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Defective platelet response to arachidonic acid and thromboxane A_2 in subjects with Pl^{A2} polymorphism of β_3 subunit (glycoprotein IIIa)

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Summary. The membrane complex $\alpha_{IIb}\beta_3$ is the major receptor for fibrinogen and is involved in platelet adhesion and aggregation. Evidence has been presented that the Pl^{A2} allele of the β_3 $Pl^{A1/A2}$ gene polymorphism might be an independent risk factor for coronary thrombosis, but the matter is still controversial. We investigated the relationship between this polymorphism and possible alterations of platelet functions *in vitro*. The platelet adhesion to fibrinogen-coated microplate wells and the aggregation induced by several different agonists were tested in 63 healthy volunteers, among them, 49 subjects with $Pl^{A1/A1}$ polymorphism, 12 subjects with $Pl^{A1/A2}$ polymorphism and two subjects with $Pl^{A2/A2}$ polymorphism. Subjects with $Pl^{A1/A2}$ polymorphism or with $Pl^{A2/A2}$ polymorphism showed significantly lower platelet responses as compared with $Pl^{A1/A1}$ subjects when either arachidonic acid or the thromboxane A_2 analogue, U46619, were used as agonists. In resting condition and after thrombin or ADP stimulation, platelet function was normal in all the subjects. An increased sensitivity to the anti-aggregatory effect of acetylsalicylic acid was observed in platelets from subjects with the Pl^{A2} allele. Finally, using a flow-cytometric evaluation and determining the β-thromboglobulin plasma levels, we did not find any evidence of a Pl^{A2} platelet hyperreactivity *ex vivo*. Our findings are not consistent with the hypothesis that the purported increase of cardiovascular risk in these subjects may be as a result of platelet hyperactivation. On the contrary, the Pl^{A2} allele is associated with a platelet functional deficiency, specifically linked to the activation of the fibrinogen receptor by thromboxane A_2.

Keywords: platelet, $Pl^{A1/A2}$ polymorphism, integrins, acetylsalicylic acid, thrombosis.

The membrane integrin complex $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) is a major receptor for platelet adhesion and aggregation. The gene encoding the subunit β_3 is polymorphic at exon 2 (position 1565). The more common allele encodes a leucine (Pl^{A1}) and the less common allele encodes a proline (Pl^{A2}). A high frequency of family members homozygous for Pl^{A2} was found in kindreds with a high prevalence of acute coronary diseases under 60 years of age (Weiss *et al*, 1995). The same authors later reported an association between acute coronary thrombosis and Pl^{A2} polymorphism (Weiss *et al*, 1996). These papers generated great interest and have given rise to various reports on this and related matters, some of which support the original findings (Carter *et al*, 1997; Walter *et al*, 1997; Zotz *et al*, 1998; Garcia-Ribes *et al*, 1998; Pastinen *et al*, 1998; Kastrati *et al*, 1999; Zotz *et al* 2000) and some of which do not (Hato *et al*, 1997; Herrmann *et al*, 1997; Ridker *et al*, 1997; Samani & Lodwick, 1997; Durante-Mangoni *et al*, 1998; Garg *et al*, 1998; Mamotte *et al*, 1998; Scaglione *et al*, 1998; Sperr *et al*, 1998; Laule *et al*, 1999). Recently, Gardemann *et al* (1998) established an association between Pl^{A2} polymorphism and coronary disease in a large population. However, they did not find a relationship between this genotype and myocardial infarction. This polymorphism was also investigated in relation to stroke and conflicting results have also been reported (Carlsson *et al*, 1997; Carter *et al*, 1998; Wagner *et al*, 1998).

