Aspirin intake in the morning is associated with suboptimal platelet inhibition, as measured by serum Thromboxane B2, during infarct-prone early-morning hours

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Aspirin intake in the morning is associated with suboptimal platelet inhibition, as measured by serum Thromboxane B_2, during infarct-prone early-morning hours

Cati Racca[1], Jeske Joanna Katarina van Diemen[1], Wessel Willem Fuijkschot[1], Karlinde Spit[1], Tobias Nicolaas Bonten[2,3], Mattijs Everard Numans[2], Johanna Gerarda van der Bom[3,4], Yvo Michiel Smulders[1], & Abel Thijs[1]

[1]*Department of Internal Medicine, Amsterdam University Medical Center, VU University, Amsterdam, The Netherlands,* [2]*Department of Public Health & Primary Care, Leiden University Medical Center, Leiden, The Netherlands,* [3]*Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands, and* [4]*JJ van Rood Center for Clinical Transfusion Research, Sanquin Research, Leiden, The Netherlands*

Abstract

Aspirin is traditionally taken once daily in the morning and considered to be effective throughout the 24h interval. Cardiovascular events occur most frequently in the early morning, suggesting that these hours are critical in terms of adequate platelet inhibition. This study therefore assed platelet function in the early morning—8.00 AM—in healthy volunteers, during a once-daily (OD) 80 mg morning in comparison with an OD evening regimen and a twice-daily (BID) 40 mg regimen. It was an open-label randomized cross-over study, comprising 12 healthy subjects. Subjects were allocated to three sequential dosage regimens: 80 mg OD at 8.00 AM, 80mg OD at 8.00 PM, and 40 mg BID at 8.00 AM and PM. Platelet function 12 and 24 hours after aspirin intake was measured by means of serum thromboxane B_2 ($sTxB_2$) levels, the collagen/epinephrine closure time (Platelet Function Analyzer(PFA)-200®) and the Aspirin Reaction Units (ARU, VerifyNow®). The results demonstrated that early morning $sTxB_2$ concentrations were 5843pg in the morning regimen, 2877pg in the evening OD regimen, and 3343pg in the BID regimen (morning- vs evening regimen $p = < 0.01$; morning- vs BID regimen $p = < 0.01$). Early morning PFA-closure time ($p = 0.12$)) as well as VerifyNow ARU ($p = 0.17$) mean values were similar for all three regimens. In conclusion, the OD-morning regimen seems to acquire the lowest level of platelet inhibition during the critical early morning window. Switching to an OD-evening or BID intake seems prudent, although further research on clinical cardiovascular outcome in patients with stable cardiovascular disease is needed.

Keywords

Cross-over trial, aspirin, platelet aggregation, circadian rhythm, chronotherapy

History

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Introduction

Aspirin is widely used as secondary prevention in patients with established cardiovascular disease [1]. The preventative effects are mostly attributable to its ability to completely inactivate cyclo-oxygenase-1 (COX-1), leading to inhibition of thromboxane A_2 (TXA_2) production by platelets [2]. Furthermore, platelets are unable to produce new COX-enzyme due to the absence of nuclei. Consequently, the inhibition of COX-1 persists throughout the platelet's full life span of 10 days. The effect of aspirin has therefore traditionally been thought to provide protection during the full 24 h dosing interval.

Patients are routinely instructed to take aspirin on awakening, with maintenance doses of 75–100 mg once daily [3]. Up to 15% of these patients develop a stroke or a cardiovascular event (CVE) under aspirin use [4]. In addition, as many as 30% of patients with coronary artery disease (CAD) treated with aspirin are associated with insufficient platelet inhibition [5]. Of these patients, some appear to maintain an incomplete inhibition of platelet aggregation even at higher aspirin doses [1].

A possible cause for CVE occurring despite aspirin use may involve aspirin's pharmacokinetic properties. Recent studies have found that a once-daily regimen is associated with progressive platelet aggregability during the subsequent 24-hour dosing interval [6–8]. A likely explanation for this could be the production of new platelets (i.e. reticulated platelets) [9]. The production and release of these reticulated platelets by megakaryocytes adheres to a circadian rhythm, with a peak release in the late night and early morning. Moreover, previous research has shown that it is during these hours that the most ischemic events preferentially occur [10,11]. Hence, these hours may be essential in terms of adequate platelet inhibition. We, therefore, propose two possible regimen adjustments to improve platelet inhibition: 1. Adherence to a once-daily evening intake regimen, as it might provide better coverage during these—critical—nightly peak releases; 2.

