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Platelet receptor gain-of-function single nucleotide polymorphisms in carotid and vertebral stenosis patients

Andrea Kopp Lugli · Martin M. Brown · Jan Steffel ·
Linda Büchi · Dorothee Förnzler · Annabelle Dupont ·
Pascale Gaussem · Marc Forestier · Juerg H. Beer

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Abstract The role of platelet receptor gain-of-function single nucleotide polymorphisms (SNP) in cardiovascular disease is controversial. We hypothesised that certain SNPs may accelerate the development of carotid artery stenosis. The intronic PAR-1 receptor intervening sequence-14 A/T (IVSn-14 A/T) polymorphism and three additional platelet

receptor polymorphisms, i.e. GPIa (807C/T), GPIb α (5T/C) and HPA-1a/HPA-1b (PI (A1/A2)) of GPIIIa were studied. The interaction of SNPs with conventional risk factors including male gender, hypertension, high cholesterol, diabetes, advanced age and smoking were investigated. The hypothesis was tested in 114 well-characterised patients with symptomatic carotid or vertebral stenosis from the British CAVATAS population and compared the results with 97 unrelated controls. The allele frequency of the platelet gain-of-function SNP was not significantly different in the CAVATAS population as compared to controls (PAR-1A/T ($P = 0.13$), GPIa C/T ($P = 0.25$), GPIIIa HPA-1a/HPA-1b (PIA1/A2) ($P = 0.66$) and GPIb T/C ($P = 0.20$)). In the subgroup of smokers, however, the prothrombotic GPIb α C mutated allele was found in a significantly higher frequency in the patient as compared to the control group ($P = 0.04$). Contrary to the primary hypothesis, the PAR-1A/T SNP as well as the other SNPs tested were not over- or underrepresented in the CAVATAS population. However, a significantly increased prevalence of GPIb- α (5C/T) was found in the subgroup of smokers and may represent an important cofactor in this patient group of our hypothesis-generating study.

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A. Kopp Lugli · L. Büchi · M. Forestier · J. H. Beer (✉)
Department of Medicine, Cantonal Hospital Baden, 5404 Baden,
Switzerland
e-mail: hansjuerg.beer@ksb.ch

A. Kopp Lugli
Department of Anesthesia, University Hospital, Basel,
Switzerland

M. M. Brown
Stroke Research Group, University College of London Institute
of Neurology, London, UK

J. Steffel
Department of Cardiology, University Hospital, Zurich,
Switzerland

D. Förnzler
Hoffmann-La Roche, Basel, Switzerland

A. Dupont · P. Gaussem
Université Paris Descartes, AP-HP, Hôpital Européen Georges
Pompidou, INSERM, Unité 765, Paris, France

J. H. Beer
Department of Medicine, University Hospital Berne, Bern,
Switzerland

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Introduction

Platelet receptor polymorphisms that result in a “gain-of-function” in platelet adhesion and/or aggregation due to increased receptor density or affinity and increased platelet activation may place carriers at increased risk of vascular events and accelerate atherogenesis. Indeed, platelet adhesion to dysfunctional endothelium and activation with subsequent degranulation are important elements of the

formation and kinetics of atherosclerotic lesions [1], which may be accelerated by gain-of-function platelet polymorphisms. Several such mutations have been described, but their contribution to clinical events remains controversial [2–7].

On this basis, we hypothesised that platelet gain-of-function polymorphisms may predispose carriers to an accelerated development of atherosclerosis, especially in combination with exogenous risk factors.

Thrombin is known to increase vascular smooth muscle cell (VSMC) proliferation and migration (i.e. crucial mechanisms of atherosclerosis and neointima formation), and PAR-1 is widely expressed in VSMC in human atheromas indicating a role of this receptor in atherogenesis [8–11]. Therefore, our primary hypothesis was that the intronic PAR-1 receptor intervening sequence-14 A/T (IVSn-14 A/T) polymorphism would enhance local thrombin growth factor effects and accelerate the development of atherosclerotic lesions. So far, the association of this mutation with clinical events has not been investigated [12].

