meijer J. Localization of the human soluble epoxide hydrolase gene (ephx2) to chromosomal region 8p21-p12. *Human genetics* 1995; **95**: 356-358.
477. Argiriadi MA, Morisseau C, Hammock BD, Christianson DW. Detoxification of environmental mutagens and carcinogens: Structure, mechanism, and evolution of liver epoxide hydrolase. *Proceedings of the National Academy of Sciences of the United States of America* 1999; **96**: 10637-10642.
478. Pacifici GM, Temellini A, Giuliani L, Rane A, Thomas H, Oesch F. Cytosolic epoxide hydrolase in humans: Development and tissue distribution. *Archives of toxicology* 1988; **62**: 254-257.
479. Sura P, Sura R, Enayetallah AE, Grant DF. Distribution and expression of soluble epoxide hydrolase in human brain. *J Histochem Cytochem* 2008; **56**: 551-559.
480. Enayetallah AE, French RA, Barber M, Grant DF. Cell-specific subcellular localization of soluble epoxide hydrolase in human tissues. *J Histochem Cytochem* 2006; **54**: 329-335.
481. Okita RT, Okita JR. Cytochrome p450 4a fatty acid omega hydroxylases. *Current drug metabolism* 2001; **2**: 265-281.
482. Cornwell PD, De Souza AT, Ulrich RG. Profiling of hepatic gene expression in rats treated with fibric acid analogs. *Mutation research* 2004; **549**: 131-145.
483. Johnson EF, Hsu MH, Savas U, Griffin KJ. Regulation of p450 4a expression by peroxisome proliferator activated receptors. *Toxicology* 2002; **181-182**: 203-206.
484. Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, et al. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in

abolishment of the pleiotropic effects of peroxisome proliferators. *Molecular and cellular biology* 1995; **15**: 3012-3022.
485. Muerhoff AS, Griffin KJ, Johnson EF. The peroxisome proliferator-activated receptor mediates the induction of cyp4a6, a cytochrome p450 fatty acid omega-hydroxylase, by clofibric acid. *The Journal of biological chemistry* 1992; **267**: 19051-19053.
486. Sharma R, Lake BG, Foster J, Gibson GG. Microsomal cytochrome p-452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver. A mechanistic inter-relationship. *Biochemical pharmacology* 1988; **37**: 1193-1201.
487. Demoz A, Vaagenes H, Aarsaether N, Hvattum E, Skorve J, Gottlicher M, et al. Coordinate induction of hepatic fatty acyl-coa oxidase and p4504a1 in rat after activation of the peroxisome proliferator-activated receptor (ppar) by sulphur-substituted fatty acid analogues. *Xenobiotica; the fate of foreign compounds in biological systems* 1994; **24**: 943-956.
488. Fang X, Hu S, Xu B, Snyder GD, Harmon S, Yao J, et al. 14,15-dihydroxyeicosatrienoic acid activates peroxisome proliferator-activated receptor-alpha. *American journal of physiology. Heart and circulatory physiology* 2006; **290**: H55-63.
489. Ng VY, Huang Y, Reddy LM, Falck JR, Lin ET, Kroetz DL. Cytochrome p450 eicosanoids are activators of peroxisome proliferator-activated receptor alpha. *Drug metabolism and disposition: the biological fate of chemicals* 2007; **35**: 1126-1134.
490. Corton JC, Apte U, Anderson SP, Limaye P, Yoon L, Latendresse J, et al. Mimetics of caloric restriction include agonists of lipid-activated nuclear receptors. *The Journal of biological chemistry* 2004; **279**: 46204-46212.
491. Kroetz DL, Yook P, Costet P, Bianchi P, Pineau T. Peroxisome proliferator-activated receptor alpha controls the hepatic cyp4a induction adaptive response to starvation and diabetes. *The Journal of biological chemistry* 1998; **273**: 31581-31589.
492. Kocarek TA, Reddy AB. Regulation of cytochrome p450 expression by inhibitors of hydroxymethylglutaryl-coenzyme a reductase in primary cultured rat hepatocytes and in rat liver. *Drug metabolism and disposition: the biological fate of chemicals* 1996; **24**: 1197-1204.
493. Shimano H. Sterol regulatory element-binding proteins (srebps): Transcriptional regulators of lipid synthetic genes. *Progress in lipid research* 2001; **40**: 439-452.
494. Hsu MH, Savas U, Griffin KJ, Johnson EF. Regulation of human cytochrome p450 4f2 expression by sterol regulatory element-binding protein and lovastatin. *The Journal of biological chemistry* 2007; **282**: 5225-5236.
495. Holla VR, Adas F, Imig JD, Zhao X, Price E, Jr., Olsen N, et al. Alterations in the regulation of androgen-sensitive cyp 4a monooxygenases cause hypertension. *Proceedings of the National Academy of Sciences of the United States of America* 2001; **98**: 5211-5216.
496. Nakagawa K, Marji JS, Schwartzman ML, Waterman MR, Capdevila JH. Androgen-mediated induction of the kidney arachidonate hydroxylases is associated with the development of hypertension. *American journal of physiology. Regulatory, integrative and comparative physiology* 2003; **284**: R1055-1062.
497. Singh H, Schwartzman ML. Renal vascular cytochrome p450-derived eicosanoids in androgen-induced hypertension. *Pharmacol Rep* 2008; **60**: 29-37.

498. Wang MH, Smith A, Zhou Y, Chang HH, Lin S, Zhao X, et al. Downregulation of renal cyp-derived eicosanoid synthesis in rats with diet-induced hypertension. *Hypertension* 2003; **42**: 594-599.
499. Zhou Y, Huang H, Chang HH, Du J, Wu JF, Wang CY, et al. Induction of renal 20-hydroxyeicosatetraenoic acid by clofibrate attenuates high-fat diet-induced hypertension in rats. *The Journal of pharmacology and experimental therapeutics* 2006; **317**: 11-18.
500. Huang H, Morisseau C, Wang J, Yang T, Falck JR, Hammock BD, et al. Increasing or stabilizing renal epoxyeicosatrienoic acid production attenuates abnormal renal function and hypertension in obese rats. *American journal of physiology* 2007; **293**: F342-349.
501. Antoun J, Amet Y, Simon B, Dreano Y, Corlu A, Corcos L, et al. Cyp4a11 is repressed by retinoic acid in human liver cells. *FEBS letters* 2006; **580**: 3361-3367.
502. Zhang X, Hardwick JP. Regulation of cyp4f2 leukotriene b4 omega-hydroxylase by retinoic acids in hepg2 cells. *Biochemical and biophysical research communications* 2000; **279**: 864-871.
503. Zhang X, Chen L, Hardwick JP. Promoter activity and regulation of the cyp4f2 leukotriene b(4) omega-hydroxylase gene by peroxisomal proliferators and retinoic acid in hepg2 cells. *Archives of biochemistry and biophysics* 2000; **378**: 364-376.
504. Johnson EF, Palmer CN, Griffin KJ, Hsu MH. Role of the peroxisome proliferator-activated receptor in cytochrome p450 4a gene regulation. *FASEB J* 1996; **10**: 1241-1248.
505. Spiecker M, Darius H, Hankeln T, Soufi M, Sattler AM, Schaefer JR, et al. Risk of coronary artery disease associated with polymorphism of the cytochrome p450 epoxygenase cyp2j2. *Circulation* 2004; **110**: 2132-2136.
506. Marden NY, Murray M. Characterization of a c-jun-responsive module in the 5'-flank of the human cyp2j2 gene that regulates transactivation. *The Biochemical journal* 2005; **391**: 631-640.
507. Wray JA, Sugden MC, Zeldin DC, Greenwood GK, Samsuddin S, Miller-Degraff L, et al. The epoxygenases cyp2j2 activates the nuclear receptor pparalpha in vitro and in vivo. *PloS one* 2009; **4**: e7421.
508. Qu W, Rippe RA, Ma J, Scarborough P, Biagini C, Fiedorek FT, et al. Nutritional status modulates rat liver cytochrome p450 arachidonic acid metabolism. *Molecular pharmacology* 1998; **54**: 504-513.
509. Honkalammi J, Niemi M, Neuvonen PJ, Backman JT. Mechanism-based inactivation of cyp2c8 by gemfibrozil occurs rapidly in humans. *Clinical pharmacology and therapeutics* 2011; **89**: 579-586.
510. Lai XS, Yang LP, Li XT, Liu JP, Zhou ZW, Zhou SF. Human cyp2c8: Structure, substrate specificity, inhibitor selectivity, inducers and polymorphisms. *Current drug metabolism* 2009; **10**: 1009-1047.
511. Ferguson SS, Chen Y, LeCluyse EL, Negishi M, Goldstein JA. Human cyp2c8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane x receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Molecular pharmacology* 2005; **68**: 747-757.
512. Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ, Maurel P. Transcriptional regulation of cyp2c9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. *The Journal of biological chemistry* 2002; **277**: 209-217.

