

## Platelets and Blood Cells

# Enhanced platelet activation by prolactin in patients with ischemic stroke

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### Summary

Prolactin and leptin are newly recognised platelet co-stimulators due to potentiation of ADP-induced platelet aggregation. Elevated leptin levels have recently been found to be a risk factor for ischemic stroke in both men and women, and especially in combination with increased blood pressure for hemorrhagic stroke in men. Until now an association between hyperprolactinemia and ischemic stroke has not been investigated systematically. We determined plasma prolactin and leptin levels as well as platelet P-selectin expression in 36 patients with ischemic stroke or transient ischemic attack and detected a significant correlation between increased prolactin values and enhanced ADP stimulated P-selectin expression on platelets. In contrast, no correlation of leptin values with platelet P-selectin expression was found. Next we determined plasma prolactin and leptin as well as acquired and congenital risk factors of thrombophilia in pa-

tients with first-ever non-hemorrhagic stroke with or without atrial fibrillation. Excluding patients with such preexisting risk factors, 21 patients with and 59 patients without atrial fibrillation were identified. Patients without atrial fibrillation revealed significantly higher plasma prolactin levels than patients with atrial fibrillation. Furthermore, the influence of aspirin or clopidogrel on prolactin stimulated P-selectin expression *in vitro* was tested, showing that aspirin was without effect, whereas clopidogrel significantly inhibited platelet P-selectin expression. In conclusion, hyperprolactinemia might be a novel risk factor for stroke mediating its thrombogenic effect through enhanced platelet reactivity, and this might correspond to a higher efficacy of antiplatelet combination therapy with clopidogrel compared to aspirin therapy alone.

### Keywords

Platelets, CD62p, stroke, prolactin, leptin

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### Introduction

Inflammation and hypercoagulability are linked to the pathogenesis of atherosclerosis and its clinical manifestations such as coronary or peripheral artery disease and stroke (1). Platelet activation is one of the central mechanisms in arterial thrombogenesis and in the pathophysiology of ischemic stroke (2–5). Recently, it has been demonstrated that patients with acute cerebral ischemia have an increase of P-selectin (CD62p) on circulating platelets as a marker for platelet activation (6–8). CD62p serves as an adhesion receptor mediating the crosstalk between platelets (“hypercoagulability”) and leukocytes or endothelia (“inflammation”). Fibrinogen, C-reactive protein (CRP) and leukocyte count are increased during stroke (9–13) and were shown to be independently associated with the risk of first-ever and recur-

rent vascular events. However, in recent studies it has been demonstrated that the increase of CD62p was regulated independently of these inflammatory markers (7, 8).

In a previous study we identified prolactin (PRL) as a novel co-factor of platelet activation (14). We demonstrated a dose dependent increase of CD62p expression and increased platelet aggregation by prolactin *in vivo* and *in vitro*. Moreover, we detected the short isoform of the PRL-receptor on human platelets and investigated the signalling during platelet activation with a focus on ADP-stimulated G-protein-regulated pathways (15). In a clinical study we showed an increased incidence of venous thrombembolism (VTE) in patients with prolactinoma, indicating that hyperprolactinemia may be an important risk factor of hypercoagulability (15). On the other hand, a further hormone – leptin – has been recently associated with platelet activation,

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suggesting a mechanism of atherothrombotic disease in obesity or diabetes (16, 17). Elevated leptin levels have recently been found to be a risk factor for ischemic stroke in both men and women and especially in combination with increased blood pressure for hemorrhagic stroke in men (18, 19). However, until now an association between hyperprolactinemia and ischemic stroke has not been systematically investigated.

Therefore, we determined plasma prolactin and leptin levels as well as CD62p expression in patients with stroke or transient ischemic attack. Furthermore, we measured plasma prolactin and acquired and congenital risk factors of thrombophilia in a cohort of 80 consecutive patients with first-ever stroke.

