Aspirin in the prevention of cardiovascular disease in type 2 diabetes

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Effects of aspirin on circulating CD34+ stem cells and CD34+/VEGFR-2+ endothelial progenitor cells in patients with type 2 diabetes: a randomized crossover trial

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Abstract

Background: In maintaining endothelial health, circulating cells of haematopoietic nature play an important role. In patients with type 2 diabetes mellitus, the number of these cells are reduced. It is unknown whether use of acetylsalicylic acid (aspirin) has an effect on the amount of circulating vasculogenic cells.

Objectives: To investigate the effect of aspirin on number of circulating $CD34^+$ stem cells and endothelial progenitor cells in patients with type 2 diabetes.

Methods: A randomized, double blind, placebo controlled crossover trial was performed in 40 participants with type 2 diabetes mellitus. Participants were randomized to receive aspirin 100 mg/day or aspirin 300 mg/day and placebo during two treatment periods. After each period, we enumerated $CD34^+$ stem cells and endothelial progenitor cells (EPCs or $CD34^+$/VEGFR-2^+ cells) using flow cytometric analysis. The effect of aspirin on *in vivo* systemic platelet activation was assessed by measuring levels of soluble P-selectin (sP-sel).

Results: The total number of EPCs was significantly reduced after aspirin treatment, on average 12 cells/mL (95%CI -23 to -2; P=0.023). The effect of aspirin 300 mg/day (-15 cells/mL, 95%CI -30 to 0, P=0.046) was more pronounced than with aspirin 100 mg/day (-8 cells/mL, 95%CI -26 to 8, P=0.27). Effects of 300 mg/day on EPC and sP-sel were significantly correlated (P<0.001, r=0.75). No significant differences were observed in the number of $CD34^+$ stem cells at the end of the aspirin treatment period compared with placebo.

Conclusions: In patients with type 2 diabetes, the number of EPC is reduced by aspirin treatment, more profound at doses of 300 mg/day. The number of circulating $CD34^+$ stem cells was not affected by the use of aspirin. These results suggest that treatment with aspirin may impair the endogenous regenerative capacity of the vasculature.

Introduction

Patients with type 2 diabetes mellitus (DM) have an increased risk for cardiovascular events. In a recent population based study in Denmark, the hazard ratio for cardiovascular death was 2.4 in men with DM compared with men without DM (1) Pathophysiological mechanisms resulting in cardiovascular disease are related to the metabolic disturbances in diabetes and include endothelial dysfunction, low-grade vascular inflammation and increased platelet aggregability(2).

Vascular health is primarily maintained by proper functioning of the endothelial cell. Hence, strategies to improve endothelial function are important targets of treatment in cardiovascular medicine. Since a decade, the concept of bone-marrow derived endothelial progenitor cells (EPC) has emerged in cardiovascular research (3, 4). Circulating cells promoting revascularization at sites of ischemia have been proposed to play an important role in maintenance and repair of the vascular endothelium. Autologous transplantation of $CD34^+$ stem cells has already been used for therapeutic angiogenesis in patients with critical limb ischemia (5).

Among patients with increased risk for cardiovascular disease, the number of circulating vasculogenic cells as $CD34^+$ stem cells and EPCs is reduced (6, 7). This has also been shown for patients with type 2 diabetes (8). Hyperglycemia, insulin resistance and oxidative stress are proposed mechanisms contributing to the limited ability of diabetic EPCs to improve vascular regeneration (9, 10). Platelet activation plays an important role in vascular repair. Activated platelets deposited at the site of a vascular injury, attract circulating $CD34^+$ stem cells and immobilize these stem cells through binding to platelet-exposed P-selectin (11-13). This was shown both in an ex vivo injury flow model and in vivo, using a carotid artery injury model in (11). Subsequent ligation of P-selectin glycoprotein-1 (PSGL-1) on $CD34^+$ stem cells leads to activation of adhesion molecules, which results in firm adhesion of the $CD34^+$ stem cells. At the site of the injury, the micro-environment of activated platelets and coagulation factors (such as fibrin) modulate the viability, proliferation and differentiation of $CD34^+$ stem cells towards endothelial-like cells. Therefore, establishment of a hemostatic plug seems to be important for the maintenance and repair of vascular injury by circulating vasculogenic cells.

