Aspirin in the prevention of cardiovascular disease in type 2 diabetes

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High levels of LDL-cholesterol and triglycerides and poor glycemic control are associated with diminished aspirin responsiveness in patients with type 2 diabetes

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Abstract

Objective: Increased platelet aggregability contributes to high incidence of cardiovascular events in type 2 diabetes. Moreover, it is suggested that failure of aspirin to inhibit platelet thromboxane A2 production or "aspirin non-responsiveness" can be found more frequently in diabetic patients. We aimed to determine patient-related factors predictive of aspirin non-responsiveness and to assess differential effects on platelet aggregation of aspirin 300 mg compared to 100 mg in type 2 diabetic patients without cardiovascular events.

Research design and methods: A randomized, placebo-controlled, double-blind, cross-over trial was performed in 40 type 2 diabetic patients. In two periods, patients used either 100 mg or 300 mg aspirin and placebo. Light transmittance aggregometry was performed in response to adenosine diphosphate 0.4 µM and 4 µM, collagen 2 µg/mL and arachidonic acid 1.5 mM. Non-responsiveness was defined as arachidonic acid-induced aggregation of >20% after aspirin therapy.

Results: Seven (18%) patients did not respond adequately to aspirin, which was predicted by high levels of LDL-cholesterol and triglycerides and poor glycemic control (HbA_{1c}>7%) in multivariate logistic regression. Five non-responders used 100 mg whereas two used 300 mg (odds ratio 3.0, 95% confidence interval 0.5-17.7). Use of 300 mg resulted in increased inhibition of collagen-induced aggregation compared to 100 mg (45% vs. 21%, *P*=0.016).

Conclusions: Prevalence of aspirin non-responsiveness is high in type 2 diabetic patients. High levels of LDL-cholesterol and triglycerides and poor glycemic control (HbA_{1c}>7%) predict non-responsiveness. An increase in dosage may improve aspirin effectiveness.

Introduction

Patients with type 2 diabetes have a 2-4 fold increased risk for cardiovascular events (1). The excess global mortality attributable to diabetes in the year 2000 was estimated to be 2.9 million deaths and is expected to increase given the growing prevalence worldwide (2,3). Several mechanisms related to metabolic disturbances in diabetes contribute to the increase in cardiovascular risk. In addition to abnormalities in endothelial function and vascular smooth muscle cells, patients with type 2 diabetes exhibit a prothrombotic tendency (4).

Platelet dysfunction resulting in a propensity for platelet aggregation contributes to the prothrombotic state (5). The excretion rate of 11-dehydro-thromboxane B2, a stable metabolite of thromboxane A2 and marker for platelet activation, is increased in patients with type 2 diabetes compared with control subjects (6,7). Hyperglycemia, insulin resistance and oxidative stress all contribute to this increased platelet aggregability (8,9).

Nowadays, use of acetylsalicylic acid or aspirin as inhibitor of platelet aggregation is an effective strategy to reduce cardiovascular events in patients at high risk. Recognizing the increased cardiovascular risk, various international guidelines advocate the use of aspirin in both primary and secondary prevention of cardiovascular events in patients with type 2 diabetes (10,11). However, evidence for a beneficial effect in primary prevention of cardiovascular events in patients with type 2 diabetes is surprisingly scarce. Various studies using low-dose aspirin (≤100 mg daily) to reduce cardiovascular risk in patients with type 2 diabetes showed no significant reduction of events (12,13).

One of the explanations of the apparently attenuated clinical benefit of aspirin in this subgroup is failure of aspirin to produce the expected pharmacological effect in patients with diabetes. This concept, aspirin non-responsiveness or resistance, is defined as failure of aspirin to inhibit platelet thromboxane A2 production or inhibit tests of thromboxane-dependent platelet function (14). Indeed, a reduced inhibition of platelet function by aspirin was found in patients with diabetes compared with controls (15). Moreover, in a cohort of 168 subjects, poor responsiveness to aspirin as determined by a platelet function analyzer (PFA-100®, Dade-Behring, Illinois, USA)

was associated with the presence of diabetes mellitus (16). Certain lines of evidence suggest that a higher aspirin dosage might overcome the inadequate suppression of thromboxane A2 production. In a crossover experiment, Hart *et al.* showed a significant decrease in serum and urinary markers of thromboxane in patients with cardiovascular disease treated with 325 mg aspirin daily compared to 81 mg daily (17). Yet, in patients with type 2 diabetes it is still unclear which patient-related factors or differences in dosage of aspirin determine the effect of aspirin on platelet aggregation.

