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Research Paper

Assessment of Aspirin Responsiveness by Light Transmittance Aggregometry in Patients with Ischemic Heart Disease – A Study from Southern Rajasthan

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ABSTRACT

The present study was conducted to evaluate the prevalence of aspirin resistance in patients with IHD living in and around Udaipur. Fifty patients of IHD (Group I) either of healed myocardial infarction (>6 months) or angina in whom ischemia can be induced by TMT, and were taking 150 mg of aspirin daily for more than 3 months were selected. Fifty healthy individuals (Group II) were selected as control to know the baseline platelet aggregation in the same age group. Platelet aggregation was assessed employing platelet rich plasma (PRP) on optical aggregometer- ELVI 840.

The study has brought that ten percent of the study population demonstrated aspirin resistance. The incidence of true resistance, that is unresponsive to both, ADP and collagen aggregating agents is 2%, while "Semi responders" constitute 8% of the study population. These patients demonstrated resistance to either of the two aggregating agents. The incidence of clinical resistance in term of recurrence of cardiac event (MI) was observed in 4% of population in whom the true resistance was observed in 2% while 2% were semi responders. Six percent of the study subjects who demonstrated aspirin resistance were semi-responders and all have inducible ischemia and did not develop infarction. Ten percent of the study subjects had recurrent episode of MI in spite of aspirin consumption. In these subjects the true aspirin resistance was observed in 20% of the subjects, twenty percent were semi-responsive, while in 60% aspirin was found to be effective in inhibiting platelet aggregation.

KEYWORDS: Aspirin treatment failure, Aspirin non-responsiveness, PFA-100 device, COX-1 inhibition

INTRODUCTION

Cardiovascular diseases account for approximately 12 million deaths annually and are the commonest cause of death globally. Previously considered to be disease of affluent; now it is increasing in developing world too in epidemic proportion. The Asian Indians living in their own country or elsewhere have much higher incidence of coronary artery disease as compared to all other ethnic group. In Indian subcontinent, from 1960's to 1990's the coronary artery disease prevalence increased two folds (from 2% to 4%) in rural and three folds (3.45% to 9.45%) in Urban Indian population[1]. Thus burden of cardiovascular disease in patients and community is enormous.

One way of reducing the burden is to reduce the platelet aggregation in people predisposed to such high risk. Cardiovascular diseases are the result of multiple complex cascade of interaction among the endogenous cells of the arterial wall, the focal haemodynamic environment, blood

components notably monocytes, lipoproteins, inflammatory processes and their mediators and various healing or reparative processes. The role of platelets in thrombosis is central for atherothrombosis.

Platelets are small (2-3 mm in diameter) non nucleated cells containing granules with constituents (e.g. 5-Hydroxytryptamine, catecholamines, and ADP) capable of influencing platelet function. They normally circulate in blood for approximately 10 days before being sequestered in the spleen. Their main function is in primary hemostasis; interacting with injured areas in the vessel wall and form a haemostatic plug that later organizes and incorporates other blood cells and components of the coagulation system[2,3].

Quiescent platelets are discoid in shape and do not adhere to intact endothelium, but a breach of the arterial lining will expose collagen in the deeper tissue layers and this substance activates platelet adhesion, when activated they become irregular and, form pseudopodia. The exposure of sub-endothelial collagen and the release of von Willebrand factor results in binding of these substances to glycoproteins IA/ IIA and IB of the platelet surface[4,5].

For platelets to interact with the endothelium, the platelet glycoproteins IIB-IIIA must first undergo a conformational change. In addition to shape change and adhesion, activated platelets release from their "dense bodies" various agonists, including serotonin and ADP, which intensify aggregation, as does thromboxane A_2 (TXA_2), besides being vasoconstrictive. TXA_2 is derived from arachidonic acid, a substance present in the phospholipids of the platelet membrane. The other inclusion system of platelets, the "alpha granules", contain P-TG (β-thromboglobulin), PF-4 (Platelet factor four), PDGF (Platelet Derived Growth Factor) and t PAI-2 (tissue plasminogen Activator inhibitor); these are also released when thrombocytes are activated and further intensify platelet aggregation.

In opposition to these aggregation promoting factors, the vascular endothelial cells synthesize prostacyclin (PGI_2) which, by stimulating CAMP, inhibit platelet aggregation and release. If endothelial cells are injured, several factors are released e.g. 11 platelet activating factor (PAF), a potent stimulus for platelet aggregation. The endothelial cells also synthesize and release "endothelium derived relaxing factors" (EDRF), one of these is nitric oxide (NO), which like $PG_{1\alpha}$ inhibits platelet aggregation and stimulates vasodilation[5]. The action is mediated by an increase of platelet cAMP and cGMP.

Platelets have been implicated as being pathophysiologically important in hypertension and ischemic heart diseases. They might contribute to coronary artery disease in at least two ways; one by thrombus formation caused by platelet activation in the presence of vascular damage and secondly as a source of mitogenic influence (platelet derived growth factor)[6,7].

In the treatment of CAD aspirin has remained cornerstone of therapy in both primary and secondary prevention of death due to CAD owing to its antiplatelet functions[8, 9]. Aspirin is being used for last 2 centuries. "Willow bark" contains salicin from which salicylic acid is derived. It was used for fever in 18th century as cheap substitute for imported cinchona bark. Its antiplatelet action has recently been recognized[10]. Today nearly all patients of CAD and peripheral vascular disease are receiving this drug.

Aspirin is a cyclo-oxygenase inhibitor thus irreversibly blocks the formation of thromboxane A_2, a potent mediator of platelet aggregation which converts arachidonic acid to prostaglandin G_2. Platelets does not contain nucleus, so once inhibited it can't form cyclo-oxygenase. The enzyme inhibition is permanent and irreversible. Other effects of cyclo-oxygenase inhibition are the block of production of prostacyclin. $PG_{1\alpha}$ that opposes platelet activation. Aspirin inhibits the release of ADP, cationic proteins, PGE_2. $PGE_{2\alpha}$ and phospholipase from the granules of platelets. The use of aspirin as anti thrombotic drug therapy started in 1983 with publication of the Veterans administration cooperative study. This study decreased 1 year event rate by 5% as compared with placebo (10.1%)[11].

