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Magnitude and time course of platelet inhibition with Aggrenox® and Aspirin in patients after ischemic stroke: the AGgrenox versus Aspirin Therapy Evaluation (AGATE) trial

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Abstract

The European Stroke Prevention Study showed greater stroke prevention for Aggrenox® than either for aspirin or dipyridamole alone. To test whether Aggrenox® has superior antiplatelet properties to aspirin alone we conducted the AGgrenox versus Aspirin Therapy Evaluation (AGATE) trial. Forty patients with prior ischemic stroke not taking aspirin for at least 30 days were randomized to Aggrenox® (2 pills/daily) or aspirin (81 mg plus matching placebo/daily) for 30 days. Platelet function was assessed at baseline, 24 h, and days 3, 7, 15, and 30 by aggregometry, flow cytometry and cartridge-based analyzers. Both Aggrenox® and aspirin provided fast and sustained platelet inhibition. Aggrenox®, however, especially after 15 days, showed significant prolongation of the closure time (P=0.04), diminished expression of platelet/endothelial cell adhesion molecule-1 (PECAM-1) (P=0.01), glycoprotein IIb (GPIIb) antigen (P=0.02), and GPIIb/IIIa activity (P=0.01) by PAC-1 C antibody, CD63 (P=0.03), as well as inhibition of Protease Activated Receptors (PAR-1) associated with intact (SPAN12, P=0.01) and cleaved (WEDE15, P=0.01) thrombin receptors as compared with aspirin. Surprisingly, GPIb expression increased, especially after aspirin. In the randomized trial of small sample size, aspirin and Aggrenox® produced fast and sustained platelet inhibition. In 25 of 90 direct comparisons, Aggrenox® was superior to aspirin, whereas in 4 of 90, aspirin was superior to Aggrenox®. In 61 of 90 direct comparisons, aspirin and Aggrenox® were equivalent. Aggrenox® was associated with a profound reduction of PAR-1 receptors, an observation that may be related to the greater clinical benefit of Aggrenox® compared with Aspirin in preventing recurrent stroke.

Keywords: Randomized trial; Aspirin; Aggrenox®; Platelet; Stroke

1. Introduction

In numerous randomized trials and their meta-analyses aspirin reduced the risk of a second stroke in patients with ischemic stroke (Antithrombotic Trialists Collaboration, 2002; Hennekens, 2002). Aggrenox®, a combination of low-dose 25 mg aspirin with extended release 200 mg dipyridamole, represents a promising combination for sustained platelet inhibition, and reduction of both arterial and venous thrombosis. In the European Stroke Prevention Study 2 (ESPS-2), Aggrenox® was twice as effective as either aspirin or dipyridamole alone (Diener et al., 1996). Experimental studies suggest that dipyridamole combined with aspirin in ratios about 7:1 or higher effectively inhibit thrombus formation, whereas a ratio of 1:1 has a little effect (Muller et al., 1990; Eldor et al., 1986; Muller et al., 1990).

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Aggrenox® combines a high dose of extended-release dipyridamole with low-dose aspirin in a ratio of 8:1. The platelet inhibition with Aggrenox® appears remarkably fast (within 30 min after oral intake), and the magnitude of such inhibition in humans is similar to that of aspirin doses of 81 and 325 mg (Serebruany et al., 2003). On the other hand, recent advances in platelet function assessment by cartridge-based analyzers and flow cytometry techniques are expanding our knowledge of antiplatelet properties beyond conventional aggregometry. Therefore, the benefits of Aggrenox® in an expanding array of clinical conditions, including ischemic stroke, may be indeed related to platelet inhibition via surface receptor blockade, despite small or negligible differences in platelet aggregation.

We designed and conducted the AGgrenox versus Aspirin Therapy Evaluation (AGATE) trial to compare the changes of several platelet activation markers over the initial treatment period of 4 weeks, to evaluate the time course in detail in order to determine to what extent additional dipyridamole treatment adds value to the antiplatelet properties of aspirin. We, therefore, performed frequent serial measurements of multiple platelet characteristics including conventional aggregometry, rapid cartridge-based platelet function analyzers and whole blood flow cytometry.

2. Methods

2.1. Patients

AGATE was a randomized trial with placebo control for aspirin, conducted at two centers (one in the USA, and one in Norway). The trial was approved by local institutional review boards. Written informed consent was obtained from all patients, who were informed of the strict compliance rules, and compensated for frequent hospital visits and blood draws. Patients aged ≥40 years were eligible if they had suffered an ischemic stroke between 2 and 6 months earlier, had received aspirin (81–650 mg/day) previously but were found to be non-compliant, and had not taken aspirin for 1 month ore more. All eligible patients were found to have atherosclerotic morphology of at least 50% vessel diameter stenosis documented by ultrasound or/and angiography. On neuroimaging, ischemic lesions of <1.5 cm have been observed in 17/40 patients. All patients exhibited clinical symptoms of typical lacunar syndrome(s). Among 434 post stroke screened patients, 46 patients were eligible for the trial participation by chart review, preliminary teleconference, and by the in person interview during the screening visit. Of these patients, six had to be excluded because of allergic reaction to aspirin (n=1), history of minor gastrointestinal bleeding (n=2), anemia (n=1), and suspected alcohol abuse (n=2). The AGATE trial algorithm is presented at Fig. 1.