513. Ferguson SS, LeCluyse EL, Negishi M, Goldstein JA. Regulation of human cyp2c9 by the constitutive androstane receptor: Discovery of a new distal binding site. *Molecular pharmacology* 2002; **62**: 737-746.
514. Chen Y, Kissling G, Negishi M, Goldstein JA. The nuclear receptors constitutive androstane receptor and pregnane x receptor cross-talk with hepatic nuclear factor 4alpha to synergistically activate the human cyp2c9 promoter. *The Journal of pharmacology and experimental therapeutics* 2005; **314**: 1125-1133.
515. Naraharisetti SB, Lin YS, Rieder MJ, Marciante KD, Psaty BM, Thummel KE, et al. Human liver expression of cyp2c8: Gender, age, and genotype effects. *Drug metabolism and disposition: the biological fate of chemicals* 2010; **38**: 889-893.
516. Rae JM, Johnson MD, Lippman ME, Flockhart DA. Rifampin is a selective, pleiotropic inducer of drug metabolism genes in human hepatocytes: Studies with cdna and oligonucleotide expression arrays. *The Journal of pharmacology and experimental therapeutics* 2001; **299**: 849-857.
517. Flockhart DA. "Drug interactions: Cytochrome p450 drug interaction table". 2007
518. Niemi M, Backman JT, Neuvonen PJ. Effects of trimethoprim and rifampin on the pharmacokinetics of the cytochrome p450 2c8 substrate rosiglitazone. *Clinical pharmacology and therapeutics* 2004; **76**: 239-249.
519. Park JY, Kim KA, Kang MH, Kim SL, Shin JG. Effect of rifampin on the pharmacokinetics of rosiglitazone in healthy subjects. *Clinical Pharmacology & Therapeutics* 2004; **75**: 157-162.
520. Jaakkola T, Backman JT, Neuvonen M, Laitila J, Neuvonen PJ. Effect of rifampicin on the pharmacokinetics of pioglitazone. *British journal of clinical pharmacology* 2006; **61**: 70-78.
521. Niemi M, Backman JT, Neuvonen M, Neuvonen PJ, Kivisto KT. Rifampin decreases the plasma concentrations and effects of repaglinide. *Clinical pharmacology and therapeutics* 2000; **68**: 495-500.
522. Bidstrup TB, Stilling N, Damkier P, Scharling B, Thomsen MS, Brosen K. Rifampicin seems to act as both an inducer and an inhibitor of the metabolism of repaglinide. *European journal of clinical pharmacology* 2004; **60**: 109-114.
523. Vormfelde SV, Brockmoller J, Bauer S, Herchenhein P, Kuon J, Meineke I, et al. Relative impact of genotype and enzyme induction on the metabolic capacity of cyp2c9 in healthy volunteers. *Clinical pharmacology and therapeutics* 2009; **86**: 54-61.
524. Miners JO, Birkett DJ. Cytochrome p4502c9: An enzyme of major importance in human drug metabolism. *British journal of clinical pharmacology* 1998; **45**: 525-538.
525. Kanebratt KP, Diczfalusy U, Backstrom T, Sparve E, Bredberg E, Bottiger Y, et al. Cytochrome p450 induction by rifampicin in healthy subjects: Determination using the karolinska cocktail and the endogenous cyp3a4 marker 4beta-hydroxycholesterol. *Clinical pharmacology and therapeutics* 2008; **84**: 589-594.
526. Tanaka H, Kamita SG, Wolf NM, Harris TR, Wu Z, Morisseau C, et al. Transcriptional regulation of the human soluble epoxide hydrolase gene ephx2. *Biochimica et biophysica acta* 2008; **1779**: 17-27.
527. Zhang D, Ai D, Tanaka H, Hammock BD, Zhu Y. DNA methylation of the promoter of soluble epoxide hydrolase silences its expression by an sp-1-dependent mechanism. *Biochimica et biophysica acta* 2010; **1799**: 659-667.

528. Oesch F, Schladt L, Hartmann R, Timms C, Worner W. Rat cytosolic epoxide hydrolase. *Advances in experimental medicine and biology* 1986; **197**: 195-201.
529. Pinot F, Grant DF, Spearow JL, Parker AG, Hammock BD. Differential regulation of soluble epoxide hydrolase by clofibrate and sexual hormones in the liver and kidneys of mice. *Biochemical pharmacology* 1995; **50**: 501-508.
530. Hammock BD, Ota K. Differential induction of cytosolic epoxide hydrolase, microsomal epoxide hydrolase, and glutathione s-transferase activities. *Toxicology and applied pharmacology* 1983; **71**: 254-265.
531. Ai D, Fu Y, Guo D, Tanaka H, Wang N, Tang C, et al. Angiotensin ii up-regulates soluble epoxide hydrolase in vascular endothelium in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104**: 9018-9023.
532. Imig JD, Zhao X, Capdevila JH, Morisseau C, Hammock BD. Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin ii hypertension. *Hypertension* 2002; **39**: 690-694.
533. Petruzzelli S, Franchi M, Gronchi L, Janni A, Oesch F, Pacifici GM, et al. Cigarette smoke inhibits cytosolic but not microsomal epoxide hydrolase of human lung. *Human & experimental toxicology* 1992; **11**: 99-103.
534. Park WY, Hwang CI, Im CN, Kang MJ, Woo JH, Kim JH, et al. Identification of radiation-specific responses from gene expression profile. *Oncogene* 2002; **21**: 8521-8528.
535. Barbosa-Sicard E, Fromel T, Keseru B, Brandes RP, Morisseau C, Hammock BD, et al. Inhibition of the soluble epoxide hydrolase by tyrosine nitration. *The Journal of biological chemistry* 2009; **284**: 28156-28163.
536. Simpson AE. The cytochrome p450 4 (cyp4) family. *General pharmacology* 1997; **28**: 351-359.
537. Palmer CN, Richardson TH, Griffin KJ, Hsu MH, Muerhoff AS, Clark JE, et al. Characterization of a cdna encoding a human kidney, cytochrome p-450 4a fatty acid omega-hydroxylase and the cognate enzyme expressed in escherichia coli. *Biochimica et biophysica acta* 1993; **1172**: 161-166.
538. Powell PK, Wolf I, Lasker JM. Identification of cyp4a11 as the major lauric acid omega-hydroxylase in human liver microsomes. *Archives of biochemistry and biophysics* 1996; **335**: 219-226.
539. Kawashima H, Naganuma T, Kusunose E, Kono T, Yasumoto R, Sugimura K, et al. Human fatty acid omega-hydroxylase, cyp4a11: Determination of complete genomic sequence and characterization of purified recombinant protein. *Archives of biochemistry and biophysics* 2000; **378**: 333-339.
540. Gainer JV, Bellamine A, Dawson EP, Womble KE, Grant SW, Wang Y, et al. Functional variant of cyp4a11 20-hydroxyeicosatetraenoic acid synthase is associated with essential hypertension. *Circulation* 2005; **111**: 63-69.
541. Aoyama T, Hardwick JP, Imaoka S, Funae Y, Gelboin HV, Gonzalez FJ. Clofibrate-inducible rat hepatic p450s iva1 and iva3 catalyze the omega- and (omega-1)-hydroxylation of fatty acids and the omega-hydroxylation of prostaglandins e1 and f2 alpha. *Journal of lipid research* 1990; **31**: 1477-1482.
542. Sharma RK, Doig MV, Lewis DF, Gibson GG. Role of hepatic and renal cytochrome p-450 iva1 in the metabolism of lipid substrates. *Biochemical pharmacology* 1989; **38**: 3621-3629.

543. Bains SK, Gardiner SM, Mannweiler K, Gillett D, Gibson GG. Immunochemical study on the contribution of hypolipidaemic-induced cytochrome p-452 to the metabolism of lauric acid and arachidonic acid. *Biochemical pharmacology* 1985; **34**: 3221-3229.
544. Hardwick JP. Cytochrome p450 omega hydroxylase (cyp4) function in fatty acid metabolism and metabolic diseases. *Biochemical pharmacology* 2008; **75**: 2263-2275.
545. Snider NT, Walker VJ, Hollenberg PF. Oxidation of the endogenous cannabinoid arachidonoyl ethanolamide by the cytochrome p450 monooxygenases: Physiological and pharmacological implications. *Pharmacological reviews* 2010; **62**: 136-154.
546. Kawashima H, Kusunose E, Kikuta Y, Kinoshita H, Tanaka S, Yamamoto S, et al. Purification and cdna cloning of human liver cyp4a fatty acid omega-hydroxylase. *Journal of biochemistry* 1994; **116**: 74-80.
547. Komen JC, Duran M, Wanders RJ. Characterization of phytanic acid omega-hydroxylation in human liver microsomes. *Molecular genetics and metabolism* 2005; **85**: 190-195.
548. Xu F, Ng VY, Kroetz DL, de Montellano PR. Cyp4 isoform specificity in the omega-hydroxylation of phytanic acid, a potential route to elimination of the causative agent of refsum's disease. *The Journal of pharmacology and experimental therapeutics* 2006; **318**: 835-839.
549. McDonald MG, Rieder MJ, Nakano M, Hsia CK, Rettie AE. Cyp4f2 is a vitamin k1 oxidase: An explanation for altered warfarin dose in carriers of the v433m variant. *Molecular pharmacology* 2009; **75**: 1337-1346.
550. Wang MZ, Saulter JY, Usuki E, Cheung YL, Hall M, Bridges AS, et al. Cyp4f enzymes are the major enzymes in human liver microsomes that catalyze the o-demethylation of the antiparasitic prodrug db289 [2,5-bis(4-amidinophenyl)furan-bis-o-methylamidoxime]. *Drug metabolism and disposition: the biological fate of chemicals* 2006; **34**: 1985-1994.
551. Kovarik JM, Dole K, Riviere GJ, Pommier F, Maton S, Jin Y, et al. Ketoconazole increases fingolimod blood levels in a drug interaction via cyp4f2 inhibition. *Journal of clinical pharmacology* 2009; **49**: 212-218.
552. David OJ, Kovarik JM, Schmouder RL. Clinical pharmacokinetics of fingolimod. *Clinical pharmacokinetics* 2012; **51**: 15-28.
553. Lee CA, Neul D, Clouser-Roche A, Dalvie D, Wester MR, Jiang Y, et al. Identification of novel substrates for human cytochrome p450 2j2. *Drug metabolism and disposition: the biological fate of chemicals* 2010; **38**: 347-356.
554. Capdevila JH, Karara A, Waxman DJ, Martin MV, Falck JR, Guenguerich FP. Cytochrome p-450 enzyme-specific control of the regio- and enantiofacial selectivity of the microsomal arachidonic acid epoxygenase. *The Journal of biological chemistry* 1990; **265**: 10865-10871.
555. Zeldin DC, Plitman JD, Kobayashi J, Miller RF, Snapper JR, Falck JR, et al. The rabbit pulmonary cytochrome p450 arachidonic acid metabolic pathway: Characterization and significance. *The Journal of clinical investigation* 1995; **95**: 2150-2160.
556. Zeldin DC, DuBois RN, Falck JR, Capdevila JH. Molecular cloning, expression and characterization of an endogenous human cytochrome p450 arachidonic acid epoxygenase isoform. *Archives of biochemistry and biophysics* 1995; **322**: 76-86.
557. Matsumoto S, Hirama T, Matsubara T, Nagata K, Yamazoe Y. Involvement of cyp2j2 on the intestinal first-pass metabolism of antihistamine drug, astemizole. *Drug metabolism and disposition: the biological fate of chemicals* 2002; **30**: 1240-1245.