## Subjects and methods

### Experimental subjects

We investigated plasma prolactin and leptin as well as CD62p expression in 36 patients with either ischemic stroke or transient ischemic attack within 24 hours after onset of symptoms. Exclusion criteria were: infections, malignancies, autoimmune diseases, acute coronary syndromes, surgery within the last twelve months, antithrombotic therapy with coumarin, aspirin or clopidogrel and intracerebral hemorrhage. Standard diagnostic measures included cranial computed tomography to exclude intracerebral hemorrhage, a duplex sonography to exclude significant stenosis of the extra- and intracerebral carotids, electrocardiogram for the detection of arrhythmias as well as echocardiography for the exclusion of an intracardial thrombus. Acute cerebral ischemias were classified according to the TOAST criteria (20, 21). The control group consisted of 15 sex- and age-matched subjects with no clinical signs of acute coronary, peripheral, or cerebral ischemia within the 12 months preceding study entrance and with a comparable atherosclerotic risk profile.

Furthermore, in a retrospective study we investigated plasma prolactin in a cohort of 80 consecutive patients with thromboembolic stroke. Blood samples were collected within 24 hours after onset of symptoms. Standard diagnostic procedures were performed as shown above. Thrombophilic congenital or acquired risk factors like factor V Leiden mutation, prothrombin G20210A mutation, antithrombin-, protein C-, and protein S-deficiency, activated protein C resistance phenomenon, hyperhomocysteinemia, anti-phospholipid antibodies and increased factor VIII levels were excluded as previously reported (15). Plasma aliquots of the patients were stored at  $-80^{\circ}\text{C}$  until prolactin determination. No patients suffered from possible hyperprolactinemia inducing diseases like: hypothyroidism, severe renal failure, liver disease, pituitary adenoma or received medical therapy like: antipsychotic drugs, dopamine receptor antagonists and opiates. The local Ethics Committee approved the study.

### Determination of plasma prolactin and leptin

The plasma prolactin values of all patients were determined with the AxSYM Prolactin Assay (Abbott, USA). This assay was performed according to the manufacturer's instructions as previously reported (14, 15). The plasma leptin was analysed with the Human Leptin RIA Kit (Linco, USA) according to the manufacturer's instructions as reported elsewhere (22).

### Flow cytometric platelet analysis and platelet aggregation

The determination of basal and ADP- and TRAP-6-stimulated CD62p expression by flow cytometry as well as platelet aggregation were performed as previously described (14, 15). Based on our previous in-vivo and in-vitro studies we incubated platelet rich plasma of healthy controls with 5,000 mU/l prolactin, which leads to maximal platelet aggregation or P-selectin expression.

For the investigation of prolactin effects on ADP stimulation of platelets during aspirin or clopidogrel therapy, citrated whole blood of healthy donors was stimulated with different concentrations of human prolactin (Sigma, Deisenhofen, Germany) *in vitro*. Volunteers were investigated four days after oral therapy with aspirin (starting dose 500 mg and further 100 mg per day) or four days after oral therapy with clopidogrel (starting dose 300 mg and further 75 mg per day).

### Statistical analysis

The Pearson correlation coefficient was calculated to analyse the correlation between prolactin or leptin and CD62p expression in patients with stroke and controls. The Mann-Whitney-U-test was used to compare the prolactin and leptin values between the patients with stroke with and without embolic stroke. To investigate the influence of diabetes as a possible confounder for stroke a multivariate analysis by a two-way ANOVA test was performed. Moreover, we compared the prolactin values between both groups with and without diabetes. P-values less than 0.05 were considered as statistically significant. Data were analysed with SPSS for Windows (released 9.0.1).