Patients were excluded for a history of bleeding diathesis, drug or alcohol abuse, prothrombin time greater than 1.5 times control, platelet count <100,000/mm^3, hematocrit <25%, creatinine >4.0 mg/dl, surgery or angioplasty for symptomatic stenosis performed within 3 months or planned for the future, known allergy to aspirin and/or dipyridamole, history of gastrointestinal or other bleeding, history of drug-induced disorders, trauma or surgery within the last 3 months, any surgery planned for the next 3 months, cancer, rheumatic diseases, or seizures. Patients participating in other investigational drug trials within 1 month of completion were also excluded. No patients had previously received thienopyridines or intravenous platelet glycoprotein IIb/IIIa inhibitors.

Using a table of random numbers by an independent statistical center, 40 willing and eligible patients were

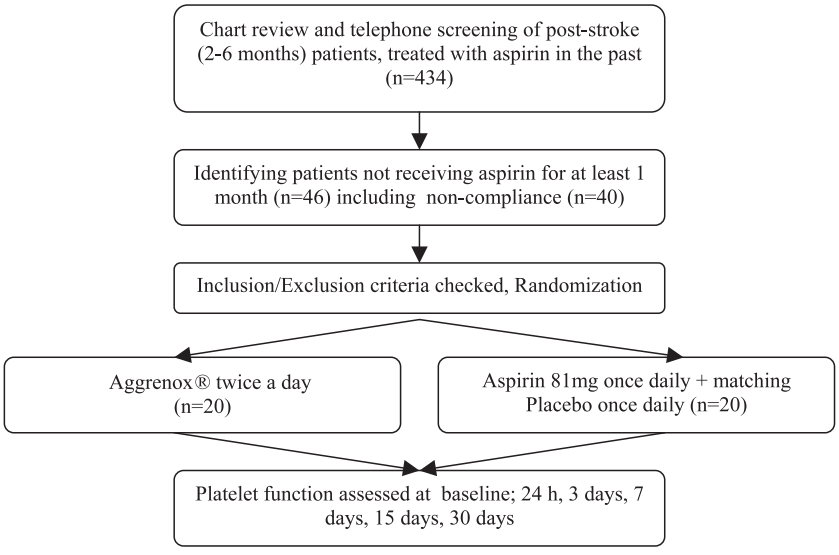

Fig. 1. AGATE trial algorithm.

assigned at random to Aggrenox® twice a day or aspirin 81 mg once daily. The follow-up visits were scheduled at days 1, 3, 7, 15, and 30 after randomization.

2.2. Samples

Blood samples were obtained with a 19-gauge needle by direct venipuncture and drawn into two 7-ml vacutainer tubes containing 3.8% trisodium citrate at room temperature. The vacutainer tube was filled to capacity and gently inverted three to five times to ensure complete mixing of the anticoagulant. The first 4–5 ml of blood were used for lipid profile analysis, or discharged. All samples were labeled with coded number and analyzed by blinded technicians. Platelet studies were performed at baseline as well as at Day 1, Day 3, Day 7, Day 15, and finally at Day 30 after treatment assignment.

2.3. Platelet aggregation

The blood–citrate mixture was centrifuged at 1200×*g* for 2.5 min. The resulting platelet-rich plasma was kept at room temperature for use within 1 h. The platelet count was determined in the platelet-rich plasma sample and adjusted to 3.5×10 8/ml with homologous platelet poor plasma. Platelets were stimulated with 5 μmol epinephrine, and 5 μmol ADP (Chronolog, Havertown, PA) and aggregation was assessed as previously described using a Chronolog Lumi-Aggregometer (model 560-Ca) with the AggroLink software package. Aggregation was expressed as the maximal percent change in light transmittance from baseline, using platelet-poor plasma as a reference. Curves were analyzed according to international standards (Ruggeri, 1994).

2.4. Cartridge-based platelet analyzers

2.4.1. Platelet function analyzer (PFA-100™)

Using the PFA-100 instrument (Dade Behring, Miami, FL), the blood–citrate mixture is aspirated under a constant negative pressure and contacts an epinephrine and collagen coated membrane. The blood then passes through an aperture that induces high shear stress and simulates primary hemostasis after injury to a small blood vessel under flow condition. The time to aperture occlusion (the closure time) is recorded in seconds and is inversely related to the degree of shear-induced platelet activation (Mammen et al., 1998).