Even though there is strong evidence that the complex $\alpha_{IIb}\beta_3$ has a primary role in the pathogenesis of acute coronary syndromes (Kleiman *et al*, 1998), the role of the Pl^{A2} polymorphism as a thrombotic risk factor is undefined and the mechanism(s) whereby this polymorphism could lead to an increased risk of thrombosis is unknown. To date,

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there is little evidence of an association of Pl^{A2} with molecular markers of abnormal haemostasis: an association between this polymorphism and higher plasma Lp(a) concentration (Joven *et al*, 1998), and a reduced plasma fibrinogen concentration (Senti *et al*, 1998) compared with Pl^{A1} subjects have been reported. This may indicate that this polymorphism plays a role in platelet post-activation events, possibly leading to increased binding of fibrinogen (Goodall *et al*, 1999). However, this hypothesis appears to be negated by the finding of no effect on fibrinogen binding (Meiklejohn *et al*, 1999) or decreased fibrinogen binding to Pl^{A2} platelets (Weiss *et al*, 1997). Investigations of the possible effects of the $Pl^{A1/A2}$ polymorphism on human platelet function *in vitro* have reported contradictory results: Feng *et al* (1999) described an increased aggregability of the Pl^{A2} platelets to epinephrine and ADP, whereas Corral *et al* (1997) reported no difference in platelet aggregation between Pl^{A1} and Pl^{A2} genotypes, and Lasne *et al* (1997) described a hypo-aggregability of Pl^{A2} platelets, compared with Pl^{A1} platelets, to thrombin and ADP.

No data have been published on the possible effect of $Pl^{A1/A2}$ genotypes on platelet adhesion, one of the earlier events by which platelets perform their functions in the haemostatic process. Therefore, further investigations are required to investigate the relationship between this polymorphism of the fibrinogen receptor and the various functions of platelets that can be assessed *in vitro*.

The objective of the present report was to evaluate possible modifications of platelet adhesion to fibrinogen and of aggregation, using stimulants that act through different activation pathways, in subjects with different Pl^{A} alleles. The starting hypothesis was that the presence of the Pl^{A2} allele was associated with a tendency to higher basal (resting) adhesion and/or higher responsiveness to agonists. We have tested this hypothesis in a well defined group of healthy volunteers.

PATIENTS AND METHODS

Subjects. A total of 63 healthy volunteers (35 men and 28 women), all non-smokers, aged from 25 years to 62 years, were studied. The participants had not taken any drugs for at least 3 weeks and were carefully interviewed at the time they donated blood to exclude known risk factors affecting platelet function and/or platelet dysfunction. All volunteers participating in this study had given their informed, written consent, according to Italian law. Blood samples were collected after overnight fasting and without venous occlusion, to avoid stasis and anoxia.

For the purpose of the Pl^{A} genotyping, 4 ml of venous blood was collected into EDTA-containing vacutainer tubes. DNA was extracted from whole blood using a standard extraction protocol (Sambrook *et al*, 1989). Pl^{A} genotypes were determined as previously described (Weiss *et al*, 1996). The sense oligonucleotide primer was 5′-TGGACTTCTCTTTGGGCTCCTGACTTAC-3′ and the antisense primer was 5′-CGATGGATTCTGGGGCACAGTTATC-3′ (M-Medical Srl) (Osborn *et al*, 1996). Template DNA was amplified in a final volume of 100 μl containing 20 pmol of each primer, 200 μmol/l of each dNTP, 2 U of *Taq* polymerase (Bioline), 50 mmol/l KCl, 1·5 mmol/l $MgCl_2$ and 10 mmol/l Tris-HCl (pH 8·3). After initial denaturation at 95°C for 2 min, amplification of DNA was performed in a thermal cycler (Perkin Elmer Cetus) for 39 cycles (denaturation: 2 min at 95°C; annealing: 1 min at 58°C; extension: 2 min at 72°C). Amplified DNA was digested by 20 U *Msp*I (16 h at 37°C). The digested products of the Pl^{A1} and Pl^{A2} alleles were electrophoresed in 4% agarose gels and visualized by ethidium bromide staining. As the negative internal control, the polymerase chain reaction (PCR) procedure was also performed utilizing amplification products in samples containing no genomic DNA. The results of the genotyping assay were analysed by at least two investigators who were unaware of the origin of the DNA. Whenever there was any ambiguity in PCR, *Msp*I digestion and scoring were repeated.