558. Goldstein JA. Clinical relevance of genetic polymorphisms in the human cyp2c subfamily. *British journal of clinical pharmacology* 2001; **52**: 349-355.
559. Ali ZK, Kim RJ, Ysla FM. Cyp2c9 polymorphisms: Considerations in nsaid therapy. *Current opinion in drug discovery & development* 2009; **12**: 108-114.
560. Lee CR, Goldstein JA, Pieper JA. Cytochrome p450 2c9 polymorphisms: A comprehensive review of the in-vitro and human data. *Pharmacogenetics* 2002; **12**: 251-263.
561. Schoch GA, Yano JK, Wester MR, Griffin KJ, Stout CD, Johnson EF. Structure of human microsomal cytochrome p450 2c8. Evidence for a peripheral fatty acid binding site. *The Journal of biological chemistry* 2004; **279**: 9497-9503.
562. Schoch GA, Yano JK, Sansen S, Dansette PM, Stout CD, Johnson EF. Determinants of cytochrome p450 2c8 substrate binding: Structures of complexes with montelukast, troglitazone, felodipine, and 9-cis-retinoic acid. *The Journal of biological chemistry* 2008; **283**: 17227-17237.
563. Zhou SF, Zhou ZW, Yang LP, Cai JP. Substrates, inducers, inhibitors and structure-activity relationships of human cytochrome p450 2c9 and implications in drug development. *Current medicinal chemistry* 2009; **16**: 3480-3675.
564. McSorley LC, Daly AK. Identification of human cytochrome p450 isoforms that contribute to all-trans-retinoic acid 4-hydroxylation. *Biochemical pharmacology* 2000; **60**: 517-526.
565. Capdevila JH, Falck JR. Biochemical and molecular characteristics of the cytochrome p450 arachidonic acid monooxygenase. *Prostaglandins & other lipid mediators* 2000; **62**: 271-292.
566. Meijer J, DePierre JW. Cytosolic epoxide hydrolase. *Chemico-biological interactions* 1988; **64**: 207-249.
567. Dietze EC, Magdalou J, Hammock BD. Human and murine cytosolic epoxide hydrolase: Physical and structural properties. *The International journal of biochemistry* 1990; **22**: 461-470.
568. Chacos N, Capdevila J, Falck JR, Manna S, Martin-Wixtrom C, Gill SS, et al. The reaction of arachidonic acid epoxides (epoxyeicosatrienoic acids) with a cytosolic epoxide hydrolase. *Archives of biochemistry and biophysics* 1983; **223**: 639-648.
569. Gill SS, Hammock BD. Hydration of cis- and trans-epoxymethyl stearates by the cytosolic epoxide hydrase of mouse liver. *Biochemical and biophysical research communications* 1979; **89**: 965-971.
570. Borhan B, Mebrahtu T, Nazarian S, Kurth MJ, Hammock BD. Improved radiolabeled substrates for soluble epoxide hydrolase. *Analytical biochemistry* 1995; **231**: 188-200.
571. Greene JF, Williamson KC, Newman JW, Morisseau C, Hammock BD. Metabolism of monoepoxides of methyl linoleate: Bioactivation and detoxification. *Archives of biochemistry and biophysics* 2000; **376**: 420-432.
572. Zeldin DC, Wei S, Falck JR, Hammock BD, Snapper JR, Capdevila JH. Metabolism of epoxyeicosatrienoic acids by cytosolic epoxide hydrolase: Substrate structural determinants of asymmetric catalysis. *Archives of biochemistry and biophysics* 1995; **316**: 443-451.
573. Moghaddam M, Motoba K, Borhan B, Pinot F, Hammock BD. Novel metabolic pathways for linoleic and arachidonic acid metabolism. *Biochimica et biophysica acta* 1996; **1290**: 327-339.

574. Spector AA, Fang X, Snyder GD, Weintraub NL. Epoxyeicosatrienoic acids (eets): Metabolism and biochemical function. *Progress in lipid research* 2004; **43**: 55-90.
575. Cronin A, Mowbray S, Durk H, Homburg S, Fleming I, Fisslthaler B, et al. The n-terminal domain of mammalian soluble epoxide hydrolase is a phosphatase. *Proceedings of the National Academy of Sciences of the United States of America* 2003; **100**: 1552-1557.
576. Newman JW, Morisseau C, Harris TR, Hammock BD. The soluble epoxide hydrolase encoded by epxh2 is a bifunctional enzyme with novel lipid phosphate phosphatase activity. *Proceedings of the National Academy of Sciences of the United States of America* 2003; **100**: 1558-1563.
577. Enayetallah AE, Grant DF. Effects of human soluble epoxide hydrolase polymorphisms on isoprenoid phosphate hydrolysis. *Biochemical and biophysical research communications* 2006; **341**: 254-260.
578. Tran KL, Aronov PA, Tanaka H, Newman JW, Hammock BD, Morisseau C. Lipid sulfates and sulfonates are allosteric competitive inhibitors of the n-terminal phosphatase activity of the mammalian soluble epoxide hydrolase. *Biochemistry* 2005; **44**: 12179-12187.
579. Edwards PA, Ericsson J. Sterols and isoprenoids: Signaling molecules derived from the cholesterol biosynthetic pathway. *Annual review of biochemistry* 1999; **68**: 157-185.
580. Liu Y, Zhang Y, Schmelzer K, Lee TS, Fang X, Zhu Y, et al. The antiinflammatory effect of laminar flow: The role of ppargamma, epoxyeicosatrienoic acids, and soluble epoxide hydrolase. *Proceedings of the National Academy of Sciences of the United States of America* 2005; **102**: 16747-16752.
581. Spector AA, Norris AW. Action of epoxyeicosatrienoic acids on cellular function. *American journal of physiology. Cell physiology* 2007; **292**: C996-1012.
582. Weinhofer I, Kunze M, Stangl H, Porter FD, Berger J. Peroxisomal cholesterol biosynthesis and smith-lemli-opitz syndrome. *Biochemical and biophysical research communications* 2006; **345**: 205-209.
583. Dos Santos EA, Dahly-Vernon AJ, Hoagland KM, Roman RJ. Inhibition of the formation of eets and 20-hete with 1-aminobenzotriazole attenuates pressure natriuresis. *American journal of physiology. Regulatory, integrative and comparative physiology* 2004; **287**: R58-68.
584. Ortiz de Montellano PR, Reich NO. Specific inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids. *The Journal of biological chemistry* 1984; **259**: 4136-4141.
585. Reich NO, Ortiz de Montellano PR. Dissociation of increased lauric acid omega-hydroxylase activity from the antilipidemic action of clofibrate. *Biochemical pharmacology* 1986; **35**: 1227-1233.
586. Xu F, Straub WO, Pak W, Su P, Maier KG, Yu M, et al. Antihypertensive effect of mechanism-based inhibition of renal arachidonic acid omega-hydroxylase activity. *American journal of physiology. Regulatory, integrative and comparative physiology* 2002; **283**: R710-720.
587. Gebremedhin D, Lange AR, Lowry TF, Taheri MR, Birks EK, Hudetz AG, et al. Production of 20-hete and its role in autoregulation of cerebral blood flow. *Circulation research* 2000; **87**: 60-65.
588. Poloyac SM, Zhang Y, Bies RR, Kochanek PM, Graham SH. Protective effect of the 20-hete inhibitor het0016 on brain damage after temporary focal ischemia. *J Cereb Blood Flow Metab* 2006; **26**: 1551-1561.

589. Miyata N, Seki T, Tanaka Y, Omura T, Taniguchi K, Doi M, et al. Beneficial effects of a new 20-hydroxyeicosatetraenoic acid synthesis inhibitor, ts-011 [n-(3-chloro-4-morpholin-4-yl) phenyl-n'-hydroxyimido formamide], on hemorrhagic and ischemic stroke. *The Journal of pharmacology and experimental therapeutics* 2005; **314**: 77-85.
590. Yu M, Cambj-Sapunar L, Kehl F, Maier KG, Takeuchi K, Miyata N, et al. Effects of a 20-hete antagonist and agonists on cerebral vascular tone. *European journal of pharmacology* 2004; **486**: 297-306.
591. Sontag TJ, Parker RS. Cytochrome p450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin e status. *The Journal of biological chemistry* 2002; **277**: 25290-25296.
592. Yamashita K, Iizuka Y, Imai T, Namiki M. Sesame seed and its lignans produce marked enhancement of vitamin e activity in rats fed a low alpha-tocopherol diet. *Lipids* 1995; **30**: 1019-1028.
593. Kamal-Eldin A, Frank J, Razdan A, Tengblad S, Basu S, Vessby B. Effects of dietary phenolic compounds on tocopherol, cholesterol, and fatty acids in rats. *Lipids* 2000; **35**: 427-435.
594. Cooney RV, Custer LJ, Okinaka L, Franke AA. Effects of dietary sesame seeds on plasma tocopherol levels. *Nutrition and cancer* 2001; **39**: 66-71.
595. Jin Y, Zollinger M, Borell H, Zimmerlin A, Patten CJ. Cyp4f enzymes are responsible for the elimination of fingolimod (fty720), a novel treatment of relapsing multiple sclerosis. *Drug metabolism and disposition: the biological fate of chemicals* 2011; **39**: 191-198.
596. Harder DR, Alkayed NJ, Lange AR, Gebremedhin D, Roman RJ. Functional hyperemia in the brain: Hypothesis for astrocyte-derived vasodilator metabolites. *Stroke; a journal of cerebral circulation* 1998; **29**: 229-234.
597. Bhardwaj A, Northington FJ, Carhuapoma JR, Falck JR, Harder DR, Traystman RJ, et al. P-450 epoxygenase and no synthase inhibitors reduce cerebral blood flow response to n-methyl-d-aspartate. *American journal of physiology. Heart and circulatory physiology* 2000; **279**: H1616-1624.
598. Zou AP, Ma YH, Sui ZH, Ortiz de Montellano PR, Clark JE, Masters BS, et al. Effects of 17-octadecynoic acid, a suicide-substrate inhibitor of cytochrome p450 fatty acid omega-hydroxylase, on renal function in rats. *The Journal of pharmacology and experimental therapeutics* 1994; **268**: 474-481.
599. Lafite P, Dijols S, Zeldin DC, Dansette PM, Mansuy D. Selective, competitive and mechanism-based inhibitors of human cytochrome p450 2j2. *Archives of biochemistry and biophysics* 2007; **464**: 155-168.
600. Lafite P, Dijols S, Buisson D, Macherey AC, Zeldin DC, Dansette PM, et al. Design and synthesis of selective, high-affinity inhibitors of human cytochrome p450 2j2. *Bioorganic & medicinal chemistry letters* 2006; **16**: 2777-2780.
601. Matsumoto S, Hirama T, Kim HJ, Nagata K, Yamazoe Y. In vitro inhibition of human small intestinal and liver microsomal astemizole o-demethylation: Different contribution of cyp2j2 in the small intestine and liver. *Xenobiotica; the fate of foreign compounds in biological systems* 2003; **33**: 615-623.
602. Shitara Y, Hirano M, Sato H, Sugiyama Y. Gemfibrozil and its glucuronide inhibit the organic anion transporting polypeptide 2 (oatp2/oatp1b1:Slc21a6)-mediated hepatic uptake and cyp2c8-mediated metabolism of cerivastatin: Analysis of the mechanism of the