2.4.2. Rapid platelet-function assay cartridge test (RPFA-ADP, Ultegra®)

RPFA-ASA (Ultegra®) is a rapid platelet-function assay aspirin cartridge test with propyl gallate used as agonist. Cationic propyl gallate is a novel platelet activator used to detect and monitor the amount of platelet inhibition induced by platelet prostanoid antagonists, including aspirin and other non-steroid anti-inflammatory drugs (Schwartz et al., 2002). The Ultegra device (Accumetrics, San Diego, CA) is a turbidimetric based optical detection system, which measures platelet induced aggregation as an increase in light transmittance. This particular test cartridge has been designed to specifically monitor antiplatelet effects of aspirin containing medications. When the platelets are exposed to the fibrinogen-coated microparticles, activated with lyophilized and resuspended propyl gallate, agglutination occurs in proportion to the number of available platelet receptors. The whole blood citrate mixture is being added to the cartridge, and agglutination between platelets and coated beads is being recorded (Smith et al., 1999). Ultegra RPFA-ASA Assay results are reported as Aspirin Responsive Units (ARU). Both PFA-100 and Ultegra assays were performed in duplicate. An electronic quality control test was performed on each instrument daily prior to performing any patient samples.

2.5. Whole blood flow cytometry

The surface expression of platelet receptors was determined by flow cytometry using the following monoclonal antibodies: CD 41 antigen (GP IIb/IIIa); CD 42b (GP Ib), CD 62p (P-selectin) (Dako, Carpenteria, CA); PAC-1 (GP IIb/IIIa activity) CD 31 (platelet/endothelial cell adhesion molecule [PECAM]-1), CD 51/CD 61 (vitronectin receptor), CD 63 (lysosome associated membrane protein or LAMP-3), CD 107a (LAMP-1), CD 151 (platelet endothelial tetraspan antigen, or PETA-3), CD154 (CD40-ligand), CD 165 (GP37) (PharMingen, San Diego, CA); CD36 (thrombospondin, or GPIV), cleaved (WEDE15), and intact (SPAN12) platelet thrombin receptors (Beckman Coulter, Brea, CA). Formation of platelet–leukocyte aggregates was assessed by dual labeling with pan-platelet marker (CD151), and then with CD14, the macrophage receptor for endotoxin lipopolysaccharides. The blood–citrate mixture (50 μl) was diluted with 450 μl Tris buffered saline (TBS) (10 mmol/l Tris, 0.15 mol/l sodium chloride) and mixed by inverting an Eppendorf tube gently two times. The appropriate primary antibody was then added (5 μl) and incubated at 37 °C for 30 min, and then a secondary antibody was applied if needed. After incubation, 400 μl of 2% buffered paraformaldehyde was added for fixation. The samples were analyzed on the FACScan flow cytometer (Becton Dickinson, San Diego, CA) calibrated to measure fluorescent light scatter as previously described (Ault, 1993). All parameters were collected using four-decade logarithmic amplification. The data were collected in list mode files and then analyzed. P-selectin was expressed as percent positive cells as previously described (Serebruany and Gurbel, 1999). Other antigens were expressed as log mean fluorescence intensity.

2.6. Statistics

The significance of differences between treatment arms was calculated by Fisher's exact tests for discrete variables,

and Wilcoxon rank-sum test for continuous variables. The significance of differences between individual flow cytometric histograms was calculated using the Smirnov–Kolgomorov test incorporated in the CELLQuest' (San Diego, CA) software. Statistical analyses were performed using SPSS/E11.5 (SPSS, Chicago, IL). To control for any baseline differences analysis of variance was used. All *P* values are two-sided.

3. Results

3.1. Patients

Forty patients were screened, and completed, in the 30-day trial. There were no deaths or serious adverse events, including symptoms attributable to cerebral ischemia and cerebral or systemic bleedings. Two patients from the Aggrenox® group and one patient from the aspirin group had headache during the first several days of therapy. Table 1 shows baseline pretreatment distribution of demographics, risk factors, clinical characteristics, and concomitant medications in both arms. Although there were no significant differences between Aggrenox® and aspirin groups, patients treated with Aggrenox® were slightly older, with a prevalence of white women. The Aggrenox® group smoked more, and had less incidence of hypertension; however, the severity of hypertension was higher than the aspirin group. Aggrenox® patients also exhibited a higher frequency of alcohol use, while concomitant medications were used fairly evenly.

Table 1
Baseline characteristics in the AGATE

Parameter	Aggrenox® (*n*=20)	Aspirin (*n*=20)
Demographics		
Age (mean±S.D.)	65.4±7.4	63.0±8.6
Male	12 (60%)	14 (70%)
White	9 (45%)	7 (35%)
Black	11 (55%)	13 (65%)
Risk factors and history		
Diabetes mellitus	7 (35%)	7 (35%)
Hyperlipidemia	13 (65%)	14 (70%)
Smoking	11 (55%)	9 (45%)
Coronary artery disease	9 (45%)	9 (45%)
Hypertension	11 (55%)	13 (65%)
Mean systolic BP (mm Hg)	143.1±25.9	139.0±23.4
Mean diastolic BP (mm Hg)	89.5±14.3	83.5±11.4
Family history of stroke/TIA	8 (40%)	8 (40%)
Current/former history of alcohol use	11 (65%)	7 (35%)
Previous history of stroke or TIA	5 (25%)	4 (20%)
Hypotensive medications	10 (50%)	13 (65%)
Statins	12 (60%)	11 (55%)
Antiglycemic	6 (30%)	7 (35%)
SSRI's	6 (30%)	8 (40%)
Location of stroke		
Right hemisphere	9 (45%)	10 (50%)
Left hemisphere	7 (35%)	6 (30%)
Cerebellar	–	1 (5%)
Bilateral	1 (5%)	–
Brain stem	3 (15%)	3 (15%)

All non-significant ($P>0.05$) between groups by Fisher's exact test.