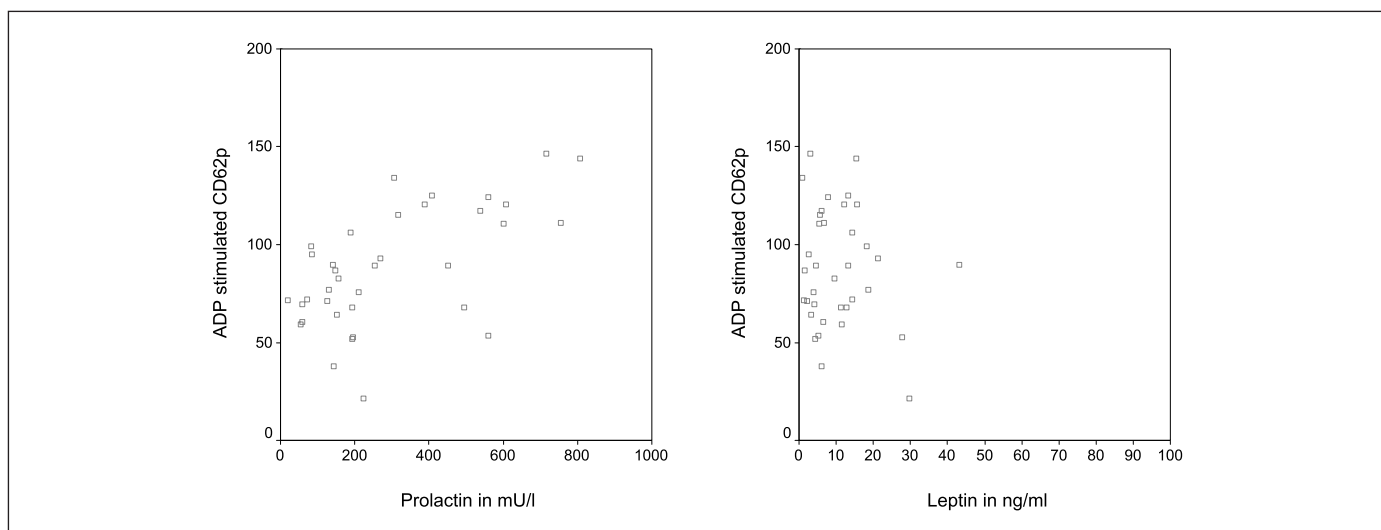
## Results

### Correlation between ADP-stimulated CD62p expression and plasma prolactin levels in patients with stroke or transient ischemic attack

Patients with ischemic stroke or transient ischemic attack revealed significantly higher ADP-stimulated CD62p expression ( $p < 0.001$ ) and prolactin values ( $p < 0.001$ ) than the healthy controls. In contrast, no significant difference was detected for leptin levels (Table 1). In patients with ischemic stroke or transient ischemic attack, prolactin values were well correlated with ADP-stimulated CD62p expression ( $r = 0.56$ ;  $p < 0.0001$ ; Fig. 1A), whereas no significant correlation was found with plasma leptin (Fig. 1B). Furthermore, we detected no differences either in PRL values or in CD62p expression between patients with stroke and transient ischemic attack. CD62p expression of TRAP-6 stimulated and non-stimulated platelets was neither influenced by prolactin nor leptin (data not shown).

### Prolactin values in patients with ischemic stroke without embolism are significantly higher than in patients with embolic stroke by atrial fibrillation

We identified 21 patients (nine female, 12 male; age:  $72.9 \pm 9.3$ , range 50–87 years) with thromboembolic stroke by atrial fibrillation and 59 patients (28 female, 31 male; age:  $62.3 \pm 15$ , range 28–88 years) without embolism. Therefore, patients with atrial fibrillation showed a significant tendency for higher age ( $p = 0.004$ ) without significant ( $p = 0.08$ ) differences in sex distribution. All pa-