Platelet isolation and in vitro functional testing. For the aggregation and adhesion tests, platelets were harvested from blood by differential centrifugation (Andrioli *et al*, 1999) and utilized within 1 h. A final volume of 20 ml of blood was drawn by venepuncture in 3·32 ml of anticoagulant solution (11·8 mmol/l citric acid, 18 mmol/l dextrose, 14 mmol/l sodium citrate) and platelet-rich plasma was obtained by centrifugation at 300 ***g*** for 10 min. The platelet-rich plasma was recentrifuged at 700 ***g*** for 15 min and platelets were gently suspended (2×10^8 platelets/ml for aggregation test and 5×10^7 platelets/ml for adhesion test) in a buffer composed of 145 mmol/l NaCl, 5 mmol/l KCl, 10 mmol/l HEPES, 0·5 mmol/l Na_2HPO_4, 6 mmol/l glucose and 0·2% human serum albumin, pH 7·4 (buffer A). The platelet suspensions were kept at room temperature and utilized within 1 h. Ten minutes before use, platelets were warmed to 37°C.

A colorimetric procedure measuring the activity of acid phosphatase was used for the determination of the adhesion of human platelets to fibrinogen-coated culture microplates (Bellavite *et al*, 1994). Briefly, 96-well microtitre plates were coated overnight with 0·2 mg/ml human fibrinogen (Sigma Chemical Company) in phosphate-buffered saline (PBS) and washed twice with physiological saline. Immediately after coating and washing, the wells were supplemented with 25 μl of buffer A containing 3 mmol/l $CaCl_2$ and 3 mmol/l of $MgSO_4$ (for assay of unstimulated cells) or with 25 μl of the tested agonists (final concentrations: 3 μmol/l ADP, 0·02 U/ml thrombin, 5 μmol/l arachidonic acid, 100 nmol/l U46619, a thromboxane A_2 analogue) in buffer A containing 3 mmol/l $CaCl_2$ and 3 mmol/l $MgSO_4$. Plates were then warmed to 37°C and 50 μl of the platelet suspension ($2·5 \times 10^6$ platelets), prewarmed to 37°C, was added to each well. The incubation was carried out for 60 min and plates were then subjected to two washing cycles with PBS at room temperature. After washing, the wells containing adherent platelets were rapidly supplemented with 150 μl of 100 mmol/l citrate buffer, pH 5·4, containing 5 mmol/l *p*-nitrophenyl-phosphate and 0·1% Triton X-100. After incubation at room temperature for 60 min, the reaction was stopped and the colour was developed by the addition of 100 μl/well of 2 N NaOH. The

Table I. General characteristics of the studied subjects. Data are means ± SD (ranges for age only).

	$Pl^{A1/A1}$	$Pl^{A1/A2}$ and $Pl^{A2/A2}$
Sex (male/female)	26/23	11/3
Age (years)	46·6 ± 4·8 (25–62)	48·2 ± 5·3 (27–59)
Body mass index (kg/m^2)	23·7 ± 1·1	24·1 ± 1·0
Blood pressure (mmHg)	119·7 ± 7·9/71·5 ± 6·5	122·4 ± 8·3/74·6 ± 5·0
Glucose (mmol/l)	4·02 ± 0·35	4·21 ± 0·29
Cholesterol (mmol/l)	4·72 ± 0·12	4·63 ± 0·16
HDL cholesterol (mmol/l)	1·01 ± 0·21	1·08 ± 0·26
LDL cholesterol (mmol/l)	3·22 ± 0·31	3·13 ± 0·27
Triglycerides (mmol/l)	1·54 ± 0·30	1·48 ± 0·24
Fibrinogen (g/l)	2·78 ± 0·39	3·01 ± 0·51
Platelet count (x 10^9/l)	237 ± 40	248 ± 31

p-nitrophenol produced by the reaction was measured with a microplate reader (Reader 400, SLT Labs Instruments) at 405 nm against a platelet-free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with a defined number of platelets from the same donor.