3.2. Platelet data

The data on platelet characteristics in the two treatment groups are presented in Table 2. At baseline, platelet activity was not remarkably elevated. Specifically, pretreatment platelet characteristics in 16/40 patients were within the normal control range (data not shown). Therapy with aspirin and Aggrenox® resulted in substantial time-dependent inhibition of platelet activity as reflected by conventional aggregometry, rapid cartridge-based platelet analyzers, and whole blood flow cytometry.

3.3. Aspirin

Treatment with 81 mg daily aspirin in patients after ischemic stroke and not taking aspirin for over a month, resulted in a rapid (within 24 h) inhibition of epinephrine-induced conventional platelet aggregation, and gradual inhibition of the adenosine diphosphate induced aggregation. Rapid platelet analyzers revealed consistently prolonged closure time by the PFA-100 instrument, and diminished aspirin reactive units by the Ultegra® device. Numerous changes in the platelet surface receptor expression after initiation of aspirin therapy, such as mild but rapid blockade of PECAM-1, P-selectin, thrombospondin, GPIIb antigen expression, and decreased GPIIb/IIIa activity assessed with the PAC-1 antibody were also observed. Aspirin diminished formation of platelet–monocyte aggregates, CD40-ligand, PETA-3, and CD165 expression; however, 1–2 weeks of treatment were required before these effects became evident. Aspirin therapy was associated with mildly diminished expression of the anti protease-activated G-protein-coupled (PAR-1) thrombin receptor measured by SPAN12 and WEDE15 antibodies. Treatment with aspirin also resulted in a paradoxical mild but consistent enhancement of GPIb receptor expression. Finally, LAMP-1, and LAMP-3 expression was not affected by aspirin.

3.4. Aggrenox®

Treatment with Aggrenox® resulted in a rapid sustained inhibition of epinephrine-induced conventional platelet aggregation, and mild decrease of adenosine diphosphate induced aggregation. Cartridge-based platelet analyzers revealed prolongation of the closure time by the PFA-100 instrument, and decreased aspirin reactive units measured by the Ultegra® device. These data suggest platelet inhibition, especially after 2 weeks of therapy. There were various phasic changes, but most notably downregulation of