Many patients at high risk for cardiovascular disease, including type 2 diabetic patients, receive aspirin, since pharmacological inhibition of platelet aggregation by acetylsalicylic acid is an effective strategy to reduce cardiovascular risk in patients at high risk (14). However, by impairing platelet aggregation, aspirin may simultaneous inhibit progenitor cell-mediated vascular regeneration. This contradiction has not been addressed before and to our knowledge, no studies on the effect of aspirin on circulating vasculogenic cells in patients with type 2 diabetes have been published. Therefore, we investigated the effect of aspirin on $CD34^+$ stem cell and $CD34^+$/ $VEGFR\text{-}2^+$ EPCs in patients with type 2 diabetes as part of a placebo controlled crossover study (15, 16).

Methods

Subjects and study design

Subjects were recruited from general practitioners affiliated to the Leiden University Medical Center (The Netherlands). Subjects were eligible if they had been diagnosed with type 2 diabetes mellitus for at least 1 year, had glycosylated haemoglobin A (HbA1c) levels < 10% and CRP levels > 1.0 mg/l, and were above 18 years old and capable to give informed consent. Subjects were excluded if they had any history of cardiovascular disease (defined as myocardial infarction, acute coronary syndrome, percutaneous coronary intervention, coronary artery bypass graft, heart failure, severe cardiac arrhythmia, cerebrovascular accident, transient ischemic attack or peripheral vascular disease) or known contra-indications to use 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, concurrent participation in other research projects or blood donation and use of non-steroidal anti-inflammatory drugs, anticoagulant medication, corticosteroid or statins. All subjects gave written informed consent and the study was approved by the Leiden University Medical Center and performed in accordance with the Declaration of Helsinki.

The study had a prospective, randomized, placebo-controlled, double-blind, crossover design. All subjects (n=40) received one period placebo and the other period aspirin. Subjects were randomly assigned to receive aspirin 100 mg/day (n=20) or 300 mg/day (n=20). 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 the Leiden University Medical Center. A computer-generated randomization code was prepared by this department. Medication was prepackaged based on 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 6-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. Non-compliance was defined as remaining pill count >10%. At each visit, blood samples were drawn from antecubital veins.

Laboratory analysis of circulating cells

A detailed description of the enumeration of circulating $CD34^+$ stem cells and EPCs ($CD34^+$/VEGFR-2^+ cells) has been published by van der Klaauw et al (17). $CD34^+$ stem cells and EPCs were determined in whole blood sampled after intervention with aspirin or placebo.

Measurement of soluble P-selectin.

The level of soluble P-selectin (sP-sel) is a measure of *in vivo* systemic platelet activation [18]. sP-Sel was measured in plasma using a commercially available ELISA (R&D Systems), according to the instructions of the manufacturers. Measurements were performed in duplicate and average values were used.

Statistics

Continuous variables are presented as mean values ± standard deviation (SD) and categorical variables as frequencies (percentages), unless otherwise stated. The numbers of circulating cells after use of aspirin or placebo, are presented as mean values ± standard error of the mean (SEM). Effects of aspirin versus placebo on circulating progenitor cells were estimated using paired samples t tests, since the differences in circulating progenitor cells between treatment with aspirin and placebo were normally distributed (data not shown). Correlations with delta sP-sel were calculated using two-tailed Spearman equations. All analyses were performed using SPSS for windows, version 16.0 (SPSS Inc, Chicago, Ill, USA) and were two-sided, with a level of significance of $P<0.05$.

Results

Patients

Subject characteristics are summarized in Table 1. No statistical differences between groups were observed. All subjects were fully compliant to study medication and there were no adverse events. Subjects had a mean age of 57±10 years and a mean BMI of 31±6 kg/m^2. Diabetes was well controlled (HbA1c, 6.1±1.2%) and most often treated by oral hypoglycemic agents only (n=27, 68%).

Circulating CD34$^+$ stem cells and EPCs

No significant differences were observed in number of circulating CD34$^+$ stem cells in the whole patient group at the end of the aspirin treatment period compared with placebo (997 ± 144 cells/mL in aspirin period versus 1084 ± 171 cells/mL in placebo period, difference –87 cells/mL (P= 0.46; 95% CI –324 to 151). Stratification of the results in used dosage (aspirin 100 mg/day or aspirin 300 mg/day) did not show a significant difference in circulating CD34$^+$ stem cells compared with placebo (difference in aspirin 100 mg/day versus placebo 16 cells/mL (P=0.90; 95% CI –249 to 281); difference in aspirin 300 mg/day versus placebo -184 cells/mL (P=0.36; 95% CI –595 to 228) (Figure 1A).

