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Evening aspirin intake results in higher levels of platelet inhibition and a reduction in reticulated platelets - a window of opportunity for patients with cardiovascular disease?

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Abstract

Cardiovascular events occur most frequently in the early morning. Similarly, the release of reticulated platelets (RP) by megakaryocytes has a peak in the late night and early morning. Which aspirin regimen most effectively inhibits platelets during these critical hours is unknown. Hence, the primary objective of this trial was to assess platelet function and RP levels at 8.00 AM, in stable cardiovascular (CVD) patients, during three different aspirin regimens. In this open-label randomized cross-over study subjects were allocated to three sequential aspirin regimens: once-daily (OD) 80 mg morning; OD-evening, and twice-daily (BID) 40 mg. Platelet function was measured at 8.00 AM & 8.00 PM by serum Thromboxane B_2 ($sTxB_2$) levels, the Platelet Function Analyzer (PFA)-200® Closure Time (CT), Aspirin Reaction Units (ARU, VerifyNow®), and RP levels. In total, 22 patients were included. At 8.00 AM, $sTxB_2$ levels were the lowest after OD-evening in comparison with OD-morning (p = <0.01), but not in comparison with BID. Furthermore, RP levels were similar at 8.00 AM, but statistically significantly reduced at 8.00 PM after OD-evening (p = .01) and BID (p = .02) in comparison with OD-morning. OD-evening aspirin intake results in higher levels of platelet inhibition during early morning hours and results in a reduction of RP levels in the evening. These findings may, if confirmed by larger studies, be relevant to large groups of patients taking aspirin to reduce cardiovascular risk.

Keywords

Aspirin, chronotherapy, circadian rhythm, cross-over trial, platelet aggregation

History

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Introduction

Since 1974, when the first published randomized controlled trial of aspirin reported a 25% reduction in cardiovascular mortality [1], aspirin has become fundamental in the secondary prevention of cardiovascular disease (CVD) [2]. These preventive effects are mostly attributable to aspirin's ability to inactivate cyclo-oxygenase-1 (COX-1), leading to inhibition of thromboxane A2 (TXA_2) production by platelets [3]. Since platelets are unable to produce new COX-enzyme, the inhibition of COX-1 persists throughout the platelet's full life span. The effect of aspirin has therefore traditionally been thought to provide protection during a 24-hour dosing interval.

Previous research has demonstrated most cardiovascular events (CVE) preferentially occur during the early morning hours [4,5]. Hence, these hours may be essential in terms of adequate platelet inhibition. However, recent studies point toward a gradual increase in residual platelet aggregability and COX activity within the 24-hour dosing interval, specifically during the early morning hours. A likely explanation for this could be the continuous daily production of new platelets (i.e. reticulated platelets), with a peak release in the late night and early morning [6,7]. Reticulated platelets (RP) are more reactive than mature platelets [8], and a high level of RPs in patients with stable cardiovascular disease has been associated with an increased CVE risk [9].

Chronotherapy involves the administration of medication in coordination with the body's circadian rhythms to maximize therapeutic effectiveness [10]. As mentioned above, the incidence of cardiovascular events adheres to such circadian rhythm, with a peak prevalence during the night and early morning. Previous research has suggested that changing the time of intake to evening or switching to a 12-hour dosing interval might result in higher levels of platelet inhibition [11–14]. Furthermore, even though megakaryocytes are able to produce new cyclooxygenase (COX), they can be inhibited by aspirin for a maximum of 12 hours [15]. Hence, it could be that after a once-daily (OD)-evening or twice-daily (BID) regimen they are – partially – inhibited during their 'peak release', and, perhaps, release fewer RPs. Hence, applying chronotherapy to antiplatelet management for the prevention of CVE seems prudent.