Table 2
Changes of platelet characteristics in the AGATE trial

Parameter (mean±S.D.)	Baseline	24 h	3 days	7 days	15 days	30 days
Platelet aggregation						
5 μM epinephrine (%)						
Aggrenox®	76.9±9.8	34.6±10.6[a]	27.8±10.2	23.4±8.8[a]	20.8±7.1[a]	25.0±10.5[a]
Aspirin	78.5±7.2	26.5±21.0[a]	25.8±20.7[a]	27.8±18.7[a]	29.5±19.3[a]	33.3±18.0[a]
P-value	0.54	0.51	0.86	0.11	0.066	0.084
5 μM ADP (%)						
Aggrenox®	72.6±10.8	60.2±12.6[a]	54.5±9.7[a]	52.3±10.0[a]	51.9±11.7[a]	53.7±10.6[a]
Aspirin	74.5±7.5	61.4±10.8[a]	50.0±11.9[a]	47.5±13.9[a]	49.15±12.4[a]	50.4±10.4[a]
P-value	0.53	0.73	0.209	0.21	0.47	0.35
Whole blood analyzers						
PFA-100™, closure time (s)						
Aggrenox®	176±40	261±47[a]	254±35[a]	251±36[a]	262±43[a]	268±43[a]
Aspirin	160±23	263±43[a]	251±49[a]	249±43[a]	242±51[a]	231±69[a]
P-value	0.12	0.9	0.82	0.85	0.2	0.04
Ultegra®, RPFA-ASPIRIN (ARU)						
Aggrenox®	652±54	464±62[a]	491±45[a]	477±48[a]	441±43[a]	458±54[a]
Aspirin	671±53	462±88[a]	488±62[a]	507±64[a]	467±75[a]	486±95[a]
P-value	0.26	0.92	0.84	0.11	0.18	0.26
Flow-cytometry						
CD31 (PECAM-1) (MFI)						
Aggrenox®	56.2±16.0	38.1±13.8[a]	34.8±11.4[a]	36.8±9.2[a]	38.5±8.7[a]	31.5±14.7[a]
Aspirin	55.1±16.4	45.1±17.7[a]	43.9±14.7[a]	54.3±12.9	44.5±15.2	50.3±17.1
P-value	0.82	0.16	0.035	0.001	0.14	0.001
CD41a (GPIIb antigen) (MFI)						
Aggrenox®	452±73	329±49[a]	346±59[a]	350±71[a]	376±73[a]	349±49[a]
Aspirin	458±56	341±57[a]	383±59[a]	424±67	429±68	424±48
P-value	0.71	0.5	0.05	0.01	0.026	0.002
PAC-1 (GPIIb/IIIa activity) (MFI)						
Aggrenox®	13.7±3.7	7.4±3.1[a]	7.6±2.5[a]	8.2±2.3[a]	7.0±2.6[a]	5.3±3.1[a]
Aspirin	13.8±3.1	7.8±3.0[a]	7.1±3.1[a]	7.0±2.8[a]	9.0±2.4	9.6±2.7[a]
P-value	0.95	0.66	0.59	0.16	0.017	0.001
CD42b (GPIb) (MFI)						
Aggrenox®	256±44	302±65[a]	292±52	270±70	253±56	248±63
Aspirin	250±30	295±32[a]	283±42[a]	316±46[a]	237±60	284±32[a]
P-value	0.63	0.66	0.55	0.021	0.41	0.032
CD62p (P-selectin), %+						
Aggrenox®	9.6±3.1	7.1±3.0[a]	6.3±2.4[a]	5.5±2.9[a]	4.8±2.7[a]	4.7±2.5[a]
Aspirin	10.6±3.3	7.4±3.1[a]	7.6±3.1[a]	8.4±3.0[a]	7.1±3.1[a]	6.4±3.3[a]
P-value	0.32	0.74	0.16	0.003	0.017	0.078
CD63 (LAMP-3) (MFI)						
Aggrenox®	8.3±2.9	8.0±2.8	7.2±3.4	6.7±3.5	5.3±3.1[a]	6.7±2.7
Aspirin	10.0±2.9	10.8±3.1	8.8±3.4	8.9±3.1	8.1±2.4	9.6±2.7
P-value	0.079	0.04	0.12	0.038	0.02	0.01
CD107a (LAMP-1) (MFI)						
Aggrenox®	4.8±1.5	5.3±1.8	5.2±1.9	4.8±1.9	4.6±2.0	5.1±1.8
Aspirin	4.9±1.7	4.3±1.2	4.1±1.1	4.8±1.3	5.4±1.2	4.8±2.3
P-value	0.8	0.028	0.032	0.98	0.16	0.6
CD151(PETA-3) (MFI)						
Aggrenox®	134.3±31.7	144.6±30.3	142.1±31.1	122.6±24.9	149.4±30	135.1±28.3
Aspirin	128.4±23.1	119.8±23.1	129.2±26.0	136.3±30.1	107.2±28[a]	122.0±25.1
P-value	0.5	0.056	0.162	0.12	0.01	0.136
CD151+CD14 (platelet–monocyte aggregates formation) (MFI)						
Aggrenox®	141.6±20.7	143.5±31.6	105±23.6[a]	90.1±30.0[a]	107.8±30[a]	93.3±26.2[a]
Aspirin	139.2±25.4	134.4±19.9	106.5±20[a]	93.9±23.6[a]	102±22.2[a]	85.9±32.2[a]
P-value	0.74	0.28	0.82	0.65	0.47	0.44
CD154 (CD40 ligand) (MFI)						
Aggrenox®	8.9±3.2	9.0±2.4	8.6±2.1	8.0±2.3	7.0±2.6	5.9±1.7[a]
Aspirin	8.8±2.6	8.2±1.9	8.1±2.9	8.6±3.4	6.6±2.6[a]	6.4±1.8[a]
P-value	0.86	0.22	0.50	0.56	0.66	0.34

(continued on next page)

Table 2 (*continued*)

Parameter (mean±S.D.)	Baseline	24 h	3 days	7 days	15 days	30 days
CD165 (GP37) (MFI)						
Aggrenox®	25.8±5.6	26.5±4.6	27.1±7.0	25.0±4.5	23.4±4.6	19.0±4.8[a]
Aspirin	26.1±5.1	22.9±5.7	25.3±7.0	25.1±6.8	22.1±4.8[a]	17.8±3.3[a]
P-value	0.89	0.37	0.45	0.99	0.41	0.39
Thrombin receptor (PAR-1) WEDE 15 (epitope of cleaved receptors) (MFI)						
Aggrenox®	21.5±4.8	12.5±4.1[a]	12.9±3.5[a]	8.0±3.2[a]	9.1±3.8[a]	9.1±3.7[a]
Aspirin	21.3±4.4	20.6±5.4	15.9±5.7[a]	17.6±4.4[a]	15.5±3.7[a]	15.7±4.3[a]
P-value	0.87	0.01	0.056	0.01	0.01	0.01
Thrombin receptor (PAR-1) SPAN 12 (epitope of intact receptors) (MFI)						
Aggrenox®	35.5±8.5	23.8±5.2[a]	15.8±5.0[a]	18.1±5.0[a]	15.3±3.5[a]	13.4±3.5[a]
Aspirin	34.0±8.8	30.8±8.0	27.3±7.3[a]	24.3±8.1[a]	23.2±6.3[a]	21.7±7.5[a]
P-value	0.61	0.01	0.01	0.03	0.01	0.002
Thrombospondin						
Aggrenox®	12.7±3.2	9.7±3.4[a]	8.2±2.2[a]	7.5±2.3[a]	7.8±2.5[a]	7.1±2.9[a]
Aspirin	13.8±3.1	9.1±3.9[a]	6.9±2.7[a]	6.1±2.0[a]	6.6±2.7[a]	6.9±3.1[a]
P-value	0.25	0.58	0.12	0.045	0.17	0.82