To test the hypothesis that use of 300 mg aspirin daily results in more profound inhibition of platelet aggregation compared to 100 mg and to determine patient-related factors predictive of platelet aggregation, we evaluated agonist-induced platelet aggregation in patients with type 2 diabetes without cardiovascular events.

Methods

Study subjects were recruited from general practitioners affiliated to Leiden University Medical Center. Subjects were eligible for inclusion if they had been diagnosed with type 2 diabetes for at least 1 year, had glycated hemoglobin A1c (HbA1c) levels <10% and C-reactive protein levels >1.0 mg/L, and were both aged above 18 years old and capable to give informed consent. Patients were excluded if they had any history of cardiovascular disease (defined as myocardial infarction, acute coronary syndrome, percutaneous coronary intervention, coronary artery bypass grafting, heart failure, severe cardiac arrhythmia, cerebrovascular accident, transient ischemic attack or peripheral vascular disease) or known contraindications to use of aspirin (defined as history of asthma, any bleeding disorder, gastrointestinal tract bleeding or known allergy to acetylsalicylic acid). Other exclusion criteria were presence of uncontrolled hypertension, severe renal or hepatic dysfunction, pregnancy, recent participation in other research projects or blood donation and use of non steroidal anti-inflammatory drugs, anticoagulant medication, corticosteroids or statins. All patients gave written informed consent and the study was approved by the Leiden University Medical

Center medical ethics committee and performed in accordance with the Declaration of Helsinki.

The study had a prospective, randomized, placebo-controlled, double-blind, and crossover design. Subjects were randomly assigned to receive aspirin 100 mg or 300 mg. The first treatment period with aspirin or placebo for 6 weeks was followed by a washout period of 4 weeks. Thereafter, those assigned to placebo in the first period received aspirin for 6 weeks and those assigned to aspirin received placebo for additional 6 weeks. Double blind study medication was prepared and stored at the department of clinical pharmacy of Leiden University Medical Center. A computer-generated randomization code was prepared by this department. Medication was prepackaged on the basis of a block size of four. Each consecutive subject was given the next consecutive randomization number and eligible subjects were assigned in a 1:1 ratio to receive the study drug or placebo.

Subjects visited the research site after an overnight fast at the beginning and at the end of each six-week treatment period. By a structured interview, we asked for compliance, possible adverse events and changes in medication. To further assess compliance, remaining pills were counted. At baseline, routine hematological and chemical variables were determined.

Blood sampling

At each visit, blood samples were drawn from antecubital veins. For aggregometry, blood was collected in tubes containing 0.105 M (3.2%) sodium citrate, in a ratio of 9 volumes of whole blood to 1 volume anticoagulant. To avoid spontaneous platelet activation the first 4 mL blood was discarded. Aggregometry was performed within one hour after blood sampling.

Platelet aggregation

Platelet aggregation was analysed at the end of each treatment period. Optical platelet aggregation was performed on a Chrono-Log 490 aggregometer (Kordia Life Sciences, Leiden, the Netherlands). Platelet-rich plasma (PRP) was prepared by centrifugation for 15 minutes at 126*g* and platelet-poor plasma (PPP) was prepared by centrifugation for 15 minutes at 2700*g,* all at 18°C. PRP is arbitrarily considered to

be 0% light transmission or 0% aggregation and PPP is considered to be 100% light transmission or 100% aggregation. The following final concentrations of agonists were used to induce aggregation: adenosine diphosphate (ADP) 0.4 µM; ADP 4 µM; collagen 2 µg/mL; and arachidonic acid 1.5 mM. Percentage aggregation was recorded after 15 minutes.