In previous research by our group in healthy volunteers evening intake of a 12-hour dosing interval showed better platelet inhibition in critical early morning hours [11]. In view of these considerations, the objective of this trial was two-fold: 1.) To

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assess platelet function in the early morning – 8.00 AM – in stable cardiovascular patients, during a 80 mg OD-morning in comparison with a 80 mg OD-evening regimen and a BID 40 mg (i.e. half a dosage) regimen; 2.) To assess RP levels after a 80 mg OD-morning regimen, in comparison with a 80 mg OD-evening - and BID-regimen.

Methods

Participants

This study is registered in the Dutch trial register NTR5114. Patients of the outpatient Cardiology department of the Amsterdam University Medical Center, location VU University were screened. Patients were eligible if they were diagnosed with stable cardiovascular disease (defined as: coronary artery disease or previous myocardial infarction) and if they were prescribed aspirin (acetylsalicylic acid 80 mg or carbasalate calcium 100 mg). Patients were excluded if they had a history of active bleeding, diabetes mellitus (in this group platelet activation is higher), gastrointestinal ulcer/bleeding/gastritis, thrombocytopenia or thrombocytosis, thrombopathy, any ischemic revascularization procedure in the previous six months, if they had alcohol intake the day before sampling (e.g. noncompliance to the protocol) and if there was recent use of antiplatelet drugs, anticoagulants or drugs that are known to alter platelet function, other than aspirin. This study was conducted in accordance with the Helsinki II Declaration. Written informed consent was obtained from all participants. The protocol was approved by the medical ethical committee of the Amsterdam University Medical Center, location VU University.

Trial Design

This was an open label, partly randomized cross-over trial. Participants were allocated to three sequential dosage regimens: OD-morning, OD-evening, and BID half dosage regimen (Figure 1). Prior to the start of the trial, the coordinating investigator created envelopes with randomization locations, without any restrictions. These sealed envelopes subsequently determined whether participants started with the morning or evening regimen. All participants received trial medication (acetylsalicylic acid, 80 mg, non-enteric-coated). For the BID half-dosage regimen, the tablets were manually split by a trained member of the research team [16]. In the OD-morning regimen, subjects were instructed to take 80 mg of aspirin at 8:00 AM. In the OD-evening regimen, subjects were instructed to take 80 mg of aspirin at 8.00 PM. In the BID-regimen subjects were instructed to take 40 mg of aspirin at 8.00 AM and PM. All intake regimens were maintained for 10 consecutive days. As the maximum life-span platelets is 8–10 days, we did not incorporate a wash-out period in between regimens [17].

Blood Collection

Blood sampling took place after the 10^{th} consecutive day of intake (Figure 1). Hence, for OD-morning regimen on the 10^{th} day at 8.00 PM and on the 11^{th} day at 8.00 AM, and for both the OD-evening - and BID regimen on the 11^{th} day at 8.00 AM & PM. Blood sampling took place at the hospital. Samples were drawn from the antecubital vein through a 21-gauge needle, first into a precursor tube, secondly into one sodium citrate tubes (BD Vacutainer® 0.109 M Buff. Na3 Citrate REF 363048) and two VerifyNow Vacuettes® (9NC Coagulation sodium citrate 3.2%), subsequently into a serum Clot Activator tubes (BD Vacutainer® Clot Activator Tube REF 368815) and last into one EDTA tube (BD Vacutainer® K2E (EDTA) 7.2 mg REF 368861).