Three additional gain-of-function platelet-receptor single nucleotide polymorphisms (SNPs) of GPIb α (5T/C), GPIa (807C/T) and GPIIIa (HPA-1a/HPA-1b (also called PIA1/A2)) served as secondary endpoints, which have been demonstrated to increase clinical events, likely due to their gain-of-function mechanisms [4, 12–15] in addition to their interaction with behavioural risk factors. The GPIb α (5T/C) SNP was of particular interest, since the A1 binding site of von Willebrand factor (vWF) may be activated to bind platelet GPIb α under high shear stress, as it occurs in stenotic atherosclerotic lesions [16–19]. So far, clinical studies investigating the implications of the GPIb α (5T/C) polymorphism have yielded controversial results [4–7, 20]. Of particular interest was the combination of these polymorphisms with smoking (an acquired risk factor) as earlier studies have reported a strong association of smoking and platelet SNP with clinical events [21, 22].

Materials and methods

Study population

In the carotid and vertebral artery transluminal angioplasty study (CAVATAS), 504 patients with high grade carotid stenosis were randomised for endovascular versus surgical treatment [23]. Patients from this international multicentre study provide a well-characterised model population with a clearly defined carotid stenosis, in which gain-of-function polymorphisms could be correlated to the human situation of advanced atherosclerosis. After obtaining written informed consent, blood samples were taken from 114

CAVATAS patients from the British cohort in London and Sheffield [23]. The CAVATAS trial enrolled patients with carotid stenosis suitable for both carotid endarterectomy and endovascular treatment. Exclusion criteria included patients thought to be unsuitable for surgery because of medical or surgical risk factors (e.g., recent myocardial infarction, poorly controlled hypertension or diabetes mellitus, renal disease, respiratory failure, inaccessible carotid stenosis, or severe cervical spondylosis). Patients were also excluded if they were unable to give informed consent, or if they had a disabling stroke with no useful recovery of function within the region supplied by the treatable artery. There was no age limit. The available partners of the patients served as unrelated controls (97 samples). All controls had a negative history for myocardial infarction, angina, claudication, heart failure, stroke/transient ischemic attack. The study was approved by the local internal review board (IRB).

While designing this hypothesis generating study we were careful to follow the standards of genetic association studies in stroke proposed by Dichgans and Markus [24]. To assess and compare the allelic frequency of platelet receptor SNP in an additional large representative control sample of a healthy Caucasian population, blood from 979 healthy Swiss blood donors was analysed.

Detection of polymorphisms

The polymorphisms were determined by the kinetic thermocycling method (KTC) [25, 26]. The single nucleotide polymorphisms (SNP) for GPIa C807T and GPIIIa HPA-1a/HPA-1b were determined by the polymerase chain reaction and melting point analysis with fluorescent hybridisation probes by using the fluorescence energy transfer principle (LightCycler System, Roche, Basel, Switzerland).

Statistical analysis

We hypothesised that CAVATAS patients with carotid stenosis have a higher frequency of the prothrombotic AA (or lower TT)-SNP of PAR-1 (primary endpoint). As secondary endpoints, the association of the other three SNPs with carotid stenosis and their interaction with smoking were evaluated in an exploratory manner.

In our previous study [21] a relative risk of about 3.3 for patients with myocardial infarction who carry the collagen GPIa/IIa polymorphism (prevalence in the general population 5%) has been found. Coronary restenoses appear to occur in the presence of the GP IIb/IIIa polymorphism (prevalence 20%) at an odds ratio of 1.5–3 [27]. As for the Kozak polymorphism (GP1b) (allele frequency 8–17% in the general population) a conservative estimate may be in

the range of a RR of 2–3. On the basis of these previous studies, expected restenosis rates [21, 27] and allele frequencies in a total of 200 (GP IIIa and GPIa) or 250 (GPIb) patients respectively will achieve a power of 80% (α two-sided 0.05).

Differences in SNP frequencies were assessed using Fisher's exact test. Additionally, a multiple logistic regression model with forward selection was fitted with the four SNPs and was adjusted for other confounding risk factors including male gender, hypertension, high cholesterol, diabetes, advanced age and smoking (only subjects with complete data for gender, hypertension, cholesterol, diabetes, age and smoking were included in this analysis). A significance level of 0.1 was used for the forward selection. For all other tests, a significance level of 0.05 was used. Statistical analyses were conducted using SAS System 9.1 (SAS Institute, Cary, NC, USA). The study was planned and conducted according the previously proposed standard criteria [24].