Poor responsiveness to aspirin was defined as 1.5 mM arachidonic acid-induced aggregation of >20% in absence of non-compliance. This method is used in other recent studies on aspirin responsiveness and is considered to be the most direct method indicating inhibition of cyclooxygenase-1 (18).

Statistical analysis

Continuous variables are presented as mean values ± standard deviation and categorical variables as frequencies (percentages). Comparisons between continuous variables were performed with independent samples *t* tests or Mann-Whitney *U* tests if not normally distributed. Categorical variables were compared with Pearson χ^2 or Fisher's exact tests as appropriate. Differences in aggregometry after aspirin and placebo were analyzed using paired samples *t* tests. To examine differences in platelet inhibition between 100 mg and 300 aspirin we compared percent inhibition of aggregation with independent samples *t* tests. To assess which patient-related factors were independently associated with aspirin responsiveness, we performed multivariate logistic regression analyses with all baseline variables as covariates which differed between aspirin responders and non-responders in univariate analyses ($P<0.10$). Analyses were performed using SPSS version 12.01 (SPSS, Illinois, USA). All analyses were two-sided, with a level of significance of $\alpha=0.05$.

Results

Subject characteristics are summarized in Table 1. No statistical differences between the groups were observed. All subjects were fully compliant to study medication and there were no adverse events.

Table 1 – Baseline characteristics

	Aspirin 100 mg (*n* = 20)	Aspirin 300 mg (*n* = 20)	*P*
Age (yrs)	59.0 ± 10.5	54.5 ± 9.6	0.161
Female sex	9 (45)	5 (25)	0.185
BMI (kg/m^2)	30.0 ± 6.3	31.5 ± 5.3	0.420
Waist circumference (cm)	103 ± 12	106 ± 11	0.420
Hip circumference (cm)	106 ± 13	107 ± 10	0.936
Systolic tension (mm Hg)	154 ± 15	150 ± 16	0.468
Diastolic tension (mm Hg)	90 ± 9	90 ± 9	0.939
Smoking	2 (10)	2 (10)	1.000
Laboratory data			
Glucose (mmol/L)	8.3 ± 3.0	7.7 ± 1.1	0.422
HbA1c (%)	6.2 ± 1.4	5.9 ± 0.8	0.426
HsCRP (mg/L)	8.4 ± 12.6	9.0 ± 9.8	0.768
Creatinin (µmol/L)	81.2 ± 15.2	85.1 ± 13.9	0.402
HDL-cholesterol (mmol/L)	1.5 ± 0.4	1.4 ± 0.3	0.253
LDL-cholesterol (mmol/L)	3.5 ± 1.0	3.8 ± 0.8	0.299
Triglycerides (mmol/L)	1.8 ± 1.3	2.0 ± 1.1	0.563
VWF:Ag (IU/mL)	1.4 ± 0.5	1.2 ± 0.5	0.260
Fibrinogen (g/L)	4.0 ± 0.7	4.2 ± 0.7	0.255
Medications			
ACEI/ARB	5 (25)	9 (45)	0.185
Diuretics	3 (15)	3 (15)	1.000
β-blockers	3 (15)	5 (25)	0.695
Oral hypoglycemic drugs	14 (70)	13 (65)	0.736
Insulin	4 (20)	4 (20)	1.000

Data are *n* (%) or means ± standard deviation (SD). ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; BMI, body mass index; HDL, high-density lipoprotein; HsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; VWF:Ag: von Willebrand factor antigen.

Effects on platelet aggregation

Both aspirin 100 and 300 mg significantly inhibited platelet aggregation compared to placebo in response to 4.0 µM ADP, collagen and arachidonic acid (Table 2, Figure

1). Use of aspirin 300 mg resulted in a more profound inhibition of collagen-induced aggregation than 100 mg (45% vs. 21%, *P*=0.016). Using other agonists, no significant differences between 100 and 300 mg were found.