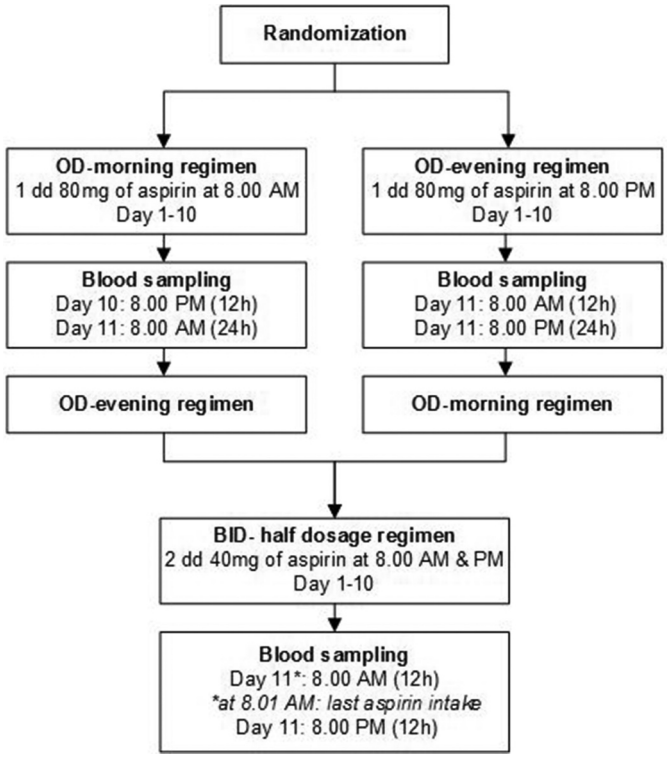

Figure 1. Flow chart of the trial protocol.

Outcome Parameters

Serum Thromboxane B_2, the Platelet Function Analyzer Closer Time, and the VerifyNow Aspirin Reaction Units were determined to be co-primary endpoint to assess the level of platelet inhibition. The level of RPs was determined to be the primary endpoint to assess the influence of chronotherapy on platelet turnover.

Serum Thromboxane B_2 ($sTxB_2$) is a major product of the metabolism of arachidonic acid (AA) by platelets. After activation via arachidonic acid, which is produced by vascular smooth muscle cells, a platelet produces TxA_2. TxA_2, however, is rapidly (t½ of 30–60 seconds) hydrolyzed non-enzymatically to form the more stable TxB_2. Hence, the level of TxB_2 is a direct measurement of COX-linked platelet activity [18]. After venepuncture, the serum tube was transported at 37°C within a travel incubator [19]. After arriving at the lab, the serum tube was incubated at 37°C for 60 minutes (minus the travel time in the incubator). Prior to storage at −80°C, the serum samples were centrifuged at 1780 g for 10 minutes. $STxB_2$ levels were measured post-hoc by enzyme immunoassay according to manufacturers' instructions (Thromboxane B2 Express, Cayman Chemicals, Ann Arbor, MI, USA) and previously described by Bonten et al [13]. Samples were analyzed in duplicate in the laboratory in order to measure the intra-assay coefficient of variance (CV), which was <10% for all measurements. The lab technician executing the measurement was blinded.

The Platelet Function Analyzer (PFA)-200 measures platelet reactivity in response to shear stress. The time needed for occlusion of an aperture cut in the collagen/epinephrine cartridge by the thrombocyte plug is called the closure time (PFA-CT). The PFA-CT has a maximum of 300 seconds. Any closure time greater than 300 seconds was reported as 301 in the data analysis.

The measurement of the PFA-CT was performed immediately after blood collection. The investigator who performed the measurement was not blinded.

The VerifyNow© measures platelet reactivity by the rate and extent of light transmission changes in whole blood, as platelets aggregate over time in response to arachidonic acid (Aspirin Test' cartridge). Within the test device wells, the instrument measures the increase in light transmittance over time. Results of the VerifyNow are expressed as Aspirin Reaction Units (ARU's). The measurement of the *VerifyNow©* was performed 30 minutes after blood collection. The investigator who performed the measurement was not blinded.

Reticulated platelets (RP) levels were initially determined by flow cytometry, using fluorescence with thiazol orange which binds to the RNA in RPs [20]. Unfortunately, mid-study the hospital switched to the Sysmex XE-2100 auto analyzer. Consequently, our reticulated platelet parameter was changed into the immature platelet fraction (IPF), measured by cell volume and RNA content which discriminate RPs from mature platelets [21]. Different articles investigated the correlation between RP% and IPF in laboratory blood tests, showing no significant difference between the two variables [22] [23]. This was in line with our own data, demonstrating no statistically significant differences between the two methods. Consequently, both methods were categorized as one during the final statistical analyzis.