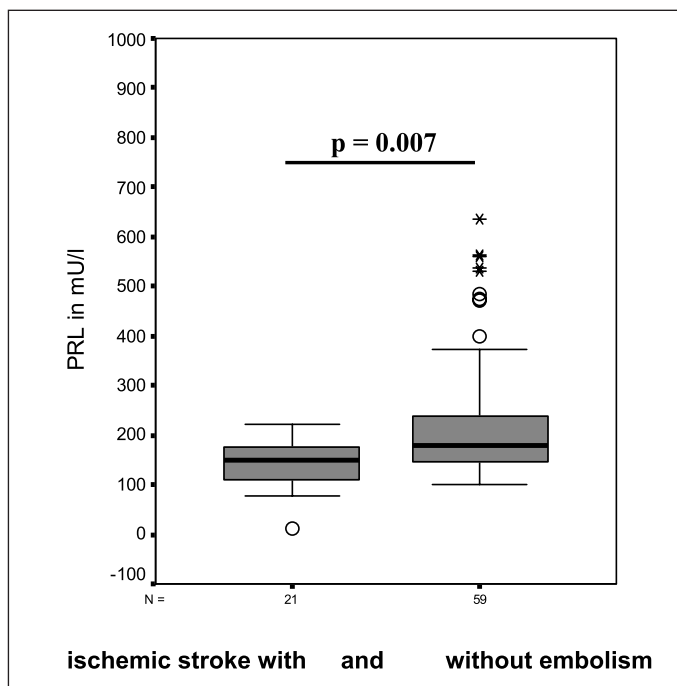
**Figure 1: Correlation between plasma prolactin or leptin and ADP-stimulated CD62p expression in 36 patients with stroke or transient ischemic attack (TIA).** In patients with ischemic stroke or TIA the correlation between prolactin (A) or leptin (B) values and ADP-stimulated CD62p expression was analysed and quantified by the Pearson correlation coefficient.

tients were free of severe accompanying diseases. Frequency of hyperlipidemia was not different between patients with (two of 21) and without (five of 59) atrial fibrillation ( $p=0.88$ ). Moreover, there were no significant differences for smoking (one of 21 vs. 10 of 59;

$p=0.27$ ) or hypertension (nine of 21 vs. 25 of 59;  $p=0.96$ ) between stroke patients with or without atrial fibrillation. Patients with atrial fibrillation showed a higher frequency of diabetes (nine of 21 vs. eight of 59) than patients without atrial fibrillation ( $p=0.004$ ).

In 21 patients with embolic stroke by atrial fibrillation the mean value of prolactin was  $142.3 \pm 50.8$  mU/l, whereas in 59 patients without embolic stroke, the mean prolactin value was significantly higher ( $268.1 \pm 336.4$  mU/l;  $p=0.005$ ; Fig. 2). Furthermore, in eight of the patients without embolic stroke, the prolactin values were above the calculated maximum of the group (prolactin levels between 471 and 2,597 mU/l; dots in Fig. 2).

To investigate the possible influence of diabetes as a possible confounder for stroke, a multivariate analysis by a two-way ANOVA test was performed. Patients with prolactin values  $> 700$  mU/l were excluded from this analysis. The significant ( $p=0.002$ ) differences in prolactin values between both groups were independent of diabetes. Moreover, we detected significant differences ( $p=0.003$ ) in prolactin values between both groups when patients with diabetes were excluded, whereas no differences ( $p=0.122$ ) in prolactin were found between patients with diabetes.