A larger number of trials including antiplatelet trialists' collaboration meta analysis, the largest trial ever conducted on aspirin efficacy has proved that aspirin in effective in reducing deaths from myocardial infarction from 25%-68%[12]. However, the antiplatelet effect of aspirin has not been observed to be uniform on all human population and relative risk of recurrent vascular events in patients receiving aspirin therapy remains high (8-18% after 2 years)[13]. Aspirin resistance has been reported to occur in 5% to 45% of general population and its detection is of clinical importance.

The initial evidence that some patients may be resistant to aspirin came from study by Mehta and associates who reported that 30% of patients had minimal inhibition of platelet aggregation after single 150 mg of dose of aspirin[14]. The significant studies by Grundmann and co-workers in ischemic stroke patients with high dose of aspirin proved aspirin resistance to be 34%[15]. Gum and associates conducted studies on 326 patients of IHD and reported a 5% incidence of aspirin resistance by optical platelet aggregation[16]. In a subgroup study of the Heart Outcomes Prevention Evaluation (HOPE) trial sample, Eikelboom and colleagues observed increased adverse events in individuals exhibiting aspirin resistance during a 5-year follow-up. As a measure of *in vivo* thromboxane production, the urinary concentration of 11-dehydrothromboxane B2 was determined. For every quartile that 11-dehydrothromboxane B2 levels increased, the adjusted chances for the composite end-point of myocardial infarction (MI), stroke, or vascular death also increased[17].

Despite consistency of such observation, the prevalence of aspirin resistance has been variable in different populations and there is lack of standardized diagnostic criteria on a single validated method of identifying affected individuals to have aspirin resistance. It has led to wide range of population estimates[18]. Prospective studies have demonstrated that the decrease responsiveness to aspirin therapy is associated with an increased risk of clinical events[19,20].

Aspirin resistance has been observed to affect patients of various categories and healthy controls without vascular disease as well[15,21,22]. Provided more than 12 million deaths caused by CAD annually, even a 5-10% prevalence of aspirin resistance affects more than a million patients[16]. Therefore, it is pertinent to take into account the aspirin resistance while

treating patients with ischemic cardiovascular diseases.

The present study, therefore, has been envisaged to study aspirin responsiveness in patients with ischemic heart disease consuming aspirin 150 mg and living in and around Udaipur.

MATERIAL AND METHOD

The study was conducted on male patients of IHD who were stable in their symptoms, attending OPD or admitted in the wards of Maharana Bhupal Hospital attached to R.N.T. Medical College, Udaipur. After informed consent a total number of 50 subjects were selected for the study in each group:

The study groups included in study are as follows:

Group I : 50 patients of ischemic heart disease (IHD) who are stable in their symptoms and taking 150 mg of aspirin daily from last 3 or more months.

Group II: 50 healthy individuals without any evidence of ischemic heart disease.

Patients with ischemic heart disease (IHD) were selected based on the following investigational criteria:

1. ECG:
   (a) Documentation of old healed myocardial infarction.
   (b) ST depression of ⩾2mm in consecutive leads with or without symptoms.
2. ECHO: Regional wall motion abnormalities (RWMA)
3. Positive TMT:
   (a) Horizontal or down sloping ST segment depression of >1 mm from previous level during TMT with or without symptoms.
   (b) Junctional depression with slowly rising ST slope that remains depressed 1.5 mm or more than 0.80 m seconds after the J point.
   (c) Slowly up sloping ST segment depression with the ST segment being depressed in excess of 2.5 mm, 80 m seconds after the J point.
   (d) Down sloping or flat, ST segment depression in excess of 2.5 mm.
   (e) Horizontal or Down sloping ST segment depression appearing during the first stage of exercise and/or persisting beyond 8 minutes in the recovery phase.
   (f) Complex ventricular, ectopic activity, including multiform ventricular ectopic beats, or runs of ventricular tachycardia or occurrence of ventricular fibrillation.

Exclusion Criteria:

The following subjects were excluded from the study-

1. Those were taking ticlopidine, dipyridamole, clopidogrel, heparin, LMWH (low molecular weight heparins) and corticosteroids and other non steroidal anti-inflammatory drugs.
2. Haemoglobin 8 gm/dL
3. History of myelo - proliferative syndrome & malignant paraproteinemias.
4. Family or personal history of bleeding disorders.
5. Patients with diabetes, hypertension.
6. Patients with peripheral vascular diseases.

Method:

Venous blood samples (9 ml) was collected in the morning in a fasting state without undue pressure of stable cardiac patients of age more than 40 yrs after brief history, physical examination and written consent. Specimens were kept at room temperature and subjected within 1 hour for estimation of platelet aggregation on ELVI-840 aggregometer and Omniscribe chart recorder.

PLATELET AGGREGATION[23]

Most important function of platelets is their role in haemostasis i.e. adhesion to the damaged tissue surfaces and cohesion to one another. This cohesion phenomenon is known as aggregation and may be initiated by a variety of substances including collagen, adenosine 5-diphosphate (ADP), epinephrine, arachidonic acid, serotonin and ristocetin. Aggregation is one of the numerous in vitro tests performed as a measure of platelet function. The described procedure is turbidimetric method of measuring the effect of collagen, ADP and epinephrine on platelets, better termed as light transmittance aggregometry (LTA).

Reagents:

1. 3.8 per cent citric acid (Trisodium salt dehydrate): Prepared by dissolving 3.8 gm citric acid in 100 ml of deionized water.
2. Tris buffer: Tris (hydroxy methyl), methylamine, 1.21 gm. (0.01M), disodium ethylene diamine tetra acetic acid 0.372 gm (0.001 M), sodium chloride 8.76 gm. (0.15 M), dissolved in distilled water adjusted to pH 7.5 with hydrochloric acid and made up to one litre with distilled water.
3. ADP reagent: Adensoine 5-diphosphate lyophilized with buffer salts (supplied by sigma diagnostics). ADP solution was prepared by reconstituting ADP reagent with 1.0 ml deionized water to yield solution containing ADP 2x1 04mol/lit. It was swirled to mix and allowed to stand at room temperature (18-26°) for 15 minutes before use. It should be kept at room temperature only for duration of testing. The reconstituted reagent is stable for one month if stored in refrigerator (2° to 6°C).
4. Collagen Reagent: Collagen (calf skin) acid soluble, approximately 2 mg lyophilized with buffer salts, Collagen solution was prepared by reconstituting a vial of collagen reagent with 1.0 ml deionized water. The, vial was allowed to stand undisturbed for at least 15 minutes at room temperature before use. Warming to 37°C may be necessary for complete dissolution. It was swirled to mix prior to each assay. It should not be vertexed. The solution should be kept at room temperature only for the duration of testing. It is usually stable for at least 2 weeks refrigerated (2° to 6° C). Stability may be extended by freezing.

