Association of UGT1A6*2 (Ser7Ala) polymorphism with therapeutic response to aspirin in ischemic stroke patients

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In

Department of Human Genetics and Molecular Medicine

By

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DECLARATION

I declare that all the changes suggested by examiners in the research project entitled **"Association of UGT1A6*2 (Ser7Ala) polymorphism with therapeutic response to aspirin in ischemic stroke patients"** have been incorporated by me in this project work for the award of degree of **M.Sc. in Life Sciences with specialization in Human Genetics** in the **Department of Human Genetics and Molecular Medicine.**

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DECLARATION

I declare that the project work entitled "**Association of UGT1A6*2 (Ser7Ala) polymorphism with therapeutic response to aspirin in ischemic stroke patients**" has been prepared by me under the guidance of Prof. Anjana Munshi, HoD [Department of Human Genetics and Molecular Medicine], [School of Health Sciences], Central University of Punjab. No part of this project has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Dharmendra kumar has prepared his project work entitled "**Association of UGT1A6*2 (Ser7Ala) polymorphism with therapeutic response to aspirin in ischemic stroke patients"** for the award of M.Sc., degree of the Central University of Punjab, under my guidance. He has carried out this work, at the Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab.

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ABSTRACT

"**Association of UGT1A6*2 (Ser7Ala) polymorphism with therapeutic response to aspirin in ischemic stroke patients.**"

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Ischemic stroke occurs due to the formation of thrombus or embolism within the arteries due to platelet aggregation. Aspirin therapy is used for the prevention of secondary stroke. The variant of UGT1A6 (Ser7Ala) gene has been found to be associated with ischemic stroke as well as aspirin resistance. We aim to study the demographic profile of ischemic stroke patients from Malwa region of Punjab and to evaluate the frequency of UGT1A6*2 Ser7Ala polymorphism and correlate it with aspirin resistance and ADRs (if any). We collected 30 samples from confirmed ischemic stroke patients from Guru Gobind Singh Medical College and Hospital in Malwa region of Punjab. DNA was isolated from blood and PCR- RFLP technique was used to evaluate the UGT1A6 gene variant in the patients. mRS value was used to classify patients as responders or non-responders. 24 patients had TT genotype and 6 patients were found to bear TG genotype. 90% of patients were aspirin responders and 10% were aspirin non-responders. Since the sample size was too low to identify significant associations, a large number of samples should be screened before coming to a conclusion. However, this preliminary study indicates that UGT1A6*2 (Ser7Ala) variant of UGT1A6 gene might be a risk factor for aspirin resistance in the studied group.

Dharmendra kumar **Prof. Anjana Munshi**

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Dharmendra kumar

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1	Trial of Org 10171 in Acute Stroke Treatment	TOAST
2	Transitory ischemic attack	TIA
3	Cyclo-oxygenase	COX
4	Arachidonic acid	AA
5	Thromboxane A2	TXA2
6	Glycoprotein	GP
7	Red blood cell	RBC
8	Adenosine diphosphate	ADP
9	Adverse drug reactions	ADRs
10	Absorption, Distribution, Metabolism and Excretion	ADME
11	Platelet antigen A1/A2	PlA1/A2
12	Human platelet antigen 1a/1b	HPA 1a/1b
13	β- hydroxymethyglutaryl coenzyme A	HMG-CoA
14	low-density lipoprotein	LDL
15	Tissue plasminogen activator	tPA
16	Cytochrome P-450 enzyme	CYP450
17	vitamin K epoxide reductase	VKOR
18	Acetylsalicylic acid	ASA

19	Non steroid anti-inflammatory drug	NSAIDs
20	Human hepatic carboxyestrase-2	HCE2
21	Xenobiotic/medium-chain fatty acid:CoA ligase	ACSM2B
22	Uridine diphosphate glucuronosyltransferase isoenzyme 1A	UGT1A6
23	Multiple drug resistance	MDR1
24	Phenol:Chloroform:Isoamyl alcohol	PCI
25	Chloroform:Isoamyl alcohol	CA
26	Ethidium bromide	EtBr
27	Milliliter	Ml
28	Centigrade	℃
29	Micro litter	µl
30	Optical density	OD
31	Nano-meter	Nm
32	Nano gram	Ng
33	Gram	G
34	Polymerase chain reaction	PCR
35	Restriction fragment length polymorphism	RFLP
36	Seconds	S
37	Modified Rankin Score	mRS

Chapter - 1

Introduction

According to world health organization (WHO) stroke is defined as “Rapidly developing clinical signs of focal (or global) disturbance of cerebral function with symptoms lasting 24 hours or leading to death, with no apparent cause other than vascular origin” (Truelsen *et al.*, 2000). It is a complex neurological disorder with multiple risk factors (Munshi and Sharma, 2015). It is a leading cause of death and disability worldwide. Globally, 15 million suffer a stroke each year. Of these, 5 million people die and 5 million are left with the permanent disability (Mackay *et al.*, 2004). In India, the estimated adjusted prevalence rate of stroke ranges from 84-262/100,000 in rural and 334-424/100,000 in urban areas. Based on the recent population study, the incidence rate has been found to 119-145/100,000 individuals (Pandian and Sudhan, 2013). Each year “29th October” is celebrated as a world stroke day by world stroke organization to spread the awareness related to stroke among the general population.

Stroke is a medical condition in which the oxygen and nutrient-rich blood supply do not reach to the part of the brain due to a halt in the cerebral blood supply. Depending on the physiology of stroke it can be classified into two types: ischemic stroke and hemorrhagic stroke. Ischemic stroke accounts for 80% of the total stroke cases. It occurs due to an obstruction or occlusion of the cerebral circulation, whereas hemorrhagic stroke accounts for 20% of the stroke cases (Munshi and Sharma, 2015). Hemorrhagic stroke results from the rupture of a blood vessel in the brain that leads to the leakage of blood in the surrounding brain tissue exerting pressure on it. A transient ischemic stroke occurs due to clot formation in the blood vessels but the symptoms are later reduced within 24 hours. It is a warning sign for a future stroke. According to “Trial of Org 10172 in Acute Stroke Treatment (TOAST)” a classification system, ischemic stroke is further classified into five subtype: 1) large-artery atherosclerosis, 2) Cardioembolism, 3) Small-vessel occlusion, 4) Stroke of other determined etiology, and 5) Stroke of undetermined etiology (H. P. Adams *et al.*, 1993).

Risk factors for a stroke include both (1) non-modifiable and (2) modifiable factors. Non-modifiable risk factors include age, race (Black and Hispanics in the USA), sex (men>women), improper birth weight (<2,500g or ≥4,000g), family history of stroke/ transient ischemic attack. Whereas modifiable risk factors include hypertension,

tobacco smoking, diabetes mellitus, obesity, physical inactivity, dyslipidemia, dietary factors, metabolic syndrome, alcohol consumption, drug abuse, and hypercoagulability (Bornstein, 2009). Hypertension contributes to 54% of the stroke cases in low-income countries followed by hypercoagulability [15%] and tobacco smoking [12%] (Pandian and Sudhan, 2013).

Stroke prevention strategies include targeting the key modifiable factors i.e. hypertension, elevated lipid levels and diabetes. Different therapeutic treatments are given to prevent a secondary stroke. These include lipid-lowering drugs, anti-hypertensive drugs, anticoagulants and antiplatelet agents. Combination of such prevention strategies have proven to be effective in reducing stroke mortality (Vasudeva *et al.*, 2017). Aspirin is considered as a golden standard in the prevention of a secondary stroke. It is an oral drug, prescribed with a dose of 75-325 mg/day. It is a non-steroidal anti-inflammatory drug (NSAIDs). Aspirin has pleiotropic effects i.e. it has multiple benefits like reducing inflammation, pain, and potent antiplatelet property (Singh and Triadafilopoulos, 1999). Despite the demonstrated benefits of aspirin, many patients suffer a secondary strokes, an observation that has led to the concept of “aspirin resistance” (AR). Several factors contribute to AR including drug-drug interaction (e.g. ibuprofen), inadequate dose of drug, and increased platelet turnover. Genetic studies in patients on aspirin treatment suggest a genetic basis for inter-individual differences in response to this drug (Catella-Lawson *et al.*, 2001) (Goodman *et al.*, 2008).

Pharmacogenetics is a branch of pharmacology that deals with the study of effect of genetic architecture on drug response. It has two distinct branches namely pharmacokinetics and pharmacodynamics. Pharmacokinetics deals with the absorption, distribution, metabolism, and elimination (ADME) of the drugs. Pharmacodynamics deals with what a drug does to the body i.e. molecular effects of a drug, receptor binding, post-receptor effect, and chemical interactions. Pharmacokinetics and pharmacodynamics help to explain the time course and intensity of pharmacological effect of drugs. Polymorphisms in the genes encoding enzymes affecting pharmacokinetics and pharmacodynamics may influence variation in drug

responses as well as adverse drug reactions (Voora and Ginsburg, 2012). In India, 48.2% of stroke patients have been found to be AR in South Indian population from Andhra Pradesh (Sharma *et al.*, 2013). Variation in more than a dozen of genes including COX-2, UGT1A6, GPIIIa, GPIIb, GPLA, and P2Y1 have been found to be associated with aspirin resistance (Voora and Ginsburg, 2012). UDP-glucuronosyltransferase 1-6 or UGT1A6 is a gene that synthesizes UDP – glucuronosyltransferase enzyme involved in glucuronidation pathway that transforms aspirin to hydrophilic molecules and excretable metabolites (Mackenzie *et al.*, 1997).

UGT gene is located on the long arm of chromosome 2 [2q37.1]. It bears 6 exons. Several variants of UGT1A6 have been reported till date but three variants of UGT1A6*2 i.e. Thr181Ala; Arg184Ser and Ser7Ala have been found to be significantly associated with drug response. However, no study has been conducted to elucidate UGT1A6*2 Ser7Ala association with aspirin resistance in ischemic stroke patients. Therefore, the present study was carried out with an aim to evaluate the frequency of UGT1A6*2 Ser7Ala and also to correlate it with aspirin resistance in ischemic stroke patients from Malwa region of Punjab.

Chapter- 2

Aims and objectives

1. To study the demographic profile of ischemic stroke patients from Malwa region of Punjab.

2. To evaluate the association of UGT1A6 (Ser7Ala) polymorphism with aspirin response as well as ADRs (if any) in ischemic stroke patients.

Chapter-3

Review of literature

3.1 Current treatments in stroke

Factors that predispose to stroke risk can be ameliorated with appropriate therapeutic agents. Post-Stroke treatment minimizes the risk of recurrent thrombotic events. Therapeutic agents like tissue plasminogen activator, antiplatelet agents, anticoagulants, and lipid-lowering drugs are given to prevent a secondary stroke (Munshi and Sharma, 2015). Early administration (<24 hours post-stroke) of aspirin and clopidogrel have been found to be associated with decreased early mortality. Aspirin is the mainstay antiplatelet agent for secondary stroke prevention (Grines *et al.*, 2007).

3.1.1 Recombinant tissue plasminogen activator (rtPA)

rtPA is called as a thrombolytic agent or more commonly referred to as “clot buster”. It is a recombinant pharmacological agent that disrupts the occluding clot within the cerebral circulation (Schielke and Lawrence, 2012). It is given during the acute phase of ischemic stroke (within 3-4.5 hours from the onset) and administered intravenously through a catheter inserted into a vein in the arm. Under normal conditions, this enzyme is found on the surface of vascular endothelial cells and secreted after a vascular injury. It has a potential serine protease activity which is responsible for the conversion of plasminogen to plasmin. Plasmin is the main biomolecule that lyses the blood clot (Home, 2013). However, safety and efficacy of r-tPA are controversial; it has been reported that 2-10% of the patients treated with this drug develop symptomatic hemorrhage and largely 40% do not even realize or do so too late and that has been proved to be fatal. Further, rtPA at increased levels may result in hyperfibrinolysis leading to excessive bleeding (R. J. Adams *et al.*, 2017).

3.1.2 Statins

Statins are the effective drug that greatly reduces the stroke risk. The relative reduction in risk for stroke is about 20%, for each 1-mmol/L decrease in low-density lipoprotein cholesterol can be achieved using statins. It prevents the secondary stroke by lowering the blood cholesterol level through the inhibition of β-hydroxymethyl glutaryl coenzyme

A (HMG-CoA) reductase enzyme (Amarenco and Labreuche, 2009). This drug has “pleiotropic effects”; its beneficial effects is not only to lower cholesterol levels but also it helps in maintenance of endothelial functions, stabilization of atherosclerotic plaque, inhibition of cell migration, and the reduction of inflammatory and oxidative stress (Huisa *et al.*, 2010).

3.1.3 Anticoagulant therapy

Warfarin is given in the treatment of thromboembolic, and cardioembolic events. It inhibits the enzyme vitamin k epoxidase reductase in the liver. Inhibition of this enzyme further inhibits the clotting factors (II, VII, IX, and X) that ultimately prevent the clotting of blood cells (Aguilar *et al.*, 2006). Warfarin is an equal mixture of two enantiomers, i.e. s-warfarin and r-warfarin. S-warfarin is 3-5 times more potent than r-warfarin (Flockhart, 2007). Studies have reported certain complications with warfarin administration. It has been found to be associated with inter-individual variation in drug response. Such a heterogeneity in response to warfarin has narrowed down its therapeutic range. Variation in the enzyme involved in warfarin metabolism might be responsible for inter-individual variation in drug responses (Oldenburg *et al.*, 2008). Thus genetic variants in cytochrome p-450 enzyme like CYP2C9 and VKORC1 that encode vitamin K epoxide reductase (VKOR) have been evaluated in several populations that revealed a positive association with reduced response (Higashi *et al.*, 2002).

3.1.4 Antiplatelet therapy

Antiplatelet therapy is effectively used in the prevention of thrombosis, myocardial infarction, and stroke (Trialists’Collaboration, 2002). Aspirin and clopidogrel are the first choice antiplatelet agents for stroke treatment. These drugs act via inhibiting platelet activation and thus prevent its aggregation (Tourmousoglou and Rokkas, 2008).