Results

Baseline characteristics and distribution of cardiovascular risk factors are summarised in Table 1. As expected, the prevalence of the cardiovascular risk factors: male gender ($P = 0.006$), hypertension ($P < 0.0001$), high cholesterol ($P = 0.004$), advanced age ($P < 0.0001$) and smoking ($P = 0.014$) were higher in the patient group as compared to controls.

The results of the distribution of the individual point mutations are summarised in Table 2. The allele frequencies of the PAR-1 SNP were similar among the patient and control groups (Fisher's exact test, $P = 0.13$). Likewise, no significant difference was observed between the two groups with respect to GPIa ($P = 0.25$), GPIIIa ($P = 0.66$) and the GPIb α SNP (Fisher's exact test, $P = 0.20$).

The multiple logistic regression model with forward selection included the factors hypertension ($P < 0.0001$), age ($P < 0.0001$), sex ($P = 0.006$), cholesterol ($P = 0.004$) and smoking ($P = 0.014$). The odds ratio estimators were 4.89 (95% CI 2.17–11.58) for hypertension, 1.14 (1.08–1.20) for age, 4.54 (2.06–10.64) for male gender, 4.24 (1.87–10.22) for cholesterol, 2.58 (0.96–7.34) for smokers versus non-smokers, and 0.35 (0.10–1.13) for ex-smokers versus non-smokers. For example, an individual with hypertension has 4.89 times higher odds of being in the patient group than an individual without; likewise, or an individual who is 1 year older has 1.14 times higher odds to be in the patient group.

When patients were separated according to smoking status (i.e. non-smokers, ex-smokers and current smokers), the GPIb α mutation was overrepresented in the subgroup of

Table 1 Baseline characteristics and distribution of cardiovascular risk factors in CAVATAS patients and controls

	Patients ($n = 114$)	Controls ($n = 97$)	P -values
Gender			
Male	73 (64.0%)	40 (41.2%)	0.006
Female	33 (29.0%)	56 (57.7%)	
n/a	8 (7.0%)	1 (1.0%)	
Age			
Mean	71.5	63.3	<0.0001
n/a	8	1	
Smoking			
Current	25 (21.9%)	17 (17.5%)	0.014
Never	73 (64.0%)	53 (54.6%)	
Ex-smoker	8 (7.0%)	20 (20.6%)	
n/a	8 (7.0%)	7 (7.2%)	
Hypertension			
Yes	81 (71.1%)	39 (40.2%)	<0.0001
No	21 (18.4%)	52 (53.6%)	
n/a	12 (10.5%)	6 (6.2%)	
Diabetes			
Yes	11 (9.7%)	12 (12.4%)	n/s
No	89 (78.1%)	79 (81.4%)	
n/a	14 (12.3%)	6 (6.2%)	
High cholesterol			
Yes	68 (59.7%)	38 (39.2%)	0.004
No	28 (24.6%)	49 (50.5%)	
n/a	18 (15.8%)	10 (10.3%)	

n/a Not available, n/s not significant

current smokers ($P = 0.044$, Table 3). In this group, the GPIb α mutation was included in the multiple logistic regression model with forward selection. The odds ratio estimators were 11.83 (95% CI 1.30–231.65) for the GPIb α mutation, 4.59 (0.90–28.47) for dyslipidaemia, and 1.17 (1.05–1.35) for advanced age. Hence, an individual with the prothrombotic GPIb α (5C) allele is at 11.8 times higher odds to be in the patient group than an individual without this allele (in the subgroup of smokers). Analyses testing interaction between SNPs and the smoking status (including current smokers, ex-smokers and non-smokers) did not reveal any statistically significant result (Tables 4, 5).