INSTRUMENTS AND MATERIALS REQUIRED

(1) Instruments:

1. Platelet aggregometer (ELVI 840).
2. Chart recorder (Omniscribe recorder dual pen type L176 2USA)

(2) Materials:

1. Cuvette 250 µl
2. Teflon coated magnetic stirring bars (micro agitators)
3. Pipettes with disposable plastic tips 50 µl and 250 µl.
4. Centrifuge machine.
5. Plastic tubes with caps.
6. Plastic transfer pipettes.

A. Specimen collection:

Blood was collected by avoiding stasis and contamination with tissue fluids into plastic tubes containing 0.1 ml buffer and 3.8 per cent sodium citrate in a ratio of blood to anticoagulant in a ratio of 9: 1.

B. Preparation of platelet rich plasma (PRP):

(i) The anticoagulant sample was centrifuged at 400 rpm, for 10 minutes.

(ii) PRP was removed carefully using a plastic transfer pipette.

(iii) PRP was expelled into a plastic tube and covered and kept at room temperature for duration of the test.

C. Preparation of platelet poor plasma (PPP):

(i) It was prepared by re-centrifuging the PRP at 6000 rpm for 10 minutes.

(ii)Supernatant was transferred to a labelled PPP tube, covered and kept at room temperature for the duration of test.

The platelet count of PRP was adjusted to the range of 4 to 5 lac/cu mm when necessary by addition of autologous PPP to PRP sample. The caution should be taken to assay platelet aggregation within 30 minutes of collection of test samples.

Aggregating agents - ADP and Collagen

ADP - ADP induced aggregation may occur in one or two phases and it may be followed by rapid disaggregation which may be seen in normal man without any hemorrhagic disease.

Collagen - Collagen induced platelet aggregation may occur in an - irreversible single phase curve or a reversible single phase curve depending on the collagen concentration in the PRP.

Procedure:

After preparing PRP and PPP the aggregation was recorded as follows:

1. Cuvette with PRP was introduced into the aggregometer.
2. The electromagnetic agitation was started by means of stiffed control after having introduced a small stiffing bar into the sample.
3. Agitation speed was maintained at 1000 rpm.
4. Baseline of the recorder was adjusted by means of the zero control.
5. The cuvette with PRP was removed and cuvette with PPP was inserted.
6. By means of gain control the maximum excursion of the pen on the recorder was adjusted.
7. Cuvette with PPP was removed and the cuvette with PRP was reinserted and it was readjusted if necessary by means of the zero control.
8. The sliding of the recorder paper was started.
9. The aggregating agents (ADP and Collagen) were added to the PRP by means of micropipette (50 µl).

The aggregation was recorded for a minimum of five minutes and results were expressed as percentage aggregation.

$$\text{Percentage Aggregation} = \frac{90 - CR}{90-10} \times 100$$

$$= \frac{90 - CR}{80} \times 100$$

CR is chart reading in terms of number of segments.

Aspirin resistance - Platelet aggregation induced by ADP and Collagen in patient receiving 150 mg of aspirin, more than 60% will be taken as aspirin resistance.

Table 1 shows the profile of 50 male patients of ischemic heart disease, selected for the study. There were 32 patients who had sustained myocardial infarction in the past and were stable in their symptoms. Their mean age was 62.53 years and they were regularly taking 150mg of aspirin daily from last 2 years or more. Eighteen patients were of ischemic heart disease, proved on TMT. Their mean age was 57.7 years and mean duration of aspirin consumption was 4 years and 4 months.

Table 1: Profile of Study Subjects (IHD)

	No. of Patients	Mean age (years)	Mean duration of Aspirin treatment
Old MI	32	62.53	2 years 10 months
IHD – TMT positive	18	57.72	4 years 4 months

MI – Myocardial Infarction, IHD – Ischemic Heart Disease, TMT – Tread Mill Test

Table 2: Platelet Aggregation in Healthy Individuals

	Platelet Aggregation (Percent)	
	ADP	**COLL**
Mean	57.00	52.87
SD ±	9.36	9.88
SE ±	1.32	1.40

Platelet aggregation profile of 50 healthy individuals (Group II), induced by ADP and Collagen have demonstrated that their mean ADP induced platelet aggregation was 57± 9.36 percent, while collagen induced platelet aggregation was 52.87±9.88 percent (Table 2).

Table 3: Platelet Aggregation in Patient with IHD taking Aspirin (150mg)

	Age	**Platelet Aggregation (Percent)**		**Duration of Aspirin Consumption (Months)**
		ADP	**COLL**	
Mean	60.80	32.23	27.43	40.22
SD ±	8.24	16.22	18.88	40.30
SE ±	1.17	2.29	2.67	5.70

Table 3 depicts the platelet aggregation of 50 patients of ischemic heart disease who were taking aspirin (150 mg) daily for more than 3 months. The age varies from 44 to 78 years. The mean duration of aspirin consumption was 40.22±40.30 months. The mean platelet aggregation was 32.23±16.22 percent and 27.43±18.88 percent induced by ADP and Collagen respectively. There are three patients each in ADP and Collagen subsets who demonstrate aggregation of more than 60 percent.

Table 4: Inhibition of Platelet Aggregation by Aspirin (150mg) in Patient with IHD

	Age	**Platelet Aggregation (Percent)**		**Duration of Aspirin Consumption (Months)**
		ADP	**COLL**	
Mean	60.80	66.78	72.58	40.22
SD ±	8.24	17.64	18.88	40.30
SE ±	1.17	2.49	2.67	5.70

The percentage inhibition of platelet aggregation by 150 mg of Aspirin in patients with Ischemic Heart Disease has been shown in table 4. On further analysis of the results, it was observed that except 3 patients (cases 2, 10 & 44) all have demonstrated more than 60 percent inhibition of platelet aggregation induced by ADP. Likewise, 3 patients (cases 18, 26 & 44) also demonstrated platelet aggregation less than 40 percent with Collagen.