Table 2 – Light transmittance aggregometry after aspirin and placebo treatment

	Aspirin 100 mg			Aspirin 300 mg			
Agonist	Placebo	Aspirin	*P**	Placebo	Aspirin	*P**	*P*†
ADP 0.4 µM	17 ± 28	5 ± 3	0.059	4 ± 4	6 ± 5	0.079	0.288
ADP 4.0 µM	67 ± 10	52 ± 9	< 0.001	61 ± 18	47 ± 13	0.002	0.821
Collagen 2.0 µg/mL	94 ± 10	73 ± 22	0.002	94 ± 10	51 ± 28	< 0.001	0.016
AA 1.5 mM	92 ± 6	24 ± 36	< 0.001	90 ± 7	13 ± 26	< 0.001	0.259

Data are means ± standard deviation of percentage of aggregation. **P*-values for differences in percentage aggregation between aspirin 100 mg or 300 mg and placebo. †*P*-values for difference in percent change between aspirin 100 mg and 300 mg. AA, arachidonic acid; ADP, adenosine diphosphate.

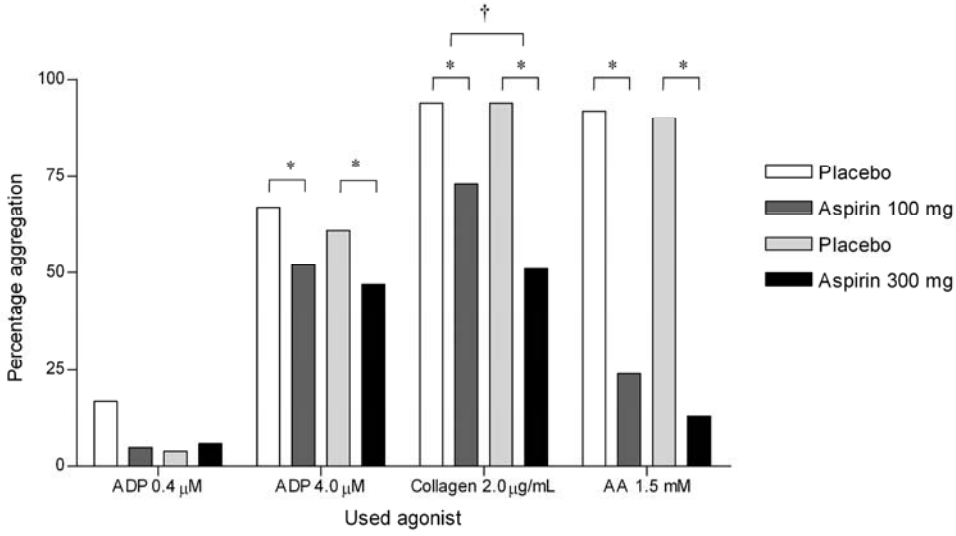

Figure 1 – Light transmittance aggregometry after aspirin and placebo treatment

* Significant difference between aggregation after placebo and aspirin therapy. † Significant difference in percent change between aspirin 100 mg and 300 mg. AA, arachidonic acid; ADP, adenosine diphosphate.

Aspirin non-responsiveness

Seven patients met the definition of aspirin non-responsiveness (18%, 95% confidence interval (CI) 7-33%). Five of them were randomized to 100 mg aspirin, whereas two non-responders used 300 mg, resulting in an odds ratio for non-responsiveness of 3.0 (95%CI 0.5-17.7). Compared to responders, aspirin non-responders established a diminished inhibition of platelet aggregation following stimuli with both collagen (7% vs. 38%, *P*=0.016) and arachidonic acid (8% vs. 95%, *P*<0.001) (Figure 2).

Subjects with poor aspirin responsiveness differed in baseline characteristics compared to those with a good response (Table 3). In non-responders higher values of LDL-cholesterol (4.3±1.1 vs. 3.5±0.7, Figure 3), triglycerides (3.0±2.0 vs. 1.7±0.9), glucose (9.3±4.3 vs. 7.7±1.4) and HbA1c (6.7±1.6 vs.5.9±1.0) were found. In a logistic regression model (Table 4) with LDL-cholesterol, triglycerides and HbA1c as covariates, both high LDL-cholesterol and triglycerides independently predict aspirin non-responsiveness (*P*-values 0.025 and 0.038, respectively). HbA1c was not significantly associated with non-responsiveness (*P*=0.065). However, when HbA1c values were dichotomized in good and poor glycemic control (cut-off value 7.0%), poor control was an independent determinant of aspirin non-responsiveness in this model (odds ratio 21, 95%CI 1-440, *P*=0.049).

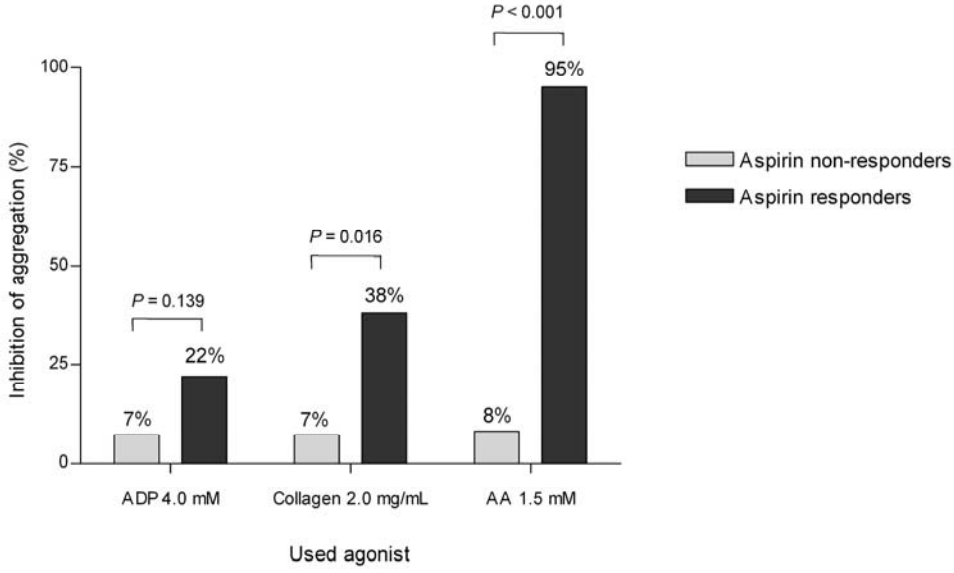

Figure 2 – Inhibition of platelet aggregation in aspirin responders and non-responders

Table 3 – Baseline characteristics of aspirin responders and non-responders

	Non-responders (*n* = 7)	Responders (*n* = 33)	*P*
Age (yrs)	61.4 ± 13.3	55.7 ± 9.4	0.183
Female sex	4 (57)	10 (30)	0.214
BMI (kg/m^2)	33.2 ± 5.1	30.2 ± 5.9	0.211
Waist circumference (cm)	108 ± 7	104 ± 12	0.356
Hip circumference (cm)	113 ± 13	105 ± 11	0.097
Systolic tension (mm Hg)	149 ± 12	152 ± 16	0.603
Diastolic tension (mm Hg)	89 ± 9	90 ± 9	0.726
Smoking	1 (14)	3 (9)	0.552
Laboratory data			
Glucose (mmol/L)	9.3 ± 4.3	7.7 ± 1.4	0.084
HbA1c (%)	6.7 ± 1.6	5.9 ± 1.0	0.093
HsCRP (mg/L)	16.6 ± 19.5	7.0 ± 7.9	0.100
Creatinin (µmol/L)	81.3 ± 16.8	83.5 ± 14.2	0.724
HDL-cholesterol (mmol/L)	1.4 ± 0.2	1.5 ± 0.3	0.904
LDL-cholesterol (mmol/L)	4.3 ± 1.1	3.5 ± 0.7	0.009
Triglycerides (mmol/L)	3.0 ± 2.0	1.7 ± 0.9	0.010
VWF:Ag (IU/mL)	1.4 ± 0.7	1.2 ± 0.4	0.392
Fibrinogen (g/L)	4.3 ± 0.5	4.1 ± 0.8	0.406
Medications			
ACEI/ARB	1 (14)	13 (39)	0.387
Diuretics	2 (29)	4 (12)	0.279
β-blockers	3 (43)	5 (15)	0.128
Oral hypoglycemic drugs	6 (86)	21 (64)	0.393
Insulin	1 (14)	7 (21)	1.000

Data are *n* (%) or means ± standard deviation. ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; BMI, body mass index; HDL, high-density lipoprotein; HsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; VWF:Ag: von Willebrand factor antigen.

Table 4 – Determinants of aspirin responsiveness in a multivariate logistic regression model

Determinant	Logistic regression coefficient	Standard error	*P*
LDL-cholesterol	2.533	1.127	0.025
Triglycerides	1.047	7.121	0.038
HbA1c	0.859	0.465	0.065

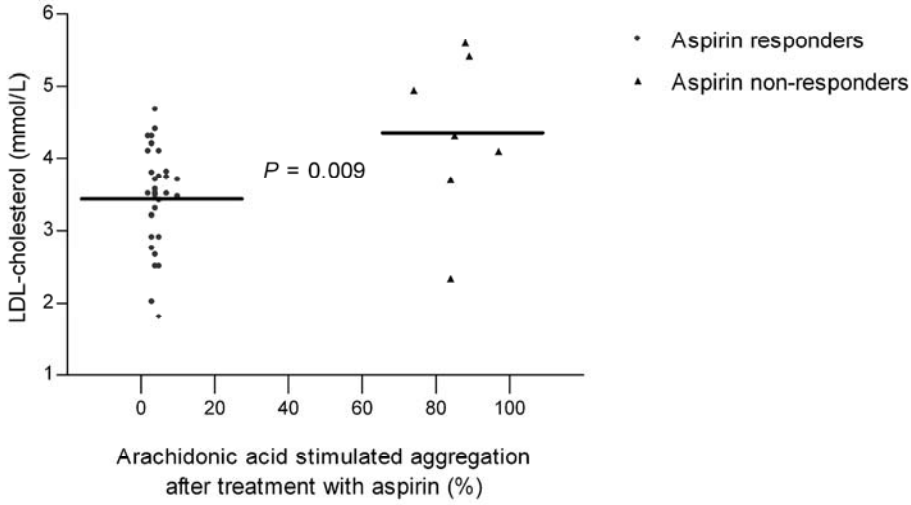

Figure 3 – LDL-cholesterol in aspirin responders and non-responders

Conclusions

This study demonstrates that 18% of patients with type 2 diabetes on aspirin therapy without cardiovascular events fail to inhibit platelet aggregation in response to arachidonic acid. High levels of LDL-cholesterol and triglycerides and poor glycemic control (HbA1c >7%) are patient-related factors that independently predict aspirin non-responsiveness. Though not statistically significant, we found more aspirin non-responsiveness in subjects using aspirin 100 mg compared to 300 mg. Moreover, use of 300 mg resulted in a more profound inhibition of collagen-induced aggregation.

The 18% prevalence of aspirin non-responsiveness we found was relatively high compared to other observations. We used light transmittance aggregometry with arachidonic acid as agonist. This method reflects biochemical action of aspirin most directly, since the main effect of aspirin on platelet activation is inhibition of the cyclooxygenase-1 dependent conversion of arachidonic acid into thromboxane A2. Other studies using this technique found prevalences varying from 0.4-12.1% among patients using aspirin for secondary cardiovascular prevention (18-22). A likely explanation of the high prevalence in our study is formed by the fact that we

studied diabetic subjects. This corroborates findings of prior studies. Watala *et al.* demonstrated a lower maximal inhibition of platelet aggregation in subjects with type 2 diabetes compared to controls (15). Furthermore, Takahashi *et al*. showed that poor responsiveness to aspirin was significantly associated with presence of diabetes (16).

Several factors related to metabolic disturbances in type 2 diabetes were predictive to this poor responsiveness to aspirin. The level of LDL-cholesterol was most strongly associated with aspirin non-responsiveness. LDL-cholesterol has been shown to enhance platelet aggregation and to increase sensitivity of platelets to various agonists via binding of apolipoprotein B100 to a receptor on the platelet membrane and via exchange of lipids to the platelet membrane (23-25). Moreover, in a cohort of 56 patients taking aspirin 325 mg daily, Friend *et al.* observed a significantly higher mean concentration of total cholesterol and LDL-cholesterol in 14 patients with poor responsiveness to aspirin than 42 patients with good responsiveness (26). Besides a quantitative relationship between LDL-cholesterol and aspirin responsiveness, a qualitative alteration of LDL-cholesterol particles effects platelet function and could subsequently influence aspirin responsiveness. In the setting of diabetes, glycation of LDL-cholesterol occurs, resulting in enhanced platelet reactivity (27,28) by an altered interaction with platelets due to compositional and structural changes in LDL-cholesterol (29).