3.1.4.1 Clopidogrel

Clopidogrel is an oral antiplatelet drug that reduces the recurrence of atherothrombotic events in patients with stroke and peripheral vascular disease. It irreversibly inhibits the P2Y12 receptor for ADP, which has a major role in platelet activation and aggregation (James *et al.*, 2009). It is a prodrug which is metabolized by hepatic cytochrome isozymes (CYP450/CYP) to an active thiol metabolite. The active metabolite of clopidogrel binds to P2Y12 receptor and irreversibly blocks the binding site of ADP and receptor activation (Markus, 2012).

3.1.4.2 Aspirin

Aspirin (C9H8O4), also known as acetylsalicylic acid, belongs to nonsteroidal anti-inflammatory drugs (NSAIDs) groups. It is the one of the most widely used antiplatelet drug globally. It is weak acid and an anti-inflammatory, antipyretic and analgesic properties (James *et al.*, 2009). It has pleiotropic benefit for which it is used to treat pain, fever and inflammatory associated complications. Aspirin is considered as a golden standard for platelet inactivation and is used as a potent blood thinning agent (Trialists' Collaboration, 2002).

3.2 Aspirin: Mechanism of action

Aspirin inhibits platelet aggregation via thromboxane pathway that has a crucial role in platelet aggregation mechanism. Aspirin is the only NSAID having the property of acetylation, and the major acetylation targets of this drug are the cyclooxygenase (COX) enzymes of platelets cells that are present in two isoforms namely, COX-1 and COX-2. It irreversibly acetylates serine residue (530) of the COX-1 enzyme. As a result, the production of prostaglandin G2 and H2 from arachidonic acid is inhibited. Prostaglandin is the substrate which gets converted by thromboxane A2 (TXA2), activating the aggregation of the platelets. TXA2 is a vasoconstrictor and activates platelets to produce more TXA2 and therefore increased platelet aggregation (Gasparyan *et al.*, 2008) (as shown in Fig. 1). Aspirin has been reported to reduce the stroke by 13%-25%. It is usually prescribed with the dose given as 75-325 mg/day.

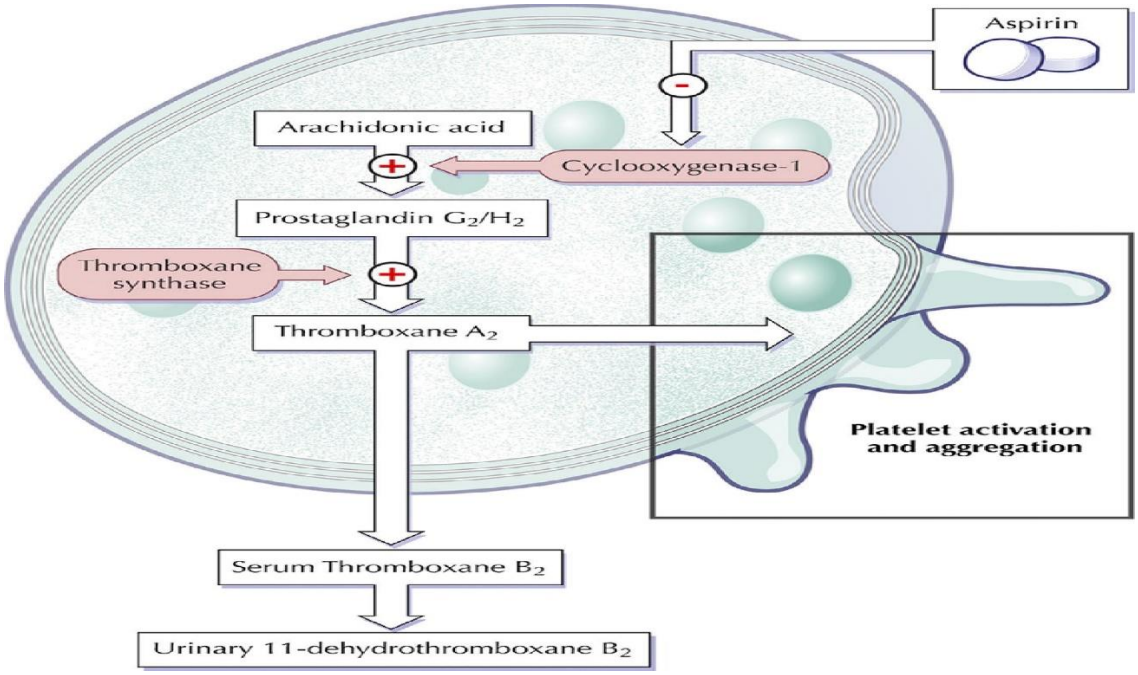

Figure 1- Mechanism of action of aspirin. (Gasparyan *et al.*, 2008)

3.3 Aspirin resistance

Aspirin therapy as an antiplatelet agent has been reported to be associated with more than one type of side effect which gets reflected mainly in the form of inter-individual variation in response. About 5-65% of patient administrating aspirin suffer recurrent thromboembolic vascular events, giving rise to the term 'aspirin resistance' (Hankey and Eikelboom, 2006). In South Indian population from Andhra Pradesh 48.2% stroke patients have been found to be affected with aspirin resistance (Sharma *et al.*, 2013). Aspirin resistance is a poorly defined term. Clinically, it can be termed as the inability to reduce the production of TXA2 which leads to platelet aggregation and activation. More appropriately, it can be defined as the inability of aspirin to i) protect individuals from thrombotic complication, ii) cause a prolongation of bleeding time, iii) inhibit thromboxane (TX) synthesis and iv) produce an anticipated effect on one or more in-vitro tests of platelets function (Hankey and Eikelboom, 2006). Moreover, some researchers have classified aspirin resistance into three subtypes:

Type 1: aspirin resistance: it is the failure of aspirin to inhibit TXA_2

Type 2: aspirin resistance: it involves the failure of aspirin to suppress TXA_2 production in-vitro and in-vivo (Pharmacodynamics)

Type 3: aspirin resistance: it involves the platelet activation despite adequate thromboxane suppression (pseudoresistance) (FitzGerald and Pirmohamed, 2011).

3.3.1 Possible causes of aspirin resistance

Several factors contribute to aspirin resistance including non-compliance to aspirin therapy, inadequate aspirin dose, increased platelet turnover, and drug-drug interactions, e.g. Ibuprofen competes with aspirin for COX-1 receptor site (Catella-Lawson *et al.*, 2001). Other factors like poor glucose control and body weight have also been reported to contribute to aspirin resistance (Cohen *et al.*, 2008). Mechanism intrinsic to the platelet that lead to aspirin resistance include the following: synthesis of TXA2 by aspirin-resistant COX-2, polymorphisms in the COX-1 gene that alter the active site conferring resistance to acetylation and genetic polymorphism affecting platelet integrin's GpIIIa and GpIIb that alter expression receptor activation (Goodman *et al.*, 2008).

3.4 Pharmacogenomics of aspirin resistance

Several investigations have confirmed that platelet response to aspirin has a genetic basis. Genetic variants that contribute to the heterogeneity in drug response might influence pharmacokinetics and pharmacodynamics and might also be associated with underlying diseases mechanisms. Regarding enzymes involved in aspirin metabolism besides nonspecific esterase; three major enzymes, namely UGT1A6, ACSM2B and CYP2C9 play a major role shown in Fig 2. Polymorphisms in the genes coding for these enzymes are likely to play a relevant role in aspirin intolerance. The effect of aspirin is due to the effect of both acetyl and the salicylate portions of the intact molecule as well as by the active salicylate metabolite. Salicylate directly and irreversibly inhibits the

activity of both types to decrease the formation of precursors of prostaglandin and thromboxane from arachidonic acid. Thus UGT1A6, AXSM2B, CYP2C9, COX-1 and COX-2 can be considered as major pharmacogenomics targets in aspirin intolerance (Agundez *et al.*, 2009).

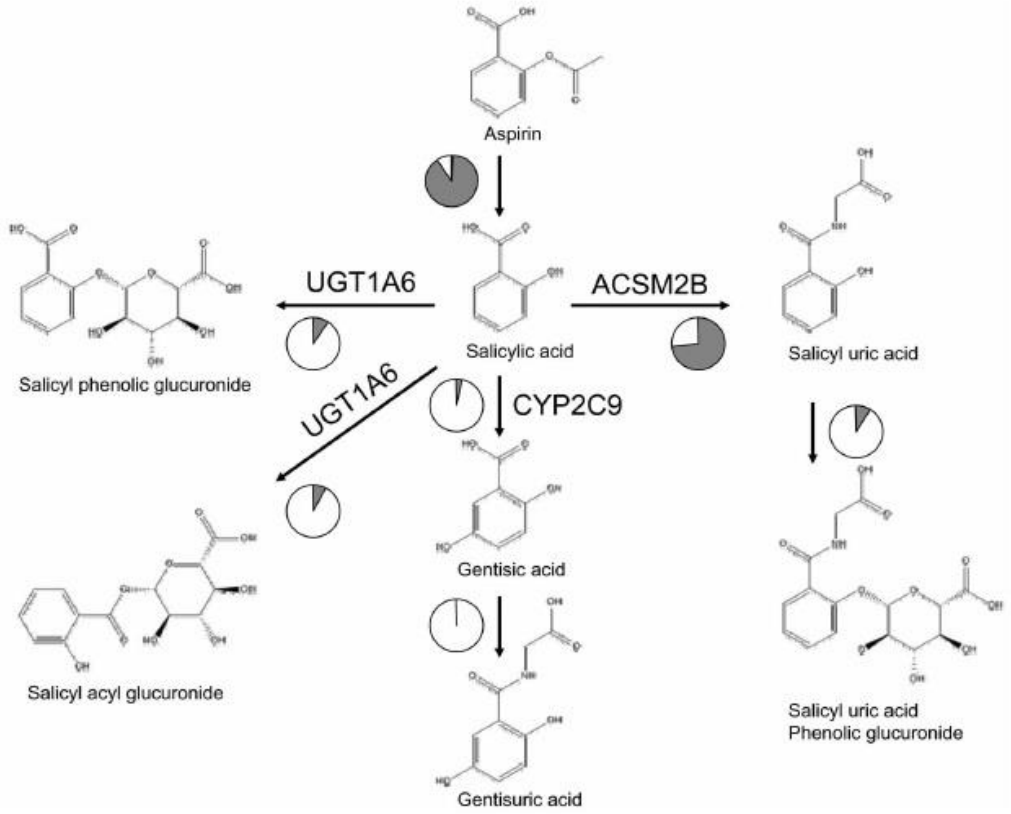

Figure 2- Scheme of aspirin metabolic pathway. The major enzymes and the average percentage of the drug (Agundez *et al.*, 2009).

3.4.1 Pharmacodynamics of aspirin

Pharmacodynamics includes pharmacological and physiological effect of the drug, and its effect on the body. It mainly focuses on action mechanism of drugs and the interaction of drugs with the biological receptors (Vasudeva *et al.*, 2017). The important receptors in aspirin pharmacodynamics include COX-1, COX-2 and glycoprotein

receptors. Genes encoding these receptors are known to play a key role in aspirin resistance. The COX-1 enzyme is encoded by cyclooxygenase -1 gene which is located on chromosome 9. It has 11 exons, approximately of the size of 22 KBs (Smith *et al.*, 2005). Gene variants of COX-1 affects the enzyme activity and its interaction with the aspirin (Maree *et al.*, 2005). Several studies have been carried out to evaluate the association of COX-1 variant with aspirin response. Till date 20 variants of COX-1 have been reported but the common SNPs associated with aspirin resistance include A-842G, C714A, G128A, C22T, and T-1676C (Goodman *et al.*, 2008). A study including 38 healthy individuals was carried out to identify the association and effect of variant of COX-1 with aspirin resistance in Caucasian population. It was found that SNPs A-842G and C50T are in complete linkage disequilibrium. They also made another finding that heterozygous (-842G/50T) haplotype was significantly associated with increased sensitivity to aspirin. Expression studies revealed that G allele (842G) provides an additional binding site to a transcriptional factor i.e. activation protein (AP2) in the promoter region, which is reported to have a repressor role thwarting the expression of COX-1 in platelets (Halushka *et al.*, 2003).

The other gene with important role in pharmacodynamics of aspirin is COX-2. The COX-2 gene is located on chromosome 1 and bears 10 exons (Sharma *et al.*, 2013). Various researchers have found its association with aspirin resistance in different ethnic groups. It has been found that COX-2 is inhibited by aspirin 170 fold less when compared with COX-1. Usually 90% inhibition of TXA2 is considered optimal for the potential antithrombotic activity of aspirin, but the increased expression of COX-2 (even in lower amounts) can influence the clinical efficacy of aspirin. Two common polymorphisms have been reported to be significantly associated with response to aspirin. It includes rs20417 (G-765C) in promoter region and the other SNPs rs5275 (T8473C) in 3'UTR. The variant -765G>C has been significantly associated with aspirin resistance (Cipollone *et al.*, 2004). A study by Sharma *et al*. included 450 ischemic stroke patients and found a significant association of C allele of COX-2 -765/C polymorphism with aspirin resistance in ischemic stroke patients. Platelet adhesion and aggregation is under the control of platelet membrane receptors known as glycoprotein receptors. The clot formation is initiated by the adhesion of platelet to the extracellular

matrix. The GPIIb/IIIa is an important glycoprotein receptor that influence platelet adhesion and aggregation (Nieuwenhuis *et al.*, 1985). It consists of two subunits i.e. alpha and beta. The alpha (92 kDa) and beta (92 KDs) subunits correspond to heavy and light chain respectively (Poncz *et al.*, 1987) (Kunicki *et al.*, 1981). Both the alpha and beta subunits are non-covalently attached to each other. Platelet activation results in conformational changes in GPIIb/IIIa receptors and consequently the shape of platelet. The GPIIb/IIIa receptors binds to fibrinogen molecules and forms bridge between adjacent platelets. This results in platelet aggregation and clot formation (Shah and Goyal, 2004). GPIIIa variant (PIA1/A2) had shown to carry risk alleles implicating elevated platelet function among the patients treated with aspirin (Vasudeva *et al.*, 2017). Caucasians have demonstrated a significant association between Leu^{33}/Pro polymorphism of GPIIIa and impaired antithrombotic effect of aspirin (Szczeklik *et al.*, 2002).

3.4.2 Genes involved in absorption, distribution, metabolism and excretion (ADME) of aspirin

Several genes are involved in ADME of aspirin. They can be classified into four classes based on their roles in the ADME process. (Van der Logt *et al.*, 2004)

i. Phase I and phase II metabolizing enzymes, responsible for the modification of functional groups and conjugation with endogenous moieties, respectively;
ii. Transporter proteins, responsible for the uptake and excretion of drugs in and out of cells (some of them include ABC family and SLCO1B1)
iii. Serum binding proteins
iv. Modifiers and transcription factors that can either alter the expression of other ADME genes or affect the biochemistry of ADME enzymes.

