AN LC-MS/MS ANALYSIS OF DRUG DOSAGE AND GENETIC POLYMORPHISM IN PATIENTS ON ANTI-COAGULANT WARFARIN TREATMENT: POTENTIAL IN BIOMARKER IDENTIFICATION

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ABSTRACT

Warfarin is considered as one of the most common anticoagulation drugs in the world. However, Because of wide dosage variation in different patients, lack of wide therapeutic index and risk of bleeding complications, treatment with this drug must be done very wisely.

Recognition of high and low dose people is of high importance in this regard. Between these two groups, low-dose are more likely to face difficulties in major bleeding complications in receiving warfarin. Optimizing dosage of warfarin has remained a challenge in the clinical setting. Among existing methods, genotyping may be considered as a suitable one in order to assess warfarin dosing. But, all of genetic polymorphisms cannot be detected in the target gene and the analysis with genotyping can be time consuming. So for more effective warfarin therapy, pharmacogenomics and pharmacoproteomics should be combined.

Specific proteins (biomarkers) present in either low- or high-dose patients may represent an added advantage. The combination of both genotyping and biomarkers may further improve the accuracy of the prognosis.

In this study, in order to find significant biomarker, 56 patients requiring low and high dose of warfarin treatment were selected. Their sera samples were then analyzed by iTRAQ-coupled LC-MS/MS analysis in three independent experiments. We found Transthyretin precursor protein which is significantly different among the patients requiring low and high warfarin dose.

1) INTRODUCTION

1.1) Warfarin

Anticoagulants are substances that prevent or delay blood coagulation. Acenocoumarol, dicoumarol, heparin and warfarin are among most widely used anticoagulants drugs. Warfarin is an oral anticoagulant drug, which is most widely used in the world. However, frequent bleeding complications during treatment with warfarin have been reported [1].

Warfarin is mostly used to prevent and treatment of venous thrombosis and embolism e.g. deep vein thrombosis (DVT), pulmonary embolism (PE), acute myocardial infarction (AMI), heart valve replacement, embolic stroke, and atrial fibrillation [1].

Warfarin effect is slow and usually appears after one week. Thus for instant effects heparin is administered simultaneously with warfarin.

Like other drugs, warfarin displays some side effect such as: haemorrhage, necrosis, joint or muscle aches, gastrointestinal (diarrhea, anorexia, etc), hypotension, and thrombocytopenia [2].

The most important adverse effect is haemorrhage. It is usually happen at higher dosage levels. The rate of haemorrhage is remarkable within the first weeks to first months of warfarin therapy [2], [3], [4].

Warfarin has a long half life of about 40 hour. After treatment it takes around 5 days for prothrombin to revert to regular amounts.

Using oral anticoagulants can be harmful and dangerous for some patients such as:

Pregnant women, patients who have haemorrhage or bleeding complications, patients with malignant hypertension or a past history of allergic reaction to warfarin derivatives. Because warfarin can cause fetal abnormalities so it should be avoided for pregnant women.

Patients with medical condition like liver failure and congestive heart failure (CHF) may have some difficulties with metabolism of the drug [5].

The anticoagulants inhibit the clotting factors Xa and IIa (thrombin) by increasing the activity of antithrombin III (AT III) and finally preventing the formation of blood clot [5].

Figure 1 summarized two different clotting pathways: Intrinsic Pathway and Extrinsic Pathway. Damage to blood vessel motivates the intrinsic pathway which is a cascade of different clotting factors. Subsequently factor X is activated in response to this cascade. Factor X in its activated form acts as an enzyme that transforms prothrombin to thrombin. The latter affects fibrinogen converting it to fibrin monomers. In the next stage factor XIII (fibrin stabilizing factor) converts fibrin to form blood clot [6].

The extrinsic pathway which is the outer pathway is activated in response to tissue damages outside the blood vessel. This stimulates thromboplastin activation which in

turn activates factor X. Factor X in its activated state modifies prothrombin to form thrombin stimulating fibrinogen to change into fibrin monomers. Finally factor XIII converts fibrin to form blood clots [6].

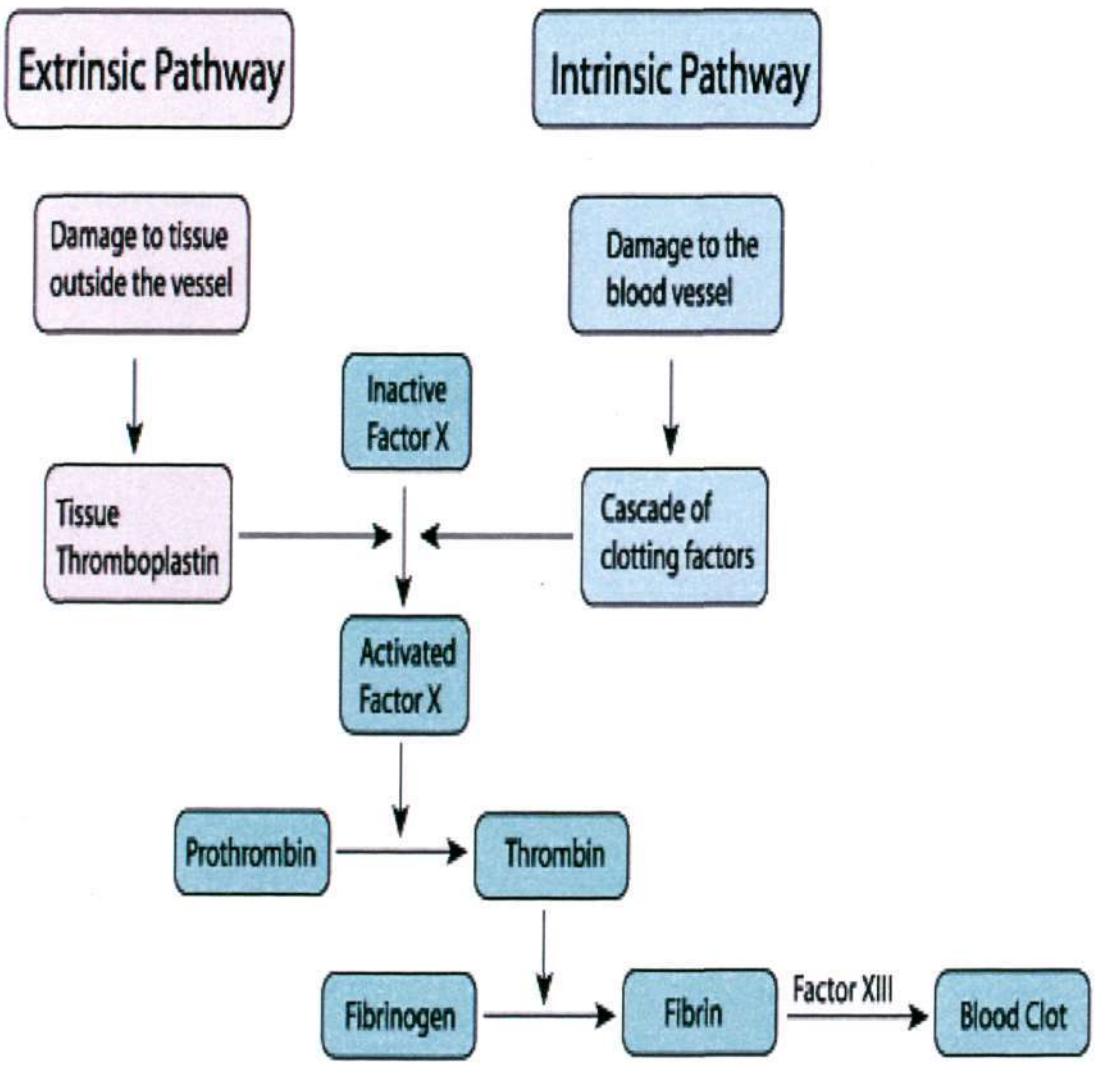


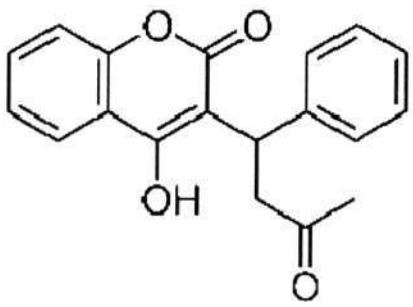
Figure 1: **Clotting Pathways** [6]

(See page 2 for detailed explanations)

Warfarin has two chemical names:

- 4-Hydroxy-3-(3-oxo-1-phenyl-butyl)- 2H-1-benzopyran-2-one
- 3-(2-acetyl-1-phenylethyl)-4-hydroxycoumarin

Figure 2 shows the two-dimensional structure of warfarin:



($C_{19}H_{16}O_4$)

Figure 2: Warfarin 2-D structure [7]

The Two enantiomeric forms of Warfarin (R-Warfarin and S-Warfarin) are resulting from the asymmetric carbon (C9) in warfarin structure. These two forms are metabolised in different ways (Figure 3).

The anticoagulation potency of (S)-enantiomer is 3 to 5 times greater than (R)-enantiomer. Two enantiomers of warfarin, S (-) and R (+) exhibit different vitamin K cycle inhibition capabilities, while it is widely accepted that the R-enantiomer is less effective than S-enantiomer. The overall anticoagulation response of S-warfarin is believed to be 60-70% and 30-40% for R-warfarin [8].

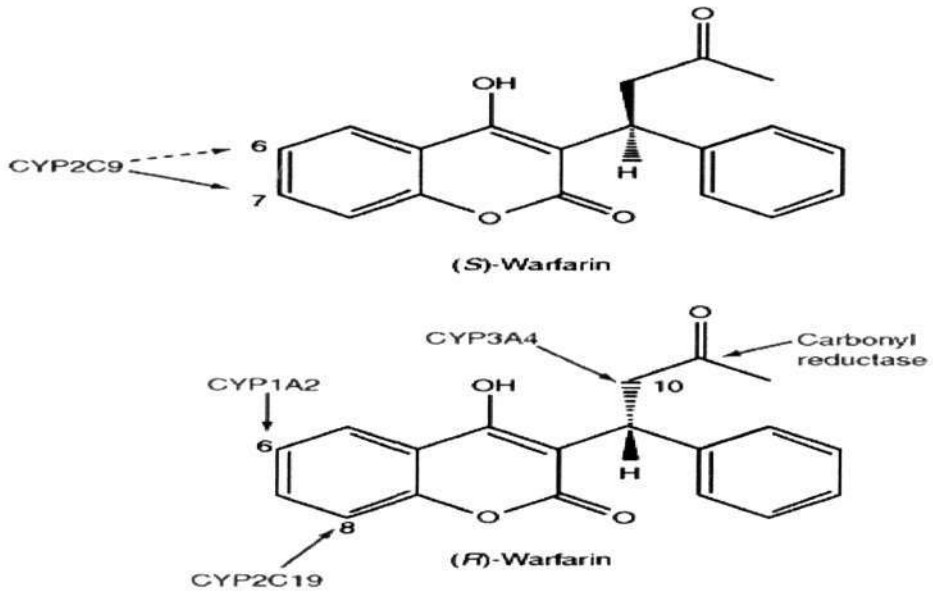


Figure 3: Enzymes involving in metabolism of warfarin in humans [9].

Warfarin is prescribed in order to achieve long-term oral anticoagulant therapy. Warfarin therapy has narrow therapeutic index as well as frequent bleeding complications and different patients may respond in different ways. In warfarin therapy the intensity of anticoagulation is very important, because it is related to serious bleeding. Therefore, warfarin therapy needs to be improved [8].

The intensity of anticoagulation can be measured by using two laboratory tests: Prothrombin Time (PT) and International Normalised Ratio (INR). PT is the test that defines the ability of blood coagulation. It shows the time needed for plasma converts to clot. The typical range for PT is about 12–15 seconds [10].

The INR is applying to standardize the PT results. INR is calculated as below [10]:

$$INR = (\text{patient PT} / \text{control PT})^{ISI}$$

ISI refers to International Sensitivity Index [10]. It is used for the analytical system. Any tissue factor which is made by each manufacturer has special ISI value. (The range of ISI is normally between 1.0 and 2.0) [10].

Normal range of INR for healthy person is between 0.9–1.3 and for people on warfarin therapy is 2.0–3.0. A high INR level means that the PT is longer than normal and the blood clotting occurs slowly, so there is a high chance for bleeding. When INR is high that is more than 3, bleeding increases significantly [11].

1.2) Cytochrome P450 Isoenzymes (CYPs) and Warfarin Metabolism

CYPs are the family of haemoprotein enzymes. Based on their function, they are mostly found on the membrane of endoplasmic reticulum [12].

Cytochrome P450 isoenzymes are mainly present in liver but are also present in intestine, lungs, kidneys, brain [12].

The main function of CYPs is to metabolise drugs, to make them more hydrophilic and then facilitate their excretion.

Cytochrome P450 catalyses many different reactions. The most important one is monooxygenase reaction: $NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + R\text{-}OH$ (Figure 4) [12].

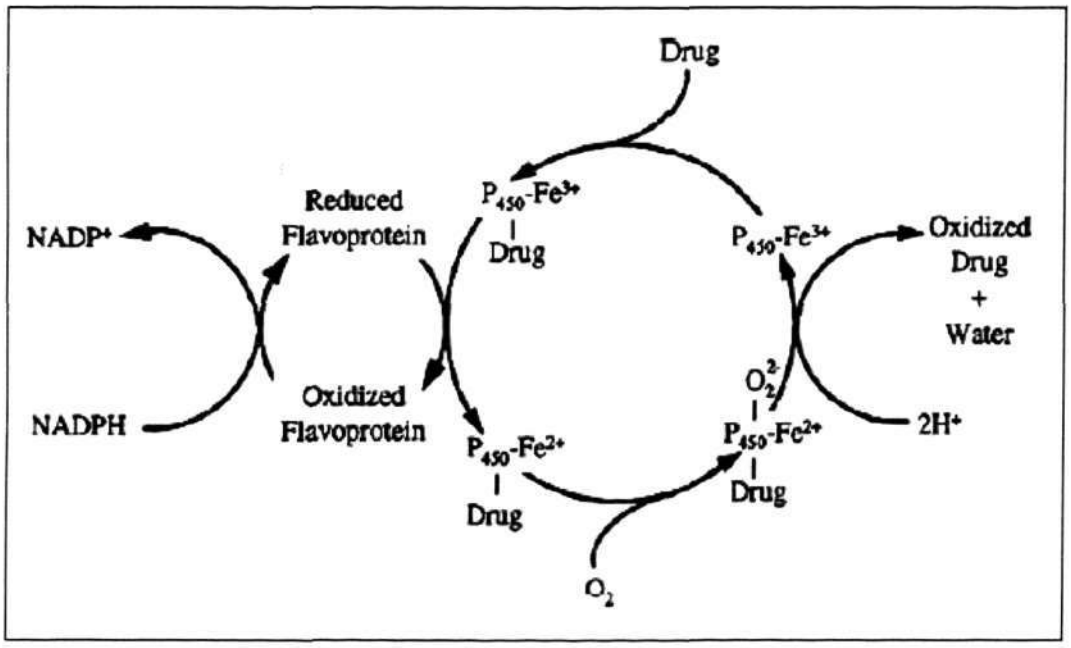


Figure 4: The Catalytic Cycle of Cytochrome P450 [12].

CYP 450 inserts one atom of oxygen into a parent drug (RH) and converts the parent drug to oxidized drug, while the other oxygen atom is reduced to water (RH is parent drug and R-OH is oxidized product) [12].

Cytochrome P450 CYP2C9 (The structure is shown in figure 5) is the main enzyme considered responsible for the metabolism of S-warfarin as well as several other drugs such as Ibuprofen, Naproxen, Piroxicam among others. CYP2C9 converts S-Warfarin to 7-hydroxywarfarin. But the metabolism of R-Warfarin is mainly catalyzed by CYP1A2 and CYP3A4. CYP1A2 catalyses the conversion of R-Warfarin to 6- and 8-hydroxywarfarin and CYP3A4 converts R-Warfarin to 10-hydroxywarfarin. Other isoforms of CYPs including CYP1A1 and CYP2C8 may also have role in the metabolism of R-warfarin [8].

Table 1 shows that CYP2C9 exhibits genetic polymorphism more than others.

Table 1: Contribution of individual CYP isoenzyme in drug metabolism and relative importance of polymorph [12].

Enzymes	Fraction of drug metabolism (%)	Genetic polymorphism
CYP2C9	10	+++
CYP1A2	5	+
CYP3A4	40-45	–

The point mutation in the CYP2C9 genes gives rise to two different alleles: CYP2C9*2 and CYP2C9*3. CYP2C9*2 carries a Cys to Arg substitution and CYP2C9*3 carries a Leu to Ile substitution [8].

Genetic polymorphisms found for *CYP2C9* have trivial effect on warfarin therapy in Asian populations. This is because of the low frequency of *CYP2C9*2* and **3* alleles in Asian patients [13].

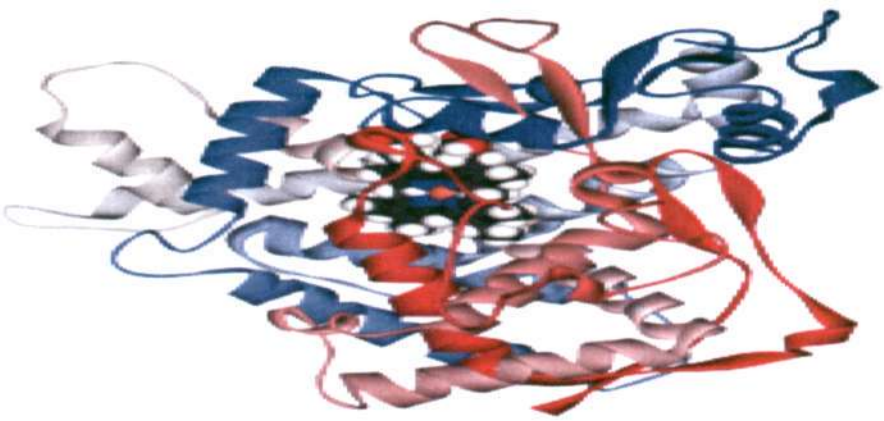


Figure 5: **Cytochrome P450, family 2, Subfamily C, polypeptide 9** [14].

Recent studies show that in the mechanism of warfarin more than 30 genes can be influenced [15].

The target genes of warfarin therapy include Vitamin K 2, 3-epoxide reductase (*VKOR*) and Apolipoprotein E (*APOE*). Genetic polymorphisms in both genes have been shown to influence warfarin therapy. Based on recent reports, it is suggested that variation in VKORC1 may show a more significant variability in warfarin requirement in comparison to other polymorphisms in warfarin target genes [15].

CYP2C9 and VKORC1 are critical genes in the pharmacokinetics and pharmacodynamics of warfarin respectively [15]. (Pharmacokinetics explains how body acts on drugs, for example mechanisms of drug absorption and distribution in body. Inversely pharmacodynamics is studying the effect of drug on the body, for example mechanisms by which drugs affect the body and the correlation between drug dose and effect.)

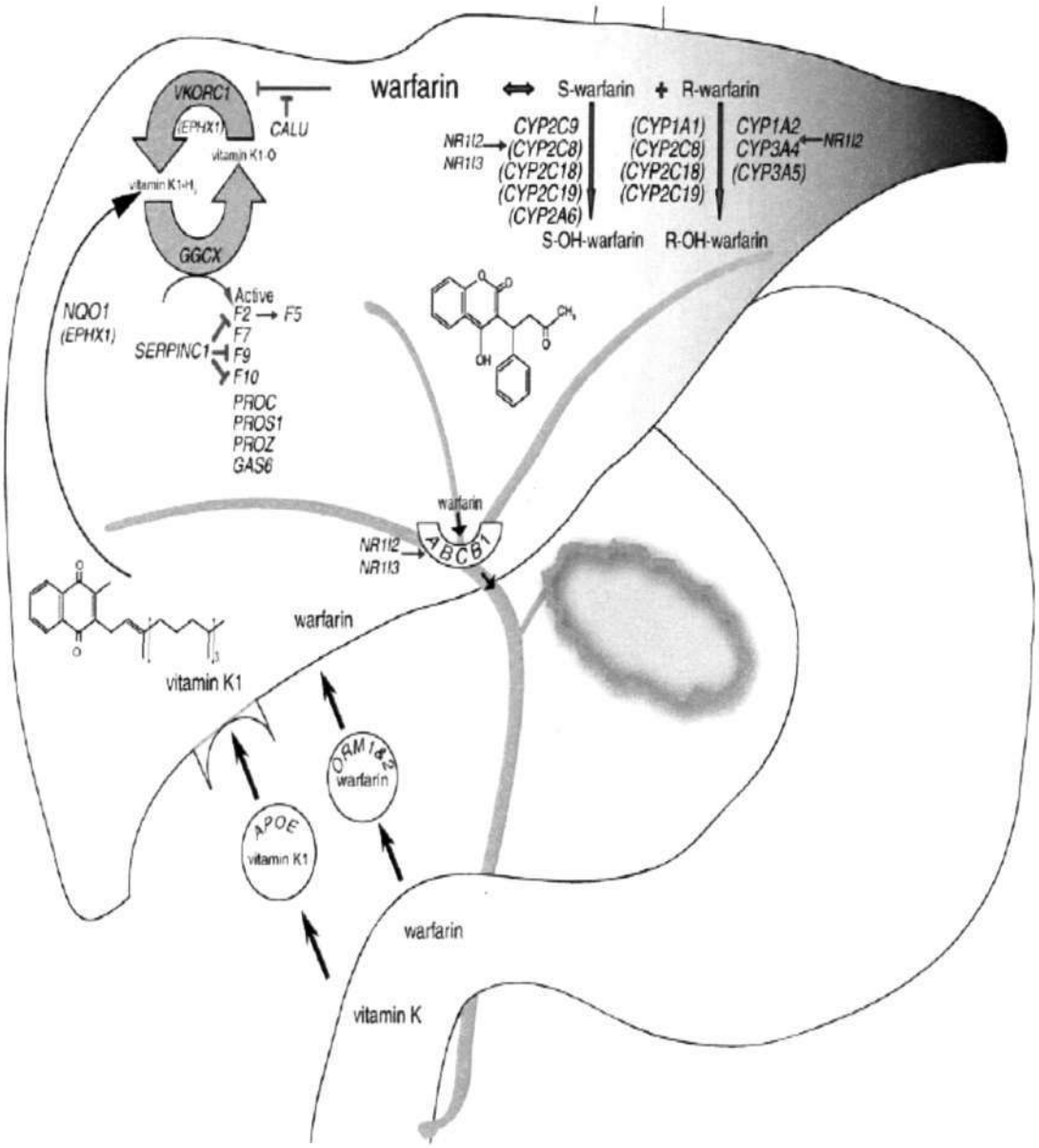


Figure 6: Warfarin biotransformation and interaction with genes.

The absorbance of warfarin occurs in the stomach. In the circulating blood warfarin is attached to albumin and alpha 1-acid glycoproteins. These two proteins are respectively encoded by OMR1 and OMR2. P-glycoprotein (encoded by ABCB1) is responsible for transportation of warfarin across the cell membrane [15].

1.3) Vitamin K and Blood Coagulation

In 1920 Vitamin K was discovered by Henrik Dam. The term means "Koagulation-Vitamin", because of its significant function in blood coagulation.Different classes of vitamin K have since been discovered and categorized as follows:

1) Vitamin K1 or phylloquinone: mainly found in green vegetable including spinach and broccoli.

2) Vitamin K2 or menaquinone: mainly found in fermented products of milk, cheese and soy.

3) Vitamin K3 or menadione: chemically synthesized and usually used for feeding the animal [16].

The structure of vitamin K is illustrated in Figure 7.

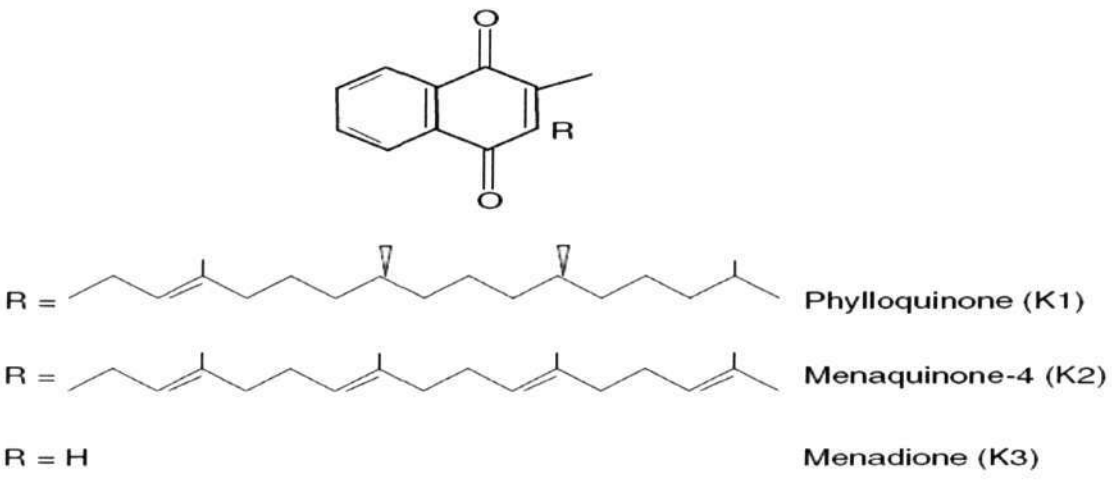


Figure 7: Different forms of vitamin K: phylloquinone (vitamin K1); menaquinones (vitamin K2): menaquinone-4; and menadione (vitamin K3) [16].

The most significant function of vitamin K is its protein carboxylation. Activation of vitamin K–dependent proteins requires the conversion of glutamate to γ-carboxyl glutamate by vitamin K. This reaction is catalyzed by the γ- glutamyl carboxylase enzyme [17].

Vitamin K has important role in blood coagulation. For this reason, an essential antagonist of vitamin K is warfarin. It is an anticoagulant which acts by interrupting vitamin K pathway. In this cycle vitamin K 2, 3-epoxide reductase (*VKOR*) is the agent that reduces vitamin K. Reduced vitamin K activates the clotting factors II, VII, IX and X as well as proteins C, S and Z with the help of γ -glutamyl carboxylase (GGCX). In this cycle, first, vitamin K is oxidized, and then recycled in the reduced form by VKOR. Warfarin is in fact the inhibitor of VKOR. Therefore it prevents blood coagulation by afflicting the synthesis of clotting factors [17].

The mechanism of action of warfarin is illustrated in Figure 8.

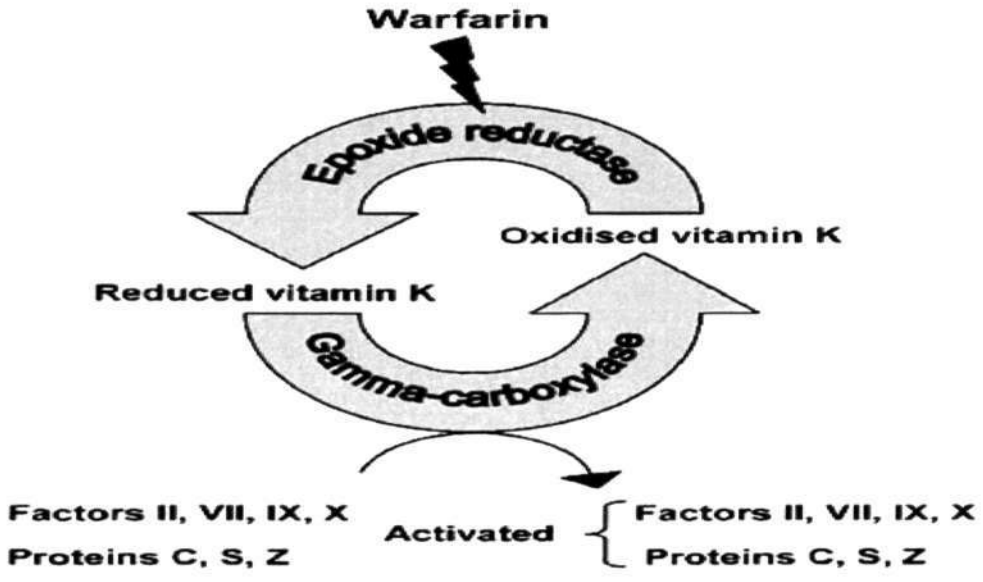


Figure 8: Cycle of vitamin K [17]

Some vitamin k-dependent proteins are shown in table 2:

Table 2: Vitamin K Protein and Peptide Family [18]

Identified or hypothetical vitamin K-dependent protein or peptide	Physiological function of protein or peptide	Species and characteristics of the protein or peptide
Protein S	Blood coagulation	Vertebrates, proenzyme
Protein C	Blood coagulation	Vertebrates, proenzyme
Protein Z	Blood coagulation	Vertebrates, proenzyme
Factor VII	Blood coagulation	Vertebrates, proenzyme
Factor IX	Blood coagulation	Vertebrates, proenzyme
Factor X	Blood coagulation	Vertebrates + snake, proenzyme
Prothrombin	Blood coagulation	Vertebrates + snake, proenzyme

1.4) Proteomics and Biomarkers

Genotyping can be considered as a good method for assessing warfarin dosing. However, not only all genetic polymorphisms can be detected in all target genes. A more comprehensive analysis, of global profile includes transcriptome and proteome. Analysis of gene expression by transcriptome determines transcript levels for thousands of genes, but it does not necessarily reflect changes in proteins. Transcriptome analysis also cannot reveal how the protein is modified post-translationally. Considering all these inefficiencies for genomics that can be solved

to more extent, sole genotyping may not be sufficient for assessing effective warfarin dosing [19].

This difficulty may be directly overcome by proteomics, which analyzes dynamic protein modifications in the stage of expression, linking both genetic and epigenetic effects. Because proteome is a much more extensive field than genome, it can also lead to discovery of valuable biomarkers. By using proteomics, new biomarkers can be generated for initial detection and speed up drug growth and development [20]. Because proteomics analysis is based on protein, the data would complete the information of genetic polymorphisms effect on drugs targets such as warfarin targets (*e.g.* CYP2C9) [20].

Biomarkers are biological molecules in the body fluids like plasma, cell lines and tissues. They are specific substances used as indicators of a biological state. Biomarkers have many applications in medicine including: monitoring the treatment, efficacy disease progression, disease diagnosis, predicts response to treatment [21].

In this study, our efforts focus on investigation of biomarkers in plasma of patients under warfarin therapy. We select, detect and quantify proteins of interest in serum samples.

The combination of these approaches, genotyping and proteomics, should lead to improve more in the accuracy of the prognosis of warfarin dosing.

Liquid chromatography-mass spectrometry (LC-MS) is a powerful method, which is highly sensitive and specific, as well as accurate. It is generally used to detect and identify chemicals in the complex mixture [22].

By reducing the limitations in mass of proteins, LC-MS is able to identify proteins with less than 1,000 Da molecular weight. The MS allows rapid identification of protein through on line database.Detection of proteins at low ng/ml rang by MS is possible but comes at a very high cost of instrument time [23].

The objective of this project is to identify biomarkers, using LC-MS/MS (Liquid chromatography- tandem mass spectrometry) analysis, in patients who require low or high dose warfarin therapy.

2) SIGNIFICANCE OF RESEARCH

Stroke which is a fast loss in function of brain, mainly as a result of inefficient job of feeding blood vessels, accounts for as the third prominent mortality factor in United States and many other countries and the main cause of adult disability in United States and Europe. It is the second cause of death worldwide and may soon become the main cause [24].

Warfarin therapy has been accepted for most of the patients who need anticoagulant drugs for many years. Warfarin decreases the chance of embolism by preventing blood clots formation [25].

People can be categorized in three groups considering required dose of drug in receiving warfarin:

1) Low-dose group which refers to patients who required 3 mg or less dose of warfarin daily.
2) Normal-dose group
3) High-dose group are patients who require more than 5 mg dose of warfarin daily.

It lacks wide therapeutic index and low inter-individual variability in patient response, so it needs to be improved [25].

Considering this basis, recognition of high and low dose people is of high importance in warfarin therapy. Between these two groups, low-dose are more likely to

encounter difficulties in receiving drugs as they may are more probable of major bleeding complications.

In fact, if we administer the drug without considering the proper dosage for the patients, life-threatening bleeding may occur instead of curing for those patients on low-dose therapy. Statistics have shown that bleeding as a side effect of warfarin occurs at a rate of 1.3 to 2.7 per hundred patient-years [8].

Therefore serum biomarkers specific to either or both groups of patients (requiring low or high dose) before administering the drug warfarin therapy will be more useful with less complications and side effects [8].

The main goal of this project is to detect specific proteins biomarkers in low or high dose patients. These biomarkers, should allow pre-treatment prognosis of patients on their closing requirement. This is particularly significant for low dose patients, as bleeding complications can be prevented.

Proteomics can be considered as study of proteins in large scale. It can be applied a lot in pharmaceutical studies. As mode of action of drugs are mostly based on proteins and not DNA/RNA, It also play critical role in drug discovery and development.

Unlike genomics studies which were involved in genes related to diseases, proteomics enable us to make the disease process more clear directly by finding groups of proteins which take part in causing the disease [26].

By the use of proteomics, disease can be recognized earlier and more accurately. Moreover therapeutic strategies, prognosis and prevention can be improved. Currently, the main focus of clinical proteomics is diagnosis and biomarker discovery. However other aspects like new therapeutic targets, drugs and vaccines are being developed. This will lead to successful disease prevention and therapy [26].

By applying LC-MS-based proteomics, we can find specific proteins, biomarkers, present in either low or high dose patients for further effective warfarin therapy. In addition, we can find which proteins are up-regulated or down-regulated in sera of the patients.

Although genotype can help in warfarin therapy, using it alone cannot be effective and the analysis in genotyping can be also time-consuming. However, the combination of both genotyping and proteomics can improve the accuracy of the treatment [27].

3) LITERATURE REVIEW

Warfarin, as a commonly prescribed oral anticoagulant with reasonable price, is highly prescribed in the case of stroke. The discovery of warfarin was taken place in Canada and the United States in the early decades of 20th century. This drug acts through prevention of blood coagulation by afflicting the synthesis of clotting factors [28].

Prescription of warfarin needs great caution because of its wide dosage variation in different patients, lack of wide therapeutic index and also the risk of bleeding complications (Figure 9).

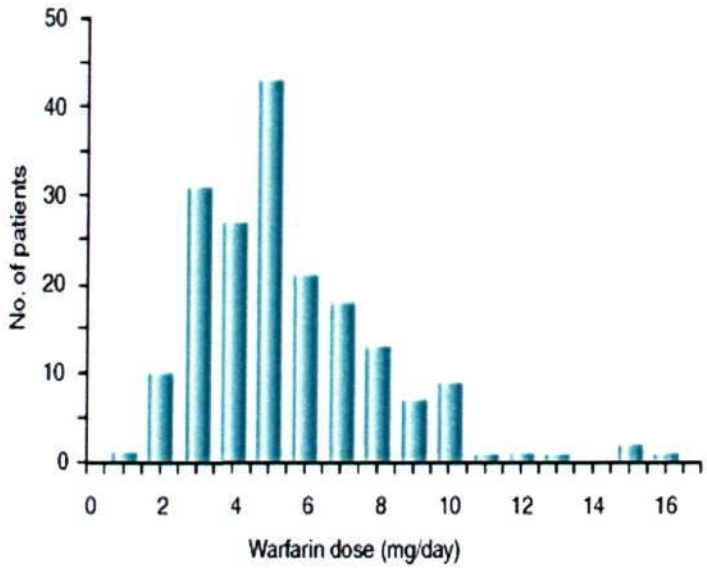


Figure 9: Warfarin dosage variability in 185 European patients [28]

Many studies have been done on the factors, which can influence different dose requirement in warfarin therapy. These have shown that both environmental and genetic factors can affect on warfarin therapy [29].

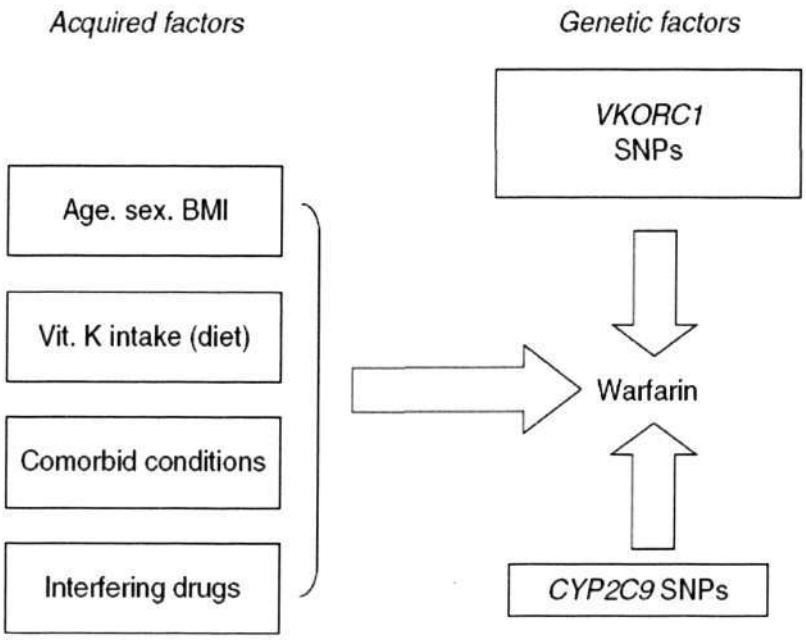


Figure 10: Environmental and genetic factors affecting the warfarin maintenance dose [16].

Some Environmental factors that affect warfarin dosage requirements are categorized as below:

1) **Hepatocellular damage**: synthesis of clotting factor is reduced by the Hepatocellular damage. So the metabolism of warfarin is reduced too. And finally the warfarin sensitivity is increased.
2) **Cardiac disease**: in this disease the abnormal liver function leads to reduced clotting factor synthesis.

3) **Drug interaction**: using some drugs can affect on the warfarin therapy. Because drug interaction can lead to adverse events with oral anticoagulant. So drug interaction can increase or reduced the warfarin dosage requirements.
4) **Food interaction**: some foods like chondroitin, garlic, Cayenne, feverfew, ginger and Gingko biloba can increase the effects of warfarin. But others such as green tea, ginseng and goldenseal reduced the effectiveness of warfarin therapy.
5) **Age**: by increasing age the liver size is decrease so warfarin dose is decrease too. Decreasing the dosage of warfarin is estimated at about 10% by decade. For example if one 30 years old patient needs warfarin 6 mg/day, in 70 years old he/she needs 4 mg/day.
6) **Dietary vitamin K intake**: A high consumption of vitamin K prevents warfarin performance. E.g. if the intake of vitamin K is more than 250 µg/day, the warfarin sensitivity is reduced. Some foods have high content of vitamin K such as: cheese, egg yolk, leafy vegetables (broccoli, cabbage, spinach and kale) and oils (peanut, corn, olive and soybean), beef and pork liver. For more effective warfarin therapy it is better that these foods are not used during therapy. And also dietary supplements of vitamin K should be avoided [30].

Other studies have shown that more than 30 genes can be influenced in warfarin mechanism [31].

Some of these genes are summarized in the tables 3, 4 and 5 as below:

Table 3: Genes Involved in Metabolism of Warfarin [31]

Protein name	Gene	Function of protein
Cytochrome P450 2C9	CYP2C9	Polymorphic hepatic drug metabolizing enzyme. Metabolism of S-warfarin
Cytochrome P450 1A1	CYP1A1	Extrahepatic oxidation, inducible. Metabolism of R-warfarin
Cytochrome P450 1A2	CYP1A2	Hepatic oxidation, inducible. Metabolism of R-warfarin
Cytochrome P450 2A6	CYP2A6	Polymorphic hepatic drug metabolizing enzyme. Metabolism of S-warfarin
Cytochrome P450 2C8	CYP2C8	Polymorphic hepatic drug metabolizing enzyme. Minor pathway for R- and S-warfarin
Cytochrome P450 2C18	CYP2C18	Found in the liver and lung. Minor pathway for R- and S-warfarin
Cytochrome P450 2C19	CYP2C19	Polymorphic hepatic drug metabolizing enzyme. Minor pathway for R- and S-warfarin
Cytochrome P450 3A4	CYP3A4	Hepatic oxidation, inducible. Metabolism of R-warfarin

Table 4: Genes Involved in Biotransformation of Vitamin K Transport [31]

Protein name	Gene	Function of protein
Apolipoprotein E	APOE	Apolipoprotein E acts as a ligand for receptors that mediate the uptake of vitamin K.
Vitamin K epoxide reductase	VKORC1	A hepatic epoxide hydrolase that catalyses the reduction of vitamin K. The target of warfarin
Epoxide hydrolase 1, microsomal	EPHX1	A hepatic epoxide hydrolase in the endoplasmic reticulum that may be complexed with VKOR
Calumenin	CALU	Binds to the vitamin K epoxide reductase complex and inhibits the effect of warfarin
Gamma-glutamyl carboxylase	GGCX	Carboxylates vitamin K-dependent coagulation factors and proteins in the vitamin K cycle

Table 5: Genes Involved in Transportation of Warfarin [31]

Protein name	Gene	Function of protein
Alpha-1-acid glycoprotein 1, Orosomucoid 1	ORM1	A plasma glycoprotein that functions as a carrier of warfarin in the blood
Alpha-1-acid glycoprotein 2, Orosomucoid 2	ORM2	A plasma glycoprotein that functions as a carrier of warfarin in the blood
P-glycoprotein, Multidrug resistance protein 1	ABCB1 (MDR1)	Transport warfarin across plasma membranes of cells

The major candidate genes which were at the center of attention are:

Cytochrome P450 2C9 (CYP2C9), subunit 1 of Vitamin K 2,3-epoxide reductase complex (VKORC1), Gamma Glutamyl Carboxylase (GGCX) and Apolipoprotein E (Apo E) genes [32].

3.1) CYP2C9 Gene

Many studies have been done on pharmacokinetics of warfarin at the molecular basis. Warfarin prescribed to patient is composed of both R- and S-enantiomers. The activity of S-enantiomer is much higher than the R-form, up to 3-5 times. After its entry into the liver, S-form of warfarin is metabolized by CYP2C9 [33].

CYP2C9 subfamily constitutes 18% of the whole CYP protein content of the human liver. It can account for the main enzyme participate in S-warfarin metabolism. CYP2C9 catalyzes the conversion of S-Warfarin to 7-hydroxy Warfarin. A high level of polymorphism has been found in the promoter region of CYP2C9 and which shows variation among different ethnic groups [32].

Point mutations in CYP2C9 gene leads to more than 50 allelic variants. The most significant of them are CYP2C9*2 and CYP2C9*3. CYP2C9*2 has 12% of wild type activity, and CYP2C9*3 shows only 5% of wild type activity [8].

Tables 6 and 7 summarize these mutations.

Table 6: Non-synonymous Mutations in CYP2C9 with Functional Effects [34]

Alleles	Nucleotide change in cDNA	Amino acid change	Enzymatic activity
CYP2C9*2	430C>T	Arg144Cys	Decrease
CYP2C9*3	1075A>C	Ile359Leu	Decrease
CYP2C9*4	1076T>C	Ile359Thr	Decrease
CYP2C9*5	1080C>G	Asp360Glu	Decrease
CYP2C9*6	del818A	Frame shift	Null
CYP2C9*8	449G>A	Arg150His	Increase
CYP2C9*11	1003C>T	Arg335Trp	Decrease

Table 7: Non-synonymous Mutations in CYP2C9 with Functional Effects [34]

Alleles	Nucleotide change in cDNA	Amino acid change	Enzymatic activity
CYP2C9*12	1465C>T	Pro489Ser	Decrease
CYP2C9*13	269T>C	Leu90Pro	Decrease
CYP2C9*14	374G>A	Arg125His	Decrease
CYP2C9*15	485C>A	Ser162X	Null
CYP2C9*16	895A>G	Thr299Ala	Decrease
CYP2C9*17	1144C>T	Pro382Ser	Decrease
CYP2C9*19	1362G>C	Gln454His	Decrease

It has been shown that previous mentioned polymorphisms in CYP2C9 significantly affects S-warfarin metabolism. Thus, there is a strong association between CYP2C9*2 and CYP2C9*3 alleles and low warfarin dose requirement. Wild type patients metabolize warfarin faster than patients with CYP2C9*2 and CYP2C9*3. Bleeding complications occurred when mutant patients received a normal dose of warfarin. The probability of serious bleeding is higher among low-dose patients during warfarin therapy [8].

Another research done in 2004 revealed that *CYP2C9* genetic polymorphisms have the least effect on warfarin therapy among Asian populations. This is because of the low frequency of *CYP2C9*2* and **3* alleles in these patients [13].

CYP2C9*2 and CYP2C9*3 alleles were found in 8-19% and 6-10% of Caucasians respectively. While in Asians (Chinese and Japanese) the frequency of CYP2C9*2 was 0% and the frequency of CYP2C9*3 was 1.7-5%.

The frequency of CYP2C9*4 allele was very low in Asian populations. In fact the CYP2C9*5 and CYP2C9*6 were absent in Caucasians and Asians [34].

It has also been proposed that CYP2C8, CYP2C18 and CYP2C19 probably are involved in S-warfarin metabolism [35].

These genes also metabolized S-warfarin to 4-hydroxywarfarin, but these are not a major pathways. Studies have shown that the role of CYP2C19*2 on warfarin therapy is insignificant [31].

3.2) VKORC1 Gene

Vitamin K epoxide reductase complex 1 (VKORC1) is the gene responsible for coding target protein of warfarin which was discovered in 2004. However, in 1970 the activity of the enzyme was first reported. The discovery of VKORC1 gene had important role in understanding vitamin K metabolism [16].

This multi-component complex, composed of lipid-protein enzymes, is located in the membrane of endoplasmic reticulum. Pharmaco-dynamic effects of warfarin are exerted by inhibiting the action of VKORC1. This complex is needed for alteration of oxidized form to reduced vitamin K [36].

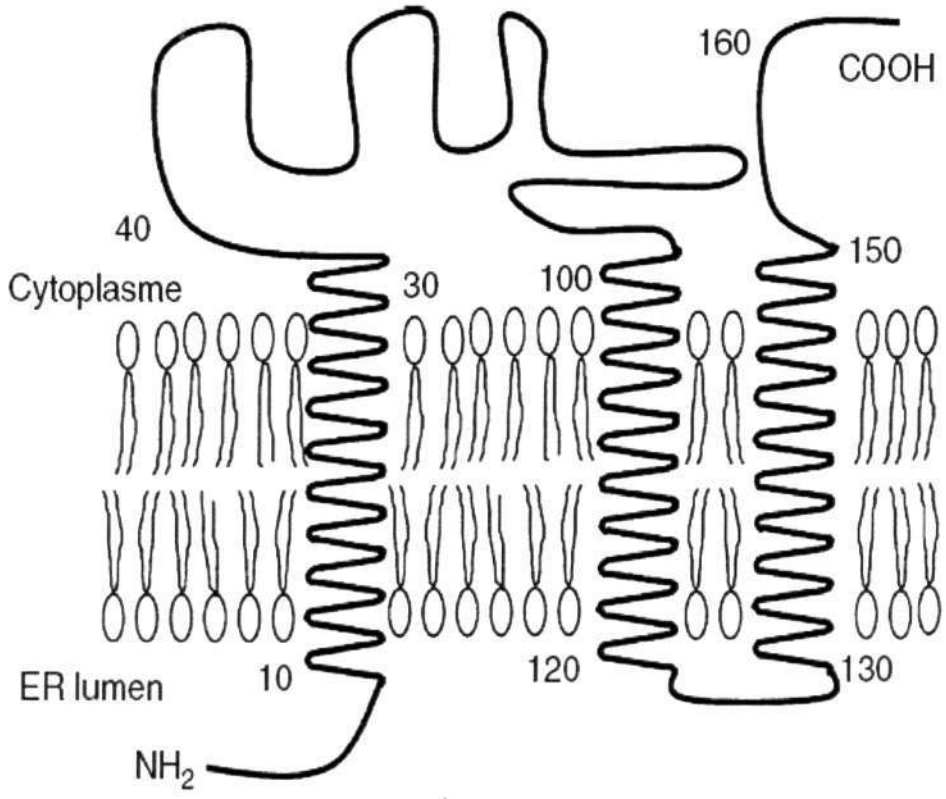


Figure 11: Structure of VKORC1 according to Stafford (2005) [16]

Reduced vitamin K is recycled by VKORC1. Reduced vitamin K activates the clotting factors II, VII, IX and X as well as proteins C, S and Z with the help of γ - glutamyl carboxylase (GGCX) [37], [38].

VKORC1 variation can be considered as guidance in determining dose need for the patients under warfarin therapy [39].

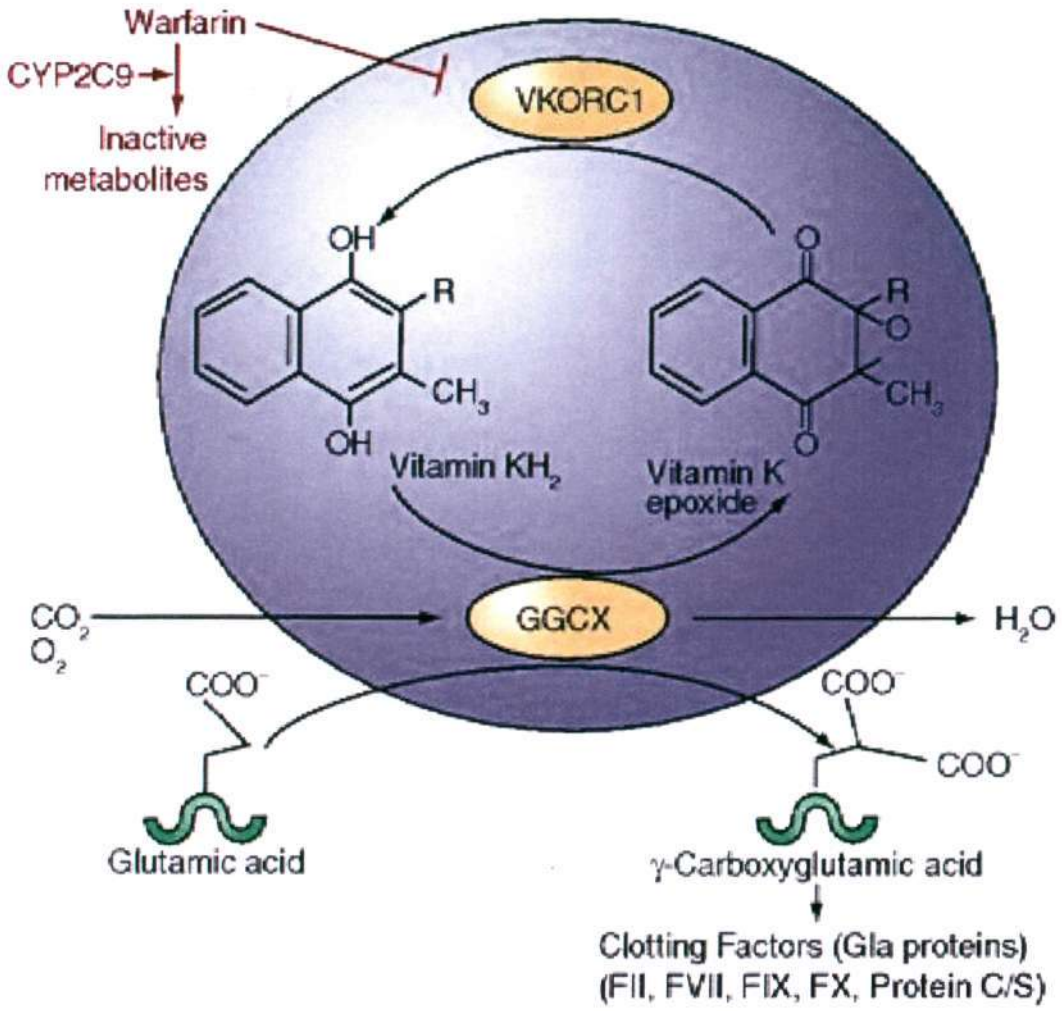


Figure 12: Effect of CYP2C9 and VKORC1 in Mechanism of warfarin. Warfarin acts as the inhibitor of VKORC1. VKORC1 is the agent that reduces vitamin K. Reduced vitamin K activates the clotting factors. Therefore warfarin prevents blood coagulation by afflicting the synthesis of clotting factors [9].

Genetic polymorphism in VKORC1 has been reported in several recent studies to have notable role on warfarin dosage [40].

In complete genotype VKORC1 gene analysis done in European patients has been shown that patients with H1 and H2 genotypes required low-dose of warfarin, and

patients with H7, H8 and H9 genotypes required high-dose of warfarin. These data configured two distinct evolutionary groups that were specified as taxonomic group A (H1 and H2) and group B (H7, H8 and H9) [40].

The overall studies carried out on genetic polymorphisms of CYP2C9 and VKORC; indicate that two thirds of variation in warfarin dosage amount is mainly caused by these genes plus environmental factors. However, more than one-third of the variability between individuals is not taken into consideration [15].

3.3) GGCX Gene

GGCX is an enzyme mostly found in Golgi apparatus and rough endoplasmic reticulum. GGCX plays an essential part in vitamin K cycle. Vitamin K–dependent clotting factors are activated by this enzyme [41].

Genetic polymorphisms in GGCX are not well clarified. It appeared that variations in GGCX gene have less effect compared with other candidate such as CYP2C9 and VKORC1 genes in warfarin therapy [32].

Figure 13 shows that how vitamin K epoxide reductase and γ-glutamyl carboxylase participate in metabolism of warfarin.

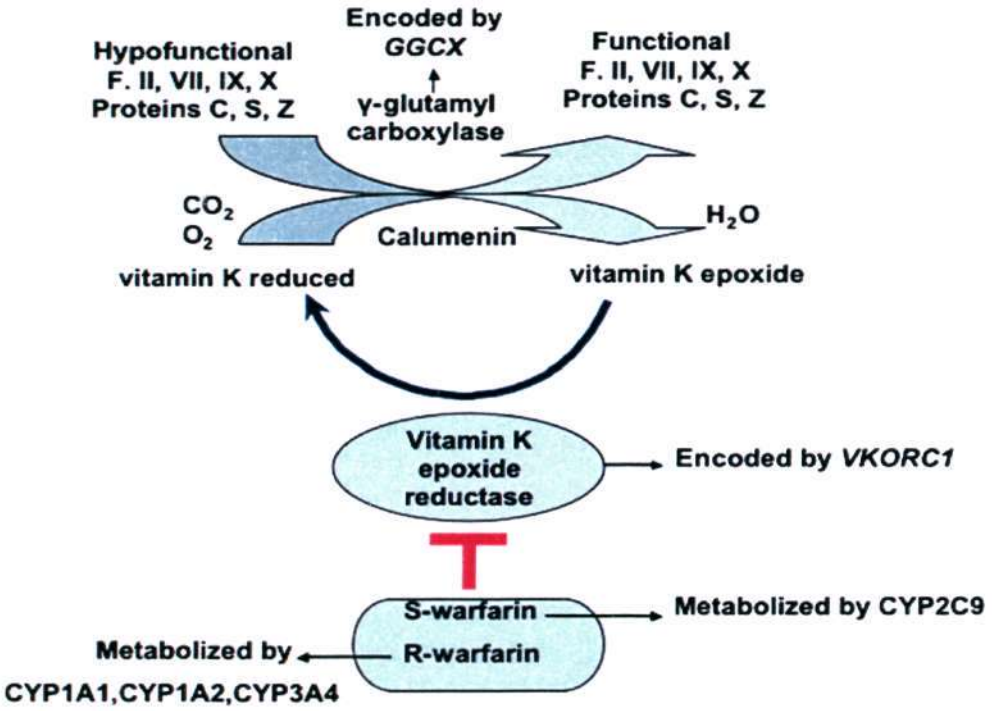


Figure 13: Warfarin Metabolism Pathway [34]

3.4) APOE Gene

Apolipoprotein E (APOE), the main apoprotein existed in the chylomicron, is essential in the optimum catabolism of triglyceride-rich lipoproteins. It binds to a specific receptor found on liver cells as well as peripheral cells. APOE receptor controls the concentration of vitamin K in plasma [42].

As vitamin K is soluble in fat, In the process of its uptake APOE should play a critical role. Lipoproteins containing APOE are considered as the main carrier of vitamin K in the blood. They bind to receptors in the membrane of cell with the help of Apo E and are absorbed into liver cells [42].

Vitamin K is reduced by vitamin K epoxide reductase in the liver. Reduced vitamin K is important factor for activation of clotting factors by γ-glutamyl carboxylase. In this stage vitamin K is oxidized but, reduced vitamin K is regenerated in the next cycle [42].

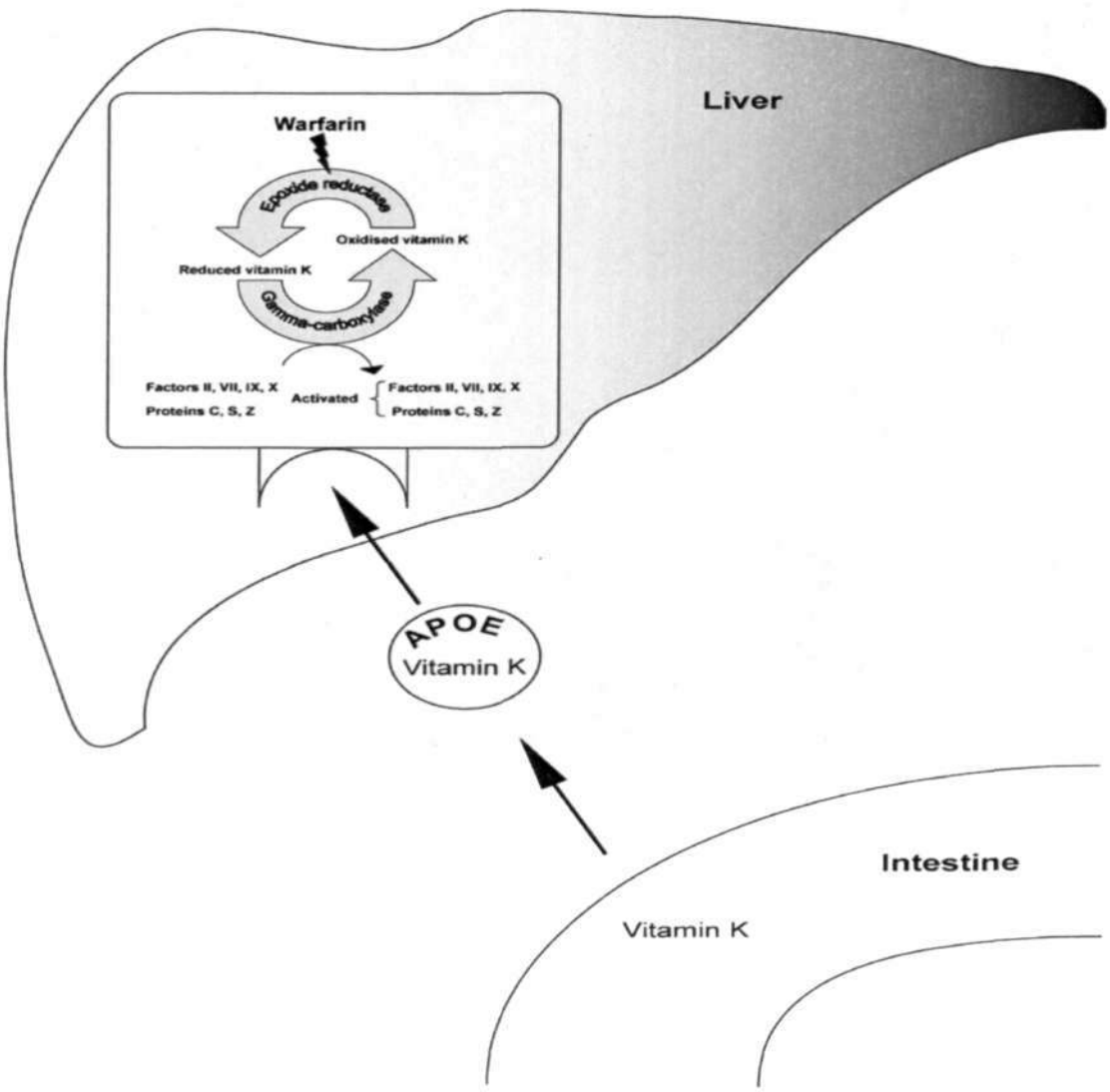


Figure 14: Vitamin K absorption through intestine to liver [42]

Vitamin K is attached to APOE receptors and carried from intestine to liver where vitamin K cycle occurs [42].

High intake of vitamin K is able to invert the warfarin performance. Vitamin K is attached to APOE receptors and carried from intestine to liver [42].

Three major isoforms of APOE is defined as E2, E3 and E4 in human. The absorption ability of different types of APOE alleles is in the following order: *E2<*E3<*E4 [13].

Individuals with APOE*E4 allele have faster liver uptake of vitamin K and hence lower concentration of vitamin K in blood. So to compensate the high content of vitamin K in their liver, they need higher dosage of warfarin [13].

According to recent study done in 2007, it is suggested that due to low frequency of the E4 allele among Asian population the APOE variants does not have much influence on warfarin therapy in this group of patients [13].

The roll of other members of CYP family is now in process to be understood. Most of the researches in proper warfarin dosing which have been done to date are involved in genotyping; however finding suitable biomarker for recognizing low and high-dose patients which let us predict in more accurate and sensitive and faster way can help medicine world a lot.

3.5) Influence of Ethnicity on Warfarin Therapy [30]

Race and ethnicity also impact warfarin sensitivity. For example, Chinese and Malays need lower warfarin dose compared to Indians patients. African-Americans require a higher dosage of warfarin. A lower dose of warfarin is required in Hong Kong Chinese patients compared with Caucasian patients.

Studies have shown that variation in CYP2C9 and VKORC1 genes results in different dosage requirement of warfarin.

CYP2C9*2 has not been reported in Chinese and Inuit populations, but it is observed in Native Canadian, Indians and Caucasian at frequencies of 0.03, 0.08 and 0.15 respectively. CYP2C9*3 is absent in Inuit people, but have been found in Native Canadian and Indians. Japanese patients have shown no CYP2C9*2 and the frequency of CYP2C9*3 is very low in this group. In East Asian populations, such as Chinese, Malays and Koreans the frequency of CYP2C9*2 and CYP2C9*3 alleles are very rare (0-2%). So these two allelic forms have minimal effect in warfarin therapy in Asian patients.

Previous studies explained that lower warfarin dose is related to CYP2C9*2 and CYP2C9*3 alleles, but the frequency of these alleles are very rare in Asian patients.

So these findings proved that the CYP2C9 variants cannot consider as a reason for lower warfarin dosage requirement in Asian ethnic groups. And defiantly there are other factors affecting warfarin dosage in this group.

Other variants of CYP2C9 have been found in some other populations. For instance, CYP2C9*5 is identified only in African-American ethnic groups.

Recent studies signify the roles of VKORC1 in warfarin therapy of different ethnic groups. For example, in Chinese patients under warfarin therapy the frequencies of VKORC1 AA genotype is 80%, AG genotype is 18% and GG genotype is 2.7%.

But these frequencies are completely different in Caucasian (AA: 14%, AG: 47%, GG: 39%).

Analysis of VKORC1 shows that the genotype frequencies in African-American compared with Chinese are significantly different. For example, frequency of VKORC1*2 in Europeans is 42%, in Chinese is 95% and in African-American is 14% [30].

All of these findings show that the VKORC1 has a key role in warfarin therapy of different ethnic groups. So far, the effects of VKORC1 and CYP2C9 on warfarin have been investigated mostly in Caucasians. Therefore, the impacts of these two genes on other ethnic groups are not obvious yet and need more investigations.

4) RESEARCH METHODOLOGY

4.1) Proteomics vs Genomics

Proteomics, study of proteome, is involved in both qualitative and quantitative assessment of proteins in large scale. In fact, it can be reflective of cell function and will help us overcome the limitations we may face in other techniques such as in genomics study. There are some limitations in genomics, which force scientists to be much more interested in proteomics, which lead to giving a much better understanding of an organism. These limitations, which are resolved in proteomics, can be categorized as below:

First, transcription level cannot give complete information about protein level which is crucial. For instance, resulted mRNA is prone to degradation or improper amount of protein may be taken as a result of inefficient translation.

Second, activities of a protein may be affected to high extent by some modifications which are very probable to take place after translation e.g. phosphorylation as one of post-translation modifications can change a protein to its active form.

Several approaches can be used to study post-translational modifications such as phosphoproteomics and glycoproteomics.

Third, there is a natural happening called alternative splicing in which many mRNA transcripts generate more than one protein.

At last, in many cases the appropriate function of proteins can be fulfilled only in relation with other proteins or other molecules such as RNAs, meaning after forming complexes [26].

Proteomics with lots of applications in practical aspects has become promising methods these last years. Proteomics studies besides genomics can be considered as useful tools in medicine for identification of new drugs in order to treatment of a disease. Finding a suitable biomarker with the help of proteomics technology is another application, which is outstanding in biomedical studies. In fact, biomarker is as a biological component which can be protein, metabolite or specific post-translational modification. Therefore, in the field of biomarker discovery, MS-based proteomics can be named as a useful tool. Indeed, protein biomarkers can cause a breakthrough in disease diagnosis which might be as a result of proteomics. It is of high importance in detecting a disease and further measuring its progression which in fact means the effect of a treatment [22], [43].

It is significant to note that a biomarker should have the capability of being easily accessible or in better words to be present within body fluids; besides, it must be sensitive and specific enough to provide accurate distinguishing between true positive, and false positive, as well as false negative. Furthermore, detection of the biomarker should clinically benefit the patient [21].

With proteomics guide, we will face new avenues to clarify unknown mechanisms for diseases. It will also help us identify new markers for diagnosis approaches as well as therapeutic aims [20].

Mainly there are two ways for studying proteins in proteomics analysis: one is determining those proteins which are modified post-translationally and the other one is finding out specific protein existence when it is in mixture with other complex proteins.

Typically, there are two steps in proteomics studies:

1) Separation of proteins done by two-dimensional electrophoresis or liquid chromatography
2) Identification of separated proteins which in turn includes characterizing post-translational modifications done by MALDI-TOF-MS or MS.

Proteomics studies have two main approaches:

a) Protein separation done by 2-dimensional electrophoresis (2-DE) in which protein are separated by two properties in two dimensions on 2-D gels, combined with identification of proteins mainly done with the help of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (abbreviated as MALDI-TOF-MS)
b) Protein/peptide dentification mainly done with help of two-dimentional liquid chromatography followed by either electrospray ionization (ESI) or tandem mass spectrometry (MS).

Although combination of separation technologies and identifications methods has also frequently been used, proteomics is still under development due to some limitations.

These limitations can also be divided to two distinct types:

I) Limitations linked to the structure of protein of interest in the mixture going to be figured out, mainly in the matter of protein expression levels.

II) Limitations concerning analysis. In proteomics, in one hand, we aim to enrich low-amount proteins to gain a valuable tracking. In the other hand, we try to apply appropriate analysis in order to have a suitable visualization of all of the existing proteins.

Note that low-abundant proteins are hard to detect, so their visualization requires enrichment steps like affinity chromatography and specific antibody [44].

From medical and also experimental point of view, plasma as body fluid is of high importance, since it contains many molecules which can provide lots of biomedical information and can be considered as indicator of the body activity. In fact, plasma is in communication with many cells of which large numbers of them release some part of their content into plasma. Although plasma contains valuable information, its proteomic analysis is laborious. We can explain it in this way that plasma which contains around 10 proteins of high abundance (totally around 98% of all existing protein. Of the rest (about 2%), may be less than 1% can be considered as main targets for identification of new biomarkers [45]. In other word, we can say more than 95% of protein concentration of plasma is due to a few proteins which are found in high abundance and form a large proportion of all plasma protein like albumin, immunoglobulins, transferrin, α1-antitrypsin and haptoglobin [46], [47].

Looking for a suitable disease biomarker in plasma is not easy and we need to get rid of minimum around two or three of these abundant proteins in plasma. One way for depleting these proteins is chromatography [48], [49].

4.2) Liquid Chromatography-Mass Spectrometry (LC-MS)

As mentioned earlier, protein identification commonly can be fulfilled by mass spectrometry technique.

Success in protein identification depends on many factors such as mass spectrometer accuracy or database availability and modifications which might take place after translation as well as other affecting factors [50].

During last 20 years, our information and understanding about protein chemistry has been increased a lot and we owe all of these progresses to advances in instrumentation of MS and related techniques. MS has been successful in finding its position in researches and experiment and has been selected to analyze samples containing mixture of proteins [23].

Use of liquid chromatography-mass spectrometry (LC-MS) would be the suitable choice in detecting low molecular weight proteins. In two-dimentional gels, low-molecular weight proteins together with heavy proteins are not detected efficiently [23].

It is interesting to know that regarding the size of molecules which go under measurement by the device; MS is considered as the smallest system [23].

During recent several years, MS has technologically improved a lot which gives the permission for its usage in the field of proteins, peptides, DNAs, carbohydrates as well as many other biological molecules such as drugs. The sources which help molecules to be ionized like matrix assisted laser desorption/ionization (MALDI) as well as electrospray ionization has made MS a distinct and vital method in biological studies [23].

Mass spectrometer plays crucial role in MS and has the ability to determine the mass of molecule such as protein by measuring m/z in which m is mass of the molecule while z indicates its charge. As we know, inducing molecules to take or lose charge will lead to form an ion, negative or positive respectively [23].

MS technique has the ability to measure m/z ratio in high accurate way and in fact this measuring provide spectra with high resolution. Developing of mass spectrometry gave rise to tandem mass spectrometry (MS/MS) providing obtaining of information from de novo protein sequence. In fact this progress (tandem mass spectrometry) caused enhancement of MS application in proteomics studies. The complexes of two mass analyzers make this machine more sensitive and accurate. Another tremendous progress in mass spectrometry was joining of high performance liquid chromatography (HPLC) to this technique which led to extraordinary and powerful technique (LC-MS) with lots of applications in which both sensitivity and accuracy are achieved [23].

Indeed, LC-MS has merged the power to physically make the molecules apart taken from LC together with separation based on mass taken from MS.

Generally there exist 4 major parts in LC-MS system including:

1) Chromatography column(s): allowing pre-separation of peptide mixtures based on one or more physico-chemical properties

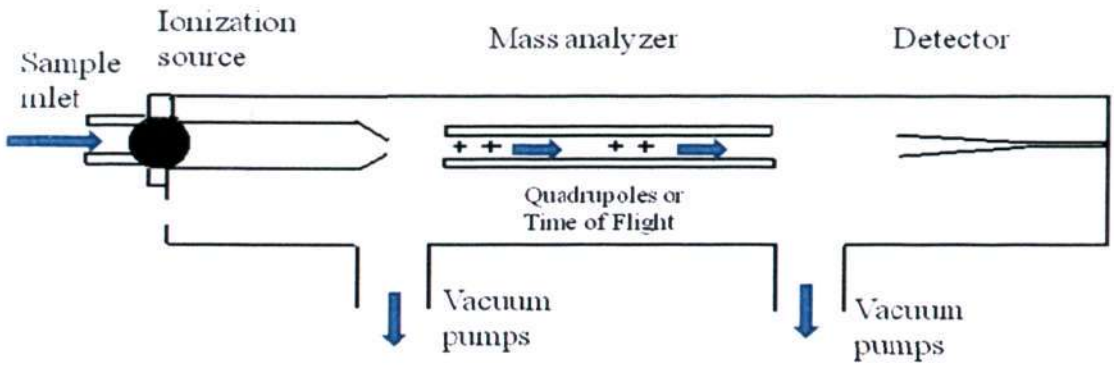
2) Ionization source: converting eluted peptides into ions in gas phase

3) Mass analyzer(s): separate ions based on m/z ratio

   Different mass analyzer are possible to be applied in LC-MS analysis, such as ion trap, single and triple quadrupole, time of flight and also quadrupole-time of flight (Q-TOF). Among these analyzers, quadrupole and time of flight (TOF) are the only two mass analyzers that applied in our two-dimentional 2-D LC-MS/MS.

4) Detector: enabling detection of the relative abundance of ions at discrete m/z. Detector has the ability to provide electrical signals from ion energy. These signals then can be transferred to a computer.

Figure 15 shows the different parts of the mass spectrometer.



Smzdak, G. (2003) *The Expanding Role of Mass Spectrometry in Biotechnology* .San Diego, California. MCC press

Figure 15: Components of a mass spectrometer [23]

In Liquid chromatography, peptides are separated based on their physiochemical characterization. Liquid chromatography includes four columns and two pumps. Two main columns are used in two-dimensional LC-MS\MS. One is a strong cation exchange (SCX) applied in the first one dimension and the other one is reversed-phased column used for the second dimension one. Moreover, it contains 2 identical columns called enrichment columns by which trapping of peptides done (ZORBAX 300SBC18 enrichment column 1 and ZORBAX 300SB-C18 enrichment column 2) [51].

High performance liquid chromatography (HPLC) which has lots of application in analytical chemistry and biochemistry is categorized as a type of column chromatography and been used for separating and identifying compounds as well as determining the quantity of them.

Two pumps with different characteristics have been applied in HPLC: one is isocratic pump associated with buffer C (95% acetonitrile, 0.1% formic acid). The other pump called nano pump and consists of two buffers: buffer A (5% acetonitrile, 0.1% formic acid) and buffer B (0.1% formic acid).

These pumps produce solvent gradient for better separation in complex mixtures of compounds [51].

In the analysis of certain classes of protein, LC–MS can be considered as a complementary technology for two-dimensional gels/MALDI-TOF-MS. In comparison between 2-D electrophoresis with mass spectrometry, we can see that it has not progressed to the same amount as mass spectrometry. Limitations of two-dimensional gels in detection of different proteins with different characteristics of pH, size and hydrophobicity have not yet been solved. Also it has possibility for overlapping proteins and limited dynamic range.

Using LC–MS, we can fulfil our goals to some extent and overcome part of these limitations. [44].

LC-MS/MS has many advantages such as:

- MS/MS information adds additional level of confirmation.
- Multiple proteins can be analyzed simultaneously with simple reversed phase LC run.
- Useful for Post Translational Modification (PTM) identification
- High coverage of proteins (30% to 90%) depending on the protein

- Good dynamic range especially for low expression level proteins
- Good for a wide range of proteins with different hydrophobicities
- MS/MS experiments performed automatically for reliable identification.
- Broader sample applicability (no sample derivatization).
- In addition to molecular mass data, tandem MS measurements are performed in real time. [23].

Also it has some limitations mainly associated with sample preparation and performance of the instrument including:

- Computationally intensive; large database searches can take hours to days; relatively slow.
- Moderate resolution separation and Ionization suppression
- It cannot measure certain classes of protein.
- Limited application shown for differential protein expression (relative quantitation) with 2D LC/MS/MS for highly complex mixtures [23]

All of these limitations have raised the need for further development in order to establishment of a strong proteomics basis [44].

4.3) Determination of Protein Concentration [52]

The description in this section was similar if not identical to reference 52. In these experiments plasma was used as a sample with the concentration of 13μgr/μlit.

We measured protein concentration of plasma by using the 2-D Quant Kit. A beneficial tool for exact measurement of protein concentration is the 2-D Quant Kit.

This analysis is generally on the basis of specific attachment of copper ions to proteins. Proteins which are precipitated are isolated and diluted in a copper containing solution and the measurement of the unbound copper is carried out with a colorimetric element. The colour density and the protein concentration related inversely. The analysis shows a linear reaction between 0 and 50 µg.

2-D quant Kit includes the following components:

Precipitant: renders proteins insoluble

Co-precipitant: Reagents and materials components of this reagent co precipitate with proteins

Copper solution: This solution is used for re-suspension of precipitated proteins

Colour reagent A: Reagent A is mixed with reagent B to indicate unbound copper amounts.

Colour reagent B: Reagent B is mixed with reagent A to indicate unbound copper amounts.

Standard Bovine serum albumin: For preparation of standard curve

We follow the protocol for determining protein concentration.

1) Preparing working solution (100 part color reagent A + 1 part color reagent B) it should be stored at 4 °C.
2) Standard procedure: We use 6 standard tubes and prepare a standard curve according to this table.

Tube number	1	2	3	4	5	6
Volume of standard solution	0 µL	5 µL	10 µL	15 µL	20 µL	25 µL
Protein quantity	0 µg	10µg	20µg	30µg	40µg	50µg

3) Add the precipitant to an amount of 500 µL, vortex them and then incubate the tubes at room temperature for 2-3 min.
4) 500 µL of co-precipitant is added to the next tube and vortex them.
5) Centrifuge the tubes at 10 000 × g for 5 min.
6) In this stage the pellet can be seen. Remove the supernatants.
7) Re-centrifuge the tubes at 10 000 × g for 2 min. use micropipette to remove the remaining supernatant. The visible liquid should not remain in the tubes.
8) 100 µL of copper + distilled H20 is added. Then vortex the tubes.
9) For each tube add 1ml of working solution and subsequently mix by inversing the tube.
10) Incubation for 15-20 min at room temperature.
11) Using the spectrophotometer and measure the absorbance at 480 nm for each samples.

4.4) Sample Preparation

First, 10 µlit of the patients' plasma (low or high dose) are added into first three tubes. The forth tube contains the normal plasma as a control. Then acetone is used in order to precipitate the proteins in plasma samples (The volume of acetone must be 5 times of volume of samples). Next step is putting samples at -20 ºC for 2hr. After this step the proteins must be precipitated by addition of the acetone. Then tubes were centrifuged at 12900 rpm for 5 min. After removing the supernatant we continued by following the protocol as below:

A) Protein Reduction and Cysteine Blockage

1. The amount of 20 µL from "Dissolution Buffer" should be added to each sample (up to four tube) following by 1 µL denaturant.
2. a. Add 2 µL Reducing reagents to each sample tube.
   b. Mix the samples well by vortexing and then centrifuge them.
   c. One hour incubation should be done at 60 ºC.
   d. Centrifugation should be done.
3. a. Add 1 µL Cysteine Blocking Reagent to each tube.
   b. Mix the samples by vortexing and then centrifuge them.
   c. Room temperature incubation should be performed for 10 min.

B) Digest the proteins with Trypsin

1. a. Use 80 µL of Trypsin Resuspension Buffer, and solve the pure Trypsin on it.
   b. Mix the samples by vortexing and then centrifuge them
2. a. Add 20 µL of the Trypsin solution to each sample tube.

b. Vortex to mix, then spin.

c. Centrifuged at 12900rmp for 1 min.

d. 37 ºC incubation should be done for 12-16 hours (overnight)

C) Protein Digests Labelling Step with Reagents of iTRAQ

1. a. Allow Put iTRAQ reagents which are needed in room temperature for a while required to reach room temperature. (iTRAQ reagents: 114,115,116,117)

b. Centrifuge the tubes.

2. a. Add 70 µL of ethanol to each iTRAQ reagent required.

b. Vortex to mix, then spin.

3. a. Mix one iTRAQ reagent contents with one tube of samples. Sample labelling is done as below:

normal = iTRAQ 114, L1 (low dose) = iTRAQ 115, L2 = iTRAQ 116, L3= iTRAQ 117.

b. Mix the samples by vortexing and then centrifuge them.

c. Room temperature incubation for one hour.

D) Mixing of Protein Digests Previously Labelled with iTRAQ Reagents

1. a. Take a new tube in which you mix all the contents of every tube which is labelled in previous step.

b. Vortex to mix, then spin.

Now the sample is ready for injecting to the machine.

Figure 16 schematically shows the procedures done in our experiments.

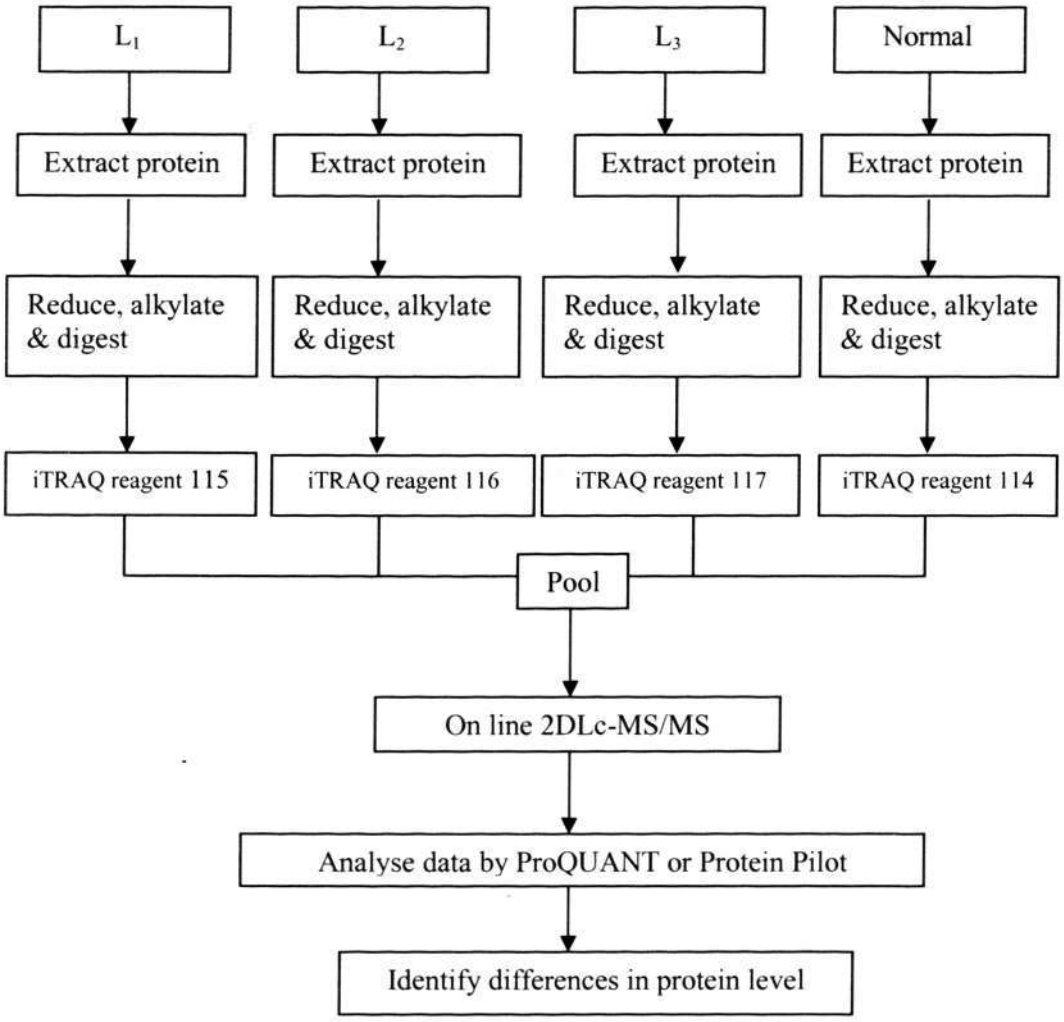


Figure 16: Workflow for the identification of changes in the proteome (using iTRAQ reagents) [51]

4.5) On-line 2-D Nano LC-MS/MS Analysis [51].

This assay was done by Agilent 1200 nanoflow LC system (Agilent Technologies) that has an interface of QSTAR XL mass spectrometer (Applied Biosystems/MDS Sciex). Primarily, separation was performed in the LC section of the system by loading 3 µL combined protein mixture to the PolySulfoethyl A SCX column. KCL salt is a component of Buffer D which is present in increasing concentrations

according to the protocol. We used this buffer by consecutive injection in order dilute peptides derived from the SCX column [51].

During the first run the 10-port valve is in position 1 (Figure 17). The flow-through containing peptides that were not binding to the SCX column was fixed on the ZORBAX 300SBC18 enriching column1. Subsequently a wash step is performed for 100 min with buffer A (containing formic acid + acetonitrile) at the rate of 0.005 mL/min) was done for getting rid of any excess reagent. Meantime, ZORBAX 300SB-C18 enrichment column 2 was changed into the path of nanopump. No peptides were trapped on the column 2; therefore, logically no peptides were expected to be observed after 100 min of running [51].

In the second run, the place of 10-port valve was put into position 2 (Figure 18). Volume of 5 µL from 10 mM KCL solution was injected into the SCX column in order to elute retained peptides to column 2, which was washed with the loading buffer according to the protocol for removing extra reagent. The nanopump was connected to Column 1 containing the unbound peptides from the first run. Then elution of buffers was carried out by buffer B (made from formic acid) and buffer C (composed of gradient of acetonitrile + formic acid). Increasing concentration of acetonitrile was used for elution of samples which were concentrated [51].

Then, additional separation was performed on Zorbax 300SB C-18 reversed-phase column [51].

After that peptides moved to the ionization source, they were ionized just before to introduction into mass spectrometer. After this step, ions were exposed to the mass

analyzers and so ions were separated based on m/z ratio. Finally, the ion energy is converted to electrical signals which will be then sent to a computer [51].

Totally, the quality of achieved profiling studies is determined by considering all of the factors like overall sensivity, detection and fragmentation efficiency and dynamic range, as well as mass resolution and accuracy [23].

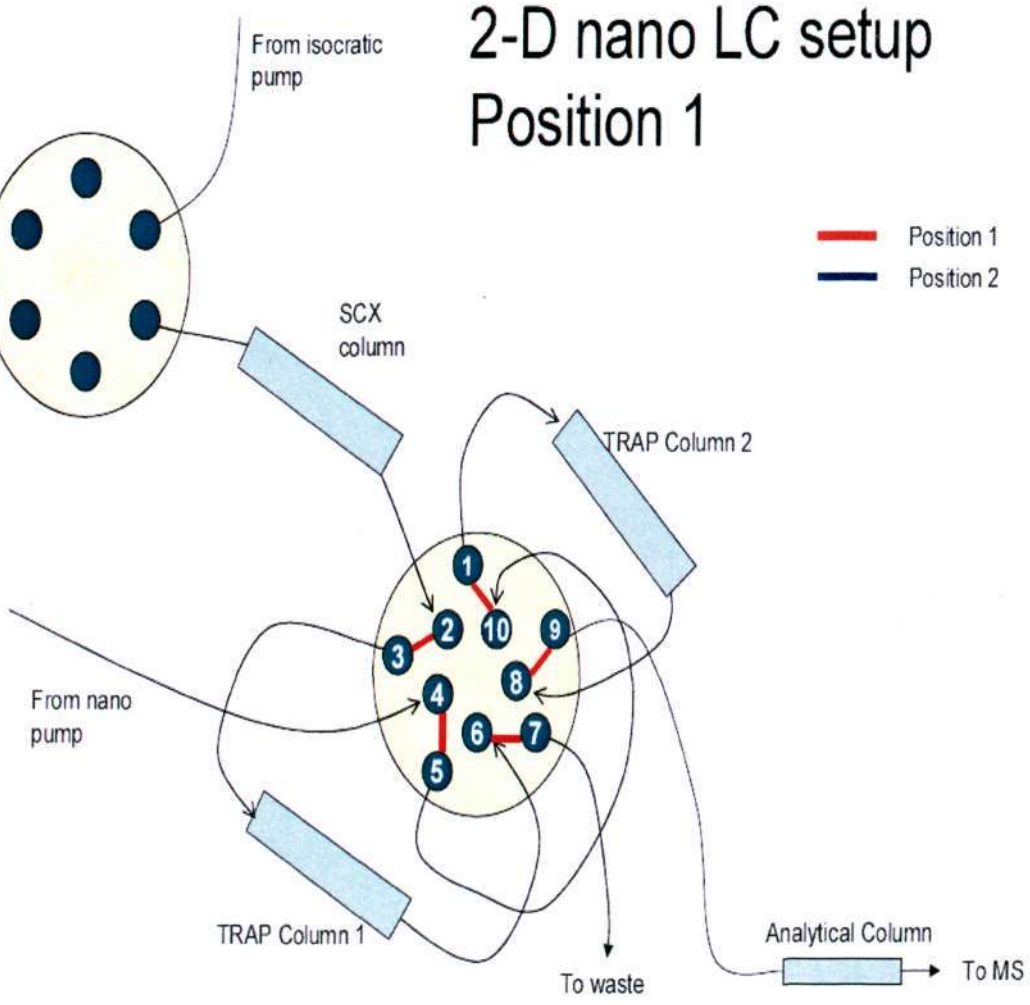


Figure 17: Position 1 in 2-D nano LC setup [51].

(See page 50 for detailed explanations)

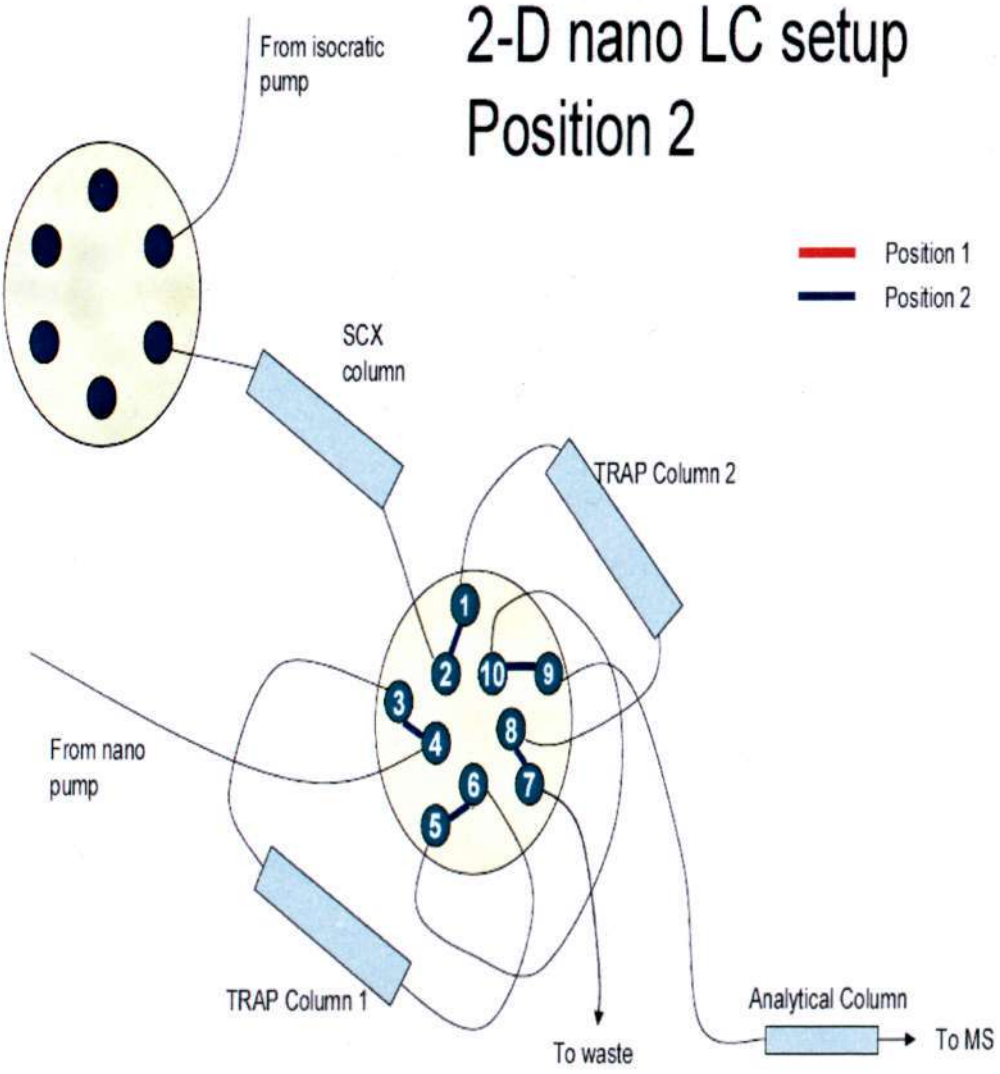


Figure 18: Position 2 in 2-D nano LC setup [51].

(See page 50 for detailed explanations)

4.6) Data Analysis and Interpretation [51]

We have used ProteinPilotTM Software 2.0 (Applied Biosystems, Software Revision 50861) to measure the relative abundance as well as peptide and protein identification.

The factors used for our research as basis are [51]:

- Some fixed modification such as that of cysteine which is labelled by methylmethanethiosulfate and also modification of iTRAQ which is related to N-terminal free amine and lysine
- Changeable modifications of iTRAQ related to tyrosine.

There are some other factors which are default by the applied software(Protein Pilot) like being unique for cleavage by trypsin as well as precursor and fragment ion mass accuracy are already default [51].

Relative concentration of peptides in the samples was analyzed by dividing the monitored peak areas at 115.1, 116.1, and 117.1 m/z by what was monitored at 114.1 m/z [51].

Subsequently the ratios of the mentioned calculated peak were corrected for their overlap in isotopic contributions, and used for the estimation of relative amounts of a peculiar protein. For proteins which had matching in more than two qualified peptide, 3 intermediate peak area ratios (specified as 115/114, 166/114, and 177/114) were calculated using the peak area ratios of the peptides originating from the same protein [51].

Sequence coverage is the ratio of number of observed amino acids / length of protein amino acid. The other factor would be error factor (EF) which tells the error in ratios [51].

Some necessary criteria must be taken into account for extra statistical analysis of protein like [51]:

- Identification of more than two unique peptides which are high confidence should be done.
- P value should be less than 0.05 and the difference also should not be more than 1.5 fold.

Finally, proteins chosen as candidates should be checked the software's protein ID. Those peptides which do not possess modifications of N-terminus free amine or modification of iTRAQ related to free amine in lysine were not taken into account in the calculation of protein ratios [51].

5) RESULTS AND DISCUSSIONS

Recognizing specific protein changes in response to drug administration in humans has the potential for the development of personalized medicine.

Optimizing dosage of warfarin has remained a challenge in the clinical setting. Routine monitoring parameters such as INR has limited value in the optimization of doses in individual patients. Target genes of warfarin therapy include Vitamin K 2, 3-epoxide reductase (*VKOR*) as well as Apolipoprotein E (*APOE*). Genetic polymorphisms in both genes have been shown to influence warfarin therapy. Although such genotyping can be advantageous for effective warfarin therapy, the analysis can be time consuming. Specific proteins (biomarkers) present in either low- or high-dose patients may represent an added advantage. The combination of both genotyping and biomarkers may further improve the accuracy of the prognosis.

The iTRAQ-coupled 2D LC-MS/MS, recently applied in the analysis of protein profile in the cell responding to incubation with different chiral drugs, such as propranolol, ibuprofen and atenolol, was extended into our investigation.

28 patients requiring low dose of warfarin and 28 patients requiring high dose of warfarin were selected in this study (totally 56 patients).

Genotyping analysis revealed distinct differences for *VKOR* gene (mostly H1/H1 in low-dose while mostly H1/H7 in high-dose) but not for *APOE* gene.

In this study most of the patients carried ε3/ε3 genotype which is in relation with clearance of vitamin K. APOE ε3/ε3 is considered as a main genotype among Asians.

Isoforms of APOE might be unimportant in Asian patients who are under treatment with warfarin, as the frequency of the E4 allele is low in this group. ε4/ε4 genotype is in relation with faster clearance of vitamin K . In individuals with APOE*E4 allele, liver uptaking of vitamin K is faster than others and its circulation is also lower. So for compensating the high content of vitamin K in their liver, they need higher dosage of warfarin [13].

Genotyping analysis of *VKOR* gene revealed that H7 genotype is in relation with high dosage in warfarin treatment but H1 is related to lower dose [13].

Their serum samples were then analyzed with iTRAQ-coupled LCMS/ MS analysis in three independent experiments. Totally 163 proteins were recognized in the first batch and 86 proteins in the second batch.

The proteins in low and high-dose patients are:

Fibrinogen alpha chain precursor, Fibrinogen gamma chain precursor, Fibrinogen beta chain precursor, Fibronectin precursor (FN), Haptoglobin precursor, Hemoglobin subunit beta , Hemoglobin subunit alpha, Apolipoprotein A-I precursor, Alpha-2-macroglobulin precursor, Alpha-1-antitrypsin precursor and Transthyretin precursor.

In the following part the function of these proteins is summarized:

Name of proteins	Function
Alpha-2-macroglobulin	A large protein of plasma which is found in the blood. It is produced by the liver. Alpha-2-macroglobulin is able to inactivate an enormous variety of proteinases. It is preventing coagulation by inhibiting thrombin [53].
Fibrinogen (gamma/beta/alpha chain)	Fibrinogen has two functions: yielding monomers that polymerize into fibrin and acting as a cofactor in platelet aggregation [54], [55].
Fibronectin	They can bind to cell surface as well as collagen, fibrin, heparin, actin and DNA. They have role in cell adhesion and its motility, also in opsonization process and keeping the cell shape [56].
Hemoglobin subunit alpha/beta	Involved in oxygen transport from the lung to the various peripheral tissues [57].
Apolipoprotein A-I	It is one of the major protein in HDL cholesterol issue which has role in transportation of cholesterol to liver as well as lipid transportation [58].
Haptoglobin precursor	Synthesised by some tissues like: skin, lung and kidney. It is mainly produced by hepatocytes. In plasma it binds to free hemoglobin and prevents the loss of iron from the kidneys [59].
Alpha-1-antitrypsin precursor	It is a plasma protein found in the blood. It inhibits the serine protease and elastase. It has affinity for plasmin and thrombin [60], [61].
Transthyretin precursor	A protein found in the plasma and cerebrospinal fluid. It is produced by the liver and the choroid plexus of the brain. Transthyretin precursor is indeed a binding protein for thyroid hormones and delivers thyroxine of blood to brain [62].

Among the protein levels investigated, Transthyretin precursor was shown to be significantly different among the patients requiring low- and high warfarin dose.

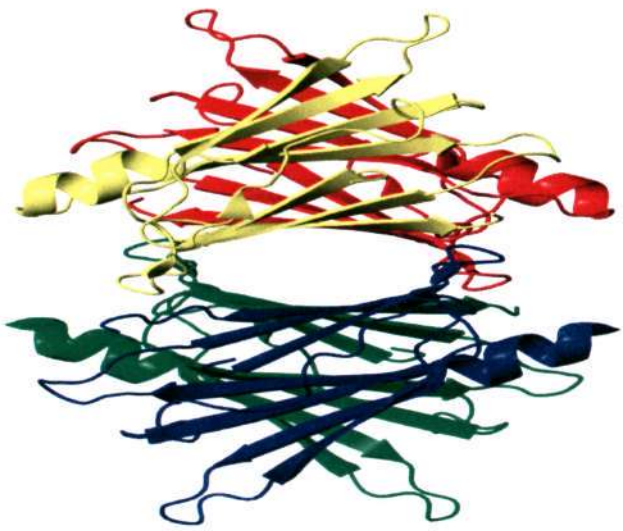


Figure 19: Transthyretin (prealbumin) [63]

Transthyretin (TTR), a protein located in plasma and cerebrospinal fluid, is primarily manufactured by the liver and choroid plexus of the brain. Its former name was prealbumin as in running on electrophoresis gels it runs faster [64].

It is indeed a binding protein which can bind to thyroid hormones and has role in delivery of thyroxine from blood to brain. Thyroid hormone (thyroxine) increases the anticoagulant efficacy. So the patients with high Transthyretin level potentially have the higher distribution of thyroxine in the system, resulting in higher anticoagulant effect [65].

This reflects the patients with high Transthyretin level require lower warfarin dose to reach effective anticoagulation compared to the patients with low Transthyretin level (Figure 20).

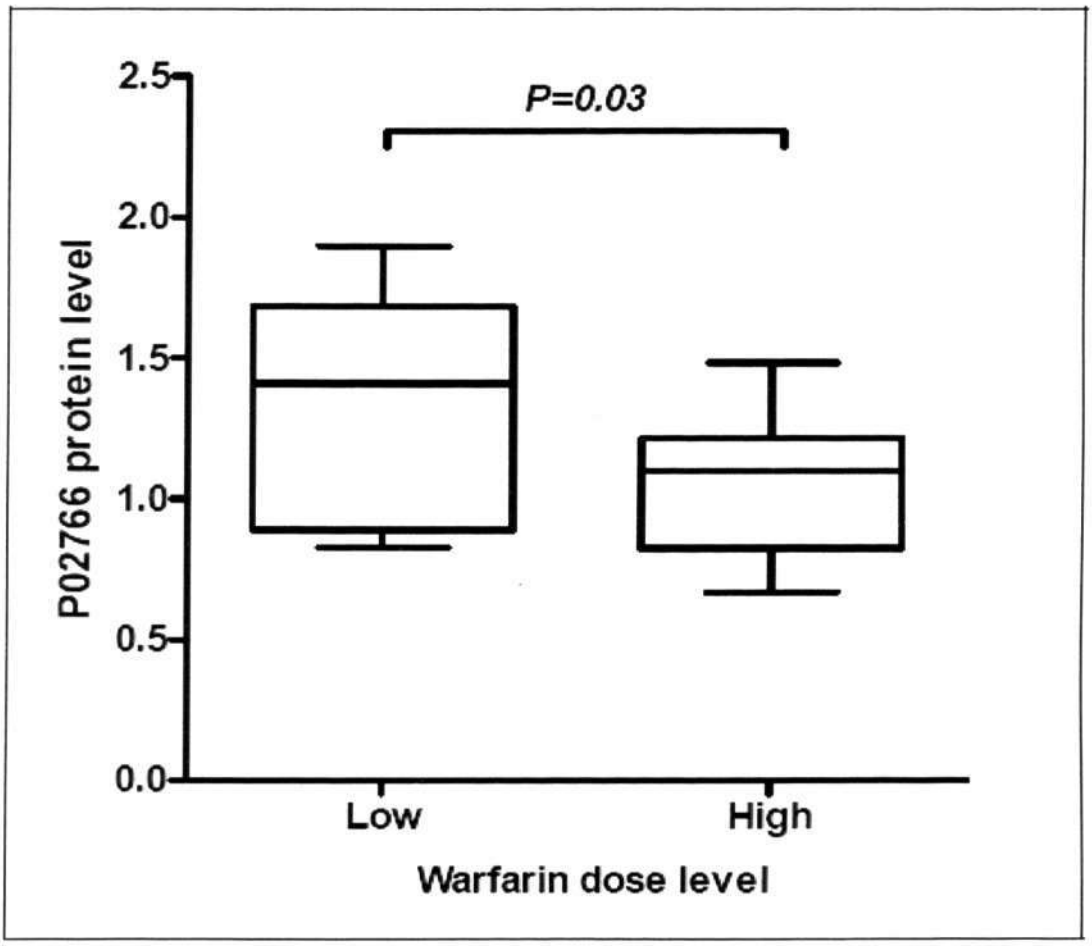


Figure 20: Identification of Transthyretin precursor as potential biomarker for warfarin treatment. Statistical analysis of LC-MS/MS data of protein profile in patients on warfarin treatment showed that the level of the Transthyretin precursor was found to be significantly higher in patients receiving low dose of warfarin ($p < 0.05$).

Table 8: Pharmacogenetic and pharmacoproteomics analysis of patients receiving warfarin. Profile of patients receiving high- and low-dose of warfarin is shown **(First batch)**.

Haplotypes in *VKORC* gene showed distinct profile in patients into low- and high-dose warfarin groups, whereas haplotype profile in *APOE* gene was similar in both groups of patients.

Low dose group samples (N =14)

Numbers	Subjects	Ethnicity	VKORC haplotype	APOE genotype	Daily Warfarin dose (mg)	Demographics Gender	Age
1	PHK 010	Chinese	H1H7	ε3/ε3	3.5	M	68
2	GHC 011	Chinese	H1H1	ε3/ε2	3	M	62
3	ETW 020	Chinese	H1H1	ε3/ε3	3	M	54
4	YKL 024	Chinese	H1H1	ε3/ε3	2	M	72
5	KSB 35	Chinese	H1H1		2	M	55
6	TKK 046	Chinese	H1H1	ε3/ε3	2	M	68
7	NKT 063	Chinese	H1H1	ε3/ε3	1.5	M	49
8	TTK 067	Chinese	H1H1	ε3/ε4	3.25	M	58
9	DSK 069	Chinese	H1H1	ε3/ε2	3	M	62
10	WDM075	Chinese	H1H1	ε3/ε3	2.5	M	63
11	LYHJ093	Chinese	H1H1	ε3/ε3	1.5	M	58
12	LLH 101	Chinese	H1H1	ε3/ε3	3	M	59
13	CGA 102	Chinese	H1H1	ε3/ε3	3	M	54
14	TSL 131	Chinese	H1H7	ε3/ε3	3	M	69

High dose group samples (N=14)

Numbers	Subjects	Ethnicity	VKORC haplotype	APOE genotype	Daily warfarin dose(mg)	Demographics Gender	Age
1	CHK 014	Chinese	H1H7	ε3/ε2	6.5	M	50
2	TCK 019	Chinese	H1H9	ε3/ε3	7.5	M	31
3	LLH 029	Chinese	H1H7		6	M	61
4	SHQ 032	Chinese		ε3/ε4	6	M	47
5	TSL 034	Chinese	H1H7	ε3/ε3	5.5	M	50
6	TYF 042	Chinese	H1H7	ε3/ε3	5.5	M	53
7	CYK 035	Chinese	H1H7	ε3/ε3	6.5	M	57
8	PHY 065	Chinese	H1H7	ε3/ε3	6.5	M	55
9	LSK 071	Chinese	H1H7	ε3/ε3	5.75	M	59
10	HEA 113	Chinese	H1H9		6	M	81
11	CSK 114	Chinese	H1H7		6.5	M	40
12	TPH 128	Chinese		ε3/ε2	5	M	55
13	TKL 129	Chinese	H1H1	ε3/ε3	5.5	M	39
14	CSY 130	Chinese	H1H7	ε3/ε3	7	M	47

Table 9: Pharmacogenetic and pharmacoproteomics analysis of patients receiving warfarin. Profile of patients receiving high- and low-dose of warfarin is shown **(Second batch)**.

Low dose group samples (N =14)

Numbers	Subjects	Ethnicity	VKORC haplotype	APOE genotype	Daily warfarin dose (mg)	Demographics Gender	Age
15	TST 028	Chinese	H1H1	ε 4/ε4	2	F	46
16	KAC139	Chinese	H1H1	ε3/ε3	1	F	66
17	LKL 009	Chinese	H1H1	ε3/ε2	3.5	F	66
18	YLK 127	Chinese	H1H1	ε3/ε3	3	F	67
19	LLH 101	Chinese	H1H1	ε3/ε3	3	M	59
20	YLK 27	Chinese	H1H1		3.5	M	53
21	KYC 054	Chinese	H1H1	ε3/ε3	2	F	73
22	TKK 046	Chinese	H1H1	ε3/ε3	2	M	68
23	LLH 015	Chinese	H1H1	ε3/ε3	2.5	M	73
24	TMF 050	Chinese	H1H1	ε3/ε3	2	F	53
25	NSH 049	Chinese	H1H1	ε3/ε3	2.5	F	70
26	PBC 108	Chinese	H1H1		2.5	M	73
27	GAT 121	Chinese	H1H1	ε3/ε2	3.5	M	72
28	LSK 123	Chinese	H1H1	ε3/ε2	3	F	75

High dose group samples (N=14)

Numbers	Subjects	Ethnicity	VKORC haplotype	APOE genotype	Daily Warfarin dose (mg)	Demographics Gender	Age
15	LSY 004	Chinese	H1H7	ε3/ε3	6	M	56
16	ACT 006	Chinese	H1H7	ε3/ε3	5	F	76
17	PON 047	Indian	H7H7	ε3/ε4	6	F	50
18	RAJA O56	Indian	H7H7	ε3/ε3	6.75	F	49
19	CCT 039	Chinese	H1H7	ε3/ε4	8.5	M	38
20	KLF 051	Chinese	H1H7	ε3/ε4	6.5	F	55
21	SUD 057	Malay	H1H7	ε3/ε3	4.5	M	29
22	SKTL 040	Indian	H7H7	ε3/ε3	10	F	64
23	VJY 041	Indian	H7H7	ε3/ε3	10.5	F	40
24	RGK 060	Indian	H7H8H9	ε3/ε3	9	F	44
25	SDJ 070	Indian	H7H7	ε3/ε3	7	F	49
26	SP 077	Indian	H7H8H9	ε3/ε3	5.75	F	58
27	BAM 083	Indian	H7H7	ε3/ε3	7	M	31
28	RBT 045	Malay	H1H7	ε3/ε3	5.5	M	47

Table 10: LC-MS/MS data (First batch)

	Low Dose Subjects Code	PHK 010	GHC 011	ETW 020	YKL 024	KSB 35	TKK 046	NKT 063	TTK 067	DSK 069	WDM 075	LYHJ 093	LLH 101	CGA 102	TSL 131	
	High Dose Subjects Code	CHK 014	TCK 019	LLH 029	SHQ 032	TSL 034	TYF 042	CYK 053	PHY 065	LSK 071	HEA 113	CSK 114	TPH 128	TKL 129	CSY 130	
Code	**Protein**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Dose**
P02671	Fibrinogen alpha chain precursor	0.559	0.632	0.598	0.636	0.613	0.513	0.61	0.641	0.585	0.618	0.6	0.584	0.595	0.494	L
		0.567	0.575	0.497	0.563	0.58	0.517	0.617	0.608	0.565	0.636	0.562	0.653	0.653	0.541	H
P02675	Fibrinogen beta chain precursor	0.643	0.666	0.662	0.725	0.691	0.604	0.63	0.661	0.601	0.628	0.593	0.662	0.667	0.563	L
		0.68	0.701	0.624	0.622	0.632	0.574	0.751	0.711	0.629	0.702	0.647	0.674	0.674	0.555	H
P02679	Fibrinogen gamma chain precursor	0.551	0.634	0.609	0.677	0.663	0.567	0.516	0.598	0.557	0.636	0.583	0.605	0.618	0.515	L
		0.601	0.624	0.535	0.592	0.652	0.569	0.609	0.625	0.534	0.624	0.563	0.581	0.634	0.486	H
P00738	Haptoglobin precursor	0.402	1.468	0.864	1.353	1.407	1.379	0.573	1.422	0.58	1.338	1.103	1.301	1.359	1.379	L
		1.003	1.251	0.955	1.117	1.612	1.46	0.992	1.408	0.415	1.614	0.423	1.386	1.899	1.658	H
P02751	Fibronectin precursor	0.378	0.324	0.392	0.437	0.399	0.39	0.531	0.483	0.5	0.47	0.554	0.407	0.374	0.358	L
		0.368	0.263	0.266	0.512	0.498	0.538	0.602	0.507	0.502	0.402	0.43	0.48	0.482	0.485	H
P68871	Hemoglobin subunit beta	0.838	0.697	0.828	0.557	0.758	0.809	0.918	0.636	1.103	0.544	0.787	0.523	0.668	0.621	L
		0.744	1.005	0.945	0.8	0.68	0.921	0.674	0.929	0.64	0.961	0.815	0.669	0.939	1.008	H
P69905	Hemoglobin subunit alpha	0.772	0.698	0.762	0.505	0.591	0.649	0.787	0.647	0.824	0.518	0.639	0.49	0.654	0.56	L
		0.544	0.709	0.62	0.683	0.613	0.744	0.532	0.659	0.526	0.79	0.634	0.509	0.726	0.838	H
P02766	Transthyretin precursor	1.7	1.459	1.712	0.86	0.849	0.828	1.601	1.53	1.358	1.671	0.997	1.08	1.897	0.921	L
		1.073	0.692	1.069	1.183	1.125	1.16	0.751	0.669	1.137	0.987	1.249	1.483	0.898	1.31	H
P01009	Alpha-1-antitrypsin precursor	1.021	0.92	0.907	1.03	1.105	1.091	1.252	1.084	1.029	1.127	1.03	1.032	1.024	0.994	L
		0.947	1.267	1.19	1.144	1.125	1.046	1	0.956	0.804	1.102	1.113	1.202	1.214	1.25	H
P01023	Alpha-2-macroglobulin precursor	0.909	0.902	0.845	0.826	0.949	0.723	0.894	0.93	0.845	0.994	0.89	0.909	0.843	1.106	L
		0.819	1.029	0.875	0.854	1.064	0.954	1.067	1.289	1.24	1.05	1.151	1.539	0.820	0.808	H
P02647	Apolipoprotein A-I precursor	0.901	1.052	0.936	0.783	1.142	0.872	0.95	1.059	0.888	1.044	1.023	0.929	0.819	1.042	L
		0.785	1.064	0.891	0.803	1.061	0.956	1.01	1.207	1.113	0.767	1.144	0.887	0.817	1.117	H

Table 11: LC-MS/MS data (Second batch)

	Low Dose Subjects Code	TST 028	KAC 139	LKL 009	YLK 127	LLH 101	YLK 27	KYC 054	TKK 046	LLH 015	TMF 050	NSH 049	PBC 108	YLK 127	LSK 123	
	High Dose Subjects Code	LSY 004	ACT 006	PON 047	RAJA 056	CCT 039	KLF 051	SUD 057	SKTL 040	VJY 041	RGK 060	SDJ 070	SP 077	BAM 083	RBT 045	
Code	Protein	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Dose
P02671	Fibrinogen alpha chain precursor	0.826	0.865	0.901	1.578	1.087	1.056	0.922	0.912	1.154	0.585	0.574	0.49	0.898	1.024	L
		1.22	1.098	1.244	0.727	1.104	1.014	0.998	1.057	1.058	0.992	1.549	0.927	0.894	1.054	H
P02675	Fibrinogen beta chain precursor	1.058	0.962	1.021	1.555	1.133	1.243	1.034	1.224	1.323	0.48	0.458	0.42	1.114	1.048	L
		1.203	1.065	1.202	0.814	1	0.843	1.153	1.019	1.054	1.075	1.471	1.057	1.086	0.993	H
P02679	Fibrinogen gamma chain precursor	0.874	0.871	0.968	1.291	0.875	0.983	0.899	0.969	0.782	0.409	0.425	0.341	0.953	0.96	L
		1.207	1.015	1.225	0.645	0.942	0.687	0.906	1.015	0.944	1.042	1.555	0.957	0.861	0.645	H
P00738	Haptoglobin precursor	0.325	0.393	0.568	0.074	0.545	0.316	0.733	0.796	0.793	0.683	0.871	0.719	0.28	0.224	L
		0.817	0.941	0.595	0.67	0.824	0.386	0.721	0.183	0.699	1.084	0.651	1.023	0.697	0.173	H
P02751	Fibronectin precursor	1.352	1.176	0.89	0.377	0.53	0.482	0.548	0.501	0.402	0.537	0.377	0.424	0.796	0.553	L
		1.176	1.291	0.633	0.407	0.387	0.382	1.005	0.906	1.492	0.569	0.511	0.512	0.48	1.269	H
P02766	Transthyretin precursor	1.557	2.107	2.55	2.663	5.36	3.833	1.518	1.898	2.857	1.007	1.065	1.28	1.654	1.648	L
		0.978	1.042	0.679	0.616	0.784	0.534	0.674	0.802	0.824	0.812	0.711	0.675	0.78	0.668	H
P69905	Hemoglobin subunit alpha	0.795	0.729	1.601	0.729	0.894	0.936	0.773	2.003	1.159	1.006	0.64	0.843	0.864	0.471	L
		1.518	0.936	0.656	0.682	0.695	0.504	0.682	0.724	0.508	0.789	0.592	0.666	0.743	0.604	H
P68871	Hemoglobin subunit beta	864	1.123	1.128	2.159	3.348	1.291	0.98	1.01	1.928	0.799	0.68	0.92	0.639	0.961	L
		1.242	0.89	0.611	1.122	2.15	4.588	0.678	0.985	0.52	0.736	0.793	1.124	1.043	0.594	H
P01023	Alpha-2-macroglobulin precursor	0.706	0.719	1.023	1.137	0.888	0.567	0.773	0.944	0.968	0.662	0.826	0.696	0.733	0.726	L
		0.787	0.74	0.86	0.791	0.756	0.522	1.065	0.824	0.628	0.758	0.769	0.941	0.823	1.003	H
P02647	Apolipoprotein A-I precursor	0.958	1.06	0.861	0.88	0.902	0.758	1.015	0.541	1.053	0.799	1.218	0.911	0.796	0.766	L
		0.907	0.879	0.633	1.058	0.999	0.776	0.988	0.806	1.013	0.759	0.722	1.015	0.892	0.848	H
P01009	Alpha-1-antitrypsin precursor	0.841	0.836	0.897	0.855	0.882	0.846	0.956	0.972	0.933	0.927	0.945	0.906	0.877	0.775	L
		0.925	0.806	0.887	0.962	1.028	1.049	0.907	0.933	0.986	0.837	0.972	0.894	0.799	0.927	H

6) CONCLUSION

Our results suggested that the transthyretin precursor may have the potential to be used as biomarker for patients who require low-dose warfarin therapy, as it was found to be significantly up-regulated patients requiring low warfarin dose compared with the level in patients on high-dose warfarin.

Because of its anti-coagulating activity, patients with high transthyretin level should require lower warfarin dose to reach effective anticoagulation compared to the patients.

Our data further suggested that proteomics analysis can provide complementary analysis to the existing genotyping for effective warfarin dosing. This combined pharmacogenomics and pharmacoproteomics approach may be applied for other target-based therapies, in matching a particular marker in a subgroup of patients, in addition to the profile of genetic polymorphism.

7) FUTURE DIRECTIONS

Despite the high sensitivity of LC-MS/MS analysis, the differences in the level of Transthyretin precursor in patients on low and high dose of warfarin therapy need to be validated by other in vitro techniques. Encouragingly, preliminary Western blot analysis appeared to support the differences detected by LC-MS/MS analysis.

As a complementary approach to the clinical samples, a cell-based system can be established in which HepG2 cells will be incubated with different dosage of warfarin prior to the analysis of secreted proteins in the culture medium by LC-MS/MS analysis. The advantages of such a cell-based system include noticeably the homogenous background as opposed to intrinsic genetic variations from one patient to another, thereby reducing the number of samples to be analyzed to make the identified proteins statistically significant.

Our project can be further expanded into metabolomics analysis to identify specific metabolites as complementary biomarkers. As compared with secreted protein biomarkers, the number of secreted metabolites which are smaller in their molecular weight should be higher and findings from metabolomics analysis should also provide helpful information on the biochemical pathway in warfarin treatment.

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