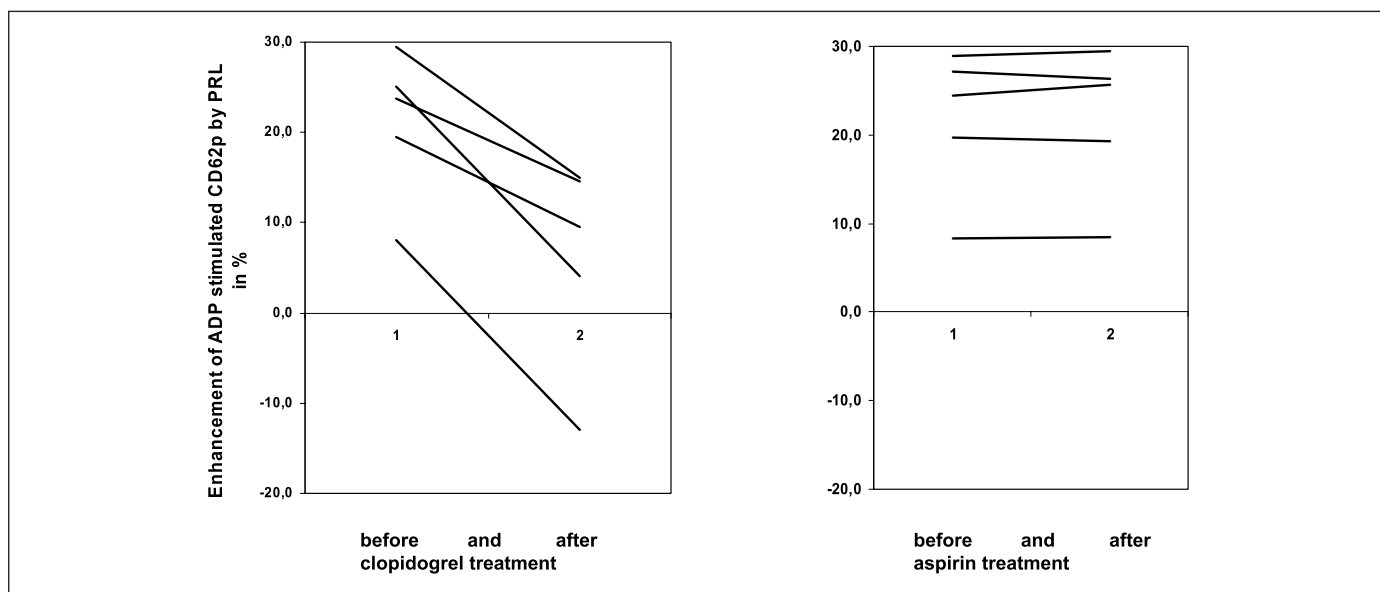
**Figure 2: Prolactin values in patients with ischemic stroke without embolism are significantly higher than in patients with embolic stroke by atrial fibrillation.** The prolactin values of stroke patients without embolism were compared to stroke patients with embolism by atrial fibrillation by the Mann-Whitney U-test. Box plots represent 25<sup>th</sup> and 75<sup>th</sup> percentiles, error bars minimum or maximum of prolactin values, the dots represent values above the calculated maximum of prolactin values within the group. P values are given above the plots.

### Influence of aspirin or clopidogrel on prolactin stimulated CD62p expression

Next we investigated the influence of aspirin or clopidogrel on prolactin stimulated CD62p expression *in vivo*. After treatment, aspirin showed no effect on prolactin enhanced ADP-stimulated CD62p expression, whereas oral clopidogrel treatment resulted in inhibition of prolactin enhanced ADP-stimulated CD62p expression in all investigated individuals (Fig. 3). Furthermore, clopidogrel completely inhibited prolactin-induced enhancement of platelet aggregation (Fig. 4).

## Discussion

Platelet activation is a crucial mechanism in arterial thrombogenesis and in the pathogenesis of ischemic stroke. An increase