According to previous published guidelines [24], we also tested if the marker genotypes were in Hardy–Weinberg equilibrium in CAVATAS patients and controls. In the CAVATAS group only PAR-1 Sn-14A/T was significant ($P = 0.04$), and in the control group only platelet GPIb α were significant ($P = 0.04$). Upon adjustment of the P -values for multiple statistical testing by Bonferroni correction, no P -value remains significant. Therefore, we may state that the marker genotypes are within a Hardy–Weinberg equilibrium [24, 28].

Table 2 Summary of the distribution of the four polymorphisms in the CAVATAS patient cohort and controls

	CAVATAS patients	Controls	<i>P</i> -value
PAR-1 Sn-14<u>A</u>/T			
AA	83 (72.8%)	68 (70.1%)	
AT	25 (21.9%)	28 (28.9%)	
TT	6 (5.3%)	1 (1.0%)	
Total number	114	97	0.13
Odds ratio: 0.19	95% CI 0.02–1.59		
Platelet GpIb-α (5T/<u>C</u>)			
CC	0 (0.0%)	0 (0.0%)	
CT	23 (20.2%)	27 (27.8%)	
TT	91 (79.8%)	70 (72.2%)	
Total number	114	97	0.20
Odds ratio: 0.65	95% CI 0.35–1.24		
Platelet GpIIa 807 <u>C</u>/T			
CC	43 (37.7%)	43 (44.3%)	
CT	57 (50.0%)	36 (37.1%)	
TT	14 (12.3%)	18 (18.6%)	
Total number	114	97	0.25
Odds ratio: 1.63	95% CI 0.76–3.47		
Platelet GpIIIa (HPA-1a/HPA-1b; PI A₁/<u>A</u>₂)			
PI A2/A2	4 (3.5%)	3 (3.1%)	
PI A1/A2	36 (31.6%)	28 (28.9%)	
PI A1/A1	74 (64.9%)	66 (68.0%)	
Total number	114	97	0.66
Odds ratio: 1.15	95% CI 0.65–2.04		

The data collectively show no difference between the patients with carotid stenosis and the British controls. The underlined allele represents “the risk allele”

The calculated *P*-values are for risk allele versus no risk allele (e.g. in A): A is the risk allele therefore, AA and AT are tested versus TT

The overall frequency of the platelet receptor point mutations in the CAVATAS population and the respective controls was similar to a large representative control cohort of healthy Swiss blood donors (data not shown) suggesting that ethnicity did not play a role in these analyses.

Discussion

Contrary to our primary hypothesis, the PAR-1 (IVSn-14) SNP was not associated with symptomatic carotid stenosis. Furthermore, the three other “prothrombotic” platelet receptor SNPs were not overexpressed in patients. However, a significantly increased prevalence of GPIb- α (5C/T) was found in the subgroup of smokers, a typical exogenous atherogenic risk factor.

The study of gain-of-function polymorphisms is justified as they potentially increase the carrier’s risk of atherosclerosis and/or vascular events. Indeed, platelet adhesion

Table 3 Distribution of the four polymorphisms in the *subgroup of smokers*

	CAVATAS patients	Controls	<i>P</i> -value
PAR-1 Sn-14<u>A</u>/T			
AA	16 (66.7%)	10 (62.5%)	
AT	7 (29.2%)	6 (37.5%)	
TT	1 (4.2%)	0 (0%)	
Total number	24	16	<i>n/s</i>
Platelet GpIb-α (5T/<u>C</u>)			
CC	0 (0.0%)	0 (0.0%)	
CT	6 (25.0%)	2 (12.5%)	
TT	18 (75.0%)	14 (87.5%)	
Total number	24	16	0.044
Platelet GpIIa 807 <u>C</u>/T			
CC	5 (20.8%)	6 (37.5%)	
CT	16 (66.7%)	8 (50%)	
TT	3 (12.5%)	2 (12.5%)	
Total number	24	16	<i>n/s</i>
Platelet GpIIIa (HPA-1a/HPA-1b; PI A₁/<u>A</u>₂)			
PI A2/A2	0 (0.0%)	1 (6.3%)	
PI A1/A2	7 (29.2%)	6 (37.5%)	
PI A1/A1	17 (70.8%)	9 (56.3%)	
Total number	24	16	<i>n/s</i>