The rate of platelet aggregation was measured according to Born (1962) by using a four-channel aggregometer (Aggrecorder II PA-3220, Daiichi). Suspensions of washed platelets (0·5 ml, 10^8 platelets) were warmed in the aggregometer at 37°C for 1 min in buffer A, in the presence of 1 mmol/l $CaCl_2$ and 1 mmol/l $MgSO_4$, with continuous stirring at 1000 r.p.m. The rate of platelet aggregation was monitored for 3 min after the addition of the agonists (10 μmol/l ADP, 0·06 U/ml thrombin, 1 μmol/l U-46619 and 15 μmol/l arachidonic acid) and was expressed as the percentage change of transmitted light.

Flow cytometry. Measurements of the expression of CD41 (complex $\alpha_{IIb}\beta_3$) and CD62P (GMP-140) in the platelets were performed by fluorescence-activated flow cytometry, as previously described (Andrioli *et al*, 1996). Immediately after collection, whole blood samples were diluted with cold (4°C) buffer A ($2·5 \times 10^5$ platelets/ml) and incubated with saturating concentrations of either anti-human CD41 monoclonal antibody (mAb) conjugated with fluoroscein isothiocyanate (FITC) and anti-human CD62P mAb (phycoerythrin, PE) or fluoresceinated and phycoerythrinated control immunoglobulin G_1 mAbs (Immunotech). The anti-human CD41 mAb is produced from clone P2 (hybridoma SP2/O-Ag.14 myeloma X Balb/C spleen cells) and reacts with the subunits α_{IIb} and β_3 in complex-inhibiting platelet aggregation induced by thrombin, collagen and ADP. The anti-human CD62P mAb is produced from clone CLB-thromb/6 (hybridoma SP2/O × Ag 1·4 × Balb/c × AJ spleen cells) and recognizes the GMP-140 expressed on the activated platelets. Incubation with mAbs was carried out for 20 min at 4°C. This temperature was selected on the basis of preliminary experiments showing a minimal platelet activation under this condition (percentage of $CD62P^+$ platelets with incubation at 4°C: 3·8 ± 0·9; percentage of $CD62P^+$ platelets with incubation at room temperature: 5·4 ± 1·1; $n = 6$). Finally, the samples were analysed immediately by a FACScan flow cytometer (Becton Dickinson) equipped with a 5 W argon laser and operated at 200 mW power at a wavelength of 488 nm. The instrument was aligned daily with 2-μm Calibrite beads (Becton Dickinson) to calibrate the light-scattering and fluorescence paramenters. Fluorescein fluorescence was detected using a 530/30 mm band pass filter and phycoerythrin fluorescence was detected with a 585/42 mm filter (Becton Dickinson). Samples were passed through the laser beam through a 70-μm nozzle at a flow rate of 500–800 blood cells/s.

β-Thromboglobulin determination. The quantitative determination of β-thromboglobulin was performed by an enzyme-immunoassay (Boehringer Mannheim). For this test, blood was collected in chilled tubes containing acid citrate dextrose and processed immediately for removal of platelets by high-speed centrifugation.

Statistical analysis. The Mann–Whitney rank sum test was used for the analysis of non-parametrically distributed data. The statistical analysis was performed using a computer program (SIGMASTAT 2·0, SPSS). Data were expressed as means ± SD. Statistical significance and the IC_{50} were calculated by a computer program (GRAPHPAD INPLOT). $P < 0·05$ was considered statistically significant.