Table 1 – Baseline characteristics

	Aspirin 100 mg (*n* = 20)	Aspirin 300 mg (*n* = 20)
Age (yrs)	59.0 ± 10.5	54.5 ± 9.6
Female sex	9 (45)	5 (25)
BMI (kg/m^2)	30.0 ± 6.3	31.5 ± 5.3
Waist circumference (cm)	103 ± 12	106 ± 11
Hip circumference (cm)	106 ± 13	107 ± 10
Systolic tension (mm Hg)	154 ± 15	150 ± 16
Diastolic tension (mm Hg)	90 ± 9	90 ± 9
Smoking	2 (10)	2 (10)
Laboratory data		
Glucose (mmol/L)	8.3 ± 3.0	7.7 ± 1.1
HbA1c (%)	6.2 ± 1.4	5.9 ± 0.8
Total cholesterol (mmol/L)	5.2 ± 0.2	5.5 ± 0.2
HDL-cholesterol (mmol/L)	1.5 ± 0.4	1.4 ± 0.3
LDL-cholesterol (mmol/L)	3.5 ± 1.0	3.8 ± 0.8
Triglycerides (mmol/L)	1.8 ± 1.3	2.0 ± 1.1
Creatinine (µmol/L)	81.2 ± 15.2	85.1 ± 13.9
Medications		
ACEI/ARB	5 (25)	9 (45)
Diuretics	3 (15)	3 (15)
β-blockers	3 (15)	5 (25)
Oral hypoglycaemic drugs	14 (70)	13 (65)
Insulin	4 (20)	4 (20)

Data are expressed as means ± standard deviation (SD). BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers.

In contrast, the number of circulating EPCs in the whole patients group was significantly reduced after aspirin treatment, on average with 12 cells/mL (P=0.023; 95% CI –23 to –2). This represents a 40% reduction (95% CI 7 to 67%). Stratification to aspirin dosage showed loss of statistical significance in the low dose aspirin group (100 mg/day, P=0.27), although the point estimate of the difference showed a reduction (difference –9 cells/mL (95% CI –26 to 8). Aspirin 300 mg/day significantly reduced EPC number compared with placebo (difference 15 cells/mL; P=0.046; 95% CI –30 to 0) (Figure 1B).

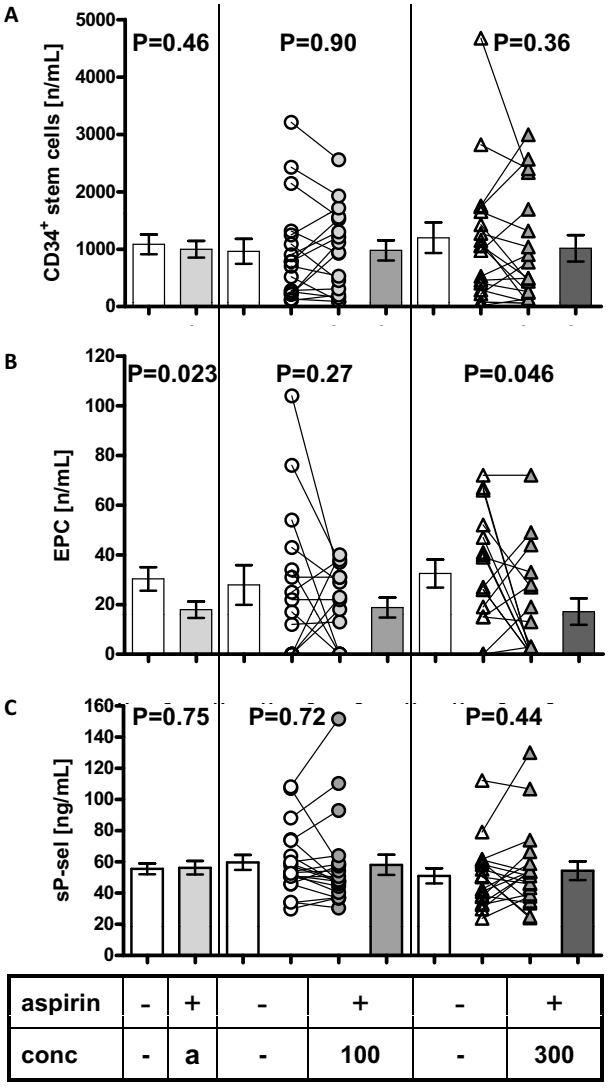

Figure 1 - Enumeration of CD34⁺ stem cells and EPCs and measurement of sP-sel.