[a] *P* values <0.05 vs. own baseline values by Wilcoxon rank-sum test. Individual flow cytometric histograms were calculated using the Smirnov–Kolgomorov test. ARU—aspirin reactive units; MFI—mean fluorescence intensity; %+—percent of positive cells.

platelet surface receptor expression after Aggrenox® therapy, such as sustained blockade of PECAM-1, P-selectin, thrombospondin, GPIIb antigen expression, and diminished GPIIb/IIIa activity. Aggrenox® reduced the formation of platelet–monocyte aggregates, CD40-ligand, LAMP-3, and CD165 expression; however, these effects were more profound at later time intervals. Treatment with Aggrenox® was associated with a rapid, threefold inhibition of both intact and cleaved PAR-1 thrombin receptors. Aggrenox® therapy was associated with an early increase of GPIb expression; however, this effect was not observed later in the course of therapy. Finally, PETA-3, and LAMP-1 expression were not affected by Aggrenox®.

3.5. Aggrenox® versus aspirin

The data for 18 different platelet markers at five different time points after the initiation of antiplatelet therapy are displayed in Table 2. Significant differences between aspirin and Aggrenox® over time are presented in Fig. 2. Platelet aggregation studies revealed that there was a strong time dependent late trend towards more inhibition of the epinephrine induced aggregation in the Aggrenox® group, although this difference did not reach significance. Adenosine diphosphate-induced aggregation did not differ between groups at any time point. Closure time was longer in the Aggrenox® group (P=0.04) at 30 days, while the Ultegra instrument readings were similar between the treatment arms. PECAM-1, and GPIIb/IIIa inhibition was more prominent in patients treated with Aggrenox® starting at Day 7, and consistently thereafter. Expression of GPIb was significantly lower for Aggrenox® at day 7, and Day 30. P-selectin, the established marker of platelet activation, was lower in the Aggrenox® group after Day 7 as well. Similar changes were exhibited by the LAMP-3 receptor, which was diminished in the Aggrenox® group at most time points. The most striking difference was found for the alpha-thrombin PAR-1 platelet receptors. Both cleaved and intact

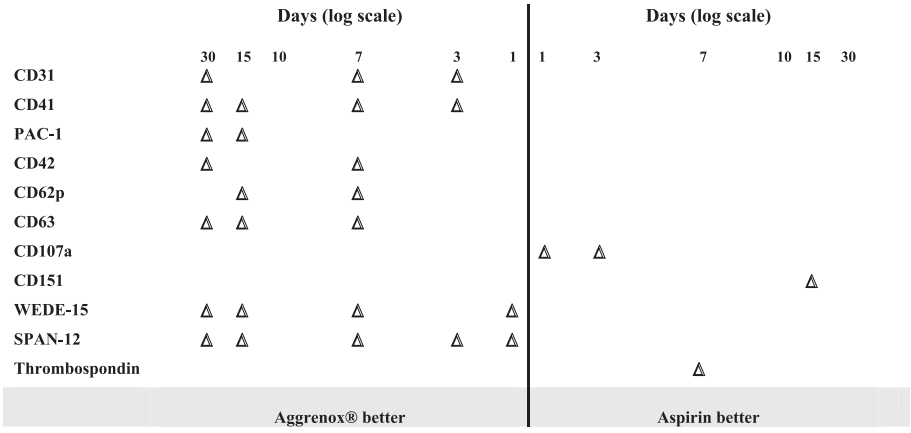

Fig. 2. Differences in platelet inhibition with aspirin and Aggrenox® in the AGATE trial. ▲—represents a statistical difference by the Wilcoxon rank-sum test between Aggrenox® and aspirin for the certain receptor during long-term platelet function monitoring. There are 25 time points when Aggrenox® downregulate platelet receptors better than aspirin, and four measures when aspirin exhibited stronger inhibition of surface receptors than Aggrenox®.

epitopes of this G-coupled protein were significantly inhibited in the Aggrenox® treated patients at all time points. CD-40 ligand, CD165, and platelet monocyte microparticles did not differ between groups. Platelet/endothelial tetraspan antigen-3, and lyzosome-associated membrane protein-1, as well as the platelet expression of thrombospondin were lower in the aspirin group.