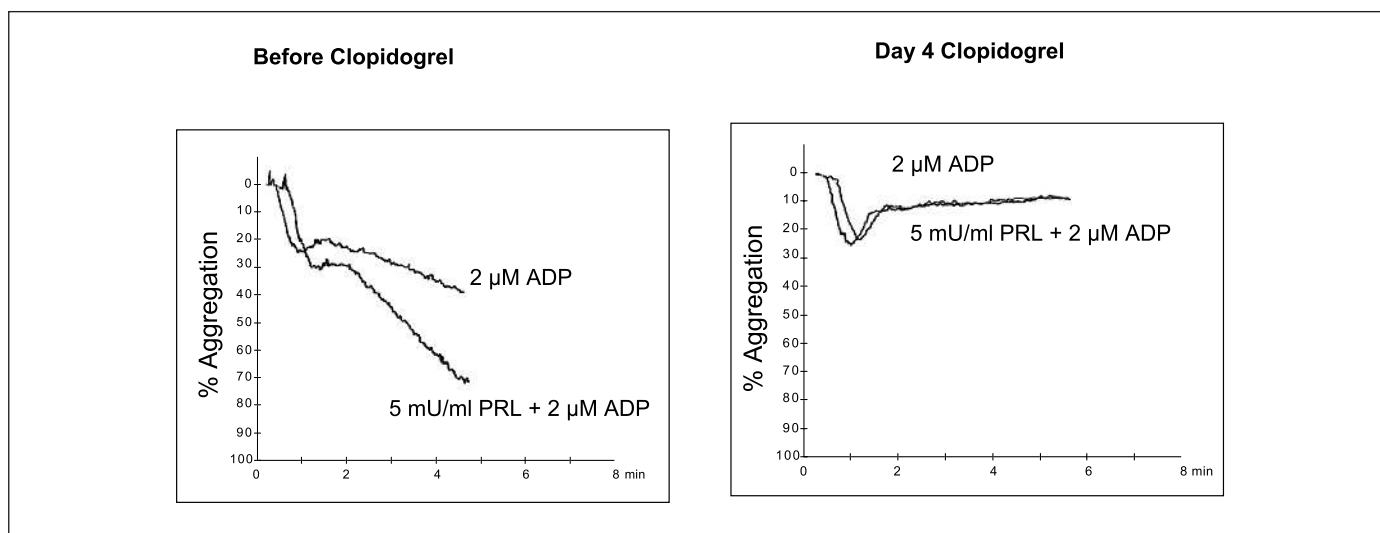


**Figure 3: Influence of clopidogrel or aspirin on enhancement of ADP-stimulated CD62p expression by PRL.** Enhancement of ADP-stimulated CD62p expression by prolactin is indicated in percent. Volunteers were investigated before and after four days of clopidogrel or aspirin treatment. Flow cytometric platelet analysis was performed in duplicate in at least two separate experiments.

of platelet activation markers such as CD62p during stroke has been reported and supports this concept (8). To the best of our knowledge, our study is the first one investigating in parallel prolactin values and ADP-stimulated CD62p expression on platelets in patients with stroke or transient ischemic attack. We found increased levels of CD62p expression in patients with ischemic stroke or transient ischemic attack (Table 1), comparable to previous reports (8). We detected significantly higher prolactin values in patients with ischemic stroke or transient ischemic attack than in matched healthy controls (Table 1). However, in these patients no difference was found in leptin levels compared to matched controls. In patients with stroke or transient ischemic

attack, the ADP-stimulated CD62p expression correlated significantly with plasma prolactin levels corresponding to previous findings that demonstrate a stronger effect on platelet activation by prolactin compared with leptin *in vitro* and *in vivo* (22). Finally, clopidogrel but not ASS was demonstrated to efficiently block prolactin-induced platelet activation.

Up to now an association between hyperprolactinemia and stroke has not been investigated. However, it is widely accepted that the risk of stroke is highly increased two days prior to giving birth and during the first six weeks postpartum (23). In parallel, prolactin has a predominant increase around delivery and during the first time of lactation (24, 25). Therefore, an association be-



**Figure 4: Influence of clopidogrel on enhancement of ADP-stimulated platelet aggregation by prolactin.** Enhancement of ADP-stimulated platelet aggregation by prolactin is indicated in percent. Volunteers were investigated before and after four days of clopidogrel treatment. Platelet aggregation was performed in duplicate in at least three separate experiments.

**Table 1: Prolactin and leptin values and expression of ADP-stimulated CD62p in 36 patients with stroke or transient ischemic attack (TIA) and 15 age and sex-matched controls.**

Comparison of ADP-stimulated CD62p expression and plasma prolactin or leptin values in 36 patients with ischemic stroke or TIA and 15 sex- and age-matched healthy subjects with no clinical signs of acute coronary, peripheral, or cerebral ischemia within the 12 months preceding study entrance and with a comparable atherosclerotic risk profile by the Mann-Whitney U test.

	Controls (n = 15)	patients stroke / TIA (n = 36)	p value
prolactin in mU/l	72 ± 22	296 ± 222	p < 0.001
leptin in ng/ml	8.9 ± 6.3	10.6 ± 8.9	n.s.
ADP-stimulated CD62p	63.2 ± 29.4	86.7 ± 28.4	p < 0.001

tween stroke and prolactin increase at delivery and during lactation can be assumed but has not been investigated so far.