Table 5: Profile of Patients Demonstrating Aspirin Resistance

S. No.	ADP ($2x10^{-4}$mol/L)			
	Age	**Diagnosis**	**Duration of Aspirin Treatment**	**Platelet Aggregation (Percent)**
1	55	TMT moderately positive	8 months	65.00
2	68	TMT strongly positive	10 years	68.75
3	58	Recurrent MI 1. Inferior Wall MI 2. Anterior Wall MI	8 years 6 months	75.00

TMT – Tread Mill Test MI – Myocardial Infarction

The profile of three patients who demonstrated aspirin resistance induced by ADP has been given in table 5.Their age was ranging from 55 to 68 years. Two patients were of IHD proved on exercise Test, while one patient was of recurrent myocardial infarction who sustained inferior and anterior myocardial infarction in spite of regular aspirin consumption for last 8 and half year. The aspirin, in this patient, was able to inhibit platelet aggregation only to the extent of 25 percent.

Table 6: Profile of Patients Demonstrating Aspirin Resistance

S. No.	Collagen (0.2µg/ml)			
	Age	**Diagnosis**	**Duration of Aspirin Treatment**	**Platelet Aggregation (Percent)**
1	64	Recurrent MI 1. Anterior Wall MI 2. Inferior MI	12 years	68.75
2	60	TMT Positive	5 years	62.50
3	58	Recurrent MI 1. Inferior Wall MI 2. Anterior Wall MI	8 years 6 months	68.75

TMT – Tread Mill Test MI – Myocardial Infarction

On analysis of profile of patients who manifested aspirin resistance based on Collagen aggregant, it was found that one patient of recurrent myocardial infarction is common in table 5 and 6. Who demonstrates true resistance i.e. both ADP and Collagen induced aggregation more than 60 percent and sustained second myocardial infarction. In remaining two patients one was of recurrent myocardial infarction and other was of inducible myocardial ischemia. In both the patients of recurrent infarction, the duration of aspirin administration was more than 8 years and both were demonstrating platelet aggregation more than 68 percent (Table 6).

Table-7: Profile of Patients with Recurrent Myocardial Infarction

Case No.	Age	Name	Duration of Aspirin Treatment (months)
5	60	Nathulal	60
16	66	Mohan Singh	30
18	64	Kachrulal	144
24	72	Lalu Ram	50
44	58	Dalpat	102

The profile of patients who had recurrent myocardial infarction inspite of aspirin therapy showed that out of 5 patients, one (case number 44) showed true aspirin resistance in whom both ADP and collagen caused more than 60% platelet aggregation. Case number 18, on the other hand, was aspirin semi-responder. Where ADP induced platelet aggregation was 33.75% but collagen induced platelet aggregation was 68.75%. Rest of three cases were aspirin responsive (Table 7).

DISCUSSION

The present study was conducted to observe the prevalence of aspirin resistance among patients of Ischemic Heart Disease (IHD) who are residing in and around Udaipur and taking 150 mg of aspirin for more than 3 months regularly.

Fifty male patients of ischemic heart disease were selected for the study and 50 healthy volunteers were also taken as control for establishment of normal platelet aggregation. All the study subjects were kept overnight fasting and venous blood samples were collected in the morning for platelet aggregation. All the blood samples were subjected for estimation of platelet aggregation using ELVI 840 aggregometer and Omniscribe chart recorder (LTA).

Platelet aggregation measurement in healthy individuals (Table 2) shows that mean aggregation induced by ADP and collagen are 57.00±9.36 and 52.87±9.88 percent respectively. Based on these limits 60% was taken as cut off point. Platelet aggregation more than 60% was taken as aspirin resistance. If both ADP and Collagen induced platelet aggregation is more than 60% than patients were labelled to have "true resistance", while, if one aggregant showed aggregation more than 60% and other less than that they were labelled as "semi responders".

Profile of 50 patient of IHD selected for the study showed that 32 patients were of healed MI and 18 patients were of Angina. All the patients were stable in their symptoms and were taking Aspirin 150 mg daily for than 2 years and 4 years respectively (Table 1).

The mean platelet aggregation induced by ADP and collagen were 32.23±16.22 and 27.43±18.80 percent which reflected good aspirin response. However, on careful analysis of observations (Table 3) 3 patients[2,10,44] showed aspirin resistance (aggregation > 60%) in ADP Induced aggregation and 3 patients[18,26,44] in collagen induced aggregation. In all these patients the percentage of inhibition of platelet aggregation by 150 mg of Aspirin was less than 40% (Table 4).

On further analysis of patients demonstrating aspirin resistance (Table 5, 6); one patient who demonstrated ineffectiveness of aspirin in inhibiting platelet aggregation by both the aggregants had true resistance. He also had recurrent MI in spite of aspirin consumption for last 8.5 years. The other 4 patients were semi-responders who demonstrated failure of aspirin activity with either of aggregants.

The present study therefore brings the fact that aspirin resistance in this area is around 10% out of which 2% is the true resistance and 8% showed semi-responsiveness. Moreover aspirin resistance and duration of aspirin consumption have proportionate relation as the majority of patients demonstrating aspirin resistance were consuming aspirin for more than five years (Figures 1and 2).

The overall prevalence of aspirin resistance in different studies varies from 8% to 45%[12,24,25]. However the dose of aspirin resistance varies in different studies from 75 mg to 325 mg /day as well as methodology used to define aspirin resistance.

Aspirin is the cornerstone of antiplatelet therapy in cardiovascular medicine. Its role in the secondary prevention of vascular events has been proven beyond any doubt. A recently published meta-analysis of 287 randomized trials of antiplatelet therapy by the Antithrombotic Trialists Collaboration has shown a significant reduction in the combined end-point of any serious vascular event in a cohort of high-risk patients with atherothrombotic diseases[26]. However, a substantial proportion of patients manifest "breakthrough" events despite regular intake of aspirin. It is estimated that one in eight high-risk patients suffers from the recurrence of a vascular event within the next 2 years despite regular daily aspirin therapy[27]. Also, by using different methods of measuring platelet activity, several studies have demonstrated marked individual variations in the response to treatment with aspirin[16,24,28,29]. Based on the clinical and laboratory evidence of reduced or absent response to treatment with aspirin in some patients, the concept of "aspirin resistance" has emerged, and has caught the attention of both professionals and the mass media[30].