RESULTS

Population characteristics

Sixty-three subjects, 35 men and 28 women, with a mean age of 47 years (range 25–62 years), were studied. In this group, we found 49 subjects with $Pl^{A1/A1}$ polymorphism (77·8%), 12 subjects with $Pl^{A1/A2}$ polymorphism (19%) and two subjects with $Pl^{A2/A2}$ polymorphism (3·2%). Owing to the small number of $Pl^{A2/A2}$ subjects, they were grouped together with the $Pl^{A1/A2}$ subjects and all these 14 subjects are referred to as Pl^{A2} subjects. The most relevant characteristics of the volunteers and their plasma parameters are summarized in Table I. As shown, no significant differences were observed between the Pl^{A1} and Pl^{A2} subjects. Metabolic indices and body mass were remarkably similar in the two groups. There was a difference in sex distribution. However, platelet activation did not differ

Table II. Pl^{A1} and Pl^{A2} platelet adhesion to fibrinogen and aggregation induced by various stimuli. Data are expressed as means ± SD.

	Resting	Thrombin	ADP	Arachidonic acid	U46619
Adhesion					
Pl^{A1}	4·9 ± 1·6	20·0 ± 3·8	16·1 ± 1·9	18·5 ± 1·4	18·3 ± 1·7
Pl^{A2}	4·8 ± 1·8	18·8 ± 1·8	16·0 ± 1·4	13·5 ± 2·9	16·0 ± 1·2
p	NS	NS	NS	< 0·003	< 0·005
Aggregation					
Pl^{A1}	–	76·3 ± 3·4	56·6 ± 8·3	65·2 ± 12·7	68·5 ± 9·4
Pl^{A2}	–	77·4 ± 2·6	57·2 ± 5·0	28·4 ± 17·2	37·9 ± 23·1
p	NS	NS	NS	< 0·01	< 0·01

between men and women in both groups. The clinical interviews and these baseline parameters excluded the presence in our studied subjects of the most known cardiovascular risk factors (smoking, hypertension, diabetes, hyperlipidaemia, increased plasma fibrinogen levels) associated with modified platelet activity.

Platelet adhesion and aggregation

The platelet adhesion on fibrinogen in the resting state and after different stimuli was tested *in vitro* under static conditions. We found no differences between the Pl^{A1} and the Pl^{A2} genotypes in resting adhesion and after 3 μmol/l ADP or 0·02 U/ml thrombin stimulation (Table II). On the other hand, using 5 μmol/l arachidonic acid or 100 nmol/l U46619 as agonists, the Pl^{A2} platelets showed a significantly reduced adhesion compared with the Pl^{A1} platelets. Platelet aggregation (Table II) was also performed with different stimuli: 0·06 U/ml thrombin, 10 μmol ADP, 15 μmol arachidonic acid and 1 μmol U46619. No differences were found between Pl^{A1} and Pl^{A2} platelet aggregation using thrombin or ADP as stimulants. Pl^{A2} platelets aggregated with a significantly reduced rate when compared with Pl^{A1} platelets using arachidonic acid or U46619.

The results of aggregation tests in response to arachidonic acid and to U46619 performed in each donor are reported in Fig 1. Two Pl^{A2} subjects were non-responders to arachidonic acid and to U46619, and four Pl^{A2} subjects were markedly low-responders (< 30% aggregation). There was a good correspondence in the aggregation responses to the two different agonists in the different individuals, with the exception of one $Pl^{A1/A1}$ subject, whose platelets showed