Moreover, pituitary apoplexy is associated with prolactinoma or hormone inactive macroadenoma, especially during TRH stimulation test (26, 27). In our previous paper we were able to demonstrate that prolactin and P-selectin showed a parallel short-term increase after TRH injection (14). Therefore, we speculate that the prolactin increase after TRH stimulation in hormone inactive macroadenoma might be triggering pituitary apoplexy via platelet activation.

A recent study demonstrated an association between atherosclerotic diseases like stroke and spontaneous venous thrombosis (28). This association was still present after adjustment for typical risk factors for atherosclerosis and thrombophilic conditions. Therefore, this study implies either that atherosclerosis can induce VTE or that both conditions share the same, presently unknown, risk factors (28). Hyperhomocysteinemia, factor V Leiden, and lupus anticoagulant have been suggested as potential risk factors for atherosclerosis and VTE (29–31). In both diseases increased fibrin turnover as well as an activation of blood coagulation, especially of platelets, is detectable (32–38). The participation of platelets in the pathogenesis of chronic atherosclerotic lesions and acute thrombotic occlusion of arteries is undisputed. This effect is due to platelet adhesive properties and their ability to respond to stimuli with rapid activation (39).

In previous studies we have demonstrated that patients with venous thromboembolism (VTE) without congenital or acquired risk factors had significantly higher prolactin levels than those with congenital risk factors or healthy controls. On the other hand, patients with prolactinoma had a significantly higher incidence of idiopathic VTE than the general population (15). Inflammation and thrombosis are intimately connected as stated above. However, until now the significance of activated platelets for the development of VTE is not systematically investigated. Beyond the well-known plasmatic factors, the pathogenesis of VTE involves complex platelet-leukocyte-interactions whereby details are not fully elucidated (40). P-selectin mediates rolling of platelets and leukocytes on activated endothelial cells. Recent data indicate that P-selectin interaction with a ligand stabilizes initial GP IIb/IIIa-fibrinogen interactions, thus allowing the formation of large stable platelet aggregates (41). Furthermore, in the Pulmonary Embolism Prevention Trial (PEP) it was de-

monstrated that aspirin can reduce VTE by at least a third throughout a period of increased risk (42). These results, along with those of a previous meta-analysis (43, 44), suggest that the activation of platelets is an important initial step in the development of VTE. Therefore, our findings indicated that hyperprolactinemia might be involved in venous and arterial thrombotic disease and that it mediates its thrombogenic effects through enhanced platelet reactivity.

Therefore, we evaluated the prolactin values in patients without congenital or acquired thrombophilic risk factors similar to our previous study on VTE (15). Patients without embolic stroke showed significantly higher plasma prolactin levels compared to patients with embolic stroke by atrial fibrillation (Fig. 2). Most of the patients with stroke without atrial fibrillation had prolactin values that were still in the upper normal range. This finding corresponds to the dose-dependent increase of platelet activation and aggregation by human prolactin *in vitro* that occurs already within the normal plasma prolactin range (14, 15). We detected no association between prolactin values and platelet counts either in patients with stroke or in our previous studies (14, 15, 22). This fact can be confirmed by other studies investigating the effect of dopamine or dopamine antagonists on prolactin values and platelet counts in parallel (45).

In univariate analysis possible confounders for stroke as hypertension, hyperlipidemia and smoking have been excluded. However, patients with atrial fibrillation showed higher frequency of diabetes, which has been demonstrated as an important risk factor for thromboembolic stroke (46). In our multivariate analysis we confirmed differences in prolactin values independent of diabetes.

The results of our study are limited by prolactin determination at the acute stage of stroke and the lack of analysis of an association between stroke severity and prolactin. Indeed it is uncertain if prolactin levels were increased before stroke, and whether prolactin is triggering or enhancing platelet activation in stroke. However, in our present study we showed an association between increased prolactin values and platelet activation measured by P-selectin expression, which is one of the central mechanisms in arterial thrombogenesis and in the pathophysiology of ischemic stroke as stated above. Therefore, we conclude that increased prolactin values might be involved in platelet activation during stroke.