Absolute numbers are shown of A) circulating CD34⁺ stem cells (n/mL), B) EPCs (n/mL) or C) sP-sel levels (ng/mL) in the placebo group and the aspirin-treated group (a), subdivided in placebo versus aspirin-100 (mg/day) and placebo versus aspirin-300 (mg/day). The symbols represent levels of individual subjects before and after treatment. Mean levels are presented in bars ± SEM and results per subjects are shown as symbols connected with lines representing placebo and aspirin-values.

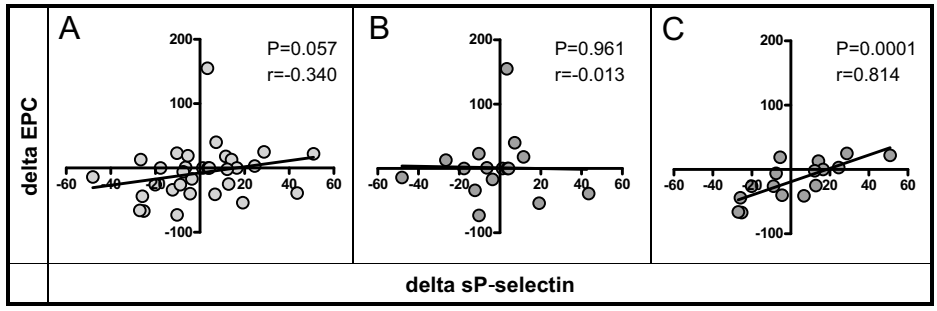

Figure 2 - Correlation between delta-sP-sel values and delta-EPC values.
Delta values were calculated of sP-sel and EPCs in A) the whole placebo group and the aspirin-treated group, B) placebo versus aspirin-100 mg/day and C) placebo versus aspirin-300 (mg/day).

Aspirin effects on the level of soluble P-selectin

Aspirin treatment did not show an effect on average values of sP-sel between the placebo- and aspirin treated groups neither at low or high dose level (Fig 1C, overall: difference 1.0 ng/mL, 95% CI -5.4 to 7.5; P=0.75; 100 mg/day: -1.5 ng/mL, 95% CI -10.4 to 7.4, P=0.72; 300 mg/day: 3.9 ng/mL, 95% CI -6.4 to 14.2, P=0.44 respectively) (Figure 1C). When looking at the subjects individually, half of the patients showed a reduction in sP-sel, both at low and high dose aspirin treatment. The other half of the subjects showed an increased sP-sel level, indicating that the sensitivity for aspirin, suggesting that actual inhibition of *in vivo* basal platelet reactivity is subject-specific.

Effect of aspirin on the number of $CD34^+$ stem cells and EPCs in individual patients

After aspirin treatment, 57% of the patients showed a reduction in $CD34^+$ stem cell-levels (Figure 1A) and 50% showed a reduction in EPC-levels (Figure 1B). Since the effects of aspirin treatment showed to be patient-specific, we calculated the delta values of sP-sel, $CD34^+$ stem cell- and EPC-levels for each patient. Delta-$CD34^+$ stem cell levels were not correlated with delta-sP-sel levels, either at low (P=0.22; r=-0.32) or high dose (P=0.21, r=0.32) of aspirin, indicating that $CD34^+$ stem cell-levels do not correspond with levels of *in vivo* platelet activation. Interestingly, delta-EPC levels were correlated with delta-sP-sel after aspirin treatment at high dose (Figure

2C, P<0.001, r=0.75), but this correlation was not observed at low dose (Figure 2B, P=0.81, r=-0.07) and was consequently less obvious when the aspirin dosage was not taken into account (Figure 2A, P=0.11, r=0.30). When *in vivo* platelet activation was inhibited to the highest degree, EPC numbers were reduced accordingly, and also, when *in vivo* platelet activation was increased, EPC-numbers were increased. These results indicate that the number of circulating EPCs correlates with *in vivo* platelet activation.

Discussion

This trial has studied the effects of aspirin on the number of circulating $CD34^+$ stem cells and circulating endothelial progenitor cells (EPCs) in patients with type 2 diabetes.

Our main finding indicates that use of aspirin significantly and dose-dependently reduces the number of EPCs. We found no significant differences in absolute $CD34^+$ stem cell numbers at the end of the aspirin treatment period compared with placebo. In order to examine possible mechanistic pathways, we tested whether the reduction in EPCs by aspirin was related to the level of sP-sel as marker of *in vivo* basal platelet activation. We compared sP-sel levels both with absolute counts and with changes in numbers of EPCs in all subjects individually. The change in EPC number correlated significantly (P<0.001) with the change in sP-sel level. In other words, when *in vivo* platelet activation was inhibited, demonstrated by a reduction in sP-sel, EPC numbers were reduced and when *in vivo* platelet activation was increased, EPC numbers were elevated. The *in vitro* cyclooxygenase-dependent aggregability of the platelets, induced by ADP, collagen or arachidonic acid, was not related to the change in EPC number (data not shown)

The conclusion that the change in EPC number is positively correlated to the change in *in vivo* platelet activation is interesting, because this finding points towards a cyclooxygenase-independent mechanism of how aspirin influences EPC number.

In vivo, platelets may also get activated through pathways independent of platelet based cyclooxygenase, for instance through the formation of thrombin. It is well

known that aspirin, especially at higher doses (>300 mg / day) suppresses thrombin formation (18, 19). Formation of thrombin is very relevant for the establishment of a pro-vasculogenic micro-environment, which is crucial for the homing of $CD34^+$ stem cells to an injury (11). We hypothesize that the number of $CD34^+/VEGFR2^+$ EPCs, which can be seen as a circulating cell differentiated more towards an endothelial phenotype as compared to the $CD34^+$ stem cell, is related to the homing of $CD34^+$ stem cells to injured endothelium. As higher doses of aspirin inhibit the homing and immobilizing of $CD34^+$ stem cells, possibly by inhibiting *in vivo* thrombin activation, the subsequent formation of EPCs is also impaired. The exact explanation for this observation needs further investigation.

Discussing the potential clinical implications of our findings, a few points can be made. First, there is still debate on the most efficacious dose of aspirin in cardiovascular protection. For maximal inhibitory effect on platelet-based cyclooxygenase, no higher doses than 100 mg / day are necessary. On the contrary, several alleged non-cyclooxygenase-mediated vasculoprotective effects of aspirin on endothelium and platelets are dose-dependent and therefore could give support to the use of higher doses. However, analysis of the ATC's database on more than 190 studies using antiplatelet therapy, did not show an incremental risk reduction at higher doses of aspirin (14). As side-effects of aspirin are more frequent in higher doses, current guidelines on chronic use of aspirin in cardiovascular prevention, advise not to uses doses above 100 mg / day. Second, so-called aspirin resistance has received much attention in the discussion concerning the optimal dose of aspirin. Among patients with cardiovascular disease, the prevalence of laboratory-defined aspirin resistance is estimated to be approximately 25% (20). The presence of laboratory-defined aspirin resistance is associated with a higher risk of recurrent cardiovascular events (21). In patients with coronary artery disease, diabetic patients have a higher prevalence of laboratory-defined aspirin resistance which could be overcome by the use of higher doses of aspirin (22). Use of a higher dose of aspirin is indeed one of the potential strategies under investigation to decrease the prevalence of aspirin-resistance. However, at higher doses of aspirin, the associated negative effect on endogenous vessel wall repair, as demonstrated in our study, may offset the putative

beneficial effect of lesser aspirin resistance. The net effect on clinical outcome could be negligible, which is compatible with the aforementioned results of the ATC's meta-analysis. Hence, the results of our study stress the importance to use clinical outcomes instead of laboratory-defined endpoints in studies designed to test the hypothesis that use of higher doses of aspirin is an effective strategy to overcome aspirin resistance.

In conclusion, we have shown that in patients with type 2 diabetes, use of aspirin significantly reduced the number of EPCs by 40%. This effect is dose-dependent. Whether this result is explained by a specific effect of aspirin on *in vivo* formed thrombin and how this relates to circulating vasculogenic cells, or whether this is a specific finding in the diabetic population remains to be determined.