clinically relevant drug-drug interaction between cerivastatin and gemfibrozil. *The Journal of pharmacology and experimental therapeutics* 2004; **311**: 228-236.
603. Ogilvie BW, Zhang D, Li W, Rodrigues AD, Gipson AE, Holsapple J, et al. Glucuronidation converts gemfibrozil to a potent, metabolism-dependent inhibitor of cyp2c8: Implications for drug-drug interactions. *Drug metabolism and disposition: the biological fate of chemicals* 2006; **34**: 191-197.
604. Baer BR, DeLisle RK, Allen A. Benzylic oxidation of gemfibrozil-1-o-beta-glucuronide by p450 2c8 leads to heme alkylation and irreversible inhibition. *Chemical research in toxicology* 2009; **22**: 1298-1309.
605. Honkalammi J, Niemi M, Neuvonen PJ, Backman JT. Gemfibrozil is a strong inactivator of cyp2c8 in very small multiple doses. *Clinical pharmacology and therapeutics* 2012; **91**: 846-855.
606. Lu Y, Won KA, Nelson BJ, Qi D, Rausch DJ, Asinger RW. Characteristics of the amiodarone-warfarin interaction during long-term follow-up. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* 2008; **65**: 947-952.
607. Siddoway LA. Amiodarone: Guidelines for use and monitoring. *American family physician* 2003; **68**: 2189-2196.
608. Catella F, Lawson JA, Fitzgerald DJ, FitzGerald GA. Endogenous biosynthesis of arachidonic acid epoxides in humans: Increased formation in pregnancy-induced hypertension. *Proceedings of the National Academy of Sciences of the United States of America* 1990; **87**: 5893-5897.
609. Shen HC, Hammock BD. Discovery of inhibitors of soluble epoxide hydrolase: A target with multiple potential therapeutic indications. *Journal of medicinal chemistry* 2012; **55**: 1789-1808.
610. Fang X, Kaduce TL, Weintraub NL, VanRollins M, Spector AA. Functional implications of a newly characterized pathway of 11,12-epoxyeicosatrienoic acid metabolism in arterial smooth muscle. *Circulation research* 1996; **79**: 784-793.
611. Gebremedhin D, Ma YH, Falck JR, Roman RJ, VanRollins M, Harder DR. Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *The American journal of physiology* 1992; **263**: H519-525.
612. Mayberg MR, Okada T, Bark DH. Morphologic changes in cerebral arteries after subarachnoid hemorrhage. *Neurosurgery clinics of North America* 1990; **1**: 417-432.
613. Fleming I. Cytochrome p450 and vascular homeostasis. *Circulation research* 2001; **89**: 753-762.
614. Harder DR, Gebremedhin D, Narayanan J, Jefcoat C, Falck JR, Campbell WB, et al. Formation and action of a p-450 4a metabolite of arachidonic acid in cat cerebral microvessels. *The American journal of physiology* 1994; **266**: H2098-2107.
615. Lange A, Gebremedhin D, Narayanan J, Harder D. 20-hydroxyeicosatetraenoic acid-induced vasoconstriction and inhibition of potassium current in cerebral vascular smooth muscle is dependent on activation of protein kinase c. *The Journal of biological chemistry* 1997; **272**: 27345-27352.
616. Gebremedhin D, Lange AR, Narayanan J, Aebly MR, Jacobs ER, Harder DR. Cat cerebral arterial smooth muscle cells express cytochrome p450 4a2 enzyme and produce the

vasoconstrictor 20-hete which enhances l-type ca2+ current. *The Journal of physiology* 1998; **507 (Pt 3)**: 771-781.
617. Kedzierski RM, Yanagisawa M. Endothelin system: The double-edged sword in health and disease. *Annual review of pharmacology and toxicology* 2001; **41**: 851-876.
618. Ellis EF, Amruthesh SC, Police RJ, Yancey LM. Brain synthesis and cerebrovascular action of cytochrome p-450/monooxygenase metabolites of arachidonic acid. *Advances in prostaglandin, thromboxane, and leukotriene research* 1991; **21A**: 201-204.
619. Ordway RW, Singer JJ, Walsh JV, Jr. Direct regulation of ion channels by fatty acids. *Trends in neurosciences* 1991; **14**: 96-100.
620. Roman RJ, Renic M, Dunn KM, Takeuchi K, Hacein-Bey L. Evidence that 20-hete contributes to the development of acute and delayed cerebral vasospasm. *Neurological research* 2006; **28**: 738-749.
621. Randriamboavonjy V, Busse R, Fleming I. 20-hete-induced contraction of small coronary arteries depends on the activation of rho-kinase. *Hypertension* 2003; **41**: 801-806.
622. Inoue R, Jensen LJ, Jian Z, Shi J, Hai L, Lurie AI, et al. Synergistic activation of vascular trpc6 channel by receptor and mechanical stimulation via phospholipase c/diacylglycerol and phospholipase a2/omega-hydroxylase/20-hete pathways. *Circulation research* 2009; **104**: 1399-1409.
623. Basora N, Boulay G, Bilodeau L, Rousseau E, Payet MD. 20-hydroxyeicosatetraenoic acid (20-hete) activates mouse trpc6 channels expressed in hek293 cells. *The Journal of biological chemistry* 2003; **278**: 31709-31716.
624. Sun CW, Alonso-Galicia M, Taheri MR, Falck JR, Harder DR, Roman RJ. Nitric oxide-20-hydroxyeicosatetraenoic acid interaction in the regulation of k+ channel activity and vascular tone in renal arterioles. *Circulation research* 1998; **83**: 1069-1079.
625. Campbell WB, Fleming I. Epoxyeicosatrienoic acids and endothelium-dependent responses. *Pflugers Arch* 2010; **459**: 881-895.
626. Kehl F, Cambj-Sapunar L, Maier KG, Miyata N, Kametani S, Okamoto H, et al. 20-hete contributes to the acute fall in cerebral blood flow after subarachnoid hemorrhage in the rat. *American journal of physiology. Heart and circulatory physiology* 2002; **282**: H1556-1565.
627. Takeuchi K, Renic M, Bohman QC, Harder DR, Miyata N, Roman RJ. Reversal of delayed vasospasm by an inhibitor of the synthesis of 20-hete. *American journal of physiology. Heart and circulatory physiology* 2005; **289**: H2203-2211.
628. Fernandez MM, Gonzalez D, Williams JM, Roman RJ, Nowicki S. Inhibitors of 20-hydroxyeicosatetraenoic acid (20-hete) formation attenuate the natriuretic effect of dopamine. *European journal of pharmacology* 2012; **686**: 97-103.
629. Cambj-Sapunar L, Yu M, Harder DR, Roman RJ. Contribution of 5-hydroxytryptamine1b receptors and 20-hydroxyeiscosatetraenoic acid to fall in cerebral blood flow after subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2003; **34**: 1269-1275.
630. Siuta M, Zuckerman SL, Mocco J. Nitric oxide in cerebral vasospasm: Theories, measurement, and treatment. *Neurology research international* 2013; **2013**: 972417.
631. Hall CN, Reynell C, Gesslein B, Hamilton NB, Mishra A, Sutherland BA, et al. Capillary pericytes regulate cerebral blood flow in health and disease. *Nature* 2014; **508**: 55-60.