The data show an increased prevalence of the GPIb C allele in the CAVATAS patients as compared to the controls. The underlined allele represents “the risk allele”

n/s Not significant

to dysfunctional endothelium and activation with subsequent degranulation are important elements of atherosclerotic lesion formation [1]. Polymorphism in the Kozak sequence of GPIb α is a major determinant of mRNA translation and, thus, the amount of GPIb present on the platelet surface, which is increased by the presence of the C allele at the –5 position [4, 29]. This receptor and its interaction with vWF is of particular interest, because it is functional at the high shear rates found in arterial stenosis. Its inhibition results in reduced generation of platelet derived microparticles, thrombin generation and procoagulant activity at high shear forces [17, 18]. Furthermore, in vitro studies have shown that platelet membrane glycoprotein polymorphisms (including the Kozak sequence) influence experimental thrombus formation at typical shear rates [30]. So far, clinical studies investigating the implications of the GPIb α (5T/C) polymorphism have yielded controversial results. In 219 cases of first-ever ischemic stroke, presence of the C allele (as evidenced by an increased frequency of the T/C genotype) was overrepresented in the stroke group as compared to healthy controls even after adjusting for conventional risk factors [4]. In a study of 1000 patients with angiographically confirmed coronary artery disease, the GPIb α (5C) allele represented a

Table 4 Distribution of the four polymorphisms in the *subgroup of ex-smokers*

	CAVATAS patients	Controls	P-value
PAR-1 Sn-14<u>A</u>/T			
AA	7 (87.5%)	13 (72.2%)	
AT	1 (12.5%)	5 (27.8%)	
TT	0 (0.0%)	0 (0.0%)	
Total number	8	18	n/s
Platelet GpIb-α (5T/<u>C</u>)			
CC	0 (0.0%)	0 (0.0%)	
CT	1 (12.5%)	6 (33.3%)	
TT	7 (87.5%)	12 (66.6%)	
Total number	8	18	n/s
Platelet GpIa 807 <u>C</u>/T			
CC	5 (62.5%)	10 (55.6%)	
CT	3 (37.5%)	4 (22.2%)	
TT	0 (0.0%)	4 (22.2%)	
Total number	8	18	n/s
Platelet GpIIIa (HPA-1a/HPA-1b; PI A₁/<u>A</u>₂)			
PI A ₂ /A ₂	1 (12.5%)	1 (5.6%)	
PI A ₁ /A ₂	2 (25.0%)	5 (27.8%)	
PI A ₁ /A ₁	5 (62.5%)	12 (66.7%)	
Total number	8	18	n/s

The underlined allele represents “the risk allele”

n/s Not significant

risk factor for thrombotic events [5]. Similarly, in a multicentre case–control study, homozygous C/C genotype carriers had a 3.5-fold increased risk for ischaemic cerebrovascular events as compared to T/T or T/C genotype carriers together (independent of atherosclerotic risk factors), indicating a role of this allele in arterial thrombosis [6]. On the other hand, young women with the C allele of the GPIb α polymorphism do not appear to be at increased risk of myocardial infarction or non-fatal stroke, even in the subgroup of smokers [7]. The discrepancies between these studies may be accounted for by different patient selection criteria and studied outcomes, population size, age and other factors. A recent meta-analysis indicated a strong association of the –5T/C polymorphism with the risk of ischaemic stroke. However, the direction of association was highly variable, suggesting that despite the presence of an association signal the true causative locus is perhaps not the Kozak –5T/C sequence itself but rather a gene region nearby [20].

The interaction of the polymorphisms in smokers was of particular interest, as we have previously demonstrated the association of two silent and linked polymorphisms (807C/T and 873G/A) within the GPIa gene with the risk of myocardial infarction, which was strongest in the subgroup