Aspirin is absorbed by passive diffusion as the dissociated salicylic acid from the stomach where pH is low, hydrolysis is minimal and from the upper small intestine. After absorption, aspirin (2-acetyloxybenzoic acid) is hydrolyzed to salicylic acid (2-

hydroxybenzoic acid) in the liver and to a lesser extent in the stomach before entering the systemic circulation by a nonspecific human hepatic carboxylesterase-2(HCE2) (Needs and Brooks, 1985). The major metabolites of salicylic acid are the glycine conjugate and salicyluric acid (2-[(2hydroxybenzoil) amino] acetic acid). In liver, conjugation with glycine proceeds in two steps which require ATP, coenzyme A and glycine. In first reaction salicylic acid and ATP react with coenzyme A to form salicylate CoA, AMP and pyrophosphate and in second reaction salicyluric acid and CoA production occur with the reaction of salicylate-CoA and glycine (Lares-Asseff *et al.*, 2004).

CYP2C9 variants have been associated with increased risk of gastrointestinal bleeding in patients on NSAID treatment. It is involved in the oxidation of salicylic acid which produces gentisic acid as a minor metabolite. Variants of this gene i.e. CYP2C9*2 and *3 with substitutions at R144C and I359L respectively has been reported to be associated with decreased activity of the respective enzyme (Pilotto *et al.*, 2007). Another study found that patients who were carriers of CYP2C9*3 allele and, to a lesser extent carrier of CYP2C9*2 alleles developed gastrointestinal bleeding with aspirin treatment (Blanco *et al.*, 2008). Two SNPs of CYP2C9 i.e. rs1799853 and rs1057910 have been documented to be involved in the gastrointestinal bleeding with the low dose of aspirin (Shiotani *et al.*, 2009).

Salicylic acid is also metabolized by glucuronidation to form acyl glucuronide and phenolic glucuronide by enzyme UGT1A6 (Uridine diphosphate glucuronosyltransferase isozyme 1A). UGT1A6*1 and UGTA16*2 are the commonest allozymes, which together accounts for 90% of all allozymes of UGT1A6 (Carlini *et al.*, 2005). UGT1A6*2 has variation in amino acids at position 7, 105, 181 and 184 while UGT1A6*2D and UGT1A6*3 exhibit variation at 93 and 7th position (Krishnaswamy *et al.*, 2005) in UGT protein. Other variants of UGT1A6 include UGT1A6*5, UGT1A6*6 UGT1A6*7, UGT1A6*8 and UGT1A6*9 (Ciotti *et al.*, 1997). Two SNPs of UGT1A6*2 namely rs2070959 and 1105879 have been reported to be involved in gastrointestinal bleeding with the low dose of aspirin (Shiotani *et al.*, 2009)

3.5 UGT1A6: role in aspirin resistance

UGT1A6 (UDP glucuronosyltransferase family -1 member A6) plays an immense role in the phase- II metabolism of aspirin (Bock and Köhle, 2005). This is located on the long arm of chromosome 2 [2q37.1]. It bears 6 exons and usually expressed in the liver, bile duct, stomach, colon, kidney and brain (Tukey and Strassburg, 2000). The catalytic reaction that employ UDP-glucuronic acid as a co-substrate for the formation of hydrophilic glucuronide from substrates like steroids, bile acid, bilirubin, hormones, dietary constituents, and thousands of xenobiotics including drugs, environmental toxicants, and carcinogens had evolved with highly specialized functions in higher organisms (Tukey and Strassburg, 2000).

3.5.1 Structure of UGT1A6

This gene is also known by many other names like GNT1, UGT1, HLUGP1, UGT1A6S, HLUGP, UDPGT, UGT1F, and UDPGT1-6 (NCBI, 2018). UGT1A6 locus in humans is approx. of the size of 160 kb and is part of UGT1A complex locus. It is a founding member of the human UGT1 family. The protein of human UGT1A6 is 531 amino acid in length (Radominska *et al.*, 1999). UGT1A6 usually functions as dimeric or tetrameric, and the dimerization domain has been mapped to the amino terminal of the UGT protein (Ghosh *et al.*, 2001). It belongs to the type I endoplasmic reticulum bound protein and the majority of this protein including its active site resides within the lumen. It has a single transmembrane domain with its short C- terminal domain projecting into the cytosol (Ouzzine *et al.*, 1999). UGT1A6 protein has three domains including an N-terminal signal sequence, a putative internal signal (141-240 amino acid long) and a 4 dilysine motif at the carboxyl-terminus. The UGT1A6 Ser7Ala polymorphism is located at N-terminal signal sequence and the variants Thr181Ala and Arg184Ser are in internal ER-localization signal region (Ouzzine *et al.*, 1999). These are the three common SNPs that induce amino acid change within the protein. Studies have demonstrated that Ala at amino acid 7 in UGT1A6 is conserved some among species but not within human UGT1A isoforms. The amino acid at 181 and 184 in UGT1A6 is not conserved between any of the species except glutamine at 184. This analysis suggests that amino acid sequence at position 184 has significant role in human

UGT1A specific phenotype (Radominska-Pandya *et al.*, 1999). The UGT1A gene and UGT1A6 protein have been depicted Fig 3.

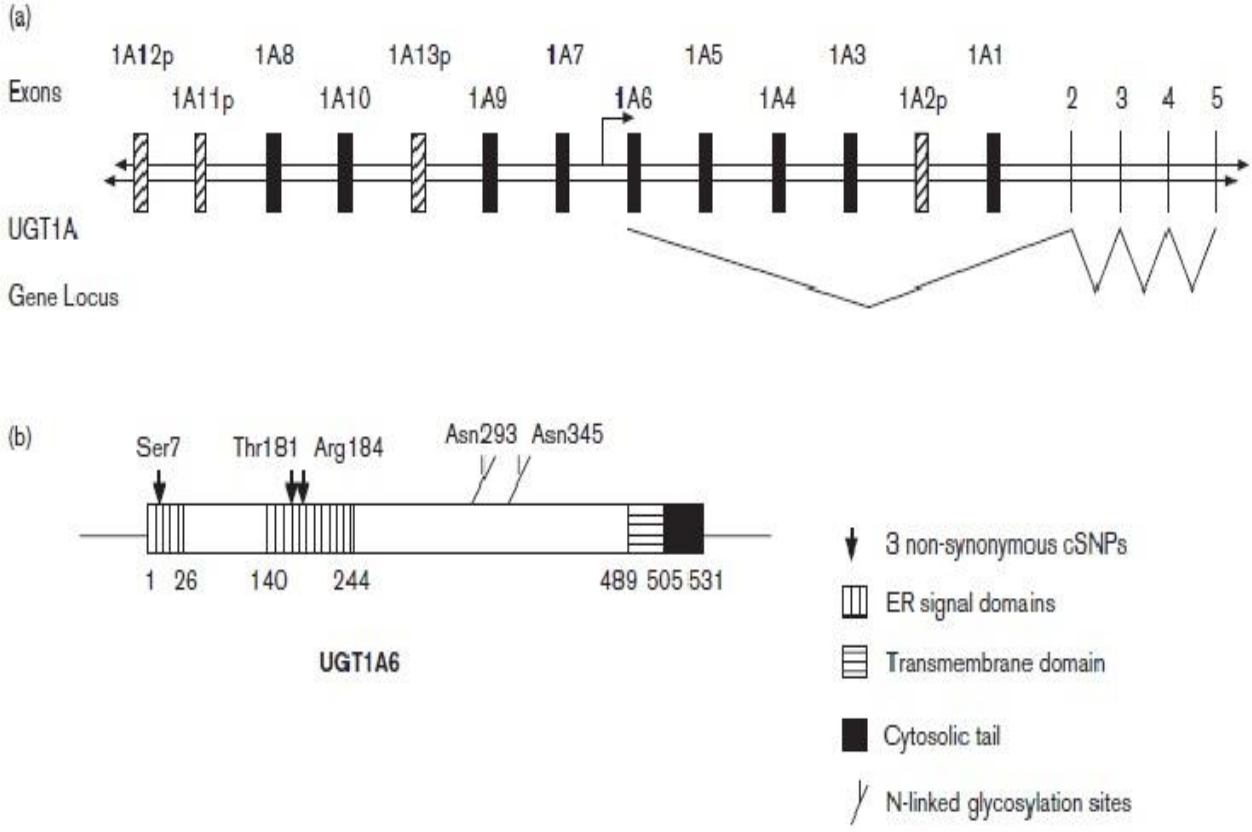

Figure 3- UGT1A6 gene locus (a) 200 KB UGT1A gene locus (b) the UGT1A6 protein structure, Source-(Nagar *et al.*, 2004).

3.5.2 Function of UGT1A6

Glucuronidation is an important process for the detoxification and elimination of a large number of endogenous (e.g. bile acids, bilirubin and steroids) and exogenous compounds (e.g. drugs, environment chemical and dietary constituents) (Tukey and

Strassburg, 2000). The biotransformation reaction is catalyzed by a multigenic family of UGTs. Glucuronidation is a major phase II metabolizing reaction that leads to the formation of hydrophilic substances with the addition of glucuronic acid on the aspirin (Tukey and Strassburg, 2000). In addition to aspirin, other common drugs which are glucuronidated by UGT1A6 like β-adrenoreceptors, antidepressants neuroleptics and other NSAIDs. UGT1A6 is also involved in other two biological functions i.e. 1) it protects the body by biochemical processes from tobacco, carcinogenic environmental pollutants and toxic dietary substances. 2) It is also involved in the maintaining the homeostasis of variety of endogenous molecules like steroids and thyroid hormones, bilirubin and biliary acids (Nowell *et al.*, 1999) (Dutton, 1980). The enzymatic property of UGT1A6 was identified by 5-HT Glucuronidation activities in yeast cell microsomes expressing human UGT1A6. No activity was detected in the negative control yeast cell microsomes with any substrate. UGT1A6 kinetic analysis was performed for 5-HT Glucuronidation. The Km values of human liver microsomes for 5-HT Glucuronidation was found to be 8.6. (Hanioka *et al.*, 2006). UGT's have more profound property in the detoxification and elimination mechanisms. Some others enzymes with conjugation property include N-acetyltransferase (NAT), sulfotransferase (ST) and glutathione S-transferase (GST).

3.5.3 UGT1A6 polymorphism with aspirin resistance

With the expanding knowledge in the field of pharmacogenomics and bioinformatics, it is evident that genetics has an indispensable role in inter-individual variation towards the drug treatment (Evans and Relling, 1999). In the case of UGTs, genetic alteration may get reflected in the form of altered glucuronidation process (Ciotti *et al.*, 1997). It may result in the diminished potential of enzymes in the glucuronidation and may lead to the adverse drug reaction. Studies on UGT1A6 gene have identified at least 4 alleles characterized by two functionally important SNPs in exon 1 of the gene. UGT1A6*1 represents the unmodified wild-type allele. UGT1A6*2 represents the missense polymorphism at codon 181((Thr181Ala; 541A/G), 184 (Arg184Ser; 552A/C) and 7 (Ser7Ala; 19T/G).

Several studies have been carried out in various populations with a demonstration of glucuronidation property of UGT1A6 and found different rate of effect of drugs like aspirin. Ciotti *et al*. (1997) demonstrated that UGT1A6*2 has a 2-fold lower salicylic acid glucuronidation compared with UGT1A6*1. Chain *et al*. (2005) performed a study on 1062 women on aspirin treatment. 616 women were carriers of mutant allele of UGT1A6. It was found that majority of these patients developed colorectal adenoma. In contrast, carriers of wild type UGT1A6 was not associated with colorectal adenoma. McGreevy *et al*. (2005) postulated that individuals inheriting the variant CYP450 and UGT1A6 have reduced ability to metabolize and eliminate the NSAIDs like aspirin and ibuprofen, and may, therefore, experience an enhanced protective effect, compared to individuals inheriting the most common functional alleles in Colorectal Cancer. Munshi *et al*. (2013) have clearly demonstrated association of the three most common nonsynonymous polymorphisms of UGT1A6*2 with therapeutic response of deferiprone in β-thalassemia major patient. They found that while Thr181Ala influences the inter-individual variability of β-thalassemia major patients in response to deferiprone, no such significant association was observed in the other two polymorphisms, i.e. Arg184Ser and Ser7Ala. The gene variant of UGT1A6*2 implicated in aspirin resistance and ADRs in different populations are shown in Table 1.