C. Racca and J.J.K. van Diemen contributed equally to this work
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Correspondence: J.J.K. van Diemen, De Boelelaan 1118 (4A-45), Amsterdam 1081 HZ, the Netherlands. E-mail: jj.vandiemen@vumc.nl

Adherence to a twice-daily half-dosage intake regimen, as it will inhibit these newly formed platelets with a 12 hr. instead of 24 hr. dosage interval.

In view of these considerations, the objective of this study was to assess platelet function in the early morning—8.00 AM—in 12 healthy volunteers, during a once-daily (OD) 80mg morning compared with an evening regimen, and a twice-daily (BID) 40mg regimen

Methods

Participants

This study is registered in the Dutch trial register NTR5114. Healthy volunteers—aged 18 to 30 years old—were included. Exclusion criteria included: a history of drug use or current medical or recreational substance use. 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 VU University Medical Center Amsterdam.

Trial Design

This was an open-label randomized cross-over study, comprising 12 healthy subjects. Subjects were allocated to three sequential dosage regimens: morning, evening, and twice-daily. Sealed envelope randomization determined whether participants started with the morning or evening regimen. In the morning regimen, subjects were instructed to take aspirin (acetylsalicylic acid, 80 mg, non-enteric-coated) at 8:00 AM on 10 consecutive days. Since the aim of the study was to investigate which regimen provides the most platelet inhibition during the last 12 hrs. of a regular dosage interval, blood samples were drawn on day 10 and 11, respectively 12 and 24 hours after aspirin intake. Similar instructions were given to participants in the evening regimen, with aspirin intake at 8:00 PM and blood sampling on day 11. In the twice-daily regimen participants took aspirin twice a day (acetylsalicylic acid, 40 mg, non-enteric-coated) at 8:00 AM and 8:00 PM on 10 consecutive days. There was no washout period between regimens. It is to be expected that, taking into account the maximum lifespan of a thrombocyte is 10 days, every thrombocyte will have been in contact with the new regimen after 10 days of intake [12]. Blood sampling took place on day 11 with instructions to take the last aspirin immediately after the 8.00 AM blood sample (Figure 1). Patients were instructed to only have a light breakfast prior to the 8.00 AM sampling, to refrain from meals associated with high fat content 2 h prior to the 8.00 PM sampling, and to not change their intake schedule of other medication. And last, compliance was checked via self-reports in research diaries, as well as via pill counting.

Blood Sampling

All venepunctures took place at the laboratory facility of the VU University Medical Center. Samples were drawn from the antecubital vein through a 21-gauge needle, first into a precursor tube, then into one sodium citrate tubes (*BD Vacutainer® 0.109M Buff. Na_3 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) 7.2mg REF 368861)*. The EDTA sample was used to measure several hemostatic values, namely platelet-, leucocyte-, thrombocyte count, hemoglobin level and percentage of reticulated platelets. All samples—expect the serum tube, which was immediately incubated for 60 minutes at 37°C—were kept at ambient temperature for a maximum of 2 hours before the assays were performed. All baseline sampling took place during the 12 hour after OD-morning intake blood sampling (i.e. 8.00P PM).

End Points and Assessments

Serum Thromboxane B_2 was determined to be the primary endpoint to assess the level of platelet inhibition. The Platelet Function Analyzer Closer Time, Platelet Function Analyzer Predictive Index, and the VerifyNow Aspirin Reaction Units were considered as secondary endpoints.

Serum Thromboxane B_2 ($sTxB_2$)

Thromboxane A_2 (TXA_2) is the major product of the metabolism of arachidonic acid (AA) by platelets. Due to its very short half-life in plasma (30–60 seconds) it cannot easily be measured [13]. It is sequentially transformed into TXB_2, which is a biologically inactive and stable product that can be measured at low concentrations in serum or urine [14]. After venepuncture, the serum tube was immediately incubated for 60 minutes at 37°C. Prior to storage at −80°C, the serum samples were centrifuged at 3000 x g for 10 minutes. $STxB_2$ was measured by enzyme immunoassay according to manufacturers' instructions (Thromboxane B2 Express, Cayman Chemicals, Ann Arbor, MI, USA) and previously described by Bonten et al. [15]. Samples were analyzed in duplicate in the laboratory in order to measure the intra-assay coefficient of variance (CV), which was < 15% for all measurements.