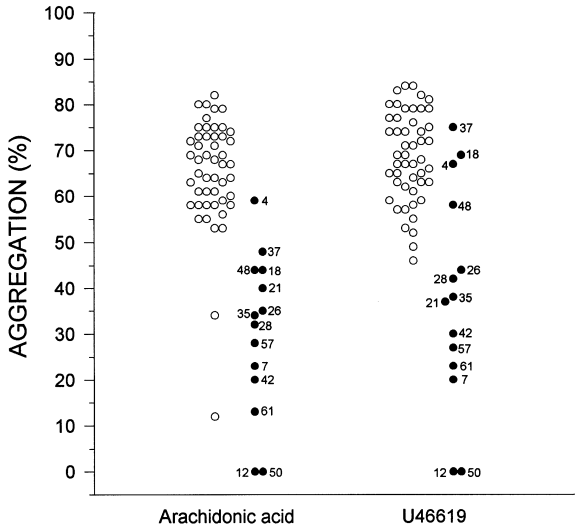

Fig 1. Distributions of Pl^{A1} (○) and Pl^{A2} (●) platelet aggregation to arachidonic acid and U46619. Each Pl^{A2} subject is identified by their identification number, in order to compare the response to the two agonists. Subjects numbers 12 and 61 were homozygous for the Pl^{A2} trait.

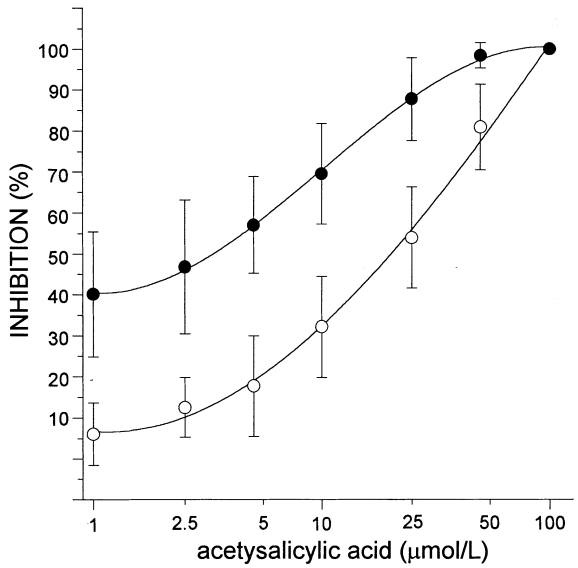

Fig 2. Effects of acetylsalicylic acid on arachidonic acid-induced platelet aggrgegation. After the incubation for 15 min of the platelet suspensions with scalar doses of acetylsalicylic acid, the aggregation test was performed as described in the Patients and methods section, using 15 μmol/l arachidonic acid. This series of assays included eight randomly chosen Pl^{A1} subjects (○) and the eight Pl^{A2} subjects (●) who showed normal or borderline responses to arachidonic acid in the absence of acetylsalicylic acid (subjects 4, 18, 21, 26, 28, 35, 37, 48 in Fig 1). Data are expressed as means ± SD.

a very low response to arachidonic acid (12%) and a normal response to U46619. Subjects numbers 12 and 61 were homozygous for the Pl^{A2} allele; probably because of the small number of subjects, this analysis did not discriminate $Pl^{A1/A2}$ from $Pl^{A2/A2}$ platelets. Figure 1 also shows that six out of seven subjects with markedly defective aggregation by arachidonic acid and six out of six subjects with markedly defective aggregation by U46619 belonged to the Pl^{A2} group.

Sensitivity of Pl^{A1} and Pl^{A2} platelets to acetylsalicylic acid

To investigate the contribution of the endogenous generation of thromboxane A_2 in subjects with different Pl^{A1} and Pl^{A2} polymorphisms, platelet aggregation in response to arachidonic acid was tested after incubation with increasing doses of acetylsalicylic acid. For technical reasons, this comparison could be performed only in Pl^{A1} and Pl^{A2} subjects that showed normal or near normal aggregation. Therefore, the six Pl^{A2} subjects with markedly reduced ($< 30\%$) aggregation responses were excluded from this series of experiments. Dose–effect curves (Fig 2) showed that Pl^{A2} platelets are significantly more sensitive to inhibition by acetylsalicylic acid than Pl^{A1} platelets. The concentration of the drug able to reduce the aggregation response by 50% was $2{\cdot}7 \pm 0{\cdot}6$ μmol/l and $23{\cdot}4 \pm 3{\cdot}3$ μmol/l for Pl^{A2} and Pl^{A1} platelets respectively ($P < 0{\cdot}005$).