Unfortunately, aspirin resistance remains a poorly defined term. There are conflicting reports on the incidence and clinical relevance of this phenomenon as this term is being used to describe a number of different phenomena. These include the inability of aspirin to either protect individuals from thrombotic complications; or failure to cause prolongation of bleeding time, or inhibit platelet aggregation ex vivo. or inhibit platelet thromboxane formation[31,32].

Perhaps a clinical definition of aspirin resistance as the failure of the drug to prevent an ischemic event despite regular intake of appropriate doses is the most relevant for practising physicians[31]. It is well known that platelet inhibition is not a uniform process, and considerable inter- and intra-individual variations exist in the antiplatelet effect of aspirin. This mandates functional and biochemical in vitro tests to individualize treatment, and possibly identify the subgroup of patients at risk for future vascular events.

Traditionally, platelet function has been assessed by measuring platelet aggregation in platelet-rich plasma using an optical aggregometer. This test is widely available, and has been used in many investigational studies. Based on this method, 5% and 24% of patients with stable cardiovascular disease on aspirin therapy (325mg/day for at least a week) were defined as "resistant" and "semi responders", respectively[31].

Recently, simpler and more rapid tests of platelet function have been developed. Whole-blood aggregometry is more user

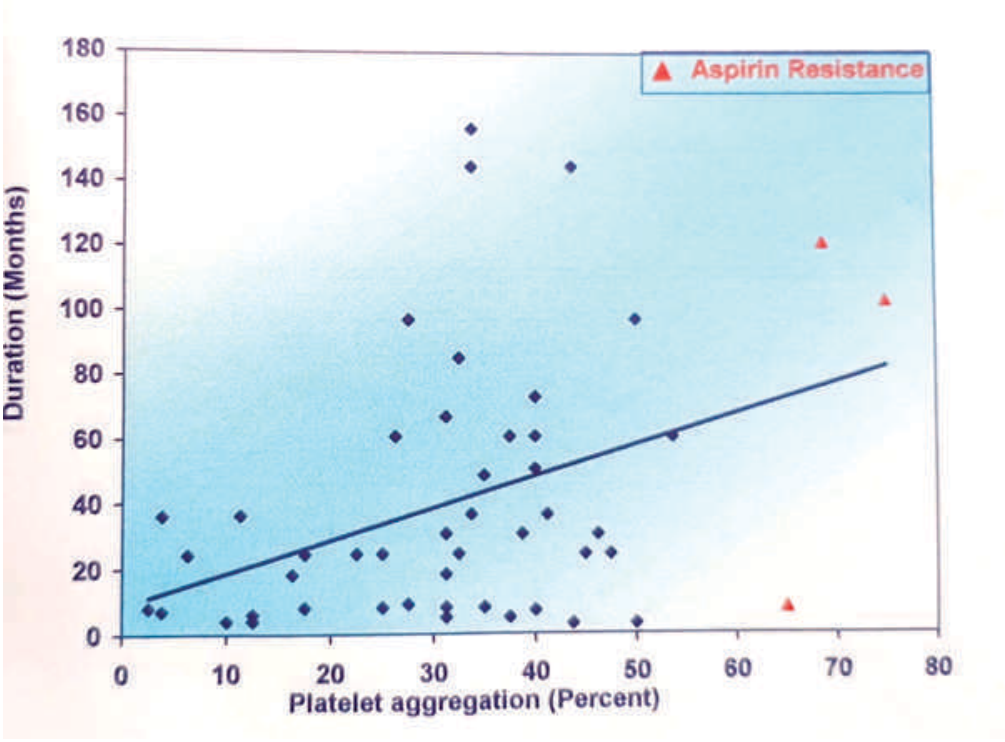

Figure 1: Correlation of platelet aggregation induced by ADP with duration of aspirin therapy

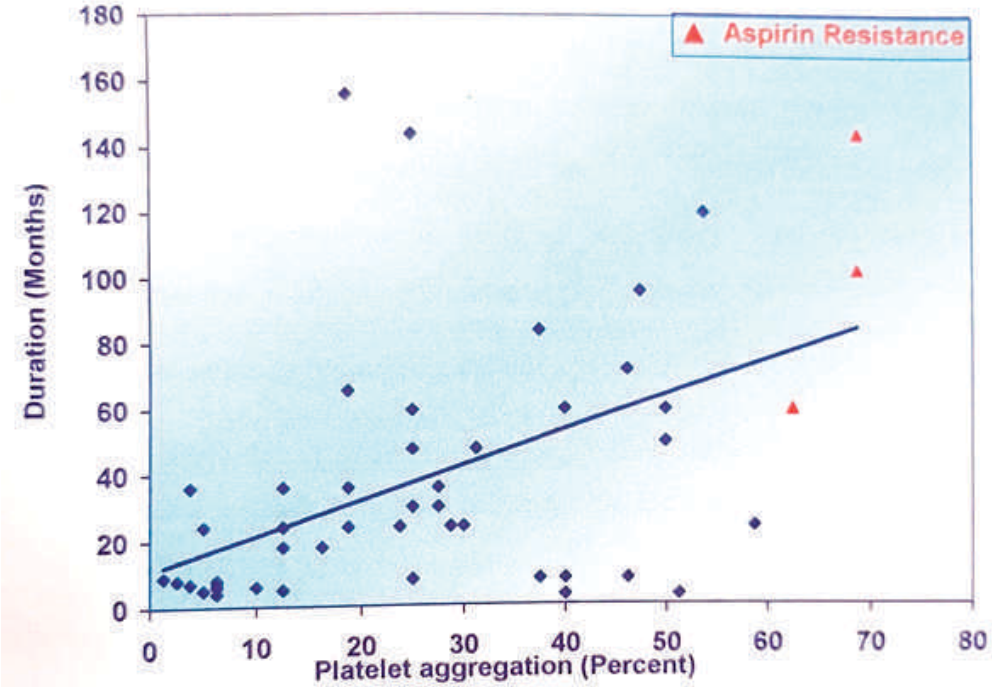

Figure 2: Correlation of platelet aggregation induced by collagen with duration of aspirin therapy

friendly as it eliminates the step of preparing platelet rich plasma. However, the results of this test have not correlated well with those from optical aggregometry[32]. In clinical practice, PFA100 (Dade Behring, Deerfield, Illinois) is the most appealing test at present for the assessment of platelet function. It is a semi-automated analyzer developed to allow the rapid assessment of platelet function using whole blood. The results are easily reproducible and correlate well with the results of optical aggregometry[32,33].