Second, we found that level of triglycerides independently predicted aspirin responsiveness. Remnant-like lipoproteins, derived from triglyceride rich very low-density lipoproteins and chylomicrons stimulate both shear-induced platelet activation (30) and collagen-induced platelet aggregation (31). One could hypothesize that high levels of triglycerides influence platelet function and aspirin responsiveness partly through this pathway. Hitherto, a relationship between remnant-like lipoprotein level and aspirin responsiveness was unknown.

Last, in our study, high HbA1c, reflecting chronic hyperglycemia, was a predictive factor for aspirin responsiveness. Hyperglycemia influences platelet function by various well-known mechanisms, among others by inducing an increase in glycated LDL-cholesterol (8). Recently, Watala *et al*. showed that glucose and aspirin compete with each other for protein free amino groups, resulting in a diminished susceptibility

of platelets to acetylation in presence of hyperglycemia (32). Other studies addressing the relationship between glycemic control and aspirin responsiveness show inconsistent results (15,33,34).

Beside these patient-related factors, used aspirin dosage might influence aspirin responsiveness. We found an odds ratio of 3.0 (95%CI 0.5-17.7) for aspirin non-responsiveness in subjects using aspirin 100 mg compared to them using 300 mg. This was not statistically significant, probably due to the small sample size of our study. Results from other studies support these findings. In a population of 102 patients with type 2 diabetes, Abaci *et al.* assessed PFA-100 closure time before and after the administration of 100 mg aspirin (35). Aspirin non-responsiveness, defined by a closure time <300s, was found in 34 of 102 patients. Prolongation of closure time >300s was obtained in 15 of these 34 patients after additional ingestion of 300 mg aspirin. In another study among 468 coronary artery disease patients, an aspirin dosage ≤100 mg independently predicted presence of aspirin non-responsiveness (36). One of the plausible mechanisms is that higher aspirin levels may shift the balance between glycation and acetylation of proteins towards an increased effectiveness of aspirin. Additionally, non cyclooxygenase-1 pathways are involved in aspirin non-responsiveness, reflected by increased collagen-induced aggregation in aspirin non-responders (37). Indeed, we found less inhibition of collagen-induced aggregation in aspirin non-responders. Interestingly, we also noted a significant difference in collagen-induced aggregation between aspirin 100 mg and 300 mg. These findings confirm the hypothesis that collagen-induced aggregation is related to aspirin responsiveness and that higher aspirin dosages might improve aspirin-mediated platelet inhibition. It is of importance to note that collagen-induced platelet aggregation was independently associated with cardiovascular events (38).

The following potential study limitation warrants comment. Theoretically, lack of inhibition of on arachidonic acid-induced platelet aggregation could also be explained by non-adherence to study medication. However, several features of our study argue against existence of non-compliance. Subjects were highly motivated to participate in this trial, medication was blinded, intervention periods were short and compliance was assessed by interview and pill count. Furthermore, responders and non-responders significantly differed in factors pathophysiologically related

to aspirin sensitivity, which we would not expect if laboratory response was only determined by non-compliance.

In conclusion, our results indicate that among patients with type 2 diabetes inter-individual variation in aspirin responsiveness can be found. High levels of LDL-cholesterol and triglycerides as well as poor glycemic control independently predict non-responsiveness. Since a lower prevalence of non-responsiveness was suggested and collagen-induced aggregation was more inhibited in patients using aspirin 300 mg compared with 100 mg, we believe that an increase in dosage may improve aspirin effectiveness in patients with type 2 diabetes. Large prospective studies are warranted to draw more conclusive answers to this issue.

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