One of the most likely explanations for increased prolactin values in patients with stroke might be stress. It is widely accepted that prolactin levels can be increased by stress factors such as pain (47, 48). However, we detected no differences either in PRL values or in CD62p expression between patients with stroke and transient ischemic attack in our cohort of 36 patients, indicating no correlation between severity/duration of ischemia and PRL levels. Probably, there are no differences in stress between both groups of patients. However, patients with stroke without atrial fibrillation showed significantly higher prolactin values than patients with atrial fibrillation. Therefore, the cause of higher prolactin values in patients without atrial fibrillation is unknown. Against the background of the association between prolactin increase and platelet activation the course of prolactin increase might be of minor importance and seems to be speculative at the moment. However, in a previous study increased

nocturnal prolactin values were observed in patients after stroke compared with healthy volunteers, whereas the rhythm of cortisol was undisturbed (49). This study implies a long-term elevation of prolactin in patients with stroke. Unfortunately, we have no prospective data on our patients yet.

Altogether, our previous epidemiological data in prolactinoma or in patients with idiopathic VTE (15) as well as our present results suggest that hyperprolactinemia might be a candidate for a novel acquired risk factor for venous as well as for arterial thrombogenesis by enhancing platelet activation and aggregation.

Several antiplatelet agents with different pharmacological mechanisms are currently available for secondary prevention of ischemic stroke. Aspirin is the best studied and most widely used antiplatelet therapy for stroke prevention. In acute coronary syndrome without ST-segment elevation, the CURE study showed a 20% relative risk reduction of cardiovascular death or an absolute benefit of 2.1% for combination therapy with clopidogrel and aspirin than for aspirin alone (50). Because of the substantial differences between cerebrovascular and cardiac patients the CURE data can not be extrapolated to cerebrovascular patients. Recently, the MATCH study addressed this question (51). This trial compared the combination of aspirin/clopidogrel with clopidogrel alone in high risk patients with a recent stroke/TIA. In MATCH, the combination of the two antiplatelet agents prevented slightly more strokes, but this effect did not reach statistical significance. However, these major vascular events are only one aspect of platelet function in stroke. Platelet attachment to endothelium, mediated by platelet P-selectin, might lead to in-

flammatory reactions promoting endothelial dysfunction and long-term cardiovascular complications (52). Therefore, combining two anti-platelet agents might indeed provide major advantages in the long run.

Our ex-vivo / in-vitro studies demonstrated that aspirin was without effect on prolactin-enhanced ADP-stimulated CD62p expression (data not shown), as documented previously in patients with acute coronary syndrome (53). In contrast, clopidogrel showed a significant inhibition of prolactin enhanced ADP-stimulated CD62p expression (Fig. 3). Our findings reconciled a previous study demonstrating an inhibition of ADP-stimulated CD62p by clopidogrel (54). This result was expected by our previous studies of the molecular mechanism of platelet activation by PRL. The stimulating effect of prolactin on platelet activation by CD62p expression and aggregation depends on a co-activation with ADP (14, 15), and clopidogrel is a specific inhibitor of ADP-receptor P2Y<sub>12</sub>, whereas ASS acts on cyclooxygenase-1, hereby blocking thromboxane A synthesis (39). Therefore, our present in-vitro data might be a facet to explain the higher efficacy of antiplatelet combination therapy with clopidogrel compared to aspirin therapy alone in arterial thrombogenesis, as suggested by the CURE study (50). In conclusion, our study demonstrates that prolactin but not leptin did correlate to platelet activation in patients with stroke.

In summary, hyperprolactinemia might be a novel risk factor for stroke mediating its thrombogenic effect through enhanced platelet reactivity, and this finding might support the higher efficacy of antiplatelet combination therapy with clopidogrel and aspirin than aspirin therapy alone.

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