Table 1: Studies showing association of UGT variants with drug resistance

Subjects	Ethnicity	Method	Association of UGT1A6 with Toxicity	References
Gilbert's syndrome	Caucasian Patients	PCR, HPLC, RFLP, Spearman rank correlation or chi-square test	Reduced activity of B-UGT causes abnormalities in glucuronidation of several drugs including aspirin due to combined effect of UGT1A1*28 and UGT1A6*2 genotypes.	(Peters *et al.*, 2003)
Colorectal Adenoma	Caucasian	Prospective, Nested Case-Control Study	Study of 1062 Women, out of them 616 women having functional variable genotype of UGT1A6. Regular use of aspirin was associated with decreased risk of adenoma among those women. In contrast, regular use of aspirin among the wild type UGT1A6 did not show significant association with adenoma.	(Chan *et al.*, 2011)
Colorectal Cancer	Caucasian	Multi Centre Case-Control Study	This study proposed that individuals inheriting certain variants of CYP450 and UGT1A6 have reduced ability to metabolize and eliminate the NSAIDs. As a result, they experience an	(McGreavey *et al.*, 2005)

			enhanced protective effect, compared to individual inheriting the most common functional alleles.	
Colorectal Adenoma	Caucasian	Follow-up colonoscopy, Genotyping PCR reactions, X^2 and t-tests, Poisson's regression, STATA	UGT1A6 variants influence colorectal carcinogenesis independent of aspirin intake and suggest that they may have clinical value in secondary prevention programs for patients diagnosed with colorectal adenoma.	(Hubner *et al.*, 2006)
Colorectal Adenoma		PCR, HPLC –UV, Win Nolin Software, Shapiro-Wilk test.	This study suggest that there were no significant differences in salivary SA pharmacokinetics parameters between the two homozygous genotype groups. The urinary metabolite data, however, suggest that conjugation of SA is more rapid in the homozygous variant UGT1A6*2/*2 than wild-type UGT1A6*1/*1.	(Chan *et al.*, 2011)

Peptic Ulcer	Japanese	QIAamp DNA blood mini kit, PCR-RFLP, ELISA kit	This study was performed on 232 patients with cardiovascular disease. They detected no significant association between UGT1A6 and CYP2C9 variants for causing peptic ulcer on aspirin treatment.	(Shiotani *et al.*, 2010)
Healthy individual	Caucasian	HPLC, Mann Whitney U tests	The study was made on female receiving acetyl-salicylic acid (ASA). UGT1A6*2 was found to be associated with lower plasma levels of SA when compared to the UGT1A6*1 homozygote genotype. The study, therefore, indicates a faster pharmacokinetics for those with UGT1A6*2 variant.	(Van Oijen *et al.*, 2009)
Cardiovascular diseases	Malaysian's population with Malay's, Indian and Chinese people.	AS-PCR, Statistical Package for Social Sciences(S PSS), Haploview	Patients with UGT1A6 variants alleles were associated with slow metabolizing capacity. On the other hand, CYP2C9*3 variant allele demonstrated to influence gastritis events when treated with aspirin.	(Jalil *et al.*, 2015)

Post-traumatic Seizures	Chinese population	Prospective cohort case-control study, PCR-RFLP, EEG	Mutant variants of UGT1A6 and CYP2C9 affected the metabolism of the anti-epileptic Valproic Acid (VPA). Male patients, over age 65 and with certain UGT1A6 variants (19T>G/ 541A>G/552A>C) show higher risk of early post-traumatic seizures.	(Sun *et al.*, 2017)

Chapter-4

Materials and methods

4.1 Patient recruitment and data collection

a) Patient eligibility and recruitment

The study was approved by the ethical committee of Central University of Punjab, Bathinda. Stroke patients were recruited from Guru Gobind Singh Medical College and Hospital, Faridkot. All patients were examined by the qualified stroke neurologist. Differentiation between ischemic and hemorrhagic stroke as well as sub-types of ischemic stroke were identified on the basis of the CT scan and MRI.

b) Data collection

Information about sex, age, social habits, family history, smoking, alcoholism and drug treatment were collected from ischemic stroke patients using a structured questionnaire. All the blood samples were collected only after obtaining the written informed consent.

c) Sample collection

Blood samples were collected from thirty ischemic stroke patients who were admitted at the Guru Gobind Singh Medical College and Hospital, Faridkot. 5ml blood sample was collected and transferred equally in two different vials i.e. one with EDTA coated and another with clot activator. EDTA coated vials were stored at -20ºC and used later for DNA isolation, whereas clot activator vials were used for serum isolation and the isolated serum was stored at -80ºC.

4.2 Isolation of genomic DNA

The genomic DNA was isolated by phenol-chloroform-isoamyl alcohol (PCI) method.

Reagents required

- Lysis Buffer I (1X)
- Lysis Buffer II (1X)
- Tris 0.5M EDTA pH 8.0
- 1M Tris-HCl
- Proteinase K
- 10% SDS
- Phenol

- Chloroform
- Isoamyl alcohol
- 3M Sodium acetate
- 100% Ethanol

Preparation of chemicals

1. Lysis Buffer I (10X)

To prepare 100ml of lysis buffer I, 8.29g of ammonium chloride, 1g of potassium bicarbonate and 0.2ml of 0.5M EDTA was dissolved in distilled water to make its volume up to 100ml.

2. Lysis buffer II (1X)

To prepare 100ml of Lysis buffer II, 0.500ml of Tris-HCl (pH 8), 1.168 g of sodium chloride and 0.200 ml of 0.5M of EDTA were dissolved in distilled water to make volume up to 100ml.

3. 0.5M EDTA (pH 8)

For 50ml of 0.5M EDTA, 9.305g of EDTA in form of disodium salt was added in distilled water and pH was adjusted by adding NaOH pellet to make its pH 8.

4. 1M Tris-HCl

6.05g of Tris base was dissolved in 40ml water, and pH was adjusted by adding concentrated HCl.

5. Phenol:Chloroform:Isoamyl alcohol (PCI)

Phenol, chloroform and isoamyl alcohol were mixed in the ratio of 25:24:1 and stored at 4ºC.

6. Chloroform: Isoamyl alcohol (CA)

Chloroform and isoamyl alcohol were mixed in the ratio of 24:1 and stored at 4ºC.

7. Proteinase K

100mg of Proteinase K was dissolved in 10ml of sterile double-distilled water and stored at -20℃

8. 10% SDS

10g of SDS was dissolved in 100ml of distilled water and stored at room temperature.

9. 70% ethanol

70ml of ethanol was added in 100ml of distilled water to make 70% ethanol.

10. 3M sodium acetate

Principle

Leukocytes are separated from red blood cells (RBCs) as only white blood cells (WBCs) contain nucleus, not the RBCs. In order to achieve this, differential lysis of RBCs is done using lysis buffers. This is followed by centrifugation of the mixture to pellet down the intact leukocytes and the lysed RBC separate out as supernatant. Enzyme proteinase K is used to digest protein and SDS is used to break sulfide bonds between protein chains. Phenol reduce the protein, chloroform facilities the separation of aqueous phase and organic phase whereas isoamyl alcohol reduces foaming during extraction. Finally, ethanol is used to precipitate the DNA and remove the remaining salts.

Procedure

Day-1 protocol

1. When blood was collected from patients it was stored at 4ºC. So blood was thawed at room temperature.

2. 2ml blood sample from EDTA tube was transferred to a pre-labelled polypropylene tube and thrice the quantity of lysis buffer I (i.e. 6ml) was added to the polypropylene tube. It was incubated for 15 minutes in ice and during the incubation period, tubes were shaken vigorously for 2-3 times.

3. At the end of 15 minutes of incubation, the mixture was centrifuged at 3000rpm for 15 minutes at 4ºC. Supernatant was removed carefully and the pellet was retained in the tube for further steps.

4. Steps 2 and 3 were repeated until pellet turned white.

5. 2ml of lysis buffer II was added to the white pellet in the polypropylene tube and vortex to get a homogenized solution.

6. 112µl of 10% SDS and 50µl of Proteinase K were added in tubes.

7. The sample was incubated in water bath at 37ºC for overnight.

Day-2 protocol

1. The sample was recovered from the water bath and equal volume of PCA (1000µl: 960µl: 40µl) was added in tubes.

2. The mixture was gently spun in rotor spin for 15 minutes and centrifuged at 2500rpm and at 27ºC for 15 minutes.

3. After 15 minutes of centrifugation, the aqueous layer was transferred carefully in new tubes with the help of wide broad tips.

4. The sample was washed by adding 2ml of CA and kept on rotor spin for 15 minutes and then the sample was centrifuged at 2500 rpm at 27ºC for 15 minutes.

5. 66 µl of sodium acetate was added to the samples.

6. 4ml of 100% chilled ethanol was also added and tubes were incubated at -20ºC for overnight.

Day-3 protocol

1. After incubation, precipitated visible DNA was transferred into 1.5ml micro-centrifuge tubes followed by washing in 70% ethanol and the pellet was allowed to dry in open.

2. 20µl TE buffer was added to it and kept at 37ºC for overnight or until pellet get dissolved.

3. DNA was labelled and stored in -20ºC.

4.3 Quantification of DNA:

The quantitative analysis of DNA sample was carried out by spectrophotometry and NanoDrop.

a) Spectrophotometry

Nucleic acids (DNA and RNA) absorb light at a wavelength of 260 nm. An optical density (OD) of 1, at 260nm, correlates to a DNA concentration of 50 ng/µl for double-stranded DNA, 40 ng/µl for single stranded DNA/RNA 20 ng/µl concentration for oligonucleotides. The ratio between absorbance at 260 nm and absorbance at 280nm provides an estimation of purity of nucleic acid. Pure DNA and RNA have a ratio of approximately 1.8 and 2.0 respectively. Ratio <1.8 indicates that the DNA is contaminated with protein and if the ratio is >2.0, indicates DNA contamination with RNA.

Requirements

- DNA samples
- TE buffer
- Spectrophotometer
- Sterile distilled water
- Cuvette

Procedure

1. 1ml TE buffer was taken in a cuvette and spectrophotometer was calibrated at 260 nm and 280nm.
2. 10µl of DNA sample was added to 2490 µl TE buffer/distilled water and mixed well and TE buffer was used as blank in other cuvette.
3. The absorbance of sample was measured at 260 and 280nm in spectrophotometer.
4. The amount of DNA was calculated by using the formula:

DNA concentration (µg/ml) = (OD260*100(dilution factor)*50 µg/ml)/1000

b) NanoDrop method

A 1µl sample is pipetted on the fiber optic cable. A pulsed xenon flash lamp provides the light source and a spectrophotometer is used to analyze the light after passing through the sample. The instrument is connected to computer or display screen with installed software in it.

Procedure

1. NanoDrop's lower measurement pedestal was cleaned with distilled water and wiped with the help of clean lint-free wipes.
2. An initial measurement was made as blank, for blank 1µl of the buffer or distilled water may be used. The measurement obtained was saved in a file.
3. Sampling arm was opened and wiped with lint-free wipes.
4.1µl Sample DNA was pipette out and loaded onto the lower measurement pedestal. Sampling arm was closed and spectral measurement was initiated using software installed specifically for NanoDrop.
5. The subsequent measurement was made for samples.

4.4 Quality estimation of DNA

The quality assessment of genomic DNA was carried out using agarose gel electrophoresis.

Agarose gel electrophoresis

Reagents

- 1X TBE Buffer
- Agarose
- For DNA estimation – 0.8%
- For amplified PCR samples- 2%
- For observing RFLP- 2%
- Ethidium bromide – 2µl

Principle

Electrophoresis is used to resolve the fragments of DNA. DNA is negatively charged due to the presence of phosphate group in its backbone. Under the influence of electric field, DNA will migrate through the gel at a speed corresponding to their size, towards the positively charged electrode. Larger molecules will move slower compared to smaller molecules. Ethidium bromide (EtBr), a fluorescent dye, is used to stain the gel

which intercalates between stacked bases. It is visualized in the presence of UV rays under UV Illuminator.

Requirements

- Weighing balance
- Conical flask
- Gel box with comb
- Gel documentation unit
- Microwave

Procedure

Step 1: Mixing the gel

1. In order to make a 50ml gel, 0.4g of agarose was weighed onto a piece of butter paper.
2. 0.4g agarose and 50ml of 1X TBE buffer was taken in a 200ml conical flask and mixed thoroughly.
3. A White mixture of was obtained.

Step 2: Melting Gel

1. The above solution was boiled in a microwave to dissolve the agarose.
2. It was boiled until the solution becomes clear like water.
3. 2µl of EtBr was added to it and mixed properly.

Step 3: Pouring the gel

1. The clear solution was poured into casting tray without creating bubbles.
2. Wait until it gets solidified.
3. The comb was removed carefully by pulling them after the gel gets partially solidified.
4. Once solidified, electrophoresis set was placed into electrophoresis tank.
5. 1X TBE buffer was poured into tank enough to cover the entire gel.

Step 4: Loading the gel

1. A piece of parafilm was placed on the bench top.
2. 1-2µl of 6X loading dye was poured on the parafilm for each sample.
3. 5µl of the product was mixed with loading dye on parafilm.
4. Samples were loaded into the particular wells.
5. Cathode and anode cords were connected to the power pack and voltage was adjusted at 80 volts and run till dye reached ¾ of gel.

Step 5: Visualizing the gel

1. The power supply was turned off and the cover was removed from the tank.
2. Using hand gloves, the gel was placed carefully inside the documentation system.
3. The gel was observed under UV light.

Analysis

After electrophoresis, the gel was illuminated with an ultraviolet lamp. The illuminator apparatus is connected with an imaging apparatus that helps in capturing the image of the gel, after illumination with UV radiation. In the presence of DNA, the ethidium bromide intercalates and exhibit fluorescence of reddish orange colour.

4.5 Polymerase chain reaction (PCR)

The target DNA sequence (gene of interest) present within the extracted genomic DNA can be amplified using an in vitro method used called polymerase chain reaction (PCR). This amplification is achieved by a three-step cycling process: initialization step, annealing and elongation step.

The region of UGT1A6 gene bearing Ser7Ala polymorphism was amplified by PCR.

Primers:

The following primers were used:

Forward primer- **5′ GATTTGGAGAGTGAAAACTCTTT3′**

Reverse primer**- 5′ CAGGCACCACCACTACAATCTC 3′**

(Munshi *et al*., 2013)

The composition of the PCR mixture used to amplify the region bearing the UGT1A6*2 polymorphic site has been given in table 2.

Table 2: PCR master mixture for amplification of UGT1A6*2 gene region bearing the Ser7Ala polymorphic sites.

Distilled water	35 µl
Taq buffer	5 µl
dNTPs	2 µl
$MgCl_2$	5 µl
Forward Primer	0.5µl
Reverse Primer	0.5µl
Taq polymerase	1µl
Template DNA	1 µl
Total	50 µl

Table 3: The PCR conditions have been given in Table 3.

Step 1	Initial denaturation	95ºC	4 min
Step 2	Denaturation	95ºC	30s
Step 3	Annealing	64ºC	60s
Step 4	Extension	72ºC	60s
Repeat step 2-4 for 37 cycles			
Step 5	Final extension	72ºC	5 min
	Hold at 4ºC for 1 Hour		

(Source: Van Oijen *et al.*, 2005)

4.6 Restriction Fragment Length Polymorphism (RFLP)

Principle

Restriction endonucleases recognize a specific sequence and cut double-stranded sequences within a specific recognition sequence to produce fragments. Change in specific recognition sequence may alter the position of cutting site for restriction endonucleases. Fragments thus produced get separated during agarose gel electrophoresis and are visualized under UV light which enables the analysis of sequence variations of a discrete region.

RFLP was carried out using Hhal fast digest enzyme supplied by Fermentas.

Equipment and Reagents:

- Water bath
- Horizontal slab gel electrophoresis apparatus
- Gel Documentation apparatus
- 0.2 ml PCR tubes
- Hhal fast digest enzymes
- Restriction buffer (10x) Distilled water
- Ethidium Bromide
- PCR products

Procedure

The amplified 237 bp PCR product was digested with fast digested with Hhal restriction [Fermentas] fast digest enzyme.