Platelet Function Analyzer (PFA)-200®

The PFA-200 measures platelet aggregation induced by shear stress. The time needed for occlusion of the aperture in the cartridge by the thrombocyte plug is called the closure time (PFA-CT). The PFA-CT has a theoretical maximum of 300 seconds. Furthermore, the PFA-200 measures the total volume of blood used in the test (PFA-TV) and the initial flow rate (PFA-IFR). The PFA-200 requires 800 µl of whole blood to run the test. Any closure time greater than 300 seconds was reported as 301 in the data analysis. Our main study parameters were the closure time (PFA-CT), and the predictive index (PFA-PI). The PFA-PI is an arithmetical calculated index of all PFA-200's parameters (i.e. the PFA-CT, PFA-TV, and PFA-IFR), technical variables apart (e.g. thrombocyte count, haemoglobin levels, pH of the liquid medium) [16]. The assay was performed using the manufacturer's guidelines. Furthermore, the Collagen/Epinephrine Test Cartridge was utilized, which has previously been described as sensitive to aspirin mediated effects [17]. In a previous study, in which the PFA-CT was measured on two different days prior to breakfast, the median coefficient of variance was found to be 9.8% [18].

VerifyNow®

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 agonists which are specific to various antiplatelet medications. Within the test device wells, the instrument measures the increase in light transmittance over time. The VerifyNow® with arachidonic

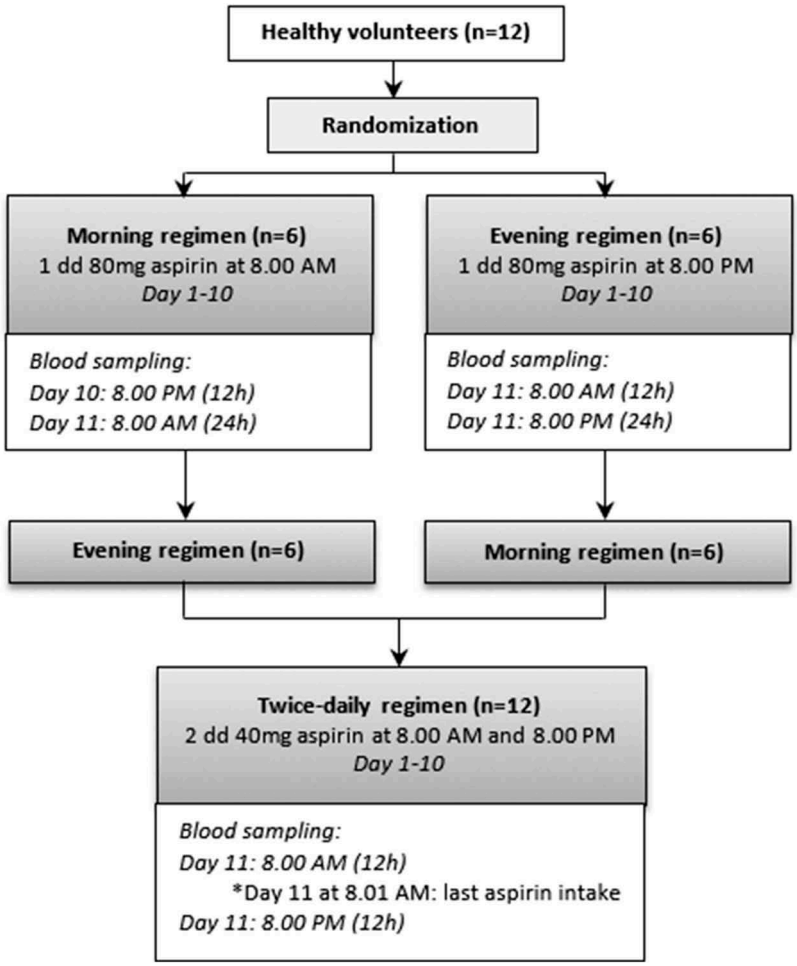

Figure 1. Flowchart of study protocol.

acid agonist (Aspirin Test' cartridge) was utilized. Results of the VerifyNow are expressed as Aspirin Reaction Units (ARU's). In a trial regarding aspirin resistance as measured by the VerifyNow Aspirin system, all duplicate analyses had a high degree of repeatability and a coefficient of variance of 3.0% during aspirin treatment [19].