A nonspecific measure of platelet function is the assessment of bleeding time[2, 27]. Other less extensively studied tests include the platelet aggregate ratio, the platelet reactivity index, and the rapid platelet function assay (RPFA)[32,34]. Recently, urinary 11-dehydrothromboxane B_2 levels (a stable metabolite of thromboxane A_2 [TxA_2]) has been used as a marker of suppression of thromboxane formation with aspirin therapy[19]. Since the levels reflect both platelet and non-platelet sources of thromboxane generation, this test lacks specificity. Collectively, these techniques identify an inadequate response to aspirin in 5-60 percent of patients with different vascular atherothrombotic diseases. It is difficult to assess which of these techniques is the most accurate and specific measure of aspirin resistance unless the results are supported by direct comparison with the clinical outcome.

Weber and co-workers classified aspirin resistance into 3 distinct types using simple biochemical tests, and functional *in vitro* studies[31].

TYPE I RESISTANCE (PHARMACOKINETIC TYPE):

When aspirin was taken orally for five days at a dose of 100 mg/day, aspirin responders showed greater than 95% inhibition of thromboxane production and of collagen-induced platelet aggregation as evaluated in vitro. Oral aspirin use for five days did not reduce either thromboxane production or collagen-induced platelet aggregation in patients with "type I resistance" (pharmacokinetic type). However, the addition of 100 µm of aspirin *in vitro* to the platelet-rich plasma significantly changed both of these characteristics. This implies that the pharmacokinetics of low-dose aspirin may vary significantly.

TYPE II RESISTANCE (PHARMACODYNAMIC TYPE):

Neither the oral aspirin consumption nor the in vitro addition of 100 µm of aspirin affected any of the platelet activities. Although the exact mechanism of this kind of resistance is unknown, it may be connected to the enzymatic pathways genetic variation and aspirin sensitivity.

TYPE III RESISTANCE (PSEUDO-RESISTANCE):

Neither the oral aspirin consumption nor the in vitro addition of 100µm of aspirin affected any of the platelet activities. Although the exact mechanism of this kind of resistance is unknown, it may be connected to the enzymatic pathways, genetic variation and aspirin sensitivity. It is possible that certain aspirin-resistant individuals have higher platelet sensitivity to collagen[35]. It's unclear whether this variation has any clinical significance. It is unknown if this change, as assessed in artificial *in vitro* settings, will correspond to a reduced aspirin`s antithrombotic action *in vivo.* 101 It has been suggested that raising the aspirin dosage may help people with type I resistance. Furthermore, additional antiplatelet medications may be beneficial for people with types II and III resistance. The classification and clinical importance has not been investigated yet, though. This problem can only be adequately addressed by prospective follow-up studies in aspirin-resistant patients and their clinical connection.

A few long-term follow-up clinical studies have suggested that aspirin resistance is indeed clinically important[25,36-38].

Grotemeyer and co-workers in a cohort of 180 patients with stroke found that nearly 30% of patients were aspirin non-responders. At a follow-up of 2 years, major clinical vascular end-points were significantly higher in this group as compared to aspirin responders (40% v/s 4.4%, $p<0.0001$). The methodology used by them was platelet reactivity; aggregation induced by blood collection[28].

Mueller and co-workers, in 100 patients undergoing peripheral balloon angioplasty reported an 87% higher risk of re-occlusion on follow-up in patients who failed to show an appropriate response to aspirin[36].

Grundmann and co-workers found that an aspirin non responder status was seen in 34% of patients with recurrent cerebrovascular ischemic events, despite regular use of aspirin for more than 60 months[15].

Buchanen and Pappas, independently conducted aspirin resistance studies on various study groups and healthy controls without vascular diseases, have also shown to have resistance by laboratory testing[21,22].

Chen and associates reported 19.2% incidence of aspirin resistance as defined by Ultra RDFA among 151 patients with coronary disease[20], using ultra rapid platelet function analyser defined aspirin resistance as ARU(ASPIRIN RESPONSE UNITS) >550.

Gum and co-workers reported 5% incidence of aspirin resistant and 23.8% were aspirin semi responders. By PFA-100 (platelet function analysis), 9.5% were aspirin resistant. They found no difference in aspirin sensitivity by race, diabetes, platelet count or liver diseases[19]. They used both optical platelet aggregation using ADP and arachidonic acid as aggregants and PFA (platelet function analyser) for determination of aspirin resistance.

Macchi and co-workers studied 160 stable cardiac patients using PFA-100 (platelet function analyser) and found aspirin resistance in these patients to be 29.2%[39]. Epinephrine closure time less than 186 sec was taken as aspirin resistance by them.

Sibi and co-workers using optical platelet aggregation studied 150 mg dose of aspirin in 75 stable cardiac patients and reported aspirin resistance to be 26% in studied patients[40].

Methodology used by them was optical platelet aggregation using arachidonic acid and ADP.

Anderson and co-worker studied 129 stable CAD patient using PFA-100 with aspirin resistance define as epinephrine closure time < 196 seconds and reported aspirin resistance of 1.35%[29].

Serum markers such as soluble CD40 ligand and P selection have also been used as markers of platelet activation with variable results[37].

Two recently published studies have highlighted adverse outcomes with aspirin resistance in a larger cohort of patients, and after a longer follow-up period.

In a subgroup analysis from the Heart Outcomes Prevention Evaluation (HOPE) trial population, Eikelboom and Co-workers (2002) found that individuals with aspirin resistance had more bad outcomes during a 5-year follow-up. As a measure of *in vivo* thromboxane production, the urinary concentration of 11-dehydrothromboxane B_2 was determined. With each rising quartile of 11-dehydrothromboxane B_2 levels, the adjusted chances for the composite end-point of myocardial infarction (MI), stroke, or vascular death rose. Individuals with insufficient suppression of TXA_2 and consequent aspirin resistance in the highest quartile were 1.8 times more likely to have composite end-points than patients in the lowest quartile. Likewise, there was a 3.5 times greater risk of cardiovascular mortality and a 2- times higher risk of MI in each group. This substantial and graded correlation between aspirin resistance in the laboratory and unfavourable outcomes was not correlated with traditional risk factors for atherothrombotic vascular diseases[41].