Flow cytometric platelet evaluation and β-thromboglobulin determination

To evaluate a possible different activated state of Pl^{A2} platelets with respect to Pl^{A1} platelets *ex vivo*, we studied the CD41 and CD62P expression on platelet membranes and determined the plasma β-thromboglobulin levels of Pl^{A2} vs. Pl^{A1} groups. The expression of the selected platelet antigens did not differ significantly in the two groups (% of $CD41^+$ platelets: Pl^{A1} 68 ± 4, Pl^{A2} 70 ± 5, NS; % of $CD62P^+$ platelets: Pl^{A1}: $4 \pm 1\%$, Pl^{A2}: 5 ± 2, NS). There was also no difference between the plasma β-thromboglobulin levels of the two groups (Pl^{A1} $26{\cdot}5 \pm 1{\cdot}8$ ng/ml Pl^{A2} subject $29{\cdot}2 \pm 2{\cdot}2$ ng/ml *p* NS).

DISCUSSION

Integrins containing a β_3 subunit include $\alpha_{IIb}\beta_3$, the most abundant and functionally important integrin on platelets, and $\alpha_V\beta_3$, which is prominently expressed on endothelial cells, monocytes/macrophages and vascular smooth muscle cells. The glycoprotein $\alpha_{IIb}\beta_3$ is the major fibrinogen receptor of the human platelet membrane and is activated after cell stimulation by a number of agonists, thus participating in adhesion and aggregation. The interest in this protein in clinical pathology was further increased when an association between the myocardial infarction with the Pl^{A2} allele of the $Pl^{A1/A2}$ β_3 gene polymorphism was reported (Weiss *et al*, 1996). However, the relationship between this molecular variance, cellular functions and pathological effects remains to be clarified.

The design of this *in vitro* study did not enable us to define whether or not the Pl^{A2} genotype is clinically relevant, because all the subjects of the population under study were of healthy status. However, our data may allow better understanding of the molecular pathophysiology of cardiovascular risk in relation to the Pl^A phenotype.

In the present study, we analysed whether β_3 genotypes have any association with two major platelet functions, i.e. adhesive and aggregative reactions. Using two different tests of platelet function and four different agonists, we provided the new finding that an abnormal platelet response to thromboxane A_2 (either exogenously or endogenously generated from arachidonic acid) exists in Pl^{A2} subjects. Thus, not only the hypothesis of a hyper-responsiveness of platelets as a possible mechanism for increased cardiovascular risk in these subjects was rejected, but a paradoxical defect in these cells was identified.

There are several possible explanations for the agonist specificity of such a defect. The most direct explanation points to the existence of (at least) two molecular 'switches' by which β_3 integrin is involved in platelet adhesion and aggregation: one modification, involving amino acid position n 1565 at exon 2, is critical for activation directly by thromboxane A_2 and indirectly by arachidonic acid, while the Pl^A polymorphism is not critical for activation by ADP and thrombin. This hypothesis is in agreement with the current knowledge indicating that different transduction pathways, proximal to the fibrinogen receptor, are utilized by different agonists (Du & Ginsberg, 1997). According to this model, the finding of increased sensitivity to acetylsalicylic acid may simply indicate that the drug unmasks a latent defect by reducing the endogenous generation of thromboxane A_2 and, thus, the β_3 integrin modification by this pathway. However, because of the complexity of fibrinogen-receptor function, it cannot be excluded that the indicated molecular modification of β_3 integrin may affect other signalling pathways involved in cellular activation. The involvement of the fibrinogen receptor in outside-in activation or amplification of signals has been already suggested (Shattil *et al*, 1997).