Sample Size Calculation

This trial was designed to investigate two main questions. 1.) In reviewing the literature, data have shown that on average the intra-assay coefficient of variance of the PFA-CT, $sTxB_2$ levels, and the VerifyNow are about of equal range. However, the PFA-CT has the highest coefficient of variance [12,24]. We therefore decided to perform our power analyzis solely based on the PFA-CT. The data were derived from a previously published trial with healthy volunteers [11]. Due to the skewed distribution of PFA-CT, the sample size calculation was based on the Wilcoxon signed-rank test. The estimated effect size of the morning vs. evening regimen was 32 seconds, with a power of 90%, and a two-sided alpha level of 5%, the number needed to include was 18. After having taking into account a possible drop-out of 10%, the total number needed to include was 20 participants (SAS version 9.2). 2.) To our knowledge, there is no previously published research regarding this effect. Hence, a pre-trial power calculation based on this endpoint could not be performed.

The initial sample size calculation of this trial, as shown in the trial register, was based on 'aspirin resistance' with the PFA-200 (i.e.closure time less than 193 seconds). The McNemar test was used to calculate a sample size. Based on a power of 80% and a two-sides alpha level of 5%, the estimated sample size for this study was 75 patients. Unfortunately, the intended sample size was not feasible.

Statistical Analyzis

Continuous variables were expressed as mean ± standard deviation (SD) if normally distributed or median and interquartile range (IQR) for non-normally distributed variables. Within-group differences were tested by the Friedman test and post-hoc Wilcoxon signed-rank test for non-normally distributed variables. Non-paired dichotomous results were compared using the Cochran's Q test and post-hoc McNemar test for non-normally distributed variables. A p-value of <0.05 was considered statistically significant. Missing values were tested for randomness, and replaced with either mean or median. Statistical analyzes were performed with SPSS statistics 22.0 for Windows (IBM SPSS Inc., Chicago, IL, USA).

Results

Baseline Characteristics

From August 2014 until December 2018, 24 patients were included in this study. A flow chart of the study patients and samples is depicted in Figure 2. The consent rate of eligible patients was 8.6%, which is extremely low. Furthermore, two patients did not complete the study: one withdrew consent, and one endured a myocardial infarction after signing consent but prior to starting trial protocol. There were no other adverse events reported. A total of 22 patients were included in the analyzis. Their baseline characteristics are summarized in Table I.

Compliance to the Protocol

According to the patient diaries and pill count, compliance with the study medication was 100%. Compliance with the exact time of intake was good in both the morning regimen (average time of intake: 8:05 hr. ± 9 min.) as well as the evening regimen (20.17 hr. ± 20 min.). For the BID regimen, the average time of intake was 8.10 hr. ± 24 min. and 20.09 hr. ± 18 min., for morning and evening intake, respectively.

Platelet Parameters as Measured by Various Methods

All parameters measured at both 8:00 AM and PM are depicted in Figure 3. At 8.00 AM, the $sTxB_2$ levels were the lowest in the OD-evening in comparison with OD-morning (mean difference −1.5 ± 2.2 ng/ml; SEM 0.5; 95% C.I. −2.5 - - 0.5; p = <0.01), but not in comparison with the BID-regimen (mean difference −0.5 ng/ml; 95% C.I. −1.8–0.7). However, the $sTxB_2$ levels measured at 8.00 PM demonstrated a higher level of platelet inhibition in favor of OD-morning in comparison with OD-evening (mean difference −2.3 ng/ml; SEM 0.6; 95% C.I. −3.5 - - 1.0; p = <0.01), but not in comparison with the BID-regimen (mean difference −0.9 ng/ml; SEM 0.6; 95% C.I. −2.2–0.4). Corresponding results were obtained for the PFA-CT, which demonstrated a statistically significant difference between the three regimens (p = .04), in favor of the OD-evening regimen. According to the PFA-CT, this increased inhibition does not seem to come at the expanse of a diminished inhibition during the evening (8.00 PM: OD-morning 182 sec. IQR: 143–301 vs. OD-evening 243 sec. IQR: 150–301 vs. R_{BID} 202 sec. IQR: 156–30; n.s.). The VerifyNow-ARU did not demonstrate any statistically significant differences between the regimens, neither at 8.00 AM or PM. And lastly, RP levels measured at 8.00 AM were similar in all three regimens (OD-morning 4.1 ± 1.9% vs. OD-morning3. 7 ± 1.7% vs. BID-regimen 3.8 ± 1.8%). At 8.00 PM, however, the RP levels were statistically significantly lower after OD-evening in comparison with OD-morning (mean difference −0.8%; SEM 0.3; 95% C.I. −1.4 - −1.2; p = .01). This was similar for the BID-regimen in comparison with OD-morning (mean difference −0.7%; SEM 0.3; 95% C.I. −1.3 - −0.1; p = .02). All results of the different regimens are shown in Table I of the supplementary material.

Discussion

The aim of this partly randomized open-label crossover trial, performed in patients with stable cardiovascular disease, was two-fold: 1.) To assess platelet function in the early morning – 8.00 AM - in stable cardiovascular patients, during a 80 mg OD-morning, in comparison with a OD-evening regimen, and a BID

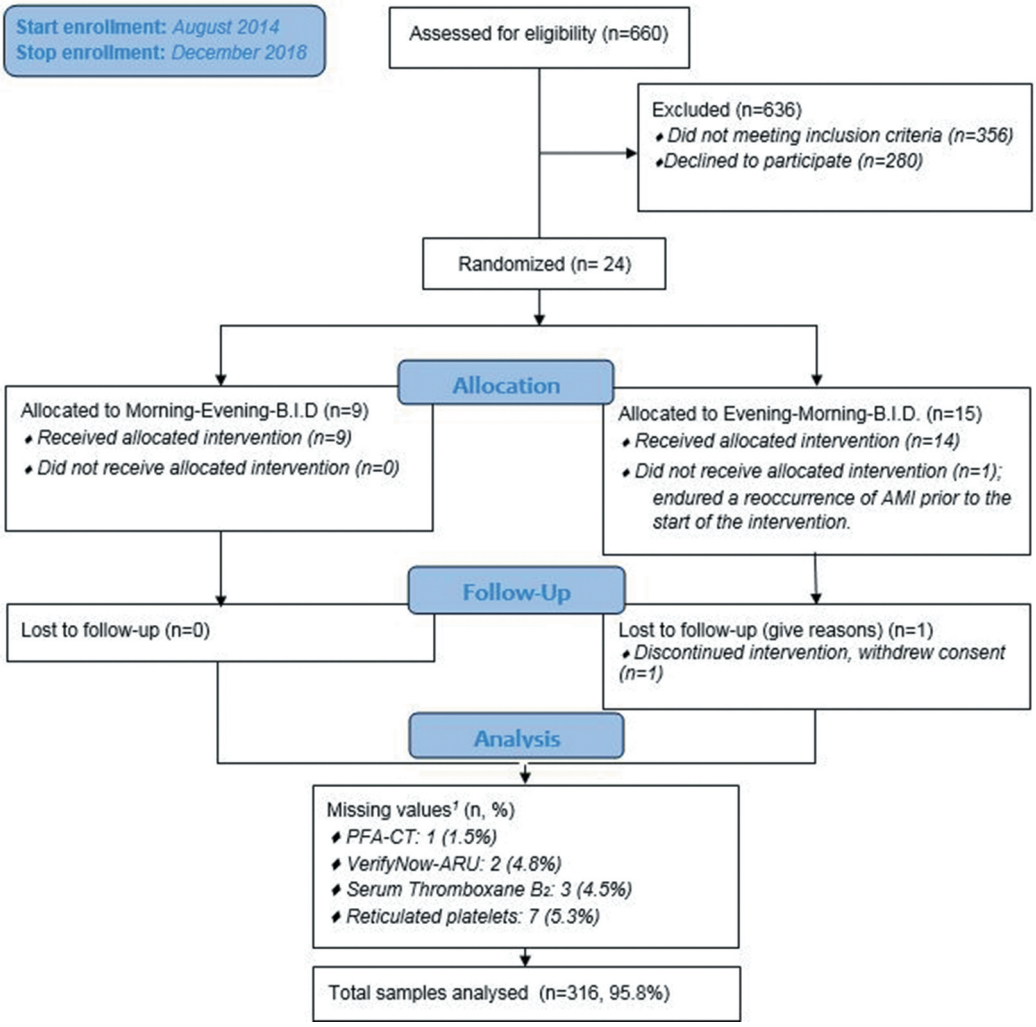

Figure 2. Flowchart of study patients and samples.

[1]The percentage of missing values was calculated over the expected total samples at both 8.00 AM and PM.

Table I. Baseline characteristics.

Characteristics	Patients (n = 22)
Male sex (n,%)	19 (86.4)
Age	66.0 ± 9.9
BMI	27.4 ± 3.5
Alcohol intake (units/week)	8.7 ± 8.1
Current smoker (n,%)	8 (36.4)
Current drug user (n,%)	1 (4.5)
Caffeine intake (units per day)	3 ± 1.8
Systolic blood pressure (mmHg)	135 ± 10.0
Diastolic blood pressure (mmHg)	80 ± 7.3
Aspirin indication (n,%)	
Myocardial infarction	16 (68.2)
Coronary artery disease	4 (18.2)
Other (Peripheral artery disease, unknown)	2 (9)
Cardiovascular medication (n,%)	
Lipid lowering drug use	20 (90.9)
β-blocker use	15 (68.2)
Other antihypertensive medication	16 (72.3)
Laboratory values	
Hemoglobin level (mmol/l)	9.0 ± 0.6
Leukocyte level (10^9/l)	6.7 ± 1.1
Thrombocyte level (10^9/l)	228 ± 43

half a dosage regimen, as measured by $sTxB_2$ levels; 2.) To assess RP levels during a OD-morning regimen, in comparison with an OD-evening and a BID half a dosage regimen. The findings of this trial are: 1.) OD-evening intake of aspirin results in statistical significantly higher level of platelet inhibition during early morning hours, compared with the OD-morning, as measured by $sTxB_2$ levels and the PFA-CT; 2.) In comparison with OD-morning, OD-evening intake and BID half a dosage intake statistically significantly reduce RP levels at 8.00 PM.

The first finding, of a superior level of inhibition after OD-evening intake of aspirin in comparison with OD-morning intake, is consistent with the results of several other studies, both in healthy subjects and patients with stable cardiovascular disease [11–14,25]. As most CVE's occur during –early morning hours [26,27], optimal platelet inhibition during these hours seems prudent. In line with studies in healthy volunteers and patients with essential thrombocytosis, a BID-regimen resulted in a higher level of platelet inhibition than a OD-morning regimen [11,28] Interestingly, both the COX-1 dependent and independent platelet function test (i.e. $sTxB_2$ levels and the PFA-CT, respectively) demonstrated a higher level of platelet inhibition by the OD-evening, in comparison with the OD-morning regimen. Despite higher levels of $sTxB_2$ at 8.00PM, this inferior level of platelet inhibition does not seem to

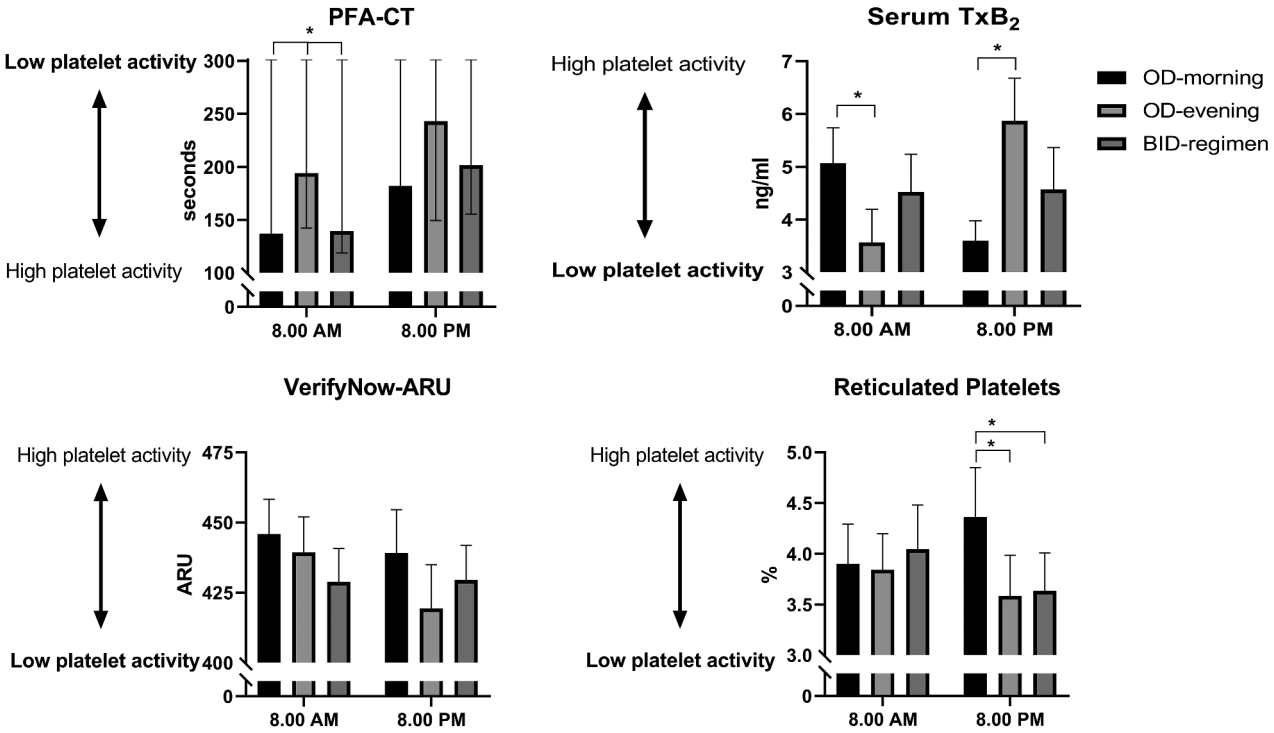

Figure 3. Results of 8:00 AM and PM measurements after the OD-morning, OD-evening, and BID half dosage regimen.
The PFA-CT results are shown as median and IQR; The VerifyNow, Reticulated Platelet levels, and $sTxB_2$ levels as mean±SEM; *, $p < .05$.

come at the expanse of a diminished level of platelet inhibition at 8.00 PM as measured by the PFA-CT. Surprisingly, the BID-regimen did not demonstrate a superior level of platelet inhibition in comparison with OD-morning. Although 40 mg of acetylsalicylic acid has been proven to provide sufficient platelet inhibition, most of this research was performed in healthy volunteers [11,29]. This is most likely due to the fact that cardiovascular patients have a higher platelet turnover, as well as more reactive platelets [30–34]. Hence, it could be that in cardiovascular patients a dose of 40 mg is insufficient and a BID-regimen with higher dosages would have provided superior levels of platelet inhibition.