During a mean follow-up period of 679±185 days, Gum and Co workers (2003)[19] emphasised the natural course of aspirin resistance in stable patients with cardiovascular disease. Aspirin resistance was linked to a significantly higher risk of composite end-points such as death, MI, or cerebrovascular accident (CVA) in this prospective, blinded study involving 326 patients when compared to aspirin-responsive patients (24 percent v/s 10 percent, respectively; p=0.03, hazard ratio 3.12)[19].

It is interesting to note that among 50 patients of IHD 32 patients were of healed MI and 5 patients demonstrated recurrent MI in spite of aspirin therapy (Table 7). In these patients of recurrent MI, one patient demonstrated true aspirin resistance, clinically as well as on laboratory study. Aspirin in this patient could not inhibit Platelet aggregation induced by ADP and Collagen. The other patient (case no 18) was aspirin semi-responder while remaining three patients were aspirin responsive. It is clear from the above data that among the patients of IHD who demonstrated recurrence of MI in spite of aspirin therapy aspirin resistance should be seriously thought of Because, as it is evident in the present study. 20% of these patients may have true aspirin resistance and need alternative or combined therapy with other antiplatelet drugs.

Aspirin blocks the formation of TxA_2, a potent vasoconstrictor and platelet agonist by irreversibly inhibiting the enzyme platelet cyclo-oxygenase (COX) (Fig.3). COX has two isoforms of clinical relevance COX-1 isoenzyme is expressed in mature human platelets. The therapeutic efficacy of aspirin in atherothrombotic vascular disease has been clearly attributed to its inhibition of COX-1 activity[38,42]. Importantly, in the low doses necessary to achieve platelet inhibition, aspirin does not inhibit endothelial cell prostaglandin synthesis, particularly prostacyclin, which is a potent vasodilator[33,34]. COX-2 isoenzyme plays a dominant role in the processes of inflammation andcancers[38]. Aspirin acts as an anti-inflammatory agent due to the inhibition of COX-2 activity at higher doses. Although much is currently known about effect of aspirin on platelets, the mechanism by which some patients are resistant to this effect has not been clearly established.

A number of extrinsic variables can alter aspirin`s capacity to deactivate platelets. Aspirin`s antiplatelet impact has been demonstrated to be influenced by smoking elevated cholesterol levels, and circumstances linked to an accelerated platelet turnover[43,44]. While full inhibition of COX-1 is anticipated with low-dose aspirin, greater doses may be needed for certain people to have the desired antiplatelet effect.

Helgason and co-workers in patients with stroke reported the effect of dose escalation of aspirin in non-responders as judged by aggregation studies. An initial 25% incidence of aspirin resistance (daily dose 325 mg) fell to 8% with dose escalation up to 1300 mg. However, a recently published meta-analysis does not support this contention, and it may not be practical in many patients due to gastrointestinal side-effects[24].

Secondary aspirin resistance may be influenced by certain medication interactions, particularly those involving non-steroidal anti-inflammatory medicines (NSAIDs). Since aspirin and NSAIDs are both frequently given medications, it is possible that many people are taking both on a long-term basis. Given that both of these medications function by suppressing the COX enzyme, there is a chance that they will interact competitively. NSAIDs, on the other hand, are reversible inhibitors of this enzyme, unlike aspirin. Aspirin`s long-lasting antiplatelet activity has been demonstrated to be blocked by NSAIDs (such as ibuprofen), which modifies the drug`s cardioprotective effects. In individuals who initially react to aspirin, this can potentially result in secondary aspirin resistance[43]. This is because an NSAID competitively inhibits the active site inside the COX-1 channel, preventing aspirin from reaching its target[44]. Furthermore, there is currently proof that this medication combination has negative long-term clinical outcomes[21,45].

MacDonald and Wein[46] reported a cohort of patients' secondary prophylaxis with aspirin, and highlighted that on concomitant administration of ibuprofen was associated with a significant increase in the all cause mortality as well as cardiovascular mortality on long-term follow-up. The absence of COX-2 in mature human platelets explains why selective COX-2 inhibitors (coxibs) do not inhibit the effects of low-dose aspirin on platelet function in comparison with ibuprofen[44,47]. These drugs would logically seem preferable to ibuprofen when patients taking aspirin for cardio protectiveness require chronic