A possible explaination of the decrease of Pl^{A2} platelet response to thromboxane A_2 could be the result of a desensitization of the thromboxane A_2 receptor following activation *in vivo*. According to this hypothesis, the Pl^{A2} platelets could be activated *in vivo* and subsequently become hypo-responsive. However, from our data obtained by flow-cytometric analysis and on determination of β-thromboglobulin plasma levels, two investigations of possible platelet activation *in vivo*, we did not determine any up-regulation of Pl^{A2} platelets with respect to Pl^{A1} platelets.

From our data obtained by *in vitro* assay of platelet function and by *ex vivo* assessment of platelet activation markers, it seems that the suggested thrombogenic effect of the Pl^{A2} polymorphism (Weiss *et al*, 1996; Carter *et al*, 1997; Walter *et al*, 1997; Garcia-Ribes *et al*, 1998; Pastinen *et al*, 1998; Zotz *et al*, 1998; Kastrati *et al*, 1999) is not related to platelet hyper-activity. This consideration is in accordance with others authors (Lasne *et al*, 1997). However, we did not observe a platelet hypo-reactivity to thrombin or ADP as described by Lasne *et al* (1997). This

discrepancy could be related to different experimental conditions (we studied washed platelets, Lasne and colleagues studied platelet-rich plasma). Basically, it cannot be excluded that the Pl^{A2} genotype is linked to another yet unidentified genetic marker, which is the true risk determinant, or to other platelet disorders, where a defect of the thromboxane A_2 receptor has been described (Higuchi *et al*, 1999). Further studies are necessary to clarify this point. It cannot be ruled out that other cellular sources than platelets are being affected, as the β_3 subunit is part of the vitronectin receptor which is expressed in endothelial cells, smooth muscle cells and a number of other cells. As this integrin receptor ($\alpha_V\beta_3$) is highly involved in angiogenesis, polymorphism of glycoprotein β_3 has also to be considered in relation to the pathophysiology of cardiac vasculature. It is known that the integrin $\alpha_V\beta_3$ could mediate platelet adhesion to the activated endothelial cells (Gawaz *et al*, 1997). More recently, in an animal model, an anti-thrombotic role for vitronectin was reported (Fay *et al*, 1999). This effect seems to be mediated by inhibiting platelet–platelet interactions and/or thrombin procoagulant activity. If these data are confirmed in humans, anomalous functioning of the anti-thrombotic role of the vitronectin receptor could be advanced to explain the role of Pl^{A2} as a cardiovascular risk factor.

Because of the small sample size of the Pl^{A2} subjects considered in this work ($n = 14$), the statistical power of the present study is low. This limitation of the study does not affect the finding of differences which are statistically significant (i.e. platelet responses to arachidonic acid and U46619). On the other hand, the negative findings (i.e. platelet responses in the resting condition or to ADP or thrombin) do not rule out the possibility that some small difference may actually exist. However, the differences between the non-significant values of the Pl^{A2} *and* Pl^{A1} groups are so small that they probably do not have a relevance at the clinical level.

The differences in dose–inhibition curves with acetylsalicylic acid are in accordance with a recent letter, Cooke *et al* (1998), showing a greater sensitivity to acetylsalicylic acid in $Pl^{A1/A2}$ platelets than in $Pl^{A1/A1}$ platelets. These authors suggested that differences in exposure of patients to the drug at the time of onset of myocardial infarction might account for some of the discrepancies amongst studies on Pl^{A} as a risk factor for ischaemic coronary events.

In conclusion, our findings are not consistent with the hypothesis that the purported increase of cardiovascular risk in these subjects may be as a result of fibrinogen receptor hyper-activation. On the contrary, the Pl^{A2} allele is associated with a platelet functional deficiency, specifically linked to the activation of the fibrinogen receptor by thromboxane A_2.

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