The second finding, of lower RP levels at 8.00 PM after OD-evening intake and BID half a dosage intake, could be of clinical relevance. As mentioned in the introduction, RPs are more reactive than mature platelets [8]. Besides, RPs have an increased ability to produce haemostatically important platelet proteins [35]. A high level of RPs in patients with stable cardiovascular disease has been associated with an increased risk of a CVE [9]. Hence, although this is highly speculative, a reduction in RPs might lead to a reduction in CVE. Furthermore, to our knowledge, this is the first study to report on a possible cause as to why OD-evening aspirin intake might result in a higher and more stable level of platelet inhibition. As mentioned in the introduction, it has been shown that although megakaryocytes are able to produce new COX, they can be inhibited by aspirin for a maximum of 12 hours [15]. Hence, it could be that after a OD-evening or BID regimen they are – partially – inhibited during their 'peak release', and, perhaps, release fewer RPs. Furthermore, as inflammation, aggregation, and thrombosis are closely intertwined [36,37], it could be that chronotherapy of aspirin influences the circadian rhythm of cardiovascular inflammation and subsequent platelet turnover (i.e. RP level). The inflammatory reaction has a significant influence on blood platelet measurements [38]. This is substantiated by evidence demonstrating that high platelet activity is associated with increased levels of cardiovascular inflammation parameters [39,40].

Strengths of this trial include the fact that all blood sampling took place under the same conditions at the same location, hereby reducing pre-analytical variables. Moreover, compliance was checked via pill count and patient diaries. Furthermore, in order to achieve the best weight uniform half tablets and the least loss of mass [16], we conducted a pre-trial investigation of the precision of four methods for the subdivision of aspirin tablets. An important limitation, apart from the small sample size, is the early termination of this trial prior to reaching the intended sample size. As is demonstrated in Figure 2, the acceptance rate of eligible participants was extremely low. In hindsight, the study design of three consecutive regimens, including three visits at 8.00 AM and three visits at 8.00 PM, seemed too demanding for most eligible patients. However, the post-hoc sample size demonstrated that we included a sufficient number of patients for our primary endpoint. Furthermore, although this is a known problem in cardiovascular research [41], this trial has an over-representation of men. Hence, we are unable to assess whether possible sex differences exist.

Conclusion

Our data suggest that OD-evening intake of aspirin results in higher levels of platelet inhibition during early morning hours, as well as a reduction in RP levels at 8.00 PM. Given the high prevalence of cardiovascular disease, already a modest relative reduction of the morning peak, in both platelet activity and RP levels, could lead to a large absolute reduction of CVE on a population level. Future trials, powered on clinically relevant endpoints (i.e. major cardiovascular events), are pending and will hopefully confirm whether chronotherapy of aspirin therapy can indeed further reduce CVE.

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laboratory of the Leiden University Medical Center & the Amsterdam University Medical Center (location VU University), respectively.

Author Contribution

J.J.K. van Diemen, MD, PhD Dr. J.J.K. van Diemen was involved in the design of the study, as well as the final draft of the manuscript. M.C. Madsen, MD, PhD-candidate Miss. Madsen played an important role in the conduction of the study. Furthermore, she was involved in the final draft of the manuscript. P. Vrancken, MD Mr. P. Vrancken played an important role in the conduction of the study. Furthermore, he was involved in the final draft of the manuscript. K. de Bie, B.Sc. Miss. K. de Bie played an important role in the conduction of the study. Furthermore, she was involved in the final draft of the manuscript. J. G. van der Bom, MD, PhD. Prof.dr. J.G. van der Bom was involved in the design of the study. Specifically, she helped with the correct implementation and execution of the platelet function testing. Furthermore, he was involved in the final draft of the manuscript. G. Veen, MD, PhD Dr. G. Veen was involved in the design of the study, as well as the final draft of the manuscript. T.N. Bonten, MD, PhD Dr. T.N. Bonten was involved in the design of the study. Specifically, he helped with the correct implementation and execution of the platelet function testing. Furthermore, he was involved in the final draft of the manuscript. W.W. Fuijkschot, MD, PhD Dr. W.W. Fuijkschot was involved in the design of the study, as well as the final draft of the manuscript. Y.M. Smulders, MD, PhD Prof.dr. Y.M. Smulders was involved with the initial design of the study and the final draft of the manuscript. A. Thijs, MD, PhD Dr. A. Thijs was involved with the initial design of the study, as well as the initial outline and final draft of the manuscript.

Disclosure Statement

The authors declare that they have no conflict of interest.

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