Table 5 Distribution of the four polymorphisms in the *subgroup of non-smokers*

	CAVATAS patients	Controls	P-value
PAR-1 Sn-14<u>A</u>/T			
AA	43 (71.7%)	36 (69.2%)	
AT	12 (20.0%)	15 (28.9%)	
TT	5 (8.3%)	1 (1.9%)	
Total number	60	52	n/s
Platelet GpIb-α (5T/<u>C</u>)			
CC	0 (0.0%)	0 (0.0%)	
CT	11 (18.3%)	16 (30.7%)	
TT	49 (81.7%)	36 (69.2%)	
Total number	60	52	n/s
Platelet GpIa 807 <u>C</u>/T			
CC	26 (43.3%)	24 (46.2%)	
CT	25 (41.7%)	18 (34.6%)	
TT	9 (15.0%)	10 (19.2%)	
Total number	60	52	n/s
Platelet GpIIIa (HPA-1a/HPA-1b; PI A₁/<u>A</u>₂)			
PI A ₂ /A ₂	2 (3.3%)	1 (1.9%)	
PI A ₁ /A ₂	22 (36.7%)	14 (26.9%)	
PI A ₁ /A ₁	36 (60.0%)	37 (71.2%)	
Total number	60	52	n/s

The underlined allele represents “the risk allele”

n/s Not significant

of smokers [21]. In the same line, polymorphisms of the GPIIb/IIIa gene and smoking were shown to affect the risk of stroke (while the SNP in itself did not) [22]. Our observation of an increased frequency of the GPIb α -5C allele in smokers with established atherosclerosis is in agreement with and extends these previous findings, not only for the ischemic events but also for the preceding development of arterial stenoses. In our present study, the odds estimator of the effect of the GPIb α (5C) allele in this subgroup was 11.83 while it was only 4.59 for hypercholesterolemia, implying an even more important effect of the GPIb α mutation as compared to this well-established atherogenic risk factor. However, given the small subgroup in which this finding was detected ($\alpha = 0.05$, $\beta = 0.775$) the significant P-value should be used as hypothesis-generating finding for further analysis in a larger population with a matched pair control group.

An accelerated development of atherosclerotic lesions in patients carrying the –5C allele may potentially occur through an altered interaction of the GPIb–V–IX complex with vWF and, subsequently, an enhanced interaction of vWF with the (sub-) endothelium as proposed in Fig. 1. As the A1 binding site of vWF may be activated to bind platelet GPIb α under high shear stress by anchoring of the