Statistical Analysis

A minimum group of 12 participants had to enter this 'two-treatment' crossover study. This was estimated based on a minimum true difference of 1.785 pg/ml $sTxB_2$ between the different treatment groups, a power of 0.8, and a two-sided 0.05 significance level. The minimum true difference was based on a calculated 1% of the reference value for non-inhibited serum Thromboxane B2 in healthy volunteers [20]. 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 using the expectation-maximization method in SPSS or left as missing values accordingly. Statistical analyses were performed with SPSS statistics 22.0 for Windows (IBM SPSS Inc., Chicago, IL, USA).

Results

Characteristics of Included Subjects

From August 2015 through January 2016, a total of 12 healthy subjects underwent randomization . The baseline characteristics of the included subjects are summarized in Table I. There was larger proportion of female participants (58,3%) . Furthermore, five of these female subjects were on—low dose—oral contraception. Nevertheless, the average blood pressure fell within the expected range of healthy subjects (DBP 72.4 ± 2.0; SBP 118.3 ± 4.6) . Lastly, of worth to note is that the reported adherence to the study protocol (i.e. time of intake) the intake regimens was best for the morning intake (OD-morning 8:04 ± 0.04; BID-morning 7:57 ± 0:19) in comparison with the evening intakes (OD-evening 20:17 ± 0:19; BID-evening 20:06 ± 0:25).

Platelet Throughout a 24-Hour Interval

As is reported in Table II, serum thromboxane B_2 ($sTxB_2$) concentrations increased from 12 hours to 24 hours after aspirin intake for both morning- (2460 vs. 5843 pg/ml; $p = < 0.01$) and evening-OD regimens (2877 vs. 5029 pg/ml; $p = 0.04$). The PFA-closure time (PFA-CT) showed a stable function for the OD-

Table I. Baseline characteristics of study sample.

	Healthy subjects (n = 12)
General characteristics	
Female sex (n, %)	7 (58.3)
Age (years)	22.3 ± 1.6
Caucasian (n,%)	11 (91,7)
BMI (kg/m2)	21.3 ± 1.4
Current smoker (n, %)	1 (8.3)
Alcohol intake (units/week)	1.2 ± 0.4
Coffee intake (units/day)	2.3 ± 1.6
Diastolic blood pressure (mmHg)	72.4 ± 2.0
Systolic blood pressure (mmHg)	118.3 ± 4.6
Reported time of intake	
Once daily morning	8:04 ± 0.04
Once daily evening	20:17 ± 0:19
Twice daily half dosage	
Morning intake	7:57 ± 0:19
Evening intake	20:06 ± 0:25
Medication usage in past month (n,%)	
Hormonal anticonception	5 (58.3)
NSAIDS	0 (0)
Antibiotics	0 (0)
SSRI	0 (0)
Hemoglobin level in mmol/l	8.5 ± 0.8
Thrombocyte count 10^9/l	255.6 ± 61.8
Leukocyte count 10^9/l	7.6 ± 2.2
Reticulated platelets (%)	1.5 ± 0.7

Data are presentated as mean and standard deviation (±).

Table II. Results of 12 and 24h after aspirin intake for thromboxane B_2, PFA-closure time (PFA-CT), PFA-predictive index (PFA-PI) and aspirin reaction units (ARU) per regimen.

	12h after intake	24h after intake	p-value
Thromboxane B_2 (pg) [1]	2460 (2076–3156)	5843 (4829–10720)	<0.01
Morning OD regimen	2877 (1903–4110)	5029 (3655–6605)	0.04
Evening OD regimen			
***PFA-200*: PFA-CT (s)**	265 (168–301)	301 (177–301)	0.25
Morning OD regimen	301 (249–301)	301 (301–301)	0.11
Evening OD regimen			
***PFA-200*: PFA-PI**	7.15 (4.06–12.86)	8.13 (4.21–12.30)	0.94
Morning OD regimen	10.23 (6.88–14.23)	11.87 (11.22–13.08)	0.31
Evening OD regimen			
VerifyNow: ARU [1]	432 (388–555)	486 (419–563)	0.03
Morning OD regimen	496 (483–527)	456 (417–556)	0.12
Evening OD regimen			

Data are presented as median and interquartile range (IQR).
[1] Data with replaced missing values using expectation-maximization method.

evening regimen (301 vs. 301 sec. $p = 0.11$), as well as the OD-morning regimen (265 vs. 301 sec.; $p = 0.25$). This was similar for the PFA-predictive index (PFA-PI), for either the morning- (7.15 vs. 8.13; $p = 0.94$) and the evening-OD regimen (10.23 vs. 11.87; $p = 0.31$). The VerifyNow results were significantly lower at 12 h compared to 24 h after intake for the morning (432 vs. 486 ARU; $p = 0,03$), but not the evening regimen (496 vs. 456 ARU; $p = 0.12$).

Table III. Results of 8 AM measurements for thromboxane B_2, PFA-closure time (PFA-CT), PFA-predictive index (PFA-PI) and aspirin reaction units (ARU) per regimen.

	Morning regimen (R_A)	Evening regimen (R_B)	2dd regimen (R_C)	p-value
Thromboxane B_2 (pg) [1]	5843 (4829–10720)	2877 (1903–4110)	3343 (1615–7380)	<0.01*
PFA-200: PFA-CT (s)	301 (177–301)	301 (249–301)	301 (261–301)	0.12
PFA-200: PFA-PI	8.13 (4.21–12.30)	10.23 (6.88–14.23)	10.74 (8.82–13.65)	0.56
VerifyNow: ARU [1]	486 (419–563)	496 (483–527)	399 (383–535)	0.17

Data are presented as median and interquartile range (IQR).
[1] Data with replaced missing values using expectation-maximization method.
* R_A vs R_B p = 0.003; R_A vs R_C p = 0.003.

Table IV. Results of average of all time points for thromboxane B_2, PFA-closure time (PFA-CT), PFA-predictive index (PFA-PI) and aspirin reaction units (ARU) per regimen.

	Morning regimen (R_A)	Evening regimen (R_B)	2dd regimen (R_C)	p-value
Thromboxane B_2 (pg) [1]	4513 (3436–7179)	3863 (3228–5609)	3383 (2573–6671)	0.05*
PFA-200: PFA-CT (s)	283 (171–301)	301 (275–301)	301 (281–301)	0.06
PFA-200: PFA-PI	8.36 (4.04–12.64)	10.84 (9.83–13.18)	11.26 (10.53–12.87)	0.27
VerifyNow: ARU [1]	472 (404–545)	487 (436–541)	440 (388–466)	0.56

Data are presented as median and interquartile range (IQR).
[1] Data with replaced missing values using expectation-maximization method.
* R_A vs R_B p = 0.050; R_A vs R_C $p = 0.041$.

The delta $sTxB_2$ concentration, defined as the difference in $sTxB_2$ values between the 12 h- and 24 h-measurement for the OD regimens and the 12 h- and second 12 h-measurement for the BID regimen, were calculated for all regimens. The delta appeared to be higher in the OD regimens than the BID regimen, with a statistically significant difference only in the comparison between the morning- and BID regimen ($p = 0.02$) (Figure 2).

Platelet Function in the Morning Hours

Early morning $sTxB_2$ levels were higher in the morning regimen (5843 pg/ml) compared to both the evening (2877 pg/ml) and the BID regimen (3343 pg/ml; $p = < 0.01$) (Table III andFigure 3). Both early morning PFA-CT (morning-OD 301 sec.; evening-OD 301 sec.; BID 301 sec.) and PFA-PI (morning-OD 8.13; evening-OD 10.23; BID 10.74) values were not statistically significantly different between all three regimens (overall $p = 0.12$ and $p = 0.56$, respectively). Lastly, verifyNow values demonstrated a lower function during morning hours within the BID-regimen, albeit not significantly (morning-OD 486 ARU; evening-OD 496 ARU; BID 399 ARU; $p = 0.17$)

Daily Average of Platelet Function

The daily average $sTxB_2$ concentrations were higher in the morning regimen (4513 pg/ml) compared to both the evening regimen

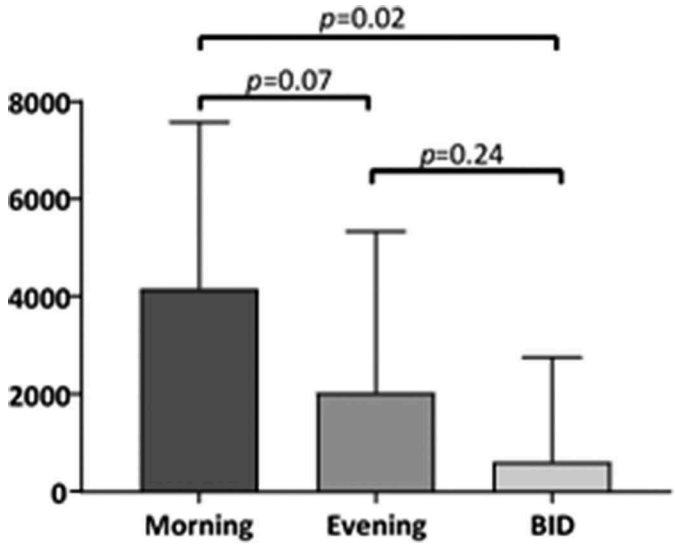

Figure 2. Difference in serum thromboxane B2 measurements (delta serum thromboxane B2) during 24h interval per regimen. Data are presented as median and interquartile range.

(3863 pg/ml; $p = 0.05$) and the BID regimen (3383 pg/ml; $p = 0.04$) (Table IV and Figure 4). The PFA-CT average values were lower in the morning regimen (283 sec.) than the evening- (301 sec.) and BID regimen (301 sec.), but the overall multiple group comparison of PFA-CT values was not statistically significant ($p = 0.06$). Also, the daily PFA-PI averages were found to be lower in the morning regimen (8.36) compared to the evening- (10.84) and BID regimen (11.26), however, not reaching statistical significance (overall $p = 0.26$). The daily average VerifyNow results were lowest in the BID regimen (440 ARU), but the multiple group comparison did not show significant differences between the three regimens (overall $p = 0.56$).

Discussion

The findings of the current study are two-fold: 1. The once daily morning intake regimen exerts the lowest level of platelet inhibition, as measured by $sTxB_2$, during the critical early morning window; 2. Both once-daily regimens showed decreasing inhibitory effects of aspirin on $sTxB_2$ concentrations between 12 and 24 h after intake.

To our knowledge, this is the first study demonstrating a better early morning platelet inhibition by a half-dosage intake regimen compared with the OD-morning regimen in healthy volunteers. These results are in line with Larsen et al. who recently also demonstrated a better suppression of $sTxB_2$ in the morning by a half-dosage aspirin regimen. This study, however, was done in patients with essential thrombocytosis [21]. Furthermore, in line with previous research, we found a better early morning platelet inhibition in OD-evening in comparison with OD-morning

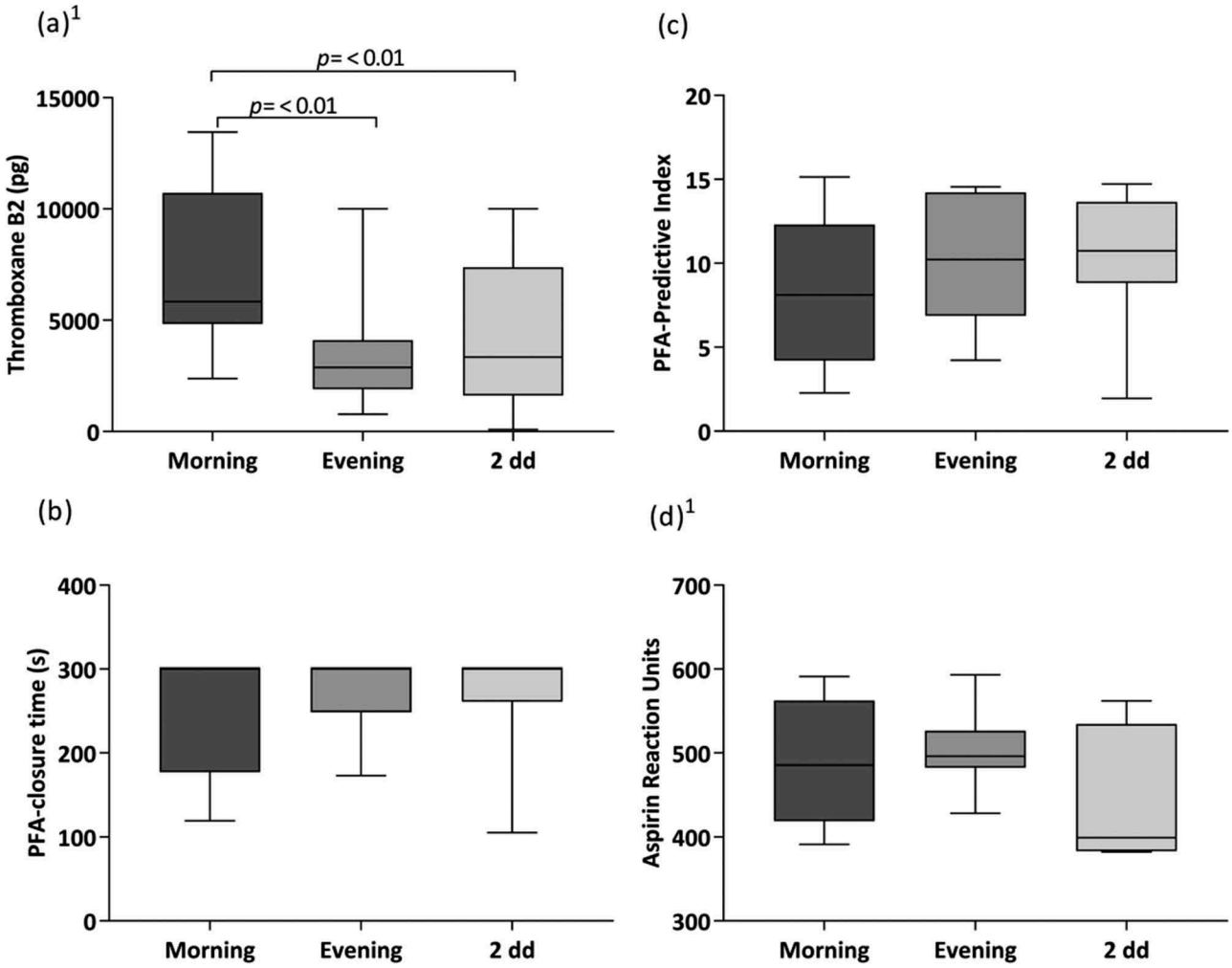

Figure 3. Comparison of 8 AM measurements for (a) thromboxane B2, (b) PFA-closure time, (c) PFA-predictive index, (d) aspirin reaction units for each regimen. Data are presented as median and interquartile range. 1 Replacement of missing values using expectation-maximization method.

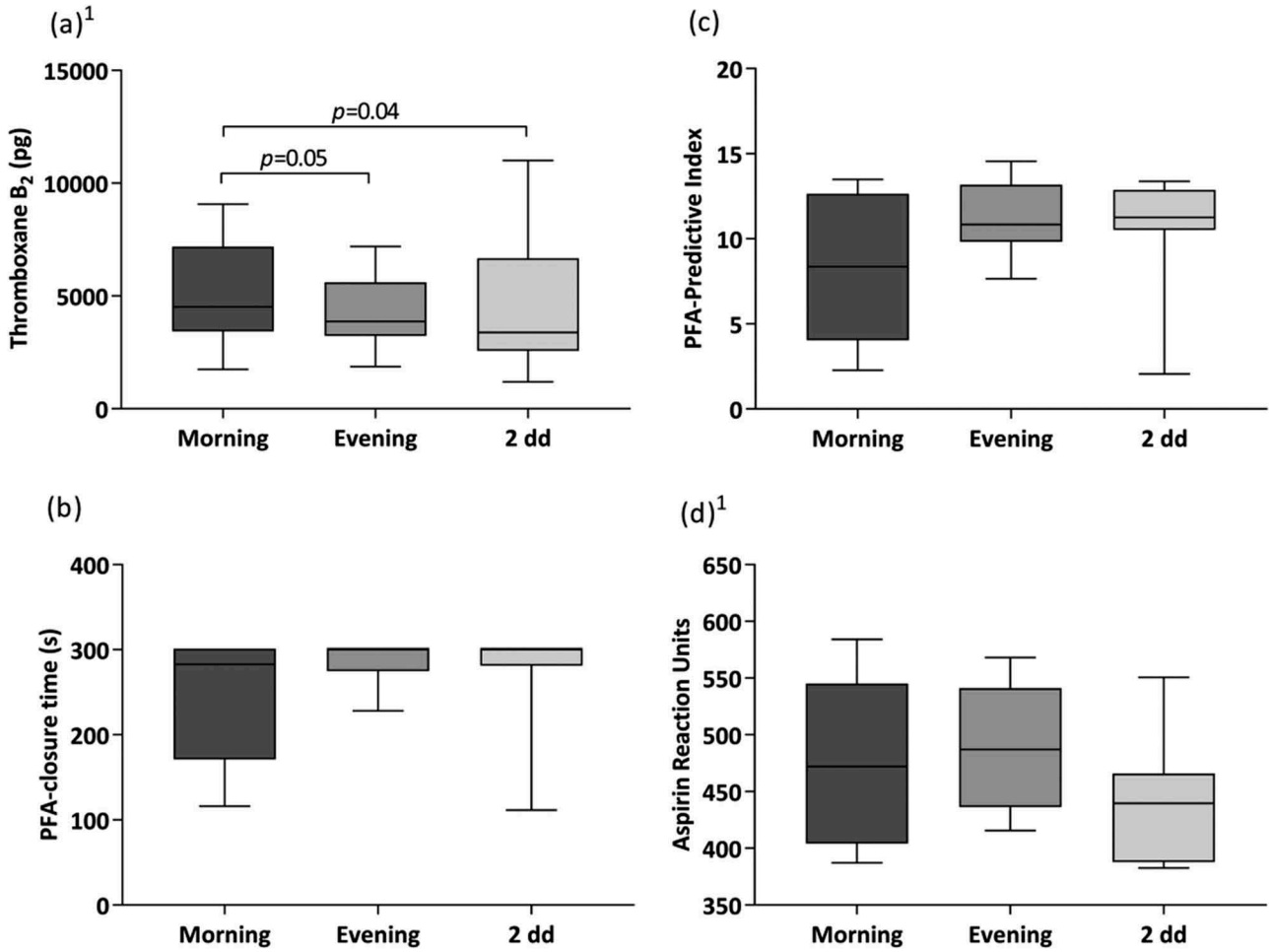

Figure 4. Comparison of average of all time points for (a) thromboxane B2, (b) PFA-closure time, (c) PFA-predictive index, (d) aspirin reaction units for each regimen. Data are presented as median and interquartile range.1 Replacement of missing values using expectation-maximization method.

[15,18,22]. Since cardiovascular events occur most frequently during late-night and early-morning hours [10], optimum platelet inhibition during these hours seems desirable.

The second finding is consistent with the results of various other studies [6–8]. The possible explanation we propose for this result is two-fold: 1. The release of reticulated platelets adheres to a circadian rhythm: most reticulated platelets are released in the late night and early morning [23]. OD-evening is more likely to inhibit these peak released reticulated platelets [9]; 2. Although megakaryocytes possess the ability to produce new cyclooxygenase (COX), they can be inhibited for a maximum of 12 hours [24]. Consequently, it could be that after OD-evening intake they are—partially—inhibited, and, perhaps, release fewer reticulated platelets. Unfortunately, our sample was too small to analyze whether OD-evening might acquire a lower percentage of reticulated platelets in comparison with OD-morning.

Surprisingly, no statistically significant differences were found between the OD-evening regimen and the BID regimen. This result may be explained by the fact that healthy individuals have a lower platelet turnover than patients with stable cardiovascular disease [25,26], thus the additional inhibition might not be visible. Perhaps, this will be beneficial in patients with an increased platelet turnover. In fact, a few other studies have demonstrated the statistically significant better platelet inhibition of a BID regimen to an OD regimen in patients [26–29]. These were mostly conducted in patients with diabetes mellitus and essential thrombocytosis, in which platelet turnover is known to be higher [2,30].

The main strength of this study lies in the strict adherence to and reported methodology. In our opinion, most previous studies do not report on a design where the two time points—12 and 24 hours after aspirin intake—are constant throughout every regimen. These fixed time points increase the comparability of every regimen. Furthermore, although not all parameters of platelet function tests showed statistically significant results—as the thromboxane production is the primary target of aspirin—$sTxB_2$ levels can be seen as the most specific test for aspirin effectivity [31]. An important limitation, however, lies in the technical properties of the Platelet Function Analyser (PFA). Due to the technical maximum of 300 seconds for the PFA-closure time, all measurements in which the orifice had not yet been occluded at the end of the test as "no closure" were reported as 301 seconds in our database. Seeing as many measurements resulted in values of > 300s it was impossible to find relevant differences between the regimens beyond these maximum values. Therefore, this might have led to an underestimation of the effect of the type of regimen on the inhibition of platelet aggregation. Furthermore, as this was only a pilot-study in healthy volunteers, more research with a larger sample of the target population—patients with stable cardiovascular disease—is needed.

Conclusion

The once daily morning intake regimen exerts the lowest level of platelet inhibition throughout the most critical window—the early morning—in comparison with the once daily evening intake, and

the twice daily half dosage intake. Furthermore, both once-daily regimens showed decreasing inhibitory effects of aspirin between 12 and 24 hours after intake, albeit only significantly for the $sTxB_2$ levels. Switching to an evening- or twice-daily intake seems prudent, although further research on clinical cardiovascular outcome in patients with stable cardiovascular disease is needed to establish the most effective intake regimen.

Conflict of interest

None.

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ORCID

Mattijs Everard Numans http://orcid.org/0000-0002-0368-5426

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