All the reagents were combined at room temperature in the order given in Table 4.

Table 4: RFLP conditions

Reagent	PCR Product
Nuclease-free water	17 µl
10X Digest buffer	2 µl
PCR product	10 µl
Enzyme (Hhal)	1 µl
Total volume	30 µl

1. The components were mixed and spun down
2. The mixture was incubated at 37ºC in a heated block for 5 minutes.
3. The restriction digested products were analyzed on 2% Agarose gel.

Result analysis:

Size of the PCR product: 237 bp

Digestion by Hhal enzyme

Normal Homozygous (TT): 165and 72 bp

Heterozygous (TG): 237, 165, 72 bp

Homozygous (GG): 237 bp

4.7 Follow up:

The follow-up of patients was conducted by the neurologist, during the visit of patients to the hospital as well as telephonically regarding their health status and improvement. This was carried out at an interval of 3 months, 6 months and 8 months. Based on the clinical outcome, patients were classified as responders and non-responders using Modified Rankin Score (mRS) scale.

4.7.1 Modified Rankin score (mRS)

It is a scale that measures disability or dependence in activities of daily life in stroke patients. Patients are grouped under responder if they have mRS Value between 0-3. If the mRS value is between 4-6, then patients are classified as non-responders.

Table 5: mRS score and symptoms

Score	Symptoms
0	Symptoms are not observed
1	No major disability despite symptoms able to carry out all common duties and activities
2	Minor disability which is unable to carry out all previous activities but able to look after own affairs without assistance
3	Moderate disability; requiring 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 requiring constant nursing care and attention
6	Dead

(Source: Van Swieten *et al.*, 1988)

Chapter-5
Results

5.1 Demographic details

A total 30 ischemic stroke patients were included in the demographic study. The mean age of patients was 65.5 years (range 26-95). The ratio of male: female was 22:8. All patients belong to Malwa region of Punjab. In our study group, 83.33 had hypertension, 30% alcohol users, 10% smokers and 26.66 % had family history of stroke [Table 6 and Figure 4]. Twenty-two patients had intracranial large artery stroke and eight had lacunar stroke [Table 7 and Figure 5]. PCR and RFLP were carried out for all the patients to check the presence of polymorphic form of UGT1A6*2 gene. The mRS values were used to classify patients as aspirin responders or aspirin non-responders.

Table 6: Demographic Data of Stroke patients

Parameters	**Patients details**
Mean age	65.55 years
Male: Female	22:8
Hypertension	83.33%
Alcohol use	30%
Smokers	10%
Diabetes	43.33%
Family history of stroke	26.66%
BMI	
a) Under weight	N/A
b) Normal	83.34%
c) Over weight	9.99%
d) Obese	6.66%

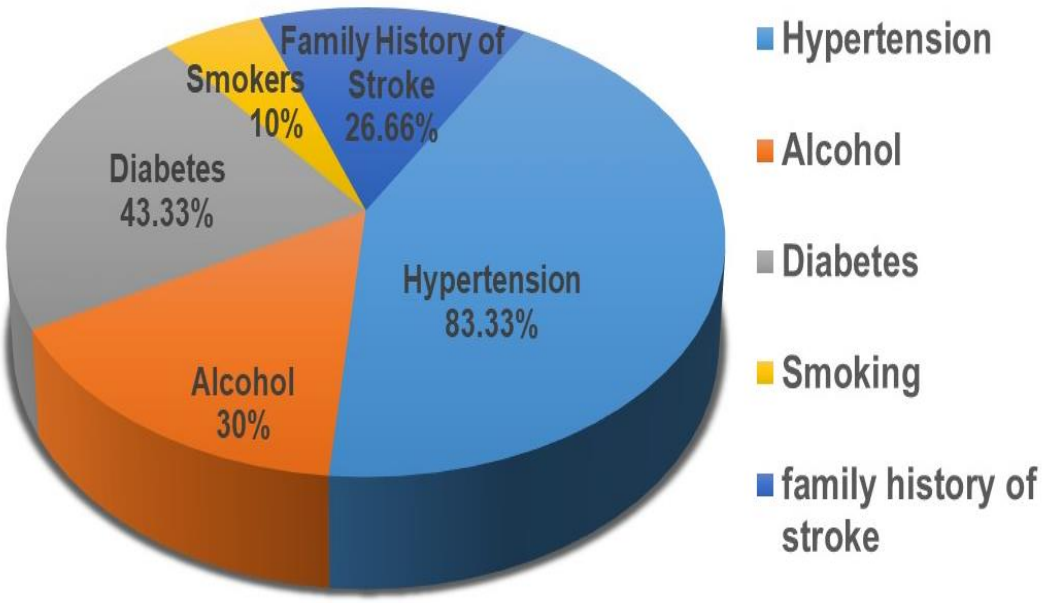

Figure 4: Risk factors associated with ischemic stroke in our study group.

Table 7: Types of stroke in given study group

S.NO.	Types of stroke	Number of Patients
1.	Large artery artherosclerosis	N/A
	a) Intra cranial arterial stroke	22 (73.33%)
	b) Extracranial arterial stroke	N/A
2.	Lacunar stroke	8 (26.67)
3.	Cardioembolism	N/A
4.	Stroke of other determined etiology	N/A
5.	Stroke of undetermined etiology	N/A

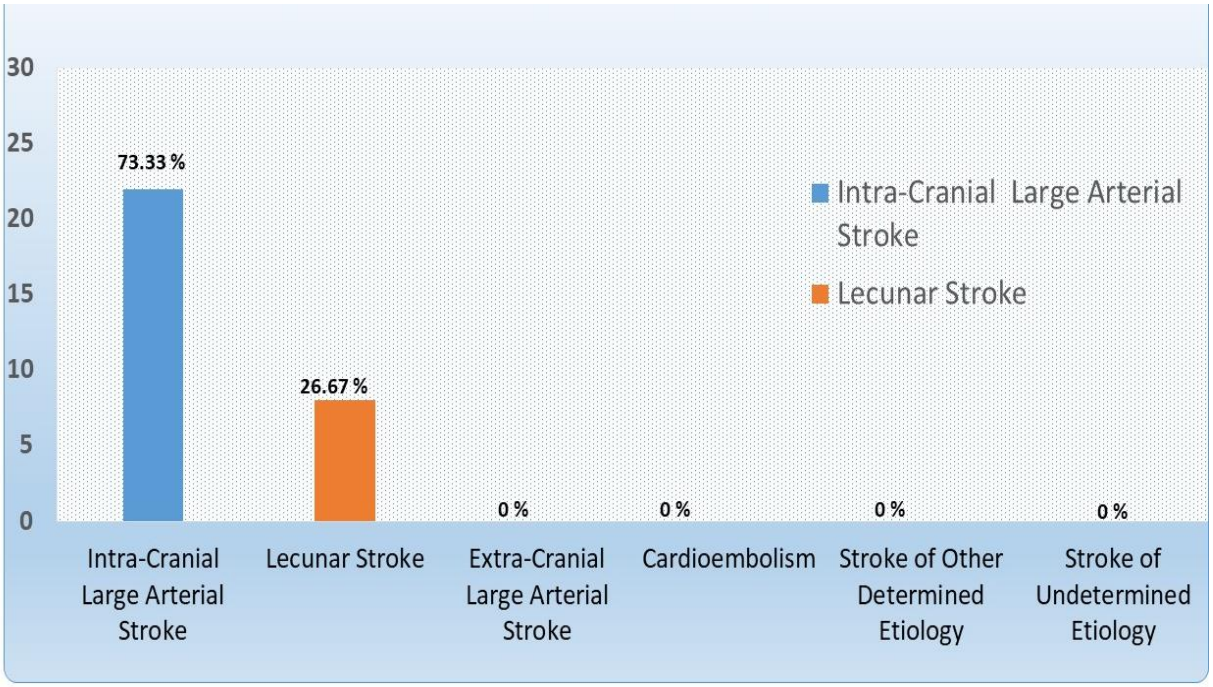

Figure 5: Subtypes of stroke in the study group.

5.2 Molecular analysis-

The DNA was isolated from the blood samples using the phenol-chloroform method. The qualitative analysis of extracted DNA was carried out on 0.8% agarose gel [Fig. 6].

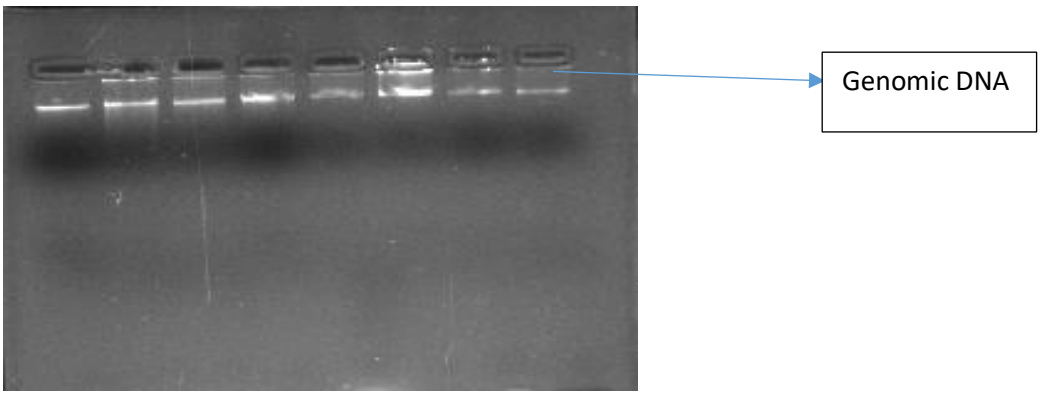

Figure 6: Agarose gel electrophoresis showing genomic DNA.

The UGT1A6*2 polymorphism was detected by the PCR-RFLP method as mentioned in [Fig.7- 8].

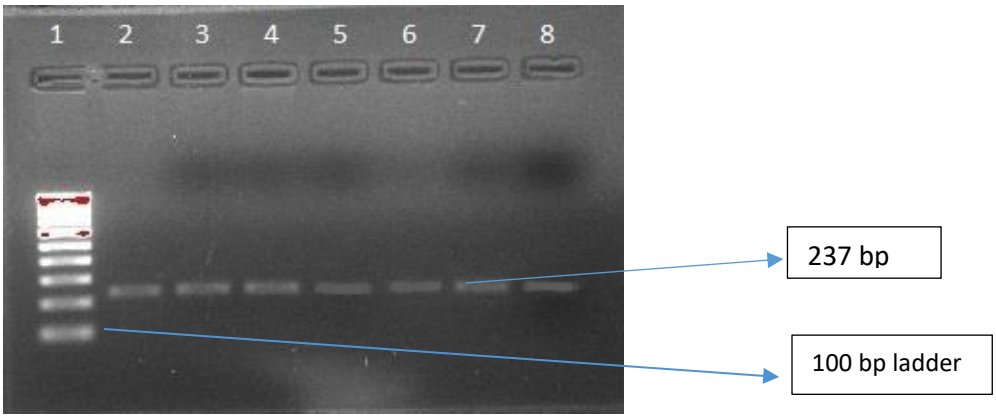

Figure 7: Amplification of region bearing Ser7Ala Polymorphic site of UGT1A6*2 gene. Lane 1 represents DNA ladder and Lanes 2-8 represents the amplified products of UGT1A6 gene bearing UGT1A6*2 Ser7Ala polymorphism.

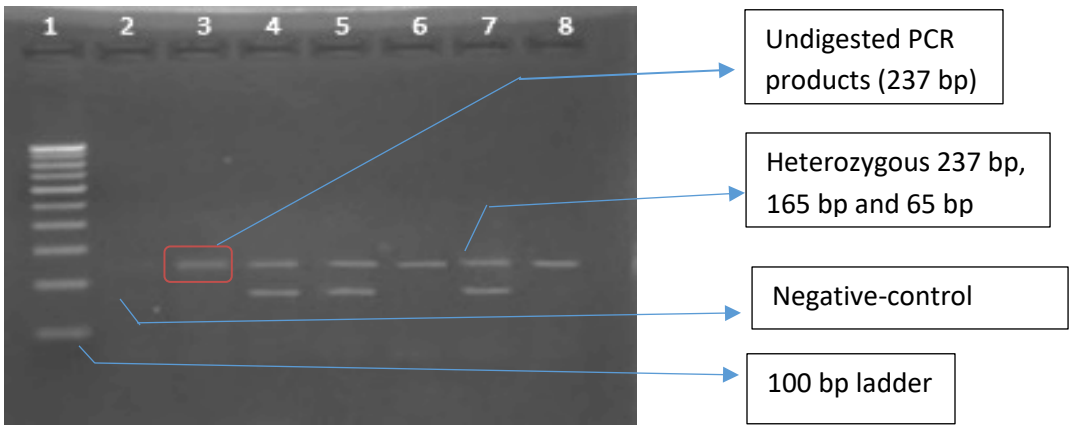

Figure 8- Digestion of PCR product using Hhal fast digest enzyme. Products of PCR-RFLP analyzed on 2% agarose gel. Lane 3 represents the normal homozygous genotype and lane 7 represents heterozygous genotypes. Lane 1 represents 100 bp ladder.

We carried out RFLP in 30 patient and found that 24 patients were carriers of TT genotype [wild type] and 6 patients were carriers of TG genotype [Heterozygous] [Table 8].

Table 8. The genotypic frequency of UGT1A6*2 gene bearing Ser7Ala polymorphism

Study group	**Genotype n (%)**			**Total**	**Allelic frequencies**		**Total**
	TT	**TG**	**GG**		**T**	**G**	
No. of patients n (%)	24 (0.80%)	6 (0.20%)	-	30	54 (0.9)	6 (0.1)	60

Twenty-seven patients found to be aspirin responders and three patients were aspirin non-responders [Table 9 and Fig. 9].

Table 9: Frequency of aspirin responders and non-responders.

Study group	**Aspirin responders**	**Aspirin Non-responders**	**Total**
No. of patients	27 (90%)	3 (10%)	30

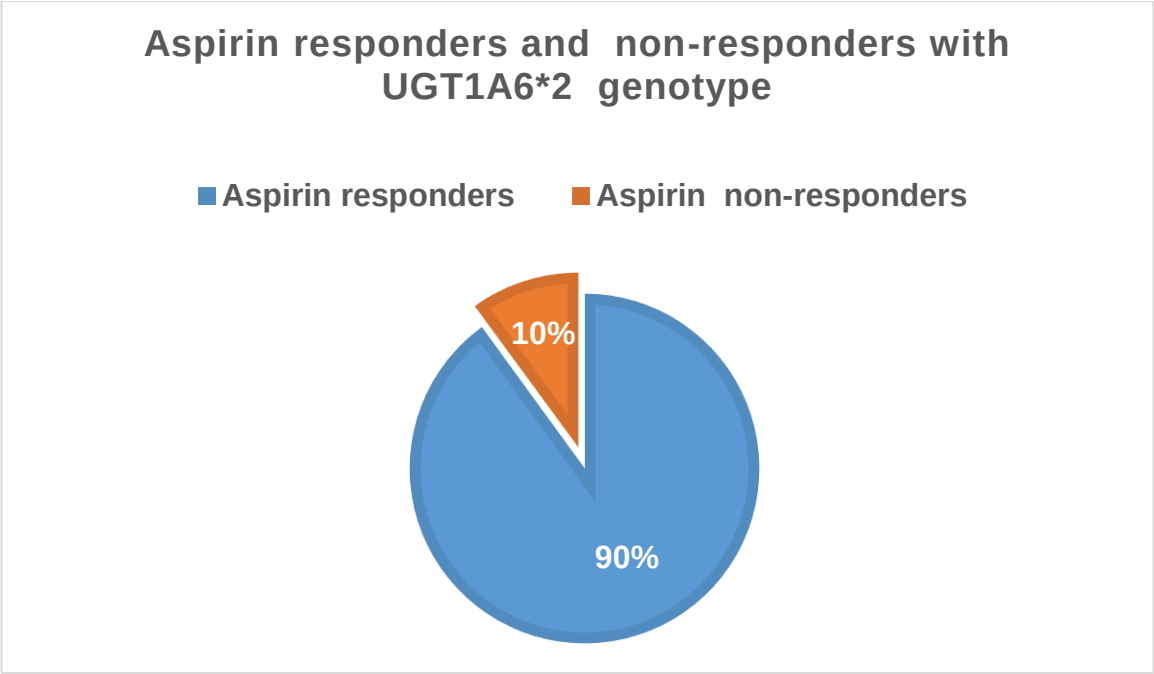

Figure 9: Aspirin responders and aspirin non-responders in our study group.

Chapter-6
Discussion

Ischemic stroke occurs due to occlusion in arteries due to platelet plug formation. Aspirin prevents the formation of platelet plug by inhibiting the function of platelet receptor. Aspirin resistance results in a chance of developing further vascular events. Heterogeneity in response to aspirin may arise due to genetic variation in drug metabolizing enzymes like UGT. Various genetic polymorphisms have been reported to be associated with inadequate platelet response to aspirin. UGT1A6 plays an important role in aspirin metabolism which may be involved in the development of aspirin resistance and adverse drug reactions. Some studies supported association of aspirin resistance on account of UGT1A6 (Jalil *et al.*, 2015) (Nagar *et al.*, 2004), (Sun *et al.*, 2017) (Hung *et al.*, 2011) (Krishnaswamy *et al.*, 2005) (Ciotti *et al.*, 1997). It has been found that UGT1A6*2 variant of UGT1A6 gene was associated with aspirin resistance and adverse drug reactions (Palikhe *et al.*, 2011) (Shiotani *et al.*, 2009).

In the present study on 30 ischemic stroke patients, we found that 27 patients (90% frequency) were aspirin responders and only 3 patients (10%) were non-responders. 24 patients belonged to TT genotypes, of which 23 were responders and 1 was non-responder. 6 patients were found to carry TG genotype, among these 4 were responders and 2 were non-responders. Since the sample size is low to identify statistically significant associations, a larger number of samples should be screened for the polymorphism before coming to a conclusion. However, this preliminary study indicates that UGT1A6*2 variant of UGT1A6 gene may be a risk factor for aspirin resistance and recurrent stroke in the studied group.

References

Adams, H. P., Bendixen, B. H., Kappelle, L. J., Biller, J., Love, B. B., Gordon, D. L., and Marsh, E. E. (1993). Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. The trial of Org 10172 in Acute Stroke Treatment. *Stroke* **24**(1): 35-41.

Adams, R. J., Cox, M., Ozark, S. D., Kanter, J., Schulte, P. J., Xian, Y., Fonarow, G. C., Smith, E. E., and Schwamm, L. H. (2017). Coexistent sickle cell disease has no impact on the safety or outcome of lytic therapy in acute ischemic stroke: findings from get with the Guidelines-Stroke. *Stroke* **48**(3): 686-691.

Aguilar, M., Hart, R., and Pearce, L. (2006). Oral anticoagulants versus antiplatelet therapy for preventing stroke in patients with non-valvular atrial fibrillation and no history of stroke or transient ischemic attacks. *Cochrane Database of Systematic Reviews*(3).

Agundez, J. A., Martinez, C., Perez-Sala, D., Carballo, M., Torres, M. J., and Garcia-Martin, E. (2009). Pharmacogenomics in aspirin intolerance. *Current drug metabolism* **10**(9): 998-1008.

Amarenco, P., and Labreuche, J. (2009). Lipid management in the prevention of stroke: review and updated meta-analysis of statins for stroke prevention. *The Lancet Neurology* **8**(5): 453-463.

Blanco, G., Martínez, C., Ladero, J. M., Garcia-Martin, E., Taxonera, C., Gamito, F. G., Diaz-Rubio, M., and Agundez, J. A. (2008). Interaction of CYP2C8 and CYP2C9 genotypes modifies the risk for nonsteroidal anti-inflammatory drugs-related acute gastrointestinal bleeding. *Pharmacogenetics and Genomics* **18**(1): 37-43.

Bock, K. W., and Köhle, C. (2005). UDP-glucuronosyltransferase 1A6: structural, functional, and regulatory aspects. *Methods in enzymology* **400**: 57-75.

Bornstein, N. M. (2009). *Stroke: practical guide for clinicians*: Karger Medical and Scientific Publishers.

Carlini, L. E., Meropol, N. J., Bever, J., Andria, M. L., Hill, T., Gold, P., Rogatko, A., Wang, H., and Blanchard, R. L. (2005). UGT1A7 and UGT1A9 polymorphisms predict response and toxicity in colorectal cancer patients treated with capecitabine/irinotecan. *Clinical Cancer Research* **11**(3): 1226-1236.

Catella-Lawson, F., Reilly, M. P., Kapoor, S. C., Cucchiara, A. J., DeMarco, S., Tournier, B., Vyas, S. N., and FitzGerald, G. A. (2001). Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *New England Journal of Medicine* **345**(25): 1809-1817.

Chan, A. T., Hsu, M., Zauber, A., Hawk, E., and Bertagnolli, M. M. (2011). The influence of UGT1A6 variants and aspirin use in a randomized trial of celecoxib for prevention of colorectal adenoma. *Cancer Prevention Research*: canprevres. 0337.2011.

Ciotti, M., Marrone, A., Potter, C., and Owens, I. S. (1997). Genetic polymorphism in the human UGT1A6 (planar phenol) UDP-glucuronosyltransferase: pharmacological implications. *Pharmacogenetics* **7**(6): 485-495.

Cipollone, F., Toniato, E., Martinotti, S., Fazia, M., Iezzi, A., Cuccurullo, C., Pini, B., Ursi, S., Vitullo, G., and Averna, M. (2004). A polymorphism in the cyclooxygenase 2 gene as an inherited protective factor against myocardial infarction and stroke. *Jama* **291**(18): 2221-2228.

Cohen, H. W., Crandall, J. P., Hailpern, S. M., and Billett, H. H. (2008). Aspirin resistance associated with HbA1c and obesity in diabetic patients. *Journal of diabetes and its complications* **22**(3): 224-228.

Dadheech, S., Rao, A. V., Shaheen, U., Hussien, M. D., Jain, S., Jyothy, A., and Munshi, A. (2013). Three most common nonsynonymous UGT1A6* 2 polymorphisms (Thr181Ala, Arg184Serand Ser7Ala) and therapeutic response to deferiprone in β-thalassemia major patients. *Gene* **531**(2): 301-305.

Dutton, G. J. (1980). Glucuronidation of drugs and other compounds.

Evans, W. E., and Relling, M. V. (1999). Pharmacogenomics: translating functional genomics into rational therapeutics. *science* **286**(5439): 487-491.

FitzGerald, R., and Pirmohamed, M. (2011). Aspirin resistance: effect of clinical, biochemical and genetic factors. *Pharmacology & therapeutics* **130**(2): 213-225.

Flockhart, D. (2007). Drug interactions: cytochrome P450 drug interaction table. *Indiana University School of Medicine* **2010**.

Gasparyan, A. Y., Watson, T., and Lip, G. Y. (2008). The role of aspirin in cardiovascular prevention: implications of aspirin resistance. *Journal of the American College of Cardiology* **51**(19): 1829-1843.

Ghosh, S. S., Sappal, B. S., Kalpana, G. V., Lee, S. W., Chowdhury, J. R., and Chowdhury, N. R. (2001). Homodimerization of human bilirubin-uridine-diphosphoglucuronate glucuronosyltransferase-1 (UGT1A1) and its functional implications. *Journal of Biological Chemistry* **276**(45): 42108-42115.

Goodman, T., Ferro, A., and Sharma, P. (2008). Pharmacogenetics of aspirin resistance: a comprehensive systematic review. *British journal of clinical pharmacology* **66**(2): 222-232.

Grines, C. L., Bonow, R. O., Casey, D. E., Gardner, T. J., Lockhart, P. B., Moliterno, D. J., O'gara, P., and Whitlow, P. (2007). Prevention of premature discontinuation of dual antiplatelet therapy in patients with coronary artery stents: a science advisory from the American Heart Association, American College of Cardiology, Society for Cardiovascular Angiography and Interventions, American College of Surgeons, and American Dental Association, with representation from the American College of Physicians. *Catheterization and Cardiovascular Interventions* **69**(3): 334-340.

Halushka, M. K., Walker, L. P., and Halushka, P. V. (2003). Genetic variation in cyclooxygenase 1: effects on response to aspirin. *Clinical Pharmacology & Therapeutics* **73**(1): 122-130.

Hanioka, N., Takeda, Y., Jinno, H., Tanaka-Kagawa, T., Naito, S., Koeda, A., Shimizu, T., Nomura, M., and Narimatsu, S. (2006). Functional characterization of human and cynomolgus monkey UDP-glucuronosyltransferase 1A6 enzymes. *Chemico-biological interactions* **164**(1-2): 136-145.

Hankey, G. J., and Eikelboom, J. W. (2006). Aspirin resistance. *The Lancet* **367**(9510): 606-617.

Higashi, M. K., Veenstra, D. L., Kondo, L. M., Wittkowsky, A. K., Srinouanprachanh, S. L., Farin, F. M., and Rettie, A. E. (2002). Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. *Jama* **287**(13): 1690-1698.

Home, F. (2013). Orange book: approved drug products with therapeutic equivalence evaluations. *USA: US Food & Drug Administration*.

Hubner, R. A., Muir, K. R., Liu, J.-F., Logan, R. F., Grainge, M., Armitage, N., Shepherd, V., Popat, S., and Houlston, R. S. (2006). Genetic variants of UGT1A6 influence risk of colorectal adenoma recurrence. *Clinical Cancer Research* **12**(21): 6585-6589.

Huisa, B. N., Stemer, A. B., and Zivin, J. A. (2010). Atorvastatin in stroke: a review of SPARCL and subgroup analysis. *Vascular health and risk management* **6**: 229.

Hung, C.-C., Ho, J.-L., Chang, W.-L., Tai, J. J., Hsieh, T.-J., Hsieh, Y.-W., and Liou, H.-H. (2011). Association of genetic variants in six candidate genes with valproic acid therapy optimization. *Pharmacogenomics* **12**(8): 1107-1117.

Jalil, N. J. A., Bannur, Z., Derahman, A., Maskon, O., Darinah, N., Hamidi, H., Gunasekaran, O. A., Rafizi, M., Azreen, N. I., and Kek, T. L. (2015). The implication of the polymorphisms of COX-1, UGT1A6, and CYP2C9 among cardiovascular disease (CVD) patients treated with aspirin. *Journal of Pharmacy & Pharmaceutical Sciences* **18**(3): 474-483.

James, S., Åkerblom, A., Cannon, C. P., Emanuelsson, H., Husted, S., Katus, H., Skene, A., Steg, P. G., Storey, R. F., and Harrington, R. (2009). Comparison of ticagrelor, the first reversible oral P2Y12 receptor antagonist, with clopidogrel in patients with acute coronary syndromes: rationale, design, and baseline characteristics of the PLATelet inhibition and patient Outcomes (PLATO) trial. *American heart journal* **157**(4): 599-605.

Krishnaswamy, S., Hao, Q., Al-Rohaimi, A., Hesse, L. M., von Moltke, L. L., and Greenblatt, D. J. (2005). UDP glucuronosyltransferase (UGT) 1A6 pharmacogenetics: I. Identification of polymorphisms in the 5′-regulatory and exon 1 regions, and association with human liver UGT1A6 gene expression and glucuronidation. *Journal of Pharmacology and Experimental Therapeutics* **313**(3): 1331-1339.

Kunicki, T. J., Pidard, D., Rosa, J.-P., and Nurden, A. T. (1981). The formation of Ca++-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. *Blood* **58**(2): 268-278.

Lares-Asseff, I., Juarez-Olguin, H., Flores-Perez, J., Guillé-Pérez, A., and Vargas, A. (2004). Pharmacokinetics and metabolic rates of acetyl salicylic acid and its metabolites in an Otomi ethnic group of Mexico. *Biological and Pharmaceutical Bulletin* **27**(5): 706-709.

Mackay, J., Mensah, G. A., and Greenlund, K. (2004). *The atlas of heart disease and stroke*: World Health Organization.

Mackenzie, P. I., Owens, I. S., Burchell, B., Bock, K. W., Bairoch, A., Belanger, A., Fournel-Gigleux, S., Green, M., Hum, D. W., and Iyanagi, T. (1997). The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics* **7**(4): 255-269.

Maree, A., Curtin, R., Chubb, A., Dolan, C., Cox, D., O'brien, J., Crean, P., Shields, D., and Fitzgerald, D. (2005). Cyclooxygenase-1 haplotype modulates platelet response to aspirin. *Journal of Thrombosis and Haemostasis* **3**(10): 2340-2345.

Markus, H. S. (2012). Stroke genetics: prospects for personalized medicine. *BMC medicine* **10**(1): 113.

McGreavey, L. E., Turner, F., Smith, G., Boylan, K., Bishop, D. T., Forman, D., Wolf, C. R., Barrett, J. H., and Group, C. C. S. (2005). No evidence that polymorphisms in CYP2C8, CYP2C9, UGT1A6, PPARδ and PPARγ act as modifiers of the protective effect of regular NSAID use on the risk of colorectal carcinoma. *Pharmacogenetics and Genomics* **15**(10): 713-721.

Munshi, A., and Sharma, V. (2015). Genetic signatures in the treatment of stroke. *Current pharmaceutical design* **21**(3): 343-354.

Nagar, S., Zalatoris, J. J., and Blanchard, R. L. (2004). Human UGT1A6 pharmacogenetics: identification of a novel SNP, characterization of allele frequencies and functional analysis of recombinant allozymes in human liver tissue and in cultured cells. *Pharmacogenetics and Genomics* **14**(8): 487-499.

NCBI. (2018). May, 19. homepage. <www.ncbi.nlm.nih.gov/gene/54578> Accessed 2018 Apr, 02.

Needs, C. J., and Brooks, P. M. (1985). Clinical pharmacokinetics of the salicylates. *Clinical pharmacokinetics* **10**(2): 164-177.

Nieuwenhuis, H. K., Akkerman, J. W. N., Houdijk, W. P., and Sixma, J. J. (1985). Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature* **318**(6045): 470.

Nowell, S. A., Massengill, J. S., Williams, S., Radominska-Pandya, A., Tephly, T. R., Cheng, Z., Strassburg, C. P., Tukey, R. H., MacLeod, S. L., and Lang, N. P. (1999). Glucuronidation of 2-hydroxyamino-1-methyl-6-phenylimidazo [4, 5-b] pyridine by human microsomal UDP-glucuronosyltransferases: identification of specific UGT1A family isoforms involved. *Carcinogenesis* **20**(6): 1107-1114.

Oldenburg, J., Marinova, M., Müller-Reible, C., and Watzka, M. (2008). The vitamin K cycle. *Vitamins & Hormones* **78**: 35-62.

Ouzzine, M., Magdalou, J., Burchell, B., and Fournel-Gigleux, S. (1999). An internal signal sequence mediates the targeting and retention of the human UDP-glucuronosyltransferase 1A6 to the endoplasmic reticulum. *Journal of Biological Chemistry* **274**(44): 31401-31409.

Palikhe, N. S., Kim, S.-H., Nam, Y. H., Ye, Y.-M., and Park, H.-S. (2011). Polymorphisms of aspirin-metabolizing enzymes CYP2C9, NAT2 and UGT1A6 in aspirin-intolerant urticaria. *Allergy, asthma & immunology research* **3**(4): 273-276.

Pandian, J. D., and Sudhan, P. (2013). Stroke epidemiology and stroke care services in India. *Journal of stroke* **15**(3): 128.

Peters, W. H., te Morsche, R. H., and Roelofs, H. M. (2003). Combined polymorphisms in UDP-glucuronosyltransferases 1A1 and 1A6: implications for patients with Gilbert's syndrome. *Journal of hepatology* **38**(1): 3-8.

Pilotto, A., Seripa, D., Franceschi, M., Scarcelli, C., Colaizzo, D., Grandone, E., Niro, V., Andriulli, A., Leandro, G., and Di Mario, F. (2007). Genetic susceptibility to nonsteroidal anti-inflammatory drug–related gastroduodenal bleeding: role of cytochrome P450 2C9 polymorphisms. *Gastroenterology* **133**(2): 465-471.

Poncz, M., Eisman, R., Heidenreich, R., Silver, S., Vilaire, G., Surrey, S., Schwartz, E., and Bennett, J. (1987). Structure of the platelet membrane glycoprotein IIb. Homology to the alpha subunits of the vitronectin and fibronectin membrane receptors. *Journal of Biological Chemistry* **262**(18): 8476-8482.

Radominska-Pandya, A., CZERNIK, P. J., LITTLE, J. M., Battaglia, E., and MACKENZIE, P. I. (1999). Structural and functional studies of UDP-glucuronosyltransferases. *Drug metabolism reviews* **31**(4): 817-899.

Radominska, A. P., CZERNIK, P. J., LITTLE, J. M., Battaglia, E., and MACKENZIE, P. I. (1999). Structural and functional studies of UDP-glucuronosyltransferases. *Drug metabolism reviews* **31**(4): 817-899.

Schielke, G., and Lawrence, D. A. (2012). Plasminogen activators in ischemic stroke. *Matrix Proteases in Health and Disease*: 127-156.

Shah, H., and Goyal, R. (2004). Glycoprotein IIb/IIIa receptor and its inhibition: A platelet-directed therapeutic strategy. *Indian journal of pharmacology* **36**(3): 133.

Sharma, V., Kaul, S., Al-Hazzani, A., Alshatwi, A. A., Jyothy, A., and Munshi, A. (2013). Association of COX-2 rs20417 with aspirin resistance. *Journal of thrombosis and thrombolysis* **35**(1): 95-99.

Shiotani, A., Sakakibara, T., Nomura, M., Yamanaka, Y., Nishi, R., Imamura, H., Tarumi, K. i., Kamada, T., Hata, J., and Haruma, K. (2010). Aspirin-induced peptic ulcer and genetic polymorphisms. *Journal of gastroenterology and hepatology* **25**(s1).

Shiotani, A., Sakakibara, T., Yamanaka, Y., Nishi, R., Imamura, H., Fujita, M., Tarumi, K.-i., Kamada, T., Hata, J., and Haruma, K. (2009). The preventive factors for aspirin-induced peptic ulcer: aspirin ulcer and corpus atrophy. *Journal of gastroenterology* **44**(7): 717-725.

Singh, G., and Triadafilopoulos, G. (1999). Epidemiology of NSAID induced gastrointestinal complications. *The Journal of rheumatology. Supplement* **56**: 18-24.

Smith, S., Judge, H., Peters, G., Armstrong, M., Dupont, A., Gaussem, P., and Storey, R. (2005). PAR-1 genotype influences platelet aggregation and procoagulant responses in patients with coronary artery disease prior to and during clopidogrel therapy. *Platelets* **16**(6): 340-345.

Sun, Y., Yu, J., Yuan, Q., Wu, X., Wu, X., and Hu, J. (2017). Early post-traumatic seizures are associated with valproic acid plasma concentrations and UGT1A6/CYP2C9 genetic polymorphisms in patients with severe traumatic

brain injury. *Scandinavian journal of trauma, resuscitation and emergency medicine* **25**(1): 85.

Szczeklik, A., Musiał, J., and Undas, A. (2002). Reasons for resistance to aspirin in cardiovascular disease. *Circulation* **106**(22): e181-e182.

Tourmousoglou, C., and Rokkas, C. (2008). Clopidogrel and aspirin in cardiovascular medicine: responders or not--current best available evidence. *Cardiovascular & Hematological Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Cardiovascular & Hematological Agents)* **6**(4): 312-322.

Trialists'Collaboration, A. (2002). Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *Bmj* **324**(7329): 71-86.

Truelsen, T., Begg, S., and Mathers, C. (2000). The global burden of cerebrovascular disease. *Geneva: World Health Organisation*.

Tukey, R. H., and Strassburg, C. P. (2000). Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annual review of pharmacology and toxicology* **40**(1): 581-616.

Van der Logt, E., Bergevoet, S., Roelofs, H., Van Hooijdonk, Z., Te Morsche, R., Wobbes, T., De Kok, J., Nagengast, F., and Peters, W. (2004). Genetic polymorphisms in UDP-glucuronosyltransferases and glutathione S-transferases and colorectal cancer risk. *Carcinogenesis* **25**(12): 2407-2415.

Van Oijen, M. G., Barthélémy, C., Janssen, M. J., Joiris, E., Peters, W. H., Laheij, R. J., Smits, P., Odou, P., and Jansen, J. B. (2009). Effect of genetic polymorphisms in UDP-glucuronosyltransferase 1A6 (UGT1A6) on acetylsalicylic acid metabolism in healthy female volunteers. *Pharmacology* **83**(4): 237-242.

Van Oijen, M. G., Huybers, S., Peters, W. H., Drenth, J. P., Laheij, R. J., Verheugt, F. W., and Jansen, J. B. (2005). Polymorphisms in genes encoding acetylsalicylic acid metabolizing enzymes are unrelated to upper gastrointestinal health in cardiovascular patients on acetylsalicylic acid. *British journal of clinical pharmacology* **60**(6): 623-628.

Van Swieten, J., Koudstaal, P., Visser, M., Schouten, H., and Van Gijn, J. (1988). Interobserver agreement for the assessment of handicap in stroke patients. *Stroke* **19**(5): 604-607.

Vasudeva, K., Munshi, A., Chaurasia, P., and Singh, S. (2017). Genetic signatures in ischemic stroke: Focus on aspirin resistance. *CNS & neurological disorders drug targets*.

Voora, D., and Ginsburg, G. S. (2012). Clinical application of cardiovascular pharmacogenetics. *Journal of the American College of Cardiology* **60**(1): 9-20.

APPENDIX A

DEPARTMENT FOR HUMAN GENETICS

SCHOOL OF HEALTH SCIENCES,

CENTRAL UNIVERSITY OF PUNJAB, BATHINDA

CONSENT FORM

I have been explained the possible risks and benefits and have understood the purpose for which blood sample from me/my children is being sought by the Department for Human Genetics, School of Life Sciences, Central University of Punjab, Bathinda.

I am free from any pressure whatsoever and herby give my own consent/consent of my children (who are under 18 years of age today) to: (i) withdrawal of sample of about .....ml blood by veni-puncture; and (ii) to all types of analysis of my blood for non-profit research purposes for acquisition of knowledge for the benefit of mankind by Department of Human Genetics or their collaborators. I will have the right to know the analyzed results for my sample (samples) and I am not giving my consent for disclosure of any personal information either direct or derived from the analysis of my sample (samples) to anyone without my further consent. I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Government agencies, and ethics committee. I understand that they may inspect my original records. I am aware of the fact that I can opt out of the study at any time without having to give any reason and this will not affect my future treatment in the hospital. I am also aware that the investigators may terminate my participation in the study at any time, for any reason, without my consent. I have been informed that my consent will be sought prior to any for-profit (including filing of patents) that may be taken by the School of Health Sciences or their collaborators on the basis of my blood sample.

Date:

Name:

Sex:

Investigator Certificate

I certify that all the elements including the nature, purpose and possible risks of the above study as described in this consent document have been fully explained to the subject. In my judgment, the participant possesses the legal capacity to give informed consent to participate in this research and is voluntarily and knowingly giving informed consent to participate.

Signature and Name of the Investigator: _____ Dated: _____

भाग :II

_____ ,पंजाब केन्द्रीय विश्वविद्यालय द्वारा जिस उद्देश्य से मुझ से व्याख्या मुझे की लाभ एवं जोखिम संभावित उसके हैं रहे जा मांगे नमूने के रक्त से बच्चों मेरे/ हूँ। चुका समझ भलीभांति उसे मैं और है गई दी कर

मैं मानव आनुवंशिकी केंद्र या इसके सहयोगियों द्वारा वेनी (i)पंचर द्वारा विधि (Venipuncture) बच्चों मेरे/मेराका लगभग .ली.मि ..... रक्त निकालने के ज्ञानार्जन में हित के मानवता (ii) और ; जिनकी) बच्चों मेरे / मेरी स्वयं की करने विश्लेषण के प्रकार सभी के रक्त मेरे से उद्देश्य के अनुसन्धान लिए आयुआज 18 वर्ष से कम है नहीं दबाव कोई का प्रकार किसी पर मुझ और ;हूँ देती/देता सहमती की (है।

मुझे अपने नमूने अपने मैं और होगा अधिकार का जानने को परिणामों विश्लेषित से (नमूनों) (नमूनों) नमूनेके विश्लेषण से प्रत्यक्ष या इससे उत्पन्न कोई भी सूचना आगे की सहमती के बिना किसी को भी प्रकट करने की सहमती नहीं दे रहा हूँ।

मैं एतदद्वारा इस अध्ययन में भाग लेने के परिणामस्वरूप मुझ से प्राप्त सूचना ,आयोजकों को (ओं) अनुसंधानकर्ताओं की करने प्रकट को समिति नीतिशास्त्र तथा एजेंसियों सरकारी ,प्राधिकरणों नियामक भलीभांत मैं हूँ। देता अनुमति कोि समझता हूँ कि वे मेरे मूल अभिलेखों का निरीक्षण कर सकते हैं।

मैं जानता हूँ कि मैं इस अध्ययन से किसी भी समय बिना कोई कारण बताए अपनी सहभागिता समाप्त कर सकता हूँ जानता भी यह मैं होंगे। नहीं प्रभावित ईलाज वाले होने में भविष्य मेरे इससे और ; अनुसंधानकर्त कि हूँा किसी भी समय किसी भी कारण से मेरी सहमती के बिना इस अध्ययन में मेरी सहभागिता समाप्त कर सकते हैं।

मुझे सूचित किया जा चुका है कि _____ केंद्र या इसके सहयोगियों द्वारा मेरे रक्त नमूने के आधार पर किसी भी लाभ लिए के (सहित करवाने पंजीकृत पेटेंट) मेरी पूर्व सहमति ली जाएगी।

तिथि_____ :

नाम_____ :लिंग _____ : आयु :(मे वर्षों)

_____

पता :

_____

अनुसंधानकर्ता प्रमाणपत्र-

मैं प्रमाणित करता हूँ कि उपरोक्त अध्ययन की प्रकृति संबंधित व्याख्या की जोखिम संभावित और उद्देश्य , के करने प्रदान सहमति सूचित की सहभागिता में अनुसन्धान इस में राय मेरी है। गई दी कर को व्यक्ति पूर्ण और से रूप स्वैच्छिक वह और है रखता क्षमता कानूनी सहभागी लिएज्ञान के साथ इसमें भाग लेने की सूचित सहमति दे रहा है।

अनुसंधानकर्ता के हस्ताक्षर एवं नाम_____ : :दिनांक

_____

ਭਾਗ ਦੂਸਰਾ:

ਮੈਨੂੰ ਸੰਭਾਵਤ ਖਤਰਿਆਂ ਅਤੇ ਫਾਇਦਿਆਂ ਤੋਂ ਜਾਣੂ ਕਰਵਾ ਦਿੱਤਾ ਗਿਆ ਹੈ ਅਤੇ ਮੈਂ ---------- ---------- ਕੇਂਦਰ, ਪੰਜਾਬ ਕੇਂਦਰੀ ਯੂਨੀਵਰਸਿਟੀ ਵਲੋਂ ਮੇਰੇ ਜਾਂ ਮੇਰੇ ਬੱਚਿਆਂ ਦੇ ਖੂਨ ਦੇ ਨਮੂਨੇ ਲਏ ਜਾਣ ਦਾ ਮੰਤਵ ਵੀ ਸਮਝ ਲਿਆ ਹੈ।

ਮੇਰੇ ਉਪਰ ਕਿਸੇ ਵੀ ਤਰ੍ਹਾਂ ਦਾ ਕੋਈ ਦਬਾਅ ਨਹੀਂ ਹੈ ਅਤੇ ਮੈਂ 1. **ਨਾੜੀ ਵਿਚੋਂ ____ ਮਿਲੀਲੀਟਰ ਖੂਨ ਦਾ ਨਮੂਨਾ ਲੈਣ ਲਈ, ਅਤੇ** 2. **---------- ਕੇਂਦਰ ਜਾਂ ਉਸਦੇ ਸਾਂਝੀਵਾਲਾਂ ਵਲੋਂ ਮਾਨਵਤਾ ਦੀ ਭਲਾਈ ਲਈ ਗਿਆਨ ਪ੍ਰਾਪਤੀ ਹਿੱਤ ਮੇਰੇ ਖੂਨ ਦੇ ਨਮੂਨੇ ਦੇ ਹਰ ਤਰ੍ਹਾਂ ਦੇ ਗੈਰ-ਮੁਨਾਫਾਕ੍ਰਿਤ ਖੋਜ ਅਧਿਐਨ ਲਈ ਆਪਣੀ ਸਹਿਮਤੀ/ਆਪਣੇ ਬੱਚਿਆਂ (ਜਿੰਨ੍ਹਾਂ ਦੀ ਉਮਰ ਅੱਜ ਦੇ ਦਿਨ** 18 **ਸਾਲ ਤੋਂ ਘੱਟ ਹੈ) ਦੀ ਸਹਿਮਤੀ ਦਿੰਦਾ ਹਾਂ।**

ਮੈਨੂੰ ਆਪਣੇ ਨਮੂਨੇ (ਨਮੂਨਿਆਂ) ਦੇ ਅਧਿਐਨ ਦੇ ਨਤੀਜੇ ਜਾਣਨ ਦਾ ਹੱਕ ਹੋਵੇਗਾ ਅਤੇ ਮੈਂ ਅਗਲੇਰੀ ਅਗਾਊਂ ਸਹਿਮਤੀ ਹਾਸਲ ਕੀਤੇ ਬਗੈਰ ਸਿੱਧੀ ਜਾਂ ਨਮੂਨੇ (ਨਮੂਨਿਆਂ) ਦੇ ਅਧਿਐਨ ਰਾਹੀਂ ਹਾਸਲ ਕੀਤੀ ਗਈ ਕੋਈ ਵੀ ਨਿੱਜੀ ਜਾਣਕਾਰੀ ਜਨਤਕ ਕਰਨ ਦੀ ਸਹਿਮਤੀ ਨਹੀਂ ਦੇ ਰਿਹਾ।

ਮੈਂ ਖੋਜ ਕਰਤਾਵਾਂ ਨੂੰ ਇਸ ਅਧਿਐਨ ਵਿਚ ਮੇਰੀ ਸ਼ਮੂਲੀਅਤ ਸਦਕਾ ਮੇਰੇ ਤੋਂ ਪ੍ਰਾਪਤ ਜਾਣਕਾਰੀ ਸਪਾਂਸਰਾਂ, ਨਿਯਮਕ ਸੰਸਥਾਵਾਂ, ਸਰਕਾਰੀ ਏਜੰਸੀਆਂ ਅਤੇ ਸਦਾਚਾਰ ਕਮੇਟੀ ਨਾਲ ਸਾਂਝਾ ਕਰਨ ਦੀ ਇਜ਼ਾਜਤ ਦਿੰਦਾ ਹਾਂ। ਮੈਨੂੰ ਪਤਾ ਹੈ ਕਿ ਉਹ ਮੇਰੇ ਅਸਲੀ ਦਸਤਾਵੇਜ਼ਾਂ ਦੀ ਜਾਂਚ ਕਰ ਸਕਦੇ ਹਨ।

ਮੈਂ ਇਸ ਤੱਥ ਤੋਂ ਜਾਣੂ ਹਾਂ ਕਿ ਮੈਂ ਕਿਸੇ ਵੀ ਸਮੇਂ ਬਿਨ੍ਹਾਂ ਕੋਈ ਕਾਰਨ ਦੱਸੇ ਇਸ ਅਧਿਐਨ ਕਾਰਜ ਨੂੰ ਛੱਡ ਸਕਦਾ ਹਾਂ ਅਤੇ ਹਸਪਤਾਲ ਵਿਚ ਹੋਣ ਵਾਲੇ ਮੇਰੇ ਸੰਭਾਵੀ ਇਲਾਜ ਤੇ ਇਸਦਾ ਕੋਈ ਅਸਰ ਨਹੀਂ ਪਵੇਗਾ। ਮੈਨੂੰ ਇਹ ਵੀ ਜਾਣਕਾਰੀ ਹੈ ਕਿ ਖੋਜ ਕਰਤਾ ਕਿਸੇ ਵੀ ਸਮੇਂ, ਬਿਨ੍ਹਾਂ ਕੋਈ ਕਾਰਨ ਦੱਸੇ ਅਤੇ ਮੇਰੀ ਸਹਿਮਤੀ ਬਗੈਰ ਇਸ ਅਧਿਐਨ ਕਾਰਜ ਵਿਚ ਮੇਰੀ ਸ਼ਮੂਲੀਅਤ ਖਤਮ ਕਰ ਸਕਦੇ ਹਨ।

ਮੈਨੂੰ ਜਾਣਕਾਰੀ ਦੇ ਦਿੱਤੀ ਗਈ ਹੈ ਕਿ ਮੇਰੇ ਖੂਨ ਦੇ ਨਮੂਨੇ ਦੇ ਅਧਾਰ ਤੇ ਕਿਸੇ ਵੀ ਲਾਭ ਵਾਲੇ ਕਾਰਜ (ਪੇਟੇਂਟ ਫਾਈਲ ਕਰਨ ਸਹਿਤ) ਨੂੰ ਕਰਨ ਤੋਂ ਪਹਿਲਾਂ ---------- ਕੇਂਦਰ ਜਾਂ ਉਸਦੇ ਸਾਂਝੀਵਾਲਾਂ ਵਲੋਂ ਮੇਰੀ ਸਹਿਮਤੀ ਹਾਸਲ ਕੀਤੀ ਜਾਵੇਗੀ।

ਮਿਤੀ:

ਨਾਮ: **ਲਿੰਗ**: **ਉਮਰ (ਸਾਲ)**:

ਪਤਾ:

ਖੋਜਕਰਤਾ ਵਲੋਂ ਪ੍ਰਮਾਣ-ਪੱਤਰ

ਮੈਂ ਇਹ ਪ੍ਰਮਾਣਿਤ ਕਰਦਾ ਹਾਂ ਕਿ ਇਸ ਸਹਿਮਤੀ ਪੱਤਰ ਵਿਚ ਦਰਜ ਉਪਰੋਕਤ ਅਧਿਐਨ ਕਾਰਜ ਨਾਲ ਜੁੜੇ ਹੋਏ ਲੱਛਣਾਂ, ਮੰਤਵਾਂ ਅਤੇ ਸੰਭਾਵਿਤ ਖਤਰਿਆਂ ਸਮੇਤ ਸਾਰੇ ਤੱਥਾਂ ਤੋਂ ਭਾਗੀ ਨੂੰ ਜਾਣੂ ਕਰਵਾ ਦਿੱਤਾ ਗਿਆ ਹੈ। ਮੇਰੇ ਵਿਚਾਰ ਅਨੁਸਾਰ ਭਾਗੀ ਇਸ ਖੋਜ ਵਿਚ ਸ਼ਾਮਲ ਹੋਣ ਲਈ ਜਾਗਰੂਕ ਸਹਿਮਤੀ ਦੇਣ ਦੇ ਕਾਨੂੰਨੀ ਰੂਪ ਵਿਚ ਸਮਰਥ ਹੈ ਅਤੇ ਸਵੈਇੱਛਾ ਨਾਲ ਅਤੇ ਜਾਣਦੇ ਹੋਏ ਜਾਗਰੂਕ ਸਹਿਮਤੀ ਦੇ ਰਿਹਾ ਹੈ,

ਖੋਜਕਰਤਾ ਦੇ ਦਸਤਖਤ ਅਤੇ ਨਾਮ: _____ ਮਿਤੀ: _____

PROJECT ON

"Pharmacogenetic study in Stroke"

Department of Human Genetics and Molecular Medicine

Central University of Punjab, Bathinda

Case Proforma

General Information:

Name of the patient		**Code No**	
Address **Phone No**			
Sex		**Age**	
Occupation		**Marital Status**	
Community		**Birth place**	
Number of brothers/Sisters of propositus		**Number of children of propositus**	
Diagnosis of disease since			

Incidence of defects and diseases in relatives

1. **Any one of your sibs affected with the same, similar or different defects/diseases?**
2. **Anyone else in your family affected ?**

PEDIGREE CHART

Patient consent to donate blood for genetic studies **Yes/No** **Signature**

CASE PARTICULARS OF PATIENT

Height		**Weight**	
BP		**Veg/Non-veg**	
Coffee/Tea		**Alcohol**	
Smoking		**Exercise**	
Age of onset		**Physical characteristics (Inabilities)**	
History of diabetes		**History of Hypertensin**	
Cholesterol		**Triglycerides**	
LDL		**HDL**	
Any other associated disease			
Any other information			

STROKE SUBTYPES

A. Intracerebral Hemorrhage

B. Ischemic stroke

1. Extracranial Large artery
2. Intracranial Large artery
3. Lacunar stroke
4. Cardio embolic
5. Other determined etiology
6. Undetermined etiology

SGOT		**Serum creatinine**	
SGPT		**Glucose random**	
Prothrombin Time		**Alk. phosphatase**	
APTT		**Na**	
Blood urea		**K**	
Serum Albumin		**Cl**	
Serum Bilirubin		**Total ser. proteins**	
VLDL			

Appendix B

Sr. No	Chemical Name	Catalogue no.	Make
1	Agarose	MB080-100G	HIMEDIA
2	Ammonium Chloride (NH_4Cl)	RM717-500G	HIMEDIA
3	Chloroform	MB109-500ML	HIMEDIA
4	Ethylenediaminetetraacetic acid (EDTA)	RM1279-500G	HIMEDIA
5	Potassium Bicarbonate($KHCO_3$)	L122521404	Lobachemic
6	Saturated Phenol	3T2063	GeNei
7	Sodium Chloride ($NaCl$)	GRM853-500G	HIMEDIA
8	Tris Base	GRM262-500G	HIMEDIA
9	Isoamyl alcohol	MB091-100ML	HIMEDIA
10	SDS	MB010-25G	HIMEDIA
11	Sodium acetate	451025	GeNei
12	Ethanol	XK-13-011-00009	Analytical
13	Potassium Chloride (KCl)	RM698-500G	HIMEDIA
14	Taq DNA Polymerase	61060240005A	GeNei
15	dNTPs	61065240004A	GeNei
16	Taq Buffer with 15 mM $MgCl_2$	602001180012	GeNei
17	Proteinase K	Amresco	0595C425

Appendix C

Buffers and Reagents

1. DNA extraction

- **Lysis Buffer I (10X):** It was prepared by adding following in 100 ml of distilled water.

  Ammonium Chloride (NH_4Cl) 8.29g

  Potassium Bicarbonate ($KHCO_3$) 1g

  0.5M EDTA 0.2 ml

  0.5M EDTA (pH-8.0) was prepared by adding 9.305g EDTA disodium salt in 50ml of distilled water.

- **Lysis Buffer II (1X):** It was prepared in total 100 ml of distilled water.

  Tris HCL 0.500 ml

  Sodium Chloride (NaCl) 1.68g

  0.5M EDTA 0.200 ml

  Tris HCl (pH-8.0) was prepared by adding 6.05g tris base to 50 ml of distilled water.

- **10% SDS:** It was prepared by adding 1g SDS to 10 ml of distilled water.
- **PCA (25:24:1):** PCA was prepared by adding phenol, chloroform and isoamyl alcohol in ratio 25:24:1.
- **CA (24:1):** CA was prepared by adding chloroform and isoamyl alcohol in 24:1 ratio.
- **Proteinase K**

Appendix D

Sr. No	Instrument Name	Maker
1	CO2 Incubator	Narang Scientific
2	Dry Bath	GeNei
3	Electrophoresis Power Supply	GeNei
4	Gel Documentation System	BioRad
5	Ice Flacking Machine	Manitowoc USA
6	Microwave	Samsung
7	Microcentrifuge	Genexy
8	Spinwinn	Tarsons
9	Thermal Cycler	Applied Biosystem
10	UV Transilluminator	GeNei
11	Vortex Shaker	Tarsons
12	Waterbath	GeNei
13	Weighing Balance Mettler	Toledo

Student Approval Form

Name of the author	Dharmendra kumar
Department	Human genetics and Molecular medicine
Degree	MSc Life Science with specialisation in Human Genetics
University	Central University of Punjab
Guide	Prof. Anjana Munshi
Project title	Association of UGT1A6*2 (Ser7Ala) polymorphism with therapeutic response to aspirin in ischemic stroke patients
Year of award	2018

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Signature of the candidate **Signature and seal of the supervisor**

Place: Bathinda

Date: May 2018

URKUND

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Sources included in the report:

https://worldwidescience.org/topicpages/c/cerebrovascular+disorders+stroke.html

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