Deanship of Graduated Studies

Al-Quds University

Adenosine Diphosphate Receptor Gene ($P2Y_{12}$) Sequence Variants among Swedish, Palestinian and Congolese Populations

Mohammad Yousef Sa'ed Asees

M.Sc. Thesis

Jerusalem - Palestine

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Thesis Approval

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Dedication

I dedicate this work to my God, my family; especially my wife Nour, my daughter Alin and my parents for their tolerance, support, inspiration and love. Also, I dedicate this to my dear supervisors, Dr. Rania Abu Seir, Dr. Camilla Hesse and Dr. Ali Reza. To all who believe in the importance of science and knowledge, I dedicate this work.

Mohammad Yousef Sa’ed Asees

Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed .....

Mohammad Yousef Sa'ed Asees

Date: 18.11.2017

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Abstract

Introduction: $P2Y_{12}$ receptor plays a central role in platelet aggregation and thrombus formation. Recently, inter-individual variations in platelet response of healthy untreated individuals were established which was explained by genetic variations in $P2Y_{12}$ receptor gene. Several single nucleotide polymorphisms (SNPs) in $P2Y_{12}$ receptor have been associated with increased platelet reactivity and risk of cardiovascular diseases. Therefore, this study aimed to evaluate the H2 haplotype (using G52T as a tag-SNP) and 18C>T polymorphisms in three different ethnic groups; Palestinians, Swedish and Congolese, in addition to evaluating the effect of these SNPs on platelet response induced by adenosine diphosphate (ADP) and thrombin receptor activating peptide (TRAP).

Methodology: The 5' end of the coding region of the $P2Y_{12}$ gene including the H2 haplotype and 18C>T SNPs were determined in conveniently selected healthy individuals from different ethnic groups (n=254). Blood samples were drawn for the aggregation and genetic studies. The whole exon-3 of $P2Y_{12}$ was sequenced and analyzed using ABI PRISM 310 Genetic Analyzer. The major and the minor allele frequencies of the $P2Y_{12}$ SNPs were determined in the study populations and the genetic differences between ethnic groups in $P2Y_{12}$ were elucidated. In addition, the frequencies of the genotypes and the haplotypes were calculated among the ethnic groups. Further, the effects of genetic variations (18C>T and 36G>T) on platelets aggregation induced by ADP/TRAP agonists were assessed in 10 healthy Swedish individuals using multiple electrode analyzer (MEA).

Results: In this study, five benign SNPs were genotyped and identified; 18C>T, 36G>T, 162G>T, 546C>T and 989A>G. The overall frequencies of each SNP in study population (n=254) was 21.9, 10.0, 0.2, 0.6 and 0.2%, respectively. The frequency of H2 haplotype among Swedish (n=55), Congolese (n=54) and Palestinian (n=145) was 23.6, 12.0, and 4.1%, respectively, while the frequency of 18C>T was 20%, 6.5% and 28.3%, respectively. There were significant differences in the frequencies of H2 haplotype and 18C>T among the ethnic groups (P<0.0001). In regard to the pathological SNPs, all of the study participants were negative. Genetic variations in $P2Y_{12}$ exon-3 (18C>T and 36G>T) had no significant effect on platelet aggregation induced by ADP/TRAP.

Conclusions: There are significant differences in the frequencies of the genetic variants of the $P2Y_{12}$ exon-3 between the study ethnic groups. Further, none of the variants 18C>T and 36G>T (H2 haplotype) had an effect on ADP or TRAP- induced platelet aggregation.

Keywords: $P2Y_{12}$, single nucleotide polymorphisms, platelets aggregation.

الاختلافات الوراثية في التسلسلات الجينية لمستقبلات البيورينرجك-$P2Y_{12}$ بين الفلسطينيين والسويديين والكونغوليين.

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ملخص:

خلفية الدراسة: تلعب مستقبلات البيورينرجك ($P2Y_{12}$) دوراً محورياً في تنشيط الصفائح الدموية وتكوين الجلطة. في الآونة الأخيرة، لوحظ وجود اختلافات في استجابة الصفائح الدموية بين الأشخاص الطبيعيين. واحدة من أهم الأسباب التي تؤدي إلى اختلاف استجابة الصفائح بين الأشخاص الطبيعيين هو وجود الاختلافات الجينية والطفرات الوراثية في التسلسل الجيني لمستقبلات البيورينرجك. حالياً، يوجد طفرتان وراثيتان غير مرضيتان (18C>T and 36G>T) في جين مستقبلات البيورينرجك والتي تعمل على زيادة نشاط الصفائح وزيادة خطر الإصابة بأمراض القلب والشرايين. تهدف الدراسة الحالية إلى تحديد نسب الطفرات الوراثية المرضية وغير المرضية بين مجموعات عرقية مختلفة (الفلسطينيين والسويديين والكونغوليين)، بالإضافة إلى دراسة تأثير هذه الطفرات على استجابة الصفائح باستخدام محفزات الأدينوسين ثنائي الفوسفات و متعددات الثرومبين.

منهجية البحث: تم تحديد الطفرات الوراثية في المنطقة المشفرة لجين مستقبلات البيورينرجك دراسة الطفرتين الوراثيتين (18C>T and 36G>T) في عينة عرضية من الأشخاص الطبيعيين ثلاثة أعراق مختلفة (ن=254)، السويديون، الفلسطينيون، والكنغوليون. تم جمع عينات دم لغرض دراسة تجمع الصفائح و الدراسة الجينية. بعد ذلك تم دراسة الأكسون-3 لجين مستقبلات البورينرجك لكل الأشخاص بدراسة تسلسل الحمض النووي ودراسة الاختلاف في توزيع الطفرات والطرز الجينية بين الأعراق المختلفة باستخدام جهاز ABI PRISM 310 Genetic Analyzer. إضافةً إلى ذلك فقد تمت دراسة تأثير الاختلافات الجينية على استجابة الصفائح الدموية لاستخدام محفزات الأدينوسين ثنائي الفوسفات و متعددات الثرومبين لدى 10 أشخاص من السويد باستخدام جهاز وظائف الصفائح متعدد الألكترود.

النتائج: بحسب النتائج التي ظهرت في الدراسة فقد تم عزل خمسة من الطفرات الوراثية الحميدة وهي: 18C>T، 36G>T، 162G>T، 546C>T، 989A>G حيث كانت النسب المئوية كما يلي: 21.9، 10.0، 0.2، 0.6، و0.2%. أما بالنسبة لنسبة النمط الفرداني H2 فقد كانت نسبته في

السويديين (ن=55)، الكونغوليين (ن=54) والفلسطينيين (ن=145) تساوي 23.6، 12.0، و4.1% على التوالي. أما بالنسبة لطفرة 18C>T فقد كانت النسب المئوية كما يلي: 20.0، 6.5، و28.3% على التوالي. وقد كان هناك فوارق ذات دلالة إحصائية في توزيع هذه الطفرات بين الأعراق المختلفة. أما فيما يتعلق بالطفرات الوراثية المرضية فكانت جميع عينات الدراسة سالبة. أما بالنسبة لتأثير الطفرتين الوراثيتين (18C>T and 36G>T) على استجابة الصفائح الدموية بين الأعراق الثلاث فكانت ليست ذات دلالة احصائية.

الاستنتاجات والتوصيات: تظهر نتائج هذه الدراسة أنّ هنالك اختلاف ذو دلالة إحصائية في توزيع الطفرات الوراثية بين الأعراق المختلفة. بالإضافة إلى ذلك، تأثير هذه الطفرات الوراثية على استجابة الصفائح كان بدون دلالة احصائية.

الكلمات المفتاحية: مستقبلات البورينرجك، تعدد أشكال النيوكليدات المنفردة، تجمع الصفائح.

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List of Abbreviations

Abbreviation	Term
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
Agarose LE	Agarose low electroendosmosis
Ap4A	Adenosine tetraphosphate
AR	Autosomal recessive
ATP	Adenosine triphosphate
AU	Aggregation
AU/min	Speed
AUC	Area under curve
BLAST	Basic local alignment search tool
Ca++	Calcium
CAD	Coronary artery diseases
cAMP	Cyclic adenosine monophosphate
CE	Capillary electrophoresis
cGMP	Guanosine monophosphate
c-Mlp	Cytokine myeloproliferative leukemia virus oncogene
COL	Collagen
CVD	Cardiovascular disease
CYP	Cytochrome P450
DAG	Diacyl glycerol
FC	Flow cytometry
GP	Glycoprotein
GPCR	G-protein coupled receptor
IP3	Inositol 3 phosphate
K+	Potassium
LTA	Light transmission aggregometry
MEA	Multiple electrode platelet aggregometry
MI	Myocardial infarction
MPC	Master Pure™ Complete
MPL	Myeloproliferative leukemia protein
Na++	Sodium
$P2Y_{12}$	Purinergic receptor subtype Y12
PAD	Peripheral artery diseases
PAR	Protease-activated receptor
PBS	Phosphate-buffered saline
PC	Platelets concentrate

PCI	Percutaneous coronary intervention
PCR	Polymerase chain reaction
PFA-100®	Platelet function assay
PGE1	Prostaglandin E1
PIP2	Phosphatidylinositol 4, 5-biphosphate
PKC	Protein kinase C
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PSL	Platelet storage lesion
PSL	Platelet storage lesion
RapIb	Ras-related protein
Rpm	Revolutions per minute
RT	Room temperature
SNP	Single nucleotide polymorphism
STEMI	Elevation myocardial infarction
TE	Tris and ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA)
TM6	Trans-membrane domain 6
TRAP	Thrombin receptor agonist peptide
TXA2	Thromboxane A2
UDP- sugar	Uracil-diphosphate-sugar
VASP	Vasodilator stimulated phosphoprotein
vWF	Von Willebrand factors

Chapter One

Introduction

This chapter includes general background, problem statement, research objectives and hypotheses of this study.

1.1 Background

Platelets are anucleated discoid cytoplasmic fragments that play a pivotal role in blood transfusion medicine. They play a key role in hemostasis, maintenance of blood vessel integrity, inflammation, thrombus formation and rapid cessation of bleeding in case of loss of vascular integrity (Maree & McRedmond, 2005). Bleeding disorders that express thrombocytopenia are generally treated by platelets concentrate (PC), which can be acquired by an apheresis technique or through the derivation of whole blood donations. In hemostasis, a series of reactions occur in response to vascular injury, the first step consists of adhesion, where the platelets come in contact with the endothelium and attach within a few seconds. The platelets then change their shape by becoming spherical and the contents of the granules, such as fibrinogen from the alpha granules and adenosine diphosphate (ADP) from the dense granules, are released. ADP secretion acts as a positive feedback that amplifies platelet response and stabilizes platelet aggregation induced by other agonist such as thromboxane A2 (TXA2), collagen and thrombin. Furthermore, ADP enhances the procoagulant effect of platelets. By this, platelets become able to aggregate via the glycoprotein (GPIIb/IIIa) receptors and can form a plug that temporarily seals a break in the vessel wall (Rondina *et al.*, 2013). ADP is considered as one of the most important mediators for platelets aggregation and thrombus formation. The action of ADP on platelet is mediated by two specific G-protein coupled receptors (GPCRs); $P2Y_1$ and $P2Y_{12}$.

The $P2Y_1$ receptor mediates weak responses to ADP and has an important role in the early steps of platelet activation induced by ADP or collagen. The role of $P2Y_{12}$ receptor completes and amplifies platelet activation and aggregation, irreversibly (Foster *et al.*, 2001). On the other hand, the importance of $P2Y_{12}$ is emphasized by the fact that its distribution is limited in the body tissues with common expression in platelets. In addition, it is the target of the thienopyridine anti-thrombotic drugs such as ticlopidine and clopidogrel (Hollopeter *et al.*, 2001). Several studies have reported differences in the $P2Y_{12}$ response between individuals. These differences were postulated to be a result of genetic variations in the $P2Y_{12}$ gene. Moreover, considerable variability in the antiplatelet effect of the antithrombotic agents especially clopidogrel targeting ADP receptors $P2Y_{12}$ has been reported. Further, defects in the $P2Y_{12}$ gene have been reported to be associated with hemorrhagic tendency in which ADP-induced platelet aggregation via $P2Y_{12}$ is impaired (Price *et al.*, 2009).

Five common benign SNPs were identified at the level of $P2Y_{12}$ receptor. Four of these SNPs were in absolute linkage disequilibrium and were designed in two phenotypic groups with two haplotypes: H1 which include: i-C139, i-T744, absence i-ins801A, and G52. H2 includes: i-139T, i-744C, presence i-ins801A, and 52T. The fifth identified SNP was C34T (18C>T) which is associated with 4-folds increased risk of ischemic stroke and/or carotid revascularization (Fontana *et al.*, 2003a; Sherry *et al.*, 2001; Ziegler *et al.*, 2005).

Thus, in this study, exon-3 of $P2Y_{12}$ gene was sequenced and analyzed among different populations (n=254). In addition, the frequencies of C34T (also known as 18C>T), H2 haplotype, (by the detection of G52T genetic variant known also as 36G>T) and other benign SNPs were identified and all study populations were tested for pathological SNPs: Arg256Gln, Pro258Thr, and Arg265Trp. Moreover, the effects of genetic variation in the $P2Y_{12}$ gene on the extent of ADP/TRAP-induced platelet aggregation were studied in healthy Swedish individuals (n=10).

1.2 Problem statement

$P2Y_{12}$ plays a crucial role in platelet function, especially in platelet activation and aggregation. Furthermore, $P2Y_{12}$ is a targeted receptor for antiplatelet drugs, in particular, thienopyredine compounds, which are used in the treatment of patients

diagnosed with cardiovascular diseases (CVDs). Indeed, platelet function and response to these drugs have shown an inter-individual variation that might be attributed to genetic variations in $P2Y_{12}$. These variations have never been studied among Swedish, Palestinian or Congolese populations. In this study, the frequencies of $P2Y_{12}$ genetic variations in different ethnic groups were studied and their effects on platelet aggregation were examined in a pilot study of Swedish population.

1.3 Objectives

The overall goal of the present study is to analyze the $P2Y_{12}$ receptor gene in different ethnic group. Our major objectives are:

1. To identify the frequencies of the common genetic variations in ADP receptor gene ($P2Y_{12}$) coding region among different populations including Palestinians, Swedish and Congolese.
2. To study the association between ethnicity, and the identified SNPs in the $P2Y_{12}$ gene.
3. To investigate the effect of the $P2Y_{12}$ allelic variants on platelet aggregation in response to ADP/TRAP agonists.

1.4 Hypotheses

1. The genetic variations in the ADP receptor ($P2Y_{12}$) coding region vary among different populations and their frequencies and haplotypes are associated with ethnicity.
2. The SNPs allelic variants affect the platelet aggregation in response to ADP/TRAP agonists.

Chapter Two

Literature Review

In this chapter, we provide an overview of the literature available on the physiology and the pharmacology of $P2Y_{12}$, focusing on molecular characterization and genetic variations of the $P2Y_{12}$ receptor gene.

2.1 Platelets and their functions

Platelets are the smallest of the circulating blood cells, or are rather in more strict sense small cytoplasmic fragments that are derived from megakaryocytes in the bone marrow. Each megakaryocyte produces approximately 2,000 to 3,000 platelets in a highly regulated process called megakaryocytopoiesis. It is regulated by thrombopoietin and its receptor; myeloproliferative leukemia protein (MPL). Platelets contain small amounts of mRNA in their cytoplasm, which give platelets a very limited capacity for protein synthesis. Platelets were discovered in 1882 by an Italian pathologist called Giulio Bizzozero. They were named platelets because they appear like small plates (Mazzarello *et al.*, 2001). These fragments of cells are anucleated and discoid, with a size of 2-4 μm in diameter and can reach up 6 micron after activation (Chang & Lo, 1998). Following their formation from megakaryocytes, platelets have a life span of 5-10 days. They play a critical role in hemostasis and thrombus formation by acting as a surface for coagulation cascade, production and secretion of factor V and VIII and consequently the formation and consolidation of primary and secondary hemostatic plug. The role of platelets in thrombus formation was established especially in the cardiovascular diseases such as myocardial infarction, where the unwanted platelet activation occurred due to an endothelial injury as a result of atherosclerosis (Badimon *et al.*, 1992). Although they act as the major role in hemostasis, they also play non-hemostatic role such as in the cases of inflammation and wound healing by helping in the leukocyte recruitment (rolling and adhesion) at the site of inflamed tissue, this action is facilitated by the p-selectin receptor at the surface of the platelets (Huang & Chang, 2012). Another role of platelets is at the level of the immune

system and allergy by the platelet itself which expresses a functional receptor for the Fc fragment of immunoglobulin E FcγRIIa (Kasperska-Zajac & Rogala, 2006) and by its mediators; such as TXA2, cysteinyl leukotrienes and lipoxins. These mediators potentiate the allergic response, and this is shown after the exposure to certain allergens that lead to platelet arachidonate pathway activation (Pitchford *et al.*, 2008). Moreover, nervous system and other body systems depend directly or indirectly on platelets and their contents. All these vital functions make platelets very important in human health. However, to fulfill their role, platelets must be adequate in function and in number (Kim *et al.*, 2012).

Platelets contain two major types of granules. The first type is alpha granules, which are the most abundant granules inside the platelets (50-80/platelet), they contain platelet specific proteins; such as: platelet factor IV and beta-thromboglobulin. Further, these granules contain coagulation factors such as FV, FXI and protein S. Type two is the dense granules, which mainly contain ADP, ATP, adenosine tetraphosphate (Ap4A), serotonin and calcium (Ca^{+2}) (Whittaker & Watkins, 1972).

2.2 Platelets and their role in hemostasis

At the level of hemostasis, platelets circulate for 5 to 10 days in the human body in non-reactive manner and stay ready for stimuli such as vascular injuries. Defects in the hemostatic system lead to serious complications such as thrombotic effect and vaso-occlusive. When vascular injury occurs, three major steps are activated and initiated. These steps are initiation, extension, and consolidation. Vascular injury exposes the negative sub-endothelial matrix, which stimulates and activates platelets by a series of events, starting with platelets adhesion with sub-endothelium substrates (collagen, fibronectin and laminin), mediated by von Willebrand factor (vWF) through GPIb-IX-V receptor complex under high shear stress (initiation step) (Kumar *et al.*, 2003). The exterior coat of platelets is called glycocalix. Glycocalix provides platelets with adhesive characteristics. Once a platelet adheres, it becomes activated, shape changes and pseudopodia formation occur (extension). This process facilitates surface intigreins, mainly GPIIb/IIIa, which is transformed from the low affinity to the high affinity form, to bind with fibrinogen. Further, platelet activation and shape changes stimulate the secretion and release of platelets granules content, such as serotonin, which induces vasoconstriction at the site of injury, and secretion of diffusible aggregating agent, in particular ADP and TXA2

(produced within platelets), that induce platelet aggregation. Following adhesion, activation and release reaction, platelets aggregate together by aggregating agent through a specific heterodimeric receptor GPIIb/IIIa and fibrinogen to form a primary plug that ultimately prevents bleeding. The release of platelets content lead to amplification and further platelet response and aggregation at the site of injury and formation of a secondary plug (consolidation) (Jackson *et al.*, 2003). Each of these functions should act rapidly and locally at the site of the injury. Platelets also provide platelet factor III (thromboplastin) and factor V and act as a surface for assembly of tenase and prothrombinase complex. Consequently, secondary hemostatic response formation and plug reinforcement occurs. Any defect in one of these functions leads to bleeding disorders (Coller, 2011).

2.3 Platelets role in transfusion medicine

The role of platelets in blood transfusion medicine is emphasized by the use of platelets for treatment of large group of patients with abnormal platelet number (thrombocytopenia) and bleeding manifestations. However, the percentage of hematological malignancies accounts from 11% to 14% of all cancer cases (Cancer Research UK, 2013). Patients with hematological malignancies and some other cancer types complain from bone marrow failure as a secondary sign for chemotherapy treatment, radiotherapy or stem cell transplantation, and consequently, most patients have thrombocytopenia. This secondary thrombocytopenia is treated with platelets concentrate (PC) to prevent serious bleeding complications such as intracranial hemorrhage. The first effective PC was demonstrated in 1910 by Duke WW (Duke, 1911). After that, in 1970s and until now, platelets have become a standard treatment for patients suffering from thrombocytopenia. In addition, PC is used as a prophylactic therapy in some surgical procedures and hematological malignancies to increase circulating platelets to the hemostatic level (Bergeron, 1989).

2.4 Platelets preparation and storage

There are several therapeutic applications for using PC with the most important in bleeding disorders that express thrombocytopenia. PC is prepared by two methods: the first one is by whole blood-derived platelet concentrates and the second procedure is by apheresis technique. In either component, platelets are suspended in an appropriate volume of the original plasma, which contains near-normal levels of stable coagulation factors that are stored at room temperature (Slichter & Harker, 1976). Apheresis platelets are stored in

additive solutions that replace plasma. One unit of platelets derived from a whole blood collection usually contains more than 5.5×10^{10} platelets suspended in 40 to 70 mL of plasma. Platelets may be provided either singly or as a pool of four leukoreduced platelet units together with platelet additive solution. On the other hand, one unit of apheresis platelets usually contains more than 3.0×10^{11} platelets and therapeutically is equivalent to 4 to 6 units of whole blood derived platelets (Simon, 1994).

PCs are stored at 20^{o}C to 24^{o}C with continuous agitation for 7 days. After 5 days, PCs are considered to be clinically useless. During storage, many changes occur in platelet structure and function. The overall changes of platelet function and structure that arise and take place from the time of blood donation to the time of platelet concentrate transfusion to the recipient are called platelet storage lesions (PSL). One of the most PSL is the decline in ADP response after storage time (Colman, 2006). The response of platelets activation and aggregation varies between healthy individuals and in patients under anti-aggregating agent therapy (Salles *et al.*, 2008). One of the underlying mechanisms of these variations is the genetic variations in receptors' genes that are involved in platelet activation (Fontana *et al.*, 2003a).

2.5 Platelets aggregation measurement

Platelets aggregation is the adhesion of platelets to each other under a variety of conditions and in the presence of a number of different agonists. It is mediated by interaction of fibrinogen and vWF with GPIIb/IIIa receptors. Platelets aggregation is considered as the test of choice for measurement of platelets function *in vitro* (Harrison, 2005a). Several congenital and acquired platelets disorders are diagnosed by spectrophotometric assays (Hayward *et al.*, 2006). These photometric assays are used also to monitor antiplatelet therapy in patients under clopidogrel therapy.

Several previous clinical studies suggested clopidogrel resistance in approximately 5-30% of the patients (Rudez *et al.*, 2009). To monitor and detect the responsiveness of patients for antiplatelets agents, in particular clopidogrel, ADP induced platelet aggregation could be carried by a number of methods including light transmission aggregometry (LTA), platelet function assay 100 (PFA-100®), VerifyNow® $P2Y_{12}$, multiple electrode analyzer

(MEA), Plateletworks® and flowcytometric measurement of vasodilator stimulated phosphoprotein (VASP) phosphorylation.

2.5.1 Light transmission aggregometry (LTA)

LTA is considered as the gold standard method for the measurement of platelets function. The principle of LTA is to measure the changes in light transmittance after adding of aggregating agents to platelet-rich plasma. The disadvantages of LTA that it's time consuming and the interpretation of the test results which requires standardization and trained laboratory staff. Before carrying the test, individuals should fast, rest and avoid smoking and drugs. The basic turbidometric method of LTA is carried by adding different aggregating agents such as ADP, collagen and epinephrine to the platelets rich plasma (PRP) at 37°C (Frontroth, 2013). PRP is acquired by centrifuging whole blood with citrate anticoagulant. First, whole blood is centrifuged at 1500 rpm for 15 minutes at room temperature to obtain PRP. After carefully removing the upper two third of PRP, the second centrifugation is done at 15,000rpm for 20 min to obtain platelet poor plasma (PPP). PPP is used to dilute PRP and to adjust the platelets count to obtain final platelet count of 250×10^9/L. Also, PPP is used as a blank to adjust the instrument optical density. LTA results are more accurate and more sensitive to monitor the thromboembolic complications than other methods such as VerifyNow and Multiplate analyzer which are point-of-care methods (Flechtenmacher *et al.*, 2015).

2.5.2 Platelet function analyzer (PFA-100)

PFA-100 is a rapid, simple and *in vitro* tool for monitoring primary hemostatic disorders through measuring high shear-dependent platelet function in citrated whole blood. It is used to detect and diagnose platelet function disorders; such as von Willebrand disease and Glanzmann's thrombasthenia. Furthermore, PFA-100 is used for pre-operative monitoring of patient with bleeding tendency before surgery and to assess the effectiveness of transfused platelets (Harrison, 2005b). The PFA-100 uses disposable test cartridges that contain a collagen/ADP (CADP) or collagen/epinephrine (CEPI) coated membrane with a small central aperture (147 µm). The test is carried under high sheer condition (5,000-6,000/s) with constant vacuum from the sample reservoir via a capillary tube to the membrane cartridge with monitoring of blood flow by aperture. After that, platelets come in contact with vWF and GPIb. This leads to platelets activation and aggregate with GPIIb/IIIa resulting in formation of platelet plug and occlusion of aperture within 3 mins

from test initiation. The time required for aperture occlusion is reported as closure time (CT) (van Werkum *et al.*, 2010).

2.5.3 VerifyNow $P2Y_{12}$ assay

This assay is a fast and standardized point of care test that widely uses ADP agonist in order to evaluate the efficacy of $P2Y_{12}$ receptor antagonists, especially clopidogrel (Price, 2009). It is a $P2Y_{12}$ specific assay that uses fibrinogen coated beads and prostaglandin E1 (PGE1). By adding the PGE1, $P2Y_1$-induced platelet aggregation is inhibited, which makes the ADP induced aggregation only by $P2Y_{12}$ receptors. After addition of ADP, it activates the platelets via $P2Y_{12}$ receptors and induces platelet-fibrinogen bed agglutination. The test has turbidimetric-based optical detection system that measures platelet-induced aggregation as an increase in light transmittance in citrate-anticoagulated whole blood. Patients with cut-off value of < 240 platelet reaction unit (PRU) are considered as good responders for clopidogrel (Marcucci *et al.*, 2009).

2.5.4 Multiple electrode analyzer

Multiple electrode platelet aggregometry (MEA) is another method for monitoring platelets function. It's an *in vitro* test to measure the aggregation of the platelets in response to specific agonists such as ADP or collagen. MEA is used to measure the platelets functions' disorders as well as platelet reactivity in response to antiplatelet drugs (Baumgarten *et al.*, 2010). It consists of five channels; each channel contains two sensors for quality control. An instrument such as multiple platelet function analyzer (Roche Diagnostic, Rotkreuz, Schweiz) is a fast and point of care testing that uses small amount of hirudin whole blood (300 µL). This method is based on aggregation with impedance technique. When whole blood or PC is added to a cuvette with special sensor, it triggers the MEA by the adhesion of activated platelets with sensors which lead to increase in the electrical impedance. By adding an agonist such as ADP, the platelets are able to aggregate and the aggregation increases the impedance between the two electrodes in a test cell. Throughout the test, the impedance is registered and three parameters are calculated: area under the curve (AUC) which is a measure of aggregation, aggregation (AU) which corresponds to the curves amplitude and the speed (AU/min) which is the maximal gradient of the curve (Wurtz *et al.*, 2014).

2.5.5 Plateletworks® assay

It is a point of care assay which is used to assess the effect of GP IIb/IIIa antagonists. The principle of this assay is based on measuring platelet inhibition percentage before and after ADP addition. The first step is to measure the baseline platelet count in K3-EDTA anticoagulated whole blood. Then, the platelet count is repeated using Plateletworks® tube containing both 50 µmol/L of D-Phe-Pro-Arg-chloromethylketone (PPACK) and 20 µmol/L of ADP agonists. In the presence of ADP agonist, platelets activation and aggregation will occur. Platelet aggregates exceed the threshold limits for platelet size (<30 fL). So, the hematology analyzer no longer counts them as platelets. The percentage of platelets inhibition is calculated by the ratio of the platelet count between the ADP and K3-EDTA tubes (van Werkum *et al.*, 2010).

2.6 Platelet receptors

After platelets activation and secretion reaction, several different agonists are released, including the agonist TXA2, ADP, thrombin and epinephrine. Each of these agonists acts on platelets through a specific receptor. These receptors modulate and coordinate the functions of platelets by complicated and interconnected mechanisms. Some of these receptors modulate their effect through the GPCR. Examples of these G-coupled receptors are purinergic receptors (P2Y) of ADP and ATP agonist, the platelet alpha-adrenergic receptor 2A (A2AR) for epinephrine and thromboxane prostanoid (TP) receptor and thrombin receptors (protease-activated receptors PAR-1/PAR-4) (de Groot *et al.*, 2012).

2.6.1 Purinergic receptors

2.6.1.1 History and nomenclature

Purines and pyrimidines nucleotides are extracellular molecules that induce huge biological effects through specific surface receptors called purine receptors. There are two major groups of purines receptors: P1 (adenosine) and P2 (ATP and ADP) receptors (Ralevic & Burnstock, 1998). P1 contains 4 distinct GPCR subtypes: A_1, A_{2A}, A_{2B}, and A_3, and each subtype has a specific amino acid homology sequence (Ralevic & Burnstock, 1998). However, P2 receptors are subdivided into two categories: the first one is ATP gated ionotropic receptors P2X. The second one is metabotropic G-coupled protein receptors P2Y depending on the basis of pharmacology, ligand characteristic and receptor sequence (Burnstock, 1996; Fredholm *et al.*, 1994). In respect to purinergic receptors

nomenclature, P2Y is the name that includes functional mammalian receptor proteins and functional non-mammalian species. In case of mammalian orphan receptors and non-mammalian functional receptors, the lower case p2y is used. The attached number following purinergic receptors (1 to n) indicates the cloning time pattern of complementary DNA. The first purinergic receptor was cloned in 1993 and the last one was in 2001 (Webb *et al.*, 1993). Cloning studies of P2 receptors and studies of transduction mechanisms in the early 1990s led to subdividing of P2 receptor into P2X and P2Y receptor families. Currently, seven subtypes of P2X receptors and eight subtypes of P2Y receptors are recognized (Mason, 1988; Westphal, 1987). Furthermore, previous studies have recognized that some P2Y receptors respond to naturally occurring extracellular nucleotide pyrimidines as well as purines. These nucleotides have relatively low bioavailability and stability *in vivo* and are widely distributed but their extracellular concentration increase in response to certain situation such hypoxia, injury, inflammation and mechanical stress (Jacobson *et al.*, 2012).

2.6.1.2 P2Y receptors' roles

Purinergic receptors P2Y are a group of guanine nucleotide-binding-protein coupled receptors (GPCR) that are widely distributed and expressed on broad types of tissues in human body. This increases their importance at physiological and pathological level. Eight distinct mammalian P2Y receptors have been cloned and recognized: the $P2Y_{1, 2,4,6,11,12,13,14}$ receptors. Moreover, P2Y receptor is further subdivided into $P2Y_1$ like subfamily and $P2Y_{12}$ like subgroup (Westphal, 1987). $P2Y_1$ subfamily includes $P2Y_1$, PY_2, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$. All of these $P2Y_1$ subfamily act by the same mechanism via Gq protein and activation of phospholipase C. Also, $P2Y_{11}$ receptor of $P2Y_1$ like subfamily has been documented to couple Gs as well as Gq in some cells to induce cyclic adenosine monophosphate (cAMP) production (Qi *et al.*, 2001). The second group is the $P2Y_{12}$ subfamily, which includes $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$. $P2Y_{12}$ subfamily act through Gi protein and thereby inhibition of adenylate cyclase enzyme (Cooper & Rodbell, 1979). There are differences between purinergic receptors among species at the level of function, occurrence, and ligand preference of the P2YRs, e.g., the absence of $P2Y_{11}$ gene in mice genome (Jacobson & Boeynaems, 2010). Each one of these purinergic receptors is coupled with a specific preferential purine and pyrimidine ligand agonists which are as following: $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ have ADP as their agonist; ATP is agonist for $P2Y_2$, $P2Y_4$ (rat, but not human), and $P2Y_{11}$; uridine diphosphate (UDP) for $P2Y_6$ and $P2Y_{14}$; uridine

triphosphate (UTP) for $P2Y_2$ and $P2Y_4$; and UDP-glucose and other UDP-sugars for $P2Y_{14}$. In some situations, the same nucleotide that activates one P2Y subtype may act as an antagonist for another subtype. For example, ATP acts as an agonist for P2X and an antagonist at the $P2Y_{12}$ receptors in platelets (Springthorpe *et al.*, 2007). One of the major challenges regarding P2Y agonists is to design a potent, specific and selective synthetic agonist that makes a key for structural and functional identification of P2Y receptors. Radio-ligand studies were able to apply and efficiently carry out at both $P2Y_1$ and $P2Y_{12}$ but it failed for other subtypes of P2Y (Abbracchio *et al.*, 2006).

The use of antibody targeted purinergic receptor plays an important role to understand the distribution and the roles of different P2 receptors in different tissues. Several previous studies emphasized the presence and the role of these receptors in different tissues such as platelets (P2X, P_2Y_1, $P2Y_{12}$), nervous system ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$), contracting/relaxing activities of gastrointestinal tract, eyes ($P2Y_4$), ears, cardiovascular, placenta and other tissues (Brass *et al.*, 2012). The central role of P2Y is seen in platelet function in hemostasis and thrombus formation. It plays a direct role by inducing platelet aggregation and indirect role by potentiating effect for other platelet agonist. In addition, purinergic receptors, especially $P2Y_{12}$, play many roles in different conditions. These roles include: thromboembolism, neuromodulation, vasodilatation, inflammatory response, cell migration and antitumor effects (Brass *et al.*, 2012).

In addition, $P2Y_{12}$ plays an important role in chemotaxis. After brain injury, the level of extracellular nucleotides is increased. This activates the $P2Y_{12}$ receptors in the brain cells and mediates the migration of primary immune microglial cells and extends the processes toward the sites of tissue damage (Haynes *et al.*, 2006).

Fibroblast is the major cell in heart muscle. It plays a very important role in structural and functional aspects of the heart. Fibroblasts are then differentiated to myofibroblasts. The myofibroblasts express all P2Y receptors. All of these receptors are functional except $P2Y_{13}$. They play a central role in myofibroblast regulation. After myocardial infarction, the nucleotide concentrations (ADP, ATP and UTP) increases with several effects at the level of cardiovascular system such as vasoconstriction induced by UTP (Wihlborg *et al.*, 2006).

The role of P2Y receptors in the status of myocardial infarction is established by enhancing the thrombus formation erosion of atherosclerotic plaque and by stimulation of endocytosis of high density lipoprotein HDL in hepatocytes. The activation of $P2Y_{13}$ in hepatocytes stimulates HDL endocytosis by inducing Ras homolog gene family, member A (RhoA) activation and its effectors' Rho-associated, coiled-coil containing protein kinase 1 (ROCK I), resulting in HDL endocytosis through cytoskeleton reorganization. Also, purinergic receptors play an unclear role in the neurological disorders such as Alzheimer disease, neuritic plaques and neurofibrillary tangles (Brass *et al.*, 2012).

The roles of P2Y as a pharmacological target expanded markedly in the past years. $P2Y_{12}$ is considered as a potent antiplatelet agent that is used efficiently in treating cardiovascular diseases.

2.6.2 Platelet purinergic receptors and their roles

Platelet has at least two different purine receptors (P2); one P2X and the second is P2Y with very important function in platelet response and aggregation. $P2X_1$, $P2Y_1$ and $P2Y_{12}$ are the major platelets receptors. Recently small amount of $P2Y_{14}$ were identified with unknown function (Cattaneo & Gachet, 1999; Moore *et al.*, 2003). Approximated number of $P2Y_1$ per platelet is 150 $P2Y_1$/platelet which account approximately to 20% - 30% of ADP binding sites on platelets (Ohlmann *et al.*, 2013; Westphal, 1987). This number is very low compared with thromboxane prostanoid receptors or with the thrombin receptor PAR-1 (1000–2000 receptors/platelet) (Westphal, 1987). On the other hand, the number of $P2Y_{12}$ receptors on platelets plasma membrane is 425 ± 50/platelet (approximately 70% of platelet ADP binding sites) (Baurand *et al.*, 2001). This is confirmed by using an antagonist radiolabeled substrate called [3H] 2-Propylthioadenosine-5- adenylic acid (1,1-chloro-1-phosphonomethyl- 1-phosphonyl) anhydride ([3H]PSB-0413) which is highly stable, accurate and specific antagonist for $P2Y_{12}$ receptors (Ohlmann *et al.*, 2013). ADP is considered to be one of the most important mediators for platelets aggregation and thrombus formation. It's the first described weak platelet aggregating agent with low molecular weight. The importance of ADP and its dense granules are proved by the fact that patients with ADP storage or secretion problems have bleeding diathesis (Daniel *et al.*, 1998). ADP is released either from activated platelets or from damaged cells at the sites of injured tissues.

ADP has a greater affinity than ATP for $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$. Also, ADP is considered as a full agonist for $P2Y_1$ while ATP is a partial agonist. Regarding $P2Y_{12}$, the ATP has an antagonism relation while ADP and its derivatives have agonist effects. At the level of $P2Y_{13}$, both ATP and ADP act as full agonist (Abbracchio *et al.*, 2006).

The autocrine – paracrine manner of ADP action in platelets is mediated by two specific G protein-coupled receptors; $P2Y_1$ receptor and $P2Y_{12}$. Thus far, $P2Y_{12}$ plays a central role in the dense granules excretion and thromboxane production which is additional important aggregating agent. Patients with $P2Y_{12}$ dysfunction have a small and loosely thrombus formation with increased bleeding time. Without $P2Y_{12}$, platelet induces shape changes in the presence of ADP but with reduced granules content and failed to inhibit adenylate cyclase enzyme. This effect is similar to $P2Y_{12}$ antagonism by thienopyridine antiplatlet drugs (Dorsam & Kunapuli, 2004).

2.6.2.1 P2X

P2X (P2X1 to P2X7) which is an ion-coupled receptor with a rapid and selective permeability for calcium (Ca^{+2}), sodium (Na^{+}) and potassium (K^{+}) within 10 milliseconds in response to ATP. The signal transduction pathway appears to be relatively fast and simple. Once P2X is activated, it leads to rapid entry of extracellular Ca^{+2} ions inside the platelets and stimulation of platelet cytoplasmic Ca^{+2} voltage channel, and consequently, increasing the intracellular Ca^{+2} ions concentration that can synergize $P2Y_1$ effects to induce platelet shape changes (Ralevic & Burnstock, 1998). The P2X receptors sequence proteins that range from 379 to 472 have a topology that includes two transmembrane domains (TMs), intracellular N- and C-termini and a large glycosylated extracellular domain (Ralevic & Burnstock, 1998). The function of P2X was obvious in different physiological processes other than platelet function such as pain sensation, nerve transmission, and immune response (Brass *et al.*, 2012).

2.6.2.2 $P2Y_1$ and $P2Y_{12}$ structure and mechanism of action

The P2Y receptors proteins range from 308 to 378 amino acid sequence and are organized in seven alpha-helix TM domains. $P2Y_1$ is a polypeptide consists from 373 amino acids that are coupled to Gq protein and partially sensitive to pertussis toxin (Ralevic & Burnstock, 1998). The presence of $P2Y_1$ receptors was confirmed by the detection and isolation of $P2Y_1$ receptor mRNA from platelets and megakaryocytes. Their role was

established by using selective agonist/antagonist and by $P2Y_1$ deficient mouse model (Hechler *et al.*, 1998). Once ADP binds with $P2Y_1$, it leads to induce an intracellular transduction pathway that activates Gq protein by α subunit and consequently phospholipase C activation. This leads to hydrolysis of membrane phosphatidylinositol 4, 5-biphosphate (PIP2) to inositol triphosphate (IP3) and diacyl glycerol (DAG). IP3 binds with IP3 receptors on endoplasmic reticulum leading to open Ca^{+2} channel and elevation of free cytoplasmic Ca^{+2}, while DAG acts by activation of protein kinase C (PKC). Also, IP3 and DAG together activate guanine nucleotide exchange factor (CalDAG-GEF1), which is responsible for activating Rap1 by inducing exchange of GDP for GTP. GTP-bound Rap1 contributes in switching on and activating GPIIb/IIIa (Bertoni *et al.*, 2002). These events lead to induce platelets shape changes and transient reversible aggregation (Gachet, 2001). $P2Y_1$ receptor has two arginine residues in the carboxy-terminal domain which play essential role for activation of Gq-pathways (Ding *et al.*, 2005).

The two receptors $P2Y_1$ and $P2Y_{12}$ should be activated simultaneously for normal aggregation since separated inhibition of each of them by selective antagonists results in dramatic inhibition of aggregation (Milic-Emili, 1990) as shown in figure 2.1.

The $P2Y_1$ knockout platelets models showed no shape changes, and no aggregation in response to ADP agonist with no effect on adenylate cyclase enzyme. This suggests the presence of another ADP receptor in platelets which is $P2Y_{12}$ (Fabre *et al.*, 1999). In addition, $P2Y_1$ receptor participates in aggregation induced by collagen, as shown by the reduced value of aggregation and the increase in the lag phase from the addition of collagen to the onset of aggregation in $P2Y_1$ knockout platelets (Mangin *et al.*, 2004). The serotonin can restore the ADP response in $P2Y_1$-deficient platelets or in the presence of $P2Y_1$ antagonist through activation of Gq. Although the serotonin alone is not able to induce platelet aggregation (Jin & Kunapuli, 1998).

$P2Y_1$ receptors also play a role in collagen-induced shape changes when TXA_2 formation is prevented. The morphological changes during platelet aggregation indicate that the $P2Y_1$ receptor is involved in the centralization of platelet granules induced by ADP and the formation of pseudopodia in platelets activated with low concentrations of strong agonists such as TXA_2 or thrombin (Mangin *et al.*, 2004).

On the other hand, $P2Y_{12}$ receptors (old names P2YADP, P2YAC, P2Ycyc or P2TAC) is a multifunctional receptors that couple G_{i2} protein to complete, amplify and stabilize the platelet response to ADP. It plays central roles in thrombus formation, activation of fibrinogen receptor, potentiating the secretion reaction and TXA2 production (Dorsam & Kunapuli, 2004; Kahner *et al.*, 2006). The action of $P2Y_{12}$ is facilitated by multiple pathways through G_{i2} coupled protein, when ADP agonize $P2Y_{12}$, multiple signal pathways are initiated, started with the inhibition of adenylate cyclase enzyme that leads to decrease cAMP production and increase in the dephosphorylated vasodilator stimulated phosphoprotein (VASP). Despite that inhibition of adenylate cyclase via $G_{\alpha i2}$ is the main mechanism for $P2Y_{12}$ stimulation, it indirectly induces platelet aggregation (Haslam, 1973). The second mechanism that is induced by $P2Y_{12}$ receptor is the stimulation of phosphatidylinositol-3 kinase (PI-3K) activity which plays a vital role in sustaining platelet aggregation, as shown in figure 1. Moreover, $P2Y_{12}$ activates small GTPase Ras-related protein (RapIb) through a PI-3K-dependent mechanism (Kauffenstein *et al.*, 2001; Trumel *et al.*, 1999). The clinical relevance of $P2Y_{12}$ is obtained when it's blocked in CVD patients with thienopyridine compounds therapy. Poor aggregation induced by ADP was seen in platelet deficient $P2Y_{12}$ with normal shape changes and intracellular Ca^{+2}. The aggregation response by ADP and in the presence of $P2Y_{12}$ antagonist is enhanced and restored by Gz stimulated via epinephrine, although the epinephrine alone is not able to induce platelets aggregation (Daniel *et al.*, 1999).

Vasodilator stimulated phosphoprotein (VASP) is an intracellular actin regulating protein. It acts as a substrate for cAMP- and cyclic guanosine monophosphate (cGMP)-dependent protein kinases (Waldmann *et al.*, 1987). After $P2Y_{12}$ activation and cAMP production inhibition, VASP dephosphorylation occurs. On the other hand, the inhibition of $P2Y_{12}$ receptors by thienopyridine compounds and the stimulation of cAMP production by vasodilator molecules such prostaglandin and nitric oxide (NO) induce phosphorylation of VASP. In case of clopidogrel resistance, measurement of VASP is needed to detect patients insufficiently protected by clopidogrel (Aleil *et al.*, 2005). So, the level of VASP dephosphorylation/phosphorylation is associated and reflects the $P2Y_{12}$ receptor activation and deactivation. The measurement of VASP by flow cytometry is considered as a reliable and specific marker for assessment of the $P2Y_{12}$ inhibition (Schwarz *et al.*, 1999).

Overall, the $P2Y_1$ receptor mediates weak responses to ADP and has a crucial role in the early steps of platelet activation induced by ADP or collagen. So, the role of $P2Y_{12}$ receptor completes and amplifies platelet activation and aggregation irreversibly**.** The $P2Y_1$ and $P2Y_{12}$ receptors also play an important role in procoagulant activity of platelets. Both receptors are indirectly involved in platelet P-selectin exposure and formation of platelet-leukocyte conjugates leading to leukocyte tissue factor exposure. In addition, $P2Y_{12}$ receptor is directly involved in the procoagulant activity of platelets through phosphatidylserine exposure at the surface of platelets (Leynadier, 1989).

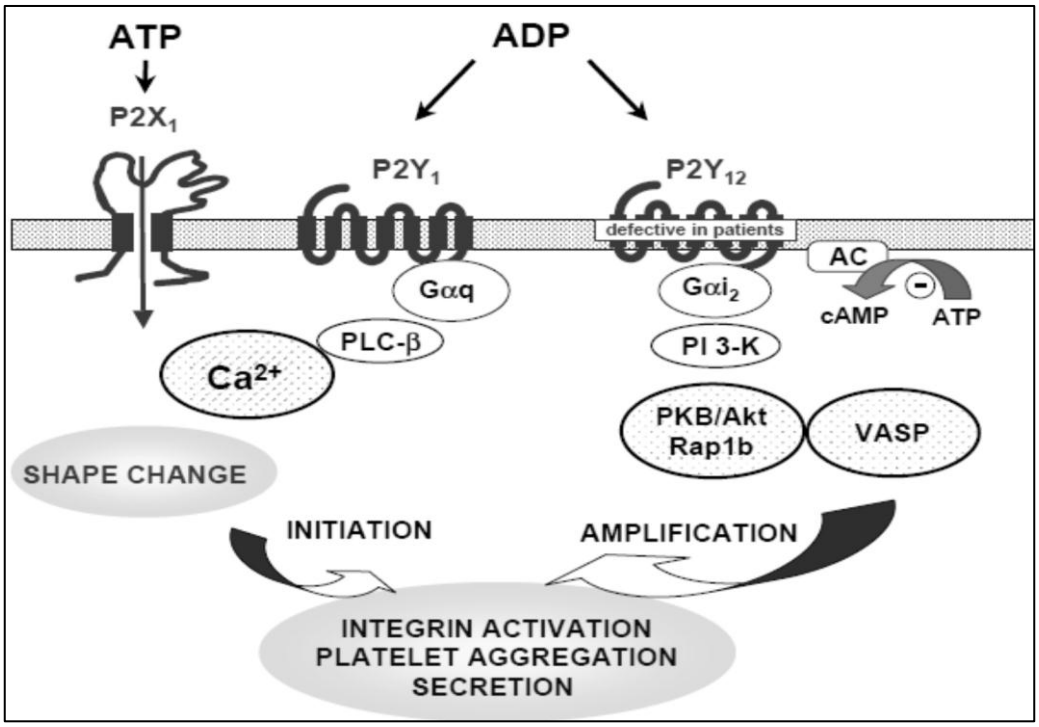

Figure 2.1: $P2Y_{12}$ receptors' structure and function. Three P2 receptors have been identified and cloned on platelets. P2X is ATP-mediated ion channel receptor that induces shape changes by rapid increase in intracellular Ca. $P2Y_1$ and $P2Y_{12}$ are ADP gated G-protein coupled receptors that induce platelets aggregation. $P2Y_1$ receptor is Gq-coupled and responsible for intracellular calcium mobilization, shape change, and initiation of aggregation; while the $P2Y_{12}$ which is Gi-coupled receptor and responsible for the completion of the aggregation to ADP and potentiating of aggregation and secretion by agents such as thrombin, serotonin, collagen, thromboxane A2, and immune complexes. The mechanism of action of $P2Y_{12}$ receptor is mediated by inhibition of adenylate cyclase enzyme and decrease cAMP leading to indirect activation of the GbIIb/IIIa integrin and subsequent aggregation. Another mechanism for $P2Y_{12}$ is dephosphorylation of the vasodilator-stimulated phosphoprotein (VASP), which negatively regulates GbIIb/IIIa, also a PI3K-dependent activation of the small GTPase Rap1B and PKB/Akt are involved in $P2Y_{12}$ receptor-mediated aggregation. The $P2Y_{12}$ receptor is the target of the thienopyridine compounds ticlopidine and clopidogrel (Harrison, 2005a).

2.6.3 P2Y regulation

Similar to most GPCRs, P2Y receptor activity is highly regulated by a number of complex mechanisms, including receptor desensitization, internalization and recycling. These mechanisms act to prevent the over stimulation of platelet response at the site of injury and thrombus formation. After platelet activation by its P2Y receptors, the regulation of P2Y receptors response is a very important and interesting point. To prevent the over stimulation and undesirable effects, once P2Y become activated by a first application of ADP, platelets become unresponsive to a second stimulation with ADP. This mechanism of activation is called refractory state of platelets to ADP and it is caused by desensitization of the $P2Y_1$ and $P2Y_{12}$ receptors (in other study the $P2Y_{12}$ receptor remains functional) with a resultant loss of shape change and aggregation (Cunningham *et al.*, 2013; Hardy *et al.*, 2005). In human platelets, both $P2Y_1$ and $P2Y_{12}$ are desensitized by different kinase dependent mechanism. $P2Y_1$ is desensitized in protein kinase dependent manner, while $P2Y_{12}$ is desensitized in G receptors kinase manner (Hardy *et al.*, 2005). Another mechanism of P2Y receptors regulation is established and called P2Y receptor trafficking. In this mechanism, $P2Y_1$ and $P2Y_{12}$ receptors are differentially regulated and relocated upon activation by agonist and recycle back to plasma membrane upon removal of agonist stimulation. Both $P2Y_1$ and $P2Y_{12}$ receptors undergo clathrin and dynamin dependent endocytosis (Mundell *et al.*, 2006), while the $P2Y_{12}$ receptor mainly stays at the plasma membrane or internalized via G protein kinase. Also, degradation of $P2Y_1$ following prolonged ADP exposure is detected as a type of receptor regulation (Cunningham *et al.*, 2013). This action may be of major consequence *in vivo*, since even in platelets refractory to stimulation by ADP, the $P2Y_{12}$ receptor would be able to ensure platelet reactivity at sites of injury, thus preventing loss of the hemostatic function (Baurand *et al.*, 2000).

In summary, both $P2Y_1$ and $P2Y_{12}$ receptors are necessary for normal hemostasis and both play a key role in arterial thrombosis. $P2Y_{12}$ receptors targeting antithrombotic drugs already exist, and new compounds have been developed and are under clinical evaluation (Gachet, 2008).

2.6.4 Pharmacology of purinergic receptors

Cardiovascular diseases are one of the most common causes of death worldwide. Ischemic vascular disease of arterial vascular bed includes three groups: peripheral artery diseases

(PAD), cerebrovascular stroke and coronary artery diseases (CAD). Moreover, CAD is further divided into two sub-groups; stable angina and acute coronary syndrome (ACS) which is the severe form of CAD which includes: an unstable angina pectoris; non-segment elevated myocardial infarction (non-STEMI), and ST-elevation myocardial infarction (STEMI) which leads to heart failure, arrhythmia and sudden death (Cannon *et al.*, 2013).

One of the most important and usable pharmacological principle for treating CVD is the inhibition of ADP induced aggregation. According to the type of vascular disease severity and duration, patients may be managed by antithrombotic agent alone or may undergo percutaneous coronary intervention (PCI) (revascularization) or surgical vascular bypass graft. Whatever the patient treatment regime, antiplatelet agent should be used in all cases to prevent and manage the thrombotic effect and re-occlusion with minimal risk of bleeding (Gachet, 2015).

Due to the important role of platelets and their receptors, especially $P2Y_{12}$ in arterial thrombosis and CVD, the anti-aggregation agent is considered as a cornerstone and drug of choice for treating CVD. The prognosis of patient with ACS is improved by PCI together with antiplatelet agents such as aspirin and/or clopidogrel (Mehta *et al.*, 2001). Aspirin was the first prescribed antiplatelet agent that acts by irreversible inhibition of cyclooxygenase-1(COX 1). This enzyme is responsible for prostaglandin H2 synthesis that is transformed inside platelet by thromboxane synthase to TXA2 which acts as a potent anti-aggregating agent and stimulates platelet release reaction and vasoconstriction (Roth & Majerus, 1975).

In contrast to other P2Y receptors, $P2Y_{12}$ has a limited distribution throughout the body. So, it is considered as an attractive target for the thieonopyredeine groups which include: ticlopidin, clopidgrel and prasugrel (Hollopeter *et al.*, 2001). Also, $P2Y_{12}$ is targeted by a group of ATP analogue ticagrelor (AZD6140), and cangrelor (AR-C69931MX) which are direct, reversible antagonist agents for $P2Y_{12}$ receptor; all of these agents antagonize $P2Y_{12}$ receptor with pharmacological differences, but with the same indirect blockage of ADP dependent activation of fibrinogen receptors GPIIb/IIIa. Thienopyridine compounds are pro-drugs that are metabolized by liver hepatocytes cytochrome P-450, especially CYP2C19 to produce active metabolites. Then, the active metabolite, which contains the thiol group, binds covalently and irreversibly with cysteine-containing sequences of $P2Y_{12.}$

This disrupts the receptor oligomer and cancels its roles in platelet aggregation and thrombus formation (Savi *et al.*, 2000).

As it has been mentioned before in chapter one, one of the most important roles for $P2Y_{12}$ receptor is that it acts as a targeted ligand for antiplatelet agent especially thienopyrdines groups. These groups of antiplatelet drug may be used alone or in combination with aspirin as a dual therapy with high efficacy to treat patient with CVD and cerbrovascular diseases as well (Williams *et al.*, 2010). The role of thienopyridine groups have become more obvious after cloning of $P2Y_{12}$ in 2001 by Hollopeter *et al.* (Hollopeter *et al.*, 2001).

Clopidogrel is the second generation drug of thienpyridine group that acts by platelet $P2Y_{12}$ receptors. The response of $P2Y_{12}$ receptors inhibitors, in particularly, clopidogrel which is measured by platelet aggregation inhibition is influenced by several factors such as: poor bioavailability, accelerated platelet turnover, smoking, interaction with other drugs, genetic and other factors that could explain the inter-individual variation. Several previous studies have emphasized the effect of SNPs on clopidogrel response in patients with vascular disorders (Bura *et al.*, 2006; Galic *et al.*, 2013). Clopidogrel is a prodrug that is activated in the liver cells by cytochrome P450 (CYP) enzymes, mainly by CYP2C19. The steady state of clopidogrel therapy is achieved after 3-4 days after taking the standard daily dose of 75 mg/day, and is considered as a disadvantage for clopidogrel therapy (Gachet, 2005). The normal platelets functions especially aggregation are returned to normal after five days of clopidogrel cessation. Once clopidogrel is activated, it binds irreversibly with ADP receptor $P2Y_{12}$ on platelet surface and prevents the secretion reaction, platelet aggregation and thrombus formation. Approximately 5-30% of patients treated with standard dose of clopidogrel therapy (75mg/daily) and 5-45% of patient under aspirin therapy have shown a wide inter-individual variability and high residual on-clopidogrel / aspirin platelet reactivity respectively (Lev *et al.*, 2007). The phenomenon of high platelet reactivity in patient under aspirin and clopidogrel therapy leads to failure in platelet aggregation inhibition ex vivo with increased risk of recurrent adverse cardiovascular diseases (Bura *et al.*, 2006).

Due to the previously mentioned factors, especially genetic ones; clopidogrel response with platelet aggregation inhibition less than 10% is considered as resistance to clopidogrel. Furthermore, when the platelet aggregation inhibition is less than 30%, it is

considered as a weak response for clopidogrel (Tang *et al.*, 2013). This low response is associated with clopidogrel resistance and increased the risk of recurrent ischemic event. Multidrug resistance protein 1 (MDR1), cytochrome p450 (CYP2C19), and $P2Y_{12}$ receptor are proteins for clopidogrel metabolism and biotransformation to active metabolite with clear evidence of association with clopidogrel response (Wiviott & Antman, 2004). Several SNPs were identified in these proteins and were correlated with clopidogrel response and platelet aggregation with considerable inter individual variation in clopidogrel response (Tang *et al.*, 2013; Ziegler *et al.*, 2005). The MDR1 gene is a coding gene for P-glycoprotein which acts as a barrier against clopidogrel absorption in intestine. C3435T is an example of a SNP in MDR1 gene that is associated with decrease in P-glycoprotein expression (Shalia *et al.*, 2013). SNPs were identified in CYP 450 include CYP2C19*1 wild type, CYP2C19*2, [G681A] with splicing defect, and CYP2C19*3 [G636A] with stop codon (Sofi *et al.*, 2011). Carriers of reduced functional variant of CYP2C19 (CYP2C19 loss-of-function alleles) have a 61% higher risk for a major adverse cardiac event compared with non-carriers. These carriers are called poor responder or metabolizer (Simon *et al.*, 2009). In contrast, people with increased functional variant of CYP2C19 (gain of functional alleles) are associated with bleeding tendency (Simon *et al.*, 2009; Williams *et al.*, 2010). SNPs at the level of $P2Y_{12}$ gene have also been studied. Two functional haplotypes are produced: H1 and H2 haplotypes. H2 haplotype has been designed as a factor that affects platelets and clopidogrel response variability. For example, 18C>T and 36G>T SNPs of the $P2RY_{12}$ gene significantly increased the risk for clopidogrel resistance and as a result, increased the risk of CVDs adverse effect (Fontana *et al.*, 2003a; Shalia *et al.*, 2013). Due to the obvious effect of SNPs in metabolizing enzyme in the case of clopidogrel, the strategy of treatment of patients with CVDs has been changed by using high dose of clopidogrel as a loading dose or using different types of antiplatelet drugs such as prasugrel. However, a combination therapy can be used to overcome clopidogrel resistance and decrease adverse effect of CVDs.

Molecules such as clopidogrel or the ATP analogs of the AR-C series selectively inhibit the G_i-coupled ADP response without any impact on the $P2Y_1$ receptor-mediated effects, whereas, $P2Y_1$ receptor antagonists inhibit ADP-induced platelet aggregation without inhibiting the effect of ADP on adenylate cyclase activity. The requirement of this receptor to complete aggregation to ADP was confirmed by the generation of $P2Y_{12}$ receptor-

deficient mice, which displayed a defect in platelet aggregation in response to ADP, although the shape change was conserved (Leon *et al.*, 1999).

Ticagrelor (Brilinta) is a novel oral non-competitive direct antagonist agent that binds reversibly with $P2Y_{12}$ receptor. It's a potent antiplatelet agent with high efficacy and high absorption rate in contrast to clopidogrel (Wallentin *et al.*, 2009). The metabolism of ticagrelor is like clopidorgrel which takes place in the liver by the action of CYP-P450 3A4 and 3A5. After metabolism of ticagrelor, it gives the active ingredient named AR-C124910XX. It has higher effects and less variability in decreasing the risk of adverse effect of CVD and cerebrovascular events compared with thienopyridine compounds. Therefore, the European Society of Cardiology (ESC) and American College of Cardiology (ACC) guidelines have recommended the use of ticagrelor instead of clopidogrel for treating and managing patients with ACS (Wallentin *et al.*, 2009). Ticagrelor has another distinct mechanism of action in which it inhibits the sodium-independent equilibrative nucleoside transporters (ENT 1/2). So, it decreases adenosine uptake by cells, leading to accumulation of adenosine outside the cell. This stimulates the GPCR and activates the adenylate cyclase enzyme. Consequently, this increases cAMP and inhibits platelet activation and aggregation (Cattaneo *et al.*, 2014). The concentration of adenosine is increased after cellular stress conditions such as hypoxia, inflammation and tissue damage. This is mediated by the action of nucleotidase enzyme which converts ADP and ATP to adenosine that acts as a vasodilator and as a potent anti-aggregating agent. However, the effect of adenosine is limited in the blood because of the fast uptake by red blood cells in a half-life of few second. The main side effects of ticagrelor are dyspnoea and ventricular pauses.

Cangrelor is a type of ATP analogue that binds competitively with $P2Y_{12}$ by intravenous injection. It acts rapidly within a short action time (3-6 min) and short offset (30-60 min). It is considered as an emergency drug for acute situations (Gachet, 2015). Another new approach for CVD treatment and management is the use of Ap4A analogue. It acts by inhibiting ADP-induced platelet activation through synergistic antagonization of ADP receptors $P2Y_1$ and $P2Y_{12}$ at the same time which prevents platelet aggregation in a reversible manner (Gremmel *et al.*, 2016).

2.6.5 Molecular and structural characterization of P2YR

There are more than 1000 members of GPCR coding by eukaryotic genome. These groups of proteins are stimulated by diverse ligands, including: amines, lipids, peptides, ions, nucleotides, or proteases. GPCRs are diverse in structure, relevant in function and lead to variety of physiological responses. GPCRs are considered as key target for 30%-60% of modern drugs (Takeda *et al.*, 2002). Based on sequence conservation, GPCR are grouped in at least 5 classes/families (GRAFS classification) with little sequence homology between each other. The largest class is group A or group 1 which is called also rhodopsin like receptors. It includes β2 adrenergic receptors, rhodopsin, P2Y receptors and others (Fredriksson *et al.*, 2003; Takeda *et al.*, 2002). To study the molecular modeling of P2Y receptors, different GPCR models such as β2 adrenergic receptor and bovine rhodopsin were used as templates for P2YR homology modeling. In 2000, the high-resolution crystal structure of bovine rhodopsin was used as a template and homology modeling was applied to all of the P2YRs at various times (Palczewski *et al.*, 2000). $P2Y_1$ was the first P2Y subtype in which molecular modeling was applied in conjunction with site-directed mutagenesis. The ligand binding site of this subtype was suggested to be located in the extracellular site within the upper third of TM 3, 6, and 7. An evidence reported that the charge of amino acid of these extracellular TM domains of $P2Y_1$ and other P2Y like subgroup were expressed as positively charge. These positively charged amino acids were responsible to neutralize the negatively charged phosphate group of the nucleotide agonist ADP ligand (Cherezov *et al.*, 2007). After that, β2 adrenergic receptors and rhodopsin were superseded and replaced by improved new alternative template with high resolution that was more identical at the level of sequence analysis homology. The new molecular modeling of peptide receptor was designed and reported in 2010 by Stevens (Deflorian & Jacobson, 2011). It was based on X-ray structures of the antagonist-bound form of the human C-X-C motif chemokine receptor 4 (CXCR4). CXCR4 is a new appropriate model template for both $P2Y_1$ like subfamily and $P2Y_{12}$ like subfamily. The overall sequence similarity between human $P2Y_{12}$ receptor and the CXCR4 chemokine receptor is 22% and 26% for the TM domains (Jacobson *et al.*, 2012).

After Hollopeter cloning and deorphanization of $P2Y_{12}$, the structure and roles of $P2Y_{12}$ had become more obvious. There were three genetic transcripts forms for $P2Y_{12}$ in human platelet resulted by alternative splicing. The common transcript in platelet was 2.4kb. Another less common 4.5kb transcript was isolated from platelet and brain. The third

transcript with 1kb in size was observed only in platelet mRNA (Hollopeter *et al.*, 2001). $P2Y_{12}$ receptor gene is composed of 4 exons and three introns located on chromosome 3q24-q25. Table 2.1 shows the $P2Y_{12}$ gene characteristic. Only exon three is encoded by 342 amino acid peptide expressed on brain tissue, megakaryocyte and platelet surface (Sattler *et al.*, 1977). The 342 amino acids protein with conserved amino acid sequence within TM domain belong to the class A rhodopsin family GPCRs. $P2Y_{12}$ polypeptide seems like other GPCR. It consists of a single polypeptide chain that forms seven α-helical TM domains, connected to each other by six varying length loops. The cleft surrounded by the TM domains is thought to act as a ligand binding site and receptor functionality. Also, extracellular and intracellular loops coordinate specific ligand binding and signal transduction pathway through a GPCR (Costanzi *et al.*, 2004). $P2Y_{12}$ peptide has two N-linked glycosylation sites (Asparagine6 and Asparagine13) that play an important role in $P2Y_{12}$ receptors expression and signal transduction pathway regulation. Also, $P2Y_{12}$ protein has 4 extracellular cysteine residues (Cys) at the positions 17, 97, 175, and 270. Cys 97 and Cys175, are bound together by a disulphide bond and are important for receptor expression and binding of thiol group of thienopyridine compounds as shown in figure 2.3 (Ding *et al.*, 2003). Molecular modeling and ligand docking of P2Y receptors reveal that the $P2Y_1$R-like and $P2Y_{12}$R-like subfamilies have 20% sequence identity. Therefore, the type of ligand and the mechanism of its binding are different for each of the subfamilies. Within each subfamily, the sequence identity is higher (Jacobson *et al.*, 2012).

Table 2.1: The $P2Y_{12}$ gene details.

Official full name	Purinergic receptor $P2Y_{12}$ (rs2046934)
Alternative names	HORK3; $P2Y_{12}$; ADPG-R; BDPLT8*; SP1999; P2T(AC); P2Y(AC); P2Y(12)R; P2Y(ADP); P2Y(cyc)
Protein family	G-protein coupled receptors
Gene type	Protein coding
Organism	*Homo sapiens*
Lineage	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo
Chromosome location	3 (151336843..151384812)
Map location	q21-q25.1
Exon count	4
Size	342 amino acids; 47970 bases; 39439 Da
Tissues expression	Brain, Platelets, Megakaryocyte
Homologs	$P2RY_{12}$ gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, zebrafish, and frog.
Function	Platelet aggregation, and is a potential target for the treatment of thromboembolisms and other clotting disorders.

*BDPL8: bleeding disorder, platelet type 8.

2.7 Abnormalities of $P2Y_{12}$

Platelets functions especially aggregation and response to antiplatelet therapy showed variability among populations. One of the underlying factors is the genetic variations at the level of platelets receptors.

2.7.1 Polymorphisms in the $P2Y_{12}$ gene

Several SNPs have been identified in $P2Y_{12}$ gene. Recently, five common benign SNPs were identified at the level of $P2Y_{12}$ receptor (Figure 2.2). According to Fontana study, two SNPs were located 139 nucleotide (nt) and 744 nt after the 5_ intron start site, consisting of a C-to-T (i-C139T) and a T-to-C (i-T744C) transition, respectively. Another polymorphism consisted of a single-nt insertion (A) at position 801 of the intron (ins801A). The remaining 2 polymorphisms were found in exon-2 and consist of a C-to-T transition (C34T), which is also called 18C>T, and a G-to-T transversion (G52T), which is called also 36G>T, but none of them modified the encoded amino acid (Asn6 and Gly12 respectively) (Fontana *et al.,* 2003a; Sherry *et al.,* 2001). Four of these SNPs were in absolute linkage disequilibrium and were designed in two phenotypic groups with two haplotypes: H1 which include: i-C139, i-T744, absence i-ins801A, and G52 with frequency 86%. H2 includes: i-139T, i-744C, presence i-ins801A, and 52T with frequency 14%. The fifth identified SNP was C34T which is associated with increased risk of adverse neurological event (4-folds) that is defined as ischemic stroke and/or carotid revascularization within a 2-year observation period than in subjects carrying the wild-type genotype (Ziegler *et al.,* 2005). H2 haplotype has been associated with increased platelet aggregation and atherothrombotic risk (gain of function SNPs) (Fontana *et al.,* 2003a).

While rare pathological mutations within the $P2Y_{12}$ gene lead to a clinical significant bleeding diathesis, common benign polymorphisms of the $P2Y_{12}$ gene may have important implications and indirect association with atherothrombosis (Bierend *et al.,* 2008). Genetic defects of human $P2Y_{12}$ gene are associated with bleeding tendency due to defect in response to ADP induce platelet aggregation. This type of bleeding disorder is called bleeding disorder-platelet type 8 (BDPLT8) and is inherited in an autosomal recessive (AR) pattern (Sattler *et al.,* 1977).

H2 haplotypes (H2/H2) are molecular variants associated with enhanced platelet responses and increased platelet aggregation (hyper responsiveness) induced by ADP, TRAP, collagen, epinephrine, and collagen-related peptide (CRP), or ristocetin (Yee *et al.*, 2005). Also, patients with H2 haplotype (H2/H2) and under anti-platelet therapy with thienopyridine group, but not Aspirin, have shown decreased response or resistance for these drugs (Bierend *et al.*, 2008; Fontana *et al.*, 2003a) . In contrast, patients carrying the H1/H1 and H1/H2 haplotypes have the same whole blood aggregation (Staritz *et al.*, 2009).

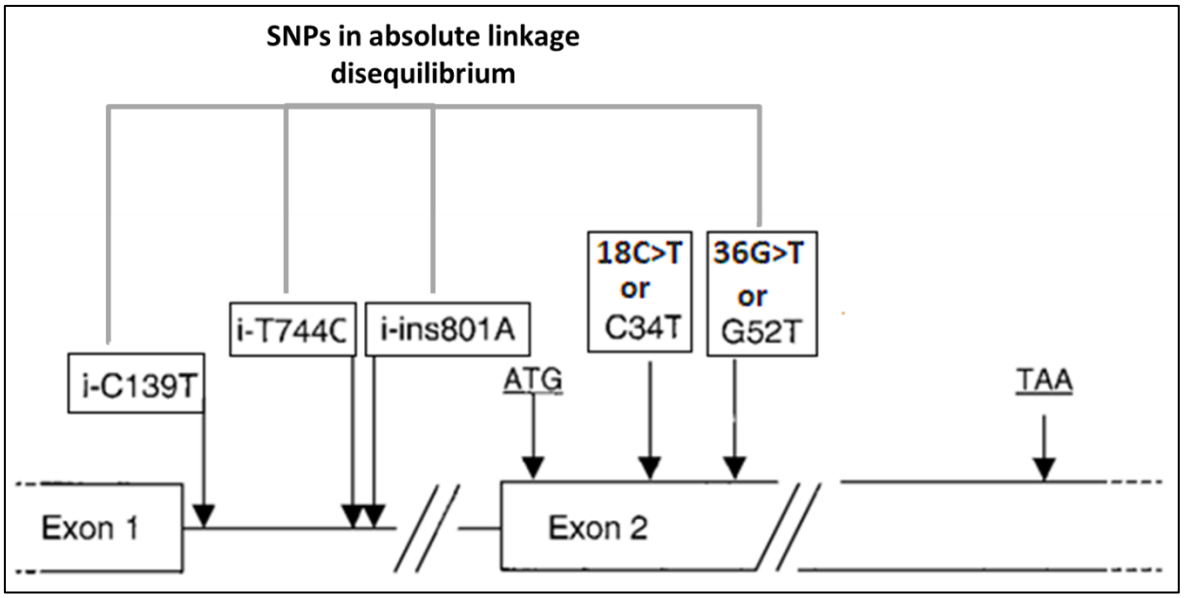

Figure 2.2: Location of the polymorphisms in the $P2Y_{12}$ gene. (Source: Fontana *et al*., 2003a).

2.7.2 Congenital deficiency of $P2Y_{12}$

It is an AR disorder characterized by severe congenital deficiency in $P2Y_{12}$ receptors. Patients with AR $P2Y_{12}$ deficiency express severely impaired platelets aggregation and longer life bleeding diathesis with bleeding time elevation up to 20 mins (Cattaneo *et al.*, 1992). It is characterized by easy bruising, mucosal bleedings, and excessive post-operative hemorrhage (Fontana *et al.*, 2009). Decreased expression of $P2Y_{12}$ receptors lead to diminished function at level of platelet function and thrombosis. The characteristic features of a homozygous patient is the failure of high concentration of ADP (>10mM) to induced irreversible platelet aggregation and the diminished potentiating effect of $P2Y_{12}$ with normal Ca^{+2} mobilization and shape changes (Cattaneo *et al.*, 1992). However, heterozygous patients with congenital $P2Y_{12}$ defect show a full response with irreversible aggregation in the concentration of ADP (>10mM).

2.7.3 Congenital dysfunction of the platelet $P2Y_{12}$ receptors

It's associated with normal $P2Y_{12}$ ligand binding site, but with dysfunctional receptors. Patients with dysfunction $P2Y_{12}$ receptor express mild bleeding symptoms due to residual amount of functional $P2Y_{12}$. The action of ADP to reduce cAMP produced by prostaglandin E1 was diminished, although the receptors site was normal when 2-methylthioadenosine 5-[33P] diphosphate agonist was used (Cattaneo *et al.*, 2003). The underlying cause of this defect is due to G to A transition that can change the codon of arginine 256 in sixth domain TM6 to Glutamine. In contrast, a C to T transition changing the codon for arginine 265 in the third extracellular loop (EL3) to tryptophan as shown in figure 2.3. Another mutation that changed codon 258 coding for proline (CCT) to threonine (ACT) (Pro258Thr) was identified and was found to affect $P2Y_{12}$ receptor hydrophobicity, size and rotational mobility (Cattaneo, 2011a; Remijn *et al.*, 2007).

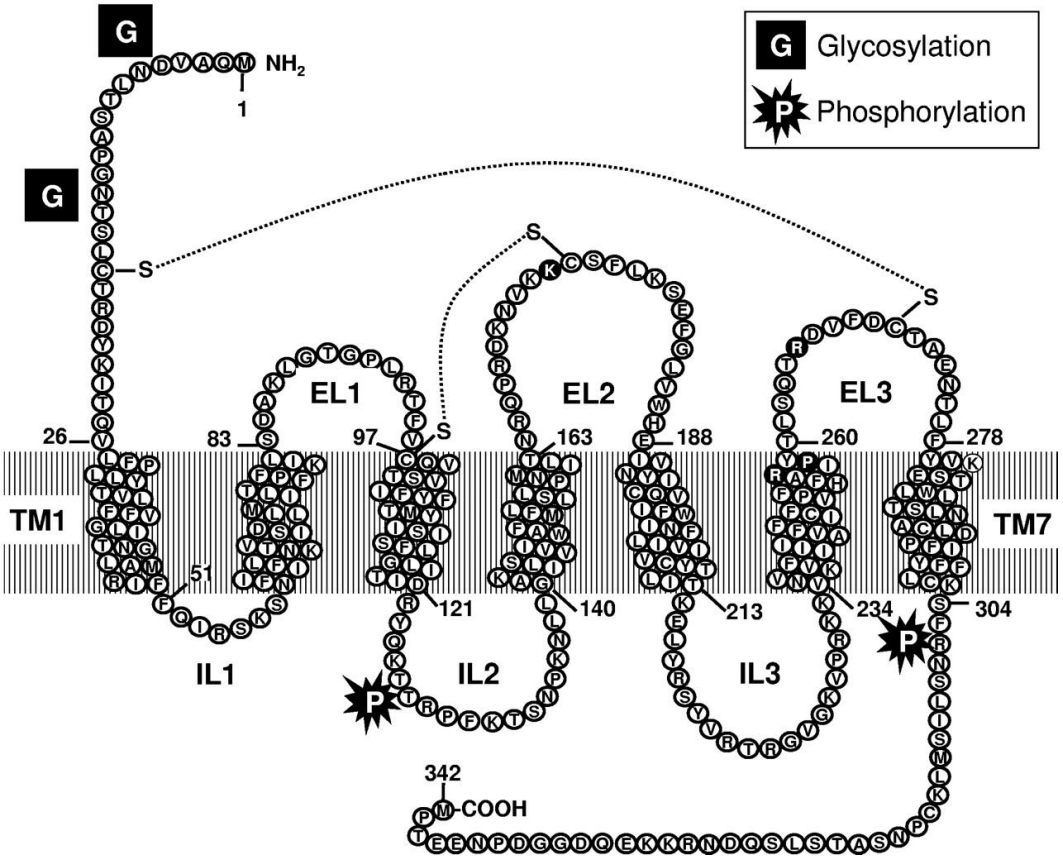

Figure 2.3: Suggested secondary structure of $P2Y_{12}$. Black circles represent the sites of amino acid substitution in patients with dysfunctional $P2Y_{12}$. TM indicates transmembrane region; EL, extracellular loop; and IL, intracellular loop. (Source: Cattaneo, 2011b).

Chapter Three

Materials and Methods

This chapter explains the methodology of the study in details. It aims to determine the frequency of the most common and significant SNPs at the level of $P2Y_{12}$ gene among Palestinian, Swedish and Congolese healthy individuals (n=254). Moreover, it covers the methodology used in exploring the effect of C34T (18C>T) and G52T (36G>T) genotypes on platelets response induced by ADP/TRAP agonists. All different materials, instruments and equipment that were used in this study are described in table 3.1.

Table 3.1a: Instruments and reagents used in the study.

Item	Components	Company
Blood collection tubes	EDTA and Hirudin vacutainer tubes.	
DNeasy Blood & Tissue Kits; purification kit for blood	1- Proteinase K. 2- DNeasy Mini Spin Columns (colorless) in 2 ml Collection Tubes. 3- Collection Tubes (2 ml). 4- Buffer ATL. 5- Buffer AL. 6- Buffer AW1 (concentrate).* 7- Buffer AW2 (concentrate).* 8- Buffer AE.	Germany
Expanded long template PCR	1- Expanded long template enzyme mix 2- Expanded long template 10x buffer 1 3- Expanded long template 10x buffer 2 4- Expanded long template 10x buffer 3	Roche; Germany
PCR Master mix 2X	1- *Taq* DNA polymerase. 2- dNTPs. 3- 1.5 mM$MgCL_{2.}$ 4- Red dye.	ThermoScientific; USA
PCR tubes	0.2 ml thin-walled reaction tubes with flat caps, Lot. 5084811.	Sarsted; Germany
Centrifuge	Eppendorfs	Germany
Spectrophotometer	Beckman; DU 530	USA
Multiplate electrode		Roche, Germany
Agonists	1- ADP 6.5 µM 2- TRAP 32 µM	Roche, Germany

Table 3.1b: Instruments and reagents used in the study.

Item	Components	Company
PCR cycle sequencing kit	1- BigDye® Terminator v1.1 Sequencing kit (BigDye Terminator v1.1 Matrix Standards) 2- BigDye® Terminator v1.1, v3.1 5X sequencing buffer.	1- Applied Biosystems California, USA 2- Warrington, UK
PCR machine	Applied biosystems; Veriti	Germany
Agarose type II	Medium EEO; Lot# SLBN2061V	Sigma-Aldrich; USA
TRIS-EDTA(5X TBE)	1- 54 g Tris 2- 27.5 g boric acid 3- 20 ml of 0.5 EDTA	
Nucleic acid gel stain	GelStar	Lonza, Basel, Switzerland
PCR product dye	6x Loading dye solution	New England; Biolab
Gel agarose standard	Gel agarose standard O Gene Ruler 100 bp (green color).	Fermentas, Waltham, Massachusetts, USA.
Power supplier	Microcomputer electrophoresis power supply (100-150 voltage).	CONSORT, Hertfordshire, England.
UV light	Dark reader	
ABI PRISM® 310 Genetic Analyzer	10x EDTA buffer, Polymerase	Applied Biosystems, California, USA
Cycle sequencing kit	BigDye® Terminator v1.1 Sequencing kit (BigDye Terminator v1.1 Matrix Standards)	Applied Biosystems, California, USA
Injection solvent for genetic analyzer	Hi-Di™ Formamide	Applied Biosystems (PN 4311320)

* Buffer AW1 and Buffer AW2 are supplied as concentrates. A 96–100% ethanol was added according to the bottle label before use to obtain a working solution.

3.1 Study population

For the genetic study, a total of 254 healthy individuals from three different populations; Swedish, Congolese and Palestinians were recruited to participate in the study. The distribution and baseline characteristics of study subjects are shown in table 3.2. A total of 145 samples were from Palestine with ages between 17-24 years and a male: female ratio of 70:75, 55 samples were from Sweden and aged between 17-55 years and 54 samples from DR Congo, all of which were females aged between 17-45 years. Furthermore, ten Swedish volunteers were recruited to participate in the aggregation study.

Table 3.2: Study population baseline characteristics.

Variable	Palestinian	Swedish	Congolese
Frequency (n)	145	54	55
Age range (years)	17 – 24	17 - 55	17 – 45
Gender (M:F)	70 : 75	---	0 : 55
Region	West bank; Hebron	Gothenburg	Bukavu

3.2 Sample collection and preparation

Subjects were recruited conveniently from healthy unrelated individuals with different blood groups from Palestine and DR Congo, while all Swedish samples were picked randomly from blood group O. The molecular part of the study was analyzed at Gothenburg University, Department of Biomedicine Science and the aggregation study was conducted at Sahlgrenska Hospital, Department of Transfusion Medicine.

For genotyping, DNA was extracted for the Palestinian samples from buffy coat EDTA blood. The DNA samples that were available were extracted according to manufacture instructions and stored at -20C°.

For the aggregation study, 3 mL of whole blood were collected from 10 Swedish individuals in hirudin coagulated tubes using a vacutainer technique for the purpose of the aggregation and direct measurement of platelet aggregation (within 2 hours) induced by ADP/TRAP using multiple electrode Analyzer (Roche Diagnostic).

3.3 Platelet aggregation study

In this study, multiple electrode analyzer (MEA) instrument was used to assess the platelet aggregation in response to 6.5 μM of adenosine diphosphate (ADP) and 32 μM of thrombin receptor activating peptide (TRAP) agonists. The purpose of the aggregation study was to correlate the effects of the identified SNPs with platelets response induced by different agonists (ADP and TRAP). Whole blood (3 mL) from 10 healthy Swedish individuals was collected and analyzed. Aggregation studies were performed within 2 hours of blood collection and aggregation was measured at 37°C. Multiple electrode analyzer instrument is a phenotypic assay that measures an intermediate clinical phenotype called platelet reactivity using different adjusted concentrations of agonists.

For whole blood, 300 µL phosphate buffered saline (PBS) (0.15 mol/L sodium chloride buffered with sodium- and calcium phosphate 0.06 mmol/L, pH 7.1) and 300µL whole blood were mixed in the test cell and incubated for 3 minutes. After the incubation, 20 µL ADP or TRAP were added as an agonist to induce aggregation and AUC was obtained after a 6 minutes measurement. AUC reflects the level of platelets aggregation response due to stimulation of the $P2Y_{12}$ receptors by ADP/TRAP agonist. In case of sufficient concentration of ADP, platelet activation and shape changes were induced. This leads to increase the impedance of electrodes during the test time until maximal aggregation occurs. The normal individual has an increasing AUC until maximal full platelets activation occurs. If a patient is under thienopyridine compounds, the AUC result is below the normal range. However, if the AUC for patients with clopidogrel resistance is close to normal range, inadequate platelet inhibition occurred.

3.4 Genetic study

3.4.1 DNA extraction

Swedish and Congolese samples: Genomic DNA was prepared from whole blood drawn in EDTA tubes using the QIAGEN DNeasy Blood & Tissue extraction Kit according to the manufacture instructions (Qiagen, 2013). Briefly, after preparation of working solution, 20 µL of proteinase K were added into the bottom of a 1.5 sterile microcentrifuge tube. Then, 200 µL of well mixed whole blood were transferred carefully to the tubes. After that, 200 µL of buffer AL (lysing buffer) were added. The tubes were mixed vigorously to yield a homogenous solution by puls vortexing for 15 seconds. After incubation for 10 min at 56 °C, the micocentrifuge tubes were centrifuged briefly to remove the drops from tube lid. Then, 200 µL of absolute ethanol were added to the tube mixture and mixed again by puls vortexing for 15 sec. Then, the tubes were centrifuged briefly to remove drops from inside of the lid. After that, the mixture was transferred to QIAamp mini spin column with a 2 mL collection tube. Tubes were centrifuged for 1 min at 8,000 rpm. After centrifugation, the QIAamp Mini spin columns were placed in clean collection tubes. Then, 500 µL of AW1 buffer were added without wetting the rim and centrifuged at 8,000 rpm for 1 min. Next, QIAamp Mini spin column was placed in a new clean 2 mL collection tube. Then, the QIAamp Mini spin columns were carefully opened, 500 µL of buffer AW2 were added and the columns were centrifuged for 3 min at full speed (14,000 rpm). After that, the

collection tubes were discarded and the QIAamo Mini columns were re-centrifuged at full speed for 1 min for drying. Finally, the QIAamp Mini columns were placed in a clean 1.5 mL microcentrifuge tubes and the DNA was eluted by adding 100 µL distilled water, incubation for 1 min and then centrifugation at 8,000 rpm for 1 min. The extracted DNA was stored at –20°C.

Palestinian samples: Genomic DNA was prepared from buffy coat using the MasterPure™ DNA Purification (Epicentre) according to the manufacture instructions (Epicentre, 2013).

3.4.2 DNA quantification

The concentration of DNA for Palestinian samples was measured spectrophotometrically by Nanodrop 2000c (Thermoscientific). For Swedish and Congolese samples, the BioPhotometer Plus (Eppendorf) was used. The instrument was adjusted using 60 µL distilled water as a blank. Most samples were measured twice to check the accuracy and precision. The protein/DNA ratio (260/280 nm) was calculated to evaluate the purity of DNA samples. The DNA concentrations for all samples were ranged from 7-114 ng/µL. The concentrated DNA samples were diluted with distilled water. In contrast, diluted DNA samples were concentrated by increasing the taken volume of template DNA. DNA samples with A260/A280 ratio less than 1.2 were not used.

3.4.3 Primer design

As mentioned before, the $P2Y_{12}$ receptor gene consists of four exons in which exon-3 is the only coding one. The most significantly reported SNPs are located in the coding region of exon-3, which includes 36G>T (H2 haplotype) and 18C>T. In this study, only exon-3 was sequenced in all samples (n=254). Four pairs of primers were designed according to the primer design software of eurofins genomics. The sequence, length, melting temperature and nucleotide position are listed in Table 3.3. Our primers' length ranged between 20-27 bp in length. They were selected with an average of GC content around 33%-60% with random base distribution. Since exon-3 is long and with high GC content, three primer pairs (3-8) with an overlap designed to cover the whole exon-3, as shown in table 3.3.

Table 3.3: Primers sequence that were used to amplify exon-3 of $P2Y_{12}$ gene.

Primer No.	Oligo[1] name	Sequence	Length (bp)	Tm[2] (^oC)	GC%
1	$P2Y_{12}$**F**[3]127532	5'-CCTTAGGCTGAAAATAACCATCCTC-3'	25	61.3	44%
2	$P2Y_{12}$**R**[4]128675	5'-GCGCTTTGCTTTAACGAGTTCTGAA-3'	25	61.3	44%
3	$P2Y_{12}$ex2.1**F**	5'-AATAACTACCTTAGGCTGAAAATAACC-3'	27	58.9	33%
4	$P2Y_{12}$ex2.1**R**	5'-TTTTAAATGGCCTGGTGGTC-3'	20	55.3	45%
5	$P2Y_{12}$ex2.2**F**	5'-CTGGGAACAGGACCACTGAG-3'	20	61.4	60%
6	$P2Y_{12}$ex2.2**R**	5'–AAGGAATTCGGGCAAAATG-3'	20	53.2	40%
7	$P2Y_{12}$ex2.2**F**	5'–AAAGAACTGTACCGGTCATACG-3'	23	58.9	43.5%
8	$P2Y_{12}$ex2.3**R**	5' –TTAGCGTTTGCTTTAACGAG-3'	21	55.9	42.9%

[1]Oligo: Oligonecleotide; [2]Tm: melting temperature; [3]F: Forward primer; [4]R: reverse primer.

3.4.4 DNA amplification by PCR

In this study, extended Long Template PCR system (LT-PCR) was used for the majority of samples. LT-PCR is a special enzyme mixture that contains thermostable Taq DNA polymerase and thermostable Tgo DNA polymerase. The Tgo polymerase has 3'-5' exoneoclease activity (proofreading activity). So, LT-PCR yields large amount of target DNA. A final volume of 50 µL PCR reaction was used and included two mixtures. The final volume of mixture one was 25 µL containing 5 µL of dNTPs, 1 µL genomic DNA (~100 ng), 1 µL of each amplification primer (10µM each) and 17 µL distilled water. The second mixture with 25 µL contains 19.25 µL ddH_2O (double distilled water), 5 µL of buffer1 10X with 17.5 mM $MgCl_2$, 0.75 µL of enzyme mix of Taq and Tgo DNA polymerase enzymes with enzyme storage buffer (20 Mm Tris-HCl, pH 7.5 at 25°C, 100 mM KCl, 1 mM dithiothereitol (DTT), 0.1 mM EDTA, 0.5% nonidet P40 V/V, 0.5% Tween 20 V/V, 50% glycerol V/V). Mixture one and two were mixed together in a PCR

tube to get a final volume of 50 µL. PCR amplification was carried out in a PCR system (Applied Biosystems, Verity 96 well thermal cycler).

A total of 32 samples of Congolese population were amplified by PCR master mix1X with the same LT-PCR program. The PCR master mix includes Taq DNA polymerase, dNTPs, 1.5 mM $MgCL_2$, and red dye. The PCR tube mixture with final volume of 25 µL contained 3 µL genomic DNA (~100 ng), 20 µL PCR buffer (buffer:75 mM Tris-HCl, pH 8.8 at 25°C, 20 mM ammonium sulfate, 1.5 mM MgCl2 and 0.625 Units ThermoPrime Taq DNA Polymerase) and 1 µL of each amplification primer (10 µM each). The amplification was performed in the thermal cycler using the PCR condition summarized in table 3.4.

Table 3.4: Thermal cycler program for exon-3 amplification by LT-PCR.

PCR program	**Exon-3 amplification by LTPCR**		
	Temp (^{o}C)	**Time**	
Initial denaturation	92	10 sec	
Denaturation	92	6 sec	35 cycles
Annealing	58	15 sec	
Extension	68	2 min	
Final Extension	72	7 min	
Keep in machine	4	24hrs	

3.4.5 Agarose gel electrophoresis

After PCR reaction and amplification of target DNA, the agarose gel electrophoresis was used for separating and analyzing the targeted DNA fragment. In neutral pH buffers, the net negative charge of phosphate backbone of DNA migrates from the cathode (negative electrode) to the anode (positive electrode). Migration is according the fragment size, whereas, the shorter DNA fragments move faster and longer.

A 1.5% agarose gel was prepared by mixing 0.9 gm of Agarose type II, with 60 mL of 0.5TBE (54g Tris, 27.5g borate, 20mL 0.5 EDTA) buffer. When the mixture had cold down to about 60°C, 1 µL Nucleic Acid Gel Stain, Gelstar, Lonza was added in order to visualize the DNA fragments after separation. Then, 5 µL of the PCR products were electrophoresed on the agarose gel. A PCR product from colorless LT-PCR was mixed with 1µL of Gel Loading Dye (6x) and then was loaded. In the first lane, 1 µL of 100 bp

DNA OGeneRuler Gel agarose marker was loaded. All amplification products were separated by high-voltage electrophoresis (150 voltages for 40 min). After separation, the gel was placed on a UV light transilluminator to visualize the DNA fragments.

3.4.6 Purification of PCR products

Purification for PCR products was performed using (QIAquick PCR purification kit) from QIAGEN company using a silica-based membrane technology in the form of spin columns. After the working reagent was prepared according to manufacturer's instructions, a 5 volume of Binding Buffer was added to 1 volume of PCR product and mixed well (250µL binding buffer to 50 µL PCR products). Then, the QIAquick column was placed in a 2 mL collection tube and centrifuged for 30-60 seconds to allow DNA to bind with the silica membrane. Next, the flow-through was discarded and 750 µL of the diluted washing buffer were added (with ethanol) to the QIAquick purification column. The column was centrifuged for 30-60 seconds and the flow-through was discarded. After that, the purification column was placed back into the same collection tube and the empty QIAquick purification column was centrifuged for an additional 1 min for completely removal of any residual wash buffer. The QIAquick purification column was transferred to a clean 1.5 mL microcentrifuge tube. Finally, 50µL of Elution Buffer (10 mM Tris-HCl, pH 8.5) were added to the center of the QIAquick purification column membrane and centrifuged for 1 minute. The QIAquick purification column was discarded and the purified DNA was stored at 4°C. The quality and quantity of purified PCR product was assessed by agarose gel electrophoresis.

3.4.7 Sequencing of PCR products

The PCR reaction for sequence was performed using applied biosystems thermal cycler instrument with a final volume of 20 µL. For each sample, three sequencing PCR were done. According to the sequencing primer (Table 3.3), exon-3 was divided into three parts with different primer sets. The PCR mixture with each primer set included 3 µL of pure PCR product, 8 µL ddH_2O, 1 µL of forward primer, 2 µL BigDye Terminatorv1.1/3.1 Sequencing Buffer and 6 µL BigDye Sequencing Buffer. This mixture was subjected to 35 three-temperature cycles. The sequencing reaction is summarized in table 3.5.

Table 3.5: Cycle sequencing reaction.

Reagent	Concentration	Volume (µL)
BigDye Terminator v1.1/3.1 Sequencing Buffer	5X	2
BigDye Sequencing Buffer	5X	6
Primer (Forward)	10X	1
DNA template		3
dd H_2O		8
Final volume		20
The samples were mixed well and spinned briefly, then, the tubes were placed in a thermal cycler and the volume was set to 20 µL.		

3.4.8 Ethanol / EDTA precipitation

After Cycle Sequencing was completed, the 20 µL PCR sequencing reactions were precipitated. A 100 µL of 99% ethanol and 5 µL of 125 mM EDTA were added to each PCR tube. The mixture was incubated for 30 min to 4 hours at room temperature, and centrifuged for 20 min at 12,000 rpm. The tube content was inverted into paper towel without disrupt the belt. The next step followed immediately and if this was not possible, the tubes were spinned for additional 2 min before performing the next step. The tube was washed with 200 µL of 70% ethanol and centrifuged for 5 min at 12,000 rpm. The tube content was inverted into paper towel without disrupt the belt for complete air drying. Finally, 20 µL HiDi formamide were used to re-suspend the single-stranded sequencing amplicons.

3.4.9 Sequencing

Swedish and Congolese samples were sequenced using ABI PRISM, 310 Genetic analyzer, Applied Bio systems, U.S, which is based on capillary electrophoresis (CE) and used POP-6 performance optimized polymer 6 and 1X buffer with EDTA. The PCR products from the Palestinian samples were sent to Eurofins Company in Germany for exon-3 sequencing.

3.4.10 Analysis of DNA sequence

Basic Local Alignment Search Tool (BLAST) searches / CDS feature (complete DNA sequence) was used to compare the DNA sequences with RefSeq (*Homo sapiens* Purinergic $P2Y_{12}$ receptor ID: 64805), RefSeq Gene on chromosome 3, Accession AK292096). Sequences were first examined for 2 benign polymorphisms in the first part of exon-3; (18C>T [p.Asn6] and (36G>T [p.GlY12]). Another two pathological SNPs were

examined in the second and third parts of exon-3; (767G>A [p.Arg256Gln] and (793C>T [Arg265Trp]). Also, all exon-3 was examined and analyzed for any other SNPs. Other programs were used to look for all $P2Y_{12}$ gene variants that have been discovered before and the role of each variant (polyphenotyping) in pathogenicity using the dbSNPs short genetic variations, ENSEMBL Genetic Variation and Exome Variant Server (EVS).

3.5 Ethical considerations

All individuals were given information about the study and then signed a written consent form for the use of the blood components in this study. An ethical approval was given by the Institutional Review Board of Sahlgrenska University Hospital and Al-Quds University.

3.6 Statistical analysis

Data was coded, entered and analyzed using SPSS version 24. Descriptive statistics for all individuals were presented as frequencies and percentages for categorical variables, and mean and standard error of mean (SEM) for continuous variables. Independent t -test was used to compare the association between the genotypes and the platelet aggregation induced by ADP/TRAP. Differences between genotype groups and population study were analyzed by using Chi-square test. Alleles and genotypes frequencies were calculated with the Hardy-Weinberg equilibrium prediction. A p-value of <0.05 was considered to be statistically significant.

Chapter Four

Results

4.1 $P2Y_{12}$ sequencing results

In this study, the $P2Y_{12}$ coding exon-3 was sequenced and analyzed. All samples (n=254) were sequenced and tested for H1, H2 haplotype (tag-SNP G52T "36G>T") and C34T (18C>T). Furthermore, all individuals were tested for two other pathological SNPs 767G>A and 793C>T. For this purpose, six primer pairs were designed for the sequencing of exon-3 of $P2Y_{12}$ gene. Swedish and Congolese samples were sequenced for whole exon-3 with the forward primers 3, 5 and 7, while the Palestinian samples were sequenced for the exon-3 with the forward primer 3. The tag-SNP G52T and C34T were located in the first part of exon-3, as shown in figure 4.1. The target exon-3 was amplified using PCR. Then, PCR products were separated by gel electrophoresis, purified, and used for DNA sequencing (Figure 4.2).

ATGCAAGCCGTCGACAA**(C/T)**CTCACCTCTGCGCCTGG**(G/T)**AACACCAGTCTGTGCACCA
GAGACTACAAAATCACCCAGGTCCTCTTCCCACTGCTCTACACTGTCCTGTTTTTTGTTGGAC
TTATCACAAATGGCCTGGCGATGAGGATTTTCTTTCAAATCCGGAGTAAATCAAACTTTATT
ATTTTTCTTAAGAACACAGTCATTTCTGATCTTCTCATGATTCTGACTTTTCCATTCAAAATTC
TTAGTGATGCCAAACTGGGAACAGGACCACTGAGAACTTTTGTGTGTCAAGTTACCTCCGTC
ATATTTTATTTCACAATGTATATCAGTATTTCATTCCTGGGACTGATAACTATCGATCGCTAC
CAGAAGACCACCAGGCCATTTAAAACATCCAACCCCAAAAATCTCTTGGGGGCTAAGATTC
TCTCTGTTGTCATCTGGGCATTCATGTTCTTACTCTCTTTGCCTAACATGATTCTGACCAACA
GGCAGCCGAGAGACAAGAATGTGAAGAAATGCTCTTTCCTTAAATCAGAGTTCGGTCTAGT
CTGGCATGAAATAGTAAATTACATCTGTCAAGTCATTTTCTGGATTAATTTCTTAATTGTTAT
TGTATGTTATACACTCATTACAAAAGAACTGTACCGGTCATACGTAAGAACGAGGGTGTAG
GTAAAGTCCCCAGGAAAAAGGTGAACGTCAAAGTTTTCATTATCATTGCTGTATTCTTTATT
TGTTTTGTTCCTTTCCATTTTGCCCGAATTCCTTACACCCTGAGCCAAACCCGGGATGTCTTT
GACTGCACTGCTGAAAATACTCTGTTCTATGTGAAAGAGAGCACTCTGTGGTTAACTTCCTT
AAATGCATGCCTGGATCCGTTCATCTATTTTTTCCTTTGCAAGTCCTTCAGAAATTCCTTGAT
AAGTATGCTGAAGTGCCCCAATTCTGCAACATCTCTGTCCCAGGACAATAGGAAAAAAGAA
CAGGATGGTGGTGACCCAAATGAAGAGACTCCAATGTAA

Figure 4.1: Screened $P2Y_{12}$ nt sequence of the transcript variant X1, mRNA for exon-3 that shows the site of SNPs C34T (18C>T) and G52T (36G>T), respectively. (Source: NCBI Reference Sequence: XM_017007069.1).

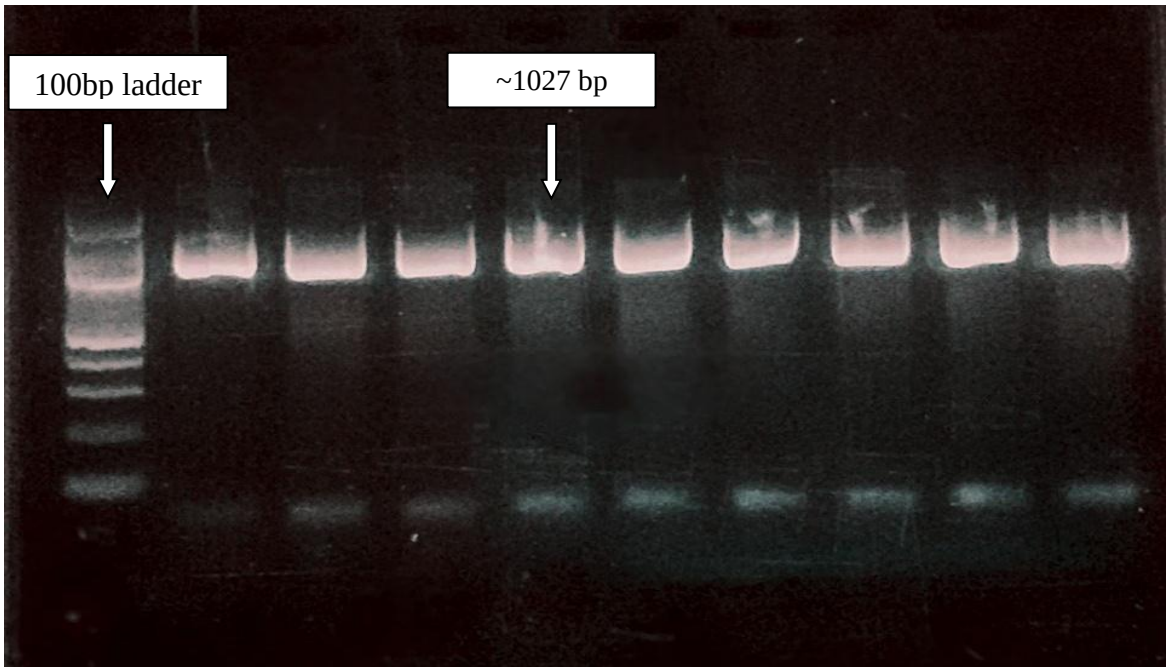

Figure 4.2: Agarose gel electrophoresis of PCR products of $P2Y_{12}$ gene exon-3 of 9 Swedish subjects.

A total of five benign SNPs were found among study population (18C>T, 36G>T, 546C>T, 989A>G, and 162G>T). In addition, the two pathological SNPs 767G>A and 793C>T were not found in the study group. The minor allele frequencies (MAF) of the five $P2Y_{12}$ gene variants are shown in table 4.1 (21.9%, 10.0%, 0.6%, 0.2 and 0.2%, respectively). Regarding the genotypes, the proportion of subjects carrying the wild type for the five genetic variants was 64.2%, 80.3%, 98.8, 99.6% and 99.6% for 18C>T, 36G>T, 546C>T, 989A>G and 162G>T, respectively. Furthermore, heterozygous genotypes for the previously mentioned variants in the same order were found in 28.0%, 19.3%, 1.2%, 0.4% and 0.4% of study subjects. Moreover, 20 subjects and only one subject had homozygous mutated alleles for the 18C>T and 36G>T variants, respectively.

Table 4.1: The frequency of $P2Y_{12}$ SNPs among study populations.

SNP	Frequency (n=254)*			Minor allele frequency** (%)
	Wild type N (%)	Heterozygote N (%)	Homozygote N (%)	
c.18C>T → p. Asn6=	163 (64.2)	71 (28.0)	20 (7.9)	21.9
c.36G>T→ p.Gly12=	204 (80.3)	49 (19.3)	1 (0.4)	10.0
c.546C>T→ p.Phe182=	251 (98.8)	3 (1.2)	0 (0.0)	0.6
c.989A>G→p.Glu330Gly	253 (99.6)	1 (0.4)	0 (0.0)	0.2
c.162G>T→p.Arg54=	253 (99.6)	1 (0.4)	0(0.0)	0.2

* Numbers may not add to 100% due to rounding.
** n=508 chromosomes.

4.1.1 Sequencing of $P2Y_{12}$ in the Swedish population

A total of 55 individuals from Swedish origin were sequenced and exon-3 was analyzed. All participants were negative for the pathological SNPs: 767G>A and 793C>T. Furthermore, all individuals were negative for other SNPs in part two and three of exon-3. Regarding part one of exon-3, only two benign SNPs were identified and genotyped C34T (18C>T) and G52T (36G>T). The rare genotype TT (H2H2 haplotype) was identified in one Swedish participant. The number of individuals and the percentage of each SNP genotypes are shown and the minor allele frequencies are shown in table 4.2. The MAF were calculated using Hardy-Weinberg Equation to be 20% and 23.6% respectively for the tow variants.

Table 4.2: The frequency of $P2Y_{12}$ SNPs among Swedish population.

SNP	Frequency (n=55)*			Minor allele frequency** (%)
	Wild type N (%)	Heterozygote N (%)	Homozygote N (%)	
c.18C>T → p. Asn6=	35 (63.6)	18 (32.7)	2 (3.6)	20.0
c.36G>T→ p.Gly12=	30 (54.5)	24 (43.6)	1 (1.8)	23.6

* Numbers may not add to 100% due to rounding.
** n=110 chromosomes

4.1.2 Sequencing of $P2Y_{12}$ in the Congolese population

Regarding the Congolese individuals, a total of 54 samples were analyzed. The two tested SNPs at the level of part 1 of exon-3, 18C>T and 36G>T, were detected with frequencies of 6.5% and 12%, respectively. In addition, for part 2 of exon-3, two additional polymorphisms were found (546C>T and 989A>G). These allele variants were found only in heterozygous manners. One of the identified SNPs was a silent mutation at position c.546C>T without any clinical significance. The other SNP was identified at position c.989A>G with a substitution in amino acid 330 from Glu to Gly. Furthermore, all participants were negative for the two pathological SNPs767G>A and 793C>T (Table 4.3).

Table 4.3: The frequency of $P2Y_{12}$ SNPs among Congolese population.

SNP	Frequency (n=54)*			Minor allele frequency** (%)
	Wild type N (%)	Heterozygote N (%)	Homozygote N (%)	
c.18C>T → p. Asn6=	50 (92.6)	1 (1.9)	3 (5.6)	6.5
c.36G>T→ p.Gly12=	41 (75.9)	13 (24.1)	0 (0.0)	12.0
c.546C>T→ p.Phe182=	52 (96.3)	2 (3.7)	0 (0.0)	1.9
c.989A>G→p.Glu330Gly	53 (98.1)	1 (1.9)	0 (0.0)	0.9

* Numbers may not add to 100% due to rounding.
** n=108 chromosomes

4.1.3 Sequencing of $P2Y_{12}$ in the Palestinian population

Regarding the Palestinians, a total number of 145 individuals were sequenced and analyzed for exon-3 using forward primer 3. The MAF using Hardy-Weinberg Equation of C34T (18C>T) and G52T (36G>T) were 28.3% and 4.1%, respectively. All Palestinian individuals were negative for the two pathological SNPs. Further, two silent SNPs at position c.546C>T and c.162G>T were detected in one individual each. The number of individuals and the percent of each genotype are shown in table 4.4.

Table 4.4: The frequency of $P2Y_{12}$ SNPs among Palestinian population.

SNP	Frequency (n=145)*			Minor allele frequency** (%)
	Wild type N (%)	Heterozygote N (%)	Homozygote N (%)	
c.18C>T → p. Asn6=	78 (53.8)	52 (35.9)	15 (10.3)	28.3
c.36G>T→ p.Gly12=	133 (91.7)	12 (8.3)	0 (0.0)	4.1
c.546C>T→p.Phe182=	144 (99.3)	1 (0.7)	0 (0.0)	0.3
c.162G>T→p.Arg54=	144 (99.3)	1 (0.7)	0 (0.0)	0.3

* Numbers may not add to 100% due to rounding.
** n=290 chromosomes

Moreover, there was a significant difference in the frequency of 18C>T genotypes between the three study populations (P <0.001) and for 36G>T genotypes (P <0.001). Examples on the sequencing results of the different SNPs are illustrated in figure 4.3 and figure 4.4. In addition, table 4.5 summarizes all SNPs/variants observed in the study populations.

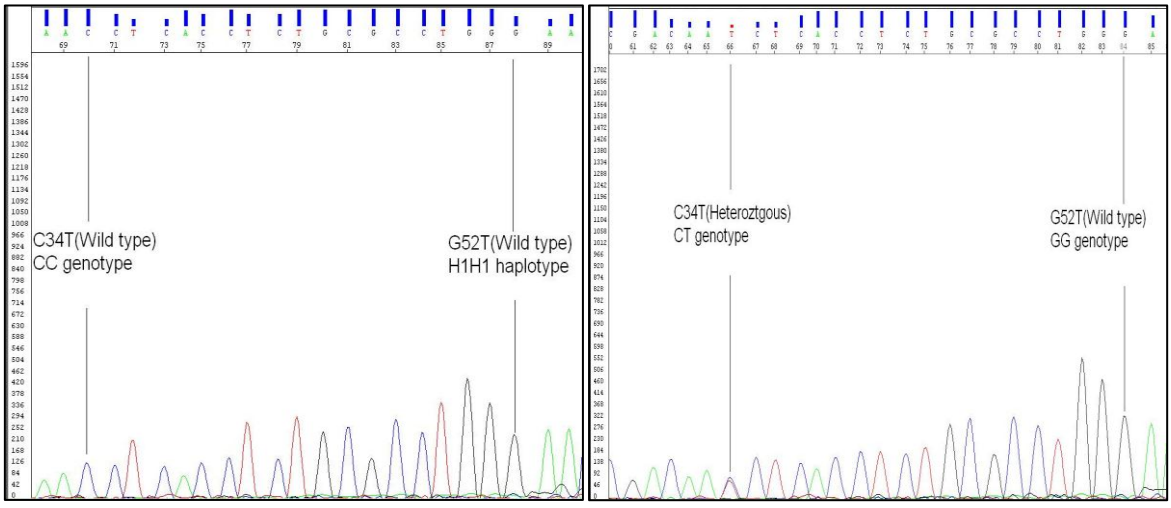

Figure 4.3: DNA sequencing result from exon-3 of the $P2Y_{12}$ gene, showing C34T (18C>T) and G52T (36G>T) genetic variants.

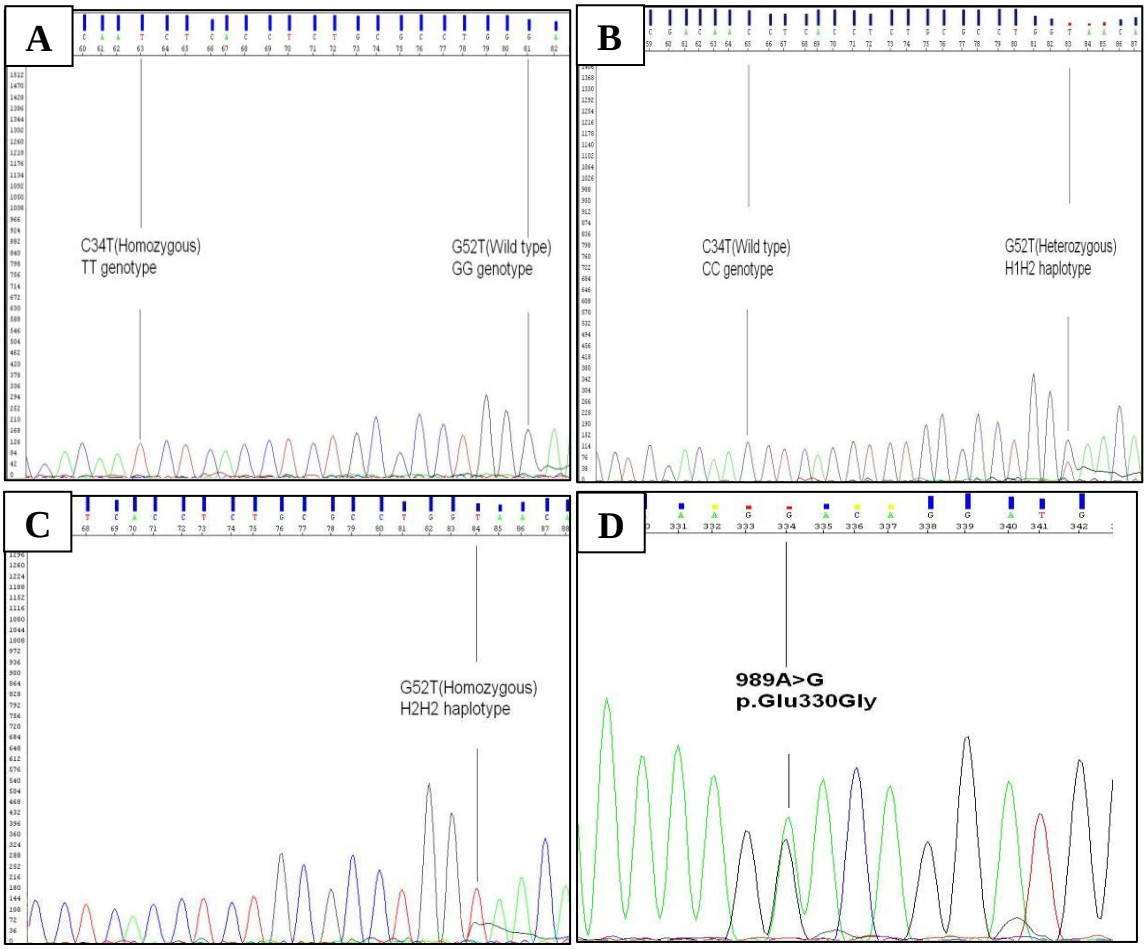

Figure 4.4: DNA sequencing result from exon-3 of the $P2Y_{12}$ gene. **A)** TT genotype of C34T (18C>T) and GG genotype (H1H1 haplotype) of G52T (36G>T). **B)** CC genotype of 18C>T and GT genotype (H1H2) of 36G>T. **C)** The rare genotype TT (H2H2) of 36G>T. **D)** The heterozygous form of 989A>G.

Table 4.5:.DNA variants observed in the study subjects. (Source: http://evs.gs.washington.edu/EVS/)

rs ID*	Variant position** (bp)	Alleles €	cDNA change	Protein change	Phenotype ¥	Allele frequency# (%)
rs6785930	151338828	G+A	c.18C>T	p.Asn6=	Benign	71% G / 29% A
rs6809699	151338810	A+C	c.36G>T	p.Gly12=	Benign	83% C / 17% A
rs121917885	151338079	C+T	c.767G>A	p.Arg256Gln	Probably damaging	100% C
rs121917886	151338053	G+A	c.793C>T	p.Arg265Trp	Probably damaging	100% G
rs16863320	151338300	A+G+T	c.546C>T	p.Phe182=	Benign	99% G / 1% A
rs16846673	151337857	T+**C**	c.989A>G	p.Glu330Gly	Benign	99% C / 1% T

* rs ID (rsids): accession number used by researchers and databases to refer to specific SNP and it stands for (Reference SNP cluster ID) or dbSNP (SNPs Database) reference SNP identifier.
** Variant position: SNV (Single Nucleotide Variant) location on the chromosome.
€ Alleles: it refers to a change from a reference allele to an alternate allele.
#Allele frequency: observed allele percentage in all populations (European American (EA) and African American (AA).
¥ Phenotype: prediction of possible impact of an amino acid substitution on protein structure and function based on Polymorphism Phenotyping (PolyPhen2) program.

4.2 Aggregation study results

Platelet aggregation in whole blood induced by ADP and TRAP agonist was measured using a multiple electrode analyzer (Roche Diagnostic) instrument. TRAP is a potent platelet aggregating agent that acts via the thrombin receptor PAR-1. The reference ranges for ADP and TRAP induced platelet aggregation for whole blood according to the manufacturer of the multiple electrode analyzer instrument are 53-122 AUC and 94-156 AUC, respectively. The ratio of ADP to TRAP induced aggregation is called relative ADP-induced aggregation (r-ADP-agg) and considered as a valuable tool for reflecting the individual degree of $P2Y_{12}$-mediated platelet reactivity.

The majority of the individuals (n=9) had a normal phenotypic group of platelet aggregation. The AUC results of all individuals were within the reference range with an aggregation profile consistence with normal platelet response for ADP/TRAP agonists. The mean ± SEM of TRAP-induced aggregation, ADP-induced aggregation and r-ADP-agg were 120.7 ± 4.8 AUC, 74.7 ± 3.43 AUC and 61.8%, respectively**.** One individual was excluded because he was under anti-platelet therapy and had a low AUC in response to ADP and TRAP, 8 and 56 AUC, respectively.

4.3 Effect of DNA sequence variants on aggregation study

Regarding the aggregation study, the frequency of G and T alleles of 36G>T in the 9 Swedish individuals were 66.7% and 33.3%, respectively. On the other hand, the frequency of C and T alleles of 18C>T were 33.3% and 66.6%, respectively. For the 36G>T genetic variant the mean ± SEM of platelet aggregation induced by ADP for 6 individuals with W.T genotype was 73.5 ± 3.7 AUC, while the mean ± SEM of 3 individuals with the heterozygous (GT) genotype was 77.3 ± 8.25 AUC. There was no significant difference in the aggregation results induced by ADP for individuals who carry WT and GT of 36G>T (P=0.632).

Regarding the effect of 36G>T SNP (H2 haplotype) on platelets' response induced by TRAP agonist, there was no significant difference between the mean AUC of the six individuals who carried the WT (121 ± 5.8) and the three individuals with the heterozygous (GT) genotype (120.3 ± 10.4) (P=0.95). On the other hand, three individuals carried the WT genotype of the 18C>T genetic variant and six individuals carried

heterozygous genotypes (CT) and homozygous TT genotypes (CT and TT were combined in the same group because only one individual had the TT genotype). The mean AUCs of TRAP-induced aggregation for individuals with the WT genotype and the (CT and TT) genotypes were 131.6 ± 4.3 and 115.5 ± 5.8, respectively, with no significant differences between the two groups of genotypes (*P*=0.113). In addition, the effect of 18C>T genotypes on ADP-induced platelet aggregation was assessed, but no significant difference was found (*P*=0.77) (Table 4.6).

Table 4.6: The effect of the SNPs C34T (18C>T) and G52T (36G>T) genotypes on the platelet aggregation response to ADP/TRAP induced aggregation (AUC).

Agonist AUC	**Genotype**											
	C34T (c.18C>T)			G52T (c.36G>T)			c.767G>A			c.793C>T		
				H1H1	H1H2	H2H2						
	CC n=3	CT n=5	TT n=1	GG n=6	GT n=3	TT n=0	GG n=9	GA n=0	AA n=0	CC n=10	CT n=0	TT n=0
ADP (AUC)	76.3	73.4	77	73.5	77.3	-	74.7	-	-	74.7	-	-
TRAP (AUC)	131.6	115.4	115	121	120.3	-	120.7	-	-	120.7	-	-

Chapter Five

Discussion, Conclusions, Limitations and Recommendations

5.1 Discussion

The purinergic receptor $P2Y_{12}$ is considered as one of the most important receptors in the human body. These receptors are particularly distributed in human brain cells and platelets with very important role in platelet aggregation and thrombus formation. $P2Y_{12}$ receptors constitute one of the major strategies for treating patients after percutaneous coronary intervention (PCI). Although $P2Y_{12}$ has a significant role in normal hemostasis and in thrombus formation, little is known about genetic variations in $P2Y_{12}$ receptor among Palestinian, Swedish and Congolese populations. The platelet aggregation response induced by ADP and its receptor $P2Y_{12}$ in healthy untreated individuals showed inter-individual variation. In addition, the response of these receptors to clopidogrel showed variation among treated patients (Galic *et al.*, 2013). One of the most important underlying mechanisms is genetic variations in the $P2Y_{12}$ gene. Genetic variations (SNPs) were shown to increase the risk of clopidogrel resistance and consequently increase the adverse effect of CVDs (Lev *et al.*, 2007). Thus, the study of $P2Y_{12}$ receptors has many beneficial aspects. First, to detect the differences in genetic variations in the $P2Y_{12}$ gene among different ethnic groups, this may explain the differences in the prevalence of clopidogrel resistance and the risk of adverse effect in CVD patients. Second, to determine the frequency of identified SNPs among the study populations and their role in platelet aggregation response.

5.1.1 $P2Y_{12}$ sequencing

In the present study, we performed a thorough analysis of the SNPs of $P2Y_{12}$ in a cross-sectional study of different unrelated ethnic groups. Different mutations were identified in the coding region of $P2Y_{12}$ gene that results in quantitative or qualitative defects in $P2Y_{12}$ receptors. For the $P2Y_{12}$ receptor defect with bleeding diathesis, we assumed that different missense mutations might be responsible such as c.767G>A and c.793C>T (Hayward *et*

al., 2006; Remijn *et al.*, 2007). Our participants were all healthy individuals which were confirmed by the absence of the pathological SNPs among study subjects. In the cases of inter-individual variations among healthy individuals, we assumed that different benign SNPs may be responsible, such as c.18C>T and c.36G>T (H2 haplotype). Moreover, these SNPs may affect thienopyridine compounds and lead to a high platelet reactivity and an increased risk of adverse effect after PCI. To identify these SNPs, we did sequence analysis of the whole part of $P2Y_{12}$ exon-3.

Sequencing results revealed that there were significant variations in the frequencies of 18C>T, 36G>T and other SNPs between three different populations. Regarding the 18C>T, there was a clear difference in the frequency of 18C>T among Palestinian, Swedish and Congolese populations (28.3, 20.0 and 6.5%, respectively). The MAFs of the genetic variants reported in this study among each of the three populations are summarized in table 5.1. Moreover, as we mentioned earlier in the literature review, carriage of the mutated allele of 18C>T was associated with clopidogrel resistance and had a 4.0-fold increased adjusted risk for neurological events compared to the wild type (Ziegler *et al.*, 2005). So, 18C>T polymorphism may act as a contributor factor to the prevalence differences in adverse effects in clopidogrel patients with different ethnicities.

Furthermore, the tag-SNP 36G>T was used to detect frequency of the H2 haplotype in the study population (n=254), which was found to be 10.0% among the overall study population and 4.1, 12.0 and 23.6 among Palestinians, Congolese and Swedish, respectively. These findings were in agreement with Fontana and Lee studies. Furthermore, different studies reported different results among different populations. For examples, among the Japanese the MAF of 36G>T was reported to be (19%), for the Chinese it was reported to be (21%, and among Caucasian populations 23%) as was reported in HapMap database (Sherry *et al.*, 2001). The H2 haplotype was more frequently found in patients with CVDs (Bierend *et al.*, 2008; Fontana *et al.*, 2003b; Ziegler *et al.*, 2005). On the other hand, inconsistent results were reported about the relation of H2 haplotype and the increase in the risk of clopidogrel resistance and cardiovascular diseases (Cuisset *et al.*, 2007; Schettert *et al.*, 2006). We observed significant differences in H2 haplotype between three ethnic groups. These differences may lead to differences in aggregation response between ethnic groups. The frequency of H2 haplotype was found to be highest among Swedish individuals compared to Congolese and Palestinians In addition, the only individual with

TT genotype (H2H2 genotype) was of Swedish origin. Due to these differences in H2 haplotype among the study populations, we assumed that Swedish patients under thienopyridine compounds are more likely to express clopidogrel resistance and an increased risk of adverse effect after PCI. Further studies are needed to confirm the differences in aggregation/ clopidogrel response between different ethnic groups and the impact of $P2Y_{12}$ genotype on individual phenotypes. Exon-3 analysis also revealed additional SNPs (c.546C>T→p.Phe182= and c.989A>G→p.Glu330Gly) which were detected in small numbers among Palestinian and Congolese populations, but not Swedish population. These SNPs were not correlated with aggregation. Table 5.1 shows a comparison between the findings of this study and what is reported by SNPs databases in regard to the prevalence of genetic variants.

Table 5.1: Prevalence of SNPs among the study populations. Values are given as the percentage of tested alleles in the study population.

Study population (n)	**SNP ID**					
	c.18C>T → p. Asn6=	c.36T>G → p.Gly12=	c.767G>A → p.Arg256 Gln	c.793C>T → p.Arg265 Trp	c.546C>T → p.Phe182=	c.989A>G → p.Glu330 Gly
Overall	21.9	10.0	0.0	0.0	0.6	0.4
Palestinian (145)	28.3	4.1	0.0	0.0	0.3	0.3
Swedish (55)	20.0	23.6	0.0	0.0	0.0	0.0
Congolese (54)	6.5	12.0	0.0	0.0	1.9	0.9
ENSEMBL Genetic Variation*/MAF (%)	2.4	9.0	0.0	0.0	4.0	2.4
EVS**/MAF$^{€}$ (%)	27.3	12.1	Not listed	Not listed	4.1	2.4

*ENSEMBL Genetic Variation available at (ENSEMBL, 2013).
**EVS: Exome Variant Server: available at (Server, 2017).
$^{€}$ MAF: minor allele frequency.

Furthermore, several previous studies have emphasized that there are both interethnic groups and intra-ethnic significant differences in the distribution of the variant alleles in $P2Y_{12}$. The frequencies of the different genotypes and the minor allele frequency of the two benign SNPs (18C>T and 36G>T) between study ethnic populations and other populations are shown in table (5.2). Regarding the 18C>T SNP, the frequency of homozygous genotype of the minor allele (TT) was highest among the Palestinian population (10.3%) in comparison to the other ethnic groups. On the other hand, for the

heterozygous genotype (CT), the highest frequency was seen among the European population (46.9%), while for the genotype (CC) the highest frequency was among the Congolese population with a percentage of (92.5%). Furthermore, comparing the MAF of the three study populations, we found huge differences between Palestinian, Swedish and Congolese populations. The highest frequency was found in the Congolese population (28.2%), followed by the Palestinian (20.0%) and the Swedish population (6.5%), respectively. However, for the other ethnic groups the highest MAF was found among Europeans (30.5%).

For the 36G>T SNP (H2 haplotype), the MAF was the highest among the Swedish population (23.6%) in comparison with the other ethnic groups. On the other hand, the Palestinians showed the lowest MAF in our study (4.2%). This percentage was close to that reported among the Sub-Saharan population (4.0%). The variation between the different ethnic groups shows that there are significant differences in the underlying genetic causes of platelets responsiveness between healthy individuals and patients with CVDs treated with thienopyridine compounds.

Table 5.2: Comparison of the distribution of the genotype frequencies and allele frequencies (%) of $P2Y_{12}$'s 18C>T and 36G>T polymorphisms among different ethnic groups.

Population	SNPs	**18C>T**					**36G>T**					Ref**
	18C>T / 36G>T (N/N)	Genotype frequency (%)*			Allele frequency (%)*		Genotype frequency (%)*			Allele frequency (%)*		
		CC	CT	TT	C	T	GG	GT	TT	G	T	
Palestinian	145/145	53.8	35.9	10.3	80.0	20.0	91.7	8.3	0.0	95.8	4.2	Present study
Swedish	55/55	63.6	32.7	3.6	93.5	6.5	54.5	43.6	1.8	76.5	23.6	Present study
Congolese	54/54	92.6	1.9	5.6	71.8	28.2	75.9	24.1	0.0	87.9	12.0	Present study
Korean	50/50	52.7	36.0	6.0	76.0	24.0	60.0	40.0	0.0	80.0	20.0	(Lee *et al.*, 2011)
European	226/266	46.0	46.9	7.0	69.4	30.5	65.4	31.8	2.6	81.4	18.5	(Sherry *et al.*, 2001)
Asian	168/172	54.7	38.0	7.1	73.8	26.1	81.3	18.6	0.0	90.6	9.3	(Sherry *et al.*, 2001)
Sub-Saharan	224/98	73.2	24.1	2.6	85.2	14.7	91.8	8.2	0.0	95.9	4.0	(Sherry *et al.*, 2001)

* Numbers may not add to 100% due to rounding.
** Ref: reference.

5.1.2 Aggregation study

In vitro platelet aggregation study is the best test to reflect *in vivo* platelet function. In our -study, *in vitro* platelet aggregation of healthy individuals induced by ADP/TRAP agonist in all of the individuals (n=9), had ADP/TRAP AUC within the normal range 53-122 and 94-156 AUC, respectively. All subjects could be considered as normal in this respect as no values were out of the reference range. The effect of the 36G>T polymorphism on the platelet aggregation response induced by ADP/TRAP was not statistically significant. Unfortunately, none of the recruited individuals in this study had H2/H2 haplotype (TT genotype), therefore, the effect of H2/H2 haplotype in the present study was not investigated. However, the aggregation results of individuals with H1/H2 showed increase in aggregation response induced by ADP (77.3 ± 8.3 AUC) compared to H1/H1 haplotype individuals (73.5 ± 3.7 AUC), but the difference was not statistically significant. This result was in agreement with a study of Fontana *et al.* (Fontana *et al.*, 2003a) and Lee *et al*. (Lee *et al.*, 2011) who found normal aggregation response to ADP in healthy untreated volunteers who were carriers of the H1 haplotype but not H2 haplotype. Moreover, our results were in agreement with Oestreich and his colleagues in which the aggregation response induced by TRAP did not show statistical differences between individuals with H1 and heterozygous H2 haplotype (Oestreich *et al.*, 2014). This means that the H1H1 and H1H2 did not affect the aggregation induced by thrombin receptors PAR-1. Regarding the 18C>T polymorphism, three individuals had mutated alleles (CT and TT) with normal aggregation results when induced by ADP in contrast to wild type individuals. For the TRAP agonist, low aggregation results induced by TRAP in three individuals with mutated alleles, CT and TT (115.5 ± 5.8 AUC) were found in contrast to wild type individuals (131.6 ± 4.3 AUC). This prompted us to suggest that the 18C>T polymorphism may affect TRAP induced aggregation but not ADP induced aggregation. This could be explained by the role of $P2Y_{12}$ receptors in potentiating the response of other agonists or may be due to other SNPs in PAR-1 receptor. Further studies should be conducted to confirm the effect of 18C>T on TRAP agonist. As for the ADP agonist and 18C>T polymorphism, it was also not associated with an increase in platelets aggregation response in our healthy Swedish individuals which is also in agreement with Fontana *et al.* and Lee *et al*. (Fontana *et al.*, 2003a; Lee *et al.*, 2011). ADP induced platelet aggregation is achieved by combination of $P2Y_1$, $P2Y_{12}$ activation and other unknown factors, thus, these receptors' genes act as prime candidate for genetic variations. Furthermore, SNPs may present at the level of

untranslated region (5' and 3' UTR) and intronic region which may also affect the platelet aggregation response induced by ADP/TRAP. The discrepancy of our study from Fontana and his colleagues may be due to the small number of individuals who were recruited for aggregation study and the absence of H2/H2 haplotype from our study, which may significantly affect the platelet aggregation induced by ADP. Thus, our result and the result of Fontana *et al.* may imply that the H2 haplotype (H1/H2 and H2/H2) are responsible for increased platelet aggregation by potentiating TXA2 formation, which leads to maximal platelet aggregation. Another discrepancy was the differences in the used aggregation method which was 4-channel aggregometry in Fontana *et al.* study.

Our findings were consistent with Fontana, Lee and Bierend studies, in which 18C>T SNP was not correlated with aggregation (Bierend *et al.*, 2008; Fontana *et al.*, 2003a; Lee *et al.*, 2011).

5.2 Conclusions

In conclusion, the populations included in this study were negative for pathological SNPs. Regarding the benign SNPs; our findings did not support previous studies where genetic variants in the $P2Y_{12}$ gene were reported to affect platelet aggregation. Furthermore, in this study we found that the frequency of genetic variants in exon-3 of the $P2Y_{12}$ gene differed by ethnicity. Further studies are needed to investigate the association between genetic variants in the $P2Y_{12}$ receptor and platelet aggregation response.

5.3 Strengths and Limitations

To the best of our knowledge, this study was the first to determine the frequency of $P2Y_{12}$ receptor gene polymorphisms among the study populations; Palestinian, Swedish and Congolese, and the effect of identified SNPs on platelet aggregation response. The first limitation in this study was the small sample size used for aggregation study and the inability to compare the aggregation response between Swedish, Palestinian and Congolese individuals. Also, the sample size of the genotyping study for the ethnic groups was small and so we were not able to detect all SNPs carried in all populations and the majority of the study population was Palestinians. In addition, the study design did not allow the examination of all the potential sources of genetic variability in $P2Y_{12}$ gene although

several identified SNPs are located in the intronic region. Moreover, genetic variations in other genes such as thrombin receptors and CYP 450 genes that were not examined in this study which may explain part of the effect of genetic variations in $P2Y_{12}$ on ADP/TRAP-stimulated platelet response in healthy individuals. The sample size for the aggregation study was very small, for better explanation and generalization; more individuals should be included in expanded studies with different haplotypes. In addition, the effect of each genotype on platelet aggregation should be studied separately. Also, other agonists such as epinephrine and collagen should be assessed for platelet aggregation in order to achieve better understanding. Finally, the unavailability of the demographic characteristics of the study subjects limited our analysis. One particular strength of this study was the analysis of the whole $P2Y_{12}$ coding region, which confirmed the absence of other novel SNPs in our study populations.

5.4 Recommendations

The role of genetic variations in $P2Y_{12}$ and their effects on platelets aggregation and thienopyridine compounds response especially clopidogrel was well established. Therefore, we recommend the examination the patients who were diagnosed with ACS and underwent PCI and stent implantation routinely for aggregation test to assess the response to clopidogrel therapy and to exclude resistance. In addition, our data prompted us to retest the full gene sequencing in patients who are under thienopyridine therapy in different ethnic groups and to correlate the effect of deferent genetic variants on drug response. So, further studies are needed to completely understand the variations between different populations and the effects of the SNPs on platelet aggregation and response.

References

1. Abbracchio, M. P., Burnstock, G., Boeynaems, J. M., Barnard, E. A., Boyer, J. L., Kennedy, C., *et al.* (2006). International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev, 58*(3), 281-341. doi:10.1124/pr.58.3.3
2. Aleil, B., Ravanat, C., Cazenave, J. P., Rochoux, G., Heitz, A., & Gachet, C. (2005). Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases. *J Thromb Haemost, 3*(1), 85-92. doi:10.1111/j.1538-7836.2004.01063.x
3. Badimon, L., Chesebro, J. H., & Badimon, J. J. (1992). Thrombus formation on ruptured atherosclerotic plaques and rethrombosis on evolving thrombi. *Circulation, 86*(6 Suppl), III74-85.
4. Baumgarten, A., Wilhelmi, M., Kalbantner, K., Ganter, M., & Mischke, R. (2010). Measurement of platelet aggregation in ovine blood using a new impedance aggregometer. *Vet Clin Pathol, 39*(2), 149-156. doi:10.1111/j.1939-165X.2009.00198.x
5. Baurand, A., Eckly, A., Bari, N., Leon, C., Hechler, B., Cazenave, J. P., *et al.* (2000). Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y1 and P2cyc receptors. *Thromb Haemost, 84*(3), 484-491.
6. Baurand, A., Raboisson, P., Freund, M., Leon, C., Cazenave, J. P., Bourguignon, J. J., *et al.* (2001). Inhibition of platelet function by administration of MRS2179, a P2Y1 receptor antagonist. *Eur J Pharmacol, 412*(3), 213-221.
7. Bergeron, M. G. (1989). The pharmacokinetics and tissue penetration of the fluoroquinolones. *Clin Invest Med, 12*(1), 20-27.
8. Bertoni, A., Tadokoro, S., Eto, K., Pampori, N., Parise, L. V., White, G. C., *et al.* (2002). Relationships between Rap1b, affinity modulation of integrin alpha IIbbeta 3, and the actin cytoskeleton. *J Biol Chem, 277*(28), 25715-25721. doi:10.1074/jbc.M202791200
9. Bierend, A., Rau, T., Maas, R., Schwedhelm, E., & Boger, R. H. (2008). P2Y12 polymorphisms and antiplatelet effects of aspirin in patients with coronary artery disease. *Br J Clin Pharmacol, 65*(4), 540-547. doi:10.1111/j.1365-2125.2007.03044.x
10. Brass, D., Grably, M. R., Bronstein-Sitton, N., Gohar, O., & Meir, A. (2012). Using antibodies against P2Y and P2X receptors in purinergic signaling research. *Purinergic Signal, 8*(Suppl 1), 61-79. doi:10.1007/s11302-011-9278-z
11. Bura, A., Bachelot-Loza, C., Ali, F. D., Aiach, M., & Gaussem, P. (2006). Role of the P2Y12 gene polymorphism in platelet responsiveness to clopidogrel in healthy subjects. *J Thromb Haemost, 4*(9), 2096-2097. doi:10.1111/j.1538-7836.2006.02113.x
12. Burnstock, G. (1996). P2 purinoceptors: historical perspective and classification. *Ciba Found Symp, 198*, 1-28; discussion 29-34.
13. Cancer Research UK. (2013). Leukaemia (all subtypes combined) statistics. Retrieved from http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/leukaemia
14. Cannon, C. P., Brindis, R. G., Chaitman, B. R., Cohen, D. J., Cross, J. T., Jr., Drozda, J. P., Jr., *et al.* (2013). 2013 ACCF/AHA key data elements and definitions for measuring the clinical management and outcomes of patients with acute coronary syndromes and coronary artery disease: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Clinical Data Standards (Writing Committee to Develop Acute Coronary Syndromes and Coronary Artery Disease Clinical Data Standards). *Circulation, 127*(9), 1052-1089. doi:10.1161/CIR.0b013e3182831a11
15. Cattaneo, M. (2011a). Molecular defects of the platelet P2 receptors. *Purinergic Signal, 7*(3), 333-339. doi:10.1007/s11302-011-9217-z
16. Cattaneo, M. (2011b). The platelet P2Y(1)(2) receptor for adenosine diphosphate: congenital and drug-induced defects. *Blood, 117*(7), 2102-2112. doi:10.1182/blood-2010-08-263111
17. Cattaneo, M., & Gachet, C. (1999). ADP receptors and clinical bleeding disorders. *Arterioscler Thromb Vasc Biol, 19*(10), 2281-2285.

18. Cattaneo, M., Lecchi, A., Randi, A. M., McGregor, J. L., & Mannucci, P. M. (1992). Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood, 80*(11), 2787-2796.
19. Cattaneo, M., Schulz, R., & Nylander, S. (2014). Adenosine-mediated effects of ticagrelor: evidence and potential clinical relevance. *J Am Coll Cardiol, 63*(23), 2503-2509. doi:10.1016/j.jacc.2014.03.031
20. Cattaneo, M., Zighetti, M. L., Lombardi, R., Martinez, C., Lecchi, A., Conley, P. B., *et al.* (2003). Molecular bases of defective signal transduction in the platelet P2Y12 receptor of a patient with congenital bleeding. *Proc Natl Acad Sci U S A, 100*(4), 1978-1983. doi:10.1073/pnas.0437879100
21. Chang, H. H., & Lo, S. J. (1998). Full-spreading platelets induced by the recombinant rhodostomin are via binding to integrins and correlated with FAK phosphorylation. *Toxicon, 36*(8), 1087-1099.
22. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., *et al.* (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science, 318*(5854), 1258-1265. doi:10.1126/science.1150577
23. Coller, B. S. (2011). Historical perspective and future directions in platelet research. *J Thromb Haemost, 9 Suppl 1*, 374-395. doi:10.1111/j.1538-7836.2011.04356.x
24. Colman, R. W. (2006). *Hemostasis and thrombosis: basic principles and clinical practice*: Lippincott Williams & Wilkins.
25. Cooper, D. M., & Rodbell, M. (1979). ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. *Nature, 282*(5738), 517-518.
26. Costanzi, S., Mamedova, L., Gao, Z. G., & Jacobson, K. A. (2004). Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. *J Med Chem, 47*(22), 5393-5404. doi:10.1021/jm049914c
27. Cuisset, T., Frere, C., Quilici, J., Morange, P. E., Saut, N., Lambert, M., *et al.* (2007). Role of the T744C polymorphism of the P2Y12 gene on platelet response to a 600-mg loading dose of clopidogrel in 597 patients with non-ST-segment elevation acute coronary syndrome. *Thromb Res, 120*(6), 893-899. doi:10.1016/j.thromres.2007.01.012
28. Cunningham, M. R., Nisar, S. P., & Mundell, S. J. (2013). Molecular mechanisms of platelet P2Y(12) receptor regulation. *Biochem Soc Trans, 41*(1), 225-230. doi:10.1042/BST20120295
29. Daniel, J. L., Dangelmaier, C., Jin, J., Ashby, B., Smith, J. B., & Kunapuli, S. P. (1998). Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem, 273*(4), 2024-2029.
30. Daniel, J. L., Dangelmaier, C., Jin, J., Kim, Y. B., & Kunapuli, S. P. (1999). Role of intracellular signaling events in ADP-induced platelet aggregation. *Thromb Haemost, 82*(4), 1322-1326.
31. de Groot, P. G., Urbanus, R. T., & Roest, M. (2012). Platelet interaction with the vessel wall. *Handb Exp Pharmacol*(210), 87-110. doi:10.1007/978-3-642-29423-5_4
32. Deflorian, F., & Jacobson, K. A. (2011). Comparison of three GPCR structural templates for modeling of the P2Y12 nucleotide receptor. *J Comput Aided Mol Des, 25*(4), 329-338. doi:10.1007/s10822-011-9423-3
33. Ding, Z., Kim, S., Dorsam, R. T., Jin, J., & Kunapuli, S. P. (2003). Inactivation of the human P2Y12 receptor by thiol reagents requires interaction with both extracellular cysteine residues, Cys17 and Cys270. *Blood, 101*(10), 3908-3914. doi:10.1182/blood-2002-10-3027
34. Ding, Z., Tuluc, F., Bandivadekar, K. R., Zhang, L., Jin, J., & Kunapuli, S. P. (2005). Arg333 and Arg334 in the COOH terminus of the human P2Y1 receptor are crucial for Gq coupling. *Am J Physiol Cell Physiol, 288*(3), C559-567. doi:10.1152/ajpcell.00401.2004
35. Dorsam, R. T., & Kunapuli, S. P. (2004). Central role of the P2Y12 receptor in platelet activation. *J Clin Invest, 113*(3), 340-345. doi:10.1172/JCI20986
36. Duke, W. W. (1911). The Rate of Regeneration of Blood Platelets. *J Exp Med, 14*(3), 265-273.

37. ENSEMBL. (2013). Transcript: P2RY12-201 ENST00000302632.3. Retrieved October 27, 2017 http://asia.ensembl.org/Homo_sapiens/Transcript/Variation_Transcript/Table?db=core;g=ENSG00000169313;oa=Orphanet:36355;ph=44272;r=3:151337380-151384812;t=ENST00000302632
38. Epicentre. (2013). MasterPure™ DNA Purification Kit. Retrieved from http://www.epibio.com/docs/default-source/protocols/masterpure-dna-purification-kit.pdf?sfvrsn=6
39. Fabre, J. E., Nguyen, M., Latour, A., Keifer, J. A., Audoly, L. P., Coffman, T. M., *et al.* (1999). Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med, 5*(10), 1199-1202. doi:10.1038/13522
40. Flechtenmacher, N., Kammerer, F., Dittmer, R., Budde, U., Michels, P., Rother, J., *et al.* (2015). Clopidogrel Resistance in Neurovascular Stenting: Correlations between Light Transmission Aggregometry, VerifyNow, and the Multiplate. *AJNR Am J Neuroradiol, 36*(10), 1953-1958. doi:10.3174/ajnr.A4388
41. Fontana, Dupont, A., Gandrille, S., Bachelot-Loza, C., Reny, J. L., Aiach, M., *et al.* (2003a). Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation, 108*(8), 989-995. doi:10.1161/01.CIR.0000085073.69189.88
42. Fontana, Gaussem, P., Aiach, M., Fiessinger, J. N., Emmerich, J., & Reny, J. L. (2003b). P2Y12 H2 haplotype is associated with peripheral arterial disease: a case-control study. *Circulation, 108*(24), 2971-2973. doi:10.1161/01.CIR.0000106904.80795.35
43. Fontana, Ware, J., & Cattaneo, M. (2009). Haploinsufficiency of the platelet P2Y12 gene in a family with congenital bleeding diathesis. *Haematologica, 94*(4), 581-584. doi:10.3324/haematol.13611
44. Foster, C. J., Prosser, D. M., Agans, J. M., Zhai, Y., Smith, M. D., Lachowicz, J. E., *et al.* (2001). Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest, 107*(12), 1591-1598. doi:10.1172/JCI12242
45. Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., *et al.* (1994). Nomenclature and classification of purinoceptors. *Pharmacol Rev, 46*(2), 143-156.
46. Fredriksson, R., Lagerstrom, M. C., Lundin, L. G., & Schioth, H. B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol, 63*(6), 1256-1272. doi:10.1124/mol.63.6.1256
47. Frontroth, J. P. (2013). Light transmission aggregometry. *Methods Mol Biol, 992*, 227-240. doi:10.1007/978-1-62703-339-8_17
48. Gachet, C. (2001). ADP receptors of platelets and their inhibition. *Thromb Haemost, 86*(1), 222-232.
49. Gachet, C. (2005). The platelet P2 receptors as molecular targets for old and new antiplatelet drugs. *Pharmacol Ther, 108*(2), 180-192. doi:10.1016/j.pharmthera.2005.03.009
50. Gachet, C. (2008). P2 receptors, platelet function and pharmacological implications. *Thromb Haemost, 99*(3), 466-472. doi:10.1160/TH07-11-0673
51. Gachet, C. (2015). Antiplatelet drugs: which targets for which treatments? *J Thromb Haemost, 13 Suppl 1*, S313-322. doi:10.1111/jth.12947
52. Galic, E., Vrbanic, L., Kapitanovic, S., Catela Ivkovic, T., Petro, D., Vukovic, I., *et al.* (2013). P2RY12 gene polymorphisms and effect of clopidogrel on platelet aggregation. *Coll Antropol, 37*(2), 491-498.
53. Gremmel, T., Yanachkov, I. B., Yanachkova, M. I., Wright, G. E., Wider, J., Undyala, V. V., *et al.* (2016). Synergistic Inhibition of Both P2Y1 and P2Y12 Adenosine Diphosphate Receptors As Novel Approach to Rapidly Attenuate Platelet-Mediated Thrombosis. *Arterioscler Thromb Vasc Biol, 36*(3), 501-509. doi:10.1161/ATVBAHA.115.306885

54. Hardy, A. R., Conley, P. B., Luo, J., Benovic, J. L., Poole, A. W., & Mundell, S. J. (2005). P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood, 105*(9), 3552-3560. doi:10.1182/blood-2004-07-2893
55. Harrison, P. (2005a). Platelet function analysis. *Blood Rev, 19*(2), 111-123. doi:10.1016/j.blre.2004.05.002
56. Harrison, P. (2005b). The role of PFA-100 testing in the investigation and management of haemostatic defects in children and adults. *Br J Haematol, 130*(1), 3-10. doi:10.1111/j.1365-2141.2005.05511.x
57. Haslam, R. J. (1973). Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. *Ser Haematol, 6*(3), 333-350.
58. Haynes, S. E., Hollopeter, G., Yang, G., Kurpius, D., Dailey, M. E., Gan, W. B., *et al.* (2006). The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci, 9*(12), 1512-1519. doi:10.1038/nn1805
59. Hayward, C. P., Rao, A. K., & Cattaneo, M. (2006). Congenital platelet disorders: overview of their mechanisms, diagnostic evaluation and treatment. *Haemophilia, 12 Suppl 3*, 128-136. doi:10.1111/j.1365-2516.2006.01270.x
60. Hechler, B., Eckly, A., Ohlmann, P., Cazenave, J. P., & Gachet, C. (1998). The P2Y1 receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *Br J Haematol, 103*(3), 858-866.
61. Hollopeter, G., Jantzen, H. M., Vincent, D., Li, G., England, L., Ramakrishnan, V., *et al.* (2001). Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature, 409*(6817), 202-207. doi:10.1038/35051599
62. Huang, H. S., & Chang, H. H. (2012). Platelets in inflammation and immune modulations: functions beyond hemostasis. *Arch Immunol Ther Exp (Warsz), 60*(6), 443-451. doi:10.1007/s00005-012-0193-y
63. Jackson, S. P., Nesbitt, W. S., & Kulkarni, S. (2003). Signaling events underlying thrombus formation. *J Thromb Haemost, 1*(7), 1602-1612.
64. Jacobson, K. A., & Boeynaems, J. M. (2010). P2Y nucleotide receptors: promise of therapeutic applications. *Drug Discov Today, 15*(13-14), 570-578. doi:10.1016/j.drudis.2010.05.011
65. Jacobson, K. A., Jayasekara, M. P., & Costanzi, S. (2012). Molecular Structure of P2Y Receptors: Mutagenesis, Modeling, and Chemical Probes. *Wiley Interdiscip Rev Membr Transp Signal, 1*(6). doi:10.1002/wmts.68
66. Jin, J., & Kunapuli, S. P. (1998). Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci U S A, 95*(14), 8070-8074.
67. Kahner, B. N., Shankar, H., Murugappan, S., Prasad, G. L., & Kunapuli, S. P. (2006). Nucleotide receptor signaling in platelets. *J Thromb Haemost, 4*(11), 2317-2326. doi:10.1111/j.1538-7836.2006.02192.x
68. Kasperska-Zajac, A., & Rogala, B. (2006). Platelet function in anaphylaxis. *J Investig Allergol Clin Immunol, 16*(1), 1-4.
69. Kauffenstein, G., Bergmeier, W., Eckly, A., Ohlmann, P., Leon, C., Cazenave, J. P., *et al.* (2001). The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin--a phosphoinositide 3-kinase-dependent mechanism. *FEBS Lett, 505*(2), 281-290.
70. Kim, Y., Kim, K., Park, Y., & Moschandreou, T. (2012). Blood Cell—An Overview of Studies in Hematology. In: Intech Croatia.
71. Kumar, R. A., Dong, J. F., Thaggard, J. A., Cruz, M. A., Lopez, J. A., & McIntire, L. V. (2003). Kinetics of GPIbalpha-vWF-A1 tether bond under flow: effect of GPIbalpha mutations on the association and dissociation rates. *Biophys J, 85*(6), 4099-4109. doi:10.1016/S0006-3495(03)74822-X
72. Lee, S. J., Jung, I. S., Jung, E. J., Choi, J. Y., Yeo, C. W., Cho, D. Y., *et al.* (2011). Identification of P2Y12 single-nucleotide polymorphisms and their influences on the variation in ADP-induced platelet aggregation. *Thromb Res, 127*(3), 220-227. doi:10.1016/j.thromres.2010.11.023

73. Leon, C., Hechler, B., Freund, M., Eckly, A., Vial, C., Ohlmann, P., *et al.* (1999). Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest, 104*(12), 1731-1737. doi:10.1172/JCI8399
74. Lev, E. I., Patel, R. T., Guthikonda, S., Lopez, D., Bray, P. F., & Kleiman, N. S. (2007). Genetic polymorphisms of the platelet receptors P2Y(12), P2Y(1) and GP IIIa and response to aspirin and clopidogrel. *Thromb Res, 119*(3), 355-360. doi:10.1016/j.thromres.2006.02.006
75. Leynadier, F. (1989). [Mast cells and basophils in asthma]. *Ann Biol Clin (Paris), 47*(6), 351-356.
76. Mangin, P., Ohlmann, P., Eckly, A., Cazenave, J. P., Lanza, F., & Gachet, C. (2004). The P2Y1 receptor plays an essential role in the platelet shape change induced by collagen when TxA2 formation is prevented. *J Thromb Haemost, 2*(6), 969-977. doi:10.1111/j.1538-7836.2004.00722.x
77. Marcucci, R., Gori, A. M., Paniccia, R., Giusti, B., Valente, S., Giglioli, C., *et al.* (2009). Cardiovascular death and nonfatal myocardial infarction in acute coronary syndrome patients receiving coronary stenting are predicted by residual platelet reactivity to ADP detected by a point-of-care assay: a 12-month follow-up. *Circulation, 119*(2), 237-242. doi:10.1161/CIRCULATIONAHA.108.812636
78. Maree, A., & McRedmond, J. (2005). Genomic and Proteomic Analysis of Platelets. In *Platelet Function* (pp. 315-331): Springer.
79. Mason, J. T. (1988). Mixing behavior of symmetric chain length and mixed chain length phosphatidylcholines in two-component multilamellar bilayers: evidence for gel and liquid-crystalline phase immiscibility. *Biochemistry, 27*(12), 4421-4429.
80. Mazzarello, P., Calligaro, A. L., & Calligaro, A. (2001). Giulio Bizzozero: a pioneer of cell biology. *Nat Rev Mol Cell Biol, 2*(10), 776-781. doi:10.1038/35096085
81. Mehta, S. R., Yusuf, S., Peters, R. J., Bertrand, M. E., Lewis, B. S., Natarajan, M. K., *et al.* (2001). Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *Lancet, 358*(9281), 527-533.
82. Milic-Emili, J. (1990). Flow resistance in anesthesia. *Acta Anaesthesiol Scand Suppl, 94*, 42-45.
83. Moore, D. J., Murdock, P. R., Watson, J. M., Faull, R. L., Waldvogel, H. J., Szekeres, P. G., *et al.* (2003). GPR105, a novel Gi/o-coupled UDP-glucose receptor expressed on brain glia and peripheral immune cells, is regulated by immunologic challenge: possible role in neuroimmune function. *Brain Res Mol Brain Res, 118*(1-2), 10-23.
84. Mundell, S. J., Luo, J., Benovic, J. L., Conley, P. B., & Poole, A. W. (2006). Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. *Traffic, 7*(10), 1420-1431. doi:10.1111/j.1600-0854.2006.00469.x
85. Oestreich, J. H., Steinhubl, S. R., Ferraris, S. P., Loftin, C. D., & Akers, W. S. (2014). Effect of genetic variation in P2Y12 on TRAP-stimulated platelet response in healthy subjects. *J Thromb Thrombolysis, 38*(3), 372-379. doi:10.1007/s11239-014-1058-5
86. Ohlmann, P., Lecchi, A., El-Tayeb, A., Muller, C. E., Cattaneo, M., & Gachet, C. (2013). The platelet P2Y(12) receptor under normal and pathological conditions. Assessment with the radiolabeled selective antagonist [(3)H]PSB-0413. *Purinergic Signal, 9*(1), 59-66. doi:10.1007/s11302-012-9329-0
87. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., *et al.* (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science, 289*(5480), 739-745.
88. Pitchford, S. C., Momi, S., Baglioni, S., Casali, L., Giannini, S., Rossi, R., *et al.* (2008). Allergen induces the migration of platelets to lung tissue in allergic asthma. *Am J Respir Crit Care Med, 177*(6), 604-612. doi:10.1164/rccm.200702-214OC
89. Price, M. J. (2009). Bedside evaluation of thienopyridine antiplatelet therapy. *Circulation, 119*(19), 2625-2632. doi:10.1161/CIRCULATIONAHA.107.696732
90. Price, M. J., Berger, P. B., Angiolillo, D. J., Teirstein, P. S., Tanguay, J. F., Kandzari, D. E., *et al.* (2009). Evaluation of individualized clopidogrel therapy after drug-eluting stent

implantation in patients with high residual platelet reactivity: design and rationale of the GRAVITAS trial. *Am Heart J, 157*(5), 818-824, 824 e811. doi:10.1016/j.ahj.2009.02.012
91. Qi, A. D., Kennedy, C., Harden, T. K., & Nicholas, R. A. (2001). Differential coupling of the human P2Y(11) receptor to phospholipase C and adenylyl cyclase. *Br J Pharmacol, 132*(1), 318-326. doi:10.1038/sj.bjp.0703788
92. Qiagen. (2013). DNeasy Blood & Tissue Kits. Retrieved from https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/dneasy-blood-and-tissue-kit/#orderinginformation
93. Ralevic, V., & Burnstock, G. (1998). Receptors for purines and pyrimidines. *Pharmacol Rev, 50*(3), 413-492.
94. Remijn, J. A., MJ, I. J., Strunk, A. L., Abbes, A. P., Engel, H., Dikkeschei, B., *et al.* (2007). Novel molecular defect in the platelet ADP receptor P2Y12 of a patient with haemorrhagic diathesis. *Clin Chem Lab Med, 45*(2), 187-189. doi:10.1515/CCLM.2007.036
95. Rondina, M. T., Weyrich, A. S., & Zimmerman, G. A. (2013). Platelets as cellular effectors of inflammation in vascular diseases. *Circ Res, 112*(11), 1506-1519. doi:10.1161/CIRCRESAHA.113.300512
96. Roth, G. J., & Majerus, P. W. (1975). The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein. *J Clin Invest, 56*(3), 624-632. doi:10.1172/JCI108132
97. Rudez, G., Bouman, H. J., van Werkum, J. W., Leebeek, F. W., Kruit, A., Ruven, H. J., *et al.* (2009). Common variation in the platelet receptor P2RY12 gene is associated with residual on-clopidogrel platelet reactivity in patients undergoing elective percutaneous coronary interventions. *Circ Cardiovasc Genet, 2*(5), 515-521. doi:10.1161/CIRCGENETICS.109.861799
98. Salles, II, Feys, H. B., Iserbyt, B. F., De Meyer, S. F., Vanhoorelbeke, K., & Deckmyn, H. (2008). Inherited traits affecting platelet function. *Blood Rev, 22*(3), 155-172. doi:10.1016/j.blre.2007.11.002
99. Sattler, T. A., Dimitrov, T., & Hall, P. W. (1977). Relation between endemic (Balkan) nephropathy and urinary-tract tumours. *Lancet, 1*(8006), 278-280.
100. Savi, P., Pereillo, J. M., Uzabiaga, M. F., Combalbert, J., Picard, C., Maffrand, J. P., *et al.* (2000). Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost, 84*(5), 891-896.
101. Schettert, I. T., Pereira, A. C., Lopes, N. H., Hueb, W. A., & Krieger, J. E. (2006). Association between platelet P2Y12 haplotype and risk of cardiovascular events in chronic coronary disease. *Thromb Res, 118*(6), 679-683. doi:10.1016/j.thromres.2005.11.009
102. Schwarz, U. R., Geiger, J., Walter, U., & Eigenthaler, M. (1999). Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets--definition and detection of ticlopidine/clopidogrel effects. *Thromb Haemost, 82*(3), 1145-1152.
103. Server, E. V. (2017). NHLBI GO Exome Sequencing Project (ESP). Retrieved October 27, 2017 http://evs.gs.washington.edu/EVS/
104. Shalia, K. K., Shah, V. K., Pawar, P., Divekar, S. S., & Payannavar, S. (2013). Polymorphisms of MDR1, CYP2C19 and P2Y12 genes in Indian population: effects on clopidogrel response. *Indian Heart J, 65*(2), 158-167. doi:10.1016/j.ihj.2013.02.012
105. Sherry, S. T., Ward, M. H., Kholodov, M., Baker, J., Phan, L., Smigielski, E. M., *et al.* (2001). dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res, 29*(1), 308-311.
106. Simon, T. (1994). The collection of platelets by apheresis procedures. *Transfus Med Rev, 8*(2), 132-145.
107. Simon, T., Verstuyft, C., Mary-Krause, M., Quteineh, L., Drouet, E., Meneveau, N., *et al.* (2009). Genetic determinants of response to clopidogrel and cardiovascular events. *N Engl J Med, 360*(4), 363-375. doi:10.1056/NEJMoa0808227
108. Slichter, S. J., & Harker, L. A. (1976). Preparation and storage of platelet concentrates. *Transfusion, 16*(1), 8-12.

109. Sofi, F., Giusti, B., Marcucci, R., Gori, A. M., Abbate, R., & Gensini, G. F. (2011). Cytochrome P450 2C19*2 polymorphism and cardiovascular recurrences in patients taking clopidogrel: a meta-analysis. *Pharmacogenomics J, 11*(3), 199-206. doi:10.1038/tpj.2010.21
110. Springthorpe, B., Bailey, A., Barton, P., Birkinshaw, T. N., Bonnert, R. V., Brown, R. C., *et al.* (2007). From ATP to AZD6140: the discovery of an orally active reversible P2Y12 receptor antagonist for the prevention of thrombosis. *Bioorg Med Chem Lett, 17*(21), 6013-6018. doi:10.1016/j.bmcl.2007.07.057
111. Staritz, P., Kurz, K., Stoll, M., Giannitsis, E., Katus, H. A., & Ivandic, B. T. (2009). Platelet reactivity and clopidogrel resistance are associated with the H2 haplotype of the P2Y12-ADP receptor gene. *Int J Cardiol, 133*(3), 341-345. doi:10.1016/j.ijcard.2007.12.118
112. Takeda, S., Kadowaki, S., Haga, T., Takaesu, H., & Mitaku, S. (2002). Identification of G protein-coupled receptor genes from the human genome sequence. *FEBS Lett, 520*(1-3), 97-101.
113. Tang, X. F., Zhang, J. H., Wang, J., Han, Y. L., Xu, B., Qiao, S. B., *et al.* (2013). Effects of coexisting polymorphisms of CYP2C19 and P2Y12 on clopidogrel responsiveness and clinical outcome in patients with acute coronary syndromes undergoing stent-based coronary intervention. *Chin Med J (Engl), 126*(6), 1069-1075.
114. Trumel, C., Payrastre, B., Plantavid, M., Hechler, B., Viala, C., Presek, P., *et al.* (1999). A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. *Blood, 94*(12), 4156-4165.
115. van Werkum, J. W., Kleibeuker, M., Postma, S., Bouman, H. J., Elsenberg, E. H., ten Berg, J. M., *et al.* (2010). A comparison between the Plateletworks-assay and light transmittance aggregometry for monitoring the inhibitory effects of clopidogrel. *Int J Cardiol, 140*(1), 123-126. doi:10.1016/j.ijcard.2008.10.046
116. Waldmann, R., Nieberding, M., & Walter, U. (1987). Vasodilator-stimulated protein phosphorylation in platelets is mediated by cAMP- and cGMP-dependent protein kinases. *Eur J Biochem, 167*(3), 441-448.
117. Wallentin, L., Becker, R. C., Budaj, A., Cannon, C. P., Emanuelsson, H., Held, C., *et al.* (2009). Ticagrelor versus clopidogrel in patients with acute coronary syndromes. *N Engl J Med, 361*(11), 1045-1057. doi:10.1056/NEJMoa0904327
118. Webb, T. E., Simon, J., Krishek, B. J., Bateson, A. N., Smart, T. G., King, B. F., *et al.* (1993). Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett, 324*(2), 219-225.
119. Westphal, H. (1987). Transgenic mice. *Bioessays, 6*(2), 73-76. doi:10.1002/bies.950060208
120. Whittaker, V. K., & Watkins, J. C. (1972). The effect of the neuronal excitant N-methyl-D-aspartate on the metabolism of mouse brain amino acids labelled from (14 C)bicarbonate and L-(U- 14 C)aspartate. *Brain Res, 43*(1), 227-234.
121. Wihlborg, A. K., Balogh, J., Wang, L., Borna, C., Dou, Y., Joshi, B. V., *et al.* (2006). Positive inotropic effects by uridine triphosphate (UTP) and uridine diphosphate (UDP) via P2Y2 and P2Y6 receptors on cardiomyocytes and release of UTP in man during myocardial infarction. *Circ Res, 98*(7), 970-976. doi:10.1161/01.RES.0000217402.73402.cd
122. Williams, M. S., Weiss, E. J., Sabatine, M. S., Simon, D. I., Bahou, W. F., Becker, L. C., *et al.* (2010). Genetic regulation of platelet receptor expression and function: application in clinical practice and drug development. *Arterioscler Thromb Vasc Biol, 30*(12), 2372-2384. doi:10.1161/ATVBAHA.110.218131
123. Wiviott, S. D., & Antman, E. M. (2004). Clopidogrel resistance: a new chapter in a fast-moving story. *Circulation, 109*(25), 3064-3067. doi:10.1161/01.CIR.0000134701.40946.30
124. Wurtz, M., Hvas, A. M., Christensen, K. H., Rubak, P., Kristensen, S. D., & Grove, E. L. (2014). Rapid evaluation of platelet function using the Multiplate(R) Analyzer. *Platelets, 25*(8), 628-633. doi:10.3109/09537104.2013.849804
125. Yee, D. L., Sun, C. W., Bergeron, A. L., Dong, J. F., & Bray, P. F. (2005). Aggregometry detects platelet hyperreactivity in healthy individuals. *Blood, 106*(8), 2723-2729. doi:10.1182/blood-2005-03-1290

126. Ziegler, S., Schillinger, M., Funk, M., Felber, K., Exner, M., Mlekusch, W., *et al.* (2005). Association of a functional polymorphism in the clopidogrel target receptor gene, P2Y12, and the risk for ischemic cerebrovascular events in patients with peripheral artery disease. *Stroke, 36*(7), 1394-1399. doi:10.1161/01.STR.0000169922.79281.a5