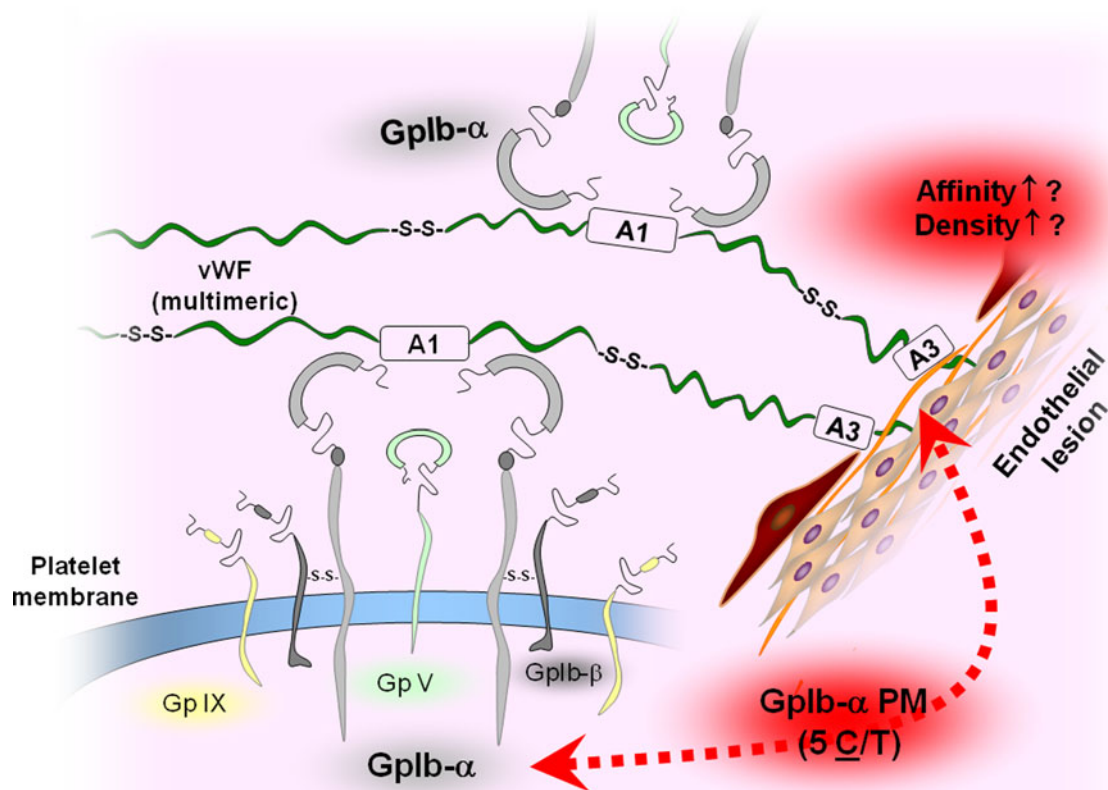


Fig. 1 Simplified overview of the interaction of GPIb-IX-V, von Willebrand Factor (vWF), and collagen. The GPIb-IX-V complex binds to the A1-binding site of vWF, while collagen, which may be exposed following endothelial injury, is bound to the A3 binding site

of vWF. Gain-of-function mutation of the GPIb-α chain may influence both the binding of GPIb-IX-V complex to vWF, as well as the interaction of vWF with the (sub-) endothelium (e.g. via an increased density and/or affinity of the collagen binding site of vWF)

downstream A3 domain to collagen [16], mutation of the GPIb α gene may, furthermore, confer an increased thrombotic risk especially in conditions with high shear forces, as they occur in high-grade atherosclerotic lesions. This could explain a potential risk for adverse events in carriers of the GPIb α (5C) mutation and in those who smoke. Platelet binding to activated endothelium and/or collagen at damaged sites will not only translate into platelet plug formation but also to recruitment of monocytes (e.g. by exposed p-selectins) and acceleration of plaque formation and arterial stenosis [8], as may be the case in the CAVATAS population of smokers.

Contrary to our initial hypothesis, mutations in the PAR-1 (IVSn-14), GPIa (807 C/T) and GPIIIa (HPA-1a/HPA-1b) gene were not overrepresented in patients with symptomatic carotid artery stenosis as compared to controls, which was also true for the subgroup analyses (data not shown). Indeed, while earlier studies implied an association of SNP in the GPIa [31] or the GPIIIa [13, 32] gene with clinical events, more recent data speak against this hypothesis [33–35], which is in accord with our data. To

our knowledge, our study is the first to investigate an association of the PAR-1 (IVSn-14) SNP with clinical events [12, 15]. However, our data do not support a role of this SNP in the development of atherosclerotic carotid artery lesions.

In summary, our data show that none of the SNPs studied are overrepresented in a general cohort of patients with symptomatic carotid artery stenosis. Thus, their roles as single atherogenic risk factors in the general population remains to be determined. Although our findings are “negative” in this regard, they nevertheless appear to be important, since publication bias towards “positive” findings can severely skew the perception of polymorphisms associated with clinical events as demonstrated in a recent large-scale meta-analysis of polymorphisms in coronary artery disease [36]. On the other hand, our analysis of smokers revealed a significantly increased prevalence for the GPIb-α (5C) allele in this subgroup (despite a relatively low number of patients), which is in accord with and extends earlier studies. Our data, nevertheless, point towards a role of the GPIb-α (5C) allele as a synergistic

“silent background risk factor”, which is functional at high shear stress and potentiated by a typical exogenous risk resulting in accelerated atherosclerotic lesion formation. As such, these findings may have important implications for the carriers of this mutation, especially in the presence of the additional exogenous and hence modifiable risk factor, smoking.

One weakness of our study is that the calculated number of patients was not achieved due to limitations of the time frame for sample size collection. Nevertheless, even considering this small patient number, the study setting detected an overrepresentation of GPIIb α in the subset of smokers. Given the exploratory nature of this study, our results should be viewed as hypothesis-generating. In view of the wealth of accumulating data, however, the synergy of arterial stenosis, high shear-favoured adhesion-polymorphism of GPIIb α and smoking resulting in an increased platelet/endothelial reactivity deserves further exploration.

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Conflict of interest The authors have no conflicts of interest to declare.

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