632. Ellis EF, Police RJ, Yancey L, McKinney JS, Amruthesh SC. Dilation of cerebral arterioles by cytochrome p-450 metabolites of arachidonic acid. *The American journal of physiology* 1990; **259**: H1171-1177.
633. Alkayed NJ, Birks EK, Hudetz AG, Roman RJ, Henderson L, Harder DR. Inhibition of brain p-450 arachidonic acid epoxygenase decreases baseline cerebral blood flow. *The American journal of physiology* 1996; **271**: H1541-1546.
634. Koehler RC, Gebremedhin D, Harder DR. Role of astrocytes in cerebrovascular regulation. *J Appl Physiol* 2006; **100**: 307-317.
635. Koehler RC, Roman RJ, Harder DR. Astrocytes and the regulation of cerebral blood flow. *Trends in neurosciences* 2009; **32**: 160-169.
636. Liu X, Li C, Falck JR, Roman RJ, Harder DR, Koehler RC. Interaction of nitric oxide, 20-hete, and eets during functional hyperemia in whisker barrel cortex. *American journal of physiology. Heart and circulatory physiology* 2008; **295**: H619-631.
637. Munzenmaier DH, Harder DR. Cerebral microvascular endothelial cell tube formation: Role of astrocytic epoxyeicosatrienoic acid release. *American journal of physiology. Heart and circulatory physiology* 2000; **278**: H1163-1167.
638. Zhang W, Koerner IP, Noppens R, Grafe M, Tsai HJ, Morisseau C, et al. Soluble epoxide hydrolase: A novel therapeutic target in stroke. *J Cereb Blood Flow Metab* 2007; **27**: 1931-1940.
639. Zhang W, Otsuka T, Sugo N, Ardeshiri A, Alhadid YK, Iliff JJ, et al. Soluble epoxide hydrolase gene deletion is protective against experimental cerebral ischemia. *Stroke; a journal of cerebral circulation* 2008; **39**: 2073-2078.
640. Liu M, Alkayed NJ. Hypoxic preconditioning and tolerance via hypoxia inducible factor (hif) 1alpha-linked induction of p450 2c11 epoxygenase in astrocytes. *J Cereb Blood Flow Metab* 2005; **25**: 939-948.
641. Koerner IP, Jacks R, DeBarber AE, Koop D, Mao P, Grant DF, et al. Polymorphisms in the human soluble epoxide hydrolase gene ephx2 linked to neuronal survival after ischemic injury. *J Neurosci* 2007; **27**: 4642-4649.
642. Poloyac SM, Reynolds RB, Yonas H, Kerr ME. Identification and quantification of the hydroxyeicosatetraenoic acids, 20-hete and 12-hete, in the cerebrospinal fluid after subarachnoid hemorrhage. *Journal of neuroscience methods* 2005; **144**: 257-263.
643. Miller TM, Donnelly MK, Crago EA, Roman DM, Sherwood PR, Horowitz MB, et al. Rapid, simultaneous quantitation of mono and dioxygenated metabolites of arachidonic acid in human csf and rat brain. *Journal of chromatography* 2009; **877**: 3991-4000.
644. Crago EA, Thampatty BP, Sherwood PR, Kuo CW, Bender C, Balzer J, et al. Cerebrospinal fluid 20-hete is associated with delayed cerebral ischemia and poor outcomes after aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation*; **42**: 1872-1877.
645. Ward NC, Croft KD, Blacker D, Hankey GJ, Barden A, Mori TA, et al. Cytochrome p450 metabolites of arachidonic acid are elevated in stroke patients compared with healthy controls. *Clin Sci (Lond)* 2011; **121**: 501-507.
646. Sugimoto K, Akasaka H, Katsuya T, Node K, Fujisawa T, Shimaoka I, et al. A polymorphism regulates cyp4a11 transcriptional activity and is associated with hypertension in a japanese population. *Hypertension* 2008; **52**: 1142-1148.

647. Ding H, Cui G, Zhang L, Xu Y, Bao X, Tu Y, et al. Association of common variants of cyp4a11 and cyp4f2 with stroke in the han chinese population. *Pharmacogenetics and genomics* 2010; **20**: 187-194.
648. Liu H, Zhao YY, Gong W, Shi JP, Fu LY, Wang J, et al. [correlation analysis and identification of g421c in regulatory region of cyp4f2 gene with essential hypertension]. *Zhongguo yi xue ke xue yuan xue bao* 2006; **28**: 143-147.
649. Liu H, Zhao Y, Nie D, Shi J, Fu L, Li Y, et al. Association of a functional cytochrome p450 4f2 haplotype with urinary 20-hete and hypertension. *J Am Soc Nephrol* 2008; **19**: 714-721.
650. Bardowell SA, Stec DE, Parker RS. Common variants of cytochrome p450 4f2 exhibit altered vitamin e-{omega}-hydroxylase specific activity. *The Journal of nutrition* 2010; **140**: 1901-1906.
651. Stec DE, Roman RJ, Flasch A, Rieder MJ. Functional polymorphism in human cyp4f2 decreases 20-hete production. *Physiological genomics* 2007; **30**: 74-81.
652. Liu PY, Li YH, Chao TH, Wu HL, Lin LJ, Tsai LM, et al. Synergistic effect of cytochrome p450 epoxygenase cyp2j2*7 polymorphism with smoking on the onset of premature myocardial infarction. *Atherosclerosis* 2007; **195**: 199-206.
653. Spiecker M, Liao J. Cytochrome p450 epoxygenase cyp2j2 and the risk of coronary artery disease. *Trends in cardiovascular medicine* 2006; **16**: 204-208.
654. Yamazaki H, Okayama A, Imai N, Guengerich FP, Shimizu M. Inter-individual variation of cytochrome p4502j2 expression and catalytic activities in liver microsomes from japanese and caucasian populations. *Xenobiotica; the fate of foreign compounds in biological systems* 2006; **36**: 1201-1209.
655. Lee SS, Jeong HE, Liu KH, Ryu JY, Moon T, Yoon CN, et al. Identification and functional characterization of novel cyp2j2 variants: G312r variant causes loss of enzyme catalytic activity. *Pharmacogenetics and genomics* 2005; **15**: 105-113.
656. Gaedigk A, Baker DW, Totah RA, Gaedigk R, Pearce RE, Vyhlidal CA, et al. Variability of cyp2j2 expression in human fetal tissues. *The Journal of pharmacology and experimental therapeutics* 2006; **319**: 523-532.
657. Gao Y, Liu D, Wang H, Zhu J, Chen C. Functional characterization of five cyp2c8 variants and prediction of cyp2c8 genotype-dependent effects on in vitro and in vivo drug-drug interactions. *Xenobiotica; the fate of foreign compounds in biological systems* 2010; **40**: 467-475.
658. Soyama A, Hanioka N, Saito Y, Murayama N, Ando M, Ozawa S, et al. Amiodarone n-deethylation by cyp2c8 and its variants, cyp2c8*3 and cyp2c8 p404a. *Pharmacology & toxicology* 2002; **91**: 174-178.
659. Soyama A, Saito Y, Hanioka N, Murayama N, Nakajima O, Katori N, et al. Non-synonymous single nucleotide alterations found in the cyp2c8 gene result in reduced in vitro paclitaxel metabolism. *Biological & pharmaceutical bulletin* 2001; **24**: 1427-1430.
660. Johansson I, Oscarson M, Yue QY, Bertilsson L, Sjoqvist F, Ingelman-Sundberg M. Genetic analysis of the chinese cytochrome p4502d locus: Characterization of variant cyp2d6 genes present in subjects with diminished capacity for debrisoquine hydroxylation. *Molecular pharmacology* 1994; **46**: 452-459.
661. Fukuda T, Nishida Y, Imaoka S, Hiroi T, Naohara M, Funae Y, et al. The decreased in vivo clearance of cyp2d6 substrates by cyp2d6*10 might be caused not only by the low-

expression but also by low affinity of cyp2d6. *Archives of biochemistry and biophysics* 2000; **380**: 303-308.
662. Bahadur N, Leathart JB, Mutch E, Steimel-Crespi D, Dunn SA, Gilissen R, et al. Cyp2c8 polymorphisms in caucasians and their relationship with paclitaxel 6alpha-hydroxylase activity in human liver microsomes. *Biochemical pharmacology* 2002; **64**: 1579-1589.
663. Smith HE, Jones JP, 3rd, Kalhorn TF, Farin FM, Stapleton PL, Davis CL, et al. Role of cytochrome p450 2c8 and 2j2 genotypes in calcineurin inhibitor-induced chronic kidney disease. *Pharmacogenetics and genomics* 2008; **18**: 943-953.
664. Dai D, Zeldin DC, Blaisdell JA, Chanas B, Coulter SJ, Ghanayem BI, et al. Polymorphisms in human cyp2c8 decrease metabolism of the anticancer drug paclitaxel and arachidonic acid. *Pharmacogenetics* 2001; **11**: 597-607.
665. Rodriguez-Antona C, Niemi M, Backman JT, Kajosaari LI, Neuvonen PJ, Robledo M, et al. Characterization of novel cyp2c8 haplotypes and their contribution to paclitaxel and repaglinide metabolism. *The pharmacogenomics journal* 2008; **8**: 268-277.
666. Jiang H, Zhong F, Sun L, Feng W, Huang ZX, Tan X. Structural and functional insights into polymorphic enzymes of cytochrome p450 2c8. *Amino acids* 2011; **40**: 1195-1204.
667. Singh R, Ting JG, Pan Y, Teh LK, Ismail R, Ong CE. Functional role of ile264 in cyp2c8: Mutations affect haem incorporation and catalytic activity. *Drug metabolism and pharmacokinetics* 2008; **23**: 165-174.
668. Hichiya H, Tanaka-Kagawa T, Soyama A, Jinno H, Koyano S, Katori N, et al. Functional characterization of five novel cyp2c8 variants, g171s, r186x, r186g, k247r, and k383n, found in a japanese population. *Drug metabolism and disposition: the biological fate of chemicals* 2005; **33**: 630-636.
669. Hanioka N, Matsumoto K, Saito Y, Narimatsu S. Functional characterization of cyp2c8.13 and cyp2c8.14: Catalytic activities toward paclitaxel. *Basic & clinical pharmacology & toxicology* 2010; **107**: 565-569.
670. Soyama A, Saito Y, Komamura K, Ueno K, Kamakura S, Ozawa S, et al. Five novel single nucleotide polymorphisms in the cyp2c8 gene, one of which induces a frame-shift. *Drug metabolism and pharmacokinetics* 2002; **17**: 374-377.
671. Zhou SF, Zhou ZW, Huang M. Polymorphisms of human cytochrome p450 2c9 and the functional relevance. *Toxicology* 2010; **278**: 165-188.
672. DeLozier TC, Lee SC, Coulter SJ, Goh BC, Goldstein JA. Functional characterization of novel allelic variants of cyp2c9 recently discovered in southeast asians. *The Journal of pharmacology and experimental therapeutics* 2005; **315**: 1085-1090.
673. Yin T, Maekawa K, Kamide K, Saito Y, Hanada H, Miyashita K, et al. Genetic variations of cyp2c9 in 724 japanese individuals and their impact on the antihypertensive effects of losartan. *Hypertens Res* 2008; **31**: 1549-1557.
674. Blaisdell J, Jorge-Nebert LF, Coulter S, Ferguson SS, Lee SJ, Chanas B, et al. Discovery of new potentially defective alleles of human cyp2c9. *Pharmacogenetics* 2004; **14**: 527-537.
675. Liu Y, Jeong H, Takahashi H, Drozda K, Patel SR, Shapiro NL, et al. Decreased warfarin clearance associated with the cyp2c9 r150h (*8) polymorphism. *Clinical pharmacology and therapeutics* 2012; **91**: 660-665.