4. Discussion

The AGATE trial provides the first randomized data of the magnitude, and detailed time course analyses, of platelet biomarkers activity after 30 days antiplatelet therapy with aspirin and Aggrenox® in post-stroke patients. The antiplatelet properties of Aggrenox® were stronger than those of the higher daily dose of aspirin especially later in the trial. Importantly, the randomized, double-blind design enhances the validity of the results, because the data are consistent regardless of the method used for assessing platelet function. Applying a wide panel of techniques minimizes the error by measuring different parameters indicative of various platelet characteristics. In the present trial, the antiplatelet activity of both agents has been documented by conventional optical aggregometry induced by several agonists, by the PFA-100 platelet analyzer assessing shear-induced activation, and by the Ultegra instrument with the propyl gallate cartridge designed specifically for aspirin monitoring. In addition, we utilized whole blood flow cytometry techniques measuring expression of multiple receptors located on the platelet surface. Considering the marked heterogeneity of platelet activity among and within groups, we used multiple tests to comprehensively assess platelet function to ensure adequate evaluation of platelets. It is well established that aspirin inhibits platelets very rapidly (Serebruany et al., 2003), but downregulation of certain platelet receptors can be observed only after chronic treatment with either aspirin or Aggrenox®. However, despite the obvious heterogeneity, at individual time points, Aggrenox® was superior to aspirin in 25 out of 90 comparisons, while aspirin yielded stronger platelet inhibition in 4 out of 90 measures (see Fig. 2 for details). In subgroup analyses, the apparent superiority of Aggrenox® over aspirin has been observed later (Day 15–Day 30) after treatment assignment. This indicates that 2 weeks of therapy may be necessary to achieve sustained mild platelet inhibition, and speculatively, optimal clinical benefit. This hypothesis, if verified, might have an important clinical impact on long-term patient care, and may explain, at least in part, the apparently null results from earlier short-term trials with dipyridamole (Fitzgerald, 1987; Gibbs and Lip, 1998).

While the major pathway of antiplatelet efficacy of aspirin is well described, the data on the effect of dipyridamole on platelet function are numerous, but less clear. The differences in the results, if real, could be attributable to vasodilatation (Akinboboye et al., 2001), antithrombotic effects (Eisert, 2001), antioxidant effects (Iuliano et al., 1995; Selly et al., 1994), oe prostacyclin (Neri Serneri et al., 1981; Costantini et al., 1990) and nitric oxide (Bult et al., 1991; De La Cruz et al., 2000) stimulating properties of dipyridamole, rather than to any direct antiplatelet efficacy. This is especially evident when platelet function is assessed exclusively by conventional plasma aggregometry. Dipyridamole may yield additional benefit by suppressing thrombus formation (Muller, 2001), and inhibiting smooth muscle cell proliferation (Singh et al., 1994; Himmelfarb and Couper, 1997) as demonstrated from in vitro and animal models.

The AGATE randomized data of small sample size suggest that adjunctive benefits of dipyridamole and low dose aspirin constituting Aggrenox® may extend beyond simply diminishing platelet aggregation. Therefore, we applied multiple platelet tests including assessment of 15 surface receptors in an attempt to prove that Aggrenox is not a “classical” antiplatelet agent targeting platelet aggregation. In fact, delicate downregulation of various activation-dependent platelet receptors may represent the unique quality of the drug, especially in the chronic post-stroke setting. It is presently becoming apparent that aggressive antiplatelet regiments may represent a threat rather than cure due to the unremarkable platelet characteristics in most post-stroke patients when compared with normal controls (Serebruany et al., 2004). However, it is plausible to speculate that based on the AGATE data, Aggrenox® may potentially override insufficient platelet inhibition and/or inadequate antithrombotic properties of monotherapy with aspirin. This hypothesis requires further research as data describing the effects of Aggrenox® on platelet function are sparse. Most of these data are derived from in vitro tests, animal experiments, uncontrolled human studies in healthy volunteers, and acute stroke patients.

Several hypotheses may be formulated from AGATE. First, both agents, and especially aspirin increase platelet glycoprotein Ib (GPIb) expression. GPIb is a transmembrane protein with approximately 25,000 copies per platelet, and is a part of the glycoprotein Ib/IX-V complex that forms the platelet von Willebrand factor (vWF) receptor (Schade et al., 2003). The vWF binding to platelet GpIb-IX-V plays a key role in the adhesion under shear flow conditions to collagen, fibronectin, and fibrinogen. Considering the fact that the peak of platelet-vWF expression occurs during alpha-granule secretion, and that aspirin impairs this event, platelets can increase surface expression of platelet-vWf receptors as a consequence of platelet shape change (Parker and Gralnick, 1989). These data are consistent with the AGATE trial finding, and are supported by the prolongation of the closure time as assessed by the PFA-100 Analyzer in our study, which is indicative of the platelet shape changes.