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References

1. Schramm TK, Gislason GH, Kober L, Rasmussen S, Rasmussen JN, Abildstrom SZ, et al. Diabetes patients requiring glucose-lowering therapy and nondiabetics with a prior myocardial infarction carry the same cardiovascular risk: a population study of 3.3 million people. Circulation. 2008 Apr 15;117(15):1945-54.
2. Mazzone T, Chait A, Plutzky J. Cardiovascular disease risk in type 2 diabetes mellitus: insights from mechanistic studies. Lancet. 2008 May 24;371(9626):1800-9.
3. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997 Feb 14;275(5302):964-7.
4. Umemura T, Higashi Y. Endothelial progenitor cells: therapeutic target for cardiovascular diseases. J Pharmacol Sci. 2008 Sep;108(1):1-6.
5. Kudo FA, Nishibe T, Nishibe M, Yasuda K. Autologous transplantation of peripheral blood endothelial progenitor cells (CD34+) for therapeutic angiogenesis in patients with critical limb ischemia. Int Angiol. 2003 Dec;22(4):344-8.
6. Fadini GP, de Kreutzenberg SV, Coracina A, Baesso I, Agostini C, Tiengo A, et al. Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. Eur Heart J. 2006 Sep;27(18):2247-55.
7. Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, Malagutti P, et al. CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation. 2004 Sep 7;110(10):1209-12.
8. Fadini GP, Sartore S, Albiero M, Baesso I, Murphy E, Menegolo M, et al. Number and function of endothelial progenitor cells as a marker of severity for diabetic vasculopathy. Arterioscler Thromb Vasc Biol. 2006 Sep;26(9):2140-6.
9. Fadini GP, Sartore S, Agostini C, Avogaro A. Significance of endothelial progenitor cells in subjects with diabetes. Diabetes Care. 2007 May;30(5):1305-13.
10. Fadini GP. An underlying principle for the study of circulating progenitor cells in diabetes and its complications. Diabetologia. 2008 Jul;51(7):1091-4.
11. de Boer HC, Verseyden C, Ulfman LH, Zwaginga JJ, Bot I, Biessen EA, et al. Fibrin and activated platelets cooperatively guide stem cells to a vascular injury and promote differentiation towards an endothelial cell phenotype. Arterioscler Thromb Vasc Biol. 2006 Jul;26(7):1653-9.

12. Stellos K, Gnerlich S, Kraemer B, Lindemann S, Gawaz M. Platelet interaction with progenitor cells: vascular regeneration or inquiry? Pharmacol Rep. 2008 Jan-Feb;60(1):101-8.
13. Langer HF, May AE, Vestweber D, De Boer HC, Hatzopoulos AK, Gawaz M. Platelet-induced differentiation of endothelial progenitor cells. Semin Thromb Hemost. 2007 Mar;33(2):136-43.
14. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. Bmj. 2002 Jan 12;324(7329):71-86.
15. Hovens MM, Snoep JD, Groeneveld Y, Frolich M, Tamsma JT, Huisman MV. Effects of aspirin on serum C-reactive protein and interleukin-6 levels in patients with type 2 diabetes without cardiovascular disease: a randomized placebo-controlled crossover trial. Diabetes Obes Metab. 2008 Aug;10(8):668-74.
16. Hovens MM, Snoep JD, Groeneveld Y, Tamsma JT, Eikenboom JC, Huisman MV. High levels of low-density lipoprotein cholesterol and triglycerides and suboptimal glycemic control predict diminished ex vivo aspirin responsiveness in patients with Type 2 diabetes. J Thromb Haemost. 2007 Jul;5(7):1562-4.
17. van der Klaauw AA, Pereira AM, Rabelink TJ, Corssmit EP, Zonneveld AJ, Pijl H, et al. Recombinant human GH replacement increases CD34+ cells and improves endothelial function in adults with GH deficiency. Eur J Endocrinol. 2008 Aug;159(2):105-11.
18. Undas A, Brummel-Ziedins KE, Mann KG. Antithrombotic properties of aspirin and resistance to aspirin: beyond strictly antiplatelet actions. Blood. 2007 Mar 15;109(6):2285-92.
19. Ratnatunga CP, Edmondson SF, Rees GM, Kovacs IB. High-dose aspirin inhibits shear-induced platelet reaction involving thrombin generation. Circulation. 1992 Mar;85(3):1077-82.
20. Hovens MM, Snoep JD, Eikenboom JC, van der Bom JG, Mertens BJ, Huisman MV. Prevalence of persistent platelet reactivity despite use of aspirin: a systematic review. Am Heart J. 2007 Feb;153(2):175-81.
21. Snoep JD, Hovens MM, Eikenboom JC, van der Bom JG, Huisman MV. Association of laboratory-defined aspirin resistance with a higher risk of recurrent cardiovascular events: a systematic review and meta-analysis. Arch Intern Med. 2007 Aug 13-27;167(15):1593-9.

22. DiChiara J, Bliden KP, Tantry US, Hamed MS, Antonino MJ, Suarez TA, et al. The effect of aspirin dosing on platelet function in diabetic and nondiabetic patients: an analysis from the aspirin-induced platelet effect (ASPECT) study. Diabetes. 2007 Dec;56(12):3014-9.