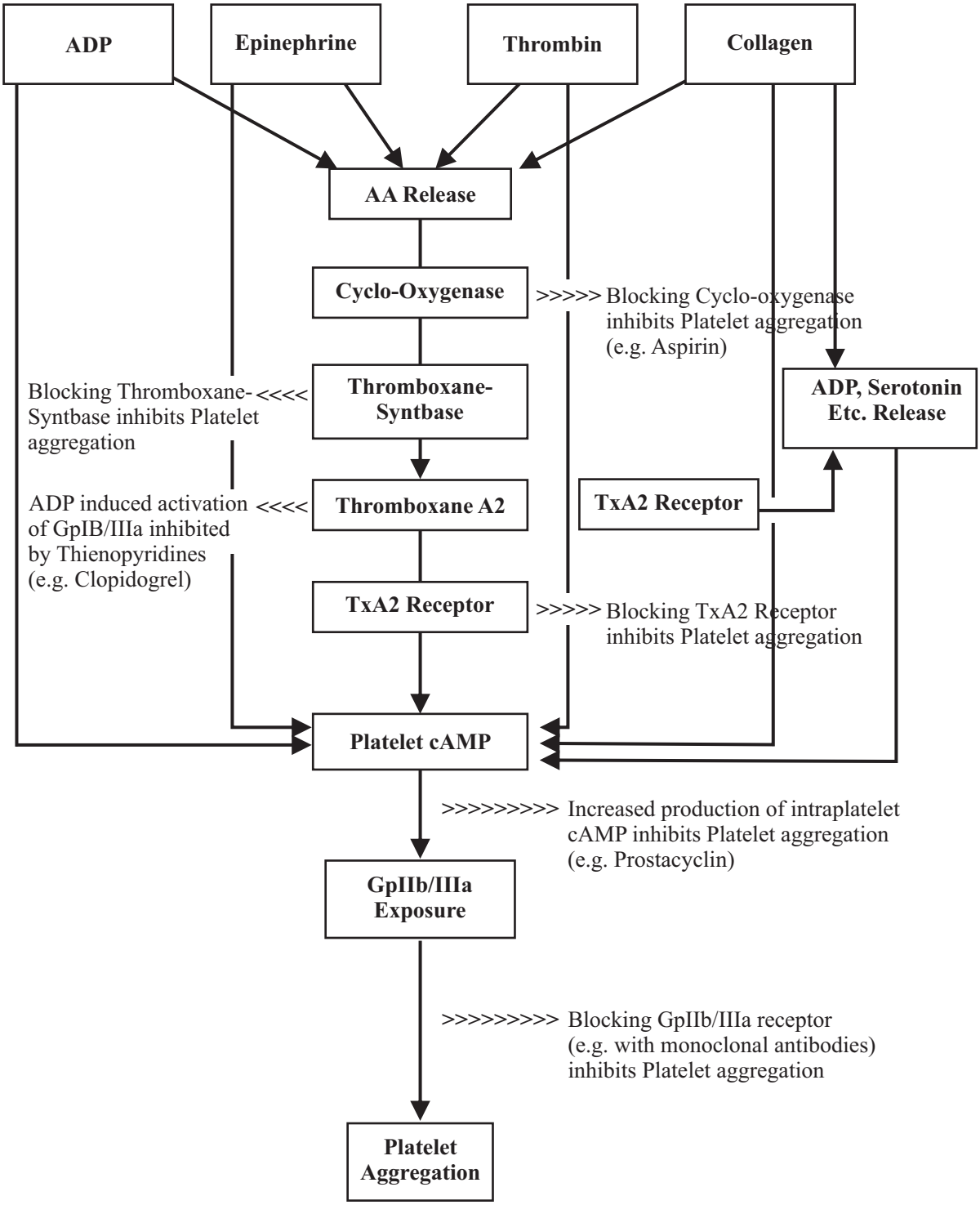

Figure 3: Platelet activation mechanism leading to platelet aggregation

treatment with NSAIDs.

A number of intrinsic mechanisms have been postulated to cause aspirin resistance. The inability of treatment to sufficiently reduce TXA_2 Synthesis is a critical factor in the pathophysiology of aspirin resistance [19, 48]. The emergence of aspirin resistance has been linked to COX-2, despite aspirin`s almost 170-fold greater potency in inhibiting COX-1. It has long been assumed that mature platelets are only COX-1 isoenzyme-containing. 99 Contemporary data, however still subject to debate, has demonstrated that COX-2 mRNA is present in platelets[48,49]. Patients differ in the extent of their COX-2 expression, and some may express COX-2 at higher levels than others, particularly under stress. Due to low dosage Aspirin`s inability to block the COX-2 enzyme, individuals on aspirin treatment may have an alternative pathway for platelet-mediated thromboxane synthesis, which might lead to aspirin resistance[16,47].

Nucleated cells, such as monocytes and macrophages, have also been linked to the processes behind aspirin resistance in addition to platelets. In terms of their capacity for synthesis, these cells are second only to platelets in terms of TxA_2 availability[50]. These cells can renew the enzyme, nevertheless, in contrast to anucleate platelets. Prostaglandins are produced by this regenerated, unrestrained COX-1 in nucleated cells, which is then transferred to the platelets to make aspirin-insensitive/resistant thromboxane, avoiding platelet COX-1. These nucleated cells have the ability to produce their own TxA_2 in addition to that which is mediated by COX-1 through COX-2, which is not blocked by aspirin at low concentrations[19,30]. In contrast to constitutively expressed COX-1, inflammatory stimuli increase COX-2 expression in nucleated cells by a factor of 10–20[19,32]. These nucleated cells may activate platelets with the help of the TxA_2 they manufacture, starting a chain reaction50. There is evidence that atherosclerotic tissue has an upregulated level of COX-2. Aspirin resistance and acute coronary syndromes may result from the macrophages in the atherosclerotic plaque contributing considerably to the pool of TXA_2 that is not inhibited by modest dosages of aspirin[48]. Studies have demonstrated that erythrocytes can increase platelet reactivity and be prothrombotic. Not all individuals experience a consistent blocking of this cell-to-cell contact by aspirin, which might offer a different route for the development of thrombus [51].

The varying effects of aspirin in different persons may also be due to genetic variances. Firstly, a genetic foundation for aspirin resistance may be provided by polymorphisms or mutations of the COX-1 gene, which renders it relatively resistant to the action of aspirin. Single nucleotide polymorphisms (SNPs) of COX-1 may occur and influence an individual`s susceptibility to aspirin's inhibitory effect.117 SNPs are thought to act as mediators of phenotypic variation and provide the genetic foundation for a drug's variable response. Second, the variable effects of aspirin in different people may potentially be due to genetic variations in the glycoprotein IIb/IIIa receptor complex. The last common route for platelet activation is the glycoprotein IIb/IIIa receptor. The PIA_1 and PIA_2 alleles are defined by a common polymorphism involving the replacement of Leu33 for Pro, respectively. The majority of research show that PIA_3 carriers are less sensitive to aspirin`s antithrombotic actions and exhibit increased platelet activation by agonists, despite contradictory data. The amount to which the glycoprotein IIb/IIIa polymorphism influences aspirin`s functions contributes to both the drugs clinical effectiveness and resistance to its effects is still unknown, though.

Despite consistency of such observation, the prevalence of aspirin resistance has been variable in different populations and there is lack of standardized diagnostic criteria on a single validated method of identifying affected individuals to have aspirin resistance. It has led to wide range of population estimates[18]. Even though, aspirin resistance should be seriously considered in patients of IHD or stroke who are taking the prescribed dose regularly but getting recurrent coronary events or stroke. These patients need supplementation or supplant of other antiplatelet drugs. Unfortunately there are reports showing that clopidogrel, a thienopyridine derivative, commonly used as an antiplatelet agent, also demonstrates resistance in many patients.

CONCLUSION

The present study demonstrated that aspirin non-responsiveness in IHD patients living in and around Udaipur is 10%, of which 2% are having true resistance and 8% are semi-responders. The real prevalence in population around the study area is thought to be much more than reflected by the present study because the present study has excluded patients with diabetes, hypertension, history of smoking and female patients in whom incidence of aspirin resistance is reported to be high. Aspirin resistance or non-responsiveness is clinically important particularly in patients of recurrent infarction in whom chances of aspirin resistance are 40%. Aspirin resistance and duration of aspirin consumption have proportionate relation as majority of patients demonstrating aspirin resistance were consuming aspirin for more than 5 years. Further large scale study is warranted including different population with different disease using other parameters of assessment of platelet function.

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