Importantly, most of the differences between aspirin and Aggrenox® with regard to platelet inhibition occur after at

least a week after therapy starts. Time-dependent increases in differences in the parameters of platelet activity at day 15 probably suggests some sort of rebound or "hostility" of young platelets attempting to resist antiplatelet agents. Clearly, the most profound differences favoring Aggrenox® happened after 2 weeks of therapy. Special interest is acknowledged for those platelet receptors which were inhibited by low dose aspirin more profoundly than with Aggrenox®, namely LAMP-1 and thrombospondin. Besides alpha- and delta-granules, platelets contain lysosomes which store mainly acid hydrolases. LAMP-1 is a highly *N*-glycosylated protein, and the constituent of lysosomal membranes. Although LAMP-1 is distributed within the cell primarily in the lyzosome, during platelet activation it is also expressed at the cell surface (Febbraio and Silverstein, 1990), and can serve as the ligand for selectins and mediate cell–cell adhesion/recognition events (Laferte and Dennis, 1989). Our data confirm previous reports that aspirin moderately, but still significantly, diminishes expression of LAMP-1 on the platelet surface (Ciferri et al., 2000), presumably via the ability of aspirin to impair hydrolase release from platelets (McKenzie et al., 2003). The matricellular protein thrombospondin is a 450-kDa homotrimeric glycoprotein that influences platelet function by modulating cell–matrix interactions (Lawler, 2000). Thrombospondin is secreted from smooth muscle cells (Majack et al., 1987), and its expression and secretion may be induced by nitric oxide (Dixit et al., 1985; Tuszynski et al., 1988). Thrombospondin accumulation was observed in atherosclerotic and restenotic arteries, including carotid and coronary vessels (van Zanten et al., 1994; Ichii et al., 2002), although the clinical significance of these findings is unclear.

On the other hand, GPIb is a high-affinity receptor for alpha-thrombin (van Zanten et al., 1998), which may be linked to the second unexpected finding in the AGATE trial. We observed the consistently time-dependent inhibition of another thrombin receptor, the anti protease-activated G-protein-coupled (PAR-1) receptor, slightly in aspirin group, and profoundly in the Aggrenox® treated patients. Protease Activated Receptor-1 (PAR-1) is a member of a novel gene family of G-protein coupled receptors (Cupit et al., 1999). PAR-1 is a single polypeptide of 66 kDa with a thrombin cleavage site located near the extracellular N terminus (Vu et al., 1991). This receptor is expressed by human platelets and is responsible for attracting alpha-thrombin to the platelet surface (Kahn et al., 1999). The monoclonal antibodies used in the AGATE trial specifically reacts with intact (SPAN12) and cleaved (WEDE 15) epitopes of PAR-1 (Brass et al., 1994). We found a consistent and significant reduction of PAR-1 platelet expression in both groups, but significantly more for Aggrenox®. Whether this finding has clinical implications is presently uncertain, but may represent a novel mechanism of antiplatelet activity by aspirin and dipyridamole. PAR-1 inhibition may represent a compensatory response of platelets to the enhanced GPIb expression, which is well described for the more potent antiplatelet agents, especially for the GPIIb/IIIa inhibitors (Xiao et al., 1999; Serebruany et al., 2000).

5. Strengths and limitations

Randomized design, comprehensive laboratory assessment, long-term monitoring, and frequent blood sampling for the platelet studies are among the strengths of this trial. There are, however, certain limitations as well. Although randomized, and compatible in size with other similar investigations, AGATE was a trial of a small sample size cohort, so chance represents a plausible alternative explanation. High frequency of use of concomitant medications may have affected the platelet characteristics; however, the use of major drugs was similar between the groups. In addition, the expression of multiple activation-dependent platelet receptors was studied but their individual roles in patients after stroke are unknown. One may argue that non-compliant patients represent an unusual and potentially different population. However, the screening in particular focused to exclude those with medical reasons for not taking aspirin. Finally, we enrolled only aspirin-free patients after recent ischemic stroke, which diminished the differences in the baseline platelet characteristics, but limited clinical relevance of our study. On the other hand, we found that >10% of patients after ischemic stroke in fact are not receiving aspirin, indicatig compliance issues.

In summary, this randomized trial suggests that aspirin and Aggrenox® produced fast and sustained platelet inhibition. In 26 of 90 direct comparisons, Aggrenox® was superior to aspirin, whereas in 5 of 90, aspirin was superior to Aggrenox®, and in 59 of 90 direct comparisons, aspirin and Aggrenox® were equivalent. Aggrenox® exhibited antiplatelet properties mostly after 15 days of therapy, and was associated with a profound reduction of PAR-1 receptors, an observation that may be part of the explanation of greater clinical benefit of Aggrenox® compared with aspirin in preventing recurrent stroke in ESPS-2. The current data show that a number of platelet markers beyond conventional aggregometry may be useful in monitoring antiplatelet therapy in the future stroke trials.

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