676. Przybyla-Zawislak BD, Srivastava PK, Vazquez-Matias J, Mohrenweiser HW, Maxwell JE, Hammock BD, et al. Polymorphisms in human soluble epoxide hydrolase. *Molecular pharmacology* 2003; **64**: 482-490.
677. Merkel MJ, Liu L, Cao Z, Packwood W, Young J, Alkayed NJ, et al. Inhibition of soluble epoxide hydrolase preserves cardiomyocytes: Role of stat3 signaling. *American journal of physiology. Heart and circulatory physiology* 2010; **298**: H679-687.
678. Sandberg M, Hassett C, Adman ET, Meijer J, Omiecinski CJ. Identification and functional characterization of human soluble epoxide hydrolase genetic polymorphisms. *The Journal of biological chemistry* 2000; **275**: 28873-28881.
679. Srivastava PK, Sharma VK, Kalonia DS, Grant DF. Polymorphisms in human soluble epoxide hydrolase: Effects on enzyme activity, enzyme stability, and quaternary structure. *Archives of biochemistry and biophysics* 2004; **427**: 164-169.
680. Wu CC, Gupta T, Garcia V, Ding Y, Schwartzman ML. 20-hete and blood pressure regulation: Clinical implications. *Cardiology in review* 2013:
681. Laffer CL, Gainer JV, Waterman MR, Capdevila JH, Laniado-Schwartzman M, Nasjletti A, et al. The t8590c polymorphism of cyp4a11 and 20-hydroxyeicosatetraenoic acid in essential hypertension. *Hypertension* 2008; **51**: 767-772.
682. Ward NC, Tsai IJ, Barden A, van Bockxmeer FM, Puddey IB, Hodgson JM, et al. A single nucleotide polymorphism in the cyp4f2 but not cyp4a11 gene is associated with increased 20-hete excretion and blood pressure. *Hypertension* 2008; **51**: 1393-1398.
683. Hu BC, Li Y, Li FH, Zhang Y, Sheng CS, Fan HQ, et al. Peripheral and central augmentation indexes in relation to the cyp4f2 polymorphisms in chinese. *Journal of hypertension* 2011; **29**: 501-508.
684. Major JM, Yu K, Wheeler W, Zhang H, Cornelis MC, Wright ME, et al. Genome-wide association study identifies common variants associated with circulating vitamin e levels. *Human molecular genetics* 2011; **20**: 3876-3883.
685. Danese E, Montagnana M, Johnson JA, Rettie AE, Zambon CF, Lubitz SA, et al. Impact of the cyp4f2 p.V433m polymorphism on coumarin dose requirement: Systematic review and meta-analysis. *Clinical pharmacology and therapeutics* 2012; **92**: 746-756.
686. Wang CP, Hung WC, Yu TH, Chiu CA, Lu LF, Chung FM, et al. Genetic variation in the g-50t polymorphism of the cytochrome p450 epoxygenase cyp2j2 gene and the risk of younger onset type 2 diabetes among chinese population: Potential interaction with body mass index and family history. *Exp Clin Endocrinol Diabetes* 2010; **118**: 346-352.
687. Gervasini G, Vizcaino S, Carrillo JA, Caballero MJ, Benitez J. The effect of cyp2j2, cyp3a4, cyp3a5 and the mdr1 polymorphisms and gender on the urinary excretion of the metabolites of the h-receptor antihistamine ebastine: A pilot study. *British journal of clinical pharmacology* 2006; **62**: 177-186.
688. Daily EB, Aquilante CL. Cytochrome p450 2c8 pharmacogenetics: A review of clinical studies. *Pharmacogenomics* 2009; **10**: 1489-1510.
689. Tornio A, Niemi M, Neuvonen PJ, Backman JT. Trimethoprim and the cyp2c8*3 allele have opposite effects on the pharmacokinetics of pioglitazone. *Drug metabolism and disposition: the biological fate of chemicals* 2008; **36**: 73-80.
690. Aquilante CL, Bushman LR, Knutsen SD, Burt LE, Rome LC, Kosmiski LA. Influence of slco1b1 and cyp2c8 gene polymorphisms on rosiglitazone pharmacokinetics in healthy volunteers. *Human genomics* 2008; **3**: 7-16.

691. Aquilante CL, Kosmiski LA, Bourne DW, Bushman LR, Daily EB, Hammond KP, et al. Impact of the cyp2c8 *3 polymorphism on the drug-drug interaction between gemfibrozil and pioglitazone. *British journal of clinical pharmacology* 2013; **75**: 217-226.
692. Kirchheiner J, Thomas S, Bauer S, Tomalik-Scharte D, Hering U, Doroshyenko O, et al. Pharmacokinetics and pharmacodynamics of rosiglitazone in relation to cyp2c8 genotype. *Clinical pharmacology and therapeutics* 2006; **80**: 657-667.
693. Kadam R, Bourne D, Kompella U, Aquilante C. Effect of cytochrome p450 2c8*3 on the population pharmacokinetics of pioglitazone in healthy caucasian volunteers. *Biological & pharmaceutical bulletin* 2013; **36**: 245-251.
694. Stage TB, Christensen MM, Feddersen S, Beck-Nielsen H, Brosen K. The role of genetic variants in cyp2c8, lpin1, ppargc1a and ppargamma on the trough steady-state plasma concentrations of rosiglitazone and on glycosylated haemoglobin a1c in type 2 diabetes. *Pharmacogenetics and genomics* 2013; **23**: 219-227.
695. Pedersen RS, Damkier P, Brosen K. The effects of human cyp2c8 genotype and fluvoxamine on the pharmacokinetics of rosiglitazone in healthy subjects. *British journal of clinical pharmacology* 2006; **62**: 682-689.
696. Tornio A, Niemi M, Neuvonen M, Laitila J, Kalliokoski A, Neuvonen PJ, et al. The effect of gemfibrozil on repaglinide pharmacokinetics persists for at least 12 h after the dose: Evidence for mechanism-based inhibition of cyp2c8 in vivo. *Clinical pharmacology and therapeutics* 2008; **84**: 403-411.
697. Niemi M, Backman JT, Kajosaari LI, Leathart JB, Neuvonen M, Daly AK, et al. Polymorphic organic anion transporting polypeptide 1b1 is a major determinant of repaglinide pharmacokinetics. *Clinical pharmacology and therapeutics* 2005; **77**: 468-478.
698. Niemi M, Leathart JB, Neuvonen M, Backman JT, Daly AK, Neuvonen PJ. Polymorphism in cyp2c8 is associated with reduced plasma concentrations of repaglinide. *Clinical pharmacology and therapeutics* 2003; **74**: 380-387.
699. Bidstrup TB, Damkier P, Olsen AK, Ekblom M, Karlsson A, Brosen K. The impact of cyp2c8 polymorphism and grapefruit juice on the pharmacokinetics of repaglinide. *British journal of clinical pharmacology* 2006; **61**: 49-57.
700. Tomalik-Scharte D, Fuhr U, Hellmich M, Frank D, Doroshyenko O, Jetter A, et al. Effect of the cyp2c8 genotype on the pharmacokinetics and pharmacodynamics of repaglinide. *Drug metabolism and disposition: the biological fate of chemicals* 2011; **39**: 927-932.
701. Garcia-Martin E, Martinez C, Tabares B, Frias J, Agundez JA. Interindividual variability in ibuprofen pharmacokinetics is related to interaction of cytochrome p450 2c8 and 2c9 amino acid polymorphisms. *Clinical pharmacology and therapeutics* 2004; **76**: 119-127.
702. Martinez C, Garcia-Martin E, Blanco G, Gamito FJ, Ladero JM, Agundez JA. The effect of the cytochrome p450 cyp2c8 polymorphism on the disposition of (r)-ibuprofen enantiomer in healthy subjects. *British journal of clinical pharmacology* 2005; **59**: 62-69.
703. Karazniewicz-Lada M, Luczak M, Glowka F. Pharmacokinetic studies of enantiomers of ibuprofen and its chiral metabolites in humans with different variants of genes coding cyp2c8 and cyp2c9 isoenzymes. *Xenobiotica; the fate of foreign compounds in biological systems* 2009; **39**: 476-485.
704. Lopez-Rodriguez R, Novalbos J, Gallego-Sandin S, Roman-Martinez M, Torrado J, Gisbert JP, et al. Influence of cyp2c8 and cyp2c9 polymorphisms on pharmacokinetic and

pharmacodynamic parameters of racemic and enantiomeric forms of ibuprofen in healthy volunteers. *Pharmacol Res* 2008; **58**: 77-84.
705. Dorado P, Berecz R, Norberto MJ, Yasar U, Dahl ML, A LL. Cyp2c9 genotypes and diclofenac metabolism in spanish healthy volunteers. *European journal of clinical pharmacology* 2003; **59**: 221-225.
706. Green H, Soderkvist P, Rosenberg P, Mirghani RA, Rymark P, Lundqvist EA, et al. Pharmacogenetic studies of paclitaxel in the treatment of ovarian cancer. *Basic & clinical pharmacology & toxicology* 2009; **104**: 130-137.
707. Leskela S, Jara C, Leandro-Garcia LJ, Martinez A, Garcia-Donas J, Hernando S, et al. Polymorphisms in cytochromes p450 2c8 and 3a5 are associated with paclitaxel neurotoxicity. *The pharmacogenomics journal* 2011; **11**: 121-129.
708. Hertz DL, Motsinger-Reif AA, Drobish A, Winham SJ, McLeod HL, Carey LA, et al. Cyp2c8*3 predicts benefit/risk profile in breast cancer patients receiving neoadjuvant paclitaxel. *Breast cancer research and treatment* 2012; **134**: 401-410.
709. Hertz DL, Roy S, Motsinger-Reif AA, Drobish A, Clark LS, McLeod HL, et al. Cyp2c8*3 increases risk of neuropathy in breast cancer patients treated with paclitaxel. *Ann Oncol* 2013; **24**: 1472-1478.
710. Henningsson A, Marsh S, Loos WJ, Karlsson MO, Garsa A, Mross K, et al. Association of cyp2c8, cyp3a4, cyp3a5, and abcb1 polymorphisms with the pharmacokinetics of paclitaxel. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2005; **11**: 8097-8104.
711. Marsh S, Somlo G, Li X, Frankel P, King CR, Shannon WD, et al. Pharmacogenetic analysis of paclitaxel transport and metabolism genes in breast cancer. *The pharmacogenomics journal* 2007; **7**: 362-365.
712. Marsh S, Paul J, King CR, Gifford G, McLeod HL, Brown R. Pharmacogenetic assessment of toxicity and outcome after platinum plus taxane chemotherapy in ovarian cancer: The scottish randomised trial in ovarian cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2007; **25**: 4528-4535.
713. Jabir RS, Naidu R, Annuar MA, Ho GF, Munisamy M, Stanslas J. Pharmacogenetics of taxanes: Impact of gene polymorphisms of drug transporters on pharmacokinetics and toxicity. *Pharmacogenomics* 2012; **13**: 1979-1988.
714. Kirchheiner J, Meineke I, Fuhr U, Rodriguez-Antona C, Lebedeva E, Brockmoller J. Impact of genetic polymorphisms in cyp2c8 and rosiglitazone intake on the urinary excretion of dihydroxyeicosatrienoic acids. *Pharmacogenomics* 2008; **9**: 277-288.
715. Guo Y, Zhang Y, Wang Y, Chen X, Si D, Zhong D, et al. Role of cyp2c9 and its variants (cyp2c9*3 and cyp2c9*13) in the metabolism of lornoxicam in humans. *Drug metabolism and disposition: the biological fate of chemicals* 2005; **33**: 749-753.
716. Allabi AC, Gala JL, Horsmans Y. Cyp2c9, cyp2c19, abcb1 (mdr1) genetic polymorphisms and phenytoin metabolism in a black beninese population. *Pharmacogenetics and genomics* 2005; **15**: 779-786.
717. King BP, Khan TI, Aithal GP, Kamali F, Daly AK. Upstream and coding region cyp2c9 polymorphisms: Correlation with warfarin dose and metabolism. *Pharmacogenetics* 2004; **14**: 813-822.

718. Lee JP, Yang SH, Kim DK, Lee H, Kim B, Cho JY, et al. In vivo activity of epoxide hydrolase according to sequence variation affects the progression of human iga nephropathy. *American journal of physiology* 2011; **300**: F1283-1290.
719. Lee CR, North KE, Bray MS, Fornage M, Seubert JM, Newman JW, et al. Genetic variation in soluble epoxide hydrolase (ephx2) and risk of coronary heart disease: The atherosclerosis risk in communities (aric) study. *Human molecular genetics* 2006; **15**: 1640-1649.
720. Edvardsen H, Brunsvig PF, Solvang H, Tsalenko A, Andersen A, Syvanen AC, et al. Snps in genes coding for ros metabolism and signalling in association with docetaxel clearance. *The pharmacogenomics journal* 2010; **10**: 513-523.
721. Sato K, Emi M, Ezura Y, Fujita Y, Takada D, Ishigami T, et al. Soluble epoxide hydrolase variant (glu287arg) modifies plasma total cholesterol and triglyceride phenotype in familial hypercholesterolemia: Intrafamilial association study in an eight-generation hyperlipidemic kindred. *Journal of human genetics* 2004; **49**: 29-34.
722. Chen B, Hu Y, Jin T, Lu D, Shao M, Zheng L, et al. The influence of metabolic gene polymorphisms on urinary 1-hydroxypyrene concentrations in chinese coke oven workers. *The Science of the total environment* 2007; **381**: 38-46.
723. Fava C, Montagnana M, Almgren P, Rosberg L, Lippi G, Hedblad B, et al. The v433m variant of the cyp4f2 is associated with ischemic stroke in male swedes beyond its effect on blood pressure. *Hypertension* 2008; **52**: 373-380.
724. Marciante KD, Totah RA, Heckbert SR, Smith NL, Lemaitre RN, Lumley T, et al. Common variation in cytochrome p450 epoxygenase genes and the risk of incident nonfatal myocardial infarction and ischemic stroke. *Pharmacogenetics and genomics* 2008; **18**: 535-543.
725. Fu Z, Nakayama T, Sato N, Izumi Y, Kasamaki Y, Shindo A, et al. Haplotype-based case study of human cyp4a11 gene and cerebral infarction in japanese subject. *Endocrine* 2008; **33**: 215-222.
726. Munshi A, Sharma V, Kaul S, Al-Hazzani A, Alshatwi AA, Shafi G, et al. Association of 1347 g/a cytochrome p450 4f2 (cyp4f2) gene variant with hypertension and stroke. *Molecular biology reports* 2012; **39**: 1677-1682.
727. Deng S, Zhu G, Liu F, Zhang H, Qin X, Li L, et al. Cyp4f2 gene v433m polymorphism is associated with ischemic stroke in the male northern chinese han population. *Progress in neuro-psychopharmacology & biological psychiatry* 2010; **34**: 664-668.
728. Fu Z, Nakayama T, Sato N, Izumi Y, Kasamaki Y, Shindo A, et al. A haplotype of the cyp4f2 gene is associated with cerebral infarction in japanese men. *American journal of hypertension* 2008; **21**: 1216-1223.
729. Zhang L, Ding H, Yan J, Hui R, Wang W, Kissling GE, et al. Genetic variation in cytochrome p450 2j2 and soluble epoxide hydrolase and risk of ischemic stroke in a chinese population. *Pharmacogenetics and genomics* 2008; **18**: 45-51.
730. Fava C, Montagnana M, Almgren P, Hedblad B, Engstrom G, Berglund G, et al. The common functional polymorphism -50g>t of the cyp2j2 gene is not associated with ischemic coronary and cerebrovascular events in an urban-based sample of swedes. *Journal of hypertension* 2010; **28**: 294-299.

731. Kaur-Knudsen D, Bojesen SE, Nordestgaard BG. Common polymorphisms in cyp2c9, subclinical atherosclerosis and risk of ischemic vascular disease in 52,000 individuals. *The pharmacogenomics journal* 2009; **9**: 327-332.
732. Funk M, Freitag R, Endler G, Lalouschek W, Lang W, Mannhalter C, et al. Influence of cytochrome p450 2c9*2 and 2c9*3 variants on the risk of ischemic stroke: A cross-sectional case-control study. *Clinical chemistry* 2005; **51**: 1716-1718.
733. Fava C, Montagnana M, Danese E, Almgren P, Hedblad B, Engstrom G, et al. Homozygosity for the ephx2 k55r polymorphism increases the long-term risk of ischemic stroke in men: A study in swedes. *Pharmacogenetics and genomics* 2010; **20**: 94-103.
734. Gschwendtner A, Ripke S, Freilinger T, Lichtner P, Muller-Myhsok B, Wichmann HE, et al. Genetic variation in soluble epoxide hydrolase (ephx2) is associated with an increased risk of ischemic stroke in white europeans. *Stroke; a journal of cerebral circulation* 2008; **39**: 1593-1596.
735. Fornage M, Lee CR, Doris PA, Bray MS, Heiss G, Zeldin DC, et al. The soluble epoxide hydrolase gene harbors sequence variation associated with susceptibility to and protection from incident ischemic stroke. *Human molecular genetics* 2005; **14**: 2829-2837.
736. Lee J, Dahl M, Grande P, Tybjaerg-Hansen A, Nordestgaard BG. Genetically reduced soluble epoxide hydrolase activity and risk of stroke and other cardiovascular disease. *Stroke; a journal of cerebral circulation*; **41**: 27-33.
737. Zhang JE, Jorgensen AL, Alfirevic A, Williamson PR, Toh CH, Park BK, et al. Effects of cyp4f2 genetic polymorphisms and haplotypes on clinical outcomes in patients initiated on warfarin therapy. *Pharmacogenetics and genomics* 2009; **19**: 781-789.
738. Merkel MJ, Liu L, Cao Z, Packwood W, Young J, Alkayed NJ, et al. Inhibition of soluble epoxide hydrolase preserves cardiomyocytes: Role of stat3 signaling. *American journal of physiology. Heart and circulatory physiology*; **298**: H679-687.
739. Nithipatikom K, Grall AJ, Holmes BB, Harder DR, Falck JR, Campbell WB. Liquid chromatographic-electrospray ionization-mass spectrometric analysis of cytochrome p450 metabolites of arachidonic acid. *Analytical biochemistry* 2001; **298**: 327-336.
740. Rivera J, Ward N, Hodgson J, Puddey IB, Falck JR, Croft KD. Measurement of 20-hydroxyeicosatetraenoic acid in human urine by gas chromatography-mass spectrometry. *Clinical chemistry* 2004; **50**: 224-226.
741. Bolcato CA, Frye RF, Zemaitis MA, Poloyac SM. Determination of 20-hydroxyeicosatetraenoic acid in microsomal incubates using high-performance liquid chromatography-mass spectrometry (hplc-ms). *Journal of chromatography* 2003; **794**: 363-372.
742. Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. *Methods in enzymology* 2007; **432**: 59-82.
743. Yue H, Strauss KI, Borenstein MR, Barbe MF, Rossi LJ, Jansen SA. Determination of bioactive eicosanoids in brain tissue by a sensitive reversed-phase liquid chromatographic method with fluorescence detection. *Journal of chromatography* 2004; **803**: 267-277.
744. Salmon JA. Measurement of eicosanoids by bioassay and radioimmunoassay. *British medical bulletin* 1983; **39**: 227-231.

745. VanderNoot VA, VanRollins M. Capillary electrophoresis of cytochrome p-450 epoxygenase metabolites of arachidonic acid. 1. Resolution of regioisomers. *Analytical chemistry* 2002; **74**: 5859-5865.
746. Grates HE, McGowen RM, Gupta SV, Falck JR, Brown TR, Callewaert DM, et al. Quantification of 20-hydroxyeicosatetraenoic acid by colorimetric competitive enzyme linked immunosorbent assay. *Journal of biosciences* 2003; **28**: 109-113.
747. Wang D, DuBois RN. Measurement of eicosanoids in cancer tissues. *Methods in enzymology* 2007; **433**: 27-50.
748. Kempen EC, Yang P, Felix E, Madden T, Newman RA. Simultaneous quantification of arachidonic acid metabolites in cultured tumor cells using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Analytical biochemistry* 2001; **297**: 183-190.
749. Zelinski V, Borlak J. Identification and quantification of metabolites of arachidonic acid from cultures of endothelial cells by hplc-ms2. *Xenobiotica; the fate of foreign compounds in biological systems* 2005; **35**: 811-823.
750. Mu Y, Klamerus MM, Miller TM, Rohan LC, Graham SH, Poloyac SM. Intravenous formulation of n-hydroxy-n'-(4-n-butyl-2-methylphenyl)formamidine (het0016) for inhibition of rat brain 20-hydroxyeicosatetraenoic acid formation. *Drug metabolism and disposition: the biological fate of chemicals* 2008; **36**: 2324-2330.
751. Yue H, Jansen SA, Strauss KI, Borenstein MR, Barbe MF, Rossi LJ, et al. A liquid chromatography/mass spectrometric method for simultaneous analysis of arachidonic acid and its endogenous eicosanoid metabolites prostaglandins, dihydroxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and epoxyeicosatrienoic acids in rat brain tissue. *Journal of pharmaceutical and biomedical analysis* 2007; **43**: 1122-1134.
752. Zhang JH, Pearson T, Matharoo-Ball B, Ortori CA, Warren AY, Khan R, et al. Quantitative profiling of epoxyeicosatrienoic, hydroxyeicosatetraenoic, and dihydroxyeicosatetraenoic acids in human intrauterine tissues using liquid chromatography/electrospray ionization tandem mass spectrometry. *Analytical biochemistry* 2007; **365**: 40-51.
753. Chavis C, Fraissinet L, Chanez P, Thomas E, Bousquet J. A method for the measurement of plasma hydroxyeicosatetraenoic acid levels. *Analytical biochemistry* 1999; **271**: 105-108.
754. Novakova L, Matysova L, Solich P. Advantages of application of uplc in pharmaceutical analysis. *Talanta* 2006; **68**: 908-918.
755. Swartz ME. Uplc: An introduction and review. *J Liq Chromatogr Related Technol* 2005; **28**: 1253-1263.
756. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on hplc-ms/ms. *Analytical chemistry* 2003; **75**: 3019-3030.
757. Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, et al. Quantitative bioanalytical methods validation and implementation: Best practices for chromatographic and ligand binding assays. *Pharmaceutical research* 2007; **24**: 1962-1973.
758. Fulton D, Falck JR, McGiff JC, Carroll MA, Quilley J. A method for the determination of 5,6-eet using the lactone as an intermediate in the formation of the diol. *Journal of lipid research* 1998; **39**: 1713-1721.

759. Yang W, Gauthier KM, Reddy LM, Sangras B, Sharma KK, Nithipatikom K, et al. Stable 5,6-epoxyeicosatrienoic acid analog relaxes coronary arteries through potassium channel activation. *Hypertension* 2005; **45**: 681-686.
760. Gallek M, Alexander S, Crago E, Sherwood P, Horowitz M, Poloyac S, et al. Endothelin-1 and endothelin receptor gene variants and their association with negative outcomes following aneurysmal subarachnoid hemorrhage. *Biological Research For Nursing* 2012:
761. Sole X, Guino E, Valls J, Iniesta R, Moreno V. Snpstats: A web tool for the analysis of association studies. *Bioinformatics* 2006; **22**: 1928-1929.
762. Crago EA, Thampatty BP, Sherwood PR, Kuo CW, Bender C, Balzer J, et al. Cerebrospinal fluid 20-hete is associated with delayed cerebral ischemia and poor outcomes after aneurysmal subarachnoid hemorrhage. *Stroke; a journal of cerebral circulation* 2011; **42**: 1872-1877.
763. Nagin DS, Tremblay RE. Analyzing developmental trajectories of distinct but related behaviors: A group-based method. *Psychological methods* 2001; **6**: 18-34.
764. Frontera JA, Fernandez A, Schmidt JM, Claassen J, Wartenberg KE, Badjatia N, et al. Defining vasospasm after subarachnoid hemorrhage: What is the most clinically relevant definition? *Stroke; a journal of cerebral circulation* 2009; **40**: 1963-1968.
765. de Rooij NK, Greving JP, Rinkel GJ, Frijns CJ. Early prediction of delayed cerebral ischemia after subarachnoid hemorrhage: Development and validation of a practical risk chart. *Stroke; a journal of cerebral circulation* 2013; **44**: 1288-1294.
766. Bhasker CR, Miners JO, Coulter S, Birkett DJ. Allelic and functional variability of cytochrome p4502c9. *Pharmacogenetics* 1997; **7**: 51-58.
767. Pratt PF, Medhora M, Harder DR. Mechanisms regulating cerebral blood flow as therapeutic targets. *Curr Opin Investig Drugs* 2004; **5**: 952-956.
768. Pfister SL, Gauthier KM, Campbell WB. Vascular pharmacology of epoxyeicosatrienoic acids. *Advances in pharmacology (San Diego, Calif* **60**: 27-59.
769. Shivachar AC, Willoughby KA, Ellis EF. Effect of protein kinase c modulators on 14,15-epoxyeicosatrienoic acid incorporation into astroglial phospholipids. *Journal of neurochemistry* 1995; **65**: 338-346.
770. Starke RM, Komotar RJ, Otten ML, Schmidt JM, Fernandez LD, Rincon F, et al. Predicting long-term outcome in poor grade aneurysmal subarachnoid haemorrhage patients utilising the glasgow coma scale. *J Clin Neurosci* 2009; **16**: 26-31.
771. Lino Cardenas CL, Renault N, Farce A, Cauffiez C, Allorge D, Lo-Guidice JM, et al. Genetic polymorphism of cyp4a11 and cyp4a22 genes and in silico insights from comparative 3d modelling in a french population. *Gene* 2011; **487**: 10-20.
772. Siler DA, Martini RP, Ward JP, Nelson JW, Borkar RN, Zuloaga KL, et al. Protective role of p450 epoxyeicosanoids in subarachnoid hemorrhage. *Neurocritical care* 2014:
773. Li N, Liu JY, Timofeyev V, Qiu H, Hwang SH, Tuteja D, et al. Beneficial effects of soluble epoxide hydrolase inhibitors in myocardial infarction model: Insight gained using metabolomic approaches. *Journal of molecular and cellular cardiology* 2009; **47**: 835-845.
774. Ren Y, Garvin JL, Falck JR, Renduchintala KV, Carretero OA. Glomerular autacoids stimulated by bradykinin regulate efferent arteriole tone. *Kidney international* 2003; **63**: 987-993.

775. Liu JY, Li N, Yang J, Li N, Qiu H, Ai D, et al. Metabolic profiling of murine plasma reveals an unexpected biomarker in rofecoxib-mediated cardiovascular events. *Proceedings of the National Academy of Sciences of the United States of America*; **107**: 17017-17022.
776. Buczynski MW, Dumlao DS, Dennis EA. Thematic review series: Proteomics. An integrated omics analysis of eicosanoid biology. *Journal of lipid research* 2009; **50**: 1015-1038.
777. Samuelsson B. Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. *Science (New York, N.Y* 1983; **220**: 568-575.
778. Arnold C, Markovic M, Blossey K, Wallukat G, Fischer R, Dechend R, et al. Arachidonic acid-metabolizing cytochrome p450 enzymes are targets of {omega}-3 fatty acids. *The Journal of biological chemistry* 2010; **285**: 32720-32733.
779. Russell FD, Burgin-Maunder CS. Distinguishing health benefits of eicosapentaenoic and docosahexaenoic acids. *Marine drugs* 2012; **10**: 2535-2559.
780. Westphal C, Konkel A, Schunck WH. Cyp-eicosanoids--a new link between omega-3 fatty acids and cardiac disease? *Prostaglandins & other lipid mediators* 2011; **96**: 99-108.
781. Konkel A, Schunck WH. Role of cytochrome p450 enzymes in the bioactivation of polyunsaturated fatty acids. *Biochimica et biophysica acta*; **1814**: 210-222.
782. Lauterbach B, Barbosa-Sicard E, Wang MH, Honeck H, Kargel E, Theuer J, et al. Cytochrome p450-dependent eicosapentaenoic acid metabolites are novel bk channel activators. *Hypertension* 2002; **39**: 609-613.
783. Yousef K, Crago E, Kuo CW, Horowitz M, Hravnak M. Predictors of delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage: A cardiac focus. *Neurocritical care* 2010; **13**: 366-372.
784. Ziegler A, Koch A, Krockenberger K, Grosshennig A. Personalized medicine using DNA biomarkers: A review. *Human genetics* 2012; **131**: 1627-1638.
785. Matsui S. Genomic biomarkers for personalized medicine: Development and validation in clinical studies. *Computational and mathematical methods in medicine* 2013; **2013**: 865980.
786. Kuhle J, Petzold A. What makes a prognostic biomarker in cns diseases: Strategies for targeted biomarker discovery? Part 1: Acute and monophasic diseases. *Expert opinion on medical diagnostics* 2011; **5**: 333-346.
787. Taylor JM, Ankerst DP, Andridge RR. Validation of biomarker-based risk prediction models. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2008; **14**: 5977-5983.
788. St Julien J, Bandeen-Roche K, Tamargo RJ. Validation of an aneurysmal subarachnoid hemorrhage grading scale in 1532 consecutive patients. *Neurosurgery* 2008; **63**: 204-210; discussion 210-201.
789. Liman TG, Zietemann V, Wiedmann S, Jungehuelsing GJ, Endres M, Wollenweber FA, et al. Prediction of vascular risk after stroke - protocol and pilot data of the prospective cohort with incident stroke (proscis). *International journal of stroke : official journal of the International Stroke Society* 2013; **8**: 484-490.
790. Saposnik G. Validation of stroke prognostic scores: What do clinicians need to know? *Neuroepidemiology* 2013; **41**: 219-220.

791. Hlatky MA, Greenland P, Arnett DK, Ballantyne CM, Criqui MH, Elkind MS, et al. Criteria for evaluation of novel markers of cardiovascular risk: A scientific statement from the american heart association. *Circulation* 2009; **119**: 2408-2416.
792. Escalante B, Omata K, Sessa W, Lee SG, Falck JR, Schwartzman ML. 20-hydroxyeicosatetraenoic acid is an endothelium-dependent vasoconstrictor in rabbit arteries. *European journal of pharmacology* 1993; **235**: 1-7.
793. Ncats re-engineering translational sciences.
794. Ncats fact sheet. 2014: