CHAPTER II

LITERATURE REVIEW

2.1. Platelets and thrombosis

Platelets are small, anucleated, discoid cells that circulate in the blood. They are 2-3 μm in diameter with a cell volume of approximately 10 fL. and circulate at a concentration of 250,000 + 100,000 cells/μL of blood. They have a life span of 8-11 days and do not normally adhere to each other or to other blood cells. On platelet surface membrane, there are a large number of receptors which specifically bind agonists that stimulate the physiological platelet response, for example, ADP, collagen, thrombin, thromboxane, epinephrine, serotonin and glycoprotein receptors. The platelet membrane also contains phospholipids, where they serve as substrates for phospholipase enzymes. Platelets contain three types of granules, namely: *dense granules*, which contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), calcium, histamine, serotonin, and epinephrine; α *granules*, which store fibronectin, von Willebrand's factor (vWF), thrombospondin, fibrinogen, platelet factors IV, V and VIII, various growth factors; and *lysosomes*, which contain hydrolase enzymes. [22, 23] Among these three granules, dense granule contents are easily secreted, α granules release requires higher agonist concentrations, while lysosomal granule secretion only occurs with potent activating agents. [24]

Under normal physiological conditions when a blood vessel is damaged and the normal endothelial-cell barrier is disrupted, platelets are quickly recruited from the circulating blood to form an occlusive plug to arrest the lost of blood. In contrast, in pathological conditions, such as atherosclerosis, arterial thrombus formation may limit the blood supply to nearby tissues, thus causing local ischemia and the progression of the atherosclerotic lesion. [24] The typical platelet response to vascular injury can be divided into three major phases. These are: *platelet adhesion*, the interaction of platelets with subendothelial matrix; *platelet activation*, a phase during which biochemical pathways are activated, platelets undergo shape change and secrete granule constituents, including ADP; and *platelet aggregation*, the interaction of platelets with each other to form platelet aggregates.

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Platelet adhesion

After blood vessels damage, platelets are exposed to a non-endothelial surface, which include collagen (most important), fibronectin and other adhesive glycoproteins, they adhere, flatten, and spread on the surface of the subendothelial matrix. The platelet surface membrane has adhesion receptors that bind to specific matrix molecules. These receptors include the glycoprotein (GP) Ib/IX complex, a receptor for subendothelial von Willebrand factor, and several of the membrane glycoproteins of the integrin superfamily GPIa/IIa, a collagen receptor, GPIc/IIa, a fibronectin receptor and GPIc/IIa, a laminin receptor. In addition, many components of the matrix, such as von Willebrand factor, thrombospondin, fibronectin, and collagen, can interact with one another as well as with platelets. Platelet adhesion to extracellular matrix is mediated via interactions with von Willebrand factor (vWF), which acts as a bridge between platelet surface receptors, glycoprotein Ib/IX/V (GP Ib/IX/V), and exposed collagen.

Platelet activation

Platelet activation can be induced by adhesion to proteins such as collagen within the subendothelial matrix; by soluble agonists, such as epinephrine, ADP, vasopressin, serotonin, and thrombin; and possibly by cell contact during platelet aggregation. The process of activation involves a redundant and complicated system of agonist receptors, phospholipases, protein kinases, and membrane transducing elements such as guanosine triphosphate binding proteins. For example, agonist binding can lead to the activation of phospholipase A_2, which liberates arachidonic acid from membrane phospholipids. This fatty acid is then converted to prostaglandin endoperoxides and thromboxane A_2, which can amplify the effect of the initial agonist by diffusing out of the platelet and binding to specific membrane receptors. Certain agonists, such as thrombin and thromboxane A_2, can also lead to the activation of phospholipase C, which converts Phosphatidylinositol-bis-phosphate (PIP_2) into diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP3). These two compounds mediate important mechanisms of platelet activation: the activation of protein kinase C and the release of ionized calcium from intracellular stores, respectively. Protein kinase C phosporylates substrate proteins, whereas the increase in

cytoplasmic calcium activates various calcium-dependent and calmodulin-dependent reactions (Figure 1). Platelet activation is accompanied by the reorganization of cytoskeletal proteins, producing a dramatic morphologic change from the disk shape to a sphere with extended pseudopods. These activation-induced metabolic processes act in concert to stimulate platelet aggregation and granule secretion. Platelet activation leads to the surface expression of a phospholipids complex, which provides a critical nucleation site for calcium and factor binding in the intrinsic clotting pathway.

Platelet aggregation

The process of platelet-platelet adherence is termed aggregation. Platelet aggregation requires agonist-induced cell activation, with conversion of membrane GP IIb/IIIa into a receptor for fibrinogen and other adhesive proteins that contain the amino acid sequence arginine-glycine-aspartic acid, such as von Willebrand factor, fibronectin, and vitronectin. The dimeric fibrinogen molecule presumably mediates cell aggregation by binding to GPIIb/IIIa on adjacent platelets in a reaction that is dependent on extracellular calcium. Fibrinogen, present in relatively high concentrations in plasma, is certainly important for platelet aggregation, and von Willebrand factor, which could mediate platelet aggregation by virtue of its multimeric structure, may also play a part.

Platelet secretion is related to platelet aggregation in several ways. First, secretion in response to certain agonists, such as ADP and epinephrine, requires initial platelet aggregation, which in turn stimulates the metabolism of arachidonic acid and production of thromboxane A2. Second, the number of fibrinogen receptors expressed on the platelet surface and, therefore, the size of the platelet plug are increased b ADP, an agonist that is secreted from dense granules. Third, the fibrinogen and von Willebrand factor secreted from alpha granules can participate as ligands in the aggregation and adhesion of platelets. Thrombospondin, also released from alpha granules, may play a part in stabilizine the platelet aggregate. Finally, an alpha-granule membrane protein, GMP-140, is detectable on the surface of platelets after granule secretion, shere it may be involved in the interaction between platelets and white cells.

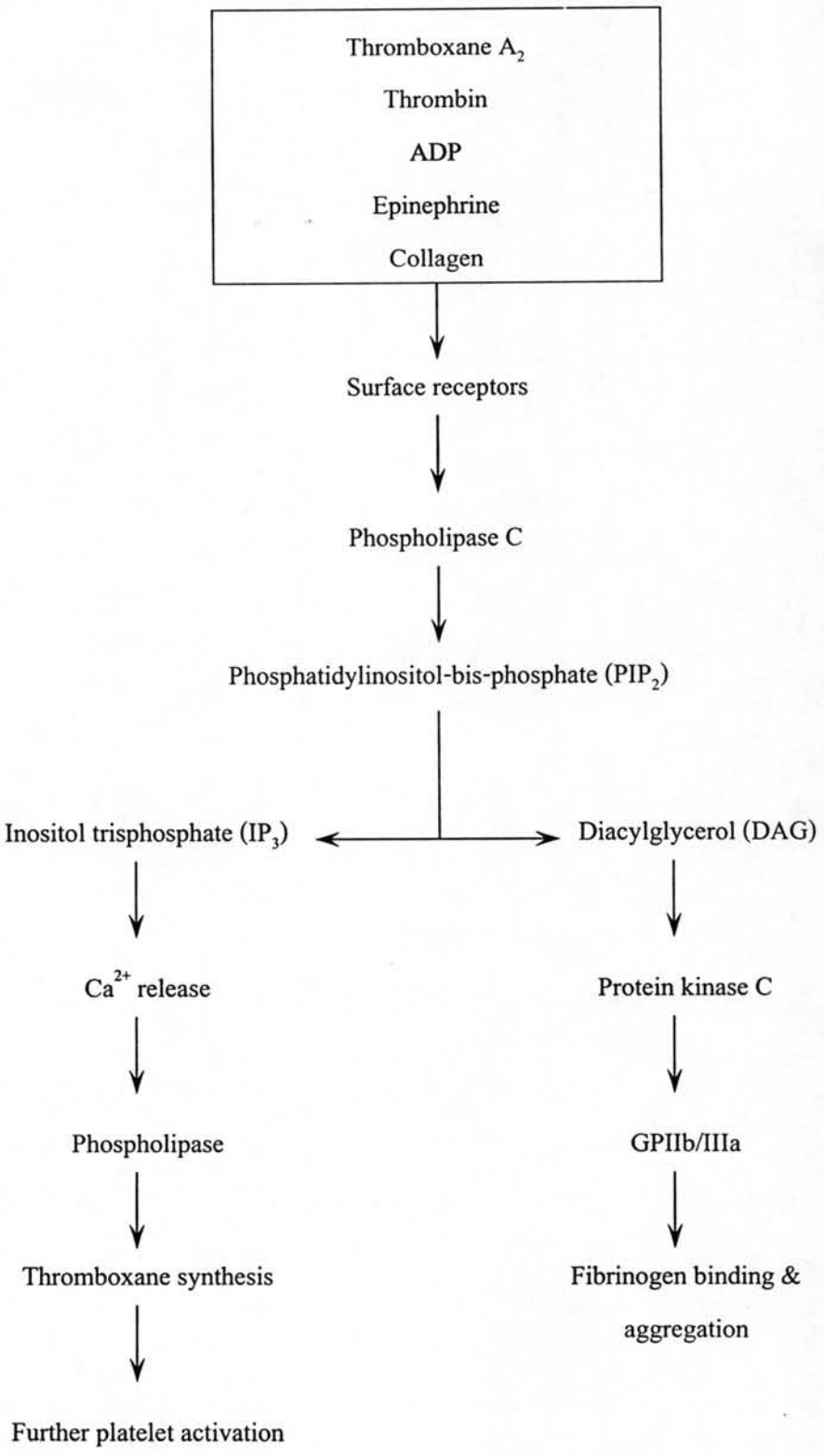

Figure1 Mechanism of platelet activation

Platelet procoagulant activity is minimal on unstimulated cells, but it increases dramatically after platelet activation by thrombin, collagen, or both. The generation of procoagulant activity requires an influx of calcium across the plasma membrane and is associated with the reorientation of acidic phospholipids (principally phosphatidylserine) from the inner leaflet of the plasma-membrane bilayer to the outer leaflet and with the shedding of membrane vesicles from the surface of the platelets and membrane vesicles expresses binding sites for specific coagulation proteins, leading to the formation of multicomponent membrane-associated enzyme complexes required for the generation of factor Xa and thrombin.

Antiaggregatory factors, namely prostacyclin (prostaglandin I_2 or PGI_2) and endothelium derived relaxing factor (nitric oxide, NO), are released by intact vascular endothelium and antagonize the effect of proaggregants so that thrombi do not form in healthy sections of blood vessels.

2.2. Evaluation of Platelet Function

Many methods have been used to measure platelet function. These include bleeding time, aggregometry and automated functional analyzers.

2.2.1. Bleeding Time

The forearm bleeding time can be useful in the diagnostic evaluation of platelet dysfunction because it specifically assesses the formation of the platelet plug in a skin wound. However, the limitations of this test must be recognized. First, the normal value is typically between 2 and 9 minutes, but technical aspects of bleeding-time measurements, such as the amount of venostasis and the direction of the forearm incision, significantly affect the duration of bleeding and the sensitivity of the test to aspirin ingestion. Second, some subjects with acquired disorders of platelet function may have deficient platelet aggregation but a normal bleeding time. Third, the bleeding time may be prolonged in patients who are anemic from any cause, possibly because red cells displace platelets toward the vessel wall or because of the augmentation of platelet activation by adjacent, metabolically active red cells. Forth, only one study has compared a standardized bleeding time with bleeding elsewhere in the body. It showed that although aspirin

prolonged the skin bleeding time, it had no effect on the duration of bleeding after endoscopic gastric biopsy. Finally, no studies have established the ability of bleeding-time measurements to predict the risk of hemorrhage in individual patients [25].

2.2.2. Optical Platelet Aggregation (Aggregometry)

The assay measures light passing through a sample of platelet rich plasma. Aggregation is usually assessed by stirring platelet-rich plasma at 37 °C in a cuvette between a light source and a detector. Aggregation is initiated by using agonists such as ADP, arachidonic acid, collagen, or epinephrine. As platelets start to aggregate there is an increase in light transmission, and results are recorded as the rate of aggregation and maximal response. Maximum light transmittance is set on the aggregometer with platelet poor plasma. Minimum light transmittance is set with an aliquot of platelet rich plasma before aggregation. These determine 100% and 0% aggregation.

Optical aggregation has been used routinely and is well accepted. There are correlations of platelet aggregation with clinical efficacy of antiplatelet agents. The technique has several disadvantages, including sample preparation. The assay is time consuming and requires that the blood samples be promptly sent to an onsite laboratory, preventing the assay from being run at the bedside. Performance of the assay requires training to a high level of technical proficiency. Additionally, methods for aggregation assays vary among laboratories. Sources of variation include adjustment of platelet concentration to some standard count, selection of agonist and agonist concentration, controlling time between sampling and analysis, and choice of centrifugation speeds in preparing platelet rich plasma [26].

Platelet aggregation is affected by a number of confounding variables. Hemolysis complicates aggregation measurements since erythrocytes contain ADP; lipemic samples obscure spectral changes due to platelet aggregation and thrombocytopenia makes platelet aggregation evaluations difficult to interpret. This test is laborious and costly.

2.2.3. Platelet Function Analyzer (PFA-100) (Dade-Behring, Miami, FL)

The platelet function analyzer (PFA) assesses platelet function in whole blood to measure platelet adhesion, activation and aggregation under conditions of high shear. A citrated whole blood sample is applied to a disposable cartridge containing a membrane coated with either collagen/epinephrine or collagen/ADP and which has a microscopic (147 um) aperture cut into it. Under high shear rates (5000-6000 s^{-1}), contact of blood with the membrane causes platelets to aggregate and occlude the aperture. The time taken to occlude the aperture or closure time (CT) is the end-point that is recorded in an analogous manner to the bleeding time. The maximal CT that can be recorded is 300 s. The technique is simple and rapid, but sensitive to a number of variables including platelet number, hematocrit, and plasma vWF levels.

2.3. Thromboxane Synthesis

The main source of thromboxanes is arachidonic acid which is found in the phospholipids. During platelet activation, phospholipase A_2, the enzyme phosphorylate phospholipids to arachidonic acid, is activated. The free arachidonic acid is then metabolized by cyclooxygenase [27] to cyclic endoperoxides, prostaglandin G_2 (PGG_2) and PGH_2 which subsequently are converse by thromboxane synthase (TX-synthase) to TXA_2. Thromoxane A_2 constricts large blood vessels and is a potent stimulus for platelet aggregation. It has a chemical half-life at body pH and temperature of 30 seconds, breaking down to the inactive TXB_2. (Figure2)

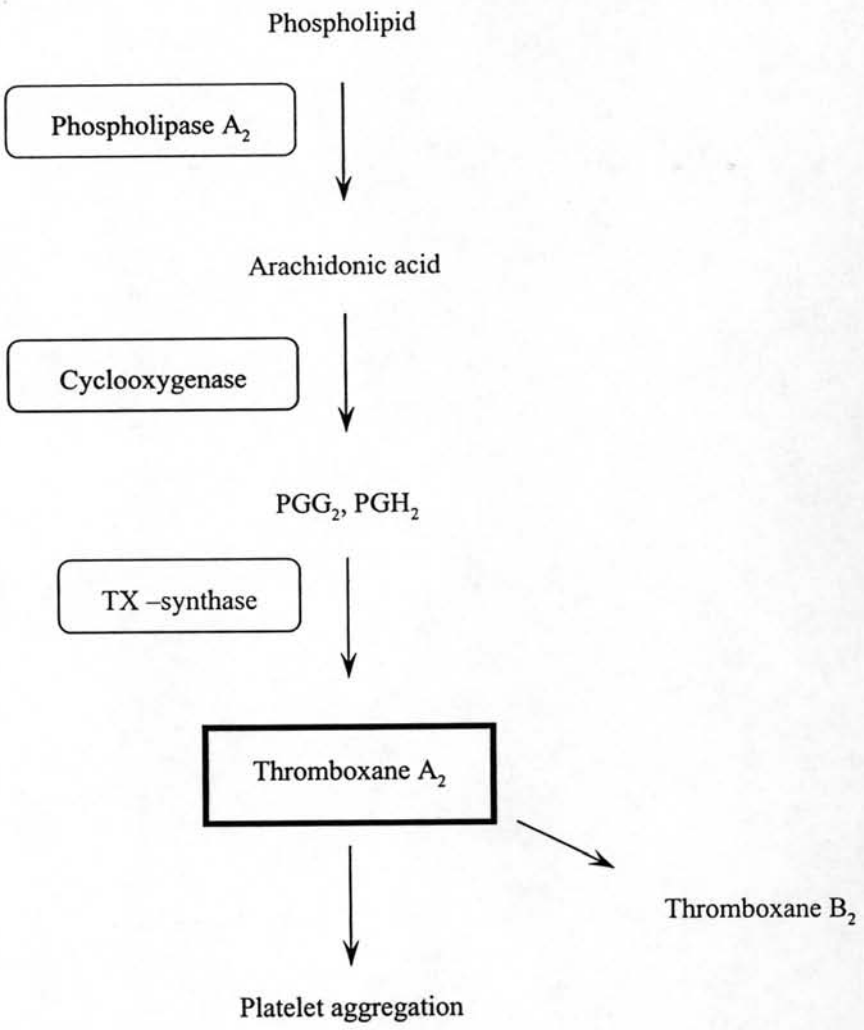

Figure 2 The biosynthesis of thromboxane A_2

2.4 Platelets in diabetes mellitus

In patients with diabetes mellitus, coronary artery disease tends to be more extensive and severe, with cardiovascular-releated deaths being three times more common than in non-diabetic patients [28]. Furthermore, diabetic patients without a document history of a previous MI have the same degree of risk for a myocardial infarction as non-diabetic patients with a previous history of infarction [2]. There is some evidences suggest that the increased risk of events in diabetes may be due to a platelet hyperaggregable state.

Platelets from patients with type II diabetes exhibit increased activation compared to platelets from non-diabetic subjects [4]. The platelet aggregation activity occur even in the time of diagnosis [5]. Diabetic platelets are found larger than those of nondiabetic patients and have increase potency to adhere and aggregate [29]. Glycoproteins in patients with diabetes show 20%-26% more than those without diabetes [30]. The increased expression of GpIIb-IIIa in platelets from diabetic subjects is consistent with the enhanced fibrinogen binding and aggregability [31]. In addition, serum fibrinogen levels are also elevated in many patients with diabetes [32].

Higher concentrations of arachidonate are found in the phospholipids membranes of DM platelets compared with normal controls [33]. Arachidonic acid metabolism is increased in platelets from diabetic patients; this leads to enhanced TXA_2 production and may contribute to increased platelet sensitivity [6]. In an animal model of diabetes, enhanced platelet aggregation and TXA_2 synthesis was detected within days of making rats diabetic with streptozotocin, before vascular disease was evident [34]. Sagel et al. found that inhibition of cyclooxygenase significantly decreased the effect of diabetes on platelets [5].

2.5 Aspirin

Aspirin is the most widely used drug in the word [35]. It is still the cornerstone of antiplatelet therapy today, more than 100 years after its initial use. Salicylic acid was first isolated from willow bark by Piria in 1838 and first synthesized in its impure form in 1853 by Charles-Frederic Gerhardt [36]. The eventual formulation of aspirin did not happen until Hoffman, a chemist at Bayer's Laboratories, took interest in 1893. Hoffman's father was treated with sodium salicylate for his rheumatism, but complained that it was intolerable because of its bitter taste. This prompted Hoffman's development of acetylsalicylic acid, which was called aspirin by the Bayer Company (A stood for acetyl and spir for spiric acid, the former name for salicylic acid) [37]. It was not realized until the 1950s that aspirin reduced the incidence of MI. This realization came about by the simple observation by Craven that his patients on aspirin were less likely to suffer an MI [35]. The Mechanism that aspirin affect on platelet was elucidated in 1967 by Weiss and Aledort [38]. Sir John R. Vane found that aspirin inhibit prostaglandin synthesis [39]. The work of Vane led to the discovery of thromboxane A_2 [40], that play an important role in the antithrombotic action of aspirin.

Aspirin pharmacology

Aspirin irreversibly inhibits cyclooxygenase-1 (COX-1) by acetylating a serine residue at position Ser529, thereby blocking the production of thromboxane A_2. COX is an enzyme responsible for the formation of eicosanoids, including thromboxane A_2 and prostacyclin (PGI_2). Thromboxane A_2 is by itself a powerful activator of platelet aggregation. When released along with thrombin and collagen, it leads to the release of ADP from platelet α granules. ADP has several functions, including effects that cause conformational changes in and activation of the GP IIb/IIIa receptor and stimulation of the arachidonic acid pathway causing further release of thromboxane A_2 [41]. Once daily, low-dose aspirin (0.45 mg/kg) suppresses serum thromboxane B_2 formation by at least 95% within about 5 days, and this level of inhibition is maintained with long-term daily administration.

Aspirin is rapidly absorbed in the stomach and upper intestine. Eighty to 100% of the drug is absorbed within 20 min to 2 hours. Peak plasma levels occur 30 to 40 min after

aspirin ingestion, and inhibition of platelet function is evident by 1 hour. Aspirin is short-lived in blood, with a half-life of approximately 15-20 min, due to rapid hydrolysis in the intestinal mucosa, liver and blood to its inactive salicylate moiety [41]. Despite the rapid clearance of aspirin from the circulation, the platelet-inhibitory effect lasts for the life span and 5 to 6 days following aspirin ingestion, the platelets function become normally [42]. Because platelets are anucleate, thus unable to regenerate active COX-1 for their life [43]. However, about 10% of circulating platelets are replaced every 24 h and up to 30% of platelets could circulate with uninhibited TxA_2 production after 48 hour without aspirin, daily administration of aspirin is necessary.

Aspirin as a therapeutic agent in cardiovascular disease

The ADA recommends the use of aspirin (81-325 mg/day) as a secondary prevention measure in diabetic patients with large vessel disease (history of MI, vascular bypass procedure, stroke or transient ischemic attack, peripheral vascular disease, claudication, and angina) [44]. Two large meta-analyses of major secondary prevention trials by the Antithrombotic Trialists' Collaboration have concluded that aspirin is protective in most patients who are at high risk for cardiovascular disease, including whose with diabetes [13, 45].

In meta analysis of 145 randomized trials of antiplatelet therapy in diabetic and nondiabetic people who had already had a major vascular event, prolonged antiplatelet therapy was definitely protective in four main high-risk categories: 1) acute myocardial infarction, 2) past history of myocardial infarction, 3) past history of stroke or transient ischemic attack, and 4) other relevant vascular history: angina, vascular surgery, angioplasty, and peripheral vascular disease. Reductions in vascular events were about 25% in each of these four categories and were separately statistically different in middle versus old age, hypertensive versus nonhypertensive patients, and diabetic versus nondiabetic patients. Dose of 75-325 mg of aspirin were most widely used. Doses throughout this range demonstrated similarly effective [45].

The Antithrombotic Trialists' Collaboration meta-analysis of 287 secondary prevention trials involved 212,000 high-risk patients who had acute or previous vascular disease or another condition that increased the risk of vascular occlusion [13]. Aspirin was the more

frequently used with doses ranging from 75-325 mg/day. A low dose of aspirin (75-150 mg/day) was found to be at least as effective as higher daily doses, although it was noted that an initial higher loading dose of at least 150 mg might be needed in acute settings. In the main high-risk groups, acute MI, past history of MI, past history of stroke or transient ischemic attack, acute stroke, and other relevant history of vascular disease, antiplatelet therapy significantly reduced the incidence of vascular events by 23%.

Aspirin is an effective antiplatelet drug reducing the risk of myocardial infarction, stroke, or cardiac death. This use is based on hundreds of randomized clinical trials and meta-analysis that show significant reduction in cardiovascular events of 20-25%. A meta-analysis conducted by the antiplatelet Trialists' Collaboration demonstrated that aspirin reduced the risk of ischemic vascular events as a secondary prevention strategy in numerous high-risk groups, including patients with diabetes. Moreover, the American Diabetes Association recommends low dose aspirin as a primary prevention strategy for people with diabetes at high risk for cardiovascular events.

Aspirin as a primary prevention strategy in diabetes

Based on collaborative trial data, the ADA recommends that enteric-coated aspirin be used as a primary prevention strategy in patients with diabetes who are classified as being at high risk for cardiovascular events on the basis of the following risk factors:

- Family history of coronary heart disease
- Cigarette smoking
- Hypertension
- Weight > 120% of ideal body weight
- Microalbuminuria or macroalbuminuria
- Total cholesterol > 200 mg/dl (LDL cholesterol > 100, HDL cholesterol < 55 in women and < 45 in men, and triglycerides > 200)

This evidence is supported by the results of the Primary Prevention Project in which low-dose aspirin (100 mg/day) was evaluated for the prevention of cardiovascular events in

individuals with one or more of the following: hypertension, hypercholesterolemia, diabetes, obesity, family history of premature MI, or being elderly (n=4,495). After a mean follow-up of 3.6 years, aspirin was found to significantly lower the frequency of cardiovascular death (from 1.4 to 0.8%; relative risk [RR] 0.56 [CI 0.31-0.99] and total cardiovascular events (from 8.2 to 6.3%; RR 0.77 [0.62-0.95]) [46].

The 5-year primary prevention trial of the U.S. Physicians' Health Study also supported the benefit of primary prevention with aspirin in diabetes. The trial was conducted in 22,701 healthy men that included 533 men with diabetes. Among diabetic men 4.0% of those treated with 325 mg aspirin every other day had an MI versus 10.1% of those who received placebo (RR 0.39)[47].

2.6 Aspirin Resistance

Platelet response to aspirin is not equal in all individuals, and a high variability in the prevalence of aspirin resistance has been reported. In addition, recent data demonstrated that patients whose platelets were not responded aspirin had a three-fold risk for death, myocardial infarction, or stroke compared with the patients who were aspirin responders. This data revealed that platelet resistance to aspirin is significantly associated with the risk of major cardiovascular events. The following studies exhibit variety responses of aspirin and aspirin resistance.

The term "aspirin resistance" has been used in a clinical and laboratory context. Clinical aspirin resistance has been used to refer to the inability of aspirin to protect individuals from cardiovascular thrombotic events such as acute MI, CVA and cardiovascular death. However, many cardiovascular events that occur in patients treated with aspirin may not be preventable by aspirin. Furthermore, the diagnosis of clinical resistance can only be made in retrospect because an ischemic event must occur before a diagnosis of clinical resistance can be considered.

Laboratory aspirin resistance refers to the failure of aspirin to inhibit platelet thromboxane A_2 production or inhibit tests of platelet function that are dependent on platelet thromboxane production.

Thromboxane A_2 production can be determined by measuring stable metabolites of thromboxane A_2 such as thromboxane B_2 in the serum or plasma and 11-dehydrothromboxane B_2 in the urine. However, measurement of serum thromboxane B_2 is labour intensive, not readily available, and might not be specific for platelet function. Urinary 11-dehydrothromboxane B_2 reflects in-vivo thromboxane production but it is not specific since renal and macrophage can produce thromboxane also.

Tests of platelet function that are dependent on platelet thromboxane production include agonist-induced platelet aggregation measured by optical transmission (turbidimetric aggregometry in platelet rich plasma), electrical impedance (whole blood platelet aggregometry), or semi-automated platelet aggregometry (e.g., platelet function analyzer (PFA-100), Ultegra rapid platelet function assay (RPFA)). The bleeding time is an in-vivo test of platelet function that also is dependent, in part, on platelet thromboxane production but is used rarely because it is highly operator dependent and poorly reproducible.

Optical transmission aggregometry measures the increase in light transmission through a platelet suspension when platelets are aggregated by an agonist such as thromboxane A_2, ADP, or collagen. This is the historical gold standard test to measure the antiplatelet effects of aspirin and remains the most widely used test for determining platelet function.

Impedance aggregometry measures the change in electrical impedance between two electrodes when platelets are aggregated by an agonist. The method is similar to light or optical aggregometry except that it can be done in whole blood, thus obviating the need for preparation of a platelet suspension.

The PFA-100 could be regarded as an in-vitro bleeding time recorder. It creates an articicial vessel consisting of a sample reservoir, a capillary, and a biologically active membrane with a central aperture, coated with collagen plus ADP or collagen plus epinephrine.

The application of a constant negative pressure aspirates the anticoagulated blood of the asmle from the reservoir through the capillary (mimicking the resistance of a small artery) and the aperture (mimicking high shear in the injured part of the vessel wall). A platelet plug forms that gradually occludes the aperture. Consequently, the blood flow through the aperture gradually decreases and ultimately stops. The time taken to interrupt blood flow, closure time, is recorded.

The Ultegra RPFA-ASA (Accumetrics, San Diego, CA, USA) is a simple rapid bedside test that measures agglutination of fibrinogen-coated beads in response to propyl gallate or, more recently, arachidonic acid stimulation. If aspirin produces the expected antiplatelet effect, fibrinogen-coated beads will not agglutinate, and light transmission will not increase. The result is expressed as aspirin reaction units (ARU).

2.6.1 Studies of Aspirin Resistance

Tohgi et al.[44] reported that 40 mg/day aspirin was able to inhibit 85% serum TXB_2 (the stable product of the hydrolysis of TXA_2) in 19 post-stroke patients. When increased the dose of aspirin to 320 and 1,280 mg/day the serum TXB_2 was inhibited 96.3% and 99.8% respectively. Platelet aggregation induced by 5 μM ADP in patients who received 0, 40, 320 and 1,280 mg/day aspirin were 72.1%, 64.9%, 59.2% and 54.5% respectively. They suggest that only 40 mg/day aspirin is able to inhibit a large proportion of TXA_2; however, higher doses of aspirin are required to attain further inhibition.

Helgason et al.[45] had measured the inhibition of platelet aggregation in 113 patients administered aspirin for stroke prevention. They found that only 80% of patients taking ≤ 325 mg/d aspirin had complete inhibition of platelet aggregation while others were partial inhibition. When increase the dose of aspirin to the latter group, most of them could switch to complete inhibition. There were only 2% of patients still had partial inhibition even taking 1,300 mg/d aspirin. This study concluded that the ability of aspirin may depend on the individual. In addition, the investigators had defined patients as aspirin resistant if despite a daily dose of 1,300 mg aspirin they never achieved complete inhibition of platelet aggregation.

Another study of Helgason et al.[46] was conducted to examine platelet aggregation at repeated intervals. Platelet aggregation test was measured and repeated every 2

weeks in patients with previous ischemic stroke who received 80-650 mg/d aspirin. The result showed that in some patients, there were fluctuations of aspirin's effect at the same dosage over time. Furthermore, these patients had recurrent stroke at a time when platelet aggregation was not completely inhibited.

Many studies revealed the prevalence of platelets non-responsiveness to aspirin or aspirin resistance in various groups of people. The results were varying due to different methods of platelet investigation and different definitions of aspirin resistance.

In 2001, Gum et al. [15] reported a prospective study in 325 patients with a prior history of coronary or cerebral vascular disease. Aspirin resistance was defined as the failure of aspirin 325 mg/d, given for a minimum of seven days before testing. The study used optical platelet aggregometry to define aspirin resistance. This technique evaluates platelet aggregation by detecting optical density changes within the platelets as they begin to aggregate. Aggregation is induced by the addition of either ADP or arachadonic acid to platelet rich plasma. Optical platelet aggregometry has the potential to be used clinically in diagnosing aspirin resistance because it is already widely available and routinely used to evaluate platelet function in other contexts. Definition of aspirin resistance by optical aggregation was a mean aggregation of $\geq$ 70% with 10 uM ADP and a mean aggregation of $\geq$ 20% with 0.5 mg/ml arachidonic acid. Aspirin semiresponders were defined as meeting one, but not both of the above criteria. The results showed that 5.5% of the patients were aspirin resistant and 23.8% were aspirin semiresponders. The investigators also used Platelet Function Analyzer (PFA-100) as a detection of aspirin resistance. PFA-100 is a newer, more rapid method. Aspirin resistance by PFA-100 was defined as having a normal collagen and/ or epinephrine closure time (< 193 seconds). The prevalence of aspirin resistant by PFA-100 was 9.5%. Of the 18 patients who were aspirin resistant by aggregation, only 4 were also aspirin resistant by PFA-100. The prevalence of aspirin resistant by optical aggregation was lower percentage than studies using alternate techniques. They also found that patients who were either aspirin resistant or aspirin semiresponders were more likely to be women (34.4% vs 17.3%, p=0.001) and less likely to be smokers (0% vs 8.3%, p=0.004) compared with aspirin-sensitive patients. There were no differences in aspirin sensitivity by race, platelet count, renal disease, or liver disease.

After two-year follow up, they found that aspirin resistance was associated with an increased risk of death, MI, or CVA compared with patients who were aspirin sensitive (24% vs 10%, hazard ratio [HR] 3.12, 95% confidence interval [CI] 1.10 to 8.90, p=0.03). Stratified multivariated analyses identified platelet count, age, heart failure, and aspirin resistance to be independently associated with major adverse long-term outcomes[17].

Eikelboom and co-workers[48], explored whether aspirin resistance, defined as failure of suppression of thromboxane generation, increases the risk of cardiovascular events in a high-risk population. Base on patients in the Heart Outcomes Prevention Evaluation (HOPE) Study, they used a nested case-control design and measured urinary 11-dehydro thromboxane B_2 in 488 cases treated with 75 to 325 mg/d aspirin who had MI, stroke, or cardiovascular death during 5 years of follow-up and in 488 matched control subjects also receiving aspirin who did not have an event. They found that, patients in the upper quartile of urinary 11-dehydro thromboxane B_2 concentration at baseline compared with those in the lowest quartile had an almost 2-fold increase in the odds of myocardial infarction, stroke, or cardiovascular death (Odds ratio [OR] 1.8; 95% CI: 1.2 to 2.7) and a more than 3-fold increase in the odds of cardiovascular death (OR 3.5; 95% CI: 1.7 to 7.4). The results indicated that in aspirin-treated patients, urinary concentrations of 11 dehydro thromboxane B_2 predict the future risk of MI and cardiovascular death.

Study of Andersen et al. [47] was conducted to investigate the response to long-term aspirin therapy in patients with a former acute myocardial infarction. Seventy-one patients were assigned to receive 160 mg/d aspirin for 4 years then evaluated platelet aggregation by the Platelet Function Analyzer (PFA-100®). This method evaluates platelet function by determining the time to occlusion of an aperture in a membrane coated with collagen and ADP or epinephrine (EPI) as citrated whole blood flows under high shear stress conditions. They defined the 95th percentile of closure time (CT/EPI) as non-responders. The result showed that 35%, 25 of 71 patients, presented as non-responders.

Fateh-Moghadam et al.[20] recently reported the prevalence of aspirin resistance in type 2 diabetic patients. This study included 172 type 2 diabetic patients with concomitant

cardiovascular risk factors that were hypertension (defined as systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg and/or antihypertensive treatment), hypercholesterolemia (blood cholesterol levels ≥ 200 mg on diet and/or treatment with statins), smoking (currently smoking), obesity (body mass index (BMI) > 30 kg/m^2) and family history (first degree relatives with symptomatic coronary heart disease). All patients were on regular aspirin treatment with 100 mg daily. The effect of aspirin was assessed using the platelet function analyzer (PFA-100®). Resistance to aspirin was defined as a normal collagen/epinephrine induced closure time (82-165 seconds). Patients with closure time ≥ 300 s were defined as aspirin responders and patients with closure time between 166 and 300 s as semi-responders. The results demonstrated that 21.5% were found to be resistant to aspirin therapy, 16.9% were semi-responders and 61.6% were aspirin responders. Diabetic patients with coronary artery disease tended to have an increased prevalence of aspirin resistance: 25.8% (16 of 62) compared with patients without coronary artery disease 16.7% (9 of 54). Univariate analysis revealed that aspirin non-responders were significantly younger (p=0.05) compared to aspirin responders. No association was found between aspirin resistance and hypertension, family history of coronary artery disease, hypercholesterolemia, active smoker, BMI, coronary artery disease and HbA1c.

Mehta el al. has conducted a study comparing frequency of aspirin resistance in patients with type 1 versus type 2 diabetes mellitus who were recommended 81-325 mg/d aspirin for primary or secondary cardiovascular protection. Ultegra Rapid Platelet Function Assay-ASA was used to assess platelet function of the subjects. The Ultegra system is a turbidimetric-based optical detection system that measures platelet-induced aggregation as an increase in light transmittance. Aspirin resistant defined as > 550 aspirin resistance units. In 111 patients with type 2 DM, 16.2% were aspirin resistance. Similar rates of resistance were found in patients with type 1 DM (21.7%). No relation between aspirin resistance was found for patients taking 81 mg/d compared with those taking > 81 mg/d in the whole group or those with either type1 DM or type 2 DM. No significant difference in the incidence of aspirin resistance was seen in subjects receiving aspirin therapy for primary or secondary cardiovascular disease. They also found that aspirin resistance was not related to age, HbA1c, total or LDL cholesterol, or a history of cardiovascular disease. Although female gender was an independent predictor of aspirin

resistance in patients with T1DM (p=0.001), gender was not associated with aspirin resistance in T2DM.

2.6.2 Mechanisms of Aspirin Resistance

There are many reasons why aspirin might not suppress production of thromboxane A_2 and activation and aggregation of platelets, and might cause laboratory aspirin resistance, and why aspirin may fail to prevent clinical atherothrombotic vascular events and cause aspirin treatment failure. Reduced suppression of platelet COX-1 or thromboxane production may be cause by poor compliance, reduced enteral absorption of aspirin, etc.

2.6.2.1 Poor Compliance

Up to 40% of patients with cardiovascular disease do not comply with aspirin therapy [49]. Poor compliance with aspirin is a common reason why aspirin is ineffective in the laboratory and clinically. It is an important potential confounder in clinical outcome studies of laboratory aspirin resistance, but is difficult to study and has not been adequately investigated to date. Two recent observational studies suggest that aspirin resistance is rare in patients with coronary artery disease who are compliant with aspirin therapy [50, 51].

2.6.2.2 Dose

Laboratory studies indicate that low-dose aspirin uniformly suppresses platelet COX-1 in healthy controls and in patients recovering from myocardial infarction [52, 53]. Moreover, systematic reviews of randomized controlled trials of antiplatelet treatment showed no significant difference in effectiveness of different aspirin doses within 75-300 mg compared with placebo, and an increased risk of adverse effects with higher doses of aspirin [13]. However, the estimates of the magnitude of the effectiveness of each dose are not precise (the 95% CIs are reasonably wide) and the comparisons are indirect. Direct comparisons of different doses are more reliable but the estimates are also imprecise (wide 95% CIs) and cannot exclude the possibility that higher doses might be more effective in some patients. Indeed, the laboratory response to aspirin can be improved by increasing the dose from 100 mg/d or less to 300 mg/d or more, but this benefit has not been correlated with a reduction in clinical events [53-55].

2.6.2.3 Reduced enteral absorption of aspirin

Aspirin is absorbed in the stomach and upper intestine with peak levels achieved within 30-40 min after ingestion of soluble aspirin and 3-4 h after enteric coated formulations [56]. Because platelet COX-1 is acetylated in the pre-systemic circulation, the antiplatelet effect of aspirin is believed to be independent of systemic bioavailability. Plain aspirin is a weakly acidic drug and rapidly crosses the gastric mucosa in its lipophilic state, where the pH is low and hydrolysis is minimal. In contrast, enteric-coated aspirin is released in the upper part of the small intestine, where the higher pH exceeds the drug's pKa and therefore, aspirin is less protonated and absorbable. Whereas soluble aspirin at doses as low as 40 mg fully inhibits platelet aggregation in healthy volunteers, it has recently been reported that low-dose enteric-coated aspirin may incompletely suppress of platelet aggregation [27, 57].

2.6.2.4 Impaired suppression of platelet COX-1

Concomitant intake of a conventional NSAID such as ibuprofen, prevents access of aspirin to the COX-1 substrate-binding site causing impaired suppression of platelet COX-1[58]. Supporting evidence for this mechanism comes from an observational study of patients with cardiovascular disease in whom the risk of all-cause mortality over 9 years of follow-up was highest in those taking aspirin plus ibuprofen, followed by aspirin plus other NSAIDs, aspirin alone, and aspirin combined with diclofenac [59].

2.6.2.5 Increased platelet turnover

Because aspirin has a very short half-life, increased platelet turnover which occurs in infection, inflammation and following major surgery can lead to an increased proportion of non-aspirinated platelets during the 24-hour dosing interval. Impaired suppression of agonist-induced platelet aggregation and thromboxane biosynthesis has recently been reported in patients undergoing carotid [60] and coronary artery bypass graft (CABG) surgery, the in-vitro addition of 100 uM aspirin but not a COX-2-selective inhibitor fully inhibited arachidonic acid-induced thromboxane formation and platelet aggregation [61], which supports impaired suppression of COX-1 as the mechanism for aspirin resistance in these patients.

2.6.2.6 Genetic polymorphisms

Epidemiologic studies suggest that one-third of the variation in laboratory response to antiplatelet drugs is genetically determined [62]. Hundreds of single nucleotide polymorphisms (SNPs) have been identified in genes involved in the thromboxane biosynthetic pathway and isolated reported have suggested that single nucleotide polymorphisms involving COX-1 and COX-2 can modify the response to aspirin and/or predict future risk of myocardial infarction or stroke [63]. However, the impact of these polymorphisms on laboratory aspirin resistance is unclear. In a recent open-label randomized cross-over trial involving 96 healthy volunteers taking aspirin 100 mg/d and clopidogrel 300 mg/d, selected polymorphism of platelet receptor genes were not associated with aspirin (or clopidogrel) responsiveness [64].

2.6.2.7 Non-platelet sources of thromboxane A_2 production

Thromboxane A_2 can be produced in monocytes and macrophages by conversion of arachidonic acid to thromboxane A_2 in a reaction that is catalysed by the enzymes COX-2 (to form prostaglandin G_2/H_2) and thromboxane synthase (to form thromboxane A_2). This reaction might be particularly likely in inflammatory states, such active atherosclerosis, when COX-2 production is upregulated [65].

Aspirin-insensitive thromboxane biosynthesis is associated with increased F2-isoprostanes (prostaglandin F2-like compounds), which are produced by lipid peroxidation of arachidonic acid in a non-COX reaction that is catazysed by oxygen free radicals. Isoprostane production is augmented by smoking, diabetes, hyperlipidemia, and unstable angina, and is associated with resistance to the effect of aspirin on platelet activation and altered response of platelets to other agonists. This effect could in part explain the mechanism of the association between conventional risk factors for cardiovascular disease and enhanced platelet activation [66].

2.6.2.8 Non-atherothromboembolic pathology

Ischemic vascular events of the heart, brain, limbs, eyes, kidneys, and other organs are not always caused by atherothromboembolism. For example, only about 50% of all recurrent ischemic strokes are due to atherothrombotic disease; about 20% arise from emboli from the heart, about 25% are due to occlusion of one of the small, deep, perforating cerebral arterial dissection, vasculitis, and infective endocarditis [67]. The pathology of cardiovascular disease is affected by complex pathways involving inflammation, endothelial function, differential responses to hemodynamic changes, and the coagulation cascade. Since atherothrombosis is a multifactorial disease, aspirin cannot be expected to prevent all